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BMS PUBLISHER

591, Galle Road,
Colombo 06.
Sri Lanka

Tel: +94 11 250 4757

For Paper Submission: bioscience@bms.ac.lk (Applied Science)

abdul.c@bms.ac.lk (Management)

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146 Pages

Contents

Page No

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Comparative Evaluation of the Effectiveness of Sunlight and Artificial Grow Lights on the Growth and Biochemical Properties of Hydroponically Cultivated Leafy Vegetables

Nilanja Ranuli Jayamanne[1], Upekkha Abhayarathne[1], Nadeera Gayan[2] and Geethika S. G. Liyanage[1*]

[1]School of Science, Business Management School (BMS), Sri Lanka

[2]AiGrow (Pvt.) Ltd., Bays 1-5, TRACE Expert City, Colombo 10, Sri Lanka

*geethika.l@bms.ac.lk

Abstract

Enhancing the nutritional quality and growth rate of leafy vegetables through different light sources could advance the rapid growth of healthier crops under greenhouse conditions. This study investigated the effects of three light sources (natural sunlight, AiGrow™ LED light, and Philips™ LED light) on the growth and biochemical properties of hydroponically cultivated Oak Red and Lollo Bionda lettuce varieties. The plants were cultivated in a Nutrient Film Technique (NFT) hydroponic system under controlled greenhouse conditions and were grown for a period of four weeks before being harvested for extract preparation. Aqueous lettuce extracts were prepared and analyzed for total carbohydrate content (TCC) using the phenol-sulphuric acid method, total protein content (TPC) using the Lowry assay, total phenolic content (TPhC) using the Folin-Ciocalteu assay, and total flavonoid content (TFC) through the $AlCl_3$ spectrophotometric method. Antioxidant activity (AA) was assessed by the DPPH assay. Qualitative phytochemical tests identified the presence of polyphenols, terpenoids, saponins, and steroids across all samples. Results indicated that Oak Red lettuce grown under sunlight exhibited the highest levels of TCC (0.094 g/100 g), TPC (0.421 g/100 g), TPhC (0.228 mg GAE/g), and AA (69.18%), while Oak Red lettuce grown under AiGrow™ LED light had the highest TFC (0.721 mg QE/g). In terms of growth performance, AiGrow™ LED light was most effective for both lettuce varieties. Thus, according to the results obtained from this study, AiGrow™ LED light, which supported a maximum flavonoid content of 0.721 mg QE/g dried weight and comparable growth performance, can be recommended as a suitable alternative to sunlight for indoor hydroponic cultivation of Oak Red and Lollo Bionda lettuce.

Keywords: Hydroponic lettuce, Sunlight, Grow lights, Nutritional quality, Antioxidant activity

1. Introduction

Hydroponics is an innovative and efficient plant-growing technique that relies on a water-based nutrient solution providing an alternative to conventional agriculture.[1] This soilless method, which may or may not incorporate a substrate for mechanical support, has gained significant attention due to its ability to produce high yields in diverse crops, including herbs, ornamental plants, and a variety of vegetables such as tomatoes, lettuce, and cucumbers.[2]

Among the different hydroponic systems available, the Nutrient Film Technique (NFT) stands out as one of the most widely used due to its simplicity and effectiveness in fostering plant growth while conserving water. Other popular systems include deep-water culture, aeroponics, wick, and drip systems, each offering unique advantages depending on the crop and environmental conditions.[3]

Hydroponic systems are generally categorized into two groups: open and closed systems. In open systems, the nutrient solution is used once and then discarded, whereas closed systems recycle the nutrient solution, minimizing waste and enhancing sustainability.[4]

The NFT system, which was utilized in this research, falls under the closed system category. This technique involves suspending plants above a continuously flowing nutrient solution, ensuring that the plant roots are exposed to an aerated stream of water, which allows the roots to absorb moisture and oxygen efficiently (Figure 1).[5] The system's design ensures that the nutrient solution flows down the channels, aided by a gentle tilt, and is recirculated to the reservoir, promoting water conservation, and reducing the overall environmental impact of the system.[6]

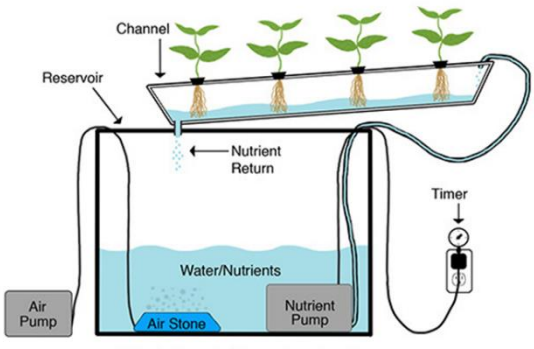

Figure 1. Nutrient film technique[7]

The NFT system is particularly suitable for growing light-demanding plants like lettuce, spinach, and strawberries, which benefit from the consistent nutrient supply and efficient water usage that the system offers.[8]

For plants to grow and develop, they require energy, which they primarily obtain through photosynthesis. Light plays a crucial role in this process, as it stimulates the plant's growth and supports its physiological functions. Light quality: specifically, the spectrum of light, greatly influences plant growth, with blue light being particularly important for vegetative growth and red light for flowering and fruiting.[9] Natural sunlight provides an ideal balance of these light wavelengths, promoting healthy plant development. However, in controlled environments such as greenhouses or indoor farms, artificial lighting is often employed to supplement or replace natural light, enabling year-round cultivation of crops. The most commonly used artificial light sources in hydroponic farming include light-emitting diodes (LEDs) and fluorescent bulbs, both of which are energy-efficient and can be tailored to provide the specific light spectrum (400-700 nm) that plants require.[10]

Studies on the impact of light intensity on tomato plant growth have shown that increased light intensity can enhance plant density, leaf thickness, and stem strength. On the other hand, insufficient light can delay critical stages of growth, such as blooming and fruiting, which can reduce yield and overall plant health.[11]

Lettuce (*Lactuca sativa*) is a popular crop in hydroponic farming due to its relatively low resource requirements and fast growth rate. It is a rich source of essential nutrients, including vitamins A and K, and is widely cultivated in a range of varieties such as romaine, butterhead, and oak leaf.[14] For this study, two lettuce varieties: red oak and leaf green lettuce (Lollo Bionda), were selected due to their popularity in hydroponic systems and their distinctive characteristics. Red oak lettuce, with its burgundy-colored leaves and mild, nutty flavor, is known for its tender texture and resistance to bitterness (Figure 2).[15] Similarly, Lollo Bionda lettuce, also known for its delicate texture and mild taste, has become a staple in hydroponic farming due to its adaptability and high nutritional value (Figure 3).[16]

Figure 2. Oak red lettuce[12]

Figure 3. Leaf green lettuce[13]

Based on the literature review, this research focused on evaluating the effects of three different light sources: Philips™ light, AiGrow™ light, and natural sunlight on the growth, nutrient composition, antioxidant activity, and bioactive compounds of oak red and Lollo Bionda lettuce varieties. By investigating the effects of different lighting conditions on the overall health and productivity of lettuce in an NFT hydroponic system, this study aims to contribute valuable insights to optimize hydroponic practices and improve crop yield and quality.

2. Methodology

2.1 Sample Collection. The seeds of the two lettuce varieties (oak red, Lollo Bionda) were obtained from CodeGen International Sri Lanka (Figure 4).

Figure 4. (A) Oak red and (B) Lollo Bionda seeds

2.2 Seed germination of lettuce. The 28 mm soil pellets were soaked in water for 10 minutes. Seeds of oak red and Lollo Bionda were then placed in soil pellets and watered daily. The seeds were left to germinate for 3 weeks before they were transferred to the NFT system (Figure 5).

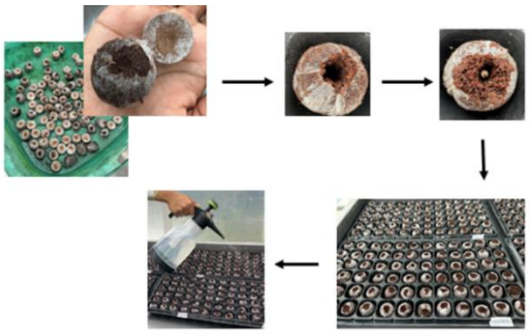

Figure 5. Steps of germination

2.3 Transferring seedlings to the NFT system and growth optimization. The pH and electrical conductivity (EC) values of the nutrient solution (fertilizer mixer) in the tank of the NFT system were balanced by adding AiGrow™ fertilizer A and B solutions at a ratio of 1:1 and KOH. Three levels of the NFT system were set up, each equipped with a different light source. Two levels of LED lighting were partially covered with a black net to avoid the effect of sunlight. The lettuce plants were then placed in the channels of the NFT system. The lettuce plants were allowed to grow for 4 weeks in the NFT system under three light sources (Figure 6).

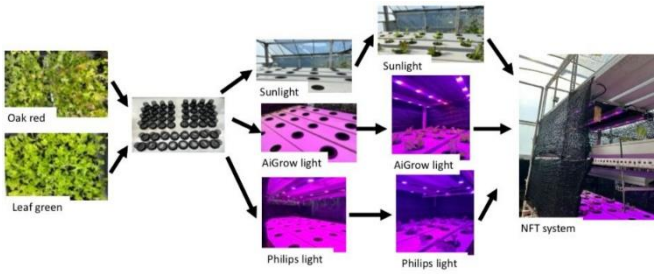

Figure 6. Transferring lettuce into the NFT system

2.4 Homogenization and Preparation of aqueous plant extracts. Once the lettuce plants were harvested after 4 weeks from the NFT system, the fresh weight of the lettuce was measured. They were then shredded and placed in the hot air oven for 48 hours at 40°C. Finally, they were crushed into a fine powder using a mortar and pestle and the dry weight was measured.

The dried lettuce powder samples were mixed with distilled water at a ratio of 1:20 in a beaker. Then the beakers were covered with an aluminium foil and placed in the hot air oven at 90°C for 15 minutes. The extracts were cooled to room temperature (RT) and filtered using Whatman No. 1 filter papers. Finally, the volumes of aqueous extracts were measured.

2.5 Total Carbohydrate Content (TCC) Analysis – Phenol Sulphuric Acid Method. For the preparation of the stock solution, 2.5 N HCl was prepared by mixing 5 ml of concentrated

HCl with 18.2 ml of distilled water. Then the measured 0.1 g of dextrose powder was mixed with 2.5 N HCl and boiled for 3 hours in a water bath. The solution was allowed to cool down at RT and neutralized with Na_2CO_3. Duplicates of a standard series were prepared by proportional dilution within the 50-250 µg/ml range (50, 100, 150, 200, 250 µg/ml) using dextrose. Then lettuce extracts were diluted 20 times and duplicates of each lettuce sample were prepared. 1 ml of 5% phenol was added to 1 ml of the standard series, blank (distilled water), and samples and they were incubated at RT for 10 minutes. After that 5 ml of 96% sulfuric acid was added to all the tubes and they were again incubated at RT for 20 minutes. Finally, the absorbance was measured at 490 nm using a UV-visible spectrophotometer.[17]

2.6 Total Protein Content (TPC) Analysis – Lowry Assay. Lowry A (2% Na_2CO_3 and 0.1 N NaOH), Lowry B (0.5% $CuSO_4$ and 1% sodium potassium tartrate), and Lowry C (Folin-ciocalteu reagent and distilled water in 1:1 ratio) solutions were prepared. A 1000 µg/ml stock solution of Bovine Serum Albumin (BSA) was prepared by dissolving 100 mg of BSA in 100 ml of distilled water. Then, duplicates of the BSA standard series were prepared using varying concentrations of 200, 400, 600, 800, and 1000 µg/ml. Extracts were diluted 20 times and duplicates of each sample were prepared. Then, the Lowry A and Lowry B mix was prepared at a 50:1 ratio. A volume of 5 ml from the mix was added to 1 ml of each standard series, blank (distilled water), and extract and they were incubated for 10 minutes at RT. A volume of 0.5 ml from Lowry C was then added to each tube and incubated for 30 minutes at RT. The absorbance was measured using a UV-visible spectrophotometer at 660 nm.[18]

2.7 Qualitative Analysis of Phytochemicals. The protocols used for qualitative phytochemical analyses are shown in Table 1.

Table 1. Qualitative phytochemical analyses test methods.[19]

Test	Methodology
Anthraquinones	2 ml of 10% ammonia solution was added and mixed with 0.5 ml of each extract.
Flavonoids	2 ml of 2% NaOH solution was added to 1 ml of each sample and 2 drops of diluted HCl was added.
Polyphenols	3 drops of diluted iodine solution were added to 1 ml of each sample.
Saponins	5 ml of distilled water was added to 1 ml of lettuce sample and vortexed for 10-15 minutes until froth was formed.
Steroids	0.5 ml of each sample was mixed with 0.5 ml of chloroform and 1 ml of concentrated H_2SO_4.
Tannins	2 ml of 5% $FeCl_3$ was added to 1 ml of each lettuce sample.
Terpenoids	2 ml of chloroform was added to each sample and 2 ml of concentrated H_2SO_4.

2.8 Total Phenolic Content (TPhC) Analysis – Folin-Ciocalteu Assay. A 1000 µg/ml gallic acid standard stock solution was made by mixing 1 mg of gallic acid powder with 10 ml of distilled water. Then a standard gallic acid series of different concentrations (20, 40, 60, 80, and 100 µg/ml) were prepared. 30 µl each of plant extract, blank (distilled water), standard solutions, 270 µl of distilled water, and 1.2 ml of 10% Folin-Ciocalteu reagent were mixed and placed in the dark for 5 minutes. Next, 1.5 ml of 7.5% Na_2CO_3 was added, and the mixture was incubated at RT for 90 minutes in the dark. Then using a UV-visible spectrophotometer, the absorbance was measured at 765 nm.[20]

2.9 Total Flavonoid Content (TFC) Analysis – $AlCl_3$ colorimetric method. A 100 µg/mL Quercetin stock solution was prepared by dissolving 1 mg of Quercetin powder in 10 mL of distilled water. Using this stock solution, duplicates of the Quercetin standard series were prepared at concentrations of 20, 40, 60, 80, and 100 µg/ml. 50 µl of each extract, blank (distilled water), standard solutions were transferred into separate test tubes. To each tube, 950 µl of distilled water was added to dilute the samples. Next, 0.2 ml of 10% $AlCl_3$ solution was added to each tube to initiate the reaction. After that, 0.2 ml of 1M potassium acetate solution was added to stabilize the complex formation. The reaction mixtures were incubated at room temperature (RT) for 30 minutes with intermittent shaking to ensure proper mixing and reaction completion. After incubation, the absorbance of each reaction mixture was measured at 415 nm using a UV-visible spectrophotometer.[21]

2.10 Antioxidant Activity (AA) Analysis – DPPH Radical Scavenging Assay. A DPPH stock solution was prepared by dissolving 40 mg of DPPH in 100 ml of methanol. The test tubes were filled with 25 µl of each leaf extract, 475 µl of distilled water, and 1 ml of DPPH working solutions. The reaction mixtures were incubated in the dark for 30 minutes. Then the absorbance was measured at 517 nm using a UV-visible spectrophotometer. The DPPH inhibition percentage was calculated using the following equation.[22]

$$Percentage\ inhibition\ of\ DPPH = \frac{Ac - As}{Ac} \times 100$$

(Ac – Absorbance of the control, As – Absorbance of the sample)

2.11 Statistical Analysis. All values are expressed as mean ± Standard Error. Microsoft Excel 2023 was used to calculate, the standard error of the mean value. IBM SPSS Statistics Version 29 was used to conduct the LSD test for multiple comparison analysis and One-Way ANOVA. A significant difference was defined as having a *p*-value less than 0.05.

3. Results

3.1 Harvested lettuce plants from the NFT system. The growth progression of the lettuce plants after 4 weeks that were harvested from the NFT system is shown in Table 2.

Table 2. Images of harvested lettuce plants after being kept in each condition for 4 weeks.

	Oak Red	Lollo Bionda
Sunlight		
AiGrow™ Light		
Philips™ Light		

3.2 Morphological parameters of the lettuce

3.2.1 Average Plant Height

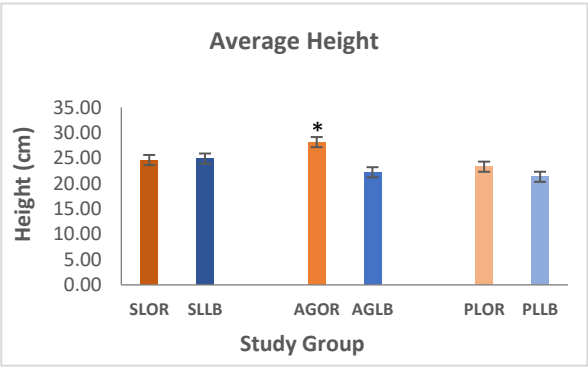

Figure 7. Average height of the harvested lettuce plants. (* represents $p<0.05$ compared to the SLOR group, SL = Sunlight, AG =

AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

The average height of the AGOR group was significantly higher compared to the SLOR group. PLOR displayed the lowest average height among the oak red plants (Figure 7).

The highest average height in Lollo Bionda was reported in SLLB and the lowest was reported in PLLB (Figure 7). However, there were no significant differences between the groups.

3.2.2 Average Leaf Number

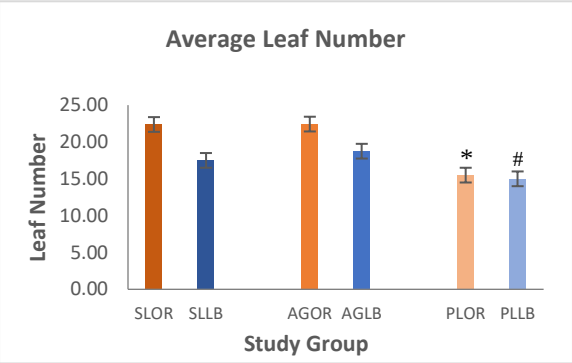

Figure 8. Average leaf number of the harvested lettuce plants. (* represents $p<0.05$ compared to the SLOR group and # represents $p<0.05$ compared to the SLLB group, SL = Sunlight, AG = AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

The average leaf number of the SLOR and AGOR groups was comparably similar. PLOR displayed the lowest average leaf number, which was significantly low compared to the SLOR group (Figure 8).

The highest average leaf number in Lollo Bionda was reported in AGLB and the lowest was reported in PLLB which was significantly low compared to the SLLB group (Figure 8).

3.3 Total Carbohydrate Content (TCC) Analysis

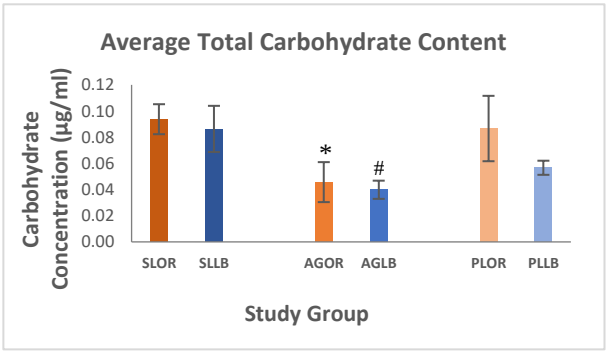

Figure 9. Average TCC analysis of the harvested lettuce plants. (* represents $p<0.05$ compared to the SLOR group, and # represents $p<0.05$ compared to the SLLB group, SL = Sunlight, AG = AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

SLOR group displayed the highest average TCC in oak red and the AGOR displayed the lowest average TCC in oak red (Figure 9).

The highest average TCC in Lollo Bionda was reported in SLLB and the lowest was reported in AGLB (Figure 9).

3.4 Total Protein Content (TPC) Analysis

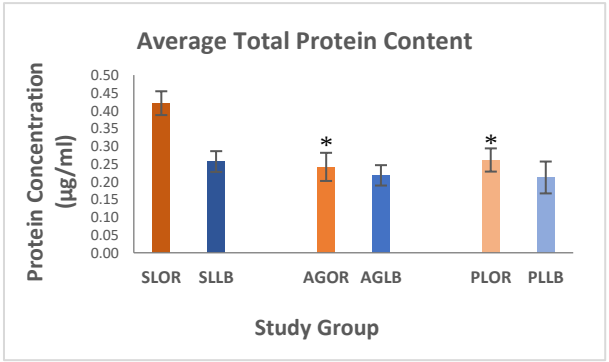

Figure 10. Average TPC Analysis of the harvested lettuce plants. (* represents $p<0.05$ compared to the SLOR group, SL = Sunlight,

AG = AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

The average TPC of the SLOR group was significantly higher compared to the AGOR and PLOR groups. AGOR displayed the lowest average TPC in oak red (Figure 10).

The highest average TPC in Lollo Bionda was reported in SLLB and there were no significant differences between the groups (Figure 10).

3.5 Qualitative Analysis of Phytochemicals

Table 3. Results of Phytochemical Analysis (√-Present; x-Absent)

Phytochemical	PLOR	PLLB	AGOR	AGLB	SLOR	SLLB
Saponins	√	√	√	√	√	√
Polyphenols	√	√	√	√	√	√
Tannins	x	x	x	x	x	x
Terpenoids	√	√	√	√	√	√
Anthraquinones	x	x	x	x	x	x
Steroids	√	√	√	√	√	√

(SL = Sunlight, AG = AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

Results showed that Polyphenols, Terpenoids, Saponins, and Steroids were present in all the lettuce samples (Table 3). However, Tannins and Anthraquinones were absent in all groups.

3.6 Total Phenolic Content (TPhC) Analysis

The average TPhC of the SLOR group was significantly higher compared to the AGOR group which displayed the lowest average TPhC in oak red (Figure 11).

TPhC in Lollo Bionda was significantly high in SLLB compared to the other two groups. The lowest TPhC was reported in AGLB (Figure 11).

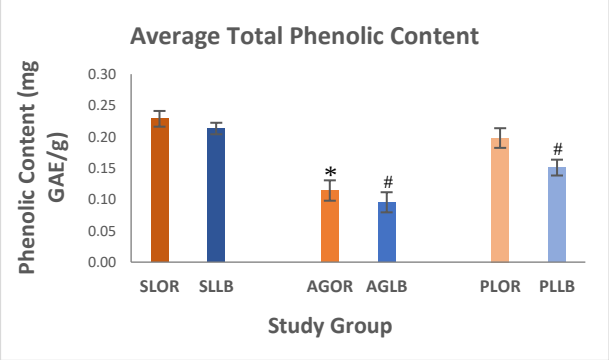

Figure 11. Average TPhC Analysis of the harvested lettuce plants. (* represents $p<0.05$ compared to the SLOR group, and # represents $p<0.05$ compared to the SLLB group, SL = Sunlight, AG = AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

3.7 Total Flavonoid Content (TFC) Analysis

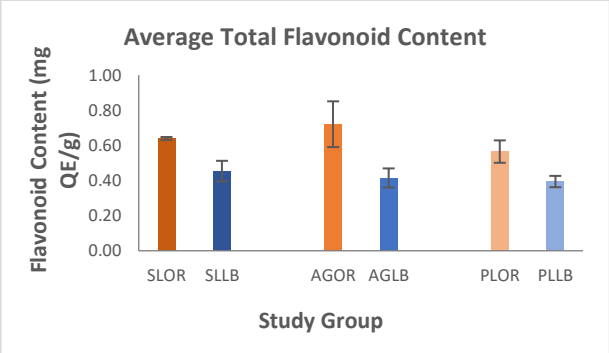

Figure 12. Average Total Flavonoids Content Analysis of the harvested lettuce plants. (SL = Sunlight, AG = AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

The average TFC of the AGOR group was higher compared to the SLOR and PLOR groups. PLOR displayed the lowest average TFC in oak red (Figure 12). However, there were no significant differences between the groups.

The highest average TFC in Lollo Bionda was reported in SLLB and the lowest was reported in PLLB (Figure 12).

3.8 Antioxidant Activity (AA) Analysis – DPPH Radical Scavenging Assay

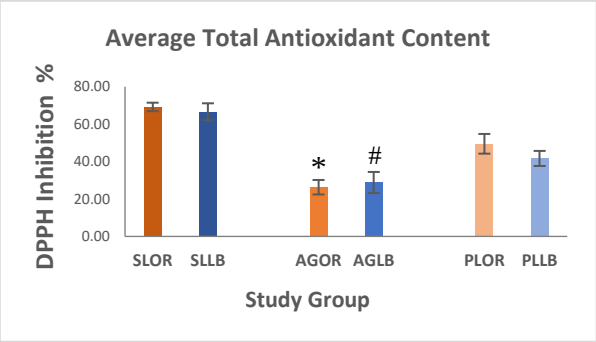

Figure 13. Average Antioxidant Content Analysis of the harvested lettuce plants. (* represents $p<0.05$ compared to the SLOR group, and # represents $p<0.05$ compared to the SLLB group, SL = Sunlight, AG = AiGrow™ Light, PL = Philips™ Light, OR = Oak Red, LB = Lollo Bionda).

The average AA of the SLOR group was significantly higher compared to the AGOR group which displayed the lowest average AA in oak red (Figure 13).

The highest average AA in Lollo Bionda was reported in SLLB and the lowest was reported in AGLB (Figure 13) which was significantly lower compared to the SLLB group.

4. Discussion

This study evaluated the effects of three distinct light sources (sunlight, AiGrow™ light, and Philips™ light) on the growth, biochemical composition, and antioxidant capacity of hydroponically grown Oak Red and Lollo Bionda lettuce using a Nutrient Film Technique (NFT) system. The primary objective was to identify the light conditions that optimize growth and nutritional benefits for these lettuce varieties, contributing to advancements in indoor agriculture.

The germination phase used soil pellets as a medium to provide consistent nutrient delivery and physical support, ensuring uniform seedling development and minimizing variability. These conditions were essential to establish healthy seedlings for hydroponic growth. The pH and EC of the nutrient solution were carefully balanced in the NFT system to optimize nutrient uptake and minimize plant stress, critical factors for achieving reliable growth outcomes. The experimental setup was designed to create distinct light environments while minimizing light interference between treatments, ensuring valid comparisons of the effects of each light source on plant performance.

Growth assessments revealed that sunlight favored the growth height of Lollo Bionda lettuce, while AiGrow™ light was the most effective for Oak Red. When considering the leaf count, both lettuce types performed best under AiGrow™ light. These findings suggest that while natural light supports some growth aspects, artificial light can be optimized to promote other growth parameters, making AiGrow™ light an ideal substitute under controlled indoor conditions, such as a temperature range of 28–30°C, relative humidity of 55–65%, and a photoperiod of 16 hours of light and 8 hours of darkness. As indoor farming expands, understanding the specific needs of each plant variety in response to artificial light becomes crucial.

Upon harvesting, the fresh weight of each plant was recorded, and leaves were processed for biochemical analyses. The study evaluated carbohydrate, protein, phenolic, flavonoid, and antioxidant content, using aqueous extracts from dried lettuce samples. In line with existing literature, lettuce exhibits minimal carbohydrate levels, a trait favorable for low-carbohydrate diets.[27] Carbohydrate content was assessed using the Phenol-Sulfuric Acid method, which utilizes sulfuric acid to dehydrate polysaccharides, forming detectable compounds when reacted with phenol.[28] The sunlight-exposed Oak Red group (SLOR) showed the highest carbohydrate levels at 0.094 g/100 g dried weight, surpassing other groups. Previous studies indicate that wavelengths within the red (620–750 nm) and blue (450–495 nm) spectrum promote carbohydrate accumulation in plants.[29] Given that sunlight provides a full spectrum (400–700 nm), these

results align with previous findings, reaffirming the role of sunlight in carbohydrate synthesis.

Protein levels were assessed using the Lowry assay method, which relies on the formation of monovalent copper ions that react with the Folin reagent to produce a blue complex, indicating protein presence.[30] The sunlight-exposed Oak Red group (SLOR) showed the highest protein levels, with a mean value of 0.421 g/100 g dried weight. Higher protein concentrations are linked to light sources with significant blue light ratios (450–495 nm), which boost soluble protein levels.[29] Sunlight encompasses this range, supporting our findings that natural light fosters protein synthesis effectively.

Qualitative phytochemical analyses revealed the presence of polyphenols, terpenoids, saponins, and steroids across all groups, while tannins and anthraquinones were absent. According to a previously conducted study, the phytochemical analysis of Red Leaf lettuce (RL) and Green Leaf lettuce (GL) revealed the presence of phenols, alkaloids, saponins, flavonoids, tannins, and terpenoids. The study further highlighted that RL contained higher amounts of phenols, alkaloids, and saponins compared to GL, while both varieties exhibited similar levels of flavonoids, tannins, and terpenoids.[31] These findings align with the qualitative phytochemical analyses in this study, emphasizing the variations in phytochemical composition across lettuce varieties. Phytochemicals play diverse roles in plants, including defense against environmental stressors, protection against pests and pathogens, regulation of growth and development, and adaptation to abiotic stress conditions such as drought and UV radiation.[32]

Polyphenols, in particular, play a crucial role in plant resilience and physiological functions, deriving from phenylpropanoid and pentose phosphate pathways.[33] The phenolic content, determined using the Folin-Ciocalteu method, was also highest in the SLOR group, with a mean value of 0.228 mg GAE/g dried weight. According to previous studies, the broad spectrum of sunlight (400–700 nm) facilitates phenolic synthesis, aligning with existing studies on the role of red and blue light in phenolic accumulation.[34]

In evaluating flavonoids, a secondary metabolite critical to plant color, aroma, and stress defense,[35] $AlCl_3$ colorimetric method was used, which binds to hydroxyl groups on flavonoids to form stable complexes detectable at specific wavelengths.[36] The highest flavonoid concentration was observed in the AiGrow™ light-exposed Oak Red group (AGOR), with a mean value of 0.721 mg QE/g dried weight. Interestingly, these results diverged from previous findings where blue LED light (450–495 nm) maximized flavonoid content.[37] This could be due to the unique spectral composition of the AiGrow™ light, which emphasizes UV-A wavelengths (315–400 nm).

To assess antioxidant activity, the DPPH radical scavenging assay was employed, measuring the ability of lettuce to neutralize free radicals.[38] Antioxidants donate electrons or hydrogen atoms to stabilize free radicals, reducing potential cellular damage.[39] The SLOR group demonstrated the highest antioxidant capacity with a 69.18% inhibition. This finding is consistent with a previous study showing that full-spectrum sunlight maximizes antioxidant activity in green leafy vegetables by activating compounds responsive to UV and visible light.[32]

5. Conclusion

This study demonstrates that sunlight is the most effective light source for maximizing growth, biochemical content, and antioxidant potential in hydroponically grown Oak Red and Lollo Bionda lettuce using an NFT system. AiGrow™ light served as a viable alternative, supporting substantial growth and bioactive compound synthesis, though slightly less effective than sunlight in promoting bioactive compounds. In contrast, Philips™ light, with its limited spectrum, was the least effective across all parameters, underscoring the critical role of spectrum and intensity in optimizing plant health. While this study provides valuable insights, it was limited by the controlled

experimental setup, which may not fully reflect real-world farming conditions. Additionally, the study only explored two lettuce varieties and a limited range of artificial light sources. Future research could explore the integration of multiple light sources, varying light intensities, and other crop species to further optimize hydroponic farming systems. These findings pave the way for advancements in light-source optimization and system design, ultimately contributing to improved crop outcomes and nutritional quality in hydroponic agriculture.

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Investigation of Abiotic Stress Responses of *Centella asiatica* in Sri Lanka

Abishan Arumugathasan[1] and Neranja Sandamini[1*]

[1]School of Science, Business Management School (BMS), Sri Lanka

[*]neranja.s@bms.ac.lk

Abstract

Plant stress is the term used to describe a plant's inability to grow under certain adverse conditions. This situation can occur only when the stressor exceeds a threshold, which can result in acute growth deficits, permanent damage, and lower agricultural productivity. The aim of this study was to investigate biochemical, anti-oxidant, and nutritional modifications of *Centella asiatica* (Gotu kola) in response to different abiotic stress conditions. *C. asiatica* plants were exposed to different stressors including drought, salinity, heavy metal, nutrient deficiency, and flood. Qualitative tests were performed to identify the presence of bioactive compounds and quantitative tests such as the Folin-Ciocalteu assay for total phenolic count, Aluminium chloride assay for total flavonoid capacity, Lowry assay for total protein content, and Phosphomolybdate assay for total antioxidant capacity were conducted. Additionally, the antibacterial property of *C.asiatica* was examined using antibacterial sensitivity testing for *Escherichia coli*. Based on the qualitative test results, control plants and, salinity, heavy metal, nutrient deficiency, drought, and flood-induced plants showed positive results for tannins, saponins, flavonoids, coumarins, terpenoids, cardiac glycoside, and steroids. However, quantitative test results showed that flood-induced plants had significantly higher values compared to control and other stresses for total phenolic content, total antioxidant capacity, and total protein content. The flood condition sample had higher overall significant values for total phenolic content (14.95 mg $GAEg^{-1}$), total antioxidant capacity (120.5 mg $AAEg^{-1}$), and total protein content (56.55 mg $BSAEg^{-1}$) at 10% error margin. *C. asiatica* showed a negative result for the antibacterial sensitivity test for *Escherichia coli*. This study showed that *C. asiatica* has the potential to be a source of natural antioxidants.

Keywords: *Centella asiatica*, Abiotic Stress, Nutritional modification, Phytochemical, Polyethylene Glycol 6000 (PEG-6000), *Escherichia coli*

1. Introduction

Plant stress refers to the difficulty in growing under certain adverse conditions. This condition occurs when the stressor exceeds a certain tolerance point, which can immediately lead to reduced agricultural production, irreversible damage, and growth deficits.[1]

Major environmental threats that significantly reduce crop yield can be categorized as abiotic and biotic threats. Abiotic stresses include drought, salinity, heavy metals, nutrient deficiencies, flooding, etc. Biotic stresses include various living organisms such as fungi, bacteria, viruses, nematodes and insects.[2] Abiotic environmental conditions significantly impact immobile plants by affecting their biological processes, gene expression, and cellular metabolism.[3]

Drought and high soil salinity are major abiotic stressors that reduce crop yields by causing osmotic stress, ionic toxicity, and impaired growth, metabolism, and cell function.[4] Soil metal concentrations affect plant physiology and seed germination, while flooded soils lead to oxygen loss, impaired respiration, and toxic CO_2 accumulation.[3] *Centella asiatica*, known as Gotu kola in Sri Lanka, shown in Figure 01, is a herbaceous plant in the Mackinlayaceae family, historically used for its therapeutic properties.[2] *C.asiatica* is a tasteless, odorless plant found in rocky terrain, rice fields, and swamps, with small oval fruit, white or light purple-to-pink flowers, and tiny fan-shaped green leaves.[5]

Extreme changes in climate like drought, flood, and changes in soil conditions have an impact on the agricultural aspect, resulting in a decline in crop yield and ultimately the nutritional properties of the crop. The current understanding of plant stress tolerance can be significantly improved through a thorough characterization and its contribution to stress tolerance.[6] Therefore, this study aimed to investigate biochemical, antioxidant, and nutritional modifications of *Centella asiatica* (Gotu kola) in response to different abiotic stress conditions as it is a commonly consumed leafy green vegetable in Sri Lanka.

Figure 1. *Centella asiatica* plant

2. Methodology

2.1 Abiotic stress-induction of Centella asiatica plants. Different abiotic stress conditions were induced according to Table 1.

Table 1. Induction of abiotic stress conditions

Abiotic Stress condition	Procedure
Heavy Metal	50 mL of 25 mM $Pb(NO_3)_2$ was applied twice a day for two weeks.[7]
Drought	50 mL of 10% Polyethylene Glycol 6000 (PEG-6000) solution was added to the plants once per week and plants were kept under direct sunlight for two weeks without adding water.[8]
Flood	Water was added until the root and the stem were completely submerged and maintained for two weeks.[9]
High Salinity	50 mL of 150 mM NaCl was added every morning and evening for two week.[10]
Nutrient Deficiency	Gotu kola plants were transferred to autoclaved sand and 100 mL of water was added to the plants once a day for two weeks

2.2 Sample collection and preparation of aqueous plant extract of Centella asiatica plant samples. Centella asiatica leaves were plucked and weight was measured. Leaves were then washed with distilled water and placed in the drying oven at 40 ^{0}C for 72 hours. Dried leaves were crushed into a fine powder using a mortar and pestle. Aqueous extract was prepared by adding distilled water and powder in 1:40 ratio. The tubes were kept on the roller mixture for 48 hours. Then the extract was filtrated using a Whatman filter paper through a funnel set-up. The filtrate was used in qualitative and quantitative analysis.

2.3 Determination of moisture content. Using the fresh and dry weight values, of the plant leaves, the percentage of the moisture content was calculated using the following equation

$$\text{Moisture Content \%} = \left(\frac{Fresh\ weight(Wo) - Dry\ weight\ (Wd)}{Fresh\ weight\ (Wo)}\right) \times 100\%$$

2.4 Qualitative analysis of Bioactive compounds in Centella asiatica samples. The presence of bioactive compounds was qualitatively analyzed using the protocols given in Table 2.

Table 2. *Bioactive compounds and respective tests to determine their presence in plant sample*[4]

Bioactive Compound	Tests
Alkaloid	To 1 mL of HCl, 2 mL of an extract was added, followed by 3 – 5 drops of Wagner's reagent
Tannins	A few drops of aq. ferric chloride ($FeCl_3$) were added to 1 mL of the extract

Phenolic Compounds	A few drops of 10% aqueous $FeCl_3$ and 2ml of distilled water were added to 1ml extract
Saponins	To 1 mL of plant extract, 5 m L of water was added and then shaken vigorously
Flavonoids	To 1 mL of extract, 2-3 drops (0.5 mL) of sodium hydroxide (NaOH) were added, followed by a few drops of diluted HCl
Quinone	A few drops of NaOH were added to 1 mL of plant extract
Coumarins	1 mL of NaOH was added to 1 mL of Plant
Terpenoids	2 mL chloroform ($CHCl_3$) and 3 mL of concentrated sulfuric acid (conc. H_2SO_4) was added to 1 mL of plant extract
Cardiac Glycoside	A few drops of $FeCl_3$ solution were added to 2 mL of plant extract, followed by 2 mL of glacial acetic acid with 1 mL of conc. H_2SO_4
Steroids	2 mL $CHCl_3$ was added to 1ml of plant extract, followed by 3 mL of concentrated sulphuric acid (Conc. H_2SO_4)

2.5 Determination of Total Phenolic Content. To measure the total phenolic content, 200 µl of 1:80 Gotu kola extract, 1000 µL of distilled water, and 200 µL of Folin-Ciocalteu reagent were mixed and incubated at room temperature for 6 minutes. Then, 2 mL of 7% sodium carbonate solution and 1000 µL of distilled water were added, and the mixture was incubated in the dark for 90 minutes. Absorbance was measured at 760 nm, and the total phenolic content was calculated as gallic acid equivalents (mg GAE/g) using a gallic acid standard curve.[11]

2.6 Determination of Total Flavonoid Content. To determine the total flavonoid content, 200 µL of a 1:80 Gotu kola extract, 1000 µL of distilled water, and 200 µL of 5% sodium nitrate were mixed and incubated at room temperature for 6 minutes. Then, 300 µL of 10% aluminum chloride and 400 µL of 1 M sodium hydroxide were added. Absorbance was measured at 510 nm using a UV spectrophotometer, and the total flavonoid content was calculated as quercetin equivalents (mg QUE/g) using a standard curve of 0.1-5 mg/mL quercetin.[11]

2.7 Determination of Total Antioxidant Capacity. To measure total antioxidant capacity, 200 µL of a 1:80 Gotu kola extract was mixed with 2 mL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at room temperature for 90 minutes. After cooling, absorbance was measured at 765 nm. Total antioxidant capacity was calculated as ascorbic acid equivalents (mg AAE/g) using a standard curve of 0.01-0.5 mg/mL ascorbic acid.[12]

2.8 Determination of Total Protein Content. To determine total protein content, 200 µL of a 1:80 Gotu kola extract was mixed with 5 mL of reagent C and incubated for 10 minutes at room temperature. Then, 0.5 mL of reagent was added, and the mixture was incubated for 30 minutes in the dark. Absorbance was measured at 660 nm, and total protein content was calculated as BSA equivalents (mg BSAE/g) using a standard curve of 0.2-1.6 mg/mL Bovine Serum Albumin (BSA).[13]

2.9 Antibacterial sensitivity test. The antibacterial activity of *Centella asiatica* was evaluated using the agar well diffusion method against *Escherichia coli* on Muller-Hinton agar (MHA). MHA was prepared and autoclaved, then poured into Petri dishes. A suspension of *E. coli* was evenly spread on the surface of the agar plates. Wells were then created in the agar, and to each well, 100 µL of Gotu kola extract was added. Negative and positive controls were included, with 100 µL of autoclaved distilled water used as the negative control and 100 µL of 0.5 mg/mL gentamicin as the positive control. The plates were incubated at 30°C for 24 hours, after which the zone of inhibition was measured.[14]

2.10 Statistical Analysis. All values are expressed as mean ± Standard Error. Microsoft Excel 2023 was used to calculate the standard error of the mean value.

3. Results

3.1 Morphological changes observed in stress-induced plants. Stress conditions were induced for 14 days and observed morphological changes are detailed in Table 3.

Table 3. Morphological changes observed in stress indued plants after 14 days

Conditions	Morphological Change
Drought	• Reduction in the plant height • Withered leaves • Extremely dry soil
High Salinity	• Decrease in leaf thickness • Withered leaves
Heavy Metal	• Withered leaves • Browning of the leaves
Nutrient Deficiency	• Yellowing of leaves • Stem degradation
Flood	• Plant stem elongation

3.2 Moisture content

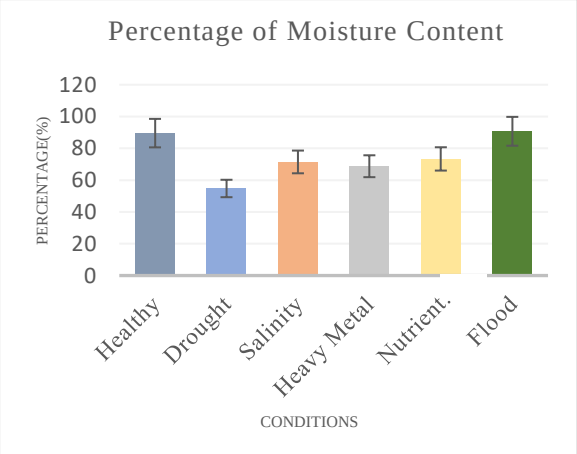

Figure 2. Moisture content percentage of control and stress induced plants. All the values are expressed as the mean ± S.E.

According to Figure 2, moisture content has significantly decreased in drought, salinity, heavy metal and nutrient deficiency induced plants at 10% error margin compared to the control plant

3.3 Bioactive compounds. Table 4 shows the presence and absence of the bioactive compounds of healthy and abiotic stress induced samples. All the samples tested positive for Tannins, Saponins, Flavonoids, Coumarins, Terpenoids, Cardiac glycoside and Steroids. Alkaloids, Phenolic Compound and Quinones were absent in them.

Table 4. Qualitative analysis of bioactive compounds

Tests	Healthy	Drought	High salinity	Heavy metal	Nutrient Deficiency	Flood
Alkaloid	Ab	Ab	Ab	Ab	Ab	Ab
Tannins	P	P	P	P	P	P
Phenolic Compound	Ab	Ab	Ab	Ab	Ab	Ab
Saponins	P	P	P	P	P	P
Flavonoid	P	P	P	P	P	P
Quinones	Ab	Ab	Ab	Ab	Ab	Ab
Coumarins	P	P	P	P	P	P
Terpenoids	P	P	P	P	P	P
Cardiac Glycoside	P	P	P	P	P	P
Steroids	P	P	P	P	P	P

P- Present, Ab – Absent

3.4 Total Phenolic Content

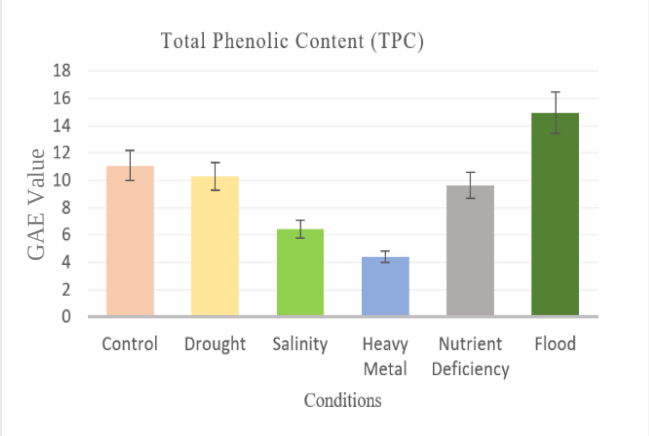

Figure 3. Total phenolic content of control and stress induced *Centella asiatica* plants.

All the values are expressed as the mean ± S.E.

The flood-induced plants had the highest total phenolic content, while the heavy metal-contaminated plants had the lowest. At a 10% error margin, the non-overlapping error bars for salinity, heavy metal, and flood-induced plants indicate statistically significant changes in phenolic content compared to control plants in Figure 3.

3.5 Total Flavonoid Content

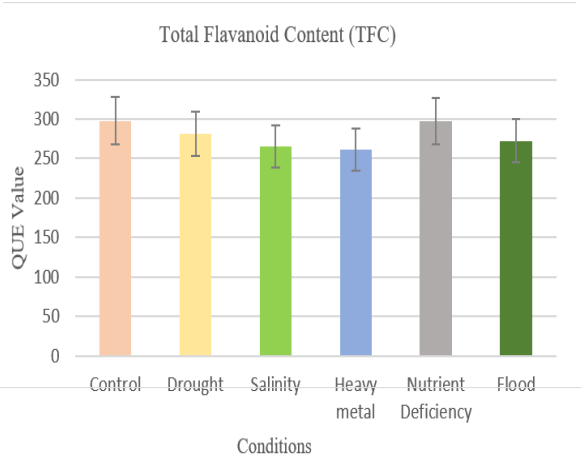

Figure 4. Total Flavonoid Content of control and stress-induced *Centella asiatica* plants. All the values are expressed as the mean ± S.E.

The control plants had the highest total flavonoid content, while the heavy metal-contaminated plants had the lowest. However, at a 10% error margin, the overlapping error bars for drought, salinity, heavy metal, nutrient deficiency, and flood-induced plants indicate that the changes in flavonoid content are not statistically significant compared to control plants in Figure 4.

3.6 Total Antioxidant Capacity.

The flood-induced plants showed the highest total antioxidant capacity, while the salinity-induced plants had the lowest. At a 10% error margin, the non-overlapping error bars for salinity and flood-induced plants suggest statistically significant changes in antioxidant capacity compared to control plants in Figure 5.

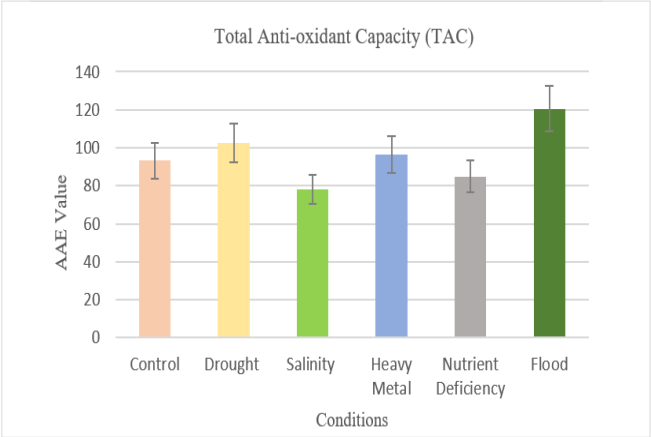

Figure 5. Total Antioxidant capacity of control and stress induced *Centella asiatica* plants. All the values are expressed as the mean ± S.E.

3.7 Total Protein Content

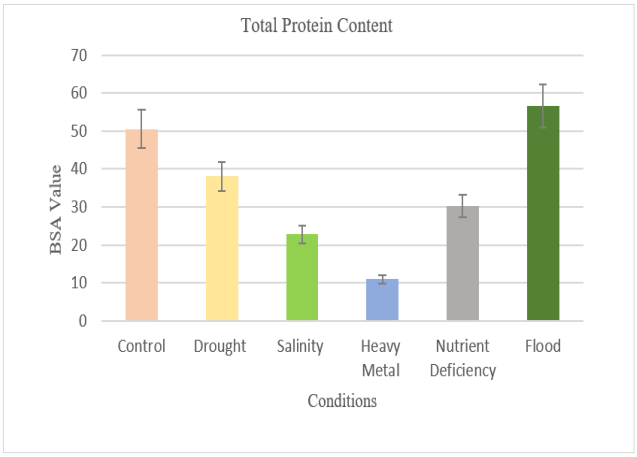

Figure 6. Total Protein Content of control and stress induced *Centella asiatica* plants. All the values are expressed as the mean ± S.E.

The flood-induced plants had the highest total protein content, while the heavy metal-affected plants had the lowest. At a 10% error margin, the non-overlapping error bars for drought, salinity, heavy metal, and nutrient-deficient plants indicate that the changes in total protein content under these conditions are statistically significant compared to control plants in Figure 6.

3.8 Anti-bacterial sensitivity test. Using the well diffusion method, only the positive control (0.5 mg/mL Gentamicin) showed an average zone of inhibition of 23 mm. Neither the negative control nor *Centella asiatica*

produced any zone of inhibition. Gentamicin effectively inhibits the growth of *E. coli*, confirming its activity and the absence of a zone of inhibition suggests that *Centella asiatica* has no antimicrobial activity against the *E. coli* bacteria at this plant extract concentration.

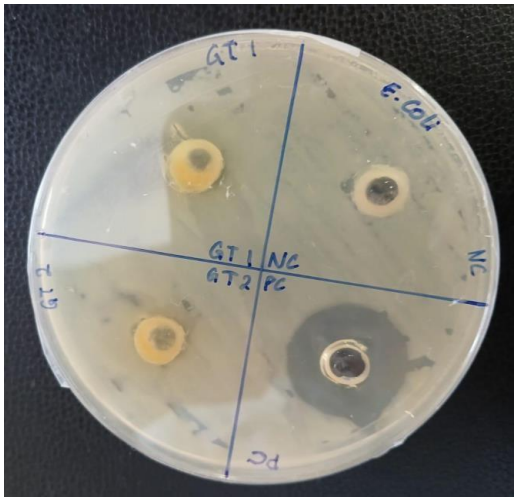

Figure 07. Anti-bacterial sensitivity test of *Centella asiatica* against *E. Coli*

4. Discussion

Centella asiatica, a plant used in traditional medicine, contains bioactive components that are influenced by various abiotic stress conditions, affecting its biochemical, antioxidant, and nutritional properties.[15] Quantitative analysis of *C. asiatica* under stress conditions revealed unique nutritional properties, including bioactive compounds with additional benefits beyond essential nutritional value.[16]

In this study of *C. asiatica* plants, qualitative analysis revealed the presence of tannins, saponins, flavonoids, coumarins, terpenoids, cardiac glycosides, and steroids, while alkaloids, phenolic compounds, and quinones were absent. Similar findings were reported by Roy M. and Krishnan in 2018, where tannins, flavonoids, terpenoids, saponins, and steroids were present, but alkaloids were absent. The results of this study align with their findings for the qualitative analysis of *C. asiatica* (Table 4).[17]

The moisture content of Gotu kola was measured, revealing that flood-induced plants had the highest moisture content at 90.72%, while drought-stressed plants had the lowest at 54.75%. These differences highlight the significant impact of water availability on the plant's hydration (Figure 02).

The Folin-Ciocalteu (FC) method, which measures total phenolic content by oxidizing phenolic compounds to produce a blue-colored reagent, was used to analyze the phenolic content in Gotu kola under various conditions. In healthy plants, the total phenolic content was 11.08 mg $GAEg^{-1}$. This aligns with Zainol *et al.* (2003), who discovered *Centella asiatica* phenolic content ranging from 3.23 to 11.7 g/100g in aqueous extracts.[18] Under abiotic stress, heavy metal exposure reduced the phenolic content to 4.417 mg $GAEg^{-1}$, and salinity stress to 6.452 mg $GAEg^{-1}$. However, flood-induced stress significantly increased the phenolic content to 14.95 mg $GAEg^{-1}$ (Figure 03). These results demonstrate the varying impact of different abiotic stresses on phenolic content, with flooding notably enhancing phenolic production.

The total flavonoid content in Gotu kola was measured using the aluminum chloride complex formation assay, with quercetin as the standard. In this study, the flavonoid content showed no significant variation between control (297.81 mg $QUEg^{-1}$) and abiotic stress conditions, including salinity (264.83 mg $QUEg^{-1}$), heavy metal (261.12 mg $QUEg^{-1}$), drought (281.55 mg $QUEg^{-1}$), nutrient deficiency (297.13 mg $QUEg^{-1}$), and flood (272.18 mg $QUEg^{-1}$). This finding contrasts with a study by Minarti *et al*. (2021), which reported a much lower flavonoid content of 9.33 mg $QUEg^{-1}$ [19,20] (Figure 4). The differences may be due to varying environmental conditions and growth factors, suggesting that Gotu kola may have an inherent resistance to abiotic stress, maintaining its flavonoid production as an adaptive response to challenging environments.

In this study, the total antioxidant capacity of Gotu kola was 97.16 mg $AAEg^{-1}$ in control plants. Under abiotic stress, flood conditions increased the antioxidant capacity to 120.59 mg $AAEg^{-1}$, while salinity slightly reduced it to 78.09 mg $AAEg^{-1}$ (Figure 05). These results align with a similar study by Rashid *et al.* (2023), which found 102.32 mg $AAEg^{-1}$ in *C. asiatica*. Despite the variations, both stress conditions produced antioxidant levels close to those of control plants,

indicating the plant's resilience and adaptive mechanisms under stress.[20]

In this study, the total protein content in healthy Gotu kola plants was 50.45 mg $BSAEg^{-1}$. Under abiotic stress, protein levels significantly varied with salinity (22.79 mg $BSAEg^{-1}$), drought (38.12 mg $BSAEg^{-1}$), nutrient deficiency (30.26 mg $BSAEg^{-1}$), and heavy metal stress (11.05 mg $BSAEg^{-1}$) induced plants, all showing notable reductions compared to control plants (Figure 6).

Infectious diseases drive global morbidity and mortality, and medicinal plants provide a promising, cost-effective alternative to antibiotics, with fewer side effects. In the antibacterial sensitivity test, *Centella asiatica* showed no antibacterial activity against *Escherichia coli*, likely due to insufficient active compounds in the extract. Gentamicin, used as the positive control, demonstrated effective antibacterial properties against aerobic gram-negative bacteria (Figure 7).

5. Conclusion

Centella asiatica under flood conditions showed higher values for total phenolic content, total antioxidant capacity, and total protein content compared to healthy samples, indicating its potential as a source of natural antioxidants.

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Green Synthesis of Silver Nanoparticles using the leaf extracts of Ginger family plants (*Zingiberaceae*) for enhanced antioxidant, antimicrobial, and photocatalytic activity.

Fathima Rizvi[1] and Mathivathani Kandiah[1*]

[1]School of Management, Business Management School (BMS), Sri Lanka

*mathi@bms.ac.lk

Abstract

Nanoscience is an expanding research field impacting nearly all the branches of science. The synthesis of metal nanoparticles is a growing field of study due to its potential to be used in the creation of novel technologies. Silver is used to synthesize nanoparticles because of their unique properties, such as shape and size-dependent optical, electrical, and magnetic characteristics. The primary objective of this research was to analyze the antioxidant, antimicrobial and photocatalytic activity of silver nanoparticles (AgNPs) synthesized using leaf extracts from ginger family plants. Changes in color and characterization by UV-Vis spectrophotometer and scanning electron microscopy (SEM) were used to confirm the synthesis of AgNPs. According to the SEM analysis, the AgNPs were spherical in shape and 40- 50 nm in size. Total Antioxidant Capacity (TAC), Total Flavonoid Content (TFC), Total Phenolic Content (TPC), DPPH (2,2-diphenyl-1-picrylhydrazyl), and inhibitory concentration50 (IC50) assays confirmed a significant difference ($P< 0.05$) in the presence of higher amounts of antioxidants in AgNPs compared to aqueous extracts of the leaves. The antimicrobial activity of the synthesized AgNPs were detected using *Escherichia coli* and *Staphylococcus aureus*, using well-diffusion technique. However, no significant difference was observed between AgNps and bacterias as ginger species too have antimicrobial activity. Out of all the photocatalytic activity, AgNP *Zingiber officinale* (ZO) was evaluated at concentrations of 267 ppm and 4000 ppm in the presence of $NaBH_4$ under sunlight, showed that Erichrome Black-T (EBT) degraded within 80 minutes at 267 ppm and within 65 minutes at 4000 ppm, as indicated by the reduction of the peak at 520 nm. Furthermore, the rate constant for the degradation were 0.0265 for 267 ppm and 0.0337 for 4000 ppm AgNP ZO, demonstrating that EBT degraded faster at the higher concentration. Therefore, AgNPs synthesized in this work have the potential for versatile applications which require antioxidant, anti-microbial or catalytic activity.

Keywords: Nanoparticles, Green synthesis, *Zingiberaceae*, Antimicrobial activity, Antioxidant

1. Introduction

Nanoscience and nanotechnology are expanding research fields that involve structures, devices, and systems with novel properties and functions because of the arrangement of their atoms on the 1–100 nm scale.

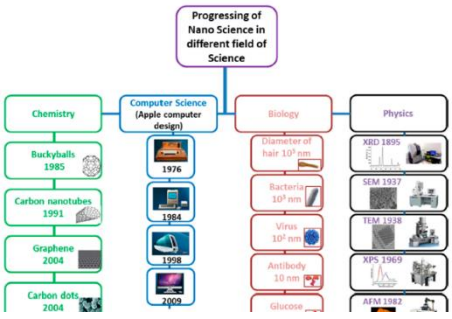


Figure 1. Progress in nanoscience and nanotechnology in different field of science.[1]

In the early 2000s, there was a growing public awareness and controversy about the field, which led to the beginning of commercial applications of nanotechnology. Nanotechnologies have an impact on nearly every branch of science, including physics, materials science, chemistry, biology, computer science, and engineering (Figure1).[1]

In 1959, American physicist Richard Feynman proposed the concept of nanotechnology thus he is regarded as the father of modern nanotechnology. Later, Norio Taniguchi, a Japanese scientist, was the first to use and define the term "nanotechnology" in 1974 as "the processing of separation, consolidation, and deformation of materials by one atom or one molecule".[1,2]

Metal nanoparticles have found wide acceptance because of their unique chemical and physical properties, higher reactivity, different shape, nondispersive size, and particularly optical properties like surface plasmon resonance (SPR).[3] AgNPs are the most thoroughly researched nanoparticles as they have immense broad-spectrum activities. AgNP research has made significant advances in nanoscience, particularly as antimicrobial, antibacterial, antioxidants, antifungal, anti-inflammatory, anticancer, and anti-angiogenic agents (Figure 2). AgNPs are small particles with unique physicochemical properties, ranging in size from 10 to 100 nm (size, shape, optical activity, electric conductivity, high surface area).[4,5]

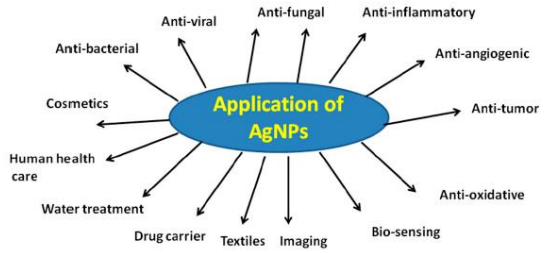


Figure 2. Various applications of AgNPs.[6]

To meet the demand for AgNPs, a variety of synthesis methods have been used such as top down and bottom methods (Figure 3). Physical and chemical techniques are widely used, but physical techniques are unaffordable; chemical techniques, on the other hand, are hazardous to the environment and living organisms. Biologically prepared AgNPs, on the other hand, have a high yield, solubility, and stability.[2] Biological methods appear to be simple, rapid, non-toxic, dependable, and green approaches for producing well-defined size and morphology under optimized conditions for translational research among several synthetic methods for AgNPs. Bio-inspired approaches based on natural products such as micro- or marine organisms, proteins, and plant extracts (PEs) have gained popularity for the synthesis of AgNPs. The cost of microbe-mediated synthesis, followed by extracellular and intracellular mechanisms, is high, and there is a risk of contamination in the microbial culture. Thus, plant extracts have received much interest because they are simple to use, cost effective and more tolerant to metal toxicity.[4,7]

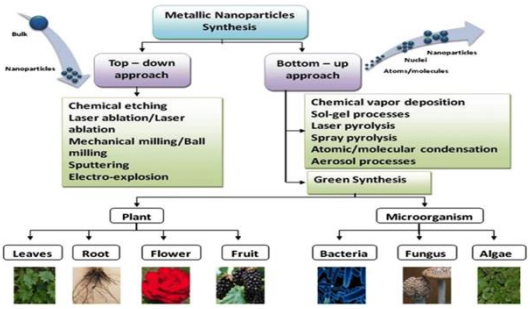


Figure 3. Types of metallic nanoparticle synthesis.[8]

Plant parts such as the leaf, stem, seed, fruit, pulp, peel, flower, and plant nectar (honey) provide a better platform to produce specific phytochemicals for the fabrication of AgNPs.[3] In this study, we used green synthesis method (Figure 4) to synthesize silver nanoparticles using leaf extract of *Zingiberaceae* species which act as a reducing and stabilizing agent. Ginger's pungent aroma comes from its volatile oil, gingerol, and other active components. These bioactive ginger constituents are known to inhibit prostaglandin and leukotriene formation by regulating blood flow, as well as to control inflammation. Ginger, on the other hand, has moderate antioxidant properties against a variety of bacterial strains.[4,9] Ginger's pharmacological activities as antifungal, antibacterial, anti-inflammatory, and antioxidant properties were primarily attributed to its active phytocompounds such as, 6-gingerol, 6-shogaol, and zingerone, in addition to other phenolics and flavonoids.[10,11]

Antioxidants are defined as "any chemical that delays, prevents, or eliminates oxidative damage to a target molecule" and are thought to be crucial to the body's defensive system against reactive oxygen species (ROS).[12]Antioxidants have a variety of physiological functions in the body because they block the process of oxidation even at low concentrations. Radical scavengers such as the antioxidant components of plant material serve to reduce the reactivity of radicals. Fruits, vegetables, tea, and other dietary sources include a variety of antioxidants that can scavenge free radicals.[13,14]

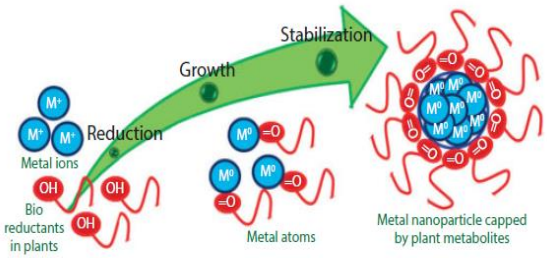


Figure 4. Green synthesis mechanism.[2]

The use of antioxidants protects the human body from cardiovascular, neurological, and carcinogenic diseases, as well as delaying chronic health problems such as cataracts.[14]

Silver has been shown to be highly toxic to a wide variety of microorganisms. Silver ions are antibacterial because they interact with the peptidoglycan cell wall and plasma membrane, and they also prevent bacterial DNA replication.[15] AgNPs' antibacterial activity is determined by the concentration of nanoparticles exposed to bacteria as well as the type of bacteria.[7]

Smaller nanoparticles have greater antibacterial activity because they have more surface exposure to the bacterial membrane. The positive charge of Ag+ interacts with the negative charge on the cell wall of bacteria, causing changes in cell wall morphology and an increase in cell permeability or leakage, resulting in cell death (Figure 5).[16]

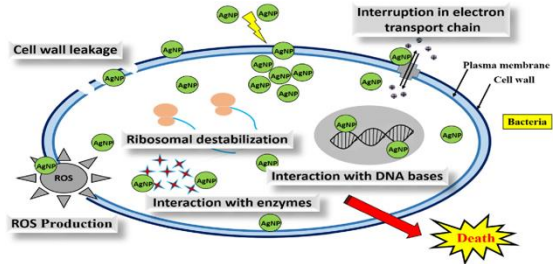


Figure 5. Mechanisms of antimicrobial activity for AgNPs .[16]

Since industrial pollutants are made up of various dyes (Figure 6) that are chemically stable, removing synthetic dyes from wastewater is a serious environmental issue that must be tackled scientifically. AgNPs are known to absorb light in the visible range of the light spectrum due to its Surface plasmon resonance characteristics. The photocatalytic nature of AgNPs is determined by the crystallographic nature, morphological structure, and size of the nanoparticles. Under ambient temperature and visible light illumination, AgNPs are highly efficient, and stable photocatalysts.[17] Degradation techniques are one of the beneficial processes that have been applied for dye removal from wastewater and pharmaceutical industries among various chemical and physical methods.[18]

Hence the aim of this study was to analyse the antimicrobial and antioxidant activity of silver nanoparticles synthesized using leaf extracts of five varieties of ginger species. The antioxidant properties were assessed by TFC, TPC, and TAC. *Escherichia coli and Staphylococcus aureus* was used to detect the antimicrobial activity performing well diffusion technique. The photocatalytic activity was assessed by using EBT dye. Thus, these AgNPs could be used in various field for the betterment of world.

Figure 6. Example for Azo dye.[18]

2. Methodology

2.1. Sample Collection. Leaves from five different species (Fig 7) of ginger family plants were collected from a plant nursery in Battaramulla.

2.2 Preparation of leaf extracts using distilled water: the samples were air-dried and ground into smaller pieces. Two grams of each ground sample were mixed with 40 mL of distilled water. The samples were incubated at 60°C for 15 min, then filtered into falcon tubes and stored at 4°C for future use.

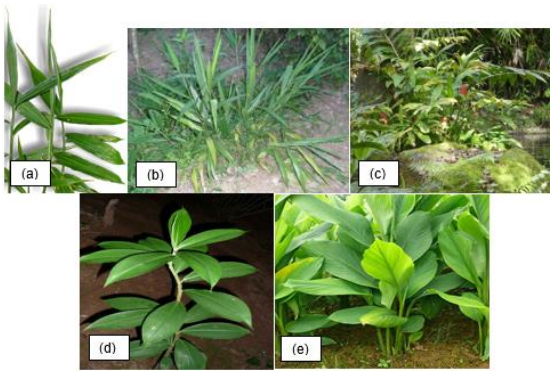


Figure 7. Varieties of ginger species used to synthesize nanoparticles*:* a) *Zingiber officinale - ZO (Ginger)* b) *Alpinia calcarata- AC (Snap ginger)* c*) Zingiber zerumbet- ZZ (Pinecone ginger)* d*) Costus speciosus- CS(Thebu)* e) *Carcumo longa- CL*

2.3 Green Synthesis of AgNPs and optimization. One milliliter of each water extract was mixed with 9 mL of 1 mM $AgNO_3$ solution and left at room temperature for 24 hours. Optimization was conducted at both 90°C and 60°C temperatures for durations of 15 min, 30 min, 45 min, and 60 min respectively. The color change of the samples was observed, and the absorbance was measured from 320 to 500 nm, using distilled water as a blank for all conditions.[19]

2.4. Dilution of the samples. AgNPs and the water extracts were diluted with a dilution factor of 1:15 and were stored at 4°C for further use.

2.5. Phytochemical Tests. The phytochemical analysis was conducted according to the methodologies shown below in Table 1.

2.6. Determination of Total Flavonoid Content. TFC was determined using $AlCl_3$ solution. For that, 1.5 mL of the sample was mixed with 1.5 mL of 2% $AlCl_3 \cdot 6H_2O$ solution and incubated at room temperature (RT) for 10 mins. The absorbance of the samples was measured at 415 nm using distilled water as the blank. Three replicates were conducted for each sample. The TFC was expressed as quercetin (mg/g) to construct the calibration curve.[11]

2.7 Determination of Total Antioxidant Activity: The phosphomolybdenum reagent was prepared by mixing equal volumes of 28 mM sodium sulphate and 4 mM ammonium molybdate and 0.6 M sulphuric acid (1:1:1). Exactly, 3 mL of the sample was mixed with 1 mL of the phosphomolybdenum reagent, and incubated at 90 °C for 90 min. The absorbance was measured at 695 nm in triplicates, using distilled water as a blank. Final concentration TAA was expressed as ascorbic acid equivalence (mg AAE/100g).[22]

Table 1. Phytochemical tests and the methodology.[20,21]

Phytochemical Test	Methodology
Carbohydrate	To 0.5 mL of sample, 1 mL of Molisch's reagent was added along with few drops of Conc. H_2SO_4
Amino Acid	Few drops of Ninhydrin solution were added to 0.5 mL sample and was kept in water bath for 10 mins
Saponins	To 0.5 mL of sample, 0.5 mL of distilled water was added and shaken vigorously for 10 mins
Tannins	To 0.5 mL of sample, 1 mL of 5% ferric chloride was added.
Proteins	To 0.5 mL of sample, few drops of Millon's reagent was added.
Terpenoids	To 0.5 mL of sample, 0.5 mL of chloroform along with few drops of Conc. H_2SO_4 was added.
Quinones	To 0.5 mL of sample, 0.5 mL of Conc. H_2SO_4 was added
Betacyanin	To 0.5 mL of sample, 0.25 mL of 2M NaOH was added and heated for 5 mins at 100◦C

2.8. Determination of Total Phenolic Content. Exactly, 0.5 mL of the sample was mixed with 2 mL of 7.5 % Na_2CO_3 and 2.5 mL of Folin–Ciocalteu reagent diluted with water in 1:10 v/v ratio. The mixture was incubated at 40 °C for 30 mins. The absorbance was measured at 765 nm in triplicates, using distilled water as a blank. The TPC was expressed as gallic acid equivalents (mg GAE/100g).[11]

2.9. Determination of Antimicrobial Activity. The antimicrobial activity was determined using two types of bacterial strains *Staphylococcus aureus* and *Escherichia coli* using the agar well-diffusion technique on Mueller-Hinton agar.The bacterial culture was evenly spread on MHA plates using a cotton swab. Three wells were created on the plates for the negative control (−) and two sample replicates (S1 and S2), as shown in Figure 8. Exactly, 1 mL of saline solution was added to the negative control, and 1 mL of the sample was added to each S1 and S2. Gentamycin discs were used as the positive control (+). The plates were incubated at 37°C for 24 hours and the

zone of inhibition diameter was measured using a ruler.[19]

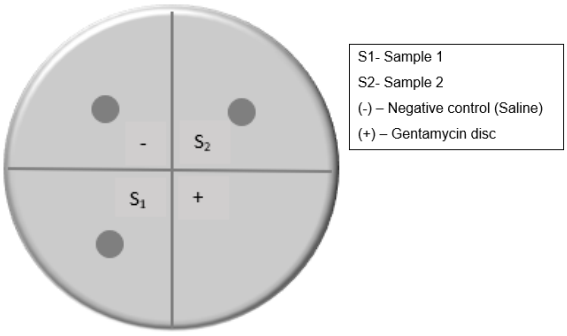


Figure 8. Labelled petri dish for antimicrobial activity.

2.10. Photocatalytic Activity. Exactly, 1 mL of 4,000 ppm of Zinc Oxide Silver Nanoparticles (ZO AgNPs) and 1 mL of 0.2M $NaBH_4$ were added to 100 mL of 1 mM EBT. A 0.5 mL aliquot of the mixture was diluted to 3 mL with distilled water, and the absorbance was measured in the range of 320 to 800 nm every 10 minutes over a period of 80 minutes, using distilled water as the blank. The same procedure was repeated for 267 ppm ZO AgNPs.[19]

2.11. DPPH assay. Exactly, 2 mL of 0.004% DPPH was mixed with 1 mL of sample and incubated for 30 mins at RT. The absorbance was measured at 517 nm in triplicates, using methanol as a blank. The following equation was used to calculate the %DPPH activity.[23]

$$\% \text{ activity} = \{(A_{(sample)} - A_{(control)})/ A_{(control)}\} \times 100$$

2.12. Determination of Median Inhibitory concentrations (IC50). Exactly, 2 mL of 0.004% DPPH was added to 1 mL of each sample and then a dilution series was prepared with concentrations of 100 %, 80 %, 60 %, 40 %, and 20 % respectively. and was incubated for 30 mins at RT. The absorbance was measured at 517 nm using methanol as a blank. The % DPPH was calculated using the same equation of DPPH assay.[23]

2.13. SEM analysis. The ZO sample was sent to Sri Lanka Institute of Nanotechnology (SLINTEC) for SEM analysis using Hitachi SU6600 SEM.

2.14. Statistical Analysis: ONE-way ANOVA test and correlation graphs were generated using the Microsoft 365 data analysis and SPSS software, respectively.

3. Results

3.1 Phytochemical Tests.

Based on the phytochemical tests conducted (Table 2), all Zingiber species contain carbohydrates and quinones.

Table 2. Results of phytochemical tests.

Phytochemical Test	CS	CL	AC	ZO	ZZ
Carbohydrate	✓	✓	✓	✓	✓
Amino Acid	✗	✗	✓	✗	✓
Saponin	✗	✗	✗	✗	✗
Tannins	✗	✓	✗	✓	✗
Proteins	✓	✗	✓	✗	✓
Terpenoids	✗	✗	✗	✓	✗
Quinones	✓	✓	✓	✓	✓
Betacyanin	✓	✓	✓	✗	✗

3.2 Synthesis of AgNPs from Zingiberaceae species.

The samples changed their color (Figure 9) to shades of yellow from colourless, indicating the synthesis of AgNPs due to reduction of Ag+ to Ag. This is further proved by spectrophotometer analysis as there was a clear peak formation from 400 nm to 480 nm in samples CL, ZO, and ZZ at 90◦C for 60 mins.

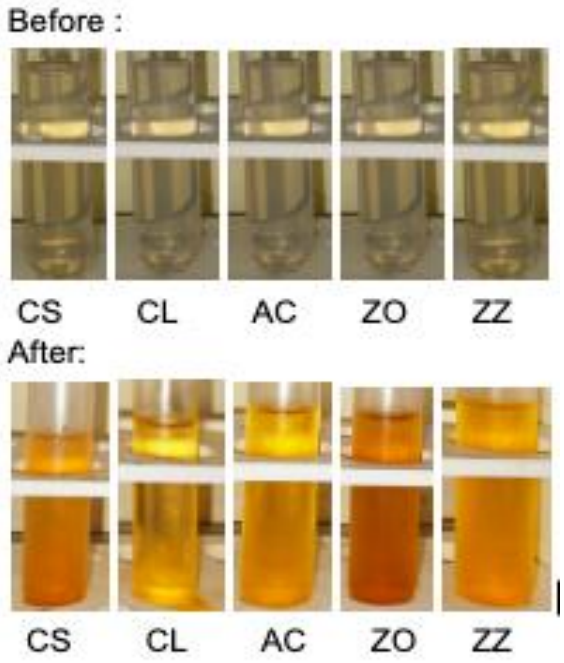


Figure 9. Synthesis of AgNPs from *Zingiberaceae* species at 90◦C for 1 hour.

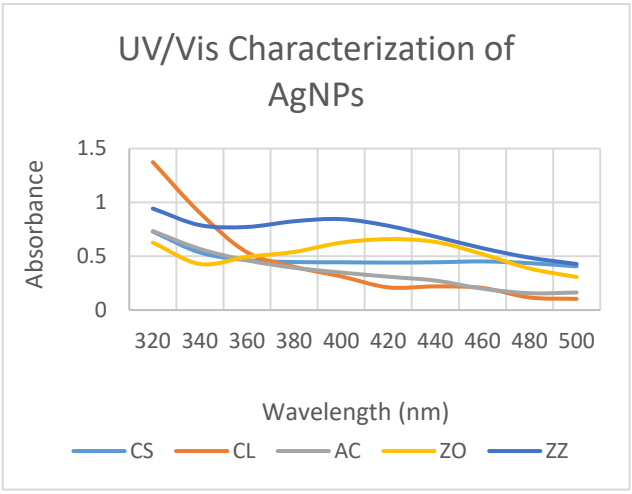


Figure 10. UV-Spectrophotometer analysis of

3.3 SEM Analysis.

According to SEM analysis the AgNPs were spherical in shape and 10 nm in size (Figure 11).

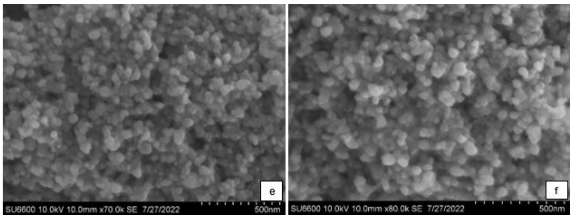


Figure 11. Electron microscopy at 500 nm.

Table 3. Optimization temperature of AgNPs

	90◦C				60◦C				25 ◦ C
	Time (mins)				Time (mins)				
Sample ID	15	30	45	60	15	30	45	60	
CS	✗	✗	✗	✗	✓	✗	✗	✗	✗
CL	✗	✗	✗	✓	✗	✓	✗	✗	✗
AC	✗	✗	✗	✗	✗	✓	✗	✗	✗
ZO	✓	✓	✓	✓	✓	✓	✓	✓	✗
ZZ	✗	✓	✓	✓	✓	✗	✓	✓	✗

3.4 Total Flavonoid Content.

TFC of AgNPs is higher than that of the water extract (Figure 12). Thus, this was further proved by the One-way ANOVA test, which showed a significance difference (P< 0.05) of (P= 0.00359) between water extract and AgNPs.

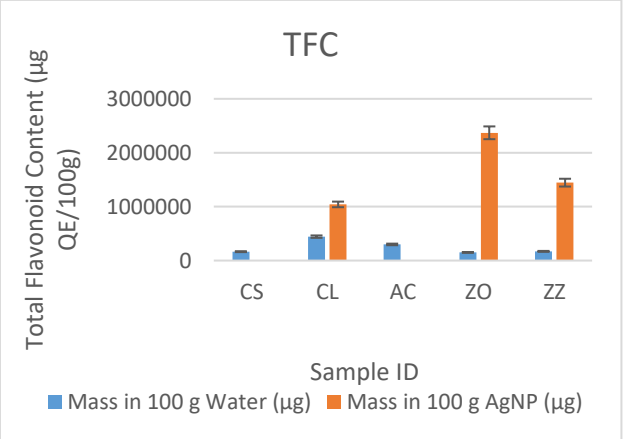


Figure 12. TFC of *Zingiber* species water extract and AgNPs.

3.5 Total Phenol Content.

TPC is comparatively higher in AgNPs than that of the water extract (Figure 13). One-way ANOVA analysis also showed a significance difference (P<.0.05) of 7.63E-08 between water extract and AgNPs.

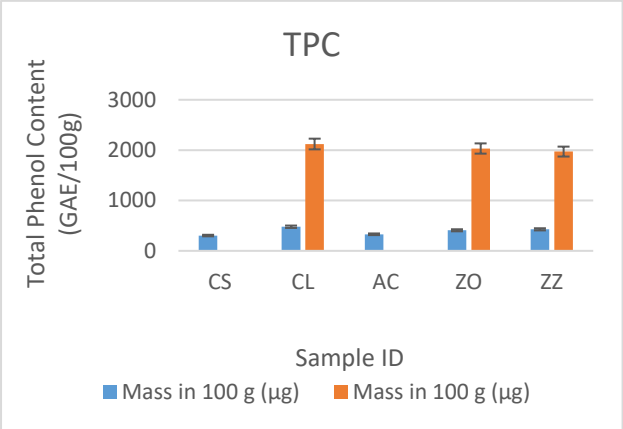


Figure 13. TPC of Zingiber species water extract and AgNPs.

3.6 Total Antioxidant Capacity.

AgNPs have comparatively higher amounts of TAC than water extracts (Figure 14) with a significance difference of 5.32E-06. AgNP ZO has the highest TAC.

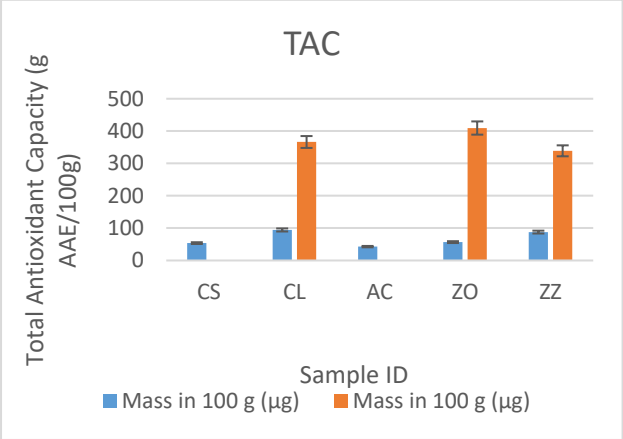


Figure 14. TAC of *Zingiber* species water extract and AgNPs.

3.7 DPPH assay.

AgNPs have higher DPPH activity compared to water extract by a minute difference (Figure 15).

3.8 IC_{50} of DPPH.

AgNPs have more antioxidants compared to water extract, as the IC_{50} value (Figure 16) is less in AgNPs than water extract (Table 4)

Table 4. IC50 of water extract and AgNPs.

Sample	Water	AgNPs
CS	11.39238	
CL	3.26094	2.949853
AC	6.122424	
ZO	4.799846	3.885004
ZZ	5.59009	3.854456

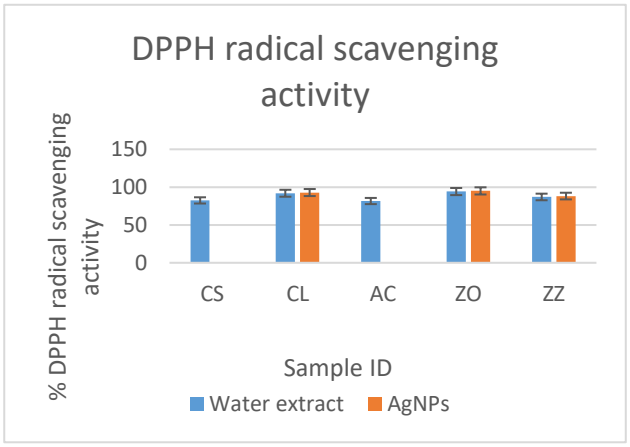


Figure 15. % DPPH radical scavenging activity for Water extract and AgNPs.

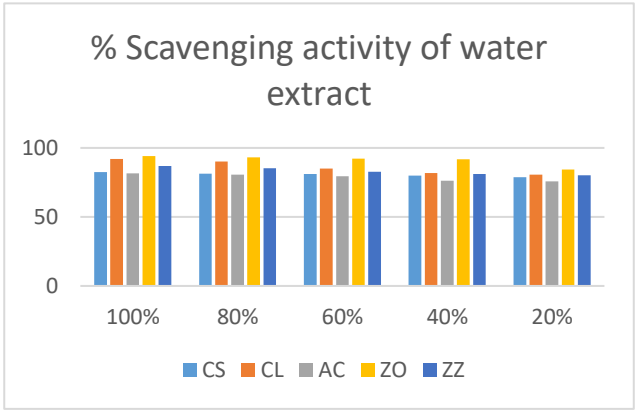


Figure 16. % Scavenging activity of AgNPs.

3.9 Photocatalytic activity of AgNP.

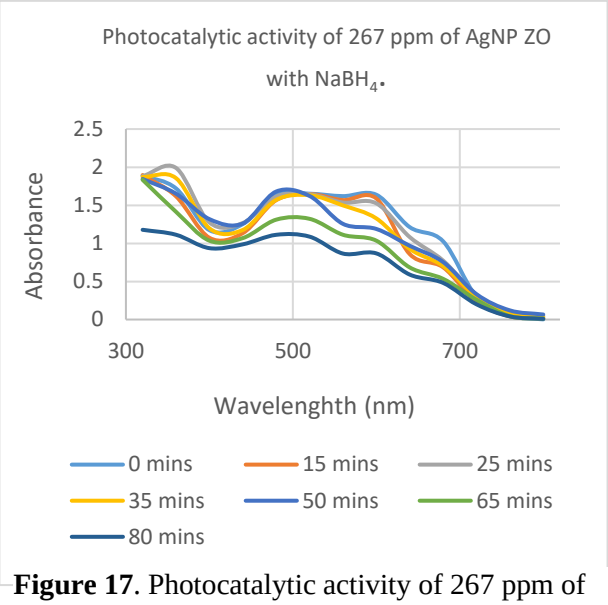


Figure 17. Photocatalytic activity of 267 ppm of AgNP ZO with $NaBH_4$ under sunlight.

EBT was degraded by 267 ppm AgNP ZO completely at 80 mins indicated by peak 520. Degradation of EBT was completed by 4000 ppm AgNP ZO at 65 mins indicated by peak 520 nm (Figure 17, 18).

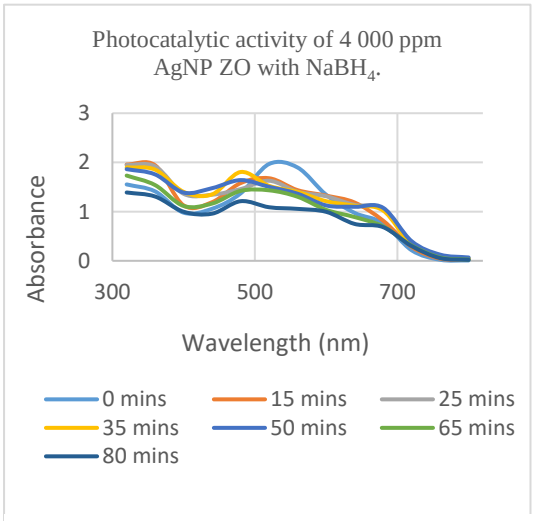


Figure 18. Photocatalytic activity of 4000 ppm AgNP ZO with $NaBH_4$ under sunlight.

3.10 Antimicrobial Activity.

ZOI of AgNP ZO and ZZ were higher than water extract (Figure 19) for *E. coli*. There was no significance (P> 0.05, P= 0.543) observed between the water extract and the AgNPs.

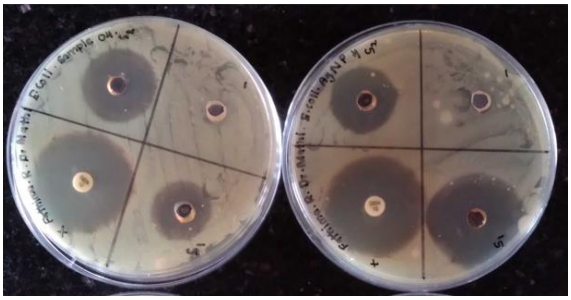


Figure 19. ZOI for *E. coli* in water extract (Left) and AgNPs (Right).

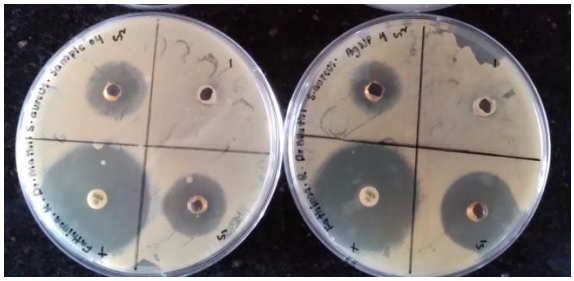


Figure 20. ZOI for *S. aureus* in water extract (Left) and AgNPs (Right).

AgNP CL and ZZ has higher ZOI compared to water extract (Figure 20) for *S. aureus*. There was no significance (0.713) seen between water extract and AgNP.

4. Discussion.

Nanoparticles with a variety of shapes, sizes, and morphologies can be synthesized using a method that is ecologically friendly, nontoxic, and reasonably priced using plant extracts. Compared to several physicochemical approaches used in production, the biosynthetic route can yield metal nanoparticles in better sizes and forms. Instead of employing microorganisms, plant extract-based nanoparticle synthesis has the benefit of not requiring multistep or complicated processes including microorganism isolation, identification, growth optimization, culture preparation, and maintenance.[16,24] In addition, plant-based synthesis is quicker than employing microorganisms, and simple to scale up for mass production of nanoparticles.[15]

Green leaves are used and preferred over other parts of plants to produce AgNPs due to their origin in photosynthesis and the availability of more Hp ions, which reduces the formation of $AlCl_3$ within the AgNPs.[7]

The extract's water-soluble components are what produces the reduction and stability of AgNPs. Thus, water was used solvent as it is less toxic and will increase the yield.[9]

Table 5. Conductivity of AgNPs.

Sample	Band Gap Energy (eV)	Classification
CS	4.32	Insulators
CL	4.51	Insulators
AC	4.73	Insulators
ZO	4.51	Insulators
ZZ	4.97	Insulators

$$E = hc \div \lambda$$

E= Band gap energy

h= Planck's Constant (6.6269×10^{-34} Js).

c= Speed of light (3×10^{8} ms^{-1}).

λ= Wavelength (400- 480 nm).

Figure 21. Planck's Equation.

It was obvious that AgNPs had formed in the reaction mixture during the early stages of reduction because the colour changed from nearly colourless to brown and due to clear peak formation from 400 to 480 nm in UV-Vis spectrophotometer analysis. With time during incubation, the colour intensity increased. The dark brown colour of the yellow solution over time may be related to both the increased concentration of AgNPs and the reduction in particle size.[25] The change of colour may be due surface plasmon resonance of AgNPs caused by an interacting electromagnetic field, the collective oscillation of free conduction electrons. The SEM analysis indicated that the AgNPs had a spherical structure with 40 nm size (Figure 11). Similar research has proven that AgNPs synthesized using ginger extract was spherical particles and polydisperse with size ranging from 3-6 nm.[24,25,26] The obtained results show that under reaction conditions, ginger leaves extract had reduced Ag+ to Ag0.[15]

One of the widely used techniques for characterizing particles and their properties is UV-Vis spectroscopy. The bandgap caused by electron transitions between the top of the valence band and the bottom of the conduction band, was used to investigate the conductivity of AgNPs. Materials can be categorized as insulators or semiconductors depending on whether the lowest energy needed for an electron transition is more than 4eV or less than 3eV respectively.[22] Accordingly, the bandgap energy of *Zingiberaceae* AgNPs was measured using the Plank's Equation (Figure 21) and it was between 4.30 and 4.98 and thus, considered as insulators (Table 5).

Ginger consists of various bioactive components, like phenolic and terpene. Gingerols, shogaols, and paradols are the primary phenolic chemicals found in ginger. Thus, it was proved by the phytochemical screening assays (Table 2).[27]

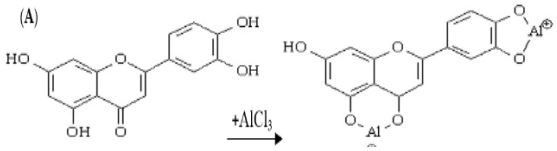


Figure 22. Total Flavonoid Content Principle.[29]

Overproduction of free radicals, such as ROS, is known to play a significant role in the development of many chronic diseases. Numerous natural products, including vegetables, fruits, edible flowers, cereal grains, medicinal plants, and herbal infusions, have been documented to have antioxidant potential. Numerous studies have revealed that ginger has a significant antioxidant capacity.[27]

TFC was measured using the $AlCl_3$ colorimetric approach. The fundamental idea behind the assay is that AlCl3 forms acid-stable complexes with flavones and flavanol C-4 keto groups as well as either C-3 or C-5 hydroxyl groups (Figure 22). Additionally, it combines with the ortho-dihydroxyl groups on the A or B ring of flavonoids to generate complexes that are acid labile.[28,29] According to this study, AgNPs (ZO> ZZ> CL) has higher amount of TFC

compared to water-extract. ONE-way ANOVA test further supported the study by showing a significance of 0.0024 (<0.05) between AgNPs and water-extract. In addition, a study has proved that AgNPs synthesized from the rhizome of ginger has more TFC than the water-extract.[30]

The most used colorimetric assay for phenolic content is Folin-Ciocalteu (F-C) assay. The F-C reagent is composed of a tungsten and molybdate combination. The phosphomolybdic acid complexes are formed when phenolic chemicals transfer electrons to them under alkaline conditions. The quantity of reactive phenolic chemicals in the sample directly correlates with the intensity of the blue colour. By measuring the sample solution's absorbance at 765 nm and comparing it to a calibration curve using gallic acid as a standard, the phenol concentration is determined. The coordinated molybdenum(V) species is assumed to be the cause of the blue colour that results from the reduction of the F-C reagent (Figure 23).[31,32] Hence, TPC was comparatively higher in AgNPs (CL> ZO> ZZ) than the water-extract. It was further proved by ONE-way ANOVA analysis which showed a significance of 7.63E-08 (<0.05) between water-extract and AgNPs. Based on the research by Jaiswal and Naik (2018), AgNPs synthesized from the rhizome of ginger has more TPC compared to water-extract.

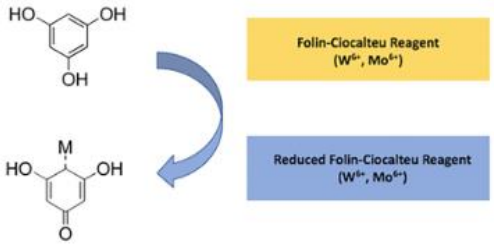


Figure 23. Total Phenol content Principle.[32]

When Mo (VI) is reduced to Mo (V) at an acidic pH, antioxidants produce a green phosphate/Mo (V) complex, which can be detected at 695 nm. This complex is used to determine TAC.[22] Thus, synthesized AgNPs (ZO> CL> ZZ) has comparatively higher amount of TAC than water-extract. In addition, it was validated by ONE-way ANOVA test between water-extract and AgNPs with a significance of 5.32E-06.

DPPH has been frequently used to assess how well different antioxidant compounds scavenge free radicals. Antioxidants can donate hydrogen in the DPPH assay, reducing the stable radical DPPH to the yellow nonradical diphenyl-picrylhydrazine (DPPH-H) (Figure 24). Based on the spectrophotometric measurement of the change in DPPH absorption at 517 nm, and a deep violet colour results from the electron's delocalization. DPPH is typically employed as a reagent to assess the free radical scavenging activity of antioxidants.[33] Methanol or ethanol is employed in the DPPH assay to maintain the hydrophobic hydroxyl radicals and phenolic test chemicals in solution while providing adequate buffering capacity.[22]

AgNPs (ZO> CL> ZZ) has a higher %DPPH activity compared to water-extract which corresponds to the result of TAC. Moreover, it has been proved in a study that AgNPs synthesized using the callus of ginger has comparatively higher %DPPH activity than water-extracts.[11]

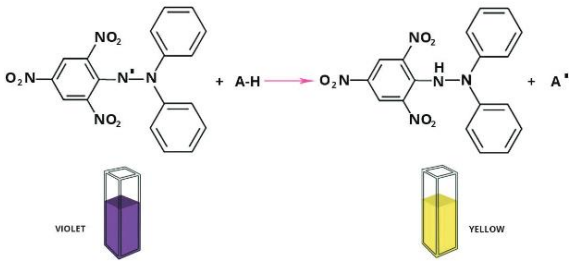


Figure 24. DPPH Assay.[10]

Low IC_{50} value values indicate strong antioxidant activity, and the reaction is preceded by a rapid drop in absorption.[34] So, it can be concluded AgNPs have a higher antioxidant activity than water-extract.

Pearson Correlation factor for all the antioxidant assays were higher than 0.8 (Figure 25). Hence it proves the correlation between all three assays.

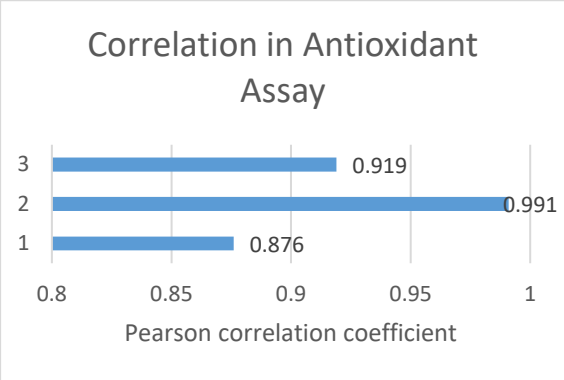


Figure 25. Pearson correlation for the antioxidant assays.

The surface electrons from the outermost sp band are excited to a higher energy state as a result of the SPR effect when AgNPs absorb visible light from the solar spectrum; these electrons are quickly accepted by the O_2 molecules to form oxygen radicals that attack and degrade the EBT. Additionally, the dye is degraded as a result of accepting electrons from the adsorbed photosensitized dye molecule to fill the holes created in the 5sp orbital.[17] Numerous photogenerated electrons are excited as a result of inter-band transition. Both oxygen radicals and hydroxyl radicals are created when these excited electrons interact with O_2 molecules and hydroxyl ions respectively. The dye is degraded as a result of the radicals' attack on the dye molecule adsorbed on the surface of the AgNPs.

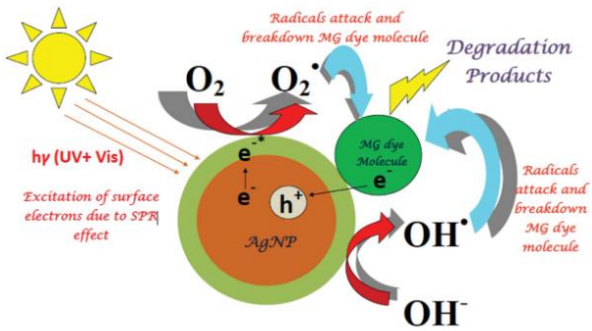
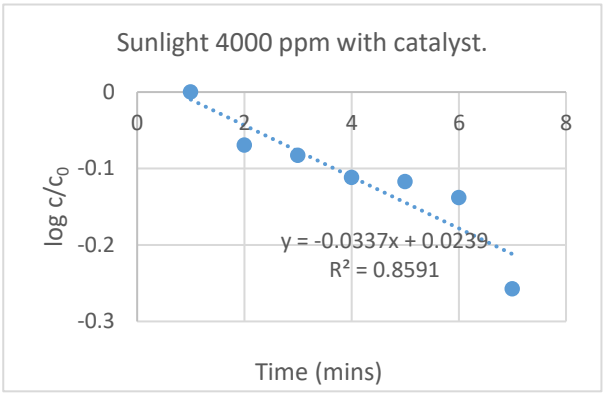


Figure 26. Photocatalytic activity of AgNPs.[17]

In addition to the radicals degrading the dyes, the holes made in the AgNPs' d orbital take electrons from the dye that has been adsorbed, further degrading the dye (Figure 26).[17,18]

While the absorbance of the dye reduces just slightly in the absence of the catalyst, suggesting a relatively slow reaction rate, it abruptly decreases in the presence of the catalyst, indicating a faster reaction rate. As an electron relay, AgNPs start the process of moving an electron from a BH_4^- to an acceptor, which reduces the dye. NPs are simultaneously adsorbed with the BH_4-, allowing for the transfer of electrons from the BH_4- to the dye.[35]

In this study, the photocatalytic activity of AgNP ZO under sunlight and UV-light without catalyst didn't show any degradation. So, the study was done using catalyst $NaBH_4$ under sunlight to degrade EBT dye. It was observed that the EBT degraded within 65 and 80 mins by 267 and 4000 ppm AgNP ZO respectively indicated by peak 520 nm. This was further justified by calculating the photocatalytic rate constant using the equation in Figure 27. 4000 ppm AgNP ZO had a higher rate constant of 0.0337 (Figure 28) than 267 AgNP (k= 0.0265) (Figure 29).



$$log\ c/c0\ =\ -kt$$

c= Concentration

c0= Concentration at 0 mins

k= Rate constant

t= Time

Figure 28. Rate constant of 4000 ppm AgNP.

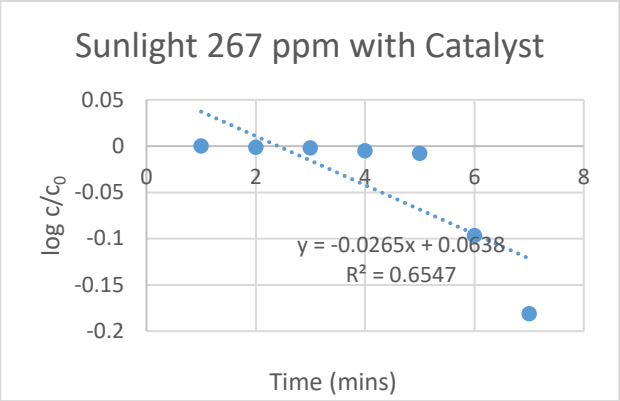


Figure 29. Rate constant of 267 ppm AgNP.

Numerous studies have detailed the use of AgNPs in a broad range of antimicrobial applications, including the treatment of wounds and the combating of clinical isolates, nosocomial, and food pathogens. The concentration of nanoparticles exposed to bacteria and the type of bacteria affect AgNPs' antibacterial activity. AgNPs have different bactericidal activities against gram-positive and gram-negative bacteria, however it is possible to distinguish which is more effective. Gram-negative bacteria are discovered to be more sensitive to AgNPs than gram-positive bacteria, according to certain research findings; nevertheless, other researchers found the opposite to be true. [16,35]

Due to their increased surface exposure to the bacterial membrane, the smaller nanoparticles exhibit more antibacterial action. It is thought that silver interacts with the thiol groups of proteins on cell membranes, preventing respiration and ultimately leading to death. (Figure 5).[16]

According to the current study it was observed that AgNP ZO and ZZ had a higher ZOI in *E. coli* than water extract; whereas AgNP CL had a less ZOI compared to the water extract. This may be due to the presence of antimicrobial activity present in ginger family. Moreover, ONE-way ANOVA didn't show significance difference (p=0.542) between AgNP and water-extract.

For *S. aureus* only AgNP CL and ZZ has higher ZOI than water extract while AgNP ZZ had a smaller ZOI. And no significant difference was observed between water-extract and AgNP (p=0.713).

In this study, it has been found more ZOI for E. *coli* than *S. aureus*. The peptidoglycan layer's different composition and thickness in the cell wall could be the most likely cause. Gram-positive bacteria have a three-dimensional peptidoglycan coating that is ~80 nm thick, which makes them less vulnerable to attack by AgNPs than Gram-negative bacteria.[36]

5. Conclusion

In conclusion, only three AgNPs (CL, ZO, and ZZ) synthesized from the leaf extracts of five species of ginger family plants exhibited spherical structures and measured 10 nm in size. All the AgNPs had higher antioxidant than water extract based on TFC, TPC, TAC, DPPH and IC_{50} assays. However, there was no significant difference between AgNPs and water-extract was observed for the anti-microbial activity of E. coli and S. aureus. Furthermore, 4000 ppm AgNP ZO degraded the EBT dye faster than 267 ppm AgNP ZO. Thus, further studies could focus on exploring microbial activity, with a particular emphasis on the potential of AgNPs for medicinal applications. Additionally, AgNPs could be utilized to help mitigate pollution caused by azo dyes, offering a promising approach to environmental remediation.

Acknowledgements

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Biodegradation of Polycyclic Aromatic Hydrocarbons in Fuel Station Soil: Impact of Immobilized Bacteria on Microbial Communities

D. Senevirathna[1], S.N.R.D.S. Jeewakarathne[1], K. Balasayanthan[1], M.S. Vanderwall[1], M.N. Fathima Shahani[1], S.M. Fernando[1], H.O.T.O. Perera[1] and R.B.N. Dharmasiri[1*]

[1]School of Science, Business Management School (BMS), Sri Lanka.

[*]nadeema.d@bms.ac.lk

Abstract

Polycyclic aromatic hydrocarbons (PAH) are made with carbon and hydrogen with two or more fused benzene rings. These substances are known to be environmental pollutants as they cause serious damage to the health of humans and other living organisms due to their carcinogenic properties. The incomplete combustion of fossil fuels and other organic substances can also produce PAHs, which are naturally occurring substances of crude oil and other petrochemical products. Forest fires and volcanic eruptions are examples of natural sources of PAH emissions. Anthracene and pyrene are two pollutant PAHs that are found in the environment. The aim of this study is to isolate and identify PAH degrading soil bacteria from polluted areas. For this analysis soil samples from three different geographical locations such as Peliyagoda, Jaffna and Gampaha were collected from fuel stations and isolated bacteria that are capable of degrading the above-mentioned PAHs. According to the primary screening and secondary screening *Microbacterium paraoxydans*, *Burkholderia multivorans*, *Staphylococcus haemolyticus* strains had above 80% degradation percentage for anthracene whereas DG2, and DJ1 had 70% degradation for pyrene. Furthermore, the Antibiotic susceptibility test demonstrates that bacteria were inhibited by certain antibiotics such as erythromycin, ampicillin, and gentamycin. Antagonistic tests proved that there is no inhibition of bacteria surrounding them which proved that it can be used in the bacterial consortium. Toxicity assay was done using mung seeds and brine shrimps proving that there is no toxicity to the environment by the introduction of these microbes. In conclusion, the bacteria identified in this study possess the potential to serve as biological agents for the effective treatment of anthracene and pyrene PAHs in contaminated soils, thus contributing positively to environmental remediation efforts.

Keywords: Polycyclic Aromatic Hydrocarbons, Anthracene, Pyrene, Degradation percentage, Toxicity assay

1. Introduction

Soil pollution has become a severe problem due to its formation from both natural and anthropogenic sources that include both industrial and agricultural activities.[1] This can further affect the soil layer strength on top of the soil reducing the fertility and the biological activity of the soil. Soil pollution is regarded as the contamination of soil with toxic substances, chemicals and certain substances that can affect the soil fertility and its quality.[2] Contaminated soil further affects the quality of groundwater as these pollutants can leach down with time, thus affecting both humans and the ecosystem.[3] The increased amount of environmental contamination is the main reason for the prevalence of Polycyclic aromatic hydrocarbons (PAHs) in soils. The power industry and large thermal companies are the most effective sources of pollution with PAHs in the environment.

The quality of agricultural production has a direct impact on human exposure, which elevates the likelihood that PAHs may accumulate in soil. To minimize the hazards of contaminated affected soils, a better understanding of the interconnected issues is crucial.[4] PAHs are toxic substances in nature that are broadly found in the aquatic systems and terrestrials. PAHs are composed of more than two fused benzene rings and are a result of

incomplete combustion of fossil fuels, coal and crude oil.[5] Over-exposure to these PAHs can lead to kidney damage, liver damage and cataracts.[6] Certain polycyclic aromatic hydrocarbons are suspected carcinogens, and prolonged exposure can lead to adverse health effects including cancers and reproductive disorders. The incomplete combustion of fossil fuels and other organic substances can also produce PAHs, which are naturally occurring substances of crude oil and other petrochemical products. Forest fires and volcanic eruptions are examples of sources of PAH emissions, but the number of manmade sources like incomplete combustion or the spilling of fossil fuels, have increased and is still a significant environmental burden. 16 PAHs were nominated as to be harmful pollutants by the US United States Environmental Protection Agency (USEPA) in 1997 based on their proven carcinogenicity and mutagenicity as well as their high of occurrence in the ecosystem.[7] PAHs are degraded by different types of bacteria by secreting enzymes such as peroxidases and oxygenases.[8] *Sphingomonas* and *Rhodococcus* are two types of bacteria capable of degrading PAHs and are responsible for the production of biosurfactants. This causes tension in water molecules to be minimized and causes the entrapment of PAHs freely available on the surface for degradation by bacteria.

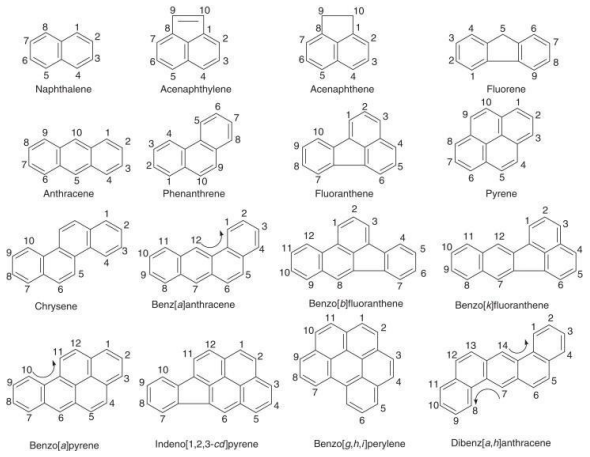

Figure 1. 16 types of priority pollutant PAHs present in environment.[9]

2. Methodology

2.1 Sample Collection. Samples were collected from three different sub-locations Gampaha (7°5'4"N 80°4'45"E), Jaffna (9°39'59.99" N 80°00'0.00" E) and Peliyagoda (6°57'21"N 79°53'10"E). These were collected into a Ziplock bag under hygiene conditions and placed in refrigerator.

2.2 HPLC environmental analysis. Each soil sample weighed around 4 g, which was then dissolved in 10 mL of hexane and shaken at 180 rpm for three minutes. After that, the supernatant was separated and allowed to evaporate. The residues were then thoroughly cleaned with 1 mL of acetonitrile. After passing through 0.22 µm nylon syringe filters, the samples were collected and placed in HPLC vials. Pyrene and anthracene concentrations of 1.25 mg were tested independently. After that, 5 mL of acetonitrile were added to the PAHs to prepare a stock concentration of 250 ppm. Pyrene and anthracene, two common PAHs, were produced at concentrations ranging from 250 ppm to 50 ppm [10].

An Agilent was used to analyze the filtered samples and assess the extract's pyrene and anthracene concentration. A 90:10 (acetonitrile: water) combination was used as the mobile phase for the PAHs at a flow rate of 3 mL per minute. The wavelength of the HPLC was 254 nm.

2.3 Soil Bacteria Isolation

2.3.1 Serial dilution. After weighing around 5 g of each sample, it was put into the conical flask and shaken for an hour at 180 rpm. After being cleaned, the soil samples were diluted to a 10^{-10} ratio.

2.3.2 Spread plate technique. An aliquot of 100 µL of each diluted sample was aseptically dispensed onto the center of a nutrient agar (NA) plate. The bacterial suspension was then evenly spread across the agar surface using a sterile glass spreader. After that, the plate was securely closed and parafilm-sealed. all the inoculated plates were incubated at room temperature for 24 hours.

2.3.3 Streak plate technique. Following incubation, bacterial colonies with distinct morphologies were chosen from the spread plates. The chosen colonies

then underwent streaking for isolation. Each of the chosen colonies underwent a 24-hour incubation period at 30°C using the same streaking approach.

2.4 Plate Assay (Primary Screening)

2.4.1 Bacterial Starvation in Bacto-Bushnell Hass (BBH) agar plates. Following the preparation of the solidified BBH agar plates, a single colony of each bacterial isolate was inoculated into 25 designated sections on separate BBH agar plates After inoculating each plate, The plates were then securely sealed with parafilm, incubated at room temperature for three days under starvation circumstances[11].

2.4.2 Starved bacteria being transferred into Anthracene and pyrene plates. First, an acetone solution containing 100 ppm of anthracene and pyrene was prepared. Subsequently, 100 µL of pyrene and anthracene solution were applied separately to solidified BBH agar plates. To evaporate the acetone, the plates were gently swabbed with a cotton applicator and left partially uncovered for a brief period. After the acetone had evaporated, the plates were inverted, and 25 equal-sized squares were marked on each plate. Each bacterial colony, which had been subjected to starvation conditions, was then carefully transferred into the corresponding square on the plate. Once all colonies were inoculated, the plates were sealed with parafilm and incubated at room temperature for 24 hours.

2.5 *Spectrophotometric analysis.* After sterilizing the test tubes, 2% methylene blue was added, and the PAH was added as a spike. An inoculation loop was used to add the corresponding bacterial colonies to the medium. The incubated samples were used for the spectrophotometric analysis. Before analysis, a blank sample was added, and negative results were recorded. Each experimental sample was examined in triplicate, and the average percentage of deterioration was calculated.

2.6 DNA extraction. Three of the finest bacteria for breaking down PAHs were subcultured, and the bacterial colonies were gathered into sterile Eppendorf tubes. After adding 1 mL of 0.9% NaCl to each tube, the tubes were centrifuged for three minutes at 13,000 rpm, and the resulting supernatant was discarded. This process was carried out three times, with the resulting supernatant being discarded. After that, 20 µL of Tris-EDTA (TE) buffer was added to each tube, and they were completely mixed by vortexing. Following freezing of the tubes on ice for 15 minutes, they were thawed in a warm water bath for another 15 minutes at 95 °C. The samples were then centrifuged for five minutes at 13,000 rpm and the acquired supernatant of each sample was put into a fresh Eppendorf tube, and each tube was well mixed with 20 µL of 100% ethanol. Then, the three Eppendorf tubes were securely sealed and stored at -25°C[11].

Table 1. PCR components, their volumes and concentrations for preparing master mix for bacterial DNA.

Component	Stock Concentration	Working Concentration	Volume required
Go taq green PCR master mix	2×	2×	12.5µL
Nuclease free water	-	-	8.5µL
Forward primer (F2 7)	100M	10M	1µL
DNA template	-	-	2µL
Reverse primer (R1 492)	100M	10M	1µL
PCR mix			25µL

Table 2. Thermal cycling conditions

Step	Temperature	Time
Initial Denaturation	94ºC	3 minutes
Denaturation	94ºC	30 seconds
Annealing	59ºC	1 minute
Extension	72ºC	1 minute
Final extension	72ºC	10 minutes

PCR amplification was carried out in a 25 µL reaction volume using a thermal cycler according to the above-mentioned PCR conditions (Table 2) which included 35 cycles of denaturation, annealing, and extension. For visualization of the PCR products, 1 % agarose gel was loaded with 2 µL of a 1KB DNA ladder and 2 µL of PCR products. The gel was set up to run for forty minutes at 55 volts. A gel documentation system was then used to visualize the PCR amplicons and to determine their sizes with reference to DNA ladder. Following the trimming of the sequencing data with the BioEdit program, the species were identified using the BLAST tool in NCBI.

2.7 Antibiotic susceptibility test. Following the preparation of NA plates, a standardized bacterial inoculum was evenly spread across the surface of each plate. The antibiotic discs were placed onto the inoculated NA plates and incubated overnight. The inhibitory zone diameter around each antibiotic disc was then measured. These readings were then compared to the standards established by the Clinical and Laboratory Standards Institute (CLSI).

3. Results and Discussion

3.1 *HPLC (*High-pressure liquid chromatography) *analysis.* The findings of the environmental analysis test using HPLC showed that the soil samples contained 50 ppm of pyrene and anthracene.

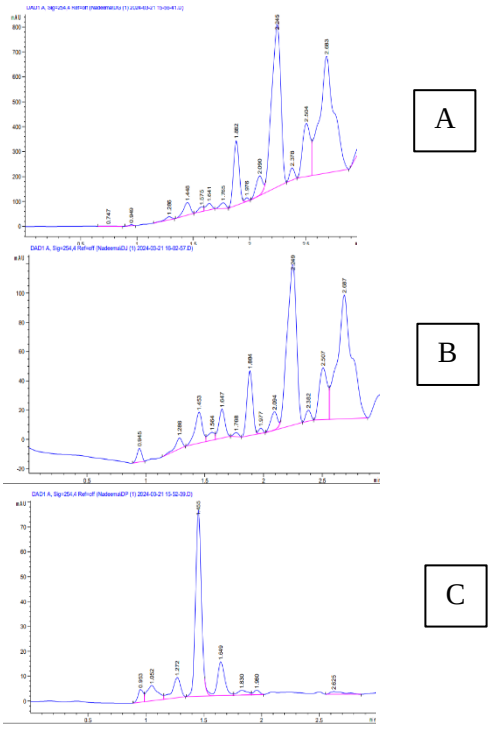

Figure 2. HPLC results for *Microbacterium paraoxydans* (A), *Burkholderia multivorans* (B), *Staphylococcus haemolyticus* (C) samples.

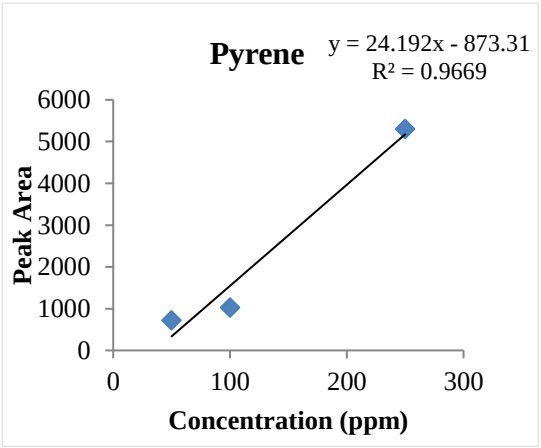

Figure 3. Standard curve of Pyrene

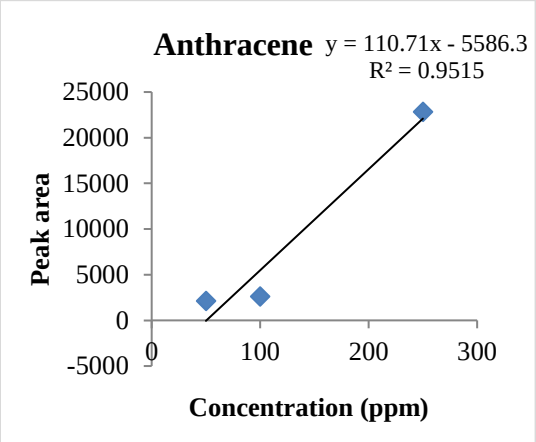

Figure 4. Standard curve of Anthracene

Table 3. Sample concentration

PAHs	DP /ppm	DG /ppm	DJ /ppm
Pyrene	49.902	75.242	45.263
Anthracene	56.749	62.199	55.752

The standard concentrations (50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm) were used to produce the standard curve using HPLC.

Following the incubation of spread plates, bacterial growth exhibited characteristic colony morphologies. Table 4 lists the several bacterial colonies that were discovered based on their morphology.

3.2 Population Density. The population density of each of the eight colonies is displayed in Table 5, with DJ 2 10^{-10} having the highest population density (6 $X10^{11}$). Single bacterial isolates of the morphologically distinct bacterial colony were obtained following the incubation of streak plates. This facilitated the identification of the bacterial colonies as distinct individual isolates that require more research.

Table 4. Different morphologies obtained from spread plate assay.

Colony	**Size**	**Colour**	**Texture**	**Elevation**	**Form**	**Margin**
DJ1 10^{-10}	small	white	smooth	convex	round	entire
DJ2 10^{-10}	small	white	smooth	convex	round	entire
DG2 10^{-10}	moderate	faded white	mucous	raised	irregular	lobate
DP1 10^{-10}	moderate	creamy white	smooth	raised	irregular	entire
DG3 10^{-5}	small	yellow	mucous	raised	irregular	entire
DG2 10^{-5}	small	faded white	mucous	umbonate	irregular	undulate
DJ1 10^{-10}	large	white creamy	smooth	convex	irregular	entire
DP2 10^{-10}	small	light orange	smooth	flat	round	entire
DJ3 10^{-5}	small	light yellow	smooth	raised	round	entire
DG1 10^{-10}	moderate	faded yellow	smooth	raised	irregular	lobate

Equation 01.

$$\text{Population Density}(\text{CFU/mL}) = \frac{\text{Number of isolated colonies}}{\text{Amount of diluted sample used in spread plate (mL) x Dilution factor}} \text{ x 1 mL}$$

Table 5. Calculation of population density

Colony samples	Number of Isolated Colonies in the sample	Dilution Factors	Population Density (CFU/mL)
DG1 10^{-10}	212	10^{-10}	2.12 $X10^{11}$
DP2 10^{-10}	2	10^{-10}	$2X10^{11}$
DJ2 10^{-10}	6	10^{-10}	6 $X10^{11}$

3.3 Primary screening technique (Plate assay)

Table 6. Plate assay results for DG1 10^{-10}, DP2 10^{-10}, DJ2 10^{-10}

Bacterial sample	Pyrene	Anthracene
DG1 10^{-10}	25/25	16/25
DP2 10^{-10}	2/25	25/25
DJ2 10^{-10}	5/25	19/25

As indicated in Table 6, all bacterial strains demonstrated positive screening results, allowing for additional screening to be carried out. Additionally, using phenanthrene and naphthalene as the only carbon sources, the replica plate findings demonstrated that over 80% of the isolated bacteria could thrive on BBH agar medium supplemented with 100 ppm anthracene and pyrene.

3.4 Spectrophotometric Analysis. Figure 5 displays the percentages of bacterial strains that degraded PAHs. This guarantees that The bacterial strains could break down PAH contaminants. These bacterial species caused a color change in the BBH broth by reducing the methylene blue indicator as a result of the oxidation of PAH. The UV-Vis spectrophotometer can detect this color shift as an absorbance value, and the overall color of the isolates change from blue to colorless suggesting their potential as hydrocarbon oxidisers.[8]

More than 80% of anthracene could be broken down by the bacterial strains DJ3 10^{-10}, DG2 10^{-5}, DG3 10^{-5} along with DG2$10^{-5}$ degraded more than 80% of pyrene.

Pyrene is broken down by bacterial strains at a lower rate than anthracene given pyrene contains four fused benzene structures, whereas anthracene has three. Consequently, the bacterial strains may readily degrade the structure of anthracene, unlike pyrene.[10]

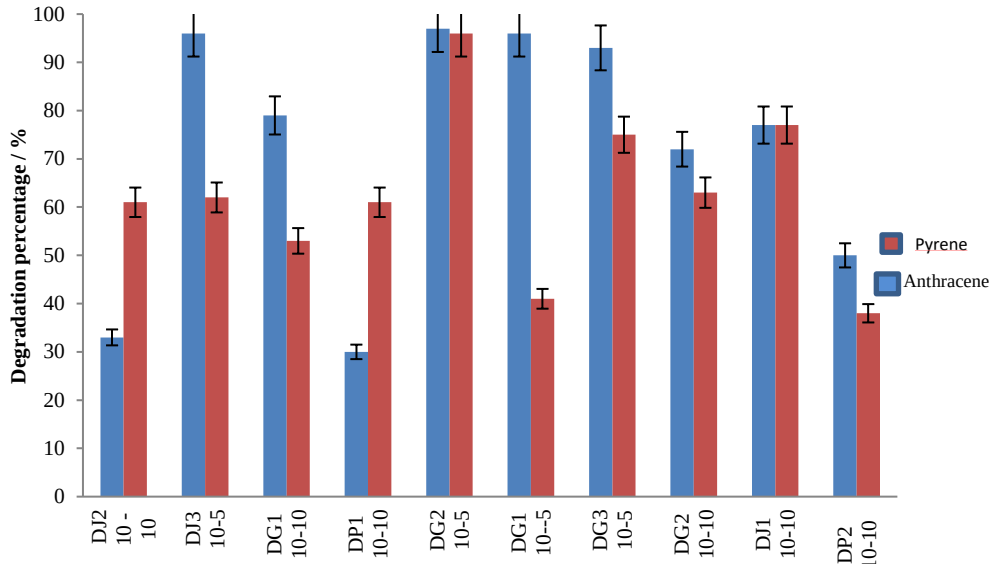

Figure 5. PAHs degradation percentage of bacteria from fuel station soil samples measured at a wavelength of 609 nm.

Table 7. Accession numbers of the PAHs

Sample name	**Bacterial strain**	**Accession number**
DJ2	*Microbacterium paraoxydans* strain	PQOO8540
DG1	*Burkholderia multivorans* strain	PQ008496
DP2	*Staphylococcus haemolyticus* strain	PQ002183

The antimicrobial susceptibility test shows that the tested bacterial strains have a high level of antibiotic resistance. Table 8 shows the larger diameters in ampicillin inhibitory zones in strains DP2, DJ2, and DG1 suggested effectiveness of the antibiotic to the bacterial strains.

Strains DP2 and DJ2 lacked Erythromycin inhibition zones, whereas strains DP2 and DJ2 further lacked Chloramphenicol and Gentamycin inhibition zones, suggesting further antibiotic resistance. Additionally, vancomycin exhibited greater zone of inhibitions in all three bacterial samples. Conversely, remaining bacterial colonies had respectable inhibition zones, suggesting that appropriate antimicrobial agents may affect their growth. The data revealed, most of the tested strains exhibited resistance to at least one antimicrobial agent highlighting the significance of selecting antibiotics with caution when treating illnesses caused by certain bacterial strains.

Table 8. Antibiotic susceptibility readings for DP2, DJ3, DJ2, DG3 samples.

ABST	DP2	DJ2	DG1
Tetracycline	2.50	3.00	2.10
Ampicillin	0.00	0.00	0.00
Vancomycin	1.90	2.40	2.40
Erythromycin	0.00	0.00	1.1
Chloramphenicol	0.00	0.00	3.00
Gentamycin	0.00	0.00	2

Table 9. Toxicity assay done for every hour

Zootoxicity					
Time	**PAH**	**DG1**	**DP2**	**DG3**	**DJ2**
11.30am	Pyrene Anthracene	10 10	9 10	9 10	10 10
12.30pm	Pyrene Anthracene	10 9	10 10	10 9	10 9
1.30pm	Pyrene Anthracene	10 9	9 10	9 9	9 9
2.30pm	Pyrene Anthracene	10 9	9 9	9 9	9 9
3.30pm	Pyrene Anthracene	10 9	9 9	9 8	9 9
4.30pm	Pyrene Anthracene	10 9	9 9	9 8	9 8

Table 10. Phytotoxicity results

Phytotoxicity			
Plant no.	**Height/cm**	**Negative/cm**	**Positive/cm**
1	11.5	28.2	8.2
2	16.6	23.9	9.3
3	19.8	25.7	9.5
4	17.6	23.0	9.0
5	19.4	27.8	9.6
6	18.2	20.3	7.1

Phytotoxicity was performed where the bacterial samples were added to the soil followed by the planting of mung seeds which were watered daily. The plant heights were measured to assess if these PAHs had an impact on the plant growth (Table 10). Growth measurements indicated that the PAHs inhibited plant development, as evidenced by reduced plant height in the experimental group compared to the positive control, which exhibited slower growth.

Additionally, the phytotoxicity of the PAHs was further evaluated using *Artemia salina* (Table 9). This was performed each of the 10-brine shrimp was monitored hourly for mortality, providing an assessment of the potential toxic effects of the PAHs *A. salina* was used in toxicity assay because of its low cost, high reproducibility, simplicity, ability they can adapt to the environment, and they can remain usable for years if stored in dried place.[12] *M. paraoxydans and B. multivorans* strains mostly survived for the time intervals as stated but *S. haemolyticus* strain had a gradual decrease in the count with time.

4. Conclusion

This study identified bacterial strains capable of breaking down pyrene and anthracene and the percentage of degradation was assessed. These results provide credence to the potential effectiveness of bacterial bioremediation of anthracene and pyrene. As a result, these strains can be employed as possible biological agents to reduce soil pollution from pyrene and anthracene, enhance soil quality to encourage plant development and reduce the risk of pyrene and anthracene exposure to humans. These findings provide a number of opportunities for further research to investigate the application of these high-efficiency degraders for effective bioremediation techniques to purify the soil. Furthermore, the current study demonstrates that the novel isolates were able to eliminate a greater amount of pyrene and other chemicals than the isolates the scientists had previously studied.

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Cytotoxicity of indigenous medicinal plants; *Salacia reticulata, Coriandrum sativum* and *Aerva lanata* on Vero kidney cell line; in-vitro study using MTT assay and SRB assay

J.K. Hewawithana[1], K.T. Dilrukshi[1,2] and M.M. Pathmalal[2*]

[1]School of Science, Business Management School (BMS), Sri Lanka

[2]Centre for Water Quality and Algae Research, Department of Zoology, University of Sri Jayewardenepura, Nugegoda, Sri Lanka.

[*]pathmalal@sjp.ac.lk

Abstract

Despite pharmaceutical advances, natural treatments are gaining popularity worldwide. Many individuals worldwide who suffer from illnesses use herbal plants to have a therapeutic effect. Use of these plants may harm patients, especially the kidneys. In this study, indigenous medicinal plants *Salacia reticulata, Coriandrum sativum, and Aerva lanata* were tested for nephrotoxicity using Vero kidney cells (Catalogue number CCL-81 TM). Four dilution series in each (5, 10, 15, and 20 grams (g), of *Coriandrum sativum*; 1, 2, 3, and 4 g of *Salacia reticulata*; 3, 6, 9, and 12 g of *Aerva lanata* in 125 milliliters (ml) of Milli-Q water) were exposed to Vero; monkey kidney epithelial cells ($5x10^3$ cells/well). Cell viability was measured using Sulforhodamine B (SRB) and (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) assays and the cell viability percentage and the cytotoxic concentration 50% (CC_{50}) values were calculated. In the MTT assay the cell viability percentages in the cells exposed to *Coriandrum sativum, Salacia reticulata* and *Aerva lanata* were ranged from 64.46 to 98.73%, 72.06 to 98.00% and 50.80 to 95.00 % respectively. The cell viability percentages in the cells exposed to *Coriandrum sativum, Salacia reticulata and Aerva lanata* in SRB assay were ranged from 69.66 to 93.33%, 76.64 to 90.98 % and 77.17 to 50.90% respectively. Significantly higher cell viability percentages were recorded in the cells exposed to plant extract than in the cells exposed to the positive control in both MTT and SRB assays ($p< 0.05$). Hence, the results of the present study indicated the water extract of the *Coriandrum sativum, Salacia reticulata and Aerva lanata* plants do not reduce the cell viability and do not cause nephrotoxicity directly. However, further studies are needed to confirm the nephrotoxicity effects of medicinal plants.

Keywords: Cell viability, indigenous medicinal plants, Nephrotoxic effects, plant extracts, Vero kidney cells

1. Introduction

More than five thousand years ago, ancient civilisations predominantly utilised plant products for healing ailments and rejuvenating their bodies.[1] Indigenous medicinal plants have been utilised for the treatment and prevention of various diseases and epidemics.[2] Certain medicinal herbs serve dual purposes as condiments, flavouring agents, colouring agents, and preservatives. Nearly all parts of plants possess therapeutic properties, with their medicinal value attributed to specific chemical active substances that elicit defined physiological effects on the human body.[3] Secondary metabolites present in medicinal plants influence various diseases and are utilised in drug formulation. Throughout history, numerous Sri Lankan plants have served as remedies with antioxidant, anti-inflammatory, anti-insecticidal, anti-parasitic, antibiotic, and anti-hemolytic properties.[4]

Coriandrum sativum, a member of the *Umbelliferae/Apiaceae* family, is widely recognised for its antibacterial, antifungal, and antioxidant properties, attributed to the unique chemical components found in various parts of the plant.[5] The leaves and seeds of plants are

extensively utilised in traditional medicine and as flavouring agents in culinary practices.

The woody climber *Salacia reticulata*, also known as Kothala Himbutu, is frequently used in the Ayurvedic medical system to treat diabetes and obesity.[6] It is also said to have anti-rheumatic effects and is used in traditional medicinal practises for a variety of skin problems.[7] *Salacia reticulata* has been extensively researched in animal models and people for its hypoglycemic and anti-obesity properties.[8]

Furthermore, *Aerva lanata* (Polpala) is frequently mentioned in Ayurveda as a diuretic with moderate analgesic, anthelmintic, antibacterial, and anti-inflammatory properties.[9] It is utilized as an antidote for rat poisoning as well as the treatment of lithiasis, cough, asthma, and headaches.

The safety and toxicity of these medicinal plants remain inadequately investigated. The utilisation of these plants may adversely affect patient health, particularly concerning renal function. Kidneys are particularly susceptible to damage caused by toxins, including harmful substances derived from certain medicinal plants. Healthy kidneys filter approximately 240 millilitres of blood per minute, generating urine from waste products and surplus water.

Nephrotoxicity is a rapid reduction in kidney function induced by medications and chemicals.[10] Nephrotoxins are chemicals that cause nephrotoxicity (10). Some drugs affect renal function in multiple ways. Most medications worsen renal failure in patients and drugs are responsible for around 20% of nephrotoxicity, a figure that is higher in the elderly.[11] Renal tubular toxicity, inflammation, glomerular damage, crystal nephropathy, and thrombotic microangiopathy can cause nephrotoxicity.[11]

In 1962, Chiba University researchers established the Vero cell line from an African green monkey kidney which is one of the most popular continuous cell lines worldwide.[12] A continuous cell lineage can divide repeatedly without senescence. This anchorage-dependent cell line has been utilised in virology research to grow and analyse intracellular bacteria and study the molecular effects of drugs and chemicals on mammalian cells.[13] Vero cells do not release interferon alpha or beta when infected with viruses, unlike normal mammalian cells. Vero kidney cells maintain contact inhibition when they achieve confluency, they stop growing and start dying, thus they must be monitored and subcultured.[13] Since Vero cells retain the attributes of normal cells, they were selected for this study.

Despite advancements in pharmaceuticals, there is a growing global interest in natural treatments. A significant number of individuals globally utilise indigenous medicinal herbs for the treatment of various ailments. The utilisation of these plants may adversely affect patient health, particularly concerning renal function. Given the absence of documented in-vitro nephrotoxicity studies, it is essential to assess the potential adverse health effects of utilising native medicinal herbs. This study investigated the nephrotoxic effects of *Coriandrum sativum, Salacia reticulata, and Aerva lanata* on Vero kidney cells.

2. Methodology

2.1 Plant crude extraction. Seeds of *Coriandrum sativum* were obtained in 5 g, 10 g, 15 g and 20 g. Powdered Stems of *Salacia reticulata* were obtained in 1 g, 2 g, 3 g, and 4 g. Leaves and stems of *Aerva lanata* were obtained in 3 g, 6 g, 9 g and 12 g. All of the plant samples were separately boiled in 250 ml of water until the water is reduced by half (125 ml). Then they were separately put into pre labeled sterile bottles.

A volume of 9 ml of each of the prepared samples were put in to thoroughly cleaned separate evaporation disks and they were placed in the dry oven which was pre heated to 40 °C. Samples were allowed to completely dry for 48 hours. After the samples have been completely dried, 3 ml of Milli-Q water was added to the residue of each dried sample in 1ml strokes. The solutions that were obtained were transferred to clean and sterilized glass bottles. Next all the solutions were filtered into separate clean and sterilized bottles using syringe filters. Milli-Q water was used as the negative control.

2.2 Preparation of exposure solutions. Each plant solution (300 µl) was transferred into 1.5 ml Eppendorf tubes, and they were topped up with 600 µl of complete media under sterile conditions.

2.3 Cell culture and exposure. The Vero cell cryovial was thawed by gently whirling in a 37°C water bath. The cap of the cryovial was kept out of the water during this to avoid any potential contaminations. Then the vial was decontaminated in a laminar flow hood by spraying with 70% ethanol. The Vero cell suspension was transferred from the cryovial into a 15 ml conical tube containing 10 ml of complete media prepared using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich,UK), 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich,USA) and strep penicillin (Sigma-Aldrich,USA). The cryo-preserving dimethyl sulfoxide (DMSO) (Sigma-Aldrich,USA) in frozen cell supplies can be detrimental to the cells. Therefore, after defrosting the cells, the DMSO was diluted and removed before transferring the cells to tissue culture flasks.[14]

The cells were palletized by centrifuging at room temperature for 5 minutes at 2600 rpm. The supernatant was removed, and the cells were resuspended in 5-10 ml filtered complete DMEM containing 10% FBS and strep penicillin. Then the Vero cell suspension was moved to a T_{25} cell culture flask with a vented top and incubated at 37 °C with 5% CO_2 for 48 hours for cell attachment.

After 48 hours of incubation the T_{25} cell culture flask containing the cells was observed under the microscope and after 75% confluence, cell passaging was done. Then carefully the media was removed from the cell flask and the attached cells were gently washed with 3 ml Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich,USA) without disturbing the cell monolayer. Next 2 ml of Trypsin EDTA (Sigma-Aldrich,USA) was added into the cell flask to detach the cell monolayer from the bottom of the cell flask, and it was incubated at 37 °C and 5% CO_2 for 2-3 minutes. Afterwards the cell flask was observed under the microscope to confirm that the cells have detached. Then the cell flask was topped up with complete media the cell suspension that was obtained was transferred into a 15 ml centrifuge tube and it was centrifuged at 1600 rpm for 5 minutes. The resulted supernatant was discarded carefully, and the cell pallet was resuspended in complete media. A micro pipette was used to break any clumps of cells.

Then 20 µl of cell suspension was mixed with 20 µl of trypan blue. Next 10 µl of the prepared mixture was loaded in the hemocytometer and a viable and dead cell count was taken by observing under the microscope. The viable cell percentage was calculated. Since the calculated cell viability percentage was above 80%, the cells were seeded in 96 well plates for cell viability assays and the cells were incubated for 48 hours at 37°C and 5% CO_2.

After 48 hours of incubation, the 96 well plates were observed under the microscope. Cells in 96 well plates were exposed to previously prepared plant extracts and cell were incubated for 24 hours at 37 °C and 5% CO_2. Triplicate samples were done for each sample. Negative and positive controls were maintained throughout the experiment and DMSO was used for the positive control. Sterile Milli-Q water was used as the negative control.

2.4 MTT assay. After 24 hours incubation, prepared MTT solution was added to each well and the plate was incubated for 3 hours. After the incubation the solution in each well of the 96 well plate was removed. Lastly the each well were filled with of 10% DMSO and the plate was shaken for 30 minutes. The absorbance was measured at 620nm.[15]

2.5 SRB assay. The cell plate treated and incubated with the exposure solutions were topped up with 50% cold trichloroacetic acid. Then it was incubated at 4 °C for 1 hour. The cell plate was carefully washed with water for 5 times, and it was airdried for few minutes. Then the cells in the wells were stained using prepared SRB solution and it was allowed to incubate for 15 minutes at the room temperature. After 15 minutes the cell plate was washed with 1% acetic acid for 5 times, and it was allowed to air dry for few minutes. Then 10 mM unbuffed tris base solution was added to each well and the plate was shaken for 30 minutes at room temperature. The absorbance was measured at 520 nm.[16]

2.6 Statistical analysis. IBM SPSS version 27 was used to do the statistical analysis. A significant difference was defined as having a *p*-value of less than 0.05.

3. Results

3.1 MTT assay for Coriandrum sativum. As the evidence suggests, the cells that were treated with the highest concentration of *Coriandrum sativum* was recorded the highest cell viability percentage whereas the cells exposed lower concentrations of *Coriandrum sativum* was recorded slightly lower percentages of cell viability. Significantly higher cell viability percentages were recorded in the cells exposed to *Coriandrum sativum* than in the cells exposed to the positive control (DMSO) accordingly ($p<0.05$) (Figure 1).

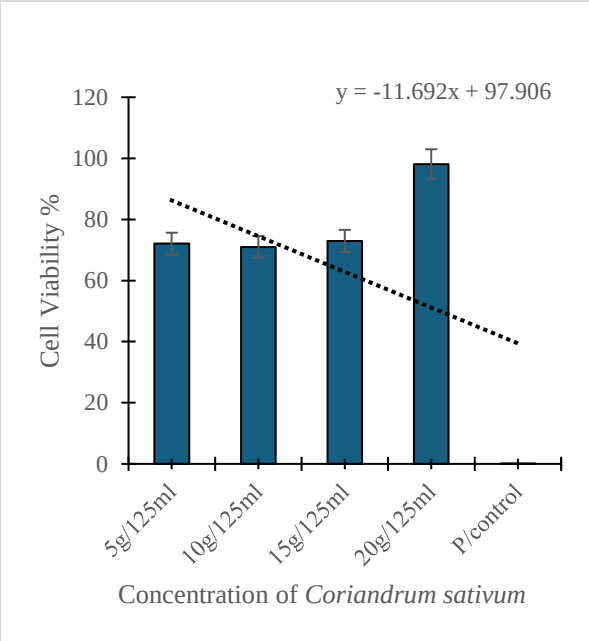

Figure 1. Variation in % viability of Vero cells exposed to different concentrations of *Coriandrum sativum.* Data are presented as mean ± SD.

3.2 MTT assay for Salacia reticulata. In the first three concentrations a decreasing trend for the cell viability percentage was observed. However, the cells that were exposed to the highest concentration of *Salacia reticulata* was recorded the highest percentage of cell viability. Furthermore, significantly higher cell viability percentages were recorded in the cells exposed to *Salacia reticulata* than in the cells exposed to the positive control (DMSO) accordingly ($p<0.05$) (Figure 2).

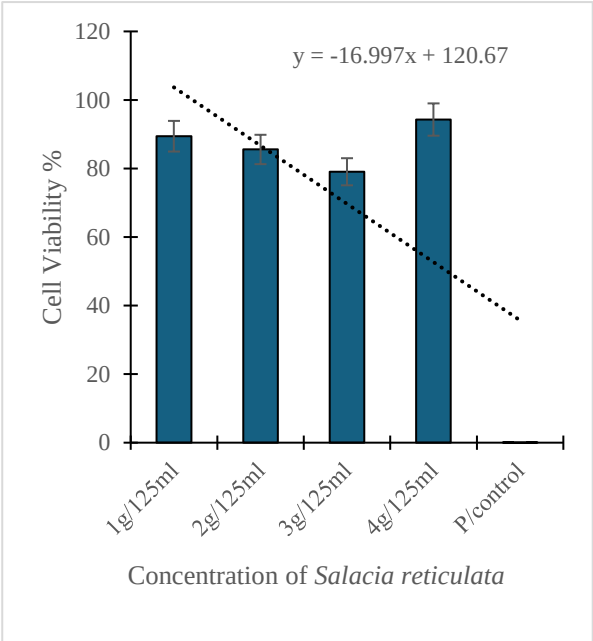

Figure 2. Variation in % viability of Vero cells exposed to different concentrations of *Salacia reticulata*. Data are presented as mean ± SD.

3.3 MTT assay for Aerva lanata. An increasing trend was seen throughout the concentration series where the highest cell viability was observed in the cells that were exposed with the highest concentration of *Aerva lanata*. Accordingly, the cell viability percentages in the cells exposed to *Aerva lanata* were significantly higher than in the cells exposed to the positive control ($p<0.05$) (Figure 3).

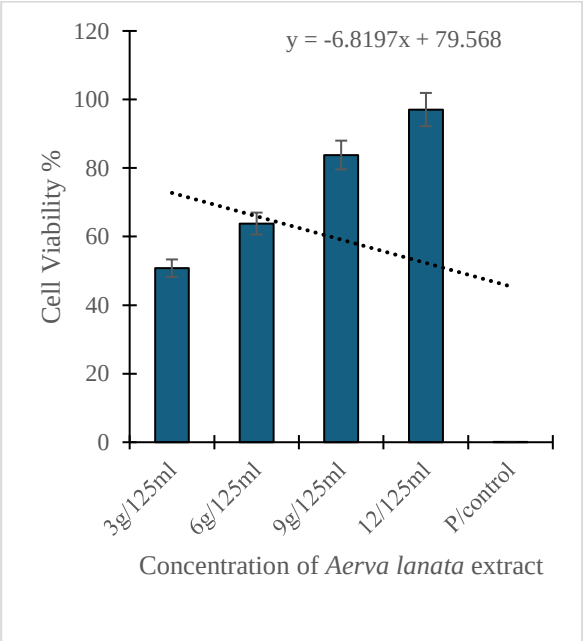

Figure 3. Variation in % viability of Vero cells exposed to different concentrations of *Aerva lanata*. Data are presented as mean ± SD.

3.4 CC_{50} value calculation for results obtained in the MTT assay. CC_{50} is the concentration of test compounds required to reduce cell viability by 50%. According to the results of percentage of viability of cells, there is no significant effect of plant extracts on cell viability. Accordingly, the CC_{50} values obtained from the present study was depicted in Table 1.

Table 1. CC_{50} values calculated according to the MTT assay results.

Plant sample	CC_{50} value
Coriandrum sativum	0.57
Salacia reticulata	-39.19
Aerva lanata	1.198

3.5 SRB assay for Coriandrum sativum. The lower three concentrations resulted in a decreasing trend of cell viability but the cells that were exposed to the highest concentration of *Coriandrum sativum* was recorded the highest cell viability. Significantly higher cell viability percentages were recorded in the cells exposed to *Coriandrum sativum* than in the cells exposed to the positive control (DMSO) accordingly (p<0.05) (Figure 4).

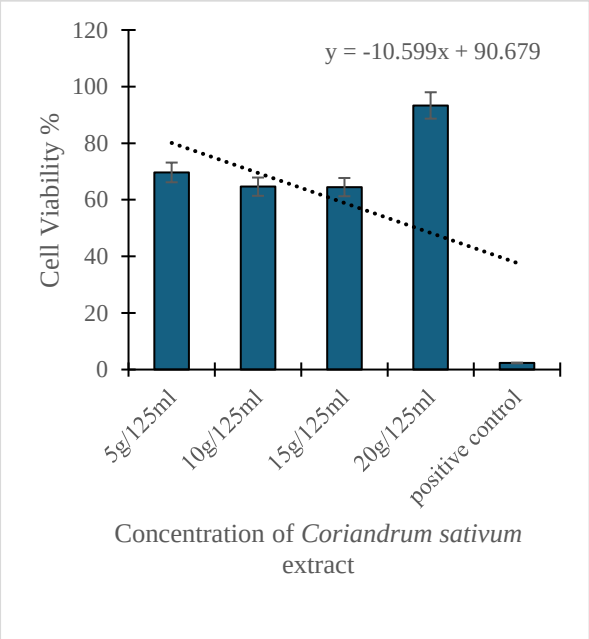

Figure 4. Variation in % viability of Vero cells exposed to different concentrations of *Coriandrum sativum*. Data are presented as mean ± SD.

3.6 *SRB assay for Salacia reticulata.* Except for cells that were exposed to the lowest concentration of *Salacia reticulata* every other cell had a cell viability percentage of more than 80%. Furthermore, significantly higher cell viability percentages were recorded in the cells exposed to *Salacia reticulata* than in the cells exposed to the positive control (DMSO) accordingly (p<0.05) (Figure 5).

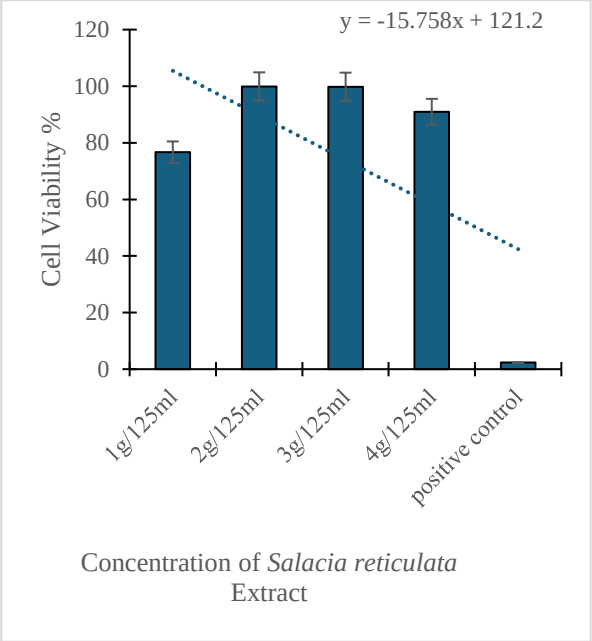

Figure 5. Variation in % viability of Vero cells exposed to different concentrations of *Salacia reticulata*. Data are presented as mean ± SD.

3.7 SRB assay for *Aerva lanata*. A decreasing trend was seen throughout the concentration series where the highest cell viability was observed in the cells that were exposed with the lowest concentration of *Aerva lanata*. However, the cell viability percentages in the cells exposed to *Aerva lanata* were significantly higher than in the cells exposed to the positive control (p<0.05) (Figure 6).

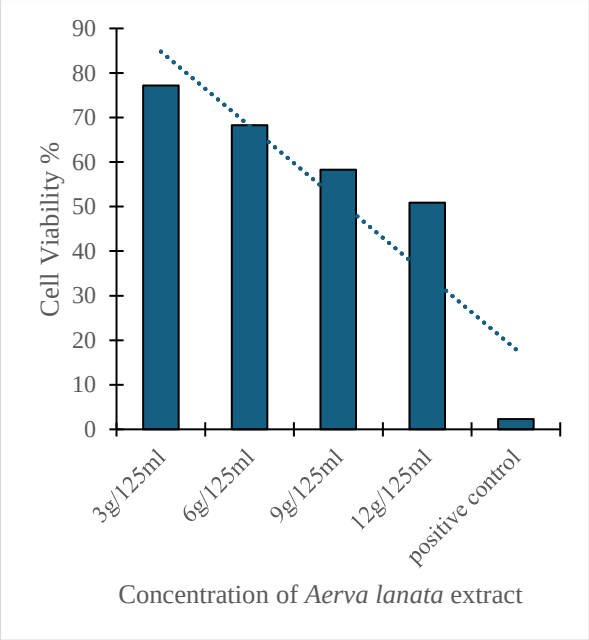

Figure 6. Variation in % viability of Vero cells exposed to different concentrations of *Aerva lanata*. Data are presented as mean ± SD.

3.8 CC_{50} value for the results obtained from the SRB assay. CC_{50} is the concentration of test compounds required to reduce cell viability by 50%. According to the results of percentage of viability of cells, there is no significant effect of plant extracts on cell viability. Accordingly, the CC_{50} values obtained from the present study was depicted in Table 2.

Table 2. CC_{50} value calculated according to the SRB assay results.

Plant sample	CC_{50} value
Coriandrum sativum	-0.752
Salacia reticulata	-7.38
Aerva lanata	4.038

4. Discussion

Some pharmacologically active compounds in medicinal plants have been related to undesirable consequences, increasing reports of adverse responses in human body. Nephrotoxicity is considered as a major side effect of usage of herbal medicines. A recent study done by Xiofen, 2020 found that over 100 herbal medicines harm the kidneys.[17] The nephrotoxicity of popular medicinal plants in Sri Lanka, *Coriandrum sativum*, *Salacia reticulata*, and *Aerva lanata* was examined in this study.

During this investigation series of concentrations were used for each plant extract. They were, the concentration prescribed by a recognized Ayurveda doctor (Dr. Nilumi Buddika, 2022), half of the prescribed concentration and double of the prescribed concentration. This was done to closely monitor the relationship between the concentration of herbal medicine and the cell viability. The plant crude extracts were prepared using only distilled water because in real life these medicines are consumed by people as water extracts.

Based on assay results, cell viability % and CC_{50} were computed. No plant extract concentration reduced cell viability, according to the results. According to the MTT assay results, *Coriandrum sativum* had a cell viability percentage between 64.46 to 98.73% throughout the series. *Salacia reticulata* had a cell viability percentage of above 80% throughout the concentration series whereas *Aerva lanata* had a percentage between 50.80 to 95.00 %. In a study that was done in 2012 by Wen et al., using plant extracts had contradicting findings to this present study where significant toxicity was observed.[18] The SRB assay findings also suggest that none of the concentrations of each plant extract caused distinct nephrotoxicity. Another study which was done by Ming, 2006 confirmed that vegetables like rhubarb caused nephrotoxicity in rats.[19]

A study, which was done by Amita and Juan, 2019 indicated that certain medicinal herbs like milk thistle can increase the concentration of ingested drugs like cyclosporin which can promote kidney inflammation and rejection in kidney transplanted patients by decreasing the immunosuppressive agent concentration.[20] Moreover, in 2021, Kiliś-Pstrusińska and Wiela-Hojeńska stated that many flavonoids present in most of the medicinal plants alter the enzyme activity, which could have potential to alter other drug metabolisms and induce nephrotoxicity.[21] Therefore, the ingestion of herbal medicines and the pharmaceutically developed other drugs *viz* antibiotics and anti-

inflammatory drugs simultaneously may cause adverse effect on kidney function.

The result of the present study is similar to a study conducted by Patel and Patel (2011), which examined the cytotoxic effect of a methanolic extract of Artocarpus heterophyllus by two in vitro cytotoxicity assays MTT and SRB.[22] Both assays SRB and MTT are applicable for assessing the cytotoxicity of herbal plants; however, each assay presents certain limitations.[23] MTT is classified as a carcinogen. Therefore, MTT waste must be properly disposed of following testing by environmental pollution control agencies.

Light contamination should be avoided during SRB staining, as light exposure can lead to the degradation of SRB. The staining process must be executed within a designated timeframe as per the manufacturer's instructions; failure to adhere to this may lead to protein loss and an underestimation of optical density.

Due to the recent significant rise in the accessibility of plant-based raw materials and the resulting decrease in the ability to regulate their usage, the existing system for monitoring the safety of medicinal products is incapable of collecting data on all adverse effects associated with the use of natural products.[24] Furthermore, due to the rising resistance against pharmacologically active compounds, the infections can be difficult to treat. Hence, investigating novel natural compounds provides an avenue for discovering new treatments that may be less prone to resistance development.

5. Conclusion

The present study indicated the water extracts of the *Coriandrum sativum*, *Salacia reticulata* and *Aerva lanata* plants do not reduces the cell viability and do not cause nephrotoxicity directly. However, further studies are needed to confirm the nephrotoxicity effects of medicinal plants.

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Isolation of Salt Tolerant Enzyme Producing Bacteria from Marine Environments in Sri Lanka for Industrial Applications

M.F. Razan[1], H.D.D. Sadeepa[1,2*] and P.M. Manage[2]

[1]School of Science, BMS Campus, 591, Galle Road, Wellawatte, Colombo-06, Sri Lanka

[2]Centre for Water Quality and Algae Research, Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka

*dilini.s@bms.ac.lk

Abstract

Microbial enzymes have been used in our day-to-day lives since ancient times. In the current world, microbial enzymes are widely used in industries because of their environmental friendly nature and cost-effective production. Halophiles are industrially important bacteria due to their high stability in high concentrations of salts. Amylases, cellulases, proteases and lipases are the most widely used halophilic hydrolytic enzymes in food production, detergent industry, pharmaceutical industry, biofuel production, paper production, etc. Each of these enzymes plays a vital role in industrial processes and world enzyme market which represents a considerable percentage of world trades. Thus the current study focused on isolation of salt-tolerant enzyme-producing bacteria from marine environments in Sri Lanka for industrial applications. The samples were collected from Negombo Lagoon and Panadura Beach in Sri Lanka and screened for the production of amylases, cellulases, proteases and lipases using plate assays. Then, the optimization of growth temperature and salt concentrations was performed for enzyme-producing bacteria. A total of 44 morphologically different bacteria were isolated, and almost 95% showed at least one enzyme activity. The optimum salt concentration for bacterial growth was in a range of 3% to 12%, while the optimum growth temperature was in the range of 30^0C and 35^0C. Thus, marine environmental bacteria can be considered as potential halophilic enzyme producer, and may be utilized in industrial settings operating under high salt concentrations.

Keywords: Halophiles, Halophilic bacteria, Industrial applications, Marine bacteria, Enzyme producing bacteria

1. Introduction

Biological reactions are being catalysed by biological catalysts called enzymes.[1] Catalysts are essential for mediating the biological reactions in living organisms. It includes the biological use of catalysts as well as the industrial use of catalysts. Use of catalysts in industrial applications would increase the rate of reaction and hence result in increased production of the desired end product.[2]

Amidst the Industrial Revolution, emerging industries utilized chemical catalysts, which produce chemical wastes.[2] Since chemical wastes can drastically affect the ecosystem, modern industries are in search of environmentally friendly catalysts, preferentially biological catalysts, as an increasing environmental concern. The use of eukaryotic enzymes as biological catalysts is often complex and involves expensive procedures.[3] Cultivation and purification of microbial enzymes were proven effective compared to eukaryotic enzymes

due to their easy access, fast growth rate and inexpensive procedures.[4]

Mesophilic enzymes produced by common mesophilic bacteria are not likely to withstand the extreme conditions of the industries, including extreme temperatures, high salinity, extreme pH levels, oxidative stress, extreme pressure, and exposure to toxic chemicals.[5,6] Hence, extremozymes derived from extremophiles are favoured due to their adaptable metabolism and unique structural alterations of their biomolecules for extreme conditions.[7]

Halophiles are salt-loving micro-organisms, preferably in Eubacteria and Archea.[8] Halophiles predominantly occupy a hypersaline environment, with a minimal microbial diversity due to its multiple extreme conditions, including high salt concentration.[9] The halo tolerance of the halophiles can be classified as slight halophiles, moderate halophiles and extreme halophiles. Slight halophiles prefer a salt concentration of 1% - 3%, which is 0.2 M to 0.5 M of NaCl, while the moderate halophiles prefer a salt concentration of 3% - 15%, which is 0.5 M to 3.5 M NaCl. Extreme halophiles are present in environments with a salt concentration of 15% - 30%, which is approximately 2.5 M to 5 M NaCl.[10,11] Halophiles have adapted to tolerate high external salt concentrations, such as accumulating salts inside the cytoplasm.[12]

The most widely wanted enzyme in the industries is the hydrolases. The major hydrolases commonly used in industries include amylases, cellulases, proteases, lipases, pullulanases, xylases, esterases, pectinases and laccases.[13] Hydrolases are preferred over chemical catalysts due to the highly specific, ecologically friendly and clean processing of the enzyme.[14]

Amylase belongs to the group of hydrolases. The primary function of amylase is to cleave glycosidic bonds of starch by using H_2O, resulting in simple sugars.[15] Amylase has a crucial role as an enzyme in most industries, including food production, paper production, textile industry, etc. For industrial purposes, amylase synthesized from fungi and bacteria is widely used.[16] Bacterial amylase is favoured due to its cheap and fast methods of extraction. Halophilic amylases are preferred due to their ability to withstand extreme conditions that occur during the process of synthesis.[17] The principal purpose of halo-tolerant amylase in industries include starch saccharification for fructose syrup production, antistaling agents in baking, laundry detergent production, textile industry, biofuel production and paper production.[18]

Cellulases are a diverse class of enzymes belonging to the group hydrolases.[19] The primary substrate for cellulase is cellulose, which is the major component of plant cell wall and is the most abundant carbohydrate. Cellulase degrades cellulose by hydrolysing the β-1, 4-glycosidic bonds.[20] Halotolerant cellulases have a wide range of applications, including the food and beverage industry, textile industry, pharmaceutical industry, detergent industry, paper processing industry, biofuel refining industry and olive oil extraction.[21-23]

Proteases, also known as proteolytic enzymes and peptidases, are a class of hydrolases that catalyse hydrolysis of peptide bonds of proteins and polypeptides forming amino acids.[13] The halophilic proteases possess stable activity at high temperatures and ionic strength in the presence of organic solvents.[24] Halotolerant proteases is widely used in the food industry involved in fish sauce fermentation, the detergent and leather industry, and the pharmaceutical industry.[25]

Lipases are a group of enzymes that catalyse the hydrolysis of lipids into fatty acid and glycerol.[26] Halotolerant lipases are efficiently utilized in food, dairy, pharmaceutical, cosmetic, agrochemical, bio-surfactant, detergent, paper industries and biofuel synthesis.[14,27,28]

Thus, the current study focuses on the isolation of salt-tolerant enzyme-producing bacteria from marine environments in Sri Lanka for industrial applications.

2. Methodology

2.1 Sampling and Enrichment of the marine bacteria. Seawater was collected from two sites: Panadura Beach (6.710 N, 79.901 E) and Negombo Lagoon (7.1584 N, 79.832 E). Approximately 250 mL of sample was collected

from each site into sterilized glass bottles. The collected samples were transported to the laboratory in ice boxes. The samples were then enriched in a quarter-diluted nutrient broth medium supplemented with 3% NaCl at room temperature for 48 hours.

2.2 Serial Dilution. After incubation, each sample was serially diluted up to 10^{-6} by adding autoclaved 0.9% NaCl solution. Briefly, 1 mL of incubated broth was transferred to a test tube with 9 mL of 0.9 NaCl solution, mixed well and labled as 10^{-1}. Then, from the first tube, 1 mL was transferred to the next tube of 9 mL of 0.9% NaCl solution and labled as 10^{-2}. The procedure was repeated sequentially up to 10^{-6}.

2.3 Isolation of Bacteria. Bacteria were isolated using the spread plate technique on Nutrient Agar plates supplemented with 3% NaCl. The autoclaved Nutrient Agar was poured into sterilized petri plates and allowed to solidify. Then 100 µL of each serially diluted sample was added onto the Nutrient Agar supplemented with 3% NaCl plates and evenly spread using a sterilized glass spreader. The inoculated plates were then incubated at room temperature for 24 hours.

Morphologically distinct bacterial colonies were isolated and were streaked onto steriled Nutrient Agar plates supplemented with 3% NaCl plates. The pure cultures were obtained by further streaking on Nutrient Agar supplemented with 3% NaCl plates. The isolated pure bacteria were used for further studies.

2.4 Primary screening for production of Amylase enzyme. The activity of the amylase enzyme was screened using starch as the substrate. Starch agar was prepared using 28 g/L of Nutrient Agar, 30 g/L of NaCl and 10 g/L of soluble starch topped with distilled water. and the media was autoclaved. Afterwards, the bacterial colonies were spotted on the starch agar plates and incubated at room temperature for 36 hours. After incubation, the colonies were flooded with Potassium Iodide (KI) reagent and observed for the formation of a clear zone around the colony, which indicates the production of amylase enzyme.

2.5 Primary screening for production of Cellulase enzyme. Carboxy Methyl Cellulose (CMC) agar was used to screen for activity of cellulase. CMC agar was prepared using 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4.7H_2O$, 1.0 g/L of $CaCl_2.2H_2O$, 1.0 g/L of K_2HPO_4, 0.2 g/L of $FeCl_3$, 2.0 g of Tryptone, 15.0 g/L of CMC powder, 15.0 g/L of Bacteriological agar and 30.0 g/L of NaCl topped with distilled water and the media was autoclaved. Then, the autoclaved CMC agar was poured into petri plates and allowed to solidify. Following this, the bacterial colonies were spotted on the petri plates and then incubated at room temperature for 36 hours. After the period of incubation, the cellulase activity was screened using 0.1% Congo Red which was flooded in the CMC agar plate and was left for 30 minutes. Then the excess stain was washed off, and 1 M NaCl solution was flooded on the plate, and after 5 minutes, the excess NaCl solution was removed, and 5% acetic acid was poured and observed for the formation of a clear zone around the colony which indicates the production of cellulase enzyme.

2.6 Primary screening for production of Protease enzyme. Protease activity was screened using Skimmed Milk Agar (SMA). Two flasks were prepared: one with 28.0 g/L non-fat milk powder in one-third of the distilled water, and the other with 2.5 g/L yeast extract, 1.0 g/L dextrose, 5.0 g/L casein hydrolysate, 15.0 g/L agar, and 30.0 g/L NaCl in the remaining water. Both were autoclaved and then mixed to form the final agar and poured into petri plates, and allowed to solidify. Subsequently, the bacterial colonies were spotted on the SMA plates and were incubated at room temperature for 36 hours. After incubation, the colonies with the enzyme protease showed a zone of clearance due to the lysis of proteins.

2.7 Primary screening for production of Lipase enzyme. Phenol Red Agar (PRA) with pH 7.4 was prepared to screen the activity of lipase activity by adding 5 g/L of peptone, 3 g/L of yeast extract, 15.0 g/L of bacteriological agar, 1.0 g/L of $CaCl_2$. 0.1 M NaOH was added to adjust pH and 30.0 g/L of NaCl was added as supplement, and the final mixture was autoclaved. Then 10 ml/L of olive oil (substrate) and 10 mg/L of phenol red dye was added. Then, it was poured into petri plates

and allowed to solidify. Afterwards, the bacterial colonies were spotted on the PRA plates and incubated at room temperature for 36 hours. The presence of lipase would break down the lipid (olive oil) into fatty acid and glycerol, changing the pH, which was depicted by the change of colour from orange to red.

2.8 Optimization of the Bacteria to deduce the optimum salt concentration required for the growth. Nutrient broth supplemented with 3% NaCl, 6% NaCl, 9% NaCl, 12% NaCl and 15% NaCl was prepared with 50 ml of Nutrient broth for each NaCl concentration using 13.0 g/L of Nutrient broth powder and appropriate NaCl, and the media was autoclaved. In the Nutrient Broth containing flasks with different concentrations, 200 µL of the equalized bacterial solutions were inoculated. Then, the flasks were incubated at room temperature for 24 hours. Then, the growth of the bacteria was identified using the absorbance obtained using a spectrophotometer at a wavelength of 600 nm.

2.9 Optimization of the Bacteria to deduce the optimum incubation temperature required for the growth. Nutrient broth of 50 mL supplemented with the respective NaCl concentration of the bacteria determined earlier was prepared and autoclaved. In the Nutrient Broth containing flasks, 200 µL of the equalized bacterial solutions were dispersed and incubated at temperatures 30^0C, 35^0C, 40^0C and 45^0C for 24 hours. Then, the growth of the bacteria was identified using the absorbance obtained using a spectrophotometer at a wavelength of 600 nm.

3. Results

3.1 *Isolation of morphologically different bacterial colonies.* A sum of 44 morphologically different bacterial colonies were isolated from both the sample sites (Figure 1). A total of 25 bacterial colonies were isolated from the Negombo Lagoon sample, and the remaining 19 bacterial colonies were isolated from the Panadura Beach sample.

3.2

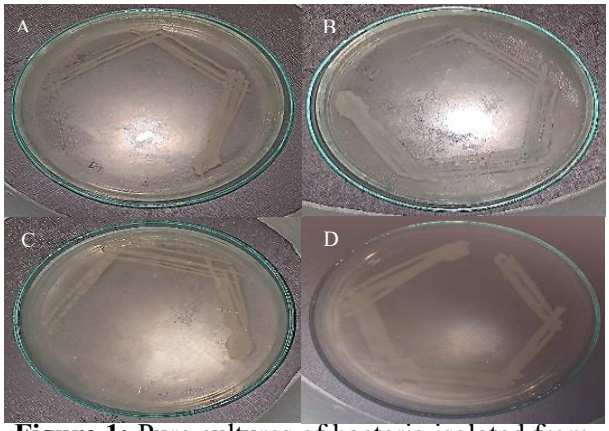
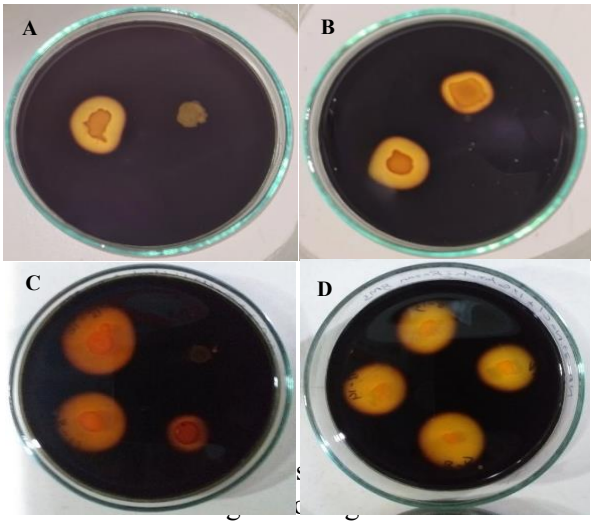


Figure 1: Pure cultures of bacteria isolated from Negombo lagoon and Panadura beach. A). NL-05 isolated from Negombo Lagoon: B).NL-16 isolated from Negombo Lagoon: C).PB-02 isolated from Panadura Beach: D). PB-18 isolated from Panadura Beach.

3.2 Screening for Salt-tolerant Amylase enzyme-producing bacteria using starch agar plate assay. A total of 21 bacterial isolates were positive for amylase production, 3 from Negombo lagoon and 18 from Panadura beach samples (Figure 2).



beach. A). NL-12 isolated from Negombo Lagoon: B).NL-09 and NL-10 isolated from Negombo Lagoon: C). PB-16, PB-17 and PB-18 isolated from Panadura Beach: D). PB-11, PB-12, PB-13 and PB-14 isolated from Panadura Beach.

3.3 Screening for Salt-Tolerant Cellulase enzyme-producing Bacteria using Congo-Red assay. A total of 26 bacterial isolates were positive for cellulase enzyme production, 9 from Negombo lagoon and 17 from Panadura beach samples (Figure 3).

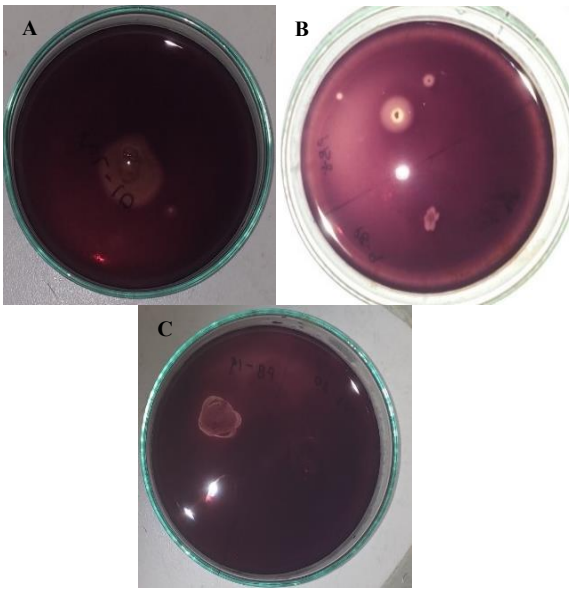
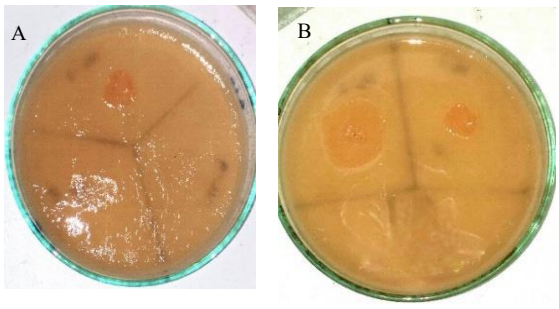


Figure 3: Cellulase positive bacterial colonies isolated from Negombo lagoon and Panadura beach. A). NL-10 isolated from Negombo Lagoon: B). PB-08 isolated from Panadura Beach: C).PB-19 isolated from Panadura Beach.

3.4 Screening for Salt-Tolerant Protease enzyme-producing Bacteria using Skimmed Milk Agar assay. A total of 30 bacterial isolates were positive for protease enzyme production, 25 from Negombo lagoon and 5 from Panadura beach samples (Figure 4).



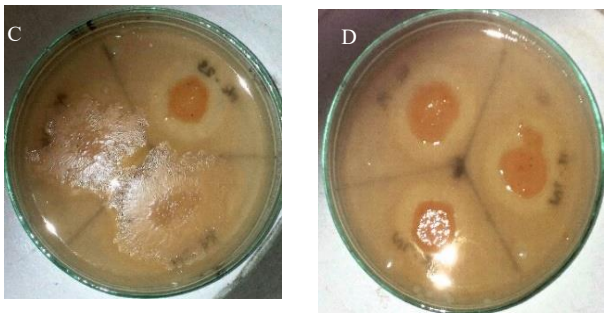


Figure 4: Protease positive bacterial colonies isolated from Negombo lagoon and Panadura beach. A). NL-07 isolated from Negombo Lagoon: B).PB-07 isolated from Panadura Beach: C). NL-25 isolated from Negombo Lagoon: D). NL-10, NL-11 and NL-12 isolated from Negombo Lagoon.

3.5 Screening for Salt-Tolerant Lipase enzyme-producing Bacteria using Phenol Red Agar assay. A total of 22 bacterial isolates were positive for lipase enzyme production, 14 from Negombo lagoon and 8 from Panadura beach samples (Figure 5).

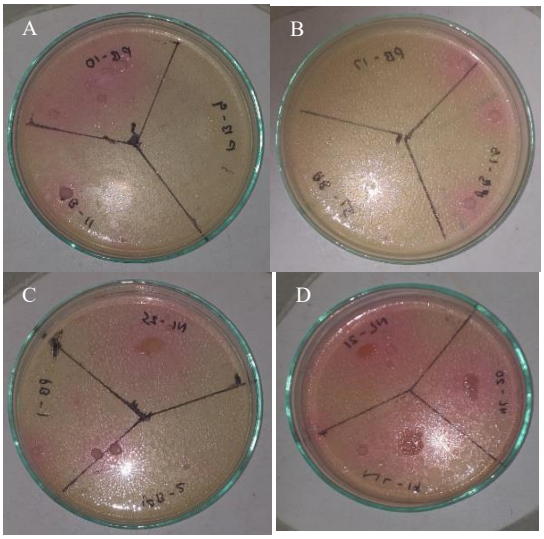
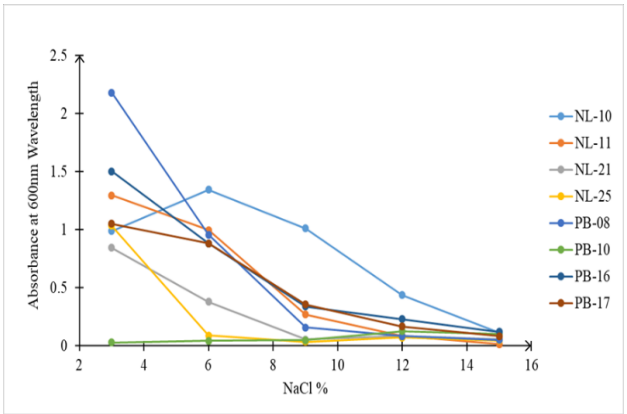


Figure 5: Lipase positive bacterial colonies isolated from Negombo lagoon and Panadura Beach. A). PB-10 isolated from Panadura Beach: B).PB-15 and PB-16 isolated from Panadura Beach: C). NL-25 isolated from Negombo Lagoon: D). NL-19, NL-20 and NL-21 isolated from Negombo Lagoon.

3.6 Optimum Salt- Concentration for the Selected Bacterial Samples. NL10 had the best growth at 6% NaCl concentration. PB-10 had an optimum NaCl concentration of 12%. NL-11, NL-21, NL-25, PB-08, PB-16 and PB-17 had the best growth at 3% NaCl concentration (Figure 6).



different salt- concentrations.

3.7 Optimum Incubation Temperature for the Bacterial Colonies at their Optimum Salt Concentration. The optimum growth temperature of NL-21 and PB-17 at 3% NaCl concentration was 35^{0}C and PB-10 also had the same optimum growth temperature at 12% NaCl concentration. The bacterial isolates PB-08, NL-11, PB-16 and NL-25 showed the optimum growth at 30^{0}C at 3% NaCl concentration and NL-10 the optimum growth at 35^{0}C at 6% NaCl concentration (Figure 7).

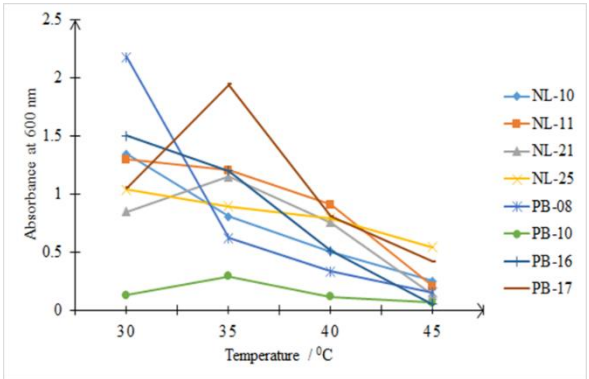


Figure 7: Growth of bacterial isolates at different incubation temperatures.

4. Discussion

Bacteria are classified in various categories due to their variations. Bacteria differ from one another in several ways including their shape, size, composition of cell wall, environment they live in, antibiotic resistance, functional enzymes produced, etc.[29] The above research determines the availability of certain enzymes in halophilic bacteria to be used in industrial applications. Even though all bacteria produce enzymes, halophilic bacteria are mainly used in enzyme assay not only due to their high concentration stability but also because of their high stability in high temperatures and organic solvents.[30]

A moderate number of bacterial isolates from the Negombo lagoon have activity of enzymes lipase and protease still, only a very few isolates from the Negombo lagoon have the enzymatic activity for amylase and cellulase. Meanwhile, the enzyme activity of amylase and cellulase were shown by almost all the bacterial isolates isolated from Panadura Beach, but only about 50%-60% of the bacterial isolates of Panadura Beach had protease and lipase enzyme activity. These differences can prove that the environment influences the growth and functionality of the bacteria.[31] The majority of the bacteria produce more than two hydrolases, while some bacteria do not produce at least one type of enzyme. Similar findings were reported in previous studies, where the majority of halophilic bacteria were found to produce multiple hydrolases.[11,32] Optimization of bacteria determines the best condition at which the bacteria grows the most. The optimum salt concentration of the bacteria is helpful in identifying the type of halophile, while the optimum incubation temperature determines the best temperature for the growth of the bacteria. Out of the isolated 44 bacterial colonies, the colonies with the most prominent enzyme activity were chosen for the optimization. Through optimization, the most promising bacteria at their optimum conditions were identified for the biotechnological usage of the enzymes for industrial applications.

5. Conclusion

Halophilic bacteria which produce amylase, cellulase, proteinase and lipase at room temperature were isolated. Hence, the marine environments can be considered as a rich source of enzyme producing bacteria. These enzymes may be used in industrial settings operates under high salt concentrations as biological catalyst and will be a better alternative for high cost environmental hazardous chemical catalysts.

Further studies are needed to determine the characteristics and suitability of these enzymes for specific industrial settings. Reaction mixture condition optimization can be carried out to determine the optimum conditions for enzyme activity.

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Detection of *kdr* mutations in a laboratory-reared *Aedes aegypti* colony

C.S. Rannettige[1], B.A.N. Mendis[2] and H.S.D. Fernando[2*]

[1]School of Science, Business Management School (BMS), Sri Lanka

[2]Centre for Biotechnology, Department of Zoology, Faculty of Applied Sciences,

University of Sri Jayewardenepura, Sri Lanka

[*]sachini@sci.sjp.ac.lk

Abstract

Insecticide resistance poses a significant challenge to vector control efforts targeting *Aedes aegypti*. This mosquito is considered the primary vector for arboviruses such as dengue, Zika and yellow fever. This study investigates the resistance status and occurrence of knockdown resistance (*kdr*) mutations present in a laboratory-reared *Ae. aegypti* colony. At present two *kdr* mutations have been discovered with increasing frequency within Sri Lankan wild *Ae. aegypti* mosquito populations. These include F1534C, a mutation converting phenylalanine to cysteine at the position 1534 and V1016G, a mutation converting valine into glycine at the position 1016. To determine the presence of F1534C and V1016G mutations within the laboratory-reared mosquitoes, ten randomly selected mosquito samples from a colony reared in the laboratory for over forty generations were obtained. DNA extraction was performed according to the modified Ballinger - Crabtree (1992) protocol, followed by allele specific PCR (AS-PCR). Resulting PCR products were visualized using a 3% agarose gel. The AS–PCR technique effectively distinguished individual haplotypes of the two mutations. Wild type, mutant and heterozygous alleles were observed in the gel images. Results revealed the presence of both cysteine and glycine mutations. The findings suggest that the original mosquito population that was reared in the laboratory may have had the mutations but due to the lack of insecticide pressure the mosquitoes have tend to reverse their mutations and change back to their wild type thus making them susceptible to insecticides.

Keywords: *Aedes aegypti*, *kdr* mutation, Insecticide resistance, Laboratory-reared mosquitoes

1. Introduction

Viruses transmitted by mosquitoes are a primary health concern leading to the loss of human life on a global scale. Diseases commonly transmitted by mosquitoes are dengue and yellow fever which have made a major global impact during the last few years.[1] Dengue is considered an acute mosquito-borne viral disease transmitted by the principal vector *Aedes aegypti*. The *Ae. aegypti* mosquito is a species belonging to the genus that breeds in close association with humans and is often responsible for transmitting various arboviruses.

Ae. aegypti mosquitoes transmit dengue fever by biting humans infected with the dengue virus and then biting uninfected humans, thus transmitting the virus.[2] This mosquito was originated in Africa and later spread to tropical and sub-tropical areas particularly in urban and semi urban areas throughout the world. *Ae. aegypti* is a small, dark colored mosquito with white markings covering the abdomen and thorax area with alternating light and dark bands on its legs. Its life cycle consists of four stages including egg, larva, pupa and adult.[3]

According to the World Health Organization, the global incidence of dengue has increased a tenfold surge over the past two decades. Currently, dengue is reported in 129 countries and is influenced by climate, population density, mosquito vector abundance and urbanization. The key factors associated with the increased risk of dengue epidemic include the increasing change in distribution of the vectors including *Ae. aegypti* and *Ae.*

albopictus in naïve countries and the lack of vaccines that help cure the disease.[4]

At present, many control methods are employed to minimize the population of *Ae. aegypti* mosquito vectors to reduce the spread of arboviruses among the human population. These methods mainly include source reduction and application of insecticides. Source reduction includes the elimination of *Ae. aegypti* mosquitoes by removing their potential breeding sites in domestic and peri–domestic areas. According to their lifestyle characteristics, these mosquitoes prefer to lay eggs in artificial water containers found around human habitation, such as discarded containers, flower pots, tires and any other water collecting materials that can serve as a breeding ground for mosquitoes[5]. Other sources include gutters and drains that can accumulate stagnant water due to improper waste management.[6] Insecticides belonging to the chemical classes of pyrethroids, carbamates, organophosphates and dichlorodiphenyltrichloroethane (DDT) are used for chemical control of mosquitoes.[7] Even though the use of insecticides helps eradicate and control vector populations, lack of planning and common usage of these insecticides has caused these mosquitoes (*Ae. aegypti*) to develop resistance towards them which is a major disadvantage.[8] Several mechanisms have been linked to the development of resistance such as altered behavior, enhanced detoxification, cuticular penetration and altered target sites.[9]

Knockdown resistance *(kdr)* is a form of target site insensitivity and is a major mechanism responsible for reduced susceptibility to pyrethroid insecticides in *Ae. aegypti*. The primary target of pyrethroid insecticides is the voltage gated sodium channel *(vgsc)*. Single or multiple mutations appearing in the *vgsc* are responsible for the decreased target site insensitivity for pyrethroid insecticide leading to the knockdown resistance (*kdr*).[10] In *Ae. aegypti*, eleven *kdr* mutations responsible for pyrethroid resistance have been identified and these mutations vary depending on the geographical spread of the mosquito, frequency of the insecticide application, and effect it has on the resistance phenotype. However, among the eleven mutations, only five have been linked to functional resistance in pyrethroids, namely, F1534C, V1016G, S989P, I1011M and V410L. At present in Sri Lanka, three *kdr* mutations have been discovered in increased frequency within *Ae. aegypti* mosquito populations including F1534C which is the conversion of phenylalanine to cysteine at the position 1534, V1016G which is the conversion of valine into glycine at the position 1016 and S989P which is the conversion of serine to proline.[11] However, it has been suggested that once the application of insecticides ceases, mosquitoes tend to reverse their mutation. The objective of the current study was to determine the insecticide resistance status and the presence of two major *kdr* mutations F1534C and V1016G in *Ae. aegypti* mosquito colonies that were reared in the laboratory for over forty generations without any insecticide pressure. Monitoring resistance to commonly used insecticides and understanding their underlying mechanisms within the *Ae. aegypti* mosquito population are critical in the management of disease transmission, resource optimization, and development of effective control methods and management strategies worldwide.[12]

2. Methodology

2.1 Mosquito samples. Ten samples were collected from Ae. aegypti colonies which were reared in the Insectary, Centre for Biotechnology, University of Sri Jayewardenepura, Sri Lanka for over forty generations without any insecticide pressure.

2.2 DNA extraction. DNA extraction was carried out according to the modified Ballinger Crabtree (1992) protocol with an additional Phenol-Chloroform step. The obtained DNA pellets were dissolved in 100µl of TE buffer.[13]

2.3 F1534C mutation. To detect F1534C mutation, allele specific PCR (AS-PCR) was carried out.[14] Each PCR reaction was performed in a total volume of 25 µl consisting of 12.5 µl Promega master mix, 2.0 µl DNA, 0.625 µl of common reverse primer (5'-TCTGCTCGTTGAAGTTGTCGAT-3'), and two forward primers; 0.625 µl F1534F (5'-GCGGGCTCTACTTTGTGTTCTTCATCAT ATT-3') and 0.2 µl of C1534F (5'-GCGGGCAGGGCGGCGGGGGCGGGGCC TCTACTTTGTGTTCTTCATCATGTG-3').

The thermal cycling conditions followed were initial denaturation for 2 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, extension for 30 seconds at 72°C with a final extension step for 2 minutes at 72°C.

2.4 V1016G mutation. To detect V1016G mutation, AS-PCR was conducted. Each PCR reaction contained a 25 µl total volume consisting of 12.5µl Promega master mix, 2.0 µl DNA, 0.625 µl common forward primer (5'-ACCGACAAATTGTTTCCC-3'), two reverse primers; 0.3125 µl G1016R (5'-GCGGGCAGGGCGGCGGGGGCGGGGCC AGCAAGGCTAAGAAAAGGTTAACTC-3') and 0.3125 µl V1016R (5'-GCGGGCAGCAAGGCTAAGAAAAGGTT AATTA-3'). The thermal cycling conditions followed for the above AS–PCR process was initial denaturation for 2 minutes at 94°C followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, extension for 30 seconds at 72°C with final extension for 2 minutes at 72°C.

Results for both F1534C and V1016G were visualized by agarose gel electrophoresis. PCR products were loaded into 3% agarose gel containing TBE buffer solution and the electrophoresis was conducted for 45 minutes at 100V with a 50 bp DNA ladder.

3. Results and Discussion

Figure 1 and Figure 2 show the visualization of the amplicons in a 3% agarose gel electrophoresis, run under 100V for 45 minutes and stained with ethidium bromide. Figure 1 shows the banding pattern obtained for F1534C mutation. Heterozygotes are presented by the presence of two bands corresponding to both the wild type and mutant alleles. Amplicons approximately close to 93bp were considered to be the wild type whereas the amplicons closer to 113bp were considered to be the mutant allele. Additionally, a 50 bp DNA ladder was included as a reference for size estimation. According to Figure 1 starting from the left contains the 50bp DNA ladder followed by L1 containing the mutant allele (C/C) and the rest of the lanes L2, L3 L4 containing heterozygous alleles (F/C), and no band was visible in the L5 lane since it is the negative control. Figure 2 Shows the banding pattern obtained for V1016G mutation. Amplicons approximately close to 60bp were considered to be the wild type, while amplicons closer to 80bp were considered to be the mutant alleles. The 50bp DNA ladder was placed in the left corner followed by four lanes L1, L2, L3 and L5 containing wild type homozygous alleles (V/V) and lane L4 containing heterozygous allele (V/G).

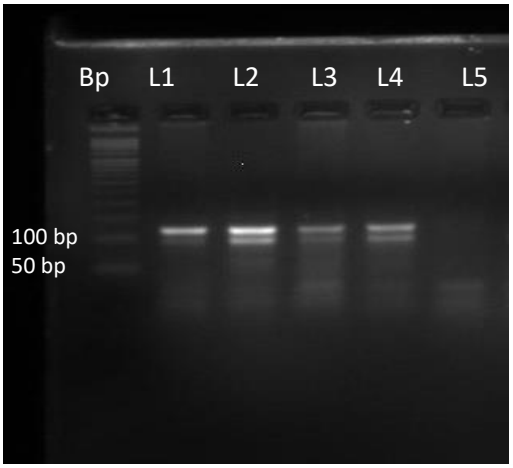

Figure 1. Gel electrophoresis results of F1534C. Two of the three genotypes are shown from left to right: mutant homozygous (C/C) in L1, heterozygous (F/C) in L2, L3, L4 and negative control on L5. The lane to the far left contains DNA ladder (bp).

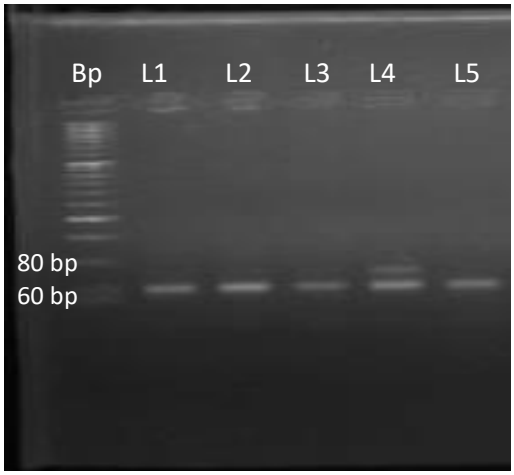

Figure 2. Gel electrophoresis results of V1016G. Two of the three genotypes are shown from left to right: wild type homozygous (V/V) in L1, L2, L3, L5, heterozygous (V/G) in L4. The lane to the far left contains DNA ladder (bp).

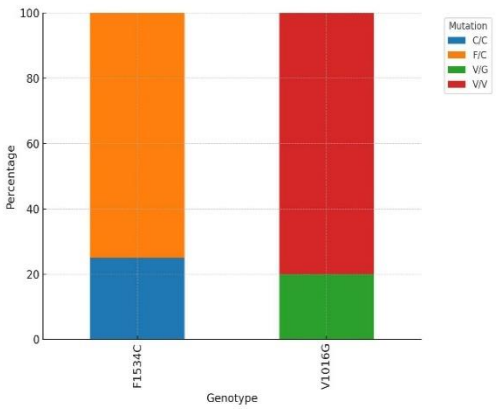

Figure 3. Percentages of haplotypes recorded in F1534C and V1016G. Two of the three genotypes are shown in F1534C; C/C mutant homozygous 25%; F/C heterozygous 75%. Two of the three genotypes are shown in V1016G; (V/V) wild type homozygous 80%; (V/G) heterozygous 20%.

According to Figure 3, among 10 samples 25% of the population had homozygous mutant allele (C/C) and 75% of the population had heterozygous allele (F/C) in F1534C mutation and in V1016G mutation, 80% of the population had wild type homozygous allele (V/V) and 20% of the population had heterozygous allele (V/G).

The resistant allele frequency was calculated using the Hardy-Weinberg equilibrium. The frequency obtained for F1534C mutation was 0.5 while frequency value obtained by V1016G mutation was 0.1. Since *kdr* mutations usually result from an evolutionary response to selective pressure from insecticide exposure, their incidence of mutations is expected to be low or absent in laboratory-reared mosquitoes that have not been exposed to any insecticides and would tend to reverse their mutations thus becoming susceptible to the insecticides.[15]

The current study was performed to identify the presence of *kdr* mutation and the occurrence of insecticide resistance in *Ae. aegypti* laboratory-reared mosquito colonies. *Ae. aegypti* is considered as the vector of many arboviral diseases such as dengue, Zika and yellow fever. Even though use of insecticides has been found to be effective in eliminating the presence of these viral mosquitoes lack of planning and common usage have caused the mosquitoes to develop resistance, making them no longer effective.

In this study ten samples of *Ae. aegypti* mosquitoes were obtained to determine the insecticide resistance status and occurrence of two *kdr* mutations such as F1534C and V1016G in laboratory-reared mosquitoes. *Kdr* mutations in *Ae. aegypti* are primarily associated with resistance to pyrethroid insecticides. Since *kdr* mutations usually result from an evolutionary response to selective pressure from insecticide exposure, their incidence of mutations is low to absent in laboratory reared mosquitoes that have not been subjected to any insecticides. However, it is not impossible for *kdr* mutations to occur spontaneously due to genetic variation or other factors but nonetheless it would occur at a very low frequency compared to mosquito populations under insecticide pressure.[17] Resistant allele frequencies obtained for F1534C and V1016G showed very low frequency levels such as 0.5 and 0.1. It could be assumed that the original mosquito population which was reared in the laboratory may have contained these two mutations, however, overtime due to zero insecticide exposure the mosquitoes have tend to reverse back to their wild type making them more susceptible for insecticides. Nevertheless, even though genetic mutations in mosquitoes may provide an advantage in surviving environments with high insecticide pressure they can also impose fitness costs on the mosquitoes.[18] Fitness cost is the adaptive process in which it leads to the survival and reproductive success of individuals exposed to a natural or induced adverse conditions such as exposure to insecticides which eventually leads to loss of biotic potential. Such changes that can be observed within *Ae. aegypti* mosquito populations are vulnerability to predation, reduced competitive potential among male mosquitoes, increased development time, decrease size of individuals, reduced flight range, reduce reproductive success including lower mating efficiency and egg production and overall reduced survival rates. Thus, in the absence of insecticide pressure, mosquitoes will reverse their resistance mechanisms.[19]

4. Conclusion

The study revealed the presence of *kdr* mutations at low frequencies in *Ae. aegypti* mosquitos that were reared in the laboratory for over forty generations. Long term rearing of the mosquitoes without any insecticide pressure has caused the mosquitoes to reverse their mutations and revert back to their wild type thus making them susceptible for insecticides.[20] Nevertheless, regular monitoring and management strategies such as use of different insecticides must be implemented to detect and prevent widespread of insecticide resistance within the laboratory-reared mosquito population.[21]

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Screening and Quantification of Selected Tetracycline (TET, OTC) and Sulphonamide (SMX, SDI) Group Antibiotics and their Resistant Bacteria in Solid Waste Dump Leachates

Rahma Arifeen[1], G.Y. Liyanage[2,3*], P.A.K.C Wijerathna[2], A.K.M.M.K. Meddage and Pathmalal M. Manage[2]

[1]School of Science, Business Management School (BMS), Sri Lanka

[2]Centre for Water Quality and Algae Research, Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka

[3]Department of Aquatic Bioresources, Faculty of Urban and Aquatic Bioresources, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka

*pathmalal@sjp.ac.lk

Abstract

Antibiotics are newly emerging contaminants (ECs) in landfill leachate that have led to antibiotic-resistant bacteria (ARB) which has become an immense threat to public health globally. Tetracyclines and sulphonamides are broad-spectrum antibiotics used in the healthcare sector, aquaculture, and veterinary medicine in Sri Lanka. The objectives of this research were to characterize the leachate samples, analyse the antibiotic residues, and isolate ARB against Tetracycline (TET), Oxytetracycline (OTC), Sulfamethoxazole (SMX), and Sulfadiazine (SDI) antibiotics. Leachates were collected into 2L glass bottles from Karadiyana and Deldorawatta open dumpsites and were characterized by adhering to APHA guidelines. Antibiotic residues were quantified using High-Performance Liquid Chromatography (HPLC). Resistant bacteria were isolated using the standard pour-plate method, with plate count agar, 48 hours after incubation at 28°C. Minimum Inhibition Concentrations (MIC) were determined from 60 to 360µg/mL concentrations of TET, OTC, SMX, and SDI using the 96-well plate method following CLSI guidelines. Recorded leachate quality parameters indicated that the leachate samples did not meet the standards for effluent discharge to inland surface waters specified by the Central Environmental Authority of Sri Lanka. The study showed that the selected antibiotic residues were not detected in the tested leachates, suggesting that they were below the detection limit (0.05 ppm) of the HPLC. However, ARB were isolated and 83.33% of the isolates from the Karadiyana open dumpsite leachate had MIC values greater than 360µg/mL against OTC. The study also found that 19.23%, 7.69%, and 26.92% of the isolates from the Deldorawatta open dumpsite leachate had MIC values exceeding 360 µg/mL for TET, OTC, and SDI, respectively. The isolates exhibited a Multiple Antibiotic Resistance (MAR) index ranging from 0.75 to 1. This study reveals that the intrinsic nature of antibiotic resistance in bacteria may allow ARB to spread even in the absence of antibiotic residues or at concentrations below detectable levels.

Keywords: Tetracycline, Oxytetracycline, Sulfamethoxazole, Sulfadiazine, Antibiotic Resistance, Leachate

1. Introduction

Due to its low cost and maintenance, landfilling is the favoured method of solid waste disposal and is used in many industrialized and developing countries.[1] Landfill leachate is the liquid leaching out of landfills due to rainwater infiltration through solid waste in dumpsites and it carries all the water-soluble and suspended fractions of waste and by-products of waste degradation.[2] Most studies have found this complex organic effluent very harmful, raising concerns for the surrounding environment[3] as landfills are a major source of water pollution,[4] and leachate may seep into ground and surface waterbodies, endangering aquatic habitats.[2] Karadiyana dumpsite is situated near major river systems and wetlands in Sri Lanka.[2] As a developing country,

protecting our groundwater resources is necessary.

Antibiotics are newly emerging contaminants (ECs) found in landfill leachate due to the unregulated disposal of municipal solid waste in landfills.[5] The landfill receives unused antibiotics through household waste,[6] antibiotic residues from hospital effluent, and used antibiotics in aquaculture, and veterinary medicine.[7] Antibiotics are natural substances produced by microbes that can inhibit/kill competing species and have been used to treat and prevent severe infections in surgical patients, cancer patients undergoing chemotherapy, and immune-compromised individuals.[8] Antibiotics have also considerably improved the health and well-being of animals[9] and have been approved to treat bacterial diseases in aquaculture.[10]

Huge selective pressures are placed on microbial communities due to heavy antibiotic use[11] giving rise to ARB which is a serious health challenge that requires early intervention.[12] Pathogenic bacteria resistant to antibiotics is a global problem linked to increased rates of morbidity and mortality.[13] Gram-positive and negative bacteria that show multidrug resistance have led to infections that are challenging and in certain instances impossible to cure with traditional antimicrobials.[13] It is estimated that by 2050, antimicrobial resistance will cause over 10 million deaths annually.[14]

The presence of high levels of ARB and Antibiotic-Resistant Genes (ARGs) in leachate has been observed.[3] When water bodies get contaminated by landfill leachate, these ARGs can spread among other bacteria in the aquatic environment, and eventually infect fish as pathogens.[15] Given its nutritional value and health benefits, fish is commonly consumed in several Asian countries, including Sri Lanka.[15] Humans are therefore at risk of being exposed to ARB and ARGs through the consumption of contaminated aquaculture food and water.[15] Tetracycline ARB and ARGs (*tetA*) has been found in fish and shellfish that restaurants and supermarkets distribute for direct or indirect consumption, such as sushi.[16]

Tetracyclines and sulphonamides are broad-spectrum antibiotics used to treat human and animal bacterial infections and are very effective against many gram-positive and gram-negative bacteria.[17,18] TET and OTC are antibiotics that fall under the Tetracycline class of antibiotics, and they inhibit protein synthesis (Figure 1A) by selectively blocking the 30S ribosomal subunit, preventing the binding of aminoacyl-tRNA to the A-site on the mRNA-ribosome complex, causing the inability of a bacterium to sustain normal functioning and proliferation.[19]

Tetracycline resistance occurs through ribosome protection, efflux pumps, modification of drug target, and enzymatic

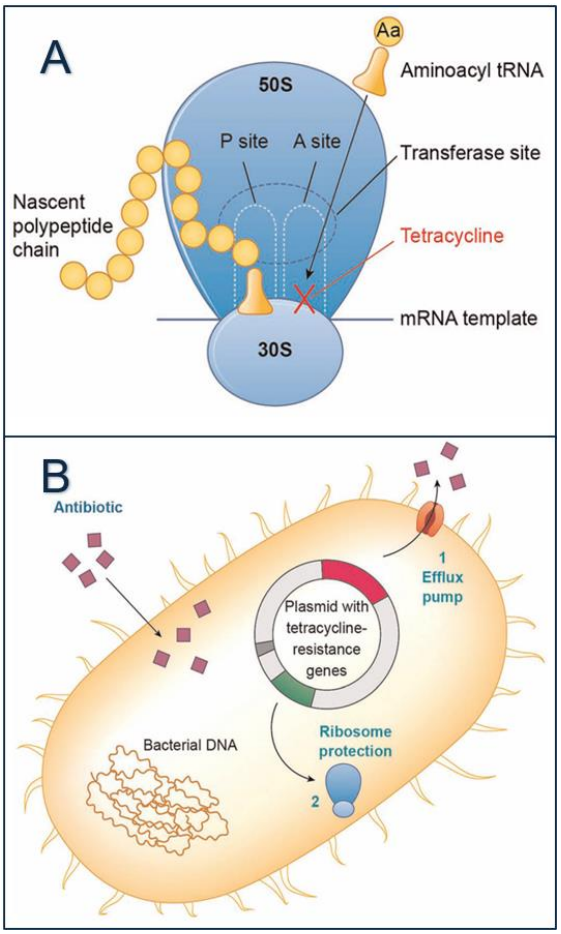

Figure 1. A-Mechanism of action of tetracyclines by inhibiting protein synthesis, B-resistant mechanisms of bacteria against tetracyclines.[20]

alteration (Figure 1B).[20] Ribosomal protection is one of the significant mechanisms of tetracycline resistance, in which ribosomal protection proteins (RPPs) bind to the 30S ribosomal subunit and displace tetracycline from the A-site. Tetracycline was the first to be found to have an efflux pump mechanism.

These specialised protein pumps actively pump out antibiotics reducing the concentration inside the cell.[21] Another type of resistance mechanism is drug target modification, which reduces the binding affinity of tetracycline to the ribosome.[22]

Sulphonamides (SDI and SMX) are a synthetic class of antibiotics.[23] They function as structural analogs and competitive antagonists (Figure 2) of p-aminobenzoic acid (PABA) that is used to synthesise folic acid, which is necessary to continue producing DNA in bacteria. Structural similarity between sulphonamide and PABA, allows sulphonamide to inhibit and replace PABA. Eventually, it can also prevent the formation of dihydrofolate and tetrahydrofolate, which is required for bacterial DNA synthesis and cell division.[18]

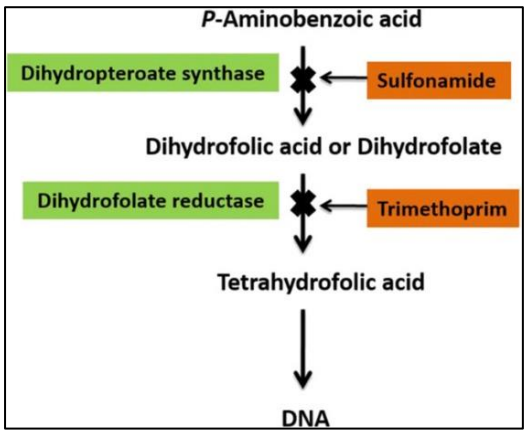

Figure 2. Mechanism of sulphonamide action by preventing the formation of dihydrofolate.[18]

The acquisition of sulphonamide resistant bacterial genes (sul) results in antibiotic resistance to sulphonamides. Two methods by which bacteria might develop resistance to sulphonamides include: intrinsic vertical gene transfer (VGT) and extrinsic horizontal gene transfer (HGT). While HGT involves the transfer of resistance genes between unrelated bacteria, VGT refers to the acquisition of resistance through spontaneous mutation within the bacterial genome that subsequently transmits to its offspring.[24]

In Sri Lanka, the contamination levels of tetracyclines and sulphonamides in different environmental samples exceeded the maximum permissible level recommended by the World Health Organization (WHO).[25-26] However, studies about antibiotic concentrations, antibiotic resistance, and ARB in solid waste dump leachate in Sri Lanka are limited. More than 260 small and large-scale landfills are found in Sri Lanka and most of them are unregulated open dumpsites.[27] The purpose of this study is to evaluate the contamination levels of tetracyclines (TET, OTC) and sulphonamides (SMX, SDI), and isolate resistant bacteria in leachate. The results of this research will provide evidence-based data to policymakers, helping them to formulate necessary environmental policies and regulations to safeguard public health and the environment.

2. Methodology

2.1 Study area and sample collection. The leachate samples were collected into 2L sterilized glass bottles from Karadiyana (6.814388, 79.902023), and Deldorawatta (6.669438, 80.022813) open dump sites (Figure 3).

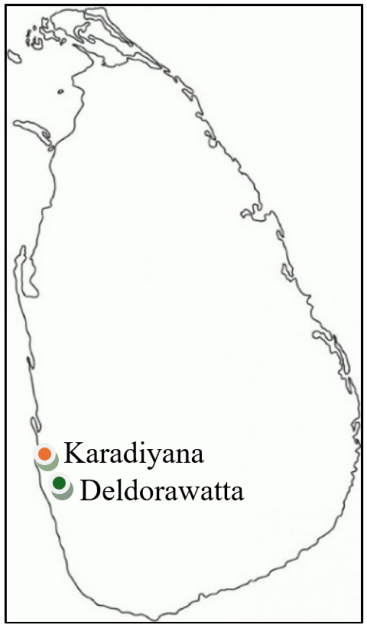

Figure 3. Sample collection points (Karadiyana and Deldorawatta open dumpsites)

2.2 Leachate Characterization. Electrical conductivity (EC) and pH were measured using a conductivity meter (340A-Set 1) and a pH meter (330 I/Set, WTW Co., Weilheim, Germany) respectively. Temperature and dissolved oxygen (DO) were measured using a multi-parameter probe. Total phosphate, ammonia (NH_3), nitrate (NO^{3-}), and nitrite (NO^{2-}) concentrations were assessed according to standard procedures specified by APHA for the Examination of Water and Wastewater.[28] The Chemical Oxygen Demand (COD) of the leachates was measured using the closed reflux method.[28]

2.3 Extraction of antibiotics from leachate samples

Antibiotics were extracted from leachates using the solid-phase extraction method by mixing 100 mL of distilled water with 100 mL of each leachate sample. Duplicates were made. The samples were sonicated for 15 minutes. A few drops of HCl were added to lower the pH down to 3. The samples were allowed to settle for 24 hours, and the supernatant was poured into centrifuge tubes and centrifuged for 10 minutes at 6800 rpm.[29]

The C18 cartridges were preconditioned with 10 mL of 100% methanol and then with 10 mL of milli-Q water. 50 mL of the centrifuged samples were passed through the cartridges. The extracted antibiotics were eluted with 3 mL of 100% methanol into HPLC vials.[15,30]

2.4 Identification and Quantification of Antibiotics in Samples

The antibiotics were quantified using Agilent 1200 series High-Performance Liquid Chromatography (HPLC) equipped with a diode array (DAD) and fluorescence detector.[29] 20µL was injected and chromatography was performed at 30ºC. 100% methanol (polar protic solvent) was pumped in the beginning at a flow rate of 4 mL/min. The column effluent was monitored by a DAD detector in the range of 200 - 450 nm. Then the identification and quantification were done by DAD. Wavelength and retention times for each antibiotic were selected.[29] Table 1 shows conditions and retention times employed for antibiotic analysis.

Table 1. Conditions and retention times employed for antibiotic analysis

Analyte	γ absorption (nm)	Retention time (min)
TET	272	14.001
OTC	280	15.851
SMX	250	4.836
SDI	238	3.034

2.5 Isolation of Antibiotic-Resistant Bacteria (ARB)

2.5.1. Total Viable Counts (TVC) of bacteria and resistant bacteria in leachate samples. TVC was measured using the standard pour plate method with plate count agar.[15] The colony forming units (CFU/mL) were counted 2 days after incubation at 28°C. Antibiotics (TET, OTC, SMX, SDI) at a final concentration of 60 µg/mL were added to each medium to take the TVC,[15] and ARB were isolated into slant bottles and incubated at 37°C and refrigerated after 24 hours.

2.6 Determination of the Minimum Inhibition Concentration (MIC). Determination of MIC was carried out using the broth dilution method following CLSI guidelines.[31] A nutrient broth culture was prepared for each isolate by inoculating a loop of bacteria. The broth cultures were incubated at 37°C for 24 hours. Cell densities were equalized with McFarland No.0.5.[29] The broth dilution method was done on 96 well plates with different antibiotic concentrations (60 to 360µg/mL). Positive and negative controls were carried out. Plates were incubated at 28°C for 24 - 48 hours. The absorbance of the wells was recorded at 595nm using an ELISA reader.[32]

2.7 Determination of Multiple Antibiotic Resistance (MAR). Liquid bacteria cultures were prepared and equalized with McFarland No. 0.5. MAR against TET, OTC, SMX, and SDI was determined at a final concentration (60µg/mL) of each antibiotic using the 96 well-plate method.[33] MAR index was calculated.

Results and Data Analysis

3.1 Leachate Characterization

Table 2. Leachate parameters (temperature, pH, EC, DO, COD, nitrate, nitrite, ammonia, phosphate levels) of Karadiyana and Deldorawatta open dumpsite leachates.

Parameter	Karadiyana sample	Deldorawatta sample
Temperature (°C)	27.0	25.8
pH	8.32	3.93
Electrical conductivity (EC) (mS/cm)	32.24	27.07
Dissolved Oxygen (DO) (mg/L)	1.13	1.23
Chemical Oxygen Demand (COD) (mg O_2/L)	2,000	36,000
Nitrate (mg/L)	99.3788	136.646
Nitrite (mg/L)	3.44	26.0521
Ammonia (mg/L)	794.4828	<Minimum Detection Limit
Phosphate (mg/L)	493.6842	6,418.947

Table 2 indicates the calculated leachate quality parameters. Temperature, pH, EC, and DO vary from 25.8°C to 27.0°C, 3.93 to 8.32, 27.07 to 32.24 mS/cm, and 1.13 to 1.23 mg/L respectively. COD values ranged from 2,000 to 36,000 mg/L. Further, nitrate, nitrite, ammonia, and total phosphate levels varied from 99.38 to 136.65 mg/L, 3.44 to 26.05 mg/L, 794.48 mg/L in Karadiyana, and 493.68 to 6,418.95 mg/L respectively.

3.2 Total Viable Counts (TVC) of bacteria and resistant bacteria in leachates. Figures 4-6 show TVC in samples. As depicted in Figure 5, the environment sample (Env.) of the Karadiyana leachate had a bacterial concentration of $2.2*10^5$ CFU/mL. No resistant bacteria were isolated against TET, OTC, SMX, and SDI. In the Deldorawatta leachate sample, a bacterial concentration of $4.2*10^5$ CFU/mL was present in Env. sample, while only $3*10^4$ CFU/mL were resistant to OTC, and $6*10^4$ CFU/mL were resistant to SMX (at 60µg/mL). No bacterial growth was present in plates with TET and SDI.

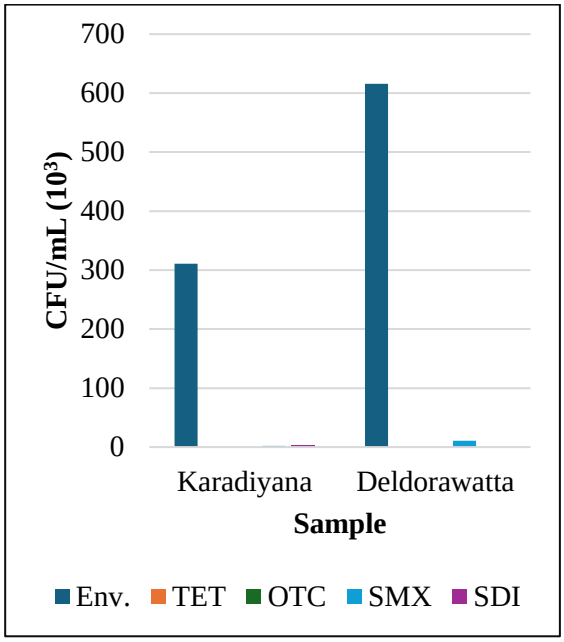

Figure 4. Concentrations of bacteria and resistant bacteria in environment sample (10^{-3})

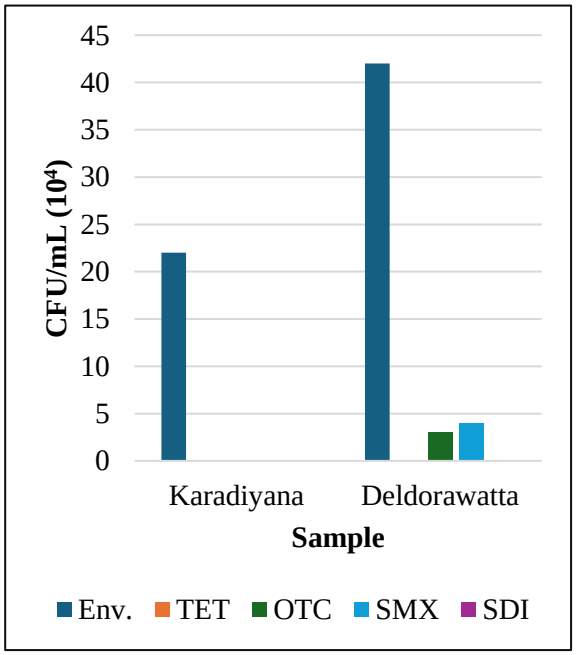

Figure 5. Concentrations of bacteria and resistant bacteria in environment sample (10^{-4})

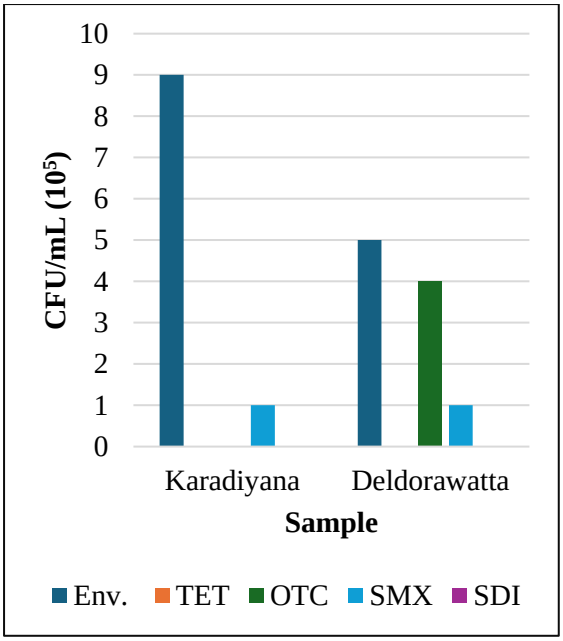

Figure 6. Concentrations of bacteria and resistant bacteria in environment sample (10^{-5})

3.3 Minimum Inhibitory Concentration (MIC) of the isolates. 1-26 were isolated from the Deldorawatta leachate sample, and 27-32 were isolated from the Karadiyana leachate sample.

As denoted in Table 3, of the isolates,

40.625% had 60 ≤ MIC >120µg/mL, 31.25% had 120 ≤ MIC >180µg/mL, 9.375% had 300 ≤ MIC >360µg/mL, and 15.625% had MIC ≥360µg/mL against TET.

12.5% had 60 ≤ MIC >120µg/mL, 18.75% had 180 ≤ MIC >240µg/mL, 25% had 240 ≤ MIC >300µg/mL, 21.875% had 300 ≤ MIC >360µg/mL, and 21.875% had MIC ≥360µg/mL against OTC.

43.75% had 60 ≤ MIC >120µg/mL, 6.25% had 120 ≤ MIC >180µg/mL, 25% had 180 ≤ MIC >240µg/mL, and 25% had 240 ≤ MIC >300µg/mL against SMX.

62.5% of the isolates had 60 ≤ MIC >120µg/mL, 15.625% had 120 ≤ MIC >180µg/mL, and 21.875% had MIC ≥360µg/mL against SDI.

3.4 Multiple Antibiotic Resistance (MAR). The resistance of all isolated bacteria to TET, OTC, SMX, and SDI antibiotics was checked at a concentration of 60µg/mL. Table 4 shows the calculated MAR indexes.

MAR index range varies from 0.75 to 1 for the isolates. 3.125% of the isolates had a MAR index of 0.75 while 96.875% had a MAR index of 1 which indicates resistance to TET, OTC, SMX, and SDI antibiotics at 60µg/mL.

Table 4. MAR index for isolates

Reference Number	MAR index
1	1
2	1
3	1
4	1
5	0.75
6	1
7	1
8	1
9	1
10	1
11	1
12	1
13	1
14	1
15	1
16	1
17	1
18	1
19	1
20	1
21	1
22	1
23	1
24	1
25	1
26	1
27	1
28	1
29	1
30	1
31	1
32	1

Table 3. MIC of isolates against TET, OTC, SMX, and SDI

Antibiotics			Reference No.																														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Tetracycline group antibiotics	TET (μg/mL)	60	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		120	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+
		180	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		240	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		300	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		360	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	OTC (μg/mL)	60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		120	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
		180	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
		240	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
		300	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
		360	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Sulphonamide group antibiotics	SMX (μg/mL)	60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		120	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
		180	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
		240	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-
		300	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		360	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	SDI (μg/mL)	60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		120	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
		180	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		240	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		300	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		360	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4. Discussion

Appropriate disposal of leachate requires a proper understanding of its physiochemical properties to prevent ecological harm and ecotoxicity.[3] Some of the basic leachate parameters measured were pH, Electrical Conductivity (EC), Dissolved Oxygen (DO), Chemical Oxygen Demand (COD), Nitrate, Nitrite, Ammonia, and Phosphate concentrations.

A young landfill (that has been in operation for less than 10 years) has a lower pH and a higher content of volatile fatty acids. pH of Deldorawatta leachate was 3.93, indicating that it might be at the beginning of the acetogenic phase in waste degradation, where the pH is often <6.6.[34] pH of Karadiyana leachate was 8.32, indicating that it might be in the methanogenic phase where pH values are >7.5, as volatile fatty acids are converted to CO_2 and CH_4 by methane-producing bacteria and are seen in mature landfills (in operation over 10 years).[34] According to a study, the Karadiyana dumpsite has been in operation for more than 20 years.[2] The pH of leachate gradually increases as the landfill gets older and more stabilized.[35]

EC is the mineralization of the analysed sample, and it increases with high biowaste fractions, inorganic components, ions, soluble salts,[36] minerals, and dissolved.[37] EC is high in young landfills.[38] However, Karadiyana leachate had the highest EC, even though it is an old dumpsite.[2]

COD measures the amount of oxygen required to chemically oxidize the organic and inorganic material present in a sample. COD is low in old landfill leachates and high in young landfill leachates due to unstable waste decomposition.[38] Deldorawatta sample recorded the highest COD, showing characteristics of a young landfill. COD increases with higher biowaste fractions, thereby, increasing the solubility of many compounds.[38]

Both leachates recorded high phosphate concentrations.[39] Phosphorus is released by organic matter during biodegradation. Agricultural fertilizers, detergents, and household and industrial waste in the landfill are a few sources of phosphates.[36] Old landfills have nitrate values <100 mg/L as seen in Karadiyana leachate.[2] Nitrate is the most oxidized form of nitrogen found in natural systems when ammonium is oxidized to nitrite and then, later, to nitrates by denitrifying bacteria.[40] The highest nitrate levels were recorded in Deldorawatta leachate (136.65 mg/L). Ammonia, a water-soluble gas, is the main reducing agent, and a significant long-term pollutant in leachates.[37] Higher NH_3 levels have been linked to eutrophication and a reduction in DO, which is evident in the results of this study.[35]

In Sri Lanka, leachate discharge is regulated under the National Environmental Act.[39] The regulations specify that the discharge of pollutants into inland surface waters must adhere to the Ambient Water Quality Standards. However, recorded pH (3.93 in Deldorawatta leachate), COD (2,000 to 36,000 mg/L), EC (27.07 to 32.24 mS/cm), DO (1.13 to 1.23 mg/L), nitrate (99.38 to 136.65 mg/L), ammonia (794.48 mg/L in Karadiyana), and dissolved phosphates (493.68 to 6,418.95 mg/L), were beyond the tolerance limits specified by these standards. Therefore, leachates must be treated before discharging them into inland waters.

Antibiotics from the leachates were extracted using the solid phase extraction (SPE) method and were quantified using HPLC. However, no TET, OTC, SMX, or SDI antibiotic residues were detected. This may indicate that the selected antibiotics were below the detection limit (0.05 ppm) of the HPLC. However, resistant bacteria were isolated from plates that contained OTC, SMX, and SDI at 60µg/mL.

OTC is used to treat respiratory, and urinary tract infections (UTIs), and in veterinary medicine, it is used to treat bovine respiratory disease (BRD) in cattle, and respiratory infections in horses.[41] TET and OTC are utilized as growth promoters in animal feed at sub-therapeutic concentrations. SMX is used to treat UTIs, chronic bronchitis, traveller's diarrhoea, and shigellosis.[42] SDI is used to treat pneumococcal, staphylococcal, and streptococcal infections as well as gonorrhea.[43]

The Minimum Inhibition Concentration (MIC) was checked for the isolates against TET, OTC, SMX, and SDI from

60 to 360µg/mL. MIC is the lowest concentration of an antibiotic that completely prevents visible growth of the isolate.[38] 15.625%, 21.875%, and 21.875% had MIC ≥ 360µg/mL against TET, OTC, and SDI respectively. It should be noted that isolates 14 and 15 had a MIC ≥ 360µg/mL for TET, OTC, and SDI.

Multiple Antibiotic Resistance (MAR) is when an organism shows resistance to two or more classes of antibiotics. The MAR index is useful when locating sources of ARB. Antibiotic usage sites are considered high-risk sources of contamination if their MAR > 0.2.[45] Calculated MAR index values ranged from 0.75-1. MAR index of 96.875% of isolates was 1, indicating resistance to all 4 selected antibiotics at 60 µg/mL.

There is an increasing interest in how sub-inhibitory concentrations of antibiotics can increase mutation rates, and HGT and continue to exert selection pressure through resistant mechanisms.[46] This may be why the isolates were resistant to certain concentrations of the tested antibiotics, even though negligible concentrations of antibiotic residues were present in the leachates.

Bacterial resistance mechanisms can be classified as intrinsic, acquired, or adaptive.[47] Resistance due to the natural abilities of the bacterium is known as intrinsic resistance. Acquired resistance is when a previously susceptible bacterium develops a resistance mechanism through a mutation or the acquisition of new genetic material from an external source.[48]

Adaptive resistance is the resistance to one or more antibiotics that arises through particular environmental conditions, such as stress, growth state, pH, ion concentrations, nutritional circumstances, or sub-inhibitory antibiotic levels. It is temporary, compared to inherent and acquired resistance. This enables bacteria to react to antibiotic challenges rapidly. When the stimulus is eliminated, adaptive resistance usually returns to its initial state.[47]

Due to the overuse and misuse of antibiotics, this natural genetic evolution of microbes to resist antibiotics has reached absurd levels in the 21st century, affecting the effectiveness of pathogen control and leading to significant medical consequences.[49] Antibiotic resistance compromises the ability to cure common infections such as the flu and typhoid, challenges the treatment of many microbial infections, and can result in treatment failure, extended sickness, permanent disability, or even death.[50] Individuals with antibiotic resistance require extended treatments and expensive medications which is a burden for low-income and developing countries.[51]

5. Conclusion

It is necessary to treat landfill leachates by adhering to Central Environment Authority guidelines before discharging them into the environment as they pose risks to surface and groundwaters. Antibiotics are newly emerging contaminants found in landfill leachate. Millions of lives have been saved by antibiotics, rendering them effective in preventing and treating microbial illnesses. However, bacteria have and will gradually evolve resistance to these antibiotics through a variety of innate and acquired mechanisms.

The study finds that although no antibiotic residues were present in the leachates, isolated bacteria showed resistance to TET, OTC, SMX, and SDI from 60 to 360 µg/mL. This suggests that the inherent nature of antibiotic resistance in bacteria may allow ARB to spread even in the absence of antibiotic residues or at undetectable levels, creating a serious risk of spread of antibiotic resistance, leading to reduced efficacy in pathogen control. Should these circumstances persist, a "post-antibiotic era" may arise in which common infections and minor injuries are the predominant causes of death.

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The effects of freezing and reheating on anthocyanin retention in beet root (*Beta vulgaris)* curry

Dilusha M. Fernando[1*] and A. Yukthika[2]

[1]Department of Biochemistry & Molecular Biology, Faculty of Medicine, University of Colombo, Sri Lanka

[2]School of Science, Business Management School (BMS), Sri Lanka,

[*]dilusha@bmb.cmb.ac.lk

Abstract

This research focuses on the impact of freezing and reheating on the retention of anthocyanin in the beetroot curry, which is a commonly consumed food by Sri Lankans. Anthocyanin is a flavonoid compound which is known for its high antioxidant properties and vibrant colours. Anthocyanins offer a range of significant health benefits, including antimicrobial and anticancer properties, anti-aging effects, and potential roles in enhancing cardiovascular and cognitive health. Additionally, they contribute to the management of obesity and the prevention of type 2 diabetes. The stability of anthocyanin can be influenced by pH, temperature, light, enzymes, oxygen, etc. In this study, three samples were made; freshly prepared beetroot curry, a sample which was frozen at 4°C overnight without reheating and a freshly prepared curry which was kept at room temperature and then reheated using a gas stove. Acidified ethanol (2% Acetic acid, 18% distilled water and 80% ethanol) was used to extract and stabilize the pigment. The concentration of anthocyanins was measured using UV visible spectrophotometry and cyanidin-3-glucoside was used as the standard. The results indicated that freshly prepared curry samples had the highest concentration of anthocyanin followed by the frozen sample and reheated samples had the lowest concentration of anthocyanin present. These findings show that multiple cycles of reheating and prolonged freezing can lead to a significant loss in anthocyanins which are sensitive to heat. This paper provides insight into the effect of different temperatures on the quantity of anthocyanins.

Keywords: Anthocyanin, *Beta vulgaris*, Freezing, Reheating

1. Introduction

Recently researchers have been conducting studies on fruits and vegetables with vibrant colors, due to their antioxidant properties etc. These vibrant colours are mostly attributed to compounds such as carotenoids and flavonoids, including anthocyanins.[1] Anthocyanins are water-soluble pigments that mainly fall under the flavonoid groups. They are highly found in purple kale, red lettuce, berries, grapes, pomegranates, beetroots, red onions etc. There are six major anthocyanin groups, cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin.[2] These groups show a variety of colours in different pH, temperature and light.[3] Cyanidin-3-glucoside is considered the most found anthocyanin in plants.[4] The basic structure of anthocyanin includes the flavylium cation which consists of two benzene rings and a pyrylium ring. Which is responsible for the range of colours expressed by the pigment by absorbing different wavelengths of UV visible light and the carbon rings provide the core structure of the pigment.[5] Therefore, the UV visible spectrophometry can be used to quantify the anthocyanins.

Anthocyanins acquire high antioxidant properties due to the presence of flavylium cation. They reduce or inhibit oxidative stress by scavenging the free radicals through the direct method by transferring a single electron or donating an H atom. Anthocyanins also activate or stimulate the synthesis of certain enzymes through indirect methods like catalase, NADPH oxidase, superoxide

dismutase (SOD) etc.[6] Anthocyanins also act as an anti-inflammatory.

Studies have shown that anthocyanins help reduce pain and inflammation in arthritis.[7] Anthocyanins facilitate the prevention of leukaemia and ovarian cancers because they have powerful cancer-fighting abilities.[8] Anthocyanins improve heart health by lowering LDL and improving HDL cholesterol levels.[9] Studies have proven that consuming anthocyanin-rich food may reduce heart risk by up to 9.[10] Anthocyanins have the potential of slowing the progression of Alzheimer's.[11]

However, anthocyanins are sensitive to extreme factors such as pH, temperature, light, oxygen levels, co-pigments, enzymes, etc. Anthocyanins show different colours in different pH. Anthocyanins are found purple in neutral environments, red in acidic and blue in alkaline nature.[12] Scientific studies show that storing anthocyanin in the range of 2-4℃ can help retain its beneficial properties. Still, prolonged storage can lead to a significant loss due to the formation of ice crystals which cause damage to the cell wall and membrane, thereby upsurging the degradation rate. At elevated temperatures, anthocyanin pigments undergo degradation through various chemical reactions. Heat can break the bond between the anthocyanidin core and its sugar moiety, forming less stable anthocyanidins. Additionally, water molecules may attack electron-deficient sites on the anthocyanin structure, leading to destabilization and loss of colour. High temperatures also promote polymerization, where anthocyanins interact with other compounds to form larger, less soluble molecules, and can cause the breakdown of the pigment's three-ring structure, producing simpler compounds like coumarin glycosides. These reactions collectively reduce the colour intensity, stability, and bioactivity of anthocyanins, highlighting the importance of temperature control during processing and storage.[12] Co-pigmentation is a specific phenomenon found in anthocyanin and, not other non-polyphenolic or polyphenol compounds.[13] It is a process where the pigments form a complex with a metal ion or a colourless compound to create or change the colour intensity. It is pH and temperature-dependent, therefore high pH and high-temperature lead to a disruption of anthocyanin structure and cause loss of colour intensity and antioxidant capacity.[14] Researchers have said that the stability of anthocyanins is highly affected due to exposure to fluorescence light.[15]

In Sri Lankan cuisine, beetroot curry is a common dish in every household. However, due to current busy lifestyles, meal preparation has significantly increased, and freezing and reheating pre-cooked curries has become very common among urban locals.The following statistical facts indicate the importance of consuming beetroot curry in Sri Lanka which helps to live a healthy life, by improving heart health, fighting against cancer, improving brain health, reducing obesity and diabetes etc.The percentage of deaths associated with cardiovascular disease increase from 24.92% to 28.05% in the years 2011 to 2019.[16] According to the World Health Organization, the adult obesity percentage in Sri Lanka has increased from 16.8% to 25.2%.[17] The prevalence of type 2 diabetes was highest in the years 2011 to 2021 (17.5%) than in the 1990s and 2000s.

2. Methodology

2.1 Beetroot Curry Preparation. Nine large beetroots were taken, cleaned and cut into small cubes to prepare the curry. Relevant spices were added while cooking. Once prepared it was divided into three portions which weighed 80g each and were labelled as fresh, frozen, and reheated samples. The frozen sample was kept in the refrigerator at 4℃ overnight, and the freshly prepared sample which was reheated using the gas stove at 45℃ the next day.

2.2 Crude extraction. Each sample was thoroughly mixed in 80mL of distilled water at room temperature. A cheesecloth was used to filter the mixture twice to obtain the crude extract. Each sample was divided into six falcon tubes and labelled. The top was secured with filter paper, and the falcon tubes were stored in the freezer at -20℃ overnight. Then,

the tubes were kept in the freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, German) for four days.

2.3 Stabilization of the anthocyanin present in the crude extraction. All three completely dried samples were obtained and dissolved in a minimum volume of distilled water. The solutions were then incorporated with acidified ethanol (2% Acetic acid, 18% distilled water and 80% ethanol) in a 1:2 ratio and occasionally stirred under a fume hood for two hours.[18] Each sample was evaporated to dryness in the rotary evaporator (Heidolph Instruments ,Germany) at 40℃ for one hour at low pressure .[19] Once the ethanol was evaporated and the volumes were diminished, they were transferred into falcon tubes and stored in the freezer at -20℃. Once the samples were frozen the lids of the falcon tubes were replaced with filter paper and kept in the freeze dryer for forty-eight hours. The dried samples were used for further assays.

2.4 Analyzing the retention of anthocyanin pigments present in all three samples. The mass of the dried samples was measured using an analytical balance. All three samples were dissolved in a minimum amount of distilled water and made into the stock solution. A serial dilution was made for each sample for screening purposes. The spectrophotometer (Brandtech Scientific, USA) was used to measure the absorbance values of samples at 520 nm to quantify anthocyanin amounts .[20]

3. Results and Discussion

The fresh beetroot curry sample had the highest mean anthocyanin concentration (0.0395 ± 0.0142 µg/mL) followed by the frozen sample (0.0285±0.0071 µg/mL). In contrast, the reheated curry sample had the lowest (0.0268±0.0053 µg/mL). This can be demonstrated by the following graph in Figure 1.

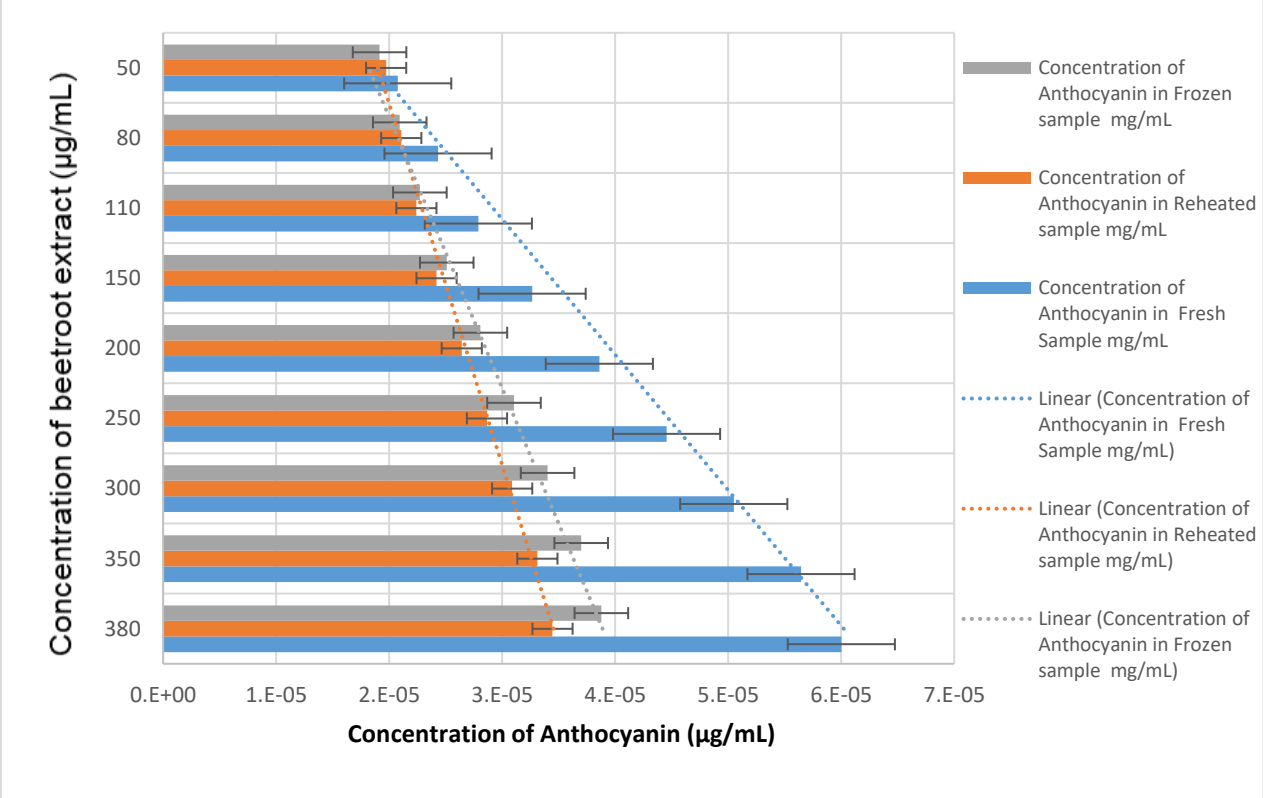

Figure 1. The concentration differences of anthocyanin present in the fresh, frozen and reheated curry samples. The graphical data is represented using the mean±SD of three separate experiments.

The finding of the anthocyanin concentration present in the freshly prepared, frozen and reheated beetroot curry sample has significant importance as anthocyanin is considered one of the main flavonoid components with high health-beneficial properties.

3.1 The effect of reheating and freezing on anthocyanin retention. This study was mainly carried out since Sri Lankans consume beetroot more often daily. The curries are preserved in the refrigerator and reheated multiple times in the Sri Lankan household. This study focuses on the effect of temperature on the retention of the anthocyanin pigment. As mentioned, the freshly prepared curry had the highest concentration of anthocyanins (0.0395 ± 0.0142 μg/mL) followed by the frozen sample (0.0285±0.00711 μg/mL) while the reheated curry sample had the lowest value (0.0268±0.00533 μg/mL). A study has been conducted on purified crude extracts and thermally processed food which proved that an increase in temperature will increase the degradation of the anthocyanin pigments while storing in low temperatures such as 2-4℃ will preserve the pigment and reduce the degradation although prolonged storage in refrigerators can induce the degradation.[21] This can be seen in the results obtained above. Studies have shown that anthocyanins degrade at high temperatures following a first-order reaction kinetics model, meaning the degradation rate depends on the amount of anthocyanin remaining.[22] Heat exposure causes significant structural changes, affecting anthocyanins' stability, colour, and antioxidant properties. Two main degradation pathways have been identified. The first involves water molecules breaking the bonds between anthocyanins and their sugar groups (deglycosylation), transforming them into unstable aglycone forms called anthocyanidins, which then degrade further into smaller compounds like methanol, chalcones, diketones, benzoic acid, and aldehyde derivatives. The second pathway involves the opening of the flavylium ring, converting anthocyanins into chalcones, which subsequently form coumarin glycoside derivatives.[23] Both processes lead to a loss of vibrant red and purple hues of anthocyanins, reduced antioxidant activity, and diminished health benefits. These findings highlight the need to carefully control heat during cooking and reheating to preserve the colour, nutritional value, and functional properties of anthocyanin-rich foods. Strategies such as shorter cooking times, maintaining an acidic environment, or using encapsulation techniques could help minimize degradation and retain the benefits of anthocyanins.

Both freezing and reheating can damage the cell wall and membrane at different levels, which provides more exposure of the anthocyanin pigments to oxygen. This causes oxidative stress and facilitates some enzymes like peroxidase and polyphenolic oxidases to act on the pigments leading to further degradation. Freezing leads to such conditions due to the formation of ice crystals within the cells, which disrupts the cell wall and membrane. The initial disruption of the cell wall and membrane was done during the cooking process, which led to the extraction of anthocyanins and other flavonoid compounds in the beetroot. The addition of spices and salt while preparing the curry leads to pH changes, which also impacts the retention of the anthocyanin pigments. Flavylium cations are more stable in acidic pH, but while cooking, freezing, or reheating the pH shifts to neutral, or alkaline which causes instability to the cation leading to degradation.[24] According to the graph obtained, it clearly shows that freezing leads to the loss of anthocyanin, but it is comparatively less than the reheating process. Therefore, reheating at a low temperature might reduce the loss of anthocyanins

3.2 Future Insights. This experiment focuses on providing critical knowledge about food preparation techniques and food storage methods by emphasizing the need for evidence-based guidelines to expand the advantages of foods rich in anthocyanin pigments by understanding how reheating and freezing impact anthocyanins' degradation and stability. Various food preservation techniques can be practiced by the food industries such as cryogenic freezing or vacuum-sealed packaging which can improve the retention of anthocyanins and other flavonoid components. Nanoliposomes made with anthocyanins, primarily containing cyanidin-3-O-glucoside and peonidin-3-O-glucoside, were created using lecithin and cholesterol in a ratio of 5:98.

These nanoliposomes successfully retained 85.60% of the anthocyanins after being stored at 25°C for 16 days, demonstrating their effectiveness in preserving anthocyanins over time.[25]

4. Conclusion

It is evident that the freshly prepared beetroot curry sample had the highest retention of anthocyanins present, followed by the frozen sample while the reheated sample had the least. This study proves that repetitive heating and prolonged freezing can cause vast amounts of degradation and loss in the functional properties of anthocyanins. Modern innovations have been introduced for stabilizing anthocyanins during freezing and heating, which adds valuable perceptions to the existing knowledge in the field.

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SCID Across Decades: Changes and Challenges in SCID Management

N. Sandamini[1*], M. Jayasinghe[1], P. Mohanraj[1], R. Kabinath[1] and N. Abinash[1]

[1]School of Science, Business Management School (BMS), Sri Lanka

[*]neranja.s@bms.ac.lk

Abstract

Severe Combined Immunodeficiency (SCID), often termed "bubble boy disease", is a rare but life-threatening group of genetic disorders characterized by profound defects in T-cell and B-cell immunity. Over the decades, advancements in understanding and managing SCID have revolutionized outcomes, yet challenges remain. The discovery of SCID in the mid-20th century marked the advent of recognizing primary immunodeficiencies as distinct medical entities. Early management relied on isolation to prevent infections, emphasizing the urgency of a definitive cure. Introduction of hematopoietic stem cell transplantation (HSCT) from matched sibling donors in 1968 transformed SCID prognosis, achieving survival rates exceeding 90% in optimal cases. Over time, donor selection expanded to include matched unrelated and haploidentical donors, addressing limitations posed by donor scarcity. The 1990s witnessed the emergence of gene therapy, offering a curative approach for SCID caused by specific genetic mutations. Initial setbacks, including treatment-related leukemia, underscored the complexities of modifying the human genome. However, refinements in vector design and delivery have revived its promise, with newer trials demonstrating remarkable efficacy. Neonatal screening, now implemented in many regions, has significantly improved early SCID diagnosis, enabling timely intervention. However, disparities in access to screening and treatment persist globally. The cost of advanced therapies, immune reconstitution variability, and long-term follow-up challenges remain critical issues. Addressing these challenges requires continued research, equitable healthcare policies, and innovations in therapy and screening. SCID management exemplifies the intersection of scientific progress and medical ethics, highlighting the need for vigilance in translating discoveries into accessible, life-saving interventions.

Keywords: Severe Combined Immunodeficiency, Primary Immunodeficiency, Lymphocyte, Immunoglobulin

1. Introduction

Immunodeficiency occurs when the immune system fails to function appropriately, leaving the body vulnerable to infections. It can be either primary (inherited) or secondary (acquired). Primary immunodeficiencies are classified based on the affected components, including T-cells, B-cells, both T and B cells, phagocytes, complement proteins, or immunoglobulin A.[1] The clinical manifestations of primary immunodeficiencies (PIDs) are diverse but commonly involve heightened infection vulnerability. These conditions can be present at any age. Many PIDs initially appear as typical infections, such as those affecting the sinuses, ears, or lungs, which can easily be overlooked in primary care.[2]

Severe Combined Immunodeficiency (SCID) is a genetic disorder caused by T and B Lymphocyte defects.[3] SCID is mainly due to mutations in genes essential for the development and function of T and B lymphocytes. While some mutations affect only T-cell function, leaving B cells intact, others impair both. Natural killer (NK) cells, which

develop independently of T and B cells and possess cytotoxic properties, are present in about half of SCID patients, offering partial protection against infections and contributing to improved outcomes. SCID immunophenotypes are classified based on the presence (T–B+) or absence (T–B–) of B cells in the blood, with both groups including forms with or without NK cells. Additionally, SCID is categorised by the functional role of the defective gene, involving cytokine signaling, antigen presentation, V(D)J recombination, T-cell receptor signaling, or basic cellular functions.[4]

According to Fischer[5], the most common type, X-linked SCID, results from mutations in the IL-2 receptor γc chain gene, impairing T and NK cell development. Adenosine Deaminase deficiency, the second most common form, leads to toxic metabolite accumulation, causing lymphopenia and neurological issues. RAG-1 and RAG-2 mutations disrupt T-cell receptor development, while IL-7R deficiency affects both T and B cells. Leaky SCID features partial T-cell presence with autoimmune tendencies, and Omenn Syndrome involves dysfunctional T cells with severe autoimmunity. Other forms include CD3 complex and JAK3 deficiencies and rare variants like bare lymphocyte syndrome and Griscelli syndrome.[6]

SCID occurs at a frequency ranging from 1 in 40,000 to 1 in 75,000 live births globally.[7] The incidence of SCID varies across different countries and is influenced by the prevalence of recessive genetic disorders and the effectiveness of case detection. Differences in detection rates are particularly pronounced based on the availability and implementation of SCID newborn screening programs (NBS), the process by which infants are screened for genetic defects. The introduction of these programs has also revealed that SCID is more common than previously estimated. In regions with high rates of consanguineous marriages, the incidence is notably higher compared to areas where such practices are less common. The median annual incidence of SCID is estimated at 4.5 cases per 100,000 live births among the Omani population[8], 1 case per 2,906 live births among the Saudi population[9], while the incidence of typical SCID, leaky SCID, and Omenn syndrome in India is approximately 1 in 58,000 live births[10] and in the USA is 1 in 58 000 infants.[11] SCID is the most common immune deficiency condition in Sri Lanka. Prevalence is significantly higher than in countries such as Europe (4.5%). However, it is lower than in other MENA region countries such as Saudi Arabia (22%).[12]

This condition constitutes a pediatric emergency, as affected infants face a high risk of severe, recurrent infections. SCID treatment involves isolating newborns to prevent infections, addressing nutritional challenges through intravenous feeding, and managing secondary infections with antimicrobial medications. Definitive therapies include hematopoietic stem cell transplantation (HSCT), gene therapy, enzyme replacement therapy (ERT), and chemotherapy, depending on the genetic cause.[9] Without timely treatment, such as stem cell transplantation or gene therapy, SCID is nearly always fatal within the first years of life.[13]

Early diagnosis through family history, clinical signs, or newborn screening is vital to improving outcomes for this life-threatening condition.[13] A thorough evaluation is required to diagnose SCID, including a detailed patient history (infections, prematurity, family history) and physical examination for signs of related conditions. Key laboratory tests include lymphocyte phenotyping, T-cell quantification, and T Cell Receptor Excision Circle (TREC) quantification to assess thymic function. Genetic sequencing is crucial for identifying SCID-causing mutations. Testing for T-cell

receptor diversity and proliferative responses may further help differentiate SCID from other disorders with low T-cell numbers. Secondary causes of T-cell lymphopenia, like HIV or prematurity, must also be ruled out.[14]

This review summarizes findings from 10 SCID case studies conducted in different countries, including developed and developing countries. The studies include clinical presentations, diagnostic approaches, treatment methods, discussion, and a prognosis. By comparing these cases, this discussion identifies patterns and differences in SCID diagnosis, management and treatment through the decades.

2. Demographics, Consanguinity and Family History

In a study done in France from 1970 to 1993, a cohort of 117 patients was studied. Most patients were placed in a sterile environment, such as the Isolator Bubble System, from 1981 onward. The mean age of diagnosis was 9.5 months. Retrospectively, family history of SCID was found in 74 of the cases.[15] 2010 to 2011, a study on 47 patients in India aimed to understand severe combined immunodeficiency (SCID). The findings focused on identifying and characterizing the disease in the region without delving into specifics like consanguinity or family history.[16]

Between 2000 and 2011, a study conducted in Brazil involving 70 patients reported 16% of cases with consanguinity. The mean age at diagnosis was approximately 6.7 months, with a median of 8 months. Eight children were diagnosed at birth or before clinical manifestations due to a previously affected sibling.[17] From 2010 to 2011, a cohort of 24 patients was studied in Taiwan.[18]

A study conducted in Saudi Arabia from 2010 to 2013, involving 502 patients, showed a consanguinity rate of 75%. A majority (93%) of patients were diagnosed during childhood. The mean age of symptom onset was 17 months.[19] From 2010 to 2014, in the United States and Canada, 118 cases were reviewed.[20]

A cohort of 10 patients was examined in Iran between 2006 and 2013. Parental consanguinity was observed in 9 of the 10 cases. Among them, seven had second-degree consanguineous parents, two had third-degree consanguineous parents, and one had non-consanguineous parents. Additionally, six patients had a history of early infant deaths among siblings, while two had relatives diagnosed with SCID.[21] Between 2013 and 2018, another study conducted in India examined 57 SCID patients. Of these, 36% had consanguineous parents, and a family history of SCID was observed in 56% of cases. The median age of diagnosis was 60 days, with two patients identified pre-symptomatically due to a strong family history.[22]

Between 2010 and 2020, 36 patients were reviewed in Oman. In 24 cases (66.7%), a positive family history of SCID was identified, and 91.7% of children were born to consanguineous parents. The median age of diagnosis was 54 days. Additionally, 33.3% of cases involved a history of sibling death[11]. From 2016 to 2019, three cases were studied in Kenya. None of the parents showed first- or second-degree consanguinity. However, all cases involved sibling deaths attributed to SCID.[23]

3. Clinical manifestations

Clinical manifestations of severe combined immunodeficiency (SCID) vary widely across countries, reflecting the diversity of immune disorders and healthcare systems. For instance, pneumonia and failure to thrive are common features globally, with additional complications like chronic diarrhea, recurrent candidiasis, and autoimmune conditions seen in countries like India, Brazil, and Saudi Arabia.[17,19,22] Unique presentations such as disseminated BCG infections in Saudi Arabia or Omenn syndrome

in the USA and France highlight the spectrum of PID manifestations and the importance of timely diagnosis and intervention.[15,19,20]

In Oman, SCID presentations paralleled those in the USA and Canada. Typical SCID was marked by severely reduced CD3 T-cell counts, impaired T-cell proliferation, and maternal T-cell engraftment in some cases. Atypical SCID exhibited reduced CD3 T-cell counts adjusted for age and partially impaired T-cell functions. Common symptoms included pneumonia, septicemia, and chronic diarrhea. This study reiterated the significance of age-adjusted immunologic criteria for diagnosing SCID.[8]

In a case study done in France, the onset of clinical symptoms occurred earlier in patients with ADA (-) SCID compared to other forms. In fact, life-threatening interstitial pneumonitis developed as early as one month after birth in ADA (-) SCID patients. Common clinical manifestations included oral candidiasis, erythema, persistent diarrhea leading to growth failure, and interstitial pneumonitis. In addition, six infants with ADA (-) SCID exhibited developmental delays, although there was no evidence of central nervous system infections. One case involved early-onset encephalopathy associated with tubular acidosis, marasmus, and abnormal fundoscopic findings. Five ADA (-) SCID patients also presented with typical skeletal abnormalities such as cupping and flaring at the costochondral junctions, as well as dysplasia of the pelvis. Furthermore, characteristics of Omenn syndrome were observed, including erythroderma with thickened skin (pachyderma), chronic diarrhea, lymphadenopathy, hepatosplenomegaly, significant eosinophilia, and elevated serum IgE levels.[15]

The study in India from 2013, involving 47 patients highlighted the diversity of immune deficiencies, with immune dysregulation observed in 29%, B- and T-cell abnormalities in 28%, predominant antibody deficiencies in 23%, well-defined immunodeficiencies in 15%, and phagocyte disorders in 4% of cases. Recurrent infections, failure to thrive, and autoimmune manifestations were common clinical features, reflecting the varied presentations of immunodeficiency disorders.[16]

In Brazil, the study of 64 patients revealed pneumonia (64.1%) as the most common symptom, followed by chronic or acute diarrhea (46.9%), candidiasis (45.3%), and sepsis (40.6%). Failure to thrive and skin conditions such as eczema or erythroderma were each observed in 35.9% of patients, while lymphadenopathy and hepatosplenomegaly were documented in 34.4% of cases. Acute otitis media was also common, indicating the widespread and severe infections associated with immunodeficiencies in the cohort.[17]

In Taiwan, two confirmed SCID cases, two suspected SCID cases, four patients with persistent T-cell lymphopenia, and five cases of chromosome 22q11.2 microdeletion syndrome were analyzed from a Chinese population. While newborns with SCID may initially appear asymptomatic, they rapidly develop severe infections. T-cell lymphopenia was a consistent finding, and patients with chromosome 22q11.2 microdeletion syndrome often exhibited congenital heart defects or cleft palate, showcasing the spectrum of presentations in primary immunodeficiencies.[18]

In Saudi Arabia, among 114 SCID patients who received the BCG vaccine, 43% developed disseminated *Mycobacterium bovis*. The mean age of symptom onset was 17 months. Common infections included lower respiratory tract infections (50%), skin infections such as cellulitis and abscesses (25%), chronic diarrhea (24%), and oral thrush (13%). Deep abscesses were noted in 6% of patients. These findings illustrate the high burden of infections and complications in SCID patients, emphasizing the need for early

diagnosis and vaccination policies tailored to this population.[19]

In the USA and Canada, SCID patients were classified into typical and atypical forms.[20] Typical SCID was characterized by extremely low CD3 levels (<300/μL), maternal engraftment, and minimal response to phytohemagglutinin (PHA). Atypical or leaky SCID showed age-dependent CD3 level reductions, no maternal engraftment, partial PHA responses, and low response to tetanus toxoid. Omenn syndrome was identified by generalized rash, a high percentage of CD45RO T cells, and distinct genetic markers. The detailed characterization of immune profiles highlighted age-related differences and the importance of genetic diagnosis.[20]

In Iran, a study of ten patients with Severe Combined Immunodeficiency (SCID) revealed pneumonia as the most common clinical presentation. In which, seven cases were pneumonia followed by failure to thrive and Lymphopenia in nine patients. The initial symptoms included chronic cough, diarrhea, gastroenteritis, and prolonged fever. A positive family history of immunodeficiency was noted in some patients. Secondary complications such as invasive pulmonary aspergillosis, encephalitis, membranous colitis, and sepsis were also documented. Unique findings included disseminated Bacille Calmette-Guérin (BCG) infections in three patients, eczematous skin rashes, and lymphadenopathy, emphasizing the heterogeneity of SCID manifestations and the need for early intervention.[21]

In the 2019 Indian case study, pneumonia emerged as the most frequent clinical manifestation, affecting 66% of patients, followed by failure to thrive in 60%, chronic diarrhea in 35%, gastrointestinal infections and oral candidiasis in 21% each, and BCGiosis in 12% of cases. Additional features included erythematous skin rash in 29%, dysmorphism and abscesses in 8% each, and hepatosplenomegaly in 3% of the patients. Immunologically, 67% of the cohort (38 out of 57 patients) exhibited absent or severely diminished T-cell counts (<300 cells/μL), while lymphopenia (<2,500 lymphocytes/μL) was observed in 63% (36 of 57 patients). Isolated T-cell lymphopenia was identified in 12% (7 of 57 patients), further highlighting the immunodeficiency spectrum in these cases.[22] This diverse clinical and immunological presentation underscores the complexity and severity of immune dysfunction in the Indian cohort.

In Kenya, three patients presented with pneumonia and reduced immunoglobulin levels, with additional unique symptoms in each case. Patient 1 included persistent irritability, seizures unresponsive to treatment, congestive heart failure, and failure to thrive. Patient 2 developed severe pneumonia progressing to Acute Respiratory Distress Syndrome (ARDS) with septic shock, severe gastroesophageal reflux disease, atopy, and failure to thrive. Patient 3 experienced severe pneumonia with rotavirus gastroenteritis, severe progressive dermatitis, and failure to thrive. Following a protracted rotavirus infection—possibly a vaccine-acquired illness—the patient experienced severe sepsis. These cases underscore the life-threatening complications associated with immune deficiencies in pediatric patients.[23]

4. Diagnosis

The diagnostic methods for severe combined immunodeficiency (SCID) vary significantly across the different countries, reflecting diverse approaches and levels of healthcare development. In the study done in Sri Lanka from 2010 to 2022, a total of 206 patients were diagnosed of inborn errors of Immunity out of which SCID was the commonest (14.9%).[12]

In Oman, a combination of microbiological and molecular techniques was employed. These included cultures for bacteria, mycobacteria, and fungi, along with PCR for

pathogens in plasma, fluids, feces, and cerebrospinal fluid. Histopathology identified virus-specific inclusion bodies, and flow cytometry was used for immune profiling. Although TREC analysis for newborn screening was unavailable, the whole exome sequencing by Centogene® facilitated genetic assessment.[8]

In France, diagnostic methods evolved over time. T-cell counts were initially determined by E-rosetting until 1981, replaced by indirect immunofluorescence. B-cell counts employed direct immunofluorescence, while serum immunoglobulin levels transitioned from radial immunodiffusion to nephelometry. PCR and Southern blotting became standard for lymphocyte analysis, enhancing diagnostic precision.[15]

In India (2013), molecular diagnostics were performed in 12 cases, with positive findings in seven. Disorders were categorized into immune dysregulation (29%), B- and T-cell abnormalities (28%), predominant antibody deficiencies (23%), well-defined immunodeficiencies (15%), and phagocyte disorders (4%). Molecular testing played a pivotal role, particularly in identifying genetic mutations in 14 hemophagocytic lymphohistiocytosis (HLH) cases.[16]

In Brazil, diagnostic methods included absolute lymphocyte CD3+ T-cell and NK cell counts, lymphocyte proliferation tests, and maternal T-cell engraftment analysis. Flow cytometry examined peripheral T-cell subpopulations, while TREC assays, adenosine deaminase, uric acid, eosinophil counts, and serum IgE levels were assessed. Chest X-rays evaluated thymic shadow, and fluorescence in situ hybridization (FISH) provided additional insights.[17]

In the Chinese population of Taiwan, newborn screening using the T-cell receptor excision circle (TREC) assay was the cornerstone of diagnosis. Modifications to the TREC assay involved adjustments in elution and RT-qPCR volumes. Infants with low or zero TREC levels underwent complete blood counts, flow cytometry, and TUPLE1 gene analysis to detect chromosome 22q11.2 microdeletion syndrome. Confirmatory genetic diagnoses employed multiplex ligation-dependent probe amplification (MLPA), ensuring precise identification of immune deficiencies.[18]

In Saudi Arabia, 26% of PID cases were diagnosed at birth through targeted screening for affected families. Genetic testing identified familial HLH mutations and a novel ELANE mutation in congenital neutropenia. Flow cytometry revealed SCID phenotypes such as $T^{-}B^{+}NK^{-}$ and $T^{-}B^{-}NK^{+}$. Molecular studies identified mutations in approximately 14.9% of cases, underscoring the importance of genetic evaluations.[19]

In the USA and Canada, diagnostic approaches included lymphocyte phenotyping, in vitro lymphocyte proliferation tests, and quantitative immunoglobulin measurements. Screening for TRECs and T-cell receptor spectratyping further aided diagnosis. Additional evaluations, such as maternal T-cell engraftment, were conducted to assess immune function comprehensively and guide therapeutic decisions.[20]

In Iran, diagnostic techniques included CT scans and Polymerase Chain Reaction (PCR). CT scans identified splenomegaly, hypodense lesions in the spleen and liver, and lymphadenopathies. PCR confirmed *Mycobacterium bovis* in lymph node and bone marrow specimens in one case. Abdominal CT scans further revealed hypoechoic lesions in the liver and spleen. Skin biopsies showed acid-fast bacilli in one patient, aiding in the diagnosis of BCGosis. Flow cytometry delineated SCID phenotypes, such as $T^{-}B^{-}NK^{+}$, $T^{-}B^{-}NK^{-}$, $T^{-}B^{+}NK^{-}$, and $T^{-}B^{+}NK^{+}$, with an average diagnostic age of 131.8 days.[21]

In India (2019), diagnostic techniques included automated complete blood counts,

lymphocyte subset analysis through flow cytometry, and serum immunoglobulin level measurement by nephelometry. Newborn screening employed TREC assays, while advanced studies like phospho-STAT5 analysis and CellTrace Violet dye were used for T-cell proliferation. Molecular diagnostics included Sanger sequencing, and ADA activity was assessed calorimetrically. These comprehensive methods provided detailed insights into immune dysfunction and deficiencies.[22]

In Kenya, chest X-rays and echocardiography were key diagnostic tools. Chest X-rays revealed pneumonia in two cases and ARDS in one, while echocardiography showed dilated cardiomyopathy in one case but was normal in another. Patients were diagnosed with $T^{-}B^{-}$ SCID and low immunoglobulin (Ig) levels based on these findings, with diagnoses occurring within 10 months to 1 month of admission.[23]

This global overview highlights the diversity in diagnostic approaches to SCID, emphasizing advancements in genetic, molecular, and immunological testing.

5. Treatment methods

Multiple techniques have been used to treat severe combined immunodeficiency (SCID), with the goal of increasing survival and quality of life. Hematopoietic stem cell transplantation (HSCT) is the treatment, with the use of matched related donors (MRD) yielding the best results.[1] When an MRD is unavailable, mismatched related donors (MMRD), unrelated donors (URD), and umbilical cord blood transfusions are viable options. Intravenous immunoglobulin (IVIG) is given on a regular basis to compensate for antibody shortages, and antibiotics, antivirals, and antifungals are used to prevent opportunistic infections.[3] In some areas, mainly North America and Europe, gene therapy has been investigated as a treatment option for specific genetic disorders.[20] Furthermore, supportive care includes the management of organ dysfunctions such as heart failure or sepsis, and the provision of nutritional support is critical for addressing complications and ensuring comprehensive patient care.

In 1993 in France, immunoglobin substitution was used for patients who were unable to produce sufficient antibodies. Immunoglobulin substitution provides the necessary antibodies to help fight infections. Subsequently, fetal liver transplant was carried out, liver contains hematopoietic stem cells (HSCs) capable of producing blood cells, including T cells and B cells. These cells can help restore normal immune function by providing the recipient with the ability to generate a functional immune system. Prophylactic antibiotics were used to prevent these infections, especially during the first few months or years of life before the immune system can be restored through treatments.[15]

The high costs of supportive care and definitive treatments pose significant barriers to improving patient outcomes. Intravenous immunoglobulin (IVIG), antibiotics and antifungals as well as definitive cure in the form of Stem Cell Transplantation are major barriers to improving prognosis of the patients. In addition, any improvement in the survival of these patients in developing countries like India would require a networked, financially sound and possibly government-backed effort to set up a national registry, improve awareness among pediatricians, and establish specific centers offering genetic diagnosis and definitive therapy.

A 2017 publication in the United States reported the use of gene therapy for X-linked SCID, which is typically caused by mutations in the IL2RG gene. Gene therapy was conducted to introduce a functional copy of the IL2RG gene into the patient's hematopoietic stem cells (HSCs), allowing these cells to produce functional T cells and restore immune function. Enzyme Replacement Therapy (ERT)

is primarily used for treating enzyme deficiency disorders where a particular enzyme is malfunctioning. ERT offers several key advantages, including direct correction of the metabolic defect, stabilization of immune function, and the ability to delay disease progression. Thymus Transplant before Hematopoietic stem cell transplantation was performed; this has several key advantages in the treatment of SCID, particularly in improving T cell reconstitution, reducing graft-versus-host disease (GVHD), and accelerating overall immune recovery. By providing a functional thymus, it enhances the success of HCT, leading to better long-term outcomes and overall survival in patients with T-cell deficiencies.[20]

In 2020, in Oman eleven children (30.6%) have received hematopoietic stem cell transplant (HSCT) with a survival rate of 73%. Hematopoietic Stem Cell Transplant (HCT) is considered the most successful and definitive treatment option for SCID. HCT is most successful when performed early in life, especially in newborns who have been diagnosed with SCID through newborn screening. Early transplantation significantly increases the chances of immune reconstitution, providing a higher likelihood of survival and preventing severe infections. One of the main advantages of HCT are the ability to use different sources of hematopoietic stem cells, such as matched sibling donors, haploidentical family members, or unrelated bone marrow donors, as well as umbilical cord blood. Advances in HLA matching and immune suppression protocols have increased the success of HCT even with mismatched donors.[8]

Hematopoietic stem cell (HSC) transduction is undergoing remarkable advancements in recent years, revolutionizing the landscape of gene therapy specifically for inherited disorders like SCID. The evolution of viral vector-based transduction technologies, including retroviral and lentiviral vectors, has significantly enhanced the efficiency and specificity of gene delivery to HSCs. Furthermore, the advent of gene editing technologies, notably CRISPR-Cas9, has empowered precise genome modification in HSCs, paving the way for targeted gene correction. These striking progresses have led to the clinical approval of medicinal products based on engineered HSCs with impressive therapeutic benefits for patients.[20]

In Sri Lanka, the treatment of Inborn Errors of Immunity (IEI) is a complex issue with significant gaps. Intravenous immunoglobulin replacement therapy is crucial for managing antibody deficiencies like Common Variable Immune Deficiency (CVID) and X-Linked Agammaglobulinemia (XLA). Hematopoietic Stem Cell Transplantation (HSCT) has been introduced for severe cases like SCID, but its availability is limited, leading to high mortality rates (83.6%). The administration of the BCG vaccine at birth often worsens the condition, with delayed diagnosis and low awareness of unexplained infant deaths. Chronic Granulomatous Disease (CGD) also faces challenges, with higher mortality rates than in Europe due to insufficient access to HSCT and limited multidisciplinary care. Genetic testing, a crucial component of IEI diagnosis, relies heavily on next generation sequencing due to the lack of local facilities. Supportive care, including antimicrobial prophylaxis and management of complications, is essential for enhancing quality of life. Expanded HSCT services, improved diagnostic capabilities, and increased awareness are needed to address these gaps in IEI care in Sri Lanka.[12]

6. Mortality

The mortality rates vary significantly depending on the region and the availability of timely interventions. In India (2013), the mortality rate stands at 51%, largely due to delays in diagnosis and limited access to HSCT.[16] In the Chinese population of Taiwan, the mortality is relatively lower at 17%, with

early detection through newborn screening and prompt HSCT improving survival outcomes.[18] Kenya reported a 100% mortality rate in a small cohort, mainly due to the lack of access to HSCT and delayed diagnosis.[23] In Oman, where consanguinity is common, and there is no national newborn screening program, the mortality rate is 69%, with late diagnosis and the complexity of the disease contributing to poor outcomes.[8] In Saudi Arabia, the overall mortality rate is 10.3%, with HSCT significantly improving survival, particularly for patients who received early treatment.[19] These findings highlight the crucial role of early diagnosis, access to advanced treatments like HSCT, and appropriate prophylactic care in improving survival rates for children with SCID. Table 1 summarizes the mortality rate and emphasizes the cause of death.

Table 1. Consolidated Mortality Data.

Region	Number of patients	Mortality Rate	Comments
India (2013)	47	51%	High due to limited HSCT and delayed diagnosis.[16]
Taiwan	24	17%	Early HSCT improved prognosis.[18]
Kenya	3	100%	Lack of infrastructure and diagnostic delays.[23]
Oman	36	69%	High consanguinity rates, no NBS program.[8]
Saudi Arabia	502 (85 SCID)	10.3%	HSCT widely available; most deaths under 5 years.[19]

7. Discussion

Severe combined Immunodeficiency (SCID) represents one of the most severe forms of primary immunodeficiency (PID) disorders characterized by impaired cellular and humoral immune responses. This report analyzes patient records from regions all over the world, which includes Saudi Arabia, United States, France, Oman, Taiwan, Iran, France, Kenya, India and Sri Lanka.

Diagnosis is crucial to establish a clear understanding of the patient's clinical presentation, medical history and other preliminary findings. This review attempts to delve into case studies, which highlight the key diagnostic tools and to uncover the evolving of diagnostic methods over the years. Majority of the patients were diagnosed from the age of 7 months to 1 year.

According to a case study conducted and published in 1993, in France, initially blood T-cell counts were determined by E-rosetting until 1981, later diagnosis was transitioned to more accurate methods like indirect immunofluorescence. Serum immunoglobulin levels, crucial for understanding immune deficiencies, were first measured using radial immunodiffusion until 1983, after which the more sensitive and automated technique of Nephelometry was introduced.[15]

Fast forward to 2005, in Iran, diagnosis significantly improved through the integration of advanced diagnostic tools such as positive serum galactomannan testing, computed tomography (CT) scans, PCR analysis and flow cytometry. Serum galactomannan testing aids in identifying opportunistic fungal infections, which are common in SCID patients due to profound immunosuppression. CT scans provide detailed imaging of lymphoid tissues, lungs, and other organs, identifying structural abnormalities or infections indicative of immunodeficiency complications.[21]

During a study conducted in Taiwan between 2010 and 2011, T-cell receptor excision circle (TREC) assay was introduced which was utilized to detect T-cell deficiencies, as particularly methods like newborn screening was used to measure complete blood counts,

which provided broader but less specific insights into immune function, the TREC assay directly assesses thymic output, making it highly sensitive and specific for identifying severe combined immunodeficiency (SCID) and other T-cell deficiencies. Its ability to detect low or absent TRECs at birth enables early identification of immune deficiencies, often before symptoms appear. TREC assay made a profound difference and was significantly advantageous over traditional diagnosis methods.[18]

By 2011, Lymphocyte flow cytometry was the commonest diagnostic technique that was used in India.[16] Lymphocyte flow cytometry is a cornerstone in the diagnosis of SCID because it allows for a comprehensive and detailed analysis of T, B and Natural Killer Cells. Similarly, Lymphocyte Phenotyping was widely used in North America which aided in characterizing immune cell populations and identifying deficiencies or imbalances in lymphocyte subsets. In vitro Lymphocyte Phenotyping, which assesses lymphocyte function, such as proliferation and cytokine production has been useful in SCID diagnosis. T-cell Receptor spectratyping is used in analyzing T-cell repertoire and identifying clonal expansions or reduced diversity. These remarkable advancements in diagnostic techniques have enabled early patient diagnosis, ensuring timely treatment delivery.[24]

By 2015, Arab populations have showed that the rate of combined immunodeficiency is higher than that in the rest of the world.[22] During the study period, 26% of the cases, comprising 132 patients, were diagnosed at birth through targeted newborn screening programs specifically designed for families with a known history of immunodeficiency disorders. Consanguineous marriages have been a major reason for mortality.[19] Inbreeding tends to reveal recessive alleles present in heterozygous carriers and to increase the frequency of homozygous individuals for a given rare allele. Therefore, it is ideal that families with an affected member could utilize preconception carrier testing and prenatal diagnosis.

By 2017, the United States and Canada had made significant advances in the early diagnosis of SCID. Among the diagnostic techniques used in USA and Canada, T cell receptor excision circle (TREC) testing stands out as the most significant, particularly in the context of early detection of T-cell deficiencies. Both countries have robust newborn screening programs that include TREC testing. Another important diagnostic technique is Transplacental Maternal Engraftment, this detects the presence of maternal cells in the infant's blood, a phenomenon known as maternal engraftment. It can be useful for diagnosing conditions like graft-versus-host disease (GVHD) or assessing the impact of maternal immune cells on the newborn's immune system.[20]

In 2019, India had made significant advances in genetic testing. Phospho-STAT5 analysis is a test that evaluates the activation of STAT5, a transcription factor critical for T-cell receptor signaling. T-cell proliferation assays evaluate the ability of T cells to proliferate in response to stimuli, which is typically impaired in SCID patients. A common method uses CellTrace Violet dye to label T cells, allowing for tracking their proliferation after stimulation with mitogens or antigens. Sanger sequencing is a method for determining the nucleotide sequence of DNA and is used to identify mutations in genes associated with SCID. This method allows for the precise identification of mutations in SCID-related genes such as IL2RG**,** RAG1**,** and RAG2**.** Sanger sequencing has been a gold standard in diagnosis for decades, as it stands out due to its high accuracy and versatility.[22]

According to a published study in 2020, Oman was introduced to a comprehensive diagnosis tool, Whole Genome Sequencing (WES) which provided a

comprehensive analysis of the exome (the protein-coding regions of the genome), allowing for the identification of mutations in any of these genes or other novel genes linked to SCID. WES revolutionized the diagnosis of SCID by enabling the rapid and detailed identification of rare genetic mutations which reduced diagnostic delays allowing for earlier interventions.[8]

By 2021, in Kenya Flow cytometry was used, which is a powerful tool used to analyze the characteristics of individual cells in a heterogeneous population, such as peripheral blood leukocytes.[23] The BD FACSCalibur™ is a flow cytometer used to analyze immune profiles by measuring specific cell surface markers on blood cells. It can provide a detailed profile of different immune cell populations, such as the enumeration of T cell subsets (CD4+, CD8+), B cells (CD19+), and NK cells (CD56+). While flow cytometry offers a cellular view, immunoglobulin testing directly evaluates the humoral aspect of immunity. This method is essential for identifying deficiencies in antibody production, which is particularly helpful in diagnosing conditions like specific Ig deficiencies.[25]

In Sri Lanka, 10 patients have been confirmed with SCID through diagnostic tests such as Full Blood Count (FBC), T Cell Proliferation Assay, and Functional Hemolytic Complement Assays. FBC identifies abnormalities in white blood cells, while T Cell Proliferation evaluates immune functionality, and complement assays assess the complement system's role in immunity. However, molecular and genetic diagnostics remain limited to X-linked SCID, XLA, and chromosomal deletion 22q11.2. Expanding diagnostic facilities could drastically reduce mortality in Sri Lanka.[26]

Diagnosis has evolved rapidly over the years, and future advancements promise to bring a great revolution. Newborn screening and Whole Genome Sequencing have brought in a drastic change in diagnosis. Apart from TREC, advanced methods of screening have been suggested in Italy.[23] TREC Assay is combined with Tandem mass spectrometry for adenosine deaminase deficiency detection (including the combination of molecular and biochemical techniques to improve early diagnosis of SCID and related conditions). Building on these advancements, multiples assays play a vital role by enabling comprehensive analysis of the genetic condition. Furthermore, advancements in automation and robotics have enabled high-throughput screening platforms by speeding up sample processing. Ongoing research and innovation in this field are crucial to enhance diagnosis of SCID on a global scale.

8. Conclusion

Severe Combined Immunodeficiency is a group of rare and life-threatening genetic disorders characterized by profound defects in the immune system, particularly affecting T cells, and often B cells and NK cells. SCID patients are highly susceptible to recurrent, severe infections, which can be fatal if not diagnosed and treated early. Early diagnosis is crucial for improving outcomes, as the condition is usually detected in infancy or early childhood. The most effective diagnostic tool for SCID is lymphocyte flow cytometry, which provides details of immune cell populations. Subsequently, TREC assay was introduced which provided detailed analysis of T cell deficiencies. Whole genome sequencing (WGS) has revolutionized the diagnosis SCID by providing a comprehensive and precise method for identifying genetic mutations. Unlike traditional diagnostic methods, which may focus on specific genes or symptoms, WGS allows for the analysis of an individual’s entire genetic makeup, enabling the detection of a wide range of mutations. Bone marrow and fetal liver transplantation, and in some cases gene therapy, offer the potential for long-term immune reconstitution and are considered curative options for SCID. Advances in

treatment have significantly improved survival rates, especially when these therapies are initiated early in life. Recent advancements in hematopoietic stem cell (HSC) transduction have revolutionized gene therapy for inherited disorders like SCID. The development of viral vector-based technologies, including retroviral and lentiviral vectors, has greatly improved the efficiency and specificity of gene delivery to HSCs. Despite the challenges, timely diagnosis and comprehensive treatment approaches, including immune system restoration and supportive care, can offer SCID patients the best chance for survival and improved quality of life.

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HIV Treatment and Cure: A Critical Review of Scientific Advances and Therapeutic Developments

Neranja Sandamini[1], Nihansa Jayathilaka[1], Lashelle Vincent[1], Aranega Ananthakumaran[1], Shahla Rasmy[1] and Yenni Debra Lee[1]

[1]School of Science, Business Management School (BMS), Sri Lanka

[*]neranja.s@bms.ac.lk

Abstract

Secondary Immunodeficiency Disorders (SID) such as HIV had claimed to have taken approximately 42.3 million lives. SIDs are defined as a transient or persistent impairment of the function of cells or tissues of the immune system, due to multiple external factors including underlying infections such as HIV, specific medications and medical conditions. HIV, being a retrovirus, specifically targets CD4 T cells, dendritic cells and macrophages. HIV-AIDS has transitioned from being a fatal diagnosis to a manageable chronic condition due to groundbreaking research, drug development and public health interventions. The most common method of treatment for HIV is antiretroviral therapy (ART). Combining drugs from different classes such as Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Non- Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs), Integrase Inhibitors , were found to suppress viral replication more effectively than monotherapy. Over 95% of individuals who adhered to combination ART (cART) achieve undetectable viral loads making it effective for preventing progression to acquired immunodeficiency syndrome (AIDS) and reducing transmission risk. In the 2000s the focus shifted to improving drug efficacy, reducing toxicity and enhancing accessibility. Around 86% of people under Highly Active Antiretroviral Therapy (HAART) achieved viral suppression preventing disease progression and transmission. INSTIs and HIV entry inhibitors further expanded therapeutic options targeting different stages of the viral life cycle. The last decade has seen further refinement of HIV treatment emphasizing long-term management and prevention including advances in functional cure research such as stem cell transplant and CRISPR/Cas 9 technology. Despite these achievements significant challenges persist. Disparities in treatment access and the emergence of drug resistance strains underscore the need for continued innovation. Continued advancements in HIV research and equitable access to treatment are essential in achieving the goal of ending the HIV epidemic.

Keywords: HIV-AIDS, Treatment, PI, NRTIs/ NNRTIs, Integrase Inhibitors, Stem Cell Transplant, CRISPR/CAS 9 Technology

1. Introduction

Immunodeficiency disorders arise primarily due to impairments in the body's immune system, making it extremely difficult to defend the immune system and overcome pathological states brought upon by foreign or abnormal cells such as bacteria, fungi, viruses and cancer cells. Hence due to the suppression of their immune system, patients are physiologically inclined to develop various disorders, common examples including immune thrombocytopenia, AIDS etc. Immunodeficiency disorders can be classified into two types: Primary Immunodeficiency Disorders and Secondary Immunodeficiency Disorders.

Primary Immunodeficiency Disorders (PID) are relatively rare, persist from birth and

are always genetically inherited. PIDs become more evident during infancy and early childhood, with certain exceptions including common variable immunodeficiency which is detected during adulthood. Alternatively, Secondary Immunodeficiency Disorders (SIDs) are conditions where the immune system is weakened or undergoes severe compromise due to extraneous factors such as underlying medical conditions, infections or due to specific medications. SIDs increase an individual's susceptibility to infections. One of the most common SIDs include human immunodeficiency virus (HIV) infection.[1]

HIV belongs to the family of retroviruses classified under two types, HIV1 and HIV2. They fundamentally target an individual's immune system. HIV invades the body and proceeds to attack T lymphocytes, decreasing their ability to defend the body against infections. HIV tends to have a higher affinity and proceeds to bind to the CD4 receptors of the T lymphocyte cell, hence causing the subsequent disintegration of the T cell. This is a major drawback as the CD4 T cell and its subtypes aid in the coordination of the adaptive immune response by promoting cell-mediated immunity, facilitating humoral immune response by producing antibodies against extracellular pathogens, enhancing inflammation to aid in combating extracellular invaders and maintaining immune tolerance by suppressing the overstimulation of immune responses thus also preventing the development of autoimmune diseases. T helper cells proliferate, resulting in the formation of memory T helper cells that retain memory and thus act as defense upon re-entry of the pathogen. Thus, when the CD4 T cells and their functions are hindered due to HIV, the patient becomes significantly more prone to the development of other diseases ultimately leading to AIDS. If not treated early on can lead to the patient experiencing grave conditions, often fatal.[2]

HIV can be transmitted via the exchange or spread of bodily fluids of the infected individuals including blood, breast milk, rectal fluids, vaginal fluids and semen specifically pre-seminal fluids. This mostly results in cases of unprotected sexual intercourse with the infected patient or due to sharing needles seen in the case of most illicit drug abusers. General symptoms of AIDS include fever, headaches, extreme fatigue, muscle and joint pain, and night sweats, whereas some individuals appear asymptomatic up till the point of diagnosis.[3]

In current-day medicine, a diverse array of tests is used to diagnose AIDS. Methods include antibody tests which can detect HIV antibodies 23 to 90 days after exposure, and rapid antigen/antibody tests which can detect HIV antibodies 18 to 90 days following exposure. Nucleic Acid Test (NAT) can detect HIV 10 to 33 days following initial exposure. In cases of initial screening, Enzyme-Linked Immunosorbent Assay (ELISA) is utilized to detect HIV antigens. Due to its high specificity and sensitivity in detecting p24 antigens, ELISA is advantageous and can be used to detect HIV only 2 weeks after exposure. Though this form of testing appears to be beneficial it cannot be solely used as a form of diagnosis and requires the use of confirmation tests such as Western Blotting, HIV differentiating tests and NAT.[4]

According to the World Health Organization, by 2023, 39.9 million individuals live with HIV globally, approximately 3 million individuals had acquired HIV and between 500, 000 to 820,000 deaths had occurred due to HIV. These figures account for all adults, both women and men as well as children. These figures highlight the significant impact of AIDS globally, affecting a large fraction of the world's existing population.

This review summarizes the mechanism of action of HIV in developing AIDS as well as the evolution and subsequent

effects of various existing and evolving treatment strategies for HIV outlining the milestones and challenges in the development of treatments and their impact on individual and public health.

2. HIV Mode of Action

HIV is a lentivirus, belonging to the retrovirus family, that primarily targets the CD4 T-cells by binding 120 viral glycoprotein to two cell surface proteins of host's immune cells, the CD4 receptor and either the CC-chemokine receptor 5 (CCR5) or the CXC-chemokine receptor 4 (CXCR4). Since the virus is a retrovirus, it can integrate its DNA into the host cell genome, thereby making the eradication of the virus exceedingly difficult with the current therapeutic strategies. HIV enters the target cells via the CD4 receptor and either the CCR5 or CXCR4 receptor through its interaction with the envelope glycoprotein. Once the virus is attached to the cell, the viral membrane fuses with the cell membrane which facilitates the entry of the virus into the cytoplasm of the target cell. Next, the viral DNA (vDNA) is transcribed by the viral reverse transcriptase enzyme (RT) into a double-stranded DNA (dsDNA) molecule. This dsDNA is then translocated to the cell nucleus where it is subsequently integrated into the cell genome as a provirus, a process that is mediated by the virus integrase enzyme. Inside the nucleus, the host enzymes mediate the transcription of the HIV DNA to viral mRNAs. These mRNAs are then exported to the cytoplasm for translation which results in the expression of viral proteins and eventually mature virions.[6] The viral proteins Tat and Nef, in addition to the intrinsic T-cell activation factors, induce the active transcription and the expression of the viral RNA and proteins to produce new virus particles. These new viral particles then exit the host cell and traverse through the tissues to infect other target cells. The CD4-independent viral entry has been demonstrated in astrocytes, B cells, and kidney epithelial cells where the replication of the virus is less likely to occur.[7] **Figure 01** illustrates the life cycle of HIV.

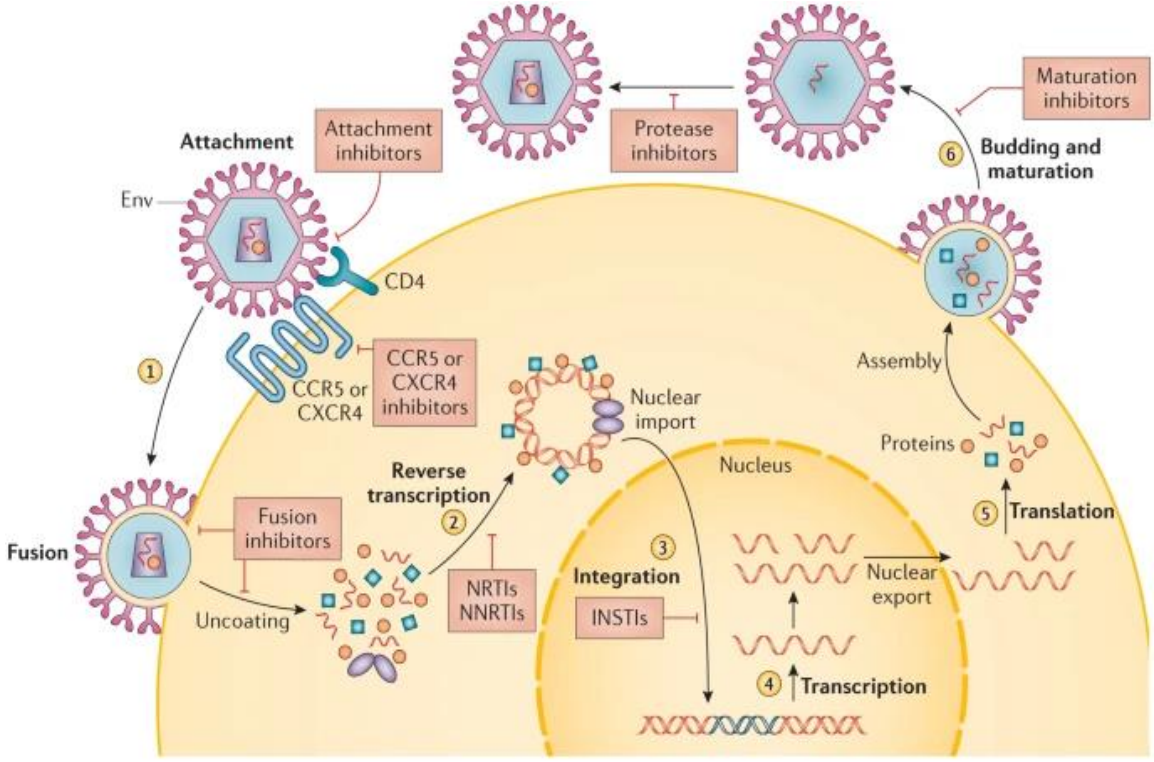

Figure 01: HIV Life Cycle.[6]

3. Treatment strategies of HIV

3.1 HPA 23

HPA-23 (ammonium 5-tungsto-2-antimoniate), a polyoxometalate (POM), was an experimental antiretroviral drug used in the early days of the HIV/AIDS epidemic. While it initially showed promise, further research revealed significant limitations and side effects. HPA-23 exhibits anti-HIV activity primarily by inhibiting critical enzymes and interfering with viral entry mechanisms. HPA-23 effectively suppresses the activity of reverse transcriptase, an enzyme crucial for the conversion of viral RNA into DNA, which is a necessary step in HIV replication. HPA-23 may also affect HIV entry by interacting with the viral envelope glycoproteins, particularly gp120, which is necessary for binding to the CD4 receptor on host cells. This disrupts the virus's ability to attach and fuse with target cells. As a member of the POM class, HPA-23 has demonstrated effectiveness against a range of viruses, but its development for HIV treatment faced challenges due to its toxicity, particularly affecting liver and kidney functions.[8]

In a study conducted in 1985 in Paris, four patients were treated with HPA 23 as part of an experimental therapy for AIDS. The patient cohort ranged from 13 years to 30 years old with underlying conditions such as cerebral toxoplasmosis, pneumocystis pneumonia, paraganglioma, oral thrush and Kaposi sarcoma (KS). HPA 23 was used as a reverse transcriptase inhibitor to reduce HIV (then referred to as LAV) replication. HPA 23 worked by inhibiting reverse transcriptase, thereby suppressing HIV replication in T-cell cultures without killing infected cells. The treatment reduced detectable HIV in peripheral blood lymphocytes during administration, though low levels reappeared in some patients' post-treatment. Side effects included temporary platelet count reductions and mild hepatic transaminase elevations, both of which normalized after treatment, and no renal toxicity was observed.[9]

3.2 Protease Inhibitors

Protease Inhibitors are a form of antiretroviral therapy (ART) and play a crucial role in treating HIV. ART slows down the progression of the virus by blocking the effect of the HIV protease enzyme. The HIV protease enzyme has a pivotal role in the maturation of the virion particles into new HIV particles due to its property of cleaving specific viral protein sites, converting large precursor proteins into smaller proteins that combine with HIV genetic information and proceed to mature into new viral particles. Protease inhibitors mimic the structure of the viral protein and bind to the active site of the enzyme instead, thereby blocking the proteases' ability to cleave the long-chained viral polypeptide by specifically preventing the enzyme from cutting the viral protein chain at the Pol and Gag sites. This is crucial as the Pol gene is necessary to mediate the assembly of the viral particle and the Gag gene plays a significant role when it comes to the replication and proliferation of the mature viral particles. Thus, when these sites are not cleaved, it results in the production of non-infectious viral particles due to them being immature. These not fully formed viral proteins cannot assemble with other viral components and hence fail to produce and release functional proteins that are completely necessary for the replication of HIV and are inhibited from infecting other healthy host cells. Therefore, protease inhibitors reduce the viral load in the patient's body and thus act as a beneficial treatment method for HIV.[10]

Protease Inhibitors can be classified according to four types. First Generation Protease Inhibitors are the earliest formulations of protease inhibitors to ever be used as a form of treatment for HIV. Examples include Ritonavir (RTV), Saquinavir (SQV) and Indinavir (IDV). These were highly associated with severe side effects and very low levels of

bioavailability and were deemed not highly effective. Second Generation Protease Inhibitors were capable of fewer side effects and comparatively more bioavailability and were predominantly designed to pharmacokinetic parameters to prevent resistance. Examples include Darunavir (DRV), Fosamprenavir (FPV), Lopinavir (LPV) and Atazanavir (ATV). For better tolerability and to obtain a very high resistance barrier, a third generation of Protease inhibitors was developed, an example includes Tipranavir (TPV).[11] In a study done by Serafino *et al*., combination ART therapy of Atazanavir and Ritonavir was administered to two patients with HIV. The male patient experienced a reduction in his viral load from 102,900 copies/mL to undetectable amounts, while the female patient also experienced a reduction in her viral load from 1,302,000 copies/mL to lowered levels post-treatment.[12]

Another study done by Vornicu *et al*. discusses a 29-year-old woman with a well-controlled HIV infection who presented with acute kidney injury and nephrotic syndrome. The protease inhibitors given to the patient included Darunavir and Ritonavir. The patient had undergone effective suppression of their HIV viral load as a response to treatment.[13]

3.3 Nucleoside and Non-nucleoside Reverse Transcriptase Inhibitors

Non-nucleoside reverse transcriptase inhibitors (NNRIs) and nucleoside reverse transcriptase inhibitors (NRTIs) are two distinct classes of viral reverse transcriptase inhibitors (vRTI), which are involved in the antiretroviral therapy (ART) regimen of HIV-AIDS patients. NRTIs are nucleoside analogues that compete with normal 2′-deoxynucleoside triphosphates (dNTPs) during DNA synthesis. Once the NRTIs are incorporated into the growing DNA chain, they cause the chain termination of vDNA transcription due to their lack of a 3′-hydroxyl group. After that, they interact directly with the catalytic site of the reverse transcriptase enzyme, specifically with residues Asp110, Asp185, and Asp186. In contrast, NNRTIs are structurally diverse compounds that bind to a specific hydrophobic pocket in the p66 subunit of reverse transcriptase, consisting of residues Tyr181 and Tyr188. This binding site is spatially distinct from the catalytic site and acts allosterically, inducing conformational changes that inhibit the enzyme's activity without mimicking nucleosides. Thus, while NRTIs block reverse transcriptase by chain termination, NNRTIs inhibit the enzyme through allosteric interference.[14] NNRTIs are prodrugs that require intracellular anabolic phosphorylation to be converted into their active form of phosphorylated NNRTI metabolites.[15] Hence, most of these drugs possess longer half-lives than their parent drug compounds. NTRIs are also prodrugs that require intracellular activation to the corresponding triphosphate (ddNTP) forms by various cellular pathways. Table 1 outlines the plasma half-life and the cellular transport mechanisms employed by the current clinically approved NRTIs. Here, it can be observed that the plasma half-life of the metabolite is longer than the intracellular half-life of the parent drug. However, NRTIs have setbacks, including the rapid development of resistance and a lack of functionality against HIV-2.

Currently, approved clinical NNRTIs are classified into two generations. The first generation includes the drug Nevirapine which has been known to be severely impacted by the emergence of drug resistance, leading to mutations in the amino acid homolog. Moreover, the first-generation drugs also lead to RT-resistant mutations such as Y181C and K103N. To address these harmful and inefficient effects of the first-generation drugs, second generation drugs such as Efavirenz, Etravirine, and Delavirdine have been discovered. These drugs target the HIV polymerase activity of the RT enzyme through allosteric inhibition and pose higher efficacy along with fewer side effects for HIV patients.[16]

Table 01 Nucleoside Reverse Transcriptase Inhibitors.[15]

NRTI Name	Plasma Half-Life	Intracellular half-life
Lamivudine (3TC)	22 h	15 to 16 h
Emtricitabine (FTC)	37 h	39 h
Tenofovir Disoproxil Fumarate (TDF)	~17h	164 h
Tenofovir Alafenamide (TAF)	~125 h	164 h
Zidovudine (AZT)	~2 h	~9 h

Despite the assumption that all the NNRTIs bind to the same hydrophobic pocket site at the HIV-RT, the different classes of HIV-1 specific RT inhibitors differ concerning which amino acids the drugs interact with at the binding site. This hypothesis is extracted from the discovery that different NNRTIs do not exactly possess cross-resistance to each other, although these drugs seem to overlap when a cocktail of drugs is prescribed.[17]

According to the research conducted by Ard *et al.* a 55-year-old man who was diagnosed with HIV-1 infection was administered with a combination of ART regimen Tenofovir Alafenamide (TAF), Emtricitabine (FTC) and Dolutegravir (DTG), out of which TAF and FTC were NRTI and NNRTI respectively.[18] After the completion of ART at its first week a slow rise in the CD4+ T-cell count was observed. Moreover, the viral load was less than 20 copies per millimeter.

In the case study conducted by Pallangyo *et al*., a 29-year-old HIV positive woman was started on antiretroviral therapy which included a cocktail of drugs namely Tenofovir, Lamivudine, and Efavirenz antiretrovirals.[19] Tenofovir and Lamivudine and NRTIs whereas Efavirenz is a NNRTI. The ART helped decrease her viral load and increase her CD4+ T-cell count which was at a low level of 316 cells/mL. However, the ART resulted in an increase in her lipid levels.

3.4 Integrase Inhibitors

Integrase strand transferase inhibitors (INSTI) are a class of antiretroviral inhibitors that specifically block the activity of the HIV-1 integrase enzyme, which is a key player in the viral replication cycle. This inhibition blocks the integration of viral DNA into the host genome, halting viral replication and infection spread. Four approved INSTIs for HIV treatment include first generation raltegravir, elvitegravir and second-generation dolutegravir and bictegravir.[20] Integrase operates within the large functional nucleoprotein complex called intasome, which includes viral DNA and components from the virus and host cell. Integrase catalyzes two key reactions within the intasome to incorporate the reverse-transcribed HIV DNA into the host DNA.[21] During 3'-processing, integrase removes two or three nucleotides from each 3' end of the viral DNA, promoting strand transfer reaction to occur after import the viral DNA into the nucleus, integrase inserts the 3' ends of the viral DNA into the host DNA. INSTIs are characterized by a metal-chelating core that binds two Mg^{2+} ions, a halogenated benzene side chain that interacts with viral DNA, and a flexible linker connecting the core scaffold to the halobenzyl side chain. INSTIs preferentially target the enzyme's active site in the intasome complex, binding to the divalent metal ions and viral DNA. This displaces the viral DNA from the active site, inactivating the intasome and

preventing the integration of reverse-transcribed viral DNA into the host genome by blocking the strand transfer reaction.[22]

In a study conducted by Peterson *et al.*, a 55-year-old woman from West Africa was diagnosed with HIV-2 in 1990. After extensive combinations of various ART cocktails, in 2010 the patient was prescribed Raltegravir with Zidovudine/Lamivudine and Ritonavir-boosted Darunavir. After 1 year of this treatment, her viral load came down to an undetectable level from 61,000 copies/mL and her CD4+ T-cell count which was 282 cells/μL rose to 428 cells/μL.[23]

The case study conducted by François *et al.,* highlights the first documented instance of Dolutegravir (DTG) resistance in ART-naive, perinatally HIV-infected infant, identified during a national HIV drug resistance (HIVDR) survey in Haiti. The infant, born to a mother on a Tenofovir/Lamivudine/Dolutegravir regimen, displayed resistance mutations affecting INSTIs, NRTIs and NNRTIs. Although INSTI resistance was low (1.1% in 143 infants), a unique case of resistance in DTG-exposed populations emphasizes the need for vigilance and further research to improve treatment strategies in low- and middle-income countries.[24]

3.5 Stem Cell Transplant

Stem cell therapy is an approach in contemporary medicine that utilizes the regenerative ability of stem cells to treat a wide range of diseases and injuries. Stem cells can develop into various specialized cell types, facilitating the repairing and restoring of damaged tissues, and organ function and combating illnesses like cancer. Stem cells can be categorized into three main types. Common characteristics include self-renewal and differentiation into other cell types. Adult Stem Cells can give rise to many specialized cell types of tissues and organs, an example includes hematopoietic stem cells (HSCs). HSCs are located in bone marrow forming all mature blood cells in the body like erythrocytes and lymphocytes cells and platelets.[25,26]

A remarkable rise in stem cell therapy in HIV occurred after the two real-life incidents of Berlin and Geneva patients. Timothy Ray Brown was the first person ever cured of HIV, commonly known as the Berlin Patient. Initially, he had a CD4+ T-cell count of 316 cells/mL which indicated a weakened immune response.[27-28] Timothy was treated with standard ART before his stem cell transplant. After receiving a stem cell transplant from a donor with the CCR5-Δ32 mutation resistant to HIV, Timothy did show improvements regarding his immune system as HIV could no longer infect his cells. In response to ART he experienced a reduction in viral load and an increase in his CD4+ T-cell count to a value of 415/mm2.[27]

According to the information analyzed by Asier Sáez-Cirión *et al.,* the Geneva patient, who was already diagnosed with HIV-1 was again diagnosed with myeloid sarcoma in 2015. The individual underwent a stem cell transplant which did not involve a donor with the CCR5-Δ32 mutation. The CCR5 receptor, present on the surface of immune cells serves as a critical pathway for HIV entry. The patient continued ART to maintain HIV suppression, as the virus remained in latent reservoirs and could rebound without treatment.[29] Finally, in 2021, all antiretrovirals were stopped following a consensual decision between the patient and his physician to evaluate the possibility of HIV remission and there was no evidence recorded of remission of HIV for 32 months despite the recurrent testing.[30] In response to the allogenic haematopoietic stem cell transplant the viral load of the patient decreased to 2.22 RNA copies / mL and was eventually undetectable.

3.6 CRISPR/Cas 9 Technology

A promising gene-editing technique for HIV/AIDS treatment is the clustered regularly

interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) systems. It can be used to target the HIV-1 genome or cellular co-factors to decrease HIV-1 infection and eradicate the provirus. It can also be utilized to trigger transcriptional activation of dormant virus in dormant viral reservoirs to eradicate the virus. In the area of gene therapy in human CD34+ hematopoietic stem and progenitor cells (HSPCs), CRISPR/Cas9 technology advanced quickly. With the help of RNA spacers, a transcript from brief segments of host DNA obtained from additional chromosomal elements, the Cas9 helicase, which is a component of the CRISPR-Cas9 machinery, can attach to RNA generated from the palindromic repeats of host DNA and cleave invasive DNA.[31] In 2013, the CRISPR/Cas9-based method was initially investigated for HIV/AIDS treatment. The target sites were the TAR sequences in the R region and the NF-κB binding cassettes in the U3 region of LTR, respectively. As a result, HIV-1 provirus transcription and replication were effectively inhibited.[32] More significantly, it is demonstrated that CRISPR/Cas9 could remove internal integrated viral genes from the chromosome of the infected host cell, indicating that it could be a useful therapy tool for HIV/AIDS. Soon after, studies on using CRISPR/Cas9 to remove the HIV-1 genome were carried out. In an HIV-1 latently infected T cell line, pro-monocytic cell line, and microglial cell line, they employed Cas9/gRNA to target conserved sites in the HIV-1 LTR U3 region.[33] This resulted in the inactivation of viral gene expression and the restriction of virus replication with minimal genotoxicity and no detectable off-target editing. It also showed that targeting multiple sites of the HIV-1 genome could increase the efficiency of excision and disruption of the non-integrated proviral genome. Furthermore, combining two potent single guide RNAs (sgRNAs) that target distinct areas of the HIV genome may stop the virus from replicating and escaping. Additionally, compared to single sgRNA-mediated SaCas9 editing, the combined SaCas9/gRNAs demonstrated greater efficacy in altering the HIV-1 genome.[34] However, a single, guide RNA (gRNA) mediated cleavage can allow the virus to escape. A combinatorial CRISPR/Cas9 gene-editing strategy can mitigate this viral breakthrough. CRISPR/Cas9 technology can be used to alter co-receptors to prevent the entry of HIV-1 via the CCR5/CXCR4 co-receptors and the CD4 receptor. Since CD4 is essential for a healthy immune system, blocking CD4 is not an ideal way to treat HIV-1 infection.[35]

According to the study done by Xu and Deng, CRISPR-edited cells were used in an HIV-positive patient by the Department of Hematopoietic Stem Cell Transplantation at the Hospital of the People's Liberation Army in Beijing.[36] The 27-year-old patient received a diagnosis of both HIV/AIDS and acute lymphocytic leukaemia (ALL). After a year, the HIV infection was under control due to antiretroviral treatment, and the virus was no longer detected in serum RNA. Once the researchers established that the HIV was CCR5-tropic, they found a male donor who had the unmutated CCR5 gene. The CCR5 locus was then edited using CRISPR. The patient was given both unedited CD34-depleted cells and newly modified CCR5 CD34-positive cells. When his CD4+ cell count rose to within the normal range and HIV RNA copies were still undetectable, the patient's antiretroviral treatment was discontinued seven months after alloHCT. The researchers report a successful allogeneic transplantation.[36]

In another study conducted by Khamaikaiwan *et al.,* patient with acute lymphoblastic leukaemia and HIV-1 infection was treated with a transplant of HSPCs with the CRISPR/Cas9-ablated CCR5 gene.[37] The sgRNAs were constructed with high cleavage efficiency to the couple at the start of the first exon of the human CCR5 gene at the Δ32 mutant location after being screened to exclude off-target potentials. After engrafting, the CCR5-knockout HSPCs showed the donor

cells' CCR5 ablation, which lasted for almost 19 months in peripheral blood. This could lead to the discovery of new sources of CCR5Δ32/Δ32 HSC donors. Hence, it can be observed that CRISPR-Cas9 technologies have shown promising potential as a treatment for HIV by offering the ability to directly edit the virus's DNA or modify human cells to resist infection.[37]

4. HIV Treatment regimen in Sri Lanka

Sri Lanka maintains a low-level HIV epidemic, with an estimated prevalence of less than 0.1%, making it one of the lowest in South Asia. By the end of 2018, approximately 3,500 individuals (range: 3,100–4,000) were living with HIV, with 350 newly reported cases that year, the highest annual increase since the first case was identified in 1987. Among key populations, such as female sex workers (FSW), HIV prevalence was reported as 0.1% in Colombo, 1.0% in Galle, and undetectable in Kandy during the 2014/2015 Integrated Bio-Behavioral Survey (IBBS). Despite 92% of FSW reporting the practice of safe sex with clients, only about one-third had undergone HIV testing within the previous year and were aware of their results, highlighting gaps in testing uptake and knowledge.[38]

In Sri Lanka, HIV treatment guidelines recommend ART initiation at a CD4 count of <500 cells/mm³, consistent with the 2013 WHO guidelines. For children aged below 1 year, ART is recommended irrespective of CD4 count. Additionally, Option B+, a program that provides lifelong ART. is implemented for the prevention of mother-to-child transmission (PMTCT), where all HIV-positive pregnant women are eligible regardless of their CD4 count. While treatment coverage and viral load testing are limited, efforts align with WHO guidelines to improve ART access and outcomes.[39]

A 34-year-old woman was diagnosed with HIV in 2011 in Sri Lanka and underwent antiretroviral therapy (ART). She had a baseline CD4 count of 295 cells/μL. Her initial ART regimen consisted of Zidovudine (AZT), Lamivudine (3TC), and Nevirapine (NVP). Her adherence to ART was inconsistent, and after a virological failure in 2016, a high viral load of 26,679 copies/mL and intermediate resistance to Efavirenz (EFV) was recorded. Subsequently, her treatment was switched to a second-line regimen comprising Tenofovir (TDF), Emtricitabine (FTC), and Atazanavir/Ritonavir (ATV/r) which gradually reduced the viral copies to 4200 copies/mL.[40]

According to the annual report by National STDs/AIDs Control Programme (NSACP) of 2023, nearly 96% of patients will be on DTG-based regimens in Sri Lanka. The most prescribed regimen for adults was Tenofovir Disoproxil Fumarate (TDF)+ Emtricitabine (FTC)+ Dolutegravir, and 86.4% of PLHIV were on that regimen. For the pediatric patients DTG 10 mg dispersible tablet was available from Global Fund (GF). PI was mostly restricted as second-line regiments and only 0.8% of patients were on PI-based regimens in 2023.[41]

5. Discussion

Although numerous treatment strategies have been implemented for treatment and management of HIV infection, they also present with their own advantages and limitations.

Protease inhibitors (PI) have multiple advantages including being able to reduce viral load and having high efficacy and bioavailability. Nonetheless, protease inhibitors also come with their set of disadvantages. One major limitation of PI is that cause a series of adverse effects, one being lipodystrophy syndrome – this is caused due to the inhibition of two proteins that play a crucial role in lipid metabolism. PI may bind to proteins, disrupting lipid metabolism, leading to fat redistribution and hence changes in the body's appearance.[42] Another adverse effect is that it can cause

hyperlipidemia, which involves a drastic increase in the patients' triglycerides and LDL cholesterol levels. The use of protease inhibitors also causes insulin resistance. This is because the drugs like Indinavir for example can lead to the disruption of the GLUT4 transport activity of glucose hindering glucose metabolism which leads to increased levels of glucose in the blood, and hence the development of exacerbate diabetes.[43] Another drawback is that they alter the cytochrome p450 enzyme system in the liver, specifically CYP3A. This presents itself as a disadvantage, due to them being able to increase or decrease the levels of co-administered drugs, like blood thinners, statins and antiarrhythmics leading to potential toxicity and low efficacy. Prolonged liver damage, with patients co-infected with Hepatitis B or C is a severe side effect.[44] Prevalent risks associated with the frequent use of PIs include- complexity when it comes to dosing regimens as not all protease inhibitors have been simplified into simple pill consumption, with some having to be still taken with food, which hinders the systemic absorption of the drug, reducing its efficacy, due to the action of GI resident enzymes.[45] Another risk includes the development of resistance to PIs - caused due to prolonged use, albeit slow resistance gradually builds over time. Another disadvantage is that protease inhibitors are a costlier means of treatment making it hard to access in resource-poor countries. PIs also impact the patient's quality of life due to the possibility of addiction.

Despite the significant findings, the limitations of the treatment methods have been increasing awareness in the medical industry. From the therapeutic standpoint, NRTIs used to treat HIV infection like Zidovudine (AZT), Zalcitabine (ddC), Didanosine, Stavudine and Lamivudine (3TC) serve as crucial medications with features that include significant long-term patient use of therapy, a growing patient population and absence of competition from preventive or curative vaccines soon. However, some reports report profound clinical side effects like mitochondrial dysfunction. Studies depict that mitochondrial toxicity (MT) is linked to impaired mitochondrial DNA (mtDNA) replication. Biochemically, this is associated with reduced levels of mtDNA, mitochondrial RNA (mtRNA), mitochondrial polypeptides, and abnormalities in mitochondrial ultrastructure, which align with micromolar mixed Kis values for dideoxy-NRTI triphosphates in various experimental models. Furthermore, NRTIs are associated with liver toxicity, hepatomegaly, steatosis and adult Reytts syndrome. Stavudine has proven links to painful peripheral neuropathy with acetyl-L-carnitine, lipodystrophy and adipocyte apoptosis. Among the frequently administered NRTIs, Lamivudine and Emtricitabine are notable for their comparatively good safety profile which is used in combination with other drugs in HAART regimens to prevent the emergence of HIV resistance mutations. While muscle toxicity associated with Lamivudine has been observed in clinical settings, there is currently no evidence of its toxicity in in vivo monotherapy studies. Emitracibine remains in observation to see if long-term toxicity is to occur in the future. Additionally, the toxicity caused by other NRTIs includes the inhibition of adenylate kinase, adenine nucleotide translocator, NADH oxidase activity, protein glycosylation, and the occurrence of a bystander effect. Nevertheless, clinical studies are not clear in extreme cases where the mortality and morbidity were severe.[46,14]

Referring to integrase inhibitors, both RAL and DTG are generally well tolerated, with low incidences of adverse effects.[47] RAL is associated with mild side effects such as headache and gastrointestinal symptoms but has minimal long-term safety concerns. DTG has shown excellent tolerability but is occasionally linked to weight gain and potential neural tube defects when used during early pregnancy, requiring careful consideration in specific populations. DTG has demonstrated superior viral suppression in clinical trials compared to RAL, particularly in patients with

prior treatment failure or integrase resistance. In addition, DTG's higher genetic barrier to resistance and pharmacokinetic profile contributes to its durability as a long-term treatment option.[48]

The Berlin and Geneva Patient case studies highlighted the importance of stem cell therapy in eliminating cancer cells and HIV viral load. The transplanted healthy stem cells establish themselves in the bone marrow, generating new blood cells.[27,29] However, stem cell therapy in HIV patients has several drawbacks that limit its widespread application. A major concern is the risk of graft-versus-host disease (GVHD) in allogeneic transplants, where donor cells may attack the patient's tissues, leading to organ damage as experienced by the Geneva patient. Additionally, there is always a possibility of cancer relapse in patients with both HIV and cancer, as the therapy might not eliminate all cancer cells. The procedure is also highly expensive and requires specialized facilities, making it inaccessible to many patients, particularly in low-resource settings. Another limitation is the intensive preconditioning required, such as high-dose chemotherapy or radiation, which can severely weaken the immune system, making the body more prone to infections. Recovery from stem cell therapy is often prolonged, with patients remaining vulnerable to complications for months or even years. There are challenging circumstances in finding compatible donors for allogeneic transplants like in Timothy's case study which required a CCR5-Δ32 mutation. The latent HIV reservoirs in the body remain a significant barrier to a complete cure. These reservoirs are unaffected by ART and can be reactivate if treatment is stopped. Ultimately, these cases provide valuable insights into the complexity of stem cell therapy for HIV cure which is believed to be resolved with advancements in future research.

Despite the cutting-edge technology of CRISP/Cas 9 systems, it poses various disadvantages to the recipients. A major concern of CRISPR/Cas 9 technology is its tendency to cause unintended gene mutations and chromosomal translocations. Significant off-target cleavage has been primarily observed in single-guide RNAs containing six or more mismatches. Strategies that have been developed to enhance the specificity and reduce the off-target cleavage include the development of dimerization-dependent RNA-guided FokI-dCas9 nucleases, limiting Cas9 expression in HIV-infected cells via a minimal HIV-1 promoter activated by Tat or delivering Cas9 as ribonucleoproteins (RNPs). However, the ribonucleoproteins delivered are known to trigger innate immune responses causing cytotoxicity. Furthermore, the escape mechanisms of HIV are conducive to reducing the long-term efficacy of CRISP/Cas 9 systems. Although CRISP/Cas 9 inhibits the viral replication of HIV, the mutations induced by non-homologous end joining repair around the cleavage sites facilitate the escape of the viral proteins[32].

6. Conclusion

Over the years the treatment for HIV has evolved to address the specific etiologies of the life cycle of the HIV. This review article discussed case studies of patients diagnosed with HIV-AIDS and their respective treatments. Amongst the treatment discussed above it's evident that so far, the most successful treatment has been the transplantation of stem cells with the CCR5Δ-32 mutation. However, medicinal drugs such as PIs, NNRTIs/NRTIs and integrase inhibitors are still prescribed during the early stages of HIV-AIDS as part of ART. Although the employment of CRISPR/Cas 9 technologies has been rare due to their complications and inaccessibility, these systems have been successful in most cases. While the complete eradication of HIV appears to be a formidable challenge, the use of proper treatment and management since the early stages of diagnosis ensures a higher quality of life and better overall well-being for the patients.

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A study of the factors that affect virtual teamwork engagement during the COVID-19 pandemic in reference to ABC (Private) Limited

Chamari Anushka[1], Khwaja Abdul-Cader[1*]and Zahra Nawfer[1]

[1]School of Management, BMS Campus, Colombo 6, Sri Lanka

*abdul.c@bms.ac.lk

Abstract

Due to globalisation and technological advancements, there is a tendency for companies to change the working environment from a physical one to a virtual one. With the Covid-19 outbreak, many organisations started conducting their businesses virtually. The employees of these organisations began to work as virtual teams by locating in different geographical areas. ABC (Private) Limited, a pioneer in the BPO sector of Sri Lanka started working virtually during the pandemic. Teamwork plays an important role at ABC and the formation of virtual teams has greatly impacted employees' engagement in terms of communication, knowledge sharing, social factors, and training. This study examined the current situation at ABC to identify the impact of working in a virtual environment on teamwork engagement. Primary data was collected by interviewing 14 employees from different hierarchical levels. Purposive sampling was used to select the employees. A qualitative study was conducted using an inductive approach. The findings of the thematic analysis conducted indicate that technology, communication, trust, accountability, and training affect virtual teamwork engagement. This study found that these factors need improvement for the smooth functioning of the teams and to improve efficiency. This study has provided recommendations on how to overcome the issues identified.

Keywords: Virtual Teams, Team Engagement, Remote Work, Teamwork, Covid-19 pandemic

1. Introduction

1.1 Background to the study

A virtual team is a group of people who work collectively by staying in different geographical locations to contribute to a common project with the support of information and communication technologies (Ale Ebrahim, Ahmed & Taha, 2009). The definition of a virtual team implies that the key requirement for virtual team formation is communication technology. Thus, with the advent of the Internet, many companies worldwide initiated a virtual workplace environment by considering its advantages to employees and employers.

During the last 20 years, virtual teamwork has shown growth due to the evolution and development of the digital era (Garro-Abarca, Palos-Sanchez, & Aguayo-Camacho, 2021). However, according to the study conducted by Brett, Behfar & Kern (2006), people tend to think that virtual communication is not as effective as physical face-to-face communication. Due to this, virtual teams were not popular among organisations and they continued with traditional work environments. However, with the emergence of the Covid-19 pandemic in 2020, many companies in the world, started using virtual workplaces considering safety, and the lockdown.

1.2 Organisational overview

ABC (Private) Limited is a business processing outsourcing (BPO) company, specialising in the Information Technology (IT) sector. ABC's vision is to support Industry 4.0 of Sri Lanka and to be a global leader in innovation. ABC has expanded throughout four service sectors: artificial intelligence (AI) solutions, data services, engineering services, and managed services, working with 11 different industries.

1.3 Research Problem

At ABC, teamwork plays a major role as every team is given a predefined target to be achieved every month. Several departments work together to achieve the given target. Hence, teamwork plays an important role in the company. Due to the pandemic in Sri Lanka, ABC started working virtually in April 2020. Though ABC is an IT-based company, its technical facilities were insufficient to support a virtual workplace. Thus, several issues and interruptions were created in the business process during the pandemic.

An employee of ABC works for 8 hours daily and ABC maintains an average of 94%-98% of output quality. Figure 1 depicts the change in work hours before and after shifting from a physical to a virtual environment. Figure 2 shows the variation in the output quality. According to the statistics, there is a drastic decrease in the output quality and an increase in the working hours. Therefore, it is important to study the reasons for this decline in quality and increase in work hours during the shift to a virtual work environment.

Figure 1. Employee work hours (May - July 2020)

Figure 2. Output quality (May - July 2020)

1.4 Research Aim

This study aims to examine the factors that affect virtual teamwork engagement during the Covid-19 pandemic in reference to ABC (Private) Limited.

1.5 Significance

Through the research findings, ABC's management can initiate strategies to improve the quantity and quality of the work output even in a virtual work environment. This research will also contribute to the limited studies on virtual teams in Sri Lanka by providing valuable information about the factors influencing effective teamwork engagement in a virtual environment.

1.6 Scope

14 employees of ABC (Private) Limited including all the hierarchical levels were interviewed to obtain their views on teamwork engagement in a virtual work environment.

2. Methodology

2.1 Research Design

The research onion model was developed by Saunders, Lewis, and Thornhill (2007) and it describes the stages that have to be completed by a researcher to develop an effective research methodology (Melnikovas, 2018; Raithatha, 2017). Due to the adaptability feature of the research onion, it can be used in developing research methodologies in different fields (Bryman, 2012). Hence, by considering these facts, the researcher is using a research onion model to develop the methodology for the research.

Saunders, Lewis, and Thornhill (2007) identified three main research approaches deductive, abductive, and inductive. An inductive approach is an approach that is moving from specific to general (Bryman & Bell, 2011). By starting from the observations, the patterns are searched within the data collected (Beiske, 2007). This study used the inductive approach.

2.2 Methodological Choice

The qualitative method uses descriptive data and aims to study the respondents' explanations of their reality (Bryman & Allen, 2011; Melnikovas, 2018).

Since this research is about teamwork engagement, it is important to gather rich data on the participants' feelings, opinions, and experiences. The researcher needs to avoid pre-judgment within the data collection process and focus more on collecting different viewpoints of the participants. This study therefore used the qualitative research method.

2.3 Research Strategy

Participants' ideas and perspectives on a situation can be explored well by conducting in-depth individual interviews with a small set of interviewees (Boyce, Neale, 2006). This study therefore used interviews as the research strategy.

2.4 Population and Sampling

The population size of ABC (Private) Limited is 1000 employees. This study used the sampling technique for the population of the study. The population was categorised into non-overlapping strata considering their job role and then, a sample of 14 was selected from each stratum.

2.5 Data Collection Method

The data collection involved face to face interviews with the participants.

2.6 Data Analysis Method

This study used thematic analysis to analyse the primary data. Thematic analysis is an analysis method used for qualitative research and it analyses the data gathered through interviews in an organised, detailed, and comprehensive manner.

3. Analysis and Findings

Interviews were conducted using a semi-structured questionnaire for the selected employees representing different hierarchical levels. The interviewees were allowed to express their ideas and suggestions freely with the assurance of not disclosing their identity or the interview results to the company. The interviews provided the researcher with valuable data due to the flexibility, and confidentiality practiced during the interviewing process. Table 1 illustrates the summary of the profiles of the interviewees.

Table 1. Profiles of the Interviewees

Interviewee	Job Role	Service Period
A	Associate Image Processor	1.5 Years
B	Associate Image Processor	4.5 Years
C	Associate Image Processor	3 Years
D	Quality Checker	5 Years
E	Quality Checker	2 Years
F	Quality Checker	1 Year
G	Team Leader	7 Years
H	Team Leader	4.5 Years
I	Team Leader	5 Years
J	Lead Business Analyst	7 Years
K	Business Analyst	9 Years
L	Business Analyst	5 Years
M	Project Manager	9 Years
N	Project Manager	6.5 Years

3.1 Technology and Virtual Teamwork Engagement

ABC has used several software applications during the pandemic period to continue the smooth functioning of the company's business process. The applications used by the teams are Email, Microsoft Teams, Telegram, WhatsApp, Zoom, and Virtual Network Computing (VNC). It allowed the employees to share the screen simultaneously while communicating

through mobile phones. The interviewees agreed that the presence of VNC allowed them to engage more as a team and reduce the occurrence of numerous unnecessary misunderstandings. This supported the view of Straus and McGrath (1994), on the similarity between technology-driven teams and teams working face-to-face on simple tasks.

According to the feedback given by the interviewees, it can be concluded that the technology has given significant support to maintain the smooth functioning of ABC's business process. However, few interviewees suggested upgrading the provided technical equipment to improve their efficiency further. Furthermore, a suggestion to conduct video conferencing was raised by the Quality Checkers and Project Managers. They believed that using video conferencing may help to keep the team more engaged.

Participant A stated, "Technology helped us a lot in getting clarifications from the Business Analysts. Screen recordings were used to store the conversation for future reference."

Participant D stated, "Technology helped us a lot when working as a virtual team. It allowed us to access software and applications easily. It was like all the required supportive tools were placed at our fingertips. It helped us to save time and to work more efficiently."

3.2 Communication and Virtual Teamwork Engagement

At ABC, team connectivity is highly required to receive a quality output. Hence, communication played a substantial part during the COVID-19 pandemic to maintain the interconnectivity among team members. Since most of the team members are youngsters, communication through social media platforms was convenient and reliable for the users. As proven by Fish, Kraut, Root, and Rice (1993), informal communication is essential for successful collaboration of a team. Furthermore, they received a chance to improve their formal communication techniques due to the virtual working environment and they appreciate the chance they received to learn professional communication platforms introduced by the company, like Zoom, Microsoft Teams, etc. Similar to the view of Shaik and Makhecha (2019), frequent formal communication has encouraged them to achieve their defined objectives.

However, contrasting viewpoints were collected from the interviews conducted with the employees in upper hierarchical levels such as Business Analyst, and Project Managers. From their interviews, it was revealed that several issues arose due to the communication conducted while working virtually. Frequent occurrences of technical failures, the absence of a proper communication platform, and the lack of technical support given by the company were the main issues generated during the pandemic period. Moreover, they encountered difficulties in contacting the team members in case of emergencies and it created an unnecessary distance between the colleagues. A few suggestions were presented by the participants during the interviews by addressing the issues they faced, due to lack of communication during the pandemic period.

Participant B suggested developing a procedure to have group discussions on making decisions whenever required to bring everyone to the same page. On the other hand, participant J suggested implementing an effective internal communication platform to increase engagement among the team in terms of virtual communication. Furthermore, participant N provided his idea of having frequent one-to-one sessions between the team members to keep everyone engaged in the achievement of team objectives.

Participant F stated, "We had a very effective communication method throughout the pandemic period and it helped us a lot to complete our work more efficiently. We never got the feeling that we were isolated. We used to have casual chats as well. So, I think that we had a very engaged team even during the pandemic period thanks to the power of communication and technology."

Participant N stated, "Though the communication channels used during the pandemic were not very advanced, we managed communicate well. Also, we can say that almost everyone's communication skills were improved due to this pandemic situation.

However, we had difficulties in contacting the team members in case of emergencies".

Participant L stated, "The absence of physical communication leads to the formation of complicated, sometimes unrealistic issues while working virtually. Maybe the trust issues among the team members have led the path for creating such useless issues."

3.3 Trust and Virtual Teamwork Engagement

Trust among the team members is important for proper team work. The interviewees agreed that team trust was good during the pandemic period. Panteli and Sockalingam (2005) found that conflict reduction is easier where the team member trust each other. The interviewees said that trust helped them to achieve their allocated targets more efficiently.

However, contrasting ideas on team trust and transparency among colleagues while working in the virtual environment were given by several other participants. The lack of transparency between departments and miscommunications resulted in the loss of trust among the team members. However, the majority of the participants agreed that there were no considerable issues that affected team engagement in terms of team trust and transparency while working virtually.

Participant I stated, "Normally our team has a greater bond between each other and I believe that I always have to maintain that trust in the team as their leader. Having such a strong, effective team bond resulted in our team achieving the allocated targets quicker during the pandemic period than when we worked physically at the office."

Participant B stated, "Actually, there were some trust issues that happened during the pandemic period. From my point of view, I think that not giving respect for each other's ideas and suggestions resulted in the occurrence of these trust issues. Some didn't respect the value of the other's job role and they tried to get their work done even by pressuring the other party."

Participant N stated, "Yes, there was a lack of transparency between the different departments while working virtually, since we weren't able to have effective communication during that period. Some miscommunication happened on several occasions and they caused some problems."

3.4 Training and Virtual Teamwork Engagement

Training employees has a considerable effect on the team performance of ABC. Proper training is essential for an employee to perform well. Thus, the effectiveness of training is an important factor for all the team members of a team. However, from the data gathered from the interviews, the lack of effective training was thought to have affected team engagement.

Lack of behavioural communication such as facial expressions affected the trainers in depicting the trainees' level of understanding of the content. Clarity of the content delivered during the training is doubtful as unlike in physical training sessions, the trainers cannot question the participants on the content discussed in virtual training effectively. The trainees also cannot ask questions from the trainers or receive instant answers from them during virtual training. Moreover, no excitement is present in virtual training as the trainers cannot do any extra activities to increase the teams' engagement.

Some positive feedback was given by some of the interviewees. As explained by a Project Manager, they were able to save energy, resources, and time by conducting the training virtually. Another point highlighted in Participant B's feedback was that unlike in physical training sessions, they were able to have recordings of the virtual training sessions for future reference. Participant B explained that the availability of recordings helped their team to go through the instructions, even after the training was concluded through group calls and team discussions.

Participant E stated, "Virtual training was not effective, because we could not see the trainees' actions or body language."

Participant K stated, "Virtual training is not effective at all. There was an incident in my project, where we were instructed to conduct a virtual training for a project to fulfill a high-level requirement of the client. However, we were not able to conduct the knowledge-transferring sessions online in an effective way, since the new recruits lacked proper understanding. Since this method was not effective at all, we decided to conduct the training in a physical environment under strict health security considering the pandemic situation."

3.5 Accountability and Virtual Teamwork Engagement

When working as a team, every person should be accountable for their actions, speech, and behaviour. Being accountable in a virtual environment is a critical requirement to maintain the smooth functioning of the process. As a company with a business process that relies on teamwork, it is necessary for ABC to have a team that values accountability. From the analysis of the feedback received from the participants, it was shown that there was a lack of accountability among the team members while working virtually during the pandemic period. Ignorance of duties was visible among the team members and the decrease in accountability resulted in issues related to trust and performance.

Every participant had negative feedback on accountability among team members and its effect on team engagement during the Covid-19 pandemic period. However, the interviewees were unable to make suggestions on how to improve the situation, although they understood the importance of accountability for effective team engagement.

Participant M stated, "I must say that it was not at a satisfactory level. Everyone raised complaints about each other during this period. So, a team without accountability has very low team engagement. The team suffered due to the lack of accountability among some team members."

Participant H stated, "I think that accountability has a connection to a person's moral qualities. As a human, they should understand that they have to be accountable for what they do and say. We have to accept the fact that there are different kinds of people in the world and have to adjust according to the situation."

3.6 Company Processes/Policies and Virtual Teamwork Engagement

The interviews revealed that working virtually considerably affected the company's general processes and procedures. Changes in the working environment have created issues related to misuse of company resources. Some team members blamed technical issues for the lack of work progress.

Participant N stated that the company had issues with securing the confidentiality of company data in a virtual environment. Generally, ABC follows a strict set of rules on data confidentiality, as they are working with a foreign client. Yet, when working virtually, the company had no option except to pass raw data to the workstations, which are located in the employees' residences."

Participant F stated, "The employees of ABC are paid according to the quality and quantity of the output provided. With the change in the working environment to a virtual one, we experienced a greater reduction in the quality of the work we received for reviews. There were situations where the work went through as much as 20 reviews and rework. So, in such situations, even though the employee had worked extra hours, he or she did not get any extra payment, due to the excess time spent on the work. As a team, we have to work collectively to avoid unwanted rework."

Participant H stated, "There was a drastic increase in the work hours of the data entry operators during the pandemic period. The reasons for this were slow connectivity, technical issues, etc. Furthermore, we had issues in monitoring the data entry operator's quality level in the virtual working environment. Furthermore, some were caught not following due process."

4. Conclusion

Working in a virtual environment was a new experience for the entire staff of ABC and they encountered several issues due to this. Due to business processes that relies mainly on teamwork, the teams must maintain a high level of engagement among each other. However, factors such as technology, communication, trust, accountability, change in company processes and policies, and virtual training influenced the teamwork engagement of ABC.

It was clear from the feedback of the interviewees that the usage of technology has significantly affected performance and work quality while working virtually. Screen sharing ability given through the VNC technology mitigated the creation of several unnecessary trust-related issues. However, due to the lack of upgraded hardware and connectivity issues in the virtual work environment, the employees of ABC faced slowness, connectivity issues, and quality drops in the video clips. It directly affected the efficiency and the team members' effectiveness creating several managerial issues on net time and quality of work.

Usage of unfamiliar applications such as Telegram, Zoom, and Microsoft Teams was a challenge. Social media platforms were popular especially among the young staff members. Regular communication reduced the feeling of isolation among the team members and helped to improve team engagement throughout the pandemic. However, technology-related issues affected the effectiveness of the communication channels used by the staff.

According to the interviewees, trust among the team members has a positive influence on the performance and team engagement of ABC. However, working in a virtual environment negatively affected trust. Lack of accountability and technical issues were identified as the causes of distrust among the team members.

Training is a frequent activity conducted for the ABC employees, due to frequent changes in instructions and requirements of the clients. According to the feedback received, unlike in physical training conducted in the past, the participants of the training sessions could not share their ideas and issues effectively in a virtual environment. In physical training, the team worked collectively in sharing their ideas and clearing doubts. Virtual training did not give the same results as physical training.

As mentioned previously, lack of accountability had a close relationship with the absence of trust among the team members of ABC. As mentioned by the interviewees, there was a drastic decrease in accountability among the team members which significantly affected teamwork engagement.

5. Recommendations

Implementing an effective internal communication platform

An effective internal communication platform has to be implemented to enhance communication among colleagues. The team members should not feel isolated, as they are working virtually and they should be able to communicate effectively as they did while working physically. In situations where the employees recognise that they are receiving information from the management, that is timely, accurate, and relevant, they will not feel vulnerable and will depend on colleagues and supervisors (Thomas, Zolin, & Hartman, 2009) Furthermore, the company should guarantee the connectivity level of the communication channels as interrupted communication platforms might create further issues rather than improving team engagement.

Regular one-to-one sessions with team members

The team members of ABC are used to working closely with their colleagues as well as their superiors. They received daily progress on their performance through their superiors and it helped the team members to trust their colleagues for their support to improve their performance. According to Gallup (2022), engagement is three times higher among the employees who are having regular meetings with their superiors than the ones who do not. With the change in the work environment to a virtual environment, they missed the chance of receiving regular feedback. Hence, to maintain the engagement among the superiors and team members, it is recommended that ABC conduct regular one-to-one sessions to mitigate the

feeling of isolation and to increase trust among the team members.

Feedback and reviews on daily production output

It is recommended that the supervisors should provide their feedback to the production team on a daily and weekly basis to guide the team on their work. Conducting these review sessions will help ABC to improve accountability of the employees by increasing productivity, motivation, and team engagement. Moreover, individual-based discussions on daily output levels help to improve mutual understanding among the team members. Mesmer-Magnus, and DeChurch (2009) have stated that collective knowledge sharing is effective in problem solving.

Video conferencing sessions for training programmes

Since the virtual work is a new experience to the employees of ABC, the lack of physical presence of the colleagues during training sessions has generated a feeling of being isolated among the team members. Zoom video conferencing creates a socially positive learning environment by allowing learners to interact with each other (Sutterlin, 2018). Hence, it is suggested that conducting Zoom video conferencing training sessions will help to improve engagement for training.

Upgrading the hardware/software

The hardware and software of ABC have to be upgraded to meet the demands of a virtual work environment. Using the latest versions of the software will help the teams to be more productive and efficient. New or upgraded hardware will reduce issues such as slowness, network errors, and connectivity issues.

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A Study of the Impact of Democratic Leadership Style on Employee Engagement at ABC Company

Jedrick N. Sellar[1] and Khwaja Abdul-Cader[1]

[1]School of Management, BMS Campus, Colombo 6, Sri Lanka

*abdul.c@bms.ac.lk

Abstract

This research aims at examining the impact of democratic leadership on engagement of employees in ABC Company with special reference to leadership behaviour including communication, honesty, integrity, and involvement of employees. The study seeks to determine the effects of these leadership characteristics on motivation, organisational commitment, and emotional attachment to organisational objectives. A structured questionnaire was used to capture the employees' perception on leadership and its impact on their level of engagement. The study also reveals that democratic leadership improves the level of engagement by embracing openness, cooperation, and respect for the employees. Managers who promote open communication, engage subordinates in decision making processes and consider their opinions foster organisational commitment, increase employees' motivation, and ensure that they are on the same page with the organisation. However, the study also reveals some areas that require enhancement: the provision of frequent constructive feedback and guaranteeing that all employees are both active and responsible. Therefore, it is crucial to understand that democratic leadership is essential for increasing the engagement level among employees. Although the leadership style enhances motivation and emotional commitment, more efforts are needed to ensure that all employees gain from these practises. The outcomes of this study are useful for organisations that want to achieve increased levels of engagement with the help of leadership that focuses on openness, communication, and employees' participation.

Keywords: Democratic Leadership, Employee Engagement, Organisational Performance, Employee Motivation.

1. Introduction

1.1 Background of the Study

The role of leadership styles in relation to employee engagement has been a topic of interest in many studies, showing a significant effect on organisational outcomes and employee well-being. Leadership, as the practice of leading and directing people towards the accomplishment of shared objectives, is a critical determinant of the work climate and organisational culture (Northouse, 2018). Of all the leadership styles, democratic leadership has received attention because of the positive impact it has on employee engagement (Jaskiewicz and Klein, 2007). Democratic leadership, which involves the engagement of employees in decision making, ensures that the employees are committed to the goals of the organisation and hence increases the level of engagement (Klein *et al*., 2013 and Berson *et al*., 2001).

Employee engagement, i.e. the willingness of the employee to invest discretionary effort to work in a manner that supports the organisation, is vital for organisational performance (Kahn, 1990). Research has revealed that employees who are dedicated to their work are more efficient, satisfied by their jobs and have a strong bond with their employers, thus improving the performance and stability of the organisation (Bakker and

Demerouti, 2007). The democratic leadership style, which involves the employees in decision making, follows the theories of empowerment and shared control that are essential for engagement (Yukl, 2013). Research has pointed out that when the leadership is democratic, the morale of the employees is enhanced, and they feel that they are part of the organisation and are responsible for the achievement of organisational goals (Klein *et al*., 2013; Jaskiewicz and Klein, 2007; Berson *et al*., 2001).

1.2 Rationale

There are different leadership styles at ABC Company. The contrast of the autocratic and democratic leadership styles within the organisation provides the basis for comparison of the two approaches. While autocratic leadership entails decision making and power in the hands of the leader, democratic leadership is more participative and involves the subordinates (Bass and Bass, 2008). Although autocratic leadership enhances fast decision-making, it has negative impacts on the morale and motivation of the employees since they are not involved in the decision-making process (De Hoogh and Den Hartog, 2008).

The research aim of this study is to test the hypothesis that democratic leadership is more effective in engaging the employees of ABC Company. The hypothesis is based on prior research, which indicates that the democratic leadership style, that is based on cooperation and inclusion, leads to the increased level of the employee's commitment (Klein *et al*., 2013; Jaskiewicz and Klein, 2007; Berson *et al*., 2001).

It is not only important to comprehend the nature of leadership and its effects on engagement of the employees at ABC company on an academic perspective but also from a practical point of view. Harter, Schmidt, and Hayes (2002) mentioned that employees that are engaged are likely to participate in innovation, quality service delivery, and customer satisfaction, which are essential for sustaining competitive performance.

Moreover, the study aims to provide actionable insights for leaders and managers at ABC Company, enabling them to adopt leadership practices that enhance employee engagement. By identifying the key attributes and behaviours of democratic leaders that contribute to higher engagement levels, the findings of this research can guide leadership development programmes and organisational policies aimed at adopting a more inclusive and supportive work environment within organisations.

1.3 Research Aim

The primary aim of this research is to analyse the impact of democratic leadership style on employee engagement at ABC Company.

1.4 Research Scope

This research will focus on 150 employees of ABC Company and their views on leadership styles. This study will focus on a particular Strategic Business Unit (SBU) operating during the night shift.

2. Methodology

2.1 Research Design

This study used the quantitative research method.

2.2 Conceptual Framework

The correlations between various leadership characteristics and employee engagement are considered. The independent variables include collaborative leadership, team player, flexibility, transparency, and communicative leadership, with employee engagement as the dependent variable as illustrated in Figure 1.

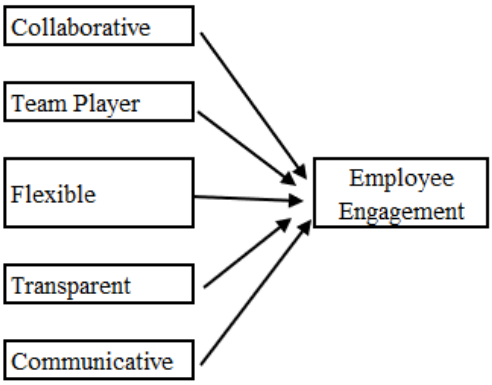

Figure 1. Conceptual Framework

2.3 Hypotheses

H1: A collaborative leader has a positive impact on employee engagement.

H2: A team player leader has a positive impact on employee engagement.

H3: A flexible leader has a positive impact on employee engagement.

H4: A transparent leader has a positive impact on employee engagement.

H5: A communicative leader has a positive impact on employee engagement.

2.4 Population and Sample

The population for this study comprises 150 employees who work the night shift across various departments in a Strategic Business Unit (SBU) within the company. This research targeted a sample size of 120 employees from the night shift population. The convenience sampling technique was used.

2.5 Data Collection

This research used an online survey using Microsoft Form. The survey used a 5-point Liker scale based structured questionnaire.

2.6 Data Analysis

SPSS software was used to analyze the respondent's feedback. Correlation and multiple linear regression analysis were conducted.

3. Analysis and Findings

3.1 Demographic Data

Table 4. The demographic data of the respondents

Criteria	Description	Percentage (%)
Gender	Male	56.60%
	Female	43.40%
Age	18-25 Years	45.90%
	26-35 Years	51.60%
	36-45 Years	2.50%
Department	Billing	24.60%
	Collections	46.70%
	Denial Management	2.50%
	Posting	4.10%
	Other	22.10%
Designation	Associate	9.80%
	Operations Analyst	20.50%
	Senior Operations Analyst	24.60%
	Team Lead/ATL	38.50%
	Manager and above	6.60%
Years of Experience	Less than 1 Year	9.80%
	Between 1-3 Years	41.80%
	Between 4-5 Years	37.70%
	Between 6-7 Years	7.40%
	8 Years and above	3.30%

3.2 Data Validity and Reliability

Cronbach's Alpha was used to measure internal reliability of the primary data. A Cronbach's Alpha value above 0.7 is considered acceptable for social science research (Hair et al., 2010).

The table below summarizes the Cronbach's Alpha values for the variables in the study.

Table 5. Cronbach Alpha

Variable	Cronbach's Alpha
Collaborative Leadership	0.792
Team Player Leadership	0.909
Flexible Leadership	0.79
Transparent Player Leadership	0.881
Communicative Leadership	0.894
Employee engagement	0.895

All variables demonstrate strong reliability, with Cronbach's Alpha values exceeding the threshold of 0.7. This confirms that the questionnaire items are measuring their respective constructs consistently, ensuring the validity of the data for further analysis.

3.5 Correlation Analysis

Pearson's correlation was used to assess both the strength and direction of the relationships between the independent and dependent variables.

Table 6. Correlation Analysis

Independent Variable	Pearson Correlation Coefficient	Sig. (5%)
Collaborative Leadership	0.583	0.000
Team Player Leadership	0.607	0.000
Flexible Leadership	0.703	0.000
Transparent Leadership	0.703	0.000
Communicative Leadership	0.704	0.000

A shown in Table 6, the significance values for all the leadership styles are less than 0. 05 (p = 0. 000), which means that there is a significant relationship between each of the leadership styles and employee engagement. This supports the research hypotheses that leadership styles have a significant effect on the level of employee's engagement at ABC Company.

The coefficients vary from 0.583 to 0.704, which indicate a moderate to strong positive correlation between the leadership styles and the level of engagement of employees.

These coefficients indicate that the most significant positive effect on engagement is provided by such leadership attributes as flexibility, transparency, and communication. This is in line with earlier studies that have pointed out the need to communicate, be transparent and be flexible to engage the employees (Macey and Schneider, 2008; Bakker and Demerouti, 2007).

3.6 Hypotheses Validation

Based on the correlation analysis, all the hypotheses formulated in this study are valid.

The findings show that Flexible Leadership, Transparent Leadership, Communicative Leadership, Collaborative Leadership, and Team Player Leadership are positively related to the level of engagement, thus supporting the hypotheses.

The correlation results of the study are in line with the previous research, especially the works of Macey and Schneider (2008), Bakker and Demerouti (2007), and Goleman (2000). The high coefficients of Flexible, Transparent, and Communicative Leadership are consistent with the literature, which points to these styles as being associated with increased engagement levels of employees. The first advantage of the approach is flexibility that enables leaders to meet the needs of the employees, secondly the aspect of transparency that fosters trust. Finally, the aspect of open communication that boosts

the motivation and commitment of the employees. Collaborative and Team Player Leadership also show positive correlation, although not as high as in the case of the other dimensions, which also corroborates Goleman's (2000) conclusion that inclusive and participative leadership enhances employee commitment, but to a lesser extent.

Therefore, all hypotheses are supported, thus affirming the contribution of leadership styles in increasing employee engagement. These results support the need to implement leadership behaviors that promote communication, flexibility, and openness to increase employee engagement.

3.2 Multiple Linear Regression

Table 7. Multiple Linear Regression Analysis

Independent Variable	Beta	Sig. (5%)
Collaborative Leadership	0.228	0.028
Team Player Leadership	-0.528	0.001
Flexible Leadership	0.27	0.045
Transparent Leadership	0.289	0.010
Communicative Leadership	0.577	0.000

The multiple linear regression analysis presented in Table 7. highlights that all the variables have a significant influence on employee engagement. All the variables, except for Team Player Leadership show a positive relationship with employee engagement. The negative relationship contrasts with other studies like those of Goleman (2000) where teamwork and cooperation had a positive impact on employee engagement. The negative Beta value can be due to the influence of confounding variables or due to multicollinearity.

3.3 Descriptive Analysis

Table 8. Degree of Satisfaction

Variable	Mean
Collaborative Leadership	3.94
Team Player Leadership	3.99
Flexible Leadership	3.91
Transparent Leadership	4.02
Communicative Leadership	4.01
Employee Engagement	3.87

Table 8 displays the mean scores for the selected leadership styles at ABC Company. Transparent Leadership has the highest mean score of 4.02, suggesting that leaders at ABC Company are considered to be transparent. Transparent communication is important in fostering trust and engagement (Men et al., 2020).

Communicative Leadership follows closely with a mean of 4.01, indicating that the leaders at ABC Company are considered to be communicative. Leaders who effectively communicate are highly valued (De Vries *et al*., 2010).

Team Player Leadership has a mean of 3.99, which means that the leaders at ABC Company are considered to be ream players.

Collaborative Leadership and Flexible Leadership, with means of 3.94 and 3.91 respectively, indicate that the leaders at ABC Company are considered to be collaborative and flexible.

Lastly, Employee Engagement has a mean of 3.87, which suggests a satisfactory level of employee engagement at ABC Company. This supports prior findings that democratic leadership directly impacts engagement levels (Shuck and Herd, 2012).

4. Discussion and Conclusion

The research findings of this study indicates that the democratic leadership style leads to higher employee engagement. The findings highlight the importance of communication, flexibility, and openness in engaging with employees. These findings support previous research on the role of democratic leadership style on employee engagement.

The employees surveyed are satisfied with the democratic leadership traits of their supervisors and display good employee engagement.

4.1 Recommendations

Based on the research findings the following recommendations can be made.

Enhance Open Communication

Leaders should promote open dialogue through multiple communication channels, including formal and informal platforms. Regular feedback sessions and open forums will foster a culture of transparency and inclusivity.

Reinforce Transparent Leadership Practices

Leaders should explain the rationale behind decisions and policies. Sharing updates on company performance and strategy will help build trust and align employees' efforts with organisational objectives.

Adopt a Flexible Leadership Approach

Leaders must be adaptable by offering tailored support, recognizing individual contributions, and fostering autonomy. This will create a more engaged and dynamic workforce.

Review the Role of Team Player Leadership

Leaders should ensure that teamwork does not undermine personal responsibility. A balance between collaboration and individual accountability will enhance both team performance and employee engagement.

Increase the Frequency and Specificity of Constructive Feedback

Leaders should provide clear, actionable, and frequent feedback. Recognising achievements while addressing areas for improvement will foster a growth-oriented culture.

Promote Ownership and Accountability

Leaders should implement programs that encourage autonomy and innovation. By fostering a sense of ownership, employees will feel more invested in their tasks and in the overall success of the organisation.

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Challenges Faced by Licensed Finance Companies in Complying with Anti-Money Laundering and Counter Financing Terrorism: A Study on Balancing Global Standards with Local Realities in Sri Lanka

H. A. L. Anthony[1], K. Abdul-Cader[1*] and U. Rajapaksa[1]

[1]School of Management, BMS Campus, Colombo 6, Sri Lanka

*abdul.c@bms.ac.lk

Abstract

International business management must address major topics like money laundering and terrorist financing, as they can significantly damage a nation's economy. Therefore, this study investigates the challenges in combating money laundering (ML) and terrorist financing (TF) within the licensed finance sector in Sri Lanka. The research aims to examine Sri Lanka's legal statutes and regulations directly related to AML/CFT, analyse the challenges faced by finance companies in implementing these measures, and offer recommendations for enhancing these methods. Utilizing an inductive approach and a case study strategy, this qualitative research involved face-to-face interviews with 10 senior management members from various Licensed Finance Companies and the Financial Intelligence Unit, employing purposive sampling techniques. Key statutes reviewed include the Financial Transactions Reporting Act, alongside the roles of regulatory authorities. Findings reveal significant inefficiencies in Customer Due Diligence (CDD) processes, manual sanctions screening, and the quality of Suspicious Transaction Reports (STRs). Notably, while thorough investigations are conducted, the lack of feedback from the Financial Intelligence Unit (FIU) limits the effectiveness of STR submissions. Recommendations include the development of a centralized customer screening system managed by the FIU, public awareness campaigns, and mandatory compliance training for key personnel. The study highlights the need for further research to address branch-level challenges and to broaden the focus across the entire banking and finance sector. This research offers actionable insights to enhance regulatory compliance and improve financial crime prevention measures in Sri Lanka.

Keywords: Anti-Money Laundering, Counter Financing Terrorism, Customer Due Diligence, Sanction Screening, Transaction Monitoring, Suspicious Transactions Reports

1. Introduction

1.1 Background of the Study

The act of illegally disguising the source of funds acquired through criminal activities including drug trafficking, corruption, fraud, or gambling by transforming them into funds from a legitimate source is known as money laundering (ML) (Isa, Sanusi, Haniff & Barnes, 2015). Likewise terrorist financing (TF) entails two types of financial activities: generating money for propaganda and training and funding terrorist actions. Therefore, these are mostly "pre-crime" actions, and because of their low monetary worth, they are hard to identify. However, ML prevention is easier than identifying TF (Clunan, 2007).

The Financial Action Task Force (FATF) provides anti-money laundering (AML) and counter-terrorist financing (CFT) recommendations, which are based on 39 member countries' national frameworks. Accordingly, compliance is assessed through mutual evaluation reports (Teichmann, 2024). In order to comply with these international requirements, Gazette Extraordinary No. 1437/24 of 23/03/2006, the Financial Intelligence Unit (FIU) of the Central Bank was

established to be the FIU for the purpose of this Act (Annual Report, 2006). Furthermore, in keeping with worldwide principles and standards, the FIU's primary goal in Sri Lanka is to fight ML, financing of terrorism, and other related crimes (FIU, 2023).

Despite the concerted efforts of Sri Lanka's FIU to align the country's regulatory framework with the standards set by the FATF, many financial institutions continue to struggle with compliance. The persistent non-compliance of these institutions poses significant challenges to the country's international financial connections and export sector. Despite the imposition of tough regulations, institutions still fail to meet the standards set by the FIU, indicating underlying issues that hinder their ability to adhere to regulatory requirements.

1.2 Industry Overview

Finance Businesses are companies that have been granted permission to do finance business by the Central Bank of Sri Lanka under the Finance Business Act, No. 42 of 2011 (FBA). Currently, 34 financing companies are listed with CBSL (CBSL, 2024). There are four categories for licensed financial firms. Which are based on their assets. (K Seeds Investments, 2024).

1.3 Rationale

Sri Lanka has emerged as a significant hub for illicit financial activities, particularly ML and terrorist financing, with an estimated annual flow of illicit funds amounting to a staggering USD 3 billion (Sivaguru & Tilakasiri, 2023). Investigations have shed light on the undercover operations of shell companies and trusts, which have facilitated the movement of over $18 million into tax havens. Additionally, revelations from Swiss leaks have implicated clients associated with Sri Lanka in transactions totalling $58.3 million (Sivaguru & Tilakasiri, 2023). Such findings underscore the gravity of the situation, compounded by Sri Lanka's dismal standing in Transparency International's Corruption Perceptions Index, where it ranks 115th out of 180 countries (Farzan, 2024). This ranking highlights the urgent need for systemic reforms. Moreover, the recent establishment of the Port City in Sri Lanka has raised apprehensions regarding potential avenues for ML, especially given the significant tax incentives offered within this enclave. Despite these concerns, Sri Lanka's justice minister has been quick to dismiss them, declaring that existing criminal laws and the FIU are sufficient safeguards against ML (Ethirajan, 2022). However, such assurances are met with scepticism, particularly in light of reports from Sri Lanka's FIU, which has rated the country's overall ML/TF risk as "medium." This assessment underscores the presence of significant threats and vulnerabilities within Sri Lanka's financial system (DailyFT, 2023). Moreover, Sri Lanka's central bank has identified high-risk informal money remitters, real estate agents, banking sector, and finance companies as high-risk in ML activity (Economy Next, 2023).

In Sri Lanka, the legal framework for combating money laundering and terrorist financing is mainly based on three legislations; the Prevention of Money Laundering Act (PMLA), No. 5 of 2006, Convention on the Suppression of Terrorist Financing Act, No. 25 of 2005 and the Financial Transactions Reporting Act (FTRA) No. 6 of 2006. These rules are consistent with delegated regulations and guidelines, which updates regularly to address global emerging issues (Jayasekara, Perera & Ajward, 2023). The existing legal framework for combating ML and TF in Sri Lanka's finance sector faces significant challenges and deficiencies in the implementation process. Despite legislative efforts, empirical evidence on the implementation and impact of these laws remains scarce. Furthermore, emerging technological advancements pose new challenges in detecting and preventing financial crimes. By conducting this research, the author seeks to provide valuable insights into the practical challenges faced by finance companies in adhering to AML and CFT regulations. Additionally, this study aims to offer recommendations for addressing these implementation gaps, thereby contributing to

the enhancement of Sri Lanka's efforts in combating financial crimes and ensuring the integrity of its finance sector.

Recent reports highlight significant non-compliance among financial institutions in Sri Lanka, particularly regarding the Financial Institutions (Customer Due Diligence) Rules, including failing to maintain updated UNSCR lists and screen customers. As per the National Money Laundering and Terrorist Financing Risk Assessment (2021/2022), deficiencies in criminal sanctions enforcement and suspicious transaction reporting were identified. Financial institutions also faced penalties for non-reporting of suspicious transactions, violating Section 9(1) of the FTRA. FATF Recommendations emphasize the importance of client due diligence and timely suspicious transaction reporting to combat money laundering and terrorist financing (FATF, 2023; NRA, 2022).

1.4 Research Aim

The research aim of this study is to identify the challenges faced by financial institutions in monitoring and preventing Money Laundering and Terrorist Financing in Sri Lanka.

1.5 Objectives

1. Examine Sri Lanka's legal framework comprehensively to identify the statutes and regulations directly related to combating Money Laundering and Terrorist Financing.

2. Investigate and analyse the challenges encountered by Finance Companies in Sri Lanka when implementing measures to combat money laundering and terrorist financing.

3. Provide recommendations to overcome the identified challenges.

1.6 Significance

By evaluating the effectiveness of the AML and CFT efforts, this study provides a valuable benchmark for these officers to assess the adequacy of their institution's compliance practices. Through a thorough analysis of the legal framework and regulatory measures, compliance officers can gain insights into the strengths and weaknesses of their institution's AML/CFT programs. Furthermore, by taking into account global viewpoints and best practices, this study provides better solutions that not only solve local difficulties but also correspond with worldwide standards.

The findings of this research will provide insights to the fundamental issues that influence the behaviour of financial institutions regarding adherence to regulations. Therefore, by identifying these factors and analysing their impact on AML CFT compliance efforts, this study can enrich the academic discourse on AML CFT compliance and offer valuable insights for future research in this field.

2. Methodology

2.1 Research Design

Inductive research allows the development of new theoretical frameworks based on empirical evidence (Bendassolli, 2013). Therefore, for this research the inductive approach is particularly valuable.

The researcher has used case study strategy to explore the challenges faced by finance companies in Sri Lanka. Furthermore, case study approach allows for a detailed and nuanced understanding of complex phenomena by examining a specific instance in depth (Koster & Fernandez, 2023).

In this study, the authors follow a qualitative method, which is characterized by an in-depth exploration of complex phenomena through non-numeric data (Hay, 2017).

This study employs both primary and secondary data collection methods and primary data is collected through face-to-face interviews using open ended questionnaires administered to stakeholders within Sri Lanka's finance companies, providing first hand insights into AML CFT framework and challenges (Rahi, 2017).

3.2 Population and Sampling

This study employs a non-probability sampling method, specifically purposive sampling. In this study, purposive sampling is crucial for targeting stakeholders within Sri Lanka's finance companies as they have direct experience with regulatory compliance framework.

The population for this study include all compliance officers as well as senior managers or department heads in all the finance companies in Sri Lanka. The author chose seven corporate managers, including four compliance officers and three senior managers. Additionally, the author included a retired compliance officer, an overseas compliance officer, and a director from FIU Sri Lanka.

3.3 Reliability and Validity

Maintaining reliability and validity is critical in a research study to maintain the consistency and correctness of findings (Noble & Smith, 2015). Thus, in this study, reliability was obtained by following standardized questionnaire administration procedures and establishing consistency in data gathering methodologies (Harris & Brown, 2019). Similarly, validity is assured by developing the questionnaire in accordance with the AML CFT framework and triangulating results with secondary data sources (Turner, Cardinal, & Burton, 2017).

3.4 Data Analysis

Castleberry and Nolen (2018) define thematic analysis as a qualitative data analysis technique that identifies, analyses, and reports patterns (themes) within data sets. In this study, thematic analysis will be utilized first to transcribe interview replies, followed by data analysis to identify flaws in the AML CFT Framework.

3. Analysis and Findings

3.1 Demographic data of the Interviewees

Table 1 outlines the diverse professional backgrounds of the participants who were interviewed.

Table 1. Participants' Background

CODE	BACKGROUND
001	Participant 001 is a Senior Assistant Director of the Financial Intelligence Unit Sri Lanka, with 14 years of experience. 001 is also a graduate from BSc Engineering, Masters in Financial Economies, Member of the Institute of Engineering Sri Lanka, and a Certified Assessor for Mutual Evaluation of the FATF.
003	Participant 003 is a Compliance Officer of a Finance Company, with 15 years of experience. 003 is also a graduate from BSc. (Hons) Town & Country Planning from University of Moratuwa, MBus. (Fin) from University of Kelaniya, Diploma in Bank Integrated Risk Management holder and a Life Member of Association of Accounting Technicians of Sri Lanka.
004	Participant 004 is a Compliance Officer of a Finance Company, with 35 years of experience. 004 is also a graduate from BSc (Col), MBA (PIM-SJP), FCMA (UK), CGMA, Dip M(UK) and Diploma in Compliance from IBSL Sri Lanka
005	Participant 005 is a Compliance Officer of a Finance Company, with 16 years of experience and a Chartered Accountant and completed the MBA from PIM.
006	Participant 006 is the Head of Branch Operations of a Finance Company, with more than 25 years of experience. 006 is also a graduate from Sri Jayawardhanapura University.
007	Participant 007 is an Assistant General Manager of a Finance Company, with 14 years of experience. 007 is also a graduate from BSc Public Management (Special) from University of Sri Jayawardanapura, an Asssociate member of The Institute of Chartered Professional Managers of Sri Lanka and Associate member of Institute of Management of Sri Lanka.

008	Participant 008 is a Compliance Officer of an Overseas Authority, with 13 years of experience. 008 is also a graduate from CIPM with CHRM, Diploma in Compliance at IBSL and CAMS Certified.
009	Participant 009 is a Head of Gold Loan Operations, with 19 years of experience. 009 is having a Post Graduate Diploma in Marketing, Advanced Diploma in Marketing Management Diploma in Marketing Management, MBA from Sri Jayawardhanapura University and Member of SLIM.

3.2 Financial Sanctions

Majority of the participants agreed that even though they have proper systems in place for sanctions screening there are still challenges that need to be addressed.

Participant 003 *stated, "It is true that our system is screening the customers against the sanction lists. However, the main challenge that we observe is updating these lists manually to our system. When we are doing that, human errors can be made."*

Furthermore, ***Participant 009*** *stated, "When the customer visits a contact point, the gold loan officers should send the request to the compliance division for clearance of the customer, who will then send an email confirmation, allowing the transaction to proceed manually and this process is really time consuming."*

In addition, ***Participant 004*** *stated, "It is true that we are providing training on sanction screening to all the staff. Even we do annual evaluations, however the problem is this area is not everyone's cup of tea. Sometimes even after many trainings, people still don't know the reason why they are requested to follow these protocols. This has a significant impact on sanction screening, as it is done by the front-line branch staff and they are moving frequently."*

Similarly, ***Participant 005*** *says, "This is an industry concern that the systems available now are very costly and doing sanction screening real time is a bit difficult."*

Likewise, ***Participant 007*** *also believes that, "a modified technological system must be available to handle screening of customers effectively, and full customer information is also required for perfect screening."*

The Regulator stated that "*developing a sanction screening system with reviewing good quality aliases, low quality aliases in the screening and adjusting thresholds is an easy task and using AI technology for this is cheap, effective, and can be done in a fraction of time and cost.*"

Further he elaborated that "*this is not a task for a compliance officer, this needs to be done by the IT staff of the financial institution. All you have to do is to hire some good IT people to the company and give them the correct guidance.*"

The Caribbean FATF (2024), has emphasized the importance of countries implementing targeted financial sanctions to align with United Nations Security Council resolutions. This is due to the consequences of terrorist attacks that are directly creating economic problems (Alvi, 2019). If those channels are not stopped, terrorists will have unrestricted access to finances for transactions, weapon purchases, bomb manufacture, and everyday expenses. That's why the United Nations Act, No. 45 of 1968 appointed as the Competent Authority to overlook the requirements related to sanctions (Competent Authority for Sri Lanka, 2014). The Financial Intelligence Unit discovered numerous instances of non-compliance among financial institutions concerning sanction screening (Annual Report, 2021). Even after deficiencies and gaps were identified by the World Bank, the NRA report indicates that "Availability and enforcement of Criminal Sanctions" needs to be enhanced (NRA, 2022).

Participant 001 stated, *"The international regulators have also criticized us for low penalty amounts where the other countries are charging millions and billions of dollars, Sri*

Lanka is only charging 1 million. Therefore, by the end of this year, the penalties will be substantially increased, causing some pain and reputational damage for companies who are not complying."

Participant 008 stated, *"The initial cost of starting a proper screening system and registering with it may be high, but in the long term, it can reduce hardships and make compliance officers' lives easier. Automated updates of details can also make the process more efficient."*

Furthermore, ***Participant 001*** stated that *"I have developed a screening application in four days as a hobby project, which turned out to be commercial grade level. The application is simple, easy, and takes little effort. It is free and I hope to give it to the Financial Institutions as well."*

Despite the aforementioned recommendations to import automated technologies for the purpose of sanctions screening, human data entry is still required at one point. The issue is that there are a lot of heavy typologies and terms in the compliance area. Therefore, branch officers are under pressure to meet their targets, even if the compliance team conducts several trainings. It is also true, as stated by ***Participant 004,*** that not everyone finds this topic to be equally digestible. Therefore, they occasionally manipulate these requirements in an effort to meet their targets. Additionally, obtaining sanction screening permission requires a lot of work and time in some companies. For instance, a consumer seeking rapid cash may visit the company for a Gold Loan; if the process takes too long, the customer would undoubtedly visit another financial institution. Because of this anxiety, branch level officers attempt to prioritize their sales over meeting requirements the majority of the time. Even though the compliance department works really hard, the entire business will be in danger if the front line made a mistake.

3.3 Customer Identification and Due Diligence

Customer due diligence (CDD) is a fundamental aspect of the AML procedures embedded within the FATF 40 Recommendations (Jun & Ai, 2009). The participants made the following comments about the CDD process.

Participant 003 *stated that, "According to the due diligence rules, it is mandatory to conduct CDD for customers and collect Know Your Customer (KYC) information. Capturing the required information helps to give the correct risk score to customers and builds their profile. Therefore, it is crucial to follow these regulations to provide excellent service and build a positive customer experience."*

Furthermore, ***Participant 004*** *stated that, "Employees receive particular compliance requirement training while they are on boarding, then after a few months and as they become familiar with the process, they receive a full-day training. Aside from that, we have a yearly certification pertaining to general compliance with these requirements."*

Aside from these positive comments, almost all the participants had a major problem with customer education.

Participant 006 *stated that, "When we ask branch staff to obtain documents related to CDD, even the address verification documents, some customers raise serious issues. Most of the customer are reluctant to provide salary slips and other documents. However, there are customers who do not like to give such documents and they look for other financial institutions. Therefore, it is more important for all the financial institutions to follow the guidelines."*

Participant 009 *mentioned that, "Customers' reluctance to divulge all necessary information, such as their employment address and positions, is an impediment. This is a problem with gold loans, especially for customers who visit institutions in order to get money right away and dislike filling out these lengthy KYC*

forms. It's also quite difficult to obtain documentation from Gold Loan clients."

According to the majority of the respondents, it is clear that one of the major problems that finance companies are facing is the lack of customer knowledge on these AML CFT regulations. Further, Aziz and Daud (2022) also highlight the challenges in implementing AML regulations in Malaysian Banks: the lack of expert staff and customer education. Likewise, Sultan and Mohamed (2023) further highlighted that the main duty of front desk staff in AML in Pakistan is to gather and validate data. The recent NRA report has identified several integrity failures, including inadequate training for compliance function employees, despite their direct customer contact within LFCs. Most of the time, bank employees gather data and enter it into a computer system without checking it, which results in inconsistent compliance. Therefore, these issues are not only limited to Sri Lanka. All the compliance officers and the operational level managers who were interviewed believe that proper customer education can lead towards better CDD and Enhanced Due Diligence (EDD) procedures in the finance companies. Notwithstanding, Financial Service Providers are mandated by the Financial Consumer Protection Regulations, No. 01 of 2023, to furnish financial customers with information pertaining to legal provisions about their financial products and services (Financial Consumer Protection Regulations, 2023). Therefore, educating customers is not only the duty of the regulator, but also lies with the finance companies as well.

Participant 005 brought another perspective saying, *"The regulator made it essential to perform EDD for high-risk customers under the rules. But they haven't made any explicit demands about that. For CDD, yes there are requirements. However, sometimes it's difficult for us to get the required documentation for EDD. Additionally, we only learn about the customer's risk once they are on-boarded. As a result, occasionally the branch does not follow up with customers to retrieve documents related to the EDDs. This is because, during the on-boarding stage, the marketing officer's or the relevant officer's get involved. However, when the consumer is on-boarded, it becomes challenging for us to have branch officers do the task."*

Similarly, a few of the participants had the same issue with the exact requirements of the Financial Institutions CDD Rules, No. 1 of 2016 in relation to EDD. Due to these grey areas in the existing rules, the compliance officers are struggling to provide the required output.

Meanwhile, when the regulator was questioned in this regard, ***Participant 001*** *stated, "Typically, the businesses carry out the CDD measures. However, they are genuinely unaware of how this will turn out. Financial Institutes may benefit from the CDD process in a variety of ways. Once the customer's profile has been created, you may sell him relevant products that fit his profile. However, creating a profile is not only taking a photograph or customer's NIC copy. You gain benefits by doing a proper CDD on a consumer. As a result, the FIs must also comprehend the requirements of the rule."*

Participant 008 who works overseas stated, "*The country where I work uses digital KYC procedures and automated processors, ensuring clients update their information and upload necessary documents during on-boarding. However, obtaining EDD related documents can be a bit challenging. When comparing to Sri Lanka, it is much easier as most of the time all the customers are aware of the requirement. But in Sri Lanka consumers with greater influence may receive different treatment and may not undergo necessary screening.*"

Kurum (2023) highlights that having KYC procedures were optimized using block chain and distributed ledgers, while artificial intelligence and machine learning are being utilized in solutions for AML compliance to enhance the efficiency of financial institutions. When the researcher inquired about the implementation of automated KYC systems and CDD processes within financial

institutions, the compliance officers highlighted the significant costs associated with adopting advanced technologies. Their responses underscored that while such systems could enhance efficiency and accuracy, the financial burden of transitioning to these technologies remains a major challenge for many institutions.

Participant 007 stated, *"System migration from a manual to an automated system is difficult due to limited information we had in our previous outdated system. During that time, the compliance requirements were not so complicated."*

As highlighted, the lack of customer KYC data on older systems poses challenges in automating customer profile creations, necessitating time and resources to cleanse old data and maintain ongoing CDD processes.

3.4 Reporting Suspicious Transactions

According to the Financial Transactions Reporting Act, No. 6 of 2006, FIs are required to report the suspicious activities of their customers (FTRA, 2006). Normally the FIs are using a transaction monitoring system to analyse customer transactions.

Participant 003 stated that, *"We currently analyse suspicious transactions using Excel-based customer transaction reports. This method is time-consuming and inefficient, as it lacks real-time alerts and operates on a post-mortem basis. This approach significantly delays the identification of potentially fraudulent activities, compromising our ability to respond promptly and effectively."*

A drawback of this method is that it allows potentially fraudulent activities to continue unchecked for longer periods, increasing the risk of financial losses and reputational damage to the institution. Moreover, delayed responses to suspicious transactions may lead to regulatory non-compliance and potential legal penalties, further exacerbating the financial and operational challenges faced by the institution (Financial Intelligence Unit, 2021; Financial Intelligence Unit, 2022).

Participant 004 revealed, *"We have an extensive monitoring system, which is similar to an international bank, provides real-time customer monitoring, risk warnings, risk ratings, and red flags. We have our own algorithms and set thresholds for on-boarding customers. When creating a CDD profile, it automatically creates the customer profile and generates alerts if any violations are detected."*

The majority of the respondents confirmed that they have real time customer transaction monitoring systems and within the day they are completing all the triggered alerts. However, the problem arises even after submitting the STRs on time to the FIU.

As per ***Participant 001,*** *"The STRs which are received by the FIU is not up to standard. According to our intelligence management, the quality is not good. It's not up to the expected level."*

Over reporting of STR, also known as "crying wolf" becomes a problem, when it fails to highlight the actual problems (Takats, 2009).

Participant 005 stated, "The investigation process is manual, with parameters set to identify unusual transactions. Transactions beyond these parameters are forwarded to product owners for clearance. A segregation of duty is in place, with one person for each product reviewing each transaction to determine, if it is suspicious before forwarding it to the Compliance Division. This ensures that transactions are cleared efficiently and accurately."

Participant 004 stated, *"The compliance assistant will conduct basic research on an alert, and if necessary, the alert will be investigated by the compliance officer. A separate team discussion will follow, and internal stakeholders will be contacted for more information. If the findings indicate suspicious activity, the STR will be sent to the FIU and Ministry of Defence."*

Overall, in spite of the various measures in place, the quality of the STRs are becoming low. Further, this has been highlighted in the

National Risk Assessment section, where the "Effectiveness of the Suspicious Activity Monitoring and Reporting" has been rated as 3, indicating areas of concern. The recommendation suggests a need to enhance the effectiveness of reporting STRs to the FIU (NRA, 2022).

Participant 004 and ***Participant 005*** complained about the difficulty in obtaining timely feedback from the FUI for the STRs submitted.

Participant 005 stated, *"Feedback is typically challenging to obtain, and we often do not receive any response at all."*

This emphasizes how crucial it is for the FIs to get timely feedback on the STRs they submit, since doing so will provide them the opportunity to reduce errors and improve efficiency.

4. Conclusion

This study highlights that while Sri Lanka's licensed finance companies are committed to adhering to global AML/CFT standards, significant challenges persist. These challenges include outdated sanctions processes, insufficient customer education, lack of uniformity in customer due diligence practices, and reliance on manual reporting tools like Excel, which hinder efficient fraud detection and reporting. The findings underline the need for better technological integration, standardized practices across the finance sector, and more robust communication with regulatory bodies like the FIU to enhance the quality of STRs. Addressing these gaps through targeted reforms and increased training can improve compliance effectiveness and mitigate associated risks.

5. Recommendations

5.1 Sanctions Screening

The Wolfsberg Group, a non-governmental association of thirteen global banks, was established in 1999 to develop frameworks and guidance for managing financial crime risks. Financial institutions are required to maintain an effective sanctions screening procedure under the Wolfsberg Guidance on Sanctions Screening. Institutions are required to use technology to handle the growing complexity and guarantee regulatory compliance (Deloitte, 2020). Therefore, in order to enhance customer screening, it is recommended to implement a centralized customer screening system managed by the FIU of Sri Lanka. This system would be suitable for all financial institutions, ensuring uniformity in screening processes and facilitating real-time updates of local United Nations Security Council Consolidated List (UNSCR) list and UN sanction lists. This is acceptable as the centralized system would automatically update with new information from the FIU, providing a consistent and up-to-date database across the financial sector. Additionally, similar to obtaining Credit Information Bureau of Sri Lanka (CRIB) clearance, this clearance should be mandatory before any individual or entity can enter into a financial institution. Furthermore, the system could include an alert mechanism that notifies all financial institutions, if a listed individual attempts to enter the financial system, enhancing collective vigilance and preventing potential threats from slipping through the cracks. Therefore, this is feasible as this integrated approach would significantly bolster the country's defences against money laundering and terrorist financing by ensuring a cohesive, responsive, and efficient customer due diligence process across all financial entities. Moreover, Huang and Trangle (2020) suggests permissioned block chain as a groundbreaking innovation in Anti-money laundering (AML) by providing a centralized version of consensus-based "truth" accessible to all relevant parties, eliminating duplicative work and back-and-forth processes.

5.2 Customer Identification and Due Diligence

The interviews identified a significant deficiency in customer education and a grey area in specific law requirements. In order to address these type of situations, Aziz & Daud (2023) suggested that all the FIs, regulators and

local media should collaborate to raise public awareness and education on financial crime to ensure successful implementation of AML. Similarly, Vineer (2020) states that financial institutions must educate the general public to explain the reasons for requesting personal and sensitive information from them. Therefore, it is important to start by addressing the most important issues, to work with other FIs and regulators, to run customer awareness campaigns explaining to consumers why financial institutions are requesting these documents and what types of documentation are appropriate to bring to the branch when making a new entry into the financial system. This is acceptable because it is being done to benefit all financial institutions and, if customers cooperate, FIs may streamline the CDD process. This will be feasible, since the majority of FIs that are having problems, are on the verge of penalties, and the regulator is preparing to raise the penalties to considerably higher amounts. Furthermore, in order to give a weight for this requirement, if the customer is reluctant there should be a way to give the notification to all the other FIs as well and therefore as mentioned earlier all the FIs will be able to maintain uniformity. If that customer is not willing to provide documents to one financial institution, he should not be accepted by any other FIs as well.

5.3 Reporting Suspicious Transactions

Based on the interviews, it is apparent that despite the regulator's concerns about the quality of Suspicious Transaction Reports (STRs), many financial institutions are conducting thorough investigations before submitting them. Therefore, to improve this process, it is recommended that the regulator provide detailed feedback on each STR submitted by financial institutions. The Hong Kong Joint Financial Intelligence Unit (2023) enhances STR awareness by providing feedback on suspicious transaction reporting issues through quantitative and qualitative analysis. Likewise, this feedback would help institutions understand the FIU perspective, enabling them to make more informed decisions about future STR submissions.

The final decision to submit an STR rests with the Compliance Officer, who must be continually updated on current ML and TF trends. In Sri Lanka, there is a lack of proper evaluation for compliance officers. To address this, a yearly certification program and annual training sessions on emerging trends and best practices in AML/CFT should be implemented. This will ensure compliance officers are well-equipped to identify and report suspicious activities effectively, enhancing the overall integrity and efficacy of the financial sector's compliance framework. Additionally, anyone aspiring to become a Key Responsible Person (KRP) within an organization must undergo comprehensive compliance training, ensuring all key personnel are knowledgeable about compliance requirements and capable of upholding the organization's integrity and adherence to regulatory standards. Mangelsdorf and Denkler (2013), stated that accredited certification is very beneficial to the firms, because the managers will be up-to-date.

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Examining How Gender Pay Gap Affects Gender Diversity: An Analysis among PMI Certified Project Professionals in Sri Lanka

C. Munasingha

University of Bedfordshire - Business School

charunikamunasinghe@gmail.com

Abstract

The gender pay gap in Sri Lanka surpasses the global average of 20% resulting in 27% of a gender pay gap despite international efforts such as the establishment of the Equal Pay International Coalition, to assist countries that make efforts to reduce the gender pay gap. Recent research conducted on the gender pay gap and its effect on gender diversity depicts that unequal pay among genders is the key barrier to women's under-representation in professions. The purpose of this study is to examine the gender pay gap's effect on gender diversity in the project profession in Sri Lanka. This study used the Human Capital Theory as the theoretical framework. The quantitative research method was employed to gather data from a target sample of 105 PMI-certified project professionals in Sri Lanka, included in the Project Management Institute Certification registry. The primary data was analysed using regression analysis. The findings indicate that the gender pay gap key drivers, specifically, job-specific experience and job-related skills, cognitive abilities have a significant relationship with gender diversity while formal education, professional qualification, and on-the-job training have no significant relationship with gender diversity. The creation of a transparent pay policy in the Sri Lankan project professional context was recommended to facilitate a fair pay policy to attract new talent and sustain existing talent in the organisations.

Keywords: Gender Pay Gap, Gender Diversity, Professional Qualification, Project Professionals

1. Introduction

1.1 Current Labor Market in Sri Lanka

There is a decline in the percentage of women in the Sri Lankan labor force. There is a decline from 36% to 24% from 2015 to 2020, while the percentage of men in the workforce remained at 76% over a span of 5 years resulting in a gender pay gap of 24% in 2020 (United Nations Women, 2020). However, compared to the 2015 gender pay gap of 31.5%, it is a considerable pay gap drop of 23.8%. At this rate, as underlined in the Global Gender Gap Report, 169 years are predicted as the period to close the economic participation and opportunity gender gap (World Economic Forum, 2023). Notably, the absence of national policies covering non-discrimination in hiring or equal pay for work of equal value can be portrayed as a major setback in closing the existing gender pay gap.

Recent research conducted by United Nations Women (2022) in Sri Lanka, portrayed gender disparities and labor market challenges for women established by Sri Lankan employers revealing major obstacles for women to enter the labor force. Notably, they pointed out major findings including employers with gender-based occupational segregation, employers' inability to finance maternity benefits, employers with stigmas of the types of jobs women can and cannot do, the reluctance of male top management who unconsciously prejudiced against women workers hired fewer women into their labor force, resulting in less gender diversity in Sri Lanka (United Nations Women, 2022).

The latest report, 'The Gender Pay Gap in Sri Lanka: A Statistical Review with Policy Implication' published by the International Labour Organization (2024), reveals that the gender pay gap in Sri Lanka surpasses the

global average of 20% with a 27% of a gender pay gap despite the international efforts such as the establishment of equal pay international coalition, to assist countries that make efforts into reducing gender pay gap. In particular, they note the inability to explain these disparities in the form of characteristics of both men and women including age or the sector; yet, using the observable factors including gender-based discrimination in the Sri Lankan labor market (International Labour Organization, 2024).

1.2 Rationale

Despite unchanged average salaries in the project management domain, the pay gap stands at 24% (APM, 2023) compared to 20% global gender pay gap declared by the United Nations. It thus becomes evident that women project professionals earn less compared to men in every surveyed country under the survey (PMI, 2023). Sri Lanka isn't a surveyed country under the recent PMI annual survey. India was surveyed and has a 15% pay gap in the project domain.

A survey conducted by APM revealed key barriers related to women entering the project profession. Accordingly, 33.4% of the female respondents pointed out unequal pay as the main barrier to entering the project management profession (APM, 2022). There is a scarcity of past research and information on gender diversity in the project profession in Sri Lanka. Globally, the overall women's representation in the project profession as a whole is at a ratio of 3:1, where the majority is dominated by male project professionals in every region as a result of the existing gender pay gap throughout the profession (PMI, 2022).

There are no of national policies covering non-discrimination in hiring or equal pay for work of equal value in Sri Lanka. Women's capacity to contribute to the economy is significant. Therefore, a study on the gender pay gap drivers is important to arrive at a potential solution for the less gender-diverse project profession domain.

This study will undertake primary research to understand the reasons for the existing pay gap and the key drivers in Sri Lanka.

1.3 Scope

This research targeted 105 PMI-certified project professionals included in the Project Management Institute Certification registry in Sri Lanka.

1.4 Research aims and objectives

The research aim of this study is to identify the key drivers that lead to gender pay gap among project professionals in Sri Lanka and how it impacts gender diversity.

Objective 1: To examine how gender pay gap impacts gender diversity in the project profession domain.

Objective 2: To find out the impact of the gender pay gap on gender diversity in the project profession in the Sri Lankan context.

Objective 3: To propose solutions on how to bridge the gender pay gap in order to improve gender diversity in the project profession in Sri Lanka.

2. Methodology

2.1 Theoretical Framework

The Human Capital Theory was used as the theoretical framework for this study.

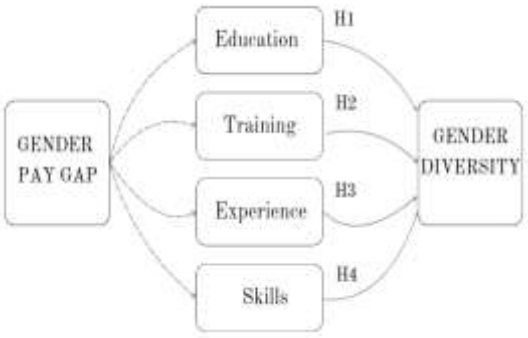

Figure 1. Theoretical Framework

2.2 Hypothesis Development

The following hypotheses were developed based on the theoretical framework.

H1: There is a relationship between Education and Gender Diversity

$H1_o$: There is no relationship between Education and Gender Diversity

H2: There is a relationship between Training and Gender Diversity

$H2_0$: There is no relationship between Training and Gender Diversity

H3: There is a relationship between Experience and Gender Diversity

$H3_0$: There is no relationship between Experience and Gender Diversity

H4: There is a relationship between Skills and Gender Diversity

$H4_0$: There is no relationship between Skills and Gender Diversity

2.3 Research Design

Positivism was adopted as the most favorable research philosophy due to the highly structured, deductive, and quantitative analysis nature of the research topic. Accordingly, primary research was carried out using mono quantitative method to gather data by deploying a survey strategy.

2.4 Population and Sampling

The population of this research was 1053 PMI-certified professionals in Sri Lanka in the Project Management Institute Certification registry. A sample of 105 was selected based on the simple random sampling method.

2.5 Data Collection

Quantitative data was collected using a structured questionnaire consisting of 24 Likert scale statements. Close-ended questions were used to determine participants' demographics, and two open-ended questions were used to gather more details.

2.6 Data Analysis Method

The quantitative data gathered using the questionnaire were analysed using both SPSS and Microsoft Excel. Descriptive, correlation, and regression analyses were conducted.

3. Findings and Analysis

3.1 Response Rate

33 respondents from the target sample of 105 PMI-certified project professionals in Sri Lanka responded to the survey, resulting in a 31% response rate.

3.2 Demographic Analysis

The sample was fairly distributed among male (55%) and female respondents (45%) respectively. 42% of the respondents were from the 25-34 years age group and the 35-44 years age group. The majority of the respondents were included in the 5-10 years tenure category. Moreover, the majority of male and female participants possess project management-related professional qualifications.

3.3 Gender Pay Gap and Gender Diversity

54.5% of the respondents were in agreement with the existence of a gender pay gap Similarly, 51.5%, affirmed the under-representation of women in the Sri Lankan Project Management domain.

The analysis of the respondents' feedback reveal that the majority believe that education and training do not have a major impact on gender pay. However, experience and skill seems to have an impact on gender pay gap. The majority of the respondents agreed that gender pay gap has an impact of gender diversity in the project profession.

3.4 Regression Analysis

The hypotheses formulated were tested using regression analysis. The results are given in Table 1.

Table 1. Regression Analysis

Variable	Coefficient	Significance
Education	-0.197	0.427
Training	-0.278	0.519
Experience	0.446	0.042
Skills	0.794	0.000

3.4.1 Education and Gender Diversity

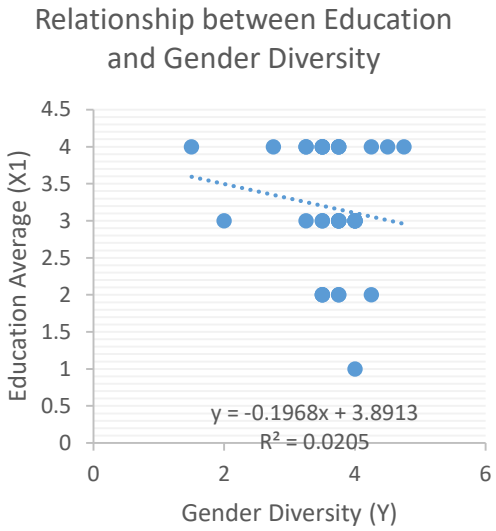

Figure 2. Education Vs Gender Diversity Scatter Plot

As evident in the regression analysis, the 0.427 P value represents a greater value than 0.05. Therefore, the alternative hypothesis (H1) is rejected and the null hypothesis ($H1_0$) is supported affirming that there is no relationship between education and gender diversity.

Additionally, the R square value being 0.02, further confirms the variable's insignificant accountability for a 2% variance in gender diversity.

A possible reason for the lack of a correlation between education and gender diversity is that many women today are highly qualified with the required education on par with the male colleagues. Therefore, in the current context, education has no impact on gender diversity in Sri Lanka.

3.4.2 Training and Gender Diversity

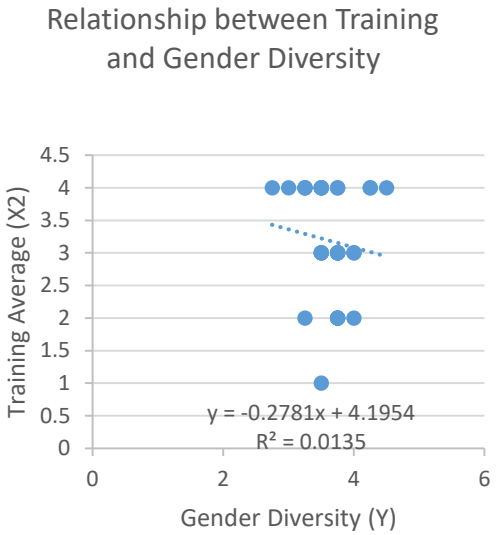

Figure 3. Training Vs Gender Diversity Scatter Plot

The 0.519 p-value which is greater than 0.05, represents an insignificant relationship between training and gender diversity. As a result, the alternative hypothesis (H2) is rejected and the null hypothesis ($H2_0$) is accepted affirming that there is no relationship between training and gender diversity.

The R square value of 0.014 means that training is only accountable for 1.4% of an insignificant variance in gender diversity.

Easy accessibility to external training programs and virtual training programs allowed women to acquire the required training as men and therefore does not have an impact on gender diversity in Sri Lanka.

3.4.3 Experience and Gender Diversity

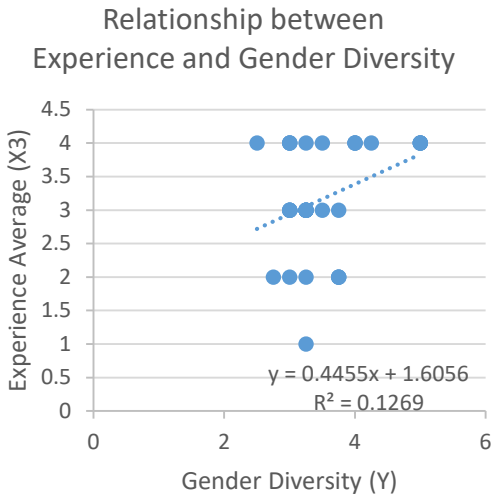

Figure 4. Experience Vs Gender Diversity Scatter Plot

As evident, a P- value of 0.042 is lower than 0.05, which means there is a significant relationship between experience and gender diversity. Hence, the null hypothesis (H0) is rejected and the alternative hypothesis (H3) is supported validating that there is a relationship between experience and gender diversity.

Furthermore, the R square value of 0.127 affirms a 12.7% significant accountability in gender diversity variance.

Experience has an impact on gender diversity. Women with more experience are more likely to get hired. Due to the availability of child care facilities and support from extended families more women are able to gain relevant work experience, which allows them to find jobs and thereby contribute to greater gender diversity in the workplace.

3.4.4 Skills and Gender Diversity

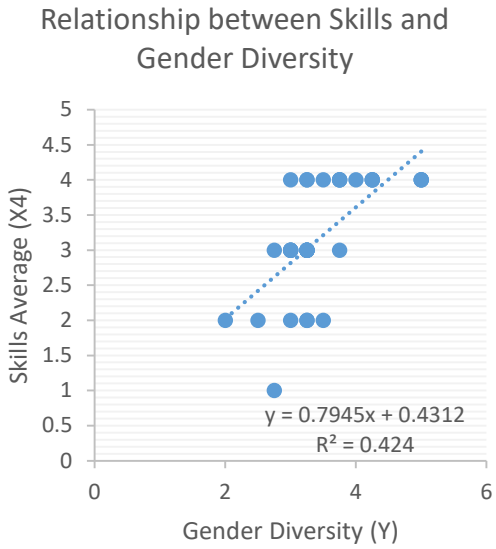

Figure 5. Skills Vs Gender Diversity Scatter Plot

As shown in Figure 5, the P-value of 0.000 which is lower than 0.05 means there is a significant relationship between skills and gender diversity. As a result, the null hypothesis (H0) was rejected and the alternative hypothesis (H4) was accepted endorsing that there is a relationship between skills and gender diversity.

Furthermore, the indicated R square value of 0.424 validates a significant accountability of a 42.4% variance in gender diversity.

Based on regression analysis, the skill variable depicted the highest accountability of 42.4% variance in gender diversity. Moreover, respondents ranked the skills variable as the second-highest key driver in gender diversity.

3.5 Summary

Table 2. Hypothesis Validation

Variable	Accepted Hypothesis
Education	H0: There is no relationship between Formal Education, Professional Qualifications, and Gender Diversity
Training	H0: There is no relationship between Training Opportunities and Gender Diversity
Experience	H3: There is a relationship between Job-specific Experience and Gender Diversity
Skills	H4: There is a relationship between Job-related Skills, Cognitive Abilities and Gender Diversity

4. Conclusion

The main drivers that impact gender pay gap and gender diversity among project professionals in Sri Lanka are experience and skills. Education and skills did not show a significant impact on gender pay gap. Women seem to be at a disadvantage in relation to experience and skills, because of less time spent on work due to family commitments and maternity leave compared to male colleagues. Women who take long career breaks struggle to return to the workforce.

5. Recommendations

The absence of a transparent pay policy in the Sri Lankan project professional context was identified. Therefore, it is recommended that organisations consider changing their practices around pay policy and adopt a transparent pay policy. According to Connell & Mantoan (2017), in recent years organisations have been legally pressured to attain pay equity by deploying strategies like transparent pay maintenance. Hence, the human resource department needs to be actively involved in action planning the change and adapt accordingly. Initiating the practices from the top management is crucial to eliminate the pay disparities and confusion of the pay to facilitate a fair pay policy to attract new talent and sustain existing talent in the organisations.

Another critical challenge to be eliminated by the organisations can be pointed out as gender bias hiring practices and accommodating gender-equal hiring practices. As evident in the literature review, Sri Lankan employers often consider women's additional responsibilities which leads to less demand, promotions, and career development for female workers due to higher costs relating to maternity leave and other security expenses. (Institute of Policy Studies of Sri Lanka, 2023). Accordingly, it is crucial to introduce national policies like achieving gender diversity in the workforce for both government and private sector organisations to reduce gender biases in the hiring process. The management needs to have confidence and believe in women project professionals in handling project management responsibilities. As discovered from the analysis, gender biases play a major role in the recognition of skills as well. Therefore, the necessity of an equal opportunity framework/ culture can be pointed out as a major milestone for organisations in Sri Lanka.

It was found that career breaks impact women in the workplace (Buehring, 2023) resulting in lower pay and less women representation (Kimhi & Hanuka-Taflia, 2019). Some women do not return to work after a career break. Returning to the workforce appears to be a challenging due to age discrimination as well (Greer, 2013). Therefore, it is critical for organisations to facilitate flexible work arrangements including remote work, and flexible working hours enabling women's work-life balance. In particular, workforce return ship programs for women need to be considered by Sri Lanakan organisations to reduce age discrimination and stigmas in career gaps.

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Impact of Technology Integration on Customer Retention at ABC Hospital

Seyed Aadil Seyed Hafeez[1], Apeksha Nirmali[1] and Mariyam Azam[1*]

[1]School of Management, BMS Campus, Colombo 6, Sri Lanka

*mariyam.a@bms.ac.lk

Abstract

In today's dynamic world, the integration of technology has been deeply rooted and plays a significant role in improving customer retention. The healthcare sector, particularly hospitals, also find the integration of cutting-edge technology essential in increasing customer retention. Therefore, this research aims to investigate the impact of technological integration, specifically, predictive analytics on personalised healthcare (PAPHC), mobile health (mHealth) application (MHA) and artificial intelligence (AI) chatbots, on customer retention at ABC Hospital, a leading private hospital in Colombo, known for its technological integration. This research employed a quantitative research method, using questionnaires, with a convenience sampling method being adopted to gather data from a sample of 120 patients. The IBM SPSS Statistical Software was utilised for data analysis. Data analysis revealed a significantly strong positive correlation between PAPHC, MHA and AI chatbots, with customer retention. The multiple linear regression analysis proved that all the factors are significant. This analysis further confirmed that PAPHC had a much higher positive impact towards customer retention than MHA and AI chatbots. Therefore, this study recommends that the ABC Hospital integrates predictive analytics, mobile health and AI technologies within existing health care systems to ensure customer satisfaction and customer retention.

Keywords: Customer Retention, Predictive Analytics on Personalised Healthcare, Mobile-Health Applications, Artificial Intelligence Chatbots

1. Introduction

1.1 Background to the Study

Technological integration within the healthcare sector has become essential for improving patient care and retaining customers (Bayramzadeh & Aghaei, 2021; Bhatia, 2021). Productivity and efficiency has improved by integrating cutting-edge technical tools and resources into organisational operations (Bajwa, Munir, Nori, & Williams, 2021). Additionally, it has been found that technological improvements not only increase patient care and quality, but also promote a more patient centered approach which helps increases satisfaction and retention (Darwish, Korouri, Pasini, Cortez, & Ishak, 2021). Mukherjee (2019) claims that technological integration also improves coordination and communication among medical professionals, which is important for improving and delivering high quality patient care. Furthermore, Nwankwo (2015) also states that hospitals must adapt to the patients' needs and improve their services to satisfy them by using technology that would help to obtain data necessary for insights towards patient behaviour and preferences.

Moreover, Consoli, Désiron and Alberto (2023) denote that from simple devices like computers and projectors to sophisticated programs like artificial intelligence and virtual reality simulations, this integration may include many different types of technology. Furthermore, Darwish et al. (2018) states that the use of telemedicine, electronic health records (EHRs), predictive analytics on personalised healthcare has improved the patient care by organising workflows and increasing diagnostic accuracy. Utilising advanced technologies such as data analytics and personalised care platforms, hospitals can better understand and respond to patient needs, enhancing their overall experience (Mukherjee, 2019). This not only strengthens patient trust and loyalty, but also helps healthcare providers maintain a competitive advantage (Liu, Bates, Wiens & Shah, 2019). Overall, as it improves customer

experience, operational effectiveness, and service quality, the strategic integration of technology in healthcare is a major factor in retaining customers (Nwankwo, 2015).

This study intends to focus on technological integration in terms of predictive analytics on healthcare, mobile health applications and artificial intelligence chatbots, to understand its impact towards customer retention. Predictive analytics on personalised healthcare (PAPHC) is used to predict health issues and give patients customisable treatment plans; while, mobile health application is a mobile optimised app where anyone from around the world can get patient support, track their health records and improve their lifestyle (Grundy, 2022; Nelson, Felgen, & Hozak, 2021). On the other hand, AI chatbots will improve patient healthcare by reducing the wait time and helps in giving timely hospital information, appointment scheduling and health tips (Martínez-Pérez, Torre-Díez, & López-Coronado, 2013). Therefore, this research will help prove how these technologies impact customer retention at ABC Hospital.

1.2 Research Problem

ABC Hospital has made significant improvements in integrating cutting-edge technologies within its facilities and services, positioning itself as a modern healthcare provider (ABC Hospital, 2024a). It has developed into a top-tier tertiary care facility inclusive of cutting-edge critical care units and modern theatre complex with state of the art intensive care units (ABC Hospital, 2024b).

Despite these advancements, the hospital has struggled with effectively utilising these technologies to their full potential, particularly in enhancing patient care and service delivery. This underutilisation of advanced technological integration has contributed to a decline in customer retention rates. The hospital serves a customer base of over 18,000 inpatients and 450,000 outpatients, yet, experienced a 32% decline in customer retention over the recent years (ABC Hospital, 2024c).

This study will help in understanding the impact that technological integration has towards retaining customers within ABC Hospital. Further to this, it has also been identified that most studies undertaken focus on investigating the impact that technological integration plays in retaining customers within industries such as the apparel and telecommunication industry. Hence, this research contributes to existing knowledge by providing valuable insights into the effectiveness of technology - driven customer retention strategies that could potentially be adapted within the healthcare sector.

1.3 Research Aim

The aim of this research is to examine the impact of technology integration on customer retention at ABC Hospital.

1.4 Scope of the Study

The study focused on the patients, inclusive of both inpatients and outpatients, of ABC Hospital, located in Colombo.

2. Methodology

This study employed a deductive approach using survey as the strategy. A mono-method quantitative research choice was adopted. A questionnaire was distributed via Microsoft Forms to obtain data from the sample of the study.

2.1 Conceptual Framework

The conceptual framework shown below was utilised to develop the hypothesis of the study.

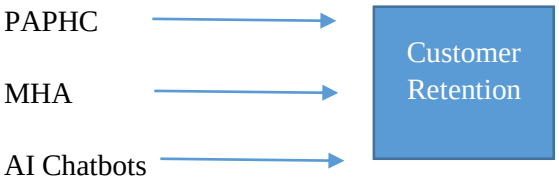

Figure 1. Conceptual Framework

2.2 Hypothesis

$H1_a$: There is a relationship between PAPHC and customer retention.

$H1_o$: There is no relationship between PAPHC and customer retention.

$H2_a$: There is a relationship between MHA and customer retention.

$H2_o$: There is no relationship between MHA and customer retention.

$H3_a$: There is a relationship between AI chatbots and customer retention.

$H3_o$: There is no relationship between AI chatbots and customer retention.

2.3 Population and Sampling

This study focused on the patients of ABC Hospital. ABC Hospital has served about 400,000 patients. A sample of 120 patients was obtained using the convenience sampling technique.

2.4 Data Collection

The study utilised a Likert scale based structured online questionnaire via Microsoft Forms to gather data relevant to the study. The questionnaire comprised of 25 closed-ended questions that included five demographic statements followed by 20 statements that assessed the independent and dependent variables of the study.

2.5 Data Analysis

The IBM SPSS statistical software was used to analyse the gathered data. Reliability, correlation, multiple linear regression and descriptive statistical analyses were carried out.

3. Findings and Discussion

3.1 Response Rate

This study obtained 111 completed responses from the 120 questionnaires that was shared. A response rate of 93% was achieved.

3.2 Demographic Analysis

Table 1 depicts the demographic data of the sample.

Table 1. Demographic Analysis

Age	
20-30	27.03%
31-40	38.74%
41-50	16.21%
51 and above	18.02%
Gender	
Male	45.05%
Female	29.73%
Other	1.80%
Prefer not to say	23.42%
Occupation	
Student	13.51%
Employed (Full-time)	54.95%
Employed (Part-time)	15.32%
Self-employed	16.22%
Unemployed	None
Monthly Income	
Below LKR 50,000	3.60%
LKR 50,001 – LKR 100,000	6.30%
LKR 100,001 – LKR 150,000	28.83%
LKR 150,001 – LKR 200,000	25.23%
Above LKR 200,000	36.04%
Use of services at ABC Hospital	
Never	0.90%
Rarely (Once or twice a year)	14.41%
Occasionally (few times a year)	32.43%
Frequently (Once a month)	35.14%
Very frequently (many times a month)	17.12%

The demographic data presented in Table 1 indicates that the majority of the respondents of the study were within the age group of 31-40 (38.74%). It also appears that the majority of the responses were from males (45.05%).

The findings also showed that most respondents (54.95%) are employed as full time employees and obtain a monthly income of LKR 200,00 or above. It is also evident that most of the respondents (35.14%) use the service of the hospital about once a month.

3.3 Reliability Analysis

A Cronbach alpha test was run to evaluate the internal consistency of the study.

Table 2. Reliability Analysis

No of Items	Variable	Cronbach Alpha
5	Predictive Analytics on Personalised Healthcare (PAPHC)	0.789
5	Mobile Health (mhealth) Applications (MHA)	0.805
5	AI chatbots	0.790
5	Customer Retention	0.792

Based on the findings, it can be concluded that all variables of the study are reliable, as each Cronbach Alpha is above the threshold value of 0.7. This denotes that there is high internal consistency within the variables (Bashir & Marudhar, 2018).

3.4 Correlation Analysis

Table 3 below gives a summary of the correlation analysis of the study.

Table 3. Correlation Analysis

Independent Variables of Technological Integration	Pearson Correlation Coefficient	Sig. Value
PAPHC	0.819	0.000
MHA	0.721	0.000
AI Chatbots	0.753	0.000
Dependent Variable: Customer Retention		

Table 3 shows a clear demonstration of the strong and significant relationship between the factors of technology integration and customer retention. All three independent variables obtained a significance value of 0.000, which is below the threshold significance of 0.05. This proves that PAPHC, MHA and AI chatbots have a significant relationship with customer retention. Further to this, the Pearson correlation coefficient of 0.819 highlights a highly strong positive correlation between PAPHC and customer retention. Furthermore, the relationship between MHA and customer retention shows a Pearson correlation coefficient of 0.721 which implies a strong relationship. Lastly, the Pearson correlation coefficient of 0.753 also demonstrates a strong impact between AI chatbots and customer retention. Thus, it is possible to conclude that these technological integrations will help in improving the customer retention rates of the hospital as they all show a strong and significant relationship with customer retention.

3.5 Hypothesis Validation

Table 4 gives the hypothesis validation based on the correlation analysis.

Table 4. Summary of Hypothesis Validation

Hypothesis		Validation
H1	**$H1_a$:** There is a relationship between PAPHC and customer retention.	Accepted
	$H1_o$: There is no relationship between PAPHC and customer retention.	Rejected
H2	**$H2_a$:** There is a relationship between MHA and customer retention.	Accepted
	$H2_o$: There is no relationship between MHA and customer retention.	Rejected
H3	**$H3_a$:** There is a relationship between AI chatbots and customer retention.	Accepted

	$H3_o$: There is no relationship between AI chatbots and customer retention.	Rejected

$H1_a$: There is a relationship between PAPHC and customer retention - Accepted

The above table indicates that $H1_a$ has been accepted showing that there is a highly strong and significant relationship between PAPHC and customer retention. The findings of this study coincides with that of Chen, Chiang, and Storey (2012) which found that the use of PAPHC is transforming hospital care and patient involvement due to personalised recommendations, medications, and lifestyle changes being offered based on patient health data. In doing so, patients tend to believe that the hospital uses such technological advancements with a patient-oriented focus, making them more likely to accept and return for their routine treatments. This also proves that predictive analytics makes it possible for the healthcare sector to become more patient-centered, effective, and efficient, eventually raising the customer retention rate (Darwish et al., 2018). Similarly, Mukherjee (2019) found that PAPHC reduces the waiting time for the patients thereby, leading to an enhanced experience giving customers a high quality service. Notably, Nelson et al. (2021) found that the use of predictive analytics results in long-term patient loyalty and retention. Thus, it is understood that patients are more likely to accept PAPHC when shown as a tool that supports their healthcare needs and their lifestyle.

$H2_a$: There is a relationship between MHA and customer retention - Accepted

Table 4 shows that $H2_a$ has been accepted. There is a strong and significant relationship between MHA and customer retention. Similar to PAPHC, this study has found that MHA is also an important factor to be considered in technology integration in order to increase customer retention. It is proven by Weinstein et al. (2014) that mHealth applications have grown into significant importance, as this resource increases the healthcare service and provides medical advice to the patients, increasing the credibility of the hospital. Additionally, Grundy (2022) shows in his research that there is a sustained patient retention due to the use of MHA, which provides a connection between the hospital and the patient. Furthermore, according to Moumtzoglou (2019) and Olla & Shimskey (2015), MHAs are used to provide patients with comprehensive patient care with a clear picture of their condition triggering the ability to take quick action contributing to a more responsive and connected healthcare experience. Thus, it can be concluded that the integration of MHA enhances patient care and customer retention within the hospital (Pires, et al., 2020; Sama, Eapen, Weinfurt, Shah, & Schulman, 2014).

$H3_a$: There is a relationship between AI chatbots and customer retention - Accepted

$H3_a$ has been validated showing that there is a strong and significant relationship between artificial intelligence (AI) chatbots and customer retention. Martínez-Pérez et al (2013) found that AI chatbots have greatly impacted and enhanced the customisation of patient interactions by employing machine learning algorithms and natural language processing to understand and react to patient requests, which in turn makes patients to mostly likely use and adopt to its technology. Additionally, Nadarzynski, Miles, Cowie and Ridge (2019) show evidence that AI chatbots give personalised health advice, reminders and active follow ups about patient visits and treatments. As a result of this, patients seem to like the adaptation of AI chatbots, since it helps them receive quick feedback, reduce waiting time and increase their satisfaction leading to an increase in customer retention for the hospital (Nadarzynski, et al., 2019; Nawaz & Gomez, 2020).

3.6 Multiple Linear Regression Analysis

Table 5 below provides the model summary of the regression analysis.

Table 5. Model Summary

Model	R	R Square	Adjusted R Square
1	.826[a]	.682	.673
a. Predictors: (Constant), AI, PAPHC, MHA			
b. Dependent Variable: Customer_Retention			

The regression analysis model has a R^2 value of 0.682, which denotes good degree of accuracy.

Table 6. Multiple Linear Regression Analysis

	Beta Coefficients	Sig. Value
PAPHC	0.642	0.000
MHA	0.569	0.000
AI Chatbots	0.598	0.000

Table 6 shows that the independent variables PAPHC, MHA and AI chatbots are significant variables that determine customer retention as they have a significance level less than 0.05 (p = 0.000).

3.7 Descriptive Analysis

This study also performed a descriptive analysis to identify the mean value highlighting the levels of agreement to the statements provided in the context of the variables. A summary of the descriptive analysis is depicted in Table 7.

Table 7. Descriptive Analysis

Descriptive Statistics	
Variable	**Mean**
PAPHC	3.5901
MHA	3.5739
AI chatbots	3.4937
Customer Retention	3.8036

Table 7 above shows that the mean value generated for PAPHC is 3.59, which means that most respondents agree that PAPHC has an impact towards the patients intending to make repeated visits to the hospital. The use of PAPHC encourages a partnership between patients and healthcare professionals as it actively involves individuals in their treatment journey (Mukherjee, 2019). MHA has a mean value of 3.57, which highlights that the majority agree that mobile health applications play a vital role in determining customer retention. The use of mHealth applications help improve patient and health professionals' communication through continuous interaction over the application creating a better and long term patient retention in the hospital (Grundy, 2022; Sama, et al., 2014). AI chatbots obtained a value of 3.49, depicting that the majority are more or less neutral about using AI chatbots. Lastly, customer retention depicts a mean value of 3.80, reflecting that most responses agree that they would make repeated visits to the hospital. These findings align with the findings of Nwankwo (2015), which concludes that the integration of technology in healthcare is a major factor in improving customer retention.

4. Conclusion

In conclusion, this research found that technological integration strongly influences customer retention at ABC Hospital. PAPHC, MHA and AI Chatbots all had a significantly strong positive correlation with customer retention. Based on the findings, this study provides recommendations to further advance the technological integrations at ABC Hospital in order to be able to retain an increased customer base.

5. Recommendations

The findings of the study proved that all aspects of technological integration, considered for the research, reflected a strong and significant impact towards customer retention.

Goodhue, Wybo, and Kirsch (2019) state that implementing a high end data governance system and standardisation policies would establish a clear set of guidelines for data collection, storage and processing to ensure that the data used by predictive analytics are accurate and complete. Moreover, Darwish et al. (2018) states that the effectiveness of predictive analytics in healthcare can significantly improve when there is a standardised approach to data management, as it enhances the outcome given through its regulated predictions. Thus, by ensuring high - quality data, healthcare providers will be able to make more accurate predictions through predictive analytics leading to better personalised healthcare and improved patient satisfaction, which in turn will lead to better customer retention at the hospital.

Isabelle (2020) states that incorporating gamification elements and personalised feedback methods into the mhealth applications would make it a more user friendly and more engaging with rewards, charts and progress

tracking for the patients. Moreover, Davies and Mueller (2020) states that gamification and personalised feedback significantly improves user experience and long-term usage of mobile health applications. By making the apps more interactive and providing users with personalised health insights and progress reports, ABC hospital will be able to increase the continuous use of these applications, thereby improving customer retention.

Lastly, the findings of this study revealed that AI chatbots have a significant and strong relationship with customer retention. Therefore, it is imperative that the hospital undertakes actions to further strengthen the impact of such chatbots on customer retention. Bajwa et al. (2021) recommends that integrating AI with existing health care systems would ensure that AI chatbots are easily integrated with systems like electronic health records (EHR) and customer relationship management (CRM) systems, which would improve and enhance their systems' effectiveness as well. Notably, Nadarzynski et al. (2019) states that this integration would allow AI chatbots to access comprehensive patient data, providing more accurate and relevant responses. Moreover, Bukowski (2020) highlight the importance of technology integration towards improving patient care and customer satisfaction and rentetion.

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591, Galle Road, Colombo 6, Sri Lanka | 094 11 250 4757 | info@bms.ac.lk

www.bms.ac.lk