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158 Pages

Contents

Page No

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Ecologically friendly synthesis of silver nanoparticles by a variation of *Dianthus caryophyllus* leaves. Analyzing their antioxidant, antibacterial, photocatalytic activities, melamine adulteration, and cytotoxicity.

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Abstract

Among the various nanoparticles that have been employed for the use in biomedical applications, silver nanoparticles (AgNP's) are one of the most significant and intriguing nanomaterials. Therefore, this study was conducted to examine their characteristics. A variety of Dianthus caryophyllus were used to make water extracts (WE) and by combining the WE and silver nitrate ($AgNO_3$), nanoparticles were synthesized. Characterization of these AgNP's were accomplished using UV-spectrophotometry and scanning electron microscopy (SEM). SEM analysis shows that the AgNP is cuboidal in shape and around 50nm in size. These WEs had their phytochemical characteristics examined, and on both the WE and AgNP's, total flavonoid content (TFC), total phenolic content (TPC), total antioxidant capacity (TAC), and 2,2-diphenyl-1-picrylhydrazylhydrate (DPPH) assays were performed. Methyl red (MR) was used to test the pink-AgNP's photocatalytic activity, and pink-AgNP was also used to identify melamine in milk. All five AgNP's and five WEs underwent antibacterial testing using *Escherichia coli* (*E. coli*) *and Staphylococcus aureus* (*S. aureus*) and with the use of brine shrimps, the pink AgNP's cytotoxicity was evaluated. The results of these tests confirmed that higher TFC and DPPH activity was seen in WE's and that higher TPC and TAC activity was seen in AgNP's. The use of sodium borohydride ($NaBH_4$) under sunlight exhibited increased degradation activity of the dye molecule. The detection of melamine using pink-AgNP was seen in 1000ppm, 100ppm, 10ppm, 1ppm and 0.1ppm of melamine, while when another method for detection using, WE and $AgNO_3$ was done, melamine was not detected in 1000ppm, the cytotoxic evaluation revealed 100% viability of the shrimps 24hrs after pink-AgNP addition. This study was useful in identifying different properties of AgNP's synthesized using Dianthus caryophyllus WE.

Keywords: Dianthus caryophyllus, Nanoparticles, Antibacterial, Photocatalytic, Melamine adulteration, Cytotoxic.

1. Introduction

Nanoparticles are small molecules between 1 to 100 nanometers (nm) in size as shown below in figure 01 and are not visible to the naked eye.[1] They are also considered the elements of nanotechnology which is the research and development at a macromolecular scale. Electronic, magnetic, and optoelectronic, biological, pharmacological, cosmetic, energy, environmental, catalytic, and materials applications are just a few of the fields in which nanoscale materials are used.[2]

There are two approaches to the synthesis of nanoparticles namely bottom-up and top-down approaches. Both these approaches are shown below in figure 02. In the bottom-up approach, atoms or/and some molecular species interact to form nanomaterials through a series of chemical processes, and in the top-down approach an energy source is supplied, the beginning material,

which is a bulk form of the desired synthetic material, is subsequently broken into smaller and smaller pieces or particles.[4]

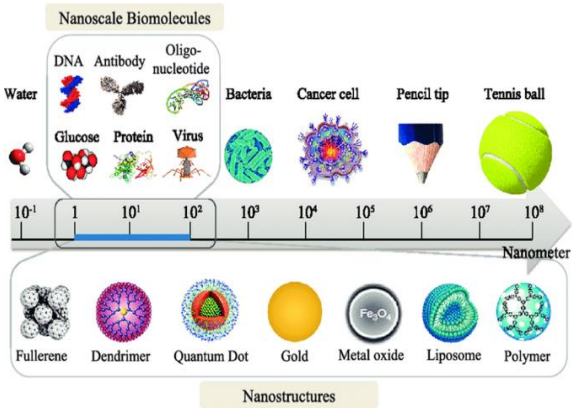


Figure 1. Nanoscale integration of nanoparticles.[3]

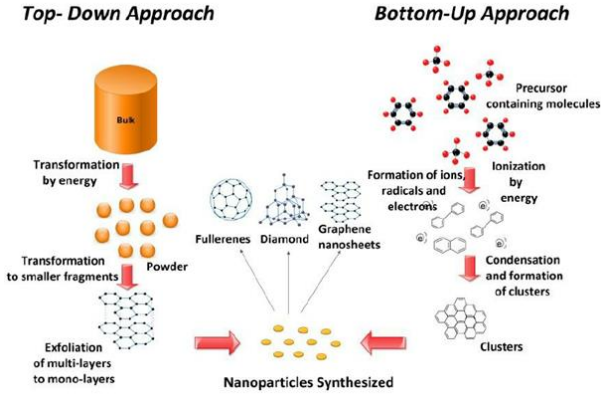


Figure 2. Bottom-up and top-down approaches.[4]

In comparison to chemical, physical, and microbiological approaches, the synthesis of metal nanoparticles using plant medicated techniques has several advantages. It is a quick, easily repeatable, ecological, and inexpensive process. Hence, using biological extracts from various plant components to create metal nanoparticles is widely encouraged.[5]

The sample used in this research is the leaves of 5 different *Dianthus caryophyllus* plants (figure 3). Research on the properties of the *Dianthus caryophyllus* leaves has not been done yet. But research on the flowers of *Dianthus caryophyllus* has been done and the phytochemical examination revealed that it included triterpenes, alkaloids, and many other chemical components. Pharmacological research also showed that the plant has anticancer, antiviral, antibacterial, antifungal, insecticidal, and antioxidant properties.[6]

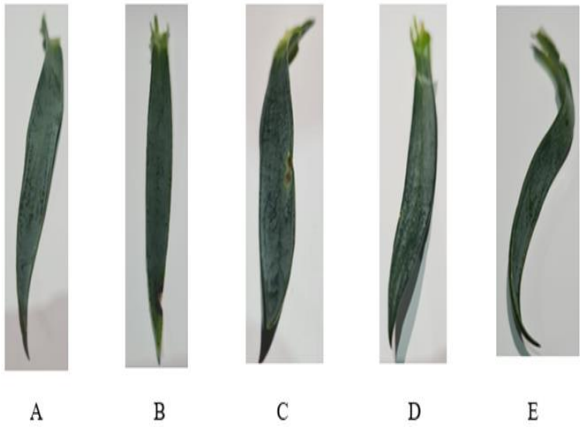


Figure 3. Variety of *Dianthus caryophyllus* used.

(A – Pink, B – Purple, C – White, D – Pink-White, E – Purple-White)

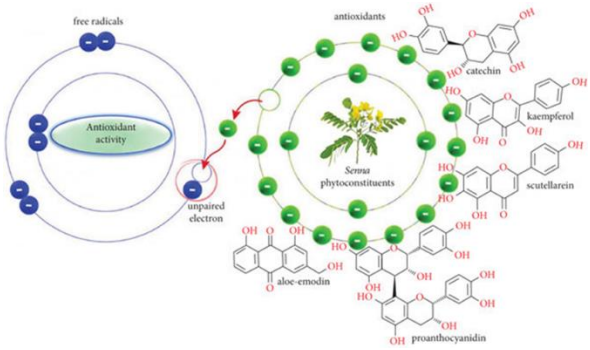


Figure 4. Antioxidant activity of bioactive compounds.[7]

Any substance that, when present in small amounts compared to an oxidizable substrate's concentrations delays or stops the oxidation of that substrate, is considered an antioxidant.[8] Figure 04 shows a brief overview of how that occurs. Antioxidants are classes of substances that stop free radicals and reactive oxygen species (ROS) from harming cells.[9] They are enzymes that fight off free radicals to

counteract any adverse effects that reactive oxygen and nitrogen species can have.[10] The ability to biosynthesize a variety of non-enzymatic antioxidants that can reduce reactive oxygen species-induced oxidative damage can be seen in plants therefore, it is more suitable for research that includes checking for antioxidant activity.[11] The development of many chronic diseases, including cardiovascular diseases, aging, heart disease, anemia, cancer, and inflammation, was significantly influenced by the presence of these antioxidants, which offer protection against the harm caused by free radicals.[9]

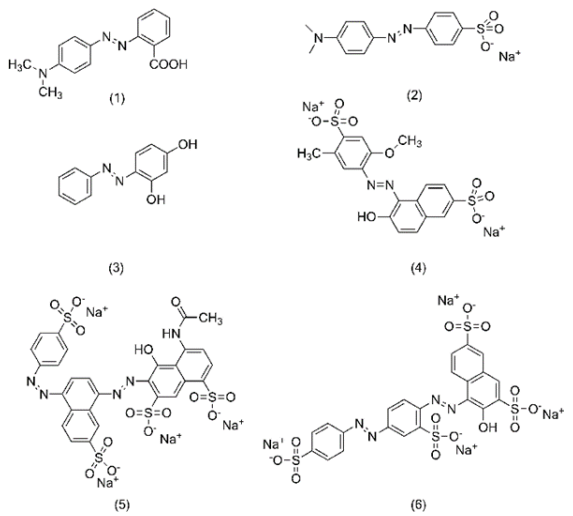


Figure 5. The azo dye structure in the following samples (1) methyl red (2) methyl orange (3) Sudan orange G (4) Allura red AC (5) Brilliant Black BN (6) Ponceau S.[12]

Before being released into the environment, organic substances found to be pollutants must be removed or destroyed.[13] By absorbing both visible and UV light from the sun spectrum, AgNPs are known to photo-catalytically break down these organic molecules like the dyes shown in Figure 05. Some azo dyes have been associated with hepatocarcinoma, splenic sarcomas, human cancer, nuclear anomalies in laboratory animals, and chromosomal abnormalities in mammalian cells.[14]

A molecule's antibacterial action is exclusively linked to substances that selectively kill the growth of bacteria without causing significant tissue damage in the surrounding area. The most crucial agents in the battle against infectious diseases are antibacterial ones.[15] The main mechanisms behind the antibacterial actions of AgNP's, according to current research, are as follows: Bacterial cell membrane disruption, ROS production, cell membrane penetration, and development of intracellular antibacterial effects, including interactions with deoxyribonucleic acid (DNA) and proteins.[16] Due to its potent biocidal impact against germs, which has been utilized for decades to prevent and treat a variety of diseases, AgNP's are well known as the most ubiquitous antimicrobial compounds.[17]

Milk is one of the best sources of the essential nutrients that both infants and adults need, including protein, fat, carbohydrates, vitamins, and minerals. Unfortunately, there is a problem with adulterated milk all throughout the world.[18] Milk is adulterated with melamine which is a synthetic chemical compound that has a high nitrogen concentration and has therefore been used to falsify the protein content of items containing protein, such as pet meals, milk, and newborn formula.[19] Kidney stones and renal failure can result from consuming this.[20] Because of their simple production, stability, and biocompatibility, AgNP's have been widely utilized for the detection of melamine. AgNP aggregates because of the interaction by hydrogen-bonding recognition, electrostatic interaction, or donor-acceptor contact, which causes a shifting of resonant excitation when melamine is added.

When identifying the potential toxicity of a test sample, such as plant extracts or physiologically active chemicals extracted from plants, cytotoxicity studies are helpful in the first step. For a pharmaceutical to be developed successfully, there must be little to no toxicity, and cellular toxicity studies are critical in this regard.[21]

The purpose of this study is to determine whether silver nanoparticles may be produced from the *Dianthus caryophyllus* leaves. Then, making use of TFC, TPC, TAC, and DPPH tests, to determine whether the AgNP's display strong antioxidant activity. Photocatalytic activity was tested using MR by leaving it under ultraviolet (UV) and sunlight (SL), a catalyst was used under SL. *E. coli* and *S. aureus* were used to test the antibacterial activities. AgNP's were tested to determine whether it could aid in melamine detection and using brine shrimps, cytotoxic evaluation was carried out.

2. Methodology

2.1 Water extract formation. The sample was shade dried for 2 weeks and then crushed using a mortar and pistol. It was then mixed with 50 milli liters (mL) of distilled water and incubated at 99°C for 20 minutes (mins). Then filtered off using Whatman filter paper 01, it was stored at 4°C for future use.

2.2 Phytochemical analysis. Water extracts were used for this analysis (table 2).

Table 2. Phytochemical analysis.

Test	**Procedure**
Saponins	To 0.5mL of water extract, 3 drops of distilled water was added and was shaken for a few minutes.[22]
Tannins	To 0.5mL of water extract, 0.8mL of distilled water was added along with 2 drops of 0.1% Ferric chloride.[22]
Sterols and Triterpenoids	To 0.5mL of water extract, 3 drops of Conc. Sulfuric acid was added and shaken well.[22]
Carbohydrates	To 0.5mL of water extract, 3 drops of molish reagent was added, then along the wall of the test tube, Conc Sulfuric acid (H_2SO_4) was added.[23]
Amino Acids	To 0.5mL of water extract, 5 drops of Ninhydrin was added, and the test tube was heated until it boiled.[23]
Alkaloids	To 0.5mL of water extract, 3 drops of Conc HCl and 3 drops of Mayers reagent was added.[23]

2.3 Synthesis of silver nanoparticles. 1mL of the water extract was mixed with 9mL of 1 milli molar (mM) silver nitrate solution and incubated at 60°C and 90°C for 15,30,45 and 60mins and at 25°C for 24hrs. They were then stored at 4°C until they were used.

2.4 Characteristics of AgNP's. Using the spectrophotometer, the absorbance rates was taken from 340-520nm. An SEM analysis was also carried out by centrifuging the AgNP's at 5000rpm for 2mins and incubating for 24hrs at 40°C. The pellet was then outsourced to SLINTECH for analysis where the Hitachi SU6600 FE-SEM (Field Emission Scanning Electron Microscope) and Oxford instruments EDX with AZtec software was used.

2.5 Dilution of Water Extract and Nanoparticles. 1mL of the sample was diluted ×15 times with distilled water and was used for the assays below.

2.6 Antioxidant assessment. WE and AgNP's were both used in these assays (table 3), and they were performed in triplicates.

Table 3. Assays for antioxidant assessment.

Total Flavonoids Content	To 1mL of the sample, 0.5 mL of 1% Aluminum Chloride ($AlCl_3$) and 0.5 mL of 1mM Potassium Acetate were added. Reading was taken at 415nm.[24] Quercetin standard curve was used to get the TFC value, and it was expressed in (µg QE/100g).
Total Phenol Content	To 125µL of a sample, 125µL of 10% Folin – Ciocalteu reagent and 2mL of 5% Sodium Carbonate (Na_2CO_3) were added and incubated at room temperature in the dark for 1 and half hours. Reading was taken at 760nm.[25] Gallic acid standard curve was used to get the TFC value, and it was expressed in (g GAE/100g).
Total Antioxidant Capacity	To 300µL of sample 2.7mL of the mixture of equal volume of 30mL 4mM Ammonium Molybdate, 30mL 28mM Sodium Phosphate, and 30mL 0.6M H_2SO_4 was added. Reading was taken at 630nm.[24] Ascorbic acid standard curve was used to get the TFC value, and it was expressed in (g AAE/100g).

2.6.1. 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay. A ×15 dilution of the AgNP's and WE's was prepared. 1mL of the sample was added to 1mL of 0.135mM DPPH. Readings were taken at 517nm at 0mins and 30mins.[26] Percentage activity was then calculated using equation 01.

Equation 01.

$$Percentage\ activity = \frac{Absorbance\ of\ DPPH - 100\%\ absorbance}{Absorbance\ of\ DPPH} \times 100$$

2.7 Photocatalytic assessment

2.7.1. Degradation MR using UV light. To 100mL of 1mM of MR dye, 1mL of 4000ppm pink-AgNP was added and kept in UV light. Readings were taken from 300-600nm every hour for 4 hours. The same procedure was carried out for 267ppm.[27]

2.7.2. Degradation of MR dye using sunlight. The same procedure as 2.7.1 was repeated with Sunlight. Readings were taken from 300-600nm every hour for 4 hours.[27]

2.7.3. Degradation of MR using sodium borohydride ($NaBH_4$) *and sunlight.* The same procedure as 2.7.1 was repeated including 1mL of 0.2M $NaBH_4$ and sunlight. Readings were then taken from 300-600nm every 15mins for 2.5 hours.[27]

2.8 Detection of melamine

2.8.1. Detection of melamine with AgNP. A series of melamine concentrations from 1000ppm, 100ppm, 10ppm, 1ppm, and 0.1 ppm were prepared. 400µL of melamine was mixed with 600µL of pink-AgNP. Readings were then taken at times o minute and time 30 minutes from wavelength 340-720nm.[28]

2.8.2. Detection of melamine in milk with AgNP. 20mL of milk was heated until it reached 90°C, and it was then cooled until it reached 60°C. 25 drops of citric acid were then added, and the mixture was centrifuged at 4000ppm for 45mins. The milk was then filtered, and the supernatant was collected. 600µL of the milk supernatant, 800µL of pink AgNP and 600µL of 1ppm melamine were mixed. Separately 600µL of the milk supernatant, and 800µL of pink-AgNP were also mixed and used. Readings were then taken at

times 0 minute and time 30 minutes from wavelength 340-720nm.[28]

2.8.3. Detection of melamine using WE and $AgNO_3$. The same series of melamine concentrations as 2.8.1 was used. 2000µL of $AgNO_3$, 40µL of pink-WE, and 800µL of melamine were mixed and incubated at 60°C for 1 hour. Readings were then taken from 340-720nm.[28]

2.8.4. Detection of melamine in milk with WE and $AgNO_3$. 20mL of milk was heated until it reached 90°C, and it was then cooled until it reached 60°C. 25 drops of citric acid were then added, and the mixture was centrifuged at 4000ppm for 45mins. The milk was then filtered, and the supernatant was collected. 800µL of the milk supernatant, 2mL of silver nitrate, 40µL of pink-WE and 800µL of 1ppm melamine were mixed. Separately 800µL of the milk supernatant, 2mL of silver nitrate, and 40µL of pink-WE were also mixed and used. They were both incubated at 60°C for 1hr. Readings were taken from 340-720nm.[28]

2.9 Antibacterial assessment. Agar was prepared by boiling the agar and distilled water and then autoclaving it. 1mL of the sample was incubated at 40°C until it evaporated. The Petri plates containing agar were streaked with *E. coli* and *S. aureus* respectively and then prepared as shown below by adding small wells in which respective samples were added as shown in figure 07. It was then incubated at 37°C for 24hrs and the zone of inhibition was checked.[29]

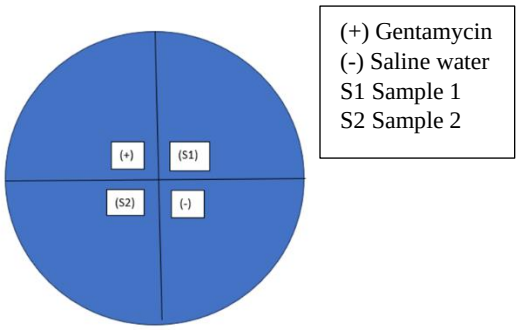


Figure 7. Illustration of the Petri plates.

2.10 Cytotoxic assessment. Capsulated brine shrimp were grown in sea water for 24hrs. Two dilutions of 40mg/ml and 10mg/ml of the pink-AgNP sample were made. Into 25 wells of the 96 well plate, 200µL of sample and 2 shrimps were added and left for 24hrs under the yellow light. 5 wells were used as a control where only sea water and shrimps were added.[30]

2.11 Statistical analysis. The obtained results were analyzed using one-way ANOVA using Microsoft excel and Pearson's correlation test to determine the correlation of TAC TPC and TFC.

3. Results

3.1 Phytochemical analysis. Water extracts were used for the Phytochemical test.

Table 4. Phytochemical results.

Phytochemicals	Pink	Purple	White	Pink & White	Purple & White
Saponins	✓	✓	✓	✓	✓
Tannins	×	×	×	×	×
Sterols	×	×	×	×	×
Triterpenoids	✓	✓	✓	✓	✓
Carbohydrates	✓	✓	✓	✓	✓
Amino Acids	✓	✓	✓	✓	✓
Alkaloids	×	×	×	✓	×

3.2 Silver Nanoparticles

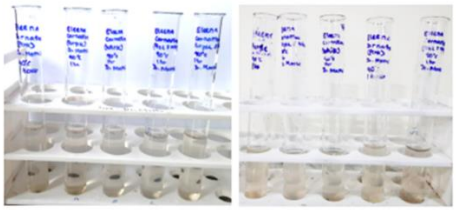


Figure 8. Before and After incubation at 60°C for 1 hour.

A slight cloudy appearance can be seen after incubation.

3.3 Silver nanoparticles graph

Peaks can be seen near 480nm. This confirms the presence of nanoparticles.

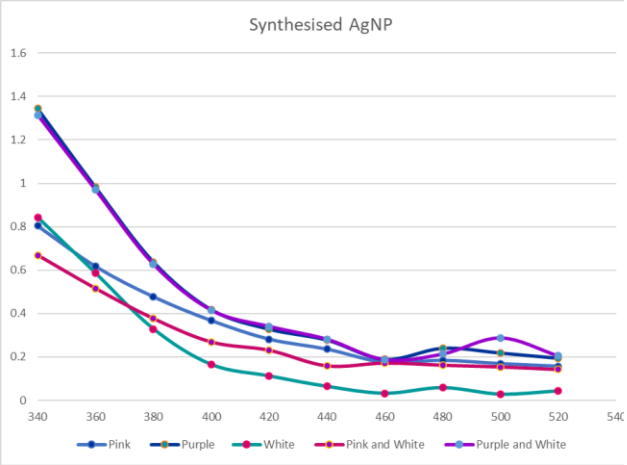


Figure 9. Graph showing nanoparticle peak.

3.4 Optimization table of AgNP synthesis

Table 5. Optimization table of the silver nanoparticles.

Temp	Time	Pink	Purple	White	Pink-White	Purple-White
90°C	1hr	✗	✗	✗	✗	✓
	45mins	✗	✗	✓	✓	✗
	30mins	✗	✗	✓	✗	✗
	15mins	✓	✗	✗	✓	✗
60°C	1hr	✓	✓	✓	✓	✓
	45mins	✗	✓	✓	✗	✗
	30mins	✗	✗	✓	✗	✗
	15mins	✗	✗	✗	✓	✓
25°C	24hrs	✗	✓	✓	✗	✗

The optimized samples are highlighted on table 5.

3.5 SEM Analysis

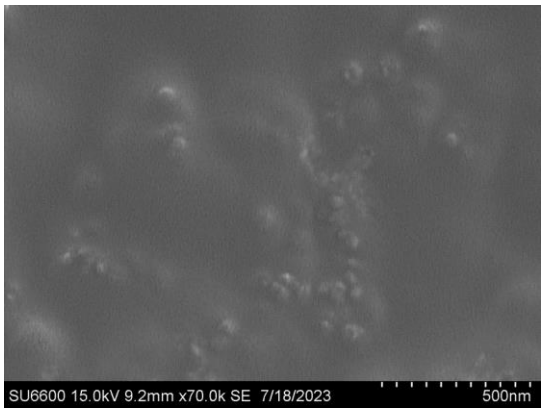


Figure 10. SEM image of pink-AgNP 15kV 9.2mm x70 k/scale - 500nm

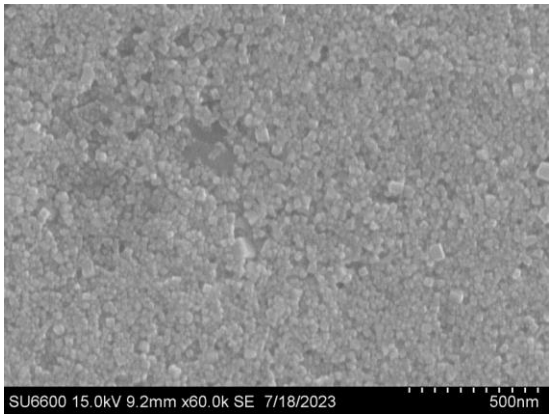


Figure 11. SEM image of pink-AgNP 15kV 9.2mm x60 k/scale -500 nm

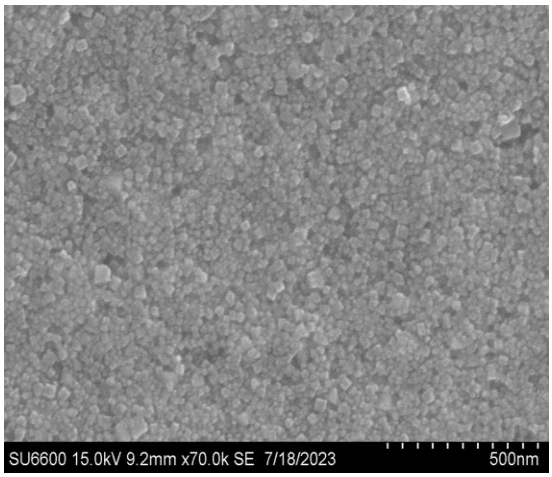


Figure 12. SEM image of pink-AgNP 15kV 9.2mm x70 k/scale - 500nm

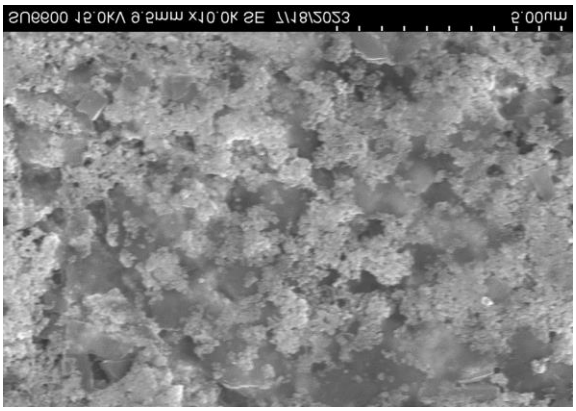


Figure 13. SEM image of pink-AgNP 15kV 9.5mm x10 k/scale - 5µm

The pink-AgNP is cuboidal in shape and has a size of around 50nm.

3.6 Total Flavonoid Content

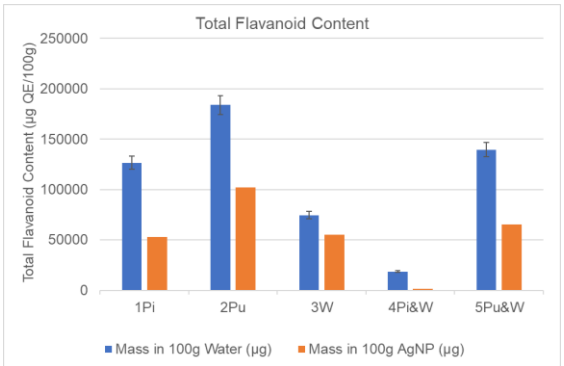


Figure 14. TFC of WE's and AgNP's.

Water Extract has a higher TFC level than AgNPs.

Table 6. ANOVA test for TFC

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6164560200	1	6.16E+09	2.012153	0.198403	5.591448
Within Groups	21381886800	7	3.05E+09			
Total	27546447000	8				

The P value is 0.198403 which is higher than the significant value 0.05.

3.7 Total Phenol Content

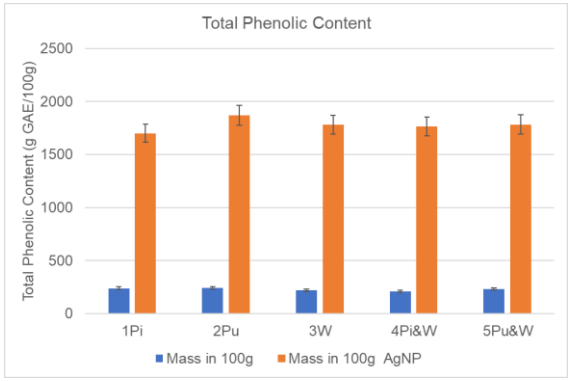


Figure 15. TPC of WE's and AgNP's.

AgNP has a higher TFC level than WE.

Table 7. ANOVA for TPC

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5481045	1	5481045	5163.164	2.65913E-11	5.591448
Within Groups	7430.969388	7	1061.567			
Total	5488475.969	8				

The P value is 2.65913E-11 which is lower than the significant value 0.05.

3.8 Total Antioxidant Activity

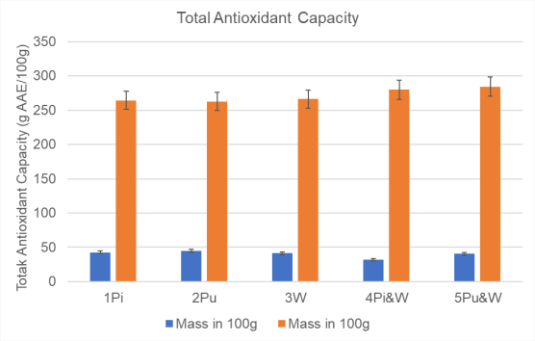


Figure 16. TAC of WE and AgNP's.

AgNP has a higher TAC level than WE.

Table 8. ANOVA for TAC

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	120736.382	1	120736.4	2023.084	7.02E-10	5.591448
Within Groups	417.7556818	7	59.67938			
Total	121154.1377	8				

The P value is 7.02E-10 which is lower than the significant value 0.05.

3.9 DPPH Activity

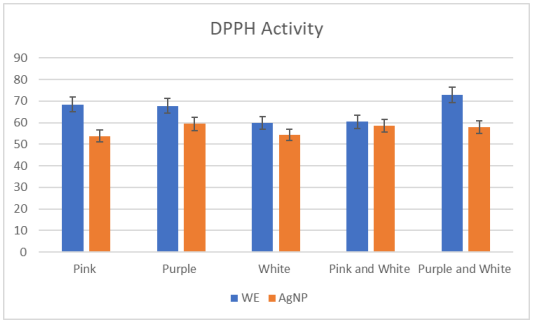


Figure 17. DPPH activity of WE and AgNP

WE's have more DPPH activity than AgNP's.

3.10 Photocatalytic of AgNP.

3.10.1 Under UV using pink-AgNP.

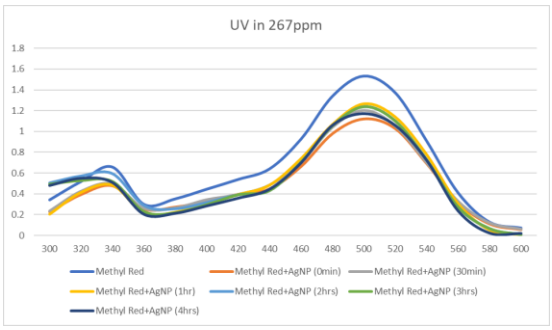


Figure 18. Photocatalytic activity of MR in the presence of 267ppm *pink-AgNP* under UV.

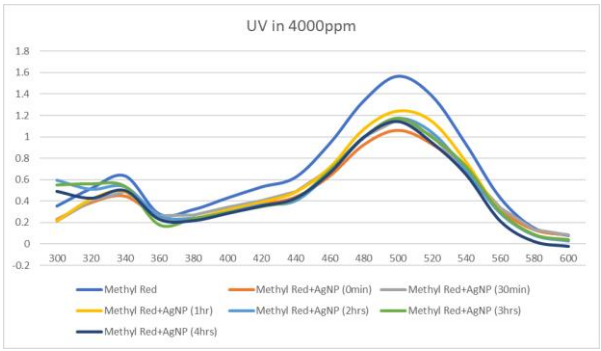


Figure 19. Photocatalytic activity of MR in the presence of 4000ppm *pink-AgNP* under UV.

There is no degradation seen under UV as shown in figures 18 and 19.

3.10.2 Under Sunlight using pink-AgNP.

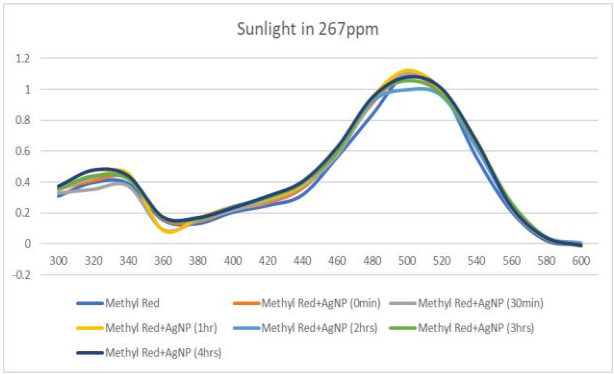


Figure 20. Photocatalytic activity of MR in the presence of 267ppm *pink-AgNP* under sunlight.

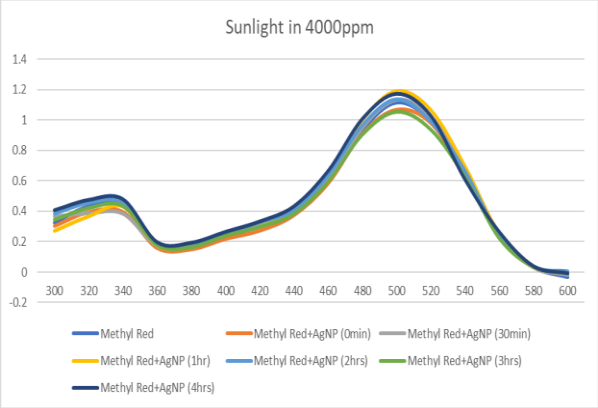


Figure 21. Photocatalytic activity of MR in the presence of 4000ppm *pink-AgNP* under sunlight.

There is no degradation seen under SL as shown in figure 20 and 21.

3.10.3 Under Sunlight with $NaBH_4$ using pink-AgNP.

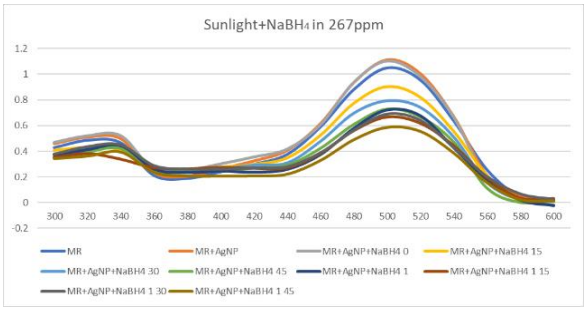


Figure 22. Photocatalytic activity of MR in the presence of 267ppm *pink-AgNP* and $NaBH_4$ under sunlight.

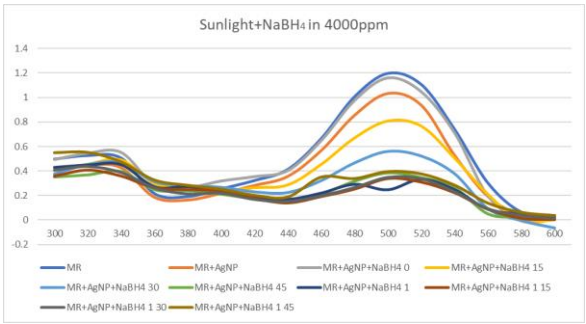


Figure 23. Photocatalytic activity of MR in the presence of 4000ppm *pink-AgNP* and $NaBH_4$ under sunlight.

Degradation of MR is shown in figure 23. The curve became flat at 1 hour and 45mins.

3.11 Melamine detection.

3.11.1 Detection of melamine with pink-AgNP.

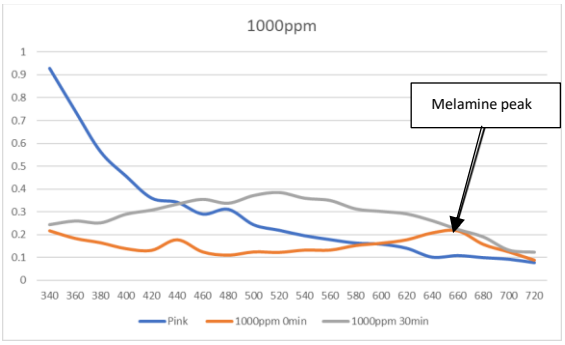


Figure 24. Melamine detection with 1000ppm pink-AgNP.

The peaks are seen near 640nm.

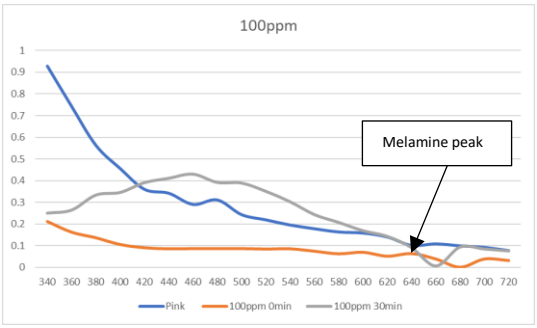


Figure 25. Melamine detection with 100ppm pink-AgNP.

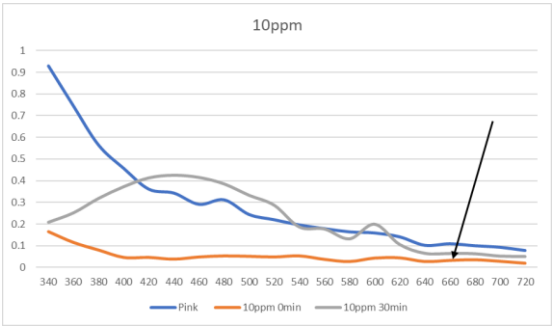


Figure 26. Melamine detection with 10ppm pink-AgNP.

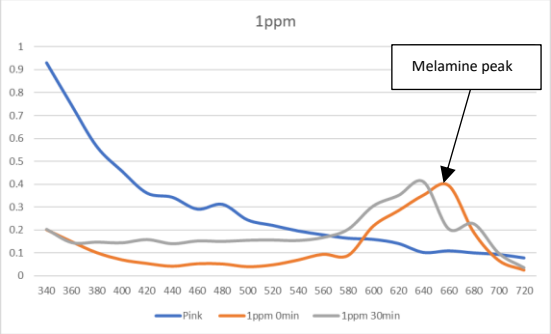


Figure 27. Melamine detection with 1ppm pink-AgNP.

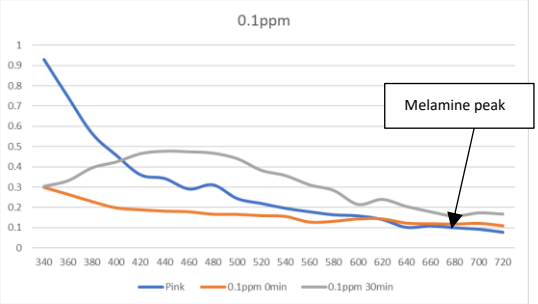


Figure 28. Melamine detection with 0.1ppm pink-AgNP.

Melamine was detected in all concentrations using this method.

3.11.2 Detection of melamine with pink-WE and $AgNO_3$

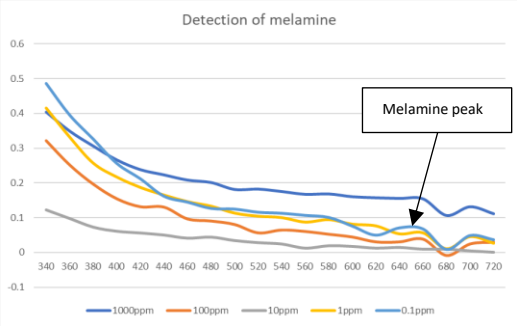


Figure 29. Melamine detection with pink-WE and $AgNO_3$.

Melamine was detected in all concentrations except 1000ppm.

3.11.3 Detection of melamine in milk with pink-WE & $AgNO_3$.

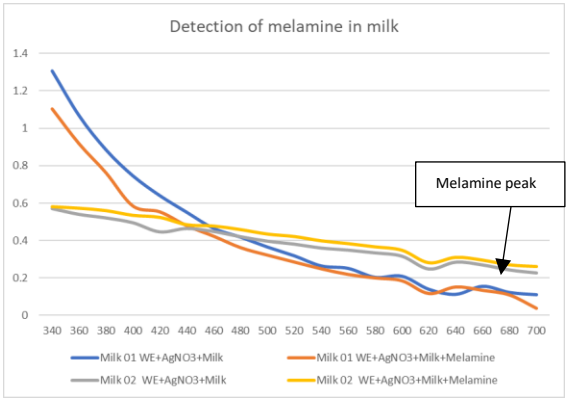


Figure 30. Melamine detection in 2 different milk samples with pink-WE and $AgNO_3$.

Melamine was detected in both milk samples.

3.12 Antibacterial assessment

Table 9. Zone of inhibition (ZOI) for *S. aureus* using WE & AgNP

Sample	**S1**	**S2**	**(+) ve control**
Pink WE	-	-	2.3cm
Purple WE	-	-	2.3cm
White WE	-	-	2.3cm
Pink-White WE	-	-	2.3cm
Purple-White WE	-	-	2.3cm
Pink AgNP	-	-	2.3cm
Purple AgNP	-	-	2.2cm
White AgNP	-	-	2.2cm
Pink-White AgNP	1.2cm	1.0cm	2.3cm
Purple-White AgNP	-	-	2.3cm

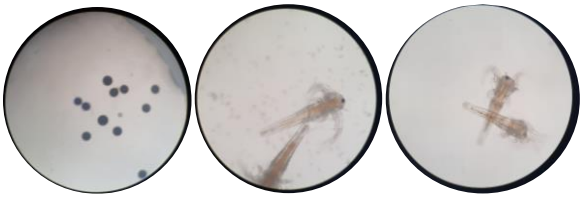
Table 10. ZOI for *E. coli* using WE & AgNP

Sample	S1	S2	(+) ve control
Pink WE	-	-	2cm
Purple WE	-	-	2cm
White WE	-	-	2cm
Pink-White WE	-	-	2cm
Purple-White WE	-	-	2cm
Pink AgNP	-	-	2cm
Purple AgNP	-	-	2cm
White AgNP	0.8cm	0.8cm	2cm
Pink-White AgNP	-	-	2cm
Purple-White AgNP	-	-	2cm

Pink-white AgNP showed a ZOI when *S. aureus* was used while the white AgNP showed a ZOI when *E.coli* was used.

3.13 Cytotoxic assessment

Control

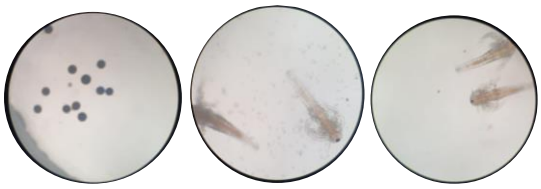


Eggs Hatchlings After 24hrs

10mg/L

Eggs Hatchlings 24hrs after AgNP

40mg/L



Eggs Hatchlings 24hrs after AgNP

Figure 31. Cytotoxic assessment using pink-AgNP.

After 24 hours all 50 shrimps used in the test were alive. Microscopic imaging can be seen (Figure 33).

4. Discussion

In this study silver nanoparticles were synthesized using leaves of 5 varieties of *Dianthus caryophyllus* flowers. This was used to check for antioxidant, photocatalytic, antibacterial, and cytotoxic activity as well as melamine adulteration in milk. In this study, the WE were synthesized by incubating the water and leaf mixture at 99°C for 1 hour, this was then mixed with AgNO3 and incubated at 60°C for 1 hour. The confirmation of the nanoparticles was done by using the spectrophotometer where a peak was seen at 480nm, as shown in figure 9, this was also confirmed using the research by Aisida *et al*., (2019) where it states that the specific surface plasmon resonance absorption peaked between 450 - 480nm.[31] A change in color was not easily observed as seen in figure 8 as the transparent mixture turned slightly cloudy white which could be due to the presence of many silver nanoparticles which give a grey and white.[32]

Among the several metallic nanoparticles used in biomedical applications, AgNP's are one of the most important and fascinating nanomaterials. They also play a crucial part in nanomedicine and nanoscience.[33] Since AgNP's are widely used for many different applications, it is inevitable that they will end up in environmental systems. Therefore, it is crucial

that research is done on AgNP's characteristics.[34] AgNP's can be easily synthesized using physical, chemical, and biological techniques. As a result of their high surface-to-volume ratio and exceptional conductivity, they have found extensive usage in chemistry and related fields.[35] Research on AgNP's is made simple due to their flexibility, which allows for their simple incorporation into a variety of media.[36]

Further analysis was carried out to check for the size and shape of the AgNP. According to the analysis, (figures 10-13) it can be confirmed that the AgNP is cuboidal in shape and is around 50nm in size.

Table 11. Confirming for the conductivity of optimized samples.

Purple	4.14E-19	2.584968	Semiconductors
White	4.14E-19	2.584968	Semiconductors
Pink & White	4.32E-19	2.697358	Semiconductors
Purple & White	4.14E-19	2.584968	Semiconductors

Equation 02.

$$\text{Conductivity equation} = \text{Plank's constant} \times \frac{\text{speed of light}}{\text{Peak wavelength}}$$

The results seen in table 11 were obtained using equation 2, this confirmed that all four AgNP samples show semiconductive properties.

The results of the phytochemical analysis seen in table 4 confirmed the presence of saponins, triterpenoids, carbohydrates, amino acids, and alkaloids which is supported using the research by Chandra and Rawat (2015) which mentions that *Dianthus caryophyllus* consists of significant levels of secondary metabolites.[37]

By indirectly increasing the activity of intracellular antioxidant enzymes or directly by interacting with free radicals, antioxidants are known to reduce oxidative damage.[38]

The TFC of the WE and the AgNP's were carried out using aluminum chloride and potassium acetate. As shown in Figure 34, the basic principle of this assay suggests that aluminum chloride forms stable acid complexes with C-4 keto groups and either C-3 or C-5 hydroxyl groups of flavones and flavanols.[40] The results (figure 14), state that purple WE have the highest levels of TFC while pink-white WE have the lowest level, and this was similar to the AgNP's. This finding is also further confirmed by the research carried out by Zhou *et al*., (2023) which suggests that the purple *Dianthus caryophyllus* WE have higher levels of flavonoids.[41] According to the results WE have a higher TFC level than AgNP's. The p–value is > than the significant value, and the F value< F crit value (table 6), therefore it can be concluded that there is no statical significant between the TFC activity of WE's and AgNP's (table 6).

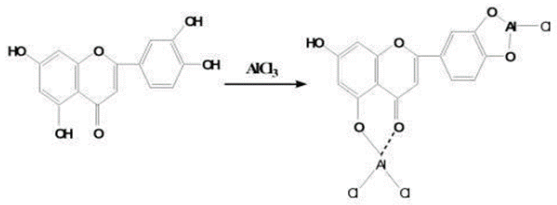


Figure 32. Principle of TFC.[39]

The TPC of the WE and AgNP's were carried out using folin – ciocalteu reagent. The production of a blue compound, which is detected at a wavelength of 765 nm, is the fundamental idea behind this technique. The heteropoly acid (phosphomolybdate-phosphotungstate) included in the folin-ciocalteau reagent will be oxidised by phenol or phenolic-hydroxy groups to produce a molybdenum-tungsten complex which is seen in Figure 35.[43] According to figure 15 the AgNP's

have higher activity than the WE, this is supported by research done by Gonçalves *et al*., (2020) confirming the presence of high penolic content in AgNP.[25] The p–value is < than the significant value, and the F value > F crit value (table 7), therefore it can be concluded that there is a statistical significance between the TPC activity of the WE's and AgNP's.

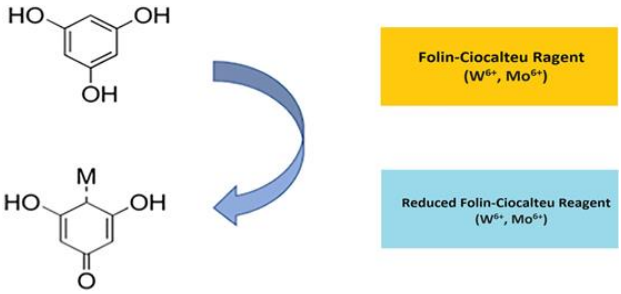


Figure 33. Principle of TPC.[42]

The TAC was carried out using phosphomolybdenum assay. As shown in Figure 36, Mo (VI) is converted to Mo (V) in the presence of extracts, forming phosphomolybdenum V complex, this is green in color and has a with a maximum absorbance at 695 nm.[45] The result (figure 16) states that AgNP's have higher TAC activity than the WE, the research by Ioana-Raluca Bunghez *et al*., (2012) supports this as it states that compared to plant extract, all silver nanoparticles displayed higher antioxidant activity levels.[46] The p–value is < than the significant value, and the F value > F crit value (table 8), therefore it can be concluded that there is a statistical significance between TAC activity of WE and AgNP's.

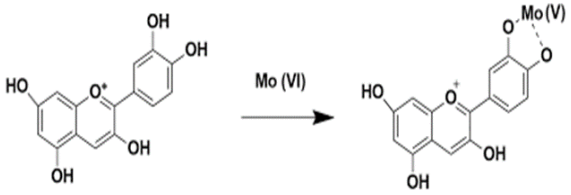


Figure 34. Principle of TAC.[44]

The accepted correlation measure in statistics is the Pearson Correlation, this displays the linear relationship between the two sets of data, and is the most popular technique for analyzing numerical variables.[47] It works by assigning a value between 0 and 1, with 1 denoting total positive correlation and 0 denoting total negative correlation.[48] The results (figure 37) concluded that there is no statistical relationship between TFC-TPC, and between TFC-TAC. But there is a statistical relationship between TPC-TAC.

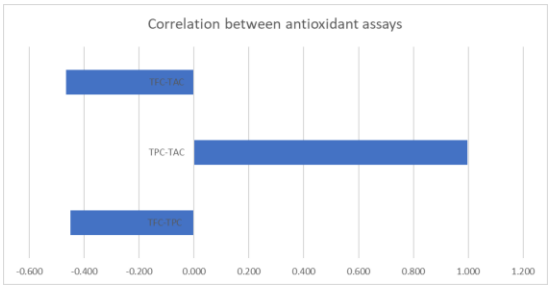


Figure 35. Correlation between antioxidant assays.

The DPPH assay was carried out to determine the free radicle scavenging activity of the WE's and AgNP's. The DPPH radical is a stable, methanol-soluble molecule with a deep violet color and a maximum absorption wavelength of 515 nm. By providing an electron or hydrogen atom, antioxidants can react with this stable radical, reducing it to 2,2-diphenyl-1-hydrazine (DPPH-H) or a substituted analogous hydrazine (DPPH-R), which is colorless or has a pale-yellow appearance, this can be easily observed using a spectrophotometer.[49] The results in figure 17 indicated that the WE's have better activity than the AgNP's, this is supported using the research by Zia *et al*., (2019) which states that low free radical scavenging activity is correlated with higher phenolic levels and overall antioxidant activity.[24]

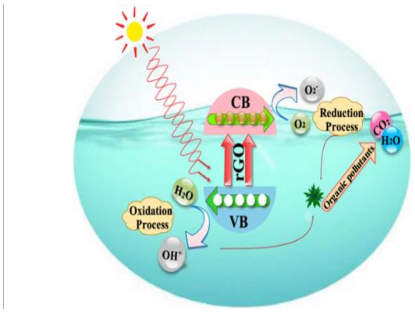


Figure 36. Photocatalytic mechanism for dye degradation.[50]

AgNP's ability to absorb UV energy from the solar spectrum results in the excitation of electrons from the 4d orbital to the 5sp orbital. Numerous photogenerated electrons are excited due to this interband transition. The oxygen molecules and the hydroxyl ion are both affected by these excited electrons, resulting in the formation of oxygen radicals and hydroxyl radicals as shown in figure 38. The dye is degraded because of radicals' attack on the dye molecule adsorbed on the surface of the AgNP's. In addition to the radicals' breakdown of the dyes, the holes created in the AgNP's d orbital take electrons from the dye molecule that has been adsorbed, further degrading the dye.[51] Confirming the photocatalytic activity of the AgNP's was carried out using the dye MR which is a synthetic azo dye that was discovered to be both mutagenic and to have serious negative effects on human health.[52] A UV spectrophotometer was used to confirm the findings and a peak was seen at 500-520nm this is confirmed using the research by Xu (2021) which stated that MR aqueous solution's UV-visible electronic absorption spectra are distinguished by the overlap of a major peak at 520±15nm.[53] The results obtained in figures 18-21, under UV and sunlight showed no significant decrease. Figures 22 and 23 illustrate how the addition of a catalyst under SL reduced the peaks; 267 ppm pink-AgNP showed less reduction than 4000 ppm pink-AgNP which resulted in a flat peak at 1 hour and 45 minutes. This is confirmed using research by Kansal *et al*., (2007) that stated that degradation of dye with the help of metal catalysts in the presence of sunlight was faster compared to other irradiation techniques.[54] Githala *et al.,* (2022) also confirmed that high concentrations of AgNP's with the help of $NaBH_4$ show full degradation of the dye molecules.[55] According to the rate constant was calculated (figures 39 and 40), the concentrated sample of 4000ppm pink-AgNP has a rate constant of 0.0709 while the diluted sample of 267ppm pink-AgNP has a rate constant of 0.0326 this confirms the concentrated sample has a faster rate of degradation than the diluted sample.

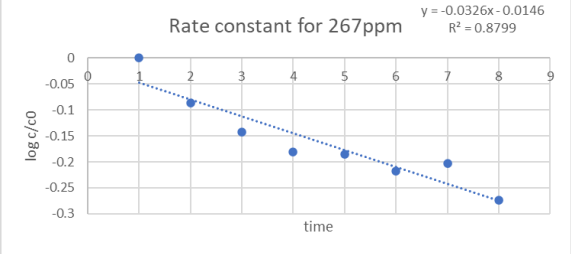


Figure 37. Rate constant for MR under SL with $NaBH_4$ at 267ppm.

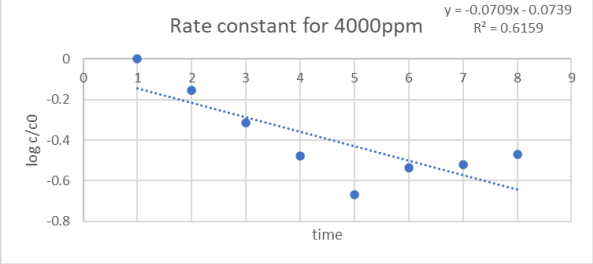


Figure 38. Rate constant for MR under SL with $NaBH_4$ at 267ppm.

Melamine detection is important to reduce melamine adulteration in milk as they cause harm to the human body. A spectrophotometer is used to confirm the findings, the melamine peaks are seen between 600-640nm this was confirmed using the research by Siddiquee *et al*., (2021) which stated that the emergence of a new absorption peak at about 600 nm was induced by melamine.[56] The

nanoparticles engage through hydrogen-bonding recognition, electrostatic interaction, or donor-acceptor interaction, and cause the nanoparticles to aggregate. Melamine's addition causes the resonant excitation to shift, which causes the solution's color to shift from bright yellow to pale red.[38] Melamine was detected in all concentrations (1000ppm, 100ppm, 10ppm 1ppm and 0.1ppm) when pink-AgNP's were used (figures 24-28). Whereas when another method was carried out by adding WE's and $AgNO_3$, melamine was not detected in 1000ppm (figure 29). The research by Ramalingam *et al*., (2017) suggested that absorbance decreases as melamine concentration increases which supports the results obtained.[57] Melamine was detected in two samples of milk, both of which showed melamine peaks as seen in figure 30. This indicated that both these milk samples are adulterated with melamine and could potentially cause harm to the people.

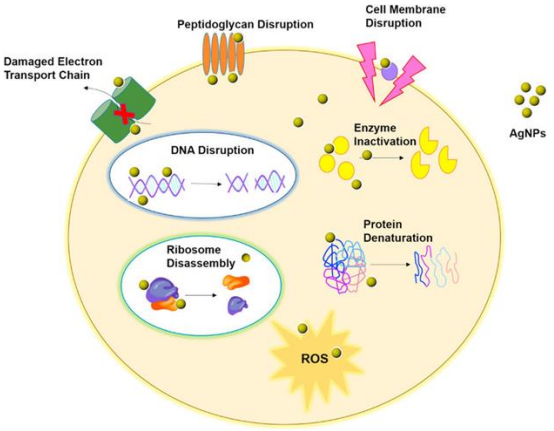


Figure 39. The mechanism of antibacterial activity of silver nanoparticles.[58]

The AgNP adhesion to the microbial cell wall and membrane along with the electrostatic attraction between the negatively charged microbial cell membrane and positively charged AgNP's as seen in figure 41 is the first step in the interaction between AgNP's and microbes. Following the interaction, the nanoparticle causes morphological changes in the membrane structure, which impair membrane permeability and respiratory processes via membrane depolarization, and ultimately destroy cell integrity and cause cell death. Proteins, enzymes, DNA, ions, metabolites, and the energy reserve all leak out of cells into the environment because of increased membrane permeability and damage to the cell wall. Thus, it is considered that the major mechanism of the antibacterial effect is the breakdown of the cell wall by the attachment of nanoparticles.[59] Antibacterial activity of the WE and the AgNPs was carried out using *E. coli* and *S. aureus*. Results were seen for the pink-white AgNP using *S. aureus* and the white AgNP using *E. coli*. Tables 9-10 presented the diameter of the zone of inhibition, this confirmed that AgNP's have better antibacterial activity than WE's.

The brine shrimp were used to test the cytotoxicity of the pink-AgNP's. They developed in a lab with saltwater, yellow light and algae food, and a 24-hour maturation period before being subjected to the test. They are used in this research since they are inexpensive, easy to handle, and needed in limited numbers.[60]

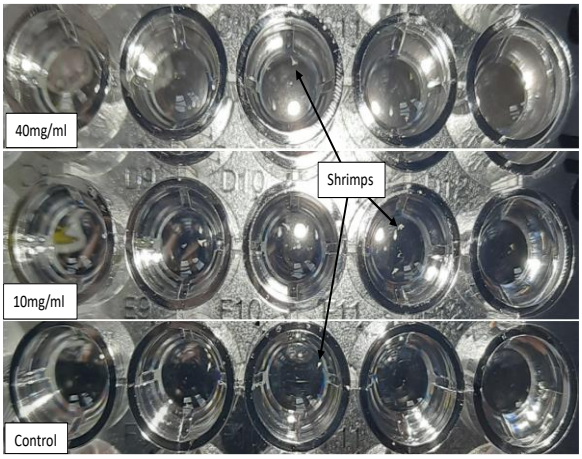


Figure 40. 96 well plates with the shrimps.

The shrimps were added in the same manner as figure 42. The percentage viability of the shrimps was obtained using equation 3, this confirmed the shrimps showed 100% viability 24 hours after the addition of the pink-AgNP. Therefore, it can be concluded that the pink-AgNP sample is nontoxic.

Equation 03.

$$\text{Percentage viablility} = \frac{\text{No of viable shrimps} - \text{No of non viable shrimps}}{\text{No of viable shrimps}} \times 100$$

5. Conclusion

In conclusion, five optimized AgNP's samples were synthesized, the optimized AgNP samples were synthesized by incubation at 60°C for 1hour. Higher TFC and DPPH results were seen in WE, while higher TPC and TAC results were seen in AgNP. When $NaBH_4$ was utilized dye degradation was visible, however it was not visible under UV and SL. AgNP allowed for the detection of melamine at all 5 concentrations (1000ppm, 100ppm, 10ppm, 1ppm and 0.1ppm), however detection via the addition of WE and $AgNO_3$ was not successful at 1000ppm, melamine was also detected in two milk samples. When *S. aureus* was used, the pink-white AgNP displayed antibacterial activity, and when *E. coli* was used, the white AgNP displayed antibacterial activity, 100% viability was also seen in the pink-AgNP. All these results indicated that the sample has high antioxidant activity, photocatalytic activity, antibacterial activity and can be very useful in the detection of melamine. This sample is also non-toxic as it showed 100% viability of the brine shrimps when AgNP's were added.

Acknowledgements

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Determination of Antioxidant Activity of *Lactobacillus* Isolated from Yoghurt

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Abstract

Lactobacillus and *Bifidobacterium* are two bacterial genera that produce probiotics, which are microorganisms that are beneficial to health and can be found in fermented dairy products. *Lactobacillus* can offer several health benefits for people who have taken antibiotics, suffer from irritable bowel syndrome or other digestive issues. Consuming a diet rich in antioxidants may help to prevent or reduce the risk of heart disease and certain cancers. Research aimed to identify *Lactobacillus* from different yogurt samples and determine its antioxidant properties. Five different commercially available yogurt samples were purchased from a local market and cultured on MRS agar. Single colonies from each sample were chosen to undergo biochemical assays such as Gram staining, catalase test, acid-fast staining, and endospore staining. Finally, the antioxidant activity of *Lactobacillus* was determined using the DPPH assay in both cell-free and intact cell samples. The presence of *Lactobacillus* in samples was indicated by small opaque, milky-white colony morphology on MRS agar, purple stained, rod-shaped bacteria in Gram staining, the lack of bubbles in the catalase test, blue rods in acid-fast staining, and red rods in endospore staining. Color change was observed for both cell-free and cell-intact samples, and the antioxidant activity was calculated accordingly. The antioxidant activity of the cell-free and cell-intact suspension is not statistically significant ($P > 0.05$). This research revealed that both cell-intact and intracellular cell-free suspensions can inhibit oxidative damage, which emphasize the importance of *Lactobacillus* as a potential source of antioxidants for food supplements.

Keywords: *Lactobacillus*, Antioxidant activity, DPPH, Cell-free, Cell-intact, Probiotic properties

1. Introduction

1.1 Probiotics. Probiotics are particularly in the focus as a consequence of the increasing interest in healthy diet, which is encouraging the food industry to develop novel products in an inventive approach. Probiotics are defined as the supplement that contain live microorganisms intended to maintain normal microflora in the body. They consist of *Saccharomyces boulardi* yeast and lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* species.[1] Fermented foods including curd, cheese, cottage cheese, and yoghurt are the main sources of probiotics. Apart from being used as a starting culture, they are sometimes added as a dietary supplement to dairy products to improve quality. A study which is carried out by Jeong[2] proposed that health benefits attributed to probiotics include enhancement of immune system, cancer prevention and reduction of inflammation and allergies. Probiotics have been viewed as one of the disease control strategies to treat some of the illnesses that might affect the human body, including inflammatory bowel disease, antibiotic-induced diarrhea and irritable bowel syndrome.[3]

1.2 Lactobacillus. This research primarily focuses on *Lactobacillus* which is the traditional probiotics utilized in the food industry. The *Lactobacillus* genus has been recognized as

having several beneficial characteristics among all lactic acid bacteria. They are important members of the healthy human microbiota. *Lactobacillus* is a genus of gram positive, rod shape, catalase negative, non-spore forming bacteria that contains more than ninety species. They are used commercially during the production of cheese, yoghurt, curd and chocolate and they are distinguished by their potential to produce lactic acid as a by-product of glucose metabolism.[5]

Lactobacilli has the ability to survive gastric conditions and colonize the intestine by adhering to intestinal epithelial cells. Thus, it was proposed as a successful probiotic according to study which is carried out by Dempsey.[6] *Lactobacillus* species appear to be promising possibilities for the treatment of intestinal diseases caused by abnormal gut microbiota and altered gut mucosal barrier capabilities. Dempsey and Corr[6] reported the presence of *Lactobacillus acidophilus* and *Lactobacillus salivarius* in human gut which have the capacity to attach to and bind to intestinal brush border tissue, which is regarded to be a critical component that prevents harmful pathogens from entering the gastrointestinal mucosa.[7]

1.3 Yoghurt as a source of Lactobacillus. Since the increased demand in *Lactobacillus*, a variety of products were proposed as supplements for probiotic microorganisms allowing customers to take huge quantities of probiotic cells for the therapeutic benefit. Yoghurt has long been recognized as a *Lactobacillus* containing product which provides the consumers with wide choice of therapeutic benefits. Yogurt is a popular food consumed all over the world today and a mainstay in many diets because of its tasty flavor and possible health advantages. It is a fermented milk product obtained from the fermentation of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*.[6] Lactic acid, which is produced during the fermentation process from lactose, acts on milk proteins to give yoghurt its distinctive flavor and texture. Yogurt is available in a variety of flavors and shapes to suit a wide range of tastes. While some yogurts are fruit-flavored or sweetened with different ingredients, others are plain. Greek yogurt, favored for its rich and velvety consistency, has become increasingly well-liked due to its increased protein content and adaptability. There are also dairy-free alternatives, such as coconut, almond, and soy yogurt, to accommodate those with lactose intolerance or dietary restrictions. Yoghurts can be high in Protein, Calcium, Phosphorus, Potassium, Zinc, Magnesium and B Vitamins Riboflavin, Niacin, Vitamin B-6 and Vitamin B-12 and live cultures which can enhance the gut microbiota.[8] It stimulates the gut microbial community as a source of probiotics that prevent intestinal infections, reduce lactose intolerance, and lower the risk of developing cancer. Yoghurt is also thought to have immune-modulating properties, which could help with the treatment of Inflammatory Bowel Disease, which includes gastrointestinal disorders like Crohn's disease. In the case of Crohn's disease, the immune system mistakenly attacks the gastrointestinal tract, leading to chronic inflammation.[11] Some research suggests that certain strains of probiotics found in yogurt may help modulate the immune response, reduce inflammation, and promote a healthier balance of gut bacteria. Yoghurt consumption improves insulin resistance, lowers blood glucose levels, and lowers the risk of Type 2 diabetes. By delaying the digestion and absorption of carbohydrates, yogurt's protein content can improve satiety and aid in blood sugar control. This may lead to a slower release of glucose into the blood, improving glycemic control.[10,11]

1.4 Probiotic properties of Lactobacillus. *Lactobacillus* may exert probiotic properties in different ways. Certain *Lactobacillus* species have been seen to deconjugate bile acids in the digestive tract through the activity of bile salt hydrolase proteins. By changing bile metabolism, detoxification, and the composition of the gut microbiota, it helps to decrease cholesterol levels.[12] Following ingestion, these beneficial

bacteria establish themselves in the intestines, where they are essential for preserving a well-balanced microbial environment. They adhere to the intestinal lining and compete with harmful bacteria for nutrients, keeping pathogenic microorganisms from gaining hold. This is how *Lactobacillus* strains help ease the symptoms of gastrointestinal disorders like diarrhea and irritable bowel syndrome (IBS) and lower the risk of gastrointestinal infections.[6] Furthermore, it has been demonstrated that *Lactobacillus* improves nutritional absorption and breakdown, which improves digestion. Certain *Lactobacillus* strains generate digestive enzymes that help break down complex proteins and carbs so the small intestine can absorb them more easily. There may be significant effects on overall health and vitality from this increased nutrient availability.[13] *Lactobacillus* enhances intestinal homeostasis by regulating immune response and promoting T-reg cell growth. Perdigon[14] reported that *Lactobacillus Casie* possesses probiotic property by regulating the host immune system. *Lactobacilli* are able to reduce intestinal inflammation through decreased toll-like receptor (TLR) expression, the production of metabolites that may block TNF-α from reaching blood mononuclear cells, and the regulation of NF-κB signaling in enterocytes. A recent study which is carried out by Diaz[15] demonstrated that *Lactobacillus casei and Lactobacillus plantrum* possess probiotic properties by suppressing intestinal inflammation. Apart from these *Lactobacillus* possessed antioxidant activity which can reduce the risk that Reactive oxygen Species (ROS) will be produced during the ingestion of food.[16] Aside from their traditional probiotic qualities, some *Lactobacillus* strains have demonstrated potential in a range of medicinal uses. As an example, some *Lactobacillus* strains have been investigated for their ability to produce lactase, the enzyme required for lactose digestion, which may help relieve the symptoms of lactose intolerance. This opens possibilities for the use of products based on *Lactobacillus* as a supplement to lactose intolerance treatment. In addition, recent studies indicate that specific *Lactobacillus* strains might affect mental health and cognitive function through modifying the gut-brain axis. These results give hope for the use of *Lactobacillus* in holistic approaches to enhance mental health, even if further research in this area is needed.[17]

1.5 Antioxidant activity of Lactobacillus. In the past ten years, it has become abundantly obvious that oxidative stress and antioxidative efficacy are the two key elements influencing the molecular regulation of cellular stress responses. The fundamental understanding of antioxidants is that they are classified as any substance which substantially inhibit the oxidation of the substrate, when present in low concentrations, compared to that of an oxidizable substrate.[18] Hence, its main role is to prevent damage from occurring to cellular components, which may cause a rise in a series of chemical reactions involving free radicals. Similarly, a free radical is defined as a molecular species that is callable of independent existence, consisting of an unpaired electron in an atomic orbital.[19] Oxidative stress is a condition in which DNA, proteins, and lipids are damaged as a result of abnormally high amounts of reactive oxygen species (ROS) production.[20] Reactive oxygen species such as superoxide anion radicals, hydroxyl radicals, hydrogen peroxide are certain aggressive oxygen free radicals, which influenced together with Reactive Nitrogen Species (RNS) causing tissue disruption, hence resulting in a condition called oxidative/nitrosative stress. Biological and pathological processes, including aging, inflammation, and carcinogenesis, have been linked, either directly or indirectly, to elevated ROS levels. Cells and organisms have created defense systems to protect themselves against the toxicity caused by oxidative stress. Antioxidants are substance that can reduce the damage and preserve the cells from free radicals.[20]

Zaharani and Shori[20] demonstrated that some *Lactobacilli* possess antioxidant activity,

which can lower the risk that ROS will be formed when food is consumed. ROS can cause oxidative stress and damage to cells if their levels are not properly regulated. The antioxidant activity of Lactobacilli may have potential health effects via lowering oxidative stress. Numerous chronic diseases, such as cardiovascular disease, neurological diseases, and several forms of cancer, have been linked to oxidative stress.[21] Therefore, the antioxidative activity of Lactobacilli may contribute to improving general health and lowering the chance of developing certain diseases. They possess antioxidant activity by chelating metal ions such as Fe^{2+} and Cu^{2+}. Duz[16] reported that *Lactobacillus plantarum* has exhibited hydroxyl scavenging ability by chelating the metal ions utilized to create the hydroxyl free radicals. *Lactobacillus Casie* possesses high antioxidant activity by chelating metal ions[22]. In *Lactobacillus helveticus CNBL1156*, Cappa, Cattiveli, and Cocconcelli[24] discovered the gene *uvrA*, which was involved in oxidative stress responses. The CtsR protein in *Lactobacillus plantarum* is a critical regulator of oxidative stress and also acts as a crucial enzyme for cell development at high temperatures, according to Bove *et al*[23]. *Lactobacillus* can exert antioxidant action via chelating metal ions, according to a study by Lee.[24]

Lactobacillus species have shown antioxidant activity by producing metabolites like folate, butyrate, and glutathione. According to a study carried by Rodriguez *et al*[25] daily consumption of yoghurt containing *Lactobacillus acidophilus* considerably increased the mean levels of plasma folate, indicating an improved level of oxidative balance. The two antioxidant *Lactobacillus fermentum* strains, E-3 and E-18, were identified by Kullisaar *et al.*,[26] to contain remarkably high quantities of Glutathione. *Lactobacillus* can produce antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) which can breakdown superoxide to bring about antioxidant activity. SOD is the important antioxidant in *Lactobacillus* sp. which can breakdown superoxide into Hydrogen peroxide (H_2O_2) and water, acting as a key regulator of ROS levels. Manganese Superoxide Dismutase (Mn-SOD) could be expressed by *Lactobacillus fermentum* strains E-3 and E-18 to protect against oxidative stress.[27] Low molecular-weight antioxidant compounds also serve as free radical scavengers in various non-enzymatic antioxidant defense mechanisms. Therefore, under normal circumstances, this antioxidant defense system protects the cells from oxidative damage, however, may become insufficient under excessive oxidant-generating conditions.

Some *Lactobacillus* offers antioxidant activity by regulating the enzymes that produce ROS like NOX (NADPH oxidase) and COX (Cyclooxygenase). Adikari[29] reported that antioxidant activity of *Lactobacillus rhamnosus* by inhibiting NOX production by downregulating the mRNA expression. Nrf2–keap 1-ARE, Mitogen activated protein kinase (MAPK), PKC and NF-κB antioxidant signaling pathway are mediated by *Lactobacillus spp.* to possess antioxidant activity. *Lactobacillus rhamnosus* was shown to produce the soluble proteins p40 and p75, which were effective in reducing the breakdown of the epithelial barrier caused by H_2O_2 by a MAPK-dependent mechanism and *Bacillus amyloliquefaciens* reduced the oxidative stress that H_2O_2-induced in IPEC-1 by decreasing ROS levels and regulating Nrf2 expressions.[29]

The main objective of this study was to determine the antioxidant activity of *Lactobacillus* isolated in yoghurt. The presence of *Lactobacillus* in yogurt is important because it can potentially offer therapeutic benefits related to its antioxidant properties. By understanding the antioxidant activity of *Lactobacillus*, researchers aim to explore their potential in treating illnesses induced by oxidative stress. Additionally, manufacturers can use this knowledge to produce high-quality yogurt products that provide consumers with a wide

range of health benefits associated with antioxidants.

2. Methodology

2.1 Sample preparation and culturing on MRS agar. Commercially available, five different yoghurt samples were obtained from local markets and stored aseptically at -4ºC to protect against contamination and deterioration. Samples were labelled as A, B, C, D and E and homogenized in 1mL of autoclaved distilled water in labelled beakers. This was done under aseptic conditions and covered with aluminum foil to avoid contamination. *Lactobacillus*-specific MRS agar medium was prepared. A mass of 3mg of Amphotericin B was dissolved in 12mL of autoclaved distilled water and added to medium. Samples were then streaked on MRS agar using the quadrant streak method under aseptic conditions and incubated at 37°C for 48 hours to form colonies. Subcultures were maintained in MRS broth.

2.2 Gram staining. A single colony of each sample was picked from a culture plate and a thin bacterial smear was prepared on a glass slide. It was allowed to air dry prior to heat setting. Crystal violet was added, and the stain was left for 1 minute. Gram iodine was then added and allowed to stand for 1 minute. The slides were stained using decolorizer and safranin and left for 15-30 seconds. After each staining step, the slides were rinsed with water and dried in the open air. Slides were blotted and observed under a microscope at 100x magnification.

2.3 Test for catalase. Bacterial colonies were picked from those that were used for Gram staining, were mixed with distilled water, and placed on slides. A drop of H_2O_2 was applied and the formation of oxygen bubbles was observed.

2.4 Endospore staining. Bacterial colonies were picked from those that were used for Gram staining, and smear was prepared for each samples separately. A filter paper was placed on the smear and malachite green was added. Then heated over a steam bath for few minutes. Filter paper was removed, and the slide was rinsed with water. Safranin was used to stain the slide for two minutes before being rinsed with water. The slide was finally blotted and observed under 100X magnification.

2.5 Acid fast staining. Slides were labeled as A, B, C, D & E and bacterial smear was prepared from each sample. Carbolfuchsin was added to the slide and heated for 5 minutes. After that 20% sulfuric acid was added to the slide & kept for 30 seconds and Methylene blue was added as a counterstain. After every staining step the slide was rinsed with water and dried in the open air. Finally, the slide was observed under 100X magnification.

2.6 Antioxidant activity using DPPH assay. Cell-free and cell-intact suspension of the *Lactobacillus* strains were initially prepared in order to determine the antioxidant activity using DPPH assay. This method was modified from Azat *et al*, (2016).

2.6.1. Preparation of cell-free suspension. A volume of 5 mL of subculture was transferred to the labelled falcon tubes and cells were harvested after centrifuging the tubes at 4000rpm for 10 minutes. Supernatant was discarded and 4mL of autoclave distilled water was added to the pellet followed by incubation at 100ºC for 20 minutes The tubes were immediately transferred to a freezer for 20 minutes at -20ºC. The intracellular cell-free extract was obtained by centrifuging for 10 minutes at 4000 rpm to remove cell debris. Then DPPH assay was carried out with extracted cell-free suspension.

2.6.2 Preparation of cell-intact suspension. For the preparation of intact cells, 5mL of subculture was transferred to labelled tubes followed by centrifugation at 4000 rpm for 10 minutes. Then 4mL of distilled water was added to the pellet and DPPH assay was performed.

2.6.3 DPPH assay. A mass of 5.9148mg of DPPH powder was measured and dissolved in 300mL of ethanol in a conical flask to prepare 50µL/mol DPPH solution. A volume of 1mL of sample mixed with 2mL of 50µL/mol DPPH solution and wrapped with Aluminum foil. Control was carefully covered in aluminum foil and stored in a dark spot. The test tubes were allowed to react for 30 minutes at room temperature (RT) in a dark environment. After the incubation absorbance was obtained at 517nm for both cell-intact and cell-free suspensions, blanks and controls. The percentage of DPPH scavenging activity was calculated using the equation below.

Equation 1: DPPH scavenging activity (Baliyan *et al.*,2022)

$$SA_{DPPH} = [1- (A_{sample} - A_{blank})/A_{control}] \times 100\%$$

2.9 Data analysis. Statistical analysis of antioxidant activity between cell-free and cell-intact suspensions was determined using the one-way ANOVA test in SPSS software. At a 5% level of significance, the p-value was determined. P-values < 0.05 were considered statistically significant.

3. Results

3.1 Isolation of bacterial colonies in MRS agar. The below figure depicts colony morphology of bacterial culture on MRS agar, after 48 hours of incubation at 37°C.

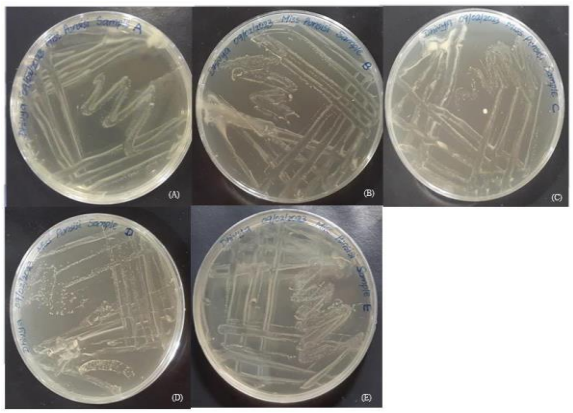

Figure 1. Colony morphology of bacterial culture on MRS agar, after 48 hours of incubation at 37°C.

As observed, until the third streak all culture samples showed growth with isolated colonies present. Creamy white, opaque, and shiny colonies with an entire edge were exhibited in all five samples and they were smooth and moist.

3.2 Gram staining. Gram staining was performed on the isolated bacterial colonies from the culture, and their morphology was observed under 100X magnification.

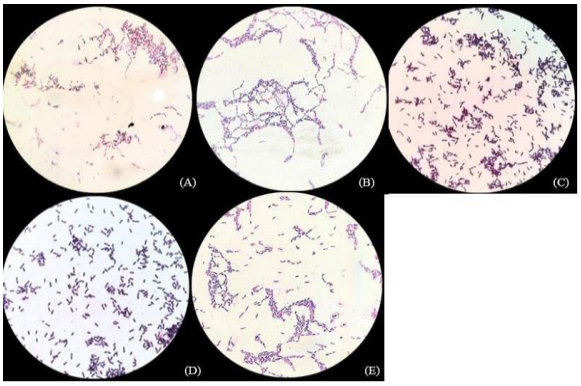

Figure 2. Gram staining of presumptive *Lactobacillus* colonies

The Gram-stain showed the presence of purple rods with round edges which were arranged in a single, double, or short chain in all five samples.

3.3 Catalase test. Figure 3 shows the catalase test results for *Lactobacillus* sample A, B, C, D & E.

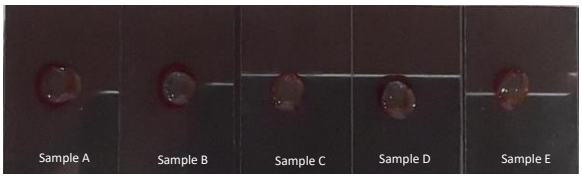

Figure 3. Catalase test for presumptive *Lactobacillus* colonies from all five samples

The colonies were randomly selected from all Petri plates for catalase test. Hence, it was noticed that none of the samples produced any bubbles, which resembled the absence of catalase enzyme.

3.4 Endospore staining. Endospore staining was performed on presumptive bacterial colonies and their morphology was observed under 100X magnification.

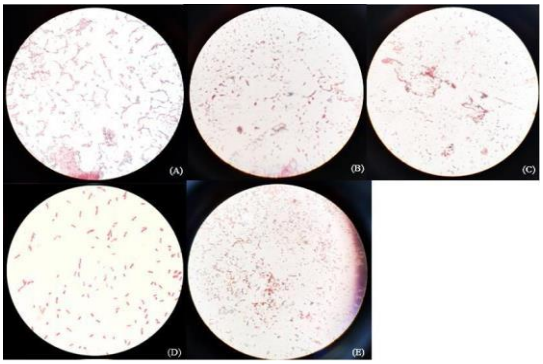

Figure 4. Endospore staining images for presumptive *Lactobacillus* colonies under 100X magnification.

The Endospore stain showed the presence of brownish red colored short rods with round edges in all five samples. Single, double, or short chains were observed as shown in figure 4.

3.5 Acid fast staining. Acid fast staining of the selected bacterial colonies observed under 100X magnification.

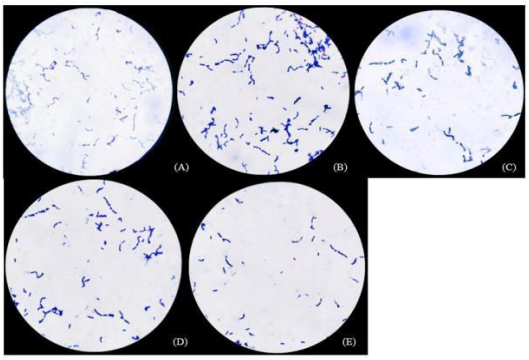

Figure 5. Acid fast staining images of selected bacterial colonies from all five samples

The Acid-fast stain showed the presence of blue colored short rods often present in chains of two or three, for all five samples.

3.6 Antioxidant Activity using DPPH Assay

3.6.1. Cell free suspension. The color of the cell-free suspensions can be observed in the images below both before and after incubation.

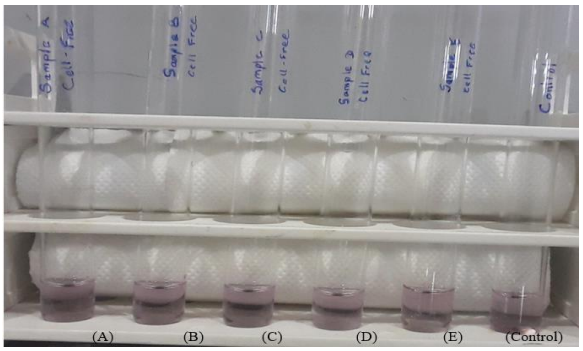

Figure 6. DPPH solution with cell free suspensions before incubation

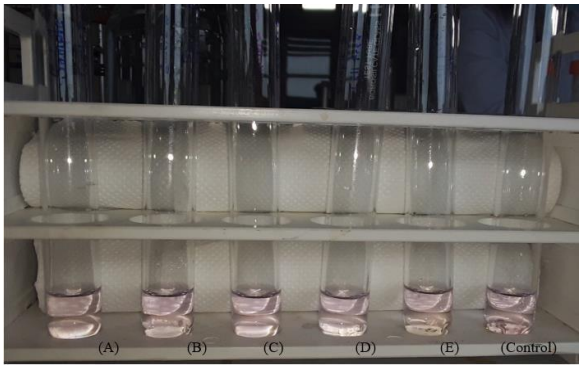

Figure 7. DPPH solution with cell free suspensions after incubation

As shown, after 30 minutes of incubation, the color of all samples changed from purple to pale yellow.

3.6.2 Cell intact suspension. The color of cell-intact suspensions before and after incubation is shown in the images below.

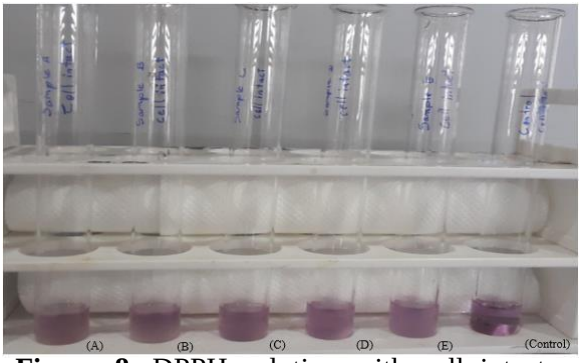

Figure 8. DPPH solution with cell intact suspensions before incubation

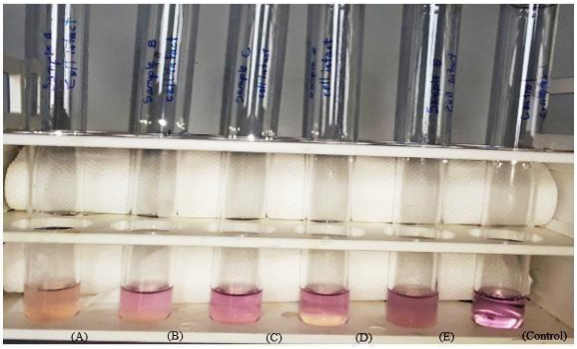

Figure 9. DPPH solution with cell intact suspensions after incubation

After 30 minutes of incubation, it was observed that all samples were changed from purple to pale yellow. However, color change was clearly observed in sample A, B and D while only slight change was observed in sample C and E.

3.7.3 Comparison of Antioxidant activity of Lactobacillus.

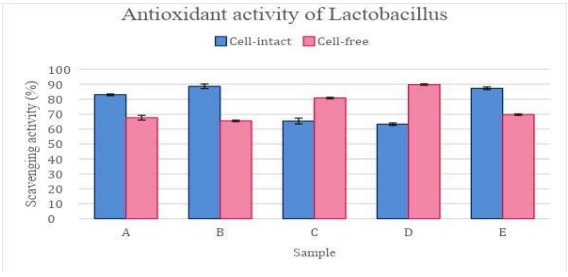

Figure 10. Comparison of antioxidant activity of *Lactobacillus*

Above figure shows the antioxidant activity of cell-free and cell-intact suspensions of Lactobacillus.

Figure 10 shows that samples A, B, and E had higher levels of scavenging activity in intact cells than in free cells, whereas samples C and D had higher levels in cell-free suspension. Comparing cell intact suspensions to cell free suspensions generally reveals higher scavenging activity.

3.8 Statistical analysis. Table 1 shows the outcomes of the one-way ANOVA analysis performed with the SPSS software to compare the antioxidant activity of cell-free and cell-intact suspensions.

Table 1. Analytical outcomes for antioxidant activity between cell-free and cell-intact suspensions

ANOVA

antioxidant activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.003	1	.003	9.525	.005
Within Groups	.008	28	.000		
Total	.010	29			

According to the outcome, the p-value is 0.05. The cell-free and cell-intact suspensions does not have statistically significant difference in its antioxidant activity.

4. Discussion

Lactobacillus spp. have received a lot of consideration for their possible probiotic effects in human health, such as regulating intestinal flora balance, lowering serum cholesterol, preventing, and lowering the risk of cancers, and revitalizing the immune system, among other things. They have been found to have antioxidative activity and can reduce the likelihood of ROS accumulation after food ingestion[30]. This research studied the antioxidant properties of *Lactobacillus* bacteria isolated from

yoghurt samples purchased from the Sri Lankan local market.

The identification of bacteria in samples was carried out using culture-based methods, which may be imprecise. Thus, the samples were first cultivated on *Lactobacillus* specific MRS agar supplemented with Amphotericin B to prevent fungal growth. Until the third streak, all cultures showed growth, with isolated colonies present. Similar to the *Lactobacillus* colony morphology described by Adikari *et al*[29]. The isolated bacteria exhibited a small circular, milky white, shiny, smooth, colony with entire margins and flat elevations when *Lactobacillus* was cultivated on MRS agar. As a result, the colonies seen in this study could be *Lactobacillus* colonies. *Lactobacillus* can be isolated, enumerated, and identified using MRS agar. Other bacterial flora is efficiently repressed by the low pH and high acetate concentrations, which promote the growth of *Lactobacillus.*[31]

Certain biochemical assays, including the Gram staining, catalase test, acid fast staining, and endospore staining, were carried out on the samples after they had been isolated in order to characterize the presence of *Lactobacillus* was present. In the Gram stain, all samples exhibited short purple rod-shaped bacteria with varying lengths (Figure 2) were detected under the microscope and it was determined that the bacteria were gram-positive. The Gram stain simply distinguishes between Gram-positive and Gram-negative bacteria based on cell wall and cell membrane permeability, thus gram-positive bacteria preserve the CV-I complex and remain purple.[31] Safranin has no effect on the color of purple gram-positive bacteria. These purple-colored rod-shaped bacteria were presumptive to be *Lactobacillus* since these observations were consistent with a previous study by Mojgani.[32] *Lactobacillus fermentum* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* with comparable morphology were found in fermented yoghurt by Tan *et al.*[33] In this study, the appearance of purple rods with rounded edges grouped into single, double, or short chain formations indicates the presence of Gram-positive bacteria in the samples. The bacteria may belong to the Lactobacillus genus, which is recognized for its rod-shaped morphology and propensity to form chains, based on their arrangement in pairs or short chains.

According to Amelia *et al.,*[34] *Lactobacilli* are selective anaerobes that favor anaerobic conditions. *Lactobacillus* is catalase negative because it detoxifies hydrogen peroxide with non-oxygen evolving peroxidase. Oxygen is occasionally used in the formation of hydrogen peroxide [H_2O_2], which is harmful to *Lactobacillus* because they lack the catalase enzyme, which breaks it down. As a result of the catalase enzyme's deficiency, *Lactobacillus* emit H_2O_2. They are released into the host's extracellular space and can be detected using proper qualitative and quantitative techniques. As a result of this study, all samples were found to be devoid of catalase enzyme (Figure 3) similar to the research carried out by Amanulla[30] in yoghurt samples for five different *Lactobacillus* spp. Thus, strains in the samples were identified as belonging to the species *Lactobacillus*.

The Endospore stain showed the presence of brownish red colored short rods with round edges in all five samples (Figure 4). Similar observation was reported in the study which was carried out by Boyanova.[35] Chamika and Weerasooriya[36] also reported the reddish rods when they were performing endospore staining on *Lactobacillus* which isolated from yoghurt. Bacterial endospores are alternative life forms that some Gram-positive bacteria develop in order to survive in unfavorable environmental conditions.[37] These endospores stained with the malachite green appear green, while vegetative cells stained with the counter dye seem red or pink, according to Rohde.[38] Therefore, the brownish red rods which were observed in figure 4 were identified as *Lactobacillus.*

The Acid-fast stain showed the presence of blue colored short rods in all five samples (Figure 5). They were observed as single, double, or short chains. According to a study which was carried out by Bayonova,[35] the observation of blue colored rods in acid fast stain indicates the acid-fast negative bacteria. Lack of lipoidal material in the cell walls of non-acid fast bacteria causes them to be quickly decolorized, resulting in colorless cells. To identify them, a counterstain, such as Methylene blue, is used, which stains the non-acid fast bacteria and allows them to appear as blue in color under the microscope. In Bergey's manual[39], *Lactobacillus* is described as an acid-fast negative. According to Chamika and Weerasooriya[40] they reported similar observation for acid-fast staining which was performed on *Lacobacillus* isolated from set yoghurt. Although *Lactobacillus* was discovered as blue colored bacillus in acid fast staining, according to Gurung *et al*[40.] Thus, the bacillus with a blue color that were seen in the acid-fast staining were identified as *Lactobacillus*. Isolated colonies from yoghurt samples were found to be gram positive, non-spore forming and catalase negative, similar to the research carried out by Mojgani, *et al.*,[32]. *Lactobacillus*-specific MRS broth promoted *Lactobacillus* growth in particular. However, this does not preclude the growth of other bacteria, alongside the *Lactobacillus* culture.

The antioxidant activities of isolated *Lactobacillus* were studied in this research using the DPPH assay. DPPH is mostly used to evaluate the antioxidant activity as it is a stable compound that can be reduced by accepting hydrogen or electrons showing color change. Figure 6, 7, 8 and 9 show the color change of cell-free and cell-intact suspensions observed before and after 30 minutes incubation under dark condition. The degree of discoloration of DPPH solution indicates the scavenging potential of the antioxidant compounds. The DPPH solution is light sensitive which turns the solution from purple to yellow in the presence of light. Therefore, it is necessary to incubate the samples in dark condition to maintain the accuracy of the result. Also, the DPPH scavenging ability of intact cells and cell free suspensions of *Lactobacillus* is shown in Figure 10. According to this, samples A, B, and E have higher scavenging activity in cell intact than in cell free suspension. Remarkably, Sample C and D showed a different result in DPPH radical scavenging activity of the cell free extract (80.78%, 89.79%, respectively) compared to that of the cell intact extract (63.34%, 65.23%, respectively). Cell free suspensions could exert higher antioxidant activity due to the enhanced accessibility of antioxidant metabolites to the substrate with oxidative properties. Cell- free suspensions exert their activity by producing various metabolites that include glutathione, butyrate, and folates or by chelation of metal ions required to initiate the oxidative stress induced oxidation.[41] Apart from these, previous studies suggest antioxidant activity of cell free suspensions is also due to the production of NADH, NADPH, Mn^{2+} and bioactive compounds. According to a study carried out by Kullisaar *et al*[26], NADH oxidase, NADH peroxidase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH) and many other internal enzymes are likely obtained after the bacterial cells are isolated and decomposed into cell-free extracts. It denotes the antioxidant capacity of bacteria.[26] In comparison to cell-free extracts, whole cells were higher at scavenging DPPH. The extracellular antioxidant components most likely to be involved are polysaccharide, peptidoglycan, and teichoic acid. The peptidoglycan layer is made up of several different glycosyl chains. These generally parallel strands are joined together by peptide bonds formed between the side chains of amino acids. The cell walls of gram-positive bacteria frequently contain the amino acid teichoic acid, which is provided. A study which was carried out by Herreros.[42] further supported the antioxidant activity of *Lactobacillus* cell-surface proteins or

polysaccharides. Previous studies suggest that cell intact suspensions could exert antioxidant activity against 4NQC, a potent carcinogen that can cause DNA damage and cytotoxicity. This proves that the presence of *Lactobacillus* bacteria as viable bacteria in food products is also essential in certain circumstances. Cell free suspensions of *Lactobacillus* can exhibit antioxidant properties in vitro and in vivo despite the acidic environment of the gut. A study by Kaizu *et al.*,[43] demonstrated that cell free suspensions of *Lactobacillus* are potent enough to exert anti oxidative effects in rats who are deficient in an anti-oxidative metabolites of cell free suspensions beyond cell intact suspensions for their anti-oxidative effect.[43] The antioxidant activities of lactic acid bacteria would be helpful in the dairy food industry. They could beneficially influence the customer by providing *Lactobacillus* with the potential of producing antioxidants during the period of growth in the intestinal tract or providing another dietary source of antioxidants.

The p-value was 0.05, according to the statistical analysis provided in table 1. This could imply that the antioxidant activity mediated by cell-free, and cell intact suspension techniques was not statistically significant. However, both suspensions showed antioxidant activity.[48] Intake of foods which contain *lactobacillus* with antioxidant properties might work against the production of free radicals to prevent harmful diseases such as cancers, cardiovascular diseases and neurologic diseases.

5. Conclusion

In conclusion, the presence of *Lactobacillus* in all yoghurt samples was demonstrated using biochemical, staining, and molecular techniques. Cell intact suspensions and cell-free suspensions contain the unique antioxidant properties of *Lactobacillus*. These antioxidant properties which serve to neutralize free radicals, can be considered as a promising treatment for oxidative stress induced diseases.

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Comparative study on the nutritional composition, antibacterial and antioxidant properties of four edible mushroom species: *Pleurotus ostreatus, Pleurotus eous, Agaricus bisporus* and *Lentinula edodes*

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Abstract

Mushrooms are a great source of protein and are commonly included in the diets of many people around the globe. Natural mushroom products have long been used as a valuable source of biologically active substances important for the treatment of numerous ailments. *Pleurotus ostreatus, Pleurotus eous, Agaricus bisporus* and *Lentinula edodes* were compared in this study and their nutritional composition, antibacterial activity, antioxidant capacity and the presence of bioactive compound levels were assessed. Qualitative tests were performed to identify the presence of bioactive compounds and quantitative tests such as phenol-sulphuric assay for total carbohydrate content, Lowry assay for total protein content, phosphomolybdenum and DPPH assays for antioxidant capacity, Folin-Ciocalteu assay for total phenolic content were performed. Additionally, their antibacterial properties were examined using antibacterial sensitivity testing for *Staphylococcus aureus* and *Escherichia coli*. Based on the qualitative test results, all the four mushroom species tested positive for carbohydrates, proteins and terpenoids. *Lentinula edodes* tested positive for tannins and *Pleurotus ostreatus* tested positive for flavonoids. Both *Pleurotus ostreatus* and *Pleurotus eous* mushrooms showed positive results for polyphenols test. However quantitative test results showed that, *Agaricus bisporus* had higher overall significant values for carbohydrates, total antioxidant content, total phenolic content, and total antioxidant scavenging capacity. *Lentinula edodes* showed positive results for the antibacterial sensitivity test for both *Staphylococcus aureus* and *Escherichia coli*. Based on the overall results it can be suggested that *Agaricus bisporus* and *Lentinula edodes* are most suitable for human consumption based on their nutritional composition and antibacterial properties respectively.

Keywords: Oyster mushrooms, Button mushroom, Shiitake mushroom, Nutritional composition, Antibacterial properties, Antioxidant activity

1. Introduction

Mushrooms are a great source of protein, which have long been regarded as an essential component of fine dining cuisine around the world. Out of the 2000 different species of mushroom found in nature, only 25 of them are commonly consumed as food and a few are commercially cultivated.[1] *Agaricus bisporus, Lentinula edodes, Pleurotus* species, and *Flammulina velutipes* are the top four most widely grown mushrooms in the world. China is currently the world's top producer of mushrooms, and this trend is continuing. Yet, the significance of wild mushrooms is growing due to their pharmacological, sensory, and mainly nutritional properties.[1] Oyster mushrooms (*Pleurotus* spp.) is an edible group of mushrooms belonging to the order Agaricales and family Pleurotaceae (Figure 1). They are widely grown due to their properties such as: high growth rate at temperatures between 10 - 30°C, secretion of enzymes that can break down the lignocellulosic biomass of substrates, potential for high yields, high nutritional value and medicinal importance, viruses and pests rarely attack their fruiting bodies and the potential to colonize substrates more quickly. As saprophytes, *Pleurotus* species draw nutrients from their environment through their mycelium to acquire elements necessary for their growth,

such as nitrogen (N), carbon (C), minerals, and vitamins.[2]

Figure 1. Taxonomic overview of the oyster mushroom *Pleurotus ostreatus*.[3]

White button mushrooms (*Agaricus bisporus*), a member of the Basidiomycetes family has gained popularity as a healthy meal due to its abundance of nutrients, including proteins, carbohydrates, lipids, fibre, minerals, and vitamins.[4] It has been shown to have positive benefits on metabolic disorder, immunological function, gut wellness, and cancer, with most evidence leading to an increase in levels of vitamin D in people.[5] Shiitake mushroom (*Lentinus edodes*) is the second-most popular edible mushroom on the commercial market, which is due to both its high nutritional content and potential therapeutic uses. *Lentinula edodes* is used medicinally to treat conditions such as cancer, allergies to the environment, fungal infections, recurrent colds and flu, bronchial inflammatory conditions, cardiac disease, hyperlipidaemia, hypertension, diabetes, and regulating urinary irregularities. These conditions including acquired immunodeficiency syndrome and conditions involving a compromised immune system.[6] Mushrooms have vegetative and reproductive phases during their lifecycle. Mushroom lifecycle begins when the spores are released from the gills. Once the conditions are favourable, a single basidiospore germinates to grow and form a mass of homokaryotic mycelium. During the vegetative phase, the mycelial growth takes place beneath and inside the substrates where they release enzymes that breakdown the lignocellulosic biomass and subsequently absorb them as nutrients.[7] From the dawn of civilization, people have been fascinated with mushrooms, who have long attracted their attention. They play a significant role in the human diet due to their abundance in protein, non-starchy carbohydrates, dietary fiber, minerals, and vitamin B as shown in Table 1. They contain a very little amount of fat and rarely contain any cholesterol.[8]

Table 1. Bioactive compounds found in mushroom and their respective health benefits.[9]

Mushroom	Bioactive compound	Health benefits
Agaricus bisporus	Pyrogallol, hydroxybenzoic acid derivatives, flavonoids, lectins	Anti-inflammatory, enhanced insulin secretion, anti-ageing property
Lentinula edodes	Lentinan, glucan, Manno glucan, fucomannogalactan, Lentin, catechin flavonoids, eritadenine	Immunomodulatory, anti-tumour, anti-inflammatory, anti-bacterial, anti-fungal, antioxidant, hypolipidemic activity
Pleurotus species	Functional proteins, proteoglycans pleuran, glucans, proteoglycan, laccase, pleurostrin	Immunomodulatory, hyperglycaemia, anti-tumour, antioxidant, anti-viral, anti-fungal

Mushrooms consist of high-quality protein, and they contain essential amino acids in abundance. They also demonstrate antiviral, antitumor, antithrombotic and immunostimulatory properties.[10] Mushrooms are rich in bioactive compounds. Bioactive compounds are phytochemicals present in food, which alter metabolic processes and improve health.[11] Bioactive compounds show positive effects such antioxidant activity, the inhibition or induction of enzymes, the inhibition of receptor functions, and the promotion and repression of gene expressions.[12] Mushrooms contain a variety of bioactive compounds that have several beneficiary effects. They have gained prominence as functional foods all over the world due to their therapeutic properties According to studies, many mushrooms have antioxidant characteristics that allow them to combat free radicals. Since the oxygen molecule is a free radical, it can cause the production of reactive oxygen species, which can harm cells. Fruiting bodies, mycelium, and culture are all sources of

mushroom antioxidant compounds. The ability to control fruiting bodies or mycelium to produce active chemicals in a short amount of time represents a substantial advantage in the extraction of antioxidant compounds from mushrooms.[13] Mushrooms require more carbon and less nitrogen for their growth. Most of the organic materials containing hemicellulose, cellulose and lignin are capable of being used as growth substrates. Common examples of such substrates include cotton seed hulls, sawdust, sugarcane bagasse, wastepaper, rice, and wheat straw etc. In this study rubber sawdust was used to grow the *Pleurotus* species (*Pleurotus ostreatus* and *Pleurotus eous*). These substrates support the growth and development of mushrooms. Adequate supply of easily accessible sawdust from various trees provides a possible source of substrate for the cultivation of mushrooms.[14]

This study focuses on comparing two lab grown oyster mushrooms: *Pleurotus ostreatus* and *Pleurotus eous*, and two commercially available mushrooms: *Lentinula edodes* and *Agaricus bisporus*, based on their nutritional and antimicrobial properties and assessing their antioxidant capacity. This study also aims to quantify and compare the bioactive compounds present in the mushroom species, ultimately determining which species of mushroom is more suitable for human consumption.

2. Methodology

2.1 Preparation of media bags. Media bags were prepared by adding the chemicals at appropriate quantity to the rubber sawdust and sealing it with aluminium foil. Prepared bags were autoclaved at 121 °C for 60 minutes. 100% rubber sawdust bags for *Pleurotus ostreatus* and *Pleurotus eous* were prepared by mixing the substrate with the following ingredients as shown in Table 2.

Table 2. Measurements of ingredients for the preparation of substrate bags.

Ingredient	For 3kg of saw dust
White rice bran	240g
Red rice bran	60g
Calcium carbonate	60g
Magnesium sulphate	60kg

Initially 3/4th of the bag was filled with rubber sawdust and corners of the bags were made. Then the bags were filled and weighed. PVC rings were used to seal the bags tightly without any visible gap. Bags were tightened with rubber bands; a piece of cotton wool was plugged in, and aluminum foil was placed over it. Prepared bags were autoclaved at 121 °C for 60 minutes.

2.2 Inoculation of Spawns. Spawns of *Pleurotus ostreatus* and *Pleurotus eous* were inoculated into the bags using a spatula under sterile conditions. Inoculated bags were transferred to the incubation room and the weekly mycelial growth was recorded.

2.3 Harvesting and drying the Oyster mushrooms. Lab grown mushrooms were harvested after the fruiting bodies were fully matured. Harvest was weighed, shredded into small pieces, and dried for 48 hours at 40°C in hot air oven. Then the dried mushroom samples were crushed into a fine powder using the mortar and pestle. Same drying procedure was followed for the store-bought *Agaricus bisporus* and *Lentinula edodes*.

2.4 Preparation of aqueous extracts of mushroom. 5 g of mushroom extract was mixed with 50 ml of water (1:10 dilution) and was kept on the roller mixer for 48 hours. Then the extract was filtered using a muslin cloth through a funnel set-up. The filtrate was used in qualitative and quantitate analysis.

2.5 Total Protein Concentration Analysis using Lowry Assay. 1 ml of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml of working standard (Bovine Serum Albumin) were pipetted out into the series of labelled test tubes. Lowry reagents A, B and C were prepared 50 ml of reagent A and 1ml of reagent B were mixed and 5ml of this solution was added to all the samples. Tubes were incubated at room temperature (RT) for 10 minutes. 0.5 ml of Reagent C was then added, and samples were incubated in the dark for 30 minutes. This procedure was repeated for all the

20X diluted mushroom extracts. Absorbance was measured using UV-Vis spectrophotometer at 660nm. A standard curve was plotted using the absorbance of the standard series and the total protein concentration of mushroom samples were derived.[15]

2.6 Total Carbohydrate Content Analysis using Phenol-Sulphuric Assay. Dextrose solution was prepared and incubated in a water bath at 100°C for 3 hours. The solution was cooled down and Na_2CO_3 was added until the effervescence stopped. Dextrose solution was then filtered using Whatman No. 1 filter paper and volume was made up to 100 ml using distilled water. 1ml of standard series was prepared with the concentrations 200-1000 µg/ml. 1 ml of 5% Phenol solution was added to each standard series and 1 ml of the mushroom extracts. The mixtures were incubated for 10 minutes at RT, vortexed and incubated again in a water bath at 27°C for 20 minutes until a reddish green colour appears. Absorbance was measured at 400 nm using the UV-Vis Spectrophotometer. A standard curve was plotted using the absorbance of the standard series and the total carbohydrate content of mushroom samples were derived.[16]

2.7 Determination of Antioxidants usin2,2-diphenyl-1-picrylhydrazyl Assay 0.004% of methanol solution of DPPH was added to five different concentrations of mushroom extracts and were incubated in dark for 30 minutes at RT. Then the absorbance was measured using UV-Vis spectrophotometer at 517 nm. The percentage of inhibition was calculated using the formula below,

Inhibition ratio (%) = $[(A_C - A_S)/A_C] \times 100$

(A_C = Absorbance of control, A_S = Absorbance of sample)

The concentration of a compound at which the percent inhibition equals 50 is known as the IC50 value.[17] It is determined by taking the mean of two or more independent trials IC_{50} values were determined using the percentage inhibition against concentration graph.[18]

2.8 Determination of Total Antioxidant Capacity Using Phosphomolybdenum Assay. 0.02 to 0.1 mg/ml of Ascorbic acid standard solutions were prepared. The reagent mixture was prepared by mixing 0.6 M H_2SO_4, 4 mM Ammonium molybdate ($(NH_4)_6Mo_7O_{24}$) and 28mM tri sodium phosphate (Na_3PO_4.) and was added to standard solutions, blank (distilled water with reagents) and 0.1 ml of mushroom extracts. The test tubes were covered and incubated in a water bath at 95°C for 90 minutes. The samples were then cooled down to room temperature and absorbance was measured at 695 nm using UV-Visible spectrophotometer.[19] A standard curve was plotted using the absorbance of the standard series and the total antioxidant capacity of mushroom samples were derived.

2.9 Determination of Total Phenolic Content of Mushroom Samples. 0.02 to 0.1 mg/ml of Gallic acid standard solutions were prepared. Then 1.2 ml of 10% Folin Ciocalteu (FC) reagent and 1.5 ml of 7.5% Na_2CO_3 was added to 300 µl of each standard solutions, blank (distilled water with reagents) and mushroom extracts. The samples were then incubated for one hour in the dark at RT. Absorbance was measured at 765 nm using UV-Vis spectrophotometer.[20] A standard curve was plotted using the absorbance of the standard series and the total phenolic content of mushroom samples were derived.

2.10 Qualitative Analysis of Bioactive Compounds in Mushroom Samples Nine qualitative tests were carried out to check the presence of bioactive compounds as shown in Table 3.

Table 3. Bioactive compounds and respective tests to determine their presence in mushroom samples.[21]

Bioactive Compound	Tests
Carbohydrates	0.25 ml of Molisch reagent and few drops of conc.H_2SO_4 was added to 0.5 ml of mushroom extract.

Proteins	0.5 ml of conc. HNO_3 and 0.5 ml of 40% NaOH was added to 0.5 ml of mushroom extract
Saponins	0.25 ml of distilled was added to 0.25 ml of mushroom extract and the sample was vortexed for 15 minutes.
Flavonoids	1 ml of 2% NaOH and 2 drops of dil. HCl was added to 1ml of mushroom extract.
Polyphenols	Few drops of diluted iodine solution were added to 0.5 ml of mushroom extract.
Tannins	1 ml of 5% Ferric Chloride was added to 0.25 ml of mushroom extract.
Terpenoids	1 ml of Chloroform and 1ml of conc.H_2SO_4 was added to 0.25 ml of mushroom extract.
Anthraquinones	1 ml of 10% Ammonia solution was added to 0.25 ml of mushroom extract.
Steroids	0.25 ml of chloroform and a drop of conc.H_2SO_4 was added to 0.25 ml of mushroom extract.

2.11 Antibacterial sensitivity test. Gram negative bacteria *Escherichia coli* and gram-positive bacteria *Staphylococcus aureus* were used to test the antibacterial sensitivity in mushroom samples. Both well diffusion and disk diffusion methods were carried out.

2.11.1 Well diffusion method. Initially Mueller-Hinton agar plates were prepared. Each microbial isolate's overnight culture was emulsified with autoclaved distilled water and the turbidity of 0.5 McFarland (108 cfu/ml) was measured through spectrophotometer at 625 nm. Sterilized pipette tips were used to create small wells in the agar plates. 100µl of each mushroom extract and bacterial culture were poured into the wells. Distilled water was used as a negative control. All the preloaded plates were incubated for 24 hours at 37°C with the appropriate extract and test organism. The zone of inhibition was measured following the incubation period.[22]

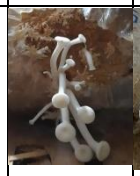
2.11.2 Disk diffusion method Plates were prepared as explained above. 100 µl aliquot of test culture was uniformly distributed across the entire surface of the solidified Mueller-Hinton agar to ascertain the antibacterial effectiveness of the fractions. 6 mm diameter sterile paper discs were placed on small Petri dishes and imprinted with 100 µl extracts solution; dried and placed carefully on petri plates. Commercial gentamicin antibiotic (10 g) was used as a positive control and distilled water as a negative control in each experiment. The zone of inhibition (mm) surrounding the discs imprinted with the extracts were measured after 24 hours of incubation at 37°C.[23]

2.12 Statistical Analysis Statistical analysis was carried out through one-way ANOVA test using Microsoft office Excel (Microsoft 365 version 2306) and the significant difference was analysed. All data are expressed as Mean ± Percentage Error Bars.

3. Results and Discussion

3.1 Mycelial Growth. Growth of lab grown *Pleurotus ostreatus* and *Pleurotus eous* oyster mushrooms are illustrated in Table 4.

Table 4. Growth of *Pleurotus ostreatus* and *Pleurotus eous* oyster mushrooms

Mushroom species	Week 04	Week 05	Week 06
Pleurotus ostreatus			
Pleurotus eous			

3.2 Bioactive compounds Qualitative tests for eight bioactive compounds were carried out for all four mushroom samples and the results were as stated in Table 5.

Table 5: Qualitative analysis of bioactive compounds in four species of mushrooms

Tests	*Pleurotus ostreatus*	*Pleurotus eous*	*Agaricus bisporus*	*Lentinula edodes*
Molisch	P	P	P	P
Xanthoproteic	P	P	P	P
Saponins	Ab	Ab	Ab	Ab
Flavonoid	P	Ab	Ab	Ab
Polyphenols	P	P	Ab	Ab
Tannins	Ab	Ab	Ab	P
Terpenoids	P	P	P	P
Anthraquinone	Ab	Ab	Ab	Ab
Steroids	P	P	Ab	Ab

P- Present, Ab - Absent

All the four species of mushrooms tested positive for carbohydrates, Terpenoids and proteins. Anthraquinones and saponins were absent in them. *Lentinula edodes* tested positive for tannins and *Pleurotus ostreatus* mushroom tested positive for flavonoids. Both *Pleurotus ostreatus* and *Pleurotus eous* tested positive for polyphenols and steroids (Table 06).

3.3 Total Carbohydrate Content

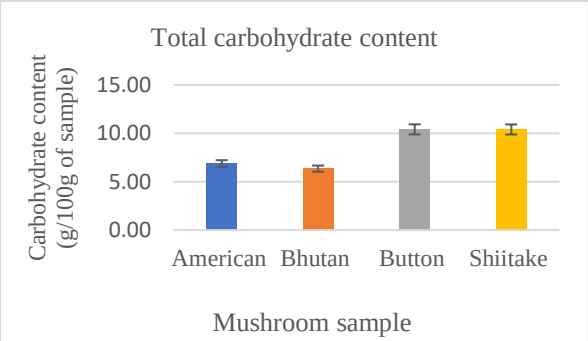


Figure 2. Total carbohydrate content of four varieties of mushroom species.

The highest total carbohydrate content was observed in *Agaricus bisporus*, and it was significantly high compared to *Pleurotus ostreatus* and *Pleurotus eous* mushrooms. The lowest total protein content was observed in *Pleurotus eous* mushrooms (Figure 2).

3.4 Total protein content

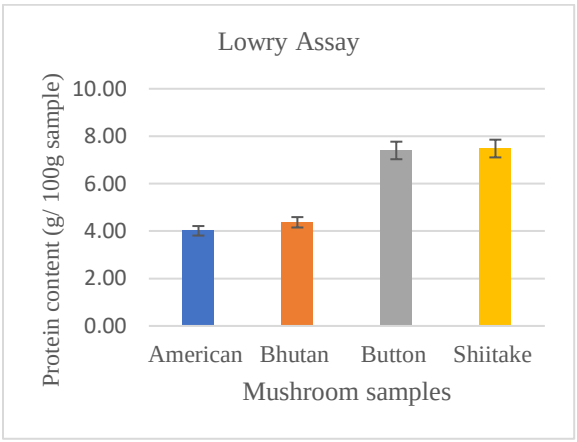


Figure 3. Total protein content of four varieties of mushroom species.

The highest total protein content was observed in *Lentinula edodes*, and it was significantly high compared to *Pleurotus ostreatus* and *Pleurotus eous* mushrooms. The lowest total protein content was observed in *Pleurotus ostreatus* mushrooms (Figure 3).

3.5 Total Phenolic content (TPC)

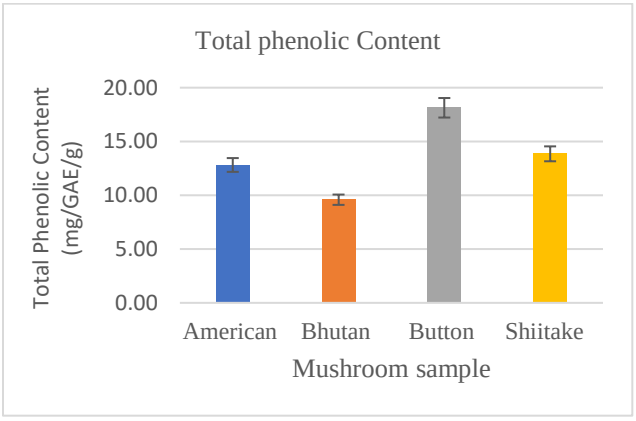


Figure 4. Total phenolic content of four different mushroom sample

The highest total phenolic content was observed in *Agaricus bisporus*, and it was significantly high compared to all the other three varieties of mushrooms. The lowest total phenolic content was observed in *Pleurotus eous*. (Figure 4).

3.6 Total Antioxidant Capacity (TAC)

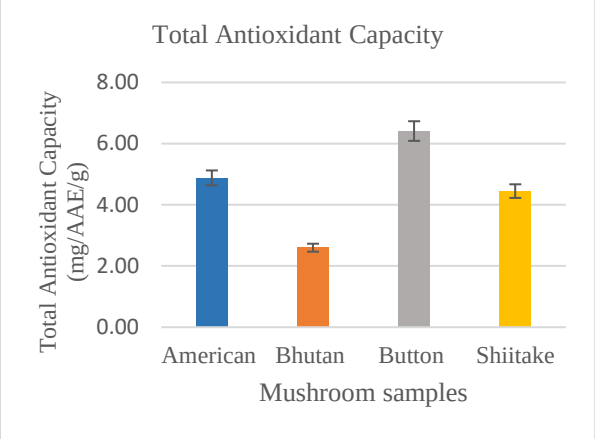


Figure 5: Total phenolic content of four different mushroom sample.

The highest total antioxidant capacity was observed in *Agaricus bisporus*, and it was significantly higher compared to all the other three varieties of mushrooms. The lowest total antioxidant capacity was observed in *Pleurotus eous*, (Figure 5).

3.7 IC_{50} values using DPPH inhibition activity

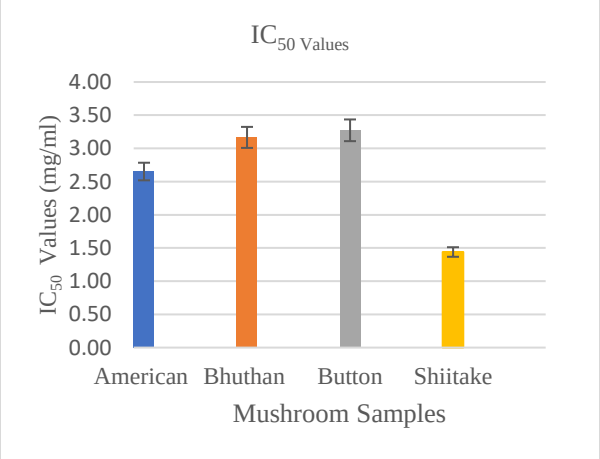
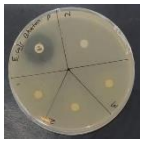
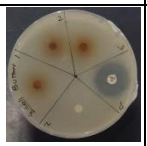
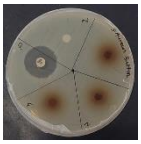
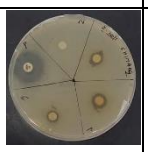


Figure 6: Antioxidant activity of four varieties of mushrooms.

The lowest IC_{50} value was observed in *Lentinula edodes*, and it was significantly lower than other mushroom varieties. (Figure 6).

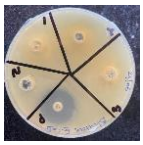
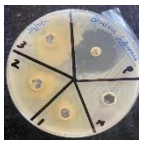
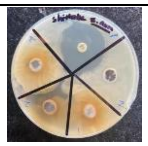
3.8 Antibacterial Sensitivity Test. Disk diffusion and well diffusion tests were performed to observe the antimicrobial activity of the four different types of mushrooms as shown in Table 6 and 7.

Table 6. Results of disk diffusion method

Mushroom species	***E. coli***	***Staphylococcus aureus***
Pleurotus ostreatus	 Negative	 Negative
Pleurotus eous	 Negative	 Negative
Agaricus bisporus	 Negative	 Negative
Lentinula edodes	 Positive	 Positive

According to the disk diffusion method only *Lentinula edodes* showed positive results against *E. coli* with an average zone of inhibition of 8 mm and *Staphylococcus aureus* with an average zone of inhibition of 11.67 mm.

Table 7. Results of well diffusion method

Mushroom species	*E. coli*	*Staphylococcus aureus*
Pleurotus ostreatus	 Negative	 Negative
Pleurotus eous		 Negative
Agaricus bisporus	 Negative	 Negative
Lentinula edodes	 Positive	 Slightly Positive

According to the well diffusion method, Lentinula edodes showed positive results against *E. coli* with an average zone of inhibition of 18.3 mm. However, in Staphylococcus aureus fungal contamination was observed and could not distinctly identify the zone of inhibition. All the other varieties showed negative results against antimicrobial tests.

4. Discussion

The present study focuses on comparing *Pleurotus ostreatus*, *Pleurotus eous Lentinula edodes* and *Agaricus bisporus* based on their nutritional and antimicrobial properties. 100% sawdust was used as the substrate to grow both the oyster mushrooms under lab conditions. This study was carried out to determine the best mushroom species with more health benefits and safe for consumption, based on its nutritional and antimicrobial properties. The four varieties of mushroom samples exhibited different properties against the quantitative, qualitative, and antibacterial sensitivity tests performed.

Bioactive compounds are substances that have a biological activity or has the capacity to influence one or more metabolic processes, hence promoting better health conditions.[11] All the four species of mushroom showed positive results for Molisch test (carbohydrate), Terpenoids test and Xanthoproteic test. The *Lentinula edodes* mushroom turned brownish green colour and showed positive results for tannins test.[24] In a similar study done by Mata *et al*. (2016), hydrolytic enzyme such as tannins were present in *Lentinula edodes*, and its activity was detected during the development of fruiting bodies. *Pleurotus ostreatus* and *Pleurotus eous* oyster mushroom extracts turned red to show a positive result for polyphenols test. *Pleurotus ostreatus* mushroom also showed positive results for flavonoids test.

Agaricus bisporus reported significantly high total carbohydrates total phenolic content and total antioxidant capacity compared to all other mushroom species. Total soluble sugars in dry *Agaricus bisporus* (0.16 mg/g) is higher than the dry *Pleurotus ostreatus* (0.62 mg/g) and they further explain that dried mushrooms have a higher concentration of soluble sugars than fresh mushrooms due to their higher moisture content.[25]

Proteins are biopolymer molecules made up of 20 different amino acids. They provide structural support to the body, act as catalysts, hormones, and enzymes.[26] The Lowry protein assay advances the biuret reaction by adding more processes and components that increase detection sensitivity. Four nitrogen atoms from peptides interact with copper to form a cuprous complex during the biuret reaction.[27] According to this study *Lentinula edodes* followed by *Agaricus bisporus* showed the highest protein content through Lowry assay. These results also align with a previous study where they concluded that *Agaricus bisporus* contained the highest

amount of protein (2.35 mg/ml) and *Pleurotus* species had the lowest amount of protein (1.20 mg/ml) based on the Lowry assay results.[28]

The DPPH assay is based on the evaluation of the antioxidants' ability to scavenge it. By adding a hydrogen atom from antioxidants to the equivalent hydrazine, the odd electron of the nitrogen atom in DPPH is lowered. Unlike most other free radicals, the spare electron in DPPH is delocalized across the entire molecule, making it a stable free radical. The deep violet hue, which absorbs ethanol solution at a wavelength of about 520 nm, is also a result of delocalization. DPPH solution changes into the reduced form and loses its violet colour when combined with an agent that can release hydrogen atoms.[29] Since having a lower IC_{50} value indicates the extracts can scavenge DPPH radicals more effectively, it could be stated that *Lentinula edodes* (71.98 mg/ml) had the highest antioxidant scavenging ability and *Agaricus bisporus* (130.91 mg/ml) had the lowest antioxidant scavenging ability. However, in a study carried out by (cite the article) the results stated otherwise.[30] They discovered that *Pleurotus ostreatus* had the highest ability to scavenge DPPH free radicals (81.3 mg/ml) than *Agaricus bisporus* (77.5 mg/ml). This deviation in results might be due to the environmental conditions, growth factors and substrates used in that study were different to that of the present study.

To prevent free radicals from harming living things, antioxidants have the essential function of neutralizing them in body cells. Superoxide dismutase is an enzyme that specifically reduces the effects of oxidative stress brought on by an excess of free radicals.[31] In Total Antioxidant capacity test Mo (VI) is converted to Mo (V) in the presence of extracts, forming a green phosphomolybdenum V complex with an absorbance peak at 695.[32] You should mention about the TAC results. The primary phenolic compounds in *Agaricus bisporus* ethanolic extract included gallic acid, protocatechuic acid, catechin, caffeic acid, ferulic acid, and myricetin and suggested that this extract had a strong antioxidant effect and might be studied as a novel natural antioxidant.[33] Another study reported that in comparison to *Lentinula edodes, Pleurotus ostreatus, Pleurotus eryngii,* and *Grifola frondosa, Agaricus bisporus* exhibited a higher antioxidant capacity.[4]

Phenolic compounds are secondary metabolites containing benzene rings with one or more hydroxyl substituents, that results from the metabolism of phenylpropanoid in pentose phosphate and shikimic acid.[34] The Total Phenolic Content test, also called the FC method, is a well-known technique that oxidizes phenolic compounds using the FC reagent. The reaction produces a blue-coloured reduced FC reagent, where the intensity of the colour corresponds with the amount of phenolics in the sample.[35] The FC method can be used to spectrophotometrically measure the total phenolic content as Gallic acid equivalents/g. According to the present analysis, the total phenolic content varied among the mushroom species as *Agaricus bisporus* (6.43 mg $GAEg^{-1}$), *Pleurotus ostreatus* (6.27 mg $GAEg^{-1}$), , and *Lentinula edodes* (4.94 mg $GAEg^{-1}$). In line with our results, a study conducted by Gan, Amira and Asmah (2013) showed that *Agaricus bisporus* contained significantly higher TPC (0.48 mg/GAE/g) in aqueous extract compared to other mushrooms of interest.[36]

In antibacterial sensitivity tests, only *Lentinula edodes* showed positive results for both *E. coli* and *Staphylococcus aureus.* However, in well diffusion method *Lentinula edodes* were contaminated with fungal growth and zone of inhibition was not clear for observations. Hearst *et al*., 2009 used aqueous extracts of *Lentinula edodes* to qualitatively test against a panel of 29 bacterial and 10 fungal pathogens for microbial inhibition.[37] Their results quantitatively depicted that *Lentinula edodes* extract had strong antibacterial effects against 85% of the examined organisms, including 50% of the yeast, mold species, including *Staphylococcus aureus and E. coli.* This is further confirmed by the research carried out by Ishikawa *et al*., 2001, where they tested the antibacterial resistance of *Lentinula edodes* against 20 foodborne pathogens and identified the zone of inhibition for 8 different bacterial species including *Staphylococcus aureus.*[38] They further explained that the stimulation of complement C3 upregulation, which enhanced resistance to infection, was the

cause of the *in vivo* impact of *Lentinula edodes* antibacterial component.

5. Conclusion

In conclusion, it can be suggested that all four mushrooms are generally rich in nutrients. Since *Agaricus bisporus* had higher overall significant values for carbohydrates, total antioxidant content, total phenolic content, and total antioxidant capacity, it could be stated that *Agaricus bisporus*s are most suitable for human consumption which could also contribute to various health benefits including boosting out immunity. However, *Lentinula edodes* can also be considered as a healthy option due to its antibacterial properties. It could safeguard against bacterial infections as it showed efficacy against both gram-positive and gram-negative bacteria. Furthermore, *Lentinula edodes* exhibited significantly higher DPPH antioxidant scavenging capacity and total protein content. Hence consuming these mushrooms regularly as part of a healthy diet may act as a natural supplement in both the prevention and treatment of several chronic illnesses.

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Isolation and identification of microorganisms in processed chicken products and determination of antibiotic susceptibility

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Abstract

On a global level, there is a rising demand for a steady supply of animal protein, and consumption of processed meat. Meat is the most perishable food type since it has the most nutrients that enables microbial development. To enhance flavor and improve shelf life, processed chicken products are produced by smoking, fermenting, curing, salting, and adding chemical preservatives. *Staphylococcus aureus, Escherichia coli,* and *Bacillus subtilis* are the most frequent bacteria found in processed chicken products. The processed chicken meat may become contaminated by a range of bacteria during meat processing facility operations. One of the largest challenges to development, food security, and global health is antibiotic resistance. Studies on the horizontal transfer of drug resistance determinants have focused mostly on the primary pathogenic and opportunistic microorganisms. The food chain can be viewed as the primary means of transmission of microorganisms resistant to antibiotics between the animal and human populations. This study aims to isolate and identify microorganisms in processed chicken products and determine the antibiotic susceptibility. Five commercially available processed chicken products from different manufacturers were collected from Sri Lankan supermarkets. Enrichment, plating methods, staining techniques, biochemical tests, DNA extraction and determining the antibiotic susceptibility using the disk diffusion method were the major procedures followed in the study. Microorganisms were predicted in all the samples using probabilistic neural network and multidimensional scaling method using MATLAB®. *Bacillus cereus, Listeria monocytogenes* and *Paenibacillus alvei* were predicted by the software. However, further confirmation using molecular biological techniques regarding the same is required to effectively conclude the results.

Keywords: Enrichment, DNA quantification, Food microbiology, Food quality and Antibiotic susceptibility

1. Introduction

Presently, there is a gradually increasing demand for a consistent supply of animal protein on a global scale, and processed meat consumption is currently expanding. Since meat contains enough nutrients to support the growth of microorganisms, it is the most perishable of all food types. Microbial infection can reduce the quality of meat, reduce its shelf life, cause financial loss, as well as present health risks. Meat is an essential component of the growth, repair, and maintenance of body cells because it is the main source of protein and important vitamins for most people in many parts of the world. Processed chicken products are made by smoking, fermenting, curing, salting, and adding chemical preservatives to improve flavor and increase shelf life. There is a worldwide interest in preservation to maintain the safety and bioavailability of processed meat for a longer shelf life.[1]

Typically, the first evidence of chicken meat dates from 4,000 to 10,500 years ago. It was claimed to have originated in India, Northern China, and Southeast Asia.[2] In 2020, Sri Lanka's population consumed 10.4 kg of poultry meat annually. This is 19.2% increase from the previous year (2019).[3] In comparison to 9.8 kg in 2000, the global per capita consumption of poultry increased to 14.8 kg in 2019. In 13 countries, the total amount of poultry consumed per person is more than doubled. In 30 of the 35

studied countries, there was a linear increase in per capita poultry consumption between 2000 and 2019.[4] In Sri Lanka, there were 7.09 kg of chicken available annually per person as meat and meat-related products and the total amount of meat produced increased from 119,620 million tons in 2004 to 185,490 million tons in 2013.[5] 130 million tons of poultry were produced globally in 2019, an increase of 3.7% from the previous year (2018). It is anticipated that this growth will continue, accounting for 45% of global production. Nearly 40% of the meat produced worldwide in 2020 was poultry meat.[6]

Microorganisms, which include bacteria, fungi, and protozoans, are defined as organisms or agents of infection that are microscopic or submicroscopic in size.[7] The common microorganisms that are present in processed chicken products are *Escherichia coli, Bacillus subtilis, Aspergillus brasiliensis, Salmonella* and *Campylobacter*.[8] Many foodborne illnesses are caused by these bacteria. *E. coli* is a typical resident of intestinal tracts and is present in dust, litter, chicken droppings, and rodent droppings.[9] An alternative to antibiotic growth promoters in the poultry industry could be *B. subtilis* as in-feed antibiotics.[10] *Salmonella gallinarum* and *Salmonella pullorum* are particularly adapted to poultry. They are responsible for the pullorum disease (*S. Pullorum*) and fowl typhoid (*S. Gallinarum*).[11] *E. coli* infections, salmonellosis and paratyphoid infections, and fowl cholera are other common infectious diseases in poultry animals.[12]

During operations at meat-processing plants, a variety of microorganisms may contaminate the processed chicken meat.[13] Microbial contamination can reduce the quality of meat, minimize its shelf life, lead to financial loss, and possibly available health hazards. Meat spoilage and afterward decomposition are caused by the practically unavoidable infection, which is carried by the animal itself, by those handling the meat, and by their tools.[14] The spoilage from bacteria is very low immediately after processing, but they can quickly grow and produce spoilage slime and odors. The primary causes of poultry products spoilage are prolonged distribution, inappropriate storage temperature and time, and high post-rigor meat pH.[15] The careful regulation and oversight of the slaughtering and processing facilities, proper handling and storage, and adequate cooking of raw and processed poultry products are all necessary for the prevention of microbial contamination.[16] Patients with weakened immunity are more vulnerable to the serious effects of foodborne infections, which can range from long-term illness to death. Typically, bacteria and their metabolites, parasites, viruses, or toxins are responsible for microbiological food-borne illnesses. Depending on the foods consumed, the methods used for food processing, preparation, handling, and storage, as well as the population's sensitivity, different food borne diseases have varying levels of importance in different countries.[17]

Antibiotic resistance (AR) is currently one of the biggest threats to development, food security, and global health. When bacteria learn to resist the medications meant to kill them, antibiotic resistance occurs. AR quickens, when bacteria are compelled to respond to the presence of antibiotics.[18] The primary pathogenic and opportunistic bacteria have received most of the attention in studies on the horizontal transfer of drug resistance determinants. The main way of transmission of antibiotic resistant bacteria between the animal and human populations can be thought of as the food chain.[19] At least 1.27 million people are dying from antibiotic resistance-related causes worldwide and this number is expected to reach almost 5 million in 2019.

Table 1. The common microorganisms in processed chicken products and antibiotic resistance.[20]

Microorganisms	Resistant antibiotics
Escherichia coli	Penicillins, aminopenicillins, and cephalosporins
Bacillus subtilis	Lincomycin, streptogramin A, and antibiotic virginiamycin M
Aspergillus brasiliensis	Polyenes, triazoles, and echinocandin
Salmonella	Ampicillin, chloramphenicol, florfenicol,

	streptomycin, sulfonamides, and tetracycline
Campylobacter	Ciprofloxacin, and fluoroquinolone antibiotic

This study is aimed to isolate and identify microorganisms in processed chicken products and determine the antibiotic susceptibility. The main techniques used in the study are plating methods, staining methods, biochemical tests, DNA extraction, and investigating the antibiotic susceptibility using disk diffusion method.

2. Methodology

2.1 Sample collection. Commercially available five processed chicken products (sausages, meatballs, ham, bacon and lingus) from different manufacturers were collected from local supermarkets in Sri Lanka. Surface sterilization of intact packaging was done using 70% ethanol to minimize environmental contamination and stored at -20 ℃. *E. coli* (ATCC 8739)*, S. aureus* (ATCC 6538) and *B. subtilis* (ATCC 6633) were used as positive controls.

Table 2. Processed chicken samples and the product type used for the study.

Sample number	Product type
1	Chicken sausages
2	Chicken meatballs
3	Chicken ham
4	Chicken bacon
5	Chicken lingus

2.2 Enrichment. Products were transferred to biosafety cabinet after surface sterilization. They were opened inside the biosafety cabinet to minimize contamination. Sterile surgical blades were used to sample the meat products. Approximately 1 g of sample was taken and transferred to sterile labelled Uricol™ containers containing 10 ml of sterile peptone water (HIMEDIA®/Ref: M028-500G). The containers were incubated at 37 ℃ for 48-72 hours. Positive and negative controls were also subjected to the same conditions.

2.3 Culturing of microorganisms

2.3.1. Streak plate method. After incubation, a loopful of samples from each container was inoculated in nutrient agar (NA) (HIMEDIA®/Ref: M57-500G) and soybean casein digest agar (SCDA) (HIMEDIA®/Ref: M290-500G) under aseptic conditions. The plates were incubated at 37 °C for 24 hours.

2.3.2 Liquid culturing. The selected colonies of bacteria were inoculated into 2 ml microcentrifuge tubes containing 1.5 ml sterile nutrient broth (HIMEDIA®/Ref: M002-500G). The tubes were incubated at 37 °C for 24-72 hours. The organisms were propagated by subculturing from time to time.

2.4 Microscopic observation. Microscopic observations were performed to identify the selected pure colony from each sample. The colonies were propagated by subculturing from time to time.

2.4.1. Gram's staining technique. The isolated colonies of bacteria were placed on microscopic slides using sterile inoculation loop. The staining was carried out following the ASM gram staining protocol.[46] The specimens were observed under the microscope.

2.4.2 Endospore staining. A loopful of bacteria were placed on the microscopic slides using sterilized inoculation loop. The staining was carried out following the ASM spore staining protocol.[46]

2.4.3 Motility test. Bacterial motility was observed using hanging drop method.

Fungus or molds that were present on the media plates were not selected for further analysis.

2.5 Biochemical testing

2.5.1. IMViC test

2.5.1.1. Indole test. A loopful from liquid culture tubes were inoculated in 10ml of tryptophan broth (HIMEDIA®/Ref: M1339-500G). The tubes were incubated for 24 to 48 hours at 37 °C.

2-3 drops of Kovacs reagent were added to it after incubation.

2.5.1.2. Methyl-red test. A loopful from liquid culture tubes were inoculated in 10ml of MR-VP broth (HIMEDIA®/Ref: M070-500G). The tubes were incubated for 24 to 48 hours at 37 °C. 2-3 drops of Methyl-Red indicator were added to it after incubation.

2.5.1.3. VP test. A loopful from liquid culture tubes were inoculated in 10ml of VP broth (HIMEDIA®/Ref: M070F-500G). The tubes were incubated for 24 to 48 hours at 37 °C. 2-3 drops of Barritt reagent were added to it after incubation.

2.5.1.4. Citrate utilization test. The selected colonies from streak plates were streaked on 10 ml citrate agar slant. The tubes were incubated for 24 hours at 37 °C.

2.5.2 TSI test. The selected colonies from streak plates were streaked on 10 ml TSI agar (HIMEDIA®/Ref: M021-500G) slant. The tubes were incubated for 24 hours at 37 °C.

2.5.3. Catalase test. A small amount of bacteria was placed onto the microscopic slides using sterilized inoculating loop. A drop of hydrogen peroxide (20% V/V) was placed on top of the bacterial smear.

2.6 DNA extraction. The pure culture plates were prepared by streaking a loopful from the liquid culture. After incubation at 37 ℃ for 48 hours, bacterial colonies were transferred to 1.5 ml microcentrifuge tubes using sterile blades and inoculation loop. 1 ml of saline water (0.9% NaCl) was added to the tubes. They were vortexed thoroughly. The tubes were centrifuged at 13 000 rpm for 5 minutes. The supernatant was discarded. The washing step was repeated once again. Residual supernatant was discarded. 20 µl of TAE buffer was added to the pellet. The tubes were vortexed again and were transferred to ice for 15 minutes. The tubes were immediately kept in 95 ℃ water bath for 15 minutes. The tubes were centrifuged at 13 000 rpm for 5 minutes. The supernatants were transferred to 1.5 ml new sterile microcentrifuge tubes. 20 µl of 100% ethanol was added to each tube containing the supernatant. The tubes were stored at -20 ℃ until further analysis.

2.7 DNA quantification

2.7.1. Spectrophotometer. Quartz cuvette was used to measure absorbance at 225 nm, 260 nm, 280 nm, 330 nm and 450 nm. Distilled water was used as the blank. Various dilutions were used to measure the absorbance.

2.8 Antibiotic susceptibility test

2.8.1 Disc diffusion method. The microbial inoculum was spread on the surface of Mueller Hinton Agar (MHA) (HIMEDIA®/Ref: M173-500G) using sterile cotton swabs. Ampicillin (SD002), Erythromycin (SD083), Vancomycin (SD163), Tetracycline (SD037) and Chloramphenicol (SD006) discs were used as antibiotics. The plates were incubated for 24 hours at 37 °C. Zone of inhibition was measured using a vernier caliper after incubation.

The organisms tested from each sample were predicted using Probabilistic Neural Network (PNN) structure and classical Multidimensional Scaling (MDS) method in MATLAB® software.[21] Annbis GUI was used for this purpose. Antibiotic susceptibility was determined after referring to the CLSI M100 guidelines.[22]

3. Results

3.1 Streak plate method. Growth was observed in all the pre-culture plates indicating the presence of organisms in all the samples. Various colony morphologies could be observed.

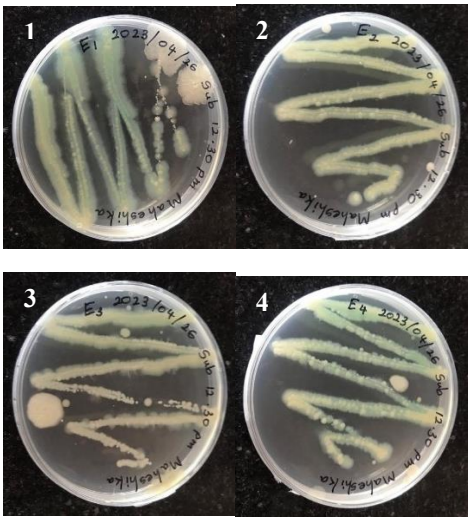


Figure 1. Third Sub-culture in Nutrient Agar.

The colonies observed had relatively similar morphologies indicated the presence of a single type of colony.

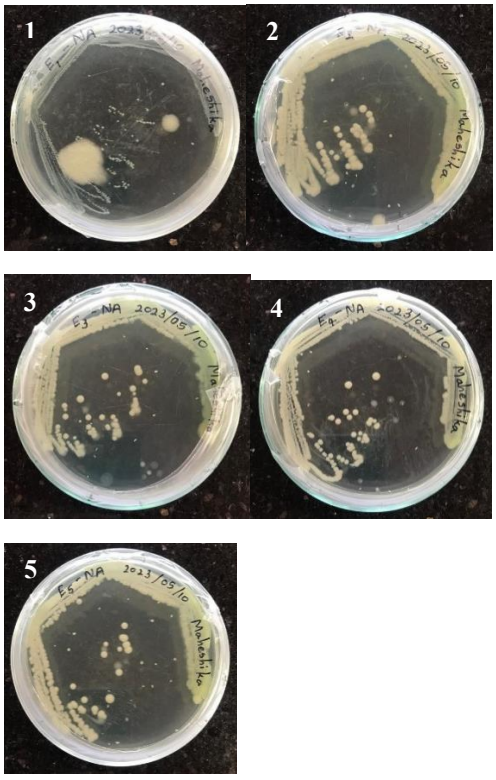
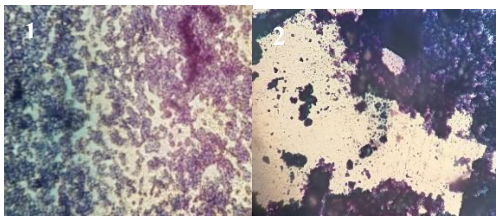


Figure 2. Fourth Sub-culture in Nutrient Agar.

After subsequent culturing pure colonies could be isolated. This was confirmed by having same colony morphologies.

3.2 Microscopic observation

3.2.1. Gram's staining technique



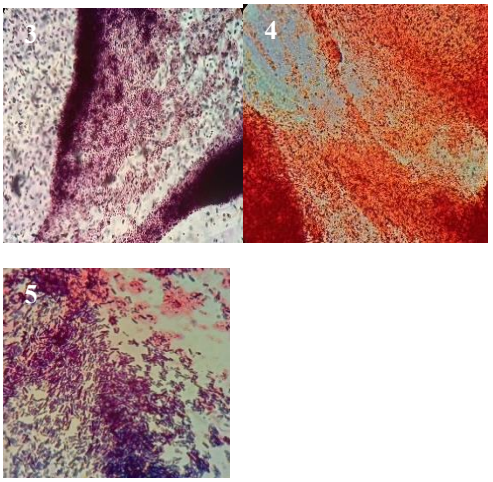


Figure 3. Gram's-stained bacteria under 100x magnification.

Gram-positive bacteria were observed in purple colour and Gram-negative bacteria in pink colour. Out of all 5 samples, 1, 2, 3 and 5 were Gram-positive and only sample 4 was Gram-negative.

3.2.2. Endospore staining

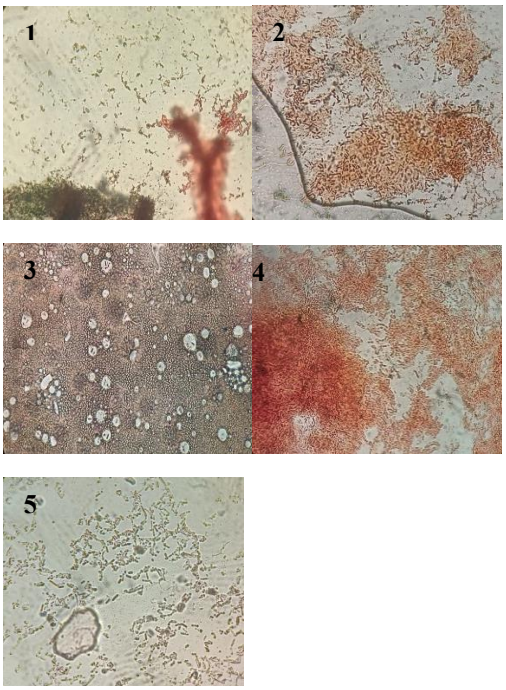


Figure 4. Endospore-stained bacteria under 100x magnification.

Bacteria containing spores were observed in green colour while vegetative bacteria were observed in red colour. Out of all 5 selected organisms, 1, 2, 3 and 5 could be

identified as endospore formers while sample 4 had vegetative cells.

3.2.3 Motility test. Out of all 5 samples, 1, 2, 3, 4 and 5 had motile bacteria. Cocci shaped bacteria were observed from 1, 2, 3 and 5 samples. Bacilli shaped bacteria were observed from 1, 3, 4 and 5 samples.

Table 3. Observations of selected organisms from samples.

Sample	Gram nature	Endospore	Motility
1	Positive	Present	M
2	Positive	Present	M
3	Positive	Present	M
4	Negative	Absent	M
5	Positive	Present	M

M- Motile

3.3 Biochemical testing

3.3.1 IMViC test

Table 4. IMViC test results.

Test	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Positive	Negative
Indole test	+	+	+	+	+		
MR test	+	+	+	+	+		
VP test	+	+	+	+	+		
Citrate utilization test	+	+	-	+	+		

(+) - Positive, (-) - negative

3.3.2 TSI test

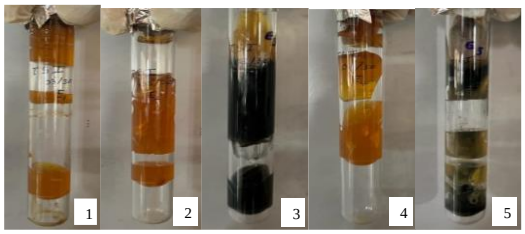


Figure 5. TSI test results.

Yellow slant and yellow butt were observed from sample 1, 2 and 4. It indicated the fermentation of glucose, sucrose and lactose. Black colour medium was observed from 3 and 5. It indicated the presence of H_2.

Table 5. The results of TSI test.

Sample	H_2 gas production (black)	Acid/ Acid reaction (Y/S, Y/B)	Alkaline /Acid reaction (R/S, Y/B)	Gas production / (H_2S)
1		√		√
2		√		√
3	√			√
4		√		√
5	√			√

Y/S: Yellow slant, Y/B: Yellow butt, R/S: Red slant

3.3.3. Catalase test

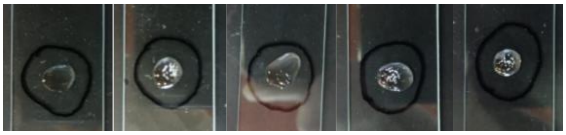


Figure 6. Catalase test results.

The positive reaction was indicated by the presence of bubbles upon addition of H_2O_2. Out of all 5 samples, 2, 3, 4 and 5 had positive results and only 1 had a negative result.

Table 6. The results of catalase test.

Sample	Positive	Negative
1		√
2	√	
3	√	
4	√	
5	√	

3.4 DNA quantification

3.4.1. Spectrophotometer

Wavelengths were measured at different wavelengths and significant amount of DNA was present in each sample.

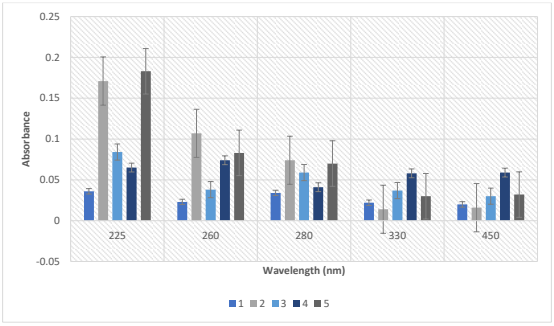


Figure 7. The readings of spectrophotometer in different wavelengths using 5 µl of DNA.

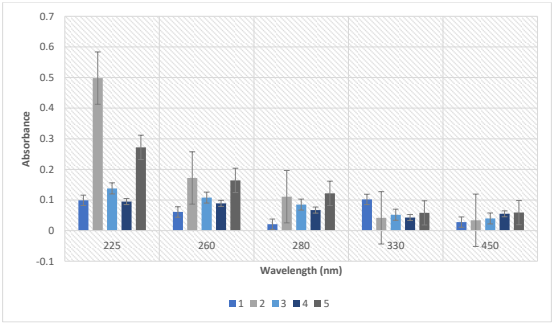


Figure 8. The readings of spectrophotometer in different wavelengths using 10 µl of DNA.

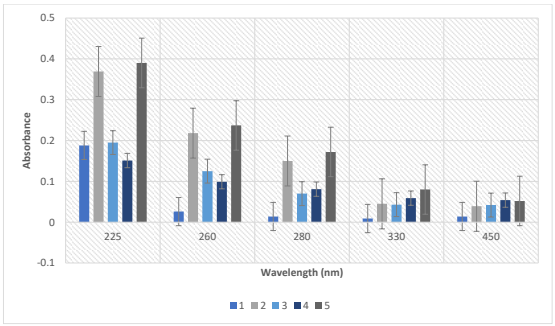


Figure 9. The readings of spectrophotometer in different wavelengths using 15 µl of DNA.

3.5 Antibiotic susceptibility test

3.5.1. Disc diffusion method

The zones of inhibition were observed in ABST.

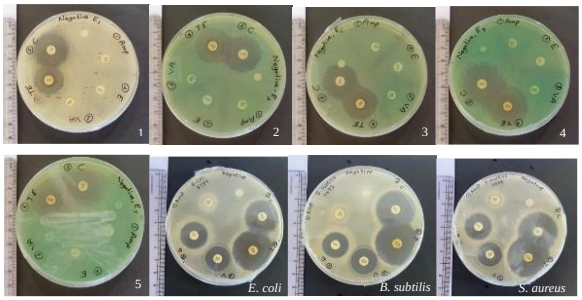


Figure 10. ABST results.

Table 7. The predicted organisms obtained from MATLAB® software by feeding biochemical characteristics.

Sample number	Predicted organism
1	*Bacillus cereus*
2	*Listeria monocytogenes*
3	*Paenibacillus alvei*
4	*Listeria monocytogenes*
5	*Listeria monocytogenes*

Table 8. The results of ABST.

Sample	Antibiotics	Diameter of the ZOI	Sensitivity
1	Amp	-	R

	Ery	-	R
	Van	-	R
	Tet	20.5 mm	I
	Chlor	27.0 mm	I
2	Amp	-	R
	Ery	-	R
	Van	-	R
	Tet	22.9 mm	S
	Chlor	26.2 mm	S
3	Amp	-	R
	Ery	-	R
	Van	-	R
	Tet	25.0 mm	S
	Chlor	26.6 mm	I
4	Amp	-	R
	Ery	15.6 mm	I
	Van	-	R
	Tet	18.7 mm	S
	Chlor	27.6 mm	I
5	Amp	-	R
	Ery	-	R
	Van	-	R
	Tet	18.6 mm	S
	Chlor	20.1 mm	I

ZOI – Zone of inhibition, Amp- Ampicillin, Ery – Erythromycin, Van – Vancomycin, Tet – Tetracycline, Chlor – Chloramphenicol, R – resistant, I – intermediate, S – sensitive.

Table 9. The susceptibility of the predicted organisms.

Sample No.	Predicted organism	Ampicillin	Tetracycline	Erythromycin	Chloramphenicol	Vancomycin
1	*Bacillus cereus*			√	√	
2	*Listeria monocytogenes*	√	√	√	√	√
3	*Paenibacillus alvei*		√			√
4	*Listeria monocytogenes*		√	√	√	
5	*Listeria monocytogenes*	√	√	√	√	√

√ = Sensitive

4. Discussion

On a global level, there is a steadily rising demand for a reliable source of animal protein, and the use of processed meat is currently expanding. Meat is the most perishable food category because it contains enough nutrients to support the growth of bacteria. One of the main pathways for the spread of antibiotic resistance between animal and human populations has come to be identified as the food chain. In fact, it has been demonstrated that commensal bacteria can serve as repositories for antibiotic resistance genes, which may be transmitted to foodborne or enteric human pathogens through the food chain .[23] This study showed the antibiotic susceptibility of isolated microorganisms in processed chicken products. Microorganisms in processed chicken products were isolated and identified using various laboratory tests including the culturing of microorganisms, microscopic observation, biochemical tests and antibiotic susceptibility test.

Enrichment was followed to provide optimal conditions to organisms that could be

present in the meat in minute amounts.[24] Because it helps with exceptionally favorable growth for an interesting organism and an unfavourable environment for any competition. This makes it possible to detect and identify microorganisms with a range of nutritional requirements.[25] After incubation, because there was turbidity in the peptone water, it was predicted that microorganisms could be present in all samples.

Numerous species of bacteria can grow on NA, which also includes many of the nutrients required for bacterial growth.[26] SCDA is frequently used for the cultivation of microorganisms from environmental sources and promotes the growth of a wide range of microorganisms.[27] The streak plate method is important for the isolation of bacteria from a large population.[28] Appropriate aseptic procedures prevent the unintended release of microorganisms into the environment and the contamination.[29] The absence of a necessary nutrient, the toxicity of the culture medium itself, or the presence of other bacteria in the sample that create chemicals that are inhibitory to the target organism are the causes of bacteria not growing in a culture plate.[30] A wide range of fastidious and non-fastidious microorganisms with no nutritional requirements can be cultivated using nutrient broth.[31] The liquid culturing was carried out to ensure the luxurious growth of the selected colony and to avid overgrowth which is usually observed on the solid agar plates.

Gram-positive bacteria were stained in purple while gram-negative bacteria were stained in pink in gram's staining technique. The process involves staining bacteria samples with crystal violet, which binds to peptidoglycan layer of both Gram-positive and Gram-negative bacteria.[32] Gram's iodine fixes the crystal violet into the bacteria's cell wall. Crystal violet inside gram positive bacteria bind to the iodine and continue to be purple. Gram negative bacteria contains secondary plasma membrane and will be washed off when treated with decolorizer.[33] Gram-negative and gram-positive bacteria can be easily separated with safranin staining. Since there is no dye available at this point, gram negative cells bind the safranin and appear pink under the microscope.[34] In this study, colonies grown on NA were used for gram's staining technique. Gram-positive bacteria were observed in 4 samples and gram-negative bacteria were observed from only 1 sample. Cocci and bacilli shaped bacteria were observed.

The cells containing spores appeared as green coloured spot containing cells while vegetative cells appeared as red colour in endospore staining technique.[35,45] In this research, the first liquid culturing Eppendorf tubes made using NB were used for endospore staining technique. The spore forming bacteria were observed from 4 samples and non-spore forming bacteria were observed only 1 sample. The motility test can determine whether the bacteria are motile or non-motile and the shape of the bacteria. The second sub-culture plates made using SCDA were used for motility test. Motile bacteria were observed from all 5 samples.

Biochemical tests were performed to identify specific bacterial species based on their metabolic activities.[36] In this research, the first liquid culturing microcentrifuge tubes made using NB were used for IMViC testing. The indole test was performed to figure out whether the bacteria could synthesize tryptophanase enzyme. The appearance of cherry red ring is considered as positive while negative is interpreted with no color change. All the samples were positive for indole test. Bacteria that have a high capacity for producing acidic byproducts during the metabolism of glucose are recognized using the methyl-red test. The appearance of red colour is considered positive while negative is interpreted with yellow colour. All the samples were positive for methyl-red test. The VP test is used to assess whether the bacterium can use the enzyme acetoin reductase to create acetylmethylcarbinol from glucose. The appearance of red colour is considered as positive while negative is interpreted with no colour change. All the samples were positive for VP test. The ability of the bacteria to use citrate as its only carbon source is assessed using the citrate utilization test. The appearance of blue colour is considered positive while negative is interpreted with no colour change. Blue colour was observed from 4 samples and no colour change was observed from only 1 sample.

The presence of sodium thiosulphate and ferrous sulphate fills the need for sulphur, and phenol red serves as an indicator of changes in the media's environment brought on by the production of acid or alkali in TSI test.[37] Gas production (H_2/H_2S) was observed from all samples. An acid/acid reaction was observed from 3 samples. Catalase-producing microbes were found using catalase test. By this enzyme, hydrogen peroxide is detoxicated by converting it to water and oxygen gas. The creation of the bubbles is the positive result of the catalase test.[38] Except for 1 sample, bubbles were observed from 4 samples.

The antimicrobials that will prevent the growth of the bacteria causing the specific condition are identified by antibiotic susceptibility testing.[39] In comparison to most other media, MH agar is a soft agar that provides for improved antibiotic diffusion. A zone of inhibition is a section of media where bacteria cannot grow because this area contains a drug that prevents the growth of the bacteria.[40] In sample 1, 2, 3 and 5, a zone of inhibition could be seen only in tetracycline and chloramphenicol. In sample 4, a zone of inhibition could be seen only in erythromycin, tetracycline and chloramphenicol. In sample 1, ampicillin, erythromycin and vancomycin were resistant and tetracycline and chloramphenicol were intermediate. In sample 2, ampicillin, erythromycin and vancomycin were resistant and tetracycline and chloramphenicol were sensitive. In sample 3 and 5, ampicillin, erythromycin and vancomycin were resistant, tetracycline was sensitive and chloramphenicol was intermediate. In sample 4, ampicillin and vancomycin were resistant, erythromycin and chloramphenicol were intermediate and tetracycline was sensitive.

The "Annbis" is a newly created graphical user interface (GUI) for bacterial identification that is powered by MATLAB®. This GUI can differentiate 270 bacterial Operational Taxonomic Units (OTUS) by comparing some optional biochemical information with probability matrices.[41] When an appropriate distance range is selected, the unknown bacterium and its allied OTUS in biochemical phenotype within the range can be displayed in three-dimensional space using the standard Multidimensional Scaling method. Statistical Neural Network structure is used to obtain the most probable result of bacterial identification.[21] The article of Osman et al., 2018 has reported that *Bacillus cereus* is highly resistant to tetracycline antibiotic.[42] According to the results of this work, *B. cereus* was resistant to tetracycline, vancomycin and ampicillin and sensitive to erythromycin and chloramphenicol. The article of Lyon et al., 2008 has reported that *Listeria monocytogenes* is intermediate to tetracycline antibiotic.[43] According to the results of this work, *L. monocytogenes* was sensitive to ampicillin, tetracycline, erythromycin, chloramphenicol and vancomycin. The article of Nthenge, Rotich and Nahashon, 2013 has reported that *Paenibacillus alvei* is resistant to ampicillin antibiotic.[44] According to the results of this work, *P. alvei* was sensitive to tetracycline and vancomycin and resistant to ampicillin, erythromycin and chloramphenicol. However, studies with increased biochemical characteristics should be fed into MATLAB® platform to confirm above predictions.

5. Conclusion

Public health professionals cannot emphasize enough how important food is in the transmission of infections. Based on the results, processed chicken meat remains a potential source in transmitting pathogenic foodborne microorganisms. It was predicted that *Bacillus cereus, Listeria monocytogenes* and *Paenibacillus alvei* bacteria are present in processed chicken meat. These isolates were resistant to ampicillin, erythromycin and vancomycin while increased sensitivity toward tetracycline and chloramphenicol was recorded. Due to the rising threat of multidrug-resistant *B. cereus, L. monocytogenes* and *P. alvei* in both humans and animals, there is a need for adequate food processing, particularly at an appropriate temperature, as well as surveillance of and good hygiene practices by meat handlers. This highlights the significance of ongoing monitoring and the requirement to implement policies in the primary sector to reduce consumer risk.

Acknowledgments

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Identification of *Lactobacillus* from commercial yogurt drink products and determination of their resistance to hydrogen peroxide

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Abstract

The genus *Lactobacillus* contains 315 species, and several of these have been identified as probiotics which are found in yogurt and yogurt-based drinks. This study was conducted to identify *Lactobacillus* genus from yogurt drink products and to determine its resistance to H_2O_2. Five yogurt drink samples were cultured in MRS agar and subjected to biochemical tests such as gram staining, endospore staining, acid-fast staining and catalase test. Thereafter, H_2O_2 resistance was determined by H_2O_2 resistance assay and it was statistically analysed using One-Way ANOVA from the SPSS Statistical Software. Creamy colour circular colonies in MRS agar plates, purple colour rod-shaped gram-positive bacteria in Gram staining, red colour non spore forming bacteria in endospore staining, non-acid-fast blue colour bacteria in acid-fast staining and absence of oxygen bubbles during catalase test confirmed the presence of *Lactobacillus* in all five samples. In H_2O_2 resistance assay, when comparing the H_2O_2 resistance of *Lactobacillus* at 0 and 6 hours, three samples indicated resistant to H_2O_2 following the 6 hours incubation period. One-Way ANOVA analysis indicated there was a statistically significant difference between 0 and 6 hours in H_2O_2 resistance. The biochemical tests confirmed the presence of *Lactobacillus* in all five samples. The H_2O_2 resistance assay confirmed the resistance of *Lactobacillus* to H_2O_2 in certain samples. Findings of this study emphasize the importance of screening *Lactobacillus* for H_2O_2 resistance to achieve its health benefits.

Keywords: *Lactobacillus,* probiotics, yogurt drink, H_2O_2 resistance

1. Introduction

The concept of probiotics evolved during the 20th century. It was based on a hypothesis presented by Elie-Metchnikoff, a Nobel Prize-winning scientist from Russia, claimed that consuming fermented yogurt products can lead to a long and healthy life. He believed that this activity could help decrease the harmful microbial population in the gut.[1] The term probiotic was first introduced by Stillwell and Lilly in 1965 to describe substances that are produced by a single microorganism and stimulate the growth of another.[2] They were then refined by Fuller in 1989 as microbial cultures that are beneficial to the host and can improve the balance of the intestinal microbial population.[3] In 2001, an expert committee of the World Health Organization and Food and Agriculture Organization of the United Nations adopted a definition of probiotic that stated they are live microorganisms that provide health benefit to the host when consumed in appropriate quantities.[4] A probiotic strain is expected to possess characteristics such as tolerance to gastric acid and bile, which is important during oral administration,[5] adherence to mucosal and epithelial surfaces (a crucial feature for effective immune modulation, pathogen competitive exclusion, and suppressing pathogen adhesion and colonization),

antimicrobial activity against pathogenic bacteria and to exhibit bile salt hydrolase activity.[6]

The microbes must be regarded as Generally Recognized as Safe (GRAS) status against pathogens in order to be used as probiotics.[7] The probiotic microorganisms that are most commonly used in human nutrition are *Lactobacillus* and *Bifidobacterium*.[8] However this research is focused on *Lactobacillus*, which falls under the GRAS criteria.[9]

The genus *Lactobacillus* is classified under phylum Firmicutes, class Bacilli, order II Lactobacillales, and family Lactobacillaceae.[10] They use carbohydrate in a fermentation process, with lactic-acid being the key product.[11] *Lactobacillus* are characterized as facultatively-anaerobic, catalase-negative, Gram-positive, non-spore-forming rods that often grow better under microaerophilic conditions.[12] *Lactobacilli* are chosen as probiotics as they exhibit a number of vital characteristics, including survival through the gastrointestinal-tract by being high tolerant to acid and bile-salts, ability to adhere to gastrointestinal surfaces to competitively exclude pathogens,[13] withstanding low acidic pH, inhibiting potentially pathogenic species (antimicrobial activity), resisting antibiotics, producing exopolysaccharides, normalizing cholesterol levels and withstand commercial manufacturing processes.[14-16]

Lactobacilli have been used in food-fermentation such as in yogurt, cheese, sour dough and pickles.[17] Yogurt drink is categorized as stirred yogurt with a low viscosity.[18] It supplies the body with liquid and nutrition in an easily absorbing form and it is high in protein, magnesium, potassium, vitamin B12 and calcium.[19-20] Kownacki *et al*[19] indicates yogurt drink kept the volunteers hydrated, reduces body temperature, sweat rate and hormonal stress. Kang, Kim and Kim, [21] observed the presence of *Lactobacillus* in yogurt drink. This research is focused in the identification of *Lactobacillus* from yogurt drink products and determination of their resistance to H_2O_2.

1.1 Importance of Lactobacilli resistance to H_2O_2. In the mammalian colon, a part of gastrointestinal-tract,[22] a single cell-thick continuous layer of epithelial cells forms the physical barrier between the body and the gut lumen, a compartment that is highly populated with environmental microbes and other foreign and potentially harmful substances. Thereby, colonic epithelial cells generate extracellular H_2O_2 in response to injury or gut microbes.[23] H_2O_2 production is also a capacity of several *Lactobacilli* such as *Lactobacillus acidophilus*, *Lactobacillus gasseri* and *Lactobacilli johnsonii*.[24] Unsuccessful colonization of *Lactobacillus* in gastrointestinal-tract and accumulation of H_2O_2 may occur as *Lactobacillus* lacks the H_2O_2 scavenging enzymes, such as catalase.[25] H_2O_2 is harmful as it can be easily converted into the highly reactive free hydroxyl radical by interacting with trace iron in the cell through Fenton reaction[26] which leads to protein, DNA, and lipid damage as well as cell death.[27] However, *Lactobacilli* has alternative mechanisms to prevent and resist H_2O_2 toxicity and reduce the occurrence of Fenton reaction to protect the cell against such failures.[26-28] Manganese containing pseudocatalase (Mn-Kat) has been discovered in *Lactobacillus plantarum*, which serves as the catalytic active site to catalyze the disproportionation of the toxic oxygen metabolite H_2O_2, into oxygen and water.[29-30] Heme dependent catalase (Heme-Kat) enzyme identified in *Lactobacillus sakei*, is able to incorporate iron atoms, thereby reducing the iron available to Fenton reaction thus preventing the formation of hydroxyl radical.[31-32] The Glutathione system in *Lactobacillus fermentum*, oxidizes Glutathione to a disulfide (by glutathione peroxidase) which then rapidly reduces back to glutathione by Gluathione reductase (GSH-r) in order to maintain a redox environment and to detoxify H_2O_2.[33]

Additionally, in *Lactobacillus plantarum* TrxB1 gene encodes for Thioredoxin Reductase (TrxR). The thioredoxin system is composed of NADPH, TrxR, and thioredoxin.[34] Thioredoxin system provides electrons to peroxidises to remove reactive oxygen species from H_2O_2.[35] Furthermore, Hydrogen peroxide resistance gene (hprA1) in *Lactobacillus casei* and *Lactobacillus paracasei* encodes hprA1 protein which binds to iron in the cell, thus preventing the formation of a hydroxyl radical (through the fenton reaction).[36] Moreover, NADH peroxidase gene (npr) encodes for NADH peroxidase which is a major H_2O_2 degrading enzyme in *Lactobacillus casei*. It reduces H_2O_2, to water and oxygen.[37] Thereby, using such alternative mechanisms *Lactobacillus* ensures its colonization and viability under oxidative stress environmental conditions such as in Gastro Intestinal Tract (GIT) and provides complete health benefits. The purpose of this study was to identify *Lactobacillus* in yogurt drink samples and analyze their H_2O_2 resistance and therefore its survival in the GIT can be identified. Further, the resistant *Lactobacillus* strains can be incorporated in yogurt drink manufacturing processes to achieve its health benefits.

2. Methodology

2.1 Sample preparation. Five different commercial yogurt drink samples were purchased from local market.

2.2 Culturing of samples. A sterile loopful of sample was cultured on De Man Rogosa and Sharpe (MRS) agar using quadrant streaking method and placed in the incubator at 37°C for 48 hours to visualize colonies.

2.3 Gram staining. Gram staining was carried out on heat fixed bacterial smear prepared from cultivated colonies from MRS agar. Few drops of crystal violet were added onto the slide and air dried for 60 seconds and the excess was washed off. Drops of Grams iodine was added and was air dried for 60 seconds. The excess was washed out. Thereafter, drops of decolourizer were added to wash the excess stain. Then it was rinsed with distilled water. Drops of Safranin were added and air dried for 60 seconds. The slide was then rinsed with distilled water, left to air dry and it was observed under the microscope at 100x with immersion oil.

2.4 Acid fast staining. A thin smear was prepared and heat fixed. The smeared slide was then flooded with carbolfuchsin and heated it using a spirit lamp until a visible steam was rising. The slide then rinsed with distilled water. The slide then was flooded with acid alcohol for 15 seconds, methylene blue for 60 seconds and rinsed with distilled water after each step. The slide was observed under microscope at 100x magnification.

2.5 Endospore staining. A thin smear was prepared and heat fixed. The smear was covered with a piece of filter paper and placed over a small beaker of boiling water. The slide was flooded with malachite green for 5 minutes. Slide then moved from the beaker, allowed to cool and the filter paper was removed. Drops of safranin were added to the slide and it was rinsed with distilled water after each step. The slide was then observed under microscope at 100x magnification.

2.6 Catalase test. Using a sterile inoculation loop a drop of autoclaved distilled water and same colony was picked, and placed onto labelled glass slide. Then, few drops of 3% H_2O_2 were added to slide, and observations were noted.

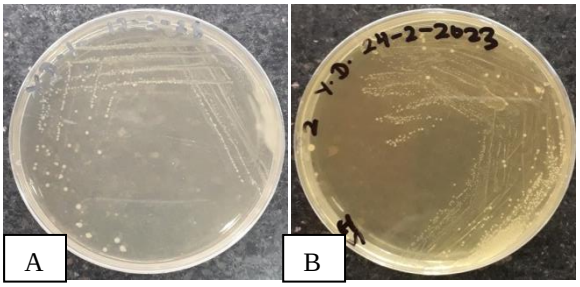
2.7 Sub-culturing. A loopful of isolated *Lactobacillus* colony from the MRS agar plates were sub-cultured in 30ml of MRS broth in a falcon tube. The sub-cultures were incubated at 37°C for 48 hours.

2.8 Hydrogen peroxide resistance assay Two centrifuge tubes were labelled for each sample as control and test. To these tubes, 3ml of MRS broth sample was added. Tubes were centrifuged for 10 minutes at 3000rpm. The supernatant was discarded and the pellet was then re-suspended in 6ml of saline solution. Cell density was checked at 600mn in UV spectrophotometer. The samples were diluted accordingly to obtain an absorbance of 0.2nm. It was centrifuged at 3000rpm for 1 minute. Saline was discarded and 10ml of fresh MRS broth was added. 36.28µL of 32mM H_2O_2 was added to the test tubes and 36.28µL of MRS broth was added to the control tubes. 3ml of the sample was added to a plastic cuvette and triplicates of absorbance reading at 600nm were recorded at 0 hours. The tubes were then placed in incubator for 6 hours and the UV spectrophotometer readings at 600nm were obtained again. [38]

2.9 Statistical/data analysis. 0 hours absorbance readings and 6 hours absorbance readings were statistically compared using one-way ANOVA in SPSS software. P value < 0.05 was considered statistically significant.

3. Results

3.1 Colony morphology. Yogurt drink samples streaked on MRS agar and incubated at 37˚C for 48 hours.



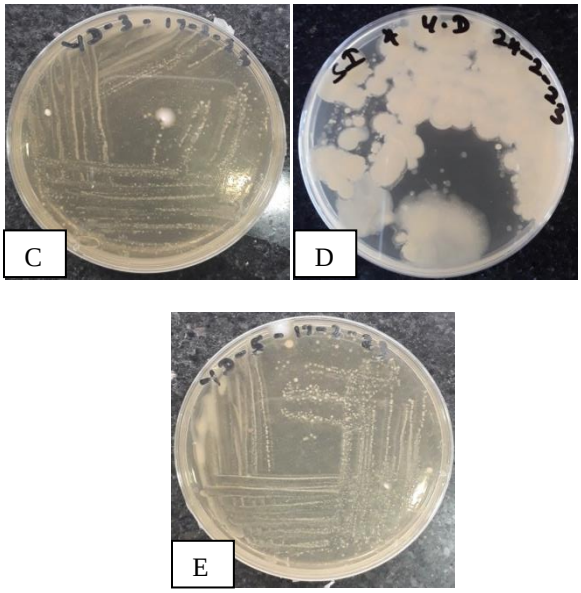
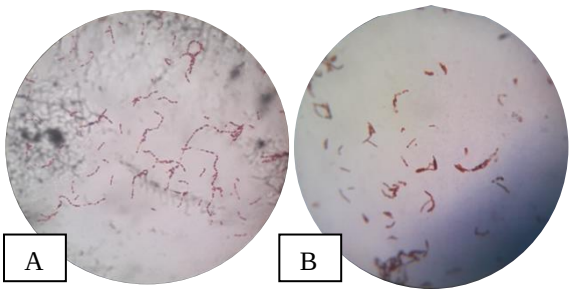


Figure 1. Bacterial growth of samples A - E on MRS agar after 48 hours incubation.

As shown in Figure 1 creamy colour, circular, entire, relatively small colonies were observed in MRS agar. Overgrowth was observed in sample D.

3.2 Gram staining. The colonies from MRS agar were subjected to Gram staining. As shown in the Figure 2 isolated bacteria were found to show morphology of rod shape and purple in colour.



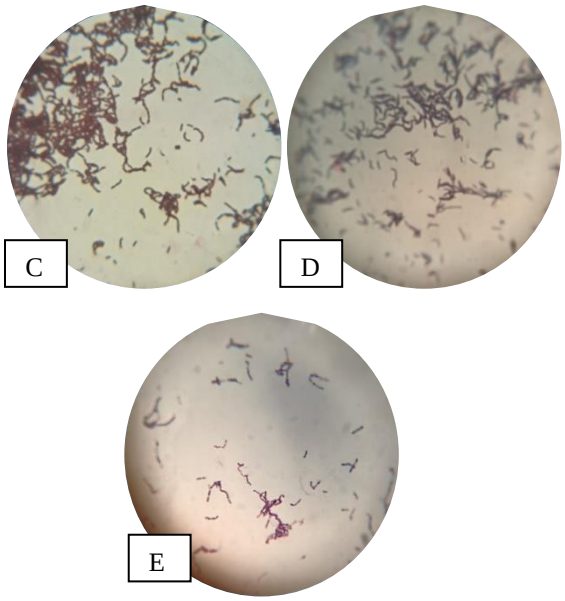
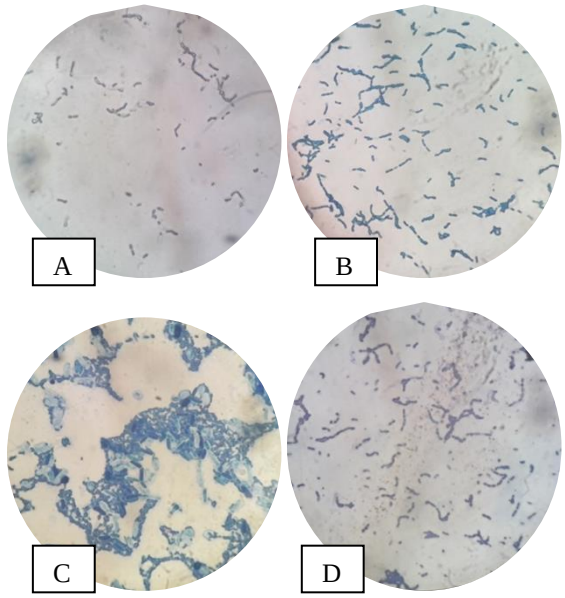


Figure 2. Bacterial identification by Gram staining images samples A - E under compound light microscope at 100 × magnification.

3.3 Acid fast staining. The colonies from MRS agar were subjected to Acid-fast staining.



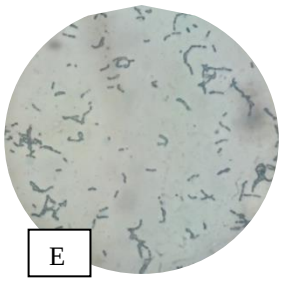


Figure 3. Bacterial identification by Acid fast staining images samples A – E under compound light microscope at 100× magnification.

The bacterial isolates appeared in blue colour rod shaped as shown in Figure 3.

3.4 Endospore staining. The colonies from MRS agar were subjected to Endospore staining.

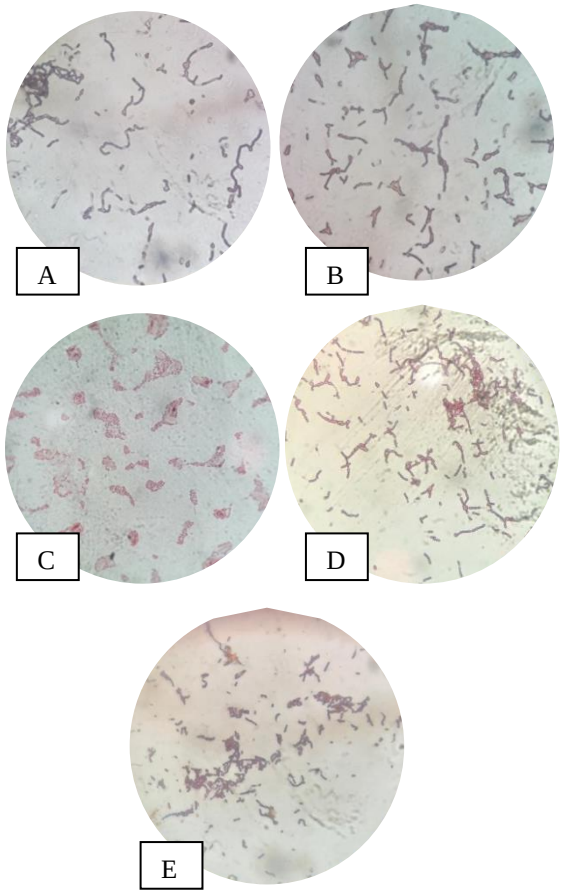


Figure 4. Bacterial identification by endospore staining images samples A – E under compound light microscope at 100× magnification.

The isolated bacteria were non spore forming, thereby appeared in red colour rod shaped as shown in Figure 4.

3.5 Catalase test. The colonies from MRS agar were subjected to catalase test detect presence or the absence of catalase enzyme.

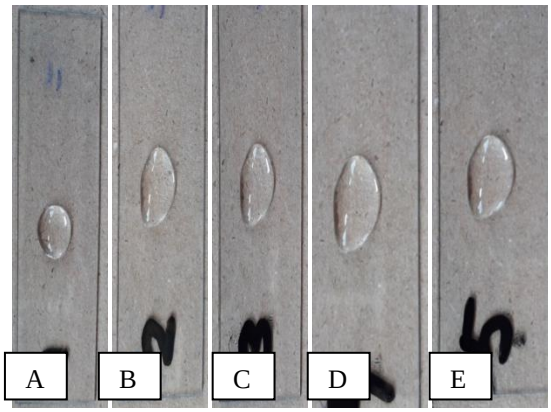


Figure 5. Catalase test results of samples A – E

As shown in Figure 5, by the absence of bubbles and effervescence, negative catalase test was observed in all the five samples.

3.6 Sub culturing. Colonies from MRS agar were sub cultured for further use.

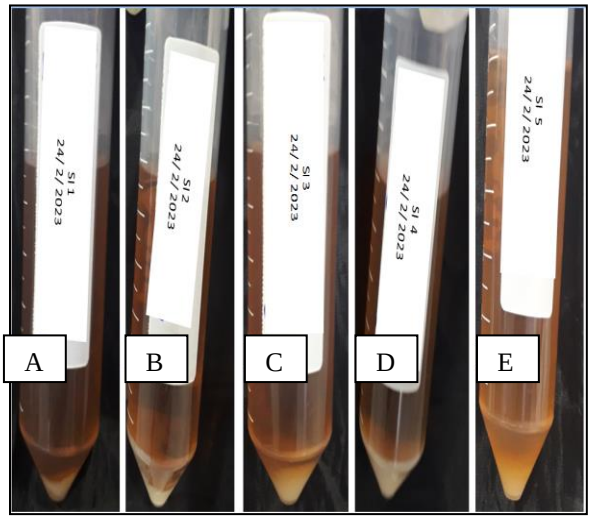


Figure 6. Bacterial sub-cultures of samples A – E on MRS broth after 48 hours incubation at 37˚C

As shown in Figure 6 high turbidity was observed in the broth and creamy colour colonies were observed in the bottom of the broth.

3.7 H_2O_2 resistance assay. The absorbance reading for H_2O_2 resistance assay is mentioned below in Table 1.

Table 1. H_2O_2 resistance of *Lactobacillus* at 0 hours and 6 hours

Sample	Absorbance readings for 0 hours (OD)	Absorbance readings for 6 hours (OD)
1	0.046 ± 0.0015	0.034 ± 0.0020
2	0.148 ± 0.0005	0.106 ± 0.0005
3	0.071 ± 0.0028	0.092 ± 0.0055
4	0.077 ± 0.0015	0.106 ± 0.0147
5	0.055 ± 0.0005	1.885 ± 0.0411

According to Table 1 sample 1 and 2 shows a decline in absorbance following 6 hours incubation period. Sample 3, 4, and 5 shows an increase in absorbance following the 6 hours incubation period.

3.8 Statistical/data analysis. Statistical significance was determined by one-way ANOVA analysis comparing 0 hours and 6 hours absorbance.

Table 2. Comparison of H_2O_2 resistance of *Lactobacillus* at 0 hours and 6 hours

ANOVA					
Hydrogen peroxide resistance					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	3.784	4	.946	4.700	.006
Within Groups	5.032	25	.201		
Total	8.816	29			

As shown in Table 2 when comparing the H_2O_2 resistance of *Lactobacillus* at 0 hours and 6 hours, it has a p – value of 0.006 which is less than 0.05. It indicates that there is a statistically

significant difference between 0 hours and 6 hours of H_2O_2 resistance.

4. Discussion

The genus *Lactobacillus* contains 315 species, and several of these bacteria have been identified as probiotics. These can be found in yogurt-based products.[39,40] The present study was focused to identify *Lactobacillus* from yogurt drink samples and to determine its resistance to H_2O_2, which aids its survival in gastrointestinal tract. As shown in Figure 1 isolates were subjected to grow on selective MRS agar media and produced round shape, off-white to cream color entire margin colonies those similar to the *Lactobacillus* grown on MRS agar media as previously reported by Chowdury *et al.*[41] Further biochemical tests such as gram staining, endospore staining, acid fast staining and catalase test were performed to confirm the presence of *Lactobacilli* as in Kumar and Kumar.[42]

As shown in Figure 2 upon Gram staining, isolates were found to be rod shaped, purple colour positive in Gram reaction which exhibited similar characteristics of *Lactobacillus* in Kumar and Kumar.[42] The principle of gram staining depends on the presence of thick peptidoglycan cell wall of *Lactobacillus* and its ability to bind to basic dyes (violet crystals) even after washing with decolourizer and counter stain safranin.[43] *Lactobacilli* are nonspore-forming.[44] Under the microscope, endospore appear light green in colour, and the vegetative cells in pink.[45]

In endospore staining in Figure 4 isolates were mainly pink stained rod shaped, the absence of green colour endospores confirms the presence of non-spore forming *Lactobacillus* in the sample.

Acid fast organisms with a high concentration of mycolic acids in their cell walls can be distinguished using acid fast stains. Non-acid fast bacteria will stain blue/green with the counterstain, while acid fast bacteria will be red. This staining was important since the acid fast bacterial cell envelope is a specialised variation of the Gram-positive cell envelope.[46,47] Isolates in Figure 3 appeared to be red coloured and rod shaped which shared similar non acid-fast staining feature of *Lactobacillus* in Khalil and Anwar, 2016.[48]

As shown in Figure 5 catalase negative test reaction was characterized by the absence of oxygen bubbles formation, that indicate the *Lactobacillus* bacteria do not produce the catalase enzyme which converts H_2O_2 to water and oxygen.[49]

As in Figure 6 pure colony of *Lactobacillus* isolate was emulsified into MRS broth to sub culture and following incubation, turbidity with creamy pellet of *Lactobacillus* were observed similar to Agbankpe *et al.*[50] Thereby, through the morphological colony characteristics and biochemical tests the samples were confirmed to contain *Lactobacillus*.

The H_2O_2 resistance of *Lactobacillus* was observed using H_2O_2 resistance assay.[38] Population density is estimated from the turbidity of the culture and is typically expressed as OD (optical density), typically at a wavelength of 600 nm.[51] As shown in Table 1 the addition of H_2O_2, following 6 hours incubation has decreased the mean OD values in sample 1 and 2, which suggest that bacteria were not resistant to H_2O_2 and thereby decreased in population density. Sample 3, 4 and 5 had shown an increase in OD value which suggest that bacteria in those samples were not affected by the addition of H_2O_2 and continued growth in MRS broth. Despite the absence of H_2O_2 scavenging enzymes, such as catalase in *Lactobacillus*,[52] certain samples were found to resist H_2O_2. Similar results were obtained by Serata, Kiwaki and Lino 2016,[53] and the study showed that *Lactobacillus casei* strain was found to be resistant to H_2O_2 due to its

advantageous hpra1 gene, suggesting the samples 3,4 and 5 possibly carrying the *Lactobacillus casei* species. Resistance property of *Lactobacillus brevi* was examined by Fang *et al.,* 2018,[54] where it was observed that *Lactobacillus casei* and *Lactobacillus paracasei* showed less resistant than *Lactobacillus brevi* in the survival resistance assay.[55] Using plating technique, incubating *Lactobacillus* treated with different concentration of H_2O_2 has indicated *Lactobacillus acidophilus* and *Lactobacillus reuter* were more resistance to higher concentration (as $30gl^{-1}$) of H_2O_2 due to the presence of NADH peroxidise. *Lactobacillus salivarius* and *Lactobacillus casei* were found to be the least resistant with no growth at a low concentration (as $20gl^{-1}$) of H_2O_2.[56]

As different strains exhibit different level of resistance towards H_2O_2, this explains the difference in results obtained for samples A – E, which indicates the presence of different strains of *Lactobacillus* in all five yogurt drink samples. Hydrogen peroxide is a weak oxidant, but it may give rise to hydroxyl radical that causes oxidative damage to the cells.[27] Other anti-oxidant enzymes such as Heme-dependent catalase,[32] glutathione peroxidase,[33] thioredoxin reductase,[35] manganese containing pseudocatalase,[30] and NADH peroxidase[37] are responsible for the varied degrees of hydrogen peroxide resistance displayed by various samples containing *Lactobacillus*. According to the Table 2 the statistical analysis was performed using one way ANOVA test. P value of 0.006 was obtained which was less than 0.05 and considered there is statistically significant difference between 0 hours and 6 hours of H_2O_2 resistance. The study was aimed to isolate *Lactobacillus* from five yogurt drink samples and checked for H_2O_2 resistance for its successful survival in gastrointestinal tract.

However, further confirmatory tests can be carried out using wider sampling to analyse the diversity of *Lactobacillus* strains present in the samples and their H_2O_2 resistance can be determined. Thereby the successful resistant strains with the highest survival rate can be identified and incorporated into yogurt drink manufacturing to maximize the health benefits.

5. Conclusion

The purpose of this study was to identify *Lactobacillus* from commercial yogurt drink products and to determine their H_2O_2 resistance. All the five samples were examined for morphological and biochemical characterization. The study demonstrated that all five samples contained rod shaped Gram positive, non-acid fast, non-spore forming and catalase negative isolates which confirmed to be *Lactobacillus.* Furthermore, during H_2O_2 resistance assay it was observed that three of the samples contained H_2O_2 resistant *Lactobacillus.* The study emphasizes that the importance of screening *lactobacillus* for H_2O_2 resistance to achieve its health benefits.

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Molecular Docking Analysis of Plasmepsin-2 Malaria Protein and Identification of Potential Ligands

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Abstract

Malaria is one of the leading causes of malignancy and death especially in tropical and subtropical countries, which is caused by parasites such as *Plasmodium* species through mosquito vectors. Despite the fact that several anti-malarial drugs have been shown to be effective, resistance towards these drugs poses a great threat to the community including developing countries like Sri Lanka. Therefore, the research aims to perform protein-ligand docking and determine the best phytochemicals with their ligand binding sites. The findings of the study revealed that Conessine is a potential drug candidate towards 1LF4 with the lowest binding energy of -9.96 kcal/mol and with a lower inhibition constant. Further, GLY-36, SER-37, TYR-77, VAL-78, SER-79, PHE-111, TYR-192 and GLY-216 were found to be the most prominent ligand binding sites towards Plasmepsin-2.

Keywords: Molecular docking, AutoDock, Binding energy, Ligand binding sites

1. Introduction

Malaria remains a major global health concern and the leading cause of death, especially in many tropical and subtropical countries including Sri Lanka. Over the past years, several renewed efforts have been made to combat the disease and several success stories have been documented in reducing the prevalence of malaria.[1] Despite these valuable efforts, the severity, and the ongoing resistance to a number of anti-malarial drugs have been a major topic of discussion in recent years. These also include several imported malaria cases in Sri Lanka with the recent transmission of locally introduced malaria cases from 2018-2019 although the country has received malaria-free certification in 2016 from the World Health Organization (WHO).[2] The Malaria World Report in 2016 reported that nearly 429,000 deaths have occurred globally since 2000. In which it is estimated that 92% of the deaths have occurred in the WHO African Region, 6% in the WHO South-East Asian Region and 2% in the WHO Eastern Mediterranean Region. Further, it was also noted that almost 99% of the deaths have resulted from the spread of *Plasmodium falciparum* malaria.[3,4] In this regard, the etiologic agents of malaria are Plasmodium species, in which, the most malignant and common forms of the cases are caused by *Plasmodium falciparum* and *Plasmodium vivax.* These parasitic agents pose threats to human lives due to their complex life cycle both inside the human host and the mosquito vector such as the Anopheles species of mosquito. The disease is caused during the intra-erythrocytic phase in which the haemoglobin of the human host is consumed completely which allows the generation of amino acids that stimulate the growth and maturation of parasites.[5]

A series of enzymes called proteases are involved in the haemoglobin degradation inside the acidic digestive vacuole of the parasite.

Meanwhile, proteases such as Plasmepsin, which belongs to a group of aspartic proteases, initiate the degradation of haemoglobin. The reaction is followed by proteolysis which breaks down the large fragments of compounds into small peptides using cysteine protease. Therefore, it highlights that the initial stage of haemoglobin degradation is an ultimate process exported to terminal degradation of the cytoplasmic exopeptides.[6] Thus, it is paramount to understand these enzymes' binding cavities, posing suitable chemotherapeutic inhibitors to combat the disease.

In addition, it is worth noting the resistance of anti-malarial drugs to treat and control malaria despite the presence of effective drugs. As a result, the emergence of resistant anti-malarial drugs has threatened the continuity of efficacy of anti-malarial drugs and raised the importance of developing novel drug targets using modern techniques.[7] Therefore, this research is intended to perform protein-ligand docking studies to find potential phytochemicals and ligand binding sites as a method to combat malarial infection and the adverse effects resulting from resistant malarial drugs.

2. Methodology

2.1 Preparation of protein receptors. The three-dimensional coordinates of the crystal structure of the Malarial protein, Plasmepsin-2 (PDB ID: 1LF4, 1.90A˚, X-Ray diffraction), a hydrolase enzyme was retrieved from RCSB Protein Data Bank (PDB) using .pdb format (https://www.rcsb.org/). The .pdb file was loaded into AutoDock (version 2.4.6) (https://autodock.scripps.edu/) to remove water molecules, hetero atoms, and ligands. Further, the exhaustiveness value was set to default with 8 Angstrom and shows the comprehensiveness of the software in finding the best docking complexes. Thereafter, AutoDock Tools (ADT) was set with AD4 type in which polar hydrogen was added to fix bond order and Kollman charges to include partial charges. The formatted proteins were saved in .pdbqt format.[8]

2.2 Preparation of ligands. Ten phytochemicals with anti-malarial properties were obtained from the previous literature as ligands. The three-dimensional structures of the phytochemicals were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) in .sdf format and converted to .pdb format using Open Babel software (http://openbabel.org). The ligands were optimized with the inclusion of aromatic carbons, rotatable bonds and setting the TORSDOF values.[9] Later, the prepared ligands were saved in .pdbqt format.

2.3 Molecular docking and visualization of docked complexes. The grid boxes were generated to cover the entire active sites of the protein.[10] The grid points X, Y and Z were set as 100, 76 and 112 respectively. The grid spacing was set to 0.531 and the grid output file was saved in .gpf format. Docking parameters were set as follows: number of genetic algorithms runs as 10 with Lamarckian algorithm, population size as 150, maximum number of energy evaluations as 2,500,000, maximum number of generations as 27,000, rate of gene mutation as 0.02 and rate of cross-over as 0.8.[8] Once the docking was completed, the docked files were saved in .dlg format and later converted into .pdbqt format. The best-docked complexes were analysed using the binding energies from the RMSD tables. Visualization of the best complexes was viewed from BIOVIA Discovery Studio (https://discover.3ds.com/discovery-studio-visualizer-download) where the ligand binding sites were determined.

3. Results and Discussion

The findings of the study revealed the interactions between ligands with anti-malarial properties and the malarial protein, Plasmepsin-2. In the present investigation, one of the ultimate requirements is to produce docking complexes with proper orientation and the need for ligands' conformation to best fit the enzyme binding sites.

In this regard, the study used optimal docking criteria to interpret the best-docked conformation such that it ensured the ligands had the lowest binding energy with Plasmepsin-2 as shown in Table 1.

Of the phytochemicals which were docked with 1LF4, Conessine had the lowest binding energy with -9.86 kcal/mol showing the high potency towards 1LF4 as a potential drug candidate. The docking pose of the best conformation of Conessine-1LF4 is shown in Figure 1 along with the identification of ligand binding sites. In addition, Conessine's lower inhibition constant (Ki) with Ki=0.058µM further describes it as a potential drug target to treat malarial diseases. For instance, according to the studies of Pratama *et al*., low inhibition constant highlights the better binding affinity of the ligand with its target site.[11] Thus, with the dry lab investigation performed in this study, it is proven that Conessine can be considered a potential drug target for malarial diseases and can be considered a new therapeutic target for resistant malarial drugs in developing countries like Sri Lanka.

Furthermore, the residues involved in ligand binding of Conessine to 1LF4 are MET-15, ILE-32, ASP-34, GLY-36, SER-37, TYR-77, VAL-78, SER-79, PHE-111, THR-114, SER-118, PHE-120, ILE-123, TYR-192, ILE-212, ASP-214, GLY-216, THR-217 and ILE-300 as shown in Figure 1. The findings of the current study are in agreement with the result obtained from the previous study of Fernando *et al*. on site-specific ligand binding sites conducted on several other phytochemicals to treat malaria.[12] It showed that higher tendency of Serpentine towards Plasmepsin-2 with a binding energy of -8.16 kcal/mol and an inhibition constant of 1.04 nm. Further, it showed GLY-36, SER-37, MET-75, TYR-77, VAL-78, SER-79, PHE-111, TYR-192 and GLY-216 as potential ligand binding sites. However, except MET-75, the remaining residues were found as potential ligand binding sites in this study as well. Hence, it can be considered that GLY-36, SER-37, TYR-77, VAL-78, SER-79, PHE-111, TYR-192 and GLY-216 act as the most prominent ligand binding sites for Plasmepsin-2.

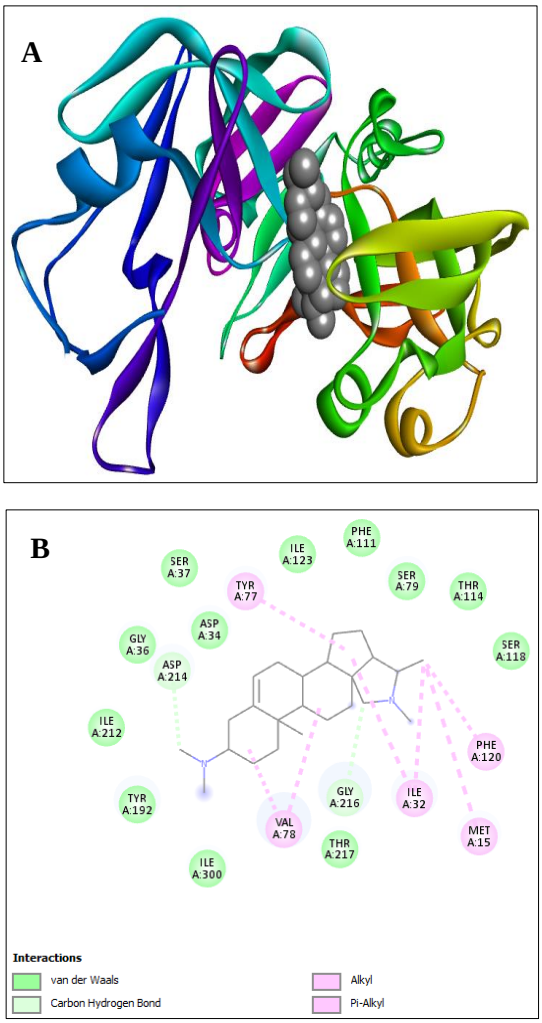
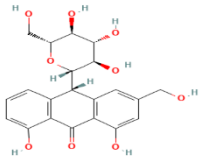
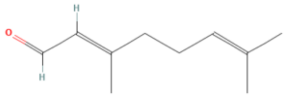
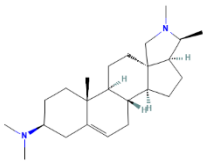
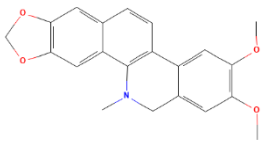
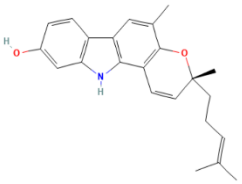
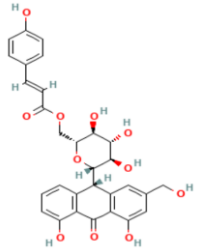
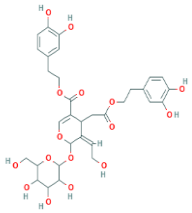
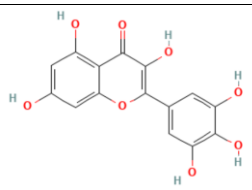
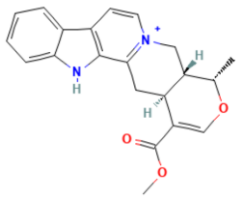
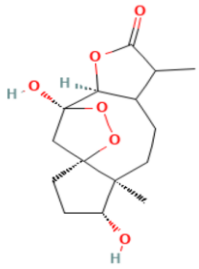


Figure 1. (A) Visualization and (B) Identification of ligand binding sites of the best-docked complex of Conessine-1LF4 using BIOVIA Discovery.

Table 1. The docking results of the phytochemicals with Plasmepsin-2.

Phytochemicals	PubChem ID	Molecular formula	Chemical structure	Binding energy (kcal/mol)	Ki (μM)
Aloin	12305761	$C_{21}H_{22}O_9$		-5.65	71.97
Citral	638011	$C_{10}H_{16}O$		-4.61	15.6
Conessine	441082	$C_{24}H_{40}N_2$		-9.86	0.058
Dihydronitidine	99641	$C_{21}H_{19}NO_4$		-6.70	12.33
Mahanine	36689305	$C_{23}H_{25}NO_2$		-7.81	1.88
Microdontin	101629131	$C_{30}H_{28}O_{11}$		-7.51	3.13
Multifloroside	14781469	$C_{32}H_{38}O_{16}$		-1.41	$91.93x10^3$

Myricetin	5281672	$C_{15}H_{10}O_8$		-5.98	41.4
Serpentine	73391	$C_{21}H_{21}N_2O_3$		-7.4	3.76
Tehranolide	6711941	$C_{15}H_{22}O_6$		-8.02	1.32

Taken together, the majority of the ligands showed encouraging selectivity against Plasmepsin-2. Further, it highlights new drug targets to treat malaria, especially in resistant malarial drugs. Although these phytochemicals showed desirable selectivity towards Plasmepsin-2, advanced validation and chemical tests and modifications need to be addressed. For instance, the current study involved rigid protein-ligand docking. However, the structure of proteins is flexible to suit the environmental conditions in real situations.[13]

4. Conclusion

In conclusion, Plasmepsin-2 is identified as the potential drug target in *Plasmodium falciparum* in treating malarial infections. Docking studies performed in this study predicted that Conessine as the potential ligand for Plasmepsin-2 with the lowest docked energy in which the interactions are stabilized by several binding residues of the protein. Therefore, this study has provided new insights into malarial interventions to the development of potent chemotherapeutic drugs to fight against the disease.

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Amplification of barcoding genes from Sri Lankan Turmeric plants.

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Abstract

Sri Lanka has many varieties of turmeric plants. They are morphologically identified with the observable differences, but they were not tested fully for any molecular differences. The present research was to check any size differences between *rbcL* and *ycf1b* genes in two varieties of Sri Lankan turmeric plants that could be detected by gel electrophoresis. Genomic DNA was extracted using CTAB method and the samples (*Curcuma domestica* MT 23, *Curcuma domestica* MT 32) were PCR amplified. The PCR program for the *rbcL* gene were as follows, initial denaturation at 95°C for 5 minutes, denaturation at 94 °C for 1 minute, primer annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute and finally, final extension at 72 °C for 10 minutes. The PCR program for *ycf1b* gene were as follows, initial denaturation at 95 °C for 5 minutes, denaturation at 95 °C for 30 seconds, primer annealing at 49°C for 30 seconds, extension at 72 °C for 30 seconds and finally, final extension at 72 °C for 7 minutes. Annealing temperatures were used accordingly for the primers. The agarose gel electrophoresis with TAE buffer showed bands proving the presence of genomic DNA and also the PCR was successful. Gel electrophoresis which was done using TBE gel revealed amplicon length polymorphism between the two samples used. At the used experimental conditions, it proved that both the varieties have the same molecular size of *rbcL* gene, but different molecular size of *ycf1b* gene. This simple and cost-effective method could be used as an alternative or a prior step to

sequencing of PCR products. As well as in supportive of sequencing results.

Keywords: Sri Lankan Turmeric, *Curcuma domestica*, *rbcL* gene, *ycf1b* gene

1. Introduction

Sri Lanka is an island nation which exhibits remarkable biological diversity and considered to be the richest country in the Asian region in terms of species concentration. For many types of flora, the country provides favorable conditions such as ecological, climatic, soil and topographical variability.

Home gardens are considered as the heart of the agricultural biodiversity. They are widespread throughout the country, existing in dry zones to wet zones.[1] On the rainfall distribution, Sri Lanka has been traditionally classified into three agricultural zones. They are the wet zone, intermediate zone and dry zone.

The following figure (Figure 1) shows the agricultural zones of Sri Lanka and the topography of Sri Lanka.

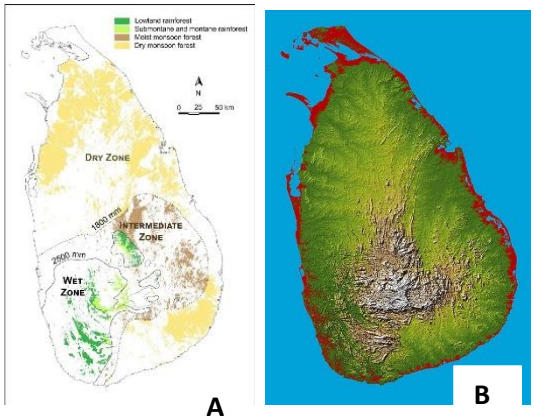

Figure 1. A) Agricultural zones in Sri Lanka[2] B) Topography of Lanka.[5]

The scientific name for turmeric is *Curcuma domestica*. Turmeric is a plant in the ginger family, which is also known as Zingiberene. Turmeric is native to Southeast Asia, and is grown commercially in that region. Its rhizome is used as a culinary spice and as a traditional medicine.[3]

Turmeric is also a common spice and a major ingredient in curry powder. Curcumin is the major component of turmeric. The activities of turmeric are commonly attributed to curcuminoids, which means curcumin and its similar substances. Curcumin also gives the yellow color to turmeric. It is the main active ingredient in turmeric. Curcumin has powerful anti-inflammatory effects, and it is a very strong antioxidant. Turmeric has a lot of health benefits. Mainly it contains bioactive compounds with medicinal properties.[3,4]

As mentioned, before there are three main agricultural zones in Sri Lanka, and turmeric is only grown in wet and intermediate zones. It is grown as a single crop and as an intercrop under coconut trees. major growing districts for turmeric are Kurunegala, Gampaha, Kalutara, Kandy, Matale and Ampara.[5]

In Sri Lanka, there are thirty-two morphological varieties described under turmeric as of 2023. *Curcuma domestica* MT 32 and *Curcuma domestica* MT 23 were researched in this project. the M stands for multiplication and T stands for turmeric in this research. (Through Personal Communication with Intercropping and Betel Research Station, Narammala, Sri Lanka.).

DNA barcoding is a system used for species identification focused on the use of short, standardized genetic region.[6] For the estimation of biodiversity, conservation of species and for ecological analysis, precise characterization of organisms is an essential fact. Typically, this has been performed by considering the visible morphological characters, but in recent years this is not universally acceptable because of cryptic morphological similarities between the species. With the advent of new technologies, “DNA barcoding” which is based on PCR was introduced to the world. This technology has emerged as an excellent tool in the unambiguous identification and improvement of their livestock and their byproducts used in several commercial entities such as food, medicine etc.[4] Figure 3 shows some examples of DNA barcoding species.

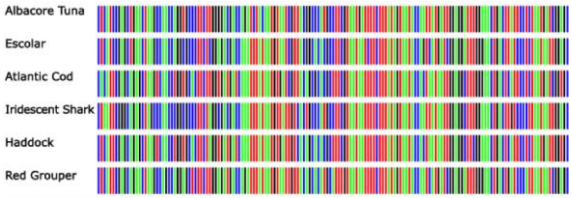

Figure 2. Examples for DNA barcoding species.[7]

In DNA barcoding most botanists use chloroplast coding regions such as *rbcL* and *mat K* with the *trnH- psbA* intergenic regions. There are several barcoding genes in turmeric. They are *mat K*, *rbcL*, *trnH-psbA*, *tmL-F* and ycf.[8]

Initiatives are taking place around the world to generate DNA barcodes for all groups of living organisms and to make these data publicly available in order to help understand, conserve, and utilize the wo’ld's biodiversity. For land plants, the core DNA barcode markers are two sections of coding regions within the chloroplast[9]. Other than these two chloroplast genes *ycf1b* is also identified as a chloroplast gene and it is the most variable plastid genome region, and this gene can serve as a core barcode of land plants.[10]

There are several accessions of turmeric with yield differences and differences in chemical composition which cultivated in Sri Lanka (Through personal communication with Intercropping and Betel Research, Narammala, Sri Lanka However, they were not proven for their differences at the molecular level. Therefore, it is important that they should be checked for any variations present among them. This is important because if the variations are being identified, it can be used for new medicinal inventions and also in food industry.

ycf1b and *rbcL* barcode genes of Sri Lankan turmeric plants were PCR amplified and checked for successful amplification, the

possible differences between amplified products were evaluated using gel electrophoresis.

2. Methodology

2.1 Sample Collection. The plant samples for this study were collected from the Intercropping and Betel research station. The information of the 2 different plant types selected are mentioned in table 1 and their morphological appearance is given in figure 3.

Table 1. Plant species of the project.

Plant species	Scientific name	Variety	Plant part used for DNA extraction
Sri Lankan turmeric plants	*Curcuma domestica*	MT 23 and MT 32	Middle part of the rhizome

Figure 3. A) *Curcuma domestica* MT 23, B) *Curcuma domestica* MT 32

2.2 Preparation of equipment. The motor and pestles used for DNA extraction was surface disinfested with 70% of ethanol and wiped with tissues, then wrapped with demy papers and were surface sterilized in the hot air oven for 2 hours at 180°C. Pipette tips, falcon tubes, Microcentrifuge tubes and distilled water were sterilized by autoclaving at 120°C under 15psi for 20 minutes. The samples were washed, and the skin was peeled off before they were used for measuring.

2.3 Plant genomic DNA extraction using CTAB method. A mass of 200 mg of both the samples were weighted into 2 different plastic holders. CTAB was preheated in the shaking incubator for 30 minutes. After adding 1ml of CTAB solution to the sterile motor, started to grind the samples with the pestle. A total of 4 ml of CTAB solution was added to the samples in order to achieve the required level of homogenization. Then the samples were incubated at 65 °C for 30 minutes in the shaking incubator. The samples were taken out and kept until it cooled down to the room temperature. After the samples were cooled down, 330 µl of 5 M potassium acetate was added and mixed it by inverting. Samples were incubated in an ice box for 45 minutes. 600 µl of chloroform-isoamyl alcohol was added. The samples were centrifuged at 12,000 g (RCF) at 4 °C for 15 minutes. Afterwards the supernatant was transferred into a separate 1.5 ml of Microcentrifuge tube. 600 µl of 30% PEG was added to the supernatants and mixed by inverting. The samples were incubated in the freezer overnight. The samples were again centrifuged at 12,000 g (RCF) at 4 °C for 15 minutes. The supernatant was removed while leaving the white DNA pellet. 500 µl of 70% ethanol was added to the samples while disturbing the pellet. The tubes were mixed by using the vortex. Again, the samples were centrifuged at 12,000 g (RCF) at 4 °C for 15 minutes. The supernatant was removed and left the white DNA pallet in the tube. Then pipetted out any residual ethanol and let the pallet air-dry. Finally, 20 µl of PCR water was added and let the samples dissolved and stored the samples at -20 °C. Afterwards spot gel method was used to check the concentration of the samples.

2.4 DNA quantification by spectrophotometer. Before measuring the absorbance values of the samples, the original DNA samples were diluted. The samples were made up to 125µl of a volume (100x diluted). The machine was calibrated, and the diluted samples were filled one by one. Then the computer was set to the optimum conditions and the samples were run. When samples were changed from the cuvettes, they were emptied and washed with distilled water.

2.5 Amplification of genomic DNA using PCR. The PCR components were taken out from the

-20°C refrigerator and was thawed on ice and given a quick spin prior to preparing the master mix. The master mix was prepared and the PCR reaction mixtures were made inside the laminar hood. Table 2 show the components and volumes of the master mix for *rbcL* primer and *ycf1b* primer.

Table 2. Components of the master mix for *rbcL* primers and *ycf1b* primers.

Components	Volume	X2	X3
PCR water	2.8 µl	5.6 µl	8.4 µl
2x buffer	5 µl	10.0 µl	15 µl
rbcL forward primer	0.3 µl	0.6 µl	0.9 µl
rbcL reverse primer	0.3 µl	0.6 µl	0.9 µl
Taq polymerase	0.6 µl	1.2 µl	1.8 µl
DNA template	1 µl	2 µl	3 µl
Total volume	10.0 µl	20.0 µl	30.0µl

A volume of 9µl of master mix was added into each tube and 1µl of DNA template from each sample was added into 2 different PCR tubes. The remaining PCR tube, which was the negative control tube, 1µl of PCR water was added instead of DNA templates.

The labeled PCR tubes were added to the PCR machine and the programs were adjusted accordingly. Following tables (Table 3 and Table 4) show the programs of the two primers.

Table 3. Stages, temperatures, and time for the PCR program for *rbcL* primer.

Stages	Temperatures	Time
Initial denaturation	95°C	5minutes
Denaturation	94°C	1minutes
Annealing	55°C	30seconds
Extension	72°C	1minutes
Final extension	72°C	10minutes

Table 4. Stages, temperatures, and time for the PCR program for *ycf1b* primer.

Stages	Temperatures	Time
Initial denaturation	95°C	5minutes
Denaturation	95°C	30seconds
Annealing	49°C	30seconds
Extension	72°C	30seconds
Final extension	72°C	7minutes

2.6 Agarose gel electrophoresis using TAE buffer. The 1% agarose in TAE gel was prepared. The PCR products were mixed with the loading dye before loading them into the gel. From each sample 3 µl was mixed with the loading dye. After mixing it well, the samples were loaded into the gel wells. The loading order was the DNA ladder, *Curcuma domestica* MT 23, *Curcuma domestica* MT 32 and finally the negative control. After loading the samples, the gel was run under the voltage of 100V for 15 minutes and 65V for 45 minutes. The PCR products were visualized following gel electrophoresis. Then the results were observed by the computer system using image lab software.

2.7 Preparation of TBE buffer. TBE buffer contains Tris, Boric acid and 0.5M Na_2EDTA. The buffer was prepared from 10x diluted buffer for an amount of 200 ml solution. The needed volumes for the solution are shown in the following table (Table 5).

Table 5. Volumes needed to prepare the TBE buffer.

Components	Needed volumes
Tris	21.6 g
Boric acid	11 g
0.5M Na_2EDTA	8 ml

2.8 Gel electrophoresis using TBE buffer. To prepare 2% of agarose gel, 3 g of agarose powder was needed. A separate conical flask was taken and 3 g of agarose powder was added into it. 150 ml of 0.5x TBE buffer was added to

the same conical flask. Then swirled gently for the powder and the buffer to mix. The solution was then heated in the microwave until the powder was dissolved in the buffer completely and no bubbles were formed. Then the solution was kept to cool down and added 3 µl of ethidium bromide. Swirled the solution gently without forming any bubbles. Then the solution was poured into the gel tray which was prepared before and kept for it to set. The samples were loaded as the same way when the samples were loaded using TAE buffer. The gel was run under a voltage of 120V.

3. Results

3.1 Plant genomic DNA extraction.

The spot gel image of the extracted DNA is indicated in figure 6. In the gel image it showed as bands and the image are shown in figure 7.

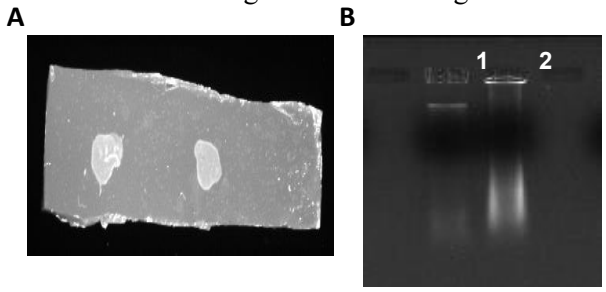

Figure 4. A) Image of the spot gel. [S1(sample 1: *Curcuma domestica* MT 23), S2 (sample 2: *Curcuma domestica* MT 32). B) Image of the gel. Lane 1: *Curcuma domestica* MT 23), lane 2: *Curcuma domestica* MT 32)].

3.2 Quantification of PCR products using Spectrophotometer. Samples were diluted with 1/100 dilution factor; the total volume was made up to 125µl.

Table 6. Quantification results of extracted genomic DNA.

Sample	A[260]	A[280]	Ratio	Nucleic Acid/diluted sample (µg/ml)	Nucleic Acid/concentrated sample (µg/ml)
Curcuma domestica MT23	0.0019	0.0006	0.9175	0.0293	2.93
Curcuma domestica MT 32	0.0002	0.0013	1.0591	0.1050	10.5

3.3 Visualization of PCR products.

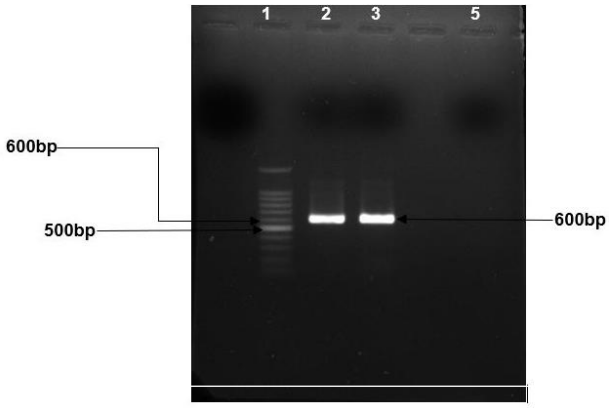

Figure 5. Gel image of PCR products with *rbcL* primers using TAE buffer. (Lane 1: 100bp ladder),(lane 2: *Curcuma domestica* MT 23), (lane 3: *Curcuma domestica* MT 32). (lane 5: Negative Control).

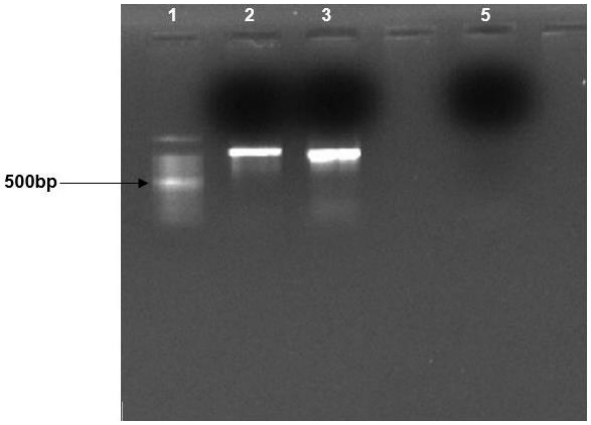

Figure 6. Gel image of the PCR products with *ycf1b* primers using TAE buffer. (lane 1:100bp ladder), (lane 2: *Curcuma domestica* MT 23), (lane 3: *Curcuma domestica* MT 32), (lane 5: Negative control.)

3.4 Visualization of PCR products using TBE buffer.

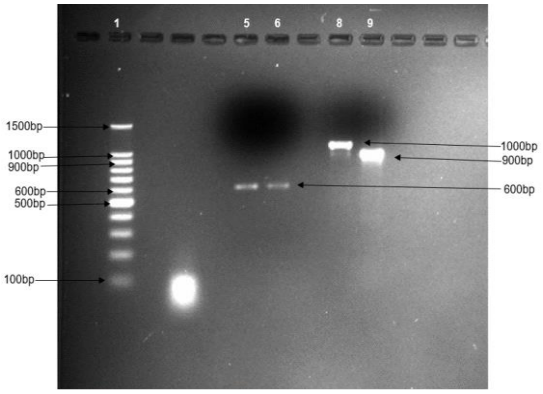

Figure 7. Gel image of PCR products using TBE buffer. (lane 1:100bp ladder), (lane 5: *Curcuma domestica* MT 23(*rbcL*), (lane 6: *Curcuma domestica* MT 32(*rbcL)*, (lane 8: *Curcuma domestica* MT 23(*ycf1b*). (lane 9: *Curcuma domestica* MT 32 (*ycf1b*).

4. Discussion.

When analyzing genes by PCR amplification, it usually depends on relatively small volumes of DNA. In this research, CTAB method was used to extract the genomic DNA from turmeric plants. Plant tissues have two classes of biomolecules which are polysaccharides and polyphenols. These biomolecules vary significantly between species and are problematic when isolating DNA. These two biomolecules can be reduced by using the CTAB method.[11] According to the spot gel image of figure 4A, the extracted plant samples contain genomic DNA in sufficient amounts. This is shown by the intense spots on the spot gel. For further confirmation, the samples were run in a gel electrophoresis, this is shown by figure 4B. According to the figure 4B, the samples contain genomic DNA by showing bands.

Polymerase chain reaction (PCR) is a common laboratory technique which is use to make many copies of an interested DNA fragment.[12] One of the essential components of PCR is *Taq* polymerase. Annealing temperatures are important when considering each primer, the temperatures can be different from one primer to another, and this is important, because if it is not in the optimal temperature non-specific products can be formed and also the yield of products can be reduced.[14] In this research, *rbcL* and *ycf1b* primers were used and the annealing temperatures for them accordingly were 55°C and 49°C. 2x PCR buffer was included with $MgCl_2$ and dNTPs. For *ycf1b*, the annealing temperatures can be used for further researches as it showed good bands without any primer dimers.

Agarose gel electrophoresis is the most effective way to separate DNA fragments in different sizes. When the current is applied, the DNA backbone is negatively charged, therefore, the negatively charged DNA was migrated from the negative end to the positive end. The results were visualized under the gel documentation system. It was shown as bands according to their molecular weights as shown in figure 5 and figure 6.

Even though as the normal routine TAE buffer was used at first, for more clarification the samples were run in an agarose gel with TBE buffer. TBE buffer is needed for a higher resolution for small DNA fragments. Furthermore, the samples were run in an agarose gel with TBE buffer too. As in the figure 7 shows the bands were very clear when the gel was observed under the UV light. The same figure shows, the PCR products were amplified using the *rbcL* primers in the same line. Both the varieties of Turmeric contain *rbcL* and both have the same molecular size *rbcL* gene, but the PCR products that contain *ycf1b* primers are not in the same line. Both the varieties of Turmeric contain *ycf1b* gene, but do not contain the same molecular size of *ycf1b* gene.[16]

5. Conclusion

In this study we, demonstrated the molecular differentiation of *rbcL* and *ycf1b* genes on the basis of amplicon length polymorphism revealed by agarose gel electrophoresis. This method will be very useful for accurate identification of plant genes' molecular sizes. This technique is simple and cost effective and could be used as an alternative or a prior step to sequencing of PCR products, The objectives which were proposed were successfully achieved. Further work includes obtaining the DNA sequence of the PCR products of the *ycf1b* primers.

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Isolation and biochemical analysis of *Enterobacteriaceae* from *Cucumis sativus*

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Abstract

The aim of this experiment was to detect the presence of pathogenic *Enterobacteriaceae* such as *Escherichia coli* and *Salmonella*, which was crucial to assess the safety of consuming raw cucumbers. Furthermore, to gain insight on the occurrence of *Enterobacteriaceae* in cucumbers. Ten cucumber samples were subjected to microbiological tests and biochemical tests. Streak plate method on MacConkey agar was used for differentiating lactose fermenters and non-lactose fermenters. The bacterial isolates were pure cultured and subjected to a series of biochemical tests according to the guidelines in the Bergey's Manual of Determinative Bacteriology. Based on microbiological test results, all cucumbers samples were tested positive for *Enterobacteriaceae*. 60% of the cucumber samples were found to contain lactose fermenters and 40% of the samples were found to contain non-lactose fermenters. A sequence of biochemical tests led to the identification of *Enterobacteriaceae* species such as *Klebsiella pneumoniae* and *Citrobacter diversus*. Out of the ten samples, *Klebsiella pneumoniae* was isolated from 5 cucumber samples whereas only 1 cucumber sample contained *Citrobacter diversus*. The other 4 cucumber samples were non-lactose fermenters which required ornithine decarboxylase test for confirmation of the *Enterobacteriaceae* species. The cucumber samples were tested negative for *Escherichia coli* and *Salmonella*, which indicated the cucumbers were safe for consumption. *Klebsiella pneumoniae* was found as the frequently occurring *Enterobacteriaceae* in *Cucumis sativus* and this result was supported by studies done in the past.

Keywords: *Enterobacteriaceae*, *Cucumis sativus*, food safety, biochemical analysis

1. Introduction

Fresh or less processed cucumbers that are whole or sliced can be suspects of microorganism contaminations, especially since they are not subjected to the steps necessary for sterilisation.[1] The probability of developing food-borne diseases can increase with the consumption of vegetables that are raw or lightly cooked.[2] Table 1 indicates the number of cases for food poisoning and dysentery in Sri Lanka from 2017 to 2020. Globally, an approximation of 550 million people fall prey to food-borne diseases annually, out of which, 230 individuals die.[3]

There was an increase in the number of food businesses in Sri Lanka and most of them were established without any confirmation on food safety. For instance, the number of venders in Sri Lanka increased after many individuals lost their jobs due to the coronavirus disease crisis in 2019.[8] In order to safeguard the health and hygiene of local consumers, it is crucial to evaluate the microbiological quality and safety of food. [9]

Table 1. Selected statistical data on food-borne illnesses documented in the Weekly Epidemiology Report (WER) Sri Lanka.[4-7]

Year	Number of cases	
	Food poisoning	Dysentery
2020	553[4]	875[4]
2019	918[5]	2229[5]
2018	1170[6]	2268[6]
2017	969[7]	2187[7]

Food-borne illnesses could arise due to contamination of food with agents such as bacteria, viruses, parasites, prions and chemicals.[3] Of the various contaminating agents, bacteria, for instance, *Escherichia coli*, *Salmonella* and *Campylobacter* have been responsible for many recurring incidents of food-borne diseases.[10]

1.1 Enterobacteriaceae associated contaminations and consequences. All bacteria that belong to the diverse *Enterobacteriaceae* family are gram negative, flagellated bacteria that are also facultative anaerobes with a size of approximately 0.3 – 1.0 * 1.0 – 6.0 µm. Figure 1 indicates some of the genera comprising the *Enterobacteriaceae* family.[11] Several bacterial species from this family have been the cause of numerous food-borne outbreaks by contaminating fresh vegetables and fruits.[12]

A serotype of *E. coli* known as *Escherichia coli* O157:H7 could trigger haemorrhagic diarrhoea and kidney failure because it produces a toxin called Shiga.[13] *Salmonella* is an organism similar to *E. coli*. *Salmonella bongori* and *Salmonella enterica* are two species of *Salmonella*.[14] *Salmonella* is known to cause salmonellosis which is also known as typhoid fever.[13]

Furthermore, a common nosocomial pathogen called *Klebsiella pneumoniae*, is known to cause Friedlander's pneumonia and rhinoscleroma. Apart from the two human specific infections, *Klebsiella pneumoniae* can also cause other infections associated with blood, urine and the respiratory system.[15]

Majority of the illnesses associated with fresh vegetables are known to be caused by *E. coli* and *Salmonella* species.[16] According to a study conducted in Sri Lanka to assess the microbiological quality of selected fresh vegetables, 6% of the leafy vegetable samples indicated presence of *Salmonella* spp. and 2% of the samples showed prevalence of *E. coli*. [17]

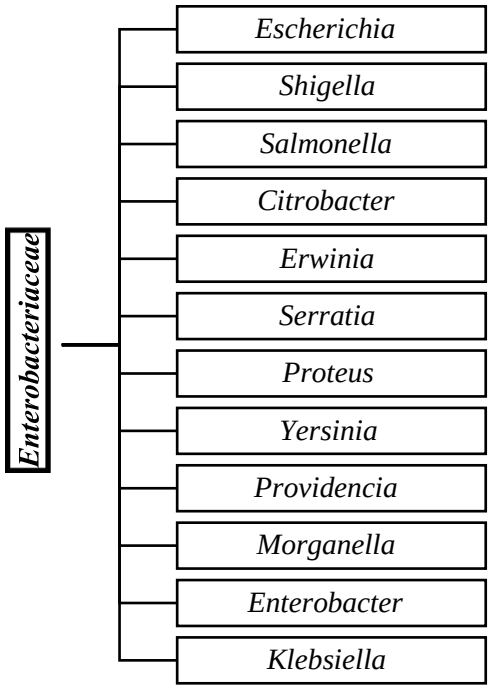

Figure 1. Classification of the *Enterobacteriaceae* family.[11]

Including Sri Lanka, a list of other countries: India, Vietnam, Philippines, USA, Mexico, Spain, Brazil, Canada and Thailand, have reported illness caused by consumption of fresh produce contaminated with *E. coli* and *Salmonella*.[13] Some of the pathogens associated with food-borne outbreaks in Sri Lanka include hepatitis A, *Salmonella*, *Listeria monocytogenes* and *Vibrio cholerae*.[18]

1.2 Contamination of C. sativus by Enterobacteriaceae. Fresh vegetables such as lettuce, cucumber, spinach, carrot and tomato are often eaten raw.[13] Figure 2 illustrates the varieties of cucumbers cultivated. It is a juicy fruit with a crisp texture, making it a highly preferred constituent of salads. Consumed in its unripe, green form, cucumber which is also known as *Cucumis sativus*, is the most cultivated member of the *Cucurbitaceae* family.[19] *Cucumis sativus* is one of the 825 species across the 118 genera comprising the *Cucurbitaceae* family.[20]

Fruits and vegetables are highly vulnerable to contamination by factors such as soil and irrigation water associated with the pre-harvesting process, and handling process after harvest.[16] Pathogenic microbes such as *E. coli* O157:H7 and *Salmonella* could contaminate the soil in cases where poultry litter is used as fertilizers for growing fruits and vegetables.[21] Fruits and vegetables could act as an intermediate for transmission of pathogens or opportunistic pathogens. Hence, over the years, there has been a positive correlation between consumption of vegetables and fruits, and human infections.[12]

Figure 2. Different cultivars of cucumber.[20]

1.3 Isolation and identification of Enterobacteriaceae. Selective media could be used to grow colonies of bacteria which in turn could be used to isolate *Enterobacteriaceae*, by subjecting them to phenotypic tests indicated in Figure 3.[22] The following research used a specific set of biochemical tests from the Bergey's Manual, as outlined in Figure 4 and Figure 5, to characterize *Enterobacteriaceae*.

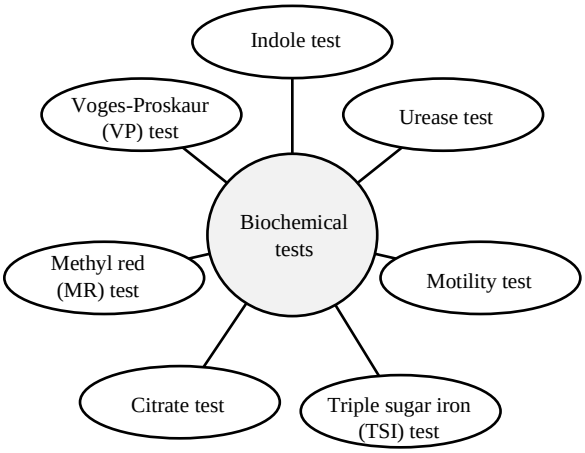

Figure 3. Selected biochemical tests used in the research for identification of *Enterobacteriaceae*.[22, 23]

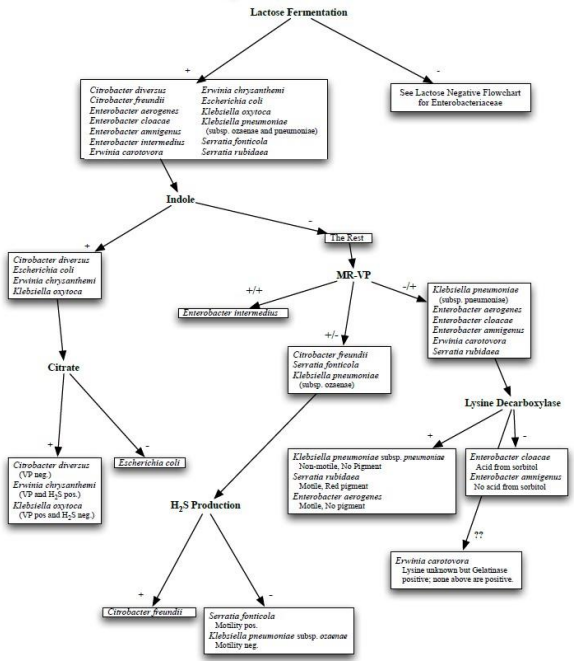

Figure 4. Biochemical tests to identify lactose fermenting *Enterobacteriaceae*.[24]

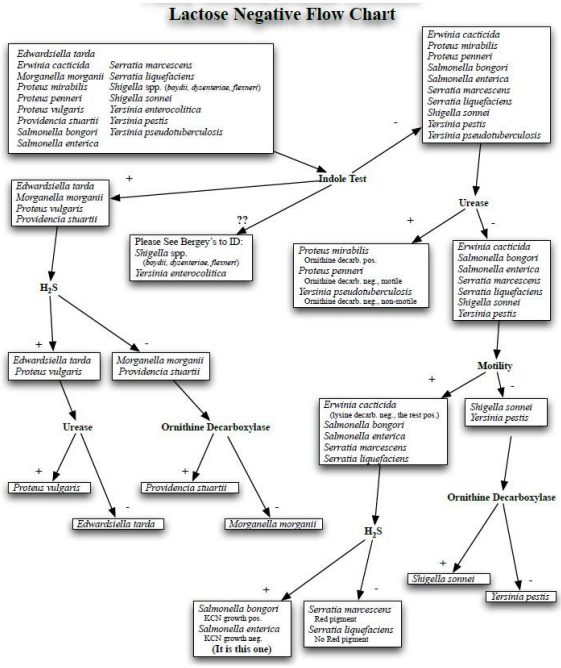

Figure 5. Biochemical tests to identify *Enterobacteriaceae* that do not ferment lactose.[24]

A tristate outbreak took place in 2016 due to the consumption of contaminated cucumbers. The FDA was able to confirm that cucumbers were the source of contamination due to the consistent investigations conducted on cucumbers to detect *Salmonella*.[25] Consistent investigations on food are essential to understand the hygienic conditions of food. This is important in Sri Lanka because there are few investigations on food hygiene.[17] Furthermore, food-borne diseases may not be detected as there are no standard diagnostic procedures to identify them in Sri Lanka.[17] Hence, scientific literatures may not cover most incidents of food-borne illnesses.[17] The occurrence of *Enterobacteriaceae* in *Cucumis sativus* in Sri Lanka can be understood from the subsequent research project. The study results can be used to understand the degree to which hygienic conditions must be refined for quality production of fresh cucumbers.[12]

2. Methodology

2.1 Sample collection. Ten fresh cucumbers were collected from different places (Table 2) within Wellawatta and Pettah, which are two zones in Colombo, Sri Lanka.

Table 2. Sample collection locations in Colombo.

Sample	Date of collection	Collection site	Location
1	18th February 2022	Vegetable market	Pettah
2		Roadside vegetable stand	Wellawatta
3		Fruit and vegetable market	Wellawatta
4		Grocery store	Wellawatta
5		Roadside vegetable market	Wellawatta
6	20th April 2022	Supermarket	Wellawatta
7		Grocery store	Pettah
8		Roadside vegetable stand	Pettah
9		Vegetable market	Pettah
10		Roadside vegetable stand	Pettah

2.2 Sample preparation. Five sterile beakers were labeled. Five fresh cucumbers were thoroughly washed using distilled water and then utilised for sample preparation. Slices of the mid region of the cucumber was cut and weighed. Approximately 100 g of the cucumber was blended to obtain homogenized cucumber sample. Sterile equipment were used for sampling.

Using a micropipette, 2 ml of the cucumber sample was transferred into sterile screw-capped containers. This was followed by the addition of 18 ml of freshly prepared peptone water. The preceding mixtures were mixed by swirling the closed container which was incubated at 37 °C for 48 hours. Thereafter, the samples were stored at -4 °C until analyzed further.

2.3 Streak plate method on MacConkey agar. The refrigerated samples were left out at room temperature. Near a Bunsen flame, five sterile petri plates were closely arranged and labeled. The surface of the biosafety cabinet was wiped with 70% ethanol and the five petri plates were appropriately aligned in a single file. Freshly prepared, MacConkey agar was poured into sterile petri plates and allowed to solidify.

Using sterile inoculating loops, quadrant streak was performed from all the samples. The preceding steps were replicated to culture another set of MacConkey agar plates to minimise errors. The streaked MacConkey agar plates were sealed with parafilm and incubated at 37 °C for 24 hours. Thereafter, the plates were stored at -4°C until analyzed further.

2.4 Preparation of pure culture. Freshly prepared sterile nutrient broth was poured into five sterile broth tubes in equal volumes and labeled. The nutrient broth tube was inoculated using a discrete colony picked from its corresponding MacConkey agar plate after incubation. The inoculated nutrient broth tubes were incubated at 37 °C for 24 hours, and then stored at -4 °C until analyzed further.

2.5 Biochemical tests. The *C. sativus* inoculums were subjected to a series of biochemical tests, as demonstrated in the results section. The test tubes involved were autoclaved at 100 °C for 45 minutes before being utilised in the biochemical tests. The test tubes were then labelled appropriately. Procedures were carried out under aseptic conditions to minimize cross contamination. For each test, two control tubes were prepared using *E. coli*.

2.5.1. Indole test. 5 ml of tryptophan broth was inoculated with pure culture. The inoculated tryptophan tubes along with the control tubes were incubated at 37 °C for 24 hours. Thereafter, 1-2 ml of Kovac's reagent was added and colour change was noted.

2.5.2. Citrate test. 5 ml of freshly prepared Simmons Citrate agar was poured into sterile test tubes and left in a slanted position for solidification. Pure culture was inoculated on the Simmons Citrate agar slants. The pure culture slants along with the control tubes were incubated at 37 °C for 24 hours.

2.5.3. Urease test. 80 ml of Christensen urea agar mixture and 4 ml of urea solution was used to produce urea slants. After solidification, 5 ml urea slants were inoculated with pure culture. The control tubes and streaked tubes were incubated at 37 °C for 24 hours.

2.5.4. Motility test. Sulphide Indole Motility (SIM) agar tubes were prepared and inoculated with pure culture by penetrating the inoculating loop half-way through the semi-solid agar. Along with the control tubes, all the test tubes were incubated at 37 °C for 24 hours.

2.5.5. Triple sugar iron test. The pure culture was streaked on its respective 5 ml triple sugar iron agar slant using a sterilised inoculating loop. The inoculated tubes with controls were incubated at 37 °C for 24 hours.

2.5.6. Methyl red test. Test tubes containing 5 ml of Methyl Red Voges-Proskauer (MRVP) broth were inoculated with their respective pure cultures using a sterile inoculating loop. The tubes were then incubated at 37°C. After 24 hours, 1 ml from the incubated MRVP tube was transferred to a clean tube and 2 to 3 drops of methyl red solution was added.

2.5.7. Voges-Proskauer test. Using a sterile inoculating loop, test tubes containing 5 ml of MRVP broth were inoculated with their respective pure cultures. After incubation at 37 °C for 24 hours, 6 drops of Barritt's reagent I and 2 drops of Barritt's reagent II was added.

3. Results

3.1 Analysis of microbiological test results. Out of the 10 samples involved in this experiment, 6 samples contained lactose fermenters (Figure 6) and 4 samples contained non-lactose fermenters (Figure 7), as indicated in table 3.

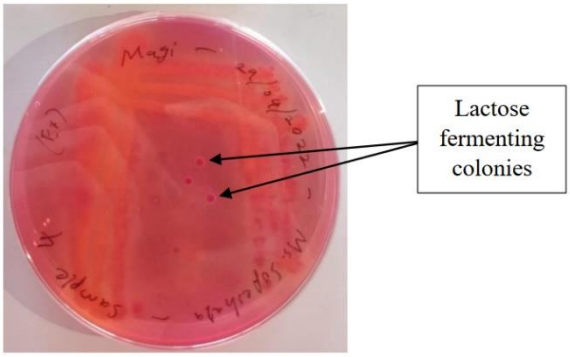

Figure 6. MacConkey agar plate with lactose fermenters.

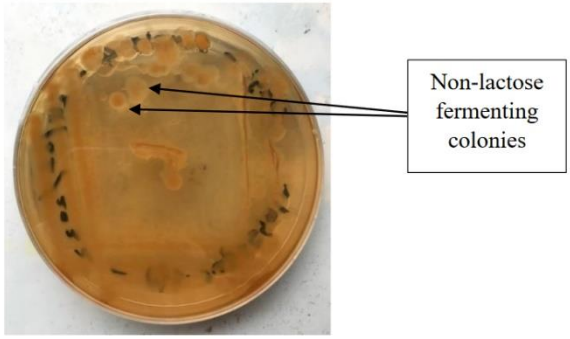

Figure 7. MacConkey agar plate with non-lactose fermenters.

Table 3. Cucumber samples assigned from 1 to 10 yielded lactose fermenters and non-lactose fermenters.

Samples						
Lactose fermenters	5	6	7	8	9	10
Non-lactose fermenters	1	2	3	4	-	

3.2 Analysis of biochemical test results. Table 4 contains the biochemical test results for all 10 cucumber samples. For making comparisons between the test results, reference microorganisms: positive control and a negative control, were involved in the experiment.[26] The bacterial species present were identified using the Bergey's Manual, shown in Figure 4 and Figure 5. Almost all tests had both a negative control (NC) and a positive control (PC).

In the MR test, the bacterial ability to ferment glucose and produce a strong acid is assessed. If the Embden-Meyerhof pathway used by bacteria led to the production of strong acid, positive results for MR test is seen, as demonstrated in Figure 8.[27] In the presence of a pH indicator such as methyl red, bacteria will induce a red colour change.[28] Negative result for MR test is indicated by a colour change to yellow.[23]

Table 4. Positive (P) and negative (N) biochemical test results for the ten cucumber samples.

Tests	**Samples**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Indole test	N	N	N	N	N	N	N	P	N	N
Citrate test	P	P	P	P	P	P	P	P	P	P
Methyl red test	-	-	-	-	P	P	P	-	P	P
Voges-Proskauer test	-	-	-	-	N	N	N	N	N	N
Triple sugar iron test	N	N	N	N	N	N	N	N	N	N
Motility test	N	N	N	N	N	N	N	N	N	N
Urease test	P	N	P	N	P	P	P	P	P	P

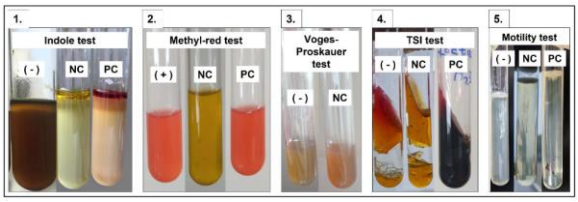

Figure 8. Biochemical tests confirming *Klebsiella pneumoniae*.

Red colour change of VP broth means a positive result.[28] The absence of colour change, as presented in Figure 8, is due to the inability of bacteria to ferment glucose and form pyruvic acid, a key compound necessary to form acetoin. Metabolic routes leading to acetoin formation is based on the bacterial type and their enzyme systems. Acetoin gives rise to diacetyl in the presence of 40% KOH and oxygen in air, and the red colouration is catalysed by α-naphthol.[29]

Constituents of Triple Sugar Iron (TSI) agar include 0.1% of glucose, sucrose and 1% of lactose.[22] The test assesses the bacterial ability to ferment these carbohydrates and produce hydrogen sulphide. Acidification is indicated by phenol red and H_2S production is indicated by the presence of ferrous sulphate.[29] The TSI positive control in Figure 8 evidences a black precipitate due to ferrous sulphide and H_2S gas generation.

The formation of carbon dioxide or hydrogen could be determined by the presence of bubbles and splits in the agar.[29]

The TSI negative result in Figure 8 is with an alkaline slant and an acid bottom, which indicates the bacteria is a glucose fermenter. Other situations that may occur is when both the slant and bottom are either alkaline or acidic, in which case the interpretations would go as, no sugars fermented or all three sugars fermented, respectively.[22]

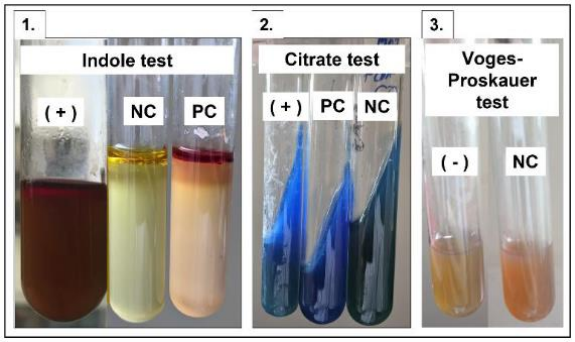

Figure 9. Biochemical tests confirming *Citrobacter diversus*.

Indole positive result witnessed in Figure 9 has a red ring which is a result of the reaction between indole and an aldehyde from the Kovac's reagent.[23] Indole test is used to understand if the bacteria is capable of using tryptophanase to generate indole, pyruvic acid and ammonia, by hydrolysing and deaminating tryptophan.[29] Indole negative result is identified by the presence of an orange-yellow ring, as evidenced in Figure 8.[22]

In the citrate test, bacteria obtain carbon and energy by degrading citrate. Bacteria produces the enzyme citritase which aids in breaking down the citrate in media into oxaloacetate and acetate. Pyruvate and carbon dioxide are produced by the further breakdown of oxaloacetate. The medium becomes alkaline due to the formation of sodium carbonate from sodium citrate, and this provokes a colour change from green to blue.[23] Bromothymol blue indicator in Simmon's citrate test will turn blue in case of a positive result, and stay green to in case of negative result, as presented in Figure 9.[15]

The positive control for motility test in Figure 8 shows signs of turbidity. This is due to the diffuse growth of bacteria, signifying its motile characteristic. The negative motility test result and the negative control show growth along the stab line. The agar was also clear, which together confirms that the bacteria is non-motile.[22]

Some of the above biochemical tests indicated the presence of *Klebsiella pneumoniae* in lactose fermenting samples: 5, 6, 7, 9 and 10. *Citrobacter diversus* was confirmed to be present in lactose fermenter sample 8.

Non-lactose fermenter samples: 1 and 3 could be either *Proteus mirabilis*, *Proteus penneri* or *Yersinia pseudotuberculosis*. The possibility of identifying one of these three species was confirmed by the negative indole results and positive urease results, as demonstrated in Figure 10. Non-lactose fermenter samples: 2 and 4 could either be *Shigella sonnei* or *Yersinia pestis*. This was concluded based on negative results for indole test, urease test and motility test, as indicated in Figure 10.

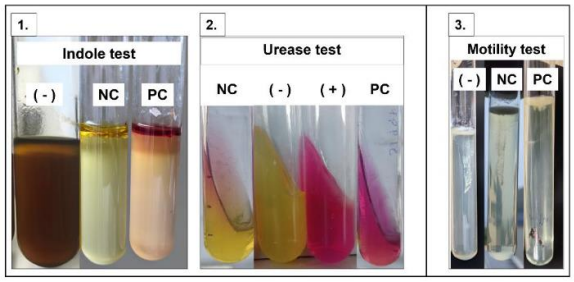

Figure 10. Indole test and urease test to specify *Enterobacteriaceae* in samples 1, 2, 3 and 4. Motility test was needed for further confirmation in samples 1 and 3.

Many bacterial species produce the enzyme urease.[29] Decomposition of urea into ammonia and carbon dioxide is catalysed by this

enzyme. Only urease-positive species are capable of triggering phenol red to turn pink by using the nutrients and pH buffers in the medium to synthesise sufficient ammonia.[28] Urease-negative species are distinguished by the absence of colour change, as visualized in Figure 10.[26]

4. Discussion

Microorganisms, for instance, *Enterobacteriaceae* could be distinguished from one another using a variety of biochemical tests.[28, 9] Biochemical testing is often restricted to essential substrates required to rule-in or rule-out the desired pathogens in resource-constrained situations. The *Enterobacteriaceae* species present in the sample could be identified by piecing together the results of the individual biochemical tests.[22]

Analysis of biochemical test results confirmed the presence of the *Enterobacteriaceae* in all ten *C. sativus*, specifically: *Klebsiella pneumoniae* (50%) and *Citrobacter diversus* (10%). In a similar study, 60% of fruits and 91% of vegetables out of the 105 fresh produces tested, were found to harbour *Enterobacteriaceae*. Along with other bacterial species such as *Enterococcus casseliflavus* and *Enterobacter cloacae*, the most abundant member after *E. coli* was *Klebsiella pneumoniae*.[12]

According to ALatawi *et al*[15], characterisation of bacteria in street stall vegetable samples using both biochemical tests and molecular tests revealed that 24 isolates were *Klebsiella* spp., and 6 isolates were *Enterobacter asburiae*. There were also isolates of other families including, three *Pseudomonas aeruginosa* isolates and two *Aeromonas caviae* isolates. Another study highlighted the prevalence of *Klebsiella pneumoniae* in which 100% occurrence was found in lettuce and 82.5% occurrence in cucumbers.[2]

As a whole, the abovementioned past studies and this research project evidence *Klebsiella* spp. as a frequently occurring *Enterobacteriaceae* member in vegetables. However, biochemical methods are time consuming and have poor specificity, therefore 16s rRNA gene sequencing; a DNA-based molecular method, can be used alternatively for highly specific identifications.[15]

One out of the ten cucumbers tested in this research project yielded a *Citrobacter* specie. Similarly, Adegun, Oluduro and Aregbesola[30] reported that only one out of the fifteen cucumber samples were contaminated with *Citrobacter* spp. However, their study findings on the percentage of Citrobacter spp. occurring in fruits and vegetables showed 68% of *Citrobacter freundii*, 24% of *Citrobacter braakii* and 8% of *Citrobacter youngae*. There is a possibility for low prevalence of *Citrobacter* species in cucumbers when compared to *Klebsiella pneumoniae*.[30]

When fresh vegetables are contaminated with microorganisms such as *Klebsiella pneumoniae*, food safety becomes a concern. People with weak immune systems are at a higher risk of developing diseases after consumption of contaminated fresh vegetables. One way to lower risk is to wash vegetables using disinfectants. This is because gentle washing will not dislodge *Klebsiella* so easily from the vegetable surface.[24] Colonisation and growth of microorganisms on vegetables, followed by metabolism of plant tissues for nutrients could be caused by the microorganisms present in the soil.[2]

The *Enterobacteriaceae* specifically inspected in this experiment were *E. coli* and *Salmonella*, but they were not found in any of the cucumber samples tested. This could be due to the small sample size. A study similar to this research project involved fifty fresh vegetable samples.[17] Along with *Listeria monocytogenes*, food-borne pathogens such as *E. coli* and *Salmonella* spp. were found to contaminate the fresh vegetables.[17, 31] In another study involving 30 ready-to-eat salad vegetables, *E. coli* was detected in 96.7% of

the samples.[32] Therefore, the chances of isolating *E. coli* are high in an experiment involving a large sample size.

On the contrary, results from Al-Kharousi *et al*[12] on cucumbers imported from Oman and UAE, showed the presence of *Enterobacteriaceae* but absence of *E. coli*. Results from Abakari, Cobbina and Yeleliere[32] highlighted *Salmonella* spp. as the least prevalent members in ready-to-eat salad vegetables. According to Erickson *et al*[33], there was no contamination found on 60% to 78% of the cucumbers, after the fruits were exposed to irrigation water infected with 3.8 log CFU/mL of *Salmonella typhimurium* and *E. coli* O157:H7.

Contamination of cucumbers may have been prevented by the cucumber plant canopy that could protect the fruits from exposure to irrigation water.[33] This way, contamination of cucumbers by pathogenic *Enterobacteriaceae* such as *E. coli* and *Salmonella* spp. may have been overcome. Poor handling by vendors and faecal contamination can lead to presence of *E. coli* in food.[32] Since *E. coli* was not detected in the ten cucumber samples tested, it could mean that the cucumbers were handled properly and were not exposed to any faecal contamination.

Each cucumber was brought from a different market or vegetable stall in order to increase the variation of *Enterobacteriaceae* members detected in this experiment. Based on results analysis, two species of *Enterobacteriaceae* members were confirmed and few other species are most likely to be present. This variation in the isolated species could be due to difference in hygiene conditions at the marketing areas and the agriculture from which the vegetables were initially sourced.[32]

Peer-reviewed literature has documented a variety of biochemical tests. Only a small fraction of substrates was found to be effective in identifying *Enterobacteriaceae*. Selective use of biochemical tests is done because, sometimes, certain substrates, though successful in identification of *Enterobacteriaceae*, are poisonous, unstable or expensive for regular use.[22] Furthermore, not all bacteria could be accurately identified using biochemical tests. For instance, since *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* share a large degree of similarity, the standard determination tests can lead to misidentification of these members.[34] Such misidentifications can be expected among the results of the experiment, especially since isolates include *Klebsiella pneumoniae* and *Citrobacter diversus*.

As biochemical reactions are simple detection methods, the medium quality can influence the precision of the tests results, and the *Enterobacteriaceae* specie identified.[27] Additional measures to prevent contamination should be taken into account.[27]

5. Conclusion

In conclusion, pathogenic microorganisms such as *E. coli* and *Salmonella* were not detected in the cucumbers tested. *Klebsiella pneumoniae* was the most prevalent *Enterobacteriaceae*. This pinpoints the risk of consuming raw cucumbers, especially by immunocompromised patients. Another lactose fermenter detected was *Citrobacter diversus*, apart from which, non-lactose fermenters were also detected. Specie confirmation of the non-lactose fermenters requires ornithine decarboxylase biochemical analysis. However, it can be concluded that *Enterobacteriaceae* were detected in all ten cucumber samples.

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Development of a qPCR method for detection of maize species

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Abstract

Maize is a widely used cereal around the world, and it is essential to know and study more about this species. Therefore, a qPCR technique was carried out to see whether the maize species were present in the sample. The gene that was used as a reference for the maize species was the SSIIb gene. First, the sample of maize was weighed accurately using an analytical balance, and the DNA was extracted by following the QIAGEN-DNeasy mericon food kit procedure. Once the DNA was extracted, it was quantified using the bio-spectrometer to determine the purity and concentration of the extracted DNA. For the amplification of this extracted DNA, specific primers, and probes specific to the SSIIb gene were reconstituted according to the volumes given in the manual, and the PCR master mix was prepared, and qPCR was performed for the 50 ng and 100 ng concentrations and PCR water. The curves obtained for the 50 ng and 100 ng concentrations were smooth curves with similar CT values, showing that the SSIIb gene was present and successfully detected. Overall, the results concluded that the SSIIb gene in maize species was successfully detected through the qPCR method.

Keywords: Maize species, SSIIb gene, qPCR, CT value.

1. Introduction

1.1 Genetically modified crops. Genetically modified (GM) is the gene transfer of organisms using laboratory techniques. These include cloning genes and inserting genes into cells to possess valuable traits such as insect resistance, herbicide resistance, disease resistance, abiotic stress tolerance, and nutritional improvement.[1] The first GM crops introduced were in the mid-1990s in the US., with GM maize being the most genetically modified crop after soybean. These adoptions have shown many benefits, such as reducing pesticide and insecticide use, increasing crop yields, decreasing the cost of crop production, and lowering CO_2 emissions. Along with all these benefits, there can be negative impacts as well, such as concerns about possible allergenicity and toxicity to human beings, potential environmental risks such as adverse effects on non-target organisms, the evolution of resistance in insects and weeds, gene flow, etc.[2] Figure 1 shows examples of genetically modified crops along with their percentage acreage in the US.

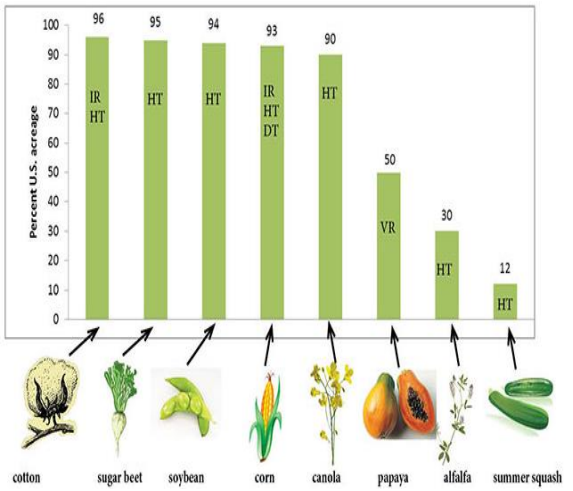

Figure 1. Currently grown GM crops in the US., traits for which they are modified, and percentage of the total acreage of the crop planted to GM varieties.[3]

IR=Insect-resistant, HT=herbicide-tolerant, DT=drought-tolerant: and VR=virus-resistant.

1.2 Maize species. Maize, *Zea mays,* a member of the family true grasses, or *Poaceae*, is a vital cereal grain used worldwide.[4] Maize is one of the most widely produced genetically modified crops. Except in Antarctica, maize can be found on every other continent. There are about 50 species of maize existing in different colours, shapes, sizes, and textures. Most cultivated maize types are yellow, white, and red.[5]

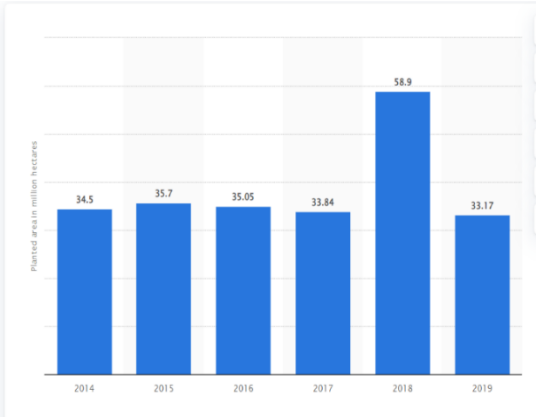

Figure 2. U.S. acreage of genetically modified corn 2013-2019.[6]

Figure 2 represents, the maize planted area in million hectares from 2014 to 2019. The benefits of GM maize include increased grain yields, decreased insect damage and high quality. In short, growing GM maize reduces health risks to consumers and increases the income of farmers.[7] Genetically modified crops are also likely to be crucial in feeding the world's growing food population and improving food security. Along with their many benefits, GM crops can also have disadvantages. Therefore, GM rules and regulations have been established.

1.3 GMO rules and regulations. GMO regulations have been established worldwide in countries, based on economic, societal, and political reasons. A score is set in the GMO index for different countries showing the country-by-country restrictiveness of GMO regulation. The score is based on six main parameters of GMO regulations: risk assessment, labelling, approval process, traceability, coexistence, and membership in international agreements.[8] The US., which is considered the global leader in the commercialization and development of GM crops, holds the global market share in agricultural biotechnology at close to 30%. The US. has no specific, overarching federal law targeted at the regulation of GMOs, unlike most countries. Therefore, GM products are assessed under safety, health, and environmental laws.[9] In Sri Lanka, according to Food Act No. 26 of 1980, no person shall store, import, transport, sell, distribute, or offer for sale any GMOs or any food produced from GMOs without any approval.[10]

1.4 qPCR technique. Many techniques, such as ELISA, multiplex PCR, etc., detect GM crops. However, qPCR testing further increases sensitivity, enabling more efficient testing for large-grain samples and processed foods.[11] The main advantages of qPCR include fast and high-throughput detection, quantification, and a lower time for amplification and visualization.[12]

There are two main methods for qPCR detection, which are the SYBR green assay (non-specific dsDNA binding) and the TaqMan assay. A DNA sequence-specific hydrolysis probe labelled with a fluorophore and a quencher is used in the TaqMan method, as shown below in figure 3. Both assays can be adapted to be absolutely or relatively quantitative, although the TaqMan method is more specific than SYBR green.[13] In this study, we use the TaqMan assay method for the detection of maize species.

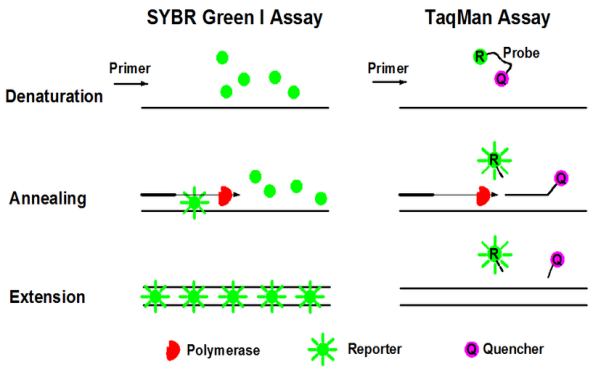

Figure 3. Method of qPCR.[14]

1.5 Significance of the Project. Maize is one of the most widely grown GM crops worldwide, with many benefits. To detect maize, qPCRis widely used, as it is a highly sensitive and quantitative technique for detecting GM crops.

The main objective of this research is to develop a qPCR method for the detection of maize species using maize specific primers. This method can be used to detect taxon specific gene in maize, which can be used to detect maize reference gene in GM maize samples. Therefore, qPCR provides a significant advantage in detecting these GM maize species.

2. Methodology

2.1 DNA extraction using the Dneasy Qiagen Mericon food kit. The workbenches were wiped with 10% bleach followed by 70% ethanol before the procedure. A 0.2006 g of maize sample provided by the biotechnology section was taken for extraction. The DNA extraction was performed according to the manufacturer's guidelines, with modifications.[15]

2.2 Quantification of DNA. The extracted maize DNA was quantified using the Eppendorf bio spectrophotometer. The concentration and purity were analyzed at 260 nm and 280 nm wavelengths. The extracted maize DNA sample was diluted to 50 µg/ml from 87.6 µg/ml. Both the extracted DNA of maize sample and the diluted DNA sample were stored at -20ºC until further use.

2.3 Amplification of maize DNA. PCR was carried out in a volume of 15 µl containing 50 ng and 100ng of extracted sample DNA PCR master mix buffer, 0.5 µM concentration of each (sense and antisense) primer; 0.2 µM probe; the balance consisted of sterile ultrapure water. PCR assays were carried out in Quant studio 5 qPCR system using a standard PCR protocol. The cycling conditions consisted of 2 minutes of UNG treatment at 50ºC, 10 min of initial denaturation at 95 °C, followed by 45 cycles consisting of initial denaturation of 95ºC for 10 minutes, 15 second denaturation at 95 ℃ and an annealing at 60°C for 1 minute.

3. Results

3.1 Quantification by Bio-Spectrometer. The concentration of extracted DNA was 87.6 µg/µl.

Purification for A260/A280- 1.89

Purification for A250/A230- 1.69

3.2 qPCR Results. As shown in figure 4 and 5, amplification curves were obtained for the SSIIb gene with 50 ng and 100 ng concentrations, which were detected and obtained through the qPCR

technique. No amplification curve for the PCR water sample was seen.

As shown in figure 4, the amplification plot was obtained, and the CT value for 100 ng concentration is 25.216.

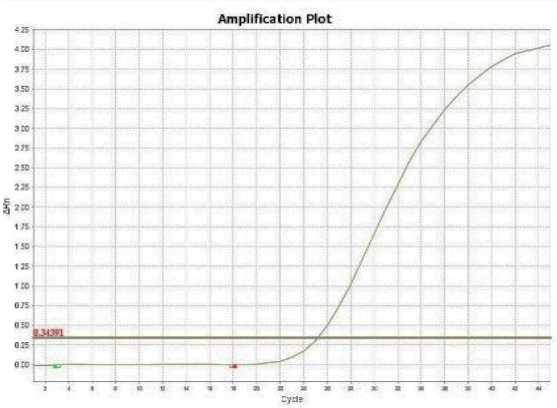

Figure 4. Amplification plot obtained for 100 ng concentration of extracted DNA.

As shown in Figure 5, the CT value obtained for a 50 ng DNA concentration is 25.411.

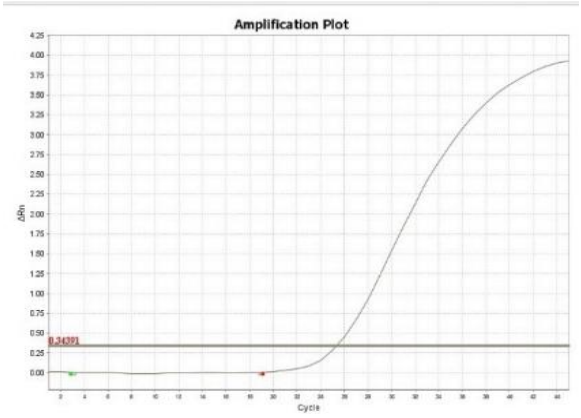

Figure 5. Amplification plot obtained for 50 ng concentration.

As shown in figure 6, no amplification plot was obtained for the sample with PCR water and no extracted DNA.

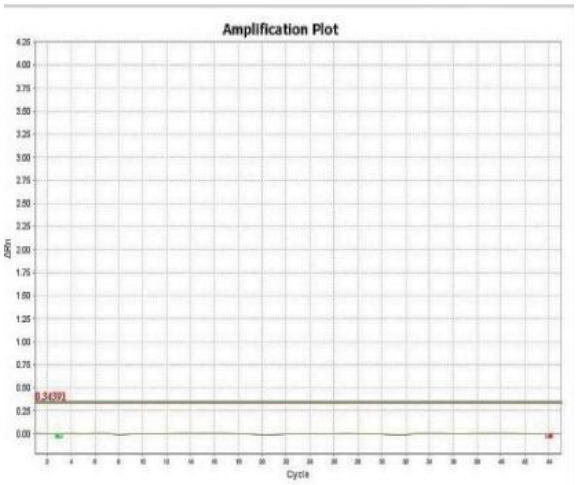

Figure 6. Amplification plot obtained for the PCR water tube.

4. Discussion

Growing maize has been demonstrated to be one of the most economical, feasible, and sustainable approaches for resource-poor farmers and to be environmentally friendly. Therefore, knowledge of the genomic and genetic resources and components of maize species is vital to guide genetic analysis and precision breeding with desirable product profiles for maize varieties.[18]

For dependable and reproducible RT-quantitative PCR (qPCR) analysis, reference genes are frequently utilized to standardize gene expression levels, to perform relative quantification and to use as housekeeping genes.[20] Therefore this study focuses on developing a qPCR-based method to detect SSIIb gene in maize as a reference gene to detect GM maize. The SSIIb gene is a starch synthase gene in maize and is involved in regulating starch content.[24]

Foods are exposed to high pressure, heat, radiation, and pH changes during processing, all of which cause DNA to break down and fragment. The DNeasy mericon Food Kit's refined chemistry was created to recover short

DNA fragments, guaranteeing that even severely fragmented DNA is effectively extracted and amplified in PCR procedures. With these properties, extraction with the DNeasy mericon Food Kit is the first generally applicable extraction technology that provides optimal and accurate results even when utilizing extremely inhibited, highly processed, fatty, acidic, high, or low DNA content foods.[25]

In DNA quantification, the concentration of DNA in the sample was 87.6 µg/µl, showing that DNA was successfully extracted.[16] The purity of the sample was 1.89 for the ratio of A260/A280 absorbance, which is between the ratio for pure nucleic acids of 1.8 and 2.0.[23] This confirms that the extracted DNA was pure and not contaminated.[17]

In DNA quantification, the concentration of DNA in the sample was 87.6 µg/µl, showing that DNA was successfully extracted. The purity of the sample was 1.89 for the ratio of A260/A280 absorbance, which is between the ratio for pure nucleic acids of 1.8-2.0.[23]

The qPCR method developed to detect the maize sample was successful and showed that the SSiib gene was present, as observed by the results. The qPCR results obtained showed that the SSIIb gene was detected for 50 ng and 100 ng sample concentrations, and no amplification curve was seen for the PCR water sample, proving no cross-contaminations or false positives have occurred.[21] However, the CT values obtained for both 50 ng and 100 ng are very similar, with the 50 ng concentration sample being slightly higher comparatively, whereas theoretically, the 50 ng DNA sample should obtain one CT value higher than the 100 ng concentration.[22] This slight variation maybe due to pipetting errors.

Therefore, with the results obtained, the objective of detecting the presence of maize species in the GM maize was successfully achieved by extracting DNA, quantifying the extracted DNA, and amplifying the extracted DNA, by the qPCR method.

This work was only carried out to develop the test method, and for future work, the number of samples used can be further increased, as in our study only 50 ng and 100 ng samples were used to confirm the presence of the SSIIb gene. A dilution series could also be used to achieve a more accurate reading and test for the presence of the SSIIb gene. Furthermore, different varieties of maize species can be used to compare and analyse the genes present in each sample.

5. Conclusion

In conclusion, the results obtained from the qPCR show that the DNA was successfully extracted and quantified. Although the CT values for both 50 ng and 100 ng concentrations obtained were similar, possibly due to a dilution error. We can successfully say that by detecting the SSIIB gene, the presence of the maize species was confirmed using the qPCR technique.

Acknowledgements

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Determination of antibacterial activity of *Syzygium aromaticum* on *Escherichia coli* and *Staphylococcus aureus*.

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Abstract

Plant extracts are widely utilized for therapeutic purposes since they are inexpensive, effective, safe and have no or very less negative effects. Spices are components of several plants that add food preparations a distinct scent and flavor. The goal of this study was to discover if *Syzygium aromaticum* bud and leave extracts have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Clove bud and clove leave extracts were made using distilled water and 100 % ethanol.Clove leave extract using ethanol extract showed maximum antimicrobial activity. The antimicrobial properties of preservation based on essential oils was also investigated by storing them for 7 days. According to disk diffusion test essential oils has exhibited antibacterial activity against *S. aureus* and *E. coli*, both alone and in combination with medicines. Storage influenced the two spices as well, with an overall decrease in activity. The ethanol extract of clove leaves had the highest activity index 38.3 against *E. coli*. To measure the Minimum Inhibitory Concentration (MIC) of essential oils broth dilution was used. The MIC was observed in 10^{-2} µl/ml concentration in both *S.aureus* and *E.coli*. For the minimum bactericidal concentration (MBC) colonies didn't appear,indicating bactericidal activity of the sample.

Keywords: *Syzygium aromaticum*, Antibacterial activity, MIC, MBC

1. Introduction

In the twenty-first century, multidrug-resistant (MDR)microorganisms pose a huge threat to human health.[1] The WHO published a report in 2017 mentioning the most dangerous MDR bacteria. Antibiotics have resulted in a massive surge in antibiotic resistance among several bacterial infections. Antimicrobial resistance (AMR) develops in microorganisms because of an infection that has a high incidence and might last for a great many years of antibiotic subjection. So, alternative approaches are needed to cure the diseases caused by these resistant organisms. New antibiotics are mostly discovered using natural compounds generated from natural sources. Researchers figured that plant extracts as an alternative to the common antibiotics.

Herbal remedies are extracted and processed for used in scientific research. When preparing medicinal plants for research purposes, must focus the timely and proper gathering of the plant, expert authentication, suitable drying, and grinding.[2] We must follow bioactive separation, stratification, and segregation of molecule, if applicable. It mainly comprises determination of the bioactive compounds. Plants have recently gained significant popularity as a source of medication due to their natural origin, availability in surrounding communities, cheaper cost, ease of administration, and maybe less bothersome nature, and acting as a different treatment strategy

for drug resistance. The extraction method is selected considering the content of the leaf tissue, the fluid employed, the acid levels of the fluid, its warmth, or the liquid to material relation.

Specially, spice medical plants are widely used for flavoring food. Most Asian countries have a long history of traditional medical systems and a diverse spectrum of spices used in therapies. Many spices have antibacterial capabilities due to presence of a variety of chemicals and metabolites present in plant body. Since ancient times, spices have long been used as condiments and additives in ceremonies. Due to their antibacterial characteristics, many spices, such as clove, oregano, thyme, cinnamon, and cumin, are being used to treat several diseases.[3] Spices are typically used to give food scent, flavor, or piquancy, as well as to season it.

Essential oils (EOs) are very promising compounds for producing new antibacterial drugs. Previous research studies have reported a strong antibacterial effect for some EOs. EOs are complex combinations of aromatic plants' secondary metabolites. EOs are known for their bactericidal, antiviral, and fungicidal capabilities, as well as medicinal and aromatic characteristics. EOs has antibacterial, analgesic, sedative, and anti-inflammatory properties. Perfumes, make-up, health, dentistry, and agricultural products all include Eos.[4] EOs can be extracted from a variety of plant organs, including flowers, leaves, fruits, seeds, barks, and roots. The composition and extracted yield of EOs are said to be dependent on the species, soil composition, extraction method, and extraction condition, according to prior study publications. Cold pressing, hydro-distillation, crude extraction, maceration and steam distillation are few methods used to extract EOs. *Syzygium aromaticum* buds and leaves essential oil were obtained using several solvent extraction procedures and it was determined which solvent was the most suitable for each sample.

Menstruum is a solution that is used to separate medicinal herbs.[5] When we are choosing the solvent; crop type, the part of the plant, the nature of the phytochemical compounds, and the solvent supply must all be considered. In addition to solvent selectivity, safety, reactivity, recovery, and viscosity.

Clove belongs to Myrtaceae family. It is commonly utilized to combat oral bacteria's infections.[6] Due to its powerful antibacterial qualities against different food-related disorders, it is utilized in the as a natural additive to improve lifespan. The antimicrobial properties of clove hydro distillation and several solvent extraction methods against *Staphylococcus aureus* and *Escherichia coli* were investigated in-vitro and in-vivo. Clove oil (CO) has biological properties. and used as an antiseptic to treat oral infections. Mold, yeast, and microbes have all been found to be suppressed by CO. In tryptone soya broth and cheese, it was efficient against *L. monocytogenes* and *S. enteritidis*. Both eugenol and the phenolic components of CO keep interaction between cell membrane as well.

The liquid medium evaporates during extraction, releasing a concentrated form of an active component. The isolates or essential oils obtained from seasonings and cuisines are determined by the type of extraction or procedure used. These extracts and oils are often evaluated for antibacterial activity using the agar disk diffusion method. The minimum inhibitory concentration (MIC) is used to determine an organism's sensitivity.[7] When compared to untreated, the MIC is defined as the antibacterial component with the lowest concentration after a particular duration of incubation at a specific temperature, usually 37^0C. According to a method demonstrated by Valgas and colleagues' various pathogens have different MICs for different extracts dilutions.

To determine which solvent was the most successful for the bacteria, essential oil from *S.*

aromaticum was extracted using different solvent extraction methods. Antibiotic sensitivity test on *E. coli* and *S. aureus* to determine susceptibility to the gentamycin.

This study looked at the influence percentage mycelial growth suppression against gram positive bacteria of herbal extracts spherical shape *S.aureus* and gram negative rod form *E.coli*.[8]

Over 80% of the global utilizes herbal treatments. Indeed, several natural products have been used as lead compounds in the development of extremely physiologically relevant chemicals, pharmacological compounds that are semi-synthetic and have a higher potency for therapeutic application, efficiency and efficacy are important.[9] The presence of tannins, saponins, and essential oils in these spices may contribute to their antibacterial properties, oils, flavonoids, and phenolic compounds. Even crude extracts of these spices are effective against multidrug resistant bacteria that have become resistant to current antibiotics.

2. **Methodology**

2.1 Sample preparation. Clove bud samples randomly collected from an ayurvedic shop were cleaned thoroughly to remove contaminants. After that, using a mortar and pestle, a good quality clove was ground.[10] 50g of each sample was weighed and processed in a spice grinder until a uniform powder was obtained. Soxhlet apparatus was used to prepare each sample within 24 hours of the essential oil.

2.2 Extraction of essential oils from clove powder using Hydro distillation method. Essential oil extraction was carried out in accordance with the procedure outlined by Muhamed *et al*. A total of 40g of material has been generated.[11] The boiling flask was placed on the hotplate and was heated to 1000^0C.Fluid remained visible after adding 150ml of distilled water. After that samples were centrifuged at 2500 rpm for 10 minutes. After that, the oil was placed in a mini centrifuge tube. The mass of the generated oil was measured to determine the yield. It was then kept at 40^0C until needed.[12]

2.3 Extraction of essential oils. 40g clove leaves were collected from a local plant nursery and was grounded using mortar and pestle. Then 100ml of 95% ethanol was mixed in 2:5 ratio (ground leaves:ethanol). The oil was then poured into a 1.5ml microcentrifuge tubes. To estimate the productivity, the bulk of the created oil was weighed using an analytical balance.[13]

2.4 Preparation of Bacterial dilutions (MacFarland standard). 1% H_2SO_4 was prepared. Then 1% $BaCl_2$ was also prepared. Then, to make the 10ml Mc- Farland standard, H_2SO_4 and $BaCl_2$ was mixed and stirred thoroughly.[14]Then using spectrophotometer absorbance was checked at 625nm. McFarland standard absorbance range could be 0.08 and 0.10.

2.5 Disc diffusion method for fresh extracted essential oil. Muller-Hinton agar plates were divided into three quadrants. Each quadrant was used for positive control (P), negative control (N) and sample (S_{01}), *E.coli* and *S.aureus* were streaked agar plates. Then filter paper discs were placed in negative and S_{01} quadrant[15]. Sterile, distilled water infused filter paper disks were used as negative controls. Gentamycin was used as positive control. After that, the inoculated petri dishes were incubated at 37^0C for 24 hours. Finally, the zone of inhibition was measured.

2.5.1. MIC broth dilution method for extracted clove leave oil. Six test tubes were prepared by adding 9ml of nutrient broth following the method described by Fauzya *et al*, 2ml of concentrated essential oil was added to the first tube and was labeled as the sample.[16] From the sample test tube 1ml essential oil was serially transferred to the rest of the tubes. Then, 2µL

S.aureus was added each test tube. For the control 9ml nutrient broth and *S.aureus* was added. Same procedure was followed for *E.coli*.[17]

2.5.2 Minimum Bactericidal Concentration (MBC) method for extracted clove leave oil. Tryptone Soy Agar was spread inoculated using 10µL from last three each dilution. Same procedure was followed for *S.aureus* dilutions.[18] Then the plates were incubated at 37^0C for 24 hours.

3. Results and Discussion

3.1 Soxhlet Extraction of *Syzygium aromaticum*. The essential oil was extracted from the clove bud using the hydro distillation. The yield was estimated by weighing the essential oil mass. (Table 1)

Table 1. Volume and yields (w/w %) of essential oils obtained by hydro distillation. [19]

Sample	Volume of oil (mL)	Mass of oil (g)	Mass of sample used (g)	Yield (w/w %)
Syzygium aromaticum (Clove bud)	2.50ml	2.708g	40g	2.708/40×100 =6.77%

3.2 Crude extraction of Syzygium aromaticum. Powdered plant materials were placed in a container. Samples were filled with menstruum. Then the falcon tubes were allowed to mix on the roller mixture for 24 hours to ensure complete extraction. After the completion of the extraction, the extract was decanted. This method is simple to implement and works well with thermo labile leaf tissue.[20]

The yield was determined by monitoring the weight of essential oil extracted from clove leaves using the crude extraction method (Table 1).[21]

Table 2. Volume and yields (w/w %) of essential oils obtained by hydro distillation.

Sample	Volume of oil (mL)	Mass of oil (g)	Mass of sample used (g)	Yield (w/w %)
Syzygium aromaticum (Clove leave)	2.50ml	1.5g	40g	1.5/40×100 =3.75%

P (+) – Positive control

N(-) – Negative control

S_y-Essential oil +Positive control

S_{01}– Sample essential oil

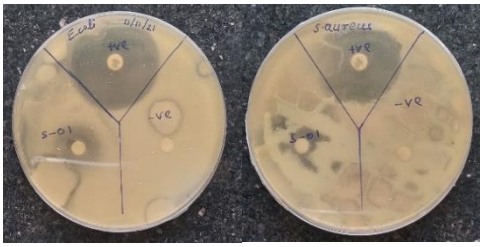
E_o- Essential oil

3.3 Antibacterial Susceptibility Test

3.3.1. Antibacterial activity of Syzygium aromaticum (Clove bud) oil

P (+) – Positive control N(-) – Negative control

S_{01}– Sample essential oil



A B

Figure 1. Zones of inhibition fresh clove bud oil trial o1 *E.coli* (A) and 1 week old clove bud oil (B) against *S aureus*

A B

Figure 2. Zones of inhibition 1 week old clove bud oil trial-02 *E. coli* (A) and 1 week old clove bud oil (B) against *S aureus*, with Gentamycin as the positive control.

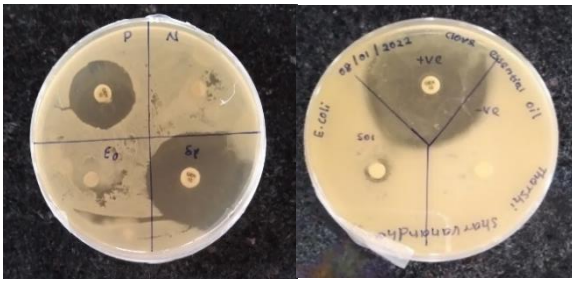
P (+) – Positive control

N(-) – Negative control

S_y-Essential oil +Positive control

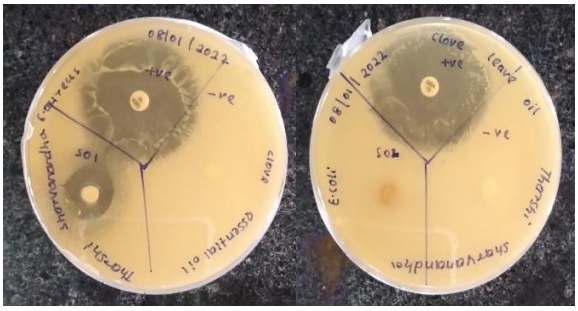
S_{01}– Sample essential oil

E_o- Essential oil



A B

Figure 3. Zones of inhibition fresh clove leave oil *E. coli* (A) and 1 week old clove bud oil (B) against *S aureus*, with Gentamycin as the positive control.



A B

Figure 4. Zones of inhibition fresh clove leave oil *E. coli* (A) and 1 week old clove leave oil (B) against *S aureus*, with Gentamycin as the positive control.

The zones of inhibition for essential oil extracted from *Syzygium aromaticum* against *S aureus* and *E coli* was observed.

86

Table 3. Zones obtained for *S aureus* antibacterial activity of Clove bud oil.

	Clove bud oil (Trial 01 mm)	Clove bud oil (Trial 02 mm)	Clove bud oil (Trial 03 mm)	Mean (mm)
Negative control	0.00	0.00	0.00	0.00
Gentamycin	32	35	35	34
Clove bud oil	14	19	18	17

Table 4. Zones obtained for *E. coli* antibacterial activity of Clove bud oil.

	Clove bud oil (Trial 01 mm)	Clove bud oil (Trial 02 mm)	Clove bud oil (Trial 03 mm)	Mean (mm)
Negative control	0.00	0.00	0.00	0.00
Gentamycin	32	35	35	34
Clove bud oil	15	17	16	16

Table 5. Zones obtained for clove leaf oil antibacterial activity against *S aureus.*

	Clove leaf oil (Trial 01 mm)	Clove leaf (Trial 02 mm)	Clove leaf oil (Trial 03 mm)	Mean (mm)
Negative control	0.00	0.00	0.00	0.00
Gentamycin	35	35	35	35
Clove leaf oil	33	23	25	27

Table 6. Zones obtained for clove leaf oil antibacterial activity against *E.coli.*

	Clove leaf oil (Trial 01 mm)	Clove leaf (Trial 02 mm)	Clove leaf oil (Trial 03 mm)	Mean (mm)
Negative control	0.00	0.00	0.00	0.00
Gentamycin	35	35	35	35
Clove leaf oil	43	44	28	38.3

Table 7. Comparison of zone of inhibition in the clove bud oil and clove leaf oil samples.

Samples	Average zone of inhibition (mm)	
	S.aureus total mean value	*E.coli* total mean value
Clove bud oil	17	16
Clove leaf oil	27	38.3

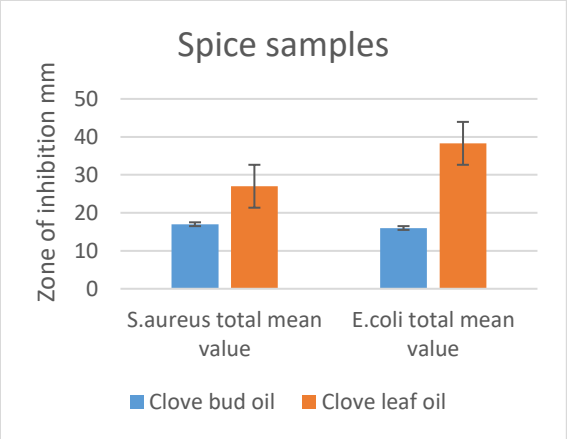


Figure 5. Graph representation of the zone of inhibition in the clove bud oil and Clove leaves oil sample.

3.4 Minimum Inhibitory Concentration

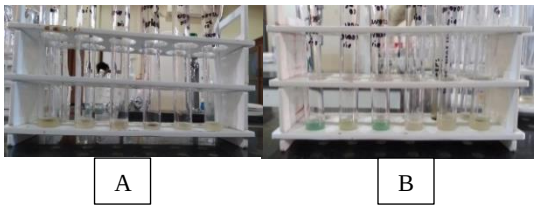


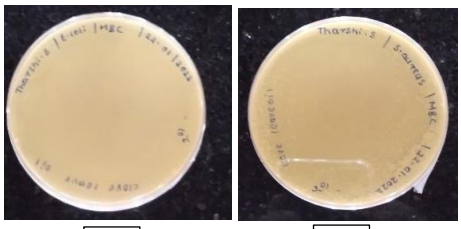
Figure 6. Minimum Inhibitory concentration of *S.aureus* dilutions (A) Minimum inhibitory concentration of *E.coli* dilutions (B).

Control-Nutrient broth and bacteria.

The MIC was observed in 10^{-2} µl/ml concentration in both *S.aureus* and *E.coli* dilutions.

3.5 Minimum Bactericidal Concentration. The clones were not observed in both samples, hence they could be bactericidal

Figure 7. MBC of fresh clove leave oil *S.aureus* (A) and 1 week old clove leave oil (B) against *E.coli*



A B

Figure 8. MBC of fresh clove leave oil *E. coli* (A) and 1 week old clove leave oil (B) against *S aureus.*

4. Discussion

Plant extracts, for example, open a new horizon for the discovery of novel medicinal molecules. Traditional medicine and medicinal herbs are widely used in most before scientific.[22] It has been commonly noticed that countries serve as a normative underpinning for maintaining good health.[23]

As a result, the antibacterial activity of clove bud and leaves was investigated against *E. coli* and *S.aureus* in this study.[24] The practical approach of utilizing disk plate assays to evaluate relative antibacterial activity based on the size of the inhibitory zone is insufficient to determine the antibacterial nature of these spices.[25] A thorough scientific investigation of traditional spices is necessary to ensure that they are effective against a wide range of bacterial species, as this would provide scientific support for their use. The solubility and velocity of diffusion in agar determine the clear zone medium and the process of volatilization.[26]

In the methodology, samples were crushed to increase the surface area. Ethanol was used in the extraction since ethanol is an effective solvent when compared with other solvents, hence can end up with high yield of essential oil.[27] Hydro distillation with powdered clove buds yielded a total amount of 2.50ml, with a return of 6.77%. Clove bud has the highest yield.[28]

Numerous research has been conducted to date in order to assess the antibacterial potential of various essential oils against a variety of microbes. Antibacterial phytochemicals included in essential oils may be useful against bacterial infections.[29] Biologists are reviving the usefulness of spices and condiments through in-vitro and in-vivo research that antibacterial activity, notably in Savita *et al*, 2017 antimicrobial activity of essential oils are used as alternative antimicrobial remedy. (*S.aureus*)[30] and (*E.coli*), can cause super skin infections and life-threatening diseases like endocarditis and sepsis, cholangitis, and urinary tract infection (UTI).[31]Antibacterial susceptibility test (ABST) is extensively used in clinical settings to assess antimicrobial resistance characteristics of test organisms, guide antibiotic treatment options, to identify antibiotic resistance.[32] The Kirby-Bauer test, also referred as disk diffusion method is a classical microbiology technique that is quite well used. The disk diffusion method for determining antimicrobial resistance is likely the most widely utilized, around the world due to its ease, efficiency, and low cost. It was carried out with the use of gentamycin as positive controls.[33]

Tables 3 and 4 of the ABST results for CO showed zones of radius of 17 and 16mm, respectively, which shows similar values in prior research studies carried out by.[34] Fresh clove oil inhibited *S.aureus* and *E.coli* in a synergistic manner. When compared to clove alone, when combination with Gentamycin 34mm.[35]

The presence of tannins, saponins, and essential oils in these spices may contribute to their antibacterial properties.[36] Oils, flavonoids, and phenolic compounds. Even crude extracts of these spices are effective against MDR bacteria that are resistant to current antibiotics. But the antibiotic effect observed in this work was limited. This study cannot predict the effect of these spices on these species in vivo.[37] The

practical approach of utilizing disk plate assays to determine relative antibacterial activity based on the size of the inhibition zone is insufficient to determine the antibacterial nature of these spices.[38] The clear zone is determined by the diffusion velocity and solubility in agar. A comprehensive scientific study of traditional spices is necessary to understand the medium and the volatilization process that results from it.[39] Must guarantee that they are effective against a wide range of bacterial species, and this would give scientific support for their use their effectiveness.[40]

ABST results observed for clove leave oil showed Table 5 and Table 6with zone of radius of 5mm,5mm respectively, which showed similarities with CLSI standard and previous studies. Based on this finding, it may be concluded that leave oil contains more eugenol anti-bacterial than bud oil.[41]

The MIC is the minimum antibacterial quantity that will suppress apparent bacterial growth during an overnight incubation period. MICs are mostly employed in labs to confirm resistance, but they are also frequently used in research in order to assess the in-silico activities of novel antimicrobials, with the results of these studies used to determine MIC breakpoints. The MIC experiments were carried out by essential oils were used in various concentrations, such as 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5}µl/ml.[42] In MIC of *S.aureus* dilutions in 10^{-2} µl/ml the turbidity was reached and *E.coli* dilution 10^{-2} µl/ml the turbidity was reached. It showed certain similarities to earlier research.[43]

The lowest antimicrobial concentration that will prevent an organism from developing following antibiotic-free medium are used for cultures known as the MBC. In tryptone soy agar plates the colonies were not visible, possibly due to bactericidal effect.[44]

5. Conclusion

Research focus on the essential oils of clove bud and clove leaves were extracted by hydro distillation and crude extraction, and the lower yields of essential oils obtained were consistent with previous research. Fresh and 7-day-old extracted essential oils were tested for antibacterial activity, and due to storage lowered activity was observed in both clove bud and clove leave oil extracts.[45] Fresh and 7-day preserved essential oils of clove bud and clove leaves have varying synergistic and antagonistic effects when combined with gentamycin against both bacterial species.[46] The action of the antimicrobial ethanolic extract of clove leaves was highest against both gram negative and gram-positive bacteria. Although all the spices studied have antibacterial properties, the degree of antibacterial activity differs by species. The ethanol extract of clove leaves had the highest activity index 38.3 against *E. coli*. The Design of experiments (DOE) study offered a fascinating and potentially useful low-cost method for combining spice and herb extracts in the ideal ratios to get the most biological impact. Thus, the DOE opened a creative space for the next generation of research and development of the best antimicrobial compounds for the food and pharmaceutical industries.[47]

Acknowledgements

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Factors influencing the employee adoption of e- procurement platforms. Evidence from ABC Bank

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Abstract

Understanding the factors that influence the adoption of electronic procurement platforms has become crucial for successful implementation as e-procurement's significance grows within organisations. The ABC bank is the focus of this study's investigation of the factors influencing the adoption of e-procurement. The research methodology used a questionnaire survey strategy, to gather data from a sample size of 50 employees. Correlation and regression data analysis were conducted on the primary data collected. According to the study's findings, compatibility and perceived relative advantages have a key role in the adoption of e-procurement within ABC bank. The study identified that the e-procurement platforms should be simple as well as compatible with the current system in order to enhance the efficacy of the overall process.

Keywords: e-Procurement, Technology Adoption, Banking

1. Introduction

1.1 Background of the study

With the purpose of enhancing their procurement as well as supply chain business operations, organisations are increasingly adopting e-procurement platforms (Hadiwidjojo, Anggraini, & Subagja, 2021; Sadeghi, Zulkifli, & Abu Bakar, 2021; Zailani, Shaharudin, Iranmanesh, & Tari, 2020; Zhou, Wu, Zhang, & Fan, 2022). Electronic procurement has demonstrated a rising trend of being used in industries as well as in government over the past ten years, with departments in the government lagging behind their industry counterparts (Huang, Zhang, & Xiong, 2020; Song, Park, & Kim, 2021). Business-business, government-business, and government-government domains are few of the areas of the applications that are included under the purview of e-procurement (Chen, Zhang, Cai, & Zhu, 2021). Government organisations differ from private enterprises since the public expects them to purchase goods as well as services that benefit society (Golshan, ShafieiNikabadi, Zarei, & Rahimi-Kian, 2019).

Recent literature indicates the policymakers' persistent influence on government procurement policies. However, new technologies, including e-procurement platforms, have completely changed the way government procurement is carried out, making it more effective, transparent, and cost-efficient (Mahmood, Asghar, Raja, & Asghar, 2021; Ramos, Pires, & Cardoso, 2021; Raza, Arif, Javaid, & Tariq, 2022). In addition, these developments have made it possible for a greater variety of suppliers to engage in government procurement, which has a positive impact on the market by fostering innovation and competition (Mahmood et al., 2021; Raza et al., 2022).

1.2 Organisational Overview

ABC bank is a leading bank governed by the government of Sri Lanka. ABC bank has switched from manual processes to an e-procurement platform to enhance productivity. However, employees of ABC bank who use the e-procurement platform still follow manual processes for sourcing, negotiating terms, purchasing items, receiving and inspecting them as necessary, and documenting each stage of the process.

1.3 Problem Statement

According to Abdulai and Hinson (2018), organisations are now procuring indirect supplies for production and sales using electronic procurement systems by facilitating direct communication with vendors. E-procurement systems shorten the buying cycle and reduce the quantity of paperwork as well as the administrative burden associated with the procurement process. However, some of the employees in the procurement division still prefer the traditional manual procurement procedures, despite the fact that all of them are required to be using the e-procurement system. Furthermore, despite the bank introducing this system to the whole Procurement Division (with a workforce of 150 employees), only a portion of them have expressed any interest in adapting and utilising it. Therefore, it is important to understand why some employees still prefer the manual system over the e-procurement platform.

1.4 Research Aim

The research aim of this study is to evaluate the factors that have an impact on employee adoption of the e-procurement platform at ABC bank.

1.5 Scope

This study surveyed 50 employees of ABC Bank to investigate the factors that influence their adoption of the e-procurement platform.

2. Research Methodology

The systematic sampling method is carried out in this mono method quantitative research. The names of the 150 employees are listed alphabetically with the third number taken into consideration. The sample size was 50.

2.1 Conceptual framework

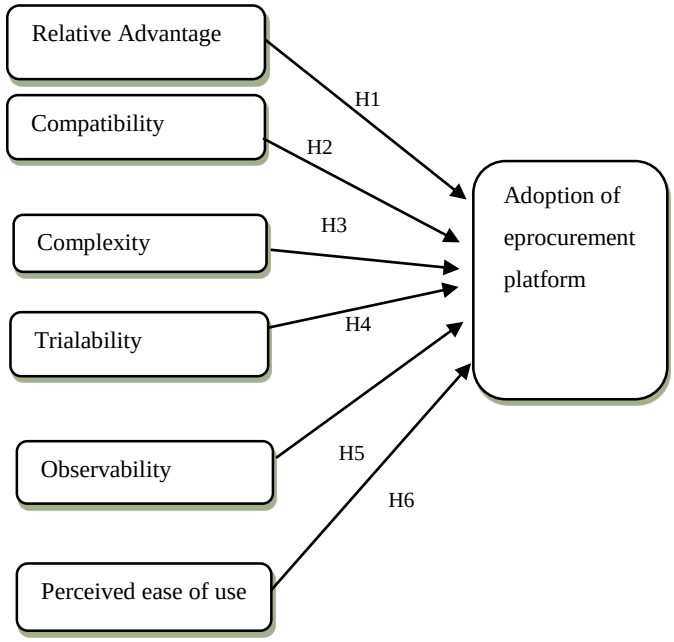

Figure 1. Conceptual framework

2.2 Hypothesis development

The following hypotheses were formulated based on the conceptual framework.

H1-There is a relationship between relative advantage and the employee adoption of e procurement platform.

H2 -There is a relationship between compatibility and the employee adoption of e-procurement platform.

H3-There is a relationship between complexity and the employee adoption of e-procurement platform.

H4-There is a relationship between trialability and the employee adoption of e-procurement platform.

H5 - There is a relationship between observability and the employee adoption of e-procurement platform.

H6-There is a relationship between the perceived ease of use and the employee adoption of e-procurement platform.

2.3 Data Collection

Primary data collection entails getting information directly from the original sources, usually through surveys and questionnaires (Saunders, Lewis, Thornhill, & Bristow, 2015). A printed questionnaire was developed for this study in order to quickly and conveniently gather data.

2.4 Sample Size

To ensure fair representation from various divisions and hierarchical levels, 50 procurement employees were chosen using the systematic sampling method.

2.5 Pilot Study

A pilot test was carried out with a small group of respondents before the questionnaire was circulated. The pilot test was conducted with 10 ABC bank employees. The pilot study was successful and the questionnaire was thereafter circulated to the entire sample.

3. Findings and Discussion

3.1. Response Rate

A 100% response rate was obtained, i.e. all the 50 chosen employees responded.

3.2. Demographic Analysis

The demographic data of the respondents are given in Table 1.

Table 1. Demographic Data

	Description	Percentage
Gender	Male	36
	Female	64
Age	21-30 years old	14
	31-40 years old	52
	41-50 years old	26
	51-60 years old	8
Work Experience	Less than 1 year	10
	1-2 years	12
	3-5 years	42
	6-10 years	24
	11-15 years	10
	More than 15 years	2

The demographic data reveals that more women (64%) participated than men (36%). The age distribution shows a wide range of individuals in different age groups. However, the majority (52%) of the respondents are between 31-40 years. The majority (42%) of the respondents have 3-5 years of work experience.

3.3 Data Reliability

When a method measures something consistently, it is said to be reliable. The measurement is viewed as reliable if the same outcome can regularly be obtained by applying the same techniques under the same conditions**.**

Table 2. Cronbach Alpha

Reliability Statistics	
Variable	**Cronbach's Alpha**
Relative advantage	0.951
Compatibility	0.931
Complexity	0.930
Trialability	0.931
Observability	0.957
Perceived Ease of Use	0.932
Adoption	0.947

The Cronbach's alpha was calculated to evaluate the reliability and the internal consistency of variables. All the variables have Cronbach Alpha values above 0.9, which makes the data highly reliable.

3.4. Correlation Analysis

Table 3. Correlation Analysis

Variables	Correlation Coefficient
Relative Advantage	0.610**
Compatibility	0.664**
Complexity	0.736**
Trialability	0.726**
Observability	0.639**
Perceived Ease of Use	0.703**

Dependent Variable – Adoption
** Significant at the level of 0.05

Table 4. Hypotheses

Hypothesis	Significance	Results
H1	0.000	Accepted
H2	0.000	Accepted
H3	0.000	Accepted
H4	0.000	Accepted
H5	0.000	Accepted
H6	0.000	Accepted

There is a correlation between the independent and dependent variables, if the significance value is less than 5% (0.05). Furthermore, the independent and dependent variables will be positively correlated, if the Pearson Correlation Coefficient is a positive value. The level of correlation based on the Correlation Coefficient is assessed under three categories such as low correlation (0.1 to 0.3), medium correlation (0.3 to 0.5) and strong correlation (0.5-1.0). Accordingly, all the variables of this study are positively correlated and the relationship is statistically significant. Therefore, all the sub-independent variables have a positive correlation with e-procurement adoption.

Ahiaga-Dagbui, Li and Love (2020) found that the adoption of e-procurement is significantly influenced positively by relative advantage, compatibility, and top management support. While the other factors are negligible, technological variety has a detrimental impact. Furthermore, according to Hsieh, Chen and Chen (2018), adoption of e-procurement is positively influenced by relative advantage, compatibility, trialability, trust, and dependency while supplier pressure, observability, and complexity have no apparent effects.

Wang, Wang and Li (2020) found that Relative advantage, Compatibility, Trialability and the Observability have a positive relationship with the adoption of e-procurement. Similarly, Yang, Gu and Zeng (2019) have suggested that there is a strong association between e-procurement adoption and the Perceived Ease of Use. Furthermore, Maresova, Drahosova and Kotaskova (2021) have found that there is a relationship between e-procurement adoption and relative advantage as well as compatibility.

3.5. Multiple Linear Regression Analysis

In the context of Multiple Linear Regression Analysis, the R square value is a statistical measure which assesses the accuracy of the model. As shown in Table 4, the R square value of the regression model is 0.637, which means the model is a good fit.

Table 5. Model Summary and ANOVA

Model Summary	
R square	0.637
ANOVA	
Model	**Significance**
Overall effect	**0.000**

The significance value is 0.000 which reflects that the model is overall acceptable.

Table 6. Significance

Significance	
Sub- independent Variable	**Sig. 5%**
Relative Advantage	**0.039**
Compatibility	**0.029**
Complexity	0.210
Trialability	0.374
Observability	0.304
Perceived Ease of Use	0.990

As shown in Table 6, the multiple linear regression analysis reveals that Relative Advantage and Compatibility are the most significant variables (at 5% Significance level).

3.6 Descriptive Analysis

Table 7 gives the mean values for the statements created for each of the variables.

Table 7. Descriptive Analysis

Relative Advantage	**Mean Value**
I find the e-procurement system is better than following traditional procurement methods.	4.2
My level of efficiency is increased by the e-procurement platform.	4.12
When compared to traditional procurement procedures, the e-procurement platform saves time.	4.5
I find the e-procurement platform as a cost-effective method.	4.48
Compatibility	
My working practices are compatible with the e-procurement platform	4.2
My organisation's other systems and the e-procurement platform integrate well.	3.72
The e-procurement platform is compatible with the requirements of the bank	3.58
The e-procurement platform is compatible with my organisation's culture.	4.02
Complexity	
The e-procurement platform is easy to understand	4.2
The e-procurement platform does not require a lot of effort to learn how to use	3.74
It's easy to learn the e-procurement platform	3.64
I find this e-procurement platform is simple to use	4
Trialability	
I had the opportunity to test the e-procurement platform	4.2
I am permitted to try out the e-procurement platform before committing	3.74
The e-procurement platform has a trial period.	3.68
Testing out the e-procurement platform is easy	3.96
Observability	
I have seen other employees use this e-procurement system	3.78
I have heard good reviews about the e-procurement platform from the other employees	4.24
I believe other employees in the bank are utilising this e-procurement platform	4.14
Perceived Ease of Use	
I find This e-procurement platform as a user-friendly system	4.2
My needs can be easily accommodated by the e-procurement platform because it is adaptable and flexible	3.74
I find it easy to become skillful at using this e-procurement platform	3.7
I find it easy to remember how to use this e procurement platform	4
Adoption	
I am currently using the e-procurement system	3.98
The e-procurement platform is something I expect to use	4.24
The e-procurement platform has been useful to me in my work	4.04

Relative advantage scored an overall mean value of 4.3, which means that the majority of the respondents are satisfied with the relative advantages provided by e-procurement platforms. Compatibility received an overall mean value of 3.9, which means the respondents find the e-procurement platforms compatible. Complexity received an overall mean value of 3.9, which means that the respondents find the e-procurement platforms easy to use. Trialability scored an overall mean value of 3.9, which suggests that trialability is important to the respondents. Observability scored an overall mean value of 4.1, which means that observability is also an important factor. Perceived ease of use received an overall score of 3.9, which means that the majority of the respondents find the e-procurement platform easy to use. Adoption intention received an overall mean score of 4.1, which means the majority of the respondents are willing to adopt the system. Overall, the respondents are satisfied with the e-procurement platform, but there is room for further improvement, since the scores are close to 4. The e-procurment platforms can be further improved to reach a score close to 5.

4. Conclusion

This study has identified seven key determinants which influence employee adoption of the e-procurement platform. This study found that all the sub- independent variables (perceived relative advantage, compatibility, complexity, trialability, observability and perceived ease of use) are positively correlated with the adoption of the e-procurement platform. Furthermore, perceived relative advantage and compatibility are the most significant variables.

These insights can be used by decision-makers to improve the adoption of the e-procurement platform by focusing on the perceived relative advantages and compatibility.

5. Recommendations

Several recommendations can be made to improve the adoption of e-procurement platform at ABC Bank based on the findings of the study. Since there is a significant positive relationship between compatibility and the adoption of e-procurement, ABC Bank should focus on ensuring that the e-procurement platform is compatible with existing processes, systems, and organisational culture. This can be accomplished by meticulously analysing the system requirements and integrating them to the organisational workflow and infrastructure. To enhance compatibility and decrease disruptions during deployment, customisation possibilities and integration capabilities require to be investigated.

The association among relative advantage and e-procurement adoption is significant, which highlights the importance of convincing staff members of the platform's advantages. The management must demonstrate how e-procurement increases effectiveness, decreases costs, boosts accuracy, and accelerates procurement processes. This can be accomplished by implementing focused training initiatives, raising awareness among the employees of ABC bank, and disseminating early adopter success stories and testimonials. Employee adoption of the platform can be encouraged by focusing on the practical advantages of e-procurement.

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Impact of Cost of Capital on Share Price: Evidence from Manufacturing Sector Companies Listed in the Colombo Stock Exchange

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Abstract

Accounting and finance researchers place great importance on capital market and behavioural studies. Investors are looking at these accounting-based capital market researches to observe and use accounting information using primary and secondary data to make investment decisions in the stock market. This study aims to outline important variables that affect share prices in the Colombo Stock Exchange (CSE). This study's primary goal is to ascertain the connection between the Cost of Capital (COC) and share prices of Sri Lankan manufacturing sector businesses in the Colombo Stock Exchange. The dependent variable in this study is the share price, whereas the independent variables are the firm's Cost of Equity (COE), Cost of Debt (COD), and Weighted Average Cost of Capital (WACC), which are all Cost of Capital components. Data was gathered from selected manufacturing sector firms, listed by the CSE. Descriptive, correlational, and regression analysis were employed and the findings showed that manufacturing businesses' Weighted Average Cost of Capital, Cost of Debt and Cost of Equity had a significant association with the share price. Therefore, investors should take these aspects into account before trading and investing funds in shares. The investors should consider the weighted average Cost of Capital and Cost of Equity of a manufacturing sector firm in predicting future trends in share prices in the capital market in order to make productive investment decisions.

Keywords: Cost of Capital, Share Price, Weighted Average Cost of Capital, Manufacturing Sector

1. Introduction

1.1 Background of the study

Financial performance is one of the measurement tools of management for directing a business. Mainly, financial management practices focus on four broader concepts: short-term investment, long-term investment, financing mix, and dividend policy. Companies can select and invest in the best alternative by considering short-term and long-term investment opportunities. Investors' perspectives always accept positive returns on the investment, in which they have already invested (Rasool & Ullah, 2020). To fulfil the above objectives of the two parties, any firm has to follow methods that maximize firm value, while minimizing the overall cost of the organization.

The capital structure shows the allocation of various long-term capital components. These choices have come to be seen as the most crucial ones that a company must make over time, because the firm's Cost of Capital, net profit, earnings per share, dividend pay-out ratio, and the liquidity situation are all impacted by the capital structure. These elements, together with several other variables, combine to influence a company's Share Price (SP). The weighted average of this return, which considers both the Cost of Debt and the Cost of Equity, is known as the COC. This COC is used by businesses as a standard for evaluating operations and placing values on investments (Baker & Wurgler, 2015). A reduced COC, similar to the COE, raises the current value of a business's future cash flows, which can lead to an increased SP. A company cannot continue if the rate of return is not greater than the COC; as a result, shareholders will move on to another company where they can reap greater rewards.

At present, there are small-scale, medium-scale, and large-scale businesses spread in the business world. The capitalization amount decides the capacity of the business, which can expand the business activities. The success of the business can be measured using the value

of the firm as a comparative figure. Most researchers have been investigating the factors which affect the SP of the firm. In general, financial costs are heavily associated with the financing mix of the company. Colombo Stock Exchange has ranked the listed companies giving priority to their market capitalization value. According to the Sri Lankan economy, higher capitalization means the company already financed their resources among various financing sources at an optimum level. Consequently, these companies can create some competition in the business environment. The idea of the SP reflects the strength to change investors' minds about whether to invest or remove the invested fund from the company. Therefore, the study is to examine the effect of COC on SP in Manufacturing Sector Companies (MSC).

1.2 Rationale

The current study will aid in identifying most of the influential factors by evaluating to what extent the COC of the firm influences the market value of the SP. This question should be answered by researching the current status of Manufacturing Sector Companies (MSC). These investigations have all been conducted in affluent nations with cutting-edge trading systems. This isn't the case in developing economies, if there is a high level of capital market imperfections (agency costs and informational asymmetries), inadequate access to internal financing, expensive financing, or inaccessibility to the capital markets. However, minimal attention has been given to this field in the Sri Lankan manufacturing sector. Furthermore, contemporary research in this area in the context of Sri Lanka has been more difficult to identify.

1.3 Research Aim & Objectives

The aim of this research is to examine the impact of the COC on Share Price with reference to the Manufacturing Sector Companies listed on the Colombo Stock Exchange.

These are the specific objectives of this study:

1. To identify the determinacies of COC and SP of a company.
2. To study the relationship between COC and Share price in the manufacturing sector.
3. To draw conclusions based on results and make relevant recommendations.

1.4 Scope

There are 289 companies listed in the CSE. 36 Manufacturing sector companies have been randomly selected for this study. The study's scope includes, how the COC's components of WACC, COE, and COD affect the SP in listed companies in the manufacturing sector.

2. Research Design

2.1 Theoretical Framework

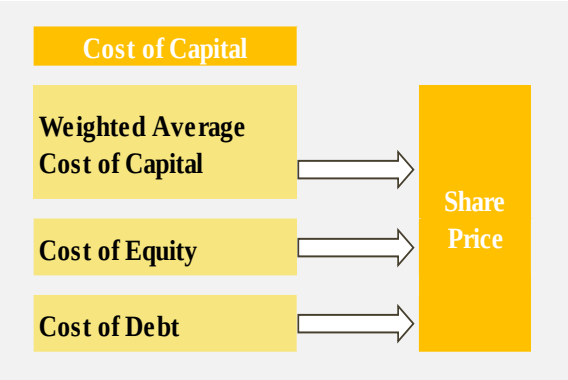

Figure 1. The Conceptual Framework.

WACC, COE, and COD of the firm are the independent variables in this study, and the dependent variable is the SP of the firm.

2.2 Hypothesis

According to Rashidirad, Soltani, and Syed (2013), the three main categories of hypotheses are positive pricing effect, negative price effect, and no-price effect. To accomplish the stated goals of the study, this research instead creates the following hypotheses based on the literature.

H1 – There exists an association between WACC and SP.

H2 – There exists an association between COE and SP.

H3 – There exists an association between COD and SP.

2.3 Methodology

2.3.1 Research Method

The quantitative mono method has been chosen as it best fits the nature of this investigation. The purpose of choosing the mono-method quantitative research method is to better understand the relationships, between the variables of this study. A survey is used as the research strategy.

2.3.2 Population and Sampling

The target population of the study is financial professionals (Finance, Accounting and Audit related staff employees) who are currently working in MSC in the listed companies in the CSE. MSC-listed companies were alphabetised and 12 companies were randomly selected. A minimum of 3 financial professionals from each company were selected from each company (including managers, executives, and staff employees). A survey requires a minimum of 30 responses in order to be valid, the data for this study was gathered using 36 responses from the listed manufacturing businesses.

2.3.3 Data Collection

A questionnaire was used to gather primary data, and the questionnaire was distributed among the randomly selected, listed manufacturing sector companies in the CSE.

2.3.4 Data Analysis

The statistical software for the research data analysis, SPSS and Microsoft Excel were used to analyse the information gathered from the sample of respondents.

2.3.5 Pilot Study

The researcher conducted a pilot survey with 16 employees of randomly selected companies. The pilot study was successful base on the respondents' feedback.

3. Analysis and Findings

3.1 Demographic Data

As shown in Table 1, most of the respondents were executives and associate analysts. 86% of the respondents were currently working in accounting or finance divisions. 39% of the respondents have an educational qualification of a Master's degree with more than 6 years of professional experience.

Table 1. Demographic Data

Demographic Characteristics		Response Frequency	As a percentage	Cumulative Percentage
Designation	Manager Level	6	16.7%	16.7%
	Executive Level	18	50.0%	66.7%
	Associate Analyst	9	25.0%	91.7%
	Assistant	3	8.3%	100.0%
Department	Finance	13	36.1%	36.1%
	Accounting	18	50.0%	86.1%
	Audit	5	13.9%	100.0%
	Supply chain	0	0.0%	0.0%
Year of Experience	0 - 2	4	11.1%	11.1%
	3-5	7	19.4%	30.5%
	6-10	12	33.3%	63.8%
	11-above	13	36.1%	100.0%
Highest Education Qualification	Masters	14	38.9%	38.9%
	Bachelors Degree	12	33.3%	72.2%
	Diploma / HND	2	5.6%	77.8%
	Charted Accounting	8	22.2%	100.0%

3.2 Overview of COC

Descriptive statistics were calculated for three independent variables and the study's dependent variable. Respondents were asked to indicate their answers on a 5-point Likert scale and the mean value and standard deviation were calculated.

3.3 Relationship between COC and SP

Correlation analysis was done to determine the relationship between the independent variables of WACC, COE and COD; and the dependent variable of SP. In this study, correlation analysis and bivariate analysis are conducted through SPSS 25 to measure the relationship between independent variables such as WACC, COE and COD, and the dependent variable, SP.

Table 2. Correlation Statistics

Correlations	WACC	COE	COD	SP
WACC	1			
COE	.384*	1		
COD	0.264473	.712**	1	
SP	.532**	.544**	.487**	1

**. Correlation is significant at the 0.05 level (2-tailed).*
***. Correlation is significant at the 0.01 level (2-tailed).*

According to Table 2, the Pearson Correlation of WACC is 0.532 which reveals a positive moderate relationship between share price and the WACC. COE shows a Pearson correlation of 0.544 which is also a moderate relationship between share price and COE. Accordingly, the Pearson correlation of COD reflects 0.487 which shows a positive moderate relationship between the COD and share price. However, the P value of the WACC and Share Price is 0.001 which reveals the relationship is significant. The P value of the COE and share price is 0.001 and the same reflects a significant relationship. Finally, the COD and share price also reflects a significant relationship as the P value is 0.003. The accepted level of P value to identify a relationship between two variables as significant is 0.05.

The results of the current study show a positive association between WACC and share price, which essentially suggests that a firm's WACC would positively affect the share price. The research results of Bhattarai (2015); Malhotra and Tandon (2013); Enow and Brijlal (2016); Sukhija (2014); Hossain, Ibrahim and Uddin, (2020) corroborate this conclusion. Additionally, the current study finds that WACC is a significant component in MS companies and findings from Geetha and Swaaminathan (2015); Sukhija (2014); Hossain, Ibrahim and Uddin, (2020) lend credence to this conclusion.

The findings suggest that the firm's COE will have a positive impact on the share price. The results of Bhattarai (2015); Malhotra and Tandon (2013); Arshad et al., (2015); Enow and Brijlal (2016); Sukhija (2014) are in favour of this conclusion. Additionally, their research indicates that the current study's COE is an important determinant. However, it was determined by Hossain, Ibrahim and Uddin, (2020) that the COE is not that significant to publicly traded companies.

This study found a positive association between COD and the share. This outcome suggests that the firm's COD will have a favourable impact on the share price. The findings of Bhattarai (2015); Valta (2012) corroborate this conclusion.

3.4 Impact of COC on SP

Regression analysis is used to evaluate the association among one or more independent variables and dependent variables (McLeod, 2019). Mainly regression analysis helps to identify the impact of WACC, COE, and COD on Share price.

Table 3. Model Summary

Model Summary[b]				
R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
.663[a]	0.44	0.388	0.35124	1.39

a. Predictors: (Constant), COD, WACC, COE
b. Dependent Variable: SP

The hypothesis that the residuals are not linearly auto-correlated is tested using Durbin-Watson's and values should typically be 2 to demonstrate the absence of auto-correlation in the data. This study's Durbin-Watson value is not equal to 2, which means there is no auto-correlation.

The explanatory power of the regression analysis, or R-square value, shows how well the dependent variable is explained by the independent variables. R-square values below 0.2 are regarded as poor explanations, between 0.2 and 0.4 as moderate explanations, and over 0.4 as a strong justification. This study's R-square is 44%, which means SP is explained at 44% by the chosen 3 variables of COC.

Table 4. ANOVA table

ANOVA[a]	Sum of Squares		Mean Square		Sig.
Regression	3.104	3	1.035	8.387	.000[b]

a. Dependent Variable: SP
b. Predictors: (Constant), COD, WACC, COE

Table 4, indicates an overall < 0.05 significant level, which means it has an overall impact of WACC, COE, and COD on SP in the manufacturing sector listed companies. The second objective of this study is satisfied from this result but the past scholars stated that there is no association of COC and SP in the service sector (Sondakh, 2019; Michael, 2016; Al-Shawawreh, 2014).

Table 5. Coefficient statistics

	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	0.378	0.71		0.532	0.598
WACC	0.403	0.151	0.381	2.662	**0.012**
COE	0.239	0.19	0.248	1.262	0.216
COD	0.233	0.209	0.21	1.114	0.274

a Dependent Variable: SP

According to Table 5, there is only one dimension that has a <0.05 significant value, which means only WACC has a relationship with SP since the significant value is 0.012. A association between WACC and SP has been proven by Jahfer and Mulafara (2016) and Asif, Arif and Akbar (2016) also supported this association from their empirical research conducted in the Pakistan stock exchange. Some past scholars Almumani, (2014); Vijitha and Nimalathasan, (2014) stated that there is no relationship between WACC and SP.

3.5 Hypothesis Testing

Table 6. Hypothesis test results

Variable	Test Value	Test Result
WACC	0.000	H_1 : Accepted
COE	0.000	H_2 : Accepted
COD	0.000	H_3 : Accepted

H_1; There is a relationship between WACC and SP

The Sig. value of the two variables is 0.000 and the relationship between the two variables is significant. Therefore, Hypothesis 1 is acceptable which means there is a relationship between WACC and SP. This outcome is supported by Renaldi, Pinem and Permadhy, (2020). Their study shows that a greater WACC can increase a firm's market worth. The cost of equity and the cost of debt are typically included in WACC. Some capital structure theories contend that WACC harms business value. But the point is that the majority of businesses get money through debt and equity. The demand for shares or any other securities increases, if the firm pays investors a higher value. Consequently, the company's SP rises on its own depending on the country’s economy, industrial changes, organisation background and other external factors. The WACC results are in contradiction with the study by Al Salamat and Mustafa, (2016). which shows that the COC and the capital structure are adversely correlated with the SP.

H_2; There is a relationship between COE and SP

According to the analysis performed in this study, the Sig value is 0.000, which means Hypothesis 2 is acceptable. The higher the equity, the lower the firm's chance of insolvency, suggesting superior financial soundness. These results support Jahfer and Mulafara, (2016) findings, which showed that the COE was significantly positively related to

the SP. Investors utilise high equity capital to determine the overall indicator of a company's financial health, decide which companies to invest in, and arrive at investment decisions. As a result, SP will rise, however, the foreign exchange fluctuations, government rules and regulations regarding the tax, and political instability affect the Sri Lankan economy and have a significant impact on the SP in listed companies in CSE.

H_3; There is a relationship between COD and SP

According to the analysis performed in this study, the Sig value is 0.000, which means, Hypothesis 3 is acceptable.

4. Discussion & Conclusion

4.1 Discusion

The three primary theories in the field of financial management are COC, Capital structure, and SP. Numerous ideas and influences exist, and these theories serve as a basis for understanding or making the best attempts to attain an ideal capital structure. However, financial management's position is always evolving, and this makes money management tasks more significant than just being a fundraiser. The ultimate goal of financial management is to increase owners' wealth, which is represented by the company's market value.

Additionally, the data analysis results showed a favourable correlation between the firm's share price and its WACC, COE, and COD. This suggests that the variables are positively correlated and related to each other and WACC, COE and COD have a significant relationship with the SP in manufacturing sector listed companies. This study found that SP is mainly affected by the debt-to-equity ratio when considering the factors of WACC. Since the equity increases, the debt-to-equity ratio also increases, which will have a favourable impact on the firm’s equity capital and SP, COE and COD have a significant impact on SP. The arguments favour accepting Hypotheses 1, 2 and 3. Finally, it can be deduced that there is a positive association between the debt-to-equity ratio (WACC) and the share price of firms in the manufacturing sector listed companies in Sri Lanka.

Previous studies have discovered that elements other than financial management actions have a substantial impact on the firm's SP. Sound business decisions should essentially increase the SP value of the company. The 12 manufacturing companies that were listed on the Colombo Stock Exchange as of April 30, 2023, were used in the study to determine the behaviour of the association between WACC, COE, COD and SP. The data analysis results indicate a favourable correlation between independent factors and a company's share price. The majority of firms, according to the data gathered, have already given the WACC, COD, and COE some thought. As a result, some businesses must pay excessive costs for their financing sources.

The CSE has 289 companies representing 19 GICS industry groups as at 30th April 2023. Out of the total population of 32 manufacturing companies the researcher only obtained data from 12 companies’ financial professionals, this means that data for 20 companies was not used in the study. This might have an impact on the validity of general population-related study outcomes.

It was proposed that the current study may be expanded to include longer periods by future research, increase firm selection and increase independent variables. The linear relationships between the variables were the main emphasis of this study, however non-linear correlations between the variables might potentially be included in future research. Additionally, other multivariate statistical forecasting models might be used to confirm the findings in future studies.

4.2 Conclusion

Finance officers of publicly traded firms in the manufacturing sector should think about measures to boost a company's profitability by considering the weighted average cost of capital, since the study has found that there is a significant association between the debt-to-equity ratio and the firm’s share price. When the firm’s equity is getting high the debt-to-

equity ratios also become high which directly impacts the firm's equity capital according to this study's findings. The investors pay attention to this factor which directly impacts the company's SP increase.

Based on the results of this study, it is recommended that investors should consider the weighted average cost of capital and Cost of Equity of a manufacturing sector firm in predicting future trends in share prices in the capital market and making productive investment decisions. In addition to the firm's WACC and debt-to-equity ratio, investors should be aware of the book value of the share, dividend cover, return on assets, and return on equity, since investors may be assured of safer investments as a result.

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Impact of product innovation on consumer brand loyalty: A study based on the Sri Lankan Tea Industry

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Abstract

Understanding the consumer perceptions on brand innovation is crucial as these perceptions of consumers impact the brand loyalty of consumers. Therefore, the purpose of this study is to identify the impact of product innovation on consumer brand loyalty in the Sri Lankan tea industry. This study identified that product quality, product variety and product price are the most common factors that have an influence on brand innovation, which impacts the loyalty of consumers when purchasing tea products of different brands. An online survey online was conducted among 100 individuals who live in the city of Colombo, Sri Lanka using a structured questionnaire to gather data quantitively. The study revealed that product quality and product price have a strong positive correlation with consumer brand loyalty, while product variety did not.

Keywords: Product Innovation, Brand Loyalty, Tea Industry

1. Introduction

1.1 Background to the Study

Companies are using innovation in the modern business world in an effort to survive and prosper in the long term (Thongsri & Chang, 2019). In this sense, companies use innovation to gain an edge over rivals in the consumer market. Larsson (2017) claimed that product innovation refers to how much a product is viewed as innovative by consumers. Winarti et al. (2021) stated that innovation could be described as the successful creation and delivery of distinctive products of high quality at a reasonable price. As per Thongsri & Chang (2019) companies must maintain high levels of efficiency and quality control through innovation in order to acquire sustainable competitive advantage in the industry.

Understanding consumer perceptions of brand innovation is crucial because these perceptions have an impact on consumer brand loyalty (Shahidan, 2020). According to Mao (2010), consumer brand loyalty refers to the preference for a particular good or service. Fernandes & Moreira (2019) define customer brand loyalty as a strong desire to continuously buy a favourite good or service. A consumer's brand loyalty is often influenced by factors such as customer satisfaction, product trust, brand image, quality and brand trust (Tabish et al., 2017).

Brands must concentrate on continuous innovation to increase consumer brand loyalty, and to remain competitive in order to capture a larger market within the industry. According to Tabish et al. (2017), customer satisfaction, product trust, brand image, quality, and brand trust all have an impact on consumers' purchasing decisions and brand loyalty. Therefore, investigating the connection between product innovation and consumer brand loyalty is crucial. Additionally, according to Winarti et al. (2021), product innovation helps companies to maintain a competitive advantage by drawing customers in through brand loyalty. Thus, one of the main goals of strategic marketing is to increase brand loyalty among consumers in order to gain a competitive edge (Shahidan, 2020).

1.2 Industry Overview

According to Ranasinghe et al. (2017), Sri Lanka's tea industry is vital to the nation's economic growth, due to the fact that Sri Lanka's tea industry is a major contributor to the country's employment generation and export revenue. As per Thasfiha et al. (2020), Ceylon Tea, the name given to Sri Lankan tea, is well-known throughout the world and held in high esteem for its exceptional quality, fragrance, and flavour. According to Kasturiratne (2015), Sri Lanka is the third-largest producer of tea worldwide. According to the Tea Exporters Association (2022), the amount of tea produced in Sri Lanka in February 2022 was 18.16 million kg, a decrease of 4.36 million kg from February 2021's output of 22.52 million kg. This is due to the fact that

Sri Lanka's tea industry is hampered by a number of problems, such as declining tea cultivation, high production costs, and a lack of labour (Thasfiha et al., 2020).

1.3 Rationale

The purpose of this study is to examine product innovations in the Sri Lankan tea industry, which has recently embraced many innovative trends. As a result, according to Heany and Koidis (2018), there are many tea brands available today, including flavoured teas with flavours like lemon, peach, cinnamon, lavender, etc. Additionally, according to Sacthivel and Goonasekere (2020), there are tea products in Sri Lanka that are used for a variety of things, including teas that aid in weight loss like green tea, matcha, and black tea. Additionally, there are numerous herbal teas made from herbs, spices, infusions, and decoctions of herbal plant material, including chamomile, peppermint, and ginger tea (Ravikumar, 2014).

Nevertheless, according to Bhattacharjee (2015), each of these teas has particular health benefits, including those for digestion, cleansing, and weight loss. Due to the high levels of antioxidants, minerals, and vitamins in these teas, people can relax, recover, and feel refreshed mentally while also living a healthy lifestyle. Since there are many different tea brands available in Sri Lanka, conducting this study is important because brand loyalty is essential for retaining customers. Although, companies spend a lot of money on product innovation to keep and win over customers, uncertainty still exists regarding the contribution of innovative products to increased brand loyalty. Numerous studies on product innovation and consumer brand loyalty have been conducted, but none of them have specifically focused on the Sri Lanka's tea industry. Hence, it is crucial to look into how brand loyalty is affected by product innovation in the Sri Lankan tea industry.

1.4 Research Aim

The aim of this study is to examine the impact of product innovation on consumer brand loyalty in the Sri Lankan tea industry.

1.5 Scope of the Study

A survey was conducted from a sample of 100 people who consume tea and reside in the city of Colombo.

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.

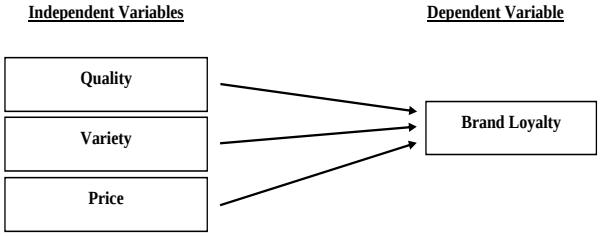

Figure 1. Conceptual Framework.

2.2 Research Hypothesis

The research hypotheses formulated based on the conceptual framework are given below.

H1: There is a relationship between innovation in quality and brand loyalty

H2: There is a relationship between innovation in variety and brand loyalty

H3: There is a relationship between innovation in pricing and brand loyalty

2.3 Operationalisation Table

The operationalisation table with the variables and the corresponding measures are given in Table 1.

Table 1. Operationalization Table

Variable	Measures
Quality	• Quality of innovative tea products • Competitiveness with other brands
Variety	• Variety of innovative tea products • Variety of innovative flavoured teas • Variety of innovative herbal teas • Variety of unique tea products
Price	• Variety of pricing options • Affordability • Good value for money
Brand loyalty	• Willingness to continue purchasing • Satisfaction • Recommendation

2.4 Population and Sampling

The Colombo city of Sri Lanka has a population of 2,480,000 individuals (Registrar General's Department, 2021). The sample size is 100 individuals. The study used the non-probability convenience 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 structured questionnaire.

2.6 Data Analysis

The data was analysed using SPSS software using correlation and multiple linear regression analysis.

3. Findings and Discussion

3.1 Response Rate

In this study, 95 out of the 100 respondents who received the questionnaire responded to it. Therefore, the total response rate is 95%.

3.2 Demographic Details

Table 2 provides the summary of the demographic data of the respondents.

Table 2. Demographic Details

Age	
18-20	23%
21-30	54%
31-40	20%
More than 41	3%
Gender	
Male	53%
Female	47%
Employment status	
Student	6%
Employed	92%
Retired	2%
Monthly income	
Less than 30,000	14%
30,000-50,000	44%
50,000-80,000	13%
More than 80,000	29%

3.3 Cronbach's Alpha Test

Cronbach's alpha test was used to evaluate the reliability of the primary data collected. The results are presented in Table 3.

Table 3. Cronbach's alpha Test

Variable	Cronbach's alpha	Reliability
Quality	0.959	Very Good
Variety	0.826	Very Good
Price	0.748	Good
Brand loyalty	0.684	Good

As per the reliability test results shown in Table 3, it can be concluded that all the variables can be considered reliable, since they are above 0.6.

3.4 Correlation Analysis

The results of the correlation analysis are given in Table 4.

Table 4. Correlation Analysis

Independent Variable	Pearson Correlation Coefficient	Significance (5%)
Quality	0.597	0.000
Variety	-0.003	0.979
Price	0.852	0.000
Dependant variable – Brand Loyalty		

As shown in Table 4, at 5% significance level both quality and price have a strong positive correlation with brand loyalty. However, it can be concluded that variety does not have a correlation with brand loyalty.

3.5 Hypotheses Validation

Based on the correlation analysis, the hypotheses can be validated.

H1: There is a relationship between innovation in quality and brand loyalty

H1 is Accepted

According to Rahmawati and Sentana (2021) product quality has a positive impact on consumer brand loyalty. Also, product innovation has helped to improve product quality, which has benefitted organisations by increasing loyal customers due to brand loyalty (Guthrie, 2021).

H2: There is a relationship between innovation in variety and brand loyalty

H2 is Not Accepted

As per Yuen and Chan (2010), even though consumers could have various preferences they might not care about product variety when it comes to purchasing. Hence, even if there is no variety in products, consumers would still continue to be brand loyal. As for them, the quality and price of a product could be the main factors that would drive them to make a purchase decision (Sonntag, 2022).

H3: There is a relationship between innovation in pricing and brand loyalty

H3 is Accepted

According to Bucklin, Gupta and Siddarth (1998) and Albari and Safitri (2018) price has a significant impact on consumer choice and purchase frequency as the purchase decisions of consumers are influenced by price. Thus, price impacts on brand loyalty, as price influences a consumer's purchasing power which in return could make a consumer brand loyal (Decker, 2022).

3.6 Multiple Linear Regression

Based on the multiple linear regression analysis given in Table 5, at 5% significance level, quality and price have a significant relationship with brand loyalty. However, variety has no significant relationship with brand loyalty.

Table 5. Multiple linear regression analysis

Independent variable	Beta	Significance (5%)
Quality	-0.395	0.000
Variety	0.009	0.845
Price	0.852	0.000

3.7 Descriptive Analysis

The descriptive analysis results based on the responses to questions regarding tea consumption and innovation are summarised in Table 6.

Table 6. Tea consumption and innovation

How long have you been consuming tea?	
Less than a year	3%
1 to 5 years	1%
6 to 10 years	6%
More than 10 years	90%
How many times a day do you drink tea	
Once a day	13%
Twice a day	49%
3 times a day	14%
More than 3 times a day	24%
Do you find the tea industry innovative?	
Yes	83%
No	17%

As shown in Table 6, the majority of the respondents (90%) have been consuming tea for a very long period of time (more than 10 years). 49% of the respondents consume tea at

least twice a day. The vast majority (83%) of the respondents consider the Sri Lankan tea industry to be innovative.

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

Quality	**Mean**
My favourite brand's quality of innovative tea products are excellent	4.05
My favourite brand's innovative tea products are of high quality	4.15
My favourite tea brand's innovativeness quality is on par with any other brand	4.87
Overall	4.37
Variety	**Mean**
My preferred brand offers me a wide variety of innovative tea products	3.58
My preferred brand offers me a wide variety of innovative flavoured teas	4.17
My preferred brand offers me a wide variety of innovative herbal teas	4.08
My preferred brand offers me a wide variety of unique tea products	4.07
Overall	3.97
Price	**Mean**
My preferred brand offers me a wide variety of pricing options	4.93
My preferred brand offers me affordable tea products	3.93
My preferred brand offers me good value for money	4.17
Overall	4.34
Brand loyalty	**Mean**
I plan to continue purchasing tea products from my preferred brand	4.62
I am satisfied with the purchases that I have made from my favourite brand	3.93
I can recommend my favourite brand of tea to my friends and family	4.33
Overall	4.29

Table 7 shows that the majority of the respondents agree with the statements given, since the mean values are close to 4 (Agree).

In terms of the quality, the overall mean value is 4.37, which implies that the respondents are satisfied with the quality of the innovative tea products.

In terms of variety, the overall mean is 3.97, which means that the respondents are satisfied with the variety of innovative tea products available in the market.

In terms of price, the overall mean is 4.34, which means the respondents are satisfied with the pricing as well.

In terms of brand loyalty, the overall mean is 4.29, which means the respondents are brand loyal.

4. Conclusion

In conclusion, when considering the responses from the survey, the findings of this study indicate that there is a significant relationship between price and quality of innovative tea products and consumer brand loyalty. However, this study found no correlation between the variety of innovative tea products and brand loyalty. This finding can be explained by the fact that the majority of Sri Lankans prefer black tea, and their brand loyalty is not impacted by the availability of other innovative tea product varieties, as long as the quality and price of black tea is to their satisfaction. Thus, companies need to ensure that the quality of black tea is of a high standard and affordable to the average consumer to ensure brand loyalty.

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Factors Affecting Financial Planning for Retirement using Provident Funds in the Private Sector

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Abstract

This study investigates the factors that influence the financial retirement planning of private sector employees who utilize provident funds. The key findings of this study reveal that attitudes towards financial retirement planning, accessibility to information, risk tolerance, financial knowledge, and the willingness to engage in retirement planning significantly impact the process. A considerable number of participants expressed positive views about financial retirement planning, indicating an increasing awareness of its value and potential benefits. The availability of information was generally good, with individuals receiving advice from various sources. Furthermore, the study found that the presence and the number of dependents, as well as educational levels, influenced participants' perceptions of uncertainty in investment decisions and their commitment to retirement planning. Individuals without dependents tended to agree more with uncertainty, suggesting a higher propensity for risk-taking. University graduates exhibited higher levels of uncertainty but also displayed a stronger dedication to planning for retirement. These findings emphasize the importance of personalized financial advice that considers family dynamics and educational backgrounds to cater to the unique retirement planning needs of individuals.

Keywords: Retirement planning, Provident Funds, Private Sector

1. Introduction

1.1 Background of the study

Financial retirement planning is essential for a pleasant and secure retirement. It entails making financial decisions and taking steps to build up assets and income that will sustain your lifestyle once you stop working. Human lifespan has increased, and the proportion of retirees to those of working age is growing. Social security policy is increasingly focusing on pensions in the unofficial sector. As a result, as the population ages, there will be a greater demand for social security or retirement programs. Employers, self-employed individuals, employees in the public sector, private sector employees, and contributing family members make up Sri Lanka's labour force. Employees in Sri Lanka's public/private sector are required to retire at age 60.

Table 1. Growth of Sri Lanka's elderly population – 2001-2040

	2000	2020	2040 (projected)
Old Age Dependency	10%	18%	27%
Dependency (both the young and the old as shares of the remaining population)	55%	63%	76%

Source: De Silva, (2003); Gamaniratne, (2007)

According to De Silva's (2003) projections, the proportion of individuals aged 60 and above in Sri Lanka, denoted as the old age dependency ratio, is anticipated to rise from its current level of 10 percent to a 20 percent share by the year 2020. This stands in contrast to the South Asian average of 13 percent. Furthermore, it is forecasted that by 2040, nearly one-third of Sri

Lanka's population will fall within the 60 and above age bracket.

When a person who has worked for a long time decides to leave their job permanently, that stage of life is known as retirement. It is a period of change in life that may be joyful for some people but painful for others. In the area of social security, retirement planning and confidence have grown to be major global concerns (Helman, et al., 2015). Planning for unknown future expenses is necessary to prepare financially for retirement while maintaining a good mind set (Hanna, et al., 2016). People who are financially literate enough will make plans for their savings and achieve financial independence in time for retirement (Azwadi, 2013). The kind of information that the public needs and can understand about pension funding varies, according to Fan, Stebbins, and Kim (2021).

Promoting long-term financial sustainability may be more successful when behavioural change is seen to have an impact on both human and financial behaviour, particularly in terms of spending and saving ideas (Turnham, 2010). Any human conduct related to money might be considered financial behaviour. According to empirical studies, financial behaviour and financial literacy are strongly correlated in the US (Hershey & Mowen, 2000). People need to understand fundamental financial concepts like interest, inflation, and risk management in order to maintain healthy financial habits. Financial literacy is associated with greater retirement wealth among those who are approaching retirement, according to various studies (Afthanorhan et al 2020; Bateman, et al., 2018; Lusardi & Mitchell, 2007).

In Sri Lanka, research on retirement planning is lacking. Retirement planning involves setting aside sufficient money for retirement. Having access to 60-80% of one's yearly preretirement income throughout retirement years is considered to be having enough retirement wealth (Hanna, et al., 2016).

1.2 Research problem

Most nations have their own retirement plans that differentiate between workers in the public and private sectors (Kim & Hanna, 2015). Public sector workers are given a monthly retirement pension. However, private sector employees are not covered by this program. Most Sri Lankans prefer to put retirement planning last because they think they can rely on their Employees Provident Fund investments. Studies related to what influences how Sri Lankan Private Sector employees approach their retirement planning are limited.

1.3 Research Aim

The aim of this study is to investigate the factors influencing retirement planning behaviour in the Sri Lankan Private sector.

1.4 Scope

The study examines the attitudes, financial knowledge, and risk tolerance of provident fund-contributing employees in the private sector, as it relates to retirement financial planning.

1.5 Significance

This study will help workers who are approaching retirement age discover investment instruments that are available for retirement planning and assess if the returns from these assets are sufficient for their retirement. This study will be very useful to insurance and financial firms who are creating retirement solutions,since it will enable them to comprehend retiree needs and design their services accordingly. Also, this study may help all EPF and ETF contributors in general better grasp how to handle their EPF and ETF funds for effective retirement investment.

2. Research Method

2.1 Conceptual Framework

The conceptual framework for this study is given below.

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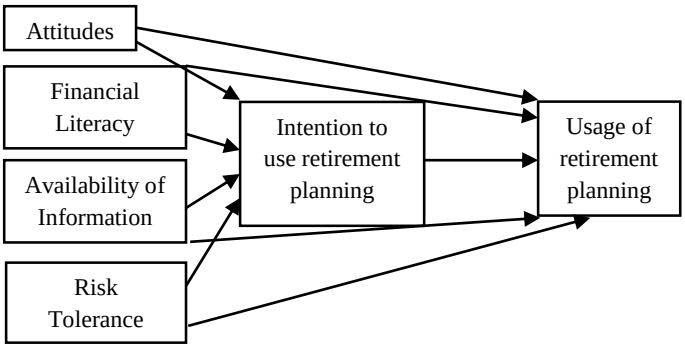

Figure 1. Conceptual Framework

2.2 Hypotheses

H1 – There is a relationship between attitudes and usage of retirement planning

H2 - There is a relationship between financial literacy and usage of retirement planning

H3 - There is a relationship between availability of information and usage of retirement planning

H4 - There is a relationship between risk tolerance and usage of retirement planning

H5 - There is a relationship between intention to use retirement planning and usage of retirement planning

H6 – There is a relationship between attitudes and the intention to use retirement planning

H7 – There is a relationship between financial iteracy and the intention to use retirement planning

H8 – There is a relationship between the availability of information and the intention to use retirement planning

H9 – There is a relationship between risk tolerance and the intention to use retirement planning

2.3 Data Collection

A structured online questionnaire was created using Google Forms to collect primary data. The questionnaire consisted of 3 sections with a total of 28 questions.

2.4 Population and Sampling

Employees aged between 40 and 50 years and working in the private sector in the Colombo area make up the population of this study. Purposive sampling was used to select a total of 100 suitable respondents.

2.5 Data analysis

SPSS software and Microsoft Excel were used for the data analysis. Descriptive statistical analysis and inferential statistical analysis (correlation and regression analyses) were conducted.

3. Findings and Analysis

3.1 Response rate

The author personally distributed and collected 100 questionnaires, achieving a commendable 100% response rate.

3.2 Demographic Data

Table 2 gives the demographic data of the respondents.

Table 2. Demographic Data

Demographic Question	Elements	Percentage
Gender	Male Female	50% 50%
Highest Educational Level	Primary Secondary Tertiary	10% 41% 49%
Monthly individual income	Less than 50,000 50,000–100,000 100,001-500,000 Above 500,000	24% 37% 32% 7%
No of Dependents	None 1 to 4 5 to 10	25% 65% 10%
Occupational	Executive	46%

Level	Non-Executive Managerial level	17% 37%

The majority of the respondents have a tertiary education, earn 50,000 to 100,000 rupees, have 1 to 4 dependents and work at an Executive level. Both genders are equally represented in the sample.

3.3 Reliability Analysis

Table 3. Cronbach's Alpha

Variable	Cronbach's Alpha
Attitudes	0.762
Financial Literacy	0.752
Availability of Information	0.746
Risk Tolerance	0.741
Intention to Use Retirement Planning	0.761
Usage of Retirement Planning	0.760

Based on Table 3, The results of the test revealed that the six variables have a high level of internal consistency, since the alpha values are above 0.7.

The high level of internal consistency enhances the validity and accuracy of the results, instilling greater confidence in researchers that the instrument adequately captures the factors impacting retirement planning.

3.4 Correlation Analysis

Table 4 gives the Significance (p Values) and the Pearson Correlation Coefficients between the independent and dependent variables.

Table 4. Significance (p-Values)

Hypothesis	Sig. (5%)	Pearson Correlation Coefficient	Results
H1	0.646	0.258	Rejected
H2	0.755	0.274	Rejected
H3	**0.000**	**0.436**	**Accepted**
H4	0.356	0.353	Rejected
H5	**0.000**	**0.533**	**Accepted**
H6	0.536	0.414	Rejected
H7	0.879	0.136	Rejected
H8	0.598	0.160	Rejected
H9	0.335	0.633	Rejected

Based on the correlation analysis results shown in Table 4, the following conclusions can be made.

H3 – "There is a relationship between availability of information and the usage of retirement planning" is valid at 5% Significance Level.

This finding aligns with the research of Duflo and Saez (2003), which emphasized the importance of information and social interactions in retirement planning decisions. This results suggests that the accessibility of information significantly influences the usage of retirement planning among private sector employees in Colombo.

H5 – "There is a relationship between intention to use retirement planning and usage of retirement planning" is valid at 5% Significance Level.

This outcome corresponds to a study by Bongini and Cucinelli (2019), that highlighted the significance of early retirement planning intention, particularly among college students. It suggests that a favourable intention to utilize retirement planning is likely to translate into

actual usage, as observed within the sample of private sector employees in Colombo.

Attitudes, financial literacy and risk tolerance did not have a correlation with the intention to use or the usage of retirement planning.

3.5 Impact of retirement planning factors on the retirement planning behaviour

Table 5. ANOVA

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	17.827	4	4.457	8.193	.000[b]
	Residual	51.679	95	0.544		
	Total	69.507	99			

The ANOVA table generated by SPSS provides valuable insights into the factors that influence financial retirement planning among employees in the private sector who utilizes the provident fund. Previous research studies have already established that variables such as attitudes, information accessibility, financial literacy, and risk tolerance play a significant role in shaping retirement planning behaviour (Gough & Niza, 2011; Tomar, Kumar, and Sureka, 2021).

The regression results in this study reinforce the findings of earlier research by demonstrating that the model employed accounts for a substantial amount of the variation observed in retirement planning utilization. This aligns with the conclusions drawn by Herrador, Hernande, and Topa (2021; Harahap et al., 2022) and Harahap et al. (2002), who have emphasized the significant influence of the independent variables considered in this study on individuals' retirement planning behaviour. The statistical significance of the F-statistic and its corresponding p-value further supports the credibility of the regression model.

To provide a more comprehensive analysis, it would be beneficial to supplement the ANOVA with a logistic regression analysis. By examining odds ratios and predicting probabilities, logistic regression can offer a deeper understanding of the relationships between the independent factors and retirement planning usage (Alkan et al., 2020; Bayar et al., 2020). This approach would enable a more nuanced examination of how attitudes, information accessibility, financial literacy, and risk tolerance impact the utilization of retirement planning among private sector employees who rely on the provident fund.

4. Conclusion

A sizable percentage of people have favourable opinions on financial retirement planning. This shows a rising understanding of the value of retirement planning and the possible advantages it may provide in assuring financial stability throughout the post-retirement era. Additionally, it was found that there is a lot of information about retirement planning, with a variety of sources and platforms offering direction and counsel (Thuku, 2013).

Participants without dependents often exhibit higher levels of agreement with uncertainty, indicating a better capacity for risk-taking, whereas participants with dependents, particularly those in the 5–10 dependents range, typically display lower levels of agreement due to more responsibility. In contrast to respondents with secondary education levels, those with university education often exhibit higher degrees of uncertainty, but a stronger commitment to retirement preparation. These findings highlight the significance of individualized financial counselling taking into account family dynamics and educational background to meet people's unique requirements in investing and retirement planning (Tamborini & Kim, 2020).

The main conclusion from the analysis of the factors influencing financial retirement planning using provident funds is that, even though attitudes, financial literacy and risk tolerance may not directly affect the use of retirement planning, the accessibility of information and people's intentions to use retirement plans both play important roles. The research findings reinforce the association

shown in other studies (Lusardi, 2008; Janor et al., 2016) by indicating that the use of retirement planning alternatives is highly influenced by the availability of information.

Furthermore, people's intentions to utilize retirement planning have a beneficial impact on their actual usage, demonstrating the value of building intention as a driving force. These findings highlight the importance of making information easily accessible and of encouraging the aim to increase the use of provident fund alternatives for retirement planning.

5. Recommendations

Based on the study's findings, a number of comprehensive recommendations may be made to improve provident fund retirement planning among 40–50-year old employees in the private sector. First and foremost, it is essential to give financial education top priority by creating focused programs that focus exclusively on retirement preparation. These programs have to address issues like risk management, long-term financial objectives, and investment methods while taking into account the educational backgrounds of the participants. People will be better able to make educated judgments about their retirement planning, if financial literacy is improved.

There is an opportunity for improvement in the accessibility of knowledge on retirement planning. Information that is thorough and accessible may be made possible by cooperative efforts between the private sector, financial institutions, and regulatory organizations. This may be accomplished by creating user-friendly websites, holding educational seminars or workshops, and disseminating informational on investment planning and guidance. Higher participation rates will result from greater knowledge of provident fund uses and their advantages.

It is critical to understand how people's views of uncertainty and commitment to retirement planning are influenced by family circumstances, such as the number of dependents. Providers of retirement planning services and financial consultants should take this into consideration individual requirement and obligations when providing guidance on retirement planning.

Additionally, fostering intention is essential since, according to this research, it has a big impact on actual usage. Employers might put in place initiatives that encourage participation in retirement planning among staff members. This may be accomplished by giving incentives like matched contributions, reminding employees on a regular basis of their retirement benefits, and sharing success stories of staff members who have successfully planned for retirement utilizing provident funds.

The success of retirement planning activities and programs targeted at private sector employees aged 40 to 50 must also be regularly evaluated and reviewed. This involves evaluating the programs' results, utilization rates, and satisfaction levels. Employers may think about conducting follow-up surveys or focus groups to get participant input and identify any obstacles or difficulties they could have in properly utilizing retirement planning alternatives.

The results of financial retirement planning for private sector employees between the ages of 40 and 50 can be improved by putting these ideas into practice. They will be more financially secure as a result, which will benefit their long-term wellbeing.

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Impact of Organisational Knowledge Sharing on Innovative Work Behaviour at ABC Trading (Pvt) Ltd

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Abstract

This research is a study of the knowledge sharing factors that influence innovative work behaviour at ABC Trading (Pvt) Ltd. ABC Trading (Pvt) Ltd is facing inefficiencies, high attrition and loss of profit due to its lack of knowledge sharing, which hampers the organisation's ability to innovate. This study has selected knowledge sharing variables that influence innovative work behaviour based on current research publications. These variables are: team work, management support, trust, knowledge-based rewards, and self-efficacy. Five hypotheses were formulated based on the chosen variables. A questionnaire was distributed to 124 employees of ABC Trading (Pvt) Ltd to obtain their feedback on how knowledge sharing influences innovative work behaviour. The primary data that was collected was used to test the hypotheses. Based on the correlation analysis results, management support and trust have a positive correlation with innovative work behaviour. Based on multiple linear regression results, trust is the most significant variable. The degree of satisfaction with the variables that influence knowledge sharing was low. Thus, this study has revealed that there is a clear problem with knowledge sharing in the organisation, which in return adversely affects innovative work behaviour. Based on the research findings, recommendations are made on how to improve knowledge sharing in order to improve innovative work behaviour at ABC Trading (Pvt) Ltd. Suggestions for future research are also presented.

Keywords: Innovative Work Behaviour, Organisational Knowledge, Knowledge Sharing

1. Introduction

Global competition on innovation, trends, technology, and consumer demands are in rapid change where organisations seek for competitive opportunities for survival through innovation. Therefore, organisations bear the responsibility of improving innovative behaviour of employees by creating value to achieve market efficiency and customer loyalty. Organisation innovation capacity and ability towards continuous innovation on product, services, and workflows is essential for an organisation to remain competitive in the market. Therefore, innovation is considered as a general learning trend for a successful organisation by focusing less on specific innovation projects and more on general innovation trends that build innovative capacities. The researchers agree that organisational innovation is the key to obtain competitive advantage and strategic innovation. Therefore, employees are an important resource for organisations to develop, respond and modify initiatives which play an important role in organisational innovation which is known as innovative work behaviour. Further, for employees to be innovative, organisations must manage and develop an internal environment and relevant knowledge that supports knowledge sharing and innovative nature of their employee behaviour. Moreover, knowledge sharing impacts a company's innovation capacity and improves the innovative working behaviour. In recent years, lack of organisational knowledge sharing reported and caused obstacles to the organization, as ABC Trading experienced a high attrition rate at different levels of the organisation. As a result, ABC Trading became less competitive in the industry, and employee performance deteriorated. Creativity, learning and innovation of employees were hampered due to this issue. In addition, employees started

to ignore the company's best problem solving experiences due to lack of guidance and knowledge. Therefore, the study is conducted based on an ongoing problem within ABC Trading under the topic 'impact of organisational knowledge sharing on innovative work behaviour at ABC Trading (Pvt) Ltd' using a sample of 124 in the operations department.

1.1 Research Problem

ABC Trading organisation is engaged in developing and implementing projects focusing on providing innovative products and services. Project-based organisations have gained reputation in the modern economy due to flexibility, innovation and well-equipped methods to overcome barriers towards organisational change, which has the ability to respond in a timely manner. Therefore, project teams possess an effective knowledge sharing and creation, as the organisation is providing the project teams the freedom and the environment to perform their tasks. Based on employee feedback, the management is not supportive and does not share knowledge. This makes employees hoard knowledge and within the past few years, the lack of efficiency, productivity and conflicts between the employees have increased. The profitability of the organisation has declined as result of lack of organisational knowledge resource sharing. This has caused obstacles to the organisation, as ABC Trading is currently experiencing high absenteeism and attrition rate at different levels of the organisation. Consequently, ABC Trading experienced a decline in competitiveness within the industry with employee grapple in areas such as creativity, learning and innovative results. In addition, employees started ignoring the company's best problem-solving experiences due to lack of guidance and knowledge.

The ABC Trading Operation team is involved in projects where they require innovative knowledge. The employees are facing difficulties in grasping knowledge from the organisation due to various barriers. Lack of knowledge sharing within ABC trading organization has caused attrition, as the organisation did not establish solutions in order to make the organisation competitive in the industry. Therefore, this research will help the current organisation to understand the reasons for the organisation's attrition and employee low performance and come up with solutions, benefiting both the organisation and the employees to achieve their career goals.

1.2 Research Aim - The research aim of this study is to understand the relationship between organizational knowledge sharing and innovative work behaviour at ABC Trading (Pvt) Ltd.

2. Methodology

This study used the quantitative research methodology.

2.1 Conceptual framework

The conceptual framework used to formulate the hypotheses for this study is given below.

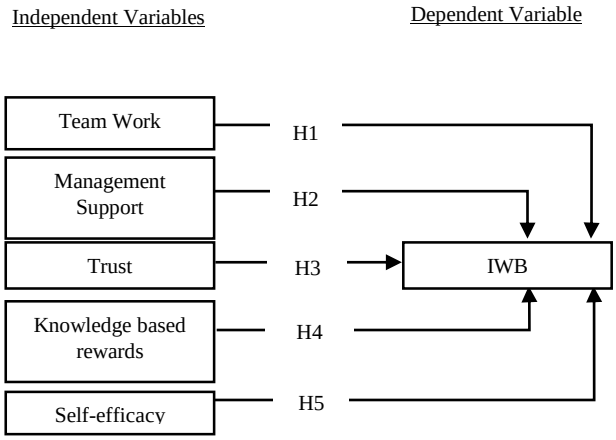


Figure 1. Conceptual Framework

2.2 Hypotheses development

H1 - There is a positive relationship between teamwork and innovative work behaviour

Team work within an organisation generates productive outputs by connecting individual knowledge, expertise and creativity to form innovative work behaviour (Parket et al., 2006) Knowledge based and innovation-based organisations engaging in complex operations require collective output and innovative work behaviour (Hauschildt & Kirchmann, 2001). Therefore, team work enables employees to brainstorm knowledge, generate multiple ideas and to identify problematic gaps from different point of views and to generate solutions for multiple problems, which can provide a unique solution (McCrae & Terracciano, 2005).

H2 - There is a positive relationship between management support and innovative work behavior

Management support encourages idea generation and provides employees training opportunities which increases employee motivation, positive behaviour, and promotes innovative work behaviour (Tallman et al., 2004). The lack of management support isolates employees without an idea as what to perform and will not be able to understand management expectations, which can cause incorrect task performance of employees, demotivation and lack of innovative work behaviour (Aulawi et al., 2005).

H3 - There is a positive relationship between trust and innovative work behavior

A high level of trust generates positive attitude on other colleagues and generates mutual understanding with colleagues which leads to innovation (Shan & Zhang, 2009). Trust creates the environment for employees to express ideas freely, willingness to share knowledge, improves commitment and employee loyalty (Chen & Hunag, 2009). Hence, a lack of trust generates thoughts of distrust and leads to knowledge hoarding.

H4 - There is a positive relationship between knowledge based rewards and innovative work behavior

Employees' positive attitude towards innovative work behaviour increases when they benefit from promotion, recognitions, monetary rewards, and learning opportunities (Lee & Ahn, 2007). Aulawi et al. (2005) state that employees being aware of the rewards available for innovative work behaviour, will encourage them to share knowledge and innovate.

H5 - There is a positive relationship between self-efficacy and innovative work behavior

Employees with high self-efficacy engage in creative practices and activities (Carmeli & Schaubroeck, 2007). Skaalvik and Skaalvik (2010) state that low self-efficacy is caused through lack of training, lack of rewards, trust, support and acceptance from other colleagues. As a result, employees are frustrated and are less motivated to perform a task, develop a lack of confidence and develops anxiety problems (Gong et al., 2009).

2.3. Operationalization Table

Table 1 gives the dimensions used to measure the independent and dependent variables.

Table 1. Operationalization table

Variable	Dimension	Source
Teamwork	Mutual communication Attitudes Willingness	(Prieto & Santa, 2014) (Kamasak & Bulutlar, 2010)
Management support	Organisation support Job training Skill development	(Wang & Wang, 2012) (Kamasak & Bulutlar, 2010)
Trust	Mutual trust Transparency Positive attitude	(Wang & Wang, 2012) (Kamasak & Bulutlar, 2010)
Knowledge based rewards	Rewards Recognitions Positive attitudes	(Patricia, 2001), (Prieto & Santana, 2014)
Self-efficacy	Capability Confidence Commitment	(Shao et al., 2015) (Chow & Chan, 2008)
Innovative work behaviour	Job-fit New ideas Innovation implementation	(Afsar, Badir & Khan, 2015) (Prieto & Sanatna, 2014)

2.4 Population and Sampling

The population of this study is 180 employees from the operations unit at ABC Trading (Pvt) Ltd. This study used the convenience sampling technique. Based on the Morgan table, for a

population of 180, the sample size should be 123, therefore a sample size of 124 was chosen.

2.5 Data Collection

To gather primary data, a Likert scale based structured questionnaire containing closed ended statements was used. The questionnaire consisted of statements to get feedback about knowledge sharing factors and demographic data of the respondents. The questionnaire was distributed using Google Forms.

2.6 Data Analysis

SPSS statistic software was used for data validity analysis, correlation analysis and multiple linear regression analysis.

3. Analysis and Findings

3.1 Response Rate

A total of 124 questionnaires were distributed to the chosen respondents. All of the respondents answered the questionnaire. Therefore, a response rate of 100% was achieved.

3.2 Demographic Data

The demographic data of the respondents are given below, gathered through the survey questionnaire.

Table 2. Demographic Data

Age	
18-20	3%
21-25	8%
26-30	73%
31-45	16%
Educational qualification	
No formal higher education	13%
Undergraduates	23.18%
Graduates	88.71%
Other	1.1%
Designation	
Intern	1%
Junior Executives	2%
Executive	72%
Senior executive level	23%
Supervisory level	2%
Years of work experience at ABC Trading	
Less than one year	5%
01-05 years	85%
06-10 years	9%
More than 10 years	1%

3.3 Reliability analysis

Table 3 provides the Cronbach's alpha values, which was used to analyze the internal consistency of the questionnaire items.

Table 3. Cronbach's Alpha Values

Variables	Cronbach's Alpha
Teamwork	0.6
Management Support	0.6
Trust	0.6
Knowledge based rewards	0.5
Self-efficacy	0.1
Innovative Work Behaviour	0.7

According to the reliability analysis, Cronbach's alpha values are acceptable, if they are 0.6 or above. Therefore, the data can be considered to be reliable (except for Self-efficacy). The Cronbach alpha value for Self-efficacy is 0.1. The majority of the respondents' rated 'Agree' as their self-rating on Self-efficacy. Self-ratings are not an accurate measure due to the tendency for respondents to show that they possess socially desirable traits

and therefore, the respondents are capable of exaggerating their skills and abilities.

3.4 Correlation Analysis

This study utilized the Karl Pearson's correlation coefficient to measure the relationship between both variables.

Table 4. Correlation Analysis

Innovative work behaviour		
	Pearson Correlation	**Sig. (5%)**
Team work	0.126	0.163
Management Support	0.219	0.015
Trust	0.266	0.003
Knowledge Based Rewards	0.044	0.627
Self-efficacy	-0.114	0.209

As shown in Table 4, the correlation analysis (at 5% sig. level) reveals that only Management Support and Trust have a positive correlation with Innovative Work Behaviour.

H1: There is a positive relationship between teamwork and innovative work behaviour

H1 is not supported by this study.

This result is supported by Xerri and Reid (2017). The study found that teamwork does not have a significant impact on innovative work behavior, however the study stated that efficiency, effectiveness and training opportunities are influential towards innovative work behaviour. Twigg et al. (2010) and Aitken et al. (2012) stated that teamwork creates less significant impact on innovative work behavior, whereas job satisfaction and the work environment were more important.

H2: There is a positive relationship between management support and innovative work behaviour

H2 is supported by this study.

The above identified relationship is supported by past literature where Yu et al. (2004) proclaims that management support influences employees to create and maintain positive principals and innovative work behaviour. Bakker et al. (2003) states that management support within an organisation is important to help employees cope with responsibilities, share knowledge and create a positive impact on organisational performance and improve innovative work behaviour. Jimmieson et al. (2009) show that management support results in employees' positive attitude towards knowledge sharing and innovative work behaviour.

H3: There is a positive relationship between trust and innovative work behaviour

H3 is supported by this study.

The above mentioned relationship has been confirmed by the studies of Dirks and Ferrin (2001), which state that trust leads to transparency and influences employees' abilities and positive attitudes on knowledge sharing. Further, several studies have shown a positive impact of trust towards innovative work behavior (Dirks & Ferrin, 2001; Reagans & Mcevily, 2003).

H4: There is a positive relationship between knowledge-based rewards and innovative work behaviour

H4 is not supported by this study.

Recent research has suggested that knowledge based rewards create less impact on innovative work behavior, as rewards and integrity in the workplace is a condition that suppresses and facilitates employee behaviour (Konovsky & Organ, 1996; Niehoff & Moorman, 1993). Milne (2001) stated that there is no positive correlation between knowledge based rewards and innovative work behaviour, because knowledge based rewards are usually more focused on knowledge retention and are less influential towards innovative work behaviour.

H5: There is a positive relationship between self-efficacy and innovative work behaviour

H5 is not supported by this study.

Henson (2001) found a weak correlation between self-efficacy and innovative work behaviour. Razak et al. (2016) suggests that self-efficacy influences individual motivation and desire to perform knowledge sharing and is influential towards innovative work behaviour.

3.5. Multiple Linear Regression Analysis

Multiple Linear Regression analysis was conducted to identify the most significant variables impacting innovative work behaviour. The results are shown in Table 5.

Table 5. Multiple Linear Regression Analysis

Variable	Beta	Sig. (5%)
Team Work	0.04	0.672
Management Support	0.07	0.561
Trust	0.266	0.003
Knowledge Based Rewards	-0.063	0.508
Self-Efficacy	-0.036	0.693

Based on the regression analysis results, at 5% significance level, Trust is the most significant variable that has a positive correlation with Innovative Work Behaviour. The regression model has a R square value of 0.07.

3.6. Average Ratings

Table 6 gives the average rating for each of the variables used in the study based on the response analysis.

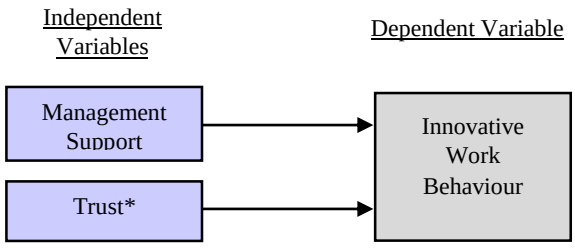
Table 6. Average Ratings

Variable	Average rating	Average value
Teamwork	Disagree	2
Management Support	Disagree	2
Trust	Disagree	2
Knowledge based rewards	Neutral	3
Self-efficacy	Agree	4

Based on the average ratings, it is clear that the average ratings given by the respondents for team work, management support, trust and innovative work behaviour was “dissatisfied”. The average rating for knowledge-based rewards was “neutral’. The average rating for self-efficacy was “agree”. Thus, it is evident that overall, the respondents are dissatisfied with the factors that contribute to innovative work behaviour.

4. Conclusion

In conclusion, as shown in Figure 2, this study has found that out of the five independent variables studied (team work, management support, trust, knowledge-based rewards and self-efficacy) only management support and trust have a positive correlation with innovative work behaviour. The other variables have no correlation with innovative work behaviour. Multiple linear regression analysis revealed that trust is the most significant variable. The average ratings given by the respondents for team work, management support, trust and innovative work behaviour was “dissatisfied”. The average rating for knowledge based rewards was “neutral”. The average rating for self-efficacy was “agree”. Thus, it is evident that overall, the respondents are dissatisfied with the knowledge sharing factors that contribute to innovative work behaviour at ABC Trading (Pvt) Ltd. These results clearly show that there is a lack of innovative work behaviour at ABC Trading (Pvt) Ltd.



Note: Trust is the most significant variable.

Figure 2. Factors influencing Innovative Work Behaviour at ABC Trading (Pvt) Ltd

5. Recommendations

Recommendations to improve trust

Each employee generates new ideas and opinions; therefore, the management should add their ideas to a common pool. This will create a foundation upon which to build a work place based on mutual trust and understanding. Employees will learn to trust one another, when they see that everyone is committed, shares their ideas and knowledge freely and works towards a common goal. As stated by Meyer and Maltin (2010), trust influences employee engagement on innovative work behaviour to produce a productive outcome. Employees should be taken out for lunch or team building

programs, as it builds trust and creates space for personal understanding and friendship. Frequent recognitions, appreciation should be awarded to the employees for collaborative efforts, which will encourage employees to trust each other and share their ideas to achieve a common goal.

Recommendations to improve management support

According to Lin (2007), teams with better management support will work better as a team and contribute to knowledge sharing more productively. Management support can be developed by creating credibility, by being respectful and making fair decisions. When employees are engaged in a task, the management should influence the employee to think innovatively by valuing their new ideas.

Recommendations to improve teamwork

According to Aulawi et al. (2009), teamwork enables employees to understand the importance of knowledge sharing with other colleagues. Plessis (2007) affirms, best practices of knowledge is shared when employees are able to collectively generate, develop, promote and implement innovative work behaviour within a team. Employees' works as a team, only if they feel comfortable to share ideas, make suggestions, speak openly, and express themselves. Therefore, creation of an open communication culture is important, where communication and information flow freely among team members. If enough freedom is not provided to a team, the team members would not work together as they expect the management to provide specific instructions, set deadlines, resolve issues and develop workflows.

Recommendations to improve knowledge based rewards

The management should implement a reward system where every employee receives positive performance evaluations based on their productivity. Based on the evaluation, the employees can be awarded a monetary or non-monetary reward by the organisation. This will help encourage employees to share knowledge in order to gain rewards and recognition. Further, the organisation can monitor individual and team performance and tie rewards with exceptional work. The management should also look beyond financial incentives by identifying what employees value other than financial rewards. Non-financial rewards may include organising lunches or dinners or social events. The management should also recognise and provide appreciations for both large and small achievements alike. According to Kanter (1988), rewards and recognition encourages and motivates employees to share knowledge within the organisation.

Recommendations to improve innovative work behavior

Khorakian et al. (2019) states that organisational support can lead towards identifying employee knowledge capabilities, trust and management involvement to improve innovative work behaviour. Therefore, promoting a knowledge sharing culture, where trust, teamwork and management support are encouraged will enhance innovative work behaviour. The management should encourage employees to keep an open mind, share ideas and to be willing to take risks. Employees from diverse backgrounds with different ideas and perspectives should be hired, who possess unique abilities and who are passionate about work. The organisational culture should create a workspace, where employees can create and share new ideas. The management can also provide breaks from routine and monotonous work to inspire employees to take part in activities that require creativity and collaboration. Employees must be allowed to participate in brainstorming sessions, where new ideas can be discussed. Every employee's opinion and suggestion must be heard and valued, not only of those holding senior positions. The management can also offer training programs and learning opportunities, as a further reward for employees who come up with new ideas.

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The Impact of Product User-Friendliness on Consumer Product Adaptation: A study on Digital Banking products offered by Sri Lankan Banks

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Abstract

The rapid development and vast improvements in technology have a great impact on the day-to-day life of everyone. Especially, the banking and finance sector depends on technology information which has brought major changes to the banking industry. Internet banking, in recent times, has become a major and strategic tool used to perform transactions; however, customers still perform over-the-counter transactions and visit the bank physically rather than using the Digital banking facility. For this reason, the Central Bank of Sri Lanka have launched campaigns from time to time to promote digital channels in Sri Lanka. Furthermore, previous studies have been conducted to identify the factors influencing customers to adapt to Internet banking. The purpose of this study is to identify the impact of product user-friendliness on consumer product adaptation in Digital Banking of Sri Lankan banks. The quantitative mono-research method was adapted, using a close-ended structured questionnaire to collect data from a sample of 100 internet banking uses within the Western Province. The SPSS software was used to analyse the data, using correlation and multiple linear regression techniques. Accordingly, it revealed that the user interface (0.793), perceived ease of use (0.715) and perceived usefulness (0.705) have strong positive correlations at a significance of 0.000 level, with consumer product adaptation in Digital Banking. Hence, it is recommended that Sri Lankan banks focus more on the user interface, ease of use or less complexity and usefulness in Digital Banking product development. Further, banks should frequently develop or add any new functionality to the existing digital products, without harming the above vital qualities of the products.

Keywords: Digital Banking, User interface, Perceived ease of use, Perceived usefulness

1. Introduction

1.1 Background of the study

The Internet was introduced in 1969, and since then information and technology (IT) has become an essential part of human life and its rapid development affects the economy, significantly (Nehmzow, 1997). The adoption of IT was a fundamental prerequisite for the business world to survive and compete with others, retain existing customers and attract new customers (Al-Khatib, 2013). Furthermore, the banking industry is most often dependent on IT and the growing usage of IT has helped to grow and differentiate the products and services (Shiraj, 2015).

User-friendliness is where the user can easily understand and navigate through the application efficiently. User-friendliness can be measured in many ways and it has many characteristics. Further, user-friendliness is important while designing a digital product because it increases user satisfaction and as a result, sales volume will increase and customer loyalty improves.

Consumer product adaptation is also highlighted as an important factor in past research. Models such as Technology Acceptance Model (TAM) and the Unified Theory of Acceptance and Use of Technology model (UTAUT) have already been developed, in order to explain the context of consumer product adaptation. The definition of digital banking is doing traditional banking activities remotely through the internet (Al-Khatib, 2013; Lang, Nolle, & Furst, 2003). It has been branded under various names, such as online

banking, digital banking, e-banking, and internet banking, and so on. This facility allows customers to do their banking activities at any time, even beyond regular banking hours.

1.2 Industry Overview

The banking sector in Sri Lanka started using IT in the 1980s (Jayasiri & Weerathunga, 2008). As a result of using IT, digital banking was first introduced in March 1999 (Jayamaha, 2008). Currently, most banks in Sri Lanka offer internet banking facilities to their customers. According to the Central Bank of Sri Lanka (CBSL) (2022), Sri Lanka currently has 24 licensed commercial banks including two state banks and 6 licensed specialist banks. Most of them are offering this facility to their customers. According to the CBSL (2022), the below figures clearly indicate the rapid growth of Internet banking usage in Sri Lanka from 2013 to 2021. As a result, from the economic point of view, internet banking can influence growth in the financial sector as it gives the facility to access from anywhere without any barriers.

Table 1. Internet-based payment

Year	Transaction Volume (000)	Transaction Value (Rs. billion)
2013	6,442.40	516.59
2021	130,312.80	7023.15

Source: CBSL (2022) Payment Bulleting

1.3 Rationale

Information and technology are rapidly changing every day and people are exposed to new technologies which result in changes in their expectations, accordingly. Initially, the internet was used as an information delivery channel by publishing information on banks' corporate websites (Tan & Teo, 2000). As of today, digital banking covers most of the conventional banking activities such as checking balances of current accounts, savings accounts, loan accounts, credit card inquiries and payments, fund transfers, utility bill payments, applying for loans and making settlement requests and value-added services. The concept of digital banking was introduced in the late 1990s and it is not as how it was then. Many previous researches have been conducted to identify the factors influencing the adoption of internet banking and some models developed to identify the factors of user acceptance of information technology (Davis, 1989; Tan & Teo, 2000). Some studies have proved that user-friendliness is a key factor that directly impacts end-user satisfaction (Pikkaraine, Pikkaraine, Karjaluoto & Pahnila, 2006). Some research has proved that there is a relationship between user-friendliness and perceived usefulness (Nayanajith, Damunupola & Ventayen, 2019). Perceived usefulness is one of the main determinants of the TAM model which is used to measure the user acceptance of technology. Hence, this study will further investigate the relationship between user-friendliness and consumer product adaptation.

Developers are working to build simple and more convenient systems which are user-friendly (Petrie & Bevan, 2009). Kuisma, Laukkanen, and Hiltunen (2007) stated that there are still some users who do not enjoy the digital banking facility due to security concerns and uncertainty, thereby still preferring to physically visit and perform banking activities. According to Jahangir and Parvez (2012), customers are very concerned about factors in adaptation to digital banking such as compatibility, convenience and communication. However, as stated by Nasreen and Lubis (2021), as people recognise the benefits of using digital banking it may become more compatible with users.

1.4 Research aim and objectives

The overall research aim is to provide detailed evidence regarding the impact of product user-friendliness on consumer product adaptation based on internet banking in Sri Lanka.

1.5 Scope

The scope of this study is users of internet banking within the Western Province.

2. Research Methodology

This study used the quantitative mono-research method using an online questionnaire.

2.1 Conceptual Framework

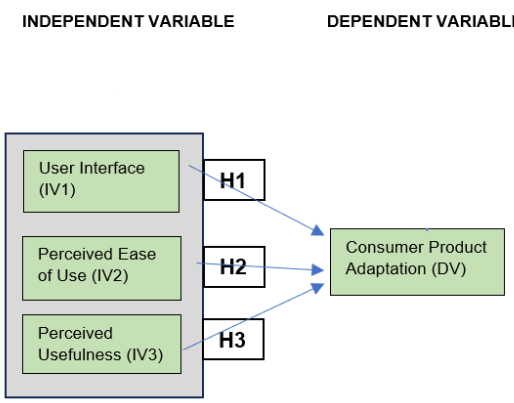

Figure 1. Conceptual Framework

2.2 Research Hypothesis

H1 – User Interface has a relationship with consumer product adaptation in digital banking.

H2 – Perceived ease of use has a relationship with consumer product adaptation in digital banking.

H3 – Perceived usefulness has a relationship with consumer product adaptation in digital banking.

2.3 Operationalisation table

The operationalisation table is given in Table 2.

Table 2. Operationalisation Table

Variable	Rationale
Consumer product adaptation	•Customer's required functions •Customer understanding •Impact of the structure and the technology used
User Interface	•Icons consistency •Colours used •Web layout/structure •Quality of the information
Perceived Ease of Use	•Complexity/flexibility •User-friendly web design •Easy accessibility •Information availability •Responsiveness
Perceived Useful	•Level of satisfaction with the available services •Effectiveness and efficiency •User expectations •User convenience •Information quality

2.4 Population and Sampling

The Western Province is the commercial hub in Sri Lanka and the population is 5,851,130 as of 2012 (Census of Population and Housing, 2012). Hence, the researcher assumed that approximately 75,000 active internet banking users are there. The sample size of the study would be 100.

2.5 Data Collection

The data was collected using Google Forms using a five-point Likert scale structured questionnaire.

2.6 Data Analysis

The data were analysed using SPSS software using correlation and multiple linear regression.

3. Findings and Discussion

3.1 Response Rate

A total of 100 responses were received out of the 100 questionnaires sent. Therefore, the response rate is 100%.

3.2 Demographic Details

Table 3 provides a summary of the demographic data of the respondents.

Table 3. Demographic Details

Age	
20 – 30 Years	48.60%
31 – 40 Years	34.60%
41 – 50 Years	11.20%
Above 50	5.60%
Gender	
Male	59.81%
Female	40.18%
Highest Education	
O/Level	2.82%
A/Level	35.51%
Degree	23.36%
MBA	17.75%
Professional Qualification	20.56%
Employment	
Unemployed	7.48%
Executive	73.83%
Manager	12.15%
Senior manager or above	6.54%

3.3 Cronbach's Alpha Test

The reliability was carried out using Cronbach's Alpha test. Hence, in the questionnaire, the results of all three independent variables and the dependent variable are shown in Table 4.

Table 4. Cronbach's Alpha Test

Variable	Cronbach's Alpha	Reliability
Consumer Product Adaptation	0.942	Very Good
User Interface	0.904	Very Good
Perceived Ease of Use	0.909	Very Good
Perceived Usefulness	0.920	Very Good

According to the above table, all the variables are above the benchmark of 0.7. Hence, it can be considered that all the variables are reliable.

3.4 Correlation Analysis

The correlation analysis of the variables is given in Table 05.

Table 5. Correlation Analysis

Independent Variable	Pearson Correlation	Significance (2-tailed)
User Interface	0.793	0.000
Perceived Ease of Use	0.715	0.000
Perceived Usefulness	0.705	0.000
Dependent Variable – Consumer Product Adaptation		

According to the above table, all the independent variables have a strong positive correlation with consumer product adaptation and the significance is 0.000. Moreover, the correlation of above 0.5 indicates all alternate hypotheses in this research are accepted.

3.5 Hypotheses Validation

H1 – User Interface has a relationship with consumer product adaptation in digital banking. – **Accepted**

This result is supported by Perera (2013), who discussed a poorly designed website could affect users and could be discouraging to use such a system.

H2 – Perceived Ease of Use has a relationship with consumer product adaptation in digital banking. - **Accepted**

This result is supported by Fernandes and Olivera (2021), who found that the perceived ease of use strongly impacts users to accept technology.

H3 – Perceived Usefulness has a relationship with consumer product adaptation in digital banking. - **Accepted**

This result is supported by Kumari and Mendis (2021), who concluded that nowadays individuals are more into technology and perceived usefulness has a very strong positive relationship with consumer product adaptation

In summary, it is evidently proved that each alternated hypothesis is accepted and has a positive relationship. Therefore, it is proved that product user friendliness and consumer product adaptation has a positive strong relationship.

3.6 Multiple Linear Regression

The R Square value is 0.641, which indicates that consumer product adaptation is explained in 64% of by-product user-friendliness dimensions. The multiple linear regression results are given in Table 6.

Table 6. Multiple Linear Regression Analysis

Independent Variable	Beta	Significance
User Interface	0.655	0.000
Perceived Ease of Use	-0.020	0.889
Perceived Usefulness	0.191	0.103

According to the regression analysis, it is evident that the most significant variables is user interface with a significance value of less than 0.05.

3.7 Descriptive Analysis

The descriptive analysis results are based on the respondent's answers to questions regarding their internet banking experience.

Table 7. Consumer Product Adaptation

I like the prompt feedback given to me by my bank's Internet banking website when I perform a transaction	
Strongly Agree	33.65%
Agree	29.91%
Neutral	20.57%
Disagree	12.15%
Strongly Disagree	3.72%
I like the way I can navigate through the menu on my bank's Internet banking website without any issues	
Strongly Agree	52.34%
Agree	25.24%
Neutral	10.29%
Disagree	10.29%
Strongly Disagree	1.84%
I can easily identify where the sub-menus are located on my bank's Internet banking website	
Strongly Agree	26.17%
Agree	35.52%
Neutral	25.24%
Disagree	10.29%
Strongly Disagree	2.78%
I can easily understand and perform any transaction using my bank's Internet banking facility, without getting help from others or without calling the hotline	
Strongly Agree	14.02%
Agree	51.41%
Neutral	22.43%
Disagree	11.22%
Strongly Disagree	0.92%
The structure and the technology used on my bank's Internet banking website attracted me	
Strongly Agree	7.48%
Agree	57.95%
Neutral	17.76%
Disagree	14.96%
Strongly Disagree	1.85%

According to Table 7, it is evident that the majority of the users feel that they are willing to adapt to digital banking products as per the findings given.

Individual Preferences

The individual preferences of the respondents in this research were gathered using the five-point Likert scale, where 1 – strongly disagree and 5 – strongly agree.

Table 8. Individual Preferences

User Interface	**Mean**
The font and size of text used on my bank's Internet banking platform are easy to read.	3.60
Icons are consistent throughout my bank's internet banking website, hence easily understood.	3.64
The colours used on my bank's Internet banking website are appropriate.	3.76
The presentation and layout on my bank's Internet banking website are pleasant.	3.63
Images and graphs on my bank's Internet banking websites are meaningful and serve a purpose.	3.36
Perceived Ease of Use	
It is easy to adopt online banking when it is less complicated.	4.05
I like to use my bank's online banking facilities because it is user-friendly.	3.50
I can easily access my bank's online banking website because it is compatible with any device.	3.74
I like to use the Internet banking facility because it meets my expectations.	3.57
I like to use my bank's Internet banking facility because I get quick responses to my inquiries.	3.45
Perceived Usefulness	

I can perform all my essential banking transactions through Internet banking without visiting the bank physically.	3.90
I like to use the Internet banking facility because it is very useful to complete my financial transactions easily.	4.07
I am satisfied with the services provided by my bank through their Internet banking facility.	3.72
I can even perform non-financial activities like requesting a balance confirmation or making a complaint through internet banking, without visiting the bank.	3.46
I like to use the Internet banking facility because I am highly satisfied with the quality of the information provided through it.	3.58

According to the above table, the rounded mean value for all the variables is 4, which is "agree" as per the Likert scale.

In terms of the user interface, the users are keen on colours used in internet banking platforms. Hence, the colours used in internet banking can be considered the most important attribute in user interface.

In terms of perceived ease of use, users are very concerned about the less complexity. Hence, it is proved that less complexity in internet banking is the most important attribute in perceived ease of use.

In terms of perceived usefulness, users are eager to perform internet banking transactions easily. Hence, performing internet banking transactions easily is the most important attribute in perceived usefulness.

4. Conclusion

The hypothesis testing revealed that the user interface has a strong relationship with consumer product adaptation. The correlation level of 0.793 indicates that the banks should focus more on the user interface to retain and attract potential customers. Moreover, the data analysis also revealed that perceived ease of use has a strong relationship with consumer product adaptation with a correlation level of 0.715 indicating that the banks should focus on implementing and developing user-friendly internet banking websites to encourage customers to use them. Finally, the data analysis revealed that perceived usefulness has a strong relationship with consumer product adaptation with a correlation level of 0.705. This indicates that the banks should focus on promoting and educating their customers.

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The purchase intention of domestic tourists toward green hotels

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Abstract

The global hospitality industry has enacted numerous sustainable measures to face the soaring green consumerism trend and entice consumer purchase intention. Sri Lanka has positioned itself as a top tourist destination in the recent past among international travellers amidst fierce competition from the region. Domestic tourists of Sri Lanka have proven to have contributed significantly to the industry during unprecedented times over the years. This study investigated the purchase intention of domestic tourists towards green hotels, using green marketing efforts applied by hotels in terms of green practices, green communications and pricing and examined these variables in the context of the theory of planned behaviour using the attitude and subjective norms as the precursors of purchase intention. The research used the quantitative method to collect data through a structured questionnaire administered online among a sample size of 100 green hotel patrons within Colombo 1 to 8 using the convenience sampling technique. The gathered data was analysed using statistical and descriptive analysis on Statistical Package for Social Sciences (SPSS). The findings of this study confirmed the assertion of the theory of planned behaviour that attitude and subjective norms are significant precursors of purchase intention whilst the variables pricing and green communications were found to be more significant than green practices and subjective norms towards inciting purchase intention. Based on the findings, the author recommended that green hoteliers include community-inclusive practices and transparent communication with the target market through electronic media to instigate the purchase intention towards green hotels.

Keywords: Green Marketing Efforts, Purchase Intention, Theory of Planned Behaviour, Domestic Tourists

1. Introduction

1.1 Background of the study

Green marketing is an ethical approach followed throughout the entire value chain of a commercial enterprise to curtail its impact on communal, global and environmental ecosystems (Sharma, 2021; Tsai et al., 2020). The degradation of the environment and the public notion towards sustainability have compelled many businesses across the globe to align their processes to inflict minimal impact on the environment (Sharma, 2021). Similarly, the hotel industry strives to comply with the green trend by enforcing processes to minimise its footprint and waste aggregation (Choi, Jang & Kandampully, 2015). As per the Green Hotels Association [GHA] (2023a), lodging enterprises that practice green marketing initiatives and manage sustainable intake and output are classified as green hotels.

Purchase intention is referred as the psychological persistence of an individual to experience a service or purchase a product to satisfy personal necessity based on subjective self-evaluation (Chanda, Isa & Ahmed, 2023). Similarly, green purchase intention is defined as an individual's mindful desire towards purchasing environmentally safe products and services (Amin & Tarun, 2020). Furthermore, Choi et al. (2015) claim that a strong intention is inclined to influence behaviour and is considered a vital tool in predicting consumer behaviour. The colossal marketing efforts enforced by green hotels in attracting first-time and repeat visitors have prompted many academics to research the drivers of purchase intention towards green hotels (Achchuthan, Prahalathan, Umanakenan & Kajenthiran, 2021; Nezakati et al., 2015; Wang, 2020). Although genuine green marketing efforts direct purchase intention towards green products, deceptive attempts may weaken the impression of the overall concept thus deliberate attention is vital to comprehend the

concept of green marketing (Juwaheer, Pudaruth & Noyaux, 2012).

1.2 Overview of the Tourism Industry in Sri Lanka

Tourism is a labour-intensive industry that is dependent heavily on destination resources and thus highly sensitive to macro-environmental changes (Nezakati et al., 2015). As a cardinal forex earner, the global tourism industry was valued at over 9,000 billion dollars with a contribution of 10.4 percent to the global gross domestic product (GDP) in 2019 (The World Bank, 2022). The tourism industry consists of lodging facilitators, destination management companies, food and beverage outlets, activities and recreation facilitators, host community, the government, international and domestic tourists (Amoako, Obuobisa-Darko & Marfo, 2021). Moreover, the industry is recognised for creating employment attributing by 10.6 percent to global employment with 330 million employees (International Labour Organisation [ILO], 2022).

Sri Lankan tourism industry contributes to the nation's economy as the third highest foreign income producer with a workforce of over 450,000 which is 11 percent of the total nation's labour force (ILO, 2020). Further, Sri Lanka has been recognised as a top travel destination over the last few years by many leading global publications acknowledging the island's natural resources, affordability and unparalleled hospitality (Sri Lanka Tourism Promotion Bureau [SLTPB], 2023). In the year 2022, the industry had catered to 791,978 international tourists which is a 270 percent increment compared to 2021 with an earning of over 1.1 billion dollars (Sri Lanka Tourism Development Authority [SLTDA], 2022). Interestingly, domestic tourists in Sri Lanka spent close to 400 percent more compared to international tourists in the year 2021 (Statista, 2021a; 2021b). Studies have proven that a sustainable approach elevates a destination's competitive advantage and the responsibility of building a sustainable destination lies with all major tourism stakeholders (Ritchie & Crouch, 2003).

1.3 Rationale of the Study

Many studies have highlighted that Asian countries lag behind in terms of awareness and availability of sustainable facilities compared to Western countries (Achchuthan et al., 2021; Wang, 2020). Although international tourists' contribution to the Sri Lankan economy is significant as one of the top forex earners, the contribution of domestic tourists during the off-season and unforeseen calamities has not been evidently recognised (Perera, 2023; Sri Lanka Tourism Alliance, 2020). Subsequent to the green trend upswing, the Switch-Asia plan initiated awareness campaigns amongst the hoteliers emanating the upspring of green hotels in Sri Lanka to lower operational costs by adopting green measures such as green certification, waste management and low energy consumption (Switch-Asia, 2014). Although a few programs have been conducted to create awareness of green hotel practices amongst international tourists, there is scant evidence of any sensitisation programs among domestic tourists on sustainability and green marketing efforts at hotels (SLTDA, 2022; Switch-Asia, 2014). Ritchie and Crouch (2003) assert that for a destination to reach its highest prospects, an apprehension of its domestic travel demands is imperative. Further, the industry's survival is closely associated with the residents' understanding of the conservation of destination resources (United Nations World Tourism Organization [UNWTO], 2020). Therefore, it is crucial that locals are made aware of the green marketing efforts applied by the hotels to comprehend the contribution a local traveller could make towards the economy (Ritchie & Crouch, 2003). Furthermore, it is vital to understand the factors that influence the purchase intention of domestic tourists when sourcing lodging facilities and the effectiveness of the green marketing efforts employed by the hotels.

Although there are limited studies conducted on green hotels relating to green marketing, green certification, hotel designs, employee behaviour, challenges and consumer behaviour, none have studied the purchase intention of Sri Lankan domestic tourists towards green hotels or related to the awareness level of the residents (Gayathri, Perera & Sumanarathna, 2016; Lee, Lee & Gunarathne, 2019; Weerakoon, Sellar & Arulrajah, 2021). Therefore, this research

aspires to contribute to the literature on this novel topic of the purchase intention of Sri Lankan domestic travellers towards green hotels and the effectiveness of the marketing efforts deployed by green hotels to attract tourists.

1.4 Research Aim

This study aims to evaluate the impact of green marketing efforts undertaken by hotels on the purchase intention of domestic tourists in Sri Lanka.

1.5 Scope

This study will employ a survey among 100 domestic tourists within the city limits of Colombo.

2. Methodology

This study employed the deductive approach along with a survey strategy through the mono-method quantitative choice and embarked on a cross-sectional time horizon. Quantitative primary data was collected via the Microsoft Forms online survey portal and the gathered data was analysed using SPSS software.

2.1 Conceptual framework

Figure 1 below outlines the conceptual framework of this study.

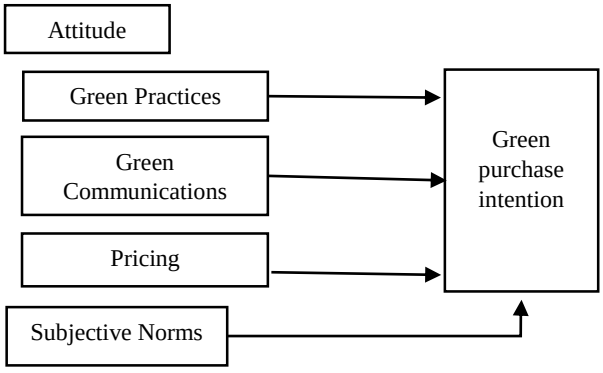

Figure 1. Conceptual Framework

2.2 Hypotheses

The following hypotheses were formed for this study.

H1: There is a relationship between green practices and green purchase intention

H2: There is a relationship between green communications and green purchase intention

H3: There is a relationship between pricing and green purchase intention

H4: The influence of family and friends impact green purchase intention

2.3 Operationalisation Table

Table 1 below outlines how the study operationalised the variables into quantifiable aspects for measuring the causal connection between the variables in designing the data collection tool.

Table 1. Operationalisation Table

Variable	Measures
Green Practices	Attitude towards the 3R Concept (Reduce, Reuse, Recycle), willingness to participate in the hotel's green practices
Green Communications	Awareness, willingness to be informed
Pricing	Perceived value, Willingness to pay a premium
Subjective Norms	Peer influence
Purchase intention	Willingness to stay at green hotels

2.4 Population and Sampling

This study selected the green hotel patrons within Colombo city limits as the population. A representative sample of 100 green hotel guests was chosen employing the convenience sampling technique to yield a reasonable response rate within the stipulated timeframe.

2.5 Data Collection

A pilot survey was carried out with 10 respondents and the questionnaire was accepted for administration based on their affirmation. The study conducted an online survey on Microsoft Forms and the survey link was distributed through WhatsApp.

2.6 Data Analysis

This study analysed the gathered responses using the SPSS (version 29) for statistical and descriptive analysis.

3. Findings and Discussion

3.1 Response Rate

This study received complete feedback from 95 respondents from the 100 questionnaires distributed among green patrons in the Colombo city limits.

3.2 Demographic Data

Table 2 below summarises the demographic findings of this study.

Table 2. Demographic Analysis

Variable	Response options	Number of respondents	Response %
Gender	Male	49	51.6%
	Female	46	48.4%
Age Group	21-30 Years	18	18.9%
	31-40 Years	36	37.9%
	41-50 Years	30	31.6%
	Above 50 Years	11	11.6%
Educational qualification	G.C.E O/L	4	4.2%
	G.C.E A/L or equivalent	13	13.7%
	Diploma	21	22.1%
	Undergraduate	15	15.8%
	Postgraduate	42	44.2%
Monthly income	Below 50,000	13	13.7%
	Between 50,000 - 200,000	47	49.5%
	Above 200,000	35	36.8%

Based on the above findings, 51.6 percent were males out of 95 respondents which highlights that males are concerned about environmental issues compared to females. Further, 37.9 percent of the respondents belonged to the age category of 31 – 40 years which signifies that millennials make the most sustainable choices compared to the other age groups influenced by technological advancement and social media. The majority of the respondents (44.2%) are postgraduates which implied that educational level has a significant influence on green purchase intention and the monthly income of the majority (49.5%) was between 50,000 to 200,000 Sri Lankan rupees which reveals that the green hotel patrons have a decent income to afford staying at green hotels.

3.3 Reliability Analysis

Cronbach's alpha was used to determine the validity of the data collected for this study.

Table 3. Cronbach's Alpha Reliability Test

Variable	No. of items	Cronbach's Alpha
Green Practices	4	0.903
Green Communication	4	0.831
Pricing	3	0.814
Subjective Norm	4	0.880
Purchase Intention	4	0.918

As per Table 3, all the variables of this study depict higher Cronbach's alpha values than the highest threshold of 0.7. Therefore, the questionnaire employed in this study satisfies the criterion and thereby confirms internal consistency and validates the research to be carried forward while anticipated to yield highly reliable results (Dwivedi, Pandey, Vashist, Pandey & Kumar, 2022).

3.4 Correlation Analysis

Table 4 depicts the correlation analysis results of this study.

Table 4. Correlation Analysis
Dependent variable – Purchase intention

Independent variable	Pearson Correlation Coefficient	Sig.
Green Practices	0.474	<0.001
Green Communications	0.537	<0.001
Pricing	0.583	<0.001
Subjective Norms	0.437	<0.001

As per Table 4, all four independent variables depict a significance value of less than 0.001 which satisfies the threshold value of 0.05 and thereby depicts that green practices, green communications, pricing and subjective norms exhibit a significant correlation with purchase intention. Further, the relationship between green practices and purchase intention is positively moderate with a coefficient of 0.474 while the correlation between green communications and purchase intention is positively strong with a coefficient of 0.537. Similarly, the correlation between pricing and purchase intention is positively strong with a coefficient of 0.583 while the correlation between subjective norms and purchase intention is positively moderate with a coefficient of 0.437.

3.5 Hypotheses Validation

This study aimed to establish the relationship between green practices, green communications, pricing, subjective norms and purchase intention. The theory of planned behaviour was applied with a focus on attitude through green pricing, green communications and pricing, and subjective norms to develop hypotheses according to the research objectives that are mentioned in Table 5 below.

Table 5. Hypothesis Validation

Hypothesis		Validation
H1	There is a relationship between green practices and green purchase intention	Accepted
H2	There is a relationship between green communications and green purchase intention	Accepted
H3	There is a relationship between pricing and green purchase intention	Accepted
H4	The influence of family and friends (Subjective norms) impact green purchase intention	Accepted

Green practices are deemed as one of the primary green marketing efforts that portray green hotels' contribution towards preserving the environment and the community (Liu, 2022). The effectiveness of green practices on customer purchase intention was measured using consumer attitude towards the 3R concept as it is regarded as a fundamental green marketing practice at hotels in order to manage resources and costs while enhancing the environmental well-being (Wickramasinghe, 2016). Further, consumers who are environmentally conscious expect and prefer to stay at hotels that practice the 3R concept, value the practices that cause less harm to the environment and are willing to take part in these practices to support sustainable measures (Choi et al., 2015; Jiang & Kim, 2015; Moise, Gil-Saura & Molina, 2021; Torres-Moraga, Alonso-Don-Santos & Carvajal-Trujillo, 2021). This study's findings validate these assertions as majority of consumers prefer hotels with green practices, inclusion of 3R concepts and consented to their willingness to participate in the measures to support the environment and the community. The acknowledgement of consumer preference and their willingness to participate in hotel green practices displays a positive attitude towards purchase intention (Jiang & Kim, 2015). Further, as per the correlation results shown in Table 4, green practices depict a correlation coefficient of 0.474 with a p-value of less than 0.001 which indicates that green practices have

a significant moderate-positive relationship with purchase intention. Thereby H1 is validated based on the previous research findings and this study's correlation analysis findings.

Green communications are a vital marketing tool in transmitting the green marketing efforts of the hotels to their clientele thereby creating awareness although the receptiveness is subject to individual perception (Sharma, 2021; Vinay & Rudresh, 2018). While direct liaisons with the hotel staff are preferred by consumers due to the personalisation, training the staff on the hotels' greening efforts is what makes it lucrative (Chan, 2014; Kim & Choi, 2013). Further, past hotel experiences are impactful in making future decisions through the knowledge gained (Wang & Wu, 2016). Moreover, whilst factual green advertisements are regarded effective than pictural skits, they also have the potential to elicit choosing green hotels despite sacrificing a few comforts to promote a healthy environment (Butt, Mukerji & Shareef, 2017; Chen, Hu, He, Lin & Matilla, 2022). The findings of this study confirm these claims as the majority are aware of their environmental responsibilities and are open to being informed about green hotel practices. Environmental awareness promotes a positive attitude toward green hotels and has the potency to influence ecological consumer behaviour and thereby instigate purchase intention of green hotels (Teng, Lu & Huang, 2018). Further as depicted in Table 4, the correlation coefficient value of 0.537 and the p-value of less than 0.001 confirm that there is a positive strong significant relationship between green communications and purchase intention. Therefore, based on the previous study findings and statistical correlation analysis, H2 is proven to be true.

Pricing of green hotels is considered the most dominating factor that leads to purchase intention and thereby influences the actual behaviour (Chan, 2014). The consumers evaluate the pricing of green hotels against their personal gains which highlights the importance of adding value-enhancing measures to justify the pricing (Jiang & Kim, 2015). Eco-conscious consumers who value environment-friendly initiatives willingly choose to stay at green hotels despite the premium pricing (Gonzalez-Rodriguez, Diaz-Fernandez & Font, 2020). Additionally, as stated in Table 4, pricing depicts a correlation coefficient value of 0.583 and a p-value of less than 0.001 which confirms a positively strong significant relationship between pricing and purchase intention. The study findings corroborate previous theoretical assertions with a majority agreeing that green hotels offer value for the price and are willing to pay a premium thereby validating the H3. However, the popular belief is that green hotels must offer discounts for the sacrifice in the level of luxury against staying at a conventional hotel in terms of reducing, reusing and recycling measures (Sharma, 2021). Alternatively, this study's findings suggest that incremental pricing creates a presupposition for an elevated value among consumers, 'more the price more value' which reemphasises the importance of the value offering (Jiang & Kim, 2015).

Prior research revealed mixed views on the impact of subjective norms on purchase intention as it is a reflection of the social influence on an individual's behaviour (Han & Yoon, 2015; Patwary, Aziz & Hashim, 2022; Wang, Wong, Narayanan, 2019). Patwary et al. (2022) asserted that subjective norms have a direct significant correlation with purchase intention and it is one of the most influential factors. As per the correlation analysis presented in Table 4, subjective norms depict a correlation coefficient value of 0.437 with a significance of less than 0.001 indicating a moderately significant relationship with purchase intention. Therefore, H4 is validated based on previous study findings and statistical findings derived from the correlation analysis of this study. However, these findings contrast Wang et al.'s (2019) findings which yielded negative results on the relationship between subjective norms and purchase intention.

Analysing the above findings in the context of the theory of planned behaviour, this study confirms that attitude plays a key role in instigating an intention which eventually leads towards a behaviour as affirmed by many researchers (Achchuthan et al., 2021; Dwivedi et al., 2022; Wang et al., 2019; Yarimoglu & Gunay, 2019). Further, subjective norms which are influenced by societal views portray a significant relationship towards instituting an

intention which has produced mixed findings in the previous research (Patwary et al., 2022; Wang et al., 2019). Thereby, this study conforms to the assertion of the theory of planned behaviour based on the tested two constructs that attitude and subjective norms initiate an intention through the prospects of individual and social perception (Ajzen, 1991).

3.6 Multiple Linear Regression

As per the regression analysis results of this study shown in Table 6, the R Square value was 0.436, which highlights that 43.6 percent of the changes in the purchase intention could be explained through the combination of the independent variables of green practices, green communications, pricing and subjective norms while pricing and green communication being constant predictors of purchase intention.

Table 6. Multiple Linear Regression

Independent variable	Significance (<0.05)
Pricing	<0.001
Green Communications	<0.001
Green Practices	0.281
Subjective Norms	0.151

The findings portrayed in Table 6 reiterate the significance of pricing and green communications towards purchase intention with a significance level of below 0.001 satisfying the criterion. However, green practices and subjective norms depict a significance of 0.281 and 0.151 respectively which is above the threshold level of 0.05, therefore both these variables are less significant towards instigating purchase intention towards green hotels compared to pricing and green communications. The lower significance of green practices could be due to the gap that has been identified by many researchers between the positive attitude towards recycling measures, willingness to participate and the actual behaviour which appears to be a challenge for green hotels (Lunde, 2018; Sharma, 2021). Alternatively, it could also be due to the lack of awareness or ineffective communication of green hotels in educating the consumers about their green practices (Wheeler, Sharp & Nenycz-Thiel, 2013). Similarly, the low significance of subjective norms could be due to the reluctance of the consumers to admit the influence of their friends and family in a survey setting or maybe due to consumers' increased activity on social media resulting in a lack of interaction with peers depicting less significance of the influence of family and friends (Mishra & Maity, 2021).

3.7 Descriptive Analysis

A descriptive analysis was performed to determine the mean value and thereby measure the central tendency of the tested variables green practices, green communications, pricing, subjective norms and purchase intention which show the average of the responses received on each variable as depicted in Table 7.

Table 7. Degree of satisfaction

Variable	Mean value
Green practices	4.38
Green communication	4.15
Pricing	3.84
Subjective norms	3.31
Purchase intention	4.15

Based on Table 7, green practices depict a mean value of 4.38 which demonstrates that the majority of consumers agree that green practices are vital in selecting green hotels for their stay. This implies that the consumers acknowledge their preference and willingness to participate in hotel green practices which in turn emphasises the importance of instigating consumer purchase intention (Jiang & Kim, 2015). Further, green communications show a mean value of 4.15 highlighting that the majority of the consumers agree that green communications help in creating awareness about environmental concerns and are willing to receive information about eco-friendly practices of green hotels. Similarly, pricing displays a mean value of 3.84 which implies that majority of consumers perceive that green hotels offer great value for their money, and are willing to pay a premium to stay at green hotels with the expectation of added value compared

to orthodox hotels. Thus, confirming that pricing and value influence green hotel selection (Verma & Chandra, 2017). However, the mean value of subjective norms of 3.31 refers that most of the consumers had a neutral view towards conforming to societal pressure in making the green hotel choice which reconfirms the mixed views of the previous research on the influence of subjective norms towards purchase intention (Wang et al., 2019). Purchase intention on the other hand depicts a mean value of 4.15 which highlights that most consumers intend to stay at green hotels and would recommend them to others.

4. Conclusion

The study findings revealed that the attitude towards green practices, green communications, pricing and subjective norms have a significant influence on the purchase intention of green hotels among domestic tourists of Sri Lanka whilst green practices and subjective norms are less significant compared to pricing and green communication. Considering the findings, the study suggested the following recommendations that would help green hoteliers enhance their product offerings based on each variable that was analysed in this study.

5. Recommendations

Green Practices

Green practices depicted less significance in initiating purchase intention in the regression analysis, although the mean value indicates that the majority of consumers depict a positive attitude towards the 3R concept and are willing to participate in green practices. These results draw attention towards implementing sensible and inclusive green marketing campaigns that promote environmental and community well-being that differentiate green hotels from the rest. While there are common green practices that would benefit the hotels, a few community-uplifting philanthropic initiatives such as donating pre-used linen, towels and napkins that are in an admissible state to village hospitals, elderly homes and children's homes, training youth on industry-specific skills, women and child welfare and an inclusive and diverse workforce would build a positive green image which is known to incite purchase intention and brand loyalty (Chung, 2019). These measures would increase employee engagement and thus reduce turnover in addition to improving the constitutionality of the organisation, increasing shareholder value and thereby inducing the purchase intention among domestic tourists to patronise a responsible brand (Kasim et al., 2022).

Green Communications

Green communications were proven a significant factor in instituting purchase intention towards green hotels through correlation and regression analysis. Therefore, it is imperative that hotels analyse their clientele based on demographics such as age group, gender, income and educational level and develop advertisements and other communications to suit the targeted consumer segment (Wang et al., 2019). Furthermore, since generations Y and Z have been identified as the most travelled segments on a global scale, social media communications are vital in communicating the hotel's green practices through these electronic mediums which have a wide reach and huge potential to influence purchase intention (Sharma, Fadahunsi, Abbas & Pathak, 2021). Moreover, Sri Lanka's internet users' percentage depicts a growing trend which emphasises the importance of designing a solid digital marketing plan including e-booking facilities (Statista, 2023). Furthermore, the social media sites, corporate websites and e-booking sites must have detailed information on the hotel's green practices, green certifications, corporate social responsibility (CSR) initiatives, community-related programs and special features along with pricing to be highlighted in order to captivate the target group (Erkan & Evans, 2018).

Pricing

The findings of this study, exhibit that the expectation of increased value corresponds with the price increase. Therefore, it is crucial that hotels set pricing models that include added value propositions that satisfy the consumers' sense of responsibility by promoting emotional benefits and the holistic outlook of the green hotel setting. Green hotels must align their communication strategies to focus on the green benefits with facts and information on the negative impacts of non-green practices to capture consumer interest and provoke environmental concern to impart effective

environmental knowledge through captivating visuals combined with facts (Gonzalez-Rodriguez et al., 2020; Jiang & Kim, 2015). These could convey an increased value to the consumers and comprehend them as their personal gain against the pricing of the hotel in inducing purchase intention (Jiang & Kim, 2015). As current Sri Lankan disposable income is affected due to various reasons, it is imperative to communicate the values built into the overall offering to convince domestic tourists of the worthiness of green hotels amidst increased price sensitivity (Athukorala, 2023).

Subjective Norms

Although subjective norms depicted a low significance towards purchase intention on the regression analysis, peer pressure is a significant factor in influencing purchase intention of premium products (Sharma, 2021). Green hotels must build an effective customer relationship management strategy to build a loyal customer base that would influence their peers and social groups towards purchasing green hotels. This could be done through periodical newsletters to patrons, targeted paid ads on social media and transparency in sharing the green practices, achievements, certifications and testimonials from guests that would evoke engagement with the brand (Wang, 2020; Wang & Wu, 2016). Additionally, green hoteliers could adopt the crowdsourcing strategy by administering surveys to obtain feedback from the targeted consumer groups to improve concerned areas by making them feel valued and advocate the brand thereby instigating purchase intention through subjective norms (Sharma, Chen, Ramos & Sharma, 2023).

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