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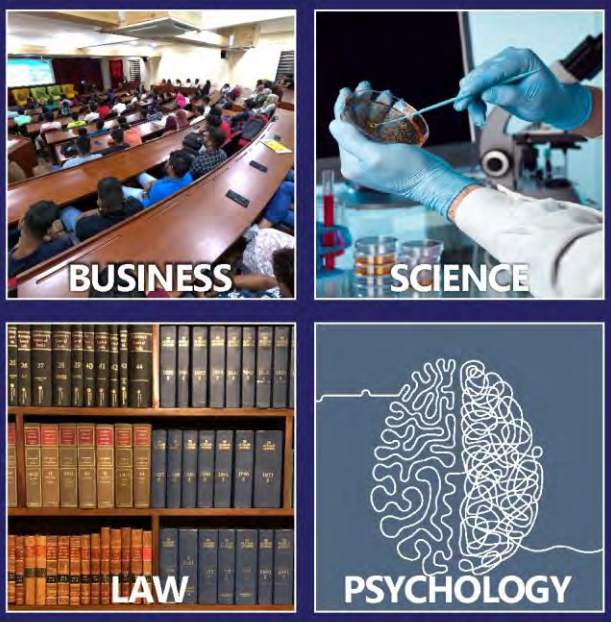

Journal of Applied Learning

Volume 1 - Issue 1 - July 2023

BMS Publications

Journal of Applied Learning

The Journal of Applied Learning is a peer reviewed journal published by the Business Management School (BMS), Colombo, Sri Lanka. The journal publishes two issues biannually in January and July. The Journal aims to publish research articles in the fields of Science and Business Management.

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BMS Digital Studio

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Contributions of a weed; Microwave assisted green synthesis of silver nanoparticles using morning glory (*Ipomoea* spp.) for the assessment of its antioxidant, antibacterial and photocatalytic activity

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Abstract

The *Ipomoea* spp. is a common weed which has untapped potential for the scientifically significant green synthesis silver nanoparticles (AgNPs). AgNPs have multiple benefits in fields including medicine and environmental clean-up. In this study, eco-friendly water-based extraction was carried out on the leaves of five different *Ipomoea* spp. The synthesis of AgNPs was attempted using the extracts. *Ipomoea lacunosa* (S3) AgNPs were successfully generated through microwave assisted synthesis. The formation of AgNPs caused the solution to deepen in colour and resulted in a peak of 420nm under UV-Vis spectroscopy. This peak corresponded to the surface plasmon resonance (SPR) peak of AgNPs. The S3AgNPs, characterized by Scanning Electron Microscopy, were spherical and of 60nm in diameter. Furthermore, the antioxidant, antibacterial and photocatalytic activity of the S3AgNPs were compared against *I. aquatica, I. cairica, I. pes-caprae* and *I. purpurea* water extracts. The S3AgNPs showed elevated activity as opposed to the water extracts in the Total Flavonoid Content, Total Phenolic Content, Total Antioxidant Content and DPPH assays. It had an IC50 of 20.3% in the DPPH assay. The S3AgNPs demonstrated potent antibacterial activity against the Gram-negative *Escherichia coli* and the Gram-positive *Staphylococcus aureus* as opposed to the water extracts. A higher zone of inhibition was observed for the *S. aureus* compared to *E. coli*. In the presence of sodium borohydride, 100ppm of S3AgNPs degraded 94% of Methylene blue dye in 240 minutes. The implications of this research are widespread and can be used to develop novel antibiotics, antioxidants and water treatment compounds.

Keywords: silver nanoparticles, *Ipomoea* spp., microwave-assisted, antioxidant activity, photocatalytic activity**,** antibacterial activity

1. Introduction

Nanotechnology is a thriving and relatively novel field of science which deals with the synthesis of particles sized between 1-100nm. The use of nanoparticles is gaining momentum as they are potent in multiple fields including medicine, engineering, electronics and environmental clean-up due to their high surface area to volume ratio. Nanoparticles are produced using two principle approaches. The top-down approach begins with larger particles which are consequently degenerated in size until they form nanoparticles. The bottom-up approach causes the proliferation of atomic and molecular particles to form larger nanosized particles. The three primary methods of synthesis are through chemical, physical and biological processes.[1]

Silver nanoparticles (AgNPs), as opposed to other metallic nanoparticles remain in the limelight due to its non-toxicity, well-documented antimicrobial activity, catalytic properties, and its great thermal and electrical conductivity. These merits contribute to AgNPs having the highest marketing value of all nanoparticles, and this trend is expected to grow exponentially.[1] It has been forecasted that by 2025, the global market value of AgNPs would reach $USD 98 billion.[2,3] As the use of AgNPs increases, much focus has been placed on synthesising them with minimal environmental impact. The green synthesis of nanoparticles

utilises plant or microorganism-based protocols, allowing the use of toxic solvents and the formation of harmful by-products to be circumvented.[4] Plant based synthesis of AgNPs gives rise to undisrupted surfaces, unlike those formed using the top-down approach and provides a more economical and simpler alternative to using microorganisms. Plant extracts reduce silver ions to metallic silver, effectively building up nanoparticles from the atomic scale until they reach the nanoscale. The extracts also contain polyphenols, amino acids and vitamins which act as capping agents that regulate the size of the particles, stabilizing them and preventing agglomeration.[5]

The primary heating techniques used in plant-mediated synthesis of nanoparticles are mild-heat and high-heat synthesis. Although the choice of conventional heating or microwave irradiation is predominantly governed by the plant material, microwave assisted heating (MAS) is energy efficient, faster, reduces agglomeration and produces nanoparticles with smaller size variation.[6]

The *Ipomoea* spp. is the largest genus classified under the family of *Convolvulaceae*, which contains flowering plants commonly known as the Morning Glory.[7] The genus grows abundantly in the tropics and some temperate regions and has been termed a weed as a result. The trumpet shaped showy flowers have been grown as an ornamental while some the seeds of some species show psychedelic properties. The leaves of *Ipomoea aquatica* is consumed in Sri Lanka, South-East Asia and China for its high nutritional value. The leaves, seeds and roots of multiple species are also well known for ethnic medicinal treatments, where they have been used as a hypoglycaemic, purgative, anti-arthritic, anti-microbial and anti-inflammatory compound among others.[8] Five different species of *Ipomoea* AgNPs are comparatively analysed in this study, *I. aquatica, I. cairica, I. lacunosa, I. pes-caprae* and *I. purpurea.*

The spread of resistant bacteria remains a threat only intensified by the emergence of multi-drug resistant super bacteria. Silver is an age known antimicrobial agent and coupling it with the efficacy of nanoparticles makes it a potential weapon against bacteria. Due to their size, AgNPs can easily gain access to cells to exert antibacterial effects and as they are not known to induce resistance, they remain an attractive choice for antibacterials.[9]

The contribution of azo dyes to environmental pollution is significant as they are released by multiple industries from textiles to printing. These dyes are persistent compounds which can accumulate in organisms and can have harmful and even carcinogenic effects.[9] Standard effluent treatments only achieve substandard efficiency and create sludge with amine residues whereas AgNPs synthesised via the green route have the additional advantage of lower risk in terms of long-term toxicity as they do not persist in the environment unlike conventional dye degrading treatments.[10–12]

The elucidation of the exact mechanisms of the photocatalytic effect of AgNPs is still in progress and the current consensus is that electrons absorb 2.7-3.03 eV of energy from the UV-Vis spectral range, exciting them from the valence band to the conductance band whilst creating holes.[13] This leads to a photo-redox reaction which reduces oxygen to oxygen radicals and oxidises hydroxide ions to hydroxide radicals.[14] The free radicals can then attack and degrade azo dyes like methylene blue. The phytoremediation expected from the *Ipomoea* extracts coupled with the potency of AgNPs could lead to synergistic effects in the combined photocatalytic potential of *Ipomoea* AgNPs.

The balance between free radicals (FRs) and antioxidants in the human body is a precarious one. FRs extend a three-pronged attack on proteins, nucleic acids and lipids leading to cell damage and ultimately cell death due to oxidative stress.[15–17]

Oxidative stress also plays a role in the progression of diabetes, cancer, cardiovascular diseases, neurodegenerative conditions and aging. Although the body is equipped to neutralise endogenously produced FRs, exposure to stressors like radiation, pollution heavy metals and processed food can disturb this fine balance as they encourage higher FR production rates.[18] The free radical scavenging activity of antioxidants takes place through the transfer of an electron or a hydrogen atom.[19,20] This ability of antioxidants to counterattack the detrimental effects of FR forms the basis for gauging antioxidant capacities of the synthesised *auoea* nanoparticles.

Previous research on the different species of *Ipomoea* have proved their high potential as precursors for AgNPs as they contain a high concentration of phytochemicals and antioxidants.[21–23] Therefore, this study focuses on the synthesis of five varieties of *Ipomoea* AgNPs for the determination of its antioxidant, antibacterial and photocatalytic activities. The assessment of antioxidant capacity would be based on the TAC, TPC, TFC and DDPH assays while the antibacterial activity will be tested using well diffusion against *S. aureus* and *E. coli*. The photocatalytic activity will be judged using methylene blue as a model dye. The outcomes of the research are expected to contribute towards the advancements in disease treatment and water effluent treatment.

2. Methodology

2.1. Preparation of leaf extracts. Leaf samples of *I. aquatica* (S1)*, I. cairica* (S2)*, I. lacunosa* (S3)*, I. pes-caprae* (S4) *and I. purpurea* (S5) were washed and shade dried. The dried leaf samples were ground to fine particles. Then 2.0g of each sample was added to 50mL of distilled water. The mixture was incubated at 95ºC in a dry hot air oven for 20 minutes. The cooled extracts were subsequently filtered using Whatman No.1 filter paper and stored at 4ºC till required.[24]

2.2 Optimization of conditions for the synthesis of nanoparticles. 9mL of 1mM $AgNO_3$ was added dropwise to 1mL of *Ipomoea* extract. The prepared solutions were then heated in a microwave at 140W, 364W and 511W for 2.5 and 5 minute intervals. The absorbance values were taken between 300-540nm using a UV spectrophotometer.[24]

2.3 Phytochemical analysis. The protocols detailed by Ravi and Krishnamurthy was followed to build a phytochemical profile consisting of alkaloids, anthocyanins, carbohydrates, coumarin, quinones, saponins, tannins and terpenoids.[25]

2.4 Dilution of the samples. The extracts and S3AgNP samples were diluted in a 1:15 ratio using distilled water.

2.5 Antioxidant Assays: The diluted water extracts and AgNPs were used in the following assays.

2.5.1 Total Flavonoid Content (TFC) Assay. To 1.5mL of sample, 0.2mL of 10% (w/v) $AlCl_3$ and 0.2mL of 1M potassium acetate were added. The mixture was left to rest at room temperature for 30 minutes, Triplicates were prepared and left at room temperature for 30 minutes. The absorbance was measured in triplicates using spectrophotometer against water as a blank at 415 nm. The concentration was expressed in equivalents of Quercetin acid in gQE/100g.[26]

2.5.2 Total Phenolic Content (TPC) Assay. To 0.4mL of the sample, an equal volume of 10% (v/v) Folin-Ciocalteu reagent was added along with 1.25mL of 7.5% (v/v) Na_2CO_3. The mixture was incubated for 2 hours at room temperature. The absorbance was measured in triplicates using spectrophotometer against water as a blank at 765 nm. The concentration was expressed in equivalents of Gallic acid in mgGAE/100g.[26]

2.5.3 Total Antioxidant Capacity (TAC) Assay. To prepare the phosphomolybdenum reagent, (0.6M Sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate in a 1:1:1 ratio)

were mixed. 0.5mL of the reagent was added to 1.5mL of the samples and incubated at 95°C for 90 minutes. The absorbance was then read in triplicates using spectrophotometer against water as a blank at 695nm. The concentration was expressed in equivalents of ascorbic acid in µgAAE/100g.[26]

2.5.4 DPPH Assay. The plant extracts and S3AgNPs were diluted with distilled water to achieve a concentration series of 100%, 80%, 60% 40% and 20%. 1mL of the sample was reacted with 2mL of 0.1mM DPPH in methanol and the absorbance was measured in triplicates using spectrophotometer against methanol as a blank. The radical scavenging activity was calculated as follows:

$$\%\ Activity = \frac{A_{DPPH} - A_{Sample}}{A_{DPPH}} \times 100$$

Where A_{sample} was the absorbance of the sample and A_{DPPH} was the absorbance of the DPPH stock solution.[26]

2.6. Antibacterial activity. Isolated colonies of *E. coli* and *S. aureus* were inoculated into 0.9% saline (w/v) to match the turbidity of a 0.5% McFarland standard. Mueller Hinton agar plates were inoculated with the two bacterial species and wells were created using a 10mm pipette tip bore. 1mL of the water extracts and the S3AgNPs was slow dried at 45°C in an oven until they evaporated. The dried samples were rehydrated by the addition of 200µL of distilled water and 50µL was loaded into each of the respective wells. For the negative control, 0.9% of saline was used while gentamicin disks were placed as positive controls. The plates were sealed and incubated upright at 37°C for 24 hours. The zones of inhibition were measured in centimetres.

2.7. Analysis of the photocatalytic activity. 10 mg/L methylene blue was prepared, and an absorbance curve was plotted between 340-780nm using a UV spectrophotometer. Dye solutions containing 10,100 and 500ppm of S3AgNPs were left under direct sunlight and absorbance readings were taken at specified time intervals. This was repeated with the addition of 10µL of 0.2M $NaBH_4$.[27]

2.8. SEM Analysis. The S3AgNPs were characterized at the Sri Lanka Institute of Nanotechnology (SLINTEC), Homagama using a Hitachi SU66600 SEM.

2.9. Statistical Analysis. Statistical analysis of the data was computed using Excel® for Microsoft 365 to obtain ONE-way ANOVA and t-tests while correlation graphs were plotted using IBM® SPSS® Statistics, version 21. All statistical tests were significant at a minimum p value of 0.05.

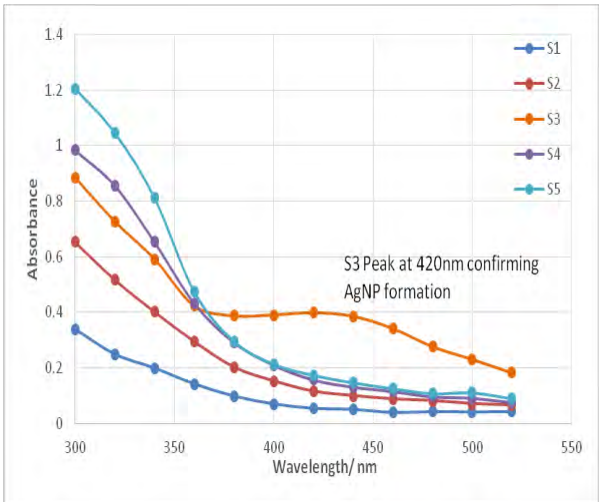
3. Results and Discussion

The colour of the solution gradually changed from pale yellow brown to deep red brown indicating the formation of AgNPs. Only S3 generated a single distinct peak at 420nm at 140W (2.5 mins), 364 (2.5 and 5.0 mins) and 511W (5.0 mins) giving a preliminary confirmation of AgNP synthesis. This peak is characteristic of AgNPs and corresponds to its surface plasmon resonance (Figure 1). The other samples had no peaks indicating non-formation of AgNPs.

The green synthesis of AgNPs has a plethora of benefits over conventional chemical methods in terms of environmentally friendliness, economy and yield. It remains a highly attractive research field with plant origins offering higher control and flexibility in terms of size and morphology of prepared particles.[28] The use of water as an alternative to organic solvents during the extraction process maintains this eco-friendliness of the protocol.[29] In this research we explored the synthesis and properties of nanoparticles from five different species of *Ipomoea*. The utilization of the *Ipomoea* species for the synthesis of nanoparticles can carry multiple advantages as the species is a pernicious weed with no competing uses. This could be an

efficient way to ensure mechanical movement of the plant and control its spread.[30]

Figure 1. Absorbance vs wavelength graph for UV-Vis characterisation of silver nanoparticles at



140W for 2.5 minutes.

The analysis of spectrophotometric curves showed that *Ipomoea lacunosa* (S3) was the only species that was able to successfully synthesise AgNPs. However, AgNPs from the other species of *Ipomoea* have been successfully synthesized in other studies and the reasons behind the absence of nanoparticles in this study can be conjectured on. This could be because S3 had more stable and higher content of reducing agents like proteins, amino acids, polysaccharides, terpenoids and vitamins.[31] In addition, S3 was the only sample which had all the phytochemicals tested. All the samples tested positive for tannins and quinones while carbohydrates were seen in none.

The microwave assisted synthesis (MAS) of AgNPs is a one pot reaction that combines the nucleation and growth phases in a single efficient reaction.[32] The alternate kinetics followed by MAS allow for a shorter heating time making the process highly efficient and even results in a higher yield.[33] Additional benefits of MAS include an increased uniformity and a greater control of nanoparticle morphology size and crystallinity.[34] Comparative studies done on noble metal NPs have demonstrated that MAS of NPs result in an elevated catalytic activity as opposed to those synthesised using conventional heat induced methods.[35]

The UV-vis spectrum, in addition to providing preliminary details of nanoparticle synthesis was also used to determine the optical properties of the AgNPs. The conductivity of the AgNPs can be gauged by calculation of the band gap energy. The band gap is defined as the minimum energy required for an electron to transition from the valence band to the conduction band. The band gap energies can differentiate between semi-conductors, which have a band gap less than 3eV, and insulators which have a band gap greater than 4eV.

To determine the band gap of the nanoparticles the following equation was used.

$$Band\ gap\ energy = \frac{h \times c}{\lambda}$$

where h is the Planck constant, c is the speed of light and λ is the absorbance peak obtained during characterization of the nanoparticles.

Based on their band energy of 2.96eV, the S3AgNPs were classified as semiconductors. This explains their photocatalytic activity as semi-conducting nanoparticles have been shown to possess intrinsic dye degradation abilities. This property also indicates that the synthesized AgNPs can be used in biosensors, light emitting devices, electronic devices, and even solar cells. The synthesized AgNPs had a mean width of 60nm and a spherical morphology (Figure 2).

The S3AgNPs outperformed the water extracts in the TFC, TPC and TAC assays and the differences were all statistically significant (Table 1).

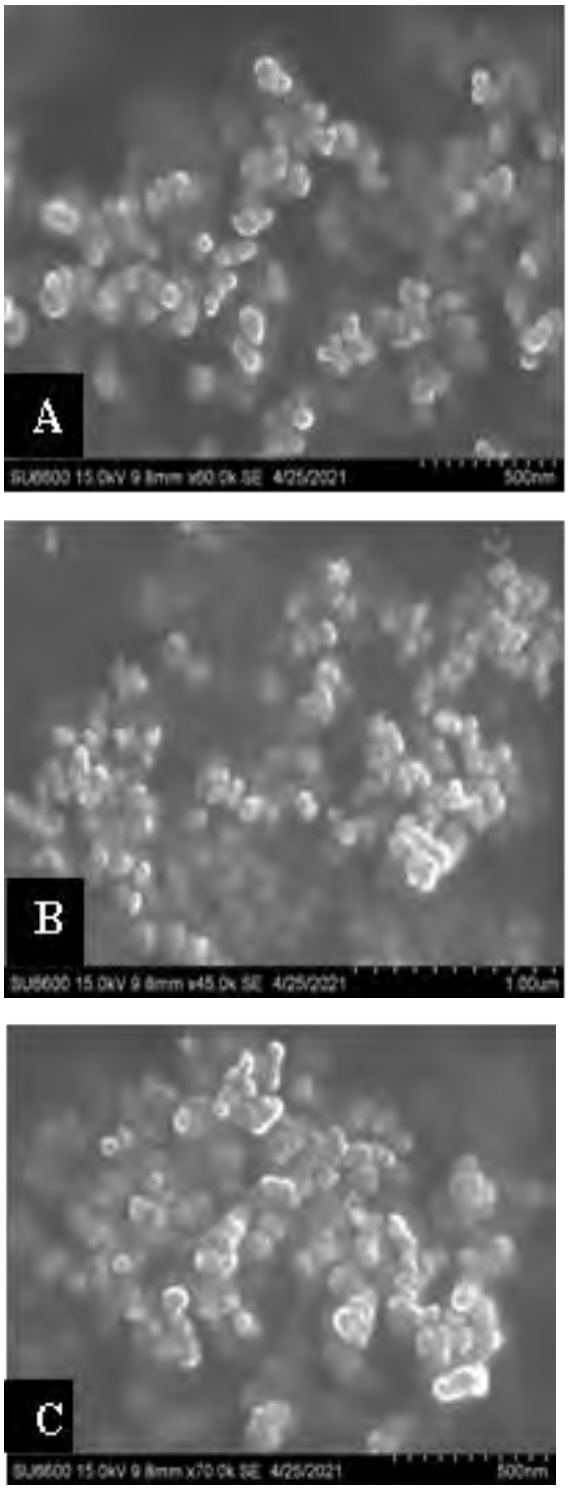


Figure 2. SEM images of S3AgNPs at different magnifications A) x60.0k B) x45.0k C) x70.0k

Table 1. The total flavonoid content, total phenolic content and the total antioxidant capacity of the leaf water extracts and the S3AgNPs.

Sample	TFC (gQE/ 100g of sample)	TPC (mgGAE/ 100g of sample)	TAC (µgAAE/ 100g of sample)
S1	0.83±0.06	48.21±6	9.89±1.4
S2	1.2±0.02	34.64±6.2	22.16±5.5
S3	1.74±0.14	42.86±8.5	16.7±2.7
S4	3.05±0.2	43.21±5.5	23.07±2.7
S5	3.69±0.05	42.14±10.7	39.77±1
S3AgNP	6.04±0.53	1561±257	97.72±20.5

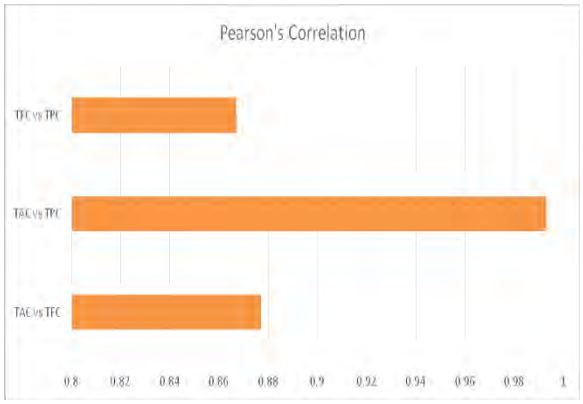


Figure 3. Pearson's correlation between the TAC, TFC and TPC assays.

The antioxidant properties of the particles were investigated using the TFC, TAC, TPC and DPPH assays. Phenols and flavonoids have optimal structures for the donation of electrons in the scavenging of free radicals making them effective antioxidants.[36] To elucidate which of the two contributed most towards the antioxidant properties seen, regression plots using the Pearson's correlation test were generated to obtain R values. The highest correlation was seen between the TAC and TPC assays which were a strong indication

that the antioxidant properties of the leaf extracts were primarily mediated by the phenols present (Figure 3). This deduction is in line with previous research conducted on the *Ipomoea* species.[37,38] The phenols predominantly contributing towards the antioxidant activity could include caffeoylquinic acid derivatives.[39] The DPPH activities were expressed as IC_{50} values (Table 2). The S3AgNPs had the lowest IC_{50} demonstrating it was the highest-ranking FR scavenger and could reach 50% of maximal activity at a concentration of 20.6%. The most potent scavenger amongst the water extracts was S1 with an IC_{50} value that was identical to previous studies.[26,36]

Table 2. IC_{50} of DPPH free radical scavenging activities of the leaf water extracts and S3AgNPs

Sample	IC_{50} (%)
S1	48.4
S2	63.6
S3	69.9
S4	75.4
S5	77.4
S3AgNPs	20.3

The S3AgNPs formed higher zones of inhibition in comparison to the leaf extracts in both *E. coli* and *S. aureus* (Figure 4). Between the bacterial species, greater antibacterial activity was observed against *S. aureus* allowing us to deduce that the *Ipomoea* species was more adept at the disruption of Gram-positive bacterial colonies. This is in agreement to other studies that have dealt with the family of *Ipomoea.*[24] Gram positive bacteria evolve resistant mechanisms easily and this potency of S3AgNPs against the species can be exploited to develop novel antibacterial remedies.

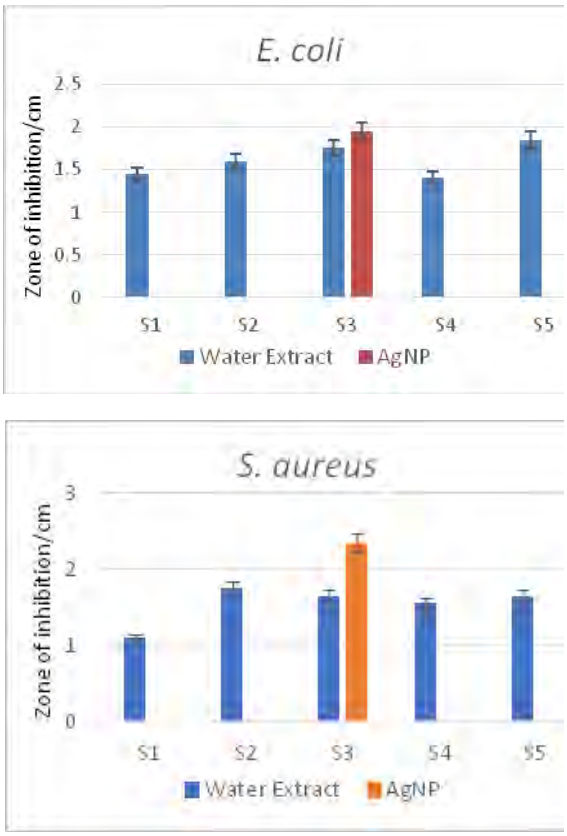


Figure 4. Diameter of the zones of inhibition in *E. coli* (left) and *S. aureus* (right) of the water extracts and S3AgNPs

In the absence of $NaBH_4$, the S3AgNPs were able to decrease the peak of methylene blue although no visible decolorisation took place. In the presence of $NaBH_4$, however, significant decolorisation was seen. Using 100ppm of AgNPs in the presence of $NaBH_4$ resulted in a 94% dye degradation in 240 minutes (Figure 5)

AgNPs inherently possess photocatalytic activity but the conjugation with plant extracts intensifies this effect. Compared to other metallic nanoparticles, AgNPs demonstrate greater photocatalytic strength due to its smaller band gap energy.[40] Furthermore, AgNPs have high absorption within the visible light spectrum. It therefore is a perfect candidate to exploit in the

goal of dye degradation. In this study, the S3AgNPs were exposed to different conditions in terms of concentration of the AgNPs and the presence or absence of a catalyst. In the absence of a catalyst, the S3AgNPs showed modest photocatalytic activity where the absorbance peak decreased but complete degradation was not observed. This validated the use of a catalyst for further analysis. The cationic $NaBH_4$ was able to cause a dramatic degradation of the dye after just 120 minutes. The kinetics of the photocatalytic activity were plotted using the equation below on the assumption that first-order kinetics were followed:

$$ln\frac{C}{C_0} = kt$$

where C was the concentration, C_0 was the initial concentration and t was the time. Values of the rate constant (k) were obtained from the slope and are listed in Table 3.

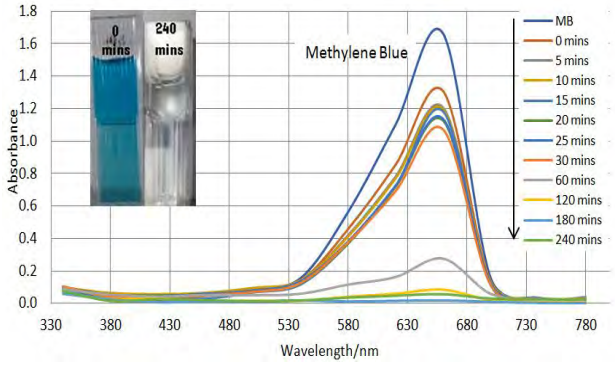


Figure 5. Photocatalytic activity of 100ppm S3AgNPs with $NaBH_4$

The highest rate constant obtained was for 100ppm of dye with $NaBH_4$ indicating that these conditions are the closest to the optimum working conditions of the synthesized S3AgNPs. In reactions which included $NaBH_4$, a higher dose of AgNPs resulted in a greater photocatalytic effect. This observation was also reported in previous studies on other *Ipomoea* species. Conversely, in the absence of $NaBH_4$, lower concentrations showed more enhanced activity. This observation could be due to the aggregation of AgNPs at increasing concentration which reduces the surface area available for catalysis.

Table 3. Rate constants of the different S3AgNPs reaction mixtures

Conditions	Rate constant (k/min^{-1})
10ppm	0.0068
100ppm	0.0059
500ppm	0.0057
10ppm with $NaBH_4$	0.0160
100ppm with $NaBH_4$	0.0187

This study is the first of its kind to describe the synthesis and activity of *I. lacunosa* AgNPs. The S3AgNPs performed remarkably in the antioxidant, antibacterial and photocatalytic assays. It is hoped that these novel findings which prove the efficacy of S3AgNPs leads to their use in biomedicine, electronics and water waste treatment.

4. Conclusion

Ipomoea lacunosa silver nanoparticles were successfully synthesized using microwave assisted heating techniques. The S3AgNPs exhibited antioxidant properties that surpassed those of the leaf extracts. Similarly, they also contained antibacterial properties and demonstrated evidence of heightened action against Gram-positive bacteria. Furthermore, the photocatalytic studies highlighted the efficacy of the S3AgNPs in the degradation of azo dyes like methylene blue.

Acknowledgements

The authors would like to acknowledge Business Management School (BMS) for the opportunity to conduct the research project and the Sri Lanka Institute of Nanotechnology (SLINTEC) for allowing the use of the Hitachi SU6600 SEM.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Effects of Rubber Sawdust and Paper waste as Substrates for Cultivating American Oyster Mushrooms (*Pleurotus ostreatus*): Their Influence on Nutrient Composition, Bioactive Compound Levels and Antioxidant Capacity

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Abstract

Pleurotus ostreatus (American oyster) species are a type of extensively cultivated mushrooms worldwide and they can be grown on a variety of lignocellulosic substrates. As an agriculture-based country, substrates like sawdust and paddy straw are commonly used to cultivate *Pleurotus ostreatus* in Sri Lanka. However, there are many other potential waste products that can be used as substrates. Thus, the main objective of this study was to analyze the use of Rubber Sawdust (SD) and Paper Waste (PW) to grow *Pleurotus ostreatus* and to determine their influence on nutrient composition, bioactive compound levels and antioxidant capacity. Mushrooms were grown in 5 different substrate combinations (100% SD, 100% PW, 50:50 SD and PW, 75% SD and 25% PW, 75% PW and 25% SD) and the water holding capacity of each substrate was determined. Aqueous extracts were prepared from the matured fruiting bodies and the total protein and total carbohydrate concentrations were evaluated using Lowry and phenol-sulfuric assays respectively. Bioactive compounds were analyzed using qualitative tests and the total phenolic content was measured. Antioxidant capacity was determined using total antioxidant assay and DPPH assay. The use of 100% SD substrate was effective in increasing the mycelial growth, yield, and antioxidant activity. Highest cap diameter, stipe thickness and protein content were observed in 75% SD, 25% PW combination. 50:50 SD, PW displayed the highest carbohydrate and phenolic contents. Qualitative results indicated that all mushrooms contained saponins, polyphenols, terpenoids and steroids. Overall, it can be concluded that 100% SD is a better substrate in terms of incubation and harvesting period and mushroom yield. Furthermore, in terms of nutrient composition and antioxidant capacity, 100% SD and SD combined with paper waste (50:50, 75:25) can be used effectively.

Keywords: *Pleurotus ostreatus*, Sawdust, Paper waste, Nutritional composition, Antioxidant activity

1. Introduction

Mushrooms are succulent, spore-bearing fruiting structures belonging to the fungus kingdom. They are saprophytic multicellular organisms that conventionally thrive above ground or on their redundant food source. There are numerous species of mushrooms, few of which are edible while some fall under the poisonous category. Examples of edible mushrooms include *Cantharellus cibarius*, *Agaricus bisporus* and *Pleurotus ostreatus*.[1]

Pleurotus species (oyster mushrooms) which belong to the class Basidiomycetes, family Pleurotaceae and the order Agaricales, feature a distinctive shell or oyster-shaped basidiocarp that develops in a variety of colors.[2,3]

They comprise over 40 species, all of which naturally grow in a wide range of temperatures.[4] Few of these include *Pleurotus purpurea-olivaceus, Pleurotus giganteus* and *Pleurotus ostreatus*.[5,6]

Figure 1. *Pleurotus species: A) Pleurotus purpurea- olivaceus. B) Pleurotus djamor C) Pleurotus ostreatus D) Pleurotus citrinopileatus E) Pleurotus giganteus.*[7]

Pleurotus species are among the most extensively cultivated edible mushrooms worldwide, particularly in parts of Asia, North America, and Europe.[1,8] This is because these species of mushrooms grow at a faster rate when compared to other edible mushrooms. In addition to this, it grows on substrates that does not need to be pasteurized thereby reducing the overall cost for its cultivation.[9,10] The production of oyster mushrooms is thought to be a highly lucrative industry because a substantial fraction of the substrates used are transformed into fruiting bodies.[11] Furthermore, these species require few environmental conditions, and their fruiting bodies are less vulnerable to disease and pest attacks. Nevertheless, *Pleurotus* species is hence considered the ideal mushrooms to be cultivated when compared to the others.[4]

Generally, all mushroom species receive their aliment by decomposing their surroundings through the production of extracellular enzymes. They are frequently observed colonizing moist wood trunks of trees and decaying organic detritus abundant in lignin and phenol degrading enzymes. As a result, farmers have been employing agricultural wastes as substrates for the cultivation of mushrooms.[6] Examples of these agro-wastes include paddy straw, wheat straw, cereal straw, sawdust, and banana leaves. This innovative approach has not only benefitted farmers but has also had a positive impact on the environment itself by reducing the quantity of agro-wastes produced globally and by narrowing the nutritional gap that exists among the populations of China, India, and Africa.[1] These remaining substrates are occasionally repurposed as fertilizers, animal feeds and in the manufacture of biogas.[12]

Pleurotus ostreatus species grow on a wide range of substrates. Some of these include wheat straw, banana leaves, sawdust, paper waste and cassava leaves.[8] Mushrooms require a high carbon source for their growth, and because paper waste and sawdust are high in carbon, they could be suitable substrates for harvesting them.[13,14] According to Tesfay *et al*., 2020, the combination of other substrates with paper waste appears to be a promising alternative to produce oyster mushrooms.[15]

In addition to having numerous positive effects on the environment and the economy, mushrooms have great nutritional value, and their cultivation is highly significant in the realm of medicine.[16] *Pleurotus* species have a high protein content which includes both essential and non-essential amino acids.[17] They are also considered as a great substitute for meat, fish, and vegetables due to their rich mineral content.[17] Additionally, they are rich in dietary fiber and vitamin C and B complexes.[18,19]

With regards to therapeutic benefits, mushrooms are highly recommended for diabetics and have been quite effective in the treatment of malignancies.[16] They are also known to contain many bioactive compounds. Bioactive compounds refer to the nutrients and non-nutrients available in the food matrix that exhibit physiological effects beyond their classical nutritional properties.[20] *Pleurotus* species contain a variety of bioactive compounds including terpenoids, phenols, steroids, and tannins.[21] Free radicals are highly reactive molecules that are

either formed in the body through normal metabolic processes or enter the body from the environment, such as pollution and other pollutants. The accumulation of these free radicals causes body harm. Antioxidants are nutrients that help the body protect itself from this damage.[22,23] The bioactive compounds found in Pleurotus species have been found to contain anticancer, antigenotoxic, antioxidant, antihypertensive, antiplatelet aggregating, antihyperglycemic, antibacterial, and antiviral properties.[6, 24, 25, 26]

Although mushroom cultivation has been increasing drastically in many parts of the world, its production has not reached to a very larger scale in Sri Lanka. This it principally due to challenges in farming and management, farmers lacking administrative and entrepreneurial abilities and pest and disease problems in mushrooms, which lead to severe losses in both its yield and profit.[27,28,29]

Therefore, it is necessary to study on better spawning techniques, effective substrates, innovative technologies, and management strategies for oyster mushroom production while avoiding significant challenges such as pest infestations. It is also crucial to find the optimal substrates that will produce mushrooms with the most yield and nutritional value. Moreover, it is essential that it remains accessible and affordable to purchase by all Sri Lankans while being profitable to farmers and economically beneficial to the country.[28]

Based on the background information, the following study was conducted to examine the efficacy of rubber Sawdust (SD) and Paper Waste (PW) as substrates for cultivating *Pleurotus ostreatus* (American oyster mushroom). The principal objective was to ascertain the impact of various substrate combinations on the nutrient composition, presence of bioactive compounds, and antioxidant capacity of these mushrooms. The ultimate aim of this study was to recommend the most suitable substrate combination to cultivate *Pleurotus ostreatus* efficiently and effectively.

2. Methodology

2.1. Preparation of substrate bags. The substrates, SD and PW were prepared by adding white rice bran, red rice bran, chemical mix, and adequate amountof water according to the ratios mentioned in Table 1.

Table 1. Preparation of substrate mix

Ingredient	Amount (per 100 kg ofsubstrate
White rice bran 8kg	8 kg
Red rice bran 2kg	2 kg
$CaCO_3$	2 kg
$MgSO_4$	200 g
Chlorinated/tap water	As required

The prepared substrate mixes were loaded into polypropylene bags. A total of 5 substrate combinations were prepared: 100% SD, 100% PW, 50:50 SD, PW, 75% SD and 25% PW, 75% PW and 25% SD. The substrate mixes were filled into bags which were all sealed using cotton wool. They were then autoclaved for 15 minutes at 121ºC.

2.2. Inoculation of substrate bags. The autoclaved bags were left to cool down for 24 hours at room temperature and were inoculated using *Pleurotus ostreatus* spawns under aseptic conditions. The bags were then transferred to the incubation room.

2.3. Harvesting. After the spawn run was complete, the bags were cut open and watered three times a day. Fully grown mushrooms were harvested, and the following parameters were recorded: no of days taken for spawn run to complete, no of days taken for first harvest from incubation, no of fruiting bodies per harvest and the parameters of the largest mushroom which included cap diameter, stipe length and stipe thickness.

2.4. Water holding capacity. 50 g of sample was prepared for each combination and mixed with 100 ml of water in a beaker. For each

substrate combination, 3 beakers were prepared. The beakers were covered using aluminum foil and were left to sit for 24 hours at room temperature. After 24 hours, the contents of each beaker were filtered, and the volume of water eluted was recorded.[30]

2.5. Preparation of mushroom extracts. 5 g of dried mushroom powder was mixed with 50 ml distilled water in a falcon tube. The tubes were left in a rollermixer for 48 hours. After 48 hours the contents of each tube were filtered using Whatman No 1 filter paper to obtain the mushroom extract.[31]

2.6. Total protein concentration analysis using Lowry assay. A standard series of 200 µg/ml – 1000 µg/ml was prepared using Bovine Serum Albumin (BSA). Lowry A, B and C chemical mixes were prepared. 1 ml of each standard, 1 ml of distilled water (blank) and 1 ml from each sample extract (20 times diluted) were taken in duplicates. 5 ml of Lowry AB mix was added to all the tubes and were left to incubate for 10 minutes at room temperature. After this, 0.5 ml of Lowry C was added to all the tubes and left to incubate for 30 minutes at room temperature. The absorbance was measured at 660 nm using a UV-Vis spectrophotometer. Protein concentrations of the mushroom extracts were calculated using the BSA standard curve.[32]

2.7. Total carbohydrate content analysis using phenol sulfuric assay. A standard curve was prepared with Dextrose in the concentrations of 200-1000 µg/ml. Then 0.25 ml of each diluted mushroom extract was added to a test tube in duplicates. To each tube, 0.25 ml of concentrated sulfuric acid and 0.25 ml of phenol was added immediately. The tubes were all heated in a water bath at 100ºC for 5 minutes and then cooled at room temperature. The samples of each tube were measured for its absorbance using a UV-Vis spectrophotometer at 490 nm along with the blanks.[33,34]

2.8. Bioactive compound analysis using qualitative test.

Table 2. Qualitative test methods.[35, 36]

Bioactive compound	Method
Saponins	To 0.5ml of each sample, 0.5ml distilled water was added and shaken vigorously.
Flavonoids	To 1ml of each sample, 2ml of 2% Sodium Hydroxide was added along with 2 drops of diluted hydrochloric acid.
Polyphenols	To 1ml of each sample, a few drops of diluted Iodine was added.
Tannins	To 0.5ml of each sample, 5% Ferric Chloride solution was added.
Terpenoids	0.5ml of each sample was mixed with 2ml of Chloroform and 2ml of concentrated Sulfuric acid.
Anthraquinones	2ml of 10% Ammonium solution was mixed with 0.5ml of each sample.
Steroids	0.5ml of each sample was mixed with 0.5ml Chloroform followed by 1 drop of concentrated Sulfuric acid.

2.9. Total phenolic content analysis. To 0.3 ml of each mushroom extract (diluted 20 times), 1.2 ml of 10% Folin-Ciocalteu phenol reagent and 1.5 ml of 7.5% saturated Na2CO3 solution was added. The sample tubes were incubated for 1 hour at room temperature and the absorbance was measured at 765 nm. Gallic acid was used at concentrations of 20-100 µg/ml to plot a standard curve to determine the total phenolic content.[37]

2.10. DPPH assay for antioxidant activity. A series of mushroom extracts were prepared in test tubes (1-5 mg/ml) to which 2 ml of DPPH solution was added. The tubes were incubated at room temperature for 30 minutes in the dark. After using methanol as a blank, the absorbance of each sample was measured using a UV-Vis

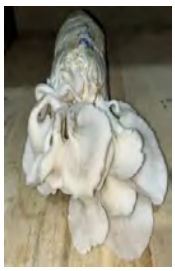
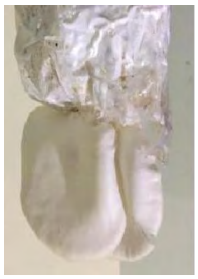
Spectrophotometer at 517 nm. The antioxidant activity was calculated using the equation below and IC_{50} (Inhibitory Concentration) values were determined.[38]

2.11. Statistical analysis. Microsoft Excel 365, Version 2302 was used to analyze the data produced. Results are expressed as mean values ± Standard Error. One-way Analysis of Variance (ANOVA) was used to determine the statistical differences. Where $p < 0.05$, the values were deemed statistically significant.

3. Results

3.1. Mushroom Harvest

Table 3. Images of Harvest Produced by Bag 1 and 2 in each Substrate Combinations

Substrate Combination	Bag 1	Bag 2
100% SD		
100% PW		
50:50 PW, SD		
75% SD 25% PW		
75% PW 25% SD		

Key: SD = Sawdust, PW = Paper Waste

3.2. Water Holding Capacity (WHC) of Substrates All the substrate combinations did not elute any amount of water upon filtration. Hence, all of them contained 100% WHC.

3.3 Spawn run

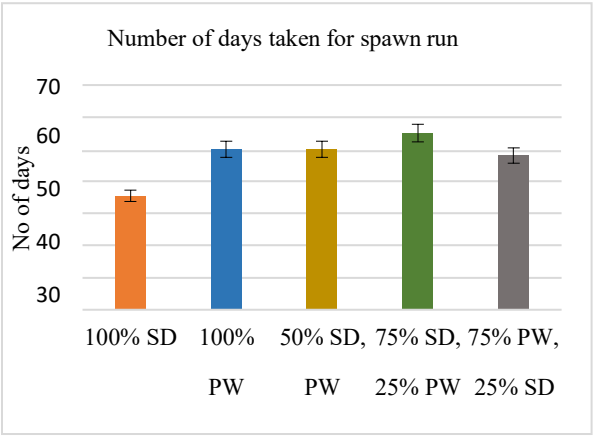


Figure 2. No of days taken for spawn run to complete.

100% SD took the least time to complete mycelial growth while 75% SD, 25% PW took the most time to complete mycelial growth (Figure. 2). A significant difference was

observed between the 100% SD group and all other groups.

3.4. Harvesting period

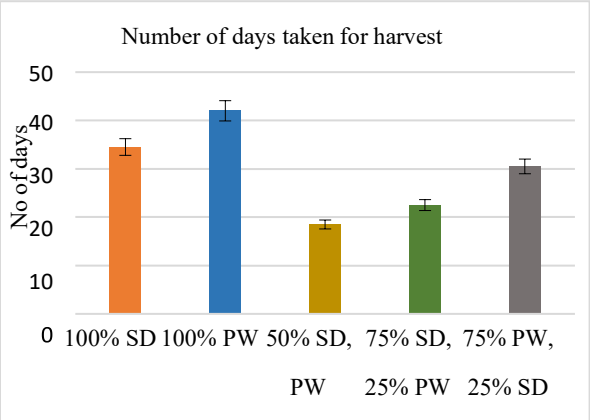


Figure 3. No of days taken for first harvest from incubation.

50:50 SD, PW took a significantly shorter period to produce fruiting bodies while 100% PW took a significantly longer period to produce fruiting bodies (Figure. 3).

3.5. Number of fruiting bodies

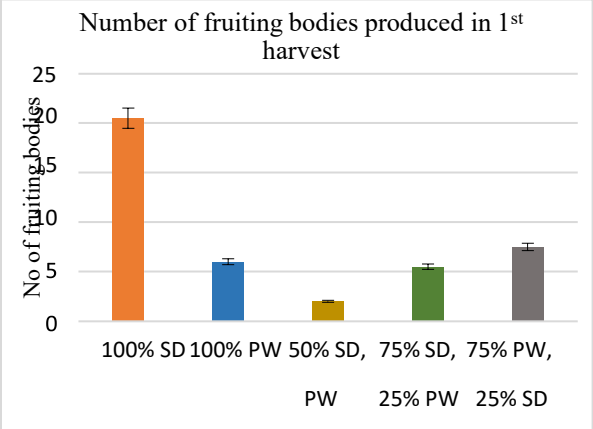


Figure 4. No of fruiting bodies produced by different substrate combinations.

100% SD produced the most fruiting bodies while 50% SD, PW produced the least number of fruiting bodies (Figure. 4). There is a significant difference between the 100% SD group and all other groups.

3.6. Fresh weight of mushrooms

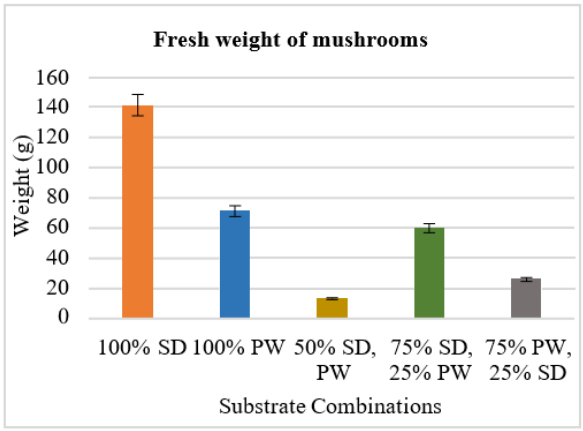


Figure 5. Fresh weight of mushrooms produced by each substrate combination.

100% SD produced fruiting bodies with the highest weight while 50% SD, PW produced fruiting bodies with the least weight (Figure. 5). A significant difference was observed between the groups.

3.7. Morphological Parameters of the largest mushroom in a bunch

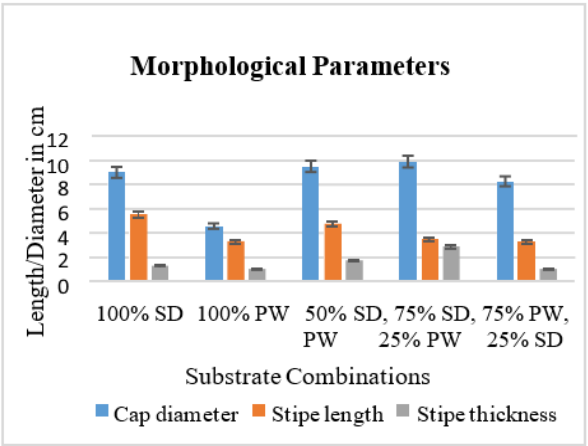


Figure 6. Parameters of the largest mushroom produced by each substrate combination in the first harvest.

A significantly high cap diameter was, and a significantly high stipe thickness was observed in

75% SD, 25% PW. The highest stipe length was recorded in 100% SD and it was significantly different compared to other groups. 100% PW displayed a significantly low cap diameter and stipe length. The lowest stipe thickness was observed in 75% PW, 25% SD (Figure. 6).

3.8 Total protein content

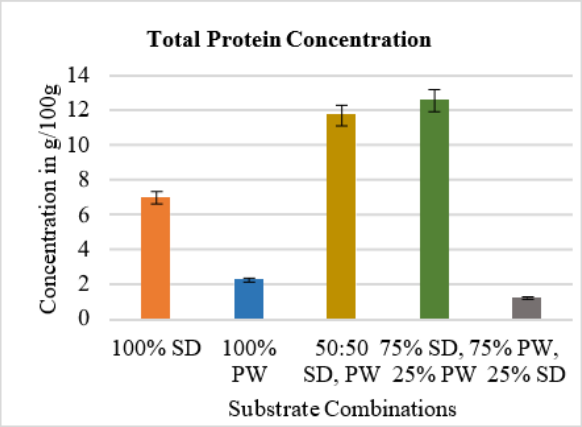


Figure 7. Protein content of *Pleurotus ostreatus* species grown in different substrate combinations.

The highest protein content was observed in 75% SD, 25% PW combination and the lowest concentration was present in 75% PW, 25% SD (Figure. 7). A significant difference was observed between the groups.

3.9 Total carbohydrate content

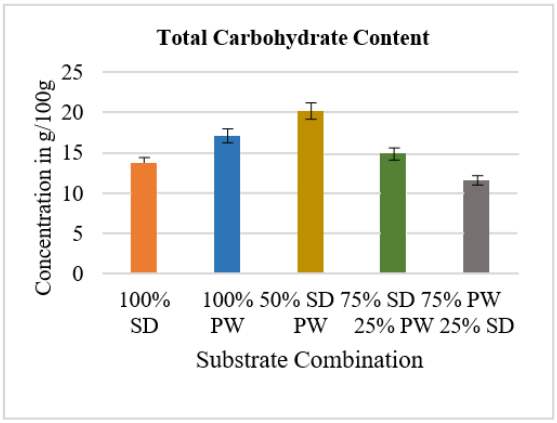


Figure 8. Total carbohydrate content in *Pleurtous ostretatus* species grown in different substrate combinations.

The highest carbohydrate content was present in 50:50 SD, PW and the lowest carbohydrate content was present in 75%PW, 25% SD (Figure. 8). A significant difference was observed between the groups.

3.10 Total phenolic content

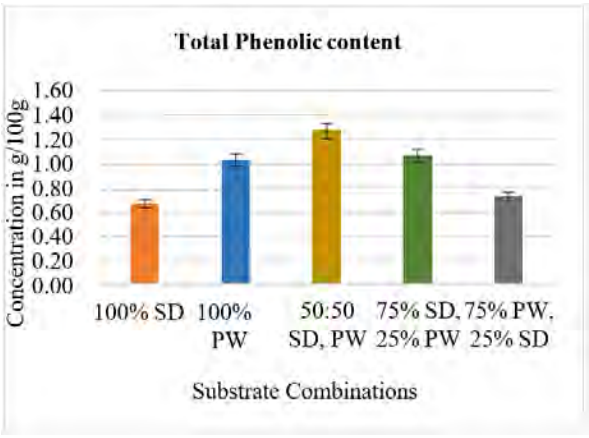


Figure 9. Total phenolic content of *Pleurotus ostreatus* species grown in different substrates.

50:50 SD, PW contained a significantly high TPC while 100% SD contained a significantly low TPC compared to other groups (Figure. 9).

3.11 DPPH Radical Scavenging Activity – IC_{50}

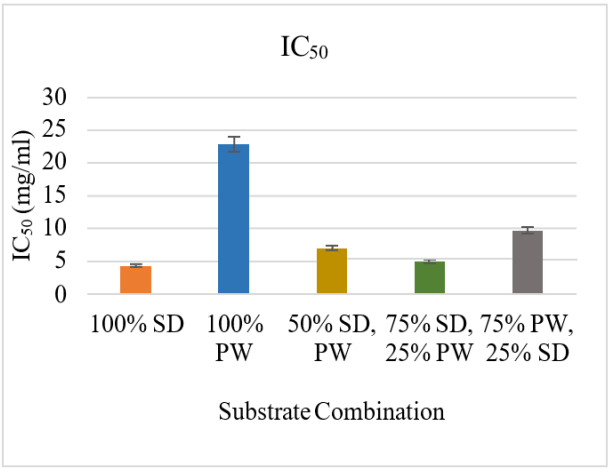


Figure 10. IC_{50} values of *Pleurotus ostreatus* species grownin different substrates.

The highest IC_{50} value was seen in 100% PW and

the lowest value was seen in 100% SD (Figure. 10). There is a significant difference between 100% PW and all other groups.

3.12 Qualitative tests for bioactive compounds

Table 4. Test results of the qualitative tests

Bioactive compound	100% SD	100% PW	50% SD, PW	75% SD, 25% PW	75% PW, 25% SD
Saponins					
	√	√	√	√	√
Flavonoids					
	X	X	X	X	X
Polyphenols					
	√	√	√	√	√
Tannins					
	X	X	X	X	X
Terpenoids					
	√	√	√	√	√
Anthraquinones					
	X	X	X	X	X
Steroids					
	√	√	√	√	√

4. Discussion

Pleurotus ostreatus species are one of the most widely cultivated mushrooms mainly due to their health benefits and ease of cultivation. The following study focused on using rubber sawdust and paper waste purchased locally to cultivate *Pleurotus ostreatus* species and to analyze the impact of these substrates on the nutrient composition, bioactive compound levels and antioxidant capacity of oyster mushrooms.

In this study, supplementing rice bran to the substrates, provides the mushrooms with added nutrients to support its growth, to increase its yield and to reduce the time taken for spawn run.[39] Moreover, the use of $CaCO_3$ and $MgSO_4$ serves as a buffer to maintain the pH of the substrate medium.[40]

The substrate combinations were all analyzed for their water holding capacity. None of them eluted any amount of water indicating that both SD and PW have a 100% WHC in both its combined and uncombined form.

As depicted in Figure 2, the fastest mycelial growth was observed in 100% SD bags (35 days) while the slowest mycelial growth was seen in 75% SD, 25% PW (55 days). These results were not in line with the results of Girmay *et al*., 2016 in which mycelial growth in 100% SD was seen within 19 days while PW without supplementing other materials, showed mycelia growth within 14 days.[41] With regards to the results obtained on the rate at which the first harvest was obtained after incubation (Figure 3); 100% PW took the longest period (42 days) while 50% SD, PW was the fastest (18 days). This can be supported with the results obtained by Tesfay *et al*., 2020 which proved that PW without supplementary materials took a longer period to show both mycelial growth and pinhead formation. These results were similar to the study conducted by Baysal *et al*., in 2003 which stated that supplementing PW with other lignocellulose rich substances like rice husk (80:20 or 50:50)

reduced the number of days for pinhead formation.[13,15]

The above results can be further supported by a study done by Oei and Nieuwenhuijzen in 2005, which found that substrates with high lignin and cellulose content start pinning more slowly than substrates with lower lignin and cellulose content.[42] In this study, combining two lignin and cellulose rich substrates like rubber sawdust and paper waste might have led to the delayed appearance of pinheads.[41] However, these results contradict with the findings of Girmay *et al*., 2016 which stated that 100% PW took about 32 days for pinhead formation.[41] Hence it was thought that the variations in every study could be due to environmental conditions and the nature of the substrate combined, in this case rubber sawdust.[43]

The number of fruiting bodies (Figure 4) and the fresh weight (Figure 5) was highest in the mushrooms produced by the 100% SD combination. The number of fruiting bodies observed in our study, very well matched with the findings of Hoa and Wang, 2015 which recorded the highest number of fruiting bodies that were grown in 100% SD. However, our findings associated with the weight of the mushrooms, did contradict with Hoa and Wang's study conducted in 2015 which stated that 100% SD produced mushrooms with lowest weight.[6]

Moreover, the results obtained for the cap diameter and stipe length (Figure 7), 75% SD, 25% PW showed the highest number. Contrary, 50:50 SD, PW in our study showed the highest stipe thickness (Figure 6) than the other combinations. These results were also relating to the findings of Hoa and Wang, 2015 which stated that the lowest cap diameter (70.62 mm) was observed in 100% SD, but 50% or 80% SD combined with 20% or 50% sugarcane bagasse or corncob, produced mushrooms with a higher cap diameter (> 80 mm). Similar findings were seen for the stipe length thickness, indicating both 50% and 80% SD combined with other substrates produced mushrooms with good stripe length and thickness.[6]

The protein content was analyzed using the Lowry method. It was noted that mushrooms grown in 75% SD,25% PW substrates showed the highest protein content (Figure 7). Again, these results were close to previous findings which identified highest protein content when 80% SD was combined with other substrates. On the other hand, in this study the total carbohydrate content and TPC was highest in the mushrooms grown using the 50:50 SD, PW combination (Figure 8 and Figure 9). These results were also matching with Hoa and Wang's study in 2015, which stated that 80% SD combination produced mushrooms with the highest carbohydrate content followed by the 50% SD combination.[6]

DPPH is an antioxidant assay which relies on the transfer of electrons to produce a violet solution.[44] In this study, DPPH assay was performed to identify the antioxidant activity of mushrooms and to recognize the IC_{50} value, the concentration of the mushroom required to scavenge 50% of the DPPH radicals.[45] Based on the results obtained from our study, 100% PW showed the highest IC_{50} values and the lowest IC50 values were seen in 100% SD (Figure 10). This implies that the mushrooms grown in 100% SD has a higher antioxidant capacity while the mushrooms grown in 100% PW has the lowest antioxidant activity. These results did not match with the study conducted by Hoa and Wang, 2015 which concluded that 100% SD showed the lowest antioxidant activity.[6]

Moreover, the bioactive compounds present in Pleurotus ostreatus were not affected by the type of substrates. Qualitative results performed indicated that all the mushrooms contained saponins, polyphenols, terpenoids and steroids. These results were somewhat similar to the ones obtained by Rahimah *et al*., 2019 which proved the presence of saponins, phenolic compounds and steroids in *Pleurotus ostreatus* species. However, this study also identified the

presence of flavonoids and tannins in the oyster mushrooms which were not identified in our study. This might have been due to the use of different protocols in the previous study.[46]

Conclusion

To conclude, with regards to the period of incubation, harvesting, number of fruiting bodies and yield; 100% SD combination appears to be an ideal substrate to grow *Pleurotus ostreatus*. However, combining it with 25-50% of paper waste, has shown to produce mushrooms with better cap diameter, stipe length and thickness. Also, the protein, carbohydrate and phenolic content appear to be greater when sawdust is combined with paper waste. Based on the antioxidant activity, 100% SD appears to be the best substrate. Therefore, it can be recommended that *Pleurotus ostreatus* grown in 100% SD and SD combined with paper waste (50:50, 75:25) can be used to have a well-balanced diet which can be used in preventing diseases and promoting better human health.

Acknowledgements

The authors would like to acknowledge Business Management School (BMS) for the opportunity to conduct the research project and Mushroom Development and Training Center in Rathmalana, Sri Lanka for providing us with the resources to cultivate the mushrooms.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Determination of the Acid Tolerance in *Lactobacillus* Isolated from Yogurt

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Abstract

Probiotics, classified as live microorganisms, are incorporated into a variety of dairy/non-dairy products such as yogurt/beer to confer a wide range of health benefits to consumers upon the consumption of adequate quantities. *Lactobacillus* is one of the most abundantly utilized genera of probiotics in food fermentation and the most commonly found bacterium in yogurt. The research study was aimed to isolate and identify *Lactobacillus* from five commercial brands of yogurt purchased from the local market and to determine its' acid tolerance. Bacteria were isolated by culturing the samples on MRS agar using quadrant streaking. Morphological characterization and four biochemical tests (Gram's staining, acid-fast staining, endospore staining and catalase test) were carried out to distinguish if the isolated bacteria were *Lactobacillus* before the identified colony was sub-cultured in nutrient broth. The acid tolerance assay was performed at pH 7.2 and 3 at 0 hours and after a 3-hour incubation period using spectrophotometry. Acid tolerance was statistically analyzed using one-way ANOVA, using SPSS statistics software. Morphological characteristics and the biochemical test results revealed that the bacteria were Gram-positive, non-acid-fast, vegetative cells and catalase-negative supporting the presence of *Lactobacillus*. No significant difference was observed according to the p-value which indicated the ability of *Lactobacillus* to tolerate acids to a certain extent. This study aided the understanding that the *Lactobacillus* species utilized by manufacturers were beneficial acid tolerant probiotics which could render the desired health benefits to the consumers.

Keywords: *Lactobacillus*, probiotics, yogurt, acid tolerance

1. Introduction

Probiotics, derived from a Greek word which means "for life", are classified as "live microbial feed supplements" that provide a beneficial effect to the host when consumed, by improving the gut microbiota.[1,2,3] Under the genera of *Lactobacillus* and *Bifidobacterium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium lactis*, *Bifidobacterium longum*, and *Bifidobacterium bifidum* are the species of beneficial probiotic bacteria.[4] Probiotics are widely found in dairy products (cheese, yogurt, milk, buttermilk, ice-cream) and in certain non-dairy products (beer, olives, pickles, cereals and chocolate).[1,2] Probiotics render several beneficial and desirable properties as demonstrated in figure 1.0. The variety of properties impart numerous health benefits to humans as mentioned by Shi *et al*[4] such as ameliorating dermal/oral health, mitigation of postmenopausal disorders, exerting antihypertensive effects.

Lactobacilli are lactic acid bacteria which are Gram-positive, non-motile, non-spore-forming coccobacilli or rods. *Lactobacillus* is catalase-negative and has a G+C content which is generally below 50 mol%. Furthermore, they are aciduric or acidophilic, aerotolerant or anaerobic and strictly fermentative (homofermentative/ heterofermentative). Lactobacilli have convoluted nutritional requirements which include vitamins, carbohydrates, salts etc.[5,6] They have an optimum pH and temperature in the range of 5.5–6.2 and 30–40 °C respectively for the effective growth of the bacteria[7]. Genus *Lactobacillus*, phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae* present the taxonomic classification of *Lactobacillus*.[8] Lactobacilli are

employed in biotechnology, pharmaceuticals, feed and food fermentation as a silage inoculant, vaccine carrier, probiotic and dairy starter.[5]

Yogurt is derived from the Turkish word "ya-urt" which means "sour milk".[9] Yogurt is a semisolid dairy product produced by the lactic acid fermentation of milk. Yogurt provides innumerable health benefits and has a high nutritive value as it provides an exceptional source of calcium, vitamin D, amino acids, potassium, riboflavin, vitamin B6/B12.[10-13] Depending on the chemical composition of yogurt, they have been classified based on the fat content into non-fat, low-fat and regular yogurt.[12] Yogurt is commonly produced with cow's milk, however, goat's milk, camel's milk, soy milk, corn milk, oat-based milk, coconut and tigernut milk may also be used to manufacture various yogurt types.[11] According to Banerjee *et al*[12], both two different groups of yogurt contain *Lactobacillus*. *Lactobacillus bulgaricus* in standard culture yogurt and *Lactobacillus acidophilus* in Bio- or probiotic yogurt. This makes yogurt an excellent and ideal source to be used to extract *Lactobacillus* for research.

Lactobacilli exhibit probiotic properties such as antioxidant activity, antimicrobial activity against pathogenic species, bile and acid tolerance and adhesion to internal surfaces.[14,15] Acid tolerance is the ability to withstand/avoid the destructive effects of an acidic environment such as in the stomach. Lactobacilli have adopted several acid tolerance mechanisms to protect themselves from gastric acids as it enables them to grow and multiply. Acid tolerance is one of the crucial criteria used to select potential probiotics.[16] F1-F0-ATPase, malolactic fermentation, ADS/ADI, AA decarboxylation (GAD system), biofilm and cell density, metabolic regulations, protection and repair of cellular macromolecules are the mechanisms utilized by *Lactobacillus* in acid tolerance.[17,18]

The F1-F0-ATPase is a proton pump that pumps out excessive H+ ions from the cytoplasm to the external environment by PMF, consuming ATP to increase the intracellular pH to promote bacterial cell survival.[19,20] Lorca and Valdez[21] justified through their research that *Lactobacillus acidophilus* CRL 639 indeed utilizes the F1-F0-ATPase as a mechanism of acid tolerance when under acidic stress. In the ADI system, ArcD initially transports arginine into the cell, which is converted to citrulline, and ammonia catalyzed by ADI. Citrulline is phosphorolysed into ornithine and carbamoyl phosphate catalyzed by OTC. Ornithine is exported out of the bacterial cell while carbamoyl phosphate and ADP are converted to ammonia, CO_2 and ATP, catalyzed by CK. Ammonia neutralizes the intracellular H+ ions whereas the ATP is made available for the F1-F0-ATPase[22]. Guo *et al* [16] confirmed the involvement of the ADI system as an acid-tolerant mechanism in *Lactobacillus plantarum* ZDY 2013 in their research findings. In the GAD system, glutamate is transported into the cells via GadC and is converted to GABA utilizing the excessive intracellular H+ ions and is catalyzed by glutamate decarboxylase. This raises the pH in the cytoplasm thus resisting the acidic stress in *Lactobacillus brevis* and *Lactobacillus acidophilus NCFM* as mentioned in the study conducted by Wang *et al*[23] and Lyu *et al*[24].

The main objective of this study was to determine the acid tolerance of *Lactobacillus* in yogurt. It is important to understand the acid tolerance ability of *Lactobacillus* which determines their ability to withstand the HCl acid in the stomach to survive and multiply, and hence provide the desired health benefits to the consumers. These study findings will be useful to educate manufacturers that using *Lactobacillus* with acid tolerance property is extremely important and beneficial as a probiotic in yogurt to elevate the overall product quality.

2. Methodology

2.1 Sample collection/preparation. Five yoghurt samples were purchased from the local market and stored at 4℃ in a refrigerator. The samples were labelled A-E respectively. Approximately 2 g of each yoghurt was homogenized in 100 ml beakers with 500 µL of autoclaved distilled water.

2.2 Isolation of Lactobacillus. A loopful of each homogenized sample was cultured on De Man, Rogosa and Sharpe (MRS) agar (with amphotericin B) using quadrant-streaking and incubated at 37°C for 48 hours.

2.3 Gram's staining. A loopful of the isolated bacterial colony from each culture was mixed with a water droplet to make a thin smear. The smear was heat fixed and flooded with crystal violet for 60 seconds, Gram's iodine solution for 60 seconds, Gram's decolorizer for 5 seconds and finally with safranin for 50 seconds. The smear was rinsed with distilled water after each step. The slide was blot dried with a tissue paper and observed under 100X oil immersion on a compound light microscope.

2.4 Acid-fast staining. A thin smear was made on each glass slide as mentioned in Gram's staining. The smears were air-dried and heat fixed. The smears were flooded with carbol fuchsin and heated until vapor rose. The slides were rinsed with distilled water. The smears were flooded with acid-decolorizer for 15 seconds and methylene blue for 60 seconds. The smears were rinsed with distilled water after each step. The slides were blot dried with a tissue paper and observed under 100X oil immersion on a compound light microscope.

2.5 Endospore staining. A thin smear was made on each glass slide as mentioned in Gram's staining. The smears were air-dried and heat fixed. Small pieces of Whatman filter paper were placed on each smear. Malachite green was added to the filter papers and the staining rack was placed over a water bath of 100°C. Filter papers were kept moist by adding drops of malachite green. Afterwards, the slides were allowed to cool down, filter papers were removed and the smears were rinsed with distilled water. The smears were flooded with safranin and rinsed with distilled water after 40 seconds. The slides were blot dried with a tissue paper and observed under 100X oil immersion on a compound light microscope.

2.6 Catalase test. A thin smear was made on each glass slide with the inoculation loop using a loopful of the isolated colony from each culture. The slides were placed over black background. 3% H_2O_2 was added to each smear and observed for effervescence.

2.7 Sub-culturing. A loopful of the isolated *Lactobacillus* colony from each culture was sub-cultured in 20 ml of nutrient broth in a falcon tube and incubated at 37°C for 48 hours. The subcultures were then stored at 4°C in the cold conditions.

2.8 Acid-tolerance assay. This method was modified from Sahadeva *et al*[25]. 5 ml of each subculture was centrifuged for 3 minutes at 4000 rpm. The supernatant was discarded. 5 ml of peptone water was added to each pellet and shaken. 2 ml of each sample solution was mixed with 0.5 µL of 37% HCl to adjust the sample pH to pH 3. pH 7.2 was used as the control. Three absorbance readings were obtained at 0 hours and 3 hour incubation at room temperature for both pH 7.2 and 3 at 600 nm wave length.

2.9 Statistical/data analysis. The acid tolerance ability was statistically analyzed using one-way ANOVA, using SPSS statistics software. ($P < 0.05$ were regarded as statistically significant).

3. Results

3.1 Cultures and colony morphology. Isolation and morphological characterization of potential lactobacilli cultures were plated on MRS agar

after an incubatory period of 48 hours at 37°C (figure 1).

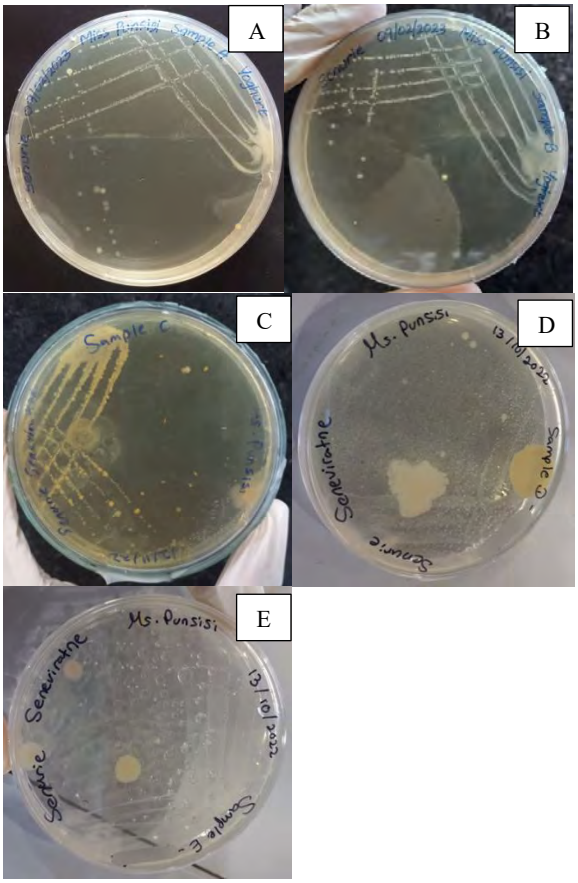


Figure 1. Colony morphology of yogurt sample cultures A-E on MRS agar after an incubation period of 48 hours at 37 °C.

The morphological characteristics of the colonies from each sample were observed to be opaque, smooth and creamy/milky white in colour, with a circular form, convex elevation and entire margin. A fungal growth was observed in culture D and E.

3.2 Gram's staining. Microscopic observation of the isolated colonies after Gram's staining were obtained (figure 2). Rod-shaped/Bacillus bacteria were observed in all the samples A-E. The isolated bacteria were observed in purple colour after the Gram's staining.

3.3 Acid-fast staining. Microscopic observations of the isolated colonies after acid-fast staining were given in figure 3. monolayer of rod-shaped bacteria was stained blue in samples A-E.

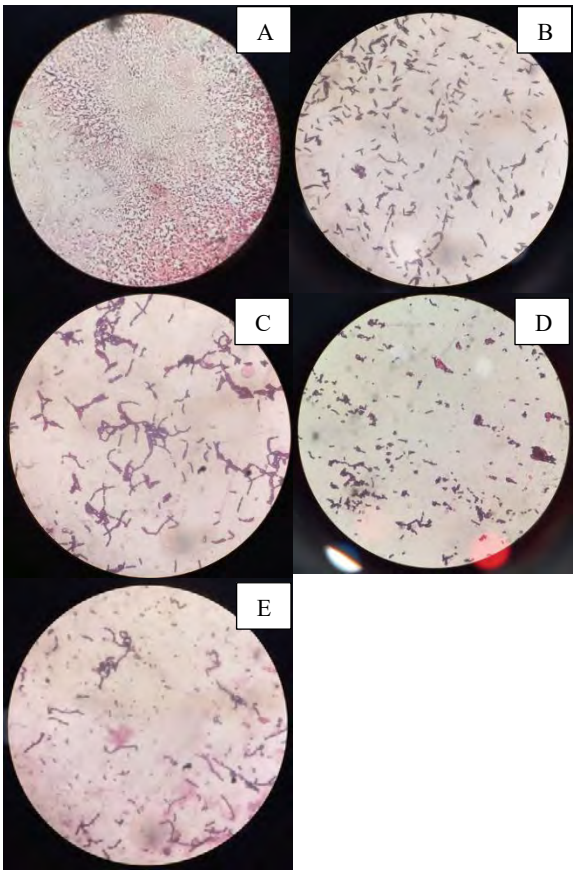


Figure 2. The Gram's stains of the isolated colony smears of samples A-E under 100x oil immersion on the compound light microscope.

3.4 Endospore staining. Microscopic observation of the isolated culture colonies after endospore staining were given in figure 4. It was observed that the bacteria in samples A-E were stained with red colour and the absence of green colour stained endospores were observed. Only vegetative cells were visible. A monolayer of bacteria was observed in samples A-E.

3.5 Catalase test. The catalase test results were observed as shown in figure 5 to determine if the catalase enzyme was present/absent from the isolated colony smears.

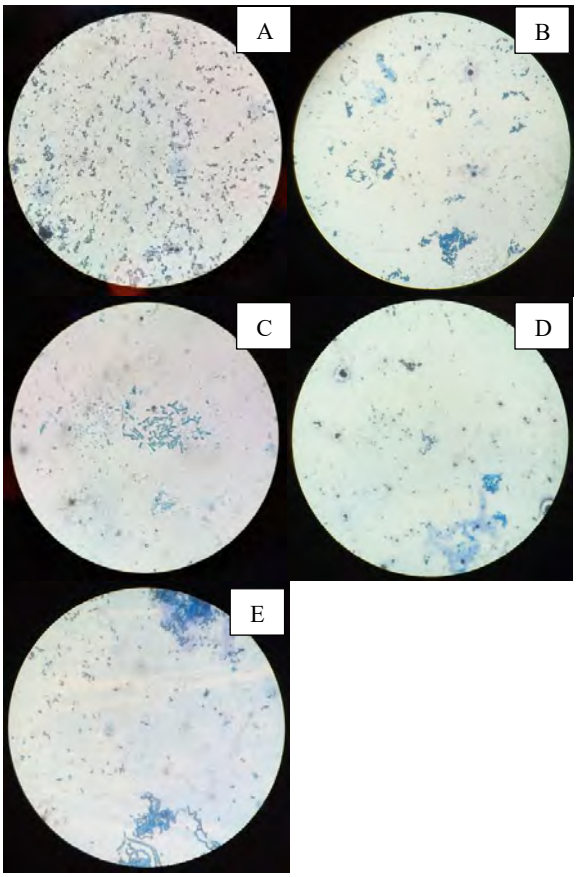
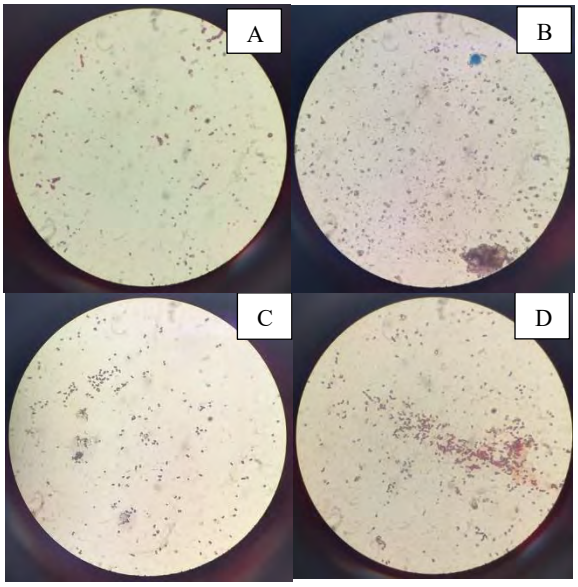


Figure 3. The acid-fast stains of the identified colony smears of samples A-E under 100x oil immersion on the compound light microscope.



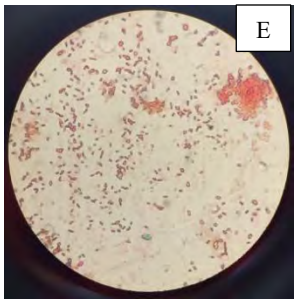


Figure 4. The endospore stains of the isolated colony smears of samples A-E under 100x oil immersion on the compound light microscope.

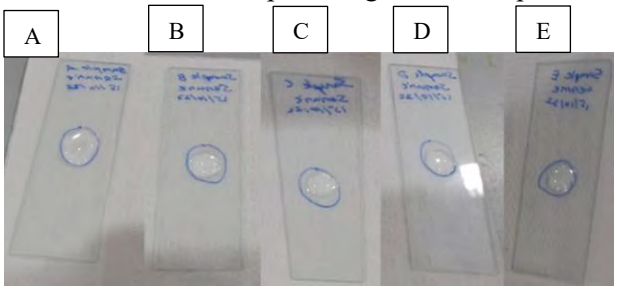


Figure 5. Catalase tests results of each identified colony smears of yogurt samples A-E.

No effervescence was observed from all samples A-E.

3.6 Acid tolerance assay. Acid tolerance of *Lactobacillus* was determined by spectrophotometry analysis by obtaining absorbance values at pH 7.2 and 3 at both 0 and 3 hours. The data was presented in two column charts as given below in figure 6 and 7. A decrease in the mean absorbance readings was noted in samples A-C while an increase in the mean absorbance readings were noted in samples D-E when the readings at 3 hours were compared with that at 0 hours at pH 3.

3.7 Data/statistical analysis. According to table 1 the obtained p-value via SPSS statistical software is 0.945 which is greater than 0.05 (5% level of significance).

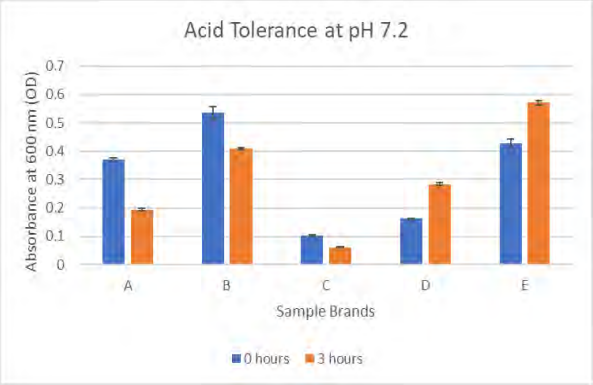


Figure 6. Column chart for the acid tolerance of *Lactobacillus* with the mean absorbance readings for samples A-E at 600 nm at pH 7.2 at 0 hours and 3 hours with the standard deviation.

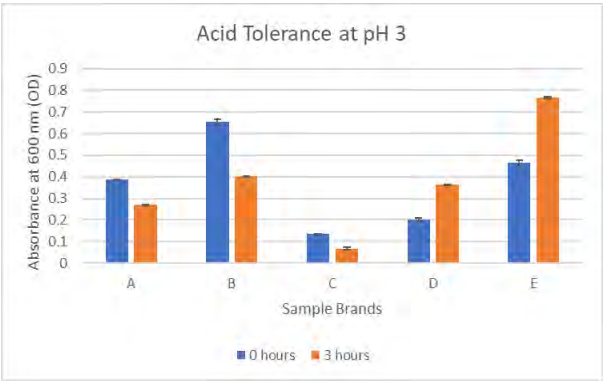


Figure 7. Column chart for the acid tolerance of *Lactobacillus* with the mean absorbance readings for samples A-E at 600 nm at pH 3 at 0 hours and 3 hours with the standard deviation.

Table 1. Statistical analysis by One-Way ANOVA for the mean absorbance readings for samples A-E at 600 nm at pH 3 at 0 and 3 hours.

ANOVA

Absorbance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	.005	.945
Within Groups	1.296	28	.046		
Total	1.296	29			

4. Discussion

This research was conducted to determine the acid tolerance of *Lactobacillus*, a bacterium commonly found in dairy products. Bacteria were isolated from five commercially available yogurt samples to determine if the yogurt contains bacteria which can survive under the acidity of the human stomach, an aseptic technique called culturing was used to isolate bacteria from yogurt samples., Morphological analysis of the colonies with a series of biochemical tests were done to confirm the presence of *Lactobacillus*. The acid tolerance assay was performed through spectrophotometry.

The yogurt samples were quadrant streaked on MRS agar and incubated at 37 °C for 48 hours to isolate *Lactobacillus* colonies for further experiments. MRS agar was used as it is a selective agar for the growth of *Lactobacillus* which inhibits the growth of unnecessary bacteria.[26,27] Morphological characteristics of the colonies were observed to determine if the bacteria isolated was *Lactobacillus*. Figure 1.0 presents the colonies formed on sample cultures A-E as creamy/milky in colour, opaque and smooth with the form as circular, elevation as convex and margin as entire. The results were similar to the observations made on the colony morphology of *Lactobacillus* by Afrin *et al* and Rabiei *et al*.[28,29] Based on the evidence it was assumed that the bacteria present in the colonies were indeed *Lactobacillus* spp. For further confirmations, Gram's staining, acid-fast staining, endospore staining and catalase testing were performed. A small fungal growth contamination was observed in sample D-E cultures. It could arise due to unskilled handling, contaminated reagents, media or equipment/glassware, from the hands/skin of the researcher, contaminated air with microorganisms found in incubators.[30] Usage of alternative antimicrobial agents such as nystatin and cycloheximide instead of amphotericin B such as in the studies conducted by Leska *et al* [31] and Nachi *et al* [32] could aid the inhibition of fungi and help prevent culture contamination.

The identified colony smears were subjected to Gram's staining as the first biochemical test to distinguish *Lactobacillus*. Purple-coloured Bacilli (rod-shaped) were seen

under the microscopic examination of the Gram's stain of samples A-E in figure 2 confirming the presence of Gram-positive bacteria. The trapped CV-I complexes inside the cell due to the shrinking of the thick peptidoglycan cell wall pores in the decolorizing step gives rise to the purple colour. Since similar results were obtained in the study conducted by Jose *et al*[33] it confirmed that the observed bacteria were *Lactobacillus*.

The identified colony smears were subjected to acid-fast staining as the second biochemical test. Figure 3 demonstrates blue-colour stained Bacilli in samples A-E thus confirming the presence of non-acid-fast bacteria. It is stated in Siegrist[34] that lactobacilli are acid-fast negative which is supportive of the observations made, thereby it confirmed that the observed bacteria were *Lactobacillus* spp.

The identified colony smears were subjected to endospore staining as the third biochemical test. It was observed that the bacteria in samples A-E in figure 4 were only stained in red and there were no visible green stained endospores. This results revealed that the cells were vegetative cells as it was consistent with the findings made by Malathi *et al*[35] and Goyal *et al* [36] who observed the same for *Lactobacillus* spp. in their research study which further supported the evidence for the presence of *Lactobacillus* in yogurt.

The identified colony smears were subjected to the catalase test as the fourth biochemical test to distinguish *Lactobacillus*. If bacteria contained the catalase enzyme, it would break down hydrogen peroxide into water and oxygen which can be visualized with the production of oxygen bubbles.[37] As shown in figure 5, the catalase test was negative with the absence of bubble formation indicating the presence of catalase-negative bacteria. The research findings of Amin *et al*[37] and Adikari *et al*[38] supports these results as they identified and concluded *Lactobacillus* spp. as catalase-negative with the absence of effervescence in the catalase test. Therefore, it can finally be confirmed that samples A-E contained *Lactobacillus* as all four biochemical tests indicated its presence.

As probiotics are incorporated into a variety of food and enters our body through the GIT, they must possess the ability to tolerate and survive the acids encountered along the way. A pH of 1 was recorded in the stomach when a person is fasting while a pH of around 4.5 is recorded after the consumption of a meal. A pH of approximately 3 was chosen as the test value, to mimic the environmental condition that the bacteria could potentially face the stomach to determine if *Lactobacillus* found in yogurt could withstand it. An incubation period of 3 hours was chosen to mimic the time the bacteria could be exposed to acids in the GIT as the ingestion of food approximately takes up to 3 hours. The assay was also conducted at pH 7.2 as a control and for comparison[39]. From figure 7, it was observed that three of the samples (A-C) had somewhat of a decrease in the quantity of bacteria present at three hours compared with that at zero hours at pH 3 as the mean absorbance had decreased slightly. However, D-E samples showed an increase in the bacterial density at the same pH indicating the growth of bacteria as the mean absorbance had increased. Absorbance was directly proportional to the quantity of bacteria present in a sample. The increase in mean absorbance in samples D-E could account for the survival of *Lactobacillus* at pH 3 similar to the study performed by Faye *et al*[40] due to the usage of either acid tolerant mechanism which pumps out H^+ ions from the cytoplasm via F1-F0-ATPase, utilization of excessive H^+ ions in the GAD system or through the neutralization of H^+ ions via ammonia generation in the ADI system thus allowing the bacterium to withstand acids to grow and multiply. The *Lactobacillus* species likely to be present in yogurt samples were *Lactobacillus casei* strains or *Lactobacillus bulgaricus* strains.[41,42] Studies were conducted on *Lactobacillus acidophilus* by Both *et al* [43] for acid tolerance presented a similar decline in the number of bacterial cells possibly due to the bacterial destruction or the growth being halted in others due to an inhibitory effect on metabolism

and reduction in its viability as a consequence of the disruption of vital elements such as DNA and proteins. due to an intolerable acidic pH.[41] Hence bacteria were unable to multiply and increase in number. However, the p-value obtained from the One-Way ANOVA table was 0.945, which is greater than 0.05 therefore it was considered that there was no significant difference between the mean absorbance readings between zero and three hours at pH 3 and the survival of *Lactobacillus* which was similar to the results obtained by Hassanzadazar *et al*[41] in their study. The decline in bacterial density in the three samples were not significant enough to conclude that they were destroyed due to the intolerability to acids but might have occurred due to a randomized human error. It can be stated that the bacteria were acid tolerant to a certain extent since the results presented a decent capacity for the bacteria to withstand acids but can only be fully confirmed by carrying out further acid tolerant assays which will aid the understanding if the strains are significantly capable of withstanding the stomach's acidity to grow and multiply and provide the health benefits or if the manufacturers need to modify the species to yield a better acid tolerance.

Conclusion

The four biochemical tests with the morphological analysis aided the identification of *Lactobacillus* in yogurt by proving that the bacteria were Gram-positive, non-acid-fast, vegetative cells and catalase-negative. It was determined that the bacteria were capable of withstanding acidity to an extent in the acid tolerant assay and further investigational analysis were necessary to comment on its acid tolerance. The research findings were of significant value as it presented the strains utilized by yogurt manufacturers as potential acid tolerant *Lactobacilli* which benefits consumers.

Acknowledgement

The authors thank BMS (Business Management School) for providing the opportunity to carry out this valuable research at the microbiology laboratory.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Determination of the antibacterial, antioxidant and photocatalytic activity of AgNPs green synthesized using extracts of 5 species of the *Diospyros* genus.

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Abstract

The use of nanoparticles in various industries have increased over the years with the improvement of the methods of nanoparticle synthesis which uses less energy at a cheaper cost through the use of eco-friendly materials such as plants and microorganisms. Silver nanoparticles (AgNPs) were synthesized in an eco-friendly method using six species of *Diospyros* leaf extracts under various temperature conditions where the optimum conditions were at a temperature of 90 °C for an hour. All species of *Diospyros* produced spherical AgNPs, with the exception of *Diospyros malabarica*, of an average diameter of 30 nm when viewed under TEM. These AgNPs were classified as semiconductors once the bandgap energy was calculated with a surface plasmon resonance at a wavelength of 420 nm. The AgNPs and the water extracts were tested for their antioxidant capabilities through Total antioxidant Capacity (TAC), Total Flavonoid Content (TFC), Total Phenolic Content (TPC), DPPH and IC_{50} assays which revealed that the AgNPs had better antioxidant activity than the water extracts with *Diospyros oocarpa* having the highest activity. The photocatalytic activity of different concentrations of the AgNPs were measured by measuring the degradation of the organic dye, Eriochrome Black T where 333 ppm AgNPs had the highest degradation rate with the use of $NaBH_4$. In addition, the antibacterial activity of the AgNPs and water extracts was measured and compared against *Staphylococcus aureus* and *Escherichia coli*, where the AgNPs had greater antibacterial activity against *Staphylococcus aureus* due to higher greater zones of inhibition. The properties of *Diospyros* AgNPs were determined and the possible uses the AgNPs can be used in several industries.

Keywords: *Diospyros*, silver, nanoparticles, antioxidant, antibacterial, photocatalytic

1. Introduction

Nanoscience is classified as the field of study of nano-structures, where nanotechnology utilises nanoscience into various applications through the manipulation of nanoparticles, that are structures of at least one dimension of length ranging from 1 to 100 nanometres.[1,2] Different nanoparticles are of different sizes and compositions ranging from liposomes and micelles to quantum dots and metallic nanoparticles.[3]

Metallic nanoparticles of various metals of gold, silver, copper, platinum and palladium, are used routinely in several applications ranging from biomedical to environmental applications.[4,5] Several properties of AgNPs such as the large surface area to volume ratio, surface plasmon resonance and the strong antimicrobial ability led to the employment of these nanoparticles in applications such as drug delivery, cell imaging, wastewater treatment, UV protection and photocatalysis,[6-9]

AgNPs are used as antibacterial agents as these nanoparticles are known to exert bactericidal action through various mechanisms by the release of silver ions, resulting in the significant inhibition of the growth of bacteria as illustrated in Figure 1.[10, 11]

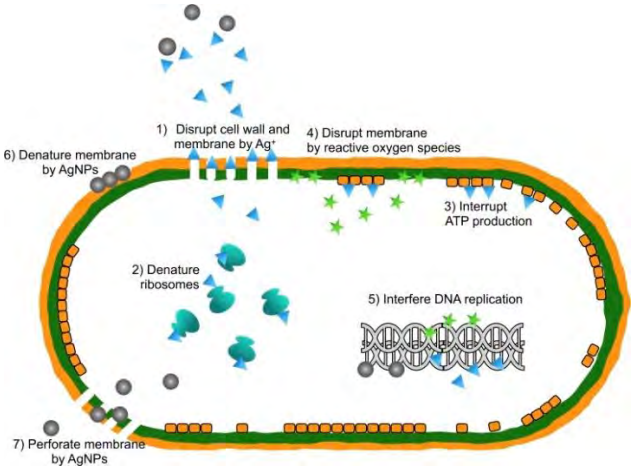


Figure 1. The antibacterial mechanisms of AgNPs.[12]

Other applications of AgNPs such as hydrogen production from wastewater and dye effluent treatment are carried out through photocatalysis. Photocatalysis is the ability of the particle to undergo oxidation when exposed to UV light, creating electron-hole pairs when an excited electron transitions from the valence band to the conduction band, forming free radicals which breakdown organic compounds as shown in Figure 2.[13,14]

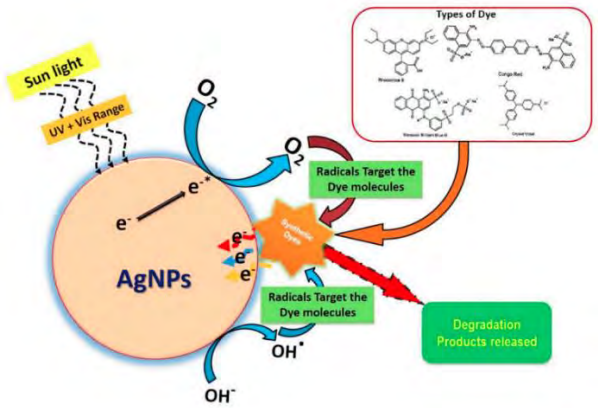


Figure 2. Photocatalytic degradation of an organic compound.[13]

In addition, AgNPs are known to be used in the treatment of cancer due to their antioxidant properties.[15] Antioxidants are compounds that inhibit oxidation reactions, preventing the formation of free radicals which cause oxidative stress (Figure 3).[16] The antioxidants in plant extracts primarily consist of flavonoids and phenols.[17, 18]

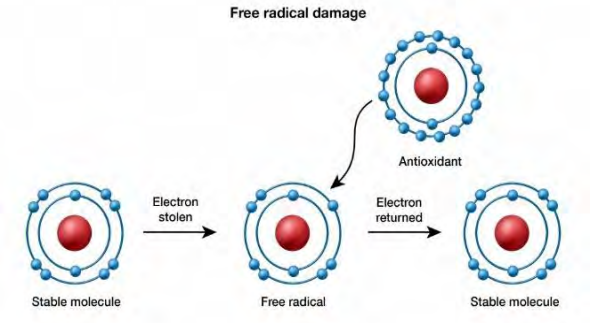


Figure 3. Mechanism of action of antioxidants.[18]

Oxidative stress in the human body can be defined as the imbalance between the number of free radicals, which is an atom with an unpaired electron, and the number of antioxidants, causing tissue damage due to the elevated levels of reactive oxygen species (ROS).[19,20] These high levels of ROS can be caused by the exogenous factors such as radioactivity and endogenous factors such as deficiencies in antioxidant enzymes, leading to harmful conditions such as macular degeneration, cardiac fibrosis, skin aging and auto-immune disorders.[21,22]

Oxidative stress is prevented in the body by antioxidants, which are primarily supplied through dietary sources such as citrus fruits.[23] AgNPs which show significant antioxidant activity can be utilized to decrease the concentration of ROS, reducing oxidative stress such as the loading of nanoparticles into hydrogels to prevent skin aging.[24]

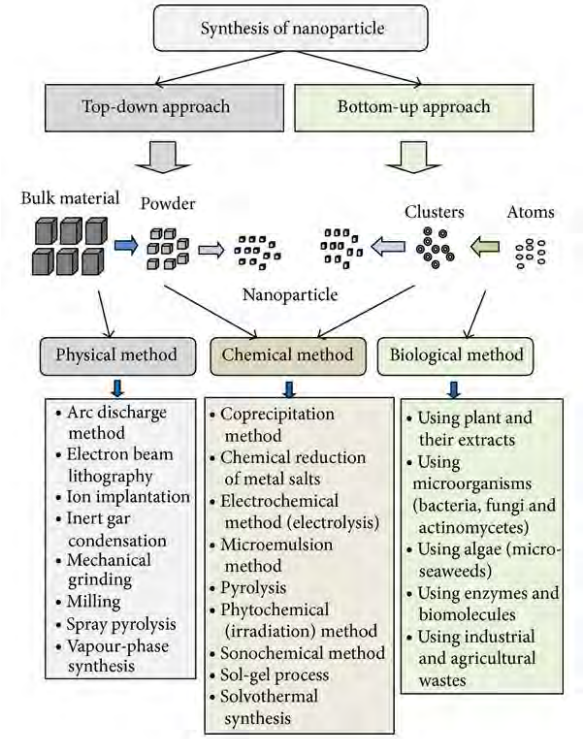


Figure 4. Approaches to the synthesis of nanoparticles.[27]

Table 1. Advantages and disadvantages of the approaches to the synthesis of nanoparticles.[30, 31]

	Advantages	Disadvantages
Top-Down	• Large scale production • Requires no purification	• Relatively expensive • Size distribution
Bottom-Up	• Relatively cheap • Able to control size and shape of nanoparticles	• Cannot be employed for large scale production

The approaches to the synthesis of nanoparticles can be divided into the Top-Down and the Bottom-Up approach (Figure 4), with their individual advantages and disadvantages (Table 1).[25, 26] The Top-Down approach involves the breakdown of large bulk material into smaller particles through physical or chemical methods until particles of the nanoscale are reached.[27, 28] Bottom-Up approach involves the build-up of nanoparticles using simpler particles such as atoms and molecules through chemical and biological methods.[27, 29] The physical synthesis of nanoparticles involves the breaking of larger materials through processes such as grinding and evaporation which require large quantities of energy and space, while being

time-consuming.[30] Chemical processes primarily use chemicals acting as reducing agents which synthesizes nanoparticles through the reduction of metallic ions, at the risk of chemical contamination.[31]

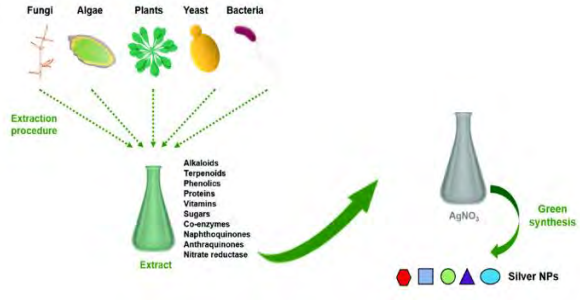


Figure 5. Green synthesis of AgNPs.[33]

The biological synthesis (green synthesis) of nanoparticles employs the use of biodegradable materials, such as plants and microbial organism, for the synthesis of metallic nanoparticles from their ionic form (Figure 5).[32, 33] In this study, leaves were preferred as a raw material due to the ease in obtaining the leaf extract, low cost and abundance in raw materials whereas microorganisms require constant culture maintenance.[34]

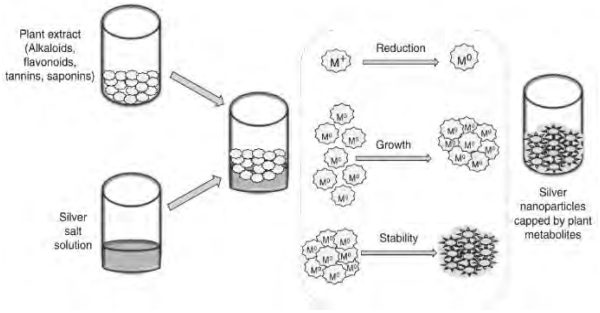
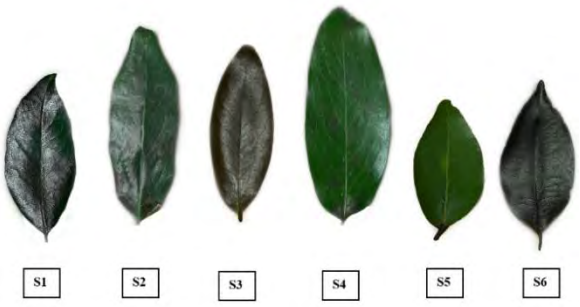


Figure 6. Formation of metallic nanoparticles.[35]

The mechanism through which metallic nanoparticles form (Figure 6) involve the mixing of the plant extracts in a metal-salt solution under certain conditions which results in the reduction of metal ions (M^+) to their metallic form (M^0), leading to nucleation, growth and stabilization of the nanoparticles.[35] Phytochemicals such as flavonoids and phenols, in the plant extracts act as reducing and stabilizing agents in nanoparticle synthesis.[36]

The green synthesis is more favourable due to the cost-effectiveness, the availability of samples, environmental friendliness, disuse of toxic chemicals and compounds, and the ability to manipulate the size and shape of the nanoparticles.[37,38]



Diospyros affinis	S1
Diospyros atrata	S2
Diospyros ebenum	S3
Diospyros malabarica	S4
Diospyros oocarpa	S5
Diospyros quaesita	S6

Figure 07. Species of *Diospyros* used

The leaf samples used in this study are of the species, *Diospyros*, from the Ebenaceae family, and is a genus of over 500 species of plants and shrubs which are distributed pantropically around the world.[39] The species of *Diospyros* used in this study are outlined in Figure 7.

Different parts of the *Diospyros* species have been used in several industries where fruit-yielding species include *D. kaki, D. lotus and D. virginiana,* whereas species such as *D. ebenum* and *D. celebica* are used for their timber.[40] In addition, a number of species are used for therapeutic purposes in traditional medicine to treat conditions such as hypertension, atherosclerosis, asthma and infectious diseases.[41, 42]

These samples were selected for this study as previous studies have concluded that titanium and selenium nanoparticles, synthesized using leaf extracts from species of *Diospyros*, possess significant antibacterial properties against gram-positive bacteria and significant antioxidant activity.[43,44]

Hence assessing the properties of AgNPs synthesized from leaf extracts of *Diospyros*, determines for which applications these nanoparticles can be used for.

The aim of this study is to synthesize AgNPs from leaf extracts of the six species of *Diospyr*os through green synthesis. The antioxidant activity of the extracts will be measured through TAC, TFC, TPC, DPPH and IC_{50} assay, whereas the antibacterial activity will be assessed using the well diffusion method with bacterial strains of *Staphylococcus aureus* and *Escherichia coli.* The photocatalytic activity of the nanoparticles will be measured through the degradation of organic dyes, and the presence of phytochemicals will be confirmed through various assays.

2. Methodology

Good laboratory practice was maintained when carrying out nanoparticle synthesis and the relevant assays to determine its properties and the relevant COOSH forms were filled out when requesting the required chemical reagents.

2.1 Sample collection. Leaves from six species of the *Diospyros* genus (Figure 7) were selected and collected from the Royal Botanic Garden in Peradeniya, Sri Lanka. The samples can be identified using the below key (Table 2).

Table 2: Sample key

Species of *Diospyros*	Code
Diospyros affinis	S01
Diospyros atrata	S02
Diospyros ebenum	S03
Diospyros malabarica	S04
Diospyros oocarpa	S05
Diospyros quaesita	S06

2.2. Preparation of leaf extracts. The leaves were air-dried in the shade for 72 hours and ground into a fine powder, from which 2 g of each sample was mixed with 50 ml of distilled water. The samples were placed in the dry oven for 15 minutes at 85 °C and filtered into a 50 ml Falcon tube using a Whatman No. 1 filter paper. The prepared leaf extracts were placed in the 4 ^{0}C for future use.

*2.3. Phytochemical analysis***.** The presence of the below phytochemicals in the leaf extracts of *Diospyro*s was confirmed using the below tests (Table 3).

Table 3. Methodology to confirm the presence of phytochemicals

Phytochemical	Methodology	Positive Result
Anthraquinones	4 - 5 drops of 10% ammonium hydroxide solution were added to 1 ml of the leaf extract.[45]	A pink colour precipitate will be formed.
Carbohydrates	1 ml of the Molisch's reagent was added to 2 ml of the plant extract along with 4 - 5 drops of concentrated sulfuric acid.[46]	A reddish-purple ring will be formed.
Proteins	A few drops of copper sulfate were added into the leaf extract along with 1 ml of 10% sodium hydroxide.[47]	A dark bluish-purple colour will be formed.
Saponins	0.2 g of the dried leaf extract was shaken with 5 ml of distilled water. The solution was heated to boiling.[45]	Foam will appear on the solution.
Steroids	1 ml of chloroform was added into 1 ml of the leaf extract along with a few drops of concentrated sulfuric acid.[47]	A brown colour ring will be formed.
Tannins	3 ml of 5% Iron Chloride was added into 1 ml of the leaf extract.[45]	A dark green-blue colour will be formed.
Terpenoids	2 ml of chloroform was added into 0.5 ml of the leaf extract along with a few drops of concentrated sulfuric acid.[45]	A reddish – brown colour will be formed at the interface.

2.3. Synthesis of AgNPs. 9 ml of 1 mM solution of Silver nitrate was mixed with 1 ml of the leaf extracts, and placed in the dry oven for 1 hour at 90 °C. The absorbance of the samples was measured at wavelengths ranging from 320 nm to 520 nm using distilled water as the blank.

2.3.1 Optimisation of AgNP synthesis. 9 ml of 1 mM solution of Silver nitrate was mixed with 1 ml of the leaf extracts and placed in the incubator at different temperatures for different periods of time which include 30 minutes at 90 °C, 1 hour at 60 °C, 30 minutes at 90 °C and 72 hours at 25 °C (Room Temperature). The absorbance of the samples was measured at wavelengths from 320 nm to 520 nm using distilled water as the blank.

2.4 Dilution of water extracts and AgNPs. The water extracts and the synthesised AgNPs were diluted into a 1:15 ratio using distilled water. The prepared leaf extracts were placed in the 4 °C for future use.

2.5. Antioxidant Assays. The diluted samples were used to assess the following assays.

2.5.1. Determination of the Total Flavonoid Content (TFC). A modified colorimetric method was carried out to determine the TFC using aluminium chloride.[48] A 1.2% aluminium chloride solution was prepared along with a 120 mM solution of potassium acetate. 1 ml of the sample was mixed with 0.5 ml of the 1.2% aluminium chloride and 120 mM potassium and left for 30 minutes at room temperature. The absorbance was measured in triplicates at 415 nm, using distilled water as the blank and the TFC was expressed as quercetin equivalents (QE).

2.5.2. Determination of the Total Phenol Content (TPC). The total content of phenols was determined through the Folin-Ciocalteu reagent test (48). A 20 N solution of Folin-Ciocalteu was diluted to a 1 N Folin-Ciocalteu solution using distilled water. 1 ml of the sample was mixed with 0.1 ml of the 1 N Folin-Ciocalteu reagent and were left for 15 minutes

at room temperature, and to which 5 ml of the saturated sodium carbonate solution was added. The test tubes were left for 30 minutes at room temperature and the absorbance was measured in triplicates at 760 nm, using distilled water as the blank and the TPC was expressed as gallic acid equivalents (GAE). [48]

2.5.3. Determination of the Total Antioxidant Capacity (TAC). The TAC was determined using the phosphomolybdenum assay derived from other studies.[49] The phosphomolybdenum reagent was prepared by mixing equal volumes of 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. 2 ml of the phosphomolybdenum reagent was mixed with 0.2 ml of the sample, and incubated for 90 minutes at 95 °C. The absorbance was measured in triplicates at 695nm, using distilled water as the blank and the TAC was expressed as ascorbic acid equivalents (AAE). [48]

2.5.4. Determination of the DPPH scavenging activity. 0.004% of the DPPH solution was prepared by mixing 0.0054 g of DPPH in 100 ml of methanol to which 1 ml of the sample was added to 1 ml of DPPH solution. The samples were incubated for 30 minutes and the absorbance was measured at 517 nm, using methanol as the blank. The DPPH scavenging activity was calculated using the below equation.

$$\text{DPPH Percentage Scavenging Activity} = \frac{\%\ \text{Activity}_{\text{Control}} - \%\ \text{Activity}_{\text{Sample}}}{\%\ \text{Activity}_{\text{Control}}} \times 100$$

2.5.5. Determination of the Median Inhibition Concentration (IC_{50}). 0.004% of the DPPH solution was prepared by mixing 0.0054 g of DPPH in 100 ml of methanol. A 100%, 80%, 60%, 40% and 20% solutions were prepared using the samples and distilled water to which 2 ml of the 0.004% DPPH solution was added. The solutions were incubated for 30 minutes at room temperature and the absorbance was measured at 517 nm, using methanol as the blank. The percentage activity was calculated using the above equation and the IC_{50} was determined.

2.6. Determination of the Photocatalytic Activity. A 2 mM solution of Eriochrome Black T (EBT) was prepared to which 1 ml of the 333 ppm AgNPs was pipetted in. The absorbance of the solution was measured at a wavelength range from 320 nm to 720 nm. The solution was placed in sunlight and the absorbance was measured at 30-minute intervals, using distilled water as the blank. The experiment was also carried out after adding 1 ml of 0.2 M solution of sodium borohydride which acts as a catalyst. The experiment once again was repeated using the 5000 ppm solutions of the AgNPs.

2.7. Determination of the antibacterial activity. The antibacterial activity of the samples was determined through the well diffusion method using bacterial strains of *Staphylococcus aureus* and *Escherichia* coli. The agar plates were produced by pouring 20 ml of Mueller-Hinton agar into each plate and left to cool. Once cooled, the plates were labelled and placed under UV light for sterilisation. The plates were separately swabbed with the bacterial strains and three wells were created on the agar for the negative control (-) and two duplicates of the sample (S1 and S2) as shown in Figure 08. The positive control (+) used was a gentamycin disc, which was placed on the agar along with 0.5 mL of saline water as the negative control (-). 0.5 mL of the sample was transferred into S1 and S2, and the plates were placed in the incubator at 37 °C for 24 hours (Figure 8). Once incubated, a ruler was used to measure the diameter of the zones of inhibition**.**

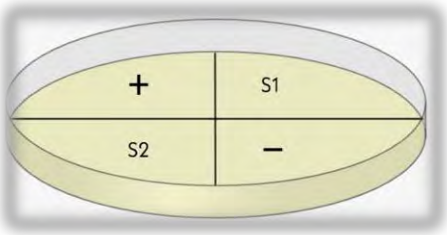


Figure 8: Labelling of agar plates

2.8. Transmission Electron Microscopy. 1 ml of the S05 AgNPs was transferred into an Eppendorf tube and centrifuged at 5000 rpm for five minutes. Once centrifuged, the Eppendorf tube was transferred into the dry oven for 48 hours. The morphology of the nanoparticles was examined at the Sri Lanka Institute of Nanotechnology using the JEM-2100 Transmission Electron Microscope.

2.9 Statistical Analysis. The statistical software, SPSS Version 25 (Statistical Package for the Social Sciences) was used to carry out the one-way ANOVA statistical test to determine a significant difference between the TFC, TPC and TAC of the water extracts and AgNPs, and the antibacterial activity of the samples against *Staphylococcus aureus* and *Escherichia coli.*

3. Results

The results of the phytochemical tests carried out on the water extracts of *Diospyros* are outlines in Table 4. Saponin is present in all the samples except in SO4. The formation of AgNPs in the extracts is indicated by the colour change from colourless to a reddish-brown solution as illustrated in Figure 9, where S01, S02, S03, S05 and S06 exhibited a colour change.

Table 4. Results of the phytochemical tests

Phytochemical	S01	S02	S03	S04	S05	S06	Positive Result
Anthraquinones	✗	✗	✗	✗	✗	✗	-
Carbohydrates	✗	✗	✗	✓	✓	✓	
Proteins	✗	✓	✓	✗	✓	✓	
Saponins	✓	✓	✓	✗	✓	✓	
Steroids	✗	✗	✗	✗	✗	✗	-
Tannins	✗	✗	✗	✗	✗	✗	-
Terpenoids	✗	✓	✗	✗	✓	✓	

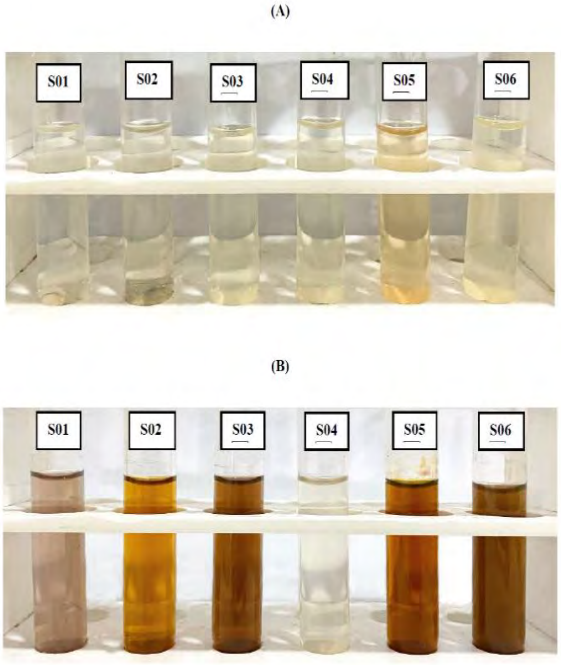


Figure 9. Colour change observed when nanoparticles are synthesized at 90 °C at 1 hour (A – Before, B – After).

The absorbance of the solutions was measured at a wavelength range from 320 nm to 520 nm, where peaks in absorbance at 420 nm indicate the formation of AgNPs (Figure 13). All the samples with the exception of S04 produced peaks at 420 nm, indicating the formation of AgNPs.

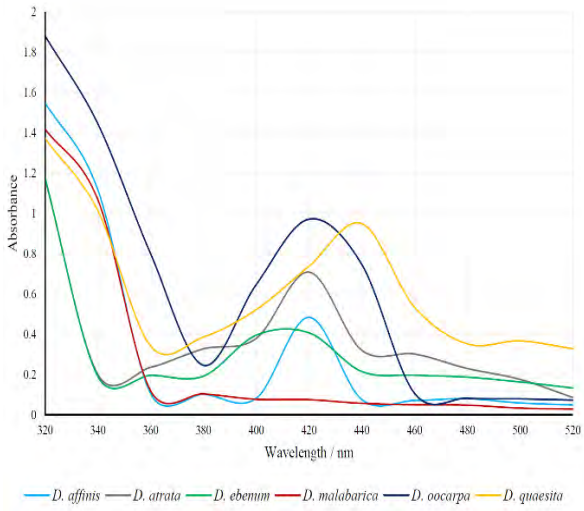


Figure 10. Visible spectrum for the optimized AgNPs synthesized at 90 °C at 1 hour.

Synthesis of AgNPs were carried out at temperatures of 90 °C ,60 °C and 25 °C (room temperature) for different periods of time where the optimum conditions were found to be 90 °C for 1 hour (Table 5).

Table 05. Conditions at which AgNPs were synthesized

Species of *Diospyros*	Conditions				
	25 °C (Room Temperature)	60 °C		90 °C	
	72 hours	30 minutes	60 minutes	30 minutes	60 minutes
S01	✓	✓	✗	✓	✓
S02	✓	✓	✓	✓	✓
S03	✓	✓	✓	✓	✓
S04	✗	✗	✗	✗	✗
S05	✓	✓	✓	✓	✓
S06	✓	✓	✓	✓	✓

When measuring the absorbance at wavelengths from 320 nm to 520 nm, the samples that had a peak at 400 nm to 440 nm are labelled with a ✓, indicating the presence of AgNPs.

Transmission Electron Microscopy was caried out to determine the morphology and size of the synthesized AgNPs. As illustrated on Figure 11, the AgNPs are spherical in shape with an estimated average diameter of 30 nm, ranging from 20 nm to 40 nm.

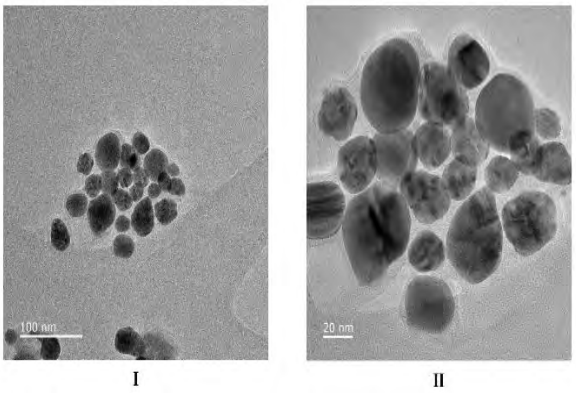


Figure 11. TEM imaging of AgNPs. I and II are in different scales.

3.1. Determination of the Total Flavonoid Content

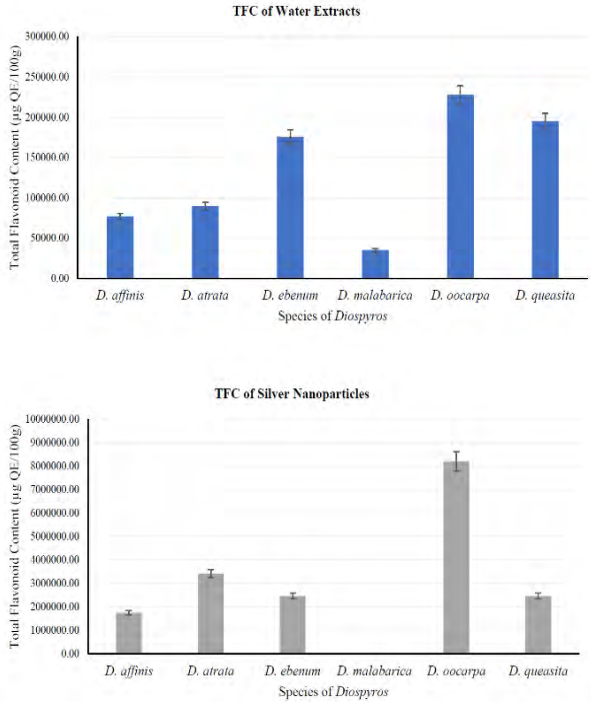


Figure 12. Total flavonoid content of water extracts and AgNPs (µg QE/100g)

As illustrated in Figure 12, the AgNPs had a higher total flavonoid content than the water extracts.

3.2. Determination of the Total Phenol Content.

As illustrated in Figure 13, the AgNPs had a higher total phenol content than the water extracts.

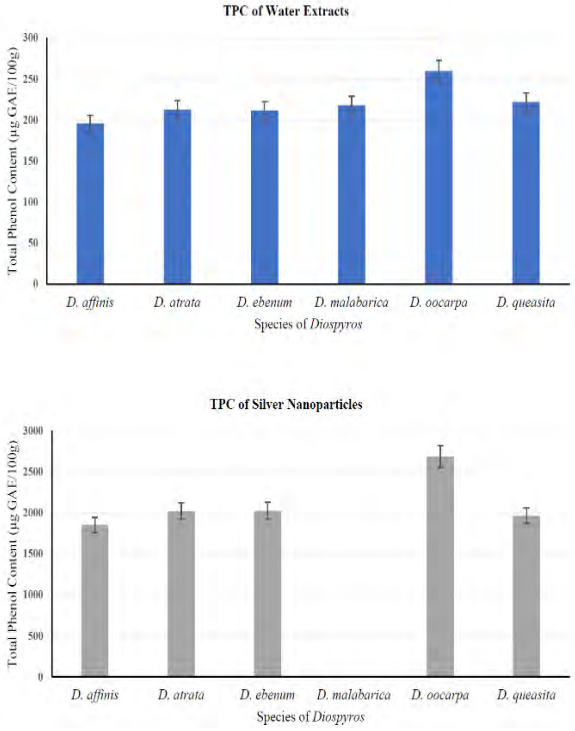


Figure 13. Total phenol content of water extracts and AgNPs (µg GAE/100g)

3.3. Determination of the Total Antioxidant Capacity.

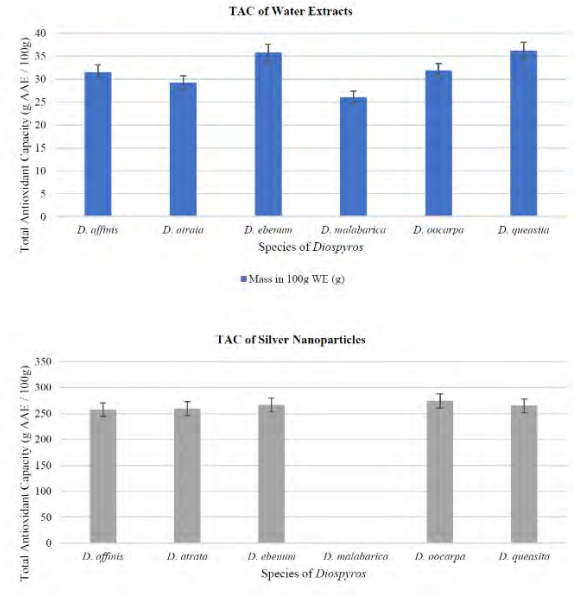


Figure 14. Total antioxidant capacity of water extracts and AgNPs (g AAE/100 g)

As illustrated in Figure 14, the AgNPs gave a higher total antioxidant capacity than the water extracts.

3.4 Statistical Analysis of TFC, TPC and TAC.

The statistical test, One-Way ANOVA was carried out to determine if there are any significant differences between the TFC, TPC and TAC of the water extracts and the AgNPs (Table 6, Table 7 and Table 8).

Table 6. One-Way ANOVA between the TFC of water extracts and AgNPs

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5423292.68	1	5423292.68	11.187367	0.008595	5.117355
Within Groups	4362924.127	9	484769.3474			
Total	9786216.807	10				

Table 7. One-Way ANOVA between the T*P*C of water extracts and AgNPs

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1560953.065	1	1560953.065	201.47957	1.82E-07	5.117355
Within Groups	69727.05701	9	7747.450779			
Total	1630680.122	10				

Table 8. One-Way ANOVA between the TAC of water extracts and AgNPs

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17070.56333	1	17070.56333	18.230835	0.0016376	4.9646027
Within Groups	9363.56646	10	936.356646			
Total	26434.12979	11				

3.5. Determination of the DPPH Radical Scavenging Activity.

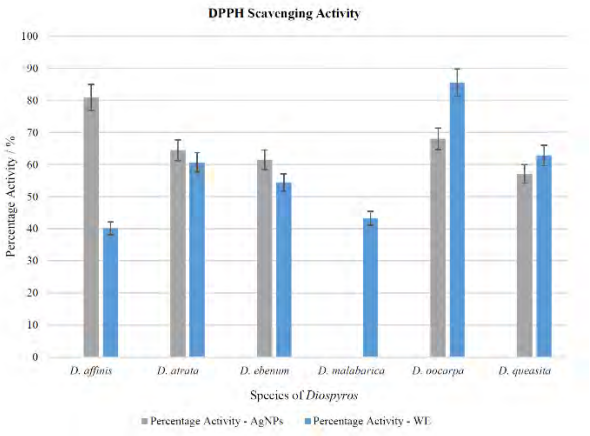


Figure 15. DPPH scavenging percentage activity of water extracts and AgNPs

The AgNPs synthesized from S01, S02 and S03 gave a higher DPPH radical scavenging percentage activity compared to the water extracts (Figure 15).

3.6. Determination of the Inhibitory Concentration (IC50) of DPPH.

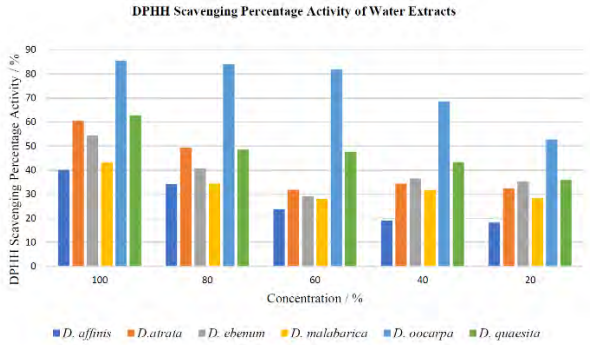


Figure 16. IC_{50} of water extracts

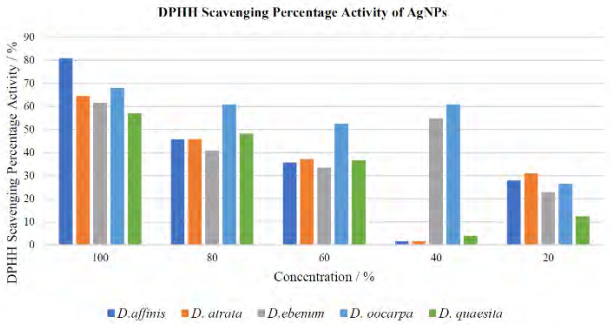


Figure 17. IC_{50} of AgNPs

Table 9. IC_{50} of the water extracts and the AgNPs.

Sample	IC50	
	WE	AgNPs
S01	137.69322	57.451875
S02	83.149342	85.155247
S03	110.91513	83.021492
S04	163.32515	-
S05	2.1448999	50.882992
S06	67.92998	62.255501

The IC50 values calculated for the water extracts of S01, S03 and S06 are greater than the IC50 values of the AgNPs whereas the IC50 values calculated for the water extracts of S02 and S05 are less than the IC50 values of the AgNPs.

3.7. Determination of the Photocatalytic Activity.

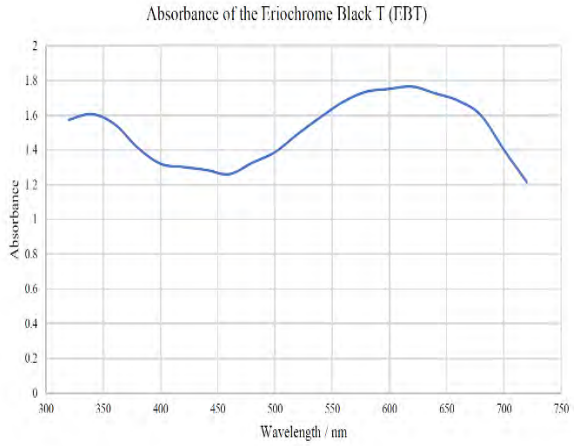


Figure 18. Absorbance of EBT from 320 nm to 720 nm

Figure 18 outlines the absorbance of EBT at wavelengths ranging from 320 nm to 720 nm, which can be used to compare the degradation of the dye by AgNPs.

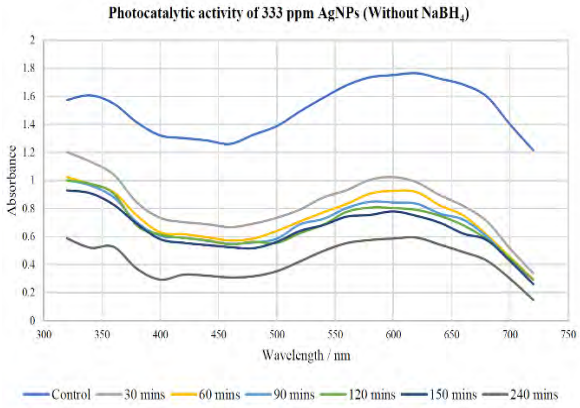


Figure 19. Photocatalytic activity of 333 ppm AgNPs (Without $NaBH_4$)

The absorbance of the dye was measured at 30-minute intervals till 150 minutes, and once again measured at the 4-hour mark (240 minutes) (Figure 19). The photocatalytic degradation rate constant was calculated to be 0.0025, using an ln (Ct/C0) against reaction time graph

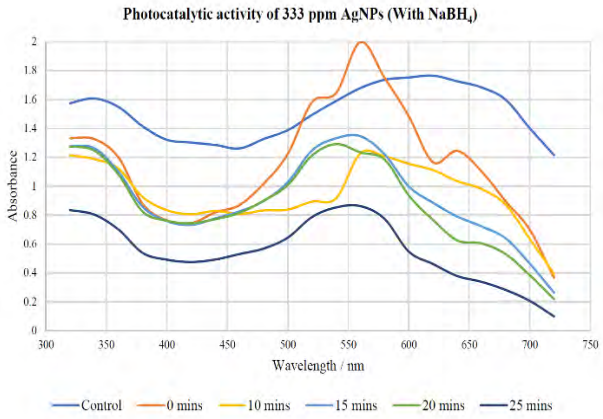


Figure 20. Photocatalytic activity of 333 ppm AgNPs (With $NaBH_4$)

The absorbance of the dye was measured at 10-minute intervals till 10 minutes, and then to 25 minutes in 5-minute intervals (Figure 20). The photocatalytic degradation rate constant was calculated to be 0.0271, using an ln (Ct/C0) against reaction time graph.

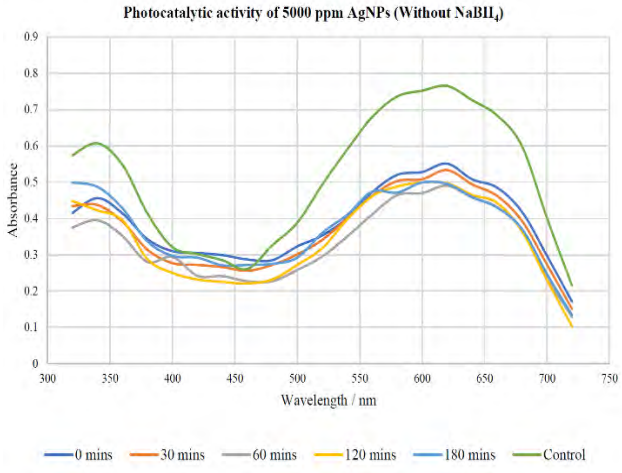


Figure 21. Photocatalytic activity of 5000 ppm AgNPs (Without $NaBH_4$)

The absorbance of the dye was measured at 30-minute intervals till 60 minutes, and then to 180 minutes in 60-minute intervals (Figure 21). The photocatalytic degradation rate constant was calculated to be 0.0005, using an ln (Ct/C0) against reaction time graph.

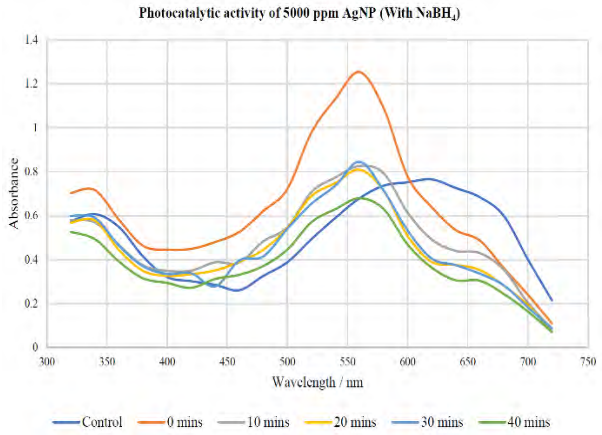


Figure 22. Photocatalytic activity of 5000 ppm AgNPs (With $NaBH_4$)

The absorbance of the dye was measured at 10-minute intervals till 40 minutes (Figure 22). The photocatalytic degradation rate constant was calculated to be 0.0111, using an ln (Ct/C0) against reaction time graph.

3.8 Determination of the Antibacterial Activity.

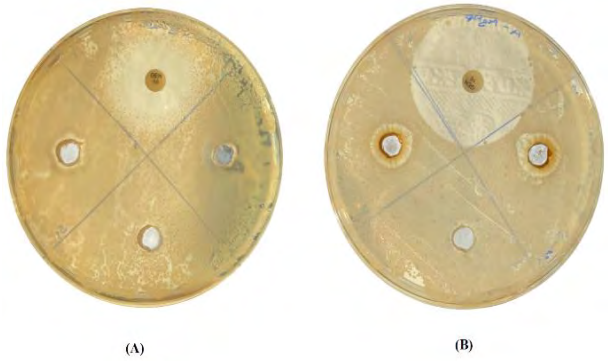


Figure 23. Antibacterial activity of the samples. (A – S04 water extract, B – S02 silver nanoparticles)

The agar plates exhibited zones of inhibition (ZOI) around the samples after being incubated for 24 hours as illustrated on Figure 23.

Figure 24 and 25 compares the zones of inhibition between the water extracts and the AgNPs concluding that the AgNPs exhibit are significantly higher ZOIs than that of the water extracts against both *Staphylococcus aureus* and *Escherichia coli.*

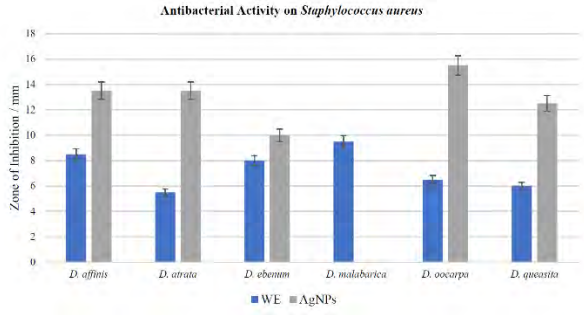


Figure 24. Antibacterial activity on *Staphylococcus aureus*

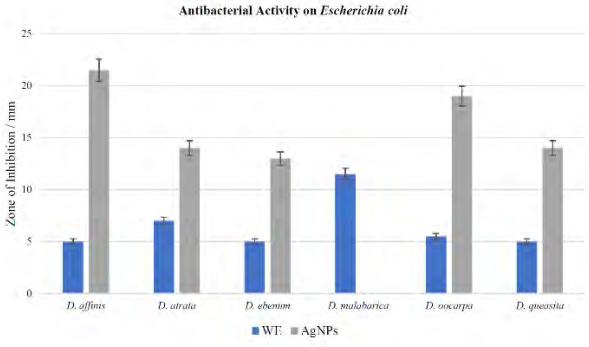


Figure 25. Antibacterial activity on *Escherichia coli*

The statistical test, One-Way ANOVA was carried out to determine if there is a significant difference between the antibacterial activity of the water extracts and the AgNPs against *Staphylococcus aureus* and *Escherichia coli* (Table 10 and Table 11).

Table 10. One-Way ANOVA between the antibacterial activity against *Staphylococcus aureus* between water extracts and AgNPs

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	87.57575758	1	87.57575758	27.818182	0.000511	5.117355
Within Groups	28.33333333	9	3.148148148			
Total	115.9090909	10				

Table 11. One-Way ANOVA between the antibacterial activity against *Escherichia coli* between water extracts and AgNPs

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	150.5208333	1	150.5208333	4.8522498	0.0521947	4.9646027
Within Groups	310.2083333	10	31.02083333			
Total	460.7291667	11				

5. Discussion

The green synthesis of nanoparticles is the most preferred form of nanoparticle synthesis as the consumption of energy in the techniques is reduced hence more cost-effective, and the absence of chemicals in the production processes removes the risk of toxicity and contamination.[37] Despite the use of both microorganisms and plants in green synthesis, the use of plant extracts is favoured over microorganisms as the use of microorganisms is more time-consuming due to the isolation and maintenance of cell cultures along with possibility of contamination.[50] Furthermore, the phytochemicals in the leaves act as reducing agents in the reduction of metal ions into nanoparticles.[51]

When selecting a solvent for leaf extraction, water over methanol or ethanol was selected as it is non-toxic, non-inflammable and removes the need for purification. In addition, the synthesized nanoparticles partially oxidize due to the presence of oxygen in water.[52]

The synthesis of nanoparticles can be affected by several external factors. When synthesizing the AgNPs from the leaf extracts of *Diospyros*, the factors of temperature and reaction time were adjusted to determine the optimum conditions for the reaction. The optimum conditions for the synthesized nanoparticles are at 90 °C for 1 hour. Whereas nanoparticles were synthesized at 60 °C and at room temperature, the increase in absorbance is greater when synthesized at 90 °C as higher temperatures result in the higher rate of formation of nanoparticles hence a greater concentration of nanoparticles leading to a higher absorbance.[53,54] However, nanoparticles formed at higher temperatures are of a smaller average size compared to nanoparticles synthesized at a lower temperature.[55]

The colour change from a light-yellowish solution to a dark reddish-brown solution, observed during the formation of nanoparticles is due to surface plasmon resonance (SPR).[56] SPR occurs when the plasmons on the surface of the nanoparticles are excited by the incident light at a specific wavelength, leading to a decrease up the intensity of the light reflected.[57,58] This decrease in reflected light causes the decrease in absorbance giving a "peak" in UV-Visible spectrum at the 420 nm wavelength, indicating the formation of AgNPs.[59] In order to characterize the nanoparticles synthesized, through TEM, the morphology of the S05 AgNPs was determined as spherical with an average diameter of 30 nm under a range of 20 – 40 nm. However, previous studies on *Diospyros montana* AgNPs of an irregular shape with an average diameter of 18 nm.[60]

Further optical properties of AgNPs were determined by calculating the band gap energy. The band gap energy can be defined as the minimum amount of energy required to excite an electron to jump from the valence band to the conduction band.[61,62]

If the bandgap energy is less than 3.0 eV, the nanoparticles are classified as semiconductors whereas if the bandgap energy is more than 4.0 eV, the nanoparticles are classified as insulators.[63] The bandgap energies were calculated using the Planck's equation given below (Figure 26).

$$E = h \times \frac{C}{\lambda}$$

$$1\ \text{eV} = 1.6022 \times 10^{-19}\ \text{J}$$

E – Bandgap Energy (J)

h – Planck's constant (6.626×10^{-34} J s

C – Speed of Light (6.626×10^{8} m s^{-1})

λ – Peak Wavelength of AgNP

Figure 26. Planck's equation

Table 12 shows the bandgap energies of the nanoparticles calculated to be less than 3.0 eV, classifying them as semiconductors.

Table 12. Classification of AgNPs based on bandgap energy

Sample	Bandgap Energy		Classification
	Joules	eV	
Diospyros affinis	4.73×10^{-19}	2.96	Semiconductor
Diospyros atrata	4.73×10^{-19}	2.96	
Diospyros ebenum	4.79×10^{-19}	2.99	
Diospyros oocarpa	4.73×10^{-19}	2.96	
Diospyros quaesita	4.52×10^{-19}	2.82	

Despite limited research on *Diospyros* AgNPs, the nanoparticles along with the water extracts were subjected to antioxidant assays to determine and compare their antioxidant capabilities.

Flavonoids are a class of polyphenolic derivatives containing at least one hydroxyl group in the aromatic ring which acts as an electron donor, providing an antioxidant effect[64]. The TFC assay was carried out to quantify the content of flavonoids in the samples through an AlCl3 colourimetric assay at a maximum absorption wavelength of 415 nm, where $AlCl_3$ binds to the C4 keto-group and the C3/C5 hydroxyl-group in flavonoids, forming an acid stable complex (Figure 27).[65, 66]

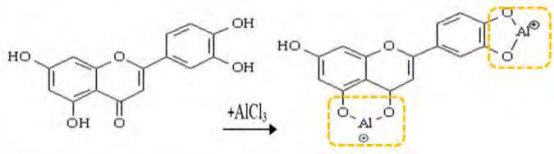


Figure 27. Formation of a flavonoid complex.[66]

The TFC of the AgNPs are significantly higher than the water extracts which can be seen in studies involving *Syzygium cumini* fruit extract due to the coating of flavonoids on the AgNPs.[67] The ANOVA test determined that there is a significant difference between the TFCs of the water extracts and AgNPs as the calculated P-value < 0.05 (0.008595), with S05 and S01 giving the highest and lowest TFCs respectively.

The TPC assay was carried out to quantify the content of phenols in the samples through a Folin-Ciocalteu colourimetric assay at a maximum absorption wavelength of 760 nm, where the Folin-Ciocalteu reagent is reduced by phenolic compounds to blue complexes (Figure 28).[68,69]

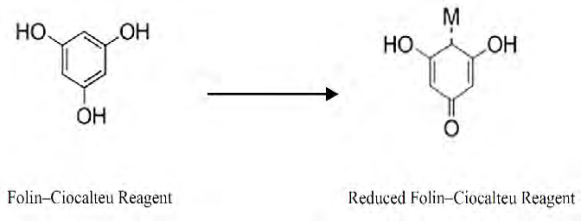


Figure 28. Reduction of Folin–Ciocalteu reagent.[69]

The TPC of the AgNPs are significantly greater than of the water extracts due to the accumulation of phenols by AgNPs (70). The ANOVA test determined that there is a significant difference between the TPCs of the water extracts and AgNPs as the calculated P-value < 0.05 (1.82×10-7) with S05 and S01 giving the highest and lowest TPCs respectively.

The TAC of the samples was determined through the phosphomolybdenum assay at a maximum absorption wavelength of 695 nm where phosphate-molybdenum VI is reduced to phosphate-molybdenum V in the presence of antioxidants forming a green complex (Figure 29).[71-73]

The TACs of the AgNPs are significantly higher than the TACs of the water extracts indicating that the AgNPs have a higher ability to stabilize free-radicals which correlates with the high content of flavonoids and phenols.[74] The ANOVA test determined that there is a significant difference between the TACs of the water extracts and AgNPs as the calculated P-value (0.001638) is less than 0.05.

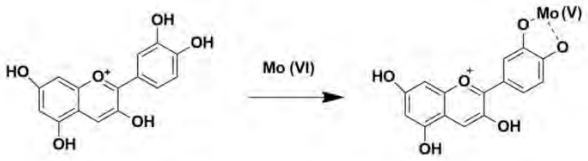


Figure 29. Reduction of Phosphate Molybdate VI.[73]

The DPPH radical scavenging assay was carried out to determine the antioxidant capabilities of the AgNPs by using a stable DPPH free radical which receives an electron from the antioxidants, stabilizing the molecules resulting in the discolouration of the violet DPPH solution.[75] The AgNPs have a greater % DPPH scavenging activity compared to the water extracts (Figure 15).

In addition to the DPPH assay, the IC_{50} of the samples was calculated to estimate the concentration of the sample required to stabilize 50% of the DPPH radicals where majority of the water extracts gave a higher IC_{50} compared to the AgNPs with the exception of S05 and S02 (Table 9).[76]

The IC_{50} values are inversely proportional to the antioxidant activity of the samples as a high IC_{50} indicates a high concentration is required to stabilize 50% of the DPPH radicals, concluding that the S02 and S05 AgNPs have greater antioxidant activities compared to the water extracts.[77]

The correlation between the antioxidant assays were analyzed and it was determined that a strong correlation existed between the assays (Figure 30). The TFC and TPC in the samples directly correlated with the calculated TAC indicating that content of flavonoids and phenols play a vital role in the TAC.

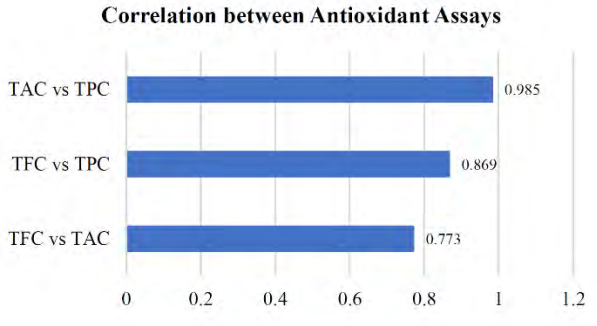


Figure 30. Correlation between antioxidant assays

The photocatalytic activity of the nanoparticles was compared by measuring the degradation of Eriochrome Black T (EBT) dye into intermediates where the N=N bond is dissolved (Figure 31) by free radicals formed by photocatalysis, resulting in a colourless solution.[78]

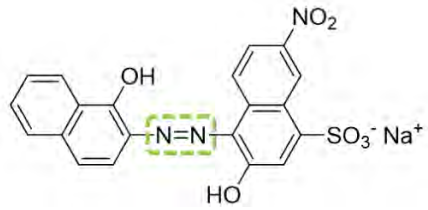


Figure 31. Chemical structure of Eriochrome Black T.[79]

The rate constant was calculated by using an ascorbic acid standard graph to calculate the concentrations at a given time and by following the below equation (Figure 32).

$$\text{Rate Constant} = \frac{\ln(\frac{C_t}{C_o})}{t}$$

C_o – Initial concentration

C_t – Peak concentration

t – Reaction time at peak concentration

Figure 32. Rate constant equation

The photocatalytic-degradation of EBT was carried out using S05 AgNPs of concentrations, 333 ppm and 5000 ppm, with and without $NaBH_4$ catalyst for a duration of 4 hours, in which the catalyzed reaction using 333 ppm AgNPs gave the highest degradation rate constant of 0.0271 (Figure 33 and Figure 34).

The addition of $NaBH_4$ significantly increases the degradation rate of the 333 ppm AgNPs from 0.0025 to 0.0271 as electrons from $NaBH_4$ are transferred to the EBT molecule by AgNPs, but the rate decreased as the AgNP concentration increased from 333 ppm to 5000 ppm possibly due to the formation of intermediates and the accumulation of excess AgNPs which cause excessive scattering of light, decreasing photocatalysis.[80-82]

A blue shift of the control peak can be seen in the graphs of the catalyzed photo-degradation of EBT (Figure 23 and Figure 25) which is caused by the formation of intermediates in the solution.[83]

When comparing the antibacterial activity, Figure 27 and Figure 28 reveals that the AgNPs have a greater antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* as the AgNPs have higher zones of inhibition (ZOI) due to the action of the Ag^+ ions (Figure 03) where S05 had the highest ZOI against *Staphylococcus aureus* with S01 against *Escherichia coli* (84). AgNPs employ Ag^+ ions to exert bactericidal activity through the proposed mechanisms in Figure 3.

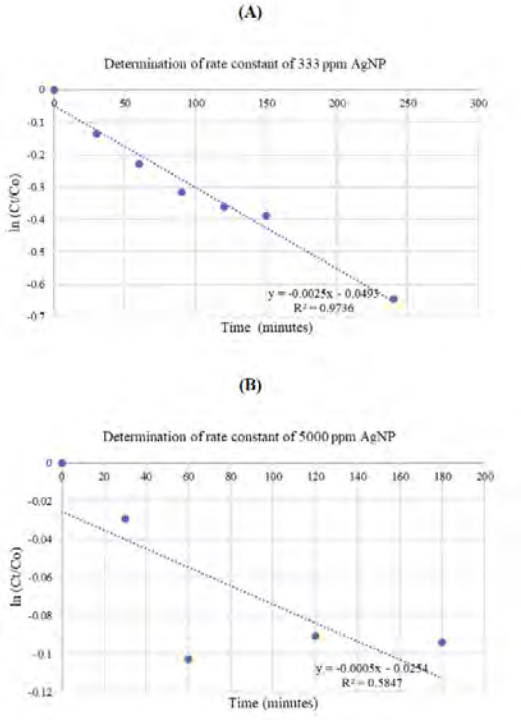


Figure 33. Rate constant graph of uncatalyzed AgNPs (A – 333 ppm, B – 5000 ppm)

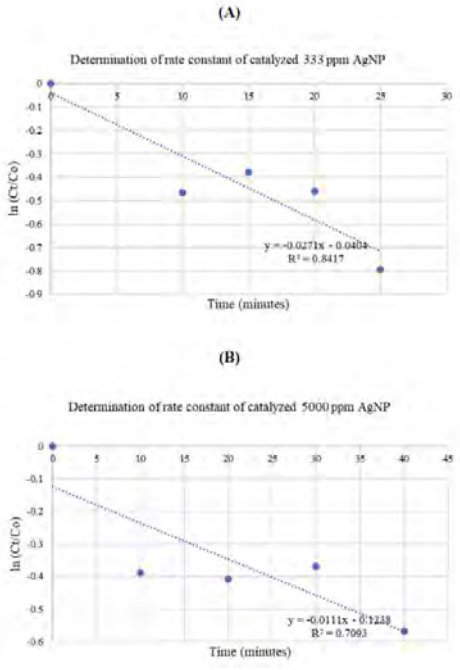


Figure 34. Rate constant graph of catalyzed AgNPs (A – 333 ppm, B – 5000 ppm)

However, when comparing the ZOI of the AgNPs, the ZOI against *Escherichia coli* are greater than the ZOI of *Staphylococcus aureus.* Similar results were obtained in studies on SnO_2 nanoparticles concluded that the difference in antimicrobial activity was due to differences in the cell wall structure where *Escherichia coli* consists of a thin peptidoglycan layer containing lipopolysaccharides and proteins allowing easy entry for Ag^+ ions and less resistance to ROS compared to *Staphylococcus aureus.*[85, 86]

The P-values calculated for the ANOVA tests stated that there is a significant difference between the antibacterial activity against *Staphylococcus aureus* between the water extracts and AgNPs (P<0.05, P=0.000511), whereas there is no significant difference between the antibacterial activity against *Escherichia coli* of the water extracts and AgNPs (P>0.05, P=0.052195). However, the P-value calculated to determine a significant difference between the antibacterial activity between *Staphylococcus aureus* and *Escherichia coli,* was greater than 0.05 (P= 0.617294), concluding there is no significant difference.

Conclusion

AgNPs were synthesized using the six species of *Diospyros* with the exception of *Diospyros malabarica* at the temperatures of 25 °C, 60 °C and 90 °C for a time periods between 30 minutes to 72 hours where the optimum temperature was 90 °C. The phytochemical analysis on the water extracts confirmed that the leaves contained carbohydrates, proteins, saponins and terpenoids. TEM analysis determined that the synthesized AgNPs were spherical in shape with an average diameter of 30 nm. The AgNPs were subjected to antioxidant assays which determined that the AgNPs had a higher antioxidant capacity than the water extracts. The photocatalytic assays concluded that the 333 ppm AgNPs showed a higher photocatalytic activity when using $NaBH_4$. The antibacterial assays determined that the AgNPs show greater antibacterial activity against the bacterial strains where the activity against *Staphylococcus aureus* was greater than *Escherichia coli* due to structural differences in the cell wall. The determination of the following properties of the AgNPs allows for further studies and analysis for its implementation of its use into industries.

Acknowledgements

Authors thank BMS for funding.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Detection and determination of effect of antimicrobial efficacy of Cumin seeds (*Cuminum cyminum*) and Indian marigold flower (*Calendula officinalis*) against *Escherichia coli* and *Staphylococcus aureus* bacteria

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Abstract

Antimicrobial resistance is the most significant threat to global health. The emergence of resistant strains is accelerated by the misuse of antibiotics. Plant metabolites beneficial in preventing antibiotic resistance due to their potential to change resistance. Plants contain secondary metabolites which possess antimicrobial activities *in vitro* and are used as an effective antibiotic for the treatment of microbial diseases. *Calendula officinalis* Linn. is an annual and versatile herb which is widely used in homeopathic remedies. To treat infectious disease, all plant components (buds, leaves, and blooms) can be utilized dry or fresh. *Cuminum cyminum* is an aromatic herb widely used as a food and flavouring additive as well as often used in alternative medicine. Due to the presence of cuminaldehyde, cumin has great antimicrobial effects against both gram positive and negative bacteria. The major objective of this study is to determine the antimicrobial efficacy of cumin seeds and Indian marigold flower against *Escherichia coli* and *Staphylococcus aureus* bacterial strains. The phytochemicals are extracted using an ethanol and methanol maceration procedure, and their antibacterial capabilities are tested and assessed using ABST testing. The results reveal that the increasing the concentration of extract causes inhibition of bacterial growth. Therefore, 50 mg/ml and 100mg/ml concentration had less zone of inhibition (ZOI) compared with highest concentration which was 150 mg/ml. When the extract concentration was increased to 150 mg/ml, the bacterial growth inhibition zone increases significantly. When compared to ethanol extraction of cumin seeds and marigold flowers, the marigold flower showed more anti-bactericidal effects compared to the cumin seeds.

Keywords: *Calendula officinalis, Cuminum cyminum,* Antimicrobial resistance, *Escherichia coli*, *Staphylococcus aureus*

1. Introduction

The medicinal plants are extensively used as drugs to treat humans and animals to cure the various diseases since the beginning of human history. Therefore, medicinal plants are considered as the backbone of holistic medicine. Bioactive components of medicinal plants are widely used to cure various diseases.[1] Extracts of the whole plant or plant parts, including barks, stalks, blooms, leaves, roots, and fruits are utilized as potent natural medications to cure various diseases, specifically caused by microorganisms.[2]

Nowadays, antimicrobial resistance (AMR) is the biggest threats to global health. Each year, it causes around 2 million infections and 23,000 fatalities.[3] The ability of bacteria, parasites, viruses, and fungi to grow and proliferate in the presence of antimicrobial medications that are ordinarily active to defense them is known as antimicrobial resistance (AMR).[4]

Plants contains phytochemicals constituents; tannins, alkaloids, flavonoids terpenoids and polyphenols. Plants utilizes these compounds as defense mechanisms against wide range of microorganisms.[5] Due to the current scenario, many ongoing research are conducted regarding plant based novel medications. Globally, 25% of prescribed drugs are plant based and more than 13000 plants are studies for last 5 years for medicinal purposes.[6]

Calendula officinalis commonly referred as 'Indian marigold' is a perennial herbaceous plant belongs to Asteraceae family. Marigolds are native to Europe, eastern and western Asia as well as in United states.[7] *C. officinalis* have angular hairy stem which can be growing up to 30-60 cm.[8] Also, it has long elliptical shape leaves. Marigold flowers are monoecious and has bright yellow to orange in color, but only dark orange color flowers have the medicinal value.[9] Flavonoids, tocopherols, terpenoids, phenolic compounds and carotenoids are the main phytoconstituents which possess pharmacological activities in *C. officinalis.*[10]

Marigold flowers are cultivated due to its nutritional, economical, and medicinal value. The petals extract has traditionally been used to dye natural fabrics (wool, silk).[11] Due to presences of saponins and essential oil, the extract was widely used for Cosmetic purposes.[12] In folk medicine, marigolds was used to treat wound healing, infectious diseases and burns since 12th century.[13] In wound healing process, the extract can stimulate the new tissues and blood vessels growth by topical application.[14]

Previous studies prove that marigold has hepatoprotective, hypoglycemic and antioxidant properties. According to laboratory experiment, *Calendula* petal extracts inhibits the human immunodeficiency virus (HIV), also neutralize the oxidative stress and liver damage followed by Aflatoxin.[14]

Figure 1. *Calendula officinalis* flower[14]

Figure 2. **A** – *C. officinalis* (young leaflet stage), **B** – *C. officinalis* (flower bud stage), **C** – *C. officinalis* (flowering stage)[15]

The past researchers have shown that under *in vitro* conditions, marigold flower and dried leaves have antibacterial properties against *Klebsiella species, Staphylococcus aureus*, and *Escherichia coli* in chloroform, methanolic and ethanol extracts.[16] Also, *in vitro* study confirms it has great inhibitory progression against skin microbiota.[17]

Marigold's antimicrobial activities were tested by antioxidant and UV induced DNA damage prevention activity.[18] DPPH and FRAP phytochemical analysis endorsed that *C. officinalis* extract has antimicrobial and antioxidant action.[19]

Cuminum cyminum generally known as 'cumin seeds' which is an aromatic herb belongs to the family Apiaceae.[20] *C. cyminum* are originated from Egypt and Turkey but due to their

special aromatic effect, the dried cumin seeds are broadly used in Asian, middle Eastern and Latin American cuisine.[21]The plant can grow up 25 cm, and has tiny whitish or pink color, umbellate formation flowers and 3-6 mm elongated, striped pattern seeds.[22]

Figure 3. Life cycle of the *C. cyminum*[23]

Cumin seeds has been in use as an alternative medicine for a long time, to cure chronic diarrhea, epilepsy, and jaundice.[24] It is considered as a carminative, antispasmodic and astringent also, cumin oil inhibits numerous pathogenic *Candida* species due to its antifungal properties.[25]

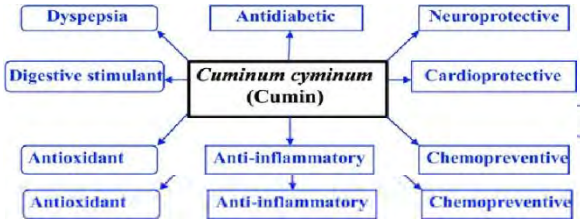


Figure 4. Medicinal values of Cuminum cyminum[26]

The plant consists of cuminaldehyde, tannin, and cymene compounds, it has potential properties in aspects such as antimicrobial, antidiabetic and hypolipidemic.[27] Therefore, due to their pharmacological characteristics, studies on *C. cyminum* roots, stems, leaves, and flowers have garnered interest.[28] Previous studies confirm that cumin extract inhibits the growth of various pathogens (*E. coli, S. aureus, Salmonella* species, and *Aspergillus niger*).[29] Antimicrobial potential of cuminaldehyde counteracts gram positive and negative bacteria respectively.[30] Cumin has been shown to inhibit the production of biofilms in *Streptococcus mutans* and *Streptococcus pyogenes.*[31] Cumin has an exceptional antibiofilm and quorum sensing inhibitory activities against gram-negative bacterial pathogens.[32]

The researchers find, plant origin antimicrobial compounds have great therapeutic potential against wide range of microorganism and have less side effects compared to synthetic drugs.[33] Therefore, plant base novel medications are effective and safe for human and animal consumption.

Gram positive and negative bacteria respectively, *Staphylococcus aureus* and *Escherichia coli*, which contribute for foodborne diseases and have increased the morbidity and mortality rate globally.[34]

Staphylococcus aureus is responsible for methicillin-resistant *Staphylococcus aureus* (MRSA) nosocomial disease, currently resistance for all type antibiotics. Due to the evolution of organisms carrying extended spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases, *Escherichia coli* develops inherent resistance to several types of β-lactamase.[35]

Both bacteria develop their resistance to antimicrobials followed by these mechanisms such as restricting acceptance of drugs, modification of drugs, enzymatic degradation of drugs and active efflux of the drugs.[36]

Due to misuse and repeated usage of antibiotics, the bacterial pathogens become resistance. Also, high consumption of antibiotics caused to escalate the side effects. Inhibiting efflux pumps and eliminating plasmids cause to increase the medicinal efficiency of antibiotics, that could help to decrease the foodborne pathogens antibiotic resistance throughout the food chain.[37]

2. Methodology

2.1 Collection of medicinal plants. Fresh healthy and disease-free *C. officinalis* flowers and *Cuminum cyminum* seeds were respectively collected from home gardens and local market of Matara district, Sri Lanka.

2.2 Preparation of plant Extracts. C. officinalis flowers and *C. cyminum* seeds were thoroughly washed with tap water then distilled water and the surface was sterilized with 70% ethanol. The cumin seeds and separated marigold flower petals and sepals were shade dried for three days. The dried plant materials were pulverized to a fine powder discretely by using mechanical grinder and passed-through a 0.5 mm mech sieve. About 200 g of marigold flower powder and 250 g of cumin seeds powder were gained after pulverization. The plant powder materials were stored in separate airtight containers for further investigation.

2.3 Ethanolic extraction of Calendula officinalis and Cuminum cyminum. Ethanolic extraction was carried out using marigold and cumin powder with 70% and 80% of ethanol respectively according to 1:10 (w/v) ratio. 2g of dried marigold powder was extracted with the combination of 14.73 ml ethanol and 5.27 ml of distilled water. 2 g of cumin powder was added to 80% of ethanol (20 ml) with the combination of 16.84 ml ethanol and 3.16 ml of distilled water.

2.4 Methanol extraction of *Calendula officinalis* and *Cuminum cyminum.* Methanolic extraction was carried out using marigold and cumin powder with 70% and 80% of methanol respectively according to 1:10 (w/v) ratio. Both ethanolic and methanolic plant extraction samples were kept in roller mixer at 27°C for 24 hrs. The macerate was filtered with Whatman no 01-filter paper, and the solvent was kept into fume hood for 48 hrs for completely evaporation. Finally, the extracted crude was reconstituted in DMSO to get stock concentration of 200 mg/ml.

2.5 Bacterial strains. Methanol and ethanol extracts of *C. officinalis* and *C. cyminum* were tested discretely against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) bacterial strains.

2.6 Inoculum preparation. 100 µL of *Escherichia coli* and *Staphylococcus aureus* were inoculated into 5 ml of LB broth in separate falcon tubes and incubated at 37°C for 24 hours. The bacterial suspension turbidity was adjusted according to 0.5 McFarland standard by using a Wickerham card in the presence of the sufficient light.

2.7 Antibiotic Sensitivity Testing. The Mueller Hinton agar (MHA) was prepared and poured into petri plates and allowed to solidify for few minutes at aseptic condition. The bacterial inoculum was swabbed by rotating the plate around for uniform distribution on the entire surface. Finally, the edge of the of the agar plates were swabbed by using sterilized cotton swab. Four wells were prepared by using sterile 1000 µL pipette tips. 50µl of reconstituted plant sample concentrations (50 and 100 mg/mL) and DMSO and Gentamicin (1 mg/mL) were used as negative and positive controls, respectively. Then solvents were introduced into separate respective wells. The plates were incubated for 24 hours at 37°C in an upright position. The diameter of the zone of inhibition was measured to the nearest millimeter to determine antibacterial activity. The experiment was done in triplicate and the average values were calculated.

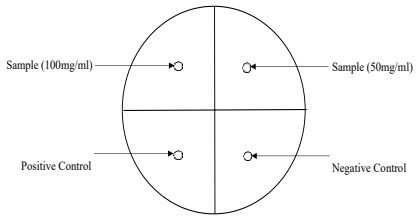
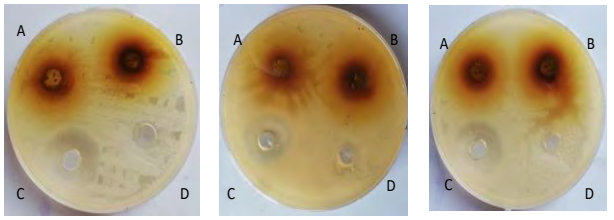


Figure 5. Outline of the petri plate for well diffusion

2.8 Statistical analysis. Results were observed and recorded in triplicates and the data were analyzed statistically to evaluate correlation among variables (antibiotic profiles) using the two- way ANOVA test using the GraphPad Prism software (version 9.1.0). The students t- test was used to evaluate the extraction efficacy between the solvents used. The statistical significance was denoted as p value ≤ 0.05 for a 95% confidence interval.

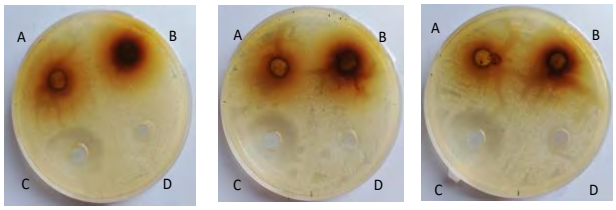
3. Results

3.1 Antibacterial Susceptibility Testing (ABST) for ethanolic extracts of Indian marigold



ABST results for *S. aureus*

R1 **R2** **R3**



ABST results for *E. coli*

R1 **R2** **R3**

Figure 6. The ZOIs produced by ethanolic extracts of Indian marigold against *E. coli* and *S. aureus*; (A)= 100mg/mL plant extract, (B)= 150mg/mL plant extract, (C)= positive control and (D)= negative control, (R= replicate).

Table 1. ZOIs measured for ethanolic extracts of Indian marigold

	100mg/mL (mm)	150mg/mL (mm)	Positive (mm)	Negative (mm)
E. coli	17.667 ± 0.577	21.000 ± 2.646	24.666 ± 1.527	-
S. aureus	18.000 ± 1.732	19.333 ± 1.55	26.333±1 .154	-

Highest ZOI was observed in 150mg/mL against *E. coli* while the lowest ZOI was in 100mg/mL against the same strain.

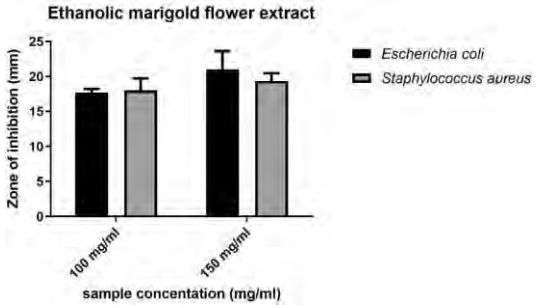


Figure 7. The antibacterial properties of 100 mg/mL and 150 mg/mL ethanolic extract of Indian marigold depicted using well diffusion test. *E. coli* is represented by the black color bars and *S. aureus* is represented by grey color bars. The bars show mean ± SD of the inhibition zones for three replicates.

Table 2. Two-way ANOVA analysis for ethanolic extract of Marigold flower

ANOVA Table	SS	DF	MS	F	P
Interaction	3.00	1	3.00	1.029	0.3402
Concentration	16.33	1	16.33	5.600	0.0455
Bacteria	1.33	1	1.33	0.4571	0.5180
Residual	23.33	8	23.33		

According to the two-way ANOVA analysis, P value for interaction has 0.34 which is higher than the 0.05. Therefore, it is not significant. Also, P value for bacteria is 0.5 which is higher than the 0.05. Therefore, it is not significant. The extract concentration is 0.04 value and it was lower than the p value, and it is significant. Therefore, increasing the concentration, the main effects are observed and they are contributing to the main effects.

3.2 Antibacterial Susceptibility Testing (ABST) for methanolic extracts of Indian marigold.

Table 3. ZOIs measured for methanolic extracts of *Indian marigold*

	50mg/mL (mm)	100mg/mL (mm)	Positive (mm)	Negative (mm)
E. coli	12.100± 0.656	12.833± 0.764	23.000± 0.00	-
S. aureus	10.000± 1.00	17.500± 0.500	20.00±0.00	-

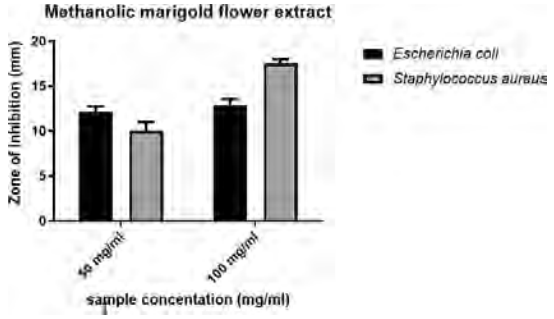


Figure 8. The antibacterial properties of 50 mg/mL and 100 mg/mL methanolic extract of Indian marigold depicted using well diffusion test. *E. coli* is represented by the black color bars and *S. aureus* is represented by grey color bars. The bars show mean ± SD of the inhibition zones for three replicates.

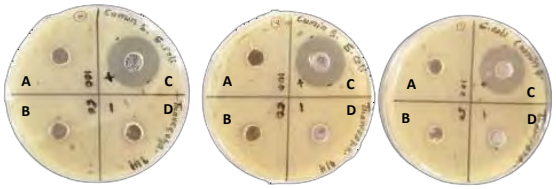
Table 4. Two-way ANOVA analysis for methanolic extract of Marigold flower

ANOVA table	SS	DF	MS	F	P
Interaction	34.34	1	34.34	60.69	0.0001
Concentration	50.84	1	50.84	89.85	0.0001
Bacteria	4.941	1	4.941	8.732	0.0183
Residual	4.527	8	4.527		

According to the two-way ANOVA analysis, P value for interaction has 0.0001 which is lower than the 0.05. Therefore, it is significant. Also, P value for bacteria has 0.0183 which is also, lower than the 0.05. Therefore, it is also significant. The concentration has 0.0001 value and it was lower than the p value, and it is significant. Therefore, all the three facts are significantly different and they are contributing to the main effects.

3.3 Antibacterial Susceptibility Testing (ABST) for ethanolic extracts of cumin seeds

ABST results for *E. coli*



ABST results for *S. aureus*

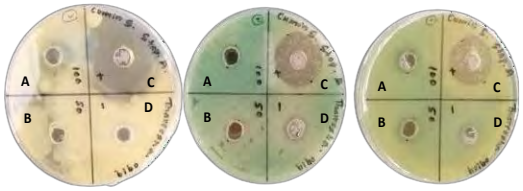


Figure 9. The ZOIs produced by ethanolic extracts of cumin seeds against *E. coli* and *S. aureus*; (A)= 100mg/mL plant extract, (B)= 50mg/mL plant extract, (C)= positive control and (D)= negative control, (R= replicate).

Table 6. ZOIs measured for ethanolic extracts of cumin seeds

	50mg/mL (mm)	100mg/mL (mm)	Positive (mm)	Negative (mm)
E. coli	9.900 ± 0.794	10.667±1.155	26.000±1.732	-
S. aureus	11.000± 0.000	11.000±0.000	20.333±1.000	-

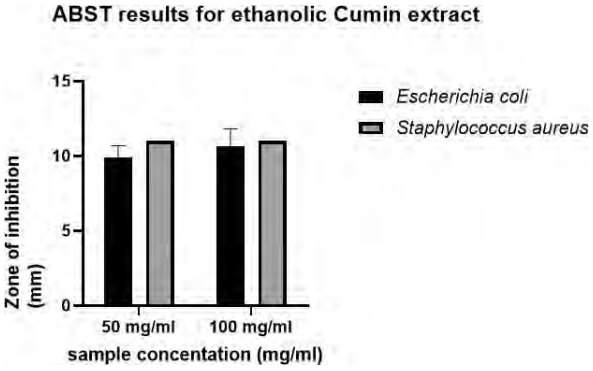


Figure 11. The antibacterial properties of 50 mg/mL and 100 mg/mL ethanolic extract of cumin seeds depicted using well diffusion test. *E. coli* is represented by the black color bars and *S. aureus* is represented by grey color bars. The bars show mean ± SD of the inhibition zones for three replicates.

Table 7. Two-way ANOVA analysis for ethanolic extract of cumin seeds

ANOVA table	SS	DF	MS	F	P
Interaction	0.4408	1	0.4408	15.17	0.3710
Concentration	0.4408	1	0.4408	1.567	0.3710
Bacteria	1.541	1	1.541	4.648	0.1144
Residual	3.927	8	3.927		

According to the two-way ANOVA analysis, P value for interaction has 0.3710 which is higher than the 0.05. Therefore, anti-bacteria effect of this concentration it is not significant. Also, P value for bacteria has 0.1144 which is higher than the 0.05. Therefore, it is not significant. P value of the concentration of the sample is >0.05. Hence, there is no significant difference between sample concentration and the antibacterial activity against the bacterial strains. However, all the three facts are not significant and they are not contributing to the main effects.

3.4 Antibacterial Susceptibility Testing (ABST) for methanolic extracts of cumin seeds

Table 5. ZOIs measured for Methanolic extracts of cumin seeds

	50mg/mL (mm)	100mg/mL (mm)	Positive (mm)	Negative (mm)
E. coli	16.033±0.850	16.867 ± 0.551	20.05 ± 0.7	-
S. aureus	18.500 ± 1.000	15.600 ± 0.854	21.5± 3.5	-

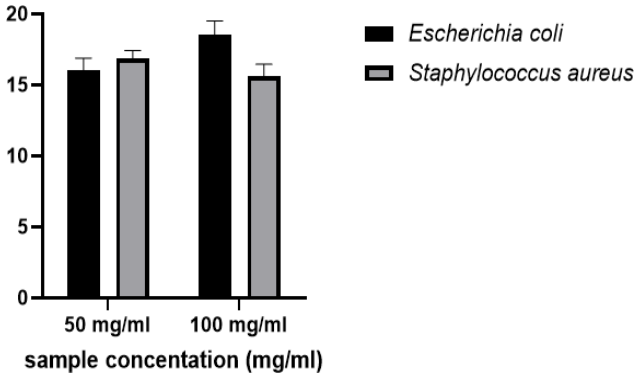


Figure 10. The antibacterial properties of 50 mg/mL and 100 mg/mL methanolic extract of cumin seeds depicted using well diffusion test. *E. coli* is represented by the black color bars and *S. aureus* is represented by grey color bars. The bars show mean ± SD of the inhibition zones for three replicates.

Table 8. Two-way ANOVA analysis for methanolic extract of cumin seeds

ANOVA table	SS	DF	MS	F	P
Interaction	10.45	1	10.45	15.17	0.0046
Concentration	1.080	1	1.080	1.567	0.2460
Bacteria	3.203	1	3.203	4.648	0.0632
Residual	5.513	8	5.513		

According to the two-way ANOVA analysis, P value for interaction has 0.0046 which is lower than the 0.05. Therefore, it is significant. Also, P value for Concentration has 0.2460 which is higher than the 0.05. Therefore, it is not significant. The Bacteria has 0.0632 value and it was not lower than the p value, and it is not significant.

4. Discussion

The selective pressure that leads to the emergence of antibiotic resistance in microorganisms is caused by human overuse of antibiotics. Emerging multidrug resistant pathogens, often known as 'ESKAPE' species represent a severe threat to global health today.[38] Medicinal plants contain phytochemical compounds which naturally associated with fighting against microbial resistance and also have antimicrobial properties. Therefore, researchers are interested in plant-based medications as an undisputable substitution for antibiotics due to high efficacy to antimicrobial activity with less side effects as well as low cost.[39]

The plant age and part are important parameters for extraction procedure. Recently matured plants are ideal for researchers. If the plant part is too old, the phytochemical compounds have less effective. When it comes to alkaloids, older plants contain significantly fewer than younger plants. Phytochemical compounds' efficacy varies depending on the stage of plant development.

Also, dried samples are better than the fresh plant samples. Fresh samples are caused to quick degradation due to water content of the sample. When the particle size is reduced, the solubility and diffusion increase, which leads to an increase in the extraction rate. As a result, the powdered sample has a greater surface area contact with the extraction solvent. Particles smaller than 0.5mm should be used for better extraction. The extraction duration, temperature, growth regions and the quality of the seeds can affect the differences of the zone of inhibition.[40] In phytochemical extractions, three types of solvents are used: polar, intermediate-polar, and non-polar, and the solvents are chosen depending on the polarity of the solute. Ethanol is a polar molecule that may dissolve in polar and non-polar solvents. It's ideal for polyphenol extraction and is safe for human consumption. Methanol has been found to be more effective at extracting polyphenols with lower molecular weights. In ethanolic extraction, a higher extraction yield was observed for *Calendula officinalis* which is 9.5% compared to the *Cuminum cyminum* which is 9.0%.

For ZOI, drug solubility, concentration, media composition and thickness, temperature, atmosphere and incubation time are all influenced. Each experiment was carried out at least three times. The results are expressed as Mean and SD.

The results revealed that each of the cumin extract concentrations tested had antibacterial action against both gram positive and gram-negative microorganisms. Comparing the results of methanolic extractions, cumin seeds showed the maximum ZOI against S. aureus which was 18.500 ± 1.000 at 50 mg/mL while showed, 15.600± 0.854mm for 100mg/ml. For *E. coli* bacteria, obtained highest ZOI observed at the concentration of 100mg/ml (16.867 ± 0.551) compared to concentration of 50mg/ml (16.033 ± 0.850). The overall results of the methanolic extract, the highest anti-bacterial activity was observed at 50mg/ml for gram positive bacteria. According to Santajit *et al*.[38], methanolic extract of cumin for *Escherichia coli* the inhibition zones were observed at 250 mg/ml which is 16.67±0.47. Compared to this study, our results obtained comparatively high values at low concentration compared to Sheikh's study.

Also, according to the Bouhenni's study, at the concentration of 100 mg/ml, *S. aureus* and *E. coli* bacterial strains showed ZOI with diameters of 21 mm± 0.333 and 12 mm ± 0.66 respectively.[41] High ZOI dimeters were found in the Bouhenni's investigation for *S. aureus*. In contrast, our investigation found 15.600 ± 0.854mm for *S. aureus*. Masood and Tariq reported that cumin extract inhibited the *S. aureus* and *E. coli* growth with the diameters 8.9±5.6 and 23.8±1.2 respectively.[42] Soniya's study found that methanol extracts of *C. cyminum* inhibited *Bacillus subtilis*, *E. coli,* and *Proteus sp*. with the maximum diameter of zones of inhibition.[43]

Comparing the results of ethanolic extracts, *S. aureus* showed high ZOIs at the concentration of both 100 mg/ml and 50 mg/ml (11.000 ± 0.000). Cumin ethanolic extract, the *E. coli* showed, 9.900 ± 0.794mm and 10.667 ± 1.155mm for 50 and 100mg/ml respectively. According to the study done by Mostafa et al[45], *C. cyminum* extract inhibit the growth of *S. aureus* at the concentration of 10 mg/ml which was 9.5 ± 0.74 but at the same concentration *E. coli* showed 0.0 ± 0.0, which means at the concentration of 10 mg/ml, *E. coli* was totally not resistant.[44] Compared to our project, Mostafa *et al*[45]. got a comparatively high ZOI for *S. aureus* at concentration of 10 mg/ml.[45] Based on the Sheikh *et al*.[41] 15.67±0.47 inhibition zones were recorded at 250 mg/ml against *E. coli.*[38] Cuminaldehyde is the active component in cumin extract, which has great antimicrobial effects against both gram positive and negative bacteria. Cuminaldehyde alters the bacterial cell's outer layer, preventing ion transfer into and out of the cell. In the end, this mechanism disrupts the action of bacterial enzymes.[46]

In this study reveals that marigold flower ethanolic extraction showed highest ZOI for gram-positive bacteria at concentration 150 mg/ml (19.333 ± 1.55mm) compared to 100 mg/ml (18.000 ± 1.732). For gram negative bacteria, higher concentrations are required for inhibit the growth of the bacteria. According to that, the maximum ZOI was measured at 150 mg/ml (21.000 ± 2.646mm) also, at 100 mg/ml 17.667 ± 0.577 mm ZOI were observed. Ethanol extract of marigold showed 14 and 18 mm of ZOI against *Staphylococcus aureus* and *E. coli* respectively at 50 mg/ml.[47]

Previous studies have shown that methanolic extracts of the marigold flower have antibacterial activity against *Klebsiella* species, *Staphylococcus aureus*, and *Escherichia coli.*[48] Comparing the results of methanolic extracts, *S. aureus* showed highest ZOI at 100 mg/ml (17.500 ± 0.500mm) compared to 50 mg/ml (10.000 ± 1.00mm). In *E. coli* samples, highest ZOI was observed at 100mg/ml (12.833 ± 0.764mm) compared to 50mg/ml (12.100 ± 0.656mm). Efstratiou *et al*.[11] reported that at 300 mg/ml in methanolic extract, *E. coli* showed ZOI 21 ± 2mm while *S. aureus* got ZOI 18 ± 2mm.[11]

The results reveal that when increasing concentration cause to inhibition of the bacteria. Therefore, 50 mg/ml and 100mg/ml concentration had less ZOI compared with highest concentration which was 150 mg/ml. When the extract concentration was increased to 150 mg/ml, the bacterial growth inhibition zone increases significantly. When compared to ethanol extraction of cumin seeds and marigold flowers, the marigold flower showed more bactericidal effects compared to the cumin seeds.

When comparing both stains, gram negative bacteria showed highest inhibitory properties at the highest concentrations. This could be due to Gram-negative bacteria having an additional outer membrane made of lipopolysaccharide, which makes them impervious to lipophilic, but Gram-positive bacteria only have an outer peptidoglycan layer, which is ineffective as a permeability barrier.

The negative control was DMSO, which had no antibacterial effect on the microorganisms examined. Gentamicin, on the other hand, demonstrated an antibacterial impact on the microorganisms tested when used as a positive control antibiotic. It inhibits protein synthesis and causes death in susceptible bacteria by attaching to the 30S component of the bacterial ribosome.

Considering results of ANOVA analysis for ethanolic extraction, the marigold flower has 0.04 value for concentration which is significant. For, cumin seeds P value of the concentration of the sample was 0.3710 which is higher than the p value (0.05). Therefore, there is no significant difference between sample concentration and the antibacterial activity against the bacterial strains in cumin seed concentration. According to the ANOVA analysis of methanolic extraction, the cumin seeds have a P value of 0.2460 for concentration which is more than 0.05. As a result, it is not significant. For marigold flower, the concentration has 0.0183 value and it was lower than the p value, and it is significant.

Conclusion

C. officinalis and *C. cyminum have* significant antimicrobial activities against both gram positive and negative bacteria. The research findings can be used to development of new plant-based drugs which has fewer side effects as well as highly effective for infectious diseases. As a result, further pharmacological and clinical research is needed to better understand the mode of action of medicinal plants and microbes, as well as the efficiency of herbal drugs in treating specific bacterial infections.

Many plant-derived medications are undergoing clinical trials, and at least 100 compounds are in the preclinical stage of research. Natural products are used to develop drugs for cancer and infections, which are the two most common treatment fields.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Protein-ligand docking study for the identification of plant-based ligands and their binding sites against Alzheimer's disease.

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Abstract

The presence of ApoE4 gene and the accumulation of beta-amyloid protein (Aβ) plaques in the brain have been identified to cause Alzheimer's disease (AD). Current drugs used for AD primarily focuses only on managing the symptoms with studies targeting amyloid formation being unsuccessful or causing severe side effects. Using plant-based compounds that are the blood-brain-barrier permeable and suppress AD-causing receptors may provide a better alternative for AD treatment with reduced side effects. In this research, existing FDA-approved drugs were used as standard. Fourteen ligands from various plant sources were retrieved from NCBI PubChem. The receptors AChE, Aβ and ApoE4 (PDB ID:4EY7, 5OQV, 1B68 respectively), were retrieved from RCSB PDB. ADMET properties of the ligands were examined using SwissADME. Docking was performed using AutoDock 4.2.6. The best pose against Aβ was observed in madecassic acid, with a binding energy of -7.77kcal/mol and Ki value of 2.0μM, for blind docking and –7.09kcal/mol and Ki of 6.37μM for site-specific. The best docked pose against ApoE4 was Asiatic acid, -6.08kcal/mol and 34.82μM, for blind docking and naringenin, -4.90kcal/mol and 256.12μM, for site-specific. The most common active residues in Aβ were GLU22, ILE31, LEU17, PHE19 and PHE20, while the common active residues in ApoE4 were ARG38, ARG142, and ARG145. In conclusion, the plant-based ligands showed potential inhibition against receptors affecting AD. However, in-vitro and clinical studies will be necessary to further determine their efficiency against AD.

Keywords: Alzheimer's disease, docking, neuroprotection, β-amyloid protein, ApoE4

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disorder that affects neuron responsible for memory and cognitive skills. One of the main types of dementia, AD is characterized by behavioural changes, memory loss, and difficulty in rational thinking.[1] Over 50 million people worldwide are affected by AD with most cases being in developing countries. With the increasing number of cases every day, it is expected to increase triple the current prevalence rate by the year 2050.[2] According to the WHO report 2020, the mortality rate of AD has shown an increase worldwide, with 5.98% of total deaths in Sri Lanka.[3] Shrinking of brain is a normal process as an individual ages and does not cause any severe damage to the neurons. However, in an individual with AD, the neurons are damaged causing loss of function and cell death. AD initially affects the entorhinal cortex and hippocampus regions, gradually affects the cerebral cortex and other regions of the brain, resulting in difficulty of functioning independently.[4] As AD can be caused due to genetic and environmental factors, it is known as multifactorial disease. Some cases of AD can be linked to family history while others sporadic. Although the cause of sporadic AD is still unclear, there are a few hypotheses, as shown in

figure 1, that has been recognized as possible causes of sporadic AD.[1]

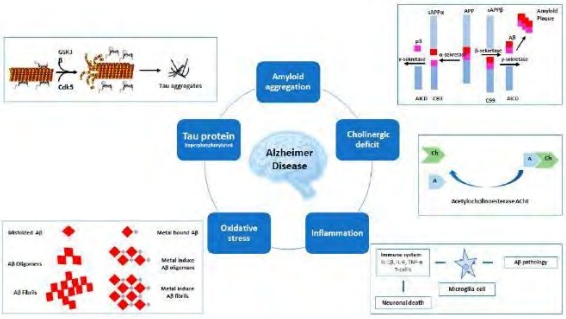

Figure 1. Alzheimer's disease hypothesis.[1]

Studies have shown that despite symptoms, such as deterioration in cognitive functions, appear at the later stage of AD, the damage to neuron begins decades before the clinical symptoms occur.[5] The pathology of AD mainly entails the abnormal build-up of beta amyloid (Aβ) plaques (amyloid hypothesis) and formation of neurofibrillary tangles (tau hypothesis) around brain cells.[5]

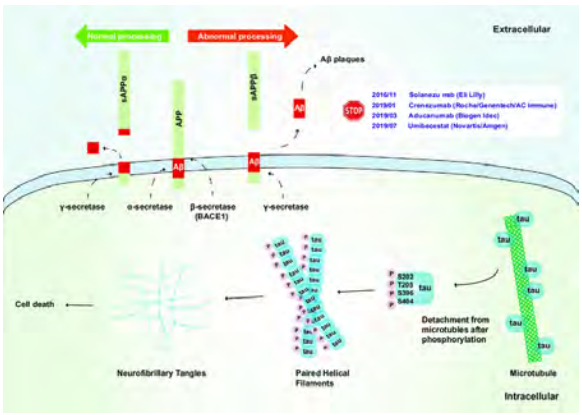

Figure 2. Diagram representation of Amyloid hypothesis and Tau hypothesis.[6]

The amyloid plaque is mainly comprised of the beta amyloid (Aβ) protein, formed by the protease cleavage of amyloid precursor protein (APP). Despite various studies conducted regarding APP, its physiological function is still uncertain.[7] The pathways of APP cleavage can be categorized as amyloidogenic and non-amyloidogenic, as shown in figure 3. During abnormal cleavage (amyloidogenic), APP is sequentially cleaved by β secretase (BACE1) and by γ secretase complex, forming insoluble Aβ.[6,7]

Among the limited AD studies conducted in Sri Lanka, increased amount of plasma homocysteine (tHcy) and Apolipoprotein E4 (ApoE4) gene were identified in individuals with AD.[8]

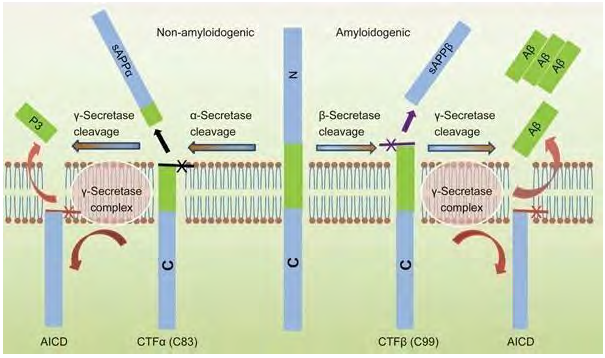

Figure 3. Diagram representation of non-amyloidogenic and amyloidogenic pathways.[9]

There are no available drugs that can cure or reverse the neuronal destruction responsible for AD. Failure to target all the elements involved in the Aβ cascade may also be a reason for the unsuccessful therapeutic trials.[10] The Food and Drug Administration (FDA) approved drugs, including donepezil, rivastigmine, galantamine and memantine, is used to treat cognitive symptoms in the early stages of AD.[11] While donepezil, galantamine and rivastigmine focuses on inhibiting acetylcholinesterase (AChE), memantine decreases the effect of NMDAR.[1] The recent FDA approved drug, through accelerated approved pathway, are aducanumab, which aims at removing amyloid plaques, and leqembi. However, there are still uncertainties regarding its clinical advantages due to adverse side effects.[12,13,14]

Protein-ligand docking is a computational method which focuses on predicting the best binding site of a ligand to the target receptor.[15,16] The procedure involved in

docking (figure 4.) is comprised of four major steps, selection of protein receptor and ligand, preparation of receptor and ligand, docking and analysis. However, additional steps may be required depending on the type of protein and ligand used. The results obtained from docking are then confirmed by experiments and can then be used in developing novel drugs.[17]

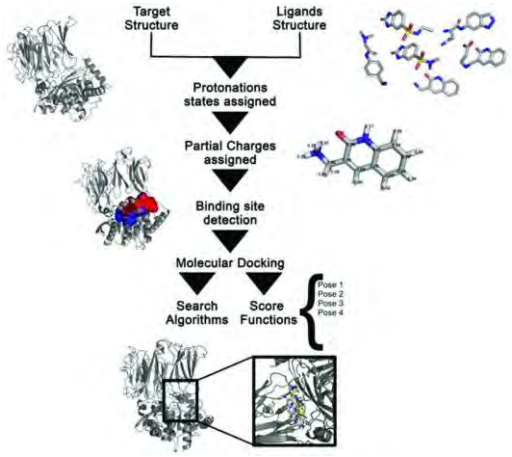

Figure 4. Steps involved in docking.[18]

The two types of protein-ligands docking techniques are blind and site-specific docking. Site specific docking is used for receptors with known binding sites, while blind docking is used when there is no prior knowledge on the active and binding sites of the receptor.[18]

Studies have shown that the presence of Aβ peptide play a major role in the reduction of hippocampal synaptic density and neuronal function during the development of AD.[19,20] Accumulation of Aβ monomers form oligomeric aggregates, which in turn forms short, protofibril aggregates. The protofibril aggregates then extend into insoluble β fibrils.[21] Numerous studies have been conducted to characterize the stability of the formed Aβ aggregates as it is associated with the severity of the disease.

Most studies that focused on reducing the formation of Aβ aggregates have shown inefficiency in terms of passing through the blood brain barrier (BBB) and severe side effects.[25]

Table 1. Structures of receptors used for docking.

Receptor name	PDB ID	Structure	
Acetylcholinesterase (AChE)	4EY7		(22)
Amyloid beta peptide (1-42) fibril	5OQV		(23)
Apolipoprotein E4 (ApoE4)	1B68		(24)

Table 2. FDA approved and clinical phase drugs.

Drug	PubChem CID	Status	Detail	
Donepezil	3152	FDA approved	Cholinesterase inhibitor	(26)
Rivastigmine	77991	FDA approved	Cholinesterase inhibitor	(26)
Galantamine	9651	FDA approved	Cholinesterase inhibitor	(27)
Amantadine	2130	FDA approved	Treatment in early stage of Parkinson's disease	(28)
Azeliragon	11180124	FDA orphan drug designation	Treatment for glioblastoma. Decrease the transport of Aβ intothe brain. Failed in phase 3 clinical trial for AD	(29)

AutoDock tool is a free, open-source, molecular docking software which can be used for protein-ligand docking with high accuracy. UCSF Chimera is another advanced software for docking and visualization of molecular

structures.[40,41] In addition to AutoDock tools, visualization software such as Biovia, and LigPlot are useful to analyse and obtain the structures of protein complex.[42,43]

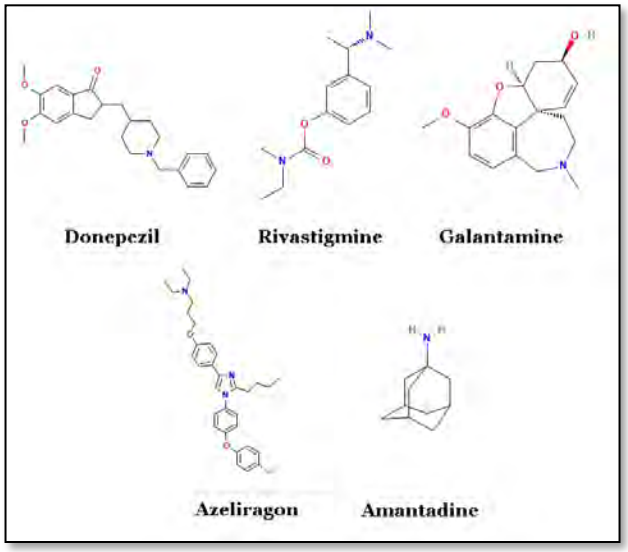

Figure 5. Structure of drugs obtained from PubChem.

Table 3. Phytochemical ligands and their plant sources.

Plant source	Phytochemical/ compound	PubChem CID	
Coffee arabica	Caffeine	2519	(29)
Curcuma longa	Curcumin	969516	(30)
Centella asiatica	Asiatic acid	119034	(31)
	Madecassic acid	73412	(31)
Cinnamomum verum	Cinnamaldehyde	637511	(32)
	Proanthocyanidin	108065	(32)
Origanum vulgare	Rosmarinic acid	5281792	(33)
Mentha piperita	Caffeic acid	689043	(34)
	Urolithin A (Ellagic acid)	5488186	(35)
Punica granatum	Kuromanin	441667	(36)
	Punicic acid	5281126	(37)
Citrus sinensis	Hesperidin	10621	(38)
	Naringenin	932	(38)
Cymbopogon citratus	Citral	638011	(39)

Validation of protein structures can be obtained using Ramachandran plots.[44] The docking protocol can be validated by redocking co-crystallized ligands of a receptor and superimposing them on the structure obtained from PDB, where a Root Mean Square Deviation (RMSD) value lower than 1.5 Å indicate a good protocol.[45]

AD has become very challenging for those who are affected, as well as their caregivers, as it hinders their day- to-day function. Moreover, considering the increasing rate of AD cases, understanding the cause, and identifying treatment methods that can inhibit AD-causing receptors has become essential. Studying protein-ligand interaction paves way in discovering new treatment methods that can prevent or cure AD at an early stage. Therefore, selecting plant-based compounds with minimal side effects and the potential to inhibit multiple pathological elements of AD would favour in developing effective, multi-target treatment method.

2. Materials

Table 4. Materials required.

Hardware	Software	Websites/ other	Samples
Windows 11	AutoDock 4.2.6	NCBI PubChem	**Receptors (PDB ID)**
4 GB RAM	MGL tools 1.5.6	RCSB PDB	4EY7 (standard)
64-bit operating system	Python 3.10.0	ADMET Lab 2.0	5OQV

Intel core i3 processor	Open Babel GUI 2.4.1	CASTp	1B68
	BIOVIA Discovery studio (DS) 21.1.0	SwissADME	**FDA-approved drugs (table 2)**
	UCSF Chimera 1.15	Clustal Omega	**Plant-based ligands (table 3)**
	LigPlot 2.2.4	Reliable internet connection	

2.1 Methodology Familiarization of software. Understanding and familiarization of the software was achieved from various literatures and user guide manuals.

2.2 Installation of software. The following software were installed using the version compatible with Windows 11, 64-bit operating system, Intel core i3 processor, and 4 GB RAM.

Table 5. Software installed and their corresponding links.

Software	Download link	
AutoDock 4.2.6	https://autodock.scripps.edu/download-autodock4/	(41)
UCSF Chimera 1.15	https://www.cgl.ucsf.edu/chimera/download.html	(46)
Python 3.10.0	https://www.python.org/downloads/	(47)
MGL tools1.5.6	https://ccsb.scripps.edu/mgltools/	(48)
Open Babel GUI 2.4.1	https://sourceforge.net/projects/openbabel/files/openbabel/2.3.1/	(22)
BIOVIA Discovery studio 21.1.0	https://discover.3ds.com/discovery-studio-visualizer-download	(22)
LigPlot 2.2.4	https://www.ebi.ac.uk/thornton-srv/software/LigPlus/download.html	(49, 50)

2.3 Selection and preparation of receptors and ligand. The 3D structures of AChE (PDB ID: 4EY7), Aβ42 fibril (PDB ID:5OQV) and ApoE4 (PDB ID:1B68) were acquired from RCSB PDB in PDB format. Multiple sequence alignment of all 9 chains of 5OQV was performed using Clustal Omega (Figure 5). (51) Based on the output only one chain of 5OQV was used for docking.

The 3D structures of the FDA approved drugs and phytochemical ligands were obtained

from NCBI PubChem in SDF format. The ligands were converted from SDF to PDBQT format using Open Babel GUI.

Figure 5. Multiple sequence alignment of 5OQV using Clustal Omega.

2.4 ADMET property analysis for phytochemicals. The absorption, distribution, metabolism, excretion, and toxicity (ADMET) property of each ligand was examined using SwissADME. (52) Using the SMILES ligand parameters, such as pharmacokinetics, drug-

likeness, water solubility and lipophilicity, was studied.

2.5 Optimization of the receptors for blind docking using AutoDock 4.2.6. The preparation of 4EY7 involved the removal of water molecules, ligands and the chains C and D. Similarly, water molecules were removed from 1B68 while 5OQV had no attached ligands or water molecules. Polar hydrogens and Kollman charges were added to the receptor and AD4 type were assigned. The receptor was then saved as PDBQT format.

2.6 Optimization of receptors for site-specific docking using AutoDock 4.2.6. The removal of water molecules and ligands, as well as the addition of polar hydrogens and Kollman charges were completed following similar steps as in blind docking with AutoDock 4.2.6. Once the Kollman charges were added, total charges on residue were checked and charge deficit was spread. Ligand file was opened in PDBQT format and Gasteiger charges were added automatically.

2.7 Blind docking using AutoDock 4.2.6. The ligands were opened in PDBQT format. Torsion tree was chosen and detected, and the ligand output was saved again as PDBQT. Grid parameters were set to cover the whole receptor and saved (table 6). The grid output was saved as a grid parameter file (GPF).

The docking step involved opening the receptor and ligand file and setting the genetic algorithm (GA) for 300 populations and 50 GA runs. The docking output was then set as Lamarckian GA and saved as docking parameter file (DPF). Autogrid and Autodock were then executed, creating GLG and DLG files respectively.

Table 6. Grid parameters for blind docking using AutoDock 4.2.6.

Receptors (PDB ID)	Centre gridbox (Points in X, Y, Z axis)	Size (Points in X, Y, Z axis)	Spacing
4EY7	-2.563 x -50.463 x 5.015	70 x 90 x 126	0.939
5OQV	38.660 x 58.104 x 52.217	82 x 94 x 34	0.500
1B68	7.830 x 15.603 x 22.838	78 x 64 x 88	0.608

2.8 Site-specific docking using AutoDock 4.2.6. To set the grid parameters, macromolecule was chosen, saved as PDBQT format, and the ligand was then chosen. The grid parameters (table 7) were set by calculating the average of binding site value, which was obtained from CASTp for

4EY7 (Figure 6), and literatures for 1B68 and 5OQV.[53] The grid output was then saved as GPF. Autogrid was then executed.

Figure 6. Binding sites of 4EY7 was obtained from CASTp.

To initiate docking, macromolecule was selected as PDBQT, and the ligand was then chosen. The GA was set for 50 GA runs and 300 populations. Output was selected as Lamarckian GA, saved as GPF, and Autodock was executed. AutoDock 4.2.6 was then closed and reopened for analysis.

Table 7. Grid parameters for site-specific docking using AutoDock 4.2.6.

Receptors (PDB ID)	Centre gridbox (Points in X, Y, Z axis)	Size (Points in X, Y, Z axis)	Spacing
4EY7	-8.967 x -44.925 x 31.054	60 x 60 x 60	0.375
5OQV	38.660 x 58.104 x 52.217	60 x 60 x 60	0.375
1B68	3.722 x 20.734 x 35.887	60 x 60 x 60	0.375

2.8 Visualization

2.8.1 AutoDock 4.2.6. The best docking pose of blind and site-specific docking were obtained using AutoDock 4.2.6. The receptor file and DLG file of the relevant docking was opened in AutoDock 4.2.6 and 3D conformation was observed. The docked ligand-receptor file (complex file) was generated in PDBQT format, by clicking on 'Write complex'.

2.8.2 Chimera. 3D structures of docked molecules were visualized using Chimera. The docked file was opened in PDBQT format and structure was examined.

2.9 Interactions. DLG file was opened in AutoDock 4.2.6 and the amino acid interactions, H-bonds of the best docking pose were identified for blind and site-specific docking of both AutoDock 4.2.6. Interactions were obtained from BIOVIA DS and LigPlot.

2.9.1 BIOVIA DS. The PDBQT format of output and complex file was opened in BIOVIA DS, and the docked 3D and 2D structures, with interactions, were viewed.

2.9.2 LigPlot. The complex files were converted from PDBQT to PDB using Open Babel GUI. The complex file, in PDB format, was opened in LigPlot and the H-bonds and hydrophobic interactions of docked molecules were examined.

2.10 Analysis. RMSD table was obtained from DLG file to identify the best docking pose for AutoDock 4.2.6. The inhibition constant (Ki) was interpreted from the conformation of the docked molecule by clicking on show information. Additionally, the Ki was also obtained from the DLG file.

2.11 Validation. The protein structures of receptors were validated using Ramachandran plots in SAVES v6.0 and BIOVIA DS. The file was opened, in BIOVIA DS, in PDBQT format for docked file and in PDB format for the receptor file. Ramachandran plot was retrieved for the receptor protein structure and the docked structure.

Docking protocol was validated by redocking co-crystallized structure with the receptor. E20 is co-crystallized in the structure 4EY7. All ligands, and chains C and D were removed from 4EY7 receptor. E20 was redocked with 4EY7 using AutoDock 4.2.6. Using BIOVIA DS, the receptor from RCSB PDB and the redocked complex were superimposed, and RMSD value was obtained.

3. Results

3.1 ADMET property analysis for phytochemicals: Factors that are used in determining the pharmacokinetic property of a drug includes the principles of Lipinski, Ghose, and Veber. According to Lipinski, a good drug should not contain more than one violation of these factors.[54] Additionally, a ligand with good drug-likeness and pharmacokinetic properties is determined based on a Topological Polar Surface Area (TPSA) >140 Å2, higher drug absorption indicated by lower C logP, GI absorption and blood-brain-barrier permeability.[55,56,57]

Table 8. Determination of ADME properties of the phytochemical ligand

Ligand	C log P	Solubility (Silicos)	MW (g/mol)	TPSA (Å²)	Lipinski's rule	Bioavailability	GI	BBB
Asiatic acid	4.41	-4.28	488.70	97.99	✓	0.56	High	×
Caffeic acid	0.93	-0.71	180.16	77.76	✓	0.56	High	×
Caffeine	0.08	-0.67	194.19	61.82	✓	0.55	High	×
Cinnamaldehyde	1.97	-2.40	132.16	17.07	✓	0.55	High	✓
Citral	2.71	-1.96	152.23	17.07	✓	0.55	High	✓
Curcumin	3.03	-4.45	368.38	93.06	✓	0.55	High	×
Hesperidin	-0.72	-0.58	610.56	234.29	×	0.17	Low	×
Kuromanin	-1.28	-0.93	449.38	193.44	×	0.17	Low	×
Madecassic acid	3.59	-3.45	504.70	118.22	✓	0.56	High	×
Naringenin	1.84	-3.42	272.25	86.99	✓	0.55	High	×
Proanthocyanidin	1.85	-4.60	592.55	209.76	×	0.17	Low	×
Punicic acid	5.26	-3.96	278.43	37.30	✓	0.85	High	✓
Rosmarinic acid	1.52	-2.17	360.31	144.52	✓	0.56	Low	×
Urolithin A	2.06	-4.14	228.20	70.67	✓	0.55	High	✓

As shown in table 8, hesperidin had the highest TPSA of 234.29 Å² while cinnamaldehyde and citral had the lowest TPSA of 17.07 Å². The lowest lipophilicity C logP was observed for Kuromanin and the highest was observed in Punicic acid. Among the 14 phytochemicals, cinnamaldehyde, citral, punicic acid and urolithin A, were permeable through the BBB and ten of the phytochemicals had high GI absorption.

3.2 Docking parameter analysis.

Table 9. FDA approved and clinical trial drugs- Results for blind docking using AutoDock 4.2.6

Receptors	Ligands	Binding energy (kcal/mol)	Ki (µM)
4EY7	Donepezil	-8.95	274.30×10^{-3}
	Rivastigmine	-6.02	38.67
	Galantamine	-8.24	906.15×10^{-3}
	Amantadine	-5.57	83.18
	Azeliragon	-6.16	30.30
5OQV	Donepezil	-6.81	10.17
	Rivastigmine	-4.91	250.57
	Galantamine	-6.20	28.64
	Amantadine	-4.54	467.2
	Azeliragon	-6.02	38.53
1B68	Donepezil	-6.01	39.35

The effective binding efficiency is determined by the lowest binding energy. Lower Ki indicates increased binding affinity, which means less amount of drug will be required to inhibit the receptor. The efficient binding energy for both receptors, as shown in table 9, were observed with donepezil. The binding energy was slightly higher than that of the standard 4EY7, indicating lower binding efficiency.

Among the 14 phytochemical ligands blind docked against 5OQV, eight had binding energy ranging between -5.0 to -8.0kcal/mol, while only three phytochemicals had a binding energy between -5.0 to -8.0kcal/mol when blind docked against 1B68. The ligand with effective binding energy and Ki against 5OQV and 1B68 (Table 10) were madecassic acid and Asiatic acid respectively.

Table 10. Phytochemicals- Blind docking using AutoDock 4.2.6

Receptors	Ligands	Binding energy (kcal/mol)	Ki (µM)
5OQV	Asiatic acid	-6.41	19.86
	Caffeic acid	-4.26	752.66
	Caffeine	-4.57	450.61
	Cinnamaldehyde	-3.96	1.26×10^{3}

	Citral	-4.15	906.07
	Curcumin	-5.69	67.11
	Hesperidin	-7.17	5.53
	Kuromanin	-5.83	53.01
	Madecassic acid	-7.77	2.00
	Naringenin	-5.26	140.42
	Proanthocyanidin	-5.30	129.43
	Punicic acid	-3.76	$1.75x10^3$
	Rosmarinic acid	-4.94	240.25
	Urolithin A	-5.17	162.47
	Asiatic acid	-6.08	34.82
	Caffeic acid	-4.26	757.68
	Caffeine	-3.93	1.32x103
	Cinnamaldehyde	-4.17	870.51
	Citral	-4.13	942.21
	Curcumin	-5.61	77.75
	Hesperidin	-2.60	12.50x103
	Kuromanin	-3.43	3.07x103
	Madecassic acid	-5.90	47.26
	Naringenin	-4.81	299.00
	Proanthocyanidin	-3.68	1.99x103
	Punicic acid	-4.38	620.07
	Rosmarinic acid	-2.86	7.94x103
	Urolithin A	-4.84	281.63

Table 11. FDA approved and clinical trial drugs- Site- specific docking using AutoDock 4.2.6

4EY7	Donepezil	-9.86	$59.46x10^{-3}$
	Rivastigmine	-7.98	1.41
	Galantamine	-9.28	$156.71x10^{-3}$
	Amantadine	-6.51	17.01
	Azeliragon	-10.11	$39.01x10^{-3}$
5OQV	Donepezil	-6.46	18.45
	Rivastigmine	-4.46	536.45
	Galantamine	-5.32	125.30
	Amantadine	-4.19	844.81
	Azeliragon	-5.97	41.87
1B68	Donepezil	-4.92	246.35
	Rivastigmine	-4.14	916.85
	Galantamine	-5.55	85.05
	Amantadine	-4.61	418.12
	Azeliragon	-3.56	$2.44x10^3$

Table 12. Phytochemicals- Site-specific docking using AutoDock 4.2.6

Receptors	**Ligands**	**Binding energy (kcal/mol)**	**Ki (µM)**
5OQV	Asiatic acid	-6.71	12.07
	Caffeic acid	-4.47	530.8
	Caffeine	-4.07	$1.04x10^3$
	Cinnamaldehyde	-3.83	$1.55x10^3$
	Citral	-3.58	$2.39x10^3$
	Curcumin	-5.40	110.65
	Hesperidin	-5.16	163.77

	Kuromanin	-4.72	345.31
	Madecassic acid	-7.09	6.37
	Naringenin	-5.13	173.08
	Proanthocyanidin	-4.27	746.18
	Punicic acid	-3.38	$3.36x10^3$
	Rosmarinic acid	-4.59	434.4
	Urolithin A	-4.97	227.82
1B68	Asiatic acid	-4.36	641.12
	Caffeic acid	-3.82	$1.59x10^3$
	Caffeine	-3.68	$2.01x10^3$
	Cinnamaldehyde	-4.25	764.97
	Citral	-4.63	402.28
	Curcumin	-4.48	518.71
	Hesperidin	-2.46	$15.64x10^3$
	Kuromanin	-1.99	$34.56x10^3$
	Madecassic acid	-4.24	775.01
	Naringenin	-4.90	256.12
	Proanthocyanidin	-1.43	$89.66x10^3$
	Punicic acid	-3.44	$3.0\ x10^3$
	Rosmarinic acid	-2.96	$6.74x10^3$
	Urolithin A	-4.66	386.96

The most effective ligands against 5OQV and 1B68, using site-specific docking (Table 12), were madecassic acid and naringenin respectively.

3.3 Best docking Poses

3.3.1 Blind docking using AutoDock 4.2.6

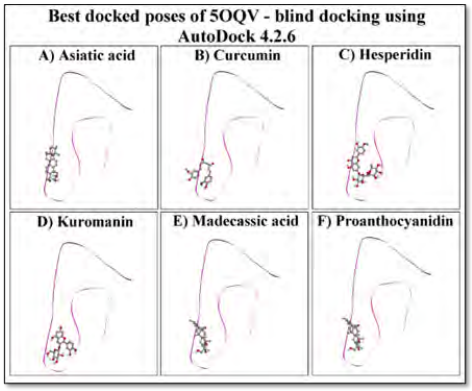

Figure 7. 5OQV - blind docking with AutoDock 4.2.6

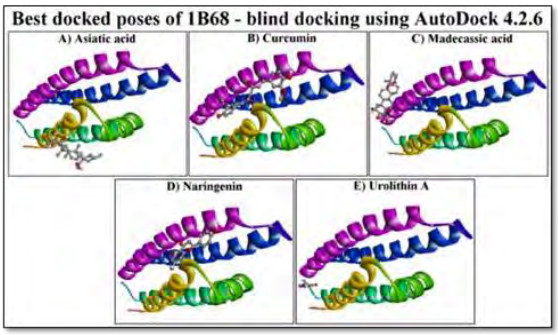

Figure 8. 1B68- blind docking with AutoDock 4.2.6

3.3.2 Site-specific docking using AutoDock 4.2.6

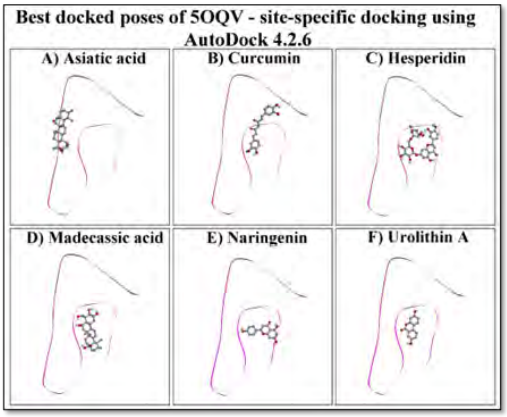

Figure 9. 5OQV- site-specific docking with AutoDock 4.2.6

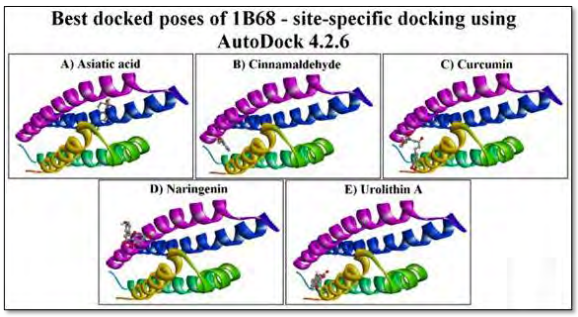

Figure 10. 1B68- site-specific docking with AutoDock 4.2.6

3.4 Interactions

3.4.1 Blind docking-AutoDock 4.2.6

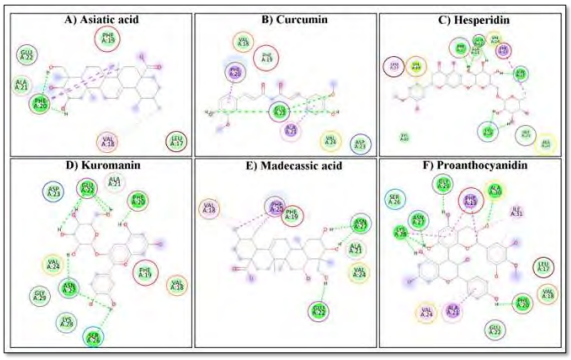

Figure 11. Interactions of ligands docked against 5OQV- blind docking (4.2.6)

ALA21, ASN27, ASP23, PHE19, PHE20, GLU22, GLY29, LEU17, LYS28, SER26, VAL18, VAL24 were common active amino acid residues identified in 5OQV with respect to h-bond.

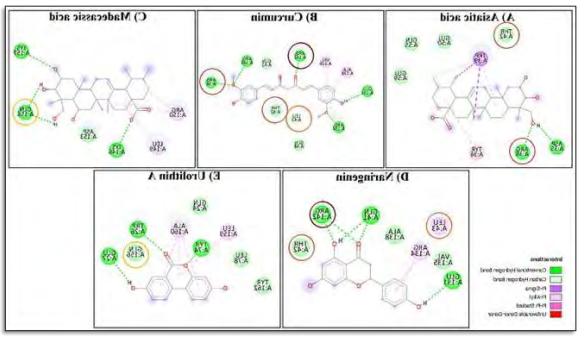

Figure 12. Interactions of ligands docked against 1B68- blind docking (4.2.6)

ARG48, ARG142and GLN156 were identified as the common active amino acid residues in 1B68 (figure 12), with respect to h-bond, while LEU43 and THR42 were identified as the common hydrophobic interactions.

3.4.2 Site-specific docking- AutoDock 4.2.6

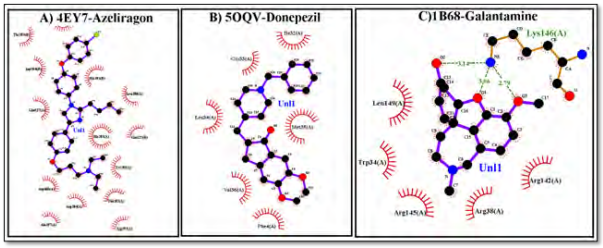

Figure 13. Interactions in FDA-approved and clinical trial drugs

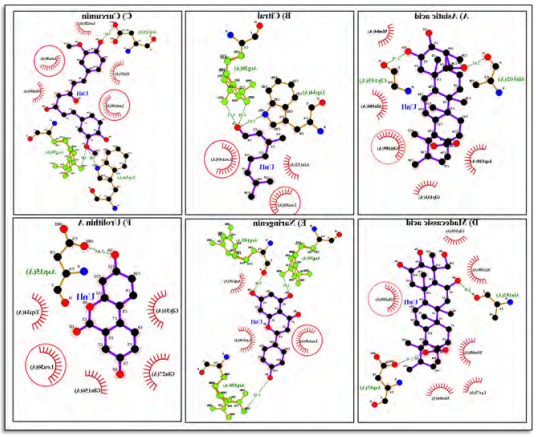

Figure 14. Interactions of ligands docked against 5OQV- site specific docking.

GLY33, GLY38, ILE31, ILE32, LEU17, LEU34, MET35 and VAL40 were identified as the common active amino acid residues in 5OQV (figure 14).

ARG38 was identified as the common amino acid residue in 1B68 (figure 15) in terms of h-bond, and GLU109, GLY31, LEU30 and LEU49 were identified as the common hydrophobic interactions.

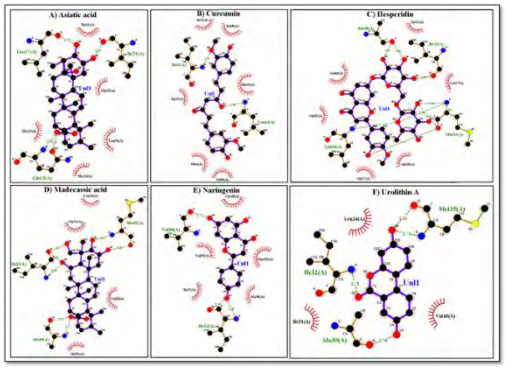

Figure 15. Interactions of phytochemicals docked against 1B68- site specific docking.

3.5 Validation

3.5.1 Redocking

Table 13. Binding energy of 4EY7 redocked with co- crystallized ligand using AutoDock 4.2.6

Receptor	Ligand	AutoDock 4.2.6	Binding energy (kcal/mol)
4EY7	E20	Blind docking	-9.15
		Site-specific docking	-9.18

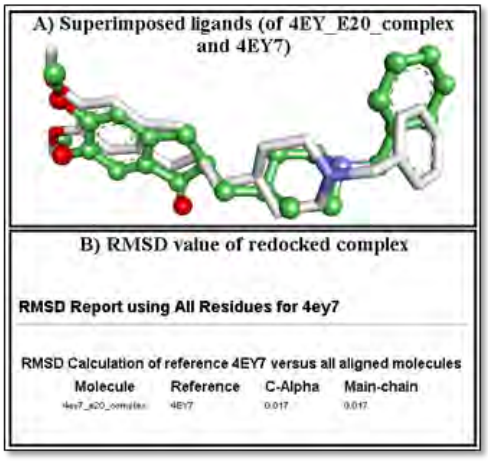

Figure 16. RMSD value for redocking and superimposition

RMSD value obtained for the superimposition of 4EY7 and 4EY7-E20 complex was 0.17, hence the docking protocol used is validated.

3.5.2 Ramachandran plot

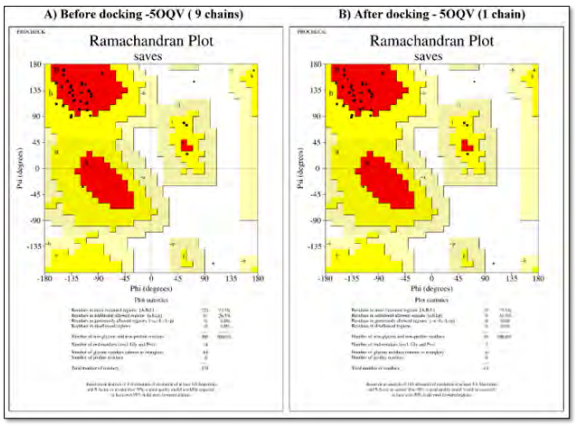

Figure 17. Ramachandran plot for 5OQV

Residues in the favoured region (indicated in red) was 73.5% and had not changed before and after (figure 17A and B) docking. This implies that there was no significant structural change after docking.

4. Discussion

Phytochemicals and macromolecules in nature are comprised of charges which are not present in the SDF and PDB structures. Therefore, addition of Kollman and gasteiger partial charges, for receptors and ligands respectively, is essential to stabilise the structures and accurate calculation.[58] Polar hydrogens were added to facilitate binding between the ligand and receptor. Torsion determines the central atom in the ligand, number of rotatable atoms and the number of active bonds.[59]

Inhibition of AD-receptors requires selection of phytochemical ligands with anti-inflammatory, antioxidant, and anti-amyloid properties. Phenolic phytochemicals such as curcumin, naringenin, hesperidin and rosmarinic acids, have been shown to contain these properties.[60,61,62]

The changes in conformation that occurs once the ligand binds with the receptor is calculated using the Root Mean Square Deviation (RMSD), where the structure of the docked receptor is compared with a reference

conformation.[63] The site-specific binding energy (table 7) was higher than what was observed in blind docking (table 9). This difference could be due to site selected for specific binding is not the binding position for that specific ligand. Determination of binding energy or affinity is based on h-bonds, hydrophobic interactions, and Van der Waal forces.[64,65]

The binding affinity obtained in a previous study for donepezil and rivastigmine against 4EY7 were -9.8 and -7.1 kcal/mol.[22] Based on the outputs of site-specific docking, as mentioned in Table 7, donepezil and rivastigmine showed higher binding affinity than that obtained from the previous study.

Amantadine is a drug that has been used to treat symptoms of Parkinsons's disease.[28] The ligand amantadine showed favourable binding efficiency against receptors 4EY7 and 1B68, indicating potential drug repurposing of Parkinson's drug for Alzheimer's disease.[66]

Ellagic acid, which is found in abundance in fruits like pomegranates, has poor absorption in human and requires the involvement of gut microbes to further metabolize it. Urolithin A is a gut-microbial polyphenol metabolite of ellagic acid that has shown to have anti-inflammation properties in the brain of AD mice model.[35,67]

Despite punicic acid's antioxidant and anti-inflammatory properties, its poor drug-likeness and ADMET properties makes it unsuitable to be used for treatment. This can be overcome through enhancing punicic acid's oral bioavailability by encapsulating Triacylglycerol-bound punicic acid with its regioisomers (TPAR) within liposomes and as a result improving its liposomal formulation.[68]

Table 14. Amino acid residues interacting in 5OQV and 1B68 from previous research.

Previous studies

Receptors	Amino acid interactions	
5OQV	PHE19, ILE31, ALA30, GLY29, LYS28, ASN27	(69)
1B68	ARG33, GLU37, GLU45, GLY49, TRP52, LEU32, LEU48, LEU167	(70)

Table 14 summarizes amino acid residues obtained for the receptors 5OQV and 1B68 from previous studies for different disease. Although there were no common amino acids with 1B68, amino acid residues of 5OQV obtained for the previous study were similar to the residues obtained in this research.

A bioavailability score determines a drug's potential in being administered orally. Ten of the phytochemicals had good pharmacokinetic property based on their bioavailability score of 0.55 or 0.56. TPSA and the number of rotatable bonds also play a role in bioavailability.[54] SwissADME evaluates the TPSA of a ligand using the polar atoms Sulphur and Phosphorous.[52] Lipinski's rule alone cannot be used to determine the drugability of a compound as the rule does not take substrate for transporters into consideration and most compounds act as a substrate for some transporter.[71]

The phytochemicals' ability to pass through BBB are still unclear, however various studies with rodents have shown permeation of phytochemicals through BBB have been achieved.[61] Although hesperidin is not BBB permeable and violates Lipinski's rule, studies have indicated that in addition to hesperidin's inhibitory properties, it can facilitate BBB permeation.[72] Similarly, despite Asiatic acid and Madecassic acid were shown to have no BBB permeation, research conducted to study the BBB permeability of compounds of Centella asiatica

showed high BBB permeation in both Asiatic acid and madecassic acid.[73]

Conclusion

Asiatic acid, madecassic acid and urolithin A were identified to have high binding efficiency with both 5OQV and 1B68. Additionally, hesperidin, kuromanin and proanthocyanidin displayed efficient binding with 5OQV while, hesperidin, kuromanin and naringenin displayed efficient binding with 1B68. Phenylalanine was the most common amino acid residue identified in 5OQV while arginine was the most common amino acid interaction found in 1B68. Based on the obtained outputs, ligands with efficient binding can be chosen to dock with AChE and BACE receptors as well. Further study can be conducted to dock multiple ligands to a single receptor, using AutoDock 4.2.6 or AutoDock vina, which can be potentially used in the future development of combinational treatment method against AD.

Acknowledgement

The authors like to thank BMS (Business Management School) for providing a platform to conduct the research.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Green synthesis of silver nanoparticles using *Oryza sativa* leaves extract and analysis of antioxidant property

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Abstract

Oxidative stress by free radicals can damage the intracellular compartments leading to a large variety of diseases. A therapy for these diseases can be identified by preventing oxidative stress through antioxidants. Plant mediated nanomaterial synthesis is a revolutionary method for extracting natural antioxidants due to the eco- friendly nature and cost-effectiveness. Silver (Ag) plays a vital role in formation of nanoparticles from plant extracts. In this study, the silver nanoparticles (AgNPs) were synthesized through water extracts of five varieties of Sri Lankan traditional *Oryza sativa* leaves include in 'Paduwas Siwuru', 'Herath Banda', 'Madathawalu', 'Mutu Samba', and 'Murungakayen'. This method allows AgNPs formation following an optimization process, which was carried out for 15, 30, 45, and 60 minutes at 60 °C and 90 °C. The formation of AgNPs was indicated by the color change of the water extracts and further characterized using a UV-vis spectrophotometer. It was further confirmed by determining the morphology analysis of AgNPs using a SEM. The observed AgNPs were spherical shaped, and the size were around 40 nm. The band gap energy was measured to confirm the nanoparticles were semiconductors. The AgNP samples were subjected to the characterization of antioxidant properties. Thus, Total Antioxidant Capacity (TAC), Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) assays were performed. It was demonstrated a higher significant for TPC and TAC and lower significant for TFC over the respective water extracts. Comprehensively, the *Oryza sativa* leaves can be used as an effectual way to find the therapeutic approaches for free radical-induced diseases.

Key words: *Oryza sativa, s*ilver, nanoparticles, Antioxidant activity

Introduction

Atherosclerosis, cardiovascular diseases, inflammatory diseases, cancer, and aging are associated with the damaging of biological molecules such as DNA, proteins, lipids and changing their structural and functional features. This is mainly due to oxidative stress.[1] An imbalance between the generation and scavenging of free radicals leads to oxidative stress. The free radicals bind with biological molecules in a healthy human cell by electron pairing when they exceed the scavenging capacity.[2] It results in lipid peroxidation, protein oxidation, and DNA fragmentation leading to multiple diseases. The studies have shown that experimenting with synthetic antioxidants exhibited an adverse effect on biomolecules.[3]

Antioxidants are stable molecules that can donate an electron to an excited free radical and inhibit free radical formation leading to reduce oxidative stress. Research on Vitamin E as a synthetic antioxidant for experimenting reproduction of mice obtained the ability to prevent lipid peroxidation by reducing oxidative stress.[4] There are a variety of synthetic antioxidants such as BHT, phenolic antioxidants, and BHA (Figure 1) which are used in the food, cosmetics, and medical industry. Due to the high-temperature instability and their carcinogenic nature at higher concentrations, studies have been shifted into finding natural antioxidants derived

from plant sources as an alternative to synthetic antioxidants. The strong natural antioxidants such as flavonoids, polyphenols, lignans, quercetin and curcumin (Figure 1) can be mainly found in berries, olives, cherries, and citrus.[5]

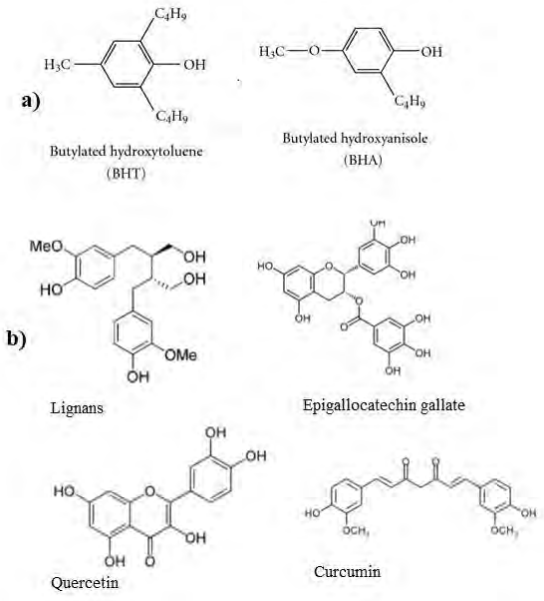

Figure 1. Aromatic structures of antioxidants. a) Synthetic antioxidants.[6] b) Natural antioxidants.[7]

Determination of natural antioxidants is commonly done by nanotechnology-based methods through synthesizing nanoparticles from plant materials. Nanoparticles contain nanoscale dimensions (1-100 nm) and a large surface area to volume ratio. Thus, they have enhanced catalytic reactivity, non-linear optical performance, thermal conductivity, and steadiness to chemicals.[8] There are top-down and bottom-up approaches for synthesizing metallic nanoparticles (Figure 2). The top- down approach involves in fractionation of large-scale particles into nanoparticles which is comparatively costly and time-consuming. Furthermore, the bottom-up approach is more advantageous since it produces nanostructures with fewer defects and homogenous chemical composition. Physical, chemical, and biological are the methods of bottom-up approach. Requirement of high temperature, energy, pressure, and the utilizing toxic chemicals suggested as the limitations for physical and biological methods. Therefore, the technology has shifted into eco-friendly methods. Considering these, biological methods are preferable over physical or chemical methods due to their eco-friendly, non-toxic, and inexpensive properties. The nanoparticles can be synthesized with controlled size, shape, and structure by controlling the reaction conditions including reducing agent, stabilizer, and different synthetic methods.[9]

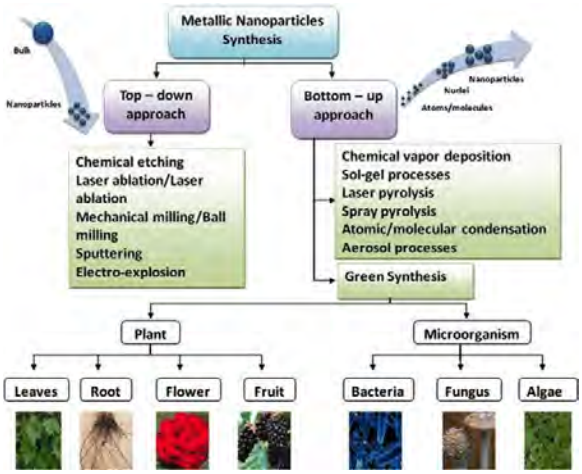

Figure 2. Synthesis approaches to the preparation of metal nanoparticles.[10]

Green synthesis is a plant or microorganisms-based production technique of biological methods. Microorganisms such as bacteria, fungi, and actinomycetes are used for the green synthesis but due to high maintenance and requirement of complex steps including sampling, isolating, and culturing plant-mediated synthesis has preferred.[11]

Generally, plant-mediated synthesis produces metal nanoparticles by the process of bioreduction. As Figure 3, secondary metabolites such as phenols, tannins, alkaloids, and minerals produced during the biological processes of the plant, results in the AgNPs synthesis.[12]

The metallic nanoparticle synthesis is explained by three steps such as activation phase, growth phase, and termination phase.[14] During the activation phase, the metal ions are reduced

into metal atoms by the secondary metabolites available and undergo nucleation. The resulting stabilized metal atoms and metabolite complex give rise to small nanoparticles which turn the separate smaller nanoparticles into larger particles through the coarsening process with increasing thermodynamic stability of nanoparticles during growth phase. This is carried out by Ostwald ripening where the nucleation and growth of nanoparticles and further metal ion reduction occurs. The shape of the synthesized nanoparticles is determined during the termination phase (Figure 4). At the termination phase, the nanoparticles gain a stronger and more energetically favourable consistency, and this process strongly influences the ability to stabilize metal nanoparticles by the plant extract.[10]

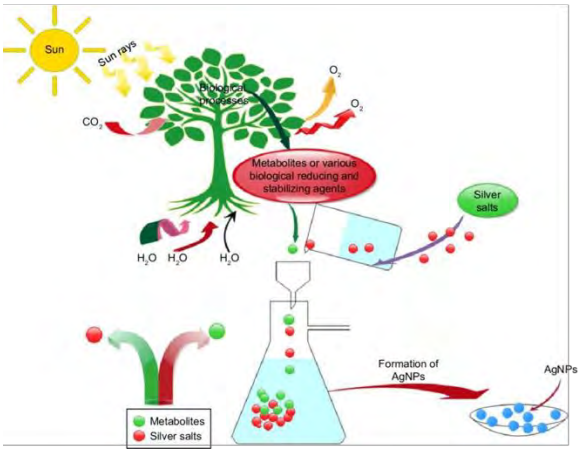

Figure 3. The process describes the formation of AgNP.[13]

For green synthesis method, a suitable plant source should be selected that is rich in metabolites. Therefore, for this study, the plant *O. sativa* was chosen, which is commonly known as rice. It is reported the seeds, husk, and bran of *O. sativa* are a rich source of natural antioxidants, but scientific information is limited for the leaves of Sri Lankan traditional *O. sativa* varieties. According to the studies of the Department of Agriculture Sri Lanka, there are over 2000 traditional varieties can be found in the country.

The use of *O. sativa* leaves is reported but for feeding, roof thatching, and broom straws, but and not for biological applications. A study of Adak *et al*., 2020 revealed rice leaves are consisting of high silica content and the AgNP synthesis was avoided earlier.[15] Antioxidants such as phenols, flavonoids, and phytosterols can be found in *O. sativa* leaves.[16] The leaves of the *O. sativa* can be found in many colors as purple rather than normal green color. According to Bianca *et al*., 2021 the reason for the color variation is due to the presence of an increased number of antioxidants.[17] In this study, pigmented Paduwas Siwuru leaves (purple genotype) (Figure 5) were introduced to synthesize AgNPs.

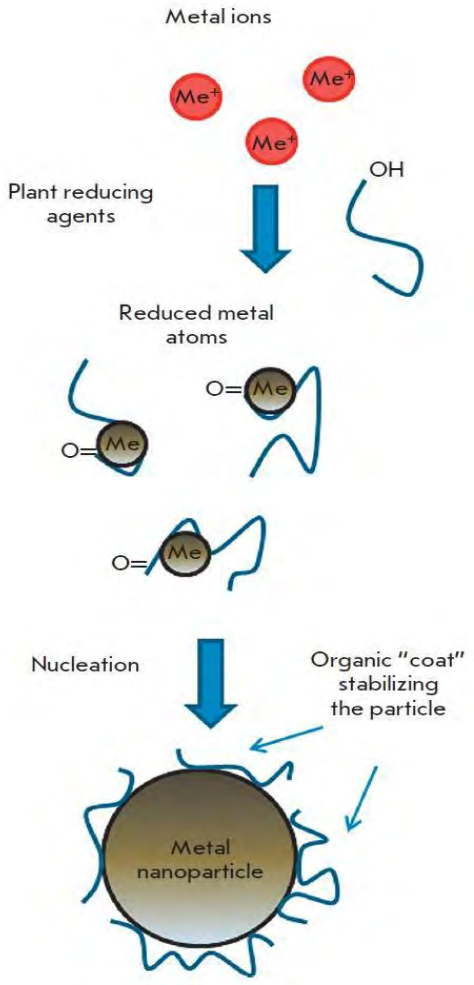

Figure 4. The metal nanoparticle synthesis in a plant extract.[14]

Figure 5. The field where Paduwas Siwuru variety was taken with purple pigmented leaves.

Therefore, this project concerns using leaves of five different Sri Lankan traditional rice varieties include in Paduwas Siwuru, Herath Banda, Madathawalu, Mutu Samba, and Murungakayen, for synthesizing AgNP using water extracts and discovering their antioxidant capacities. To determine antioxidant properties, assays such as TFC, TPC, and TAC and to characterize the synthesized AgNPs SEM analysis will be performed. Hence, the synthesized AgNPs could be explored against human diseases induced by free radicals.

2. Methodology

2.1. Sample collection. Leaves of *Oryza sativa* were collected from the Regional Rice Research & Development Centre, Bombuwala, Sri Lanka. There were five traditional varieties; Paduwas siwuru, Herath banda, Madathawalu, Mutu samba, and Murungakayen.

2.2 Extraction method. The water extract and $AgNO_3$ were prepared according to Srisawat *et al*., 2010 with some modifications. The entire leaf with leaf sheath of each sample was collected from the field and wiped using cotton to remove external contaminants.[18] The samples were air-dried by giving the same conditions for all five varieties at room temperature. 2 g of leaves in each type were measured using the balance and homogenized. 50 mL of distilled water was added to each sample and boiled for 20 minutes at 60 °C. The extracts were filtered using a muslin cloth followed by a Whatman filter paper No. 1. The prepared water extracts were kept at 4 °C until further use.

2.3. AgNP synthesis. A 9 mL of 1 mM prepared $AgNO_3$ solution was mixed with 1 mL of the prepared water extracts of each sample inside the test tubes to synthesize AgNPs. These AgNP samples were subjected to incubation. The absorbance of the synthesized AgNPs were measured using a UV- Vis spectrophotometer for the range of 320-560 nm to identify the nanoparticles. Distilled water was used as the blank for the readings. Samples were kept at 4 °C until further use.

2.3.1 Optimisation. A 9 mL of 1 mM $AgNO_3$ was mixed with 1 mL of each plant extract in separate test tubes. The resulted samples were incubated for 15, 30, 45, and 60 min at 90 $^{\circ}$C and 60 $^{\circ}$C temperatures. The absorbance was measured using a UV- vis spectrophotometer for the range of 320-560 nm to determine the synthesized AgNPs. Distilled water was used as the blank. Samples were kept at 4 °C until further use.

2.4. Dilution. A 1 mL of each plant extract and AgNP samples were diluted with 14 mL of distilled water separately and kept at 4 °C until further use.

2.5. Antioxidant Assays. Diluted samples of water extract and AgNPs were used for the antioxidant assays.

2.5.1. Total Antioxidant Capacity (TAC). TAC was determined according to Rao *et al*., 2010 with some modifications.[1] Phosphomolybdenum assay was performed to analyze TAC. 0.5 mL of prepared solution (0.6 M H_2SO_4, 28 mM Na_3SO_4,

and 4 mM $[NH_4]_6Mo_7O_{24}.4H_2O$ in 1:1:1 ratio) was added to 1.5 mL of each diluted sample separately and incubated at 90°C for 90 mins. This assay was carried out in triplicates. The absorbance was measured using the UV- vis spectrophotometer at 695 nm using distilled water as blank. The results were expressed in g Ascorbic Acid Equivalents per 100 g (g AAE/100 g).

2.5.2. Total Phenolic Content (TPC). Determination of TPC was conducted as per the method in Srisawat *et al*., 2010 with some modifications.[18] Folin- Ciocalteu assay was executed to analyze phenolic content. 0.2 mL sample was added to the mixture of 1 mL of 10% Folin- Ciocalteu reagent and a 0.8 mL of 7.5 % Na_2CO_3 solution. This assay was carried out in triplicates. The samples were incubated by giving dark conditions for 1 h. The absorbance of the incubated samples were measured using UV- vis spectrophotometer at 750 nm using distilled water as the blank for readings. The results were expressed in g Gallic Acid Equivalents per 100 g (g GAE/100 g).

2.5.3. Total Flavonoid Content (TFC). TFC was analysed along with several modifications. An aluminium colorimetric method was performed to analyze TFC. 1.25 mL sample was mixed with 0.15 mL of 10 % w/v $AlCl_3$ and 0.075 mL of 5% $NaNO_2$ and incubated for 30 min at room temperature. The assay was carried out in triplicates and the absorbance was measured using the UV- Vis spectrophotometer at 510 nm. Distilled water was used as the blank for the readings. The results were expressed in µg Quercetin equivalents per 100 g (μg QE/100 g).[18]

The graphs that indicate absorbance vs wavelength for each assay (Total Antioxidant Capacity, Total Phenolic Content, and Total Flavonoid Content) were analyzed using Microsoft Excel software. The optimization of AgNPs was carried out both at 60 °C and 90 °C for 15, 30, 45, and 60 min by using Microsoft Excel software. One-way ANOVA tables were performed to obtain statistical information of the samples using Microsoft Excel software. The correlation of TPC vs TFC, TPC vs TAC, and TAC vs TFC were analyzed by using IBM® SPSS® software.

3. Results and Discussion

There are different approaches such as medical, pesticides, and food industry that have been occupied with the parts of *O. sativa* include in the husk, bran, and seeds by synthesizing nanoparticles due to antioxidant activity.[19] But there are limited researches that have been carried out on synthesizing nanoparticles using leaves of Sri Lankan traditional varieties of *O. sativa*. Since the leaves of this plant are not used for any purpose other than being used as straws, it may have various biological properties. Hence, this study was carried out to synthesize AgNPs using *O. sativa* leaves extracts and determine their antioxidant capacity.

To extract the bioactive compounds of *O. sativa* leaves, water was used as the solvent. Despite the other solvents of extraction including alcohol, chloroform, ether, and ionic liquid,[20] water is the most polar, nontoxic, and non-flammable solvent. It allows the dissolution of large varieties of polar organic compounds such as phenols, flavonoids, and phytochemicals.[21] To concentrate the extracts, 60°C temperature was used to avoid hydrolysis and degradation of the samples. It was observed by the study of Ngo *et al*., 2017 the highest extractable solid was obtained by absolute methanol followed by ethanol, methanol, and acetone.[22] The results of water extract were determined as half of the extractable solids compare to the absolute methanol. It also suggested that a yield with a high level as possible when using 50% (v/v) water with ethanol, methanol, and acetone.

Ag is a toxic and strong antibacterial that can damage the cell structure, growth, and metabolism. It inhibits protein synthesis by binding with DNA and proteins of the cell leading to cell death. Nanoparticles can be synthesized using Ag. Zn and Cu are alternative, and the reactivity is higher in Ag than Zn and Cu.[23]

UV-Vis spectrophotometry was used to gain the structure of the AgNPs based on the surface plasmon resonance (SPR). When the AgNPs are excited by a specific wavelength, the conduction electrons undergo collective oscillation which is known as SPR. The SPR peaks of Figure 6 determine the yield and the distribution by height and width respectively. The peaks with shorter wavelengths indicate the decreased size of the AgNP.[24] Since the peaks of the samples centered in 460-500 nm wavelength and the lower height of the peak denoted the increased size and the and the lower AgNP yield respectively. As Makarov *et al*., 2014 reported, the formation of high AgNP yield is possible when increasing the reaction temperature.[14] The ratio of 9:1 of $AgNO_3$: water extract is the most appropriate for achieving hydrodynamic size while the dilution has increased the lead to slow AgNP formation. It also can increase the AgNP yield by increasing the concentration of the extraction. [15]

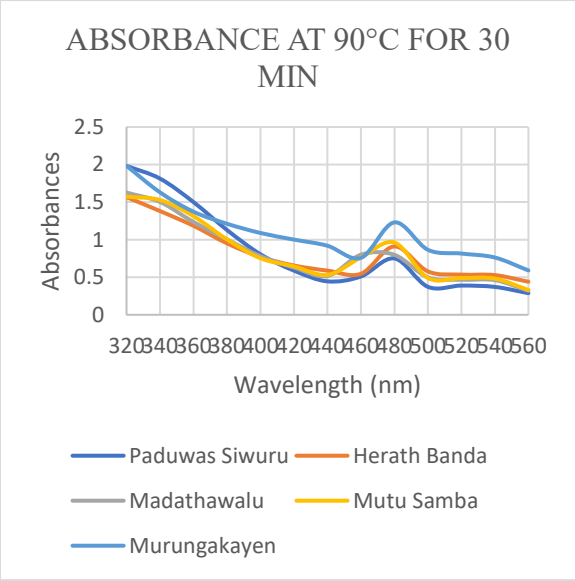

Figure 6. Absorbance at optimization temperature using UV-vis spectrophotometry. The peaks centered in 460-500 nm wavelength for all samples.

The AgNPs were optimized to obtain accepted temperature and duration. It exhibited a color change and the peaks for absorbance, indicating the formation of AgNP at 90°C for 30 min and 45 min for all samples. The color of the samples was changed after incubating the plant extracts with $AgNO_3$ from colorless (Figure 7.a) to yellowish brown due to excited surface plasmon vibration (Figure 7.b). The reduction of Ag atoms into Ag^+ ions on the effect of heat leading to the formation of AgNPs.[25] The study of Adak *et al*., 2020 indicated that between 36-48 h period of incubation leads to constant absorbance of AgNPs and when increasing the temperature improves the ability to the formation of AgNPs.[15] Hence, 90°C was the optimum temperature for the AgNP synthesis and the samples were taken for further study. The samples which were incubated for 48 h at room temperature obtained AgNPs for Paduwas Siwuru.

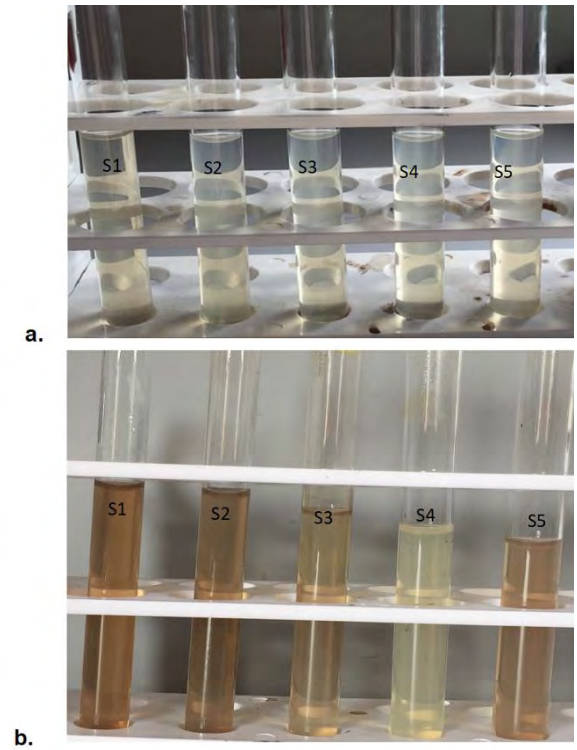

Figure 7. Multi paneled figure (a, b) showing five varieties of *O. sativa* before and after incubation with $AgNO_3$. a) Leaf extracts mixed with $AgNO_3$ before incubation. Absorbance at optimization temperature. b) Leaf extracts mixed with $AgNO_3$

after incubation at 90°C for 30 min. (S1 - Paduwas Siwuru, S2 – Herath Banda, S3 – Madathawalu, S4 – Mutu Samba, S5 – Murungakayen).

According to the Zielinska *et al*., 2009 different reducing agents including $NaBH_4$ can be used to obtain the AgNP with controlled size. Also providing Polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) as stabilizing agents can protect the synthesized colloids of Ag.[26]

The optical properties were analyzed by performing UV–Visible absorbance spectra. The conductivity of the nanoparticles was determined to characterize them by measuring the bandgap energy. The energy difference between the conduction band and the valance band of the molecular orbitals is indicated by bandgap energy. The transfer of electrons from conductance band to valance band required minimum bandgap energy. When the bandgap <3 eV is referred to as semiconductors while >4 eV is referred to as insulators.[27] According to the calculation the synthesized AgNPs at optimum temperature were classified as semiconductors (table 1).

$$E=\frac{h \times c_{light}}{\lambda}$$

E=Band gap Energy

h= 6.626×10^{-34} Js

c_{light}= Speed of light= 3×10^{8} ms^{-1}

λ= Wavelength peak of AgNP synthesis (nm)

Table 1: AgNP classification according to band gap energies.

Synthesized AgNP	**Band Gap Energy (eV)**	**Classification**
Paduwas Siwuru	2.48	Semiconductor
Herath Banda	2.03	Semiconductor
Mutu Samba	1.78	Semiconductor
Madathawalau	1.87	Semiconductor
Murungakayen	1.25	Semiconductor

It is well known that free radicals play a specific role in various pathogenic expressions. To avoid these free radical activities antioxidants, can act against them. Both scavenging activity and the defence mechanisms perform a vital role for them. The phosphomolybdate method was used to obtain the TAC where the reduction of Mo (VI) into Mo (V) green color complex (Figure 8) was determined by antioxidant compounds at acidic pH which give a maximum absorption at 765 nm. They neutralize the free radicals forming decomposed peroxides.[28]

Figure 8. Reduction of Mo during antioxidant activity.[29]

As Figure 9 indicated, AgNPs of Madathawalu express higher antioxidant capacity while Paduwas Siwuru expresses lower antioxidant capacity. It was observed a significant difference ($p \leq 0.05$) between the water extract and the AgNPs of the leaves samples during this study (Table 2). According to Abeysekara *et al*., 2012 the bran extract of *O. sativa* revealed a significance ($p \leq 0.05$) between water extract and AgNPs for TAC.[30] Also, Rao *et al*., 2010 was revealed the TAC content from methanolic extracts of rice bran showed a significant difference from water extracts and it can be changed by increasing the concentration of the extracts.[1]

Phenols are involved in the inhibition of different oxidative enzymes and the stabilization of lipids against peroxidation. Hence, they are increasingly used in the food industry, to improve the quality of the food. Phenolics compounds obtain a scavenging ability by their hydroxyl group. The modified Folin– Ciocalteu reagent

technique was used to determine the TPC in *O. sativa*.[15]

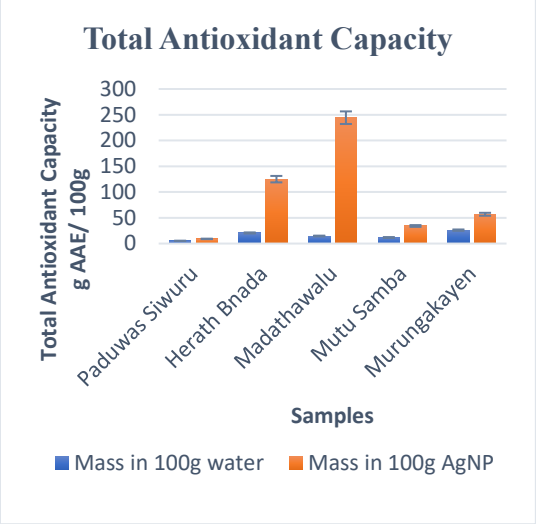

Figure 9. TAC of water extract and AgNP samples expressed as the g AAE/100 g. AgNPs of all five varieties exhibited a higher antioxidant capacity than their respective water extracts.

During the mechanism, phenols act as hydrogen donors and a reducing agent that undergo oxidization. The blue color phosphotungstic acid and phosphomolybdic acid complex were produced after the reduction reaction of the yellow color Folin-Ciocalteu reagent (Figure 10). The conditions are given by the Na_2CO_3 form phenolate by disassociation of phenolic compounds.[31]

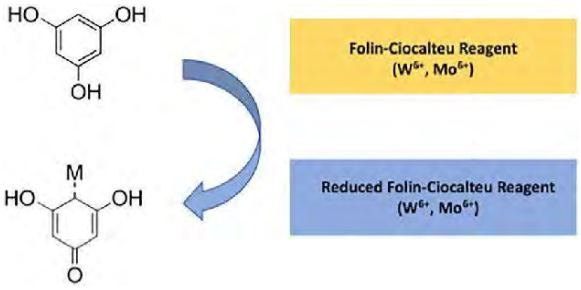

Figure 10. Reduction of Folin-Ciocalteu reagent during phenol oxidation.[32]

The phenol yield of the AgNP was higher than the water extract as given in Figure 11. Among, AgNP samples, the highest phenol content was obtained by Madathawalu whereas the lowest was obtained in Murungakayen. The phenolic yield was comparatively lower and equal in water extracts. A study by Panunto *et al*., 2010 revealed the water extract of rice bran was obtained a significant difference ($p \leq 0.05$) for samples showing the probability to have heat-liable phenols, and concatenation of the initial extract could be affected to the phenolic yield of the leaves extract.[3] It can be increased when increasing the incubating temperature.

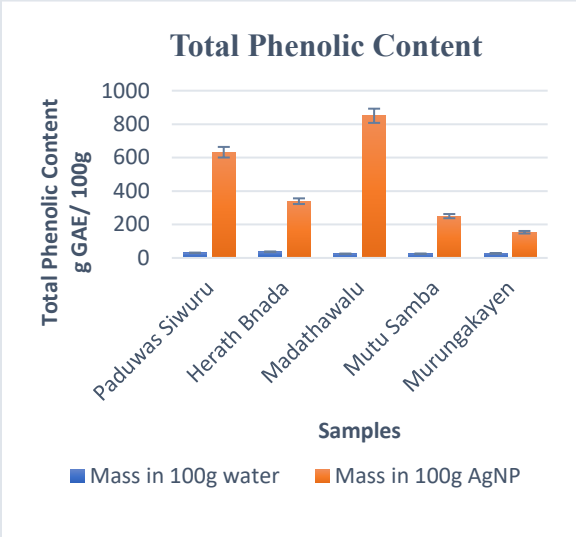

Figure 11. TPC of water extract and AgNPs expressed as g GAE/100 g. The phenolic yield was comparatively much higher in AgNPs than in the respective water extracts.

Flavonoids contribute antioxidant activity as same as phenols. They provide a positive effect on health via anti-inflammatory, antibacterial, antiviral, and anticancer activities. They perform as a scavenger for free radicals and oxidizing molecules.[28] During the mechanism of the Aluminum Chloride Colorimetric method (Figure 12) Al^{3+} reacts with OH groups of flavonoids result in the formation of acid-stable flavonoid-Al^{3+} yellow color complex. It contains flavonoids with the C-4 keto group and either C-3 or C-5 hydroxyl group of flavonoid maximum abruption at 510 nm. Besides, it contains an acid

liable complex with the ortho-dihydroxyl groups in the A- or B- ring of flavonoids. The absorption is not affected by the other phenolic content. The intensity of the flavonoid-Al^{3+} the complex is directly proportional to the flavonoid concentration.[14] The research of Amorim *et al.*, 2008 revealed that using this method can identify flavonoids and tannins and it helps to use plants for pharmacological studies.[33]

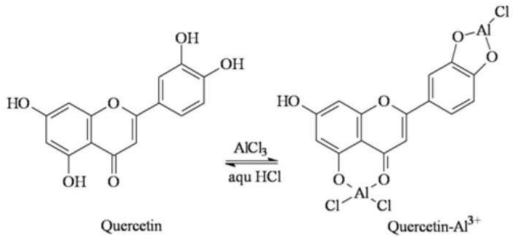

Figure 12. Mechanism of action of Aluminium chloride colorimetric method.[33]

As the Figure 13, the extraction of *O. sativa* samples exhibited comparatively higher flavonoid yield for AgNP samples but indicated as significantly lower ($p \geq 0.05$) than water extracts. The highest flavonoid yield was observed in Herath Banda while the lowest was observed in Murungakayen within the AgNP samples. Despite the pigmented variety, (Paduwas Siwuru) non-pigmented varieties (Herath Banda, Madathawalu, Mutu Samba, and Murungakayen) also exhibited AgNPs. According to Wen, 2015 the high flavonoid content can be observed in pigmented varieties and suggested non-pigmented varieties also gain flavonoids due to genetic variations, growth factors, and errors in the extraction method.[34]

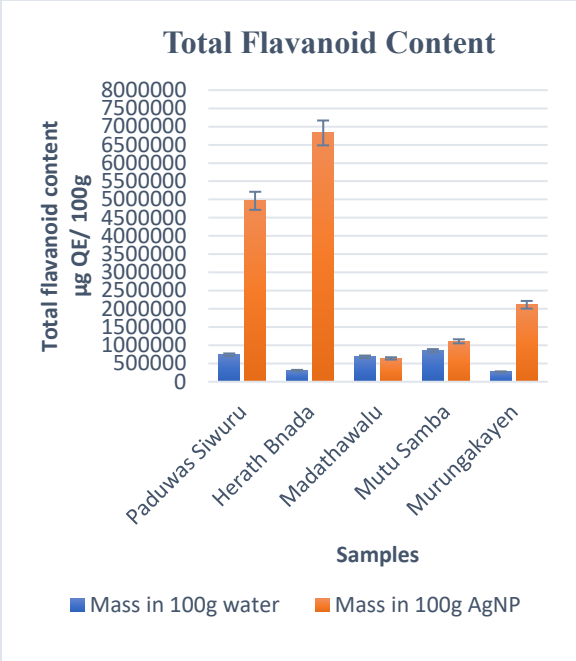

Figure 13. TFC of water extract and AgNP samples determined in g QE/100 g. Flavonoids of AgNPs attain higher compare with the respective water extracts.

Also, the strong correlation of TPC and TAC can be occurred due to antioxidants present in the leaves including phenols and other phytochemicals contribute to the antioxidant activity against reactive oxygen species rather than flavonoids. This can be also provided the weak correlation between TAC and TFC that can be due to the radical scavenging of both antioxidants and flavonoids (Figure 14).

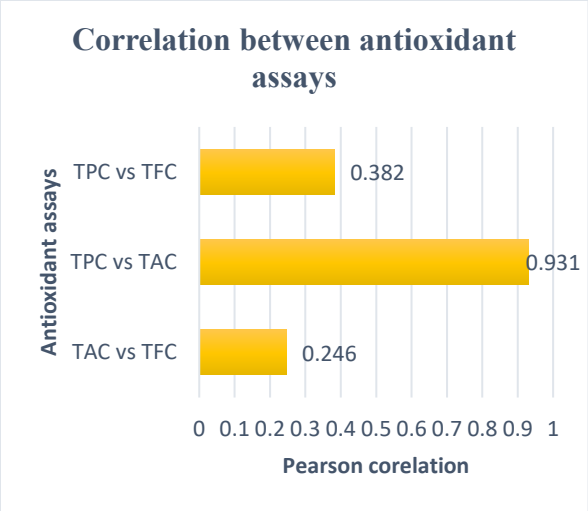

Figure 14. Correlation between antioxidant assays showing a strong positive correlation in TPC and TAC (0.93) while a weak positive correlation in TAC and TFC (0.246) compare with the correlation of TPC and TFC (0.382).

Conclusion

In conclusion, the formation of AgNPs was observed for all five varieties which incubated at 90 °C for 30 mins. The diameter of the AgNPs was obtained around 40 nm and categorized as semiconductors based on the calculated band gap energy.

Antioxidant assays showed decreased flavonoid content and increased antioxidant where the phenolic content for AgNPs over their respective water extracts. Hence, the nanoparticles can be extracted from plant-mediated synthesis methods and can be used for biomedical applications such as discovering drugs for diseases and other industries due to their eco-friendly and non-toxic properties.

Acknowledgements

Authors thank BMS for funding.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Screening for antimicrobial activity of *Coriandrum sativum* against potential enteric pathogens

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Abstract

The aim of this study was to understand the bioactivity and phytochemical study of methanol, ethanol and chloroform extracts of *Coriandrum sativum* (coriander) against bacteria including *Salmonella typhi*, *Staphylococcus aureus* and *Escherichia coli*. Coriander fruit is an annual herb originating from the Mediterranean countries. This herb is recommended in urethritis, urinary tract infection, and also for enteric diseases caused by bacteria. In the present study cold maceration technique was used to extract phytochemicals which are known to exhibit antimicrobial properties. The antimicrobial activity was tested using disk diffusion and macro broth dilution methods in order to determine the antibiotic sensitivity of the different extracts and the minimum inhibitory concentrations (MIC) of each extract against the pathogens. Moreover, minimum bactericidal concentration (MBC) was also determined. According to the test results, methanol extract of coriander showed significant antibacterial properties compared to the other two extracts. It was able to identify antibiotic properties against *Salmonella typhi* at higher concentrations (60mg/ml) of methanol extract but other two extracts were unable to show significant results against this bacterium. Moreover, methanol and ethanol extracts were able to show antibiotic properties against *Staphylococcus aureus* for all three concentrations (20mg/ml, 40mg/ml, and 60mg/ml). Therefore, it is clear that methanol showed significant results most precisely due to the presence of phytochemicals with higher antibacterial properties.

Keywords: *Coriandrum sativum, Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli,* MIC, MBC

1. Introduction

Coriandrum sativum (coriander) is a member of the Apiaceae family and it's a widely used medicinal plant. South-East Asian countries are known to grow coriander as a culinary herb and the large-scale production of coriander is mainly exist in India, Southern Russia, the Ukraine and other East European countries[15]. Coriander mainly consists of fiber (23%–36 %,), carbohydrates (20%), fatty oil (16%–28%) and protein (11%–17%) [2]. The phytochemical screenings have shown that coriander contains essential oils, tannins, terpenoids, reducing sugars, alkaloids, phenoles, flavonoids etc. Phytochemicals are bioactive non-nutrient plant compounds which exhibit antimicrobial properties against pathogenic microorganisms. It was found that several components are responsible for the antimicrobial activity of herbs/spices[6]. Plant antimicrobials are phytochemicals which are generally divided into phenolic compounds (phenols), terpenoids, essential oils, lectins, alkaloids and polypeptide [20.] Mainly phenolic compounds have shown highest antimicrobial activity compared to other antimicrobial components such as alcohols, aldehydes, ketones, ethers and hydrocarbons. Research has found that this is due to the presence of hydroxyl (-OH) groups in phenolic compounds. Similarly, linalool (major component of EO), α-pinene, p-cymene, γ-terpinene, limonene and linalyl acetate present in coriander oil has also proved to be effective against several bacterial species[1]. Enteric pathogens are known to cause various diseases among people including enteric infections and diarrhoeal diseases [8]. Hence, it is important to

understand the possible treatments to overcome these health problems. Microbial pathogens can produce toxins which can directly damage the host and can perform signal triggering host inflammation[8]. Microbial enteric pathogens, mostly rotaviruses, *Vibrio cholerae*, *Shigella spp*., *Salmonella spp* (eg: *Salmonella typhyrium*), enteropathogenic *Escherichia coli* (EPEC), and enteroaggregative *Escherichia coli* (EAEC) can induce acute diarrhea by ingesting contaminated food or water[16]. Moreover, enterotoxins produced by *Staphylococcus aureus* can result in food poisoning soon after its ingestion, [20] while *Escherichia coli* is responsible for producing toxins which induce diarrheal diseases.

In Sri Lanka typhoid fever is a major health concern compared to other enteric diseases, which is caused by *Salmonella enterica enterica typhi*. In Sri Lanka from 2000 to 2008, there was a decreasing trend of enteric fever cases, but in 2009 the notifications of typhoid are increasing. Increased numbers of infections are reported from the districts located in the dry zone (eg: Vavuniya, Mannar, Jaffna, Nuwara Eliya and Puttlam districts) due to lack of water in these areas. Therefore, the consumption of the contaminated water and food is abundant [5]. In Sri Lanka, to overcome these health problems studying of antimicrobial agents especially, natural compounds is important.

Nowadays the food industry uses preservatives such as benzoic and sorbic acids which are weak acids. These chemical preservatives are used to maintain the stability and safety of the food product on its whole shelf life. However, such components can result in microbial resistance against pathogenic bacterial species[10]. As substitutes natural products can be used because they are innately better tolerated in the human body and provide several advantages for the food industry [10]. Recently there has been a high demand for natural antimicrobial products as food preservatives and as medicines such as spices including *Coriandrum sativum*. These spices are known to contain significant antimicrobial and antifungal properties and have the ability to decrease the possibility of food poisoning and to increase the food safety and shelf-life of products [10].

Bacterial species such as *Staphylococcus aureus* and *Escherichia coli* are multidrug resistant and disinfectant bacterial types. The emergence of these species has increased rapidly, causing the increase of morbidity and mortality resulting in foodborne diseases [10]. Incidence of antibiotic resistance within bacterial species has increased since the commercial use of antibiotics became widespread. As examples, resistant to chloramphenicol, ampicillin, and trimethoprim by *Salmonella typhi* has resulted in many outbreaks in countries in the Indian subcontinent, Southeast Asia, and Africa [19]. Similarly *Staphylococcus aureus* has the ability to respond quickly to each new antibiotic with the development of resistance mechanisms [14]. Treatments for *Escherichia coli* infections have been increasingly challenging due to its resistance to most first-line antimicrobial agents [17]. From the present study, these microorganisms were tested for their antibiotic resistance against the natural antimicrobial compound; *Coriandrum sativum*, and the presence of phytochemicals were also analysed. Three extractions of *Coriandrum sativum* were obtained using the solvents; methanol, ethanol and chloroform. The extractions were carried out from *Coriandrum sativum* seeds using cold maceration technique. Antimicrobial activity of *Coriandrum sativum* was analysed against potential enteric pathogens including *Salmonella typhi*, *Escherichia coli* and *Staphylococcus aureus*, using the disk diffusion method and to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Coriandrum sativum* extracts, using broth dilution and spread plate methods, respectively. Since enteric diseases have become a common health issue to world, studying natural antibiotic compounds is important since such bacteria has become resistant to common antibiotics. Introducing new antibiotics may not provide a solution for the

antibiotic resistance but in presence of a disease alternative treatment options are important in case if the standard drug does not show any significant result against the pathogen. This study provides the idea of developing natural pharmacological compounds that are highly effective and less expensive. This can be obtained by extracting these synthetic compounds and introducing improved novel drugs to varieties of enteric infections.

2. Methodology

2.1 Extraction of *Coriandrum sativum.* The powdered sample of *Coriandrum sativum* (coriander) was obtained (10g) and extracted with 80% of methanol, ethanol and chloroform (40ml) using the cold maceration technique. For phytochemical tests, a 100mg/ml stock solution was prepared by mixing 1.0g of the extract with 10ml of 50% solvent.

2.2. Phytochemical screening of coriander extractions

2.2.1 Test for carbohydrates. 2ml of the extract was mixed with 2ml of Molisch's reagent. Then, a few drops of concentrated (conc.) H_2SO_4 were added along the test tube wall. The formation of a purple ring was observed as positive results.

2.2.2. Test for flavonoids. 1ml of the extract was added to a test tube. Then few drops of 10% NaOH were added to the tube. Yellow/red/brown colour formation was observed as positive results.

2.2.3. Test for tannins. 2ml of the extract was mixed with a few drops of 1% FeCl3 in a test tube. Green/blue/solid black colour formation was observed as positive results.

2.2.4. Test for saponins. 2ml of distilled water was mixed with 2ml of the extract. The tube was shaken vigorously. If saponins are present, formation of froth was observed as positive results.

2.2.5. Test for phlobatannins. 1ml of sample was mixed with few drops of 2% HCl. Formation of a red colour precipitate was observed as positive results.

2.2.6. Test for coumarin. 1ml of extract was mixed with few drops of 10% NaOH. Yellow colour formation was observed as positive results.

2.2.7. Test for phenols. 1ml of the extract was mixed with a few drops of 5% $FeCl_3$ in a test tube. Green colour formation was observed as positive results.

2.2.8. Test for oils. 1ml of sample was mixed with few drops of Sudan III. Pink droplets were observed as positive results.

2.2.9. Test for quinones. 1ml of the extract was mixed with 1ml of conc. H_2SO_4. Red colour formation was observed as positive results.

2.2.10. Test for terpenoids. This test was only done for methanol extract. 1ml of the sample was mixed with 2ml of 100% chloroform. Then 1.5ml of conc. H_2SO_4 was added to the mixture. The formation of a red colour ring was observed as positive results.

2.3. Testing the antimicrobial activity of *Coriandrum sativum*. As per the agar media, initially Nutrient, MacConkey and Muller hinton (MH) agar plates were made. Subcultures of *Salmonella typhi*, *Escherichia coli* and *Staphylococcus aureus* were prepared using streak plate method. MacConkey agar was used for both *Salmonella typhi* and *Escherichia coli* whereas nutrient agar was used for *Staphylococcus aureus*.

Disk diffusion was carried out for three extracts of coriander for the three microorganisms. 10ml of saline solution was inoculated with each microorganism using a sterilized loop. The turbidity was compared with the 0.5 McFarland solution using a Wickerham card. After saline solution reached 0.5 McFarland standard spread plating was done using MH agar.

Initially, 200µl of the saline suspension was added to the labelled agar plate and spread evenly on the agar. Then the stock solutions of the coriander extracts were diluted into 20mg/ml, 40mg/ml and 60mg/ml concentrations. First, gentamycin disks were placed on the agar plate followed by the filter paper disks which were dipped in negative control, 20mg/ml, 40mg/ml and 60mg/ml concentrated solutions for one minute. The zone of inhibition was measured after incubation.

Finally, the extractions which gave zone of inhibitions for each bacteria at all three concentration (20mg/ml, 40mg/ml and 60mg/ml) were subjected to broth dilution. Therefore, methanol and ethanol extractions of *Coriandrum sativum* were used to determine MIC against *Staphylococcus aureus.* After determining the MIC value, diluents which showed a clear appearance without turbidity were subjected to MBC.

3. Results and Discussion

Following table indicates the presence of phytochemicals in each extract of *Coriandrum sativum* (table 1)

Table 1. The presence of phytochemicals

Phytochemical	M	E	Chl
Carbohydrates	(+)	(+)	(+)
Saponins	(+)	(-)	(-)
Tannins	(+)	(-)	(+)
Flavonoids	(+)	(+)	(-)
Phlobatannins	(-)	(-)	(-)
Coumarin	(+)	(+)	(-)
Phenols	(+)	(+)	(+)
Oils	(+)	(+)	(+)
Quinones	(+)	(+)	(+)

Methanol extract (M), ethanol extract (E), chloroform extract (Chl), positive results (+), negative results (-).

80% methanol, ethanol and chloroform can extract a higher amount of phenols by creating a moderately polar medium. Due to impurities, water was not used as a single solvent because this interferes with phenol identification [13]. The highest extraction yield was obtained for chloroform compared to other extracts. It is strongly believed that higher molecular weighted solvents with low polarity, enables the easy extraction of substances with the same molecular weight solutes such as condensed tannins [13]. Increased molecular weighed components increase the sample yield. Therefore, higher molecular weight of the chloroform which also has a least polarity have contributed to highest extraction yield compared to others. Antimicrobial properties of coriander depend on different phytochemicals. Major antibacterial phytochemicals like phenols and oils were found to be positive in all three samples, which indicated higher antimicrobial properties. Compared to all three extracts methanol was able to give positive results for most of the phytochemicals showing that the most polar solvent with the least molecular weight extracts more phytochemicals. Chloroform showed negative results for flavonoids since it contains a hydroxyl group which can form hydrogen bonds with polar solvents like methanol and ethanol [7].

The presence of many phytochemicals in methanol compared to ethanol is due to its higher dielectric constant [12], or the higher water concentration in the ethanol extract due to less evaporation. This results in less concentration of phytochemicals which will not give visible results. During the Sudan III test, compared to methanol and ethanol extracts, chloroform extract gave significant results for oils. Since oils are hydrophobic and non-polar substances, as a non-polar solvent chloroform can effectively extract oils. Oils can be slightly soluble in polar solvents like methanol and ethanol due to attached polar molecules [3].

Disk diffusion results revealed zone of inhibition (7.1mm) for *Salmonella typhi* at only 60mg/ml concentration of methanol extract (Figure 1). For *Staphylococcus aureus* all three concentrations of methanol extract gave zones of inhibition;

20mg/ml (11.66mm), 40mg/ml (22.33mm) and 60mg/ml (23.33mm) (Figure 2). For chloroform extract of no inhibition zones were observed for all three microorganisms.

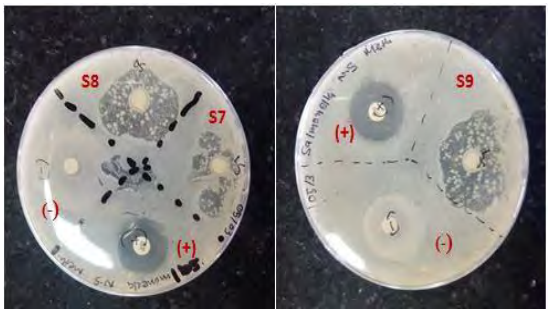

Figure 1: Disk diffusion results for Methanol extract against *Salmonella typhi* at 60mg/ml (S7, S8 and S9) concentration

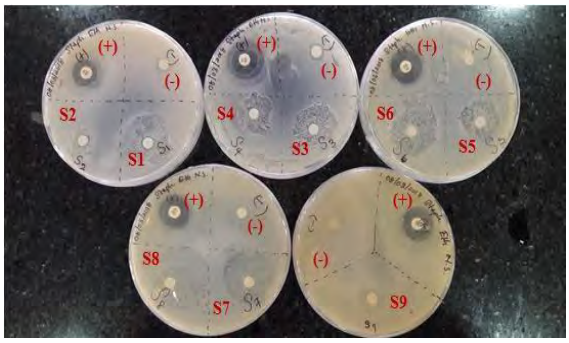

Figure 2: Disk diffusion results for Methanol extract against *Staphylococcus aureus* at 20mg/ml,40mg/ml and 60mg/ml concentrations

According to results, all three extractions did not give inhibitory zones for *Escherichia coli*, probably because as a gram-negative bacterium it has higher tolerance toward antibiotics. This even agrees with the inhibition of *Salmonella typhi* only at high concentration of methanol extract, which is also a gram-negative bacterium. The outer membranes of bacteria promote antimicrobial resistance and have the ability to interpret signals from antibiotics. Hydrophilic antibiotics (methanol/ethanol extracts) cannot cross the outer membranes of gram-negative bacteria. These bacteria show resistant to commonly used antibiotics, therefore only a few antibiotics are used to target these bacteria [11].

Excluding the chloroform extract, methanol and ethanol extracts exhibited zones of inhibition against *Staphylococcus aureus* (gram positive bacteria). Hydrophilic antibiotics use porin channels to enter into gram positive bacteria because they cannot diffuse across hydrophobic layer [9]. Similar mechanisms may have been used by methanol and ethanol extracts against *Staphylococcus aureus.* The results showed higher inhibitory zones for all three concentrations for ethanol extract, compared to methanol extract. This could be due to the presence of an antimicrobial agent present only in the ethanol extract which can significantly inhibit the growth of *Staphylococcus aureus*, or the agar medium and environmental factors have affected the bacterial growth. Compared to the positive control (gentamycin), 60mg/ml concentration of methanol extract has shown larger inhibition zone whereas ethanol extraction showed larger inhibition zones for all three concentrations. This indicates that both of the extracts show higher antimicrobial activity against *Staphylococcus aureus*.

Efficacy of the disk diffusion method always depends on the MIC value because it does not give a definitive value for the antimicrobial activity of the agent. In order to obtain the MIC results of methanol and ethanol extractions for *Staphylococcus aureus*, colony forming units (CFU) were initially determined as 1.18 x 10^6CFU/ml. Then broth dilutions were carried out where no turbidity was observed at 8mg/ml, 16mg/ml and 32mg/ml concentrations. Hence, minimum concentration that showed no turbidity was observed at 8mg/ml concentration for both methanol and ethanol extracts, the MIC value (x) was assumed to be at ($8 \geq x > 4$) mg/ml concentration. Therefore, the lowest concentration of the extract that inhibits the

growth of the bacterium is in between ($8 \geq x > 4$) mg/ml concentrations [18].

Since broth dilution results were observed for both methanol and ethanol extractions at 8mg/ml, 16mg/ml and 32mg/ml concentrated samples, they were subjected to determine the MBC value. For both extracts, MBC value was determined to be present at ($8 > x > 4$) mg/ml concentration. The determination of MBC is used to estimate bactericidal activity [4]. According to the MBC results for both of the extracts, the value should be present at ($8 > x > 4$) mg/ml concentration. This will be the concentration of the extract that kills >99.9% of the bacteria [18]. Since this kills the bacteria other than inhibiting the growth at MIC, this is the least concentration that gives highest antibacterial activity for a particular antibacterial agent.

Conclusion

According to the study, methanol and ethanol extracts can act as potential antibiotics against *Staphylococcus aureus*. Study shows that there is considerable antibacterial activity of methanol extract against *Staphylococcus aureus*, compared to other two extracts. According to the test results, methanol extract of coriander showed significant antibacterial properties compared to other two extracts. Also, it was found that antibiotic properties against *Salmonella typhi* at higher concentrations (60mg/ml) of methanol extract were effective against this bacterium. Based on the findings, this study may contribute future researches to develop new methodologies where medicinal plants like *Coriandrum sativum* to be used in alternative treatment methods to treat infections caused by common enteric pathogens.

Acknowledgements

The authors would like to express their sincere gratitude towards all the staff members of Business Management School, Colombo, Sri Lanka.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

One pot Biological Synthesis of Silver Nanoparticles using *Dracaena* Plant Leaf Varieties and assessing their Antioxidant, Photocatalytic, Antimicrobial Properties, and Melamine Adulteration.

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Abstract

Nanotechnology, which is a result of a multidisciplinary research concept is a general term that refers to all advanced technologies in the field of working with nanoscale. Energy storage, optical engineering, biotechnology, biomedical and drug delivery are some of the applications of nanotechnology. In this study, Silver Nanoparticles (AgNPs) were synthesized using *Dracaena* leaf varieties such as *Dracaena sanderiana, Dracaena reflexa, Dracaena warnecki, Dracaena reflexa 'variegata',* and *Dracaena surculosa*. Scanning Electron Microscopic (SEM) analysis confirmed that the synthesized Nanoparticles (NPs) are spherical in shape and about 50 nm in size. These AgNPs were used to assess their antioxidant, photocatalytic, antimicrobial properties, and melamine adulteration. Phytochemical analysis was carried out for each water extract (WE) and the total flavonoid content (TFC), total phenolic content (TPC), and total antioxidant capacity (TAC) were measured using both WEs and synthesized nanoparticles. AgNPs showed higher TFC, TPC, and TAC compared to WEs. The DPPH radical scavenging assay revealed that the AgNPs have the higher DPPH percentage activity compared to the WEs. The photocatalytic activity was measured using both 267ppm, and 4000ppm AgNPs with Malachite green as the model dye. The dye degraded faster after the addition of $NaBH_4$. Five melamine concentrations were used to detect melamine using DRE AgNPs and Melamine was detected in 2mM solution. *Staphylococcus aureas* and *Escherichia coli* strains were used to determine the antibacterial activity of AgNPs. AgNPs showed higher Zone of inhibition (ZOI) compared to the WE. However, this study confirms the vast number of properties of synthesized nanoparticles such as drug delivery, radiosensitivity treatments, and for diagnosis of the diseases like CVD, and cancer.

Keywords: Silver Nanoparticles, *Dracaena*, Antioxidants, DPPH, Melamine.

1. Introduction

Nanotechnology is a result of a multidisciplinary research concept that was performed during the past few years. Further, it is a general term that refers to all advanced technologies in the field of working with nanoscale.[1] Usually, the purpose of nanoscale dimension is about 1 nm to 100 nm (Figure 1). Nanotechnology is a fast-growing technology that can synthesize nanoparticles using numerous systems and their applications (Figure 2). In this research, the silver nanoparticles were synthesized using *Dracaena* plant leaf varieties. Silver is chosen to synthesize AgNPs over other metals due to their unique physical and chemical properties such as optical, electrical, and thermal, high electrical conductivity, and biological properties.[2] There are two basic methods for synthesizing nanoparticles: top-down and bottom-up (figure 3). Breaking down the bulk material into nano-sized structures or particles is a top-down approach. The bottom-up approach is also intended when nanoparticles are created through chemical reactions between atoms, ions, and

molecules. In the bottom-up approach, different techniques can be used to synthesize nanoparticles: physical, chemical, and biological.

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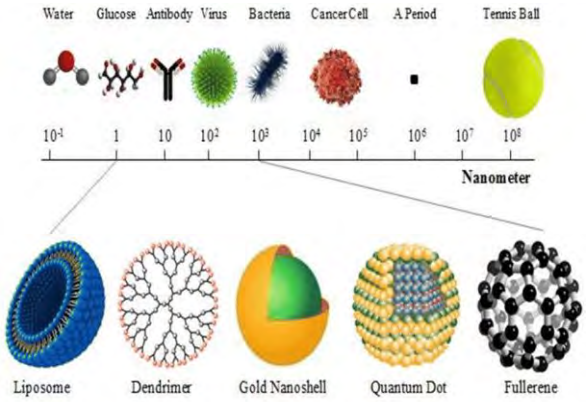

Figure 1. Nanoscale.[3]

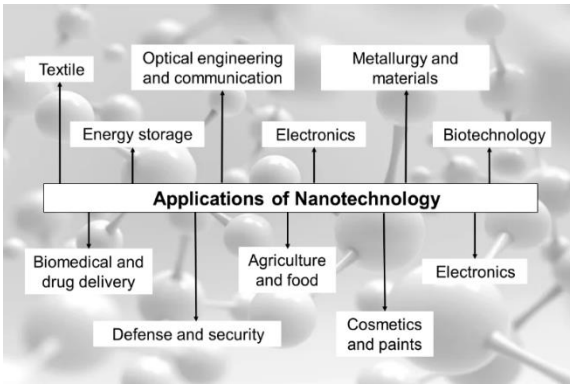

Figure 2. Applications of Nanotechnology.[4]

There are two basic methods for synthesizing nanoparticles: top-down and bottom-up (figure 03). Breaking down the bulk material into nano-sized structures or particles is a top-down approach. The bottom-up approach is also intended when nanoparticles are created through chemical reactions between atoms, ions, and molecules. In the bottom-up approach, different techniques can be used to synthesize nanoparticles: physical, chemical, and biological.[6] The physical method consists of two approaches such as evaporation condensation and laser ablation which are responsible for the production of nanoparticles in high

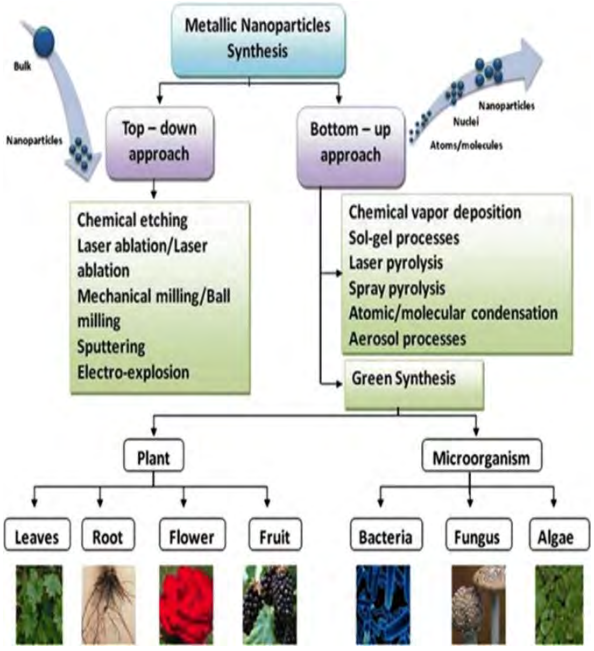

Figure 3. Metallic Nanoparticle Synthesis.[5]

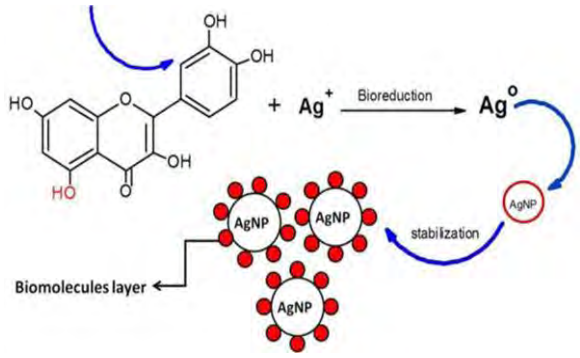

Figure 4. Nanoparticle Synthesis Mechanism.[9]

concentration. In the chemical method, different reducing agents (such as sodium citrate) are used to reduce Ag^+ in solutions both aqueous and nonaqueous [7]. The biological method refers to microorganisms, bio-templates, and plant extracts-assisted biogenesis. [8] In comparison to other biological processes, plants are the finest synthesizers due to the vast availability of resources. Additionally, the presence of nontoxic compounds in plants offers a better platform for the synthesis of AgNPs.

Plants also lower the cost of improving culture medium and isolating microorganisms. In green synthesis, the phytochemicals (proteins, flavonoids, phenols, and enzymes) act as capping agents to stabilize the silver nanoparticles (Figure 4). The sample selected for the research was *Dracaena* which is an indoor plant native to the old-world tropics, Africa, southern Asia, and northern Australia (Figure 5). It has about 120 species. *Dracaena* is a good air purifier, increases concentration and sharpens focus, increases humidity, low maintenance, and absorbs lead. Furthermore, they contain numerous antioxidants [10]. Five varieties of dracaena leaf were collected from Henarathgoda Botanical Gardens, Gampaha, Sri Lanka for this research.

As mentioned above, these Dracaena plants contain numerous antioxidants such as phenols and flavonoids. Antioxidants are molecules, either natural or artificial, that can stop, or delay cell damage brought on by oxidants like Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), free radicals, and other unstable molecules.[11] An atom with an unfilled outer shell (a free radical) is unstable and reacts with other substances fast to balance out the number of electrons in the outer shell. When oxygen

Figure 5. Dracaena Plant.[14]

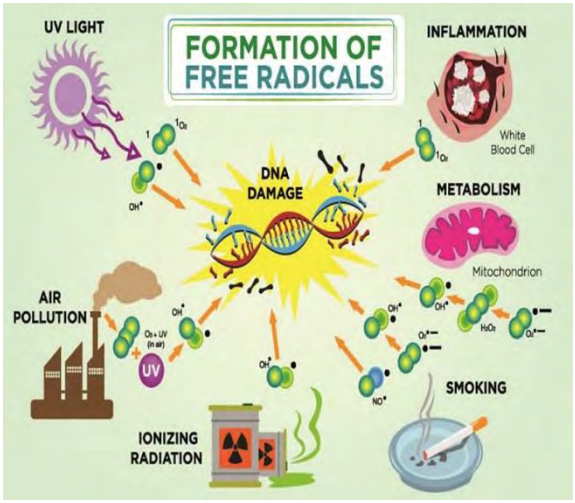

Figure 6. Formation of free radicals.[11]

molecules break into single unpaired electron atoms, the resulting free radicals are unstable and seek out other atoms or molecules to form bonds with (Figure 6). As a result, oxidative stress starts to occur. Free radicals are neutralized by antioxidants.[12] Thus, antioxidants serve as scavengers (Figure 7). Adverse effects of synthetic antioxidants may cause DNA damage,[13] hence interest in natural antioxidants with plant origins is growing.

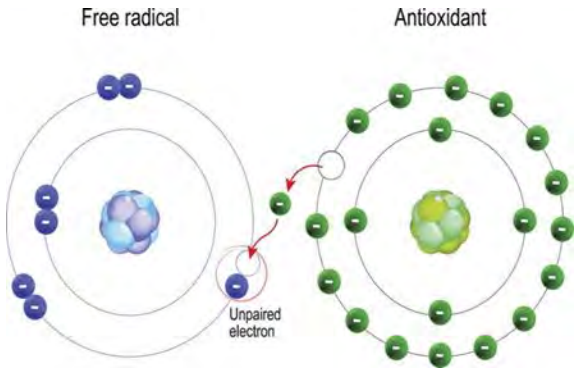

Figure 7. Antioxidant defense mechanism.[12]

DPPH can be used to determine whether many strategies for lowering pollution levels and is regarded as a very effective, environmentally antioxidants are stabilized in a sample that is rich in antioxidants. It is based on the evaluation of antioxidants' ability to scavenge it.[15]

AgNPs release Ag^+ ions during their antibacterial activity, which can build up on the cell walls and membranes of microorganisms and then move into their cytoplasm. Reactive Oxygen Species (ROS), which are the primary cause of antimicrobial activity and which are produced inside of cells by Ag^+ ions, include (1) inhibition of DNA synthesis, (2) inhibition of mRNA synthesis, (3) destruction of cell membranes and leakage of cell components, (4) inhibition of protein synthesis, (5) inhibition of cell-wall synthesis, (6) mitochondrial damage, and (7) inhibition of the electron transport chain (Figure 08). Cell death is the result of these effects. Thus, AgNPs can destroy bacteria.[16]

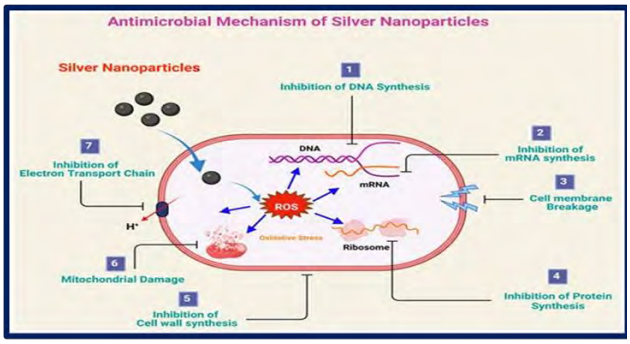

Figure 08: Antibacterial mechanism of silver nanoparticles [16].

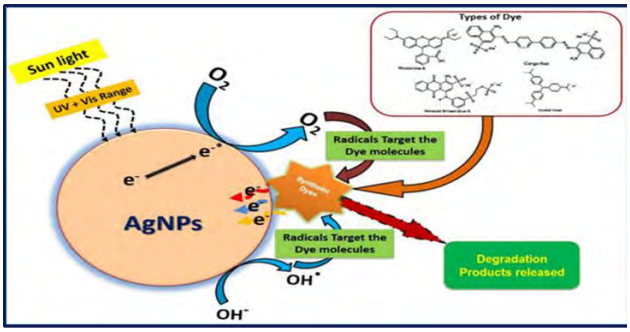

Figure 09: Photocatalytic mechanism [19]

Photocatalysts are regarded as a green method of cleaning the environment and play a critical part in the degradation of contaminants in the environment. Photocatalytic activity is one of many strategies for lowering pollution levels and is regarded as a very effective, environmentally friendly, and economical strategy.[17] Due to the visible and UV regions of the solar spectrum (due to surface plasmon resonance) and inter-band transition properties of AgNPs, they are used as photocatalysts.[18] During the photocatalytic activity, valence band electrons absorb energy from sunlight and then get excited into the conduction band. These electrons are involved in the formation of free radicals from molecules like water and oxygen. Free radicals will attack azo bonds in the synthetic azo dyes causing the degradation of compounds forming colorless solutions (Figure 9).[19]

Melamine is a chemical that is high in Nitrogen content. It gives rise to the conditions such as uric acid stones.[20] When submitted to a test for protein levels that is based on nitrogen content, melamine gives the impression that normal protein levels have been added to diluted milk. The silver nanoparticles become aggregated when melamine is added to them.[21] Due to their lower cost, safer by products and better extinction coefficient, AgNPs are more frequently used in the detection of different analytes than conventional methods.[22]

The principle objectives of this research are to synthesize silver nanoparticles using five species of *Dracaena* plant leaf and to determine their morphology by SEM analysis, evaluate antioxidant properties using total flavonoid content (TFC), total phenolic content (TPC), total antioxidant capacity (TAC), DPPH radical scavenging assay, and photocatalytic activity using Malachite green dye, antimicrobial properties using *Escherichia coli* and *Staphylococcus aureus* and melamine adulteration in milk using silver nanoparticles. After achieving the objectives of this study, AgNPs can be an effective method for treating diseases caused by free radicals, and creating an environment free of toxic substances like azo dyes.

2. Methodology

2.1. Extraction of Dracaena leaves using water. The leaves were cleaned with tissue paper to remove dust and impurities. The cleaned leaves were shade dried on different trays with labels without exposing them to direct sunlight. After a few days, the leaves were kept inside the oven at 40ºC to dry in the lab. The dried leaves were finely cut, and 2 g of each sample was weighed and placed in different beakers. 50 mL of distilled water was added, and the beakers were covered with Al foil. The beakers were placed in the oven at 80ºC for 20 minutes. The boiled aqueous solutions were left to cool down and the extracts were separated by filtration using Whatman No.1 filter papers. They were stored at 4ºC until further use.

2.2. Sample Collection. Dracaena leaf varieties were collected from the Botanical Garden, Gampaha, Sri Lanka (Figure 10)

Figure 10: Samples collected for the research (A. Dracaena sanderiana - DSA, B. Dracaena reflexa - DRE, C. Dracaena warnecki - DWA, D. Dracaena reflexa 'variegata' - DRV, E. Dracaena surculosa - DSU).

Table 2. Test, methodology, and expected result of phytochemical analysis.[23]

Test	Procedure
Test for Proteins	0.5 mL of each extract was added to 5 test tubes separately and a few drops of Millons reagent were added to them.
Test for Carbohydrates	0.5 mL of each extract was added to 5 test tubes separately. 2 drops of Molisch reagent were added to them and conc. H_2SO_4 was added along the wall.
Test for Saponins	2 mL of distilled water was added to the test tubes along with the extracts (0.5 mL) and they were shaken vigorously.
Test for Tannins	A few drops of 2% $FeCl_3$ were added to the test tubes containing the extracts (0.5 mL).
Test for Flavonoids	A few drops of NaOH were added to the test tubes containing the extracts (0.5 mL). Few drops of dil. H_2SO_4 were added to them.
Test for Steroids	1.5 mL chloroform were added to the test tubes containing extracts (0.5 mL) and 1.5 mL 0f H_2SO_4 were added to them.
Test for Anthraquinone glycoside	A volume of 1 mL ammonia was mixed with extracts (0.5 mL) and the mixtures were shaken vigorously.
Test for Phenols	To the 1 mL of the filtrates, a few drops of $FeCl_3$ were added.

2.3. Qualitative Analysis of Phytochemicals. Phytochemical tests were performed on 0.5 mLof each extract following the procedure in table 02.

2.4. Synthesis of Silver Nanoparticles (AgNPs). 1 mM aqueous $AgNO_3$ solution was prepared. Next, 1 mL of each extract and 9 mL of $AgNO_3$ solution were added into test tubes separately and covered with Al foil. One from each sample (from prepared solutions) was kept at 90ºC and 60ºC for 15-minute intervals until 1 hour. Another set of samples was kept at room temperature for 24 hours. The absorbance was measured from 320nm to 520nm and distilled water was used as the blank.

2.5. SEM Analysis. 1 mL of DRE AgNPs was diluted with 2 mL of distilled water. It was centrifuged at 13,000rpm for 3 minutes. It was repeated until the pellet occurs. Then the supernatant was discarded, and the rest was kept drying. After that, it was sent for SEM analysis at the Institute of Nanotechnology (SLINTEC).

2.6. Quantitative Analysis of Phytochemicals. Water extracts and AgNPs were diluted 15 times and used for the following Quantitative analysis.

2.6.1. Determination of Total Flavonoid Content (TFC). 2 mL of sample was mixed with 0.1 mL of 10% aluminum chloride hexahydrate, 0.1% mL of 1M potassium acetate. After 40 minutes of incubation at room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415nm. The determination of total flavonoids in the extracts and AgNPs was carried out in triplicates. Distilled water was used as the blank. Results were expressed in µg Quercetin equivalents per 100 g (µg QE/100 g).[10]

*2.6.2. Determination of Total Phenolic Content (TPC).*0.5 mL of the sample was mixed with 1.25 mL of 10% Folin-Ciocalteau phenol reagent. After 5 minutes, 2 mL of 7.5% Na_2CO_3 solution was added to each tube, and they were incubated in the dark for 90 minutes at room temperature after fully covering them with foil paper. The absorbance of the extracts and AgNPs was measured at 750nm with a UV visible spectrometer. The determination of TPC was carried out in triplicates. Distilled water was used as the blank. Results were expressed in g gallic acid equivalents per 100 g (g GAE/100 g).[10]

2.6.3. Determination of Total Antioxidant Capacity (TAC). 1 mL of extracts and AgNPs at 90ºC for 15 minutes were added to the tubes and 3 mL of reagent solution (0.6M Sulfuric acid, 28Mm Sodium phosphate and 4Mm Ammonium molybdate in 1:1:1 ratio) was added to them. Solutions were incubated at 95ºC for 90 minutes. The absorbance of the reaction mixtures was measured at 695nm using a spectrometer. TAC was determined using triplicates. Distilled water was used as the blank. Results were expressed as g ascorbic acid equivalents per 100 g (g AAE/ 100 g).[10]

*2.6.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity.*2 mL of 0.1 mM DPPH were added to 1 mL of the samples. They were kept in dark for 20 minutes. After that, the absorbance was measured at 517nm and the %DPPH scavenging activity was calculated using the bellow equation (Figure 11). Methanol was used as the blank.[10]

$$\%DPPH = A(control) - \frac{A(sample)}{A(control)} \times 100$$

Figure 11. Equation for %DPPH radical scavenging activity.

2.7. Melamine Detection. Melamine detection was carried out according to the following methods.

2.7.1. Melamine Detection using DRE AgNPs. Melamine solutions were made with different concentrations (1 mM, 2 mM, 4 mM, 6 mM and 8 mM). 500 µL of AgNPs were added to the test tubes. 300 µL of melamine were added to them. After that, the tubes were kept for 5 minutes. The absorbance was measured from 320nm to 700nm for 10-minute intervals until 40 minutes. Distilled water was used as the blank.

2.7.2. Melamine Detection on Spike Milk. The milk sample was pre-treated with 300 g/L trichloroacetic acid and the mixture was vortexed

until a white precipitate was observed. The mixture was filtrated using Whatman No.1 filter paper. The pH of the filtrate was adjusted to 7.0 by adding 3 M NaOH. It was converted into 2 different samples such as milk without melamine (control) and milk with melamine (1 mM). Lastly, the absorbance was measured for 30-minute intervals until 1 hour from 320nm to 700nm after adding AgNPs. Distilled water was used as the blank.

2.8. Photocatalytic Activity. 450 µL of Malachite Green was added to 100 mL of distilled water. The absorbance was measured from 320nm to 800nm. Following which, 267ppm of AgNPs were added and the absorbance was measured from 320nm to 800nm for 30-minute intervals until 150 minutes. This was carried for 4000ppm AgNPs also. After that, $NaBH_4$ was added and the absorbance was measured from 320nm to 800nm for 5-minute intervals until 15 minutes. Distilled water was used as the blank. This was carried out with both 267ppm and 4000ppm AgNPs.

2.9 Antimicrobial Activity. The Kirby-Bauer method was carried out. *Staphylococcus aureus* and *Escherichia coli* bacterial cultures were evenly spread on the MHA plates. Three wells were created on the MHA (negative control, sample 1, sample 2) plates (Figure 12). Saline was added as the negative control (1 mL) and a gentamycin disc was added as the positive control. 1 mL of the sample was added to S_1 and S_2. The plates were incubated at 37ºC for 24 hours and the diameter of the zone of inhibition was measured in cm.

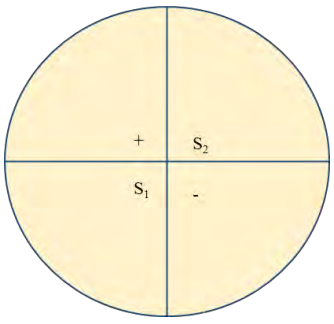

Figure 12. Petri plate labeling

2.10. Statistical Analysis. The one-way ANOVA statistical analysis was done using Microsoft® Excel 2016 software and the correlation coefficient was analyzed using IBM SPSS Statistics software.

3. Results

3.1. Phytochemical Analysis. The existence of phytochemicals in the *Dracaena* samples was revealed by phytochemical analysis as shown in Table 3.

As shown in the above-mentioned table, all the samples showed positive results for proteins, carbohydrates, saponins, tannins, phenols, flavonoids, and steroids except for anthraquinone glycoside.

3.2. Synthesis of AgNPs

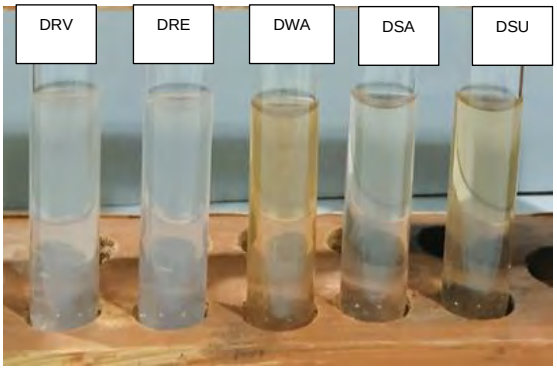

Figure 13. Before Synthesis AgNPs.

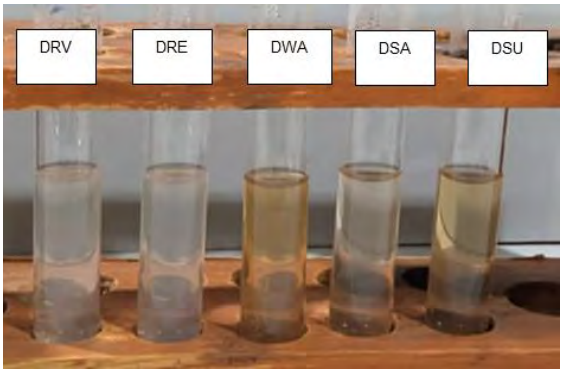

Figure 14. After Synthesis of AgNPs

A darker color was observed from the samples after the synthesis of AgNPs and the optimized sample's conditions were 90ºC for 15minutes.

Table 3. Phytochemical analysis for *Dracaena* water extracts.

Test	*Dracaena sanderiana*	*Dracaena surculosa*	*Dracaena reflexa*	*Dracaena reflexa 'variegate'*	*Dracaena warneckii*
Test for Proteins	+	+	+	+	+
Test for Carbohydrates	+	+	+	+	+
Test for Saponins	+	+	+	+	+
Test for Tannins	+	+	+	+	+
Test for Phenols	+	+	+	+	+
Test for Flavonoids	+	+	+	+	+
Test for Steroids	+	+	+	+	+
Test for Anthraquinone glycoside	-	-	-	-	-

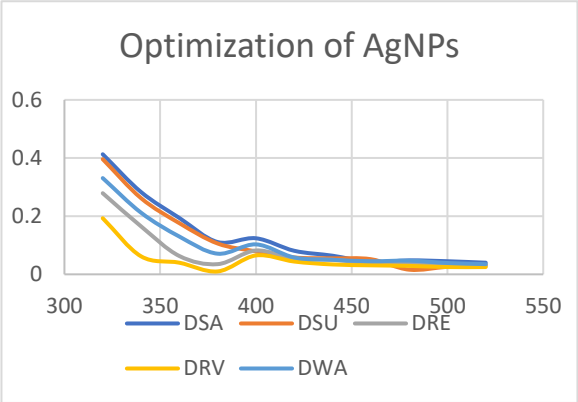

Figure 15. Optimization of AgNPs.

Table 4. Optimization Table

Temperature and Time		DSA	DSU	DRE	DRV	DWA
60ºC	15min	-	+	+	+	-
	30min	+	+	-	+	+
	45min	-	-	-	+	+
	60min	-	-	+	+	+
90ºC	15min	+	-	+	+	+
	30min	+	+	+	-	+
	45min	-	+	+	+	-
	60min	-	-	-	-	-
RT	24hour	-	+	+	+	-

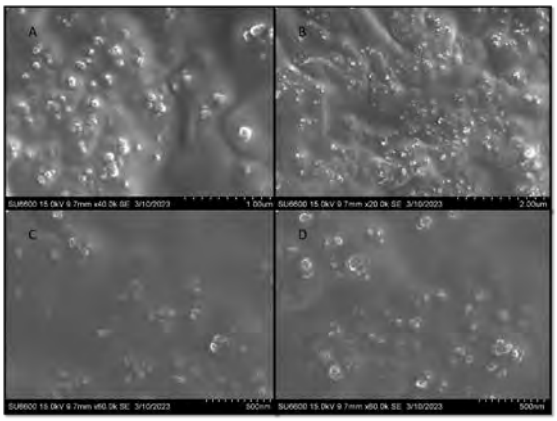

Figure 16. Images of SEM analysis. A) 15.0kV 9.7mm x 40.0k. 1µm. B) 15.0kV 9.7mm x 20.0k. 2µm. C) 15.0kV 9.7mm x 60.0k. 500nm. D) 15.0kV 9.7mm x 60.0k. 500nm.

According to the results, AgNPs were spherical and about 50nm in size

3.4. Antioxidant Assay

3.4.1. Total Flavonoid Content

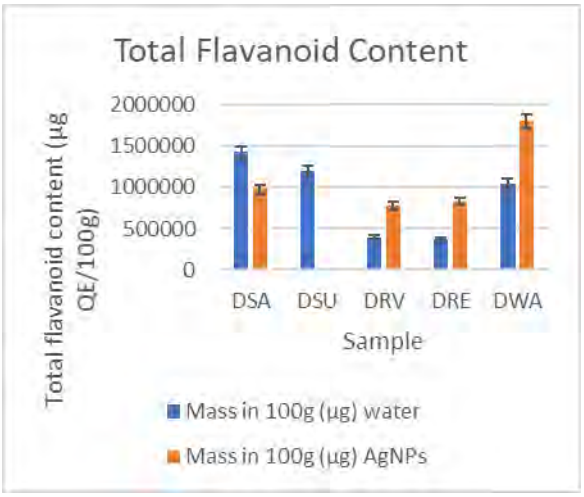

Figure 17. Total Flavonoid Content.

The TFC of DRV, DRE, and DWA AgNPs was higher compared to the water extracts of them.

3.4.2. Total Phenolic Content

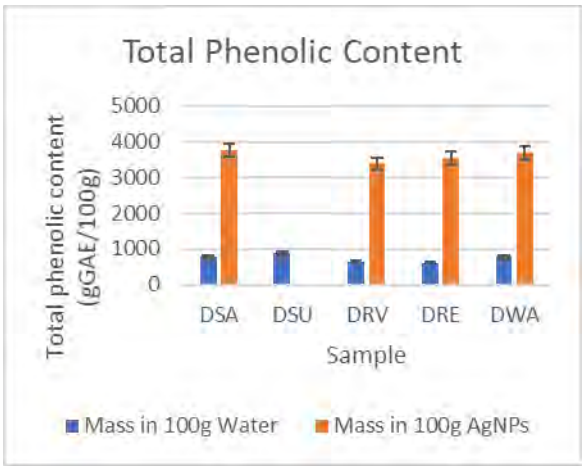

Figure 18. Total Phenolic Content

The total phenolic content of AgNPs was higher compared to the water extracts of them.

3.4.3.Total Antioxidant Capacity

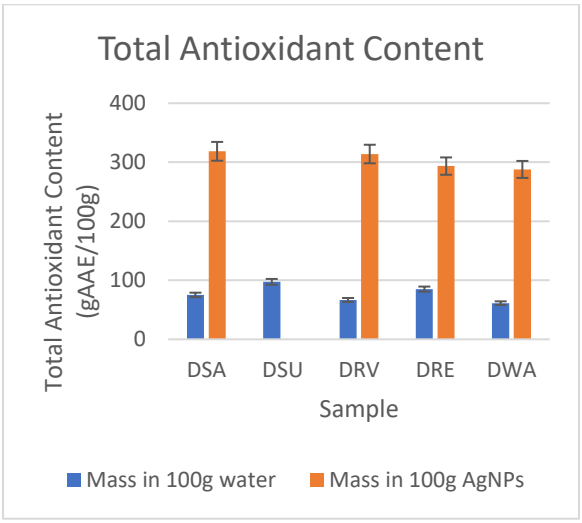

Figure 19. Total Antioxidant Content

The total antioxidant content of AgNPs was higher compared to the water extracts of them.

3.4.4. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity

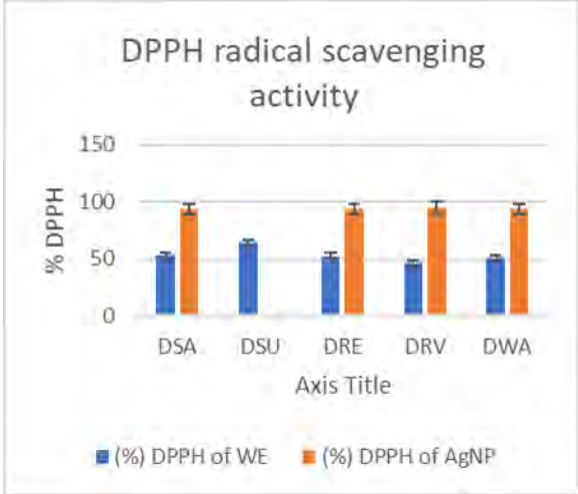

Figure 20. DPPH radical scavenging activity.

The DPPH percentage activity of AgNPs was higher compared to the water extracts.

3.5. Photocatalytic Activity under Sunlight

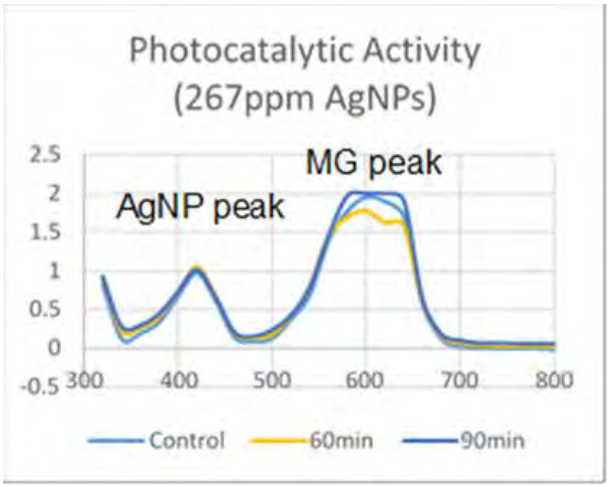

Figure 21. Photocatalytic activity (267ppm AgNP without $NaBH_4$).

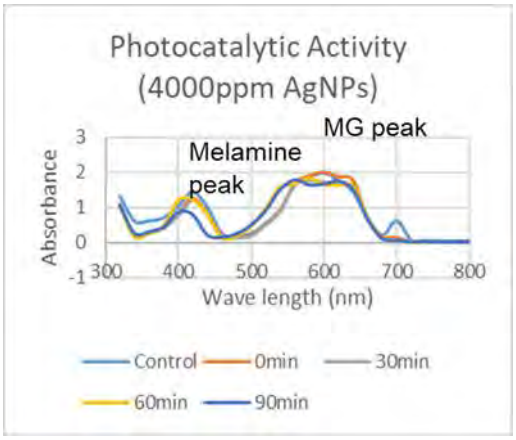

Figure 22. Photocatalytic activity (4000ppm AgNP without $NaBH_4$).

No degradation was observed even after 90 mins (Figure 21 and 22)

Figure 23. Color change of 267ppm AgNP after adding $NaBH_4$

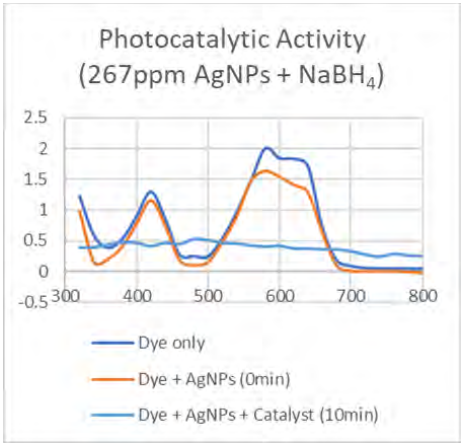

Figure 24. Photocatalytic activity (267ppm AgNP with $NaBH_4$)

Malachite green was degraded in 10minutes after adding $NaBH_4$

Figure 25. Color change of 4000ppm AgNP after adding $NaBH_4$.

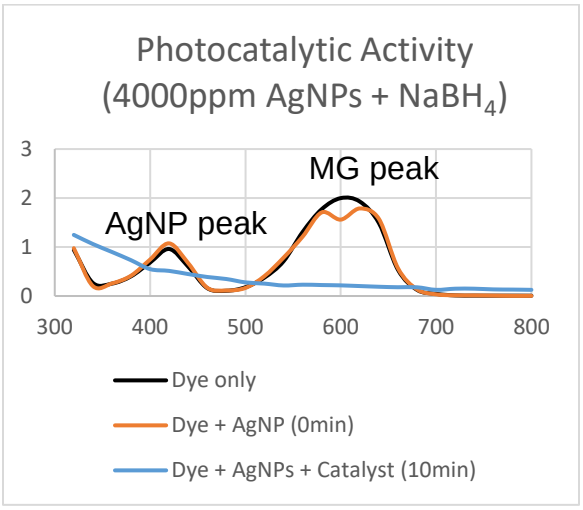

Figure 26. Photocatalytic activity (4000ppm AgNP + $NaBH_4$)

Malachite green was degraded in 10minutes after adding $NaBH_4$.

3.6. Melamine Detection

3.6.1. Melamine detection using DRE AgNPs.

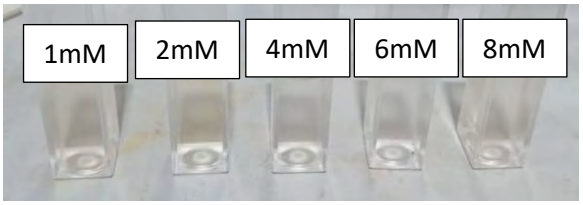

Figure 27. Melamine + DRE AgNPs (0minutes).

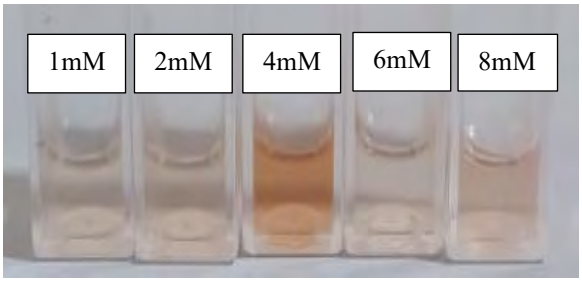

Figure 28. Melamine + DRE AgNPs (40minutes).

As shown in the figures the color change was observed after 40 minutes.

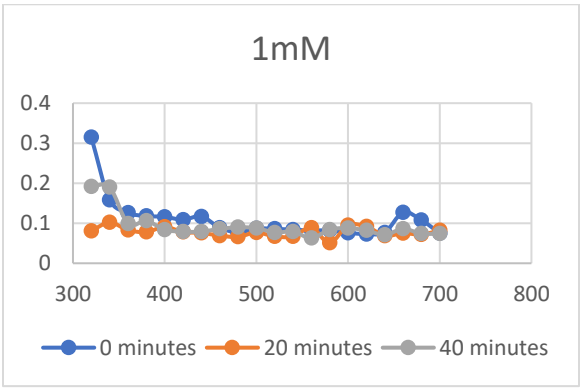

Figure 29. 1mM melamine detection using DRE AgNPs.

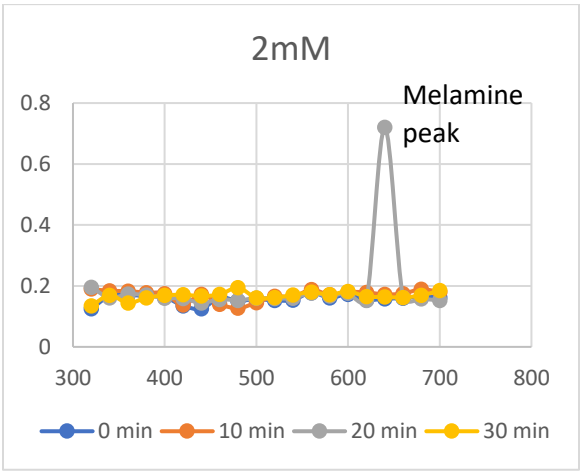

Figure 30. 2mM melamine detection using DRE AgNPs.

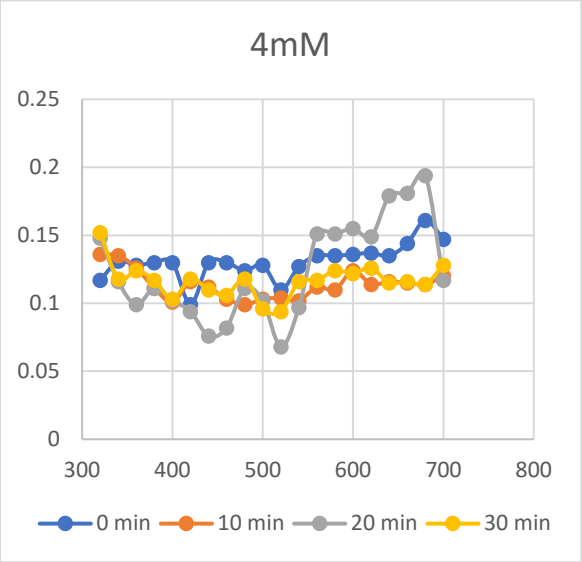

Figure 31. 4mM melamine detection using DRE AgNPs.

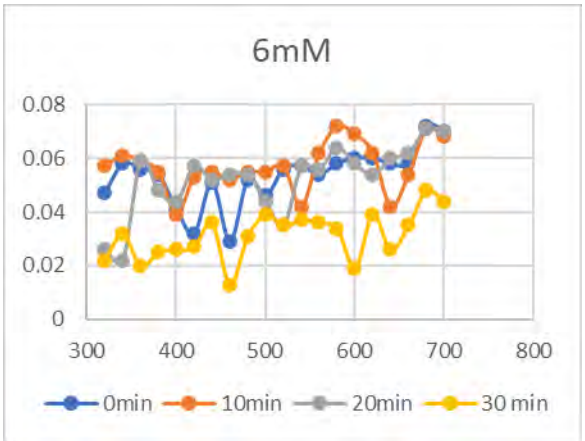

Figure 32. 6mM melamine detection using DRE AgNPs.

Melamine was detected in 2mM melamine solution.

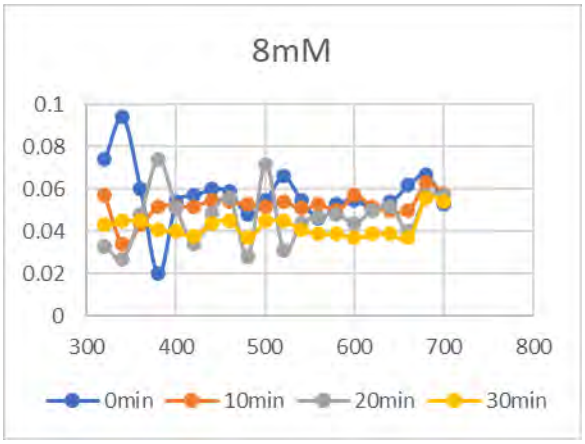

Figure 33: 8mM melamine detection using DRE AgNPs.

3.6.2. Melamine detection on spike milk.

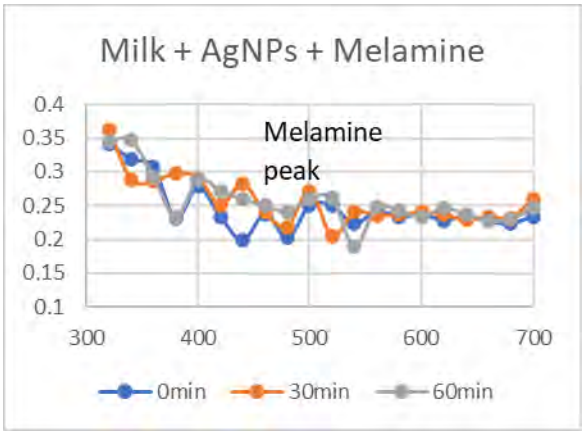

Figure 34. Melamine detection in milk using DRE AgNPs.

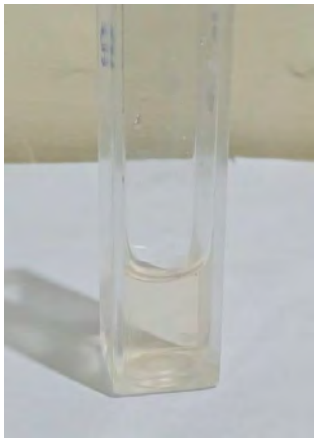

Figure 35. Melamine detection in milk using DRE AgNPs (0 minutes).

Melamine was detected at 520nm and there was a slight color change.

Antibacterial Activity

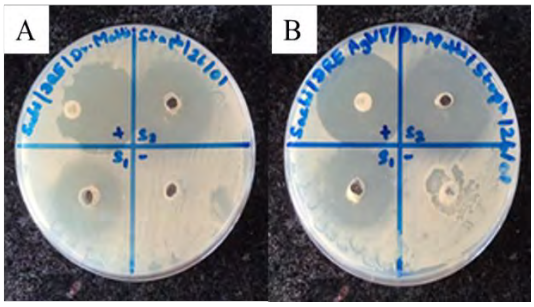

Figure 36. ZOI of *S. aureus*. A) WE and B) DRE AgNPs.

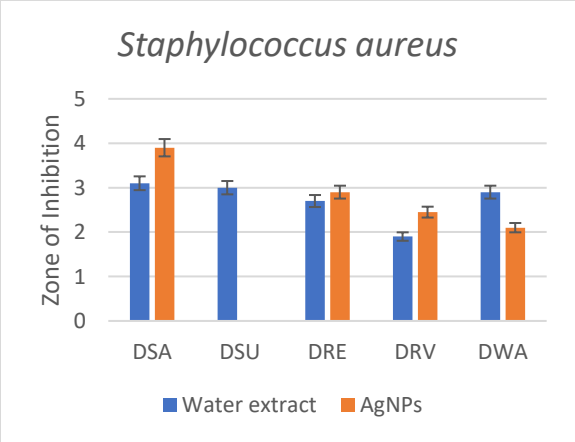

Figure 37. ZOI of S. *aureus*

DSA AgNPs showed the highest ZOI.

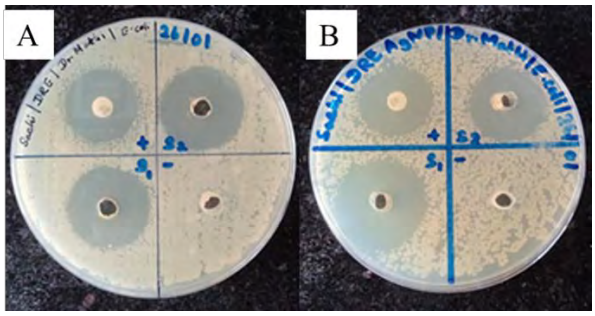

Figure 38. ZOI of *E.coli.* A) WE and B) DRE AgNPs.

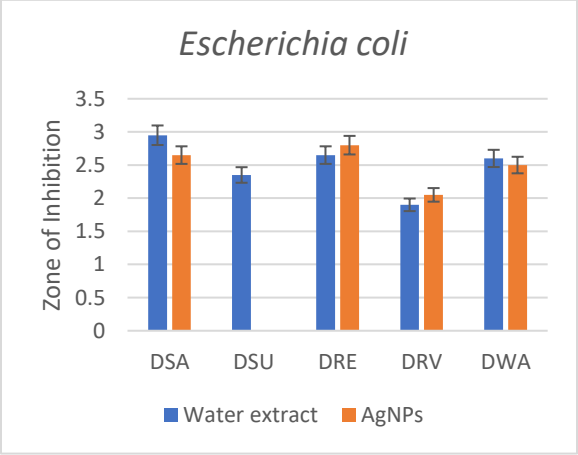

Figure 39. ZOI of *E.coli.*

DRE AgNPs showed the highest ZOI.

According to the ONE-way ANOVA, the p-value of *E.coli* was higher than *S. aureus* and the F-value of *S. aureus* was higher than *E.coli.*

4. Discussion

In this study, nanoparticles were synthesized using *Dracaena* leaf varieties with the biological method which uses nontoxic and environmentally benign materials in conjunction with green technology and is therefore eco-friendly and more acceptable than traditional methods.[30] Plant-based materials seem to be the best candidates and they are suitable for large-scale 'biosynthesis' of nanoparticles. The key active agent in some of these syntheses is believed to be polyphenols which are antioxidants. These

antioxidants have a wide range of biological effects, such as anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer.[24] In this study, water was used as the green extraction solvent due to its non-inflammability, nontoxicity, and opportunities for clean processing and pollution prevention.[25] Greener synthesis of nanoparticles provides advancement over other methods as it is simple, cost-effective, and relatively reproducible and often results in more stable materials.[26]

The presence of phytochemicals (proteins, carbohydrates, saponins, tannins, phenols, flavonoids, and steroids) in the *Dracaena* samples was confirmed by phytochemical analysis (Table 01).

According to the results, the synthesis of AgNPs was confirmed by the color change observed (Figure 01). This is due to the excitation of the SPR and SPR band which both play an important role in the confirmation of AgNP formation.[27] The peaks were observed at 400nm from DSA, DRE, DRV, and DWA samples except for the DSU sample. This can be due to the slow formation and incomplete bioreduction.[28] During this process, phytochemicals and enzymes present in the plant extract, reduce Ag^{+} to Ag^{0}. Unstable Ag^{0} will be stabilized by the active compounds present in the plant extract.[29] AgNPs synthesized at 90ºC in 15 minutes were taken as the optimized samples. Temperature is an important parameter that affects the synthesis of nanoparticles using all three methods (physical, chemical, and biological).[30] In most cases, the synthesis of nanoparticles using green technology requires temperatures less than 100°C or ambient temperature.[31]

SEM analysis which provides high-resolution imaging revealed the morphology of AgNPs as spherical and 50nm in size.

The measurement of the band gap of materials is important in the semiconductor, nanomaterial, and solar industries. The term “band gap” refers to the energy difference between the top of the valence band to the bottom of the conduction band, electrons are able to jump from one band to another. In order for an electron to jump from a valence band to a conduction band, it requires a specific minimum amount of energy for the transition, the band gap energy. The band gap energy of insulators is large (> 4eV), but lower for semiconductors (< 3eV).[32] According to the synthesized *Dracaena* AgNPs, all the peaks were observed at 400nm and 3.10eV resulted as the energy band gap of all AgNPs. It was calculated using the following equation (Figure 40).

The obtained band gap energy shows that all the AgNPs are semiconductors.

The mechanism of antioxidant actions involved either hydrogen atom transfer, transfer of a single electron, sequential proton loss electron transfer,

$$\boldsymbol{E = h \times \frac{C}{\lambda}}$$

h	Planks constant (6.626×10^{-34} Joules sec)
C	Speed of light
λ	Wavelength shown in the UV visible spectrometer

Figure 40. Planks Equation.

and chelation of transition metals.[33] Polyphenols are the natural antioxidants present in plants.[24] Phenolic compounds act as an antioxidant by reacting with a variety of free radicals.[33] Flavonoids act as antioxidants due to their strong capacity to donate electrons or hydrogen atoms.[34] However, the studies confirmed the presence of antioxidants in *Dracaena* samples.

The TFC was determined using the Aluminium chloride colorimetric method. The C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols create acid-stable

complexes with aluminium chloride, according to the basic idea behind the method mentioned. Additionally, it combines with the ortho-dihydroxyl groups on the A or B ring of flavonoids to create complexes that are acid labile.[35] According to the results at 415nm, the highest flavonoid content was observed in DWA AgNPs and the lowest was observed in DRV AgNPs (Figure 4). Similar findings have been found by Vasudevan, Kumar and Sabu in 2019.[10] According to the ONE-way ANOVA, the p-value was 0.45 ($p>0.05$) which means there is no statistical significance. F-value was 0.6 and the F-crit value was 5.99 ($F<Fcrit$) which means there is no significant difference between samples.

The Folin-Ciocalteu reagent technique was used to calculate the TPC. The creation of complex blue compounds that can be detected at a wavelength of 750 nm is the fundamental idea behind this technique. In order to convert the heteropoly acid (phosphomolybdate-phosphotungstate) present in the Folin-Ciocalteau reagent into a molybdenum-tungsten compound, the phenol or phenolic-hydroxy groups in the reagent will be oxidized.[36] According to the results, the highest phenolic content was observed in DSA AgNPs and the lowest was observed in DRV AgNPs (Figure 05). Similar findings have been found by Vasudevan, Kumar and Sabu in 2019.[10] According to the ONE- way ANOVA, the p-value was 7.28E-08 ($p<0.05$) which means there is statistical significance. F-value was 969.89 and the F-crit value was 5.99 ($F>Fcrit$) which means there is a significant difference between samples.

The TAC was determined using the phosphomolybdenum method. This is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid.[10] According to the results, the highest antioxidant content was observed in DSA AgNPs and the lowest was observed in DWA AgNPs (Figure 06). A similar finding has been found by Li, Chen and Xiao in 2021.[37] According to the ONE-way ANOVA, the p-value was 2.53E-07 ($p<0.05$) which means there is statistical significance. F-value was 638.38 and the F-crit value was 5.99 ($F>Fcrit$) which means there is a significant difference between samples.

The correlation was carried out to figure out the contribution of phenols and flavonoids in the total of antioxidants.

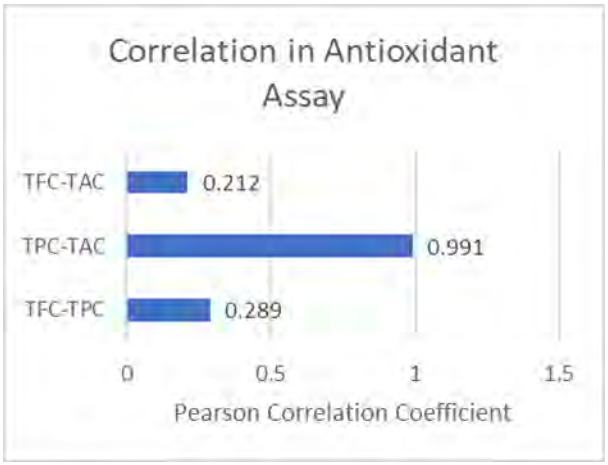

Figure 41. Pearson Correlation Coefficient.

A weak correlation was indicated between TFC and TAC assay which is 0.212 when compared to the correlation between TPC and TAC assay which is 0.991 (Figure 41). Therefore, TFC-TAC is weakly correlated, TPC-TAC is strongly correlated and TFC-TPC is weakly correlated. This can be due to the different antioxidant activities of different phenolic, and flavonoid structures present in the sample.[38] A similar finding has been found by Vasudevan, Kumar and Sabu in 2019.[10]

The radical scavenging activity of the water extracts and AgNPs was determined by using DPPH radical scavenging activity. During this assay, violet color DPPH solution is reduced to a yellow-colored product, diphenylpicryl hydrazine, by the addition of the extract in a concentration-dependent manner.[29] According to the results, the highest observed was DRV AgNPs and the lowest was observed in DSA AgNPs (Figure 20). A similar finding has been found by Tyagi *et al*. in 2021.[39]

AgNPs have been imposed as an excellent antimicrobial agent being able to combat bacteria in vitro and in vivo causing infections. The antibacterial capacity of AgNPs covers Gram-negative and Gram-positive bacteria, including multidrug-resistant strains.[40] The death of bacteria may be attributed to the silver nanoparticles' ability to continuously discharge silver ions. The bacterial envelope may be damaged as a result of the adhering ions increasing the cytoplasmic membrane's permeability. Respiratory enzymes may be inactivated following the import of free silver ions into cells, leading to the formation of reactive oxygen species but not adenosine triphosphate. Cell membrane breakdown and DNA alteration can both be triggered by reactive oxygen species. The toxicity of silver, including nanoparticles of silver, to humans, is generally low.[41]

According to the results, ZOI for *S. aureus* was shown higher in the DSA, DRE, and DRV AgNPs than in the water extracts of them, and ZOI for against *E. coli* was shown in the DRE and DRV AgNPs than in the water extracts of them. This can be due to the small size with the large surface area of AgNPs can be attached to the cell membrane or break through it.[42] Similar findings have been found by Palanivelu *et al*. in 2015.[43] According to the ONE- way ANOVA, in *S. aureus*, p-value was 0.79 ($p>0.05$) which means there is no statistical significance. F-value was 0.08 and the F-crit value was 5.59 ($F<Fcrit$) which means there is no significant difference between samples. In *E.coli*, p-value was 0.97 ($p>0.05$) which means there is no statistical significance. F-value was 0.002 and the F-crit value was 5.59 ($F<Fcrit$) which means there is no significant difference between samples.

Photocatalytic activities harness light energy to excite the photo-induced electrons.[44] The dye must first bind to the surface of the NPs in order to generate an electron-hole when exposed to sunlight. Oxygen and the superoxide anionic radical react with the electron in the conductance band. A hydrogen ion and a hydroxyl anion are created when the hole in the valence band interacts with water molecules. Another hole produces hydroxyl radicals when it interacts with water molecules at the same time. In parallel, dye is triggered by sunlight absorption and simultaneously releases electrons to make dye while producing dye. When the dye combines with two extremely unstable species, hydroxyl radicals and superoxide anionic radicals, it creates CO_2 and water as breakdown products (Figure 42).[45]

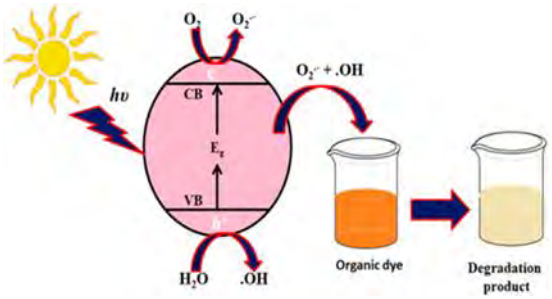

Figure 42. Mechanism of Photocatalytic activity.[4]

In this study, the azo dye used was Malachite Green in order to degrade and achieve complete mineralization.[46] The degradation process of MG confirmed the pseudo-first-order kinetics as shown below (Figure 43).[44]

$$\ln (C_0/C_t) = kt$$

C_0	The initial concentrations
C_t	The concentration at time t
k	Pseudo-first-order rate coefficients
t	Time

Figure 43. Pseudo first-order kinetics equation.

The photodegradation of MG dye assessed the photocatalysts' performance under UV irradiation. The catalyst used was $NaBH_4$ to give more degradation. The role of $NaBH_4$ is to produce $BH4^{-1}$ which acts as an electron relay to provide AgNPs with more electrons to produce more radicles which is why AgNPs have been reported to degrade organic dyes in a short period.[47]

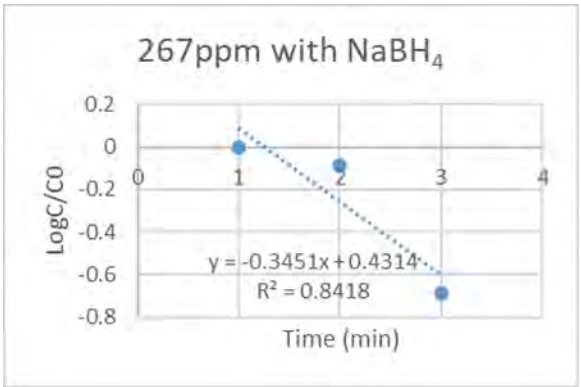

Figure 44. Rate Constant (267ppm AgNPs + NaBH4).

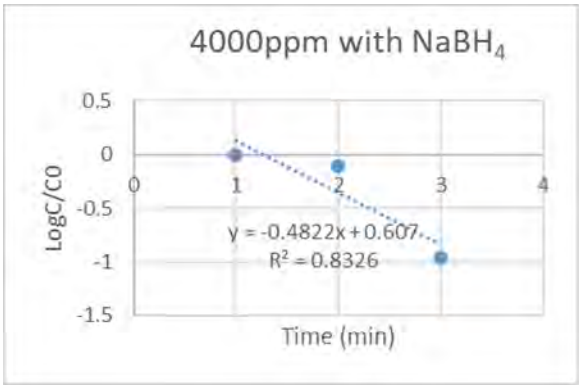

Figure 45. Rate Constant (4000ppm AgNPs + NaBH4)

According to the results, the color change was observed from dark blue to colorless after the addition of $NaBH_4$ (Figure 23 and 25). In 267ppm AgNPs, the MG absorption peak at 580nm was shown to be decreasing with time. The rate constant for 267ppm showed 0.3451. The degradation was completed in 10 minutes. In 4000 ppm, the MG absorption peak at 600 nm was also shown to be decreasing with time and the degradation was completed in 10 minutes. The rate constant for 4000 ppm showed 0.4822. It can be concluded that 4000 ppm can degrade the dye more efficiently.

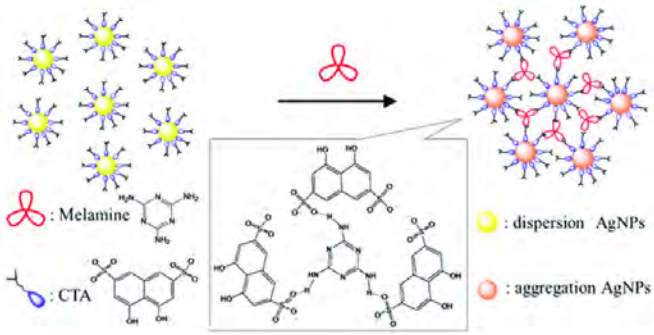

Figure 46. Mechanism of Melamine detection.[48]

Nanotechnology is used in melamine detection. Melamine causes the aggregation of AgNPs and the yellow color changes to red. Change in the color of AgNPs is the basis of the colorimetric detection method (Figure 46).[36]

According to the results, color change was observed within 40minutes in 2mM melamine concentrations (Figure 27, 28). The lowest melamine concentration (2mM) was used for the melamine detection in milk and a color change was observed (Figure 35).

Conclusion

Five *Dracaena* plant varieties were used in order to synthesize AgNPs and the optimized conditions were 90ºC for 15minutes. Synthesized AgNPs were spherical and 50nm in size according to SEM analysis. According to the antioxidant assay results, AgNPs showed a higher antioxidant activity than the water extracts of

them. Furthermore, high antimicrobial activity was shown by the AgNPs against *E. coli* compared to *S. aureus* which means that the AgNPs' effectiveness against gram-negative bacteria is higher. The photocatalytic activity was more efficient in 4000ppm AgNPs compared to 267ppm AgNPs and the degradation of the dye was faster after the addition of $NaBH_4$. Melamine was detected using DRE AgNPs in both 2mM melamine solution and spike milk. AgNPs play major roles in anti-inflammatory, anti-cancer, anti-proliferative, and biosensing and imaging processes. Cardiovascular, and neurological disorders are also treated using AgNPs.

Acknowledgements

Authors thank BMS for funding and SLINTEC for SEM analysis.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Green synthesis, antioxidant, antibacterial and photocatalytic activity of *Ixora coccinea* flower extract mediated silver nanoparticle

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Abstract

Nowadays, water resources are commonly polluted which consist of hazardous organic matters due to draining of industrial effluents. This affects the environment, aquatic life and human health such as azo dyes. Silver nanoparticles (AgNPs) is the known commercialised nano material as it is non-toxic to human and is eco-friendly. Five varieties of *Ixora coccinea* were obtained to assess the presence of phytochemicals. AgNPs were synthesized from *Ixora coccinea* extract using green synthesis at condition of room temperature (RTP) for 42 hours but it was absent for 50˚C for 15 minutes. The presence of a brown solution and formation of peaks between 420-480 nm at the optimisation of AgNPs. In addition, SEM images showed AgNPs of spherical in shape and approximately 50 nm of size confirmed the AgNPs formation. Antioxidant assays and Free radical assays conducted showed that all AgNPs showed higher antioxidant activity compared to water extracts. AgNPs have similar or lower IC50 compared to the water extracts. Photocatalytic activity was assessed by using Methylene Blue (MB) in which 100 ppm showed the highest photo degradation with and without the catalyst compared to 10 ppm and 500 ppm. AgNPs showed higher antimicrobial activity compared to water extracts on both *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). *E. coli* showed a higher antimicrobial activity compared to *S. aureus.*

Keywords: *Silver nanoparticles, Ixora coccinea, Antioxidant, Photocatalytic activity, Antimicrobial activity, Escherichia coli, Staphylococcus aureus*

1. Introduction

Currently, water resources show dominance of pollution that consist of hazardous organic matters that is caused by draining of industrial effluents. The contaminated water showed harmful effects to the environment, aquatic life, and human health such as azo dyes which are toxic due to their visibility and carcinogenicity.[1] Physical and chemical techniques that are used for treatment of dye effluent are toxic, less effective, costly and time consuming. In 1981 Richard Feynman discovered the development of nanotechnology, and it's been used to synthesise metal nanoparticles (NP) synthesized from plants extracts to degrade azo dyes efficiently and eco-friendly.[2] Nanoparticles show high catalytic potential due to its large surface: volume ratio and smaller structure. This is a better alternative for dye removal and degradation.[3,4]

According to Keerawelle and his co-workers nanotechnology is known as science, engineering and technology conducted in a nanoscale of 1-100 nm.[5,6]. Nanomaterials consist of various applications such as drug and gene delivery, detection of protein, hyperthermia and tissue engineering.[7] Volume ratio, size, distribution and morphology are the different physiochemical nanomaterial properties compared with bulk materials that gives the ability to perform in various applications. Nanoparticles are synthesized by two approaches, the top-down or bottom-up approach. Top-down approach bulk material is physically broken down into nanoscale structures which are used in chemical etching, laser ablation and milling. In bottom-up approach nanoscale structures are formed from the atoms which are used in sol-gel processes, spray pyrolysis and green synthesis.[8]

Physical, biological and chemical methods are various types of nanoparticle synthesis. Physical and chemical methods are extremely costly and uses toxic, hazardous chemicals. Biosynthesis or green synthesis decreases usage of toxic chemicals and environmental impact using materials like fungi, bacteria and plant extracts. Plant mediated synthesis of NP is used commonly since it is non-toxic, biocompatible, simple and consist of phytochemicals like flavonoids, saponins and tannins that enhances the synthesis of NP.[9-12]

According to Natsuki and his co-workers silver nanoparticles (AgNPs) is the main commercialized nano-material among other metallic nanoparticles.[13,14] Antimicrobial effects, surface plasmon resonance properties, antioxidant properties non-toxic to mammals and eco-friendly are the following properties in AgNPs which are of interest.[15,16] The plant mediated of silver nanoparticles synthesis are synthesized in 4 steps (i) nucleation, (ii) coalescence, (iii) growth and (iv) capping.[17] Presence of phytochemicals in the plant extract reduces the Ag^{+1} ion to Ag^{0} atom and also, they act as a capping agent and provide stability to nanoparticles.[18]

According to Baliga and Kurian *Ixora coccinea* is well-known as Jungle of Geranium that belongs to family Rubiaceae.[19,20] Originally the plant is native to Sri Lanka and India. Nowadays it is found in subtropical and tropical climates of the world, which have naturalized to Florida, Nigeria and Puerto Rico.[21] The plants are found to grown in dry area with slightly acidic pH. There are wide varieties of flowers that produce red, red-orange, pink, white, yellow, pink flowers. Phytochemical analysis has shown various phytochemicals present such as alkaloids, tannins, terpenoids, flavonoids, saponins, essential oils.[22] Flowers are mainly used in medicine to treat dysentery, catarrhal bronchitis, skin diseases and chronic ulcers. The shaded dried flowers are heated in coconut oil and are applied externally to reduce eczema.[23] It also consists of following pharmacological properties such as antioxidant, antimicrobial,

anti-inflammatory, hepatoprotective and chemoprotective properties.[19,24-26]

As mentioned AgNPs consist of antibacterial properties and it is toxic to bacterial cells that has the ability to disrupt bacterial cell wall, inhibit bacterial cell growth.[27] It releases Ag ion that disrupt cell metabolism by interacting the macromolecules (eg: protein and DNA), preventing protein synthesis, which decreases the permeability of the membrane leading to cell death. AgNPs is known to have stronger antibacterial properties since it's more reactive chemically than Ag in bulk.[28, 29]

Cell damage caused by oxidants such as free radicals. Reactive oxygen species (ROS) consist of an unpaired electron in an atomic orbital. These radicals are highly reactive; and unstable. Free radicals are generally produced in biological oxidation reaction. The radicals are reactive and start a chain reaction causing cell death by attacking macromolecules. ROS are prevented by 'Antioxidants' which is known as free radical scavengers are natural or synthetic substances by binding with the free radicals (oxygen or nitrogen) thereby terminating the chain reaction.[30-33]

MB, rhodamine B and Methyl orange (MO) are common water pollutants.[34] MB is a thiazine cationic azo dye that is generally used in textiles and printing industry which is toxic and consists of C=N bond.[35] Accumulation of MB in wastewater leads to breathing difficulties, vomiting, eye burns and nausea.[36] Thus, some treatment should be given prior to the removal of MB from waste water. Techniques such as adsorption, irradiation and membrane filtration are used frequently to remove MB from aqueous solutions. These techniques are costly and requires various tools. These methods can accumulate MB and is unable to cover the dye into harmless compounds.[37-39] Plant mediated AgNPs have the ability to photo degrade the azo bonds which are released to the environment, thus producing nontoxic by products.[40]

AgNPs absorb visible light the surface electrons get excited from the valence band to conduction band due to SPR effect. These excited electrons react with oxygen and hydroxyl molecules to form oxygen and hydroxyl free radical. These radicals attack the dye molecule adsorbed on surface of AgNPs and degrade the dye. Holes generated in 5sp orbital are filled with electrons which are known as 'electron holes' from the photo synthesized dye molecule oxidized dye to reactive intermediates thereby degrading the MB dye molecule.[41, 42]

The aim of the study focuses on the green synthesis AgNPs from *Ixora coccinea* flower extract using five different varieties which are Pink, Orange, White, Red and Orange-Pink hybrid and assessing their antioxidant, antimicrobial and photocatalytic activity. Antioxidant assays such as Total Flavonoid Content (TFC), Total Phenolic Content (TPC), Total Antioxidant Capacity (TAC) and the reducing ability of antioxidants will also be measured by conducting 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity and Median Inhibitory Concentration (IC50). Antimicrobial activity by using. *E. coli* and *Staphylococcus aureus* and Photocatalytic activity of AgNPs on MB under sun light and catalyst will be assessed. Thereby, AgNPs can be used in future studies to find out various treatment for diseases and to prevent environmental issues.

2. Methodology

All the Experiments were carried out by following good lab practice and using personal protective equipment.

2.1. Sample Collection. Flowers of *Ixora coccinea* of colours Pink (M1), White (M2), Red (M3), Orange-Pink hybrid (M4) and Orange (M5) were obtained from Negombo and Nugegoda, Sri Lanka (figure 1).

Figure 1. Diversity of Ixora coccinea (M1: Pink, M2: White, M3: Red, M4: Orange-Pink, M5: Orange)

2.2. Sample Processing. The obtained samples were washed and subjected for shade drying. Flowers were crushed using a motar and pestle.

*2.3. Extraction of Flower extract.*To each 2 g of crushed sample, 50 mL of distilled water was added. Then they were heated at 60˚C for 20 minutes using an oven and were left to cool followed by filtration using a Whatman 1 filter paper and gauzed into a 50 mL falcon tube. Then they were stored at 4˚C until further use.[43]

2.4. Synthesis of Silver nanoparticles using Silver Nitrate.
1mL of extract for each sample and 9 mL of 1 mM $AgNO_3$ was added to a test tube. The test tubes were covered with Aluminium foil and were left at room temperature for 42 hours. Optimization was carried out for all samples, under these conditions; 50˚C for 15 minutes, 90˚C for 30 minutes and 90˚C for 60 minutes and the corresponding absorbance across 300-550 nm were measured.[44]

*2.5. Dilution of the samples***.** A series of 1:15 dilutions was carried out by dissolving 14 mL of distilled water for each water extract and synthesized AgNPs and kept it at 4ºC until further use.

2.6. Determination of Antioxidant Activity. Diluted water extract and AgNPs were used for further assays.

2.6.1. Total Flavonoid Content (TFC). Total flavonoid content was founded by aluminium chloride colorimetric method where 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1 M Potassium acetate, and 1.5 mL sample were added together into a test tube. This was kept under room temperature for 30 minutes. The absorbance for each sample was measured in triplicates at 420 nm using distilled water as the blank and were expressed as mg Quercetin equivalents per 100 gram (mg QE/ 100g). [45]

2.6.2. Total Phenolic Content (TPC). 200 µL of Folin-ciocalteu (FC) reagent, 1160 µL of distilled water, and 600 µL of Na_2CO_3 was added to 40 µL of sample. The mixture was incubated at room temperature for 2 hours and the absorbance was measured in triplicates at 765 nm using distilled water as the blank and were expressed as mg Gallic acid equivalents per 100 gram (mg GAE/ 100g).[46]

2.6.3. Total Antioxidant Capacity (TAC). 1.5 mL of sample was added with 0.5 mL of the phosphomolybdenum mixture (6 mL of 0.6 M H_2SO_4, 6 mL of 28 mM sodium phosphate and 6 mL of 4 mM ammonium molybdate in the ratio of 1:1:1) and was incubated at 95˚C for 90 minutes. The absorbance for each sample was measured in triplicates at 695 nm using distilled water as the blank and were expressed as mg Ascorbic acid equivalents per 100 gram (mg AAE/ 100g).[46]

2.6.4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity. 20 µL of sample was mixed with 2 mL of 0.004% DPPH solution and incubated for 30 minutes at room temperature. The absorbance for each sample was measured in triplicates at 517 nm using methanol the blank. DPPH scavenging activity was calculated using the following equation and was expressed as a percentage inhibition; $I\ (\%) = (A_{control}\text{-}A_{sample}/A_{control}) \times 100$, where $A_{control}$: initial absorbance of DPPH reagent, A_{sample}: sample absorbance.[47]

2.6.5. Median Inhibitory Concentration (IC50). 2 mL of 0.004% DPPH solution was added to 20 µL five varying concentrations (100%, 80%, 60%, 40% and 20%) and was incubated for 30 minutes at room temperature. The absorbance for each sample was measured in triplicates at 517 nm using the spectrophotometer against methanol as a blank.[47]

2.7. *Phytochemical Analysis.* Methodology of phytochemical analysis was adapted from Nair and his co-workers (Table 1).[48]

Table 1. The methodology for phytochemical

Phytochemical	Procedure	Expected results
Tannin	0.5ml of extract was added into 5 different test tubes. Few drops of 10% $FeCl_3$ was added.	Formation of red ppt (positive result).
Saponins	0.5ml of extract was sample was added into 5 test tubes respectively to each sample. 1.5ml of distilled water was added and shaken vigorously.	The formation of froth
Alkaloids	0.5ml of each extract was added into each test tubes respectively. 0.5ml of Wagner's reagent was added.	Formation of reddish-brown ppt
Terpernoids	0.5ml of each extract was added into each test tubes respectively. 1ml of chloroform was added into 3 drops of conc. H_2SO_4.	Formation of emerald green colour
Carbohydrates	0.5ml of each extract was added into each test tubes respectively. 0.5ml of conc. H_2SO_4 and few drops of Molisch's reagent.	Formation of purple's ring indicates the presence purple ring
Quinones	0.5ml of extract was added into each test tubes respectively. 0.5ml conc. HCL was added.	Formation of yellow ppt
Anthocyanin	0.5ml of extract, 0.5ml NH3 and few drops of conc. HCL.	Appearance of bluish violet colour
Coumarin	0.5 ml of extract and 0.75ml 10%NaOH was added to each test tube.	Appearance of yellow colour

2.8. Determination of Photocatalytic activity using MB with silver nanoparticle extract. Photocatalytic activity procedure was adapted from Roy, Sarkar and Ghosh 188 µL of dil. AgNPs of M2 sample was added to 50 mL of MB solution which was stirred for 15 mins and was left under direct sunlight.[49] Absorbance across 340 - 780 nm was noted for intervals of 30 mins. The procedure was repeated for 100 ppm and 500 ppm M2 AgNPs sample. The same procedure was repeated with the addition of 20 µL of 1mM $NaBH_4$ and left under sunlight.

2.9. Determination of Antimicrobial Activity. Antimicrobial activity of the sample was assessed using *Escherichia coli* and *Staphylococcus aureus* by the well diffusion technique on Muller-Hinton agar and three wells were punctured into the agar. One well for the negative control and two wells for the samples. Using a cotton swab the bacteria was streaked onto the Mueller Hinton Agar plate. Saline was added for the negative control and for the positive control Gentamicin discs were used (Figure 2). This was conducted for all the water extracts and AgNPs samples and incubated at 37˚C for 24 hours. The diameters of the zone of inhibition were then measured using a ruler for each sample.[47]

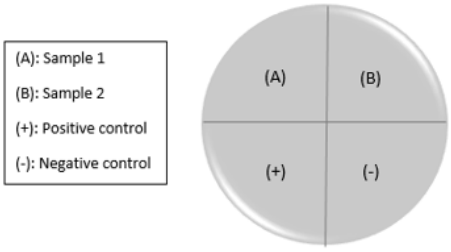


Figure 2. Culture plate layout of samples by well diffusion technique

2.10. Statistical Analysis. One-way ANOVA was performed using Microsoft Excel 2016 software in which graphical representation of data was created and correlation graphs were developed from IBM SPSS Statistics 23 Software.

2.11. SEM Analysis. Scanning Electron Microscope analysis was performed on M2 AgNPs from Sri Lanka Institute of Nanotechnology (SLINTEC) using Hitachi SU6600 SEM.

3. Results and Discussion

3.1. Phytochemical analysis. Phytochemical analysis in which the presence of chemical components was analysed qualitatively (table 2). The results of *Ixora coccinea* extract expressed the presence of quinone, quinone, saponins, terpenoids, tannins and coumarin and the absence of anthocyanin, carbohydrates. Phytochemicals are well-known to play a major role in silver ion (Ag^+) reduction to silver metal (Ag^0) and it prevents agglomeration by capping thereby, confirming the Ag particle formation.[50] As stated below level of phytochemicals in the parts of the plant are shown; Flower extract > Leaf extract > Stem extract. Water extract showed higher phytochemical activity compared to ethanol extract Water extracts are used in synthesis of AgNPs as it is eco-friendly and non-toxic.[48]

Table 2. Phytochemical analysis results and observation

Phytochemical	Result					Observation
	M1	M2	M3	M4	M5	
Alkaloids	✓	✓	✓	✓	✓	
Anthocyanin	×	×	×	×	×	
Carbohydrates	×	×	×	×	×	
Coumarin	✓	✓	✓	✓	✓	
Quinone	✓	✓	✓	✓	✓	
Saponins	✓	✓	✓	✓	✓	
Tannins	✓	✓	✓	✓	✓	
Terpenoids	✓	✓	✓	✓	✓	

3.2. Synthesis of Silver nanoparticles using Silver Nitrate and its Optimization. The synthesized AgNPs can be confirmed by the colour change of AgNPs synthesized extract from red-brown colour to a grey colloidal solution indicates the formation of AgNPs and reduction of $AgNO_3$ (figure 3). This is due to the Surface plasmon resonance (SPR) effect, which is a collection of oscillation of free electrons of the AgNPs that resonate with the frequency of light wave that interacts with AgNPs. Therefore, the SPR peak formed shows the presence of AgNPs.[51]

3.3. Synthesis and Optimization of AgNPs.

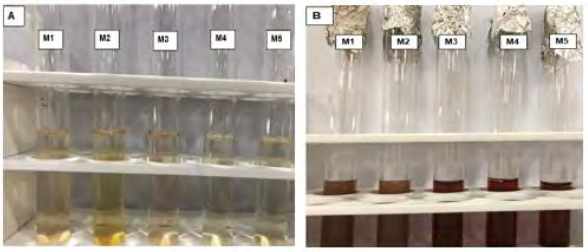


Figure 3. Observation of synthesized AgNPs (A: Before AgNPs synthesis; B: After AgNPs synthesis)

By the U/V spectrophotometer analysis the absorption maxima and stable peaks were formed for all optimization except at 50˚C for 15 mins (table 3), the best conditions of AgNPs synthesis were noted under RTP for 42 hours as shown in figure 4. Broadening of the peak can be due to formation of polydisperse AgNPs in the mixture and a well-defined peak between 400-480 nm corresponds to the surface plasmon resonance.[52,44]

Table 3. Interpretation of synthesis and optimization of AgNPs results

Sample	RTP for 42 hrs	50˚C for 15mins	90˚C for 30mins	90˚C for 60mins
M1	✓	×	×	✓
M2	✓	×	✓	✓
M3	✓	×	✓	✓
M4	✓	×	✓	✓
M5	✓	×	×	✓

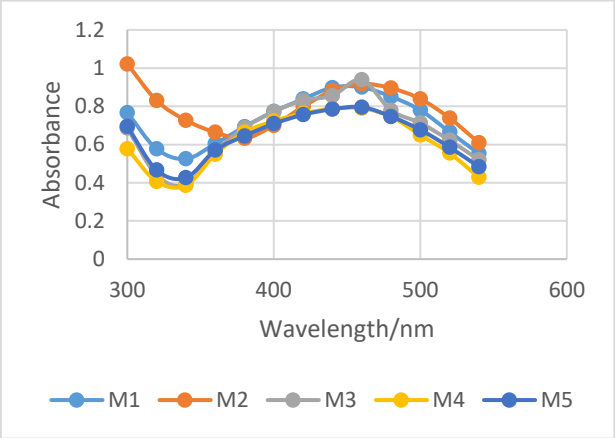


Figure 4. U/V spectrophotometer analysis of AgNPs at RTP for 42 hours

The band gap energy is the difference between the top valence band and bottom conduction band which electrons jump from one band to another and is calculated by the equation stated in figure 5.[53]

$$E = h\nu = \frac{hc}{\lambda}$$

Figure 5. Band gap energy equation

Levels of conductivity can be differentiated into semi-conductors and insulators. Semi-conductors are known to have a band gap energy $\leq$ 3.0 eV and insulators $\geq$ 3.0 eV. Bandgap of materials is important in conclusion of photo activity and conductance of the material. The light in visible spectrum is not absorbed by substances with wider band gaps.[54,55] Table 4 shows the band gap energy and conductivity properties of the samples.

Table 4. Conductivity property of samples

Sample	Wavelength/nm	Band gap energy/ eV	Conductivity
M1	460	2.7	Semi-conductive
M2	460	2.7	Semi-conductive
M3	460	2.7	Semi-conductive
M4	460	2.7	Semi-conductive
M5	460	2.7	Semi-conductive

3.4. SEM Images. The particles are spherical in shape, agglomerated crystals and AgNPs were 50 nm of size that lies within in the nanoscale thereby confirming the formation of AgNPs (figure 6).

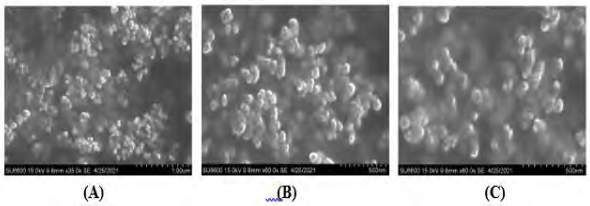


Figure 6. SEM image of biosynthesized AgNPs (A) – x35.0k / scale - 1 µm , (B) – x60.0k / scale - 500nm, (C) – x80.0k / scale - 500 nm)

3.5. Antioxidant Assays. Antioxidant Activity of the sample was analysed by the following techniques.

3.5.1. Total Flavonoid Content. Total Flavonoid Content (TFC) which is determined by using the aluminium chloride colorimetric method. $AlCl_3$ react with the keto or hydroxyl groups of flavonoid compounds forming an acid-stable complex with these groups. AgNPs showed higher TFC than water extracts. AgNPs of M1, M2 and M3 showed the highest TFC values than M4 and M5 (figure 7). One- way ANOVA that was conducted showed us that the p value 6.01E-06 is lesser than 0.05 and F > Fcrit (F- 109.6608, Fcrit- 5.317655) thereby, suggesting that there is a significant difference between the water extract and AgNPs.[56,57]

3.*5.2. Total Phenolic Content.*
Total Phenolic Content (TPC) the commonly used colorimetric technique is by Folin-Ciocalteu (FC). FC functions by the electron transfer from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes under alkaline conditions. This electron transfer facilitates a colour change that is detected at 760 nm due to the reduction of FC

reagent. AgNPs showed higher TPC than water extracts. AgNPs of M1, M2, M3 and M4 showed the highest and M5 showed the lowest TPC content (Figure 8). One- way ANOVA that was conducted showed us that the p value 7.9E-07is lesser than 0.05 and F > Fcrit (F- 186.8618, Fcrit- 5.317655) thereby, suggesting that there is a significant difference between the water extract and AgNPs.[58, 59]

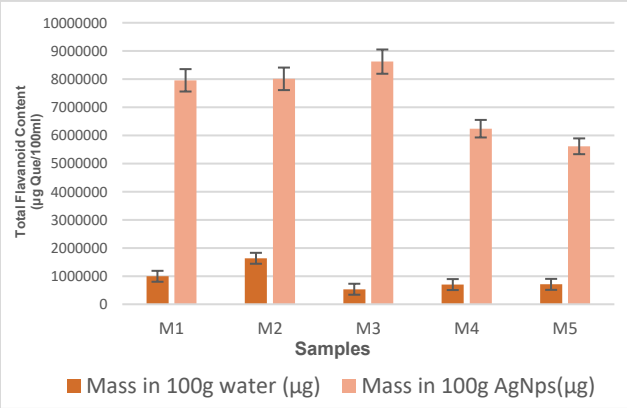


Figure 7. TFC analysis of AgNPs which are expressed as Quercetin equivalents

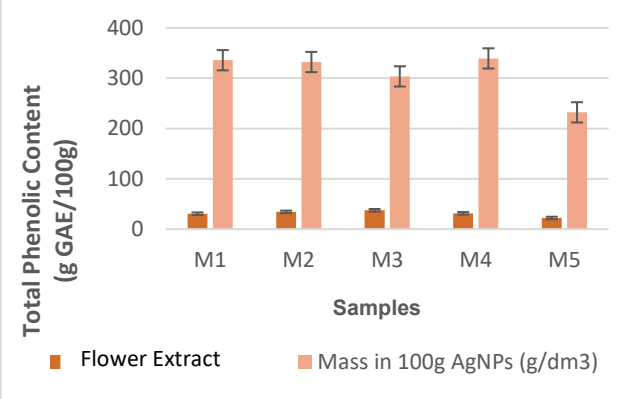


Figure 8. TPC analysis of AgNPs which are expressed as Gallic acid equivalents

3.5.3. Total Antioxidant Capacity.

Total Antioxidant Activity (TAC) mainly depends on chemical mechanisms such as single electron transfer (SET), hydrogen atom transfer (HAT) or chelating transition metals. SET involved in a redox reaction with the oxidant known as the radical that act as the indicator of the endpoint of reaction. Phosphomolybdenum method is used in TAC. A greenish blue complex is formed under acidic conditions due to the reduction of ammonium molybdate into an oxide known as 'keggin ion' $[H_3PO_4(MoO_3)_{12}]$ and this ion reduces into $[H_4PMo_8^{VI}Mo_4^{V}O_{40}]^{3-}$. AgNPs showed higher TAC than water extracts. AgNPs of M1, M2 and M5 showed the highest and M4 showed the lowest TAC content (figure 9). One- way ANOVA that was conducted showed us that the p value 0.00503 is lesser than 0.05 and F > Fcrit (F- 31.48948, Fcrit- 5.317655) thereby, suggesting that there is a significant difference between the water extract and AgNPs. [46,58,60]

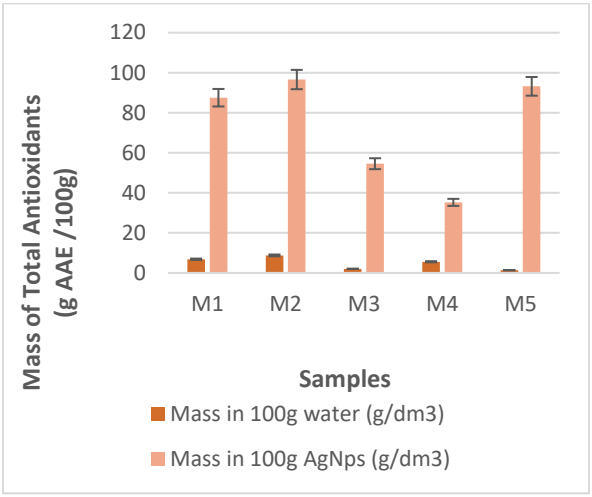


Figure 9. TAC analysis of AgNPs which are expressed as Ascorbic acid equivalents

Correlation analysis were carried out to find the interaction among the antioxidants. High correlation values among TPC, TAC and TFC indicates the contribution of phenols and flavonoids to the antioxidant activity (figure 10). Similar research was carried on *Ixora coccinea* flowers, leaves and stems by Torey and his co-workers[46], stated that all the antioxidant assays exhibited higher antioxidant activity on flower extracts compared to compared to leaves and stems.

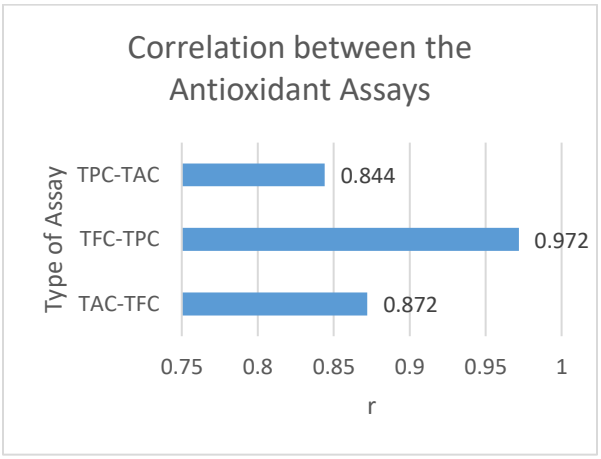


Figure 10. Correlation between antioxidant assays

3.5.4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity.

Free radical scavenging activity of the AgNPs and water extracts were tested by the DPPH technique. It assesses the interaction between antioxidants in the extracts with the stable free radical DPPH that is a major factor in biological damage

caused by oxidative stress, it has a deep violent colour and turns into yellow colour after the reaction. The scavenging potential of the sample is determined by the degree of discolouration and its absorbance values.[61-63] One- way ANOVA that was conducted showed us that the p value 3.86E-07 is lesser than 0.05 and F > Fcrit (F- 224.896, Fcrit- 5.317655) thereby, suggesting that there is a significant difference between the water extract and AgNPs. AgNPs showed higher DPPH Activity than water extracts, M5 showed the highest among other AgNPs (figure 11) and the finding is correlates with the TAC analysis from this study.

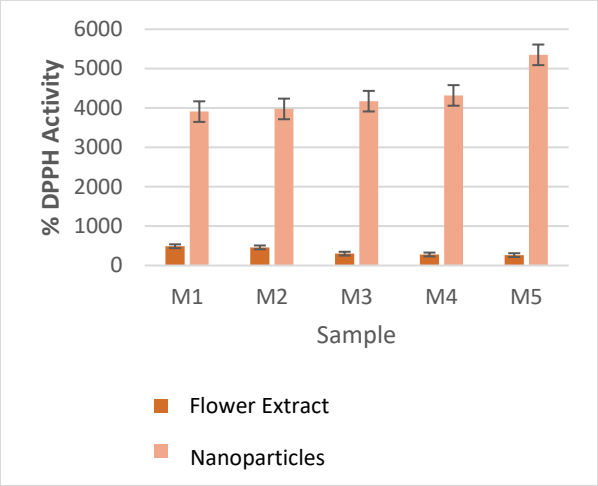
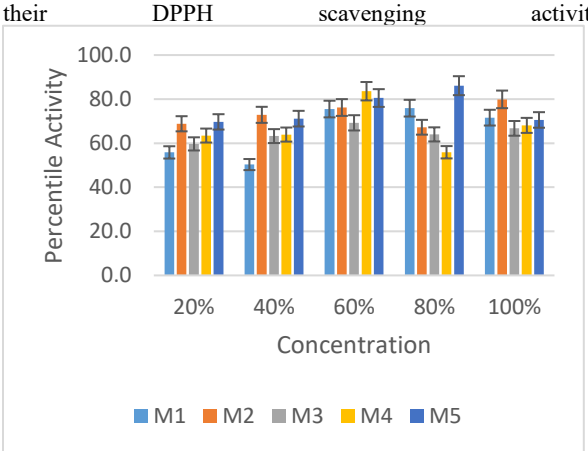


Figure. 11 % DPPH activity of synthesized AgNPs and Water extract

3.5.5. Median Inhibitory Concentration (IC50. IC50 determines the sample concentration that is required to scavenging the 50% of free radicals present. AgNPs have similar or lower IC50 compared to the water extracts (figure 12). Similar finding has been reported by Torey and his co-workers[46] for Stem and leaves. Previous studies conducted found that *Ixora coccinea* flowers and leaves have a strong reducing power abilities and free radical scavenging[64] and there is a good correlation between phenolic content of different parts of plant extract and their DPPH scavenging activity.



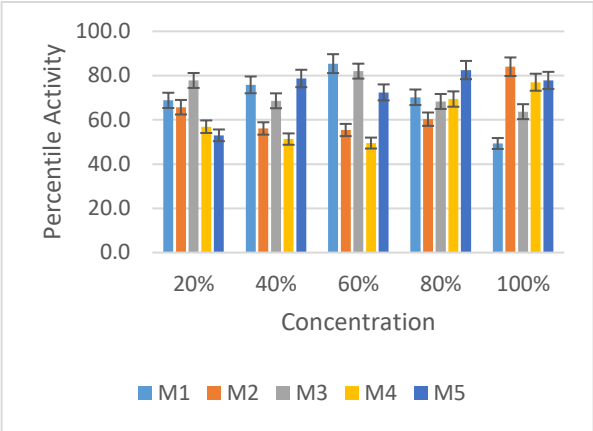


Figure 12. IC_{50} analysis of a) Water extracts b) AgNPs

AgNPs have a lower IC_{50} compared to the water extracts except in M1 and M3 this shows that AgNPs have higher radical scavenging activity in M2, M4 and M5 compared to the water extracts (table 5).

Table 5. IC_{50} for water extracts and AgNPs

Sample	Median Inhibitory Concentrations (IC_{50})	
	Water extract	AgNPs
M1	68.8	69.9
M2	73.0	64.3
M3	64.6	72.1
M4	67.0	60.8
M5	75.6	72.9

3.6. Photocatalytic Activity. Photocatalytic degradation of MB was carried out by using synthesized AgNPs under sunlight. Dye degradation was initially identified by colour change of deep blue colour into light blue (figure 13).

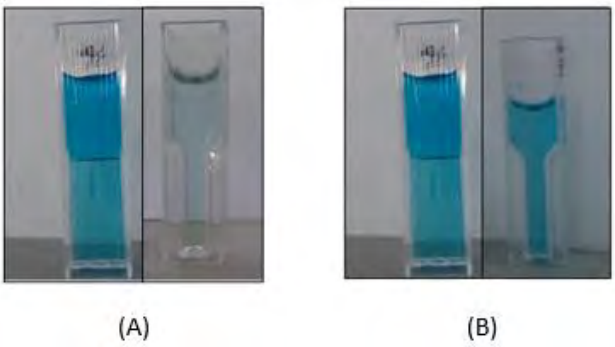
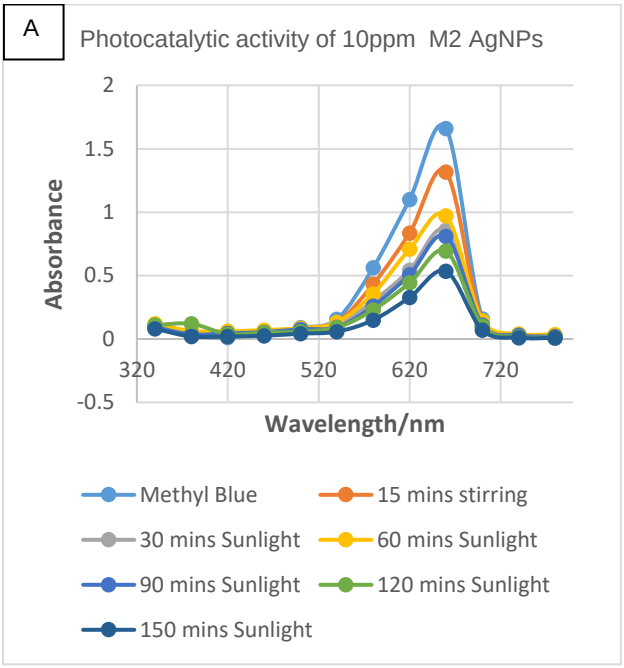


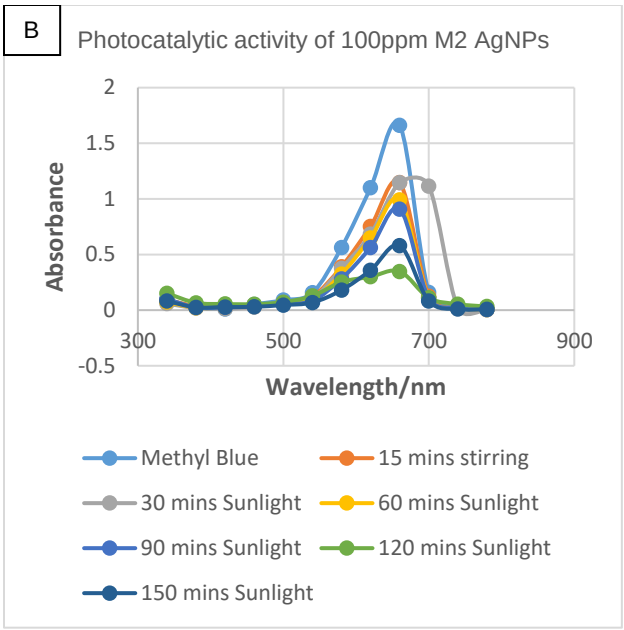
Figure 13. Colour change of MB A) with catalyst, B) without catalyst

AgNPs acts as an electron acceptor, it accepts electrons obtained from the extract and it also acts as an electron donor for MB. Therefore, AgNPs acts as a 'redox catalyst', this effect is called as 'electron relay effect' [65, 66]. Methyl dye degraded well and much faster with the presence of a catalyst in comparison to the concentrations in the absence of the catalyst.

Methyl dye degraded well under sunlight than in U/V. Highest photocatalytic is shown under sunlight in the presence of catalyst.

At 10 ppm, 100 ppm and 500 ppm it showed a peak at 660 nm with a faster degradation due to decrease in absorbance (figure 14). At 100ppm peak at 30 mins and 500 ppm peak at 60 mins have been shifted to the right-hand side which is known as 'blue shift'. The blue shift at this peak can be due the formation of N-demethylated intermediates in the degradation process. MB becomes lighter due to the removal of auxochromic groups (methyl or methylamine) that play a role in photocatalytic degradation. Hence, degradation of the dye in presence of sunlight is due to demethylation process.[40,67]





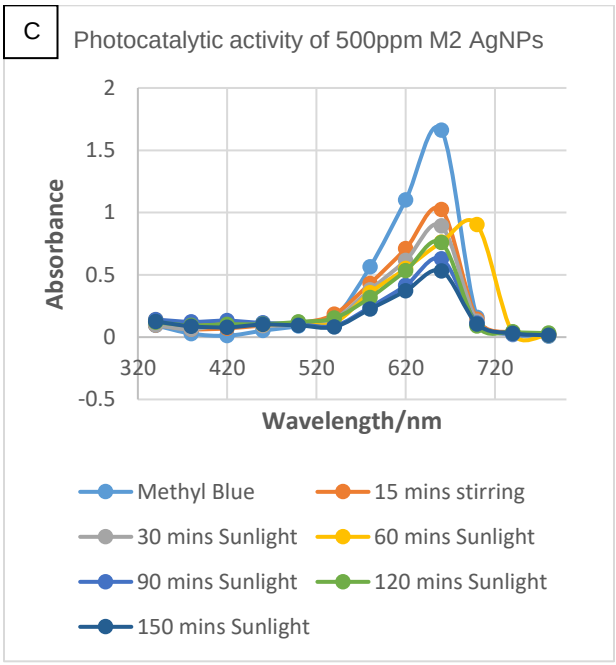
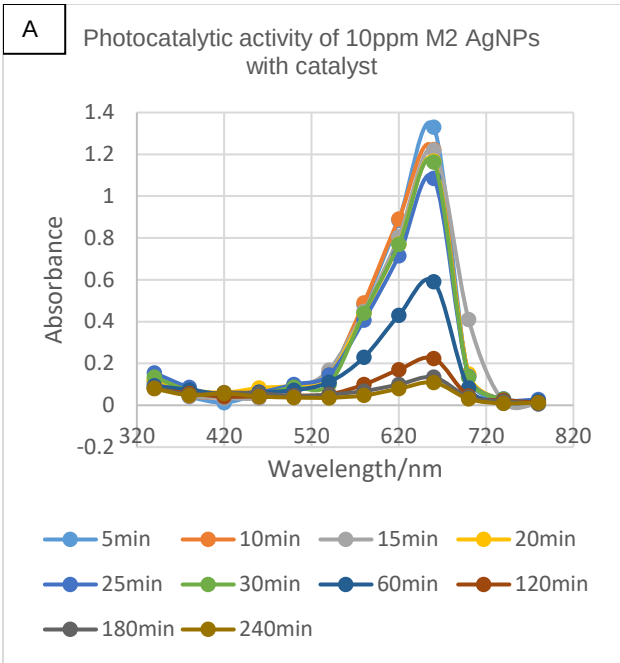


Figure. 14 Photocatalytic activity of A) 10 ppm and B) 100 ppm C) 500 ppm M2 AgNPs on MB

At 10 ppm and 100 ppm with $NaBH_4$ (catalyst) showed a peak at 660 nm (figure 15) with a much faster degradation compared to without catalyst in a period of 240 mins, the peak is almost flattened. A completely flattened peak is observed 100 ppm in a period of 180 mins but in 10 ppm a partial flattened peak is observed in a period of 240 mins. Observation of the reduction in absorbance peak suggests that AgNPs have the potential in MB degradation.[66]



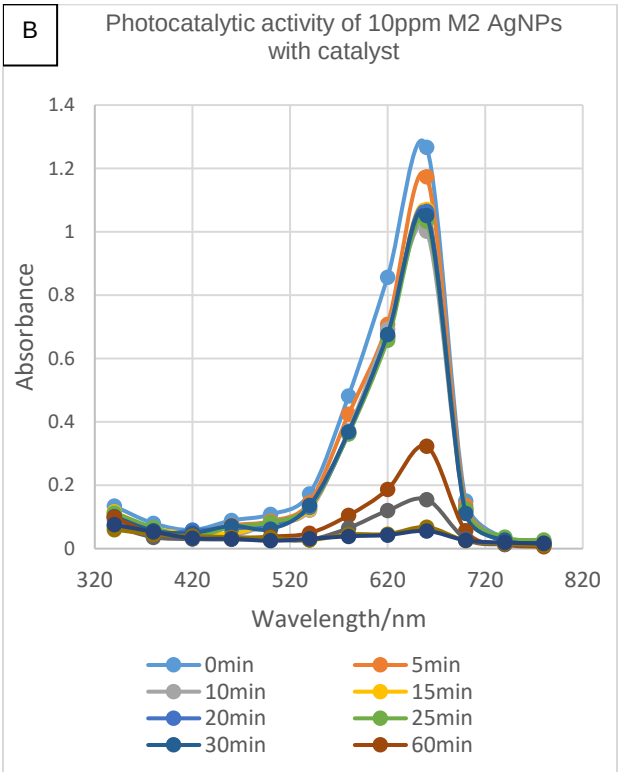


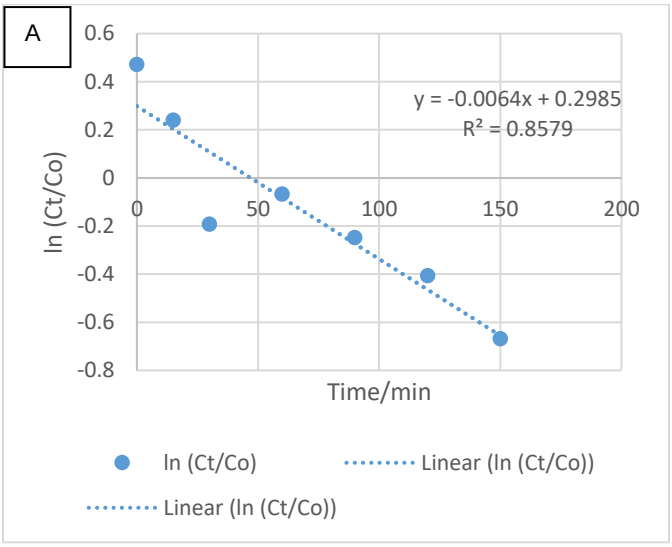
Figure 15. Photocatalytic activity of A) 10 ppm and B) 100 ppm M2 AgNPs on MB with catalyst

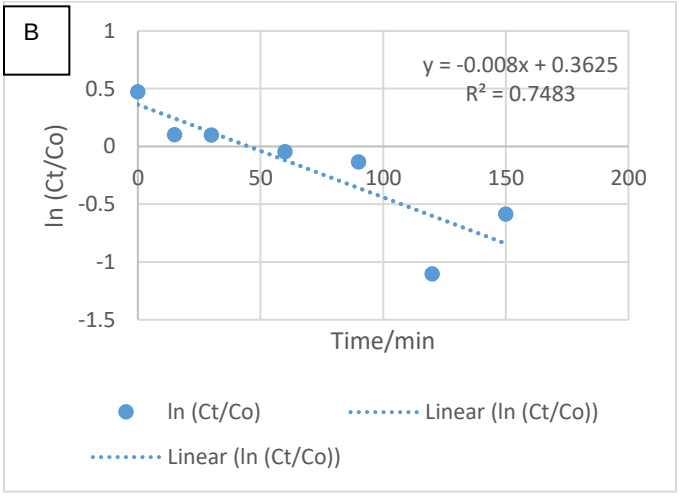
Rate constant indicates the relationship between the rate of chemical reactions and its concentration of the reacting substances. Rate constant values of catalysts are much higher compared to values without catalyst (table 6).

Table 6. Rate constant values

Concentration of M2 AgNPs	Rate constant values
10ppm	0.0064
100ppm	0.008
500ppm	0.0058
10ppm with $NaBH_4$	0.0123
100ppm with $NaBH_4$	0.0151

100 ppm showed the highest photo degradation with and without the catalyst. The degradation of the dye is increased with increase concentration of $NaBH_4$ (figure 16 and 17). With increased AgNPs concentration there is a high availability of active site onto AgNPs catalyst. Therefore, large number of MB dye molecules interact with the active sites and get degraded.[68] Photocatalytic activity can be enhanced by using polyvinylpolythene (PVP) capped nanoparticles. PVP act as a capping agent, the removal of MB dye will be increased efficiently.[69]





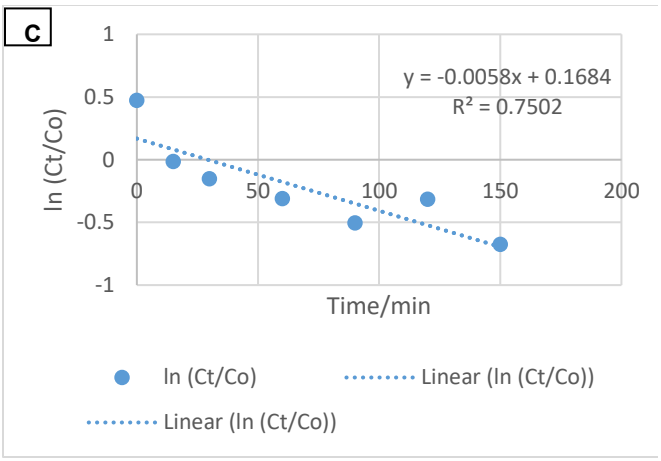
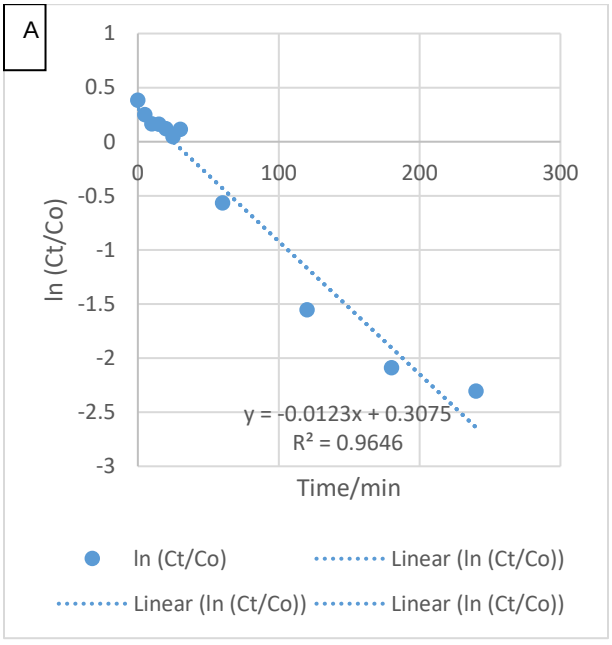


Figure 16. Rate constant graphs for A) 10ppm and B) 100 ppm C) 500ppm M2 AgNP samples



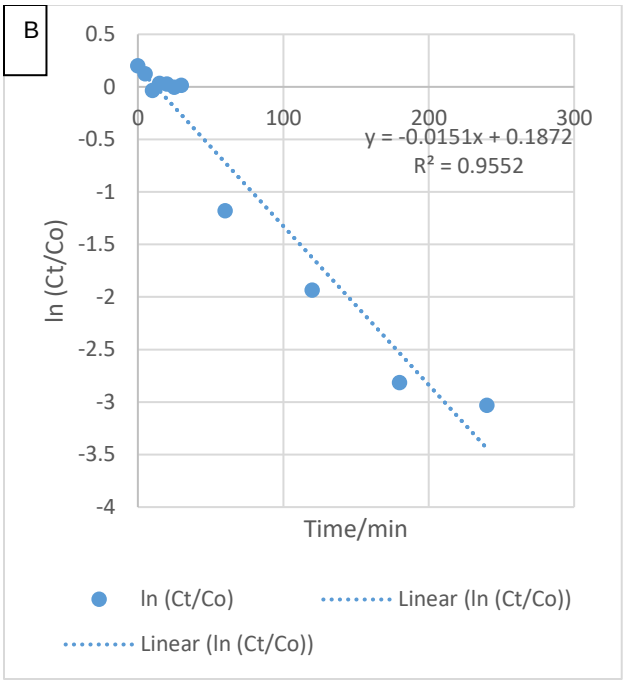


Figure 17. Rate constant graphs for A) 10ppm and B) 100 ppm with catalyst M2 AgNP samples

3.7. Antimicrobial Activity. Antimicrobial activity of the green synthesized AgNPs was studied by using the well disc diffusion method. As the cell membrane is negatively charged and the silver ion is positively charged it can cross the membrane easily and degrade the cell by reacting with thiol groups present on the membrane.[43]

The maximum zone of inhibition was recorded against *Ixora coccinea* followed by *S. aureus* (gram positive) and *E. coli* (gram negative). *E. coli* showed a higher zone of inhibition compared to *S. aureus.* Therefore, antimicrobial activity is higher in *E. coli* (figure 18 and 19).

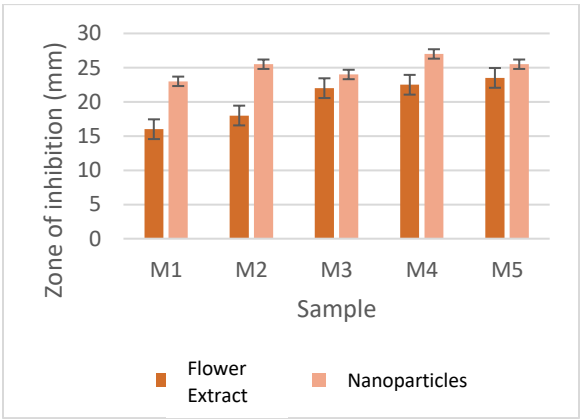


Figure 18. Anti-microbial analysis of AgNPs extract vs. Water extract using *E. coli*

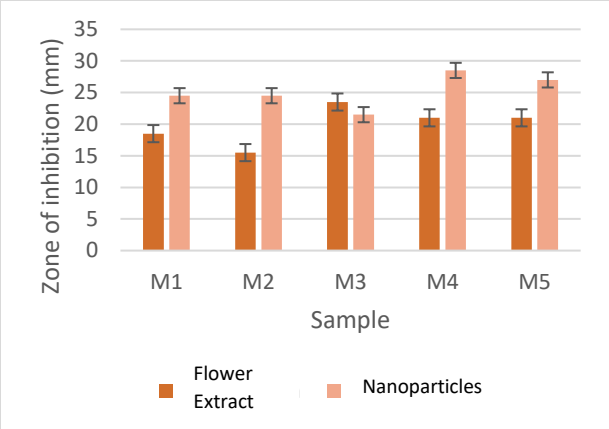


Figure 19. Anti-microbial analysis of AgNPs extract vs. Water extract using *S. aureus*

AgNPs showed a higher zone of inhibition compared to water extracts therefore, AgNPs showed higher antimicrobial activity (figure 20 and 21).

As noted by Hamid and his co-workers.[44] The AgNPs has a higher potency against gram-negative organism compared to gram-positive but Vinay and his co-workers[43] states that *S. aureus* has a higher antimicrobial activity compared to *E. coli*. Nalvolthula, Mrugu and Rudra[70] noted that, *S. aureus* with 5 µL shows a higher zone of inhibition compared to *E. coli* with of AgNPs but with 10 µL of AgNPs *E. coli* shows a higher zone of inhibition. One- way ANOVA that was conducted for *E. coli* showed us that the p value 0.02067 is lesser than 0.05 and F > Fcrit (F- 8.265625, Fcrit- 5.317655) thereby, indicating that there is a significant difference between the water extract and AgNPs. One- way ANOVA that was conducted for *S. aureus* showed us that the p value 0.019037 is lesser than 0.05 and F > Fcrit (F- 8.577099, Fcrit- 5.317655) thereby, indicating that there is a significant difference between the water extract and AgNPs.

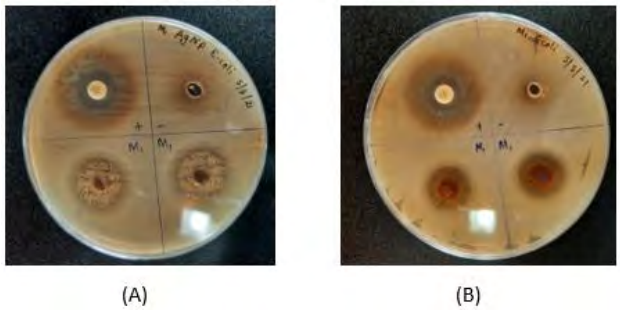


Figure 20. Zones of inhibition of M1 A) AgNPs B) Water extracts using *E. coli*

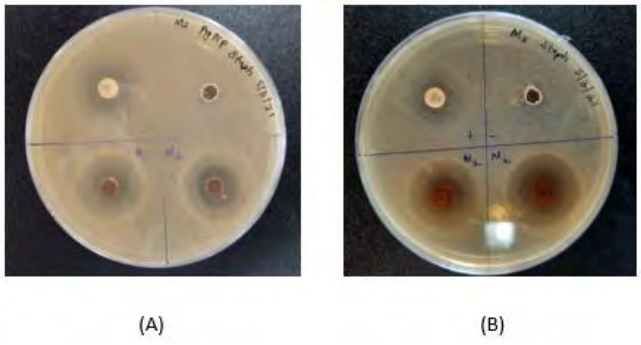


Figure 21. Zones of inhibition of M3 A) AgNPs B) Water extracts using *S. aureus*

Conclusion

In conclusion, all five *Ixora coccinea* synthesized AgNPs showing the presence of all phytochemicals except anthocyanin and carbohydrates. Presence of brown solution and formation of a peak between 420–480 nm for the optimization of AgNPs except 50˚C for 15 minutes and SEM images confirmed the presence of AgNPs. All AgNPs showed higher antioxidant activity and DPPH activity compared to water extracts. AgNPs have similar or lower IC_{50} compared to the water extracts. 100 ppm showed the highest photo degradation with and without the catalyst. AgNPs showed higher antimicrobial activity compared to water extracts. *E. coli* showed a higher antimicrobial activity compared to *S. aureus.*

Acknowledgements

The authors would like the acknowledge Business Management School (BMS) for funding and the Sri Lanka Institute of Nanotechnology (SLINTEC) for permitting the use of Hitachi SU6600 SEM.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Determination of Enterobacteriaceae (*Escherichia coli*, *Salmonella*) and *Vibrio cholerae* in *Centella asiatica* (Gotukola) samples

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Abstract

Healthy diets should include leafy green veggies. The usage of leafy greens, such as *Centella asiatica* (Gotukola), has increased in recent years as a result of suggestions for a healthier life. Nevertheless, as it is typically eaten raw or barely cooked, gotukola is prone to spreading foodborne illnesses. Controlling bacterial contamination during the pre-harvest and post-harvest stages is needed to avoid foodborne disease outbreaks. The purpose of this study was to detect the presence of *Escherichia coli*, *Salmonella*, and *Vibrio cholerae* in Sri Lankan gotukola leaves. Ten fresh gotukola samples were collected for this study from supermarkets and open air markets in Western, Southern, Central and North - Western provinces of Sri Lanka. All samples were evaluated using MacConkey agar culture, TCBS agar culture and biochemical assays for further bacterial confirmation of *Escherichia coli*, *Salmonella*, and *Vibrio cholerae*.

Keywords: *Escherichia coli*, *Centella asiatica*, Gotukola, antimicrobial activity

1. Introduction

The recent worldwide trend of switching from synthetic to herbal medication is known as "return to nature." As a result, both developing and developed countries have boosted their demand for plant-based medications. Non-toxicity, great effectiveness, and affordability are few reasons for this current shift to herbal treatments.[1] *Centella asiatica* is a medicinal plant that has therapeutic properties. It is a flowering plant in the Apiaceae family that grows as a perennial herbaceous plant.[2] *Centella asiatica*, often known as gotukola in Sri Lanka, can be found in household gardens. India, Sri Lanka, Thailand, China, Indonesia, and Madagascar, as well as other tropical and subtropical Asian countries, are home to this species.[3] It is generally used as a culinary vegetable as well as a medicinal herb and can be used in a variety of ways. Essential nutrients, carbohydrates, proteins, vitamins, and minerals, as well as potent antioxidants and plant-based bio chemicals, are abundant in *Centella asiatica*.[4] Gotukola is a blood purifier that helps the circulatory system's veins and capillaries stay healthy. It's also widely used to aid children with hyperactivity and memory problems.[5] Additionally, Ayurvedic treatment makes use of gotukola juice, powder, herbal oil, tea and tablets.[6]

Foodborne pathogens are bacteria that can infect humans due to the consumption of contaminated foods.[7] This is seen as a major public health threat that affects both developing and industrialized countries. The onset of foodborne pathogen-associated illnesses is influenced by host sensitivity, pathogen evolution and adaptation, changes in lifestyle, and pre and post-harvest stages of food production and manufacturing process.[8] Fresh vegetable consumption is becoming increasingly linked to human foodborne illnesses.[9] Because these are taken uncooked, there is a chance that germs could be present, posing a health risk.[10] During harvesting, handling, processing, and packing, fresh vegetables are at risk of contamination from chemical fertilizers, polluted water, unsanitary conditions and other sources.[11]

The purpose of this study was to use biochemical and microbiological techniques to determine the presence of *Escherichia coli, Salmonella* and *Vibrio cholerae* in Sri Lankan gotukola samples. Isolation and identification of bacterial colonies using MacConkey and TCBS agar medium, followed by a series of

biochemical assays depending on the bacteria's ability for lactose and non-lactose fermentation are the objectives of this study. In addition, the microbiological quality of the *Centella asiatica* samples were compared with different locations based on their geographical and storage conditions. *Enterobacteriaceae* is a vast bacterial family that includes numerous well-known members such as *E. coli*, *Salmonella*, *Shigella, Citrobacter* and *Klebsiella*.[12] They are Gram-negative, rod-shaped, facultatively anaerobic, motile bacteria that are commonly utilized for research due to their ease of handling and adaptability. They can be classified based on their ability to ferment lactose.[13]

Vibrio cholerae is a Gram-negative, facultative anaerobe bacterium with a comma-shaped morphology.[14] A flagellum is located at one of the bacterium's poles, and pili can be found all over its cell surface.[15] MacConkey agar culture media is used to isolate and distinguish gram negative lactose fermenting and lactose non-fermenting bacteria, specifically those from the *Enterobacteriaceae* family.[16] The MacConkey agar contains peptones, which are necessary nutrients for microbial growth.[5] Bile salts and crystal red hinder the growth of gram-positive bacteria, resulting in bacterial selection.[17] Neutral red acts as a pH indicator, turning pink when the pH falls below 6.8.[18] To isolate *Enterobacteriaceae* colonies, samples are grown on MacConkey agar media in the current study. To isolate and cultivate *Vibrio cholerae* and other *Vibrio* species from samples, the selective differential medium TCBS Agar is utilized.[19] Depending on whether *Vibrio* spp. can ferment sucrose, they either create yellow or green colonies on TCBS agar culture.[20] When sucrose is fermented, *Vibrio cholerae* produces yellow colonies, but *Vibrio parahaemolyticus* produces green colonies in TCBS agar medium.[21]

Biochemical assays are also carried out to establish the presence of *Enterobacteriaceae* in the cultured sample. Biochemical analysis of each sample is carried out in accordance with Bergey's manual. Because the study's main focus was on *E. coli, Salmonella,* and *Vibrio cholera* the lactose fermentation indole positive pathway and lactose non-fermentation indole negative pathway are also investigated. The capability of an organism to use citrate as its main source of energy is measured using the citrate test.[22] The capability of an organism to synthesize and maintain acid end products from glucose fermentation is assessed by the methyl red test.[23] Hydrogen sulfide test is mostly used to detect members of the *Enterobacteriaceae* family. This test aids in the recognition and classification of *Enterobacteriaceae* members.[24] The indole test determines whether or not an organism can digest tryptophan and create indole. The motility test is used to evaluate whether or not an organism is motile. The urease test identifies the formation of microorganisms that can hydrolyze urea to create ammonia and carbon dioxide.[25] The VP test is used to detect acetone in bacterial broth cultures. Alpha-naphthol and potassium hydroxide are added to bacteria-inoculated VP broth to perform this test.[26]

2. Methodology

2.1. Sample Collection. The *Centella asiatica* plant's fresh, undamaged, infection free leaves were collected from ten different geographical locations in Sri Lanka (Table 1). All samples were collected in ziplock bags and delivered to the lab within 24 hours, kept at room temperature before being analysed.

2.2 Sample Preparation. Collected samples were gently grinded using mortar and pestle and transferred to beakers. Peptone water was prepared, and was added to small beakers and mixed well (5 g of each gotukola sample was mixed with 20 ml of peptone buffer). Samples were filtered into labeled sterile plastic containers and kept in the incubator for 24 hours at 37°C and finally, refrigerated.

Table 1: Sample collection data sheet

Sample code	Date	Province	Location	Type of store
A_1	18/02/2022	Western	Aluthgama	Open air market
A_2	23/02/2022	Central	Dambulla	Open air market
B_1	19/02/2022	North-Western	Ganewatta	Supermarket
B_2	23/02/2022	Western	Kalutara	Open air market
C_1	18/02/2022	North-Western	Galgamuwa	Open air market
C_2	23/04/2022	North-Western	Kurunagala	Supermarket
D_1	18/04/2022	Western	Beruwala	Open air market
D_2	23/04/2022	Western	Panadura	Open air market
E_1	19/04/2022	Western	Matugama	Open air market
E_2	22/04/2022	Southern	Ambalangoda	Supermarket

2.3 MacConkey Agar Culturing. MacConkey agar was prepared. Inside the biosafety cabinet, MacConkey agar was poured into petri plates and kept until the culture medium solidified. Under aseptic conditions, a loop of prepared sample was streaked on MacConkey agar using the quadrant streaking technique. Incubated for 48 hours at 37°C and refrigerated[27].

2.4 TCBS agar culturing. Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS) agar was prepared. Inside the biosafety cabinet, TCBS agar was poured into petri plates and kept until the culture medium is solidified. Using the quadrant streaking technique, a loop of each prepared sample was streaked on TCBS agar under aseptic conditions. Incubated for 48 hours in 37°C and refrigerated[28].

2.5 Nutrient broth preparation. Nutrient broth was prepared. 35ml of nutrient broth was transferred into the falcon tubes. A distinct colony was isolated from a MacConkey agar plate and dissolved into its nutrient broth. This procedure was done for all the remaining samples near the bunsen flame and incubated for 48 hours at 37°C[29].

2.6 Biochemical tests. Bergey's manual was followed for performing biochemical tests on each sample. The lactose fermentation indole positive pathway and lactose non-fermentation Indole negative pathway were investigated because the study was focused on bacteria. Biochemical tests were performed under aseptic conditions near the bunsen flame and work benches were cleaned previously with 70% ethanol. A set of biochemical tests such as Indole test, Citrate test, Hydrogen sulfide test, Urease test, Motility test, Voges - Proskauer test and Methyl - Red test were performed to detect bacteria in each sample.

2.7 Indole test. Indole test was performed on both fermented and non-fermented samples. Tryptophan broth was prepared. In Tryptophan broth, isolated pink colonies from sub cultured agar plates of each gotukola sample were incubated at 37°C for 48 hours[30]. After the incubation period, 1-2 ml of Kovac's indole reagent was added, and the color changes were noted and recorded.

2.8 Citrate test. For all Indole positive results, Simmons citrate agar was prepared. Colonies from each sub cultivated gotukola sample were stab cultured on citrate agar slants. After, the agar slants were incubated for 48 hours at 37°C, and the color variations were noted and recorded.

2.9 Hydrogen sulfide test. Triple iron sugar agar was prepared for the Indole positive samples. 5mL of solution was kept at a slant form. Loop full of colony was taken and inoculated into the medium and incubated for 48 hours at 37°C[31]. The color changes were noted and recorded after 24 hours.

2.10 Urease test. For Indole positive results, Christensen's urea was prepared and mixed with 40% urea solution. 5mL of agar medium was added to test tubes in slanting position. Loop of isolated colony was taken and inoculated on the urea surface. Finally, test tubes were incubated at 37°C for 48 hours[32]. The color change was noted and recorded after 24 hours.

2.11 Motility test. Sulphide Indole Motility (SIM) agar was prepared for the Indole negative samples. On motility semi-solid agar, precise colonies from the subcultured samples were stab inoculated to the depth of ½ inch and

incubated at 37°C for 24 hours[33]. Results were noticed and recorded after 48 hours.

2.12 Voges-Proskauer test. The samples that tested negative for Indole performed Voges-Proskauer test. MRVP broth, Alpha-Napthol solution and KOH solution was prepared. 1mL of each MRVP broth with the bacterial growth was transferred into labeled sterile test tubes. Then 6 drops of Alpha-Napthol solution was added and 2 drops of KOH solution was added[34]. Color changes were observed and recorded after 20 minutes.

2.13 Methyl Red test. Methyl Red test was done for the samples negative for Indole test. MRVP broth and Methyl Red indicator were prepared. 5mL of the broth was introduced to the labeled sterile test tubes. Each sample of nutritional broth contained a bacterial colony that was transferred to sterile test tubes and incubated for 48 hours at 35°C to 37°C.[35] Then, 4 - 6 drops of Methyl Red indicator were added and color changes were observed and recorded.

3. Results

3.1 Microbiology analysis results. Lactose-fermenting bacterial colonies are pink, while non-lactose-fermenting bacterial colonies are colorless (Figure 1). Dark pink colorless dome-shaped colonies were prioritized when choosing colonies for biochemical tests.[1]

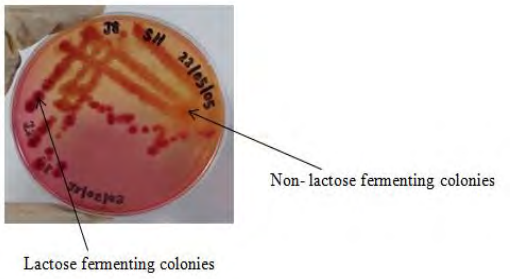


Figure 1. MacConkey agar colonies

Six of the ten samples used, formed pink colonies, accounting for 60% of the total, and lactose-fermenting bacteria were discovered in all six samples. And five samples (50% of the total) produced colorless colonies. D_2 sample was contained both pink and colorless colonies. After isolation of gram negative bacteria, they were tested for the presence of lactose and non-lactose fermenting bacteria.

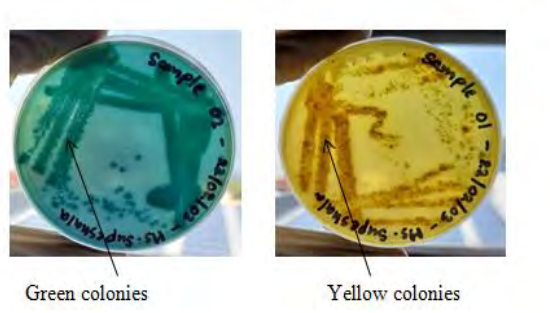


Figure 2. TCBS agar colonies

As shown in Figure 2, green colonies contained *Vibrio parahemolyticus* while yellow colonies contained *Vibrio cholera.*[36] Only 4 (Sample A_1, B_1, E_1, D_1) out of the 5 samples or 80% of the entire sample produced the yellow colonies that represented *Vibrio cholerae*.

3.2 Biochemical analysis results

Table 2. Biochemical test results interpretation

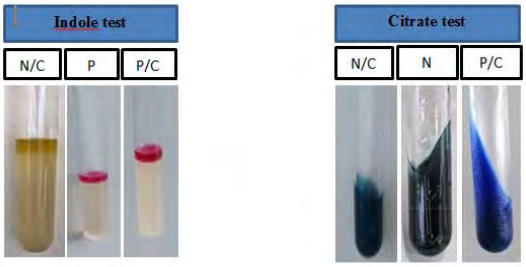
Biochemical test	Positive test result	Negative test result
Indole test	Cherry red ring	Yellow ring
Citrate test	Blue slant	Green slant
Urease test	Pink	Yellow
MR test	Red color	Remained yellow
VP test	Cherry red	Yellow brown
Motility test	Diffuse, hazy growths that cover the medium	Sharply confined to the stab line
H_2S test	Blacking precipitate	No black precipitate

3.3 Summary of the results obtained from microbiological and biochemical analysis. Based on the results from biochemical analysis for each sample organisms in each sample was identified as follows.

Among the 11 samples, *E. coli* was observed in 4 samples (36.37%) (Samples A_1, B_2, C_1, E_1). Lactose fermentation ability of the bacteria in these samples were confirmed using MacConkey agar growth results and

biochemical data for Indole positive and Citrate negative bacteria (Figure 3).

Figure 3. *E.coli* confirmatory biochemical test



result

There was evidence of *Klebsiella oxytoca* in one of the 11 samples (9.09%) (Sample D_1). Gram negative lactose fermenting which tested positive for Indole, Citrate, and VP test but negative for H_2S test (Figure 4) showed for the presence of *Klebsiella oxytoca*.

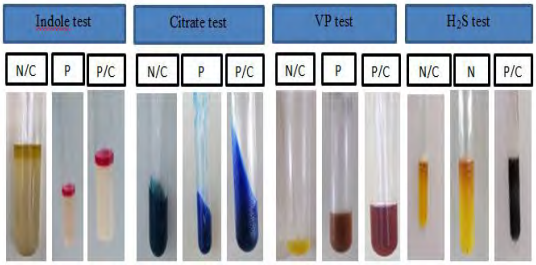


Figure 4. *Klebsiella oxytoca* confirmatory biochemical results

Out of the 11 samples only D_2 sample was confirmed the presence of *Citrobacter freundii* (9.09%). Gram negative lactose fermenting Indole and VP negative but MR and H_2S positive bacterial colonies indicated the presence of bacteria (Figure 5).

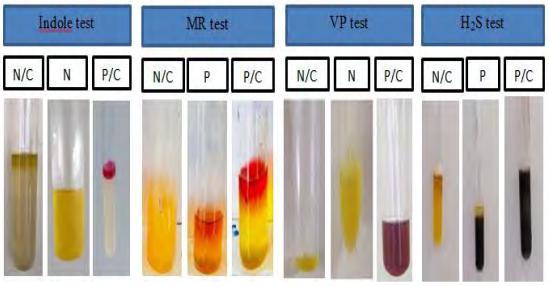
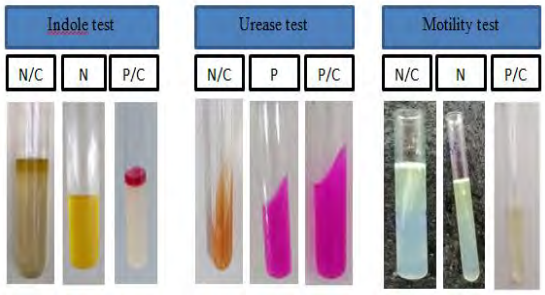


Figure 5. *Citrobacter freundii* confirmatory biochemical test results

Yersinia pseudotuberculosis was found 2 samples out of 11 samples (Samples E2 and D2) in 18.18%. These samples had colonies which were gram-negative and non-lactose fermenting and they tested negative for Indole and Motility test but positive for Urease test (Figure 6).

Figure 6. *Yersinia pseudotuberculosis*



confirmatory biochemical test results

Gram negative, non-lactose fermenting colonies, which tested negative for Indole and Urease and positive for Motility, and H_2S test (Figure 07) found in 2 out of the 11 samples, verified the presence of salmonella (18.18%) (Samples B1, C2).

Out of the 11 samples only A_2 sample was confirmed the presence of *Proteus vulgarius* (9.09%). This sample showed gram negative, non-lactose fermenting colonies which positive for Indole, Urease and H_2S tests (Figure 8).

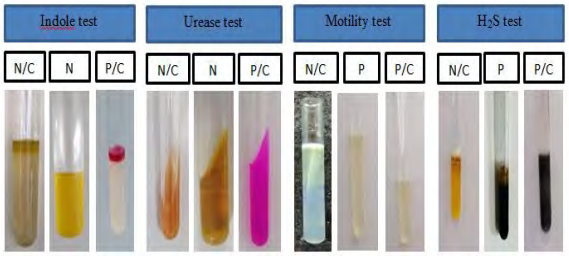


Figure 7. *Salmonella* confirmatory biochemical test results

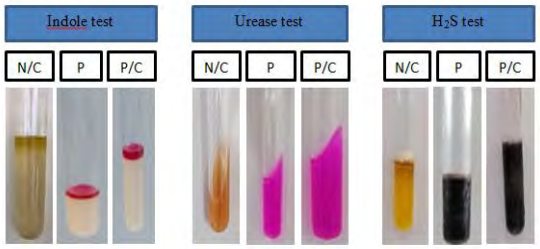


Figure 8. *Proteus vulgarius* confirmatory biochemical test results

Table 3. Summary of the results

Sample	MacConkey agar	Indole test	Citrate test	Urease test	VP test	MR test	Motility test	H_2S test	Confirmed Organism
D_2	Pink colonies	(-)			(-)	(+)		(+)	*Citrobacter freundii*
A_1		(+)	(-)						*E.coli*
B_2		(+)	(-)						*E.coli*
C_1		(+)	(-)						*E.coli*
E_1		(+)	(-)						*E.coli*
D_1		(+)	(+)		(+)			(-)	*Klebsiella oxytoca*
E_2	Color less colonies	(-)		(+)			(-)		*Yersinia pseudotuberculosis*
D_2		(-)		(+)			(-)		*Yersinia pseudotuberculosis*
B_1		(-)		(-)			(+)	(+)	*Salmonella*
C_2		(-)		(-)			(+)	(+)	*Salmonella*
A_2		(+)		(+)				(+)	*Proteus vulgarius*

Summary of the results obtained for all 10 samples were as follows (Table 3). D2 sample was obtained both pink and colorless colonies. Therefore, D2 was taken as two samples.

As shown in figure 9, the gotukola samples from the four provinces contained *Escherichia coli* (36.37%), *Citrobacter freundii* (9.09%), *Klebsiella oxytoca* (9.09%), *Yersinia pseudotuberculosis* (18.18%), *Salmonella* (18.18%), and *Proteus vulgarius* (9.09%).

Bacteria had a substantial distribution in the Western and North - Western provinces. Also, Western province demonstrated a wide distribution of bacteria due to the heavily polluted, largely urbanized, and unsanitary conditions (Figure 10).

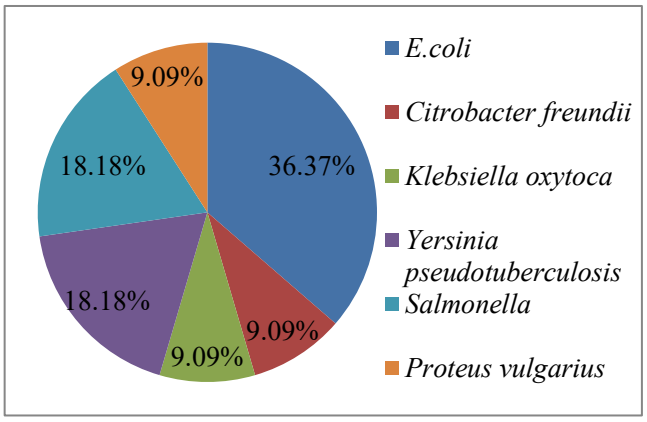


Figure 9. Microbiological and biochemical analysis results of gotukola

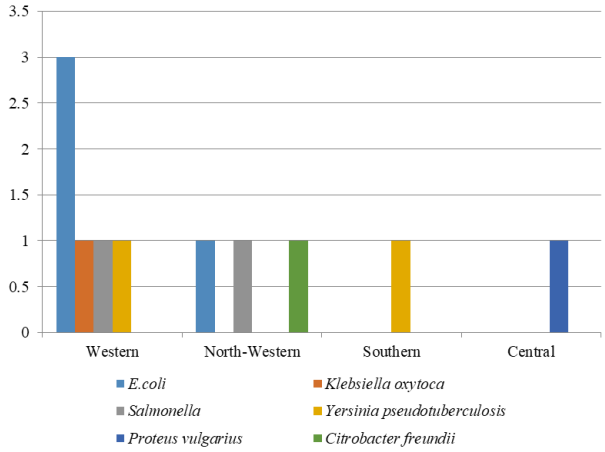


Figure 10. Regional distribution of bacterial contamination in *Centella asiatica*

4. Discussion

Centella asiatica (gotukola) leaves were collected from various regions of Sri Lanka to detect the presence of *Escherichia coli, Salmonella, and Vibrio cholerae* in gotukola samples representing different conditions and geographical locations. Young, undamaged, and infection-free leaves were chosen for the sample collection, as shown in the methodology. The goal of the current study was to determine the presence of bacteria *E. coli, Salmonella, and Vibrio cholera* in gotukola leaf samples from Western, Southern, Central, and Northern-Western Provinces of Sri Lanka. Samples were stored under different conditions upon buying from open air markets and supermarkets. Considering that they were collected from several regions, there is a high

probability for many samples to be contaminated with faeces[40].

In the current investigation, only 4 of the 5 samples (Samples A_1, B_1, E_1, and D_1), or 80% of the overall sample, formed the yellow colonies that represented *Vibrio cholera* during the TCBS agar culture examination (Figure 02). The green colonies represented *Vibrio parahemolyticus*, were only grown in one sample (Sample C_1), which accounted for 20% of the whole sample. Samples A_1, B_1, C_1, D_1, and E_1 were cultured on TCBS agar. After analysis, it was proved that *E. coli* contamination rate of gotukola samples from open air markets (36.37%) was greater than those from supermarkets. Many local markets are open air markets where food is exposed and handled room temperature. The dispersion of bacteria was significantly high in all four provinces. Hence, this study also showed that highly populated, largely industrialized areas in developing countries were more vulnerable to bacterial contamination of food due to the polluted water and unhygienic conditions.

Despite the fact that consuming green leafy vegetables like gotukola is crucial for maintaining good health, this study indicates that these foods can harbor a variety of food-borne pathogens such as *Escherichia coli, Salmonella, and Vibrio cholera*. This is due to the fact that several bacteria types were identified in all the samples. *Escherichia coli* showed that greatest dispersion in samples from the Western and North-western Provinces (36.37 %), whereas the bacteria had the least distribution in samples from the Southern and Central provinces.

When compared to the Western and North-Western provinces, Southern and Central provinces are less urbanized with low overall pollution. This may have caused the lower spread of bacteria.

Salmonella species was present (18.18%) in the samples from Western and North-Western Provinces, but was absent from the samples from southern and Central provinces. This might be due to the fact that, the respective samples were taken from areas with a lower level of microbial population and less population.

Green leafy products are prone to microbial contamination during both pre and post harvesting periods[39]. The risk of direct contamination rises during post- harvesting, when pathogenic bacteria get established on growing crops, prior to harvest[41]. Due to irrigation water pollution, contaminated containers and fertilizers containing animal manure, leafy crops are susceptible to contamination during pre-harvesting[13]. Especially, the widespread use of untreated irrigation water and sewage fertilizer in Sri Lankan agricultural practices is a major contributor to the pre-harvest microbial contamination of leafy vegetables[14].

According to the studies, *Klebsiella oxytoca* is seldom isolated from organic materials like vegetables[41]. Infected water is often where *Klebsiella oxytoca* is found. Also, it is possible that contaminated water used to wash the green leaves led to the presence of *Klebsiella oxytoca* in sample D_1 from the western province's seaside Beruwala region from open-air market. Certain investigation, imply that the contamination was directly caused by water infected with pathogenic *E. coli* that was sprayed on gotukola leaves[25]. The water sprinkled on green leaves may have been polluted and might be even contaminated with bacteria[36]. Bundling and storing gotukola with other vegetables may also increase the exposure to contamination of pathogens[25,35].

Moreover, it has been found that contaminated soil also can be infected the gotukola plant[17]. This indicates that post-harvest washing greatly affects the microbial contamination of leafy greens. This study was able to detect a noticeable dissemination of bacteria despite the samples being from local open air and super markets with diverse storage conditions. A study conducted in Thailand, discovered that samples of leafy green vegetables from open air markets and supermarkets respectively had 44% and 15%, bacterial contribution[28]. This is a notable difference in percentages. Therefore, it may be assumed that varied environmental and sanitary

conditions would have a substantial impact bacterial establishment. The findings demonstrate that *Proteus vulgarius* was detected in sample A_2 from the open air market in Dambulla, Central province. It is highly likely that gotukola samples were either directly or indirectly exposed to faecal contamination, as *Proteus vulgarius* was found in water and leafy vegetables. The poor hygiene of the merchants may also be responsible for this contamination[42].

In the samples taken from Aluthgama, Kalutara, Beruwala, Panadura and Mathugam, *Escherichia coli, Klebsiella oxytoca, Yersinia pseudotuberculosis*, and *Salmonella* were discovered. This could be due to the western province being one of the most populated and industrialized regions in the country, which shows a greater susceptibility for contamination of food products. The Western province has a broader bacterial distribution than the other provinces. The vegetable cages were surrounded by a lot of flies and market places were close to drainage lines carrying polluted water in the Western province. Additionally, insects are yet another potential cause of bacterial contamination. Certain studies show that bacteria can be directly transferred from contaminated files to crops.[7]

According to the current study, in order to prevent food-borne illnesses, gotukola should be properly cleansed with fresh water and disinfectants before consumption. During cooking, people frequently use turmeric water, vinegar, and salt solutions to wash green leafy vegetables.[12] But past studies have demonstrated that such disinfectants are poor at eliminating faecal coliform bacteria36. Given that gotukola is frequently consumed raw, it is important to consider washing procedures before consumption, as all the studied gotukola samples confirmed the presence of pathogenic bacteria.[43]

New advanced technologies can be suggested for future perspectives in order to detect the pathogens within a short time period accurately. Therefore, advanced methods are introduced in the molecular detection of microorganisms for further detection in this area of study. The most recent next-generation technology being utilized for the quick identification and categorization of microorganisms is Matrix-Assisted Laser Desorption/Ionization (MALDI). Its foundation is the use of brief laser pulses to ionize microbial cells, followed by the use of an electric field to accelerate the particles in a vacuum system[11]. Standard molecular techniques for detecting bacteria are 16S ribosomal RNA gene sequencing, restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) [27]. These techniques are used in both clinical and laboratory circumstances. The 16S rRNA gene is a prime target for identification since it is unique to each bacterial species[44].

Ribotyping, which is an rRNA, based phylogenetic analysis, flow cytometry, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) can also be used to identify the morphological characteristics of any pathogen of interest[45]. Nucleic acid-based detection techniques like Real-time PCR and microarray technologies like DNA micro array can be used to detect pathogens accurately[46]. The creation of microbe detection limits will continue to be an important step in microbiology and combination of these approaches and technology will develop the capacity for pathogen identification.

Conclusion

According to results of the microbiological and biochemical analysis, *Escherichia coli* (36.37%), *Citrobacter freundii* (9.09%), *Klebsiella oxytoca* (9.09%), *Yersinia pseudotuberculosis* (18.18%), *Salmonella* (18.18%), and *Proteus vulgarius* (9.09%) were substantially contaminated in the gotukola samples from the all four provinces.

Further research can be done to determine whether other bacterial species are present in gotukola. It is still possible to find out whether other bacterial species are present in gotukola by conducting further research. Hence in conclusion, gotukola leaves should not be consumed in their raw state due to the presence of pathogens in all of the samples

analyzed. Furthermore, follow washing treatments prior to the consumption and cooking it on a low flame will lessen the danger of infected with food borne diseases.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Plant mediated synthesis of silver nanoparticles using five different Lantana leaf extract and assessing their antioxidant, antibacterial and photocatalytic properties.

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Abstract

Silver nanoparticles (AgNPs) are used in a wide range of applications due to their antimicrobial, antioxidant, and photocatalytic activity. Cost-effective and eco-friendly methods for AgNP synthesis was thoroughly researched with available technologies. In this research varieties of Lantana leaves were used to synthesise AgNPs and determine antioxidant, antibacterial, and photocatalytic activity. The AgNPs was optimized at room temperature as overnight incubation. To characterize AgNPs UV-Vis spectroscopy and SEM were used. Phytochemicals such as tannin, saponins, alkaloids, terpenoids, carbohydrates, quinones, anthocyanin and coumarin were analyzed. The antioxidant capacity of AgNPs was measured and compared using Total flavonoid content (TFC), Total phenolic content (TPC) and Total antioxidant capacity (TAC). The water-soluble characterization of flavonoids in leaves were able to spectacularly reduce AgNPs. Their free radical scavenging activity was measured using DPPH and IC_{50}. The significant photocatalytic activity was demonstrated by K5NP through the degradation of Erichrome Black-T with sodium borohydride. *Escherichia coli* and *Staphylococcus aureus* were used to determine antimicrobial activity, where AgNPs showed significant effects. All the results were statistically analyzed using Microsoft Excel 2016 and one-way ANOVA statistical analysis was performed using IBM SPSS Statics 23 software. In this study green synthesis of AgNPs was studied significantly to provide valuable information to many fields for the betterment of the environment.

Keywords: Nanoparticles, Lantana leaves, Nanotechnology, phytochemicals, EBT

Introduction

Nanotechnology is one of the most active substantial research areas in modern science where it provides an understanding of the fundamental properties of objects. It is a study of manipulating materials at their atomic level by a combination of chemical, biological, and engineering approaches, and the synthesis of nanoparticles.[1] There is a rising commercial for nanoparticles because of their wide applications such as chemistry, energy, electronics, and medicine. There are two main types of nanoparticles as organic and inorganic nanoparticle.[2] Metal nanoparticles are the most widely studied nanoparticles due to their wide range of applications and easier to synthesize. In nanotechnology, different types of metals are being used but silver is the most preferred metal due to its properties such as chemical stability, catalytic activity, good conductivity, nontoxic, and antimicrobial activity. Silver has the ability to inhibit bacterial growth by damaging bacterial cell walls and disrupts cell metabolism.[3]

Two general approaches for the synthesis of nanoparticles are the top-down approach and the bottom-up approach. The top-down approach involves the formation of nanosized structures by breaking down the bulk materials. The bottom-up

approach involves building up material from the bottom: atom-by-atom, molecule-by-molecule, or cluster-by-cluster. Physical, chemical, and biological methods are used to synthesis nanoparticles.[4] However physical and chemical methods involve the usage of harmful chemicals which leads to a requirement of ecological and nontoxic nanoparticles. The biosynthesis method uses environmentally affable materials like bacteria, plant extract, and enzymes which appeared facile, cohesive for different biomedical application and better alternative for complex chemical synthetic methods. Compared to microorganism, the leaf extract is eco-friendly and compatible.[5]

Biological synthesis eliminates the usage of high pressure, energy, temperature, toxic chemicals and release environment-friendly products and byproducts. However, plant-mediated AgNps are non-hazardous and can be easily synthesized compared to microorganism mediated AgNPs. Plant extract can be obtained in various way since water-based extraction is ecofriendly compared to other chemical methods. Biological synthesis combines metal salts and plant extract to obtain nanoparticles. Nanoparticles synthesized using medicinal plants shows more benefits and AgNP enhance the antibacterial activity. Natural functional groups like amine, hydroxyl, carboxyl, and carboxylic groups are non-toxic reducing agents which facilitate the formation of metal ion complexes and reduces metal ions by oxidization. Plant extract mediated nanoparticle synthesis is influenced by factors such as time of reaction, pH and temperature.[6,7]

Lantana is a genus under the Verbenaceae family which is mostly known as a medicinal and ornamental herb. It is mostly found in tropical, subtropical countries and temperature regions.[8] *Lantana camara, Lantana involucrate, Lantana montevidensis, Lantana horrida and Lantana trifola* are the species that are studied under this project. Phytochemicals are natural bioactive compounds found in parts of plants and play important roles in the stabilization and reduction of nanoparticles. The leaves extract exhibit antimicrobial, antitumor and insecticidal activities and contain verbascoside, which possesses antimicrobial, immunosuppressive, and antitumor actives.[9]

According to Kalita *et al*. (2011), lantana leaves are rich in various phytochemical constituents such as triterpenes, steroids, glycosides, flavonoids, and essential oils.[10] It has various medicinal properties and used in the traditional medicinal system to cure various diseases like skin disease as dermatitis, cuts, itching, scabies, leprosy, rheumatisms, and chickenpox. Lantanoside, linaroside and camarinic acid are isolated from lantana which is investigated as potential nematicides.[11]

The function of an antioxidant is to trap free radical species, intercepting radical chained reactions and repairing oxidative damage. Free radicals are uncharged molecule having unpaired valency electron which is highly reactive and short lived. Free radicals and reactive oxygen species (ROS) are generated by normal metabolic actions. Oxidative stress is caused due to the imbalance of ROS and antioxidants which are an important risk factor in numerous pathogeneses of chronic diseases.[12]

Oxidative damage mediated by ROS to macromolecules incriminates within the pathogenicity of diseases like cancer, ageing, autoimmune diseases, rheumatoid arthritis, and cardiovascular disease.[13] There is increasing interest in natural antioxidants due to their wide-ranging activity compared to synthetic antioxidants. Antioxidant compounds such as polyphenols, phenolic acids and flavonoids scavenge free radicals inhibits the oxidative mechanisms that lead to degenerative disease. Since ancient times medicinal plants are considered to possess good antioxidant.[14,15] Organic dye is one of the major pollutants widely used in textile, medicine, and many other industries. Carcinogenic or toxic dye and their N-

substituted aromatic biotransformation products can be harmful to humans and the environment. The presence of aromatic rings and nitrogen bonds makes azo dyes non-degradable.[16]

The water bodies are accumulated with these dyes which cause eutrophication, toxic effects on aquatic life form by hindering the infiltration of sunlight and reduction of reoxygenation capacity. It also causes harmful effects on humans like allergic, some dyes are carcinogenic and tumor formation in the kidney, bladder, and liver. In wastewater treatment, the dominance of photocatalytic degradation by nanoparticles is due to its advantages over traditional methods, such as rapid oxidation, the absence of polycyclic materials and the oxidation of contaminants.[17] Recent studies show metal nanoparticles degrade dyes as an efficient photocatalyst at ambient temperature with visible light illumination. Biosynthesis nano catalysts are now commonly used for the effective removal of dye pollutants.[18,19]

The present study aims to synthesis AgNPs using five different types of Lantana leaves, characterization of AgNPs and assess their antioxidant, antibacterial and photocatalytic activity. Antioxidant activity will be assessed using TFC, TPC, TAC, DPPH and IC_{50} assays. Erichrome black-T will be used in determining photocatalytic activity. *Staphylococcus aureus* and *Escherichia coli* strains will be used to assess the antimicrobial activity of AgNPs. This research data is expected to be used in relevant future studies for the advancement of green mediated nanoparticle synthesis and betterment of prevailing environmental issues.

2. Methodology

The five different lantana plants (*Lantana camara, Lantana horrida, Lantana involucrate, Lantana trifola* and *Lantana montevidensis*) were collected from Rajagirya area. The leaves were washed, and shade dried. The leaves were labelled according to Table 1. Dried leaves were crushed using mortar and pestle. 2 g of each powdered sample was added into 50 ml distilled water (d.H_2O) separately and heated in a dry oven at 95°C for 20 min. Then it was let to cool and filtered into 50 ml falcon using Whatman filter paper No. 1. The falcons were labelled and stored at 4°C for further use.

Table 1. Labelling of samples.

Label	Sample
K1	*Lantana camara*
K2	*Lantana horrida*
K3	*Lantana involucrate*
K4	*Lantana trifola*
K5	*Lantana montevidensis*

2.1. Phytochemicals testing. Water extracts have been used to test for the presence of phytochemicals (Table 2).[20]

Table 2. Procedure for phytochemicals.

Phytochemicals	Procedure
Tannin	0.5 ml of leaf extract and few drops of 10% $FeCl_3$ were added into the test tube.
Saponins	0.5 ml of leaf extract and 1.5 ml of d.H_2O were added into a test tube and shaken vigorously.
Alkaloids	0.5ml of leaf extract and 0.5ml of Wagner's reagent were added into a test tube
Terpenoids	0.5ml of leaf extract, 1 ml of chloroform and 3 drops of conc. H_2SO_4 were added into the test tube.
Carbohydrates	0.5ml of leaf extract, 0.5 ml of conc.H_2SO_4 and few drops of Molisch reagent were added into the test tube.
Quinones	0.5ml of leaf extract and 0.5ml of conc. HCl were added into a test tube.
Anthocyanin	0.5ml of leaf extract, 0.5ml of NH_3 and few drops of conc. HCl was added to the test tube.
Coumarin	0.5 ml of leaf extract and 0.75ml 10% NaOH were added into a test tube.

2.2. Synthesis of silver nanoparticle (AgNP) using five different lantana species leaf extract. To 1 ml of each leaf extract, 9 ml of 1mM $AgNO_3$ was added into labelled test tubes. Then it was covered with a foil and stored overnight in a dark place at room temperature (RT). Colour change was observed. Samples were shaken and mixed well. The absorbance was measured at the wavelength of 300-540 nm. Optimization was carried out at 90°C for 30 min and 60 min using 1 ml of leaf extract and 9 ml of prepared AgNO3. Then the absorbance was measured at the wavelength of 300-540 nm.

2.3. Scanning Electron Microscopy (SEM) analysis. 2 ml of K5 AgNP was added into Eppendorf and centrifuged at 10 RPM for 2 min. The supernatant was discarded, and it was repeated till the pellet is observed. Then the pellet was dried at 40°C in the dry oven for 3 hours. SEM analysis was carried out at the Sri Lankan Institute of Nanotechnology (SLINTEC), Homagama, using Hitachi SU6600 SEM.

2.4. Dilution of samples. 1 ml of each leaf extract and AgNPs was added with 14 ml with d.H_2O to have 1:15 dilution. Diluted samples were stored at 4°C for further use.

2.5. Antioxidant assays. Diluted water extracts and AgNPs were used for the following assays.

2.5.1. Analysis of Total flavonoid content (TFC). 1.5 ml of sample was added into a test tube and 0.2 ml of 10% $AlCl_3$ and 0.2 ml of 1M potassium acetate was added. Then it was let to incubate for 20 min at room temperature (RT) and absorbance was measured in triplicates at 420 nm using a spectrophotometer with d.H_2O was used as a blank. TFC was expressed in equivalents of Quercetin in µg QE/100g.[21]

2.5.2. Analysis of total phenolic content (TPC). To 0.5 ml of dilute sample, 2.5 ml of 10% Folin-Ciocalto reagent and 2 ml of 7.5% sodium carbonate were added into each tube. Then it was let to incubate at RT for 30 min. The absorbance was measured in triplicates at 765 nm using a spectrophotometer with d.H_2O was used as a blank. TPC was expressed in equivalents of Gallic acid in mg GAE/100g.[21] (John et al., 2014).

2.5.3. Analysis of total antioxidant content (TAC). To 1.5 ml of dilute sample, 0.5ml reagent solution (mixture of 28mM Sodium phosphate, 0.6M H_2SO_4, 4mM Ammonium molybdate in 1:1:1 ratio) was added into tube. It was let to incubate at 95°C for 90 min. The absorbance was measured in triplicates at 695 nm using a spectrophotometer with d.H_2O was used as a blank. TAC was expressed in equivalents of Ascorbic acid in mg AAE/100g.[21]

2.5.4. Analysis of 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity. To 1 ml of 0.004% DPPH, 10 µl of the sample was added. Then it was let to incubate in a dark place for 30 min at RT. Absorbance was measured in triplicates at 517 nm using UV-Vis spectrophotometry with methanol as a blank. TAC was expressed in equivalents of Ascorbic acid in mg AAE/100g.[22]

2.5.5. Analysis of medium Inhibition concentration (IC_{50}). To 1 ml of 0.004% DPPH, 1ml of samples and d.H_2O were added in series of concentration (Table 3). Then it was incubated in a dark place for 30 min at RT. Absorbance was measured in triplicates at 517 nm using UV-Vis spectrophotometry with methanol as a blank.[22] DPPH scavenging activity was expressed in percentage inhibition. It was calculated using the following equation:

$$\text{Inhibition (\%)} = [(A_{CONTROL} - A_{SAMPLE}) / A_{CONTROL}] \times 100$$

Where $A_{CONTROL}$ is the initial absorbance of DPPH reagent, and A_{SAMPLE} is the absorbance of the sample.

Table 3. Concentration of the sample.

Concentration	Sample	d.H_2O
100	10 µl	0
80	8 µl	2 µl
60	6 µl	4 µl
40	4 µl	6 µl
20	2 µl	8 µl

*2.6. Analysis of antibacterial property***.** 13.3 g of Muller-Hinton agar was dissolved in 350 ml d.H_2O and 9.8 g of nutrient agar was dissolved in 350 ml d.H_2O. It was autoclaved and poured into Petri plates. *E. coli* and *S. aureus* were inoculated from the mother culture. The plates were labelled and streaked with *E. coli* and *S. aureus* respectively. 1 ml of leaf extract and AgNP was dried in the watch glass at 90°C for 5 min. Three wells were made in the plate using clean pipette tips for sample (2 replicant) and negative control (saline). Gentamycin was used as a positive control. The procedure was done in a fume hood. All the plates were incubated at 37°C overnight. The inhibition zone was measured by using a ruler in cm.[8]

2.7. Analysis of photocatalytic activity. To 50 ml of 2 mM Erichrome black-T (EBT) and 0.188 ml of 10 ppm, K5 AgNPs was added, and the absorbance was measured from 340-780 nm for 210 min using a spectrophotometer. The same procedure was carried out for 100 ppm and 500 ppm K5 AgNPs. To 50 ml of 2 mM EBT, 0.188ml of 10 ppm K5 AgNPs sample and 20µl of NaBH4 was added and the absorbance was measured from 340-780 nm for 210 min. The same procedure was done for 100ppm K5 AgNPs.[23]

2.8 Statistical analysis. Using Microsoft Excel 2016 One-way ANOVA statistical analysis was performed and using IBM SPSS Statistics 23 software correlation graphs were generated. $p<0.05$ was used as the significance cut-off for the analysis.

3. Results

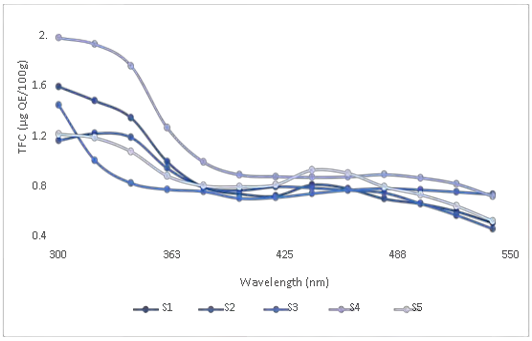

Figure 2. Spectrometric analysis of AgNP at RT.

Figure 3. Results of phytochemicals

Table 5. Spectrophotometry analysis of AgNP for optimization

Sample	30min (90C)	60min (90C)
K1	Absent	Absent
K2	Absent	Absent
K3	Absent	Absent
K4	Absent	Present
K5	Absent	Absent

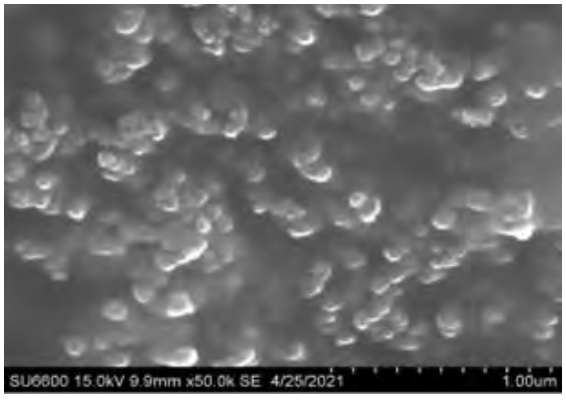

Figure 4. SEM imaging at 15.0kV 9.9mm x50.0k

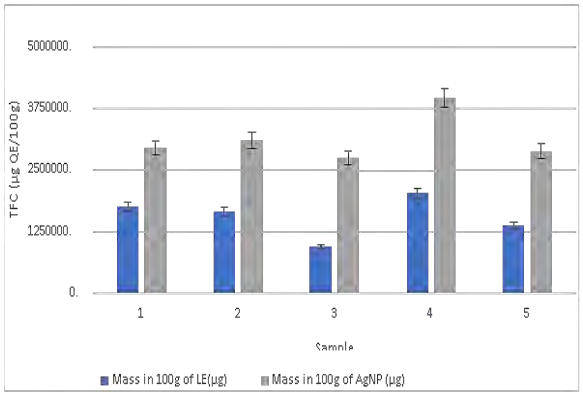

Figure 5. TFC of leaf extract and AgNPs.

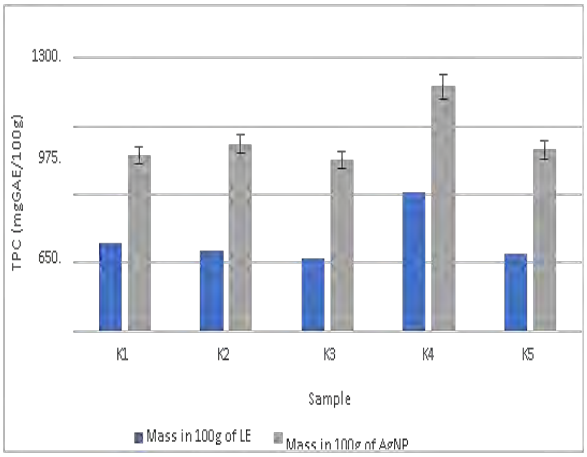

Figure 6. TPC of leaf extract and AgNP

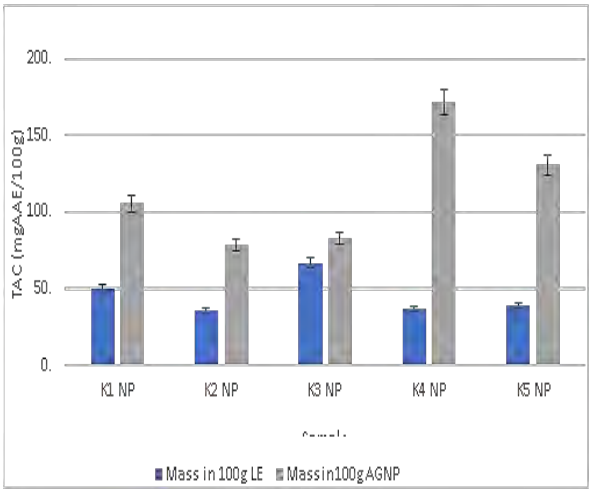

Figure 7. TAC of leaf extract and AgNPS.

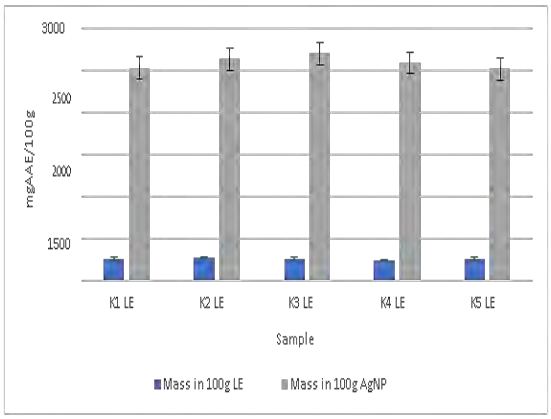

Figure 8. DPPH inhibition activity of leaf extract and AgNP.

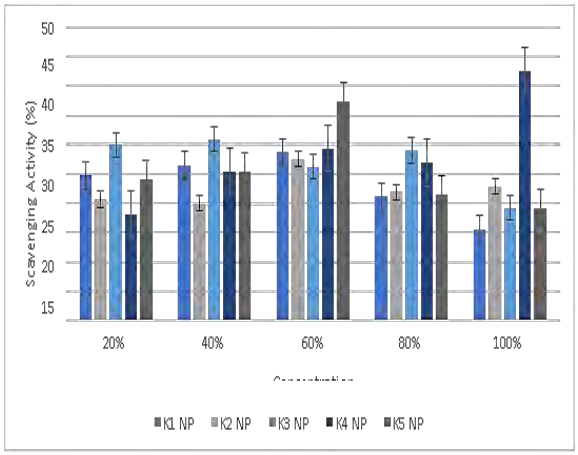

Figure 9. Inhibition concentration of the leaf extract.

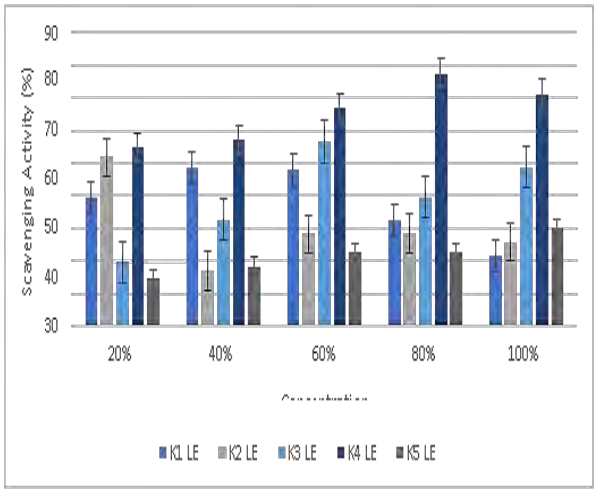

Figure 10. Inhibition concertation of the synthesized AgNP.

According to Figure 9 and 10, the highest IC50 was observed in K4LE and K4NP.

Table 6. IC50 of the samples expressed in the specific activity (%).

Sample	Leaf extract	AgNP
K1	66.231	24.543
K2	52.847	22.313
K3	36.279	28.265
K4	62.843	25.982
K5	20.146	13.154

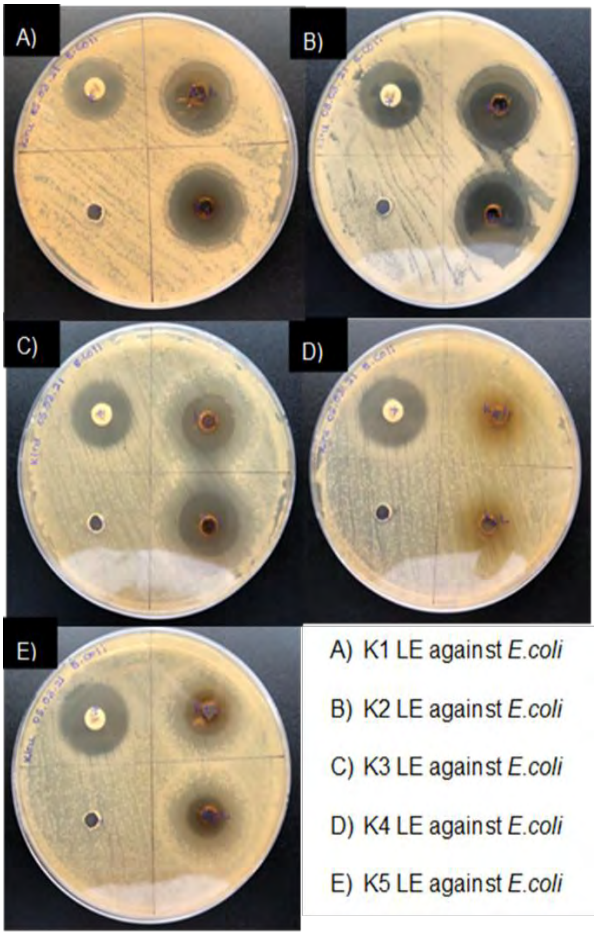

Figure 11. Antibacterial activity of leaf extract against *E. coli*.

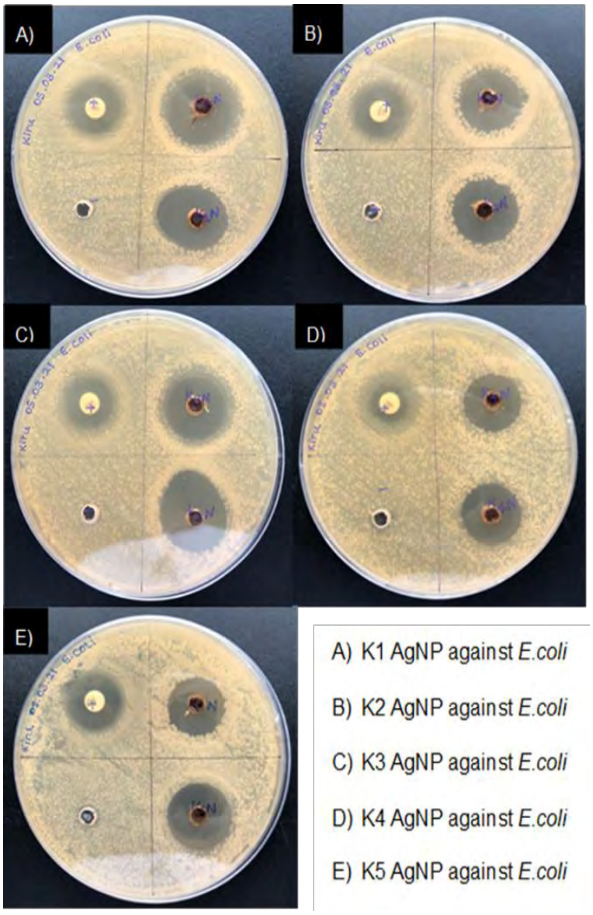

Figure 12. Antibacterial activity of AgNP against *E.coli.*

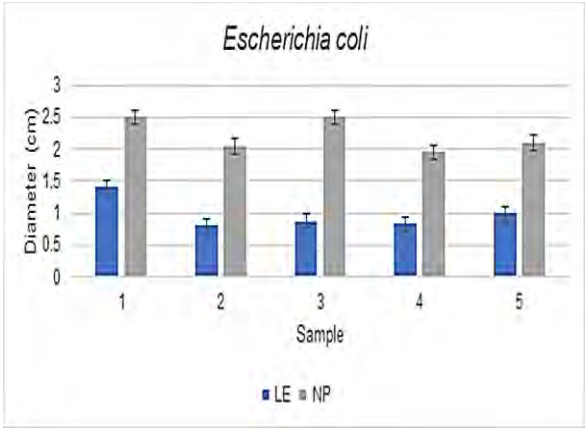

Figure 13. Antimicrobial activity against *E.coli* using leaf extract and AgNPs.

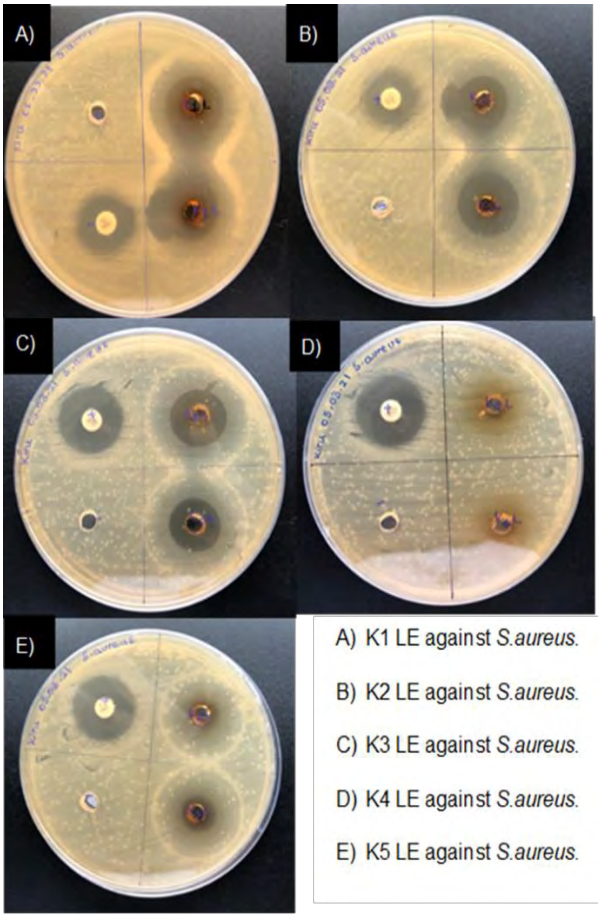

Figure 14. Antibacterial activity of Leaf extract against *S. aureus***.**

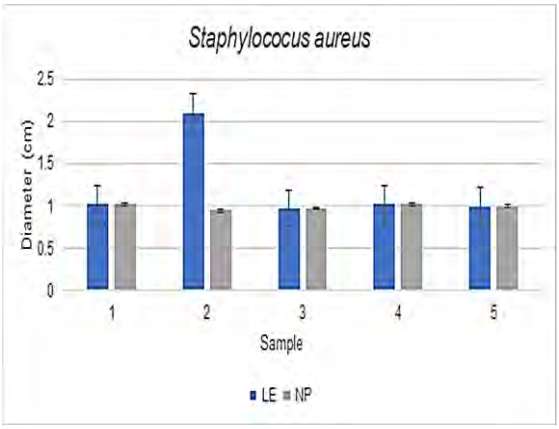

Figure 15. Antimicrobial activity against *S. aureus* using leaf extract and AgNPs

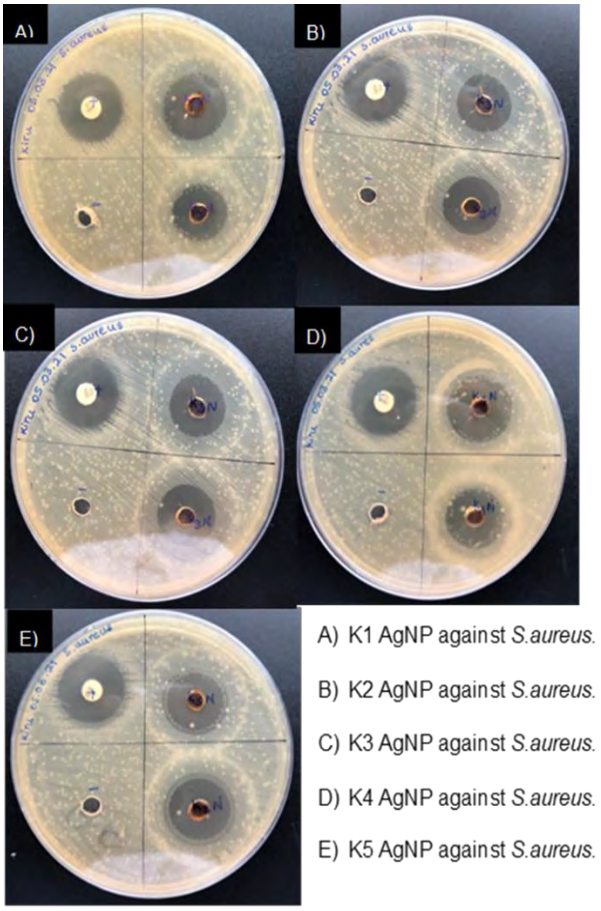

Figure 16. Antibacterial activity of AgNPs against *S. aureus*.

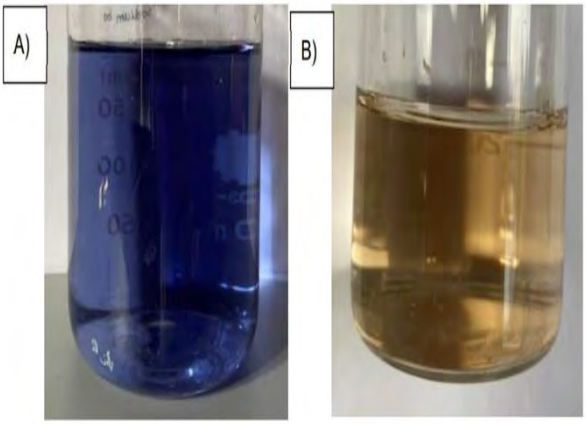

Figure 17. Photocatalytic degradation of EBT by K5NP. A) Before the addition of K5NP. B) After the addition of K5NP at 210min.

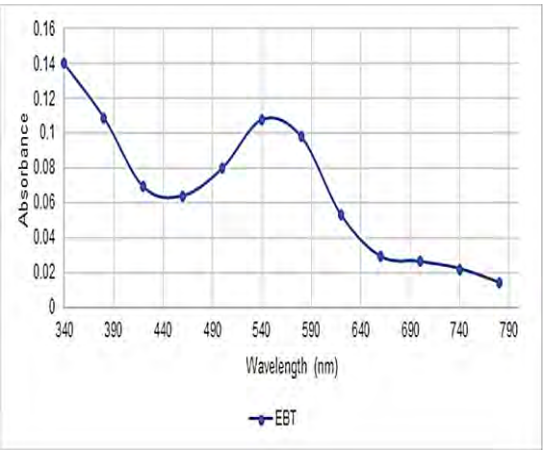

Figure 18. EBT dye absorbance.

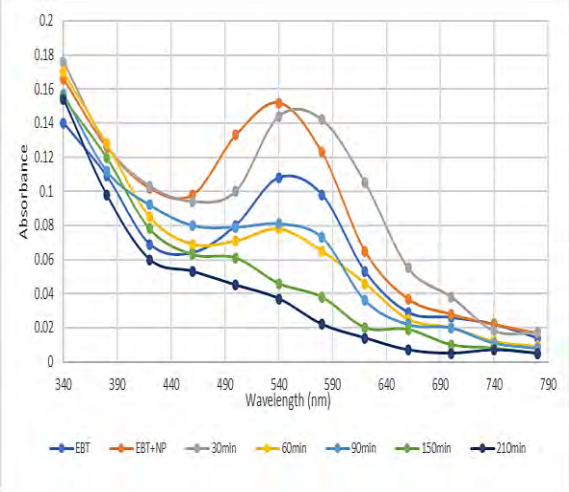

Figure 19. Photocatalytic activity at 10ppm of K5 AgNP

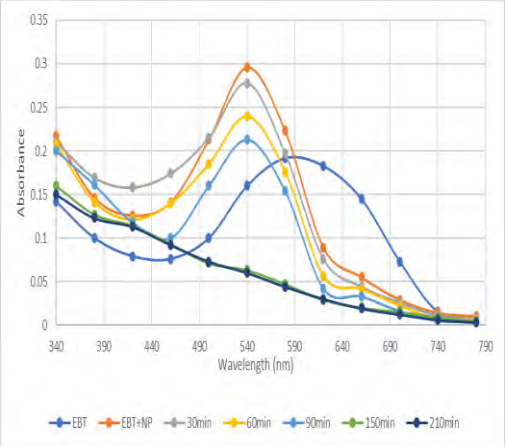

Figure 20. Photocatalytic activity at 10ppm of K5 AgNP with sodium borohydride.

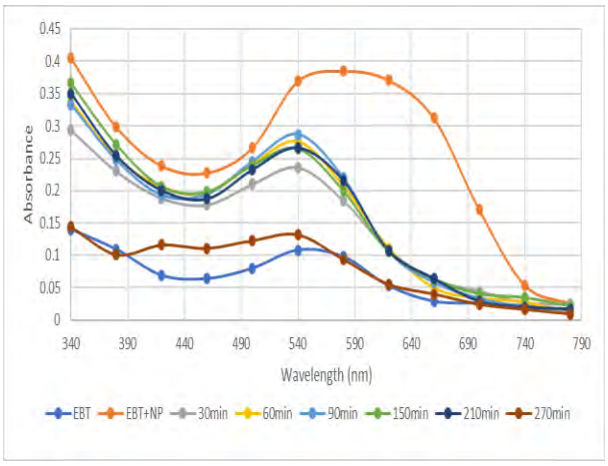

Figure 21. Photocatalytic activity 100ppm of K5 AgNP.

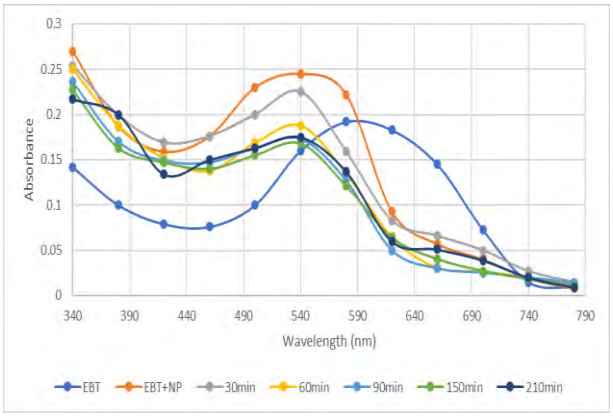

Figure 22. Photocatalytic activity 100ppm of K5 AgNP with sodium borohydride.

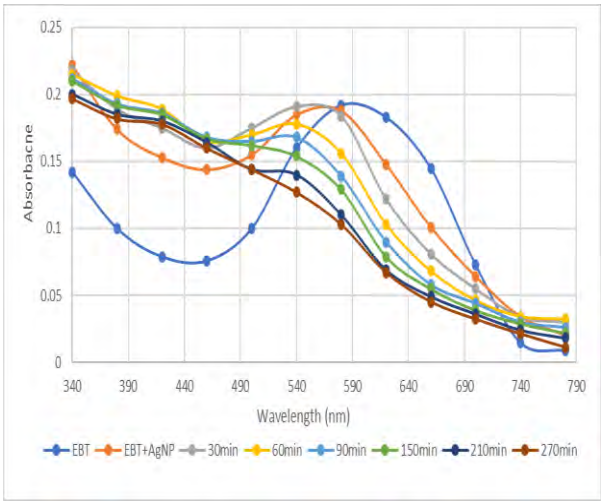

Figure 23. Photocatalytic activity at 500ppm of K5 AgNP.

4. Discussion

Nowadays the development of NP has become an attribute of the development of Richard Feynman laid down the concept of nanotechnology. The green synthesis of nanoparticle is a promising method to replace more complex physiochemical synthesis since it's free from toxic chemicals and hazardous byproducts.[2] Lantana species was hypothesized to be an efficient way to synthesize and stabilize NPs. AgNPs are the significantly used nanomaterial due to their physical, chemical, and biological characteristics and their superiority stem mainly from the size, shape, composition, crystallinity, and structure of AgNPs. In this study, leaf extracts were extracted using distilled water due to their ecofriendly nature. Phytochemical tests which were carried out on the Lantana sample gave positive results for tannin, saponin, alkaloid, terpenoids, carbohydrate, quinone, anthocyanin and coumarin (Figure 3). The presence of these reducing agents in the plants acts as natural capping agents for the stabilization of AgNPs.[24] However, Kalita *et al.* 2011, review of literature done with other solvent indicate the absence of these compounds. This difference might be due to the different polarity of the solvents.[10]

In this study, five lantana species leaf extract formed AgNPs successfully which was confirmed by primary indication, colour change of leaf extract to reddish after overnight incubation at RT with $AgNO_3$ solution. The colour change is due to the reduction of Ag+ ions to form Ag(O). Subsequently conducted spectroscopic studies for AgNPs confirmed this finding due to the unique optical properties of AgNPs making them strongly interact with a specific wavelength of light. AgNP showed an absorption peak in the visible region between 420-480nm, corresponding to surface plasma resonance (SPR) of K5NP shows elevated absorbance compared to other samples (Figure 2). Depending on the particle size, dielectric medium and chemical surrounding the absorption of AgNPs varies.[25]

The effect of temperature and time on the formation of AgNPs was analyzed using UV-Vis spectroscopy by heating AgNPs at 90°C for 30min and 60min. It was observed that there was no appearance of the peak which led to the conclusion of the absence of AgNPs except K4 at 60min (90°C) (Table 5). Due to prolonged exposure at 90°C, K4 sample was able to produce AgNP by stabilizing it with the phytochemicals. The AgNPs physiochemical properties are important for their bio-distribution, behaviour, safety, and efficacy.[26] Therefore, it has been characterized using scanning electron microscopy (SEM) in order to evaluate the functional aspects of the synthesized particles. SEM imaging results show surface morphology, agglomeration of nanoparticles and size was around 40 nm with spherical shape (Figure 4).[27,28.]

The SPR absorption band is formed due to the collective oscillation of the free electrons of AgNPs in resonance with a light wave. The conduction band and valence band of AgNPs lie very close to each other in which electrons move freely. The energy difference between the top of the valence band and the bottom of the conduction band in insulators (>4eV) and semiconductors (<3eV) is referred as the band gap.[29] According to the results, all the synthesized AgNPs are semiconductor (Table 7).

$$\text{Band gap energy } (E) = h \times C/\lambda$$

where h (Planck's constant) = 6.629 x10-34Js, C (Speed of light) = 3 x 108 m/s, and λ (Cutoff wavelength) which ranges from 420-460nm of the samples.

where h (Planck's constant) = 6.629 x10-34Js, C (Speed of light) = 3 x 108 m/s, and λ (Cutoff wavelength) which ranges from 420-460nm of the samples.

The classification of the samples is shown in Table 2 calculated using band gap energy.[30]

Table 7. Classification of conductance in synthesized AgNPs.

Sample	Band gap energy	Conductance
S1	2.82	Semiconductor
S2	2.95	Semiconductor
S3	2.58	Semiconductor
S4	2.58	Semiconductor
S5	2.82	Semiconductor

By using the aluminum chloride colourimetric method, TFC (QE) was estimated. Al^{+3} reacts with OH groups of the flavonoids establishing a stable flavonoid-Al^{+3} complex with a yellow colour where the intensity is proportional to the concentration of flavonoids.[31] The TFC of AgNPs was observed higher than leaf extract with AgNP of K4 being the highest among AgNPs followed by K2NP≈K1NP≈K5NP>K3NP and leaf extract followed by K4LE>K1LE≈K2LE≈ K5LE>K3LE where K4LE shows higher TFC among leaf extract (figure 5). The ONE- way ANOVA shows F > Fcrit (F-30.63463, Fcrit- 5.317655) indicating there is statistical significance between the groups. TFC in Lantana leaves study conducted by El-Sayed *et al*. in 2016 showed lesser value which might be due to the geographical difference, plant season and the extraction method.[32]

Using colourimetric assay is used to determine TPC (GAE) using Folin- Ciocalteu reagent which is a mixture of phosphomolybdate and phosphotungstate. The Folin-Ciocalteu reaction is a redox reaction where phenolic groups transfer an electron to phosphomolybdic and phosphotungstic acid compounds, in an alkaline medium. Sodium carbonate is the alkali that extents an optimum pH. The reducing acids change colour from light yellow to blue colour (reduced state) depending on the number of reacting phenolic groups.[33] The TPC of AgNPs was observed to be higher than leaf extract with AgNP K4NP was highest and other AgNPs have shown similar TPC (Figure 6). K4LE showed higher TPC among leaf extract followed by K1LE≈K2LE≈K5LE>K3LE. The ONE- way ANOVA shows F>Fcrit (F-30.66322, Fcrit-5.317655) indicating there is statistical significance between the groups. A similar study conducted by Kumar, Sandhir and Ojha in 2014 showed similar TPC in Lantana leaves.[13]

The TAC (AAE) of leaf extract and AgNPs were depicted and showed AgNPs have higher TAC compared to leaf extract. TAC in leaf extract is mainly constituents of the redox potential of Phyto constitutes, which satiating singlet and triplet oxygen and nullifying the free radicals. Therefore, the higher antioxidant activity of AgNPs might be due to preferential absorption of the antioxidant material from the extract onto the surface of the nanoparticles.[11] The overall highest TAC was observed to be higher than in K4NP among followed by K5NP>K1NP> K3NP≈K2NP. K3LE showed higher TAC among leaf extract followed by K1LE>K2LE≈K4LE≈K5LE (Figure 7). The ONE- way ANOVA shows F>Fcrit (F-14.12283, Fcrit-5.317655) indicating there is statistical significance between the groups. The recent study conducted by Kim and Lee in 2020 showed similar TAC results in Lantana leaves.[34]

Pearson's correlation coefficient was applied to evaluate the relationship between the antioxidant assays, including TFC, TPC and TAC. The correlation between TFC versus TAC was higher than the rest of the correlation (Figure 24). Flavonoid content is more responsible for the antioxidant property compared to phenolic content. A similar finding was recorded by Aryal and his coworkers in 2019.[35] The phenolic and flavonoid compounds have been reported to have high potent antioxidants because they possess the ability to neutralize free radicals. This possibly suggests that Lantana leaf extracts have higher antioxidant activity.[35]

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a stable compound that is reduced by accepting hydrogen/electrons. Antioxidants have the ability to donate an

electron to DPPH free radical leading to colour change from purple to yellow. The reducing activity of Lantana species leaf extract and AgNPs was quantified using spectrophotometer. DPPH scavenging activity of all the leaf extract was similar as well as the AgNP (Figure 8). Due to the encapsulation of bioactive molecules on the spherical surface of AgNPs through the electrostatic attraction between negatively charged bioactive compounds and neutral or positively charged NPs the antioxidant efficacy is higher in AgNPs compared to leaf extract. The AgNPs showed higher DPPH scavenging activity compared to leaf extract with AgNP being highest in K3NP.

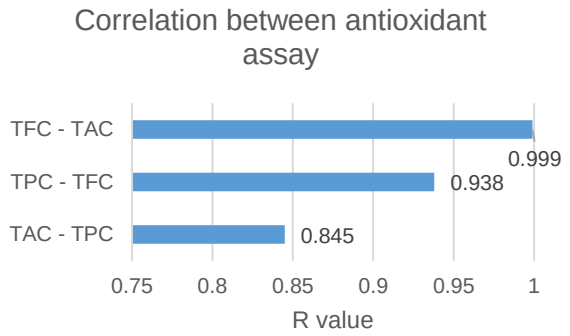

Figure 24. Correlation between antioxidant assays.

The ONE- way ANOVA generated for leaf extract vs AgNPs shows F>Fcrit value (F-4645.267, Fcrit-5.317655) indicating there is a significant difference between leaf extract and AgNPs.[36]

The IC_{50} value was intended to determine the concentration of the sample required to inhibit 50% of radicals. The lower the IC_{50} the higher the antioxidant activity of the sample (Figure 9 and 10). IC_{50} of the samples shows that leaf extract has a higher value compared to AgNPs followed by K1LE>K4LE>K2LE>K3LE>K5LE (Table 6). The highest IC_{50} was observed in K5NP followed by K3NP>K4NP>K1NP>K2NP. Leaf extract shows higher specific activity (%) than AgNPs. A similar study in 2013 by Khan and his coworker showed higher results, due to the geographical difference and different extraction procedure.[37]

AgNPs seems to be the potential antibacterial agent among the several promising nanomaterials due to their large surface to volume ratios and crystallographic surface structure. AgNPs have various mechanisms to inhibit the growth and proliferation of gram-positive and gram-negative bacteria. AgNPs damages the bacterial cell membrane, disrupts protein synthesis and the formation of ROS which lead to bacterial death. The leaf extract and synthesized AgNPs were able to inhibit the growth of *E.coli* and *S. aureus* to an extent. Compared to leaf extract, AgNPs showed a better inhibition zone. Samples showed higher inhibition in *E.coli* compared to *S. aureus* (Figures 11-16)

The ONE- way ANOVA generated for leaf extract vs AgNPs shows F>Fcrit value (F-59.45288, Fcrit-5.317655) indicating there is a significant difference between leaf extract and AgNPs. The recent study conducted by Mansoori and his co-worker in 2020 shows similar results. AgNPs indicate stronger antibacterial capabilities due to the antibacterial property of leaf extract enhanced with Ag ions.[38]

The catalytic activity of AgNPs was assessed by using EBT dye. It's a known fact that AgNPs shows greater catalytic activity in the reduction and removal of dye. Mallick *et al*. studied the catalytic activity of AgNPs synthesized using Lantana leaves on the reduction of phenosaffarin dye and Ajitha *et al*. studied the reduction of methylene blue by green aqueous extract of Lantana mediated AgNPs.[6,39]

In this study, green synthesized Lantana AgNPs is reported for EBT reduction. EBT was recorded to have maximum absorbance at 530 nm. In addition to sample at various concentration, EBT was reduced within a period of time but a remarkable decrease in absorbance

peak was observed at 10 ppm. Higher concentration 500 ppm was not able to fully degrade EBT in 270 min. At 10ppm K5NP was able to degrade EBT within 210 min but with the help of sodium borohydride, it degraded at 150 min (Figure 17-23).

At 100ppm K5NP degrade EBT within 270min however the addition of sodium borohydride didn't enhance the degradation at 100ppm. On the other hand, there was a blue shift observed at 540 nm, due to the decrease in particle size and accumulation of byproducts. Sodium borohydride ($NaBH_4$) is a reducing agent which acts as a catalyst in the degradation of dye. Since BH_4- ions are nucleophilic (donor) nanoparticles accept the electron and transfer to electrophilic nature dye (acceptor).[40] In 2021 a similar study conducted by Surendra and his coworker showed similar results.[41]

The rate constant was analyzed for further confirmation. According to Table 8, a comparison of each rate constant reveals that 10 ppm with sodium borohydride shows a better rate constant. This reveals that the lower concentration is the best concentration to degrade the EBT dye.[42]

Table 8. Rate constant of AgNPs degrading EBT at different concentration.

Concentration	Rate constant
10 ppm	0.2986
10 ppm + $NaBH_4$	0.3587
100 pm	0.103
100 ppm + $NaBH_4$	0.0755
500 ppm	0.0677

Conclusion

The present study demonstrates that Lantana leaves contain most of the phytochemicals which Phyto stabilized the Ag ions for the formation of AgNPs. All the variety of Lantana leaves were able to synthesize AgNPs. The water-soluble characterization of flavonoids in leaves were responsible for the spectacular reduction process of AgNPs. SEM analysis revealed the spherical shape of the AgNP. AgNP contains higher TAC compared to leaf extract. As well as AgNPs shows higher antibacterial activity compared to LE.

They have higher antibacterial activity against *E.coli* compared to *S. aureus*. Photocatalytic degradation of EBT by K5NP at lower concentration with the catalyst shows greater activity within a short period of time. It shows natural renewable and eco-friendly reducing agent used for the synthesis of AgNPs exhibits excellent photocatalytic activity and can be used in dye effluent treatment. Leaf mediated synthesis of AgNPs by using Lantana shows more compatible, eco-friendly, low cost, and less time-consuming approach.

Acknowledgements

Authors would like to thank BMS for funding.

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ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

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Determination of complex dye decolourization level by fungi.

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Abstract

Textile dyes, when released to the environment, are difficult to decolorize and removed. Chemical and physical methods of textile dye decolourization are considered expensive. On the other hand, the biological methods of textile dye decolorization are environmentally friendly and effective. This research was aimed at studying the textile dye decolourization ability of the fungus *Trametes versicolor* using three textile dyes (Dianix Royal Blue CC (Dye 1), Dianix Blue XF (Dye 2), and Dianix Red CC (Dye 3)). The mushrooms were trimmed to about 1cm^2 pieces and placed on the centre of the PDA plates. After 7 days of incubation they were subcultured on a new plate to obtain pure cultures. Both solid and liquid media were used to study dye decolorization. Inoculation from pure cultures was done under aseptic conditions on solid media. Inoculums were subcultured onto dye incorporated plates and were incubated at room temperature (25 ºC) under exposure to light for three days. The inoculums were incubated at 25°C for 3 days in liquid media. Surface sterilization was successful as no contamination was observed. The fungus showed a fast growth, with a growth rate of 0.145 cm/day. Dye decolorization was observed in both solid and liquid media. The fungus was most effective in decolourizing Dianix Blue XF in both liquid and solid media. The procedure can be further improved by controlling the temperature and the light intensity. The surface disinfestation procedure can be further used. The procedure has the possibility of further use in studying textile dye decolorization by fungi with further improvements and replicates.

Keywords: *Trametes versicolor,* decolorization, textile dyes, fungus

1. Introduction

Dyes are widely used in industries like textile colourization and printing processes[1,2]. Commercial colours and dyes are resistant to degradation, light, and heat. Intense utilization of these synthetic chemicals has caused severe problems, such as contamination, environmental pollution, and toxicity[3]. Several treatment technologies are used nowadays for the removal of dye from industrial wastewater including coagulation, adsorption, biological methods, advanced oxidation processes (AOPs), membrane technology and electrochemical methods[4]. Dye wastewater is treated physically and chemically by flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation, ozonization and Fenton's oxidation[5-8]. *Trametes Versicolor* (common name: Turkey Tail), a member of the family Polyporaceae, is one of the most popular medicinal macro fungi[5].

Figure 1. *Tremetes Versicolor* mushroom[9]

Wastewater from textile industries can be refined by applying biological, physical, and chemical approaches. However, there are

disadvantages such as high costs, toxic side produces, excessive energy consumption, concentrated mud formation and difficulties in adapting to different wastewater resources[1].

Biological methods of bioremediation are more economical and have no destructive effects on the environment. There have been many studies on the fission and decolourization of dye using microorganisms and, found that there are microorganisms that possess the capacity[1,5]. The enzyme laccase which is secreted by *Trametes Versicolor* has been used in the pretreatment of lignocellulosic biomass, bioremediation, triclosan biodegradation, blue wastewater biodegradation and dye decolourization[5,11].

Due to the use of living organisms capable of natural bioremediation in biological systems, they are more adapted to environmental conditions. Recent studies have shown that use of white-rot fungus in the decolourization process was very effective[3]. In the field of waste and environmental biotechnology, white rot fungi are widely used[3]. This group of fungi can be used in several industrial applications, such as pulp delignification, textile dye bleaching, effluent detoxification, biopolymer modification and bioremediation[2,12]. The successful rescue of wild strains and their ability to produce fruiting bodies under artificial cultivation conditions have been highlighted[5].

2. Methodology

2.1 Fungus isolation and pure culture preparation. The fresh fruiting bodies of *Trametes Versicolor* were collected from the environment with the descriptors for *Trametes Versicolor* identification using the articles and the internet. To prepare the potato dextrose agar (PDA) medium, the potato was cut into small pieces and boiled till the potato pieces could be smashed. Then it was filtered using a cheesecloth to obtain potato infusion without any potato pieces, and after cooling down, 4 g of Dextrose was added, and mixed. The pH measured was 5.5 (Standard range 5.6 ± 0.2). Then the agar was added and closed the mouth of the conical flask using a cotton plug and was covered with aluminium foil. Then autoclaved at 60°C and 121 psi. After autoclaving the media was poured into the sterile petri dishes and was allowed for solidification. Then the mushroom piece was washed using running tap water for about 10 minutes. This step removes any dust particles, and then the mushroom piecewas soaked with liquid soap and rinsed with distilled water three times. Then the mushroom piece was put into a sterile beaker, and transferred to laminar flow.

2.2 Surface sterilization and pure culture isolation. The mushroom piece was washed with sterile distilled water. Then it was washed with 70% ethanol (made using 25 ml bydissolving 17.5 ml of 100% alcohol and 7.5 ml of water) for 1 minute and again washed with sterile distilled water. Next, it was washed with 5.0% Sodium hypochlorite (Clorox) (made using 12.5 ml of 10% Clorox and 12.5 ml deionized water) for 5 minutes and washed with sterile distilled water. Then the mushroom piece was kept drying for about 5 minutes. Then it was cut into (1–2 mm diameter) plugs of the fruiting body using a sterile blade and cultured on a PDA plate using sterile forceps and this was done under aseptic conditions. After inoculating for seven days at 28°C, the mycelial disks (6 mm diameter) from the peripheral region in the PDA were inoculated on a fresh PDA and incubated at 28 °C for five days and a pure culture was obtained.

2.3 Preparation of Potato Dextrose Broth (PDB) media. A volume of 300 ml of PDA media was made using 60 g of potatoes, 6 g of sucrose, and 4.5 g of agar. Before adding agar, the pH was measured (6.4) which should be in between 6.5±0.2. Then the agar was added and poured into three conical flasks (100 ml each) and autoclaved at 121 ºC and 15 psi. Then the flasks were transferred to the lamina flow and the dyes were added. 11 mg of each dye was added to each flask and mixed well before pouring onto the plates. Then poured into the plates and labeled and refrigerated at 40ºC after solidification.

2.4 Preparation of PDB media. A volume of 500 ml of PDB media was prepared using 100 g of potatoes and 10 g of sucrose. Then the pH was measured (6.5) which should be at 6.5±0.2. The mixture was poured into 5 conical flasks, 100 ml each; and 1.5 g of agar was added toeach, before autoclaving. 11 mg of each dye was added to conical flask and 100 ml PDB media was added and mixed. The mixture was separated into four falcon tubes. each containing 25 ml of PDB. To this solution 3 mg of each dye was added. The dyes in the falcon tubes were used as controls to check the

colour changes with the conical flasks. PDA was poured into plates and allowed to set. Inoculation was carried out on solidified PDA plates.

2.5 Inoculation of dye plates. Fungal colonies grown in the sub cultured plates were cut into small pieces and were placed in the centre of the dye plates using sterile blades and forceps under aseptic conditions. Three petri plates were used for each dye, and the controls were PDA without dye but with the fungus, PDA with dye but without fungus and PDA without dye but with agar piece for each dye.

2.6 Inoculation on liquid media. Five equal sized pieces of fungus from the subculture plates were added to each dye PDB media and was shaker incubated for three days. Controls used were PDB + dye 1, PDB + dye 2, PDB + dye 3 and PDB + agar piece each containing 25ml of PDB media in falcon tubes.

2.7 Measuring UV absorbance. The radius of the fungus growth on the dye plates were measured and recorded for three days. After 24 hours of incubation 4 ml of solution was transferred into falcon tubes. And centrifuged (sigma 3 – 16 PK) at 3000 g for 30 minutes. Then the supernatant was taken to cuvette and put into the first well of Agilent Technologies Cary series UV – Vis spectrophotometer for each dye containing the fungus and controls. The graphs were observed to check whether there are any shifts in the graphs with the controls. This was done and recorded for three days.

3. Results

3.1 Isolation of Trametes Versicolor from the environment

Figure 2. Mushroom used for the experiment

3.2 Isolation, culturing, and subculturing of the fungus

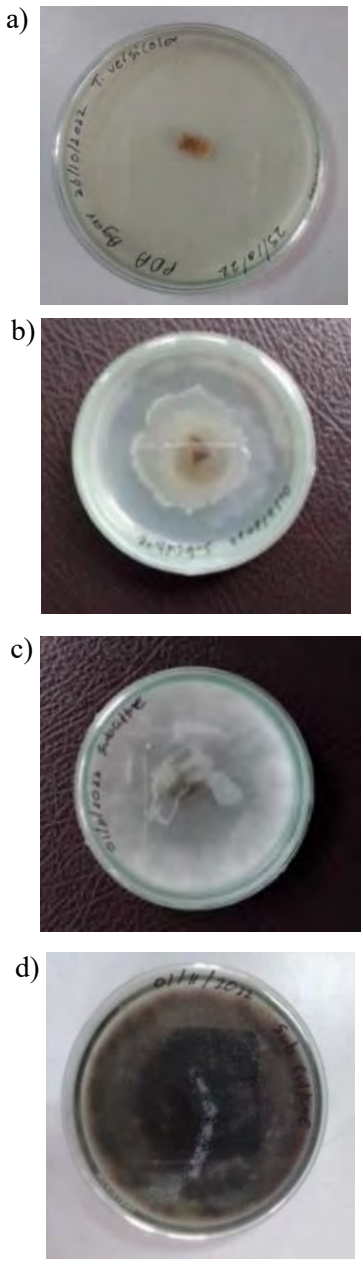


Figure 3. Isolation, culturing, and subculturing of the fungus a) Cultured mushroom piece in the PDA media b) Subcultured mushroom piece c) Fungal growth of subcultured mushroom piece d) After three days of subculturing.

3.3 Subculturing on the dyed plates and liquid media to observe the decolorization of the dye.

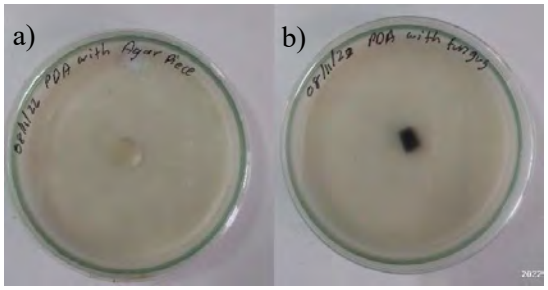


Figure 4. a) PDA with only the agar piece (control) b) PDA with only the fungus (control)

Table 1. Dye incorporated plates with the fungus and the controls from day 0 to day 3

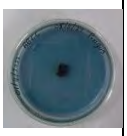
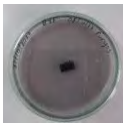
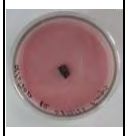
Day	Dyed plate with fungus			
	Control	Dianix Royal Blue CC	Dianix Blue XF	Dianix Red CC
Day 0				
Day 1				
Day 2				
Day 3				

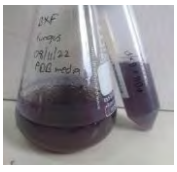
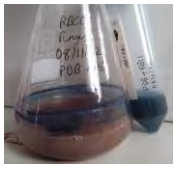
Table 2. Radius and the growth rate of the dye incorporated plates from day 1 to day 3.

Radius of the dye incorporated plates			
	Dianix Royal Blue CC	Dianix Blue XF	Dianix Red CC
Day 1	1.5 cm	1.5 cm	1.8 cm
Day 2	3.3 cm	3.3 cm	3.3 cm
Day 3	3.5 cm	3.5 cm	3.5 cm
Growth rate	0.048 cm	0.048 cm	0.052 cm

Table 3. Dye-incorporated liquid media with the fungus and the controls.

Day 0				
	Dianix Royal Blue CC	Dianix Blue XF	Dianix Red CC	Agar piece
Control				
Potato Dextrose Broth media with fungus				

Table 4. Dye-incorporated liquid media with the fungus and the controls from day 1 to day 3.

Control and the Potato Dextrose Broth media with the fungus			
Day	Dianix Royal Blue CC	Dianix Blue XF	Dianix Red CC
Day 1			
Day 2			
Day 3			

3.4 Absorbance graphs of each dye in the liquid media with the controls.

Table 5. Absorbance graphs of Dianix Royal Blue CC dye in the liquid media from day 1 to day 3.

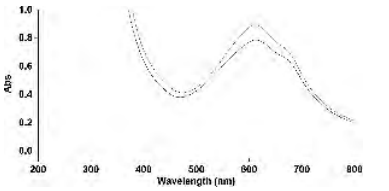
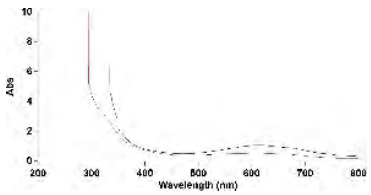
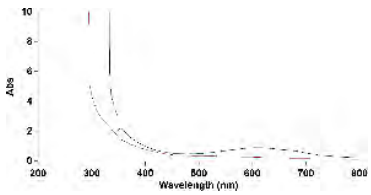
Dianix Royal Blue CC		
Day 1	**Day 2**	**Day 3**
		

Table 6. Absorbance graphs of Dianix Blue XF dye in the liquid media.

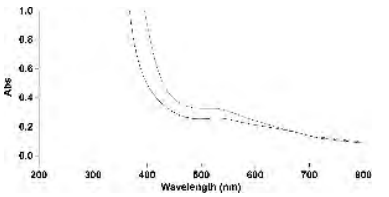
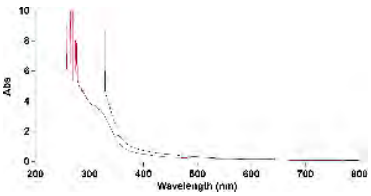
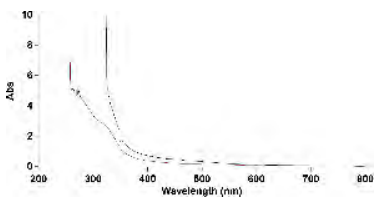
Dianix Blue XF		
Day 1	**Day 2**	**Day 3**
		

Table 7. Absorbance graphs of Dianix Red CC dye in the liquid media.

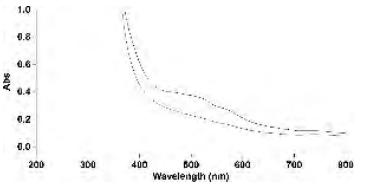
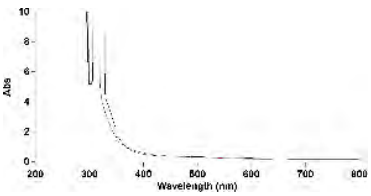
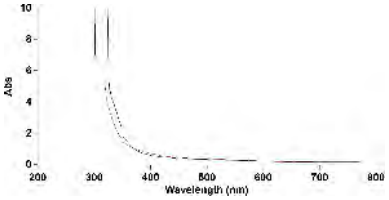
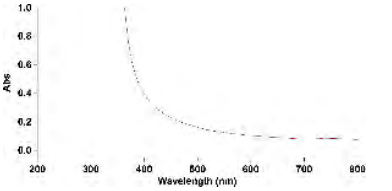
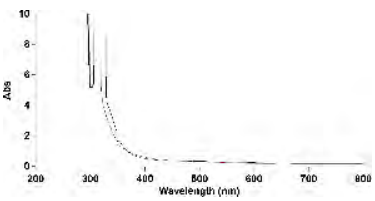
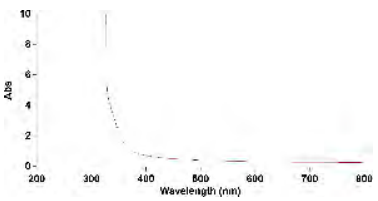
Dianix Red CC		
Day 1	**Day 2**	**Day 3**
		

Table 8. Absorbance graphs of the control in the liquid media.

Control		
Day 1	**Day 2**	**Day 3**
		

4. Discussion

Trametes Versicolor is a wood rotting fungus. It was collected from the environment by observing special features, structure, and color that aligns to the published articles and images. This mushroom has a specific band pattern. Isolated mushroom (Figure 2) piecewas cultured in PDA media plates (Figure 3a). After the growth it was cut with the agar piece and placed onto another petri plate to obtain pure culture (Figure 3b). The fungal growth is denoted in Figure 3c) and sub cultured fungus in figure 3d. The radius of fungal growth was measured to compare the growth rate of the fungus. Dye incorporated plates with the fungus piece Dianix Royal Blue CC, Dianix Blue XF, and Dianix Red and the control from day 0 to day 3 is mentioned in Table 2. The fungal growth can be clearly seen from day 0 to day 3 (Table 2). The growth rate of fungus is higher. Table 4 shows the controls used in the liquid media and they are PDB + Dianix Royal Blue CC, PDB + Dianix Blue XF, PDB + Dianix Red CC, and PDB + Agar piece and the dyed liquid media with the fungus pieces for day 0. Table 5 shows the PDB containing Dianix Red CC with the fungus pieces and the control, PDB containing Dianix Royal Blue CC with the fungus pieces and the control, and PDB containing Dianix Blue XF with the fungus pieces and the control from day 1 to day 3. A clear colour change is observed by naked eye, but absorbance graphs indicate no difference. This may be due to the formation of the pigment from the fungus. The mushroom selected for the study is a pigment producing species. There is a notable difference in the absorbance graphs (Table 5,6,7) compared to the controls (Table 8). But significant difference is not observed among day 2 and day 3 absorbance graphs, when considering the visual difference observed by naked eye. The black color graph shows the control absorbance while the red color graph shows the dye incorporated liquid media with the fungal pieces. These graphs are used to measure and to compare the best dye which can decolorize the most.

Conclusion

In conclusion, fungal growth and decolorization was successful in both methods; however, fungal growth and decolorization is more effective in the liquid media than the solid media. The change of color in the PDB could be clearly detected in the liquid media which can also be observed with the naked eye. This should be done under aseptic technique and the surface sterilization should be done properly to avoid contamination. Further study and optimizations are required regarding controlling the temperature and light intensity. Decolorization can be measured by providing both light and dark conditions to the samples to measure which suits better for the process. Also, screening is required to select *Trametes* strains which do not produce pigments that could interfere with absorption spectra and readings.

Acknowledgement

The authors wish to thank Eng. Ms Gayathri Liyanage of Environmental Technology Section, Industrial Technology Institute for providing the textile dyes for the experiments.

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ISSN 2989-0705 (Online) | Vol 1 | Issue 1 | July 2023

Surviving in the Creative Industry – Analysis of Innovation, Challenges, and Opportunities Faced by Small Art and Craft Enterprises in Sri Lanka

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Abstract

The creative industries are experiencing steady growth and increasing popularity, particularly in Sri Lanka. Research has shown that handicraft businesses play a significant role in the economy of both developing and developed countries. As the creative sector becomes more integrated into the economy, creative firms face various challenges and opportunities. However, there is limited research on the challenges and opportunities faced by small art and craft enterprises in Sri Lanka. This study aims to address this gap by examining how these small enterprises survive in the creative industry through innovation practices, and by identifying the challenges and opportunities they encounter. The researcher identified that although the creative industry is flourishing in Sri Lanka, it is far behind its peers. Hence, this study aims to shed light on the functioning of small art and craft enterprises within the Sri Lankan creative industry. To gain a deeper understanding of the industry, a qualitative research method was utilised, including interviews with eight handicraft business owners in Sri Lanka. Several themes emerged through these interviews, including the importance of digital presence exposure, leveraging social networks for innovation, understanding consumer purchase intention, closed innovation practices, purchasing constraints, short-term vision, and creative value chain. Finally, the study offers key recommendations to help art and craft businesses thrive in Sri Lanka such as building relationships and networks to expand business reach, improving marketing campaigns to attract a larger customer base, and emphasising trust-building and brand development.

Keywords: Art and Craft Enterprises, Creative Value Chain, Innovation, Creative Industry

1. Introduction

1.1 Background of the Study

Landoni et al. (2020) defined the creative industry as a sector that utilises innovative and creative practices of individuals, offering potential job opportunities. On the other hand, Potts, and Cunningham (2008) describe creative industries as segments of the industry, where creativity is the input and intellectual property (copyrights and trademarks) is the output. The creative industry comprises fifteen subsectors including advertising, art market, craft, apparel, video filming and photography, interactive games, design, music, architecture, performing arts, computer and software

services, publishing and printing, TV, radio, and culinary (Herawaty & Raharja, 2018; Townley et al., 2009). However, variations in the definition of creative industries are diverse in different contexts (Berg & Hassink, 2014). In Europe, for instance, the creative industries are categorised into two groups: the 'core' creative industry, encompassing visual art and craft activities, and the 'partially' creative industry, which includes fields such as marketing, architecture, and media (O'Connor, 2007). Relatively, this study will examine innovation practices, opportunities, and challenges encountered by small visual artists and crafters in Sri Lanka, aiming to enhance their success in the industry. The art and craft sector are commonly referred to as the handicraft sector.

According to Jayawarna (2020), Sri Lanka's creative industry has shown consistent growth, from $433.63 million in 2010 to $845.41 million in 2014. The crafting sector employs around 62% of the creative industry workforce, contributing 5.3% to the country's GDP in 2020 (Hirimuthugodage, et al., 2020). There are approximately 83 established artists and 1000 crafters and encompasses three main sectors: Arts and culture, design, and media (Hirimuthugodage et al., 2020; Jayawarna, 2020). Micro firms, typically managed by a single owner and not seeking significant growth, are prevalent in the creative industries, with the majority employing less than 10 individuals (Jaouen & Lasch, 2015).

1.2. Problem Statement

Sri Lanka lacks creativity compared to its peers, as indicated by the Global Creativity Index (GCI) rankings (Hirimuthugodage et al., 2020). This highlights the significant challenges in harnessing the country's potential in the creative industry. Most existing studies in this field have primarily used quantitative research methods, with limited in-depth exploration through qualitative research. Therefore, the author intends to conduct qualitative research to examine the challenges and opportunities faced by the handicraft enterprises in Sri Lanka. Although the creative industry in Sri Lanka is experiencing growth and demand, small businesses in this industry still face significant obstacles in terms of survival, including the high-risk nature of accessing potential customers and the lack of financial, technical, and government support (Hirimuthugodage et al., 2020; Jayawarna, 2020). The findings of this study may benefit other researchers conducting similar studies and enhance their research based on these findings.

1.3 Research Aim

The research aim of this study is to examine the challenges and opportunities in terms of innovation faced by small art and craft enterprises in Sri Lanka.

2. Methodology

This research was conducted using a mono method qualitative research approach. The research process was commenced through the collection of primary data through semi-structured online interviews aimed at small art and craft business owners in Sri Lanka.

This study involves a sample of 8 handicraft business owners who can represent and provide a detailed discussion on the creative industry in Sri Lanka. Moreover, the purposive sampling technique was utilised in the selection of the sample. The data analysis procedure was conducted using thematic analysis.

3. Findings and Discussion

The details of the small enterprises are given in Table 1. The participants in this study were predominantly female artists and entrepreneurs in the art and craft sector in Sri Lanka. They were motivated by passion and sought personal satisfaction and gratification through their creative endeavours. While some initially started their businesses without a focus on profits, others aimed to earn money from their passion. The participants specialised in various areas such as acrylic and oil canvas paintings, traditional art, digital art, greeting cards, posters, and wedding favours. Most operated as micro-firms without employees, although one participant had three employees. All participants were founders and CEOs of their businesses, with industry experience ranging from 2 to 6 years.

Table 1. Details of the small enterprises

Code	Area of specialisation	No of employees	Years in business
01	Acrylic painting	1	2
02	Commission paintings	1	5
03	Canvas painting, string art, greeting card, shadow box	1	2
04	Oil painting, selling art supplies	3	5
05	Acrylic and oil painting, calligraphy	1	5
06	Craft, cards, painting, digital art, posters	1	5
07	Digital art, wedding favours	1	2
08	Acrylic painting, framed watercolour wedding favours	1	6

Digital Presence

Consistent with Scheepers et al. (2022), the findings of this study revealed that most of the participants in the art and craft sector are leveraging digital tools and platforms to enhance their business reach. The participants reported using popular digital and social media platforms such as Instagram, TikTok, and Facebook to establish an online presence and attract customers. These platforms served as valuable tools for promoting their work and adding value to their businesses.

A respondent stated, "*I started from Instagram, and it has benefitted me big time. I have reached some people who I thought I would not reach. So, that is like the biggest benefit of going digital for me".*

In line with the findings of Putrivi (2020), and Shult (2015), the participants in this study affirmed that online platforms have played a crucial role in increasing demand and expanding their customer base. They reported that utilising social media platforms has significantly improved their business reach compared to offline methods. Furthermore, participants emphasised the importance of digital tools in editing and marketing their products which helped raise awareness of their business.

A respondent stated, *"I think in my business, for everything except creating the actual artwork, I use the help of platforms. Whether it is, you know, taking a picture, or editing it or posting it or even for advertising. Even communicating with my customers is through social media platforms. So, I mean, it is very convenient and cheap at the same time and with all the digital tools that you have today, it has never been easier."*

Additionally, in line with the findings of Guha et al. (2021) and Shafi et al., (2019), the participants of this study highlighted the cost-effectiveness of digital platforms for marketing their businesses. Small art and craft firms opt for word-of-mouth and social media strategies to create business awareness, instead of expensive marketing methods. This resonates with the findings of Guha et al. (2021), that small artisans who often face financial constraints, rely on intermediaries for resources.

A respondent stated, *"I mostly use Instagram and Facebook as my main channels and it is not heavy on my wallet, in terms of marketing and reach, which I am happy about. On Instagram, we have a whole section dedicated to supporting small businesses which I feel is a very great and cost friendly way to market my work".*

Moreover, Bianco et al. (2019) found a growing demand for digital artwork, which participants recognised as a significant opportunity. Participants believed their focus on digital art made them innovative, cutting costs by providing soft copies instead of physical materials.

A respondent stated, *"I mostly take on softcopies so, I don't have the hassle of printing*

the artwork and framing it, compared to other businesses that invent and sell goods, I would say that my business is a pretty good, because I do not have the hassle of purchasing tangible goods and stuff. So, I think in that way, digital art is an opportunity that I have".

Scheepers et al. (2022) noted that going digital in the art industry can be mentally draining due to technological complexities. Few participants reported challenges in software maintenance, platform engagement, and experienced stress in managing their businesses.

A respondent stated, *"I think one of the challenges I faced was to put in a capital. Although I do digital art, you must pay for platforms to purchase them and to maintain them. We need to upgrade the software and all of that."*

A respondent stated, *"The amount of physical and emotional labour that comes with art is honestly not talked about enough. It's just so tiring sometimes to repetitively do paintings and stuff… one downside to using Instagram is that the algorithm is not always easy to deal with. I have come across many instances where I have noticed that my friends could never see my posts on their feeds. This is a tricky business, but I am trying hard to figure it out".*

Social and Personal Networks

Boren and Young (2011), Turner (2023) and Gundolf et al. (2018) emphasised the importance of broad networks for artists in the creative industry to gain exposure. However, the findings contradict this notion, as most artists prefer working in isolation and rely on their own marketing efforts for audience reach.

A respondent stated, *"I do not have an art network., mainly because I prefer working alone and I find it difficult to collaborate with someone who is not in the same wavelength as me. My art style may not match another artist and I feel that's a disadvantage for me and my business".*

As stated by Hirimuthugodage et al. (2020), most artists prefer to work in isolation, because they believe that there are limited networking opportunities in Sri Lanka.

A respondent stated, *"I do not have an art network mainly because I believe that artists have poor exposure to the world, and because the government does not seem to provide much of a benefit to artists in the country so there is limited exposure".*

Boreng and Young (2011) noted that artists often collaborate with their existing and known social networks; some participants in this study expressed their preference for working with known networks. A few participants mentioned having personal connections within their social network and enjoying collaborations to combine talents and generate new ideas.

A respondent stated, *"I do not really have a huge external network, but I do have personal networks. So, I work with known businesses who I have already worked with in the past. So that is convenient for me, so I don't have to feel*

awkward working with new and unknown people. So, it's fun to work with different people because it brings in new ideas".

A respondent stated, *"When we collaborate with another artist, it helps share our audience and you can basically integrate both your art styles. This provides a greater audience, and you can create new ideas".*

Consumer Purchase Intention

Guha et al. (2021) found that consumer purchase intention and buyer attitude significantly impact small businesses in the handicraft sector. Similarly, Koswatte (2020) and Kalubowila and Rajapakse (2021) noted that Sri Lankans have a poor sense of brand consciousness and spend relatively little on art and craft. Additionally, Koswatte (2020) observed that premium pricing negatively affects buyer attitude, unless consumers develop an emotional attachment to the product. This study's findings align with these observations, as most artists face challenges in selling their products in Sri Lanka, due to the purchasing attitudes of the local population towards art and craft products.

A respondent stated, *"I cannot focus on the long-term because most people in Sri Lanka do not have a mind-set to purchase artwork. I realised that nobody is going to be willing to purchase even if there was no economic problem in the country right now".*

Another respondent stated, *"I think pricing and costs are major challenges for me because customers in Sri Lanka have little to no basic awareness on the costs that comes with an art business and are always trying to bargain and be a cheapskate which is annoying. They clearly are undermining the seriousness and effort it takes into creating such labouring work.*

Closed Innovation

Snowball et al. (2021) and Putrivi (2020) emphasised the importance of innovation for survival in the creative industry, as it requires adapting to changes in demand and supply. Similarly, Chollisni et al. (2022) and Liu (2021) highlighted the significance of open innovation, collaborating with other firms to add value. However, the findings contradict this notion as most participants prefer and practice closed innovation and conduct their own market research to identify demands.

A respondent stated, *"I feel like anything that you do is innovation. I feel like, even a stroke that you make on a canvas is innovation like I said, I'm not doing this business full-time, so I don't really collaborate or anything. I just do my own research and browse through the internet to identify what people want".*

Another respondent stated, *"I conduct my own research because based on demand only we are supposed to create the products. So, I would say doing my own research helps me to be more innovative".*

Purchasing Constraints

According to Landoni et al. (2020), artists and crafters in the industry lack a steady income and prefer wide profit distribution. Similarly, participants face financial hindrances, such as purchasing materials, locating supplies, and poor financial grants in Sri Lanka (Kalubowila & Rajapakse, 2021). The findings indicated that locating and purchasing supplies and raw materials in Sri Lanka is a major financial constraint for most participants. This is due to fluctuations in the economy, transportation difficulties in the country, and taxations in the country.

A respondent stated, *"I face financial challenges in purchasing materials. Especially these days, the prices of everything have gone high… it's extremely difficult to purchase products from other countries due to increased taxes and import restrictions. On top of that it takes a long time to bring them down to Sri Lanka".*

Short-term Vision

Small art and craft enterprises often operate short-term due to the reasons for starting the business. Owners aim to build a career through their passion and may lack skills and capital funding (Choudhary & Mishra, 2022; Yang et al., 2018). Market vulnerability in Sri Lanka further contributes to the short-term focus (Romein & Trip, 2011). Although the findings of this study indicate the importance of a long term business plan, most participants have a short-term business plan, indicating short-term investment in the business.

A respondent stated, *"I would say I'm focused on short-term at the moment. I'm trying to meet my short-term needs and I don't really have a long-term vision because I do this because it's my passion, and with the way things are going right now in the country, I'm facing more issues in the short-term rather than in the long-term".*

Another respondent stated, *"A long-term business plan is very important, if I had it, I think I would be elsewhere in the business. I wouldn't be having very few followers and still be in this level if I had a long-term plan".*

A respondent stated, *"I guess I'm not really focused on the long run right now, because I cannot manage my time with my personal life. So, I just have a short plan, based on the current situation".*

Creative Value Chain

Bhatiasevi and Dutot (2014) noted the variation of the creative value chain in different business sectors. While Madudova (2017) and Wang (2018) observed that art and craft firms operate in small quantities. Similarly, the findings indicate that participants conducted production in small quantities due to resource limitations and order-based earnings. Moreover, the primary activities in the value chain varied among participants. Some focused on receiving orders, production, packaging, shipping, and selling, while others included idea presentation, customer preferences, materials purchase, and

Instagram marketing within their value chain. Hence, the primary activities in the art and craft value chain differ significantly among businesses.

4. Conclusion

This paper has provided an overview and evidence of the opportunities and challenges faced by small art and craft industries in Sri Lanka, along with the innovation practices used to survive in the industry. This research has argued that it is important to build networks to enhance business reach and building trust is important in branding the business to a broader audience. Key challenges identified were purchasing constraints due to unavailability of supplies, poor consumer purchase intention, short-term vision limiting capital and funding opportunities, and building social networks. Likewise, the identified key opportunities included digital presence exposure, closed innovation practices, and specialisation.

5. Recommendations

Building relationships and networks: According to Santoro, Bresciani, and Papa (2020), networks and alliances foster innovation in the creative industry. However, most handicraft businesses in this study practiced closed innovation, facing branding and awareness challenges. Therefore, collaborating and improving networking activities can enhance competitiveness, and expanding the customer base. Forming team networks and seeking low-cost operations are valuable for building long-term profitable relationships (Kuhn & Galloway, 2015).

Improve marketing campaigns*:* The research findings revealed that participants lacked marketing activities and funding, while being unaware of government support in Sri Lanka. However, the Arts Council and the Laksala institute provides promotion and aid to handicraft sectors (Teo et al., 2020). Obtaining help from such institutes and government credit facilities can enhance marketing, help reach a larger customer base, and improve business efficiency while reducing costs (Silva, 2019).

Brand and trust building*:* Brand building and trust are crucial for small art and craft businesses (Kuhn & Galloway, 2015). Consumers in Sri Lanka have a negative buying attitude towards art and craft products, often requiring an emotional connection to the product, to make a purchase (De Silva & Hemachandra, 2012; Kalubowila & Rajapakse, 2021; Koswatte, 2020). Therefore, it is crucial to foster two-way interactions between businesses and customers, to strengthen relationships. Utilising social media platforms would effectively establish two-way communication, foster trust, and create awareness about the business. Creating value in products and incorporating cultural values can improve buyer attitudes in Sri Lanka.

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ISSN 2989-0705 (Online) | Vol 1 | Issue 1 | July 2023

A Thematic Analysis: Influence of Consumer Ethnocentrism on Consumer Purchase Intention of Recession-bound Sri Lankan Millennials in the Food Industry

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Abstract

Consumer ethnocentrism is an essential concept that plays an important role in marketing management as it helps resonate a person's purchase intentions towards foreign and local products. The purpose of this thematic study is to investigate the influence of consumer ethnocentrism on consumer purchase intentions among Sri Lankan millennials in the food industry, during the Sri Lankan economic crisis. A survey was conducted in Colombo, Sri Lanka, amongst ten millennial residents, working in Sri Lanka for more than five years. The survey was carried out through in-person interviews. Socio-psychological traits like cultural openness, patriotism, dogmatism, collectivism and consumer cosmopolitanism were chosen antecedents of consumer ethnocentrism. The participants of the survey were found to be only somewhat ethnocentric.

Keywords: Consumer Ethnocentrism, Consumer Purchase Intentions, Food Industry, Economic Crisis

1. Introduction

1.1 Background of the study

Consumer ethnocentrism is an important consideration of manufacturers as it forecasts consumer behaviour towards foreign or local products (Nguyen et al., 2022). Consumer purchase intention indicates the purchasing behaviour of consumers concerning specific products. Consumer ethnocentrism has influential demographic and socio-psychological antecedents such as income, education, age, education, patriotism, openness to foreign culture and ethnic pride, while antecedents for consumer purchase intention include brand trust, perceived quality, perceived value and brand image (Jain & Jain, 2013; Kreckova et al., 2012; Nguyen et al., 2022).

Kreckova et al. (2012) found that there is a significant influence of consumer ethnocentrism on consumer purchase intention during an economic crisis. Further, Akbarov (2022) and Miguel et al. (2022) concluded that consumer ethnocentric customers have a negative perception towards foreign products and a positive perception towards local products.

1.2 Overview of the Sri Lankan Food Industry

The Sri Lankan diet traditionally consists of wholesome foods like legumes, tuber roots, fruits, whole grains and bread, however, this has changed due to the commercial and demographic transformations in the island (Bandara et al., 2021). Sri Lankan food manufacturers provide a large variety of food items ranging from processed foods to fresh produce (Sri Lanka Export Development Board, 2022). Overall, the industry is segmented into milk by-products, poultry, fish and seafood, fresh perishables, meat, bread and grain-based products, greases and fats, dips and spreads, convenience food, confections and snacks, infant food and pet feed (Sri Lankan Export Development Board, 2017).

The agri-food sector contributes to 7.4% of the GDP, of which 1.3% is derived from fisheries and 0.9% is accounted for by the livestock sector (International Trade Administration, 2021).

1.3 Problem Statement

Consumer ethnocentrism is an important concept that enterprises take into consideration to forecast consumer behaviour. However, studies on consumer ethnocentrism are sparse in the Sri Lankan context (Silili & Karunarathna, 2014). Presently, due to the prevailing economic crisis, the Sri Lankan government has banned imports of various food items and other goods, encouraging people to purchase locally produced products. It is evident that the import market is heavily affected by the current situation. Businesses that are import-oriented are either forced to go out of business or face extreme consequences. The impact of consumer ethnocentrism in the current economic crisis will allow local businesses to comprehend the role of ethnocentrism in consumer purchasing behaviour and allow them to strategise accordingly in order to remain competitive.

1.4 Research Aim

The research aim of this study is to examine the influence of consumer ethnocentrism on consumer purchase intentions among Sri Lankan millennials in the food industry during the economic crisis.

2. Methodology

A survey was conducted in Colombo, Sri Lanka, amongst ten millennial residents, working in Sri Lanka for more than five years. The survey was carried out through in-person interviews. Thematic analysis was used to analyse the data collected.

3. Findings and Discussions

3.1 Demographic Composition

Age, income, degree of education and gender are significant factors that affect consumer ethnocentrism in a given region (Agarwal, 2019; Alam et al., 2022). The demographic data of the participants is given in Table 1.

Table 1. Respondents' demographic details

	Gender	Age	Occupation
A	Female	37-41	Lecturer
B	Female	26-30	Bank Teller
C	Female	26-30	Academic Administration Officer
D	Female	26-30	Academic Administration Officer
E	Female	37-41	Administrative Officer
F	Male	37-41	Customer Relationship Manager
G	Male	31-35	Operations Executive Officer
H	Male	26-30	Waiter
I	Male	31-35	Project Manager
J	Male	31-35	Lecturer

3.2 Consumer Ethnocentrism and Consumer Purchase Intention during an Economic Crisis

The political state of a country plays a vital role in the level of consumer ethnocentric tendencies expressed by consumers (Dogi, 2015; Jain & Jain, 2013). It was found that all the respondents agreed that their purchasing power or intentions had changed drastically due to the economic crisis, mainly due to low quality of food items, lack of availability and inflated costs.

All the respondents expressed that they preferred foreign food items but were forced to switch to locally produced foods due to financial constraints. Their preference of foreign food items was due to the general belief that foreign-produced foods were tastier and better quality-wise. This aligns with Karoui and Khemakhem's (2019) findings, where several developing countries expressed reverse ethnocentrism, wherein foreign cultures are considered superior to local cultures. Further, the researcher also found that all respondents were in agreement that it was necessary for them to step-up and purchase local food products to aid the economic growth of the country.

Respondent 'F' stated, "*at a time when the whole country is facing a crisis, I believe that we should support our own businesses to help our economies and uplift the life of small business owners. I'm a hard-core believer of that and usually support local*".

Respondent 'J' further provided examples of fast-food outlets, implying that he would prefer to buy from local outlets than international franchises. Further, respondent 'A' was disheartened to express that parents were unable to buy powdered milk for their children due to inflated prices and she also expressed concern over the poor quality or nutritional value of locally produced alternatives.

Moreover, these findings align with previous studies on the effects of consumer ethnocentrism and consumer purchase intention during an economic crisis in developing countries, due to worry regarding their country's economic strength (Dogi, 2015;

Karoui & Khemakhem, 2019; Onin et al., 2022).

3.3 Socio-psychological Consumer Ethnocentrism Antecedents Affecting Consumer Purchase Intention

Consumer ethnocentrism has the following antecedents: cultural openness, dogmatism, patriotism, cosmopolitanism and collectivism (Jain & Jain, 2013; Prince et al., 2019; Shankarmahesh, 2006).

3.3.1 Influence of Cultural Openness on Consumer Purchase Intention

Lee and Robb (2022), Nesaei and Shahtahmasbi (2022), and Rybina (2021) found that cultural openness influenced consumer purchase intention.

The respondents are generally open to trying foods that typically originate from foreign cultures like Ramen from Korea, Sushi from Japan, Kebab from the Middle East, Croissants from France, and much more. The young-millennials and mid-millennials are more open towards purchasing foreign-originated foods, whereas the older millennials were less culturally open and preferred locally originated food items.

The younger and mid-millennials seemed to express more cosmopolitan traits as they did not define their preferences or identity as restricted to a single nation. Further, culturally open individuals also highlighted that the economic crisis played a definite role in their reduced purchase of foreign-originated foods.

Respondent 'B' stated, "*I am so tired of Sri Lankan cuisine, always having to eat rice and curry. My favourite cuisine is Italian, and the crisis played a major role in preventing the purchase of my favourite cuisine and it made me depressed to not be able to fulfil my cravings for such a long time*". However, Respondent 'A', who was among the older millennials had stated, "*I'd switch pasta and burgers any day for yellow rice and papadam and string hoppers, potatoes, cooked in a traditional way*".

It was found that culturally open individuals express lesser consumer purchase intention towards local products, indicating low consumer ethnocentric tendencies (Jain & Jain, 2013; Sharma, 2015).

3.3.2 Influence of Patriotism on Consumer Purchase Intention

Costa et al. (2018) and Wel et al. (2015) state that patriotism has an impact on consumer purchase intention and consumer ethnocentricism. Shankarmahesh (2006) concluded that the impact of patriotism on consumer ethnocentric tendency varies from one country to another.

This study found that highly patriotic individuals are not very open to other cultures and open-minded consumers are not particularly patriotic. Three respondents were highly patriotic, while four respondents stated that they were completely unpatriotic. The remaining respondents identified themselves as neither patriotic nor unpatriotic. Highly

patriotic individuals had strong likings towards purchasing typically Sri Lankan food items. While unpatriotic individuals were very much open to purchasing food items from other cultures, however, mid-level patriots preferred purchasing any kind of food, regardless of origin.

Further, there were no distinct demographic implications on the effects of patriotism on consumer purchase intention and consumer ethnocentrism. Interestingly, the mid-level patriots made it a point to imply that most of their purchase decisions do not include patriotism as a consideration. Respondent 'I' stated that it was unfair to question the implication of patriotism on economic decisions during a crisis period. Responded 'D' stated, "*I wouldn't exactly say I'm patriotic, it's more like a survival tactic with consideration to everything going on economy-wise*".

Similar to the findings of Jain and Jain (2013), Pentz et al. (2017), and Rybina et al. (2010), this study was able to conclude that patriotism influences consumer purchase intention. More specifically, patriotic individuals tend to express more consumer ethnocentric tendencies and purchase local food items, while unpatriotic individuals showed lesser consumer ethnocentric tendencies and purchased food regardless of production origin (Costa et al., 2018; Čvirik, 2021; Wel et al., 2015). On the contrary, respondents did have conflicting views regarding their purchase of local food items due to patriotic reasons during an economic crisis. Shankarmahesh (2006) highlighted how such conflicting repercussions could occur on a regional basis.

3.3.3. Influence of Dogmatism on Consumer Purchase Intention

Caruana (1996), and Nesaei and Shahtahmasbi (2022) found that highly dogmatic consumers are more ethnocentric than those who are not.

Considering the concept of dogmatism, this study questioned respondents on whether recommendations of other people towards food items affected their purchase decisions during the crisis period. The findings revealed that the majority of males considered recommendations towards food items, while females preferred to trust their gut feelings when purchasing local food items. Noticeably, it was discovered that the females expressed higher dogmatic traits than the males. This finding coincides with the studies of Deocampo et al. (2021), Egler (2021), and Zmigrod et al. (2021), that found that dogmatic behaviour was influenced by demographic variables like gender and age.

3.3.4 Influence of Collectivism on Consumer Purchase Intention

Chatman et al. (2015), and Faqih and Jaradat (2015) and found that demography influences collectivism.

Sri Lanka is well-known for its tight-knit communities and the ability to form networks (Nelson & Nordin, 2020). This study inculcated the elements of collectivism by specifically questioning if a person's purchase decisions were a result of empathy towards the wellbeing

of local sellers and their families during the crisis. Out of the ten respondents, the researcher found that only two individuals did not take into account the feelings of local vendors, nor did they empathise with their situations. The remainder of respondents mentioned that during the peak crisis, they were keen on helping out local vendors whenever possible, as they empathised with their daily struggles and also the economic impact on local vendors.

Considering demographics, there were no distinct constructions of demographic factors on collectivistic traits of the respondents, which was conflicting with the findings of Chatman et al. (2015), and Faqih and Jaradat (2015) as they identified that demography influenced collectivism. Respondent 'D' stated, "*Local vendors are immediate partakers of the economy. If we do not purchase from them, then local production would reduce, affecting livelihood and also crippling the economy as local production and income drop, and also for some local vendors, it is their only source of income, so I'll be contributing to their personal livelihood as their income enables them to afford daily necessities*''.

Additionally, respondent's 'B', 'F' and 'G' added that during the economic crisis, they have given extra tips and donations to support local vendors. For instance, Respondent 'F' helped a local vendor set up a small food stall in Nuwaraeliya, close to a major tourist attraction, where he was able to earn a better livelihood. These findings show that collectivism has a definite impact on consumer purchase intention of local food items. Further, these results align with the research findings of Erkaya (2019), Pentz et al. (2017), wherein it was concluded that collectivism influences consumer ethnocentric tendencies as collectivistic people were more conscious of how their purchase of local food items affected society.

3.3.5 Influence of Consumer Cosmopolitanism on Consumer Purchase Intention

Prince et al. (2019) found that consumer cosmopolitanism plays a mediating role in consumer ethnocentrism, based on the moral implications and demographic background of the individuals.

Many individuals identify themselves as foodies or jest that food is their best friend. Through this identification, many individuals also expose consumer cosmopolitanism in form of their preferences and personalities. Further, this study identified that cosmopolitan traits of individuals are relative to their levels of cultural openness. This study inquired if the respondents' decision to purchase local or foreign foods came as a result of their personality and international exposure. The researcher discovered that almost all the respondents had strong personal preferences towards purchasing foreign brands as a result of their international exposure. Further, some respondents stated that personality traits like frugalness (price), attentiveness to detail (quality or quantity) and health consciousness

(nutrition factors) were qualitative factors that affected their purchase decisions for local or foreign food items.

Individuals during the crisis period had ideally preferred foreign foods but were forced to switch to locally produced food items. The respondents consider themselves global citizens but had to switch to local food items due to the economic crisis.

Respondent ‘A’ claimed that she would rather buy foreign-produced milk powder than local-produced due to quality and taste concerns. Respondent ‘C’ stated that price is a decisive factor in her purchase decision of daily foods like rice, where she further elaborated that locally harvested rice and imported rice are very similar, but that locally harvested rice is much cheaper. Respondents' ‘H’ and ‘F’ mentioned that nutrition factors played a role in their preferences for local food items, as they used lesser preservatives, were fresher and also closer to organic.

Furthermore, there were not many demography-based variations in the responses obtained which implies a moderate influence, similar to the outcomes of Prince et al. (2019). This study found that cosmopolitan individuals expressed consumer ethnocentric tendencies during the crisis period, due to personal characteristics, similar to studies conducted by Abrar et al., (2019).

4. Conclusion

The selected socio-psychological ethnocentric antecedents that were studied were cultural openness, patriotism, dogmatism, collectivism and cosmopolitanism. A summary of the research findings is given below.

Cultural Openness

Younger (26 – 30 years) and mid-millennials (31 – 36 years) expressed more cultural openness and typically purchased foreign foods during the crisis. Older millennials (37 – 40 years) were less culturally open and had higher consumer ethnocentric tendencies during the crisis.

Patriotism

Highly patriotic individuals expressed more consumer ethnocentric tendencies and preferred purchasing local food items. Unpatriotic individuals had low consumer ethnocentric tendencies and preferred purchasing foreign foods. Mid-level patriots did not have specific preferences and purchased regardless of origin.

Dogmatism

Females were highly dogmatic and had low consumer ethnocentric tendencies. Males were not very dogmatic, and their consumer purchase intention of local foods remained unaffected.

Collectivism

Collectivistic people are conscious of how their purchases affect society. The respondents

displayed collectivistic tendencies and supported local vendors whenever possible.

Consumer Cosmopolitanism

Almost all respondents had strong personal preferences towards purchasing foreign brands. However, they still switched to purchase of local foods due to the economic crisis. Demography did not play a significant role in the levels of cosmopolitanism of individuals.

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ISSN 2989-0705 (Online) | Vol 1 | Issue 1 | July 2023

The impact of Metaverse Virtual Reality towards Improving Customer Engagement: A Study Based on Online Shopping Clothing Businesses in the Clothing Industry of Sri Lanka

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Abstract

Online shopping is becoming more widespread in Sri Lanka due to its convenience. However, there are still limitations to online shopping, since the two dimensional (2D) images, do not allow customers to experience the level of in-depth experience that physical stores offer. Therefore, the purpose of this study is to examine how the metaverse technology will impact customer engagement for online shoppers in the clothing industry in Sri Lanka. This study identified social factors, interactivity, technological convenience, and motivational factors as the most common independent factors that have an influence on customer engagement when shopping online. These factors were used to study how customer engagement will be impacted by using metaverse technology. A survey was conducted among 100 individuals from Colombo using a structured questionnaire to gather their opinions on using metaverse technology when shopping online. The study revealed that all the factors have a strong positive relationship with customer engagement in a metaverse. The most significant factors are social factors and motivational factors. The individual preferences of the respondents indicate a willingness to try metaverse technology when shopping online.

Key words: Metaverse Virtual Reality, Customer Engagement, Online Shopping

1. Introduction

1.1 Background to the study

Developments in digital technology, including the emergence of the internet, have transformed commercial activities, allowing for new methods of conducting business known as electronic commerce. The concept "online shopping" represents the type of electronic commerce which includes the process of buying products that are sold online (Gabriel, Ogbuigwe & Ahiauzu, 2016). Businesses have pursued to sell their products to consumers who surf the internet since the dawn of the World Wide Web which helps consumers to purchase from the comfort of their own homes while sitting in front of the computer (Kaur, 2013).

Clothing shopping on the internet is becoming increasingly popular (Kim & Kim, 2004). It has been stated that the clothing boom has been spurred by the recent integration of apparel manufacturers towards the continued

encroachment of established shops into the online channel (Schaeffer, 2000). As a result, many consumers prefer to shop online due to its various conveniences (Kaur, 2013). Further, secure digital technologies have resulted in a highly competitive economy with more shopping options than ever before for customers (Kim & Kim, 2004).

However, despite all of the benefits of online shopping, there are also several drawbacks, such as the lack of a physical environment and ambience in which customers may experience and converse with the salesman face-to-face. To combat these issues, online stores have created real-time chat rooms and virtual fitting rooms (Yang, Zhao & Wan, 2010). As a result, the nature of purchasing is changing as technology changes. Not only has the internet changed the way people shop, but so have new improvements in smart and linked devices (Kim et al., 2013). The Metaverse Virtual Reality (MVR) is such a technical innovation that has a significant impact on the shopping experience (Swilley, 2015). The Metaverse is an immersive 3D world based virtual atmosphere where it enables users to interact with one another in a realm powered by technology (Shen et al., 2021). Digital avatars like humans allow them to socialise, shop, and interact more. Three-dimensionality in metaverse retailing allows for a richer purchasing experience across the three virtual channels that represents the real retail establishments (Cagnina & Poian 2009).

With the goal of increasing customer engagement in Sri Lanka's online clothing industry, this dissertation will gain more insight into the relationships between the theories of online shopping and metaverse virtual reality.

1.2 Overview to the Online Clothing Industry of Sri Lanka

In recent years, there has been a huge expansion in the number of Sri Lankan (SL) online-shopping platforms where SL e-commerce platforms grew by 34% in 2018, and the country's e-commerce business sector is expected to reach USD 400 million by 2022. (Ginige & Mahima, 2021). In the context of SL online-shopping platforms, clothing industry is expected to be the future of e-commerce. As stated by statistics and researchers, clothing companies are now paying particular attention to their online presence (Ginige & Mahima, 2021).

SL's internet shoppers, on the other hand, are still conversant with metaverse virtual reality. According to reports, metaverse is becoming the biggest trend in Sri Lankan fashion, with brands developing clothes that exist partially or entirely in virtual space (Fashionating World, 2022). Local multinationals such as MAS, Brandix, Norlanka, and others, which have evolved to be high-value in the Sri Lankan apparel industry, are now increasing their design capabilities with new digitalisation investments. It could even choose to create fashion for the metaverse as a following step (Fashionating World, 2022). Additionally,

telecommunications businesses such as Dialog Axiata PLC, Ideamart, and Innovation Foundry, in partnership with the Google Developer Group Sri Lanka, recently staged the highly anticipated metaverse tech event for the first time in South Asia on May 28. (The Island, 2022). According to Morgan Stanley, the metaverse will add roughly 25% to the entire earnings of the Sri Lankan fashion industry by 2030. (Fashionating World, 2022). In 2021, it would be nearly ten times Sri Lanka's garment export revenues. Nevertheless, the metaverse, according to Yohan Lawrence, Secretary General of the Joint Apparel Association Forum (JAAF), will determine the next decade of fashion in Sri Lanka (Fashionating World, 2022).

1.3 Problem Statement

Most facets of modern society are heavily influenced by technology, and the fashion business is no exception (Sina & Wu, 2019). Social networks and other online media are excellent engagement and two-dimensional communication tools, however, given the nature of this 2D communication, there is the possibility for serious risks associated with 2D static environments, due to its inability for customers to feel a product (Sina & Wu, 2019). But nevertheless, it was asserted that 3D settings provide a physical distribution of information relating to product, texture, motion, and other features. These informational spatial distributions give customers a greater sense of presence in those settings. Thus, compared to 2D static images, 3D virtual displays have a greater local presence and product impact, which affects consumers' impulsive purchasing behaviour (Sina & Wu, 2019). However, Sri Lankan online shopping has yet to adopt 3D settings, and the use of 3D environments in the fashion industry is scarce in the place. Hence, metaverse's vivid, immersive, engaging online shopping experience will encourage more people to buy online. This is true for the clothing industry as well. Therefore, it is important to understand the interest of Sri Lankan online shoppers to adopt to the MVR in their online shopping experience. As a result, this study will look at how desirable it is for online customers to have an engaging 3D-based online shopping experience with metaverse VR.

1.4 Research Aim

The research aim of this study is to examine how the metaverse virtual reality factors impact customer engagement in the clothing industry in Sri Lanka.

1.5 Scope

The survey was conducted among individuals from Colombo city who shop for clothes online.

2. Research Methodology

This study used the quantitative mono research method.

2.1 Conceptual Framework

The Conceptual Framework of this study is given in Figure 1.

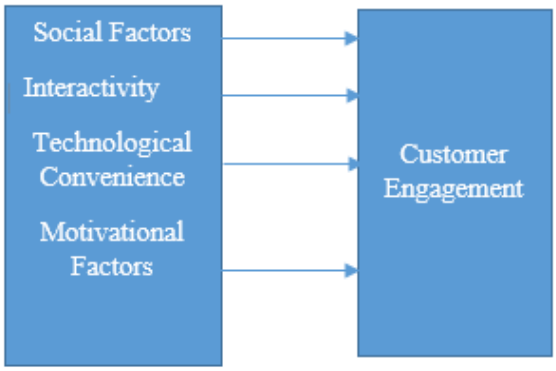

Figure 1. Conceptual Framework

2.2 Research Hypothesis

H1 – There is a positive relationship between social factors and customer engagement in metaverse virtual reality.

H2 – There is a positive relationship between interactivity and customer engagement in metaverse virtual reality.

H3 – There is a positive relationship between technological convenience and customer engagement in metaverse virtual reality.

H4 – There is a positive relationship between motivational factors and customer engagement in metaverse virtual reality.

2.3 Operationalization Table

The Operationalisation table is given in Table 1.

Table 1. Operationalisation Table

Independent Variables	Measures
Social Factors	Sharing the shopping experiences, knowledge with others
	Giving recommendations
Interactivity	Stronger emotive involvement
	Digital environment of stores
	Drive cognitive and emotional states
Tech. Convenience	Service quality and system quality
	Real-time information
	Responsiveness, compatibility and usability
Motivation Factors	Utilitarian motivation and Hedonic motivation
	Customers repurchase of goods and services
	Enjoyment derived from the highly pleasurable sources

The dependent variable, customer engagement was measured using interaction and communication.

2.4 Population and Sampling

The population of this research is 626,000 individuals who reside in the Colombo City (Macrotrends, 2022). The sample size is 100 individuals. The study used the non-probability snowball sampling method by taking into account its convenience and inexpensiveness.

2.5 Data Collection

The data was collected using Google Forms using a Likert based structure questionnaire.

2.5 Data Analysis

The data was analysed using SPSS software using correlation and multiple linear regression analysis.

3. Findings & Discussion

3.1 Response Rate

A total of 93 valid responses were received out of the 100 questionnaires sent. Therefore, the response rate is 93%.

3.2 Demographic Details

Table 2 provides the summary of the demographic data of the respondents.

Table 2. Demographic Details

Age	
18 - 24 yrs.	45%
25 - 34 yrs.	47%
35 - 44 yrs.	4%
45 - 55 yrs.	3%
Gender	
Male	50%
Female	50%
Highest Education	
O/L	5%
A/L	31%
Bachelors	42%
Masters	15%
Employment	
Student	24%
Employed	71%
Unemployed	5%
Occupation	
Private sector	65%
Public sector	2%
Self-employed	11%
Unemployed	20%

3.3 Cronbach's Alpha Test

The reliability of the variables was examined using Cronbach's Alpha. Thus, in this questionnaire, four independent variables and one dependent variable have been analysed using Cronbach's Alpha Test. The results are shown in Table 3.

Table 3. Cronbach Alpha Test

Variable	**Cronbach's Alpha**	**Reliability**
Social Factors	0.801	Very Good
Interactivity	0.865	Very Good
Technological Convenience	0.674	Good
Motivational Factors	0.765	Good
Customer Engagement	0.903	Very Good

As per the reliability test results shown in Table 3, all the variables can be considered reliable, since they are above 0.6.

3.4 Correlation Analysis

The correlation analysis results are given below.

Table 4. Correlation Analysis Results

Independent Variable	**Pearson Correlation Coefficient**	**Significance (2- tailed)**
Social Factors	0.622	.000
Interactivity	0.523	.000
Technology Convenience	0.502	.000
Motivational Factors	0.653	.000
Dependent Variable – Customer Engagement		

As shown in Table 4, all the independent variables have a strong positive correlation with customer engagement, since the significance is 0.000 and the correlation coefficients are above 0.5. This results indicates that all the four hypotheses formulated in this study are valid.

3.5 Hypotheses Validation

H1 – There is a positive relationship between social factors and customer engagement in a metaverse virtual reality.

H1 is accepted.

This result is supported by Busalim et al. (2019), who stated that there is a relationship between social factors and customer engagement.

H2 – There is a positive relationship between interactivity and customer engagement in metaverse virtual reality.

H2 is accepted.

This result is supported by Utami, Ekaputra, Japutra & Doorn (2021) who concluded that there is a relationship between interactivity and customer engagement.

H3 – There is a positive relationship between technological convenience and customer engagement in metaverse virtual reality.

H3 is accepted.

This result is supported by Busalim et al. (2019) and Kumar et al. (2017), who found a relationship between technological convenience and customer engagement.

H4 - There is a positive relationship between motivational factors and customer engagement in metaverse virtual reality.

H4 is accepted.

This result is supported by authors such as Xu, Ryan, Prybutok & Wen (2012) and Kim et al. (2013), who concluded that there is a relationship between motivational factors and customer engagement.

3.6 Multiple Linear Regression

The multiple linear regression results are given in Table 5. The R square value is 0.490 which means that 49% of the total variance of customer engagement in metaverse is predicted by social factors, interactivity, technological convenience and motivational factors. This also means that 52.2% of the variance is impacted by other factors.

Table 5. Multiple Linear Regression analysis

Variable	Beta	Sig.
Motivational factors	0.429	0.000
Social factors	0.337	0.001
Interactivity	0.018	0.456
Technological Convenience	0.166	0.693

Based on the regression analysis results given in Table 5, it becomes evident that at 5% significance level, the most significant variables are motivational factors and social factors.

3.7 Descriptive Analysis

The descriptive analysis results based on the the respondents' answers to questions regarding their online shopping experience are summarised in Table 6.

Table 6. Online Shopping Experience

How often do you use internet every day?	
Less than one hour	1%
1-2 hours	12%
2-3 hours	13%
More than 3 hours	72%
I believe online shopping is secure and safe	
Strongly agree	31%
Agree	46%
Neutral	19%
Disagree	2%
Strongly disagree	1%
I am comfortable in using online shopping	
Strongly agree	46%
Agree	31%
Neutral	19%
Disagree	2%
Strongly disagree	2%
I am aware about the risks when using online shopping	
Strongly agree	38%
Agree	47%
Neutral	11%
Disagree	0
Strongly disagree	1%

As shown in Table 6, the majority of the respondents feel that online shopping is safe and secure and they are comfortable using online shopping. They are also aware of the risks when using online shopping.

Individual Preferences

The individual preferences of the respondents in relation to the factors examined in this study were gathered using a 1-5 Likert Scale, where 1 – Strongly Disagree and 5 – Strongly Agree.

Table 7. Individual Preferences

Social factors	**Mean**
The use of online shopping in my community influences my decision to purchase online	3.91
I share my shopping experience with others via social commerce platforms (Instagram, Facebook, Pinterest, Snapchat, TikTok and much more)	3.76
I like to get to know others experience from online shopping purchases on social platforms	4.42
The sharing of shopping experience on social platforms encourages me to engage more in online shopping	4.23
Interactivity	**Mean**
I like a vivid (realistic) online shopping experience	4.46
I like an engaging online shopping	4.28
I like an immersive (3D virtual displays) online shopping experience	4.32
I like to be able to control my online shopping experience	4.34
Technological convenience	**Mean**
I like to be able to get valuable information about the products I buy	4.69
I like to be able to talk to a digital agent when shopping	4.38
I like a stable and seamless (without delays) online shopping experience	4.70
I like the availability of the purchases at any time of the day in online shopping (24/7 availability)	4.55
Motivational factors	**Mean**
I like to collect information before I make an online purchase	4.62
I find online shopping enjoyable	4.19
I find online shopping convenient	4.32

I am satisfied with my last online shopping experience	4.26
Customer engagement	**Mean**
I would like to be able to interact with other shoppers in the metaverse virtual reality	4.15
I would like to be able to interact with agents in the metaverse virtual reality	4.30
I would like to be able to exchange my ideas and opinions with the community members in the metaverse virtual reality	4.23
I would be highly engaged in the online shopping provided in a metaverse setting	4.26
3D Virtual Displays	**Mean**
I would like to be able to shop for products with 3D virtual displays	4.39
I believe the 3D setting of a product in online shopping would have a greater sense of presence	4.44
The 3D setting of a product would highly encourage my purchasing behaviour in online shopping	4.37

Table 7 shows that the respondents on average, agree with the statements given, since the mean values are 3.9 and above. In terms of the social factors, the respondents are keen on getting to know the online shopping experiences of other shoppers, so that they could make better purchase decisions.

In terms of interactivity, the respondents are very keen on a vivid, realistic online shopping experience, which a metaverse promises to bring.

In terms of technological convenience, the respondents want a seamless online shopping experience without technical issues and lag time.

In terms of motivational factors, the respondents are eager to collect information prior to making their purchase decisions.

In terms of customer engagement, the respondents would like to conduct their online shopping in a metaverse virtual reality in order to improve the interaction and communication with other shoppers.

In terms of 3D Virtual Displays, the respondents would like to shop with 3D Virtual Displays when using online platforms, which they believe will give them a better sense of presence and would probably encourage purchases.

4. Conclusion

In conclusion, the survey results of this study indicate that there is a significant demand for the use of metaverse technology in relation to online shopping in the clothing industry. The respondents are keen on trying the 3D and VR technology in the metaverse to experience a more realistic look and feel for the products, which they believe will help customer engagement, which in turn will lead to better purchase decisions.

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ISSN 2989-0705 (Online) | Vol 1 | Issue 1 | July 2023

Journal of Applied Learning | BMS make learning happen

Impact of Digitalisation on Consumer Awareness of a SME Mobile Enterprise

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Abstract

Digitalisation is crucial for success in today's business world, providing a competitive edge and keeping companies on par with competitors. However, small and medium-sized enterprises (SMEs) in Sri Lanka have been slow to adopt digitalisation. This study focuses on the impact of digitalisation on customer awareness in SME enterprises and identifies key areas for improvement. The study examined the impact of interactive marketing, personalisation, and comment reviews on customer awareness. A survey was conducted with 100 employees of A & G Enterprises, a SME in the mobile phone business. The findings indicate that interactive marketing and comment reviews have a greater influence on customer awareness compared to personalisation. Based on the research findings, recommendations are provided on how to improve customer awareness through digitalisation. These recommendations include incorporating 24/7 chatbots for inquiries, offering targeted recommendations, implementing a comment section, and including a star rating system on their websites.

Keywords: Digitalisation, Consumer Awareness, Small and Medium Enterprises

1. Introduction

1.1 Background of the study

The mobile phone industry is witnessing a rapid growth globally, with 6.6 billion user's worldwide, accounting for 83.32% of the global population (Statista, 2022). In Sri Lanka, there are 32.29 million mobile connections, growing by 1 million from the previous year (Kemp, 2021). Samsung leads the Sri Lankan market with a 43.35% share, followed by Huawei and Apple (StatCounter Global Stats, 2022). Digital transformation is vital for companies to stay competitive (Kraus et al., 2021). SMEs should prioritise digitalisation and e-commerce to increase customer awareness, boost sales, gain market share, and tap into new segments (Kergroach, 2020; Montenegro, 2021; Yacob et al., 2021). It is recommended to emphasise digitalisation in interactive marketing, personalisation, and customer reviews (Dilham et al., 2018).

1.2 Company Background

The study focuses on A & G Enterprises, a small-scale partnership established in 2011 in the mobile industry. It employs six workers and two area managers. A & G Enterprises serves as the exclusive distributor for Zigo, Akai, Etel, Joyroom, Bird, and IPro brands in the Kalutara and Colombo districts. Their product portfolio

includes feature phones, tablets, touch phones, headphones, speakers, and other mobile accessories. Their current interests are digitising operations despite ongoing challenges (Lakshman, W. K., Personal Communication, July 16, 2022).

1.3 Problem Statement

The economic downturn in Sri Lanka has hampered sales and profits. USD scarcity and fuel concerns have prompted customers to shift to online platforms, negatively impacting A & G Enterprises' sales (as shown in Figure 1), resulting in revenue that falls short of expectations (Lakshman, W. K., Personal Communication, July 16, 2022). Currently, A & G Enterprises seeks to understand how digitalisation can enhance customer awareness and increase customer retention/loyalty (Subhashini, M. H., Personal Communication, July 16, 2022).

The declining sales of A & G Enterprises can be addressed by digitalisation as indicated in studies conducted by Dwivedi et al. (2021), Hien and Nhu (2022), and Lähteenmäki, et al. (2022), which indicate that digitalisation enhances customer awareness and helps boost sales.

Various global studies have explored the impact of digitalisation on customer behaviour (Dimova, 2021; Hofmann, 2021). However, there is limited research conducted on its impact specifically on SME mobile enterprises, especially in Sri Lanka. Thus, conducting a contemporary study is crucial to gain insights into how digitalisation can help improve customer awareness in the SME mobile industry in Sri Lanka.

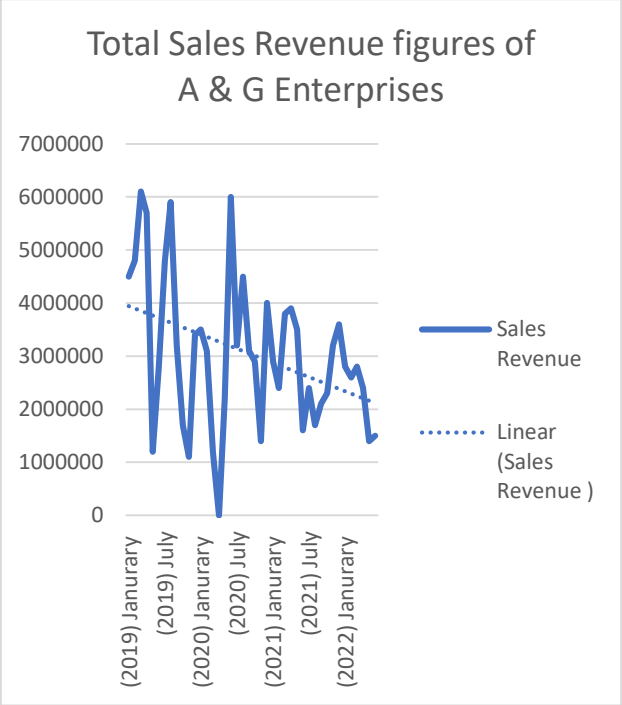

Figure 1. Total Monthly Sales Volume (A & G Enterprises, 2022)

1.4 Research Aim

The aim of this study is to identify the impact of digitalisation on customer awareness of customers of A & G Enterprises.

1.5 Scope

A & G Enterprises' target consumer base is estimated to be around 700,000 (Kasun, G. B., Personal Communication, July 16, 2022). This study will survey 100 customers of A & G enterprises in the Kalutara and Colombo district.

2. Research Methodology

This study used a mono method quantitative research approach.

2.1 Conceptual Framework

The conceptual framework of this study is given in Figure 2.

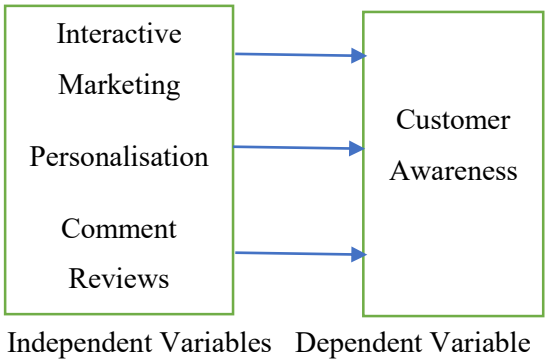

Figure 2. Conceptual Framework

2.2 Hypotheses

The hypotheses formulated in this study are given below.

H1: There is a relationship between interactive marketing and customer awareness.

H2: There is a relationship between personalisation and customer awareness.

H3: There is a relationship between comment reviews and customer awareness.

2.3 Operationalisation Table

The operationalisation Table is given in Table 1.

Table 1. Operationalisation Table

Variable	Measure
Interactive Marketing	Customer Engagement
	Interactivity
	Experience
Personalisation	Customisation
	Targeting
	Categorisation
Comment Reviews	Reduce uncertainty
	Customer impression
Customer Awareness	Customer Knowledge
	Brand recognition

The entire questionnaire used the 5-point Likert scale.

2.4 Population and Sampling

Considering the fact that A & G Enterprise's target market exceeds 100,000 individuals, the researcher selected 100 clients as a representative sample due to cost and time limitations. The convenience sampling technique was used.

2.5 Data Collection

A pilot study involving ten participants was conducted. The questionnaire was approved based on the positive feedback.

The research used Google Forms to collect the data. The invitations were distributed via email and WhatsApp.

2.6 Data Analysis

The collected data was analysed using SPSS. Cronbach's alpha, correlation and multiple regression analyses were conducted.

3. Findings and Analysis

3.1 Response Rate

A total of 96 responses were received from 100 distributed questionnaires to A & G Enterprise customers.

3.2 Demographic Data

The demographic data of the respondents are given below.

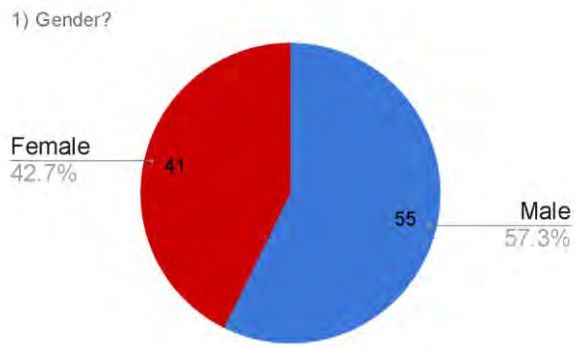

Figure 3. Respondents' Gender

It is evident from Figure 3, that 57.3% of the 96 participating customers were male, while 42.7% were female.

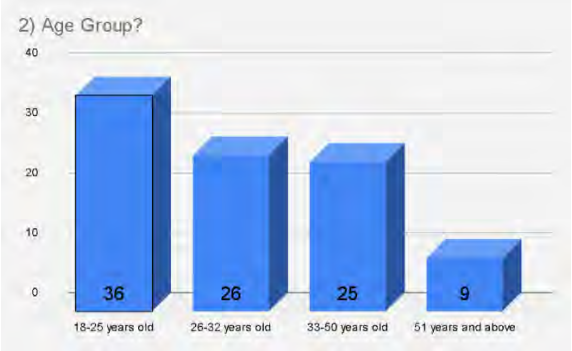

Figure 4. Respondents' Age

The majority of A & G Enterprises' customers, as shown in Figure 4, fall within the 18 to 25-year-old age group.

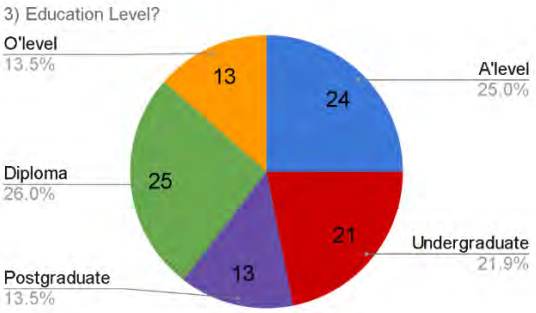

Figure 5. Respondents' Education Level

The Figure 5 shows a significant proportion of A & G Enterprises' customers have a sound education, indicating their intellectual capacity to adapt to digitalisation.

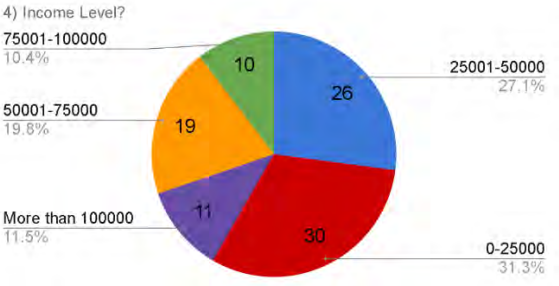

Figure 6. Respondents' Income level

Figure 6 indicates that a majority of respondents fall within the income bracket of 0-

25000 rupees. This finding provides guidance for A & G Enterprises in prioritising their product catalogue during their digitalisation journey. Based on these results, it is advisable for them to focus on establishing an online customer base for their lower-priced products before targeting their higher-end products.

3.3 Data Reliability

The data reliability was assessed using Cronbach's alpha test.

Table 2. Reliability Analysis

Variable	Cronbach's Alpha
Interactive Marketing	0.712
Personalisation	0.759
Comment Reviews	0.702
Customer Awareness	0.716

The reliability analysis, presented in Table 2, indicates that all the variables are reliable, since the Cronbach alpha values are higher than 0.70, indicating acceptable reliability (Tavakol & Dennick, 2011).

3.4 Correlation Analysis

The Correlation analysis results are given in Table 3.

Table 3. Pearson Correlation Analysis

Variable	Correlation Coefficient	Significance
Interactive Marketing	0.508	0.000
Personalisation	0.406	0.000
Comment Reviews	0.569	0.000

Note: The dependent variable is customer awareness.

Table 3 shows a moderate positive correlation between interactive marketing and customer awareness, with a Pearson correlation coefficient of 0.508 and a significance of 0.000. Similarly, personalisation and customer awareness exhibit a moderately favourable relationship, with a correlation coefficient of 0.406 and a significance of 0.000. Additionally, comment reviews and customer awareness have a moderate association, with a correlation coefficient of 0.569 and a significance of 0.000.

Overall, the correlation analysis demonstrates positive relationships between the independent variables and the dependent variable, supporting the research hypotheses.

3.5 Hypothesis Validation

H1: There is a relationship between interactive marketing and customer awareness

H1 is accepted

This study's findings support previous research by Halvadia and Menon (2021), and Sharma et al. (2022). According to Giombi et al. (2022), despite its lower click-through rates, interactive marketing leaves a lasting impression. However, Bóveda-Lambie and Hair (2012) discovered no evidence of stronger self-brand connections through social media engagement. Furthermore, Ismagilova et al. (2020), cautioned against excessive inquiries due to slower response times and high costs.

Additionally, studies by Junco and Mastrodicasa (2007), and Shankar et al. (2010) revealed that millennials swiftly embrace interactive marketing that suit their social lifestyle but resent corporate intrusion in their personal online space and are concerned about data protection.

H2: There is a relationship between personalisation and customer awareness

H2 is accepted

The study shows a positive association between personalisation and customer awareness. This finding is supported by Dong et al. (2019), Segijn and Ooijen (2020), Shanahan et al. (2019), and Siddiqui and Warraich (2021). However, Ramnarayan (2011) found privacy concerns and only a partial correlation. Fridh and Dahl (2019) highlighted the importance of timely and authentic personalisation for effective customer awareness.

Conversely, Kharouf et al. (2018), and Mahajan (2015) highlighted the positive impact of personalisation on brand experience and value co-creation. Tyrväinen et al. (2020), confirmed the relationship with customer experience and loyalty. Jackson (2007) suggests integrating personalisation into a broader strategy for business success.

H3: There is a relationship between comment reviews and customer awareness

H3 is accepted

This study confirms a positive relationship between comment reviews and customer awareness. This is consistent with previous studies such as Battha and Zina (2022), Chakraborty and Bhat (2017), and Changchit et al. (2020). Moreover, Saavedra et al. (2015) revealed that positive reviews impact lesser-known brands, while negative comments have a greater impact on well-established brands.

3.5 Multiple Linear Regression

The multiple linear regression results are given in Table 4. The regression model has an R-squared value of 0.398, indicating that 39.8% of the variability in customer awareness can be explained by the independent variables of interactive marketing, personalisation, and comment reviews.

Table 4. Multiple Linear Regression Results

Variable	Unstand. B	Sig. 5%
Interactive Marketing	0.301	0.004
Personalisation	0.074	0.429
Comment Reviews	0.401	0.000

The regression analysis revealed that the most significant variables that impact customer awareness are comment reviews (significance of 0.000) and interactive marketing (significance of 0.004).

Overall, these findings highlight the importance of interactive marketing and comment reviews in increasing customer awareness, suggesting that personalisation may have a lesser impact.

3.6 Descriptive Analysis

The degree of importance of the variables are given in Table 5.

Table 5. Degree of Importance

Variable	Mean
Interactive marketing	4.8
Personalisation	4.9
Comment Reviews	4.8
Customer Awareness	4.9

Note: 1 – Low Importance, 5 – High Importance

Based on the descriptive statistics analysis, interactive marketing was perceived positively by potential customers, with a mean score of 4.8. The responses indicate a preference for humorous interactions and marketing. These findings align with previous research by Groening et al. (2020), Jenneboer et al. (2022), and Yun and Park (2022) supporting the positive perception of interactive marketing.

Regarding personalisation, participants showed a positive perception, with a mean score of 4.9. The findings revealed a willingness to allow websites to store cache/cookies for an improved shopping experience. These findings align with studies by Scientific American (2022) and Strycharz et al. (2021), emphasising the benefits of using cookies for personalised and seamless experiences.

Comment reviews were also positively perceived, with a mean score of 4.8. The findings indicate that customers are influenced by online recommendations from friends and family. This finding is consistent with research by Dellarocas et al. (2014), Kaushal (2022), Venkatesakumar et al. (2020), and Wang and Wang (2020) emphasising the significance of word-of-mouth recommendations.

Regarding customer awareness, potential customers demonstrated a positive perception, with a mean score of 4.9. The findings indicate that customers were already aware of A&G Enterprises' trademarks and logo. This finding aligns with previous research by Najat (2017), and Noorlitaria et al. (2020), emphasising the importance of brand recognition and differentiation in influencing purchasing decisions.

4. Conclusion

In conclusion, this study has revealed that interactive marketing, personalisation and comment reviews have a positive impact on customer awareness in a digital online shopping platform. The study also revealed that interactive marketing and comment reviews have a greater impact than personalisation.

Based on the research findings, it is clear that A & G Enterprises' customers show a strong preference for digitalised operations and online purchasing. In light of these findings, the following recommendations are given for A & G Enterprises to consider in their digitalisation efforts.

Interactive Marketing

To thrive in the digital era, businesses must prioritise online communication and enhance customer experience (Maroengsit et al., 2019; Suwono & Sihombing, 2016). Utilising chatbots can boost customer loyalty by offering an additional communication channel and proactive responses to inquiries and concerns (Brandtzaeg & Følstad, 2017).

A significant majority of respondents (88.6%) prefer having a 24/7 chatbot feature for addressing product inquiries, highlighting their preference for immediate answers. Moreover, customers express interest in interacting with a digital agent to gather product information. Thus, incorporating an AI-powered chatbot on the A & G Enterprises website can enhance the customer experience and reduce the workload of customer service representatives (Ameen et al., 2021). By utilising natural language processing (NLP) technology, the chatbot system can provide real-time assistance without human intervention, improving efficiency (Adamopoulou & Moussiades, 2020). This leads to positive customer experiences, increased sales, and reduced response time and support costs (Jenneboer et al., 2022; McLean & Wilson, 2016). Implementing a chatbot should be considered by A & G Enterprises to enhance customer satisfaction and operational efficiency.

Implement a Wishlist feature on the website with an automated notification system for discounts. Online wish lists have gained popularity among consumers and offer retailers various benefits, including measuring product popularity, predicting sales, optimising product assortments, and enabling personalised advertising (McMullen, 2019; Miller et al., 2010; Zhao et al., 2016). Wish lists can influence consumer choices and increase sales by providing touchpoints for retailers to engage with consumers (Bradford & Sherry, 2013).

The majority of the respondents (86.5%) showed interest in having a Wishlist feature on the website that automatically notifies them of discounts related to their Wishlist items through social media or email. By integrating the Wishlist with an automated notification system, customers can receive mobile alerts about discounts for their desired items. This feature

enhances the overall shopping experience, increasing customer satisfaction and the likelihood of repeat purchases. It also contributes to a sense of psychological ownership and perceived value for consumers (Groening et al., 2020; Peck & Shu, 2018; Petit et al., 2019). Thus, incorporating this feature into the A & G Enterprises website can be a strategic move to boost sales and improve customer awareness.

Personalisation

Personalised recommendation agents play a crucial role in increasing customer satisfaction and gaining a competitive edge for organisations (Lye et al., 2020; Shen, 2014).

According to the findings of this study, 87.5% of respondents prefer websites that offer recommended searches and receive personalised emails with relevant product suggestions and special deals based on their viewed products. To leverage this preference, A & G Enterprises can utilise machine learning algorithms to personalise recommendations according to customer shopping history and preferences. They can also send tailored promotional emails with relevant discounts (Dwivedi et al., 2021; Zhou & Han, 2019). By implementing these strategies, A & G Enterprises can enhance customer convenience, improve the online shopping experience, and drive sales (Lambillotte et al., 2022). Personalised recommendations based on customer behavior have been proven to enhance the customer experience, increase satisfaction, and boost sales (Basu, 2021; Hoyer et al., 2020).

Comment Reviews

Customers are increasingly relying on social media and virtual communities to understand brands, with online reviews significantly influencing purchasing decisions (Chakraborty & Bhat, 2017; Fagerstrøm et al., 2016; Zhao et al., 2013). The importance of credibility has led to increased information-seeking and sharing behaviour among customers (Changchit et al., 2020).

A substantial number (90.6%) of respondents prefer reading product reviews before making online purchases. To enhance customer trust and product promotion, A & G Enterprises can encourage customers to leave reviews and feedback on their website, social media pages, and third-party review platforms (Dellarocas et al., 2014; Nasr et al., 2014). Leveraging popular social media platforms such as YouTube, Instagram, Facebook, and Twitter can facilitate effective marketing campaigns, generate buzz, and encourage customers to share reviews (Mudambi & Schuff, 2010).

The importance of leveraging social media is further supported with 74.5% of respondents stating they are likely to purchase a product if recommended by friends and family on social media. Additionally, this study highlights that 89.6% of respondents desire an online store with a star rating system for products. Implementing a star rating system alongside the

review system, A & G Enterprises can empower customers to make informed choices and foster trust in both the products and the organisation (Kostyk et al, 2017; Venkatesakumar et al., 2020).

Acknowledgments

We are grateful to the employees of A & G Enterprises for granting us access to the company and providing us with invaluable information. We would like to offer a special thank you to those who participated in this study.

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ISSN 2989-0705 (Online) | Vol 1 | Issue 1 | July 2023

The Impact of the COVID-19 Pandemic on Airline Cabin Crew Motivation

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Abstract

The Covid-19 Pandemic has taken a tremendous toll on employees in the airline industry. Many airline companies have suffered huge financial losses, which have led to downsizing of the staff. Safety concerns among employees have led to increased work stress and resignations. This study aims to understand the impact of the Covid-19 pandemic on the motivation of the cabin crew of a leading airline. A survey was conducted among the cabin crew of the airline using a structured online questionnaire. The questionnaire was answered by 105 cabin crew members. Maslow's hierarchy of needs was used to study the motivation of the cabin crew. The impact of physiological, safety, love and belonging, self-esteem and self-actualization needs on the cabin crew motivation were analyzed using correlation and multiple linear regression analysis. The correlation results revealed that all the five needs (physiological, safety, love and belonging, self-esteem and self-actualization) have a positive correlation with cabin crew motivation. Multiple linear regression results revealed that self-esteem and self-actualization were the most significant variables affecting cabin crew motivation. The cabin crew were least satisfied with their safety needs and most satisfied with their self-esteem needs. Employee motivation is critical to ensure proper work standards are maintained, especially during times of crisis. The impact of the Covid-19 pandemic on employee motivation of airline cabin crew is of utmost importance, in order to understand how the Covid-19 pandemic has affected employee motivation and to identify steps that can be taken to improve employee motivation of the cabin crew.

Keywords: Employee Motivation, Covid-19 Pandemic, Cabin Crew, Airline Industry

1 Introduction

1.1 Background

The Covid-19 pandemic has had a tremendous impact on the airline industry. The pandemic brought airports around the globe to a virtual halt. This resulted in huge financial losses to many airlines. Financial losses compelled airlines to severely downsize its staff. Safety concerns forced many staff members to resign from their jobs. The Covid-19 pandemic has adversely affected the mental health of the airline cabin crew. The cabin crew during their flights are exposed to health risks (Görlich and Stadelmann, 2020). Cabin crew members typically suffer from more sleep problems,

depression, anxiety, and fatigue than the average person. Irregular working hours, due to shift work and night work was found to increase physical and psychological problems. The cabin crew's health is also affected by work related fatigue, jet lag, and physical and mental workloads (McNeely et al., 2018). The Covid-19 pandemic has increased their health risk, which has caused cabin crew members to resign from their jobs or to feel very anxious about getting sick with Covid-19. This has affected the motivation of the airline cabin crew. Therefore, it is important to study how the Convid-19 pandemic has affected the motivation of the cabin crew, in order to ensure the concerns of the cabin crew are taken into consideration and to ensure the smooth functioning of the airline.

1.2 Problem Statement

The Covid-19 pandemic has increased the health risk and adversely affected the job security of the airline cabin crew. This has caused the airline cabin crew to experience anxiety and stress, which in turn has affected employee motivation. It is vital to ensure that the cabin crew is motivated in order to ensure their mental and physical wellbeing. This is important to maintain the smooth functioning of the airline operations.

1.3 Research Aim

The aim of this study is to evaluate the impact of the COVID-19 pandemic on the employee motivation of the airline cabin crew of a leading international airline.

1.4 Scope

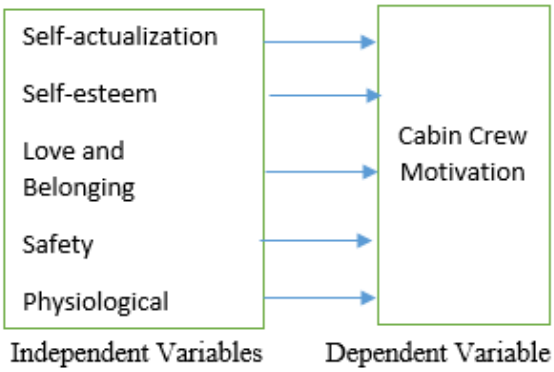
The cabin crew members of a leading international airline were surveyed about their level of satisfaction with their basic needs based on Maslow's hierarchy of needs theory. A total of 105 cabin crew members were surveyed in this study.

2. Research Design

2.1 Theoretical Framework

The theoretical framework used in this study (Figure 1) is based on Maslow's hierarchy of needs theory (Maslow, 1943). This theory was used to study how the needs of the cabin crew members are being met and how they affect employee motivation.

Maslow's model has an immense influence on the field of psychology and organizational behavior, and it continues to be cited widely in modern textbooks (Kenrick et al., 2010).



Source: Maslow (1943)

Figure 1. Theoretical Framework

According to Maslow (1943), five basic needs influence an individual's motivation. The physiological needs include food, shelter,

clothing; safety needs include health, personal security, job security; love and belonging needs include intimacy, trust, acceptance; self-esteem needs include self-respect and respect from others and self-actualization needs include the need to reach one's full potential.

2.2 Hypotheses

The following hypotheses were formulated based on Maslow's hierarchy of needs (Maslow, 1943).

H1: There is a positive relationship between self-actualization needs and cabin crew motivation

Gopinath (2021) states that self-actualization is positively correlated to the ability to enhance employee commitment level, which positively contributes to enhanced organizational performance.

Kumar, Liu and Hossain (2020) revealed that increased self-actualization helps employees take charge of their work and improves their creativity and job performance.

According to Shahrawat and Shahrawat (2017), self-actualization and self-esteem are the most critical needs that need to be satisfied for the employee motivation to be achieved.

H2: There is a positive relationship between self-esteem needs and cabin crew motivation

Li and Wen (2018) identified that positive self-esteem directly impacts decision-making, relations, emotional health, and overall well-being, which in turn affects employee motivation.

A study conducted by Kim and Beehr (2018) revealed that positive self-esteem makes employees feel better about themselves, which in turn helps them to better focus on their work, reduce absenteeism, and help them get along with their co-workers.

H3: There is a positive relationship between love and belonging needs and cabin crew motivation

Ștefan, Popa and Albu (2020) stated that when love and belongingness needs are met satisfactorily, feelings of isolation and depression are not felt and therefore employees cooperate well with each other and are motivated to perform well at work.

H4: There is a positive relationship between safety needs and cabin crew motivation

Chen, Chen and Wu (2018) ranked employee job security as the most relevant motivational factor affecting employees. Further, Lorincová et al. (2019) stated that job security of the employees contributes to an improved feeling of relaxation in their working environment. This reduces employee turnover and improves overall efficiency. Therefore, job security significantly increases employees' efficiency and overall productivity. Cabin crew members have to cope with unique occupational health and safety implications, including radiation exposure, cancer, mental ill-health, musculoskeletal injury, reproductive disorders,

and symptoms from cabin air contamination (Griffiths and Powell, 2012). These safety concerns will impact cabin crew motivation.

H5: There is a positive relationship between physiological needs and cabin crew motivation

A study conducted by Yılmaz and Toylan (2021) revealed that when the physiological needs of employees are met, they are motivated to be more productive at work.

2.3 Methodology

An online survey was used with close ended questions using a 1 - 5 Likert scale to assess the airline cabin crew's satisfaction with their basic needs and how it impacts employee motivation. The pilot test was successful, and no changes were made to the questionnaire. The questionnaire was sent to 120 cabin crew members and 105 responses were received. The response rate was 87.5%. The airline employs a total of 420 cabin crew members. The demographics details of the respondents are given in Table 1.

Table 1. Demographic Data

Demographic Data		**Percentage**
Gender	Female	73.3%
	Male	26.7%
Work Experience	Less than 3 years	14.3%
	3-6 years	29.5%
	7-10 years	24.8%
	Over 10 years	31.4%

The data collected from the 105 cabin crew members was analyzed using the Statistical Package for Social Sciences (SPSS version 20).

3. Analysis and Findings

3.1 Cronbach's Alpha

Data validity was computed using Cronbach's Alpha for all the variables in the study. All the Cronbach's Alpha values were above 0.8, and therefore are deemed reliable.

3.2 Correlation Analysis

Correlation analysis using Pearson's Correlation Coefficient was used to test the relationship between Maslow's basic needs and cabin crew motivation. The correlation analysis results are given in Table 2.

Table 2. Correlation Analysis

Independent Variable	**Pearson's Correlation Coefficient**	**Sig.**
Self-actualization	0.723	0.000
Self-esteem	0.776	0.000
Love and belongingness	0.693	0.000
Security	0.543	0.000
Physiological	0.538	0.000

The correlation analysis reveals that all the independent variables have a positive correlation with cabin crew motivation (at 5% significance level). Therefore, the five

hypotheses formulated in this study are supported, as shown in Table 3.

Table 3. Hypotheses Validation

Hypotheses	Supported
H1: There is a positive relationship between self-actualization needs and cabin crew motivation	Yes
H2: There is a positive relationship between self-esteem needs and cabin crew motivation	Yes
H3: There is a positive relationship between love and belonging needs and cabin crew motivation	Yes
H4: There is a positive relationship between safety needs and cabin crew motivation	Yes
H5: There is a positive relationship between physiological needs and cabin crew motivation	Yes

The correlation analysis results reveal that all the five Maslow's needs have a positive correlation with cabin crew motivation.

3.3 Multiple Linear Regression

Multiple linear regression results revealed that the most significant variables are self-actualization and self-esteem (at 5% significance level), as shown in Table 4.

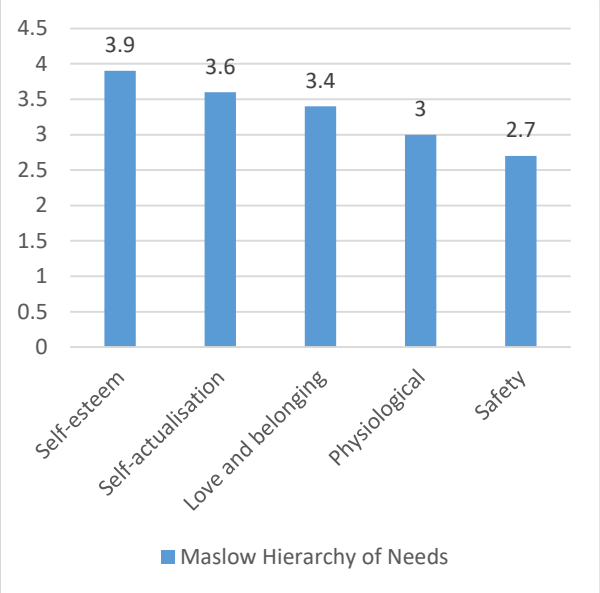
Table 1. Multiple Linear Regression Analysis

Independent Variable	Beta	Significance
Self-actualization*	**0.287**	**0.005**
Self-esteem*	**0.547**	**0.000**
Love and belonging	0.184	0.056
Safety	0.083	0.287
Physiological	0.137	0.059

The regression model has an R square value of 0.632.

3.4 Degree of Satisfaction with Maslow's needs

The cabin crew rated their degree of satisfaction with Maslow's basic needs using a Likert Scale. The results are given in Figure 2.

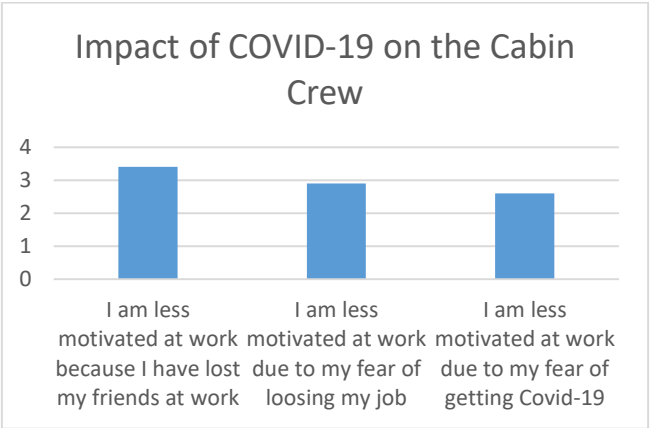


Note: 1-Higly Dissatisfied to 5-Highly Satisfied

Figure 2. Degree of Satisfaction with Maslow's Needs

The ratings reveal that the cabin crew are most satisfied with their self-esteem (3.9) and self-actualization needs (3.6) and they are least satisfied with their physiological (3) and safety needs (2.7). Love and belonging got a rating of 3.4.

The Covid-19 pandemic has increased the health risk and work-related stress. It has also negatively impacted the cabin crew's job security and remuneration/bonuses. This has in turn impacted their degree of satisfaction with their physiological and safety needs. Love and belonging needs have also been impacted, since many cabin crew members have not been able to visit their family due to travel restrictions. They have also lost colleagues and friends due to downsizing and resignations. Their self-esteem and self-actualization needs are being met to a reasonable degree. Working during the pandemic has proven to be a big challenge. The cabin crew are taking risks and continuing with their work in challenging times, which has gained them admiration and respect, which has positively influenced their self-esteem and self-actualization needs.



Note: 1-Strongly Disagree to 5- Strongly Agree

Figure 3. Impact of Covid-19 on the Cabin Crew

As shown in Figure 3, some of the main causes that affected the cabin crew members' motivation include losing colleagues/friends due to downsizing and resignations, fear of losing their jobs and the fear of getting sick with Covid-19. Some crew members experienced severe depression due to these reasons.

4. Conclusion

In conclusion, this study has revealed that all the five Maslow's basic needs have a positive correlation with cabin crew motivation. The most significant needs are self-esteem and self-actualization needs. The cabin crew are most

satisfied with their self-esteem and self-actualization needs and they are least satisfied with their physiological and safety needs. The fear of getting sick with the Covid-19 virus, fear of losing their jobs, the fear of losing their friends/colleagues due to downsizing and resignations, the inability to visit their families due to travel restrictions, the lack of bonuses and the general uncertainty about the future were the main causes that affected employee motivation of the cabin crew members.

The airline has to address the concerns of the cabin crew adequately to ensure that the cabin crew are motivated to perform their job effectively. Some of the steps that the airline can take are: ensure proper Covid-19 related health standards are maintained consistently, provide psychiatric consulting to help the cabin crew cope with their anxieties and fears, reward the cabin crew with bonuses for their commitment, keep a close eye on employee burnout and fatigue, allow sufficient time for rest and socializing with their friends and family.

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ISSN 2989-0705 (Online) | Vol 1 | Issue 1 | July 2023

The impact of transformational leadership on remotely working employees' motivation

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Abstract

Working remotely is becoming more and more popular due to several reasons. Some of the reasons are improved work-life balance, avoiding long commutes to work and back and increased work flexibility. The Covid-19 pandemic has forced millions of employees worldwide to work from home. This transformation from working in the office to working from home will continue due to the advantages it offers. It is important for the company leadership to be able to effectively manage their employees who work remotely. This study examines the impact of transformational leadership on the motivation of remotely working employees. A survey was conducted among remotely working employees from various industries using a structured online questionnaire. The questionnaire was answered by 150 remotely working employees. Six transformational leadership traits were hypothesized to impact the motivation of remotely working employees. The primary data collected was analyzed using correlation and multiple linear regression analysis. The correlation results revealed that all the six transformational leadership traits (goal oriented, supportive, inspiring, encouraging, relationship oriented and creative) have a positive correlation with remotely working employee's motivation. Multiple linear regression results revealed that creative and supportive traits were the most significant traits influencing the motivation of remotely working employees. It is vital to ensure that employees are motivated to perform their job well. This is especially true of remotely working employees who are isolated from their work colleagues. This isolation may affect their motivation due to challenges in interacting with their peers and the leadership. The Covid-19 pandemic has pushed millions of employees all over the world, to work remotely. The transformational leadership style has been identified to be suitable during times of crisis, such as the Covid-19 pandemic. Therefore, a study on the impact of transformational leadership on remotely working employees' motivation is of utmost importance.

Keywords: Transformational leadership, Remote working, Employee motivation, Covid-19 pandemic

1. Introduction

1.1 Background

The Internet and mobile connectivity have made working remotely very popular. Being able to facilitate employees to work remotely instead of reporting to an office poses significant benefits to both employers and employees. Some of the advantages are cost reduction due to low overheads and wider access to human resources for the employers and saving travel time & cost and improving work life balance for the employees (Russel, 2020).

Donati et al. (2021) emphasizes that employers should sustain remote working employee motivation since being restricted to electronic communication can cause isolation and reduces frequent informal interaction among employees. Maintaining employee motivation is important to ensure mental wellbeing as well as job satisfaction levels are improved (Molino, 2020). Remotely working employees are scattered across geographies collaborating through digital communication means. A leader is needed to synergize their work for optimum output while maintaining employee loyalty and admiration. Therefore, in a remote working environment, the leadership style performs a crucial part in maintaining motivation levels of employees (Andersen, 2018). Sinclair (2021) states that Transformational Leadership is an appropriate and robust type of leadership which suits leading a remote workforce.

1.2 Problem Statement

As organizations came to a standstill due to Covid-19 related lockdowns and international border closures, organizations rapidly moved to converting employees to remote workers in order to sustain business operations. Gartner (2021) estimated that the global remote working population was likely to reach 32% by the end of the year 2021. Furthermore, a survey by QuantumWorkplace (2021) on a sample of 32,000 remote working employees observed 77% of participants reporting an increase in productivity, while working remotely over working from a physical office location.

However remote working has several disadvantages, such as employee isolation, anxiety and doubt, lack of motivation in the long term, less emotional connections among colleagues as well as severe limitations in physical encounters between colleagues (Grant et al., 2013). Hence, for any organization, motivation and engagement with their remote working employees is necessary and should be a top priority to ensure that they can fulfill their job responsibilities well.

The lack of maturity and expertise in managing remote working employees as well as insufficient leaders to direct a workforce which was suddenly switched from on premises duties to remote working roles owing to the ongoing Covid-19 pandemic, has led to new challenges in people management (Donnelly and Johns, 2021). Finding methods to ensure workers are well motivated and grievances resolved to

perform their day-to-day duties has now become a common challenge to most organizations due to remote working employees.

Remotely working employees can also suffer from lack of motivation to perform their jobs effectively, due to being separated from their peers and the company leadership and due to other challenges, such as poor connectivity and technology related issues. Therefore, it is important for the leadership to be able to motivate their remotely working employees to perform their jobs well, in order to ensure consistent desirable work output. The transformational leadership style has been identified as a suitable leadership style to manage remote working employees (Sinclair, 2021). There is limited research on the impact of remote working on employee motivation, especially in Sri Lanka. Therefore, a study on the impact of transformational leadership style on the motivation of remotely working employees will contribute to fill this research gap.

1.3 Research Aim

The aim of this study is to examine the impact of the transformational leadership style on the motivation of remotely working employees.

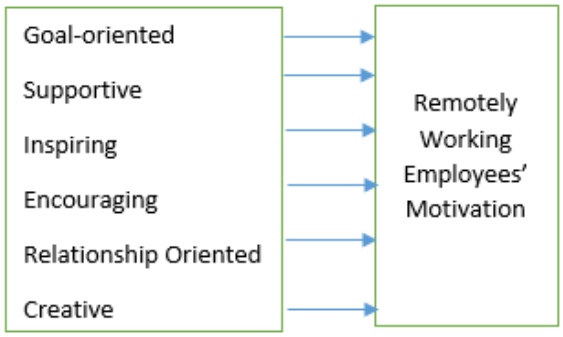
1.4 Scope

A total of 150 remotely working employees from various industries were surveyed in this study. The survey was conducted in Colombo, Sri Lanka.

2. Research Design

2.1 Theoretical Framework

The theoretical framework used in this study is shown in Figure 1. It is based on six transformational leadership traits, which have an influence on employee motivation based on empirical research.



Independent Variables Dependent Variable

Figure 1. Theoretical Framework

2.2 Hypotheses

The following hypotheses were formulated based on the conceptual framework.

H1: A goal orientated leader will have a positive impact on remotely working employees' motivation

Dragoni (2005) stated that positive employee motivation was attained when leaders were goal

orientated. Locke and Latham (2006) highlighted that a goal orientated manager has a high level of influence on employee motivation. Apart from motivating their followers, goal-oriented leaders can build positive interpersonal relationships with their team members (Porter et al., 2016).

H2: A supportive leader will have a positive impact on remotely working employees' motivation

Lam et al. (2007) stated that supportive leaders were able to enhance healthy work relationships with subordinates. Supportive leaders created a friendly work atmosphere and build supportive relationships with their employees thereby enhancing employee well-being (House, 1996).

H3: An inspiring leader will have a positive impact on remotely working employees' motivation

Avolio (1999) and Top et al. (2020) observed that leaders who inspire their followers were able to build and sustain a motivated workforce. By creating an inspirational atmosphere, a leader empowers their followers to be more open and creative.

H4: An encouraging leader will have a positive impact on remotely working employees' motivation

Pink (2009) observed that encouraging remote workers to operate autonomously in their day-to-day activities resulted in a positive level of employee motivation. Encouraging leaders who support employees to perform better through moral boosting coaching sessions are able to keep employees highly motivated (McDonald, 2020).

H5: A relationship orientated leader will have a positive impact on remotely working employees' motivation

Basford and Offermann (2012) highlighted that positive relationships between leaders and followers resulted in motivated employees as well as provided further incentive to continue serving in the same organization. Men (2011) observed that a relationship-oriented leader promoted employee empowerment and created a highly motivated workforce. Such leaders were able to create self-confidence through empowerment within their followers and thereby encouraged open communication, which resulted in a motivated team (Herrmann and Felfe, 2012).

H6: A creative leader will have a positive impact on remotely working employees' motivation

Blaskova and Trskova (2017) concluded that a highly creative leader is able to better motivate their employees to be creative. Generation Z employees were found to be more motivated by creative and inspiring leaders over financial remuneration (Vernooij and Wolfe,2014).

2.3 Methodology

An online survey was used with close ended questions using a 1 - 5 Likert scale to assess the impact of transformational leadership traits on

remotely working employees' motivation. The pilot test was successful, and no changes were made to the questionnaire. The questionnaire was distributed to 150 employees and 103 responses were received. The response rate was 68.7%. The demographics details of the respondents are given in Table 1.

Table 1. Demographic Data

Demographic Data		**Percentage**
Gender	Male	59%
	Female	41%
Work Experience	15 years	24%
	16 - 20 years	51%
	More than 20 years	25%
Industry	Business Finance	46%
	Education	12%
	Information Technology	8%
	Entertainment (Media)	6%
	Construction	6%
	Other	22%

Statistical Package for Social Sciences (SPSS version 20) was used to analyze the primary data collected.

3. Analysis and Findings

3.1 Cronbach's Alpha

Data validity was computed using Cronbach's Alpha for all the variables in the study. All Cronbach's Alpha values were 0.9 and above, and therefore are deemed reliable.

3.2 Correlation Analysis

Correlation analysis using Pearson's Correlation Coefficient was used to test the relationship between transformational leadership traits and remotely working employees' motivation. The correlation analysis results are given in Table 2.

Table 2. Correlation Analysis

Independent Variable	**Pearson Correlation Coefficient**	**Significance**
Goal Oriented	.679	.000
Supportive	.754	.000
Inspiring	.778	.000
Encouraging	.745	.000
Relationship Oriented	.769	.000
Creative	.791	.000

The correlation analysis reveals that all the independent variables have a positive correlation with employee motivation (at 5% significance level). Therefore, the six hypotheses formulated in this study are supported, as shown in Table 3.

Table 3. Hypotheses Validation

Hypotheses	Supported
H1: A goal orientated leader will have a positive impact on remotely working employees' motivation	Yes
H2: A supportive leader will have a positive impact on remotely working employees' motivation	Yes
H3: An inspiring leader will have a positive impact on remotely working employees' motivation	Yes
H4: An encouraging leader will have a positive impact on remotely working employees' motivation	Yes
H5: A relationship orientated leader will have a positive impact on remotely working employees' motivation	Yes
H6: A creative leader will have a positive impact on remotely working employees' motivation	Yes

The correlation analysis results reveal that all the six transformational traits have a positive correlation with remotely working employees' motivation.

3.3 Multiple Linear Regression

Multiple linear regression results revealed that the most significant transformational traits are being supportive and creative (at 5% significance level), as shown in Table 4.

Table 1. Multiple Linear Regression Analysis

Independent Variable	Beta	Significance
Goal Oriented	0.028	0.772
Supportive	**0.367**	**0.000**
Inspiring	0.189	0.139
Encouraging	0.006	0.961
Relationship Oriented	0.204	0.084
Creative	**0.515**	**0.000**

The regression model has a R square value of 0.684.

3.4 Degree of Satisfaction with Maslow's needs

The respondents rated their degree of satisfaction with transformational leadership traits of the senior management using a Likert Scale. The results are given in Table 5.

Table 5. Degree of Satisfaction with Transformational Leadership Traits

Leadership Trait	Average Rating
Goal Oriented	3.8
Supportive	3.7
Inspiring	3.7
Encouraging	3.7
Relationship Oriented	3.9
Creative	3.8

Note: 1- Highly Dissatisfied to 5- Highly Satisfied

The ratings reveal that the respondents are satisfied (average ratings of 3.7 to 3.9) with the transformational leadership traits of the senior management during remote work. Employee motivation also received an overall rating of 4.1, which means the remote working employees are motivated to perform their job, even though they work remotely without direct contact with peers and the management.

4. Discussion

4.1 Goal Orientation

Goal orientation has a strong positive correlation with remotely working employee motivation. Therefore, the leadership must display goal orientation leadership traits. By expressing their goal orientation, leaders succeed in getting employees focused and motivated (Dragoni, 2005). However, goal orientation can result in employee dissatisfaction, if the leaders' goal orientation is towards self-promoting or those which increase work pressure. As such, goal-oriented leaders should ensure that their goals are in accordance with the organizational strategy and vision, clearly communicate their goals to subordinates, ensure that the goals pursued by the leader do not negatively affect the subordinates.

4.2 Supportive

The supportive leadership trait has a strong positive correlation with remotely working employee motivation. It is also the most significant leadership trait that impacts employee motivation (along with the creative trait). Therefore, a leader must display qualities of empathy, compassion and assistance and support the employees to achieve their work goals and maintain good mental and physical well-being.

4.3 Inspiring

The inspiring leadership trait has a strong positive correlation with remotely working employee motivation. Thus, an inspiring leader can motivate employees to exert themselves to achieve exceptional results. However, some leaders may tend to pursue initiatives which are more beneficial to their own career growth rather than the overall goals of the team or the company.

4.4 Encouraging

The encouraging leadership trait has a strong positive correlation with remotely working employee motivation. Leaders who encourage employees to openly share their ideas and suggestions are able to motivate them to do well at work. Certain leaders might downplay weak or unfitting ideas from subordinates during problem solving. However, allowing team members to explain themselves and politely reasoning out why the ideas are being excluded creates a feeling of openness and encouragement for the rest of the team to come forward with their thoughts due to the lack of being rejected or humiliated.

4.5 Relationship Oriented

The relationship-oriented leadership trait has a strong positive correlation with remotely working employee motivation. Research suggests that a key trigger of enhancing employee engagement is through effective communication between leaders and followers (Karanges et al., 2015). Therefore, a leader who is approachable and communicates well with employees is capable of motivating them to perform well at work.

4.6 Creative

The creative leadership trait has a strong positive correlation with remotely working employee motivation. It is also the most significant leadership trait that impacts employee motivation (along with the supportive trait). Transformational leaders ensure that their creative traits are propagated to their subordinates by facilitating a supportive environment (Pieterse et al., 2009; Amabile and Khaire, 2008). Leaders should ensure that organizational bureaucracy is kept to the minimum and a relaxed and welcoming work environment is sustained so that the subordinates can express themselves freely and be influenced by the creative traits of a transformational leader.

5. Conclusion

In conclusion, this study has revealed that the six transformational leadership traits (goal oriented, supportive, inspiring, encouraging, relationship oriented and creative) have a positive correlation with the motivation of remotely working employees. The most significant transformational leadership traits that have an impact of employee motivation of remote workers are supportive and creative. Overall, the respondents were satisfied with the leadership traits of the management. The respondents were also satisfied with their level of motivation.

Empirical studies have shown that transformational leadership traits have a positive influence in successfully motivating employees. This study has shown that this is true, when it comes to remotely working employees as well. The respondents belong to different industries (business finance, education, information technology etc.). Therefore, transformational leadership traits

are effective in motivating employees from different industries.

Since transformational leadership traits improve employee motivation among remote working employees as well, the management should encourage the leadership to be goal oriented, supportive, inspiring, encouraging, relationship oriented and creative when working with the teams under their supervision.

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