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122 Pages

Contents

Page No

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Microbiological Analysis of Phenanthrene and Naphthalene Degrading Soil Bacteria Isolated from Landfills and Filling Stations: Bioremediation Approach for a Green Environment

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Abstract

Polycyclic Aromatic Hydrocarbons (PAH) are organic compounds with two or more fused benzene rings produced by the incomplete combustion of anthropogenic sources. Due to their ubiquitous nature, contribution to pollution and prominent health risks such as carcinogenicity and genotoxicity, they have been considered as persistent organic pollutants. With 90% of PAHs settling on soil, the primary purpose of this study is to isolate, identify and select the best phenanthrene and naphthalene degrading bacteria from contaminated soil of landfills and filling stations and study their degradation percentages. In this study, via quadrant streaking, a total of 3 morphologically different strains that can degrade the PAHs of interest above 25%, confirmed via plate assay and spectrophotometry were identified as potential biological degraders towards bioremediation. Via this study, a total of 3 morphologically different strains that can degrade the PAHs of interest above 25% were identified as potential biological degraders towards bioremediation.

Keywords: Bacterial Degraders, Bioremediation, Naphthalene, Phenanthrene

1. Introduction

Soil is one of the key environmental components that sustain most living organisms and maintain biodiversity, directly and indirectly.[1] However, soil fertility is rapidly declining amidst steady global population increase and persistent disposal of pollutants while failing to provide appropriate care. With the rapid global industrial advancement, this has created pressure on sustaining soil quality due to the resulting soil toxicity changing its integral properties.[2] Essentially, soil pollution is the presence of xenobiotics (pollutants or contaminants) in soil at a concentration higher than accepted levels which can cause adverse effects on flora and fauna diversity of the ecosystem.[3] Similarly, exceeding the accepted levels of naturally occurring soil contaminants is also considered as soil pollution. One of the major components that is primarily responsible for soil pollution is anthropogenically produced aromatic hydrocarbons (AHs).

Currently, AHs or arenes are considered as a primary pollutant that majorly contributes to environmental pollution. They are a class of unsaturated, cyclic and planer compounds that represents a six-carbon ring moiety termed benzene (C_6H_6) in electronic configuration and chemical behavior in its simplest form.[4] In a nutshell, AHs can be defined as any cyclic compound composed only of hydrogen and carbon atoms that fulfills the Hückel's Rule.[5] Monocyclic Aromatic Hydrocarbons (MAHs) consists of a central benzene ring with six carbon and hydrogen atoms and three double bonds.[6] Among MAHs, benzene, toluene, ethylbenzene, and the three xylene isomers (ortho-, meta-, and para-xylene) (BTEX), produced via substitution of the hydrogen atoms and considered volatile organic

compounds (VOCs).[7] MAHs are often produced in gasoline service stations, motor vehicle exhaust and fuel evaporation, the burning of coal and oil, and various other sources including natural sources such as volcanoes and forest fires.[8]

On the other hand, Polycyclic Aromatic Hydrocarbons (PAHs) are a wide class of ubiquitous organic compounds that consist of two or more fused benzene rings in linear, cluster or angular arrangements and are classified as per their sources of emissions and molecular weight.[9] PAHs with four or fewer aromatic rings are considered low-molecular-weight (LMW) and often originates from petroleum sources, while those with five or more rings are considered high-molecular-weight (HMW) PAHs which originates from pyrogenic (combustion) sources.[10] They are rapidly released into the environment due to incomplete combustion of natural sources which attributes to PAH background values and accumulation via anthropogenic activities with the emergence of industrialization and progressive technologies contributing to the majority of PAH contamination as they occur naturally in coal, crude oil, and gasoline.

PAHs have a very strong stable index in soil as they are highly hydrophobic, have low volatility and vapor pressure while being thermodynamically stable due to their strong negative resonance energy.[11] Approximately, 90% of PAHs deposit in soil as a final reservoir which creates a path to the food chain. PAHs have been reported to alter grain size, porosity and hydrophilicity of soil resulting in adverse effects on the diversity and population of its habitual microbes required for its replenishment.[12] The effects of PAHs on soil are further exacerbated in combination with the impacts of global warming. Interestingly, reports evidence that PAHs could have played a vital role in the development of biological processes involved in the emergence of life.[13]

Human exposure is often via breathing contaminated air, automobile exhaust, consuming contaminated food, and cigar smoke. The effects of PAHs on human health greatly will differ with the extent of exposure, concentration, toxicity, and way of exposure.[14] Short-term, exposure to high amounts of PAHs mixed pollutants can result in eye irritation, nausea, vomiting, diarrhea, and confusion; while chronic impacts of PAH exposure on living organisms have been reported as carcinogenic, mutagenic, immunotoxic, genotoxic and teratogenic at different levels of development.[14]

Naphthalene can be primarily found in moth repellents, cigarettes, and deodorants. Its main exposure routes are dermal, inhalation and ingestion, commonly through clothing. For humans, primary exposure for phenanthrene is via inhalation of tobacco smoke apart from ingestion and dermal contact.[15]

Several remediation techniques attempt reduction to safe levels via transformation and degradation. Most physical methods such as soil washing and electrokinetic remediation, do not structurally change the compounds but simply transfer PAHs and are time-consuming. Alternatively, chemical methods such as ozone oxidation and photocatalysis predominantly involve redox reactions which could disturb soil quality due to the possibility of harmful secondary compounds apart from being costly and complex.[16] Hence, biological methods that are environment-friendly and efficient are favored.

The study was conducted to identify bacteria capable of degrading naphthalene and phenanthrene, select the best bacteria with effective degradation, and study their degradation percentage in order to investigated the cocktail effect of bacteria to address PAH contamination with PAH-degrading bacterial consortia, isolated in a porous bed medium. The final product will be a compost medium that contains bacteria that can degrade PAHs. With this, the ultimate goal is to utilize eco-friendly method such as bioremediation to address soil pollution by PAH contamination.

2. Methodology

2.1. Sample Collection. Approximately 50g of soil samples were collected from landfills and

filling stations located in 6 different locations (Table 1) of Sri Lanka, which are considered highly contaminated areas with PAHs, due to regular occurrence of oil spills, leakages, automobile exhaust, and waste disposal and incineration from frequent urban activities.

Table 1. Sample collection sites

Landfills	filling stations
Negombo, Gampaha 7°6'59.924"N, 79°52'28.989"E	Wallawatta, Colombo 6°51'58.4"N 79°51'46.0"E
Meethotamulla, Colombo 6°56'12.592"N, 79°53'24.781"E	Kurunegala, Kurunegala 7°28'58.7"N 80°21'13.0"E
Kolonnawa, Colombo 6°54'8.375"N, 79°54'7.205"E	Beruwela, Kaluthara 6°28'27.3"N 79°59'02.4"E

2.2. Environmental Analysis. Accurately, 4g of each sample was dissolved in 20ml of Hexane and redissolved in acetonitrile followed by nylon filtration. Standard solutions 500, 250, 100 and 50 ppm were used for phenanthrene and naphthalene. They were then analyzed using a High-Performance Liquid Chromatography (HPLC) (Agilent 1100 series Agilent Technologies, Waldbronn, Germany) and vacuum-filtered acetonitrile and distilled water was used as the mobile phase. The obtained results were then compared to the human hazardous levels available for Naphthalene and Phenanthrene.

2.3. Isolation of Bacteria. Five grams of soil collected from each location was measured and centrifuged at 13000rpm with saline water and the supernatant was collected and diluted from 10^{-1} to 10^{-10}. 10^{-1}, 10^{-5} and 10^{-10} from the dilution series were spread on Nutrient Agar (NA) and incubated for 24 hours at 25℃.

After the incubation period population density for each morphologically different strain identified from the spread plates was calculated using below equation.[17]

$$\text{CFU/ml} = \frac{\text{No.of Colonies}}{\text{Vol.of Sample Used x Dilution Factor}} \text{ x } 1\text{ml}$$

Morphologically different bacterial isolates were quadrant streaked onto NA plates under aseptic conditions and incubated for 24 hours and 25℃. The Gramm's Staining[18] was performed to observe the Gramm's positive and negative bacterial isolates.

2.4. Primary and Confirmatory Degradation Screening. In preparation for Plate Assay, the bacterial isolates were starved for 3 days. After which, BBH Agar petri plates supplemented with 25 mg of Naphthalene and Phenanthrene were divided into 25 equal quadrants. The isolates were placed onto each quadrant and incubated for 7 days at room temperature (25℃).[19]

For the spectrophotometric analysis each strain was inoculated in 25 mg of spiked PAHs in 10 ml of distilled water with 2-3 drops of methylene blue (MB). The tubes were incubated for 24 hours at 25℃ and the average absorbance was measured at 609nm following 7 days.[17,20] The percentage degradation of each strain was calculated using the following equation.[17]

$$\text{Degradation Percentage} = \frac{\text{Actual Absorbance} - \text{Sample Absorbance}}{\text{Actual Absorbance}} \text{ x } 100$$

2.5. Molecular and Biological Identification of the Selected Strains. In order to extract rDNA, the sub-cultured samples and 1 ml of 0.9% NaCl were centrifuged for 5 minutes at 13000 rpm. To the supernatant collected, 20 µl of TAE buffer was added and placed in ice for 15 minutes. Next, the samples were transferred to a 95℃ water bath for another 15 minutes. After which, it was centrifuged again at 13000rpm for 5 minutes. The supernatant was transferred to new Eppendorf tube with 20 µl of 100% ethanol

and stored at -20^{0}C. PCR amplification (Table 2) was performed to isolate 16s rDNA using Bacterial Universal Primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) in 5'-3' direction.

Table 1. PCR reagents and their respective volumes.

Reagents	Amount (Volume)
DNA Template	2 µl
27F primers	1 µl
1492R primer	1 µl
GoTaq® Green Master Mix (M7122, Promega, USA)	12.5 µl
Nuclease-Free Water	8.5 µl
Total	25 µl

Table 3. PCR Procedure; temperature and time duration of each step.

Step	Temperature	Duration
Initial denaturation	94 °C	
Denaturation	94 °C	30 seconds
Annealing	52 °C	1 minute
Extension	72 °C	1 minute
Final extension	72 °C	

2.6. Agarose Gel Electrophoresis. In the agarose gel electrophoresis process, 1% agarose gel was made by mixing 1g of agarose powder with 100ml of 1x TAE buffer solution. The molecular weight marker of a 1 kb ladder was used. The gel was run at 55 V for 1 hour and the PCR bands were visualized using UV image analyzer.

The PCR products were purified via NGS bead purification prior to being sequenced via Sanger sequencing at GeneLabs, Sri Lanka. The sequences were trimmed between 550 - 680 bp and the best matching strains were identified as the respective species via NCBI BLASTn tool and the sequences were submitted to NCBI-GeneBank to accession numbers were obtained. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 11 software and the pairwise alignment was conducted using EMBOSS watersmith online alignment tool.[20]

2.7. Statistical Analysis. Using Statistical Product and Service Solutions (SPSS 29.0 Command Syntax Reference, USA), the average percentages and means of the confirmatory screening results were analyzed via one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

3. Results and Discussion

3.1. Environmental Analysis (HPLC). The results obtained by HPLC analysis; the total PAH concentration ranged from 20.24 ppm to 66.37 ppm in landfills (Figure 1) and 22.32 ppm to 43.31 ppm in filling stations (Figure 1). The concentration level of PAHs vary depending on the location and how polluted the sites are. This was further confirmed by a study conducted by Oketola and Oyeleke (2017) that reported LMW PAH between 18.0 - 28.9% from landfills in Nigeria while Nganje *et al.,* (2006), demonstrates, that in soil samples collected from Petroleum Handling Facilities in Calabar (including filling stations), the total PAH concentration ranged from 1.80 mg/kg to 334.34 mg/kg (1 ppm: 1 mg/kg).[21,22] The results observed in this study demonstrate a higher occurrence of naphthalene in landfills (131.12ppm) however, a higher prevalence of phenanthrene was observed in filling stations (129.04ppm).

For humans, the hazardous exposure levels of Phenanthrene have been determined to be between 100 ppm orally and 300 ppm dermally.[23] For naphthalene, it was 250 ppm.[24] HPLC environmental analysis of soil samples

collected from landfills and filling stations determined values (Figure 1) were lower than the above mentioned levels however if pollution continues without effective remediation, they could rise exponentially within a few years.[22]

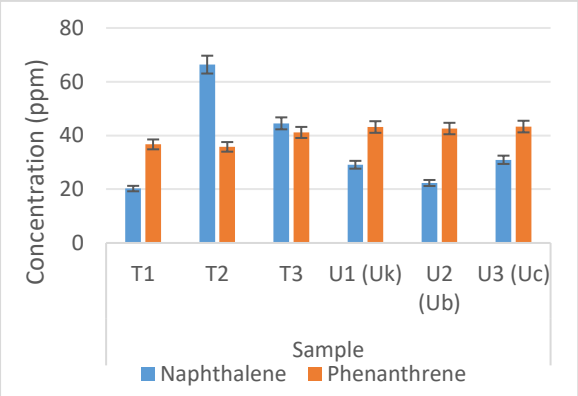

Figure 1. Concentration (ppm) of PAHs present in the soil samples collected as per HPLC analysis conducted (T=Landfills, U=Filling Stations)

3.2. Primary Screening. A total of 9 bacterial isolates were isolated from landfills and 8 isolates were isolated from filling stations.

Table 4. Plate Assay Results obtained for land fills

Strain	Naphthalene	Phenanthrene
ST1-1	25/25	25/25
ST1-2	3/25	4/25
ST2-3	7/25	25/25
ST2-4	25/25	19/25
ST3-5	5/25	1/25
ST3-6	25/25	11/25
ST3-7	9/25	14/25
ST3-8	2/25	3/25
ST3-8/a	25/25	25/25

Table 5. Plate Assay Results obtained for Filling Stations

Strain	Naphthalene	Phenanthrene
SS2-U2	25/25	19/25
SS2-U3	25/25	10/25
SS3-U4	25/25	24/25
SS3-U5	25/25	25/25
SS2-U6	20/25	11/25
SS2-U7	25/25	25/25
SS2-U8	23/25	09/25
SS2-U9	24/25	25/25

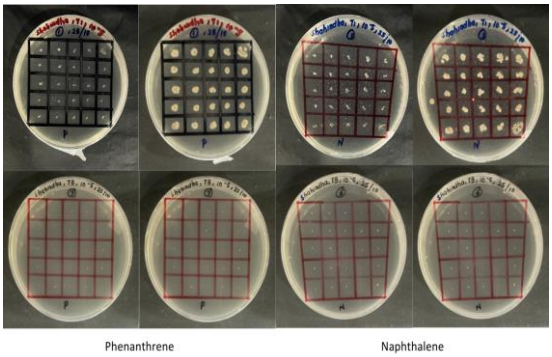

Figure 2. Following 3 days of starvation (Left) and following 7 days of incubation (Right)

Transferring individual colony isolates to BBH medium supplemented with PAHs allow bacterial growth by degrading PAHs. Each strain was able to successfully degrade both naphthalene and phenanthrene, but it shows some strains have higher proficiency than others (Table 4 & 5, Figure 2). From the strains isolated from landfills, a total of 127/225 squares on phenanthrene spiked plates and 126/225 squares on naphthalene spiked plates exhibited degradation. Meanwhile, all the strains collected from filling stations exhibited growth on over 20 squares for naphthalene unlike phenanthrene. It can be determined from the primary screening that the bacterial strains isolated from filling stations are better adapted towards PAH degradation and that strains collected from landfills are better adapted

towards degrading phenanthrene while for the strains that belonged to filling stations are better opted towards naphthalene degradation.

To analyze PAH degradation of each isolate, two screening processes were conducted using BBH mediums, due to a lack of carbon source – bacterial growth become inhibited, and enter a state of stress-induced dormancy, which can enhance their ability to degrade PAHs.[25]

In 2022, Dharmasiri *et al.*, reported *Bacillus sp.* and *Bacillus megaterium* isolated from ornamental plants around urban areas of Sri Lanka, that demonstrated growth on 25 squares for phenanthrene.[26] Walton and Buchan in 2023 also reported *Bacillus* strains isolated from contaminated marine environments that developed clear zones indicative of phenanthrene degradation.[27] Additionally, Ibrahim *et al.*, reported *Staphylococcus aureus*, isolated from oil polluted soil in Dammam, Saud Arabia that can degrade PAHs in lysogeny broth (LB) liquid medium.[28]

3.3. Secondary Screening. The above results were further elaborated by the spectrophotometry analysis in secondary screening, using the cationic dye MB sensitive to 609nm. The degradation was corroborated by measuring absorbance however, considerable decolorization of MB was also observed in all the tubes. Since the bacterial biodegradation of PAHs are generally initiated by oxidization, MB is reduced to colourless leuco-MB in this process.[29-30]

As per the results demonstrated by Figure 3, from landfills, ST1-1, ST8-8 and ST3-8/a demonstrated high degradation ability above 40%. ST1-2 and ST3-5 demonstrated similar degradation for naphthalene and ST3-6 for phenanthrene. The rest of the strains demonstrated moderate to low degradation below 30%. The statistical analysis generated a p-value of 0.332, which is higher than 0.05. Therefore, it was concluded that there are no significant differences between degradation percentages of both PAHs of interest.

From filling stations, majority of the strains demonstrated high degradation ability above 50%, with SS3-U5 and SS2-U9 demonstrating the highest ability for phenanthrene and naphthalene respectively. Strain SS3-U4 and SS2-U8 demonstrated the lowest degradation at 15% and 33% for phenanthrene and naphthalene respectively. With a p-value of 0.592, which is higher than 0.05 it was concluded that there are no significant differences between degradation percentages of both PAHs of interest.

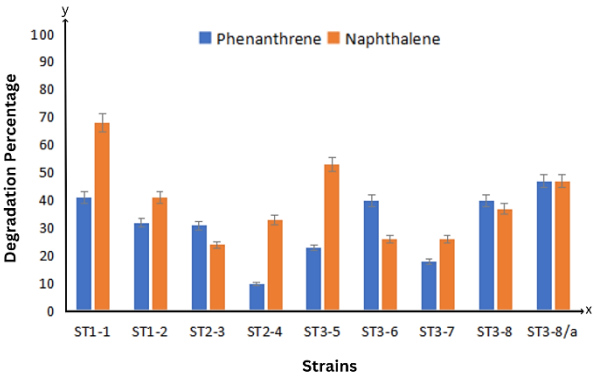

Figure 3. Percentage Degradation Rates for Landfills (T) and Filling stations (U) determined via Spectrophotometry

Previously conducted studies substantiate the results reported above. For instance, Sharma *et al.*, (2023) isolated *Providencia rettgeri* VMP5, *Bacillus tropicus* VMP4 and *Bacillus sp.* VMP2 that can degrade phenanthrene at a rate of 98.63%, 89.9% and 82.63% respectively from mined soils.[31] These strains presented biosurfactants and more defence compounds which could assist in their adaptability and degradation potential. Additionally, Dharmasiri *et al.*, (2019) reported isolation of 5 species of *Bacillus* from phyllosphere in Sri Lanka, that can degrade phenanthrene above 20% which were analyzed via UV-Vis spectrophotometry and HPLC.[32] Similarly, Kiamarsi *et al.*, (2018) reported *Bacillus subtilis, Staphylococcus pasteuri* and *Bacillus atrophaeus* isolated from contaminated soils that can degrade PAHs at rates of 55.5%, 39.0% and 49.9% respectively.[33] Chen *et al.*, (2023) identified a phenanthrene degrading consortium that consisted of *P. seudomonas sp., Stenotrophomonas sp., Delftia sp., Pseudomonas sp., Brevundimonas sp., Curtobacterium sp.*, and *Microbacterium sp.*, with an individual capacity of 15%, 12%, 16%, 38%, 13%, 8% and 21%, respectively.[34] They were isolated from long term phenanthrene

exposed soils in China and the degradation was assessed via GC analysis.

3.4. Molecular Analysis. Isolates ST3-6, ST3-8 and SS2-U3 were selected for further analysis considering their degradation kinetics.

Following sequencing (Table 6), ST3-6 was identified as *Bacillus anthracis* strain B3 with an accession number of PP340948. Belonging to the *B cereus* group, they are rod-shaped Gram-positive, aerobic, spore-bearing strains that are highly resistant to harsh treatment such as dehydration, elevated temperature, toxic chemicals, and enzymatic digestion.[35] ST3-8 was identified to be *Ureibacillus manganicus* strain SS4R-24 with accession number PP340961. It has not been widely investigated except for its basic details. It is an aerobic gram-positive, rod-shaped spore-forming bacteria that belongs to the *Caryophanaceae* family.[36]

SS2-U3 was identified as *Staphylococcus hominis*, with accession number PP340951. *Staphylococcus hominis* strain SS2-U3 is a coagulase-negative member of the bacterial genus *Staphylococcus*, aerobic, spherical shaped, consisting of Gram-positive, spherical cells in clusters.

Table 6. Identification results for selected strains

Sample	Strain	Accession Number
ST3-6	*Bacillus anthracis* strain FS-B3	PP340961
ST3-8	*Ureibacillus manganicus* strain SI-SS4R-24	PP340948
SS2-U3	*Staphylococcus hominis* strain SS2-U3	PP340951

4. Conclusion

The following study was conducted to improve the efficacy of bioremediation as a solution for PAH contamination. This is the first wet laboratory research in Sri Lanka to address PAH contamination. From landfills, Bacillus anthracis strain B3 (PP340948) and *Ureibacillus manganicus* strain SS4R-24 (PP340961) were identified as Phenanthrene (both at 40%) and Naphthalene (26% and 37%) degraders. From filling stations, *Staphylococcus hominis* strain SS2-U3 was identified with a 48.78% of phenanthrene degradation percentage and a 50.68% naphthalene degradation percentage. With a degradation percentage of over 25% these strains, offer an eco-friendly bioremediation solution.

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Low cost and eco-friendly green synthesis of silver nanoparticles using *Citrus reticulata* leaf extract: Analysis of their antioxidant properties

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Abstract

This study explores the green synthesis of silver nanoparticles (AgNps) utilizing *Citrus reticulata* (mandarin) leaves as a sustainable and eco-friendly approach. In the current study, three varieties of *Citrus reticulata* leaves such as heen naran, yaki naran, and nas naran were used and AgNps were formed through bioreduction of silver nitrate to a colloidal solution of AgNps. Water was used as the solvent for AgNp synthesis. Nanoparticle formation was identified with colour change in samples from yellowish-brown to reddish-brown and by measuring the absorbance at 320-560 nm range in UV visible spectroscopy. Optimization was carried out at 90ºC and 60ºC for 15 minutes, 30 minutes, 45 minutes, and 1 hour, also at room temperature for 72 hours. Room temperature was considered as the optimum temperature for silver nanoparticle synthesis. Optical properties were analyzed by calculating the bandgap energies. All nanoparticles from three varieties showed semiconducting properties. The antioxidant capacity of AgNp was determined with antioxidant assays such as Total Flavonoid Content (TFC), Total Phenolic Content (TPC), and Total Antioxidant Capacity (TAC) assays. Silver nanoparticles showed higher TFC, TPC, and TAC, compared to water extracts. Statistical analysis was performed with single-factor ANOVA analysis. Based on the results, a significant difference was observed between AgNp and water extracts in TFC and TAC while no significant difference was observed between AgNp and water extracts in TPC. TAC was found to be strongly correlating to TPC while a weak positive linear correlation was observed between TAC and TFC, and in between TFC and TPC assays.

Keywords: Silver nano particles, *Citrus reticulata* leaves, Green synthesis, Antioxidants

1. Introduction

The world is facing a major problem associated with human health due to free radicals induced diseases such as neurodegenerative disorders, cardiovascular diseases, diabetes mellitus, and various respiratory diseases. Free radical is a molecule that is capable of independent existence with a free unpaired electron in the outer shell, which causes the molecule to become highly reactive and attack the macromolecules such as lipids, proteins, and nucleic acids, leading to homeostatic disruption and cell damage.[1]

Reactive Oxygen Species (ROS) can be produced due to various factors such as smoking, pollution, radiation, processed food, heavy metals, and UV light. Antioxidants can defend against ROS by donating an electron and inhibit oxidative damage due to their free radical scavenging property. Antioxidants are available as flavonoids, enzymatic antioxidants, unprocessed food, and vitamins.[2] .

Natural antioxidants are mainly present in medicinal plants. Polyphenols act as the major antioxidants in plants as they are good hydrogen donors, and these are accepted by highly reactive radicals to become stable.[3] . Major polyphenols in plants are flavonoids, anthocyanins, chalcones, stilbenes, lignans and phenolic acids (Figure 1).

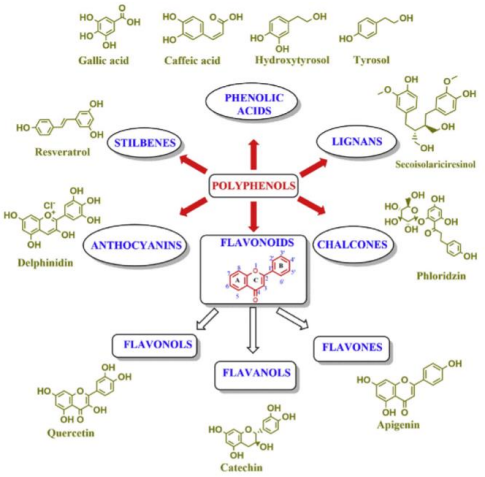


Figure 1. Classification of Polyphenols containing antioxidant property.[4]

Synthetic antioxidants, such as BHT and BHA, are widely used in the food industry, therapeutics, and cosmetics, but they pose potential risks to human health. As a result, there is a high demand for an alternative for synthetic antioxidants. Several studies have recently been reported about the use of AgNp synthesized from natural sources that can reduce the use of synthetic antioxidants.[5]

Nanobiotechnology has emerged as integration between biotechnology and nanotechnology, offering various applications in the biological field including green synthesis of nanoparticles.[6] Due to the tremendous growth in nanobiotechnology, so far various noble metal nanoparticles have been synthesized using Au, Cu, Zn, Pt, and Ag. However, more than other noble metal nanoparticles, AgNp has gained attention due to their unique properties such as good conductivity and chemical stability and most importantly non-toxic, cost-effective and ecofriendly. Thus, biocompatible AgNps have extensive applications in many fields such as nanomedicine, cosmetics, food and feed, optics, drug-gene delivery, catalysis and photo-electrochemical applications.[7]

Nanofabrication occurs in two major ways. They are the top-down approach and bottom-up approach. In the bottom-up approach, nanoparticles are fabricated from atomic-scale using chemical and biological methods while in the top-down approach the bulk material is broken down into fine particles to produce nanoparticles using the physical method which includes milling, grinding, and arc discharge method. Among these, chemical methods have been frequently used in large-scale production. Unfortunately, most of the physical and chemical methods for nanoparticle synthesis use various hazardous chemicals and high energy requirements.[8]

The need for an eco-friendly, non-toxic, and cost-effective method for the synthesis of nanoparticles leads to increasing demand for biological approaches. Thus, a method called ‘‘Green synthesis’’ was used by scientists to synthesize metal nanoparticles using microorganisms and plant extracts. Several parts of the plant such as the leaf, fruit, stem, and seed can be utilized in plant extract mediated synthesis (Figure 2). This method is potentially advantageous over microorganisms to reduce the cost when isolating and culturing microorganisms, and use of microbes can be hazardous. The rate of reduction of metal ions is much faster, and stable nanoparticles can be formed in plant extract-mediated synthesis.[9] Thus, in the current study *Citrus reticulata* leaves were used to synthesis AgNp from green synthesis. *Citrus reticulata* is commonly called mandarin orange, belonging to the Rutaceae family. Citrus leaves are rich in bioactive components such as flavonoids, ascorbic acid, phenolic compounds, and citric acid which are involved in the bio-reduction of metal ions and stabilization of synthesized nanoparticles by adhering to the surface of AgNPs (Figure 2) and results in high antioxidant capacity in AgNp

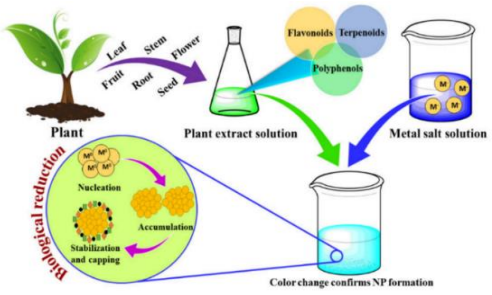


Figure 2. Mechanism of plant extracted mediated green synthesis.[10]

Green synthesis of nanoparticles was first reported in a study conducted by Gardea-Torresdey and his colleagues.[11] Many researchers have focused on the green synthesis of nanoparticles from plant extracts in the past decade. Scientists have also used fruit peel of *Citrus limon*, *Citrus sinensis*, and *Citrus tangerina* for the synthesis of nanoparticles and to examine their antibacterial activities,[12] and also AgNps have been synthesized from *Citrus reticulata* juice[13] and peel extracts to analyze antioxidant and antibacterial properties.[14] However, less research has been carried out for AgNp from *Citrus reticulata* leaf extract.

More than 60 types of flavonoids have been identified in citrus fruits. Flavonoids are polyphenols with a C6-C3-C6 structure containing two phenolic rings which carry one or more hydroxyl groups connected by a chain with 3 carbons. Citrus flavonoids can be classified as flavanols, flavanones, flavones, and isoflavones. [15]

Citrus plants consist of mainly glycosylated flavanones and poly-methoxylated flavones.[16] A study carried out by Dalia and others in 2016, identified that *Citrus reticulata* leaf oil contains linalool (21.20%) and sabinene (23.10%) as a major component and in the fruit peel oil, limonene (79.64%) was found to be the most abundant component. Limonene is a major flavonoid contributing to antioxidant activity while linalool and sabinene are also bioactive components that possess antioxidant activity.[17]

This study aims to synthesize AgNp in an ecofriendly and cost-effective manner using five varieties of *Citrus reticulata* leaf extracts. *Citrus reticulata* leaves were chosen because synthesis of nanoparticles with leaves can be used as an effective tool in management of waste. In Sri Lanka, *Citrus reticulata* leaves are utilized in ayurvedic medicine due to their extensive medicinal properties and nanoparticles were synthesized from these leaves to increase the efficiency of the medicinal value of *Citrus reticulata* leaves.[18] In the current study, antioxidant activity was determined by using TFC, TAC, and TPC assays. Statistical analysis was performed with SPSS and ANOVA.

2. Methodology

2.1 Sample collection. Citrus reticulata leaves of all five varieties were collected from Fruit Crops research and Development Centre, Horana, Sri Lanka. They are Heen naran, Yaki naran, Nas naran, Jama Naran and Maha naran.

2.1 Methodology.

COSHH forms and ethical consideration forms were prepared according to the reagents and materials used to carry out the research.

2.1.1 Preparation of aqueous leaf extracts. Five varieties of fresh mandarin leaves were air-dried under the same conditions for several days. Dried leaves were finely cut into small pieces and grounded with a motor and pestle. Leaves were weighed using an analytical balance and 2 g from each leaf variety was taken. To 2 g of each grounded sample, 50 mL of distilled water was added, and leaves were immersed in beakers containing distilled water. The samples were incubated at 80ºC for 20 min in the dry oven. The samples were filtered using a Whatman filter paper No.1 into 50 ml falcon tubes. The water extracts obtained were stored at 4ºC in the refrigerator until further use.

2.1.2Preparation of silver nitrate solution. Using an analytical balance, 0.0425 g of silver nitrate was measured. Measured silver nitrate was mixed with distilled water in a 250 mL volumetric flask. Distilled water was added to top up. The volumetric flask was covered with aluminum foil to maintain dark conditions.

2.5.1 *Synthesis of silver nanoparticles by green synthesis.* 1 mL of water extract was mixed with 9 mL of 1 mM aqueous $AgNO_3$ solution in a 250 mL volumetric flask and was kept in the dry oven at 60ºC and 90ºC for 1 hr, 45 min, 30 min, and 15 min under dark conditions. Dark conditions were maintained by covering the test tubes and with aluminum foil. The same procedure was followed at room temperature for 72 hrs.

2.5.2 *Characterization of silver nanoparticles.* A UV visible spectrophotometer was used to identify the formation of silver nanoparticles. Distilled water was used as the blank. The absorbance of samples was

measured within the 320-560 nm range for samples obtained during optimization temperatures with varied time intervals.

2.5.4.1 Determination of antioxidant activity. Diluted silver nanoparticle samples and aqueous extracts were prepared by adding 14 mL of distilled water to 1 mL of each silver nanoparticle and aqueous extract sample. The diluted samples were stored in the refrigerator at 4ºC until further use. Each analysis was carried out in triplicates.

2.5.4.2 Determination of total flavonoid content (TFC). The aluminum chloride colorimetric method was used to analyze total flavonoid content. From each of the previously prepared 10 % w/v aluminum chloride and potassium acetate solutions, an amount of 0.1 mL was added into each 2.8 mL of dilute aqueous samples separately and incubated at room temperature for 40 min. The same procedure was performed on each diluted silver nanoparticle sample. Subsequently, the absorbance of the samples was measured at 415 nm using distilled water as the blank. The total flavonoid content was expressed in µg Quercetin equivalents per 100g (µg QUE/100 g).

2.5.4.3 Determination of Total Phenolic Content (TPC). Folin-Ciocalteu reagent was diluted by adding 10 mL of the reagent with 90 mL distilled water. To 0.5 mL of the diluted aqueous extract samples, a mixture of 2.5 mL of Folin-Ciocalteu reagent and 2 mL of 7% Na_2CO_3 were added. The same procedure was followed for diluted silver nanoparticle samples. The samples were incubated at room temperature for 30 min under dark conditions. The absorbance was measured at 765 nm using distilled water as the blank. The results were expressed in g Gallic acid equivalents per 100g (g GAE/100g).

2.5.4.4 Determination of total antioxidant capacity (TAC). Phosphomolybdenum reagent was prepared by using equal volumes of 28 mM sodium sulfate, 4 mM ammonium molybdate and 0.6M sulfuric acid. To 1 mL of phosphomolybdenum reagent, 3 mL of diluted aqueous extract sample was added.

The samples were incubated at 90°C for 90 min. The same procedure was followed for diluted silver nanoparticle samples. The absorbance was measured at 695 nm by using distilled water as the blank and total antioxidant capacity was expressed as g ascorbic acid equivalents per 100 g (g AAE/100g).

2.5.4.5 Statistical analysis. Microsoft Excel 2013 software was used to generate graphs for total flavonoid of water extracts and silver nanoparticles expressed as Quercetin equivalents, total phenolic content of water extract and silver nanoparticles expressed as gallic acid equivalents, and total antioxidant capacity of water extracts and silver nanoparticles expressed as ascorbic acid equivalents.

Single factor ANOVA was performed to identify the statistical significance difference between silver nanoparticles and aqueous extracts in total flavonoid content, total phenolic content, and total antioxidant capacity. Using SPSS, correlation tables were generated and correlations between antioxidant assays were identified with Pearson coefficient.

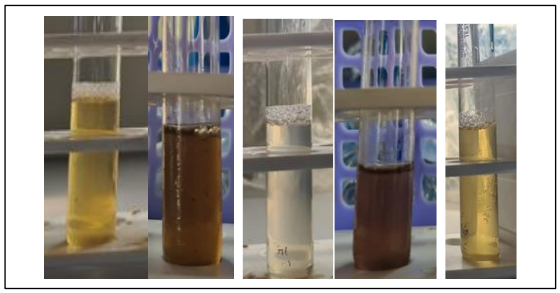
3. Results

3.1 *Identification of* AgNp.

In this study, AgNps were synthesized from *Citrus reticulata* leaf extract by using the green synthesis method to access antioxidant properties. This method acts as a reliable, sustainable, and eco-friendly procedure and aims at reducing the generated waste. In several studies, researchers have shown that mandarin leaves are rich in phenolic compounds, vitamin C, and flavonoids which significantly contribute to the antioxidant capacity.[20]

Synthesis of AgNp using plant leaf extract of *Citrus reticulata* was observed visually with colour change from light brown to dark brown (Figure 3). Several studies have reported that AgNp show striking colours from light yellow to reddish-brown due to the excitation of surface plasmon vibrations in AgNp.[21] A wide range of various nanoparticles can be synthesized by changing several factors like incubation temperature, time, pH, and

solvent used in nanoparticle synthesis, and also the yield of nanoparticles can be increased by adjusting the above parameters.[22]



(S1) (S2) (S3) (S4) (S5)

Figure 3. Colour change of aqueous leaves extracts. (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

The polarity index of the reaction medium affects the size of nanoparticles. Smaller and spherical-shaped nanoparticles are produced with a higher polarity index while larger nanoparticles are produced with a lower polarity index. Various organic solvents with high polarity are mainly used in the synthesis of silver nanoparticles, such as water, ethanol, methanol, isopropanol, or mixtures containing water and alcohol.[23] Water was used as the solvent due to its eco-friendly and non-toxic nature and also acts as a reducing agent and non-reactive with any components in the plant extract.

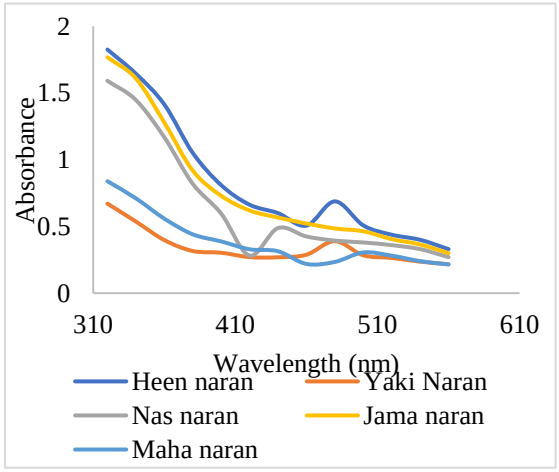


Figure 4. Absorbance at optimization temperature (room temperature) using UV-vis spectrophotometry. Three peaks were observed in heen naran, yaki naran and nas naran.

Colour change from yellowish-brown to reddish-brown indicates the formation of AgNp in samples. UV-Visible spectroscopy was used to further confirm the formation of AgNp. In this study, optimization of AgNp was performed with incubation temperatures such as 60ºC and 90ºC for 15 min, 30 min, 45 min, and 1 hr, and at room temperature for 72 hrs. Higher incubation temperatures were not used as it can degrade the phenolic and other active components in the plant samples.[23] As shown in (Figure 4), the optimized temperature for AgNP formation was at room temperature. AgNPs were not synthesized at 60ºC during any time interval but at 90ºC for 15 min, nanoparticles were formed for two samples: Heen naran and yaki naran. For all five samples, a peak was obtained at 340 nm range when the condition was at 60ºC for 15 minutes. This can be due to the presence of unreacted silver in the solution which can cause an increase in absorbance. Synthesis of AgNp for the highest number of samples was observed at room temperature for 72 hrs with absorption peaks within the 440-480 nm range (Figure 4). Thus, the optimum temperature for the synthesis of AgNPs was considered as room temperature for 72 hrs and these samples were used for further analysis. The formation of nanoparticles took 3 days to complete at room temperature due to the slow bio-reduction process of Ag ions to Ag nanoparticles.[24] In a study by Leenus and his coworkers in 2020, AgNps were synthesized with *Citrus reticulata* leaves at room temperature confirms the results of the current study.[25]

The optical properties of AgNps were analyzed with UV Visible spectroscopy. AgNps have various optical properties such as absorption and emission.[7] The conductivity of nanoparticles can be measured by using the bandgap. The bandgap is the energy difference between the valence band (VB) and conduction band (CB). The minimum energy required for electron transmission from VB to CB is known as the bandgap energy which is specific to each type of nanoparticle. Nanoparticles with bandgap energy less than 3eV are considered semiconductors while nanoparticles with more than 4eV are considered insulators. All three AgNp samples were classified as semiconductors as depicted in table 1. Band gap

energies were measured using the following equation,

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

where E= bandgap energy: h= 6.626 x 10^{-34} Js: c_{light}=speed of light= 3 x 10^{8} ms^{-1}: λ= wavelength peak of AgNP synthesis (nm).

Table 1. Classification of silver nanoparticles based on bandgap energies.

Synthesized AgNPs	Band gap energy (eV)	Classification
S1	2.58	Semiconductor
S2	2.70	Semiconductor
S3	2.82	Semiconductor

Bandgap energy is inversely proportional to the size of the nanoparticles. Based on the bandgap energies, AgNPs of S3 is the smallest in size while S1 is the largest of all three AgNPs samples.

3.2 Antioxidant assays

3.2.1 Total Flavonoid Content.

Plants contain polyphenolic compounds which can act as natural antioxidants and function as free radical scavengers and reducing agents.[26] Flavonoids are the most common polyphenolic compounds. Pandey and his coworkers in 2019 carried out research using leaves, fruits, and peels of 3 varieties of citrus plants and showed that leaves of citrus species contain high TPC and TFC compared to citrus peel and fruits.[27] Flavonoid solubility depends on the polarity and chemical nature of the solvent.[28] A study by Khettal and his coworkers in 2016,[18] stated that regardless of the citrus species, TFC and TPC are high in aqueous leaf extract compared to methanolic extracts.

A study by Zhang and others have evaluated the antioxidant capacity of mandarin fruit tissues by determining the TFC and TPC. The highest number of antioxidants were present in the peel when compared to seeds, pulp residues, and juice, with the highest TFC and TPC, suggesting that that the use of fruit waste such as peel is ideal for the eco-friendly synthesis of silver nanoparticles.[29]

TFC was analyzed with $AlCl_3$ colorimetric method. $AlCl_3$ forms acid-stable complexes with the keto group of 4th carbon and either the hydroxyl group of 3rd carbon or 5th carbon of flavanols and flavones. Also, with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids, acid-labile complexes can be formed.[30] The maximum absorption was measured spectrophotometrically at 415 nm. TFC in all AgNp samples was high compared to aqueous extracts. In (Figure 5), there is no flavonoid content in S2. This can be due to the degradation of flavonoids during the extraction procedure. The p-value was 5.41E-05 and the F value was greater than the Fcrit value: 102.4012> 5.987378, indicating a statistically significant difference between AgNp and water extracts. Naz and his coworkers in 2017,[30] conducted research for AgNp synthesis from diluted and concentrated kinnow peel extract, which is a variety of *Citrus reticulata*, and stated that TFC was higher in AgNp when compared to the peel extract.

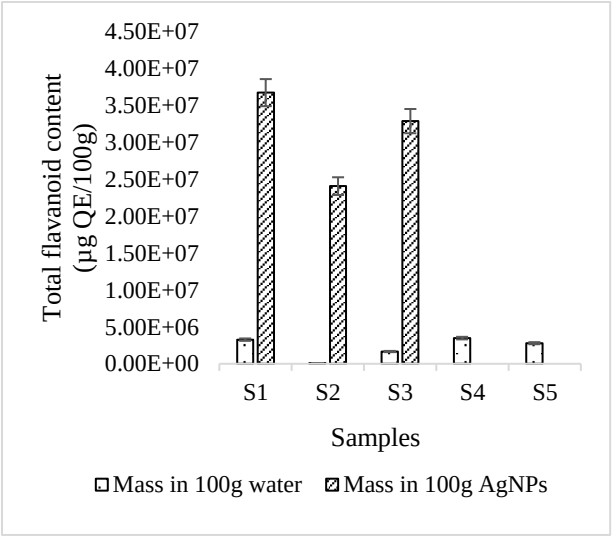


Figure 5. Total flavonoid content of water extracts and AgNPs expressed as Quercetin equivalents (QUE). (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

As depicted in (Figure 5), TFC in AgNp is high compared to the TFC in the water extract. In AgNp extracts, the TFC for S1 and

S3 is the same while S2 has the lowest flavonoid content.

3.2.2 Total Phenolic Content.

TPC was estimated with the Folin-Ciocalteu reagent method.

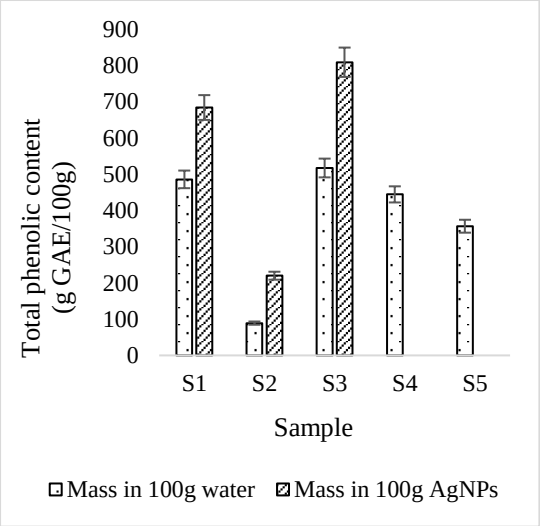


Figure 6. Total phenolic content of water extract and AgNPs expressed as gallic acid equivalents (GAE). (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

In the presence of phenolics, the Folin-Ciocalteu reagent is reduced resulting in the formation of blue oxides of molybdenum and tungsten, with the maximum absorption measured at 765 nm. Even though TPC in all AgNp samples was high compared to their relevant water extract samples, there was no significant difference observed between AgNp and water extracts because the p-value was 0.29185 and F crit value was greater than F value: 5.987378>1.334986. TPC was significantly higher in AgNp when compared to *Citrus reticulata* peel extract.[30] As depicted in Figure 6, AgNp has a high number of phenols when compared to water extract as S3> S1> S2. In the water extract the phenolic content varies as S3>S1> S4 > S5 >S2.

3.2.3 Total Antioxidant Capacity

As depicted in the above (Figure 7), AgNp contained the highest TAC compared to water extracts. When the TAC of AgNp was considered, S3 and S1 were identified with the highest TAC while the lowest TAC was observed in S2. In water extracts, S1, S3, and S4 had the highest antioxidant capacity while S2 was observed as the sample with the lowest antioxidant capacity.

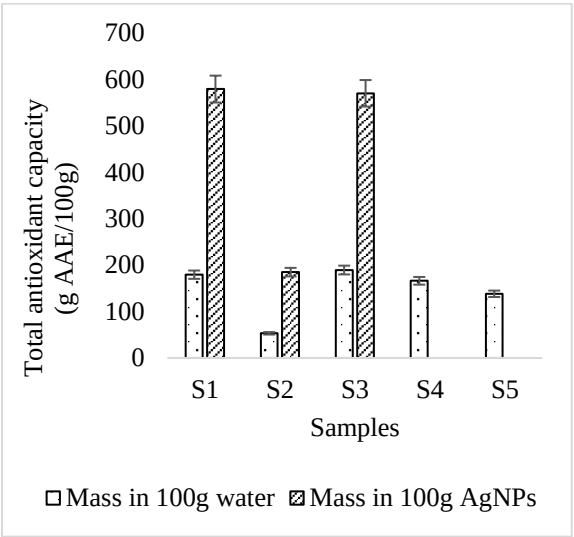


Figure 7. Total antioxidant capacity of water extracts and AgNp expressed as ascorbic acid equivalents (AAE). (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

TAC was estimated with phosphomolybdenum assay. The main principle of the assay is the reduction of Mo (VI) to Mo (V) to form a green phosphate, by the plant extract containing antioxidants with the maximum absorption of 695nm.[31] TAC in all three AgNp was high compared to water extracts. P-value was 0.024558 which indicates a significant difference between AgNp and water extracts. F value was also greater than the F crit value: 8.895163> 5.987378. In a study by Naz and his coworkers stated that TAC was higher in AgNp synthesized from diluted *Citrus reticulata* peel extract than concentrated peel extract and overall, TAC in AgNp was higher than both diluted and concentrated peel extracts.

3.2.4 Correlation

As shown in (Figure 8), the R-value between TAC and TPC was 0.995 which indicates a strong positive linear correlation while the R-value between the weak between TAC and TFC was 0.164, which indicates a weak positive

linear correlation. A weak positive correlation was also observed between TFC and TPC assays with an R-value of 0.131.

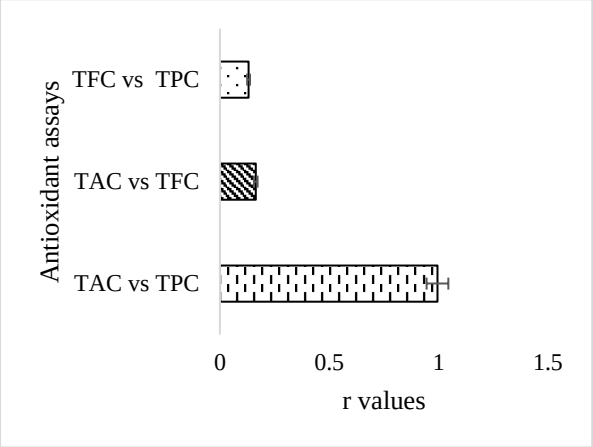


Figure 8. Correlation between antioxidant assays.

Pearson correlation analysis was performed to determine the association between TFC, TPC, and TAC. A higher positive correlation was observed between TAC and TPC while a weak positive linear correlation was shown between TAC and TFC. This can be due to the presence of phenolics as the major component contributing to TAC in *Citrus reticulata leaves*. A study by Khettal and others in 2016,[18] showed that regardless of the solvent used in extraction, the polyphenols in *Citrus reticulata* leaves contribute to 87% of the antioxidant ability. A weak correlation between TAC and TFC indicates that various phenolics and phytochemicals such as tocopherol, ascorbic acid, and other pigments contribute to TAC other than flavonoids. A study by Yang and his coworkers in 2018,[28] showed that flavonoid contribution is less for antioxidant activity in Citrus peels. A weak positive correlation was also observed between TFC and TPC assays due to less flavonoid contribution to polyphenol content in mandarin leaves.

4 Conclusion

In conclusion, AgNps were synthesized from three varieties of *Citrus reticulata* leaves by green synthesis at RT for 72 hrs. The particle size of AgNp was around 40 nm which was analyzed with SEM and bandgap energies. All AgNp samples showed semiconducting properties. The antioxidant property of AgNp was analyzed with TFC, TPC, and TAC antioxidant assays. TAC and TPC showed higher correlation while the lower correlation was in TAC and TFC, also in TFC and TPC. Based on the results, it can be concluded that mandarin leaves are a good source of naturally occurring antioxidants and can be applied in various fields as an eco-friendly, cost-effective, and non-toxic method.

Acknowledgements

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Iron Biofortification of Hydroponically Grown Widely Consumed Leafy Greens in Sri Lanka

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Abstract

Iron (Fe) deficiency is a prevalent nutritional concern in Sri Lanka, compelling innovative approaches to enhance dietary iron intake. This research explores the biofortification of iron in hydroponically grown Green Thampala (*Amaranthus viridis*) and Mukunuwenna (*Alternanthera sessilis*), which are widely consumed leafy greens in Sri Lanka. The effects of varying Fe concentrations on growth parameters, fresh yield, nutrient contents, antioxidant activity, total phenolic, and flavonoid content of hydroponically grown leafy greens under controlled conditions by administering $FeSO_4$ in Albert's solution at concentrations of 162.5 ppm (control), 200 ppm, and 240 ppm were assessed in this study. The Phenol-Sulfuric assay, Lowry assay, Folin-Ciocalteu assay, $AlCl_3$ spectrophotometric method, and DPPH assay, were used to estimate the Total Carbohydrate Content (TCC), Total Protein Content (TPrC), Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and total antioxidant activity of Fe-fortified plants, respectively. Moreover, the Fe content of Fe-fortified plants was determined using the Atomic Absorption Spectrophotometry (AAS) technique. The plants were tested positive for saponins, tannins, polyphenols, terpenoids, and steroids and they were tested negative for anthraquinones. The 200 ppm of Fe showed the highest Fe content in both plants, along with growth parameters such as significantly increased height in both plants, the highest leaf count in Green Thampala, and optimized fresh yield in Mukunuwenna. In both plants, the 240 ppm of Fe had the highest nutritional content. Both 200 ppm of Fe and 240 ppm of Fe concentrations appeared as successful for fortifying Fe in Green Thampala and Mukunuwenna plants, since they showed a higher Fe content than control, which had a concentration of 162.5 ppm of Fe.

Keywords: Iron biofortification, Green Thampala, Mukunuwenna, Coco peat grow bags, Albert's solution

1. Introduction

Iron (Fe) is widely recognized as a necessary mineral element for humans, as it plays a role in the synthesis of both hemoglobin and myoglobin.[1] Beyond its role in oxygen transport, iron is essential for immune system function, neural systems, homeostasis, energy metabolism, exercise, and overall human health maintenance.[2] The Recommended Daily Allowance (RDA) for Fe is 8–18 mg, which is the amount that the human body needs each day; particularly, pregnant mothers necessitate 27 mg of iron per day. However, the tolerable Upper Intake Level (UL) of Fe in adults is 40 mg per day.[3] Insufficient iron consumption through diets can lead to iron deficiency anaemia, posing severe health risks, as heart and lung failure, restless legs syndrome, pregnancy complications, and developmental delays in children.[4] Two billion people worldwide are anaemic, and according to the World Health Organization (WHO), the major reason is Fe deficiency in human diet. 11.2% of 6 to 11month-old babies in Sri Lanka was reported to have an iron deficiency in 2022.[5] But occasionally, the diet falls short of the minimum requirements, leading to micronutrient deficiencies and the phenomenon referred to as "hidden hunger".[6]

Biofortification is a method of addressing hidden hunger by increasing the nutritional content of plant edible parts during their vegetative life cycle.[7] To accomplish biofortification, techniques including as breeding, mineral fertilization, and biotechnological approaches can be applied.

All these approaches, though, have drawbacks. For instance, excessive fertilizer application might contaminate the soil or precipitate insoluble mineral forms; high concentrations of minerals can stress the plants. An alternate strategy to these approaches is to use hydroponic technique to increase the target minerals in food crops through biofortification.[8]

In hydroponics, plants are grown without the use of soil. They are planted in inert growing media as mentioned in Figure 1, and given access to water, oxygen, and nutrient-rich solutions.

Figure 1. Inert growing media for hydroponics.[9]

Hydroponics offers quicker growth, larger yields, and superior quality. A plant's roots are always looking for the nutrients to survive when it is grown in soil. A plant does not require energy to survive when it gets water and nutrients straight through its roots. The way hydroponic systems function is by enabling fine control over environmental parameters such as pH and temperature balance, electrical conductivity (EC value), and optimal exposure to water and nutrients. The typical hydroponic pH range is 5.5 to 6.5, and ideal EC range is between 1.5 to 2.5 ds/m.[10]

Various hydroponics systems are available including Nutrient Film Technique (NFT), deep water culture, drip irrigation and aeroponics.[11] Each technique has unique features and advantages. The NFT system is a recirculating hydroponic system among hydroponic techniques. It suspends the plant above a continuously flowing stream of nutrient solution that covers the ends of the root system, and it reduces the nutrient loss and effectively increases the nutrient absorption.[12] The sterility of the coco peat grow bags set it apart from other growing media. Additionally, it effectively retains water, ensuring plant hydration and reducing the likelihood of dehydration.[13]Biofortification of leafy greens can be carried out in hydroponics by adding higher concentrations of target minerals in the nutrient solution.[14] Moreover, Fe, which presents a low solubility in the soil, a hydroponics system can be a good option to increase micronutrient availability, since it facilitates the pH management in the nutrient solution.[15] The effectiveness of biofortification depends on the chemical form of micronutrients. In the case of Fe absorption, roots absorb Fe^{2+} which is oxidized to Fe^{3+}, chelated, and transported to the plant top.[16] Fe is essential for respiration, photosynthesis, and enzyme reactions. The concentration of Fe in plant leaf tissues varies depending on the species of the plant, but it usually falls between 50-500 ppm. Toxic effects could be observed at concentrations of Fe greater than 500 ppm. In general, if the concentration is less than 50 ppm, there are signs of deficiency.[17]

The reduction of Fe deficiency in Sri Lanka can be achieved through the implementation of strategies designed to raise the Fe content of food. A balanced diet can be completed with the help of healthy foods like green leafy vegetables. They are often high in fibre and minerals and low in calories and fat. Leafy vegetables have long been used in traditional medicine for their medicinal and therapeutic properties.[18] Two of the mostly consumed leafy greens in Sri Lanka are Mukunuwenna (*Alternanthera sessilis*) and Green Thampala (*Amaranthus viridis*) (Figure 2). Biofortifying Fe in these leafy greens can be a solution to iron deficiency since Green Thampala contains 207.38 µg of Fe in 100 g of leaves and Mukunuwenna contains 55.16 µg of Fe in 100 g of leaves.[18]

Figure 2. A) *Alternanthera sessilis* B) *Amaranthus viridis*

Based on the background information, this study was designed to address iron deficiency in Sri Lanka by biofortifying Fe in hydroponically grown Mukunuwenna, and Green Thampala, as hydroponic biofortification has never been attempted in Sri Lanka before. In this study, we selected two different concentrations of Fe (200 ppm and 240 ppm) to increase the Fe content in those plants, and Fe was added to the Nutrient solution as $FeSO_4$, based on the plant tolerance level of Fe range of 50 ppm – 500 ppm. Moreover, this study aimed to evaluate the effects of varying Fe application rates within the nutrient solution on growth parameters, fresh yield, mineral composition, antioxidant activities, and the phenolic and flavonoid profiles of leafy greens in the NFT system and coco peat grow bags under controlled conditions.

2. Methodology

2.1 Selection of seeds and nutrient solution. Commercially available seeds of Green Thampala and stem cuttings of Mukunuwenna, were collected (Figure 3). To support plant growth in a hydroponic system, Albert's solution, a commercially available nutrient solution, was utilized (Figure 4, Table 1).

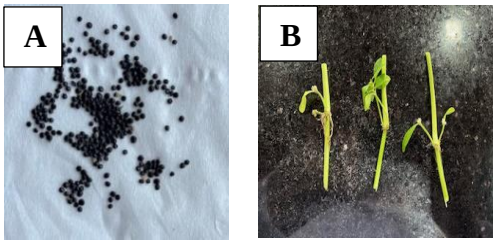

Figure 3. A) Green Thampala seeds, B) Mukunuwenna stem cuttings.

Figure 4. Albert's solution.

Table 1. Nutrient content in Albert's Solution

Macro nutrients	Percentage (%)	Micro nutrients	Percentage (%)
N	10.5	S	1.00
H_3PO_4	9.1	B	0.003
K	16.4	Zn	0.014
Mg	0.86	Cu	0.0004
Ca	9.5	Fe	0.065
		Mn	0.012
		Mo	0.0019

2.2 Germination of the plants. Coconut coir pellets were soaked in water for 10 minutes before placing the seeds of Green Thampala into the pellets. The plants were watered and kept in the dark for three days. Meanwhile, the stem pieces of Mukunuwenna were placed in water until new roots formed.

2.3 Preparation of hydroponics system.

2.3.1 Method 1: NFT System. According to the user manual, the parts of the NFT system were assembled (Figure 5A). A 50% concentration of Albert's solution was prepared and used as a control. After that, 0.25 g and 0.5 g of $FeSO_4$(s) were added to the 50% Albert's solution, creating iron concentrations of 325 ppm (control), 350 ppm, and 375 ppm. The pH and EC values of the nutrient solutions were measured. The motor was then installed in the system. Mukunuwenna stems, which had been kept in water for six days, were then placed into the holes of the system.

2.3.2 Method 2: Coco peat grow bag method. 10 kg of coco peat were soaked in water for 24 hours. The polythene bags were then filled with the coco peat. Green Thampala and Mukunuwenna stems were planted in the coco peat grow bags (Figure 5B). A 25% concentration of Albert's solution was prepared and used as a control. Subsequently, 0.375 g and 0.775 g of $FeSO_4$(s) were added separately to the 25% Albert's solution, resulting in iron concentrations of 162.5 ppm (control), 200 ppm, and 240 ppm. The pH and EC values of the nutrient solution were measured, and it was poured onto the plants in the morning, and water was sprayed on them in the evening on a daily basis.

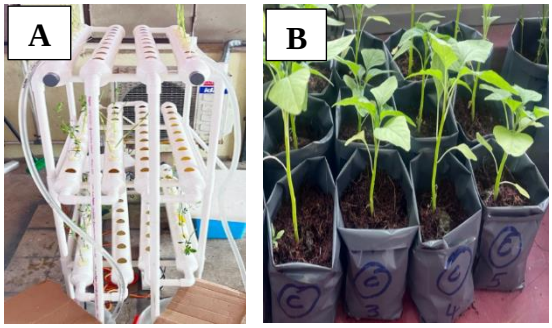

Figure 5. A) NFT system B) Coco peat grow bags

2.4 Homogenization and Preparation of aqueous plant extracts. Fully hydroponically grown plants were harvested after 5 weeks, and fresh weight was measured. Then the plants were shredded and kept in the hot air oven at 40°C for 48 hours. The dried plants were ground using a mortar and pestle. The powdered samples were diluted to 1:20 using water and the solutions were kept in the oven at 70°C for 10 minutes. After that the aqueous extracts were filtered, and the filtrates were refrigerated in falcon tubes for qualitative and quantitative analyses.

2.5 Determination of total carbohydrate content: Phenol- sulfuric acid method. A 0.1 g of dextrose powder was measured using an analytical balance and mixed with 2.5 N HCl. This mixture was placed in a water bath at 100°C for 3 hours. After cooling to room temperature, the mixture was neutralized with Na_2CO_3 and topped up to 100 ml in a volumetric flask to create a dextrose stock solution (1000 µg/ml). Using this stock solution, a dextrose standard series of known concentrations (50-250 µg/ml) was prepared. 1ml of diluted plant extract (1:20) was mixed with 5 ml of 96% H_2SO_4 and 1 ml of 5% phenol. The test tube was shaken for 10 minutes and then placed in a water bath at 25-30°C for 20 minutes until the solution turned green. Finally, the absorbance was measured at 490 nm using a UV-visible spectrophotometer.[19] A standard curve was plotted using the absorbance of the standard series. The total carbohydrate content of plants samples was derived by comparing their absorbance values to the standard curve.

2.6 Determination of total protein content: Lowry Assay. A BSA standard series (200-1000 µg/ml) was prepared. 1 ml of standard series and 1 ml of diluted (1:20) plant extract was added to the test tubes. 5 ml of Lowry A and B mixture was added to each test tubes and incubated at RT for 10 minutes. Then 0.5 ml of Lowry C was added to each tube and incubated at RT for 30 minutes. Finally, the absorbance was measured at 660 nm using a UV-visible spectrophotometer.[20] A standard curve was plotted using the absorbance of the BSA standard series. The total protein content of plant samples was derived by comparing their absorbance values to the standard curve.

2.7 Determination of total flavonoid content: $AlCl_3$ Spectrophotometric method. A quercetin standard series (20-100 µg/ml) were prepared. 1 ml of diluted Plant extract (1:10) and standards were mixed with 0.2 ml of 10% $AlCl_3$ and 0.2 ml of 1M potassium acetate. Then the mixture was incubated at RT for 30 minutes with intermittent shaking. Finally, the absorbance measured at 415 nm using a UV-visible spectrophotometer.[21] A standard curve was plotted using the absorbance of the quercetin standard series. The total flavonoid content of plant samples was derived by comparing their absorbance values to the standard curve.

2.8 Determination of total phenolic content: Folin-Ciocatleu assay. A standard series of gallic acid (20-100 µg/ml) was prepared. 0.3 ml of both standard and diluted sample (1:10) were mixed with 1.2 ml of 10% FC reagent and 1.5 ml of 7.5% Na_2CO_3. Then the mixture was shaken and incubated for an hour in the dark at RT. Finally, the absorbance was measured at 765 nm using UV-visible spectrophotometer.[21] The total phenolic content of plant samples was derived by comparing their absorbance values to the standard curve.

2.9 Determination of Fe content: Atomic Absorption Spectrophotometry (AAS). 10-20 g of the samples were dried at 100°C, and the samples were ashed using a programmable furnace. The temperature gradually increased to 450°C for8 hours. Ashing was repeated until the product turns white or grey. Then HCl was added to the ash, and it was allowed to evaporate. Then the residue was dissolved in HNO_3. The solution was transferred to a plastic

bottle, and blank was treated in the same way. Then Fe content of the samples was determined by flame AAS.[22]

2.10 Qualitative analysis of phytochemicals. Qualitative tests were carried out to check the presence of the phytochemicals as shown in Table 2.

Table 2. Qualitative tests for phytochemical screening.

Phytochemicals	Procedure
Saponins	2 ml of distilled water was added to 2 ml of extract. The mixture was then shaken for 15 minutes using a vortex until foam formed.[23]
Tannins	2 ml of 5% $FeCl_3$ solution were added to 1 ml of extract. The presence of tannins was confirmed if the color changed to greenish black.[23]
Polyphenols	3 drops of diluted iodine solution were added to 1ml of sample.[24]
Anthraquinones	2 ml of 10% Ammonia solution was mixed with 0.5 ml of plant, formation of red precipitate was indicative of anthraquinones.[24]
Terpenoids	0.5 ml of each plant sample was mixed with 2ml of chloroform and 2 ml of conc. H_2SO_4.[25]
Steroids	0.5 ml of each plant sample was mixed with 0.5ml of chloroform and 1 ml of conc.H_2SO_4.[25]

2.11 Determination of antioxidant activity: DPPH assay. 1 ml of 0.004% DPPH radical solution (100 µM in methanol) was mixed with 1ml of sample. Then the mixture was incubated at 37°C for 30 minutes. The absorbance was measured at 517 nm using UV-visible spectrophotometer. Then DPPH radical scavenging activity was measured using the equation given below.[25]

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

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

2.12 Statistical analysis. All graphs were generated using Microsoft Office 365 Excel. IBM SPSS Statistics Version 28.0.0.0 was used to perform the statistical analysis and all the values are expressed as mean ± SE. Statistical analysis was conducted by one-way ANOVA followed by LSD test for multiple comparison analysis. A p value less than 0.05 was considered to have a significant difference and $0.05 < p$ value < 0.1 was considered to have a tendency for a significant difference.

3. Results

3.1 Growth of plants (Plant height and Leaf number)

3.1.1 Growth of Green Thampala (GT). On the day of harvest (5^{th} week), the growth of GT, shown in Figure 6, was assessed under three conditions: A (200 ppm), B (240 ppm), and C (control).

Figure 6. Growth of GT on the day of harvest (5th week). A-200 ppm, B-240 ppm and C-control

3.1.2 Growth of Mukunuwenna (MK). On the day of harvest (5th week), the growth of MK was evaluated under three conditions: 200 ppm (A), 240 ppm (B), and control (C), as shown in Figure 7.

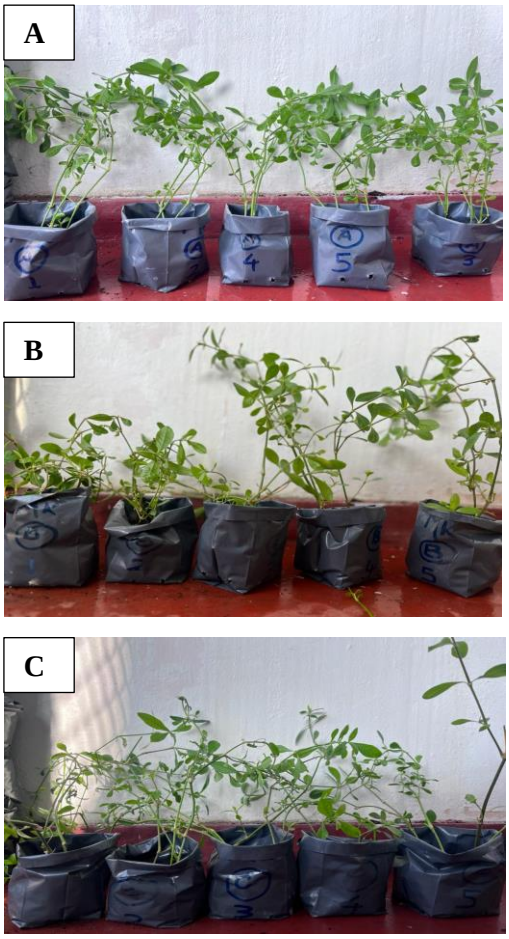

Figure 7. Growth of MK on the day of harvest (5th week), A-200 ppm, B-240 ppm and C-control.

3.2 Fresh yield of plants

3.2.1 Fresh yield of Green Thampala

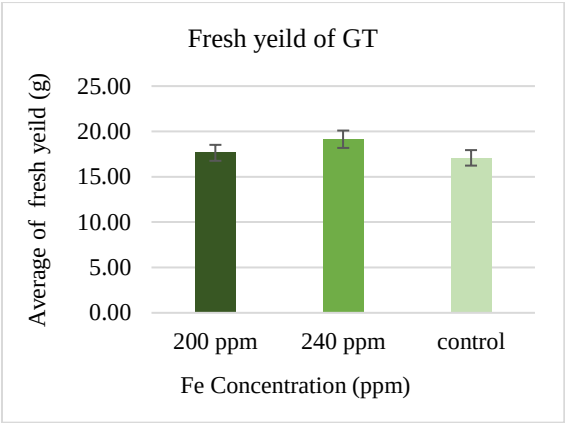

Figure 8. Average fresh yield of GT in different Fe concentrations.

GT grown at 240 ppm of Fe concentration had the highest fresh yield and control had the lowest fresh yield (Figure 8).

3.2.2 Fresh yield of Mukunuwenna

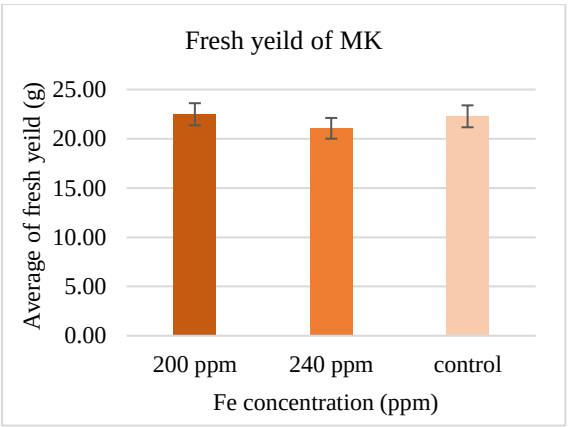

Figure 9. Average fresh yield of MK in different Fe concentration.

MK grown at 200 ppm of Fe concentration had the highest fresh yield and 240 ppm had the lowest fresh yield (Figure 9).

3.3 Height of plants

3.3.1. Height of Green Thampala

At the time of harvest, GT grown at 200 ppm of Fe concentration had the highest height during the growth period, and it was significantly high compared to GT grown at 240 ppm (Figure 10).

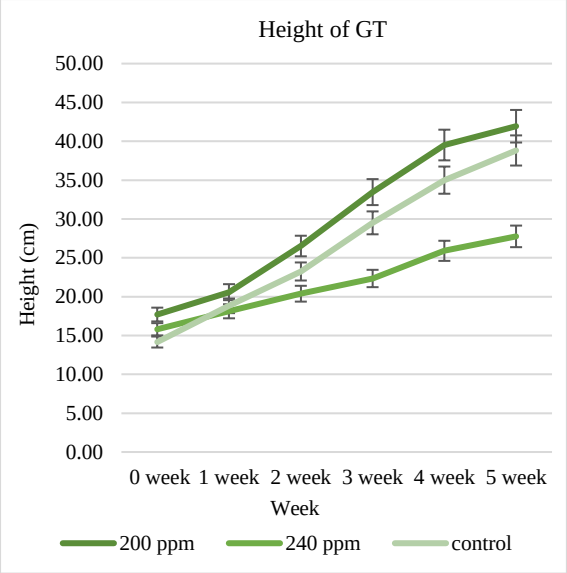

Figure 10. Average height of GT from week 0 to week 5.

3.3.2 Height of Mukunuwenna

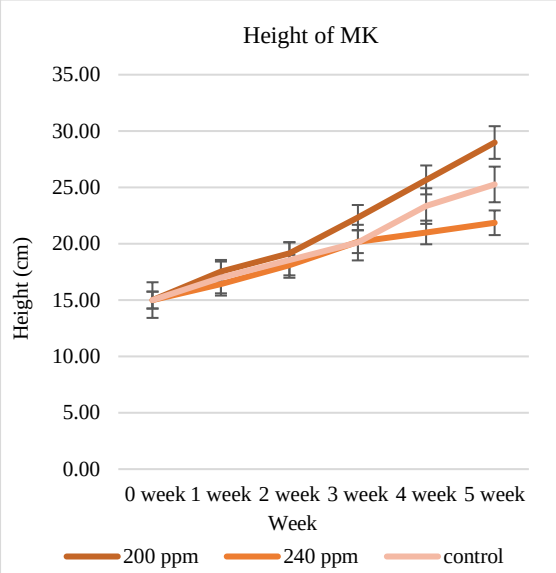

Figure 11. Average height of MK from week 0 to week 5

At the time of harvest, MK grown at 200 ppm of Fe concentration had the highest height during the growth period, and it was significantly high compared to MK grown at 240 ppm (Figure 11).

3.4 Leaf count of Plants

3.4.1. Leaf count of Green Thampala

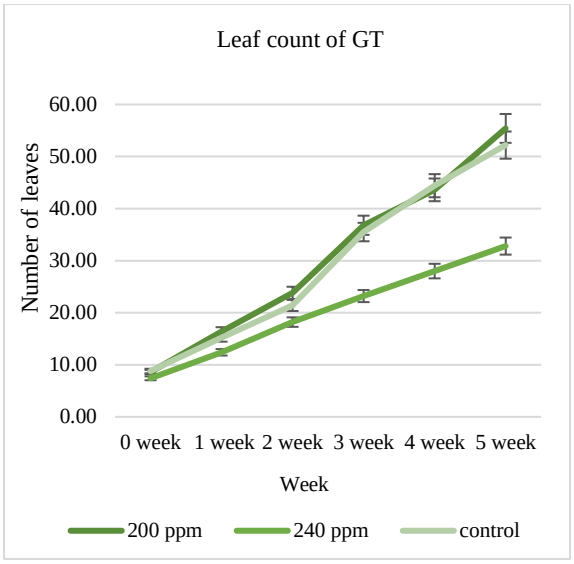

Figure 12. Average leaf count of GT from 0th week to 5th week.

GT grown at 200 ppm Fe concentration had the highest number of leaves during the growth period, and 240 ppm had the lowest number of leaves (Figure 12).

3.4.2 Leaf count of Mukunuwenna

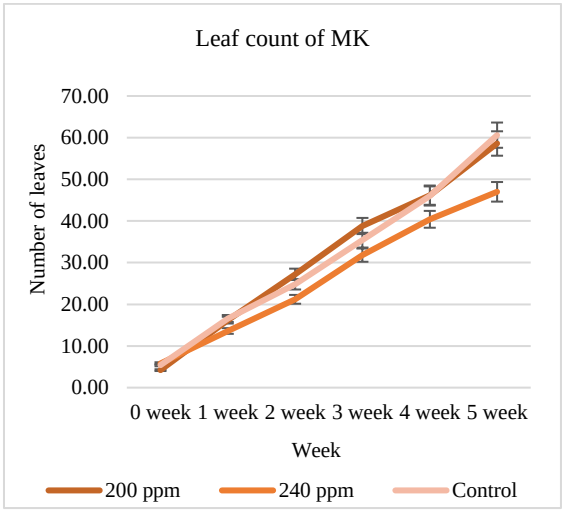

Figure 13. Average leaf count of MK from week 0 to week 5.

Mk grown at 200 ppm of Fe concentration, had the highest number of leaves during the growth period and 240 ppm had the lowest number of leaves (Figure 13).

3.5 Total Carbohydrate Content (TCC) of Plants

3.5.1. TCC of Green Thampala

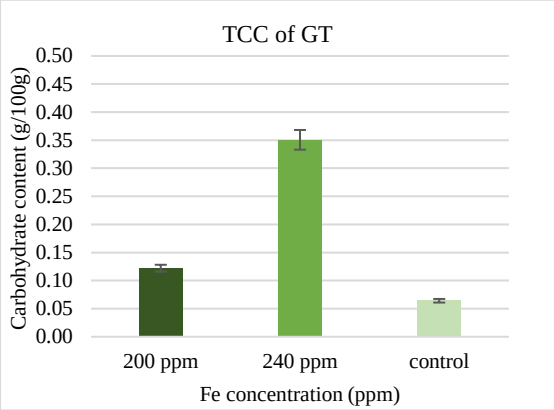

Figure 14. Total carbohydrate content of GT in different Fe concentrations.

GT grown at 240 ppm of Fe concentration had the highest content of carbohydrate and control had the lowest content of carbohydrate (Figure 14).

3.5.2 TCC of Mukunuwenna

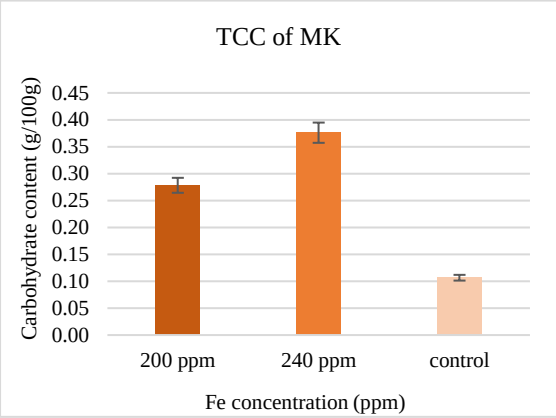

Figure 15. Total carbohydrate content of MK in different Fe concentrations.

MK grown at 240 ppm of Fe concentration had the highest carbohydrate content, while control had the lowest carbohydrate content (Figure 15).

3.6 Total Protein Content (TPrC) of Plants

3.6.1 TPrC of Green Thampala

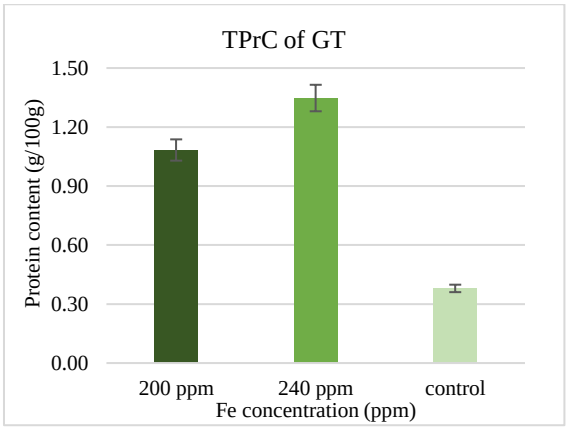

Figure 16. Total protein content of GT in different Fe concentration.

GT grown at 240 ppm of Fe concentration had the highest content of protein and control had the lowest content of protein (Figure 16).

3.6.2 TPrC of Mukunuwenna

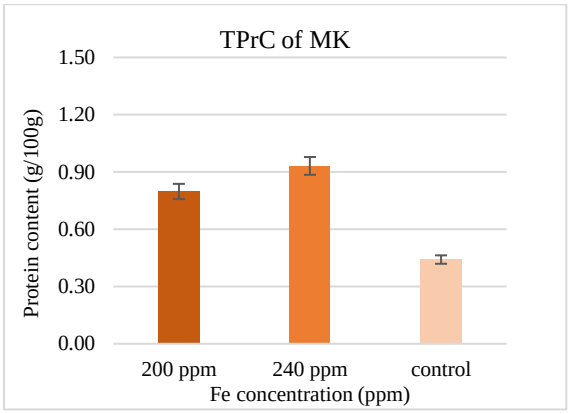

Figure 17. Total protein content of MK in different Fe concentration.

MK grown at 240 ppm of Fe concentration had the highest protein content, while control had the lowest protein content (Figure 17).

3.7 Total Flavonoid Content (TFC) of Plants

3.7.1 TFC of Green Thampala

GT grown at 240 ppm of Fe concentration had the highest content of flavonoid, and 200 ppm had the lowest content of flavonoid (Figure 18).

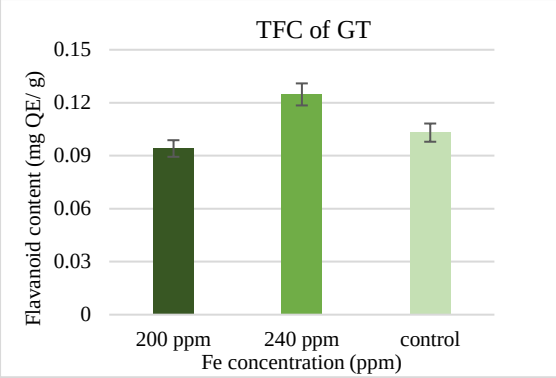

Figure 18. Total flavonoid content of GT in different Fe concentration.

3.7.2 TFC of Mukunuwenna

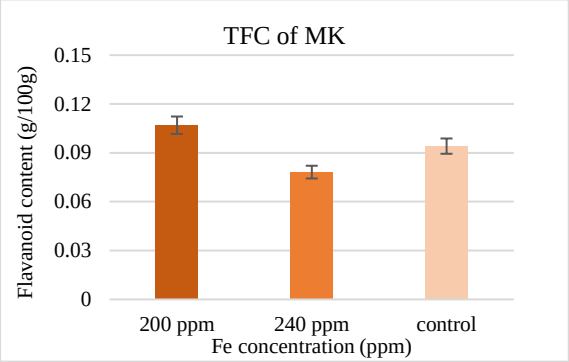

Figure 19. Total flavonoid content of MK in different Fe concentration.

MK grown at 200 ppm of Fe concentration had the highest content of flavonoid and, 240 ppm had the lowest content of flavonoid (Figure 19).

3.8 Total Phenolic Content (TPC) of Plants

3.8.1 TPC of Green Thampala

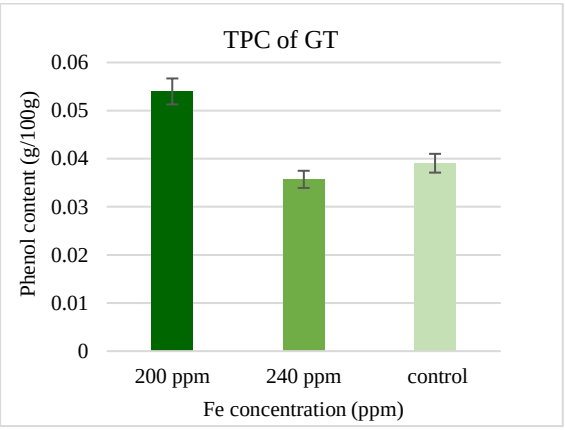

Figure 20. Total phenol content of GT in different Fe concentrations.

GT grown at 200 ppm of Fe concentration had the highest content of phenol, and 240 ppm had the lowest content of phenol (Figure 20).

3.8.2 TPC of Mukunuwenna

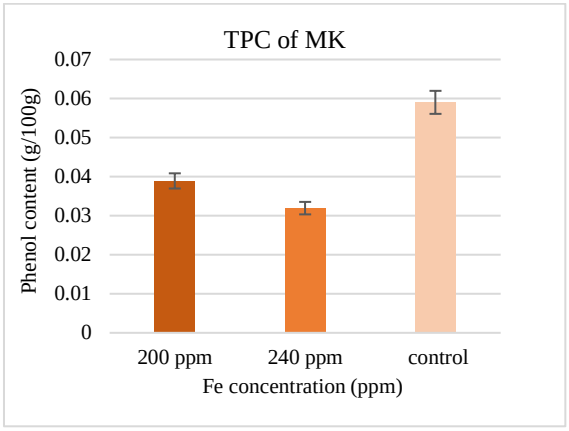

Figure 21. Total phenol content of MK in different Fe concentration.

MK grown in control of Fe concentration had the highest content of phenol, and 240 ppm had the lowest content of phenol (Figure 21).

3.9 Fe content of Green Thampala and Mukunuwenna

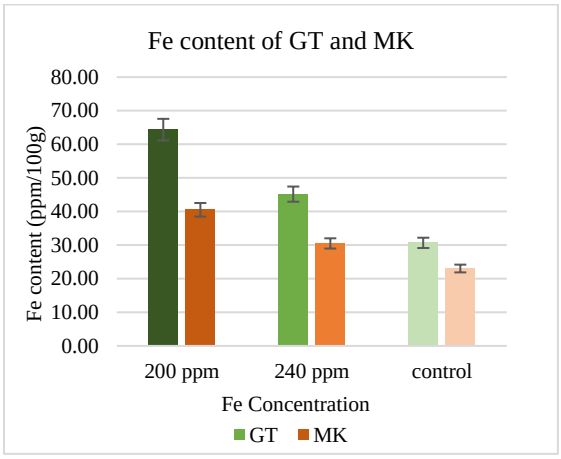

Figure 22. Fe content of GT and MK in different Fe concentration.

MK and GT grown at 200 ppm of Fe concentration had the highest content of Fe, and control had the lowest content of Fe (Figure 22).

3.10 Qualitative Analysis of Phytochemicals

Qualitative tests for phytochemicals were conducted across different Fe concentrations of Green Thampala and Mukunuwenna. The results of these tests are stated in Table 3.

Table 3. Results of the qualitative analysis (√- Present; x-Absent)

Bioactive compound	GT-200 ppm	GT- 240 ppm	GT- control	MK-200 ppm	MK-240 ppm	MK- control
Saponins	√	√	√	√	√	√
Polyphenols	√	√	√	√	√	√
Tannins	√	√	√	√	√	√
Anthraquinones	x	x	x	x	x	x
Steroids	√	√	√	√	√	√
Terpenoids	√	√	√	√	√	√

The results suggest that saponins, polyphenols, tannins, terpenoids, and steroids were present, while anthraquinones were not found in Green Thampala (GT) and Mukunuwenna (MK) across different Fe concentrations.

3.11 Antioxidant activity of plants

3.11.1 Antioxidant activity of Green Thampala

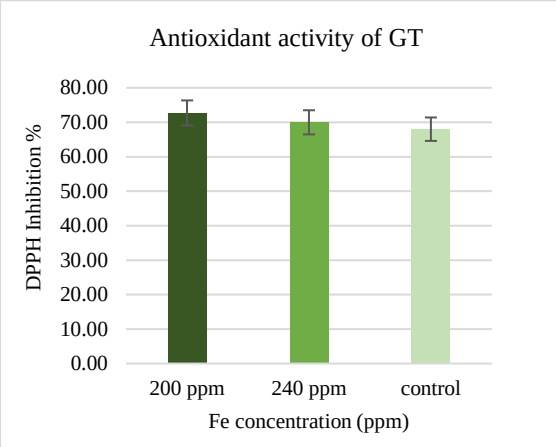

Figure 23. Antioxidant activity of GT in different Fe concentrations.

GT grown at 200 ppm of Fe concentration, had the highest percentage of inhibition, and control had the lowest percentage of inhibition (Figure 23).

3.11.2 Antioxidant activity of Mukunuwenna

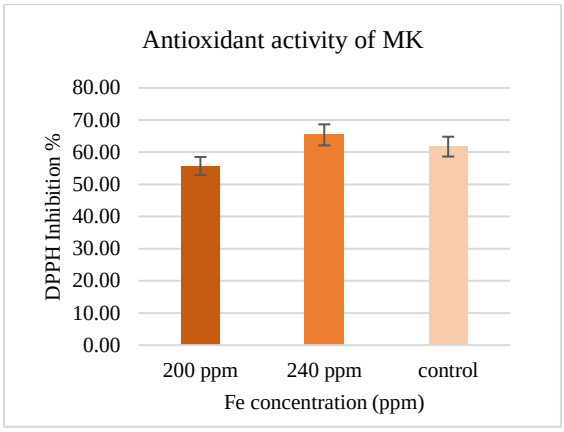

Figure 24. Antioxidant activity of MK in different Fe concentration.

MK grown at 240 ppm of Fe concentration had the highest percentage of inhibition, while 200 ppm of Fe concentration had the lowest percentage of inhibition (Figure 24).

4. Discussion

In this study, iron biofortification of Green Thampala and Mukunwenna was carried out using hydroponic techniques such as NFT and the coco peat method. Throughout the growth period, various parameters including height and leaf count, were recorded. Also, the total protein and carbohydrates of Fe-fortified plants were determined. Several qualitative tests were carried out to identify the presence of phytochemicals in GT and MK. Moreover, DPPH assay was carried out to determine the antioxidant activity. Furthermore, the TPC and TFC of plants were utilized to analyse the phenolic content and flavonoid content of Fe-fortified plants.

Albert's solution (AB solution) was used as a nutrient solution in the NFT system. It contains 650 ppm of Fe. Initially, 100% concentration of the AB solution was used as a control. MK plants were dead after 5 days of transferring to the NFT system due to the high concentrations of Fe. Normally, plants absorb 50–500 ppm of Fe; if it increases more than 500 ppm, it causes toxicity; if it is lower than 50 ppm, it will raise Fe deficiency in plants.[17] Moreover, 50% of the AB solution gives higher plant growth than 100% of the AB solution.[26]

Therefore, a 50% AB solution was used as a control in the next trial. However, this concentration was also not successful, possibly due to overwatering and over fertilizing. Overwatering may be prevented by submerging the tip of the root in a nutrient solution, and over fertilizing may be prevented by diluting the AB solution by 25%. Considering the drawbacks, the coco peat grow bag method was used as an alternative technique since it reduces overwatering and promotes plant growth.

When looking at the height and number of leaves of GT and MK in the 5th week, both showed the highest results in 200 ppm and the lowest in 240 ppm. Also, when comparing the fresh yield of GT at different Fe concentrations, the highest yield was obtained at 240 ppm, the lowest at control. These results agree with those of Buturi *et al*. 2022, where Fe deficiency stress inhibits the growth of plants while Fe efficiency induces the growth of plants.[14] Moreover, different parameters of the plant, such as height, weight, number of leaves, and root shoot ratio, increase with Fe concentration, but when the plant meets the threshold value of Fe, it suppresses the elevation of the parameters.[14] In this research, MK grown at 240 ppm of Fe concentration showed the lowest height, number of leaves, and fresh yield compared to MK grown at control and 200 ppm. Hence it can suggest that the threshold value of Fe for MK may fall between 200-240 ppm.

Carbohydrates produced during photosynthesis in plants are widely known as energy sources and carbon skeletons for organic molecules and storage components.[27] The phenol-sulfuric acid technique was employed in this investigation to identify TCC. In this method, disaccharides, oligosaccharides, and polysaccharides are broken down into monosaccharides by concentrated sulfuric acid. Following their reaction with phenol, these compounds provide a yellow-gold coloration. [28] When comparing the TCC of GT and MK in different concentrations of Fe, both showed the highest result at 240 ppm and the lowest in the control. These results were not in agreement with the findings of a previous study by Buturi *et al*.,2022, since excessive levels of Fe can increase Reactive Oxygen Species (ROS), causing cell damage and disrupting several metabolic events, including lowering the rate of photosynthesis. A reduction in the photosynthesis rate significantly reduces the carbohydrate content.[14]

Protein is a high-molecular weight bioactive compound. The Lowry method was used to determine the TPrC. Under alkaline conditions, cupric ions (Cu^{2+}) chelate with nitrogen atoms in peptide bonds, resulting in a reduction of Cu^{2+} to cuprous ions (Cu^{+}). Folin-Ciocalteau reagent reduces the Cu^{+} to produce tungsten blue.[29] When comparing the TPrC of GT and MK in different concentrations of Fe, both showed the highest protein content at 240 ppm and the lowest content in the control. The results were not in agreement with the findings of previous study by Ramzan *et al*., 2020. Protein content of maize was affected by the treatment applications; however, the highest protein content (14.37%) was found in control while lowest protein content (12.63%) was found in 1% of Fe foliar application.[30]

Chemical substances that are found naturally in plants are known as phytochemicals. There are many different parts of the plant that contain essential phytochemicals, such as terpenoids, flavonoids, phenolics, tannins, saponins, and steroids. In addition to providing the plant with color, scent, and flavor, they shield it from illnesses, pollution, stress, and UV radiation.[24] When considering the phytochemical analysis of GT and MK in different concentration of Fe, both showed positive results for saponin, tannins, terpenoids, steroids, and polyphenols, but they showed negative for anthraquinones.

Flavonoids are plant secondary metabolites with a polyphenolic structure.[31] In this study, TFC was performed using an $AlCl_3$ spectrophotometric assay. $AlCl_3$ forms acid-stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl groups of flavones and flavanols.[32] When looking at the TFC of GT and MK in different concentrations of Fe, GT grown at 240 ppm had the highest TFC, and 200 ppm had the lowest TFC. But the MK showed the highest TFC in 200 ppm and the lowest at 240 ppm. These results are in line with the previous study of Giordano *et al*. (2019), where a subgroup of flavonoids was

significantly increased in 2.0 mM Fe compared to 1.0 mM and 0.015 mM Fe.[33]

Phenolics are thought to have the highest ability to neutralize free radicals.[34] In this study, the Folin-Ciocalteu method was used to determine the TPC. The assay involves reducing the FC reagent with phenolic compounds in an alkaline medium. The reaction produces a blue chromophore composed of a phosphotungstic-phosphomolybdenum complex with the maximum absorption of the chromophores depending on the alkaline solution and the concentration of phenolic compounds.[35] When looking at the TPC of GT and MK in different concentrations of Fe, GT grown at 200 ppm had the highest TPC and the lowest at 240 ppm, but the MK showed the highest TPC in control and the lowest at 240 ppm. The result of GT is in line with a previous study by Buturi *et al*.,2022 and MK is not in agreement with these findings where 1 mM and 2mM of Fe application has increased the production of phenol whose main role is to control ROS production.[14]

Antioxidants play a crucial role in minimizing oxidative stress in biological processes by neutralizing free radicals, which can cause damage when combined with essential cellular elements like DNA and proteins.[36] Using the DPPH test, the antioxidant activity was assessed. When antioxidants interact with DPPH, they convert it to DPPH-H, which decreases absorbanc.[37] A higher inhibition percentage indicates the greater antioxidant activity of the sample compound. In this study, GT grown at 200 ppm had the highest inhibition percentage and control had the lowest while MK grown at 240 ppm had the highest and 200 ppm had the lowest. The results agree with the previous study of Arreola *et al.*, 2015, where an antioxidant activity of common beans was significantly increased in 50 µmol Fe compared to 25 µmol of Fe.[38] When comparing the Fe content of GT and MK in different concentrations of Fe, both plants showed the highest content at 200 ppm and the lowest content in control. Fe content increases until the plant reaches its optimum level; when it crosses the optimum level, Fe content decreases.

In interpreting the findings of this study, it is important to acknowledge several limitations. Controlled environmental conditions are essential for ensuring reliable results in future studies. Variations in factors like temperature and humidity can significantly impact outcomes, highlighting the necessity for precise control over these variables. The nutrient solution concentration was adjusted based on observed symptoms, but more precise initial trials with varied concentrations could help determine optimal levels more accurately. The study duration may not have been sufficient to observe long-term effects, suggesting that extending the duration would provide insights into the sustained impacts on plant health and nutritional content. Additionally, while qualitative tests identified the presence of phytochemicals, a quantitative analysis would offer a deeper understanding of their concentration and impact. By addressing these limitations and incorporating the recommended improvements, future studies can achieve more robust and reliable results, enhancing the overall quality and impact of the research.

5. Conclusion

Both 200 ppm and 240 ppm Fe concentrations were effective in fortifying Fe in both Mukunuwenna and Green Thampala, as evidenced by higher Fe content compared to the control group. Fe-fortified Mukunuwenna and Green Thampala also demonstrated varying levels of antioxidant activity, total flavonoid content, total phenolic content, and fresh yield. Based on the findings of the study, it can be recommended that 200 ppm of Fe can be utilized for increasing Fe content along with plant development, while 240 ppm of Fe may be preferred for enhancing Fe content along with nutrient enrichment.

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Phytochemical analysis and the evaluation of antioxidant and antimicrobial activities of five Sri Lankan Cucurbitaceae varieties

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Abstract

Medicinal plants of the Cucurbitaceae family include a variety of edible fruits and vegetables. Various parts of these plants show different pharmacological activities such as anticancer, antidiabetic, hypolipidemic, immunomodulatory, anti-inflammatory, and antimicrobial activities. Five Sri Lankan Cucurbitaceae varieties: Cucumber (*Cucumis sativus*), Pumpkin (*Cucurbita maxima*), Watermelon (*Citrullus lanatus)*, Snake gourd (*Trichosanthes cucumerina*), and bitter gourd (*Momordica charantia*) were chosen and the leaves of the selected varieties were analyzed for their phytochemicals, antioxidant and antimicrobial activities, after extraction using the maceration technique. Total phenolic content, total flavonoid content, total antioxidant capacity and the free radical scavenging activity were determined by the Folin-Ciocalteau method, Aluminum Chloride colourimetric assay, Phosphomolybdenum method, and the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay respectively. Antibacterial susceptibility tests were also done to find the antibacterial activity of the extracts using the disc diffusion and well diffusion methods. Qualitative analysis showed the presence of flavonoids, terpenoids, phenols, proteins, saponins, and reducing sugars in all the extracts. Snake gourd had the highest phenolic content and antioxidant capacity, while watermelon had the greatest flavonoid content and the highest free radical scavenging activity among the rest of the samples. This study was useful in identifying the antioxidant and antimicrobial activities in the selected Cucurbitaceae leaf extracts and using them as potential antioxidants.

Keywords: Cucurbitaceae, phytochemicals, antioxidant, antimicrobial, plant leaves

1. Introduction

The use of plants as a source of remedies for the treatment of diseases dates back to prehistory, and people from all continents still follow this ancient practice. Since they play such a significant role in community safety nowadays, medicinal plants are receiving more attention from research institutions.[1] Research on medicinal plants, including toxicological and pharmacological evaluations, is key for drug research and development. Some of the drugs that are, obtained from plants are morphine, colchicine, quinidine, aspirin, tubocurarine, artemisinin, digoxin, ephedrine, physostigmine, reserpine, pilocarpine, quinine, paclitaxel, vincristine, atropine, and vinblastine.[2] Because of their easy availability, affordability, and accessibility, as well as their promising potency in contrast to the general high cost and negative side effects of general synthetic drug agents, their use is currently on the rise.[3] According to Future Market Insights (FMI), a "trend shift from conventional medicines to traditional medicines," along with a more simpler regulatory environment and growing global productive capacity, will result in a progressing compound annual growth rate (CAGR) of 7.6% over the reported 10year

forecast span (2017–2027) in the global market for plant based medicinal medicines.[4]

Herbal medicines have a solid traditional or conceptual foundation and the ability to be effective drugs in terms of safety and efficacy, leading to the treatment of numerous diseases. The present research was on a family of plants known as the Cucurbitaceae also called the gourd family, figure 1 shows a classified list of the genera and tribes of the Cucurbitaceae family food plants including the five plants used in this research. This family includes both wild and domesticated species and is consumed in different ways,[5] many different species with medicinal value belong to the family Cucurbitaceae. It is a family of about 130 genera and about 800 different species. They are one of the most significant plant families that provide useful fibers and edible products to people.[6]They are climbers with stores in their roots that are widely distributed in the tropics, Africa, Madagascar and central South America, in addition to warm temperate areas of South, Southeast, and East Asia. Traditional medicine uses all parts of the Cucurbitaceae plant (leaf, stem, root or tuber, fruit, and seeds).[7]

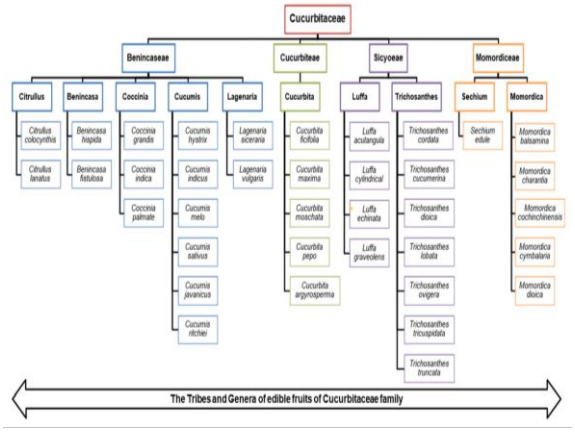


Figure 1. Cucurbitaceae family[8]

Many studies have been conducted on various Cucurbitaceae plants by researchers around the globe. Table 1 shows data from research done across the globe, on potential pharmacological effects of some plants of the Cucurbitaceae family.

Table 1. Worldwide research data on potential pharmacological effects of some plants of the Cucurbitaceae family

Plant	Pharmacological Activity
Cucumis sativus	Anticancer potential against HepG2 cell.[9]
Cucurbita moschata	Anticancer potential and increased ribosomeinactivating protein activity.[10]
Citrullus lanatus	Antidiabetics.[11]
Cucurbita pepo	Anti-inflammatory, antioxidant, anticarcinogenic, antiviral, anti-proliferative antimicrobial, and analgesic properties.[12]
Iberis amara	Anti-inflammatory.[13]
Cucumis melo	Anticancer, analgesic, antimicrobial, antiinflammatory, antidiabetic, hepatoprotective, antioxidant, antiulcer, diuretic and immunomodulatory properties.[10]
C. Grandis	Antidiabetic and antioxidant properties.[14]
Coutarea hexandra	Anticancer, anti-inflammatory, antimalarial and antidiabetic properties .[15]
Rubus chingii	Anticancer, anti-inflammatory, antioxidant, antidiabetic, and anti-ageing properties.[16]
T. cucumerina	Antidiabetic, and antioxidant properties.[14]
Kageneckia oblonga	Anti-inflammatory, analgesic, and antipyretic properties.[17]

Plants from this family are widely used as traditional herbal treatments for a range of illnesses. They have shown anti-inflammatory, anti-fungal, anticancer, antiviral, anti-bacterial, antidiabetic, cardiovascular, hepatoprotective and immunoregulatory properties. The fact that members of this family are rich sources of proteins and have a variety of biological properties, has generally led to their consideration as research subjects. Several phytochemicals, including flavonoids, sterols, phenols and alkaloids, are also known to be present in this family.[18]

Most of the protective effects of these plants has been linked to phytochemicals, which are the non-nutrient plant compounds

such as alkaloids, carotenoids, phenolic acids, flavonoids and isoflavonoids. Although many phytochemicals found in food have been discovered, many more remain unidentified. Numerous phytochemicals have been discovered to possess a range of activities.[19] Due to their widespread presence in the diet and apparent minimal toxicity, phytochemicals can influence disease risk and human health at the population level.[21] Many phytochemicals function as antioxidants, stabilizing free radicals and removing their ability to cause damage.[22] Figure 2 shows the classification of the main phytochemicals, the phytochemical families are shown and examples of phytochemicals are shown in light grey.

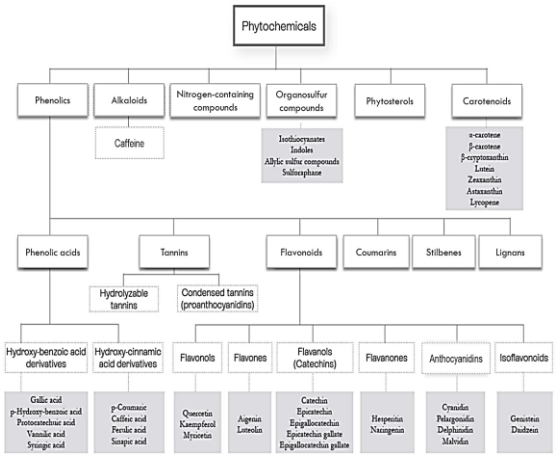


Figure 2. Classification of the main families of phytochemicals[23]

An antioxidant can be defined as "any substance that, when presented at a less amount compared to an oxidizable substrate (carbohydrates, lipids, proteins and DNA), prevents or significantly delays the oxidation of that substrate". Antioxidants' primary job is to shield the body from the damage that free radicals can cause. Free radicals might be produced in cells and tissues as a result of weakened protective capacity or from internal (metabolism, diseases or inflammation) or external (pollution, drugs, food or irradiation) sources as shown in figure 3. In any event, an increase in the production of free radicals can cause oxidative damage.[24] Due to the numerous negative side effects of synthetic antioxidants, natural forms of antioxidants are now being focused upon. Additionally, there is a need to look for novel antimicrobial agents due to the rise in antibiotic resistance.[25]

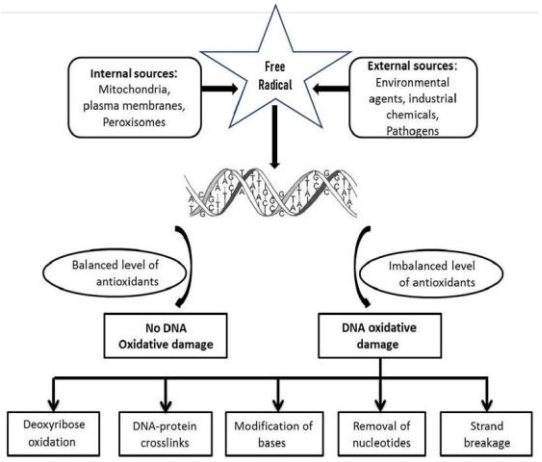


Figure 3. Free radical formation[26]

Researchers are now considering the use of other natural products with antibiotic actions, such as medicinal plants, due to the global concern over the rapid development of bacterial resistance to synthetic antibiotics.[27] Higher plants have also been a source of antibiotics, even though the majority of the clinically used antibiotics are made by soil microorganisms or fungi. Antimicrobials derived from plants are a vast untapped supply of potential medicines. Research on plant-based antimicrobials must continue and be expanded upon. Antimicrobials derived from plants have a great deal of therapeutic potential. They effectively cure infectious diseases while also minimizing a number of the side effects frequently connected to synthetic antimicrobials.[28]

The aim of this research was to determine the phytochemicals, antioxidant and antimicrobial activity in the leaf extracts of cucumber, bitter gourd, watermelon, pumpkin and snake gourd.

2. Materials and Methodology

2.1. Materials

2.1.1. Plant Materials. Leaves of *Cucumis sativus* (cucumber) (C), *Momordica charantia* (bitter gourd) (B), *Citrullus lanatus*

(watermelon) (W), *Trichosanthes cucumerina* (snake gourd) (S) and *Cucurbita maxima* (pumpkin) (P) plants were used in this study.

2.2. Methodology

2.2.1. Preparation of plant materials and extracts. Leaves (50g) from each species of Cucurbitaceae were collected in February from the western and central province of Sri Lanka. They were then cleaned and left to shade dry for a week (Figure 4), the dried leaves were then powdered using a mortar and pestle, after which 2g of the powder was measured using an analytical balance and mixed with 100mL of distilled water (DW), this was placed into a roller mixer and left for 48 hours for extraction, after which the mixture was filtered using muslin cloth and filter paper to get the filtrate which was stored at 4°C.[29]

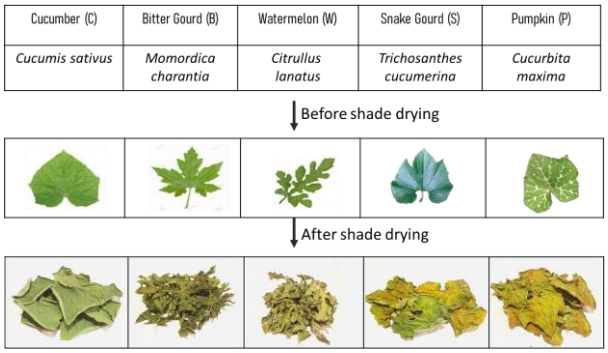


Figure 4. Sample collection and shade drying

2.2.2. Qualitative tests for phytochemicals. The below tests were carried out for all five samples.

2.2.2.1. Test for flavonoids. A few drops of NaOH were added to the aqueous extract (AE) (1mL), followed by the addition of a few drops of sulfuric acid.[30]

2.2.2.2. Test for terpenoids. Chloroform (2mL) and conc H_2SO_4 (3mL) was carefully added to the AE (1mL).[31]

2.2.2.3. Test for phenols. A few drops of 5% ferric chloride solution were added to the AE (1mL).[32]

2.2.2.4. Test for proteins. Millon's reagent (1mL) was mixed with the AE (2mL), and gently heated.[33]

2.2.2.5. Test for saponin. DW (2mL) was added to the AE(1mL) and shaken vigorously.[33]

2.2.2.6. Test for reducing sugars. Benedict's solution (2mL) was added to the AE (1mL) and boiled.[33]

2.2.3. Quantitative analysis of phenolics, flavonoids and antioxidants. The following tests were carried out for each sample in triplicates.

2.2.3.1. Determination of the Total Phenolic Content (TPC). 1mL of 1:10 diluted Folin-Ciocalteu reagent was mixed with 200 µL of AE. 800 mL of saturated sodium carbonate solution (75 g/L) was added after 4 minutes. The absorbance of the AE and standard solutions at 765 nm was measured in triplicates after 2 hours of incubation at room temperature, shielded from light. The standard curve was constructed using gallic acid (0–150 µg/mL) following said procedure. The TPC was expressed as mg gallic acid equivalent (mg GAE)/g dry weight of plant extract.[34]

2.2.3.2. Determination of the Total Flavonoid Content (TFC). 2mL of DW and 0.15mL of 5 % sodium nitrite solution was added to 0.5mL of AE, after 5 minutes, 0.15mL of 10% aluminum chloride was added, at the 6th minute,1mL of 1 M sodium hydroxide was added and mixed well. The absorbance of the AE and standard solutions at 510 nm was measured in triplicates against a blank containing DW. The standard curve was constructed using quercetin (100-1000 µg/mL) following said procedure. The TFC was expressed as milligram quercetin equivalent (mg QE)/g dry weight of plant extract.[35]

2.2.3.3. Evaluation of the Total Antioxidant Capacity (TAC). 1mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added to 100 µL of AE. Tubes were sealed and incubated for 90 minutes in a water bath at 95°C. The tubes were allowed to cool at room temperature. The absorbance of the AE and standard solutions at 695 nm was measured in triplicates against a blank containing 1 mL of reagent and 100 µL of DW. The standard curve was constructed

using ascorbic acid (20-200 µg/mL) following said procedure. The TAC was expressed as milligram ascorbic acid equivalent (mg AAE/g) dry weight of plant extract.[36]

2.2.4. Quantitative evaluation of the DPPH free radical scavenging activity. The following tests were carried out for different concentrations of each sample in triplicates.

2mL of DPPH solution (0.1mM in methanol) was added to 1mL of AE of different concentrations (0.2 – 6 µg/mL), mixed well and incubated at room temperature for 30 minutes in the dark. The absorbance at 510 nm was measured in triplicates against methanol as a blank and DPPH as a control.[37] The percentage inhibition (PI)was calculated using the following formula:[36]

% Inhibition = [(Control absorbance - Sample absorbance) / (Control absorbance)] × 100

The dose response curve was constructed with PI against the concentration, and IC_{50} was calculated for each sample.[38]

2.2.5. Evaluation of antimicrobial activity

2.2.5.1. Well Diffusion Method. Muller-Hinton-agar was cultured with a standard 0.5 McFarland (1.5 × 108 CFU/mL; Absorbance at 625 nm: 0.08–0.13)[39] inoculum of each microorganism (*E. coli*, *S. aureus*) in two different plates for each sample. Wells were made in the agar (9 mm in diameter), one standard (gentamycin) antibiotic disk (positive control), DW (100 µL) (negative control) and sample (100 µL x 3 for triplicates) was placed onto 5 wells in the inoculated plate (Figure 5), the plates were then incubated in 37 °C for 24 h. The diameter of inhibition zone against the tested organisms was measured by a calliper to find the antimicrobial activity.[40] This procedure was repeated for each sample.

2.2.6. Statistical Analysis

All tests were done in triplicates, the results were expressed as mean ± standard deviation (SD). One-way ANOVA on mean values were used to analyse the significance of differences between means and Pearson's correlation analysis was done using SPSS and a $p< 0.05$ was considered to be statistically significant.[41]

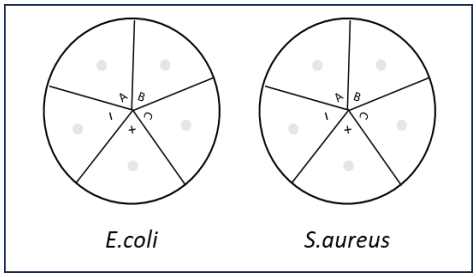


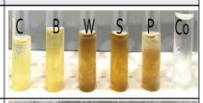
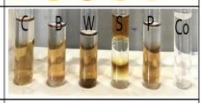
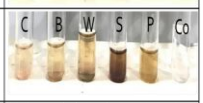
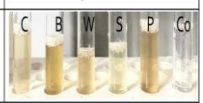
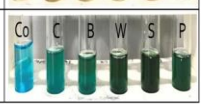
Figure 5. Plate separation and wells made for both the microorganisms for each sample

A: Sample, B: Sample, C: Sample, -: Negative control, +: Positive control

3. Results

3.1 Qualitative tests for phytochemicals. Phytochemical analysis of the extracts revealed that flavonoids, terpenoids, phenols, proteins, saponins, and reducing sugars were present in all the extracts.

Table 2. Qualitative phytochemical analysis of leaf extracts from selected plants.

	Cucumber(C)	Bitter Gourd(B)	Watermelon(W)	Snake Gourd(S)	Pumpkin(P)	Results
Flavonoid	++	++	+++	+++	+++	
Terpenoid	+	+	++	+++	+	
Phenol	+	+	++	+++	++	
Protein	+	+	+	+++	++	
Saponin	++	++	+	+	+++	
Reducing Sugars	+	+	++	++	+++	

Key = + Slightly positive, ++ moderately positive, +++ Strongly positive; Co- Control

The results are shown in Table 2**,** snake gourd was found strongly positive for flavonoids, terpenoids, phenols and proteins, while pumpkin was found strongly positive for

flavonoids, saponins and reducing sugars and watermelon was found strongly positive for flavonoids.

3.2 Total Phenolic Content. TPC was measured using the Folin–Ciocalteu Reagent (FCR) in each extract. The results were calculated from a calibration curve of gallic acid and expressed as gallic acid equivalents (GAE) per gram dry extract weight (Figure 6) mg GAE/g ± Standard Deviation (SD).

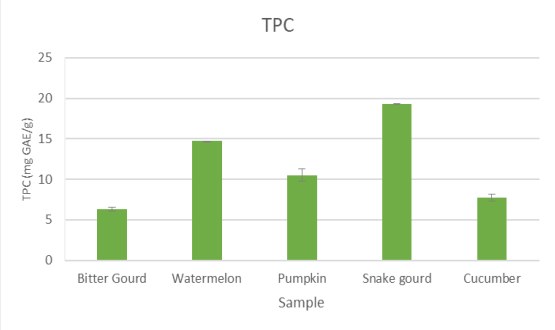


Figure 6. Total phenolic content of plant leaf extracts. Values are the mean ±SD

The content of phenolic compounds in AE ranged from 6.3±0.2 to 19.3mg GAE/g. Snake gourd had the greatest phenolic content and bitter gourd had the least. The TPC of all the samples were significantly different from each other (p<0.05).

3.3 Total Flavonoid Content (TFC). TFC was determined using the aluminium chloride colorimetric assay. The results were calculated from a calibration curve of quercetin and expressed as quercetin equivalents (QE) per gram dry extract weight (Figure 7) QE± SD.

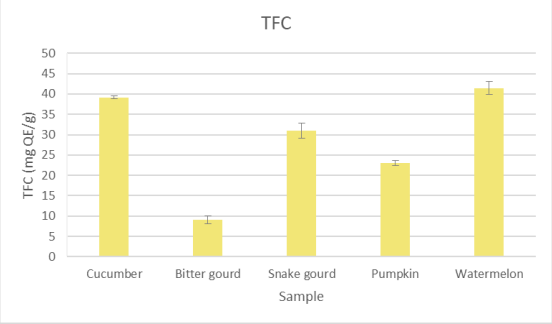


Figure 7. Total flavonoid content of plant leaf extracts. Values are the mean ±SD

The content of flavonoids in AE ranged from 9.1±1.0 to 41.4±1.6 mg QE/g. Watermelon had the greatest flavonoid content and bitter gourd had the least. The TFC of all the samples were significantly different from each other (p<0.05).

3.4 Total Antioxidant Capacity (TAC). TAC was evaluated using the Phosphomolybdenum method in each extract. The results were calculated from a calibration curve of ascorbic acid and expressed as ascorbic acid equivalents (AAE) per gram dry extract weight (Figure 8) AAE± SD.

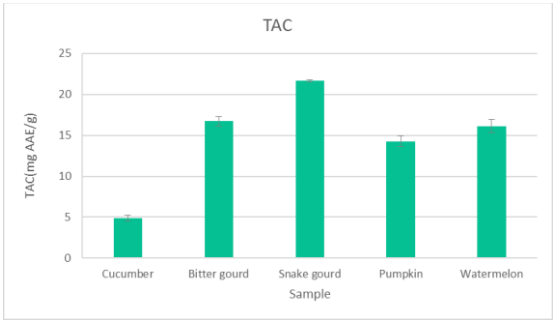


Figure 8. Total antioxidant capacity of plant leaf extracts. Values are the mean ±SD

The content of the TAC in AE ranged from 4.9±0.4 to 21.7± 0.1 mg AAE/g. Snake gourd had the greatest TAC and cucumber had the least. The TAC of all the samples were significantly different from each other (P<0.05).

3.5 DPPH Assay. The DPPH free radical scavenging activities of the selected plants are presented in Figure 9.

All the AE showed concentration-dependent increases in free radical scavenging capacity.

The greatest DPPH radical scavenging potency, with a minimum IC_{50} value was recorded for watermelon (0.6±0.1µg/mL), and the least, with a maximum IC_{50} value was recorded for cucumber (3.1±0.1µg/mL). The free radical scavenging activity of all the samples were

significantly different from each other (p<0.05).

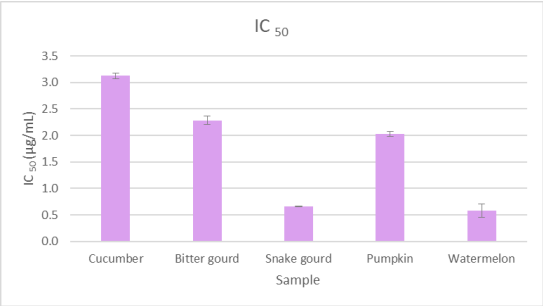


Figure 9. IC_{50} of each plant leaf extract. Values are the mean ±SD

3.6 ABST. ABST was done to find the antimicrobial activity of the AE against *E. coli* and *S. aureus.*

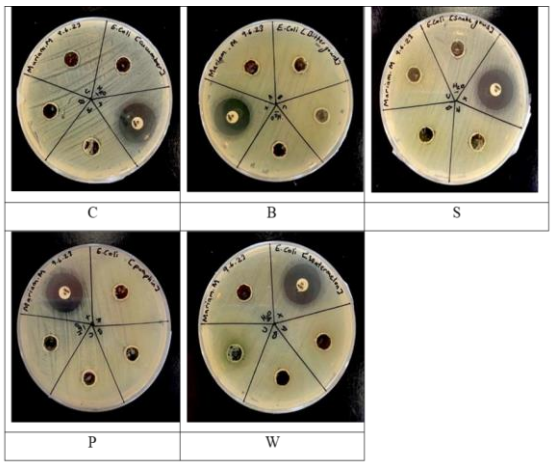


Figure 10. Antimicrobial activity of AE against *E. coli* (C- *Cucumis sativus*, B- *Momordica charantia*, *S- Trichosanthes cucumerina*, *P- Cucurbita maxima*, W-*Citrullus lanatus)*

All the AE showed negative antimicrobial activity against *E. coli* (Figure 10) and *S. aureus* (Figure 11). A larger zone of inhibition was observed for the positive control (gentamycin) against *S. aureus* (3.1cm) compared to *E. coli* (2.6cm).

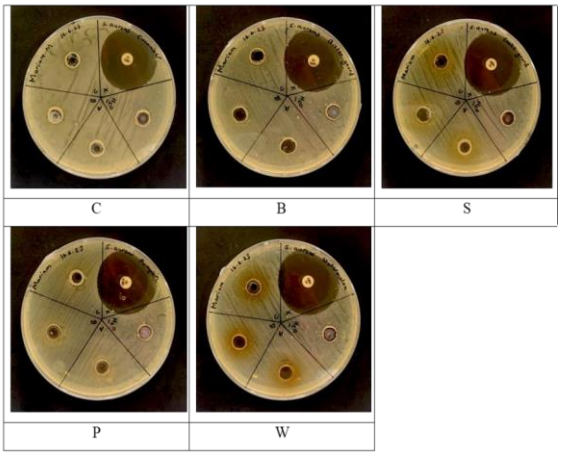


Figure 11. Antimicrobial activity of AE against *S. aureus* (C- *Cucumis sativus*, B- *Momordica charantia*, *S- Trichosanthes cucumerina*, *P- Cucurbita maxima*, W-*Citrullus lanatus*)

3.7 Statistical Analysis. Different assays were compared to find the linear association between them.

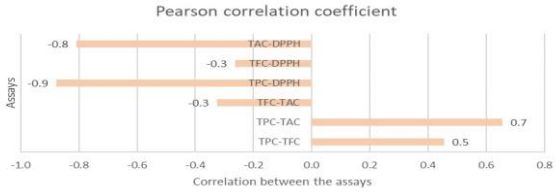


Figure 12. Pearson correlation between the different assays

A strong negative correlation was seen between TPC-DPPH and a strong positive correlation was seen between TPC-TAC (Figure 12).

4. Discussion

The Cucurbitaceae family of plants is a great source of bioactive functional elements with a range of therapeutic applications. The exploration of new biomolecules to be used directly by the pharmaceutical and agrochemical industries or to serve as a lead molecule to synthesis more potent molecules requires the extraction of bioactive components from plants and their quantitative and qualitative measurement.[42] The discovered metabolites are extremely beneficial due to their wide biological activity.[8] In this research a conventional extraction technique called maceration was performed, this involves

soaking the powdered leaf in a solvent at room conditions for at least three days with intermittent agitation,[43] the critical factor influencing extraction efficiency is the solvent's polarity,[44] water was used as the solvent as it is the most polar solvent and it dissolves a wide range of substances, it is also cheap, non-flammable and nontoxic.[45] After the extraction was completed, the mixture was filtered through filter paper and muslin cloth, a stock concentration of 0.02 g/mL was obtained and the extract was left at 4°C, as Cheng et al., (2022) showed that storage at temperatures below 5°C remarkably improved the retention of the major constituents of the extracts.[46]

It is interesting to note that the Cucurbitaceae leaf extracts showed the presence of many phytochemicals through qualitative tests, flavonoids, terpenoids, phenols, proteins, saponins and reducing sugars, these were found in various amounts through the qualitative phytochemical tests. Similar results were seen in AE's of bitter gourd, ethanolic extracts (EE) of snake gourd and methanolic extracts (ME) of cucumber, watermelon and pumpkin.[47-51]

Flavonoids play a crucial role in a wide scope of pharmaceutical, nutraceutical, and medicinal applications because they have a wide range of therapeutic effects due to their ability to influence important cellular enzyme activities as well as their anti-inflammatory, antioxidative, anti-carcinogenic, and anti-mutagenic capabilities.[52] The majority of terpenoids, despite structural variations, are biologically active and are utilized worldwide for the treatment of many ailments. Many terpenoids suppress various human cancer cells and are used as anticancer treatments, such as Taxol and its variants.[53] Plant phenols are recognized as powerful natural antioxidants that play a significant role in a variety of biological and pharmacological properties, such as anti-inflammatory, anti-cancer, antiviral, antimicrobial, antithrombotic, antiallergic, hepatoprotective, and many more.[54] The health benefits of plant-based proteins include anticancer, antioxidant, hypoglycemic, antibacterial, and hypolipidemic effects, according to numerous publications.[55] Saponins are naturally occurring sugar-conjugated compounds that have a variety of biological properties, such as therapeutic effects and antibacterial and antiviral activity.[56] Against this background, our work on Cucurbitaceae proves to be quite interesting due to the presence of all the above-mentioned important classes of bioactive phytochemicals in the selected leaves.

Quantification of phenols was done, the technique of measuring TPC in plant materials is based on the color change reaction between the FCR and polyphenols[57] leading to the formation of complex blue compounds that can be measured at a wavelength of 765 nm. The heteropoly acid (phosphomolybdate-phosphotungstate) in the FCR will be reduced into a molybdenum-tungsten complex by the oxidation of phenol or phenolic-hydroxy groups by FCR[58] (Figure 13). In this experiment gallic acid is used as the standard solution since it is a natural and stable phenol that is also relatively inexpensive compared to the others. It is also a constituent of the phenolic compound derived from hydroxybenzoic acid, classified as simple phenolic acid.[59]

The results showed snake gourd had the highest (19.3 mgGAE/g) TPC and bitter gourd had the least (6.3±0.2 mg GAE/g), the phenolic content of previous research differed from the present research. The present research had a lower TPC for bitter gourd and snake gourd compared to the previous research ,[60,61] while it had a higher TPC for cucumber compared to the previous research,[62] and a higher TPC for watermelon compared to the previous research on watermelon methanolic seed extract,[63] and a higher TPC for pumpkin compared to the previous research on pumpkin aqueous fruit extract.[64] Differences in the phenolic composition of the same species reported in different studies may be caused by the variance in growth conditions. This suggests that phenolic content may vary with variation in geographical location and climatic circumstances.

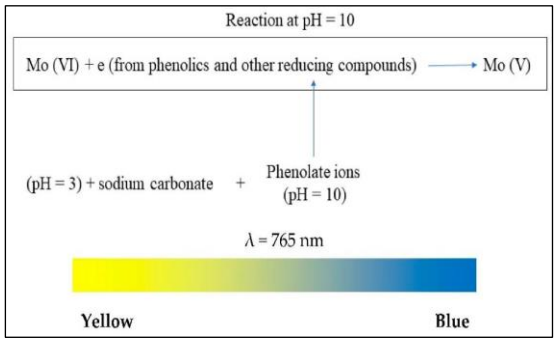


Figure 13. Folin–Ciocalteu method[65]

Quantification of flavonoids was done, the TFC content was determined using the $AlCl_3$ colorimetric assay. This method is based on the formation of chelates of Al(III)-flavonoids (Figure 14***)***. Due to their numerous hydroxyl and oxo groups, flavonoids show a strong affinity for binding metal ions like Al(III), typically at a 1:1 ratio, depending on experimental conditions. Experimentally, yellow-colored Al(III)-flavonoid complexes are formed, upon the addition of AlCl3, and their absorbance is subsequently measured at 510nm.[66]

The results showed watermelon had the highest (41.4±7.3 mg QE/g) TFC and bitter gourd had the least (9.1±1.8 mg QE/g), the flavonoid content of previous research's differed from the present research. The present research has a lower TFC for bitter gourd and snake gourd compared to the previous research, [61,67] while it had a higher TFC for cucumber compared to the previous research,[62] and also a higher TFC for watermelon compared to the previous research on watermelon methanolic seed extract,[63] and a lower TFC for pumpkin compared to the previous research on pumpkin fruit AE.[64] Differences in the flavonoid composition of the same species reported in different studies may be caused by the variance in growth conditions. This suggests that flavonoid content may vary with variation in geographical location and climatic circumstances.

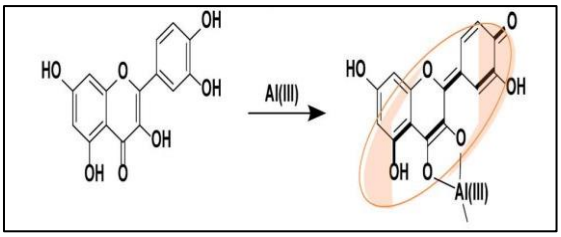


Figure 14. Al(III)-quercetin chelate[66]

Due to the abundance of phenolic and flavonoid components they contain, they are said to have strong antioxidant action. The antioxidant capacity of the sample was assessed using the phosphomolybdenum method with a spectrophotometer, which is based on the reduction of Mo (VI) to Mo (V) by the plant sample and the subsequent generation of blue-green phosphate/Mo (V) compound with a maximum absorption at 695 nm ***(***Figure 15***)***. In terms of the reduction of molybdate ions, the phosphomolybdenum technique provides a quantitative way to assess antioxidant activity. Since ascorbic acid is used to generate a standard curve, the antioxidant activity is expressed in terms of ascorbic acid equivalents.[68]

The results showed snake gourd had the highest (21.7±0.1 mg AAE/g) TAC and cucumber had the least (4.9 ±0.4 mg AAE/g), the TAC of previous research's differed from the present research. The present research had a lower TAC for snake gourd compared to the previous research,[61] while it had a higher TAC for bitter gourd compared to the previous research,[60] the present research also had lower TAC for cucumber compared to the previous research on cucumber ME,[69] it also had a lower TAC for watermelon compared to the previous research on watermelon methanolic seed extract,[63] and a higher TAC for pumpkin compared to the previous research on pumpkin fruit EE.[70] The variation of the TAC between the studies may be because variable amounts of antioxidants are found in the same species when it is grown under diverse environmental conditions and in different regions.

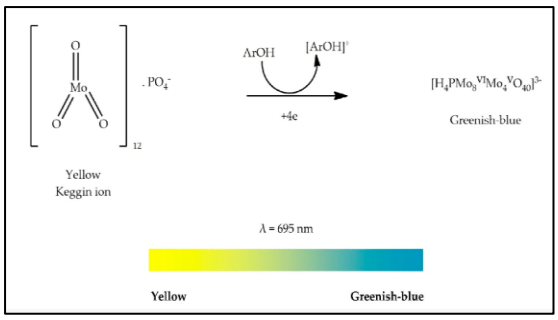


Figure 15. Phosphomolybdenum method[65]

The most rapid and popular approach used to assess the antioxidant activity of plant materials is DPPH radical scavenging. The free radical DPPH, which generates a violet solution in ethanol, is stable at room temperature. The IC_{50} value, which represents the concentration of each sample needed to scavenge 50% of DPPH free radicals, was used to express the DPPH radical scavenging activity. A lower IC_{50} value indicates a greater capacity to scavenge DPPH radicals.[71]

The results showed watermelon had the lowest (0.6 ±0.1 µg/mL) IC_{50} and cucumber had the highest (3.1 ±0.1 µg/mL). The IC_{50} values of previous research's differed from the present research. The present research had a lower IC_{50} for cucumber and bitter gourd , compared to the previous research on cucumber and bitter gourd ME.[72,73] It had a lower IC_{50} for snake gourd compared to the previous research, [61] it also had a lower IC_{50} for pumpkin compared to the previous research on pumpkin fruit AE[74] and a lower IC_{50} for watermelon compared to the previous research on watermelon seed ME .[63] This can be due to modifications to the measurement parameters, such as reaction time ,concentration, solvent, pH and the presence of inorganic salts or metal ions. These factors need to be closely monitored because they have an impact on the reaction's kinetics and equilibrium, thereby altering the IC_{50} values.[75]

The antimicrobial activity of the leaf extracts were evaluated against *S.aureus* and *E.coli* , no observations were seen for either of the bacteria in a concentration of 0.02 g/mL of the extract. Previous research done on cucumber ME (10 µg/mL) showed, a zone of inhibition of 10mm for *S.aureus* and 11mm for *E.coli* , and no zones of inhibition for acetone extract, this could be due to the various solvents used.[76] *E.coli* has a smaller zone of inhibition to gentamycin compared to *S.aureus*. In general, gram-negative bacteria are more resistant than gram-positive due to their phospholipidic membrane and lipopolysaccharide components.[20]

The Pearson correlation showed a strong negative relationship between TAC-DPPH and TPCDPPH, the higher the phenolic and antioxidant content the stronger the scavenging activity of the extract. A weak negative relationship was seen between TFC-DPPH and TFC-TAC, and a relatively strong relationship between TPC-TFC and TPC-TAC, the phenolic content had a significant effect on all antioxidant activities while the flavonoid content didn't contribute significantly.

5. Conclusion

In this research, the results of qualitative assays showed the presence of flavonoids, terpenoids, phenols, proteins, saponins, and reducing sugars in all tested samples. Snake gourd had the highest phenolic content of 19.3 mg GAE/g, and antioxidant capacity of 21.7 mg AAE/g, while watermelon had the greatest flavonoid content of 41.4 mg QE/g and the highest free radical scavenging activity (IC50 value of 0.6 µg/mL), among the rest of the samples. Among each variable, the correlation between TPC and TAC provided the strongest positive correlation ($r = 0.7$, $p < 0.05$) in aqueous leaf extracts.

This research suggested that cucumber, pumpkin, watermelon, snake gourd and bitter gourd leaves are a reliable source of phytochemicals and have shown significant antioxidant activities. Further, these results will support the curative claims of various ethnomedical uses of this plant family in Ayurvedic medicine of Sri Lanka.

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Isolation of Endophytes with Antimicrobial Activity from Selected Indigenous Medicinal Plants against Amoxicillin-Resistant Environmental Bacteria

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Abstract

The continuous discovery of novel antimicrobial compounds and antibiotics-producing microorganisms is an obligatory process to overcome the antibiotic resistance of pathogenic bacteria. Antibiotic resistance arises as a consequence of the misuse of antibiotics. Thus, the main objective of this study is based on the isolation of endophytic bacteria and fungi with antimicrobial activity against amoxicillin-resistant environmental bacteria. The endophytes were isolated from the selected indigenous medicinal plants, namely *Acalypha indica* (Kuppameniya) and *Cyanthillium cinereum* (Monarakudumbiya). Fresh samples of the selected plants were collected from their natural habitats, and the plant parts were surface-sterilised using 70% ethanol. The endophytic bacteria and fungi were isolated on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) media respectively at room temperature. The isolated endophytic bacteria and fungi were screened for their antimicrobial activity against previously isolated amoxicillin-resistant (AR) environmental bacteria, namely *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterobacter ludwigii* and *Enterobacter pyrinus*, on Mueller-Hinton Agar (MHA) media. As per the results, a prominent inhibition of the AR bacteria *Staphylococcus aureus* was exhibited by four endophytic isolates (KpR-02B, MnS-01B, KpS-01F and MnR-04F). The AR bacteria *Enterobacter ludwigii* was prominently inhibited by two endophytic isolates (MnS-01B and MnR-04F), while *Enterobacter pyrinus* was also observed to be prominently inhibited by two endophytic isolates (MnF-03B and MnR-04F). Therefore, the indigenous medicinal plants, *Acalypha indica* (Kuppameniya) and *Cyanthillium cinereum* (Monarakudumbiya), can be considered as a potential source of antimicrobial compound-producing endophytes against amoxicillin-resistant environmental bacteria, which can be utilised to develop a novel antibacterial drug against antibiotic-resistant pathogenic bacteria.

Keywords: Antimicrobial compounds, Medicinal plants, Endophytes, Antibiotic-resistance

1. Introduction

Endophytes are microorganisms that colonise the endospheric domain of the plant microbiome. Endophytes are present in the intracellular regions of the roots, stems, leaves, and flowers of plant tissue.[1] Endophytes have a mutualistic relationship with the host plant.[2] Endophytes can be either bacteria, actinomycetes, or fungi. Most endophytic bacteria have been found to belong to the phyla Proteobacteria, Actinobacteria, and Firmicutes, while most endophytic fungi belong to the phyla Ascomycota and Basidiomycota.[3,4]

Endophytes are essential in maintaining the quality of the soil as they can solubilize nutrients without disturbing the microbial community of the soil, remove pollutants from the soil, and are also involved in soil mineral cycling.[5] Endophytes also produce metabolic compounds which benefit the survival of the host plant.[6] Endophytes promote plant growth by increasing the uptake

of nutrients (nitrogen, phosphorus, and iron), producing plant growth promoters (PGP) such as auxins, ethylene and gibberellins.[7]

Endophytes help to defend the host plant by providing resistance against biotic and abiotic stresses of the environment.[8] They protect the host plant from pathogenic microorganisms by synthesising various bioactive compounds, such as alkaloids, peptides, phenols, terpenoids and aliphatic compounds.[9] The bioactive secondary metabolites produced by endophytic bacteria include substances that possess antibiotic, antifungal, antiviral, anticancer, antiarthritic and antidiabetic properties.[10] Therefore, these secondary metabolites can be utilised to develop novel drugs for therapeutic purposes.

Antibiotic resistance has posed a significant threat globally in recent years.[11] Antimicrobial resistance is the ability that microorganisms such as bacteria and fungi develop to overcome the mechanisms of antibiotics designed to kill or inhibit their growth.[12] Some of the most common antibiotic-resistant (AR) bacterial strains include MRSA (Methicillin-resistant *Staphylococcus aureus*) and VRE (Vancomycin-resistant *Enterococcus faecalis*).[13] The misuse and overuse of antibiotics have greatly contributed to the development of AR bacteria, leading to challenging consequences in the agricultural, medical and pharmaceutical sectors.[14]

Amoxicillin is a broad-spectrum antibiotic that is commonly used to treat bacterial infections in both humans and animals.[15] Amoxicillin is rapidly degraded in the water by biotic and abiotic environmental stress factors, resulting in different intermediate compounds which are more toxic and resistant to degradation.[16] Amoxicillin cannot be completely removed from the environment as it is hydrophobic and lipophilic.[17] Over time, the bacterial strains present in the environment develop antibiotic resistance genes (ARGs) and can eventually lead to disease outbreaks.[18] Therefore, antibiotic contamination in wastewater has significantly increased the risk of environmental bacteria developing resistance to antibiotics.[19]

Based on previous studies conducted, medicinal plants and crops have been targeted as the most potential source to screen for novel secondary metabolites produced by endophytic microbes.[20,21] The antimicrobial compounds produced by endophytes are eco-friendly and non-toxic to humans.[22] Hence, there is an immense potential for having an endophytic microbial flora which produces important secondary metabolites associated with these medicinal plants.

Several antimicrobial compounds are produced by endophytic bacteria. Ecomycins and Pseudomycins have antifungal properties, while Munumbicins, Kakadumycin, and Xiamycins have effective antibiotic activities against several bacterial strains such as MRSA, VRE, and MDR-TB (multidrug-resistant tuberculosis).[23] The continuous discovery of novel antimicrobial compounds against AR bacteria has attracted immense interest among scientists. Therefore, the natural bioactive compounds produced by endophytes, which exhibit antimicrobial activity against amoxicillin-resistant environmental bacteria, can be beneficial in developing novel drugs to combat the issue of antibiotic resistance.

Acalypha indica, commonly known as Kuppameniya, is a plant traditionally known to have medicinal properties which have made it useful in treating rheumatoid arthritis and respiratory problems, and also help in wound healing.[24] *Acalypha indica* was found to possess various secondary metabolites, such as flavonoids, saponins, alkaloids, catechols and phenolic compounds, with antimicrobial, antioxidant, anti-inflammatory, anti-diabetic and anti-cancer properties.[25] Therefore, the Kuppameniya plant is proven to be a valuable medicinal plant to screen for secondary metabolites with pharmacological benefits.

Cyanthillium cinereum, which is known as Monarakudumbiya, is a plant traditionally used to treat arthritis, rheumatism, and conjunctivitis, and also helps in wound healing.[26] *Cyanthillium cinereum* is known to

possess therapeutic properties against various maladies such as asthma, cough, diarrhoea, cholera, malaria, cancer, and night blindness.[27] According to previous studies that were conducted, this plant is known to possess bioactive compounds such as alkaloids, flavonoids, sterols, esters and terpenoids.[28,29] Hence, Monarakudumbiya is a plant of significant medicinal importance with possible therapeutic potential against AR microorganisms.

Thus, the main objective of this study is to isolate endophytic bacteria and fungi from selected indigenous medicinal plants, *Acalypha indica* and *Cyanthillium cinereum*, with potential antimicrobial activity against previously isolated amoxicillin-resistant environmental bacteria.

2. Methodology

2.1 Collection of samples. Whole plants (containing roots, stems, leaves and flowers) of *Acalypha indica* and *Cyanthillium cinereum* were collected from their natural habitats (home gardens and roadsides). The plant samples were placed in clear plastic zip-lock bags and were transported to the laboratory within 24 hours of sampling under refrigerated conditions.

2.2 Enrichment of plant endophytes. About 1g of each plant part (roots, stems, flowers and leaves) of both plants were separately surface sterilised using distilled water and 70% ethanol. Each of the disinfected plant parts was separately ground aseptically in 10ml of 0.9% NaCl solution using a sterile mortar and pestle. About 5ml of each crude extract was transferred aseptically into quarter-diluted nutrient broth (NB) media flasks. The flasks were incubated at room temperature for about 24 to 48 hours on the shaking incubator at 120 rotations per minute (rpm) speed.

2.3 Preparation of serial dilutions. After incubation, each of the enriched NB cultures was serially diluted up to 10^{-6} dilution by adding sterile 0.9% NaCl solution.

2.4 Isolation of endophytic bacteria.

An aliquot of 100µl of the serially diluted cultured broth of each sample was transferred onto separate nutrient agar (NA) media plates and inoculated evenly via spread-plate technique. Following incubation at room temperature for about 24 to 48 hours, the morphologically distinct bacterial colonies were identified and isolated by further inoculation on NA plates via quadrant-streak plate technique to obtain pure cultures.

2.5 Isolation of endophytic fungi. Surface-sterilised plant parts were aseptically cut into small pieces of approximately the same size and separately placed on potato dextrose agar (PDA) media plates. Following incubation at room temperature for about 72 hours, the morphologically distinct fungal colonies were identified and isolated by further inoculation on new PDA plates to obtain pure cultures.

2.6 Preparation of AR environmental bacterial cultures. The previously isolated AR environmental bacteria, namely *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterobacter ludwigii* and *Enterobacter pyrinus*, from the stock cultures were enriched by aseptically mixing a loopful of the bacterial inoculum into separate NB media flasks. The flasks were incubated at room temperature for about 24 to 48 hours on the shaking incubator at 120rpm speed. After incubation, a loopful of each NB culture was inoculated on new NA plates via the quadrant-streak plate technique. The plates were then incubated at room temperature for about 24 to 48 hours.

2.7 Antimicrobial assay for endophytic bacteria. Equalized AR environmental bacterial solutions were prepared for the absorbance value of 0.35 at 395nm wavelength. The equalized solutions of AR environmental bacteria were evenly spread on Mueller-Hinton agar (MHA) plates using sterile cotton swabs to prepare the bacterial lawns. Then each endophytic bacterial isolate was separately inoculated at the centre of AR environmental bacterial lawns. Following incubation at room

temperature for about 24 to 48 hours, the plates were observed for clear zones around each endophytic bacterial colony and the results were recorded. Likewise, the antimicrobial assay was separately performed for each AR environmental bacteria.

2.8 Antimicrobial assay for endophytic fungi. Equalized AR environmental bacterial solutions were prepared for the absorbance value of 0.35 at 395nm wavelength. The equalized solutions of AR environmental bacteria were evenly spread on MHA plates using sterile cotton swabs to prepare the bacterial lawns. Then each endophytic fungal isolate was separately inoculated at the centre of AR environmental bacterial lawns. Following incubation at room temperature for about 72 hours, the plates were observed for clear zones around each endophytic fungal colony and the results were recorded. Likewise, the antimicrobial assay was performed for each AR environmental bacteria.

3. Results

3.1 Isolation of endophytic bacteria.

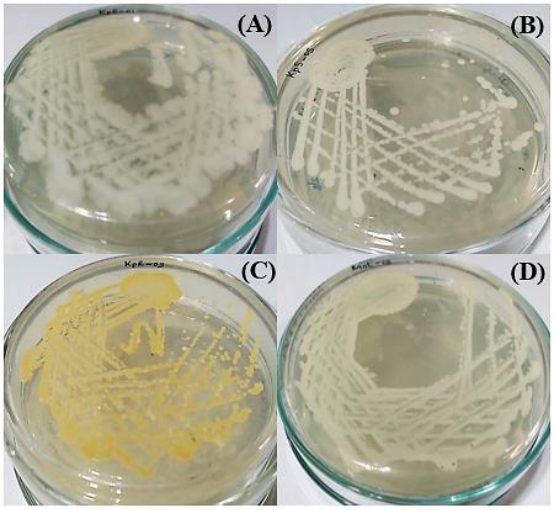

Figure 1. Pure cultures of endophytic bacteria. (A) KpS-01B isolated from the stems of *Acalypha indica* plant. (B) KpS-05B isolated from the stems of *Acalypha indica* plant. (C) KpR-03B isolated from the roots of *Acalypha indica* plant. (D) MnL-05B isolated from the leaves of *Cyanthillium cinereum* plant.

Growth of bacterial colonies was observed after incubation for 24 to 48 hours at room temperature. The bacterial colonies appeared to be white, yellow or cream coloured, with smooth shiny surfaces. Endophytic bacteria with different morphological characteristics were observed, with entire, undulate or lobate margin, circular or irregular form, and raised, flat or convex elevation (Figure 1).

3.2 Isolation of endophytic fungi.

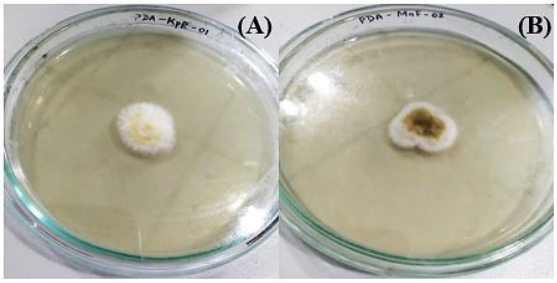

Figure 2. Pure cultures of endophytic fungi. (A) KpR-01F isolated from the roots of *Acalypha indica* plant. (B) MnF-02F isolated from the flowers of *Cyanthillium cinereum* plant.

Growth of fungal colonies was observed after incubation for about 72 hours at room temperature. The fungal colonies were observed to be of different colours, including white, grey, yellow, pink, green, blue, black and purple. Endophytic fungi with different morphological characteristics were observed, with woolly, powdery, cottony or granular texture (Figure 2).

3.3 Antimicrobial assay for endophytic bacteria.

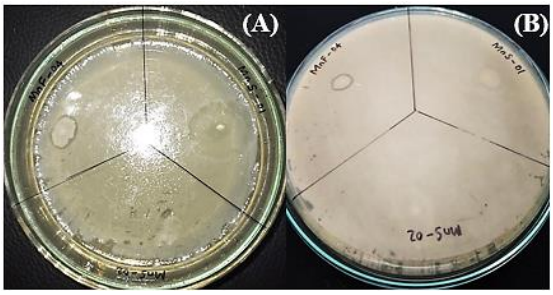

Figure 3. Antimicrobial activity of endophytic bacterial isolates. (A) MnS-01B isolated from

the stems of *Cyanthillium cinereum* plant against *Enterobacter ludwigii*. (B) MnF-04B isolated from the flowers of *Cyanthillium cinereum* plant against *Enterobacter pyrinus*.

Endophytic bacterial isolates, namely KpR-02B, MnS-01B and MnF-03B, showed prominent inhibition against the tested AR environmental bacteria after incubation for 24 to 48 hours at room temperature (Table 1).

3.4 Antimicrobial assay for endophytic fungi.

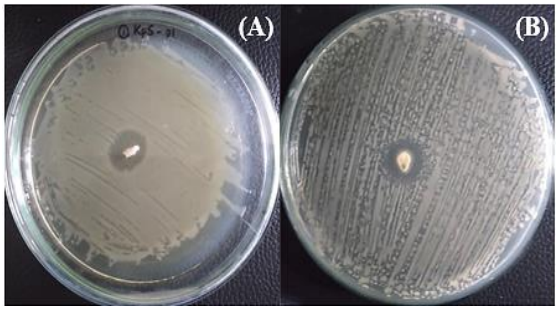

Figure 4. Antimicrobial activity of endophytic fungal isolates. A) KpS-01F isolated from the stems of *Acalypha indica* plant against *Staphylococcus aureus*. B) MnR-04F isolated from the roots of *Cyanthillium cinereum* plant against *Staphylococcus aureus*.

Endophytic fungal isolates, namely KpS-01F and MnR-04F, showed prominent inhibition against tested AR environmental bacteria after incubation for about 72 hours at room temperature (Table 1).

Table 1. Prominent antimicrobial activity.

AR environmental bacteria	Endophytes that exhibited inhibition
Staphylococcus aureus	KpR-02B MnS-01B KpS-01F MnR-04F
Enterobacter ludwigii	MnS-01B MnR-04F
Enterobacter pyrinus	MnF-03B MnR-04F

4. Discussion

Equalising the concentration of each bacterial solution to a specific absorbance of 0.35 before preparing the bacterial lawn ensured that fair results were obtained when screening the isolated endophytes for antimicrobial activity. However, other factors, such as the incubation period, the volume of bacterial solution spread on each MHA plate and the amount of each endophytic isolate inoculated on the AR environmental bacterial lawn, may also affect the results obtained by the antimicrobial assay.[30,31]

The presence of zones of clearance around the endophytic isolates indicated the inhibition of the growth of the AR environmental bacteria (Table 1). The growth of AR environmental bacteria was suppressed due to the production of antimicrobial compounds by the endophytic isolates.[32] Weak inhibitory zones may be due to small quantities of antimicrobial compounds produced by the endophytes.[33] Phytochemicals from the plant extracts exert inhibitory effects against the AR bacteria by various mechanisms of action, such as impairing the function of the bacterial cell membrane, inhibiting the synthesis of bacterial cell walls and disrupting the synthesis of nucleic acids.[34] Polyphenols are considered as the phytochemicals with the most potent antibacterial and antifungal activities.[35] The mechanisms of action by which AR bacteria can resist antibiotics are by producing enzymes which chemically modify, deactivate or destroy the antimicrobial compounds, by modifying the cellular target sites, and by reducing the intracellular accumulation of the antimicrobial compounds.[36,37]

Prominent inhibition by the endophytic isolates was not observed against the amoxicillin-resistant bacteria *Acinetobacter baumannii*, although some positive results were obtained. This implies that of the four different amoxicillin-resistant environmental bacteria which were screened, *Acinetobacter baumannii* exhibited the most resistance to the antimicrobial activity of the endophytic

bacterial and fungal isolates. *Acinetobacter baumannii* is known to possess various resistance mechanisms against antimicrobials, such as hydrolysis of β-lactamases, overexpression of efflux pumps, reduction of porin permeability, mutations in target genes and modification of antibiotic targets.[38,39]

The endophytic isolates displayed the highest number of prominent zones of clearance against the amoxicillin-resistant bacteria *Staphylococcus aureus*. This demonstrates that of the four different AR environmental bacteria which were analysed, *Staphylococcus aureus* exhibited the highest susceptibility to the antimicrobial activity of the endophytic bacterial and fungal isolates. Previous studies have also reported findings of endophytes displaying antagonist action against *Staphylococcus aureus* by inhibiting its protein expression and nucleic acid synthesis.[40,41,42]

Furthermore, two endophytic isolates (MnS-01B and MnR-04F) exhibited prominent antimicrobial activity against the AR bacteria *Enterobacter ludwigii*, and two endophytic isolates (MnF-03B and MnR-04F) also showed prominent inhibition of the AR bacteria *Enterobacter pyrinus*. Hence, the results of this study indicate that these endophytic isolates have the potential to inhibit the activity of AR environmental bacteria.

The previous studies conducted on endophytes isolated from *Acalypha indica* and *Cyanthillium cinereum* plants also reported several antimicrobial compound-producing bacteria and fungi against human pathogenic bacteria and antibiotic-resistant bacteria. The endophytic fungi *Trichoderma harzianum* isolated from *Acalypha indica* is known to have antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella*.[43,44] The endophytic fungi *Colletotrichum cobbittiense* isolated from *Cyanthillium cinereum* has been found to produce bioactive substances, such as saponins, terpenoids and flavonoids, which have effective antimicrobial activity.[45,46]

Based on this study, the endophytic bacterial isolate which exhibited the most prominent antimicrobial activity was MnS-01B, which was extracted from the stems of *Cyanthillium cinereum* plant, and the endophytic fungal isolate which exhibited the most significant antimicrobial activity was MnR-04F, which was extracted from the roots of *Cyanthillium cinereum* plant. This proved *Cyanthillium cinereum* to be an excellent source to isolate bioactive compounds with effective antimicrobial activity against specific AR environmental bacteria. According to findings from a previous study, the bioactive compounds in the crude extract of *Cyanthillium cinereum* were known to have significant antibacterial activity against AR bacteria such as *Escherichia coli* and *Staphylococcus aureus*.[26] Several phytochemical compounds such as phenols, saponins, flavonoids and tannins present in *Cyanthillium cinereum* were responsible for the effective antimicrobial activity.[47]

The endophytic isolates from *Acalypha indica* also provided prominent results, proving it to be a beneficial source for extracting novel antimicrobial compounds. However, the difference in results for *Acalypha indica* compared to *Cyanthillium cinereum* could have been due to experimental errors. Therefore, further evaluation of the antimicrobial activity of endophytes isolated from *Acalypha indica* is suggested. According to findings from previous studies, the phytochemicals in the crude extract of *Acalypha indica*, including alkaloids, glycosides and phenolic compounds, were found to exhibit prominent antibacterial activity against various AR bacteria, such as *Staphylococcus aureus* and *Salmonella typhi*, as well as against several other gram-positive bacteria.[48,49]

As future perspective, the microbial isolates which showed antimicrobial activity against AR environmental bacteria need to be identified using conventional and molecular biological methods. The biochemical characterisation of the endophytes can be carried out by performing biochemical tests such as catalase test, endospore staining, capsule staining, gelatinase test, Simmon's citrate test and triple sugar iron test.[50,51] The

microscopic characterisation can be done using simple staining and Gram's staining techniques.[52] The molecular biological characterisation can be done by sequencing of 16S rRNA gene of potential bacterial isolates and 18S rRNA gene sequencing of potential fungal isolates.[53]

Moreover, the antimicrobial compounds produced by the endophytes can be identified and quantified by separation techniques, such as LC (liquid chromatography), GC (gas chromatography) or CE (capillary electrophoresis), coupled with detection systems, such as MS (mass spectrometry), NMR (nuclear magnetic resonance) or FTIR (Fourier transform infrared spectroscopy).[54] Further metabolomic studies can help to determine the metabolic stability, therapeutic efficacy and toxicological profiles of the compounds.[55] Therefore, these antimicrobial compounds can be designed and developed into novel antibiotic agents against pathogenic amoxicillin-resistant bacteria.[56]

5. Conclusion

This study aimed to isolate endophytes with antimicrobial activity from *Acalypha indica* and *Cyanthillium cinereum* plants against Amoxicillin-resistant environmental bacteria. As per the results of this study, prominent inhibition against *Staphylococcus aureus* was exhibited by four endophytic isolates (KpR-02B, MnS-01B, KpS-01F and MnR-04F), while *Enterobacter ludwigii* was prominently inhibited by two endophytic isolates (MnS-01B and MnR-04F) and *Enterobacter pyrinus* was also inhibited by two endophytes (MnF-03B and MnR-04F). Therefore, *Acalypha indica* and *Cyanthillium cinereum* plants can be considered as a good source of antimicrobial-compound producing endophytes against AR environmental bacteria.

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Development of a qPCR method for the detection of *Staphylococcus aureus* species

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Abstract

Rapid detection of *Staphylococcus aureus* is crucial because it causes food poisoning in living beings. *Staphylococcus aureus* bacteria are present on the skin surface and the mucous membranes of humans and animals. Quantitative PCR (qPCR) can be utilized to give fast and specific results for detecting *S. aureus*. Therefore, this research aimed to develop a qPCR method for detecting *S. aureus* species, in both non enriched and enriched medium. The DNA of pure *S. aureus* culture was extracted using the QIAGEN mericon food kit, followed by measuring the concentration of DNA using a fluorometer and performing qPCR using TaqMan chemistry for nuc gene of *S. aureus*. Artificially spiked meat samples were incubated for an 18-hour enrichment period. The collected samples both pre-enriched and enriched samples were then subjected to DNA extraction using the QIAGEN mericon food kit, followed by measuring the concentration using fluorometer and qPCR using TaqMan chemistry for the *S. aureus* nuc gene. This process was done in order to evaluate the accuracy of the qPCR method for the quantitative detection of *S. aureus*. The concentration of extracted DNA of *S. aureus* culture was 6.6 ng/µL. The concentration of the extracted DNA of pre-enriched sample and the enriched sample was 6.8 ng/µL and higher than the standard respectively. The five-times diluted enriched sample produces a DNA concentration of 42 ng/µL. In the qPCR results, *S. aureus* culture gave a Ct value of 16.961. The pre-enrich meat sample gave a Ct value of 27.119, the enriched meat sample gave a Ct value of 21.374. The aim of this study was achieved as the enriched sample produced a Ct value lower than the pre-enriched sample indicating the development of a qPCR method for the detection of *S. aureus* species.

Keywords: *Staphylococcus aureus*, pre-enrichment, enrichment, qPCR, Fluorometer, Ct value

1. Introduction

Staphylococcus aureus species are a division of the *Staphylococcus* genus which are anaerobic, non-motile, gram-positive cocci which colonize on the surface of the skin and the mucous membranes of humans and animals.[1] Figure 1 shows an image of the *S. aureus* species. *Staphylococcus aureus* is the most common coagulase-positive Staphylococci (CoPS) and is the foremost virulent.[2] The infection of *S. aureus* can cause most hospital- acquired diseases such as Methicillin Resistant *Staphylococcus aureus* (MRSA). Therefore, there's an urgent need to establish a rapid method to detect *S. aureus* species present in food.[3]

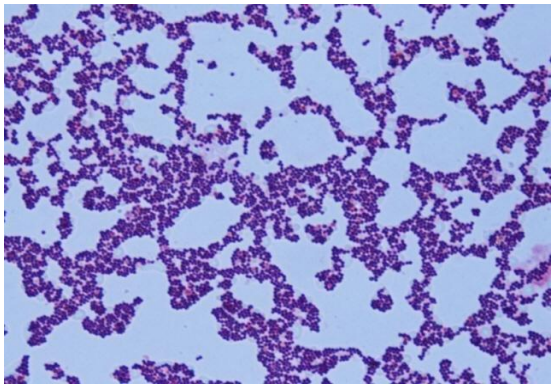

Figure 1. Gram staining of *S. aureus* species[4]

These organisms can grow in both aerobically and anaerobic conditions. They can also grow under diverse conditions such as heat. *Staphylococcus aureus* contamination can be minimized by heat treatment of foods. Symptoms of *S. aureus* include redness, swelling, and pain at the site of infection.[5] Approximately 30% of the human population in the world is colonized with *Staphylococcus aureus.*[6] In Sri Lanka, over 42% - 67% of hospitals are infected with MRSA. Figure 2 shows the percentage of MRSA isolates, by country, in recent years from 2011 to 2014.

qPCR is quantitative real-time PCR which is a PCR-based method in which the amplification of a target DNA sequence is combined with quantification.[14]

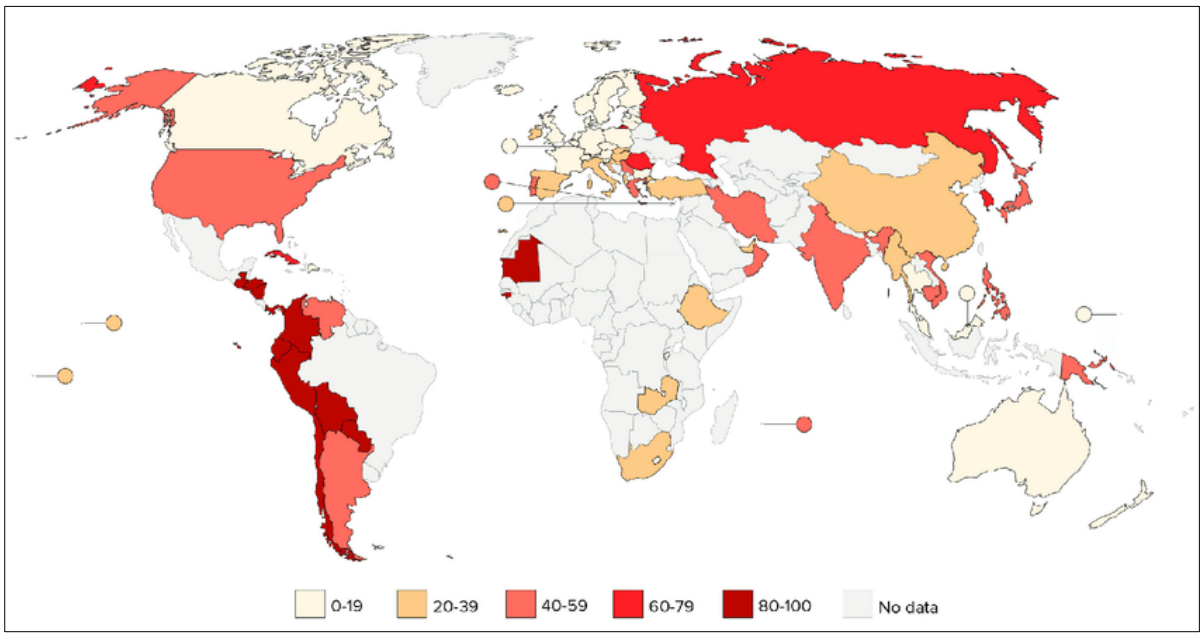

Figure 2: Percentage of MRSA isolates by country in recent years[7]

Staphylococcus aureus is detected using culture-based methods, ELISA and Aptasensors.[8] The disadvantages of using culture- based methods are they are time consuming and have a higher chance of contamination from occurring.[9] For the ELISA method an isolated pure culture is needed to obtain an accurate result.[10,11] Aptasensors also have disadvantages as the reagents are difficult to store as they have a poor stability, the catalytic activity can be affected by different conditions such as pH.[12] Therefore, this research focuses on the development of qPCR for the detection of *Staphylococcus aureus* species as this method is specific, takes less time compared to other methods and have a low chance of producing false positive results.[13]

This technique makes it possible to calculate the initial template concentration, making it a frequently utilized analytical tool when assessing the DNA copy number.[14,15] Methods of qPCR uses fluorescent dyes such as SYBR Green or DNA probes containing a fluorophore, such as TaqMan. Figure 3 shows the TaqMan-based assay chemistry.

The advantages of the TaqMan probe are they are labelled with different dyes, which allows amplification and detection of two distinct sequences in one reaction tube. Post-PCR processing is eliminated, which reduces material costs.[17] The primary disadvantage of TaqMan is that the synthesis of different probes is required for different sequences. The advantages of SYBR dyes are,

they can be used to monitor the amplification of any double-stranded DNA sequence, and no probe is needed.[17,18] The primary disadvantage of SYBR dye is that it may generate false positive signals.

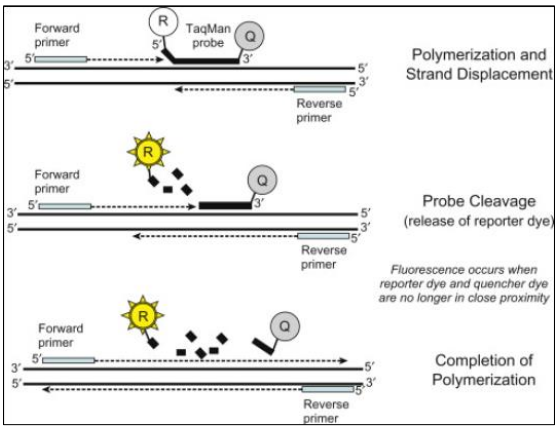

Figure 3. TaqMan-based assay chemistry[16]

Staphylococcus aureus is the most common CoPS and is considered the foremost virulent because it is the most typical hospital-acquired infection. Therefore, the detection of *Staphylococcus aureus* is crucial. qPCR can be used as a less time- consuming method to detect *S. aureus* species.[13] qPCR presents a fast and reliable method for the detection and quantification of *S. aureus* species.[19] Hence, this study aims to develop a qPCR method to detect *Staphylococcus aureus* species in both non enriched and enriched medium.

2. Methodology

2.1. Detection of S. aureus in the culture. *Staphylococcus aureus* culture was prepared by inoculating 5 mL of nutrient broth with *S. aureus* glycerol stock and was incubated at 37℃ overnight.

2.2. Extraction of DNA of S. aureus. Extraction of DNA from the overnight grown *S. aureus* culture was performed using QIAGEN DNeasy mericon Food Kit. Pelleted 1 mL of homogenized culture was placed in a 2 mL microcentrifuge tube then 1 mL of food lysis buffer and 2.5 µL of proteinase K solution was added and mixed thoroughly by using the vortex. Then the sample was incubated in a water bath at 60℃ for 30 minutes with constant shaking of about 1000 RCF. The sample was centrifuged at 2500 RCF for 5 minutes then 500 µL of chloroform was added to the microcentrifuge tube. Then 700 µL of clear supernatant obtained from earlier was added to the microcentrifuge tube containing chloroform. The microcentrifuge tube was vortexed thoroughly, then centrifuged at 14000 RCF for 15 minutes. 350 µL of buffer PB (Binding buffer) was pipetted into a fresh 2 mL microcentrifuge tube and then 350 µL of the supernatant were added and mixed thoroughly by using the vortext. Then the solution was pipetted into a QIAquick spin column placed in a 2 mL collection tube. The spin column is then centrifuged at 17900 RCF for 1 minute and the flow through is discarded and the collection tube is reused in the next step. 500 µL of buffer AW2 (Wash buffer 2) was added to the QIAquick spin column and the tube was centrifuged at 17900 RCF for 1 minute and the flow through was discarded. The collection tube is reused to centrifuge the sample again at 17900 RCF for 1 minute to dry the membrane.[18] 50 µL of elution buffer was used rather than 150 µL. The sample was stored at -20℃ until further use.

2.3. Quantification of DNA using Fluorometry. The blank sample, standard sample and DNA sample of extracted *S. aureus* culture was prepared according to the manufacturer's instructions.[20] The fluorometer was calibrated, and the concentration of the DNA was measured.

2.4. Amplification of DNA using qPCR. The PCR master mix was prepared with 0.25 µM of nuc forward primer, 0.25 µM of nuc reverse primer. 0.1 µM of the nuc probe and 1X applied biosystems master mix and 25 ng of DNA template. The nuclease free water was added as the negative control. The experiment was performed in dark conditions. The RT PCR computer software was set up using the following information in table 1 along with the PCR conditions given in table 2.

Table 1. The computer software set-up

Probe	NUC	
	Forward primer	Reverse primer
	5'-GGGTTGATACGCCAGAAACG-3'	5'-TGATGCTTCTTTGCCAAATGG-3'
Reporter	FAM	
Quencher	BHQ	
Fluorescence	TaqMan Reagents	
Run Mode	Standard	

Table 2. qPCR cycle

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95 °C	10 minutes	
Denaturation	95 °C	0.15 seconds	40 cycles
Annealing	60 °C	1 minute	
Extension	60 °C	1 minute	

2.5. Detection of S. aureus in the pre-enriched and enriched sample.

2.5.1 Spiking and Homogenization of the sample. A chicken piece of 25 g was measured using a weighing machine, and the chicken piece was added to the sterile filter bag along with 125 mL of nutrient broth and spiked with 1 mL of the *S. aureus* culture inside the laminar flow. The sample was homogenized using a stomacher blender at a speed of 4. Soon after the sample was homogenized, 2 mL was transferred to a microcentrifuge tube. This sample was labeled as pre-enrichment sample the remaining homogenized meat sample was incubated for 18 hours at 37℃. A volume of 2 mL of the homogenized enriched sample was obtained to microcentrifuge tube which was labelled as the enriched sample.

2.5.2 Extraction of pre-enriched and enriched sample DNA. Extraction of DNA was performed using QIAGEN DNeasy mericon Food Kit. Pelleted 1 mL of homogenized sample was placed in a 2 mL microcentrifuge tube. Then 1 mL of food lysis buffer and 10.0 µL of proteinase K solution were added and mixed thoroughly by vortexing. Then the sample was incubated in a water bath at 60℃ for 30 minutes with constant shaking of about 1000 RCF. The sample was centrifuged at 2500 RCF for 5 minutes then 500 µL of chloroform was added to the microcentrifuge tube. Then 700 µL of clear supernatant obtained from earlier was added to the microcentrifuge tube containing chloroform. The microcentrifuge tube was vortexed thoroughly, then centrifuged at 14000 RCF for 15 minutes. 350 µL of buffer PB was pipetted into a fresh 2 mL microcentrifuge tube then add 350 µL of the supernatant and mix thoroughly by using the vortex. Then the solution was pipetted into a QIAquick spin column placed in a 2 mL collection tube. The spin column is then centrifuged at 17900 RCF for 1 minute and the flow through is discarded and the collection tube is reused in the next step. 500 µL of buffer AW2 was added to the QIAquick spin column and the tube was centrifuged at 17900 RCF for 1 minute and the flow through was discarded. The collection tube is reused to centrifuge the sample again at 17900 RCF for 1 minute to dry the membrane.[18] 50 µL of elution buffer was used rather than 150 µL. The sample was stored at -20℃ until further use.

2.5.3. Quantification of DNA using Fluorometry. The samples (pre-enriched and enriched) were prepared according to the manufacturer's instructions.[20] The DNA from the enriched samples was diluted 5 times in order to get a measurable value for DNA concentration. The concentration of DNA was measured.

2.5.4. Amplification of DNA using qPCR. The PCR master mix was prepared with 0.25 µM of nuc forward primer, 0.25 µM of nuc reverse primer. 0.1 µM of nuc probe, 1X applied biosystems master mix and 25 ng of DNA template. The DNA from *Salmonella* was added as the negative control. The experiment was performed in dark conditions.

The RT PCR computer software was set up using the following information in table 3 along with the PCR conditions given in table 4.

Table 3. Computer software set up

Probe	NUC	
	Forward primer	Reverse primer
	5'-GGGTTGATACGCCAGAAACG-3'	5'-TGATGCTTCTTTGCCAAATGG-3'
Reporter	FAM	
Quencher	BHQ	
Fluorescence	TaqMan Reagents	
Run Mode	Standard	

Table 4. qPCR cycle

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95 °C	10 minutes	
Denaturation	95 °C	0.15 seconds	40 cycles
Annealing	60 °C	1 minute	
Extension	60 °C	1 minute	

3. Results and Discussion/Analysis

3.1 Quantification of DNA

The *S. aureus* culture demonstrated a value of 6.6 ng/µL. The pre- enriched culture produced a value of 6.8 ng/µL, the enriched culture produced a value higher than the standard. The five times diluted enriched culture produced a value of 42 ng/µL in the fluorometer.

3.2 Amplification of DNA

According to the amplification plot, the amplification of *S. aureus* can be confirmed using the qPCR method (Figure 4,5,6,7 and 8). Summary of the Ct Values are stated in the table 5.

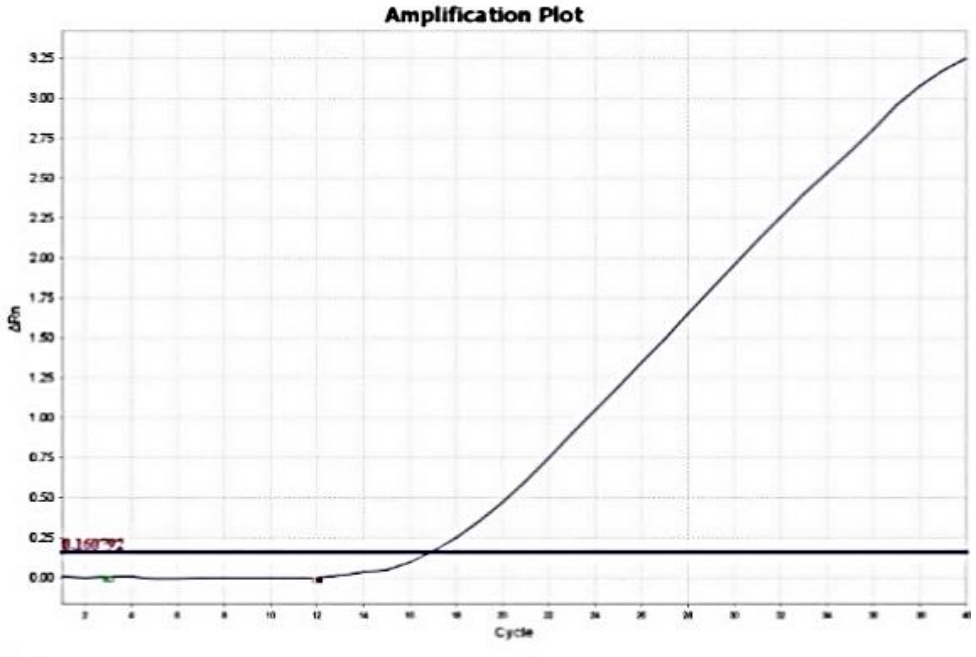

Figure 4. The amplification plot of *S. aureus* (Ct value – 16.961)

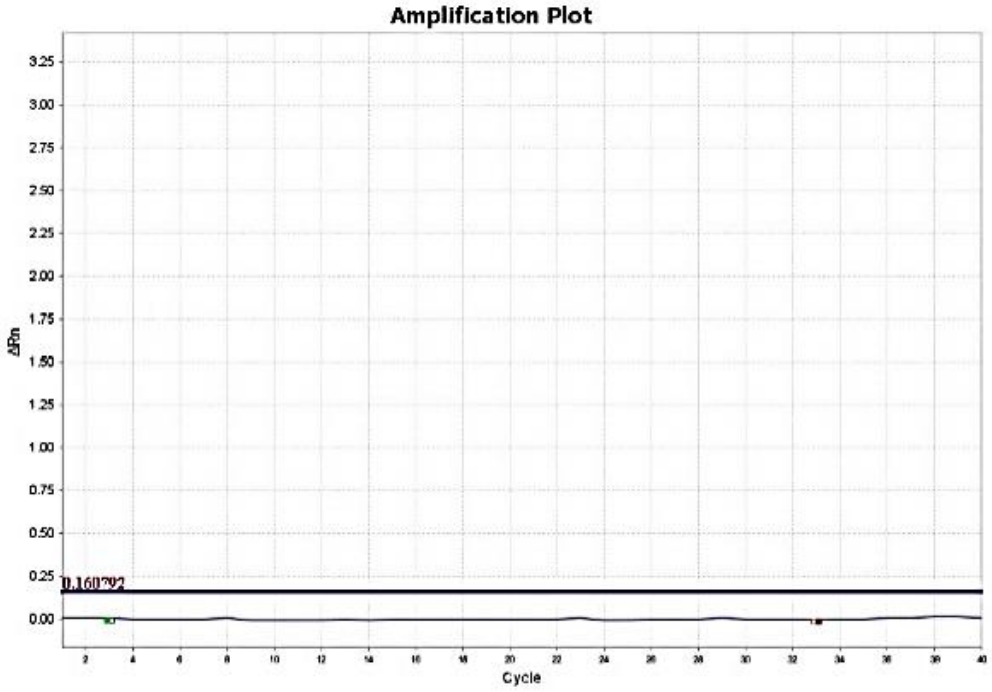

Figure 5. The amplification plot of the negative sample (nuclease free water)

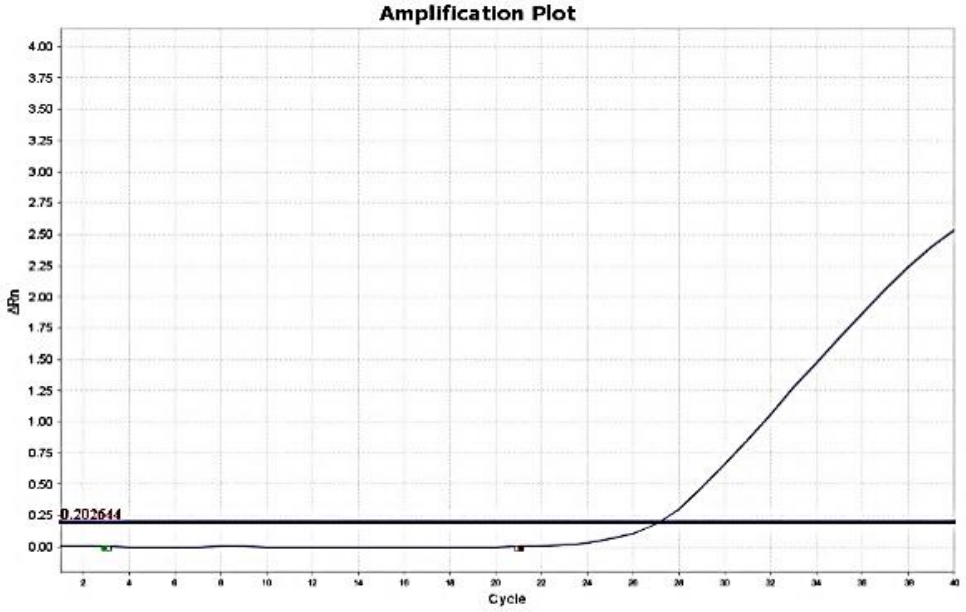

Figure 6.: The amplification plot of the pre-enriched sample (Ct value – 27.119)

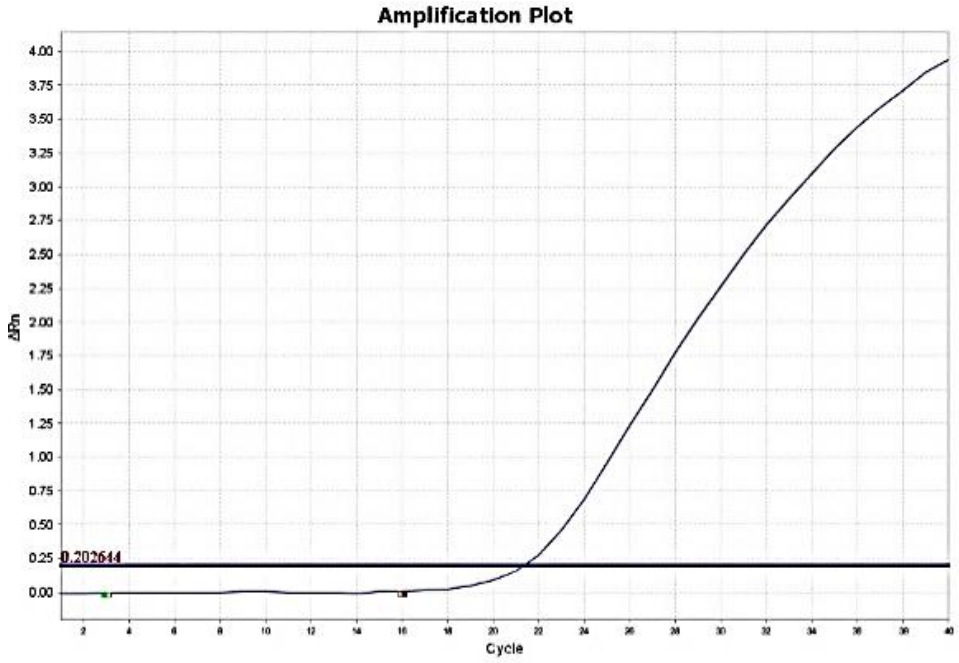

Figure 7. The amplification plot of the enriched sample (Ct value – 21.374)

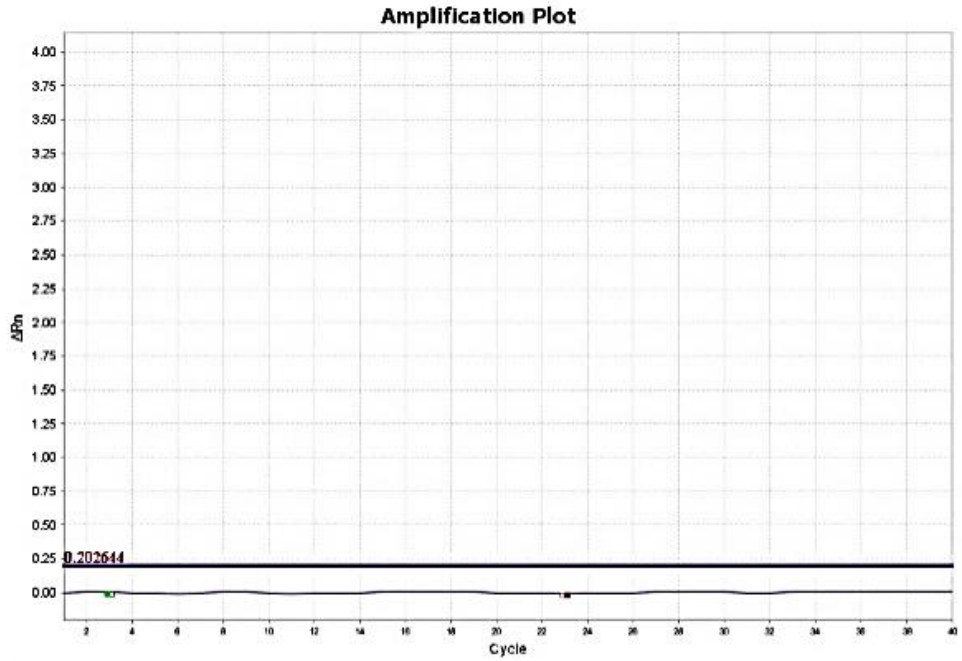

Figure 8. The amplification plot of the negative sample containing *Salmonella* DNA

Table 5: Ct values of the samples

	S. aureus **Positive sample**	***S. aureus*** **Negative sample**	**Pre-enriched sample**	**Enriched sample**	**Negative sample containing *Salmonella* DNA**
Ct value	16.961	Not determined	27.119	21.374	Not determined

Staphylococcus aureus is a gram-positive bacterium. This causes most hospital- acquired infections in the world. Therefore, the detection of *Staphylococcus aureus* is crucial. qPCR presents a fast and reliable method for the detection and quantification of *S. aureus.*[19] This study aimed to develop a qPCR method to detect *S. aureus* species in both non enriched and enriched medium. In the fluorometer results, *S. aureus* has a concentration of 6.6 ng/µL because *S. aureus* is gram- positive, so the breakdown of the cell membrane was difficult; as a result, a low yield of DNA was obtained. Pre-enriched samples have a concentration of 6.8 ng/µL because the sample was obtained before the incubation. The DNA concentration of the enriched sample was higher than the standard due to two factors: first factor is increase of *S. aureus* cells after the incubation period, second factor is homogenized sample includes DNA from meat in addition to *S. aureus*. Due to the higher DNA concentration, the DNA of the enriched sample was diluted five times so the DNA concentration can be measured. NUC is the gene which is detected in the qPCR when detecting *S. aureus* species in food. NUC decodes for thermostable nuclease of *S. aureus* and is a nuclease gene.[21] NUC is a gene marker used to identify the presence of *S. aureus* as the bacteria can function in the presence of heat resistant nuclease gene which is related to the production of enterotoxin.[22,23] The Ct value of *S. aureus* culture was 16.961. The Ct value of the pre-enriched sample was 27.119 and the Ct value of the enriched sample was 21.374. The Ct value of the enriched sample is lower than the pre-enriched sample because the concentration of DNA is more significant in the enriched sample than in the pre-enriched sample. This indicates that the *S. aureus* has grown in the enriched sample after incubation

indicating viable cells of *S. aureus* can be detected by this method.

4. Conclusion

In conclusion, the DNA extraction was successful in both methods; pre- enriched and enriched. However, considering the concentration of the DNA, enriched sample has a higher concentration than the pre-enriched sample. Because the enriched sample contains more DNA than the pre-enriched sample, the amplification plot indicates that the enriched sample has a lower Ct value compared to the pre-enriched sample. This shows that the live *S. aureus* cells could be detected by this method. This suggests that the aim of this study has been achieved, which is the development of a qPCR method to detect *S. aureus* species.

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Characterization and Identification of Anthracene and Pyrene Degrading Soil Bacteria: An Environmentally Friendly Approach for Enhance Bioremediation Efficacy

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Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of organic pollutants that are highly hazardous to the environment and public health due to their genotoxic, mutagenic, and carcinogenic properties as well as their widespread occurrence. The current study's goal was to isolate soil bacteria that are highly capable of degrading pyrene and anthracene from soil that was being inoculated into compost medium. And the bacteria's capacity for degradation was evaluated. The Samples of soil were taken from various urban roadside and coastal locations. This study methodically described how aerobic bacterial strains biodegraded pyrene and anthracene. These bacterial strains were recognized and isolated using molecular and microbiological methods. The results of the spectrophotometric analysis tests show that the SA5 bacterial strain degrades anthracene by more than 70%, while the BW01, BJ3, SA4, SA7, and SJ1 bacterial strains degrade it by more than 50%. Moreover, BWO1, SA5 colonies degrade more than 50% of pyrene. These results imply that the selected bacterial strains could be used as potential biological agents to break down anthracene and pyrene. After the molecular analysis the bacterial samples were recognized as *Brevundimonas vesicularis* sp., *Massilia* sp., *Bacillus* sp., and *Brevundimonas* sp. as the bacterial species that break down anthracene and pyrene.

Keywords: Bioremediation, Pyrene, Anthracene, Antibiotics, High performance liquid chromatograph

1. Introduction

Polycyclic Organic Matter (POM) includes diverse compounds with three or more fused aromatic rings, typically composed of carbon, hydrogen, oxygen, nitrogen, and sulfur.[1] While millions of POM variants are theoretically possible, only about 100 have been identified and studied, with PAHs being the most prevalent subset, containing only carbon and hydrogen.[2] PAHs are ubiquitous environmental pollutants known for their carcinogenic, mutagenic, and genotoxic properties, posing significant health risks.[3] They are produced by the combustion of carbonaceous materials and can be found in both outdoor and indoor environments, originating from activities such as smoking, cooking, and domestic heating.[4]

Pyrene and anthracene are examples of polycyclic aromatic hydrocarbons (PAHs), which are important for science and industry because of their distinct chemical structures and characteristic.[4]

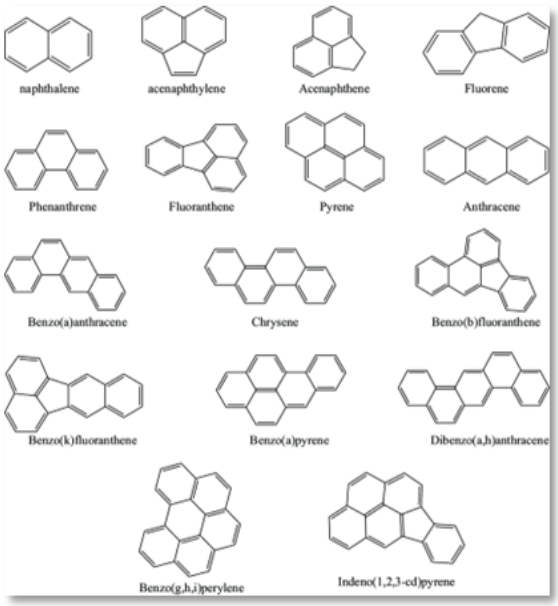

Figure 1. Molecular structure of PAHs[4]

Because of its strong fluorescence and four fused benzene rings, pyrene is useful in fluorescence spectroscopy and environmental monitoring. Because of its photochemical qualities, anthracene—which is made up of three linearly fused benzene rings—finds use in organic electronics, dyes, and radiation detection. As persistent organic pollutants and possible carcinogens, PAHs pose risks to the environment and human health despite their beneficial uses.[5] Therefore, to fully utilize their properties for technological advancements, it is imperative that we comprehend and manage their presence in the environment. Because PAHs are both useful and toxic, it is crucial to conduct more research and implement regulations to properly utilize their advantages.[6] PAHs can undergo degradation through photolysis, reactions with environmental pollutants, or metabolism by fungi and microorganisms. Due to their hazardous nature, effective bioremediation strategies are essential.[7] Through a process called biodegradation, soil bacteria can effectively break down polycyclic aromatic hydrocarbons (PAHs), such as pyrene and anthracene. This process consists of several steps, the first of which is the addition of oxygen to the PAH molecules by means of enzymes such as dioxygenases, which are produced by bacteria such as *Mycobacterium* and *Pseudomonas*. The product of the first oxidation is dihydrodiol. Subsequent enzymatic reactions involving these molecules lead to ring cleavage and the formation of simpler compounds such as catechols. These intermediates eventually fully mineralize into biomass, carbon dioxide, and water after being further broken down into smaller molecules that can enter central metabolic pathways. The soil's pH, temperature, microbial communities, and nutrient content are some of the factors that influence how quickly polycyclic aromatic hydrocarbons deteriorate. By converting toxic compounds into non-toxic end products, soil bacteria aid in the natural attenuation process and lessen the environmental effects of PAH pollution. Bioremediation employs microorganisms to degrade, confine, or alter pollutants, aiming to mitigate environmental contamination. Pyrene and anthracene can be degraded by soil bacteria such as *Rhodococcus, Arthrobacter*, and *Bacillus* which enhances bioremediation techniques and cleanup of sites.[8] As natural scavengers, have evolved to break down organic pollutants, making them valuable in biotechnological applications for environmental cleanup.[9] In particular, the study has concentrated on bacteria that can destroy the PAHs, like pyrene and anthracene, because of their potential to combat PAH pollution.[10] The objective of the study is to isolate PAHs degrading bacteria from urban roadsides and coastal areas and evaluate their PAHs degradation percentages and develop a consortium to overcome PAH pollution.

2. Methodology

2.1 Sample Collection. First, polluted 6 soil samples were collected from urban roadsides and busy coastal areas which depicts 3 different districts in Sri Lanka including Akkaraipattu (Ampara district), Karainagar (Jaffna district) and Wellawatte (Colombo district). Jaffna - 9° 44′ 0″ N, 79° 52′ 0″ E, Ampara - 7° 13' 0" N, 81° 51' 0" E, Colombo - 6° 52′ 28.76″ N, 79° 51′ 37.74″ E, Wellawatta beach (6° 52′26.4"N79°51′26.9"E), Jaffna beach (9°45′47.9N79°53′17.1"6) Addalaichenai (7°14′0"N81°51′0"E). These polluted sample (handful or approximately 100g) were gathered within sterile zip lock bags and closed strongly. Each sample was labelled transported to the laboratory. Then the samples were storednder -ºC in the refrigerator until use.[6]

2.2 HPLC environmental analysis

2.2.1 Sample preparation. Approximately, 4 g of each soil samples were measured and dissolved with 10 mL of hexane and allowed to shake at 180 rpm for 3 minutes. Then the supernatant separated and kept for evaporation. After that 1 mL of acetonitrile was added and completely washed the residues. The samples were filtered through 0.22 µm nylon syringe filters and collected to HPLC vials.

2.2.2 Standard preparation (pyrene and anthracene). 1.25mg of pyrene and anthracene was measured separately. then 5ml of acetonitrile was transferred to the PAHs to preparing 250 ppm stock concentration. According to C1V1= C2V2 formula 250ppm to 50ppm concentration

standard PAHs (Pyrene and anthracene) were prepared.

2.2.3 HPLC analysis. Filtered samples were analyzed and pyrene and anthracene content of the extract were determined using an Agilent 1100 series HPLC equipped with an Agilent 1200 Diode array detector. A ZORBAX ECLIPSE Plus C18 column from Agilent Technologies, USA (4.6 × 100 mm × 3.5 µm particle size) maintained at room temperature was used for this. The PAHs were mobile phased using a 90:10 (acetonitrile: water) mixture at a flow rate of 3 ml/ min. HPLC wave length was 254 nm.[7]

2.3. Isolation of soil bacteria

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2.3.1 Serial Dilution. Approximately 5 g of each sample was weighed and was transferred into the conical flask and shake it for 1 hour at 180 rpm. The washed soil samples were diluted until 10^{-10} factor.[11]

2.3.2. Spread plate technique. For this, 100µL of each diluted sample was placed on the center of the nutrient agar plate. Then the glass spreader was used to spread the bacterial sample throughout the agar plate. Then the plate was closed tightly and was sealed using parafilm. All the inoculated plates were incubated at room temperature for 24 hours.[12]

2.3.3 Streak plate technique. Morphologically different bacterial colonies were selected from the spread plates after incubation. These selected colonies then proceeded with streaking for the isolation purpose. All the selected colonies followed the same streaking technique and was incubated at room temperature for 24 hours.[12]

2.4 Plate Assay (Primary Screening)

2.4.1 Bacterial Starvation in Bacto-Bushnell Hass (BBH) agar plates. First the solidified BBH agar plates were prepared and then a single colony of selected bacterial isolates were transferred into 25 squares drawn BBH agar plate accordingly. After inoculating all the plates, they were closed tightly and sealed using a parafilm. The inoculated plates were allowed to starve at room temperature for 3 days.[13]

2.4.2 Transferring starved bacteria into Anthracene and pyrene plates. First 100 ppm of Anthracene and pyrene in acetone solution was prepared. Then 100 µL of anthracene and pyrene solution was added into solidified BBH agar plates separately. Then it was spiked throughout the plate using a cotton swab and was kept half closed for few seconds to eliminate acetone.

Then the prepared anthracene and pyrene plates were inverted, and 25 equal squares were drawn on each plate and was labeled appropriately. Then each starved bacterial colony was carefully transferred into each square on the plate accordingly. After transferring all the starved bacterial colonies, they were closed tightly and sealed using a parafilm and was incubated at room temperature for 24 hours.[13]

2.5 Spectrophotometric analysis. The sterilized test tubes were filled with 2% of methylene blue and was spiked with the PAH. Afterwards, respective bacterial colonies were added to the medium by using an inoculation loop for both anthracene and pyrene. The spectrophotometric analysis was done with the incubated sample. A blank sample was placed and negative adding was noted prior to analysis. Triplicates were done for each sample and the average degradation percentages were taken.[14]

2.6 DNA extraction. The subcultures of 3 best PAH degrading bacteria were taken and the bacterial colonies were collected into sterile Eppendorf tubes. Then 1 ml of 0.9% of NaCl was added into each tube and centrifuged at 13,000 rpm for 3 minutes and the obtained supernatant was discarded, and this step was repeated 3 times. obtained supernatant was discarded, and this step was repeated 3 times. Then 20µL of Tris-EDTA (TE) buffer was added into each tube and was vortexed to mix them thoroughly. The subcultures of 3 best PAH degrading bacteria were taken and the bacterial colonies were collected into sterile Eppendorf tubes. Then 1 ml of 0.9% of NaCl was added into each tube and centrifuged at 13000 rpm for 3 minutes and the obtained supernatant was discarded, and this step was repeated 3 times. Then 20µL of Tris-EDTA(TE) buffer was added into each tube and was vortexed to mix them thoroughly. Then all the tubes were freeze on ice for 15 minutes and then was thawed at 95ºC using

a heated water bath for 15 minutes. Then it was centrifuged at 13000 rpm for 5 minutes. Then the obtained supernatant was transferred into new Eppendorf tube and 20 µL of 100% ethanol was mixed completely in each tube. Then all 3 Eppendorf tubes were closed tightly and was stored in -25ºC until further use.27 following the volumes and the order as shown in Table 1 inside the biosafety cabinet.[14]

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

Components	**Stock Concentration**	**Working Concentration**	**Volume required**
Go tag green PCR master mix	2×	2×	12.5µL
Nuclease free water	-	-	8.5µL
Forward primer(F27)	100M	10M	1µL
DNA template	-	-	2µL
Reverse primer(R1492)	100M	10M	1µL
PCR mix			25µL

And then the prepared PCR mix was amplified using thermal cycler following the thermal conditions mentioned in Table 2. Also 35 cycles were maintained to reach the detectable level. After the PCR was run using an electrophoresis apparatus, the product was visualized on a 1% agarose gel using a 1 KB DNA.

Table 2. Thermal cyclic conditions for amplification

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

2.8 Visualization of PCR product. To analyze the results of the PCR, 1% agarose gel was used in gel electrophoresis. The gel wells samples were then examined with the aid of a gel documenting system. The BLAST tool was used to identify the species after the sequencing data were trimmed using the BioEdit tool. The accession number was then acquired by using the NCBI submission tool.[14] were filled with 2µL of PCR products and 2µL of a 1KB DNA ladder. The gel was programmed to operate at 55 volts for forty minutes

2.9 Antibiotic susceptibility test. After nutrient agar was made, the surface of the plate was properly filled with a standardized bacterial inoculum. Antibiotic disks were gently placed on the agar plate after an overnight incubation period. Next, the diameter of the inhibitory zone surrounding each antibiotic disk was measured. The Clinical and Laboratory Standards Institute's (CLSI) standards were then contrasted with these measurements.

3. Results and Discussion

3.1 HPLC analysis. The high-pressure liquid chromatography environmental analysis test results revealed that the soil samples were contains pyrene and anthracene at 50 ppm concentration (Figure 3 and 4). PAH concentrations revealed that this study is related to low levels of PAHs because their low concentrations confirmed that these PAHs were below 50 ppm (Figure 3 and 4) which did not exceed the maximum tolerable level of PAHs (68 ppm) for humans.[14]

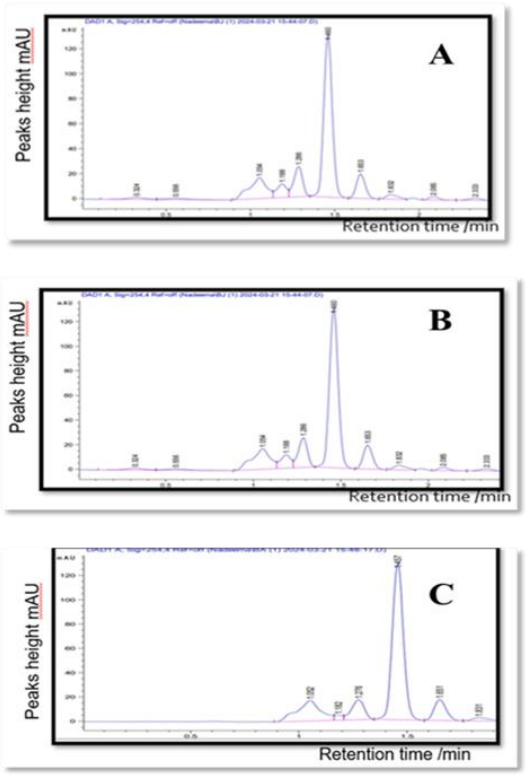

Figure 2. HPLC chromatogram of the samples with peak heights and retention time (A, B, C). A: BW soil sample, B: BJ soil sample, C: BA soil sample

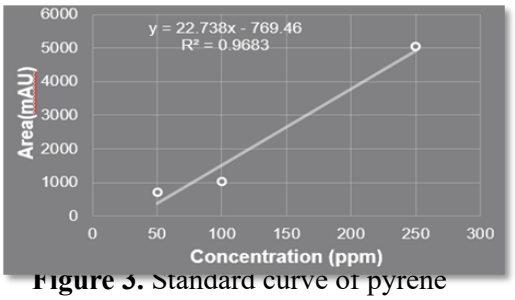

Figure 3. Standard curve of pyrene

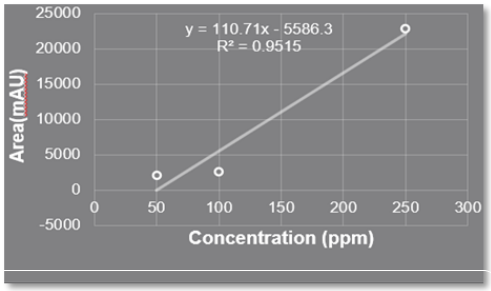

Figure 4. Standard curve of anthracene

Some research studies revealed that these ppm levels are changing due to the addition of PAHs to the environment due to more soil pollution.

Table 3. Standard PHAs retention time

PAHs	Retention time range (min)
Anthracene	1.875 to 2.1
Pyrene	2.366 to 2.5

Table 4. Sample concentration

	BA	BW	BJ
Pyrene (ppm)	42.49	48.37	42.50
Anthracene (ppm)	55.58	56.77	55.59

The standard curve was created using the standard concentrations (50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm) by using the chromatogram peaks to calculate the concentrations of each sample. and standard phase (pyrene and anthracene). Following that, the concentrations of the samples were computed through comparison with the standard curve.[14]

3.2 Spread plate technique. After the incubation of spread plates, the bacterial growth resulted as shown in figure 3 where morphologically different bacterial colonies were identified as given in Table 5.

These identified colonies were named using a unique code such as BJ02, BW01, BW02, BW03, SA4, SA5, SA7, SJ1 for its identification.

Table 5. Spread plates assay observation of samples BJ02, BW01, BW02, BW03, SA4, SA5, SA7 and SJ1.

Colony	Size	Color	Texture	Elevation	Form	Margin
BJ02	Moderate	Yellow	smooth	convex	Round	Entire
BW01	small	Orange	Smooth	Flat	Round	Entire
BW02	Moderate	White	Viscid	convex	Round	Entire
BW03	Large	Yellow	Smooth	Flat	Round	Entire
SA4	Medium	Creamy	Smooth	Convex	Circular	Entire
SA5	Medium	Creamy	Smooth	Flat	Irregular	Entire
SA7	Small	Yellow	Mucoid	Convex	Punctiform	Entire
SJ1	Medium	Creamy	Smooth	Flat	Concentric	Entire

3.3 Population Density

Equation 1.

$$\text{Population Density}(\text{CFU/mL}) = \frac{\text{No. of isolated colonies}}{\text{Amount of diluted sample used in spread plate (mL) x Dilution factor}} \text{ x 1 mL}$$

Table 6. Population density observation of BJ02, BW01, BW02, BW03, SA4, SA5, SA7 and SJ1 colonies.[10]

Colony	Number Of Isolated Coloies	Dilution Factor	Population Density (CFU/mL)
BJ02	1	10^{-5}	1 x 10^{6}
BW01	1	10^{-5}	1 x 10^{6}
BW02	4	10^{-5}	4 x 10^{6}
BWO3	3	10^{-5}	3 x 10^{6}
SA4	1	10^{-10}	1 x 10^{11}
SA5	2	10^{-10}	2 x 10^{11}
SA7	14	10^{-10}	1 4x 10^{11}
SJ1	1	10^{-5}	1 ×10^{6}

The table 6 shows the population density of all 8 colonies and the higher population density containing colony is SA7 with (1 4x 10^{11}).

3.4 Streak Plate technique. After the incubation of streak plates single bacterial isolates of the morphologically different bacterial colony was obtained. This helped in clear identification of the bacterial colony as single isolates that can be further studied.

3.5 Primary Screening (Plate Assay).

Table 7. In the initial screening PAH degradation ability out of 25 squares in bacterial Colonies: BJ02, BW01, BW02, BW03, SA4, SA5, SA7, and SJ1, on BBH agar.

Bacterial code name	Pyrene (number of squares)	Anthracene (number of squares)
BJ02	24/25	25/25
BW01	22/25	25/25
BW02	19/25	23/25
BW03	22/25	25/25
Sa4	25/25	25/25
Sa5	25/25	25/25
Sa7	22/25	25/25
SJ1	25/25	25/25

All bacterial strains showed positive screening as shown in the table 7 which can be further proceeded with secondary screening. Furthermore, the replica plate results showed that more than 80% of the isolated bacteria were able to grow in 100 ppm anthracene and pyrene supplemented BBH agar medium utilizing the phenanthrene and naphthalene as the sole source of carbon.

3.6 Spectrophotometric Analysis. PAH degradation percentages of bacterial strains were obtained as shown in figure 6. this ensures that the bacterial strains are capable of degrading PAH pollutants. Due to the oxidation of polyaromatic hydrocarbon, these eleven specific bacterial species reduced the methylene blue indicator, causing a color shift in the BBH broth. The isolates' total color change from blue to colorless corroborates their potential as hydrocarbon oxidizers and the UV-Vis spectrophotometer can measure this color change as an absorbance value.[8]

Negative control: Anthracene: 1.458

Pyrene: 1.2

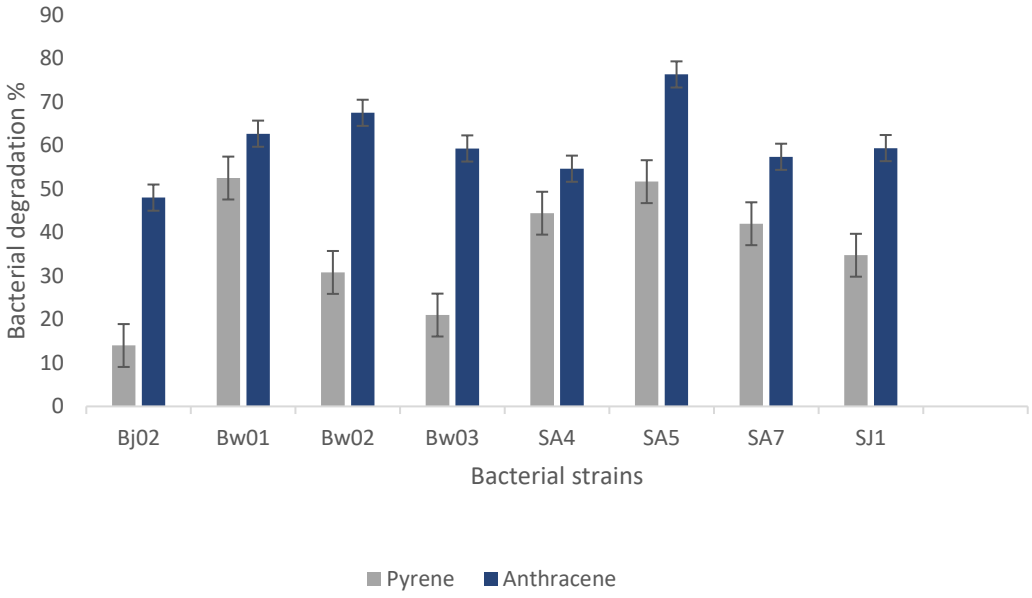

Figure 5. The percentage of pyrene and anthracene that are degraded by the BJ02, BW01, BW02, BW03, SA4, SA5, SA7 and SJ1 bacterial colonies is measured at a wavelength of 609 nm.

The bacterial strains BW01, BW02 and SA5 were capable of degrading anthracene above 60%. Bacterial strain BW01 was able to degrade pyrene by more than 50%. Phenanthrene, fluoranthene, and pyrene could all be degraded by bacterial strains isolated from the mangrove rhizosphere: *Rhodococcus* HCCS, Sphingomonas MWFG, and *Paracoccus* SPNT.[15] Their respective rates of degradation were 90%, 40%, and 69%.25 Compared to anthracene, pyrene degrades at a lower percentage by bacterial strains. Because anthracene has three fused benzene structures while pyrene has four. Therefore, in contrast to pyrene, the bacterial strains can easily break down the structure of anthracene.[2]

Table 8. Identified bacterial species name and their query cover with accession number by using BLAST tool software analysis and NCBI submission tool.

Bacterial name	**Query cover**	**Accession number**
Brevundimonas vesicularis SP.	100%	PQ008462
Bacillus SP.	100%	PQ007442
Massilia SP.	100%	PQ013039
Brevundimonas SP.	100%	PQ013040
Brevundimonas SP.	100%	PQ002185

Table 9. Antibiotic susceptibility readings of BJ02, BW01, BW02, BW03, SA4, SA5, SA7, and SJ1

ANTIBIOTIC SUSEPTIBILY TEST RESULTS (cm)		
Antibiotic disc	**Colony**	**Average zone of inhibition**
Ampicillin	BWO1	-
	BWO2	-
	BWO3	1.2
	BJO2	1.43
Vancomycin	BWO1	2.53
	BWO2	2.1
	BWO3	2.9
	BJO2	2.1
Tetracycline	BWO1	2.56
	BWO2	1.46
	BWO3	2.8
	BJO2	-
Erythromycin	BWO1	-
	BWO2	-
	BWO3	3.53
	BJO2	-
Gentamycin	BWO1	2.53
	BWO2	1.73
	BWO3	2.8
	BJO2	-
Chloramphenicol	BWO1	3.33

	BWO2	2.86
	BWO3	3.6
	BJO2	-
Ampicillin	SA4	1
	SA7	1.3
	SA5	-
	SJ1	-
Vancomycin	SA4	2.3
	SA7	1.9
	SA5	1.8
	SJ1	-
Tetracycline	SA4	2.5
	SA7	2.5
	SA5	2.1
	SJ1	-
Erythromycin	SA4	1.8
	SA7	-
	SA5	2.1
	SJ1	-
Gentamycin	SA4	2
	SA7	1.9
	SA5	2.5
	SJ1	1.2
chloramphenicol	SA4	1.4
	SA7	1
	SA5	3.3
	SJ1	

The tested bacterial strains exhibit significant resistance, according to the results of the Antimicrobial Susceptibility Testing (AST). Strains BW01, BW02, SA5, and SJ1 showed no ampicillin inhibition zones, indicating resistance. Tetracycline inhibition zones were absent from strains BJ02 and SJ1, and erythromycin inhibition zones were absent from strains BW01, BW02, BJ02, SA7, and SJ1, indicating resistance to these antibiotics as well. Furthermore, strains BJ02 and SJ1 did not exhibit an inhibition zone for gentamicin or chloramphenicol, respectively.

All other bacterial colonies, on the other hand, showed reasonable inhibition zones, indicating that they were susceptible to the appropriate antimicrobials. The results demonstrate that most of the strains that were tested had at least one antimicrobial resistance, with strain SJ1 demonstrating resistance to four different antibiotics. This emphasizes how important it is to choose antibiotics carefully when treating infections brought on by these bacterial strains.

4. Conclusion

In this study, bacterial strains that can degrade pyrene and anthracene were identified and their degradation percentages were evaluated. These findings support that bacterial bioremediation of pyrene and anthracene could be effective. Therefore, these identified strains can be used as potential biological agents to mitigate pyrene and anthracene pollution and improve soil quality to promote plant growth and avoid unnecessary passage of pyrene and anthracene toward humans. These research data open several avenues for future experiments to find out the possibility of these best degraders to use as efficient bio remediators to clean the soil. In comparison to the isolates the authors previously tested, the results of the current investigation show that the new isolates were able to destroy a higher quantity of pyrene and anthracene. investigation show that the new isolates were able to destroy a higher quantity of pyrene and anthracene.

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Isolation and biochemical characterization of bacteria in processed chicken meat balls and determination of antibiotic susceptibility

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Abstract

In emerging countries like Sri Lanka, urbanization, religious beliefs, and health influence meat consumption. Meat contains essential amino acids and high protein content. Meat has a short shelf life and spoils quickly if preservation methods are not followed properly. Pre-slaughter handling and post-slaughter handling of meat affect the quality of the meat product. Therefore, meat processing is important to increase the shelf life. Temperature, storage atmosphere, water activity and pH level affect how quickly meat spoils. Proper hygiene procedures should be followed to prevent the emergence of antibiotic-resistant bacteria and microbial contamination of food. The objective of this research study was to isolate and characterize bacteria in selected five different chicken meatball brands in local market and determine antibiotic susceptibility. This sampling consisted of five different chicken meatball brands in local market. After the enrichment process, several subculture procedures were used to obtain single colonies from the culture plates. Motility test, gram's staining and endospore staining was done to observe the bacteria morphology. Indole, methyl-red, Voges-Proskauer (VP), citrate, catalase, and Triple Sugar Iron (TSI) biochemical tests were performed for biochemical characterization. Finally, the antibiotic resistance of the isolated organisms to ampicillin, erythromycin, vancomycin, tetracycline, and chloramphenicol was evaluated by Kirby Bauer's disc diffusion method. Evidence of microbial growth was detected in all five samples. The organisms from each sample were predicted as *Vibrio nigripulchritudo*, *Bacillus cereus*, *Aeromonas salmonicida*, *Paenibacillus alvei* by MATLAB®. However, 16S sequencing needs to be performed to accurately determine the organisms present in the samples. Regardless of standards, poor hygiene controls, poor heat treatment, contaminated minor ingredients, can lead to bacterial contamination. For this, the responsible institutions should check and update the quality assurance processes.

Keywords: Food microbiology, Food quality, Biochemical tests, Antibiotic susceptibility

1. Introduction

Processed meat is defined as meat that has been salted, cured, fermented, smoked, or given another flavoring.[1] Meat and animal products, particularly well-known meat products like sausages, ham, salami, meatballs, etc., are highly valued in many cultures all over the world.[2]

Processed poultry meat is made by treating and processing fresh poultry meat to extend its shelf life and enhance flavor. Some treatments employ chemical preservatives like sodium nitrite.[2]

Processing refers to the mechanical recovery of chicken flesh from a chicken carcass or the mixture of chicken flesh and skin. There are numerous unique forms of preparations that can be done on different meat products. Any meat that has gone through a procedure like drying, curing, maturing, or precooking is referred to as transformed meat.[3]

In Asian countries, chicken meatballs are a common chicken meal. As with red meatballs, the chicken can simply be substituted for the red meat. The most crucial concept is that they are an exquisite food product.[3]

The poultry industry continued to develop and industrialize throughout the world, it was primarily responsible for the increase in global meat production for the last five years. The Food and Agriculture Organization (FAO) reports that 119 million pounds of poultry meat were produced worldwide.[4]

The fastest-growing agricultural sub-sector in emerging nations, particularly, is the chicken industry, which plays a crucial role in nutrition. Future expansion of the sector is anticipated to be influenced by variables like population increase, income level growth, and urbanization. The poultry industry, with a market size of $ 310.7 billion in 2020, is anticipated to increase by 3.8% annually to reach $ 322.55 billion in 2021.At a 7% Compound Annual Growth Rate, the market is anticipated to reach $ 422.97 billion in 2025.[5]

Poultry was the most widely consumed form of meat in the world in 2021 with an estimated 132.3 million tons consumed. By 2031, 153.85 metric kilotons of poultry meat are anticipated to be consumed globally.[6] In 2020, Sri Lanka's population consumed 10.4 kg of poultry meat annually. This is an increase of 19.2% from the prior year. In the past, Sri Lanka's consumption of poultry meat per person reached an all-time high of 10.4 kg in 2020 and a record-low of 0.630 kg in 1961.Regarding the interest rate on poultry meat intake per capita, Sri Lanka is rated 107th out of the 161 nations.[7]

The contamination of processed chicken products with pathogenic microorganisms, particularly bacteria, is one of the most challenging problems facing the worldwide food industry.[8] The carcasses, the cuts made from them, and processed meat products are all contaminated during and after slaughter by the atmosphere of the slaughterhouse, the instruments used, and the microbes from the animal microbiome.[9] While food is being prepared and kept, some of these bacterial contaminations might emerge or continue to exist. Contamination of equipment surfaces by bacteria can occur early in the process. This includes rubber fingers that are used to remove feathers or conveyor belts as sources of bacterial contamination. Even new rubber fingers can harbor bacteria and be a source of contamination for carcasses. Cross-contamination can occur between cuts or carcasses through contact with a contaminated surface or direct contact. Air, manipulators, and equipment surface are the main sources of contamination during subsequent processing processes such as cutting, deboning, mixing, and mincing, for the production of meat-related food items. Transformation processes expand the surface area of meat in contact with air and work surfaces. As a result, there is a higher level of bacteria in the transformed products than in the primary cuttings.[9] *Escherichia coli*, *Bacillus subtilis*, *Campylobacter*, and *Salmonella* sp. are typical bacteria found in chicken products.[10]

These bacteria are resistant to some antibiotics. Antibiotic resistance occurs when bacteria acquire resistance to antibiotics meant to kill, stop their growth. When exposed to antibiotics, antibiotic-resistant bacteria are capable of proliferating, expanding, and infecting the host. Antibiotic resistance becomes an obstacle in the treatment of bacterial infections. The end result is that some antibiotics can no longer be used to treat some bacterial infectious diseases.[11] Antibiotic resistance mechanisms can be divided into four categories. The first involves beta-lactamases to modify the antibiotic's enzymes, thereby breaking the beta-lactams ring of penicillin and cephalosporins. Other enzymes (e.g., AmpC), hydrolyze most beta-lactams. Alteration of the target site of the bacterial cell wall is the second mechanism of antibiotic resistance. This involves the production of modified penicillin-binding proteins that reduce the target affinity for the antibiotic. Third, bacteria can use efflux pumps to prevent the accumulation of various antibiotics in bacterial cells by pumping out from the cell. Finally, some antibiotics (e.g., sulfonamides) work by inhibiting the synthesis of molecules essential for bacterial survival, such as folic acid.[12]

The general objective of this study was to biochemically characterize the microorganisms present in five processed meat products available in the local market and to test their antibiotic sensitivity. To achieve this objective, microorganisms were cultured under necessary conditions and staining techniques were performed to observe the morphology. After that, isolated organisms were tested against selected antibiotics to assess sensitivity.

2. Methodology

2.1 Sample collection. Commercially available, chicken meatballs from five (05) different brands were purchased from local supermarkets in Sri Lanka.

Table 1. Meatball sample details

Sample ID	Packaging size (g)	Standards used for the production
CM1	200	SLS
CM2	200	HACCP
CM3	200	SLS
CM4	200	HACCP
CM5	200	SLS
Positive Controls	*S. aureus* (ATCC 6538) *E. coli* (ATCC 8739) *B. subtilis* (ATCC 6633)	
Negative Control		

2.2 Sample Enrichment. The products were surface sterilized before transferring to the bio safety cabinet (BSC) (HPsafe-1200LC/ Class, Type: II, A2). Packages were opened and approximate 01 g of each sample was weighed in to 10 ml sterile peptone water added Uricol™ containers. Aseptic conditions were maintained during the transfer.

For negative control 10 ml of peptone water (HIMEDIA®/ Ref: M028-500G) was used. The containers were incubated at 37 °C for 24 hours.

2.3 Screening and isolation of microorganisms

2.3.1 Streak plate method using Nutrient Agar (NA). The streak plate technique was used and incubated at 37 °C for 24 hours. Well isolated colonies were selected for further analysis. Gram staining, motility testing, and endospore staining were performed on these selected colonies from each sample.

2.4 Microscopic analysis

2.4.1 Motility test (hanging drop method). A loopful of isolated colony inoculum from each sample was examined under a microscope.

2.4.2 Gram's staining. Gram staining was carried out according to the ASM protocol. [36]

2.4.3 Endospores staining. Endospore staining was carried out following the Schaeffer-Fulton procedure.[37]

2.5 Biochemical tests

2.5.1 IMViC test

2.5.1.1 Indole test. A loopful of pure culture inoculum was inoculated in tryptophan broth (HIMEDIA®/ Ref: M1339-500G) 37°C for 24 hours. 2-3 drops of Kovac's reagent were added into each test tube, and results were obtained after 15 minutes.

2.5.1.2. Methyl-red test. MR-VP broths (HIMEDIA®/ Ref: M070-500G) were inoculated and incubated at 37 °C for 24 hours. 2-3 drops of methyl-red indicator were added into each test tube, and results were taken after 15 minutes.

2.5.1.3 VP test. As sample ID, five test tubes containing VP broth (HIMEDIA®/ Ref: M070F-500G) were inoculated and were incubated at 37 °C for 24 hours. 2-3 drops of Barritt's reagent were added into each test tube, and results were taken after 15 minutes.

2.5.1.4 Citrate test. Test was performed using citrate agar slants (HIMEDIA®/ Ref: M099-500G) Bacterial colonies were streaked throughout the test tubes. The test tubes were incubated at 37°C for 24 hours.

2.5.2 TSI test. 5 slanted test tubes with TSI agar (HIMEDIA®/ Ref: M021-500G) were used. Bacterial colonies were streaked throughout the test tubes. The test tubes were incubated at 37°C for 24 hours.

2.5.3 Catalase test. Catalase was tested using drop of hydrogen peroxide (20% V/V) on a bacterial smear.

2.6 Hi-chrome E. coli agar inoculation. A loopful of the bacterial inoculum was straked on Hi-chrome (HIMEDIA® /Ref: M1295-500G) to check whether the isolated organism was *E. coli*. Plates were incubated at 37°C for 24 hours.

2.7 Streak plate method using TCBS agar. The streak plate technique was then used and incubated at 37°C for 24 hours.

2.8 DNA extraction. Pure colonies were transferred to 1.5 ml tubes. 1ml of saline water (0.9% NaCl) was added and vortexed. It was centrifuged for 5 minutes at 13000 rpm. The supernatant was discarded. The preceding stages were carried out twice. The pellet was vortexed after 20 µl of TAE buffer was added. The tubes were placed on ice for 15 minutes before being placed in a 95°C water bath for 15 minutes. It was then centrifuged for 5 minutes at 13000 rpm. The supernatants were transferred to fresh microcentrifuge tubes. A total of 20 µl of 99% ethanol was added. The tubes were then placed on ice.

2.9 DNA quantification. Quartz cuvette was used to measure optical density of extracted DNA samples. At 225 nm, 260 nm, 280 nm, 330 nm, and 450 nm.

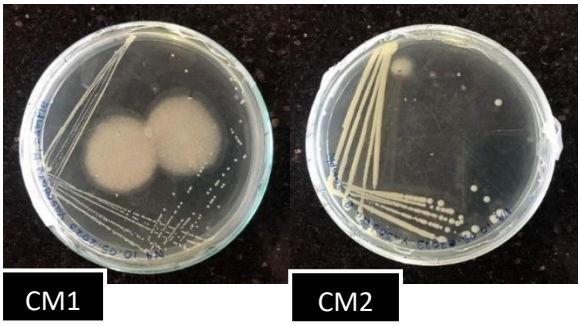
2.10 Antibiotic susceptibility test

2.10.1 Disc diffusion method. A loopful of pure isolated colony from each sample was swabbed on Mueller Hinton Agar (MHA) (HIMEDIA®/ Ref: M173-500G) plates with sterile cotton swabs. Selected antibiotic discs were placed on the plates using sterile forceps and incubated at 37°C for 24 hours.

2.11 Organism prediction using MATLAB. Observed biochemical test results were fed into MATLAB annbis algorithm interface to generate predictions.[32]

3. Results

3.1 Streak plate method. The colony morphologies observed indicated the presence of a single type of organism. In some of the plates pigment formation could be observed.



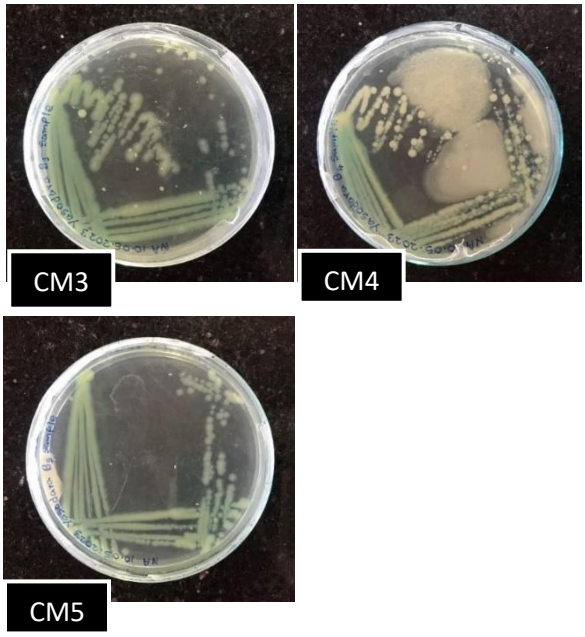


Figure 1. Results of 3rd subculture nutrient agar plates

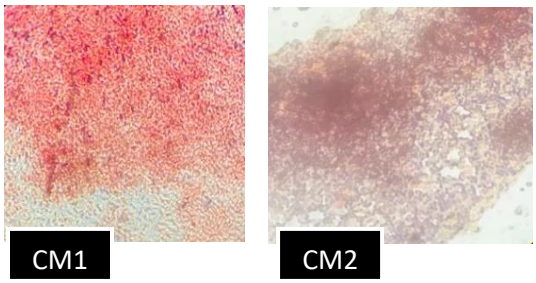
3.2 Microscopic analysis

3.2.1 Motility test. The results of the motility test are summarized in table 2. Selected bacteria from each sample were motile in all the samples, and rod-shaped bacteria could be observed in sample CM3 while others were cocci.

Table 2. Motility test results

Sample ID	Shape
CM1	Cocci
CM2	Cocci
CM3	Bacilli
CM4	Cocci
CM5	Cocci

3.2.2. Gram's staining



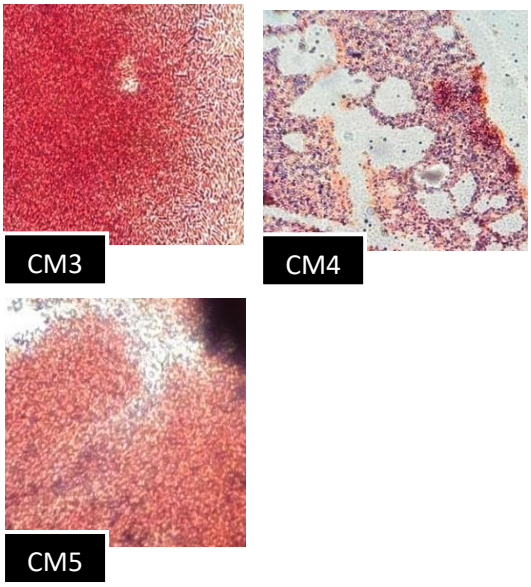


Figure 2. Gram's-stained bacteria under 100X magnification

When observed under the microscope (10X 100X1), samples CM1, CM3, CM5 appeared pink in color indicating Grams negative nature, while samples CM2 and CM4 appeared purple in color indicating Grams positive.

3.2.3 Endospore staining

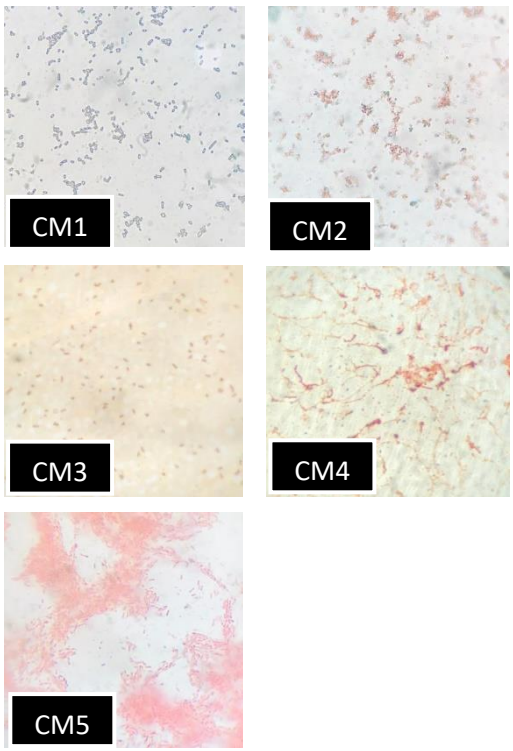


Figure 3. Endospore-stained bacteria under 100X magnification.

When observed under the microscope (10X 100X1), the spores of CM1 sample were appeared green and the other samples except CM1 was appeared red. The results of the endospore's staining test are summarized in the table 3.

Table 3. Observation of selected organisms from sample

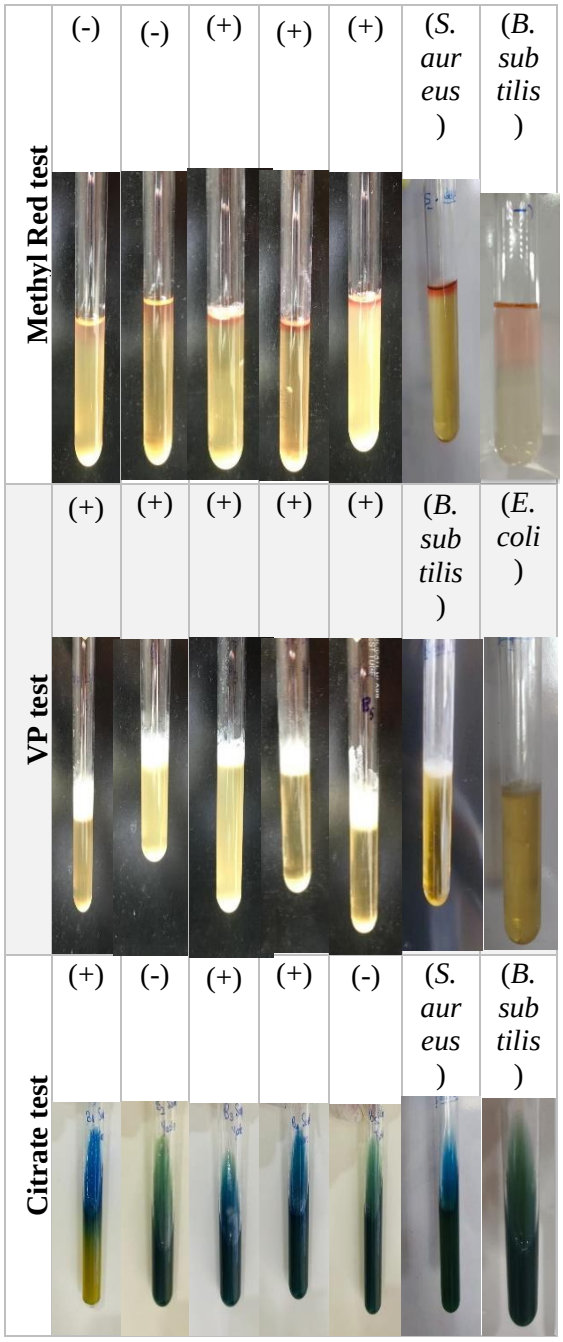
Sample ID	Gram nature	Endospore	Motility
CM1	G-	observed	Motile
CM2	G+	Not observed	
CM3	G-	Not observed	
CM4	G+	Not observed	
CM5	G-	Not observed	

3.3 Biochemical Test results

3.3.1 IMViC test

Table 4. IMViC test results

Test	CM1	CM2	CM3	CM4	CM5	Positive	Negative
Indole test	(+)	(+)	(+)	(+)	(+)	(*E. coli*)	(*B. subtilis*)
							



Positive Results- (+), Negative Results- (-)

3.3.2 TSI test

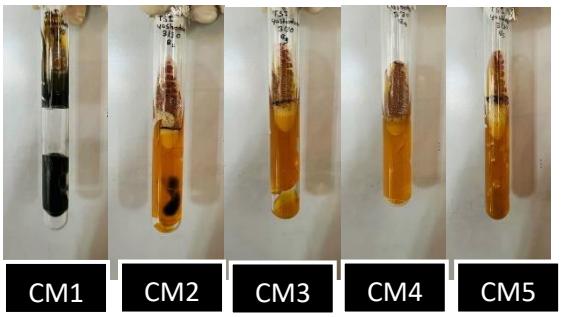


Figure 4. Results of TSI test

Table 5. TSI test results

Sample ID	Slant	Butt	Gas production	Blackening of the medium/ black pigments
CM1			✓	✓
CM2	R	Y	✓	✓
CM3	R	Y	✓	
CM4	R	Y	✓	
CM5	R	Y	✓	

R- Red, Y- Yellow

3.3.3 Catalase test

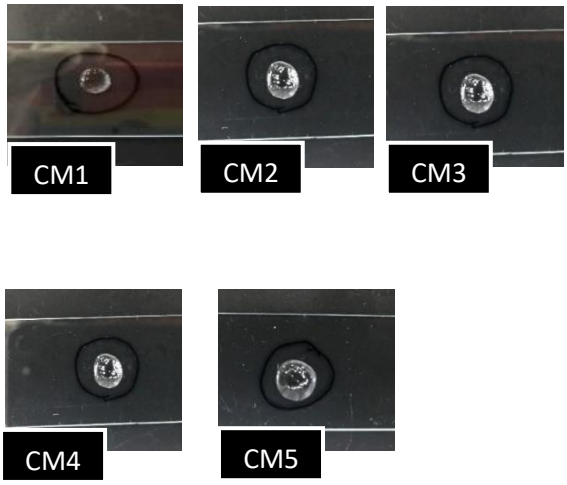


Figure 5. Results of catalase test

Bubbles appeared in all the samples except CM1.

3.4 Isolation on Hi-chrome E. coli agar

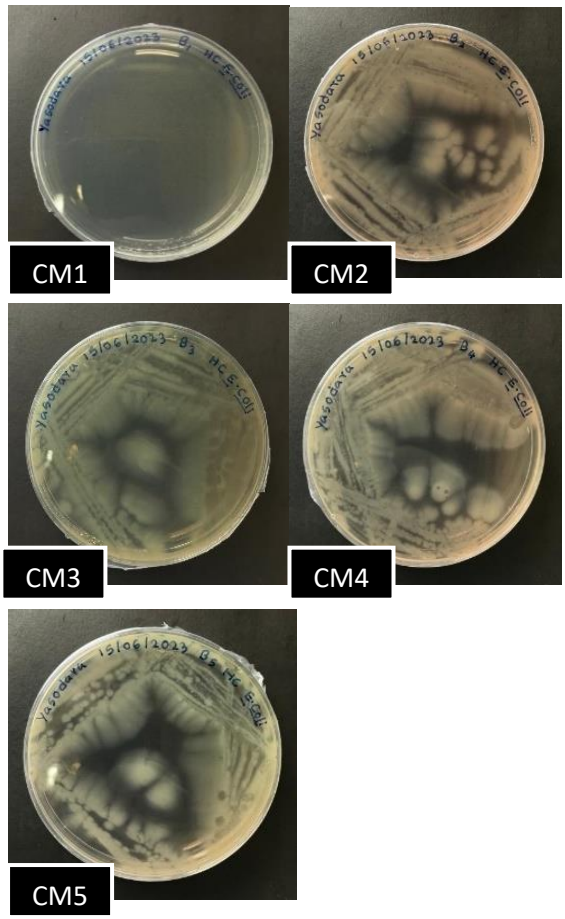
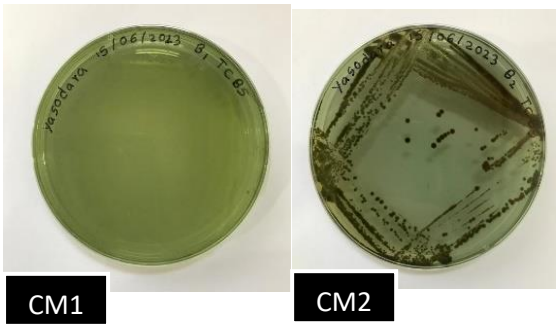


Figure 6. Hi-chrome *E. coli* agar test results

Table 6. Results of Hi-chrome *E. coli* agar test

Sample ID	Blue green appearance	colorless
CM1		✓
CM2		✓
CM3		✓
CM4		✓
CM5		✓

3.5 Inoculation on TCBS agar



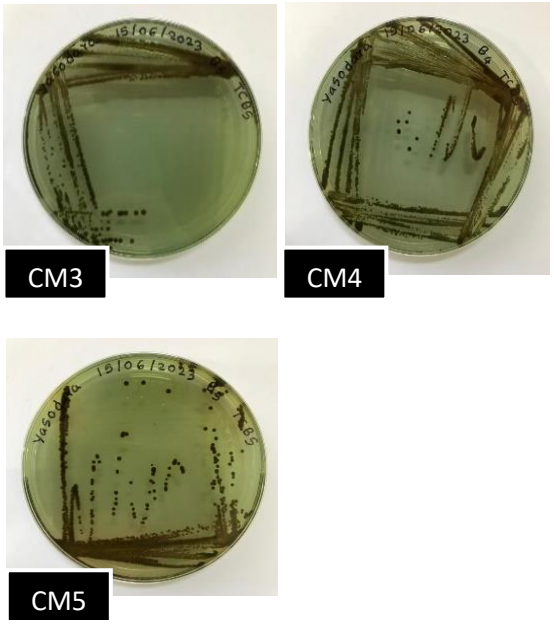


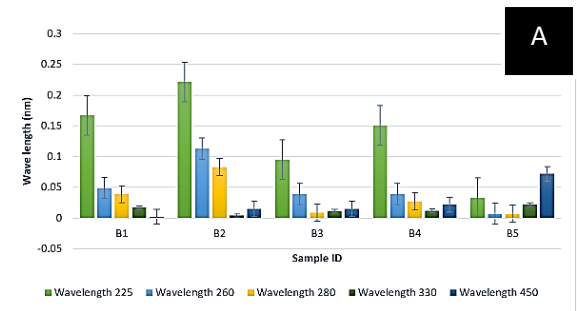
Figure 7. TCBS agar test results

Table 7. Results of TCBS agar test

Sample ID	Growth inhibited	Bacterial Growth	Colony color
CM1	✓		
CM2		✓	Dark green
CM3		✓	Dark green
CM4		✓	Dark green
CM5		✓	Dark green

3.6 DNA quantification

3.6.1 Spectrophotometer



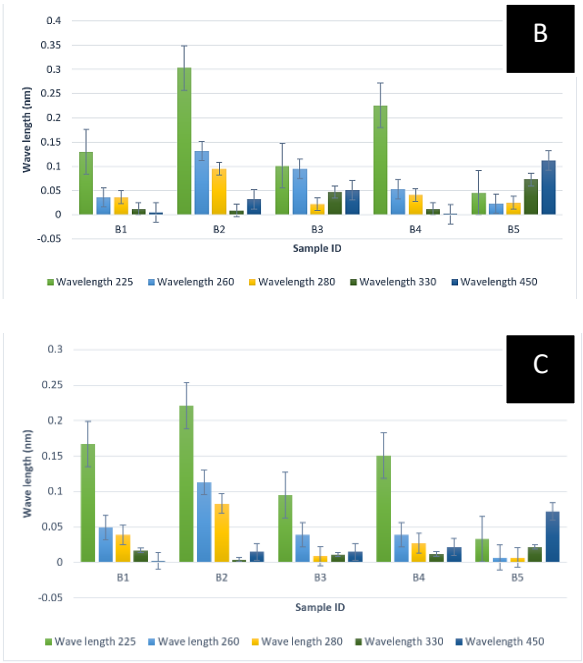
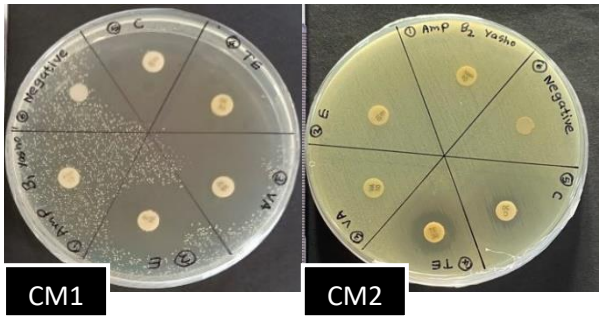
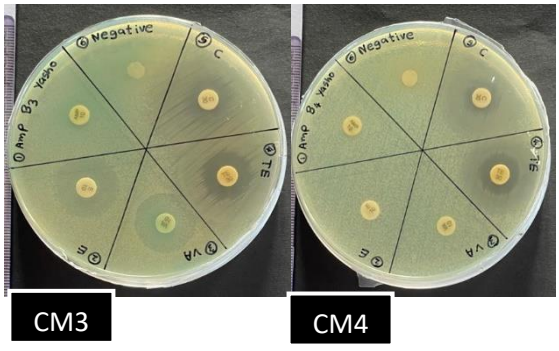


Figure 8. DNA OD measurement using 5 µl (A), 10 µl (B) and 15 µl (C).

3.7 Antibiotic susceptibility test

3.7.1 Disc diffusion method





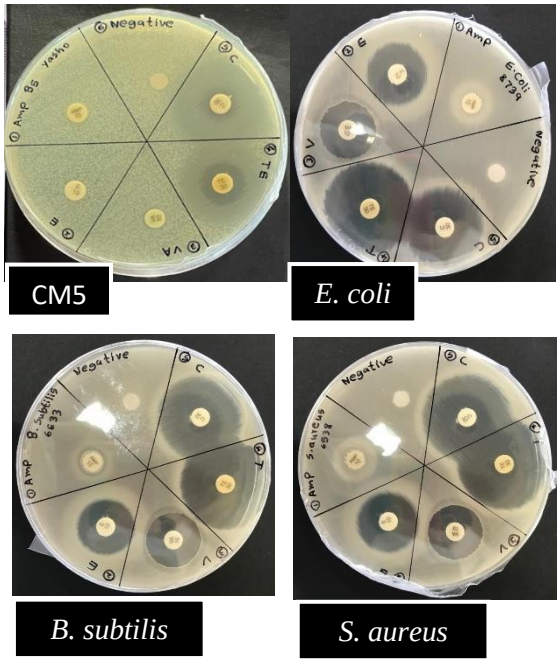


Figure 9. ABST on MHA the zone of inhibition (ZOI), incubated at 37 °C for 24 hrs.

Table 8. ZOI of inhibition (mm).

Sample ID	Amp	Ery	Van	Tet	Chl
CM1	-	28.4	24.8	39.5	36.5
CM2	-	-	-	21.8	31.5
CM3	-	11.6	18.2	28.5	35.3
CM4	-	-	-	24.8	22.3
CM5	-	-	-	22.3	12.5

Amp – Ampicillin, Ery – Erythromycin, Van – Vancomycin, Tet – Tetracycline, Chl – Chloramphenicol

Table 9. Organism prediction using MATLAB®

Sample ID	Organism	Evaluation	Similarity
CM1	*Bacillus cereus*	Good identification	VP (+), IND (+)
CM2	*Aeromonas salmonicida subsp. Masoucida*	Acceptable identification	VP (+), IND (+), CIT (+)
CM3	*Vibrio nigripilchritudo*	Acceptable identification	VP (+), IND (+), CIT (+)

CM4	*Vibrio nigripilchritudo*	Acceptable identification	VP (+), IND (+), CIT (+)
CM5	*Paenibacillus alvei*	Good identification	VP (+), IND (+), CIT (+)

Table 10. Susceptibility of the predicted organisms to the selected antibiotics according to Bergey's Manual.

Sample ID	**Predicted Organism**	**Amp**	**Ery**	**Van**	**Tet**	**Chl**
CM 1	*Bacillus cereus*	R	R	S	R	S
CM 2	*Aeromonas salmonicida subsp. Masoucida*	R	I	R	R	S
CM 3	*Vibrio nigripilchritudo*	R	R	R	S	I
CM 4	*Vibrio nigripilchritudo*	R	R	R	S	I
CM 5	*Paenibacillus alvei*	R	R	S	S	S

R - resistant, S - sensitive, I – intermediate

4. Discussion

The purpose of this study was to isolate and biochemically characterize the bacteria present in selected processed chicken meat ball products and ascertain whether or not they were susceptible to antibiotics. The samples were collected from the Kalubowila area in Sri Lanka. After the enrichment process, the turbidity was observed in the sample containers indicating the presence of microorganisms. Overgrowth could be seen in some culture plates. A series of biochemical tests were performed to characterize the bacteria present in the samples.

The hanging drop method was used to study bacterial motility along with the size, shape, and arrangement of bacteria.[13] According to research performed by Supriya in 2023, if there are motile bacteria, the bacterial structure, shape, and number of bacteria can be predicted to some extent. When there is no movement, the result indicates absence of flagella or other locomotive structures.[14] Selected organisms from B1 to B5, bacteria swam erratically with the kinetic energy held by the fluid molecules surrounding the microparticles in the fluid. Therefore, those bacteria were motile in CM1, CM2, CM4, and CM5, bacteria appeared in the form of spheres, so the bacterial shape were recognized as cocci.

Gram staining was performed to determine the ability of bacterial cell walls to retain crystal violet dye during solvent.[15] Microorganisms selected from samples CM1, CM3, and CM5 stained pink and were gram-negative. Gram-negative bacteria have a thin peptidoglycan layer and an outer lipid membrane. In CM2, CM4 samples the bacteria stayed purple therefore they were gram positive. Presence of a thick cell wall, peptidoglycan layer and no outer lipid membrane, those bacteria are capable of retaining crystal violet and iodine complex when decolorized by ethanol, which appeared as blue or purple. The CM3 sample was rod-shaped and was identified as bacilli. All the other 4 samples were round-shaped and were identified as cocci. According to study done by WU and YANG in 2020, the quality of the bacterial cell wall determines whether the organism will be Gram-negative or Gram-positive.[16] Fresh bacteria cultures were used for staining.

Endospore staining test helps to identify endospore-containing bacteria.[17] According to a research performed by Hussey and Zayaitz in 2007, the endospore retains malachite green color and appears green. The cells could appear red-brown or pink in the absence of spores.[17] Samples CM2, CM3, CM4, and CM5 appeared red-brown in color. Therefore, it can be concluded that spores were absent in those bacteria during the time of testing. The CM1 sample appeared green and it was concluded that they were spores. However, the bacterial samples were not specifically stressed to induce spore formation prior to the endospore staining.

The indole test is used to measure the ability of an organism to degrade the amino acid

tryptophan and produce indole.[18] *E. coli* was used as the positive control and *Bacillus subtilis* was used for the negative control. A cherry red ring appeared at the top of the positive control medium and no color change in the negative control. A pink to red was formed in the treatment layer above the medium in all the samples (CM1 to CM5) indicating positive results for the indole test. This concludes that all isolated bacterial samples were capable of producing indole.[19]

The Methyl-red test was performed to test the ability of the organism to produce, maintain stable acids as an end product of glucose fermentation and to overcome the buffering capacity of the system.[20] For Methyl-Red test, *S. aureus* was used for positive control and *B. subtilis* was used as the negative. The culture medium of the positive control turned red. This is because the pH was 4.4 or lower due to glucose fermentation. No color change occurred in the negative control. CM1 and CM2 did not exert any color change, but CM3, CM4, and CM5 samples turned red indicating positive reaction. It was concluded that the selected microorganisms in samples CM3, CM4, and CM5 have the ability to produce and maintain stable acids as the end product of glucose fermentation. Microorganisms selected in CM1 and CM2 samples are not able to do so. According to a research performed by Shanmugaraj, Anokhe and Kalia in 2021, two (02) of the three (03) bacterial samples they had studied was negative for Methyl Red, and one sample was positive.[21]

VP test determines whether organisms produce acetylmethyl carbinol by fermenting glucose.[22] *B. subtilis* and *E. coli* was used for positive and negative controls for VP test. When the results were analyzed it could be concluded that all 5 samples were positive.

The primary purpose of the Citrate test is to determine the ability of an organism to use citrate as a sole source of carbon for metabolism with alkalinity.[23] In the citrate test, *S. aureus* was used as the positive control and *B. subtilis* was used as the negative control. The color of the positive control slant changed from green to dark blue. The negative control did not show any color change. The color of samples CM1, CM3, and CM4 changed from green to dark blue after incubation at 37 °C for 24 hours. Therefore, CM1, CM3, and CM4 samples were positive and it was concluded that the selected microorganisms can use citrate as a carbon source. But there was no color change in CM2 and CM5 samples, so those samples were negative. It was concluded that those bacteria are not able to use citrate. The CM1 sample appeared in blue color above and yellow color below. The reason for this is the pH difference and the presence of anaerobic bacteria. According to a paper by Salauddin *et al* published in 2019, citrate testing has been done for two (02) samples of bacteria.[24]

The TSI test was performed to determine an organism's ability to produce hydrogen sulfide and to ferment glucose, sucrose, and lactose.[25] CM1 and CM2 samples blackened the medium after incubation at 37 °C for 24 hours. Hence the selected microorganisms in those samples were capable of producing H_2SIn samples CM2, CM3, CM4, and CM5, the slant appeared red and the butt appeared yellow. Therefore, it can be concluded that these samples showed alkaline/acid conditions. Hence it was concluded that organisms fermented glucose in the medium. Cracks could be observed in all sample tubes. It was concluded that all 5 samples produced gas. According to a study done by Sultana *et al* in 2022, the TSI test has been done for 5 samples.[26]

Catalase test was performed to identify organisms that produce catalase enzyme. This enzyme breaks down hydrogen peroxide into oxygen and water.[27] No bubbles occurred in sample CM1 indicating a negative result. Other samples were positive for catalase test. It was concluded that the organisms in these samples produce catalase enzymes. According to a research performed by Khairullah *et al* in 2022, the catalase test has been done for *Staphylococcus aureus* and a positive result has been obtained.[28]

Hi-chrome *E. coli* agar test is used to detect *Escherichia coli* and total coliforms simultaneously.[29] There was no growth of colonies in the CM1 sample, while colonies were observed in the rest of the samples. However, desired colour change (Bluish-green colony appearance) could not be observed. It was concluded that there was no *E. coli* in the enriched samples. According to a research

performed by Antony in 2018, there were *E. coli* positive and negative samples they had analyzed.[29]

TCBS agar test was done to check whether *Vibrio* species were present in the samples.[30] CM1 sample did not show any growth on TCBS, while the rest of the samples were capable of making colonies on TCBS agar. Since growth was observed on the plates. It can be concluded that *Vibrio* presence in CM2 to CM5 samples. But, based on the manufacturer reference the colonies on the plate may probably not *Vibrio cholerae, Vibrio parahemolyticus* or *Vibrio alginolyticus*, and could be another subspecies of Vibrio, which needs to be confirmed by further genetic testing.

According to the obtained biochemical results Aeromonas salmonicida, Vibrio nigripulchritudo, Paenibacillus alvei in the samples were further predicted using the mentioned MATLAB platform. However, this datapoint has to be confirmed with further DNA sequencing. [30,32] The predictions obtained from MATLAB platform may not be completely accurate since the later versions of MATLAB does not support the annbiss algorithm. Predictions could not be further confirmed with genetic testing.

The antibiotic susceptibility test determines the sensitivity or resistance of bacteria to specific antibiotics.[31] *E. coli, B. subtilis*, and *S. aureus* were used as positive controls. The sensitivity to the ABST test was analyzed with reference to MATLAB® predictions. CLSI standards were used to determine the zone diameter around the predicted organism. The CM1 sample was *Bacillus cereus* and the CM2 sample was *Aeromonas salmonicida subsp. masoucida*, samples CM3 and CM4 were identified as *Vibrio nigripulchritudo* and sample CM5 as *Paenibacillus alvei*.[32] All samples from CM1 to CM5 were resistant to Amp. CM1, CM3, CM4 and CM5 samples were resistant to erythromycin. The CM2 sample was intermediate for Ery. CM2, CM3, and CM4 samples were resistant to vancomycin and CM1 and CM5 samples were sensitive. CM3, CM4, and CM5 samples were sensitive to tetracycline, and CM1 and CM2 samples were resistant. CM1, CM2, and CM5 samples were sensitive to chloramphenicol, and CM3 and CM4 samples were intermediate.[33] Studies conducted in 2019 show that *Bacillus cereus* is resistant to Erythromycin, and Tetracycline. The ABST test conducted for CM1 also gave similar results to the study.[34] A 2021 study found that *Aeromonas* is resistant to ampicillin. The ABST test conducted for the CM2 sample also obtained similar results to the study.[35]

The antibiotic sensitivity of this work was based on the MATLAB annbis algorithm predictions which should be further confirmed with DNA sequencing. However, it was confirmed that there are microorganisms capable of growing on hi-chrome and TCBS agar and carrying out various other biochemical reactions as mentioned in the context. It is suggested that the quality of processed meat products produced in Sri Lanka should be further investigated.

5. Conclusion

In conclusion, by examining the results of the tests conducted, it can be concluded that all five (05) samples of processed chicken meat ball products contained bacteria with various biochemical capacities. Although chicken meat balls are processed under food safety conditions, this work evidenced the presence of microorganisms in those products.

Acknowledgements

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Phytochemical screening, antioxidant and antimicrobial activities of the leaves from Parsley (Apiaceae) family plants grown in Sri Lanka

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Abstract

Plants that are classified as part of the Parsley (Apiaceae) family are aromatic and flavorful culinary herbs. They are distributed worldwide as good sources of raw materials for the development of pharmaceuticals. Some pharmaceutical properties of plants in Apiaceae family include antioxidant, anti-inflammatory and antitumor activities. In this investigation, the phytochemical profile was assessed, and the antioxidant and antimicrobial activities of leaf extracts were evaluated from five plant species of the Parsley family: *Anethum graveolens* (Dill), *Apium graveolens* (Celery), *Coriandrum sativum* (Coriander), *Foeniculum vulgare* (Fennel), and *Petroselinum crispum* (Parsley). Leaves were extracted by the maceration technique using water as the solvent. Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Antioxidant Capacity (TAC) were quantified using Folin-Ciocalteu, Aluminum Chloride colorimetric and Phosphomolybdenum methods respectively. The free radical scavenging activity was analyzed using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay while the antimicrobial activity was determined by well diffusion technique using *Escherichia coli* and *Staphylococcus aureus* as test micro-organisms. Dill showed the highest content of phenols (0.016 mg GAE/g), flavonoids (0.0034 mg QE/g), antioxidants (0.039 mg AAE/g) as well as the highest free radical scavenging activity (IC_{50} = 1.18 µg/mL). All samples exhibited no antimicrobial activity at a concentration of 0.2mg/mL. Thereby it can be concluded that, besides its nutritional value the intake of plant-based food such as leaves from the Apiaceae family can play a beneficial role in the prevention of certain infections and also provide sources of natural antioxidants.

Keywords: Phytochemicals, Antioxidant activity, Antimicrobial activity, Parsley leaves

1. Introduction

Since immemorial times, nature has been explored for several sources of medical agents for the development of drugs.[1] Herbal medicine plays a major role in the synthesis of alternative drugs as well as to treat various diseases. Similarly, drugs originated from natural resources has resulted in the success of modern medical science due to the ability of natural medicines to boost health.[2] Phytochemicals are bioactive, non-nutritive components found in plants that has health curative and disease preventive properties (Figure 1).[3] Thereby, plants are considered to be rich sources of natural phytochemical classes for the discovery of pharmaceutical compounds and medicines. Moreover, these natural compounds are used for commercial purposes in medicine, cosmetics as well as in food products.[4]

In addition, edible plants that are rich in secondary metabolites have been paid special attention recently due to the presence of phytochemicals in diet.[5]

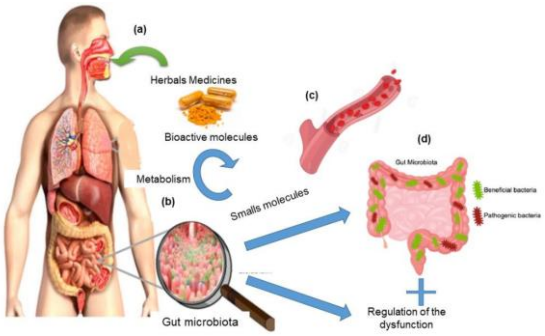


Figure 1. Oral administration of bioactive molecules.[6]

Phytochemicals are phytonutrients with biological activity that are naturally produced

by plants. Some of the natural phytochemicals include alkaloids, phenols, flavonoids, saponins and tannins.[7] The availability of these compounds, especially phenolic acids and flavonoids in plants indicate the presence of antioxidant and pharmacological properties which are recognized to exhibit many health benefits such as antitumor, anti-inflammatory, antihepatotoxic, antioxidant, and antimicrobial activities.[8,9] The properties of these natural compounds also play an important role in plants as a defense mechanism against the UV radiation, temperature, mechanical damage, as well as a chemical defense against insects.[10] Furthermore, phytochemicals show a major impact in exhibiting microbial activity by interfering with the transport of nutrients that are important to their function. In which the interference as a result causes the inhibition of microbial growth.[10]

Globally, resistance to antibiotics has become a huge concern, and in recent years the occurrence of multiple resistances in humans has been growing significantly due to indiscriminate consumption of pharmaceutical antibiotics which are commonly administered for the management of infectious diseases. Thereby, this has drawn increased attention to scientists in search for new antimicrobial substances from different sources like the medicinal plants.[1] For instance, plants with alternative mechanisms of action that have been searched for bioactive compounds has also been triggered due to the increasing occurrence of antibiotic-resistant microorganisms and several chronic and degenerative pathologies of humans caused by Reactive Oxygen Species (ROS). These bioactive compounds help neutralize harmful microorganisms and natural antioxidants which are capable of defending the body from oxidative stress and free radical-induced damage. In addition to that, any disruption to the natural phytochemical compounds that contributes to various antimicrobial properties in plant extracts can result in causing disruption to the cell membrane, resulting in cell death.[5]

As a vital part of normal physiology, free radicals are naturally produced in the human body and food systems.[11] However, due to external factors such as UV radiation, ionizing radiation, or air pollution (Figure 2), it can result in the event of numerous degenerative and chronic diseases such as respiratory, cancer, neurodegenerative, and digestive diseases that is caused due to the overproduction of ROS leading to oxidative stress.[5] Moreover, the pathogenesis of many diseases including heart disease, cancer, atherosclerosis, Alzheimer's disease and in the aging process was found to be implicated by the free radical action thus can be blocked by the natural antioxidants found in plants (Figure 3).[11] Furthermore, due to health risks and toxicity caused by synthetic phenolic antioxidants, they have been replaced with natural antioxidants which can neutralize the free radicals.[12]

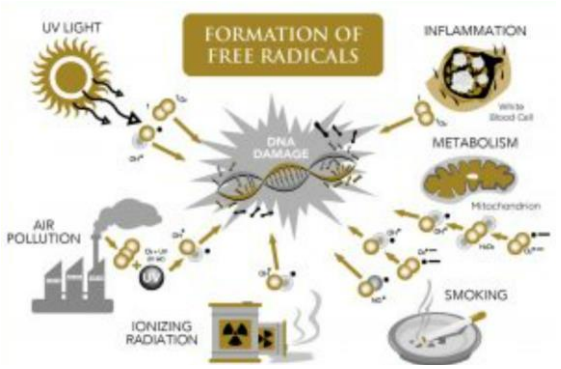


Figure 2. Formation of free radicals.[13]

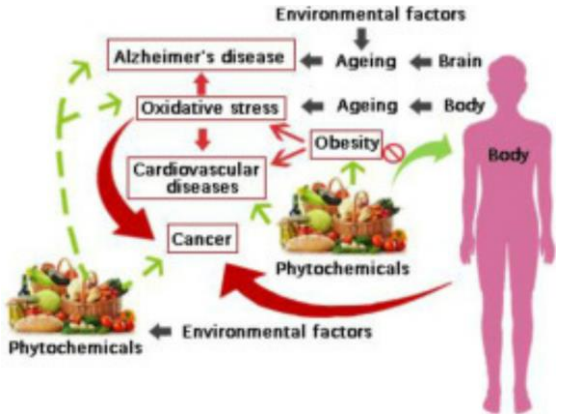


Figure 3. How phytochemicals help to mitigate oxidative stress and human diseases.[14]

The Parsley (Apiaceae) family is widely distributed throughout the world and is said to be one of the most significant groups of flowering plants, which consists of 3780 species in 434 genera.[15] The Parsley family consists of aromatic and flavorful flowering plants that has drawn attention to researchers as good sources of raw materials to industries such as the food, cosmetic, perfumery and most

importantly the pharmaceutical industry where it has been used as a treatment to cure different illnesses associated with digestive, endocrine, reproductive, and respiratory systems.[15-17]

Anethum graveolens (Dill), *Apium graveolens* (Celery), *Coriandrum sativum* (Coriander), *Foeniculum vulgare* (Fennel), and *Petroselinum crispum* (Parsley) are members of the parsley family that possess certain medicinal properties. These plants have hypoglycaemic and hypolipidemic activities as well as anticancer properties thereby are used for the prevention and treatment of many disorders via food supplements.[18] Moreover, it has also been documented that its value as a potential source of natural agrochemicals and biological activities such as anti-inflammatory antimicrobial, anti-tumour, diuretic, analgesic, radical scavenging, gastrointestinal and anti-obesity properties was revealed from previous studies on the Apiaceae family plant materials.[15] In addition, the most frequently researched plant parts were bark and seeds followed by leaves and aerial parts.[19]

The current study was conducted to evaluate the phytochemical profile and to determine the antioxidant and antimicrobial activities of leaf extracts from the Parsley family plants made using the maceration extraction technique.

2. Methodology

2.1 Plant material collection. The selected five plant species of the Apiaceae family were purchased from a local market from Western province, Sri Lanka in the month of March in 2023. The leaves of each fresh plant samples were cleaned, segregated and, shade dried for 18 days. Then they were finely powdered (Table 1).[21]

2.2 Preparation of extracts. The extraction was conducted by the maceration technique using water as the solvent, by mixing 2g of each finely powdered sample with 100mL distilled water.[22] The samples were left to run in the roller mixer for 48 hours, then filtered using the Whatman No1 filter paper, and the filtrates were collected and stored at 4°C for further analysis.[23]

Table 1. Sample collection and preparation

Sample	Shade Dried (18 days)	Powdered sample
Dill		
Celery		
Coriander		
Fennel		
Parsley		

2.3 Analysis of phytochemical content

2.3.1 Qualitative assays

Qualitative tests were executed to detect the presence of phytochemicals based on colour or precipitation reactions. Froth test, Alkaline reagent test, Salkowski's test, Ferric Chloride test, and Millon's test were conducted to assess the phytochemicals: saponins, flavonoids, terpenoids, phenols and proteins respectively.

2.3.1.1 Froth test. 1mL extract was mixed with 1mL distilled water and shaken for few minutes.[24]

2.3.1.2 Alkaline reagent test. 1mL of 2N NaOH was added to 1mL extract.[25]

2.3.1.3 Salkowski's test. 2mL chloroform was added to 1mL extract, followed by 3mL concentrated H_2SO_4.[24]

*2.3.1.4 Ferric Chloride test.*1mL extract was mixed with few drops of 5% $FeCl_3$.[26]

2.3.1.5 Millon's test. 1mL extract was mixed with few drops of Millon's reagent and heated.[27]

2.3.2 Quantitative assays

2.3.2.1 Determination of TPC (Total Phenolic Content). The TPC of samples were analysed using the Folin-Ciocalteu method carried out by

El-Sayed *et al* (2018)[28] with slight modifications. 200µL of sample was mixed with 1000µL Folin-Ciocalteu (diluted 10 times). After 5 minutes, 7.5% Na_2CO_3 (800µL) was added followed by a 1-hour incubation at room temperature. Gallic acid was used as the standard with dilutions (20-140µg/mL) prepared from 1mg/mL stock solution.

Absorbance was measured at 765nm for each sample and gallic acid concentrations in triplicates. The calibration curve was plotted using mean absorbance of gallic acid concentrations, and TPC of samples were expressed as Gallic Acid Equivalent (mg GAE/g).[15]

2.3.2.2 Determination of TFC (Total Flavonoid Content). TFC of extracts were determined using the Aluminum Chloride colorimetric method.[29] 500µL of extract was mixed with 5% Sodium Nitrite (150µL). After five minutes, 10% aluminium chloride (300µL), 1M sodium hydroxide (1000µL), and 2000µl of distilled water were added and shaken vigorously. The solution was incubated for 15 minutes at room temperature and the absorbance were measured at 510nm in triplicates. Lastly, the TFC of each sample was calculated and expressed as Quercetin Equivalents (mg QE/g) using the Quercetin standard.[30]

2.3.2.3 TAC (Total Antioxidant Capacity) Assay. Based on the Phosphomolybdenum method, the TAC for each sample was evaluated.[31] L-ascorbic acid was used as the standard (20-140µg/mL). 200µL of the sample was mixed with 4000µL Phosphomolybdenum reagent (0.6M sulfuric acid, 28mM Sodium Phosphate, and 4mM ammonium molybdate) followed by 90 minutes of incubation in a water bath at 95 °C.

Samples were cooled and the absorbance of each sample in triplicates were measured at 695nm.[32] The calibration curve was used to express TAC of each sample as Ascorbic Acid Equivalents (mg AAE/g).

2.3.2.4 DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) Free Radical Scavenging Activity. DPPH radical scavenging assay is a rapid, reliable assay that was used to assess the antiradical activities in the plant samples.[33] Firstly, a range of concentrations (0-10µg/mL) of each extract was prepared and to each, 500µL of sample extract, 0.1mM DPPH solution (2500µL) was added and stored in the dark for 30 minutes at room temperature. Absorbance was measured at 517nm for different concentrations of each sample triplicates.

Radical scavenging activity (%) = $[(A_C–A_S)/A_C)] \times 100$

Where, A_C= absorbance of control and A_S= absorbance of sample. From the above-mentioned formula, the radical scavenging activity (%) was calculated. Sample concentration required to scavenge 50% of DPPH free radical (IC_{50}) was calculated from the dose response graph of radical scavenging activity against the concentration of extracts.[34]

2.4 Evaluation of Antimicrobial activity. The antimicrobial activity of extracts was evaluated using the well diffusion technique. Each petri plate was sectioned to five equal parts labelled: positive control gentamycin (+), negative control autoclaved distilled water (-), and A, B, C as triplicates for each sample (Figure 4).

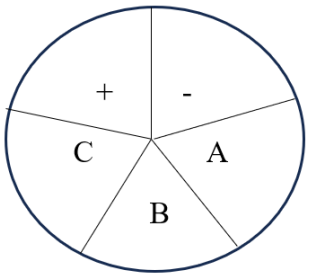


Figure 4. Petri plate sectioned to 5 parts

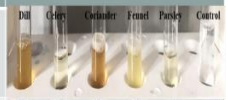
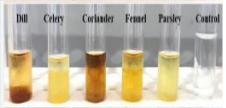
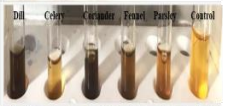
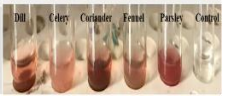
0.5% McFarland turbidity standard was prepared and visually compared using the test suspension turbidity to standardize the approximate number of bacteria in the suspension. The spread plate technique of test organisms was performed on the Muller Hinton agar plates prepared under aseptic conditions. [35] 50µL of an extract was dispensed into the wells (1cm in diameter) labelled A, B, and C. 50µL of distilled water was used as the negative control (-) and gentamycin disc (10mcg) as positive control (+). The plates were left for 15 minutes for pre-diffusion followed by incubation at 37°C for 24 hours. Lastly, the diameter of zones was measured.[36] Above procedure was repeated for all extracts.

2.5 Statistical Analysis. Statistical analysis was performed using the SPSS software. All the analysis were performed in triplicates with results expressed as mean ± SD. Data was analyzed using t-test and Analysis of Variance (ANOVA) at confidence level $p < 0.05$. Pearson's test was used to find the correlation between TPC, TAC, TFC, and DPPH from the studied plant extracts.

3. Results

Results obtained for the qualitative analysis of the phytochemicals: Saponins, Flavonoids, Terpenoids, Phenols and Proteins are shown in Table 2 below.

Table 2. Results of qualitative analysis of phytochemicals

Sample → / Test ↓	Dill	Celery	Coriander	Fennel	Parsley	Results
Froth test (Saponins)	++	+	++++	+++	+	
Alkaline reagent test (Flavonoids)	++++	++	+	+++	+	
Salkowski's test (Terpenoids)	+++	++	++++	+	++	
Ferric Chloride test (Phenols)	+++	+	++++	++	++	
Millon's test (Proteins)	++	+	++++	+	+++	

[+ < ++ < +++ < ++++]

least positive most positive

As shown in Table 2, all samples revealed positive results for each qualitative tests indicating the presence of Saponins, Flavonoids, Terpenoids, Phenols and Proteins. However, both Dill and Coriander showed the most positive results for the qualitative phytochemical tests, whereas Celery showed the least.

3.1 TPC of samples

The TPC of each sample was quantitatively evaluated and expressed as GAE that was executed using the Folin-Ciocalteu method.

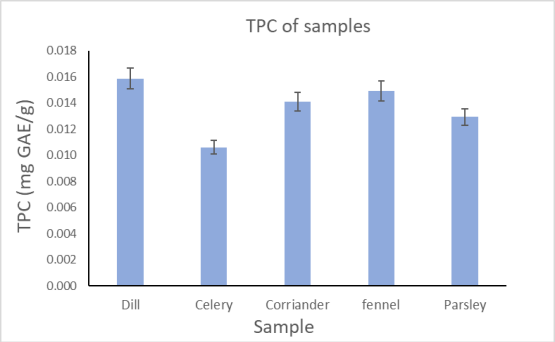


Figure 5. TPC of samples

The results obtained from the quantitative analysis of TPC for each plant extract (Figure 5), showed that Dill (0.016 mg GAE/g) expressed the highest TPC while Celery (0.011 mg GAE/g) showed the least. TPC results expressed a significant difference ($p < 0.05$) between all samples.3.2 TFC of samples

The TFC of each sample was quantitatively evaluated and expressed as QE using the Aluminum Chloride colorimetric method.

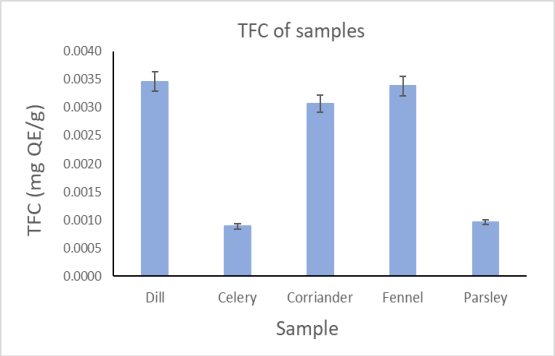


Figure 6. TFC of samples

The results obtained from the quantitative analysis of TFC (Figure 6) for each plant extract, showed that Dill (0.0034 mg QE/g) expressed the highest TFC while Celery (0.0009 mg QE/g) showed the least. TFC results expressed a significant difference ($p < 0.05$) between all samples.

3.3 TAC of samples

The TAC for each sample was quantitatively evaluated and expressed as AAE that was executed using the Phosphomolybdenum method.

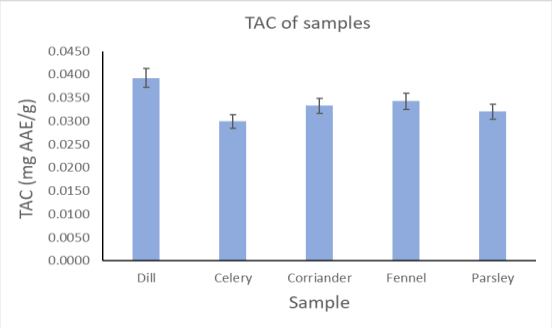


Figure 7. TAC of samples

The results obtained from the quantitative analysis of TAC (Figure 7) for each plant extract, showed that Dill (0.039 mg AAE/g) expressed the highest TAC while Celery (0.030 mg AAE/g) showed the least. TAC results expressed a significant difference ($p < 0.05$) between all samples.

3.4 DPPH of samples

The free radical scavenging activity of each sample was expressed as IC_{50}, that was executed using the DPPH assay.

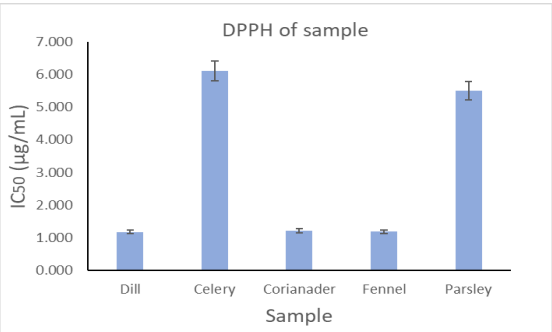
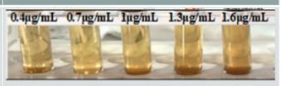
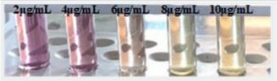
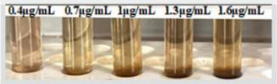
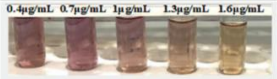
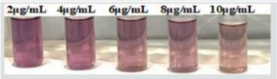


Figure 8. DPPH free radical scavenging activity

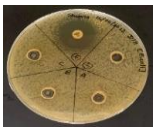
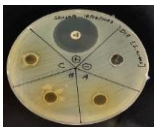
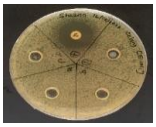
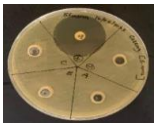
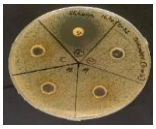
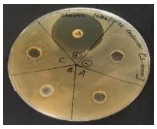
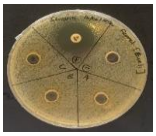
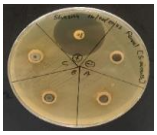
The results obtained from the analysis of DPPH for each plant extract (Figure 8), showed that Dill (IC_{50}= 1.18 µg/mL) expressed the highest free radical scavenging activity while Celery (IC_{50}= 6.10 µg/mL) showed the least. DPPH results expressed a significant difference ($p < 0.05$) between all samples. The colour changes of DPPH free radical scavenging activity for each plant extract of the Apiaceae family is as shown in Table 3.

Table 3. Free radical scavenging activity for each plant extracts

Sample	Result
Dill	
Celery	
Coriander	
Fennel	
Parsley	

3.5 Antimicrobial activity of samples

Table 4. Results of Antimicrobial activity using well diffusion.

Sample	Test-microorganism	
	Escherichia coli	*Staphylococcus aureus*
Dill		
Celery		
Coriander		
Fennel		
Parsley		

The results for the antimicrobial activity of the plant samples were assessed from the well diffusion method using *Escherichia coli* and *Staphylococcus aureus* as shown in Table 4. As the positive control, gentamycin showed a zone

of inhibition, however no zones of inhibition were observed in any of the samples that were tested with both the test microorganisms at a concentration of 0.2mg/mL.

3.6. Data Analysis

The Pearson correlation coefficient results obtained between the assays, TPC, TAC, TFC, and DPPH are shown below (Figure 9).

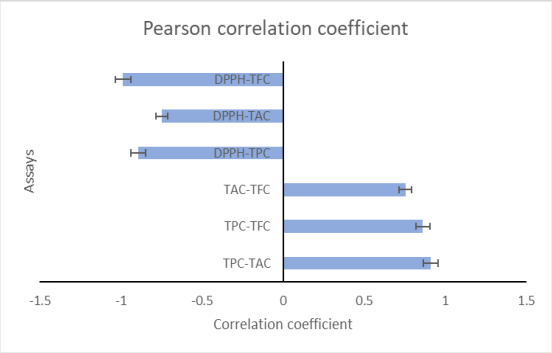


Figure 9. Correlation coefficient between assays

The correlation coefficient between TPC and TAC was positive and highly significant ($p < 0.05$), while TAC and TFC was insignificant ($p > 0.05$). Similarly, DPPH and TFC presented a strong, negative correlation that was highly significant ($p < 0.05$) compared to DPPH and TAC ($p > 0.05$).

4. Discussion

In this study, leaves from the Parsley (Apiaceae) family were chosen for the evaluation of phytochemical screening and the determination of antioxidant and antimicrobial activity. These culinary herbs are readily available as consumables and have been widely used as a garnish due to its great aroma and flavour. In addition, the plants of the Apiaceae family are believed to have health beneficial properties that can help heal and treat various diseases.[8] Once the samples were air dried in room temperature and powdered using the mortar and pestle, they were extracted using the maceration extraction technique. Water was used as the solvent due to its beneficial properties such as: highly polar, soluble, inexpensive, nontoxic, and non-flammable.[22] This investigation was also executed using plant leaves as they were found to have a greater abundance of phytochemicals than other parts of the plant.[37]

Despite the use of various non-conventional methods recently, which are much costly, conventional methods such as the maceration extraction was used due to its inexpensiveness, simple procedure, and lower solvent consumption.[38] Furthermore, during the maceration extraction, the sample solution was ensured to run in the roller mixer for 48 hours until a homogenized sample solution was obtained.

Phytochemicals are natural bioactive components produced by plants via primary or secondary metabolites that is used as a remedy for various diseases. A wide range of medicinal properties such as providing protection against various diseases was found to be possessed in different phytochemicals.[39] Some important primary bioactive components include phenolic compounds, flavonoids, terpenoids, tannins, steroids and carbohydrates.[40] The aqueous plant extracts of the Parsley family were analyzed qualitatively for the presence of phytochemicals using phytochemical tests such as: Froth test, Alkaline reagent test, Salkowski's test, Ferric Chloride test, and Millon's test. In this investigation the phytochemicals: saponins, flavonoids, terpenoids, phenols and proteins showed presence in all sample extracts. Moreover, these phytochemicals found to have beneficial properties such as antioxidant, antiviral, anti-inflammatory, and anticancer effects.[41] Dill and Coriander showed the most positive results while celery showed the least for the qualitative tests. However, celery also showed presence of more flavonoids and terpenoids than other phytochemicals. Similarly, the presence of flavonoids and terpenoids in celery was found to exhibit pharmacological effects including anti-microbial, anti-oxidant, and cardiovascular protective effects.[42]

As the most abundant secondary metabolites, phenolic compounds are ubiquitous constituents in plants that play a crucial factor in promoting growth and protection against the harmful effects of pathogens, parasites, and UV rays.[15] In addition, Folin-Ciocalteu assay being a fast and rapid assay was used for the assessment of phenolic content. In the Folin-Ciocalteu assay, after the samples were left for incubation, a

colour change from yellow to blue was observed. This is due to the presence of phenolic compounds in the aqueous plant extract that caused the Folin-Ciocalteu reagent to be reduced and resulting in the formation of a blue coloured complex.[43] Thus, darker the intensity of the blue coloured complex, greater the phenolic compound present in the sample. Therefore, based on the colour intensity observed and the calculated results, Dill was found to have the highest TPC (0.016 mg GAE/g) indicating high amounts of phenolic compounds while celery showed the least (0.011 mg GAE/g). The TPC results in the current study were shown to be varied when compared with the results obtained from Derouich et al (2020)[44], who measured the highest TPC in parsley (21.63 ± 1.81 mg GAE/g DW) and the least TPC in Coriander (13.72 ± 1.13 mg GAE/g DW). Thus, the occurrence of these variations in total polyphenols level may be due to certain genetic differences, conditions of cultivation, extraction time or type of solvent used for extracting solvent. Moreover, TPC of Dill was highly significant ($p < 0.05$) to Parsley, however showed insignificance ($p > 0.05$) to Celery, Coriander and Fennel.

Flavonoids are natural secondary metabolites that are most abundant in plants, fruits, vegetables, seeds as well as in wine and tea.[45] The TFC assay was carried out using Aluminium Chloride colorimetric method which resulted in the formation of a yellow-coloured complex.[29] In the presence of Aluminium Chloride stable complexes with keto group and hydroxyl group of flavones were formed.[46] Thus, based on the colour intensity observed and the calculated results obtained from TFC, Dill displayed the highest concentration (0.0034 mg QE/g) at 510 nm while Celery (0.0009 mg QE/g) showed the least.

The TAC assay was conducted using the phosphomolybdenum method, which resulted in the formation of a yellow-green complex due the reduction of molybdate ions.[31] Therefore, based on the results, Dill was found to have the highest TAC (0.039 mg AAE/g) indicating high antioxidant capacity while Celery showed the least (0.030 mg AAE/g). Dill showed a significance ($p< 0.05$) between Coriander and Parsley, however, was highly insignificant between the other plant extracts.

The DPPH assay was carried out in order to determine the scavenging activity of each plant extract. From a range of concentrations of each plant extract, the dose response curves were plotted and IC_{50} values for each sample extract was calculated. From the results obtained, Dill (1.18 µg/mL) showed the least IC_{50} value while Celery (6.10 µg/mL) showed the highest. This indicates that, lower the IC_{50} value the higher free radical scavenging activity and vice versa. [34]

Results from the Pearson correlation coefficient showed strong positive correlations between TPC and TAC, TPC and TFC, as well as TAC and TFC. While strong negative correlations were observed between DPPH and TPC, DPPH and TAC, as well as DPPH and TFC. This is because plant extracts with increasing TPC, TAC, or TFC show reduced DPPH and vice versa. In addition, a previous study on medicinal herbs such as *Adhatoda vasica* Nees, *Bergenia ciliata* (Haw) Sternb, *Phyllanthus emblica* Linnaeus, *Terminalia bellirica* (Gaerth) Roxb, *Terminalia chebula* Retzius and *Vitex negundo* Linnaeus demonstrated a positive correlation between radical scavenging activity and total phenolic content, in which the extract having the highest phenolic content showed the lowest $IC_{50.}$ [34]

In recent years, bacterial resistance to antibiotics and antimicrobials has increased due to changes in major mechanisms that allow bacteria to resist them. Some of the changes include: changes to active drug efflux systems, mutations that alter cell permeability, cellular degradation of antimicrobials, and changes in cellular targets. Thereby, this has resulted in the rise of bacterial resistance to antibiotics and antimicrobials in recent years.[47]

In this current study all five of the plant extracts were tested for antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* using the well diffusion technique. From the results obtained from each of the samples, the positive control (Gentamicin) was found to be more sensitive against *Staphylococcus aureus* than compared to *Escherichia coli*. Thus, large, and clear zones of inhibition were observed for the positive control of each plant extracts against *Staphylococcus aureus* (3.4 cm

in diameter) while clear and smaller zones of inhibition was observed for *Escherichia coli* (2.2 cm in diameter). In fact, *Escherichia coli* being a gram-negative anaerobic bacterium, there has been a rise in the rates of resistance among *Escherichia coli* around the world.[48] Thereby. this has confirmed that the gram-positive bacteria (*Staphylococcus aureus*) were more sensitive to gentamicin than the gram-negative bacteria (*Escherichia coli*). The reason for this susceptibility difference between the two test microorganisms is because of the Gram-negative cell wall acting as a barrier to many compounds, such as antibiotics, due to changes in their outer membrane structure.[49]

Furthermore, it is ensured that the antimicrobial susceptibility testing is carried out under aseptic conditions to avoid any contamination that would affect the results. In addition, a previous study on the seeds of 6 plants of the Apiacea family: Caraway (*Carum carvi* L.), anise (*Pimpinella anisum* L.), coriander (*Coriandrum sativum* L.), dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.) and cumin (*Cuminum cyminum* L.) showed a strong antimicrobial activity against a wide range of pathogens.[18] However, no zones of inhibition were observed in any of the plant extracts against both the test organisms in this present study. Moreover, it has been found that in various literature studies the antimicrobial activities have a direct relationship to the concentration of extract used.[50] Thus, by increasing the concentration of the plant extract placed in the wells, a positive result may be observed. Similarly, the use of ethanolic plant extracts showed a greater impact than the use of aqueous extracts.[49] Both *Escherichia coli* and *Staphylococcus aureus* are also considered normal intestinal flora that are harmless commensal, that plays a major role in preventing and fighting infections, therefore the intake of certain plant-based food such as leaves from the Apiaceae family that has no antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* can play a beneficial role in the prevention of certain infections.[51]

5. Conclusion

In the present study, Dill (*Anethum graveolens*) showed the highest total phenolic content, total flavonoid content, total antioxidant potential as well as the highest free radical scavenging activity along with moderately high amounts of other phytochemicals from the qualitative tests. Thus, demonstrated potent anti-inflammatory properties along with other beneficial implications in human health such as the treatment and prevention of cancer, cardiovascular disease and other pathologies via inhibition of the oxidation of lipids and the propagation of oxidative chain reactions.

Further analysis can be conducted on leaf extracts of Apiaceae family of their pharmacognostic properties for the development of natural drugs.

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A Study of the Factors that Influence the Usage of Credit Cards in Sri Lankan Banks

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Abstract

A revolutionary change has taken place in the global financial service landscape due to the digitalisation of payment systems. This shift has been accelerated by the outbreak of the COVID-19 pandemic, leading to the widespread adoption of cashless transactions, as the safest form of transaction method. Out of the cashless transactions in the Banking Industry, the credit card market is a particularly active segment with a high degree of competitiveness. Therefore, understanding the precise variables influencing credit card usage behaviour would provide the issuer with a strategic advantage. Hence, this study used the UTAUT2 model to examine factors influencing Sri Lankan customers' use of credit cards. This study used a quantitative method to conduct primary research, gathering data from 200 credit card users in Sri Lanka using a convenience sampling technique. Data was analysed using descriptive statistics and inferential statistical analysis. The results of the study show that social influence, habitat have the most impact on the intention to use credit cards. Performance expectations, effort expectations, and perceived financial cost also have a significant impact on intention to use credit cards. Moreover, due to the pandemic and the rapid development of the required infrastructure for cashless transactions in Sri Lanka, non-cash transactions are expected to take the lead, including the use of credit cards.

Keywords: Credit Cards, Banking Industry, Usage Behaviour

1. Introduction

1.1 Background of the study

The Central Bank of Sri Lanka (CBSL) holds the exclusive authority to issue currency, with a total value of 901bn LKR in circulation as of Q1 2021 (Payment Bulletin, 2021). Cash remains the predominant form of currency in Sri Lanka (Piyananda, Dhanushka, Aluthge & Chandana, 2020). The physical currency has been a significant drag on the economy, with an estimated 1.5% of the GDP allocated for maintaining physical currency circulation, and this has prompted CBSL to initiate a number of efforts over the years to move towards a cashless system (Lugoda, 2020).

In Sri Lanka, both high-value and retail payment systems offer non-cash payment options, encompassing various methods such as Cheques, SLIP System, Credit and Debit cards, Mobile and Internet Payments, Tele Banking, Postal Instruments, and LANKAQR (Payment Bulletin, 2021). While cheques and SLIP payments dominate the retail payment system, constituting 50% and 15% of the transaction value as a percentage of GDP (Payment Bulletin, 2021), the credit card market is gaining popularity due to its ability to provide benefits not offered by cash or cheques. Furthermore, with technological advancements and its transformative characteristics, the credit card market has evolved into a symbol of lifestyle (Dianto et al., 2020).

As of Q1 2021, there were 1,996,279 cards in use, with a total transaction value of 221.8bn LKR, averaging 10,121 LKR per capita transaction value. Fourteen authorised Commercial Banks and three licensed finance companies were offering credit card services at the completion of Q1 of 2021 (Payment Bulletin, 2021). Table 1 and Table 2 show the usage of currency and credit

cards over time, reflecting widespread adoption in Sri Lanka. A World Bank survey in 2018 revealed that more than 75% of credit card users in Sri Lanka prefer cards over cash (Daily FT, 2019). In Q1 2021, credit card transactions increased in value by 27% compared to Q1 of 2020 (Payment Bulletin, 2021).

Table 1. Currency Circulation from 2018-2020

Indicator	2018	2019	2020
Currency in circulation as a % of GDP	4.5%	4.5%	5.6%
Per capita currency in circulation value (LKR)	29,577	31,095	38,086
Currency held by the public as a %of GDP	3.3%	3.3%	4.3%

Source: Payments Bulletin (2021)

Table 2. Credit Card use from 2018-2020

Indicator	2018	2019	2020
Per capita CC transaction value (LKR)	11,158	12,714	10,121
No of credit cards in use	1,710,671	1,854,103	1,984,525
Value of transactions (LKR. Bn)	241.8	277.2	221.8

Source: Payments Bulletin (2021)

Based on Table 2, the usage of credit cards indicates a positive growth.

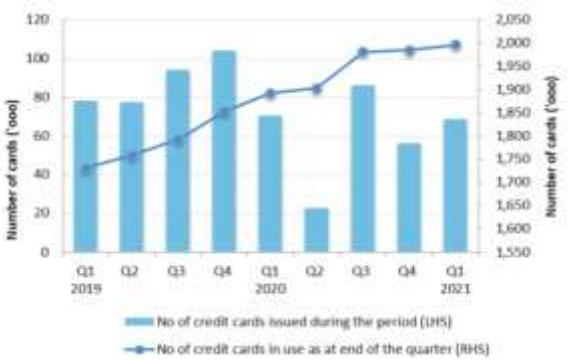

Figure 1. Number of credit cards in use and cards issued.

Source: Payments Bulletin (2021)

As shown in Figure 1, there is an increase in card ownership. Furthermore, recent findings indicate that more than three-fourths of credit card users prefer to use cashless payment methods (Abdeljawad, Hashem & Rashid,2022). However, despite Sri Lanka's substantial progress in digital financial infrastructure, overall adoption remains low. Issuers are vying for the same tiny group of customers, providing the same person a fourth or fifth credit card (Daily FT, 2019) and just about 2m people use them which is only 10% of the population. This is in sharp contrast to developed countries, where credit cards account for virtually all consumer expenditure (Ketepearchchi, 2021). Additionally, credit card usage for retail transactions remains limited, with fewer than 10% of Sri Lankans utilising them (Jayasinghe, 2020).

1.2 Rationale

The COVID-19 pandemic accelerated the adoption of digital payment methods in Sri Lanka, encouraged by financial institutions. This led to increased awareness and usage among consumers who had not previously used digital financing. According to CBSL, QR payments, particularly, saw a remarkable 400% growth since January 2020, starting from nearly zero usage. However, during the pandemic, the use of credit cards experienced a decline despite the online and digital payment methods being widely used. This was primarily due to reduced household expenditure, resulting in decreased value and volume of credit card spending in the

second quarter of 2020 (Jayasooriya, 2021). The decrease in total volume and value of credit card transactions is shown in Figure 2.

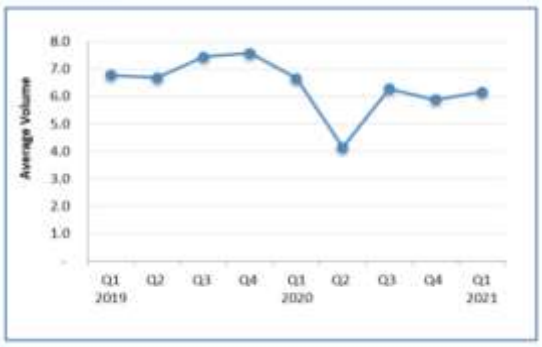

Figure 2. Average Volume of transactions per credit card

Source: Payments Bulletin (2021)

Credit card holders benefit from cashless transactions and settling debts immediately, leading to a shift in societal attitudes towards debt where debt is no longer considered taboo. Using credit cards as a payment method offers practicality and security benefits, particularly when cash is scarce. However, improper credit card use can drive excessive consumption and lead to users becoming trapped in risky debt habits, particularly when their income is unstable. Therefore, in addition to economic factors, various studies have indicated the role of behavioural issues in driving excessive credit card usage (Trinh, Tran, & Vuong, 2021; Anastasia & Santoso, 2020; Wickramasinghe & Gurugamage, 2009).

Hence, this study aims to understand the factors influencing credit card usage. Knowing the precise factors that influence credit card usage behaviour, would offer a significant advantage in understanding consumers' purchase intentions towards credit card usage. Furthermore, as customers tend to switch card issuers due to intense rivalry, recognising consumer purchase intention is critical for increasing market share and card utilisation. Hence, this study will focus on discovering the factors influencing credit card usage using the UTAUT2 model.

1.3 Research Aim

The aim of this study is to examine the factors that impact consumer usage of Credit Cards in Sri Lanka.

1.4 Scope

This study surveyed 200 credit card holders who own a credit card issued by a licensed Commercial Bank in Sri Lanka, and the study considered only the present cardholders and individuals who have done a transaction within the past 12 months. The study was conducted to understand consumer perceptions of credit card usage.

2. Research Design

2.1 Research Method

The quantitative research method was used for this study. An online structured questionnaire was distributed to the participants. The questionnaire was discributed to 200 credit card holders using the convenience sampling method.

2.2 Conceptual Framework

The UTAUT2 model, shown in Figure 3, was used to provide the theoretical foundation for presenting the conceptual model.

Performance expectation (PE), effort expectancy (EE), social influence (SI), habitat (HT), and perceived financial cost (PFC) were presented as the main drivers of consumers' intention, as illustrated in Figure 3.

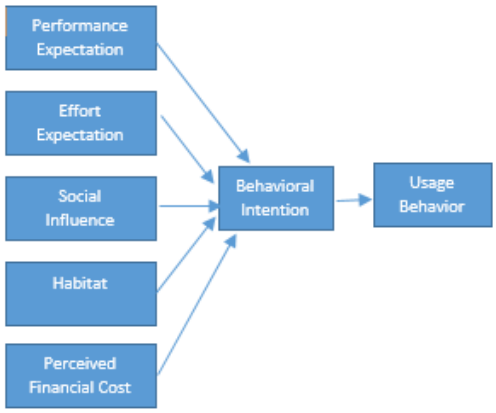

Figure 3. Conceptual Framework

2.3 Hypotheses

The following hypotheses were formulated based on the conceptual framework.

H1: Performance Expectation has an effect on behavioural intention to use credit cards.

H2: Effort Expectation has an effect on behavioural intention to use credit card services.

H3: Social Influence has an effect on behavioural intention to use credit cards.

H4: Perceived Financial Cost has an effect on behavioural intention to use credit cards.

H5: Habitat has an effect on customers' behavioural intention to use credit cards.

H6: Behavioral intention has an effect on the usage behaviour of credit cards.

3. Analysis and Findings

3.1 Demographic Data

Table 3. The demographic data of the respondents

	Description	Percentage (%)
Gender	Male	58
	Female	42
Age	20-30 years	27
	30-40 years	36
	40-50 years	23
	50 years +	14
Experience	Less than 1 year	5
	1-2 years	20
	3-5 years	17
	6-10 years	58
Number of Cards	1	62
	2	23
	3	8
	4	3
	5	3
	More than 5	1
Length of Ownership of Cards	1 -2 years	14
	2-5 Years	30
	5-10 Years	22
	> 10 Years	34
Highest Education Level	O/L	0
	A/L	17
	Graduate	36
	Postgraduate	39
	Other	8
Monthly Income (Rs.)	Less than 45,000	9
	45,000 – 70,000	23
	75,000 – 150,000	38
	150,000 – 250,000	16
	Above 250,000	14

As shown in Table 3, 62% of those surveyed have one card, 23% have two cards, and 15% have three or more cards. Furthermore, 34% of the respondents has used credit cards for over ten years. The majority of the respondetns have an monthly income of between 75,000 LKR-150,000 LKR.

3.2 Reliability Analysis

Table 4. Reliability Statistics

Variables	Cronbach's Alpha
Effort _Expectancy	0.6
Performance_Expectancy	0.6
Usage_Behaviour	0.7
Behavioural_Intention	0.6
Perceived_Financial_Cost	0.6
Habitat	0.5
Social_Influence	0.6

As shown in Table 4, the reliability of the variables was computed using Cronbach's Alpha. Cronbach alpha values of 0.6 and above are considered to be reliable.

3.3 Correlation Analysis

Table 5. Pearson Correlation Analysis

Variable	Correlation Coefficient with Behavioural Intention
Performance Expectancy	.589**
Effort Expectancy	.487**
Social Influence	.304**
Habitat	.651**

Perceived Financial Cost	.244**
Variable	**Correlation Coefficient with Usage Behaviour**
Behaviorial Intention	0.072**

** Correlation is significant at the 0.01 level.

As shown in Table 5, the Correlation Analysis results reveal that Performance Expectancy, Effort Expectancy, Social Influence, Habitat and Percieved Financial Cost have a positive correlation with Behaviorial Intention (to use Credit Cards) at 1% significance level. Behaviorial intention has a positive correlation with Usage Behavior (at 1% significance level).

3.4 Hypothesis Validation

Based on the correlation analysis results, all the hypotheses formulated in this study are valid, as shown in Table 8.

Table 6. Hypothesis Test

Hypotheses	Result
H1: PE has an effect on behavioural intention to use credit cards	Valid
H2: EE has an effect on behavioural intention to use credit cards	Valid
H3: SI has an effect on behavioural intention to use credit cards	Valid
H4: PFC has an effect on behavioural intention to use credit cards	Valid
H5: HT has an effect on behavioural intention to use credit cards	Valid
H6: Behavioral intention has an effect on the usage behaviour of credit cards.	Valid

Perofrmance Expectancy has a significant relationship with behavioural intention, which supports the findings of Makanyeza and Mutambayashata (2018). Moreover, the findings reveal a significant impact of Effort Expectancy on Behavioural Intention, supporting the findings of Trinh and Vuong, (2019); Alwahaishi and Snášel (2013).

It was found that Social Influence has a significant impact on Behaviorial Intention, as supported by Venkatesh et al., (2003); Hancock, Jorgensen, & Swanson (2012) and Anastasia, Santoso (2020).

Further, Perieved Finacial Control has a significant effect on Behavioural Intention, which supports the findings of Lydia and Gan (2005).

Moreover, there is a significant relationship between Habitat and Behavioural Intention, supporting the findings of Makanyeza and Mutambayashata (2018) and Harsono and Suryana (2014).

3.5 Multiple Linear Regression Analysis

According to Table 7, Social Influence is the most significant variable that impacts Behavioural Intention (at 5% significance level).

Table 7. Regression Analysis

Model	Unstandardised Coefficients B	Sig.
Social_Influence	.137	**.002**
Habitat	-.090	.058
Perceived Financial Cost	-.049	.387
Performance_Expectancy	.027	.659
Effort_Expectancy	-.040	.568

Dependent Variable: Behaviorial Intention

The model has an R square value of 0.108.

4. Results and Discussion

In conclusion, this study found that Performance Expectancy has a significant impact on behavioural intention and intention mediates between Performance Expectancy and Usage Behaviour. Past research found that Performance Expectancy has an influence on the usage of credit cards due to its ability to be used for various purposes (Alwahaishi & Snáel, 2013; Makanyeza & Mutambayashata, 2018; Venkatesh et al., 2003). Alwahaishi and Snáel, (2013); Trinh and Vuong (2019) found that convenience of use has an impact on the usage of credit cards. Customer satisfaction is influenced by quality and expectation, which leads to greater retention (Foscht et al., 2010). Thus, credit card

businesses must meet or exceed expectations in order to acquire a competitive advantage.

This study found that Effort Expectancy has a significant impact on behavioural intention and Intention mediates between Performance Expectancy and Usage Behaviour. Credit cards are used as a convenient tool to pay without carrying cash. Hence, convenience of use has an impact on the intention to use credit cards.

Further, research findings show that Social Influence is the strongest motivator, having a significant positive impact on credit card usage, supporting the findings of Hancock et al. (2012). Consumers view credit cards as a tool that may lead to a better financial situation leading to a better lifestyle, as evidenced by findings from Khare (2012).

Furthermore, this study discovered that Perceived Financial Cost has a significant impact on Behvaiorial Intention, and Behviorial Intention mediates between Perceived Financial Cost and Usage Behviour, supporting the results of Khalid et al. (2013). Mallat (2006) found that Perceived Financial Cost had a deleterious impact on behavioural intention. Thus, the cost involved in using a credit card plays a significant role in behaviorial intention.

In addition, it was found that there is a significant impact on Behvaiorial Intention by Habitat, as identified by Makanyeza and Mutambayashata (2018) and Harsono and Suryana (2014), who found that Habitat positively impacts behavioural intention. As argued by Wickramasinghe and Gurugamage (2009), if customers form a practice of using credit cards, it can result in greater purchasing.

Most Sri Lankan consumers do not settle their credit card debt on time (Wickramasinghe and Gurugamage, 2009). The overall demand for loans and non-performing loans increased in Q1 2021 (Credit Supply Survey, 2021). Hence, it can be argued that in a country where debt has become more socially acceptable, customers perceive credit cards as a convenient method to spend now and pay later.

Dewri et al. (2016) identified that the majority of credit card users are from the age group of 20- 40 years. This is in line with past research, which found that usage diminishes with age, since the more senior consumers are more comfortable with cash payment (Khare,2012).

Manning (2000) found that consumers with high earning capacity are more likely to use credit cards.

Furthermore, this study revealed that most of the Sri Lankan consumers are using only one credit card. This supports the findings of Gan et al. (2008), where the majority of respondents in developing countries used just one credit card. This is in contrast to a developed economy where the average number of cards exceeds four (Robb & Sharpe, 2009).

5. Conclusion

Although Sri Lanka has made significant progress in developing innovative digital financial infrastructure, adoption of credit cards remains low. Further, despite the gradual increase in card ownership, Sri Lanka remains essentially a cash economy, with only 38% of the population using even a debit card (Jayasinghe, 2020). It was also evident that although the number of cards has gradually risen, an outbreak of COVID-19 has caused a downward trend in Sri Lankan card spending due to the slowdown in household expenditure (Jayasooriya, 2021).

In order to popularise the use of credit cards, social media platforms can be used to promote their usage. Moreover, it is recommended that credit card issuers focus on promoting their credit cards using social media influencers. Credit card issuers can also introduce a referral system, with suitable rewards.

The Banks can also conduct more awareness programmes educating the citizens about the benefits of using credit cards.

Acknowledgements

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A study of the fast food delivery service quality during the fuel shortage in the Colombo district

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Abstract

This research paper studies the ramifications of the fuel shortage on the fast-food delivery service quality within the confines of the Colombo district. This research paper delves into the repercussions of the crisis, which has disrupted the operational capacities of fast-food delivery companies, leading to delayed deliveries and compromised service quality. This study explores how tangibility, responsiveness, reliability, assurance and empathy have impacted the service quality of fast-food delivery services amidst the fuel scarcity. The primary data collection involved a structured questionnaire administered to a sample of 130 participants who had utilized fast food companies in the Colombo district over the past year. Statistical analyses, including correlation, and multiple linear regression, were conducted using SPSS software to identify the significant factors influencing service quality. The research findings revealed that tangibility, responsiveness, reliability, assurance and empathy have a positive correlation with service quality, with tangibility and assurance having the most impact. The overall service quality was given a rating of "neutral", which means the respondents were not satisfied with the service quality. Recommendations are given on how to mitigate the impact of the fuel shortage on the service quality of fast-food delivery services.

Keywords: Fuel Shortage, Fast Food Delivery Services, Service Quality

1. Introduction

1.1 Background of the Study

Sri Lanka presently finds itself ensnared within one of the most severe economic crises in recent memory. The adverse effects of this crisis have been acutely felt. During the peak of the crisis, millions of Sri Lankan citizens were plunged into interminable queues for fuel, profoundly hampering their daily lives.

Amidst this turmoil, companies like Uber Eats and PickMe Food have grappled with a protracted period of stagnation over the past year, predominantly due to the omnipresent fuel shortage afflicting Sri Lanka. This crisis has critically impacted the quality of fast food delivery services (FFDS), particularly in the bustling Colombo district. The gravity of this situation cannot be overstated, as substantiated by Jayasinghe (2022), who underscored that the fuel crisis catalyzed an unprecedented economic contraction of 8.4% in the June quarter of 2022, a precipitous decline witnessed over a mere three-month period. Escalating fuel costs have wrought havoc on the agricultural sector, leading to a scarcity of essential fertilizers and culminating in a perilous confluence of fuel and food supply chain disruptions, culminating in soaring food prices (Matthias, Govindapala & Jaysinghe, 2023).

In the midst of this crisis, fast-food delivery service quality has been negatively impacted. The paucity of fuel has severely constrained the operational capacities of these delivery companies, rendering them unable to meet the demands of a populace in dire need. Restaurants and fast-food establishments, especially those situated in densely populated commercial hubs in the Colombo district, continue to grapple with the arduous task of timely and efficient food distribution (Jayasinghe, 2022).

Marian (2022) has stated that the increase in essential commodity prices is directly attributable to the soaring fuel costs. Sectors vital to the nation's economy such as agriculture, tea exports, tourism, and the food industry have all been grievously impacted. Fast food delivery services have been especially impacted by the fuel crisis. The impact has been more prevalent in urban centers, where 60%-75% of the populace relies on fast-food, as opposed to rural areas, where this figure stands at 25%-30% (Jayatissa, Wickramasinghe & Piyasena, 2014). In the ongoing crisis, the issue of food security has emerged as a major issue, posing significant hurdles to fast food delivery service companies (Wijesignhe & Yogarajah, 2022).

1.2 Research Aim

The aim of this study is to analyse the impact of the fuel shortage on the fast-food delivery service quality in the Colombo district.

2. Research Methodology

This study used the quantitative mono method. Correlation and multiple linear regression analyses were conducted using SPSS software to identify the significant service quality variables that impacted the fast food delivery outlets. A sample of 130 respondents were chosen based on the convenience sampling method.

2.1 Conceptual Framework

The conceptual framework of this study is given in Figure 1.

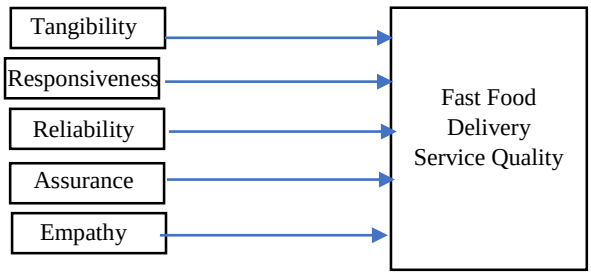

Figure 1. Conceptual Framework

The conceptual framework is based on the SERVQUAL model. The SERVQUAL model is used to determine whether the service quality of fast food outlets deteriorated during the Sri Lankan fuel crisis due to challenges in delivery and other service quality aspects.

The definitions of the variables are given below based on Parasuraman, Zeithaml and Berry (1988).

Tangibility - The willingness to help customers and to provide prompt service.

Assurance - the knowledge and courtesy of employees and their ability to convey trust and confidence.

Reliability - The ability to perform the promised service dependably and accurately

Empathy - The provision of caring, individualized attention to customer.

Responsiveness - The willingness to help customers and to provide prompt service

2.2 Research Hypotheses

The hypotheses formulated in this study are given below.

H1: There is a relatıonsnip between Tangibility and Service Quality

H2: There is a relationship between Responsiveness and Service Quality

H3: There is a relationship between Reliability and Service Quality

H4: There is a relationship between Assurance and Service Quality

H5: There is a relationship between Empathy and Service Quality

2.3 Operationalization

Table 1 gives the statements that were used to measure the service quality of the fast food outlets based on the SERVQUAL model.

Table 1. Operationalization Table

Variable	Statements
Tangibility	I am comfortable with the appearance and condition of the food packaging when it is delivered to me
	I think the delivery staff presented themselves well in terms of their attire and personal grooming
	I found it easy to track the delivery status of my order, such as the estimated delivery time and the delivery person's location
	I am comfortable with the completeness of my order and the transaction of the order
	I am satisfied with the packaging.
Responsiveness	I feel that the fast-food delivery service promptly addresses my concerns or complaints
	I am promptly informed of any delays or changes in the delivery time by the fast-food delivery service
	I receive adequate assistance and answers to my questions from the fast-food delivery service
	I am satisfied with how the fast-food delivery service fulfills my specific requests or requirements
	I feel that the fast-food delivery service is responsive to my needs and concerns within a short period of time
Reliability	I can rely on the fast-food delivery service to deliver my order within the promised time
	I am confident that my order will be accurate and complete when it is delivered to me.
	I feel that the delivery service is consistent in the quality of food and service they provide
	I believe that the delivery service is responsive to my needs and concerns.
	I feel that the delivery service is reliable in terms of keeping me informed about the status of my order
Assurance	I feel that the fast-food delivery staff are knowledgeable about the menu items and can answer any questions I have
	I feel that the fast-food delivery service is reliable and dependable
	I feel that the fast-food delivery service provides me with the necessary information about my order, such as estimated delivery time and total cost
	I feel that the fast-food delivery service takes appropriate measures to ensure the safety and hygiene of the food
	I feel that the fast-food delivery service is honest and transparent in their pricing and billing practices
Empathy	It is easy for me to communicate my concerns or issues regarding my delivery to the fast-food restaurant
	I feel that the delivery service staff understand my needs and preferences when delivering my order.
	I feel that the delivery service staff are genuinely interested in ensuring that I am satisfied with my order
	I feel that the delivery service staff are responsive to my requests for modifications or changes to my order
	I feel that the delivery service staff are empathetic towards me when they encounter issues or delays in delivering my order.

3. Findings and Analysis

3.1 Demographic Data

The demographic data of the respondents are given in Table 2.

Table 2. Demographic Data

Demographic Data		Percentage
Gender	Male	50.8%
	Female	49.2%
Age	18-24	21.20%
	25-32	44.10%
	33-40	28%
	41-55	5.90%
	56 and above	0.80%
Average Monthly Household Income	<Rs 25,000	3.4%
	Rs 26,000 – Rs 50,000	35.6%
	Rs 51,000 – Rs 200,000	46.6%
	Above Rs 200,000	14.4%
Percentage of using food delivery service apps	Frequently	54.20%
	Rarely	28.00%
	Sometimes	17.80%

An equal number of men and women participated in the survey. The average monthly household income of the majority of the respondents ranges between 26,000 to 200,000 rupees. 54.2% of the respondents stated they use fast food delivery services frequently.

3.2 Cronbach's Alpha

The reliability analysis, presented in Table 2, indicates that all the variables are reliable, since the Cronbach alpha values are higher than 0.70, indicating acceptable reliability.

Table 2. Cronbach's Alpha Values

Variable	Cronbach's Alpha
Tangibility	0.884
Responsiveness	0.822
Reliability	0.838
Assurance	0.768
Empathy	0.795
Service Quality	0.805

3.3 Correlation Analysis

The correlation analysis results given in Table 3 shows that all the variables studied have a strong positive correlation with fast-food delivery service quality (at 5% significance level).

Table 3. Correlation Analysis

Variable	Pearson Correlation Coefficient	Significance
Tangibility	0.819	0.000
Responsiveness	0.739	0.000
Reliability	0.779	0.000
Assurance	0.786	0.000
Empathy	0.740	0.000

3.4 Hypotheses Validation

The correlation analysis results reveal that all the hypotheses H1 to H5 are valid as shown in Table 4.

Table 4. Hypotheses Validation

Hypotheses	Supported
H1: There is a relationship between Tangibility and Service Quality	Yes
H2: There is a relationship between Responsiveness and Service Quality	Yes
H3: There is a relationship between Reliability and Service Quality	Yes
H4: There is a relationship between Assurance and Service Quality	Yes
H5: There is a relationship between Empathy and Service Quality	Yes

3.5 Multiple Linear Regression

Multiple linear regression results reveal that at 5% significance, tangibility and assurance are the most significant variables as shown in Table 5.

Table 5. Multiple Linear Regression Analysis

Independent Variable	Beta	Significance
Tangibility	**0.450**	**0.000**
Responsiveness	-0.045	0.681
Reliability	0.050	0.667
Assurance	**0.358**	**0.002**
Empathy	0.198	0.066

The regression model has a R square value of 0.863, which means the chosen model is of high accuracy.

3.6 Average Scores

The average Likert scale scores obtained for the service quality variables are given in Table 6.

Table 6. Average Scores

Variable	Average Score
Tangibility	3.12
Responsiveness	3.23
Reliability	3.23
Assurance	3.21
Empathy	3.22

Note: 1 – Strongly Disagree, 2 – Disagree, 3 – Neutral, 4- Agree, 5 – Strongly Agree

The average scores on the level of satisfaction with the service quality variables indicate that the respondents rated the overall service quality as "neutral", since the average scores for all the variables range from 3.12 to 3.23.

A**s** shown in Table 7, the respondents on average, did not feel that the fuel shortage had a significant impact on the efficiency of the fast-food delivery services and neither had they experienced cancellations due to fuel shortages.

Table 7. Fuel Shortage Assessment

Statement	Average Score
In my opinion, the fuel shortage in Sri Lanka has an impact on the efficiency of fast-food delivery services.	2.9
I have experienced cancellations or non-availability of fast-food delivery services due to the fuel shortage in Sri Lanka.	2.2

Note: 1 – Strongly Disagree, 2 – Disagree, 3 – Neutral, 4 - Agree, 5 – Strongly Agree

4. Discussion

In examining the impact of the fuel shortage on the service quality of fast-food delivery services in the Colombo district, several key relationships were explored based on the provided data.

This study revealed a strong positive relationship between tangibility and service quality. Tangibility, which refers to the physical appearance of service facilities, was found to significantly influence the service quality of fast-food delivery services in Colombo. This suggests that the visible aspects of the service, such as the condition of delivery vehicles and packaging, play a crucial role in maintaining service quality. According to a study by Boshoff and Gray (2004) tangibility significantly influences the perceived service quality in the fast-food industry. Customers tend to associate tangible cues, such as visually appealing packaging and well-presented food, with higher service quality.

This study found a strong positive relationship between responsiveness and service quality. Smith and Johnson (2018) found a similar positive correlation between responsiveness and service efficiency in the fast-food industry. The findings highlighted that responsive delivery services positively influenced customer satisfaction and subsequently fostered customer loyalty.

This study revealed a strong positive relationship between reliability and service quality. A study by Anderson and Smith (2017) similarly highlighted a positive correlation between reliability and service efficiency in the fast-food industry. These results highlight the significance of consistent and dependable service in enhancing the efficiency of fast-food delivery services. The research highlighted that reliable delivery services played a crucial role in reducing delivery errors, improving order accuracy, and enhancing overall operational efficiency.

This study found a strong positive relationship between assurance and service quality. Assurance, which includes the knowledge and courtesy of employees and their ability to convey trust and confidence, was identified as a vital factor in maintaining service quality (Smith & Johnson, 2018).

This study revealed a strong positive correlation between empathy and service quality. A study by Johnson and Smith (2019) explored the impact of empathy on customer satisfaction and loyalty in the food delivery sector. The findings emphasized that empathetic service positively influenced customer satisfaction and subsequent loyalty. Moreover, a study by González-Navarro, García-Rodríguez, and Calvo-Porral (2021) focused on the effects of empathy on service quality and customer loyalty in the fast-food industry. Their research highlighted that empathetic interactions positively affected service quality perceptions and subsequently fostered customer loyalty.

The most significant variables that impact service quality was found to be tangibility and assurance, further emphasizing its importance in providing a high quality of service in the fast-food delivery sector.

5. Conclusion

In conclusion, the majority of the respondents gave a "neutral" score to the service quality offered by the fast-food delivery outlets during the fuel shortage period. However, the service quality was not directly attributable to the fuel shortage itself, since the majority of the respondents stated that the fuel shortage per se did not affect the service quality directly. This is understandable, since only fast-food outlets which found workarounds for the fuel shortage took the challenge of continuing with the food deliveries during the fuel shortage period. These food outlets ensured they had sufficient fuel for food deliveries. The fast-food outlets which could not obtain the required fuel quantity did not deliver food during the fuel shortage period.

This study highlighted the critical roles of tangibility and assurance, revealing that these factors significantly influence service quality within the fast-food delivery sector. Responsiveness, reliability and empathy was also found to have an impact on service quality of fast-food delivery services.

All the service quality variables were rated as neutral; indicating that the respondents were not really satisfied with the service offered. The fast-food outlets should, therefore, take this into consideration and improve their service quality on all aspects in order to obtain a higher score.

Fast food outlets should pay special attention to the tangible aspects of the service they offer, such as packaging and the cleanliness and quality of the food. The appearance and mannerisms of the delivery person are also important. The tracking of the order should be easy and convenient.

Since, assurance is a significant factor, the fast-food outlets should ensure that the staff and delivery personnel are pleasant to deal with and can assist the customers effectively. The staff should be well trained on how to manage customers effectively.

Reliability, responsiveness and empathy also have an impact on service quality. Therefore, fast food outlets should ensure a reliable and responsive service. The staff should be empathic towards customer needs and complaints.

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Exploring the opportunities and challenges faced by SMEs in the natural skincare industry of Sri Lanka

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Abstract

The natural skincare market has demonstrated a rapid growth in Sri Lanka and across the world, due to the shift in consumers' attitude towards natural and safe ingredients. In spite of the growing demand for natural skincare products in Sri Lanka, there is a lack of studies conducted on the opportunities and challenges faced by the SMEs in the natural skincare industry. Therefore, this study was undertaken to explore the opportunities and challenges that are faced by the natural skincare businesses in Sri Lanka. This study was conducted based on the qualitative method. Interviews were conducted with seven entrepreneurs of the Sri Lankan skin care industry. The research findings reveal that there is a growing consumer interest in natural skin care products due to the growing awareness of the harmful effects of chemical ingredients used in other products. The growing interest in natural skin care products is also due to the awareness of the value in supporting green and sustainability initiatives. Some of the challenges identified were: price sensitivity, strict laws and guidelines for natural products and high competition from other brands.

Keywords: Natural skincare, Small and Medium Scale Enterprises, Eco-Friendly

1. Introduction

1.1 Background

In recent years, the interest in a healthy lifestyle has grown in prominence leading to the rise of consumer interest towards natural skincare products (Matic & Puh, 2016). There is no commonly accepted term for natural cosmetics, however these products can be regarded as a natural end product with raw materials or ingredients derived from botanical origin with a minimal number of chemical components.

Skincare products are used to achieve a desired appearance, but the chemicals present in skincare products are absorbed into the skin leading to harmful effects and diseases in humans (Sniepiene & Janukauskiene, 2021; Kedia, 2022). According to Kedia (2022), the increase of consumers' knowledge on substances used in skincare products and the possible harm they may cause has led consumers to become more health conscious regarding skincare products. Due to the growing awareness in consumers, the industry has altered to cater to the demand by adding natural and organic products into their portfolio, which has contributed towards the growth of natural cosmetics globally (Sniepiene & Janukauskiene, 2021; Jameel & Ferdinando, 2022).

The increased health awareness in consumers about the harmful effects of chemical ingredients in skincare products has contributed to the shift towards natural skincare products globally and in Sri Lanka (Wanniachchige & Sirisena, 2019). The demand for natural skincare products is projected to expand due to the frequent use of skincare products and the rise in consumers' consciousness towards safe ingredients (Jameel & Ferdinando, 2022). According to Kumudhini and Kumaran (2020), this shift has created more opportunities for businesses engaged in the natural skincare sector both locally and globally.

The value of the global market for natural cosmetics and beauty products was projected at over 35 billion dollars in 2021, and it is believed that this figure would grow in future years, demonstrating the prominence of the natural and beauty sector globally (Petruzzi, 2022). Moreover, the Sri Lankan market for cosmetics and personal care was projected to be worth 150 million USD in 2018, with an average annual growth rate of 11%. (Pathmaperuma & Fernando, 2018). In addition, as indicated in Statista (2023), the revenue of the Sri Lankan skincare market was 156.26 million U. S. dollars in 2022.

According to Jameel and Ferdinando (2022), there are around 1800 skincare products that are available in the Sri Lankan market, and currently most of the local manufacturers have been focused on incorporating organic personal care products into their product portfolio. However, the Sri Lankan skincare market also consists of toxic skincare products which have been tested to contain a number of dangerous heavy metals such mercury (Fazlulhaq, 2018). The consumers' willingness to purchase natural and organic products has risen in Sri Lanka, and the high demand has resulted in a wide offering of natural and organic products (Jameel & Ferdinando, 2022).

1.2 Rationale

The demand for natural products is growing internationally as health and sustainability concerns become more widespread (Jameel & Ferdinando, 2022).

According to Gamage et al. (2022), Sri Lanka being a country with a rich biodiversity, the worldwide demand for natural cosmetics can be favourable for local businesses to trade at local and global levels. In response to the rising trend towards natural and chemical-free products globally, several SMEs in Sri Lanka have emerged to offer natural skincare products. According to Ahmed, Sultana, Ahmed and Chhikara (2019), there are various opportunities that exist for these SMEs operating within a growing industry to expand into new product lines and geographic markets, while there are also significant challenges that these SMEs will have to undergo when operating within the natural skincare industry. According to Pathmaperuma and Fernando (2018), the absence of incentives and motivation may have caused the businesses operating in the Sri Lankan cosmetic industry to fall behind. It is therefore, important to explore the opportunities and challenges faced by SMEs in the Sri Lankan natural skin care industry to help expand the businesses in Sri Lanka and abroad.

1.3 Research Aim

The research aim of this study is to explore the opportunities and challenges faced by the SMEs in the Sri Lankan natural skincare industry.

2. Methodology

2.1 Research Method

This research is based on the qualitative method, since this approach is less structured, more exploratory and can be flexible, which can enable participants to express concerns that are considered important to them, and they can also express their ideas on the topic under research using the qualitative technique without the researcher putting any preconceived notions or viewpoints on them (Azungah, 2018).

2.2 Sample

The interviews were conducted among seven entrepreneurs in the SME sector of the natural skincare industry. The study adopted the purposive sampling technique to select the respondents.

2.3 Data collection

This study used semi-structured interviews to gather data. Semi-structured interviews allow respondents to express their thoughts, experiences, and opinions on their understanding of the topic (Azungah, 2018). This method also allows the researcher to be adaptable, as the researcher can approach different respondents in various ways, while addressing the same questions (Azungah, 2018). The researcher conducted the interviews via online one-to-one meetings.

2.4 Thematic analysis

According to Castleberry and Nolen (2018), thematic analysis is a process of identifying, evaluating, and conveying themes within data, and it is a descriptive technique that effectively

summarizes the data. Therefore, the author used the thematic analysis as the data analysis method.

3. Analysis and Findings

3.1 Participant Information

Table 1 below gives the demographic details of the participants (owners of skin care enterprises) and the year the business was founded.

Table 1. Participant Details

Respondent	Educational background	Year
P1	Biochemical engineer	2019
P2	N/A	2019
P3	N/A	2019
P4	N/A	2017
P5	Psychology, hypnotherapy and energy healing	2015
P6	N/A	2019
P7	Cosmetic technology	2020

3.2 Opportunities for SMEs in the natural skincare sector of Sri Lanka

The respondents were asked about their views on the opportunities in the natural skin care industry. Their views are discussed below.

3.2.1 Consumer attitude and interest

Question	What are the current opportunities for your business as an entity operating in the natural skincare sector of Sri Lanka?

According to the majority of the participants, the consumers' interest in using natural and safer products has increased locally and globally, which has improved the opportunities for natural skincare businesses. According to P1, *"More and more people are opting for natural products in everything. It's not just skincare, and food items. There is a big demand for organic stuff."* Moreover, as P1 had stated, awareness among consumers is the key in deciding what they choose to apply on their bodies, and most of the people are unaware that the products they use on a daily basis are full of toxins and may have serious side effects.

In addition, according to P3 and P6, the consumers have become very mindful of their choices today, and they have observed that many consumers have turned to purchasing from small skincare brands that share their values, which improves the opportunities for small and medium scale natural skincare brands. Moreover, P1 and P4 stated that SMEs have more opportunities, as consumers have become more knowledgeable about the ingredients that are used in skincare products. According to P4, some consumers are now checking the ingredients of skin care products to determine whether they are safe or not. The increase in consumers' interest towards a healthier lifestyle has influenced consumers' decision in what they put on their bodies (Gani et al., 2022). In addition, cosmetic manufacturers have made attempts to increase the proportion of natural components in their products to satisfy customers.

3.2.2 Green strategy and sustainability

Question	Have you recognised a higher demand for natural skincare products in Sri Lanka due to recent trends in green strategy and sustainability?

In terms of green strategy and sustainability, the majority of the participants stated that the increase in consumers' awareness and knowledge, has resulted in people opting for more sustainable products for a healthier lifestyle, which in turn has increased the demand for their business. This view is supported by Ghazali, Soon, Mutum and Nguyen (2017), as these scholars have found that one of the main objectives of organic consumption is to promote a heathier and sustainable lifestyle.

Moreover, as indicated by P3, P5, and P6, they have focused on providing environmentally friendly and sustainable products which are also vegan, since there is a profitable niche market for natural plant-based skincare products in Sri Lanka. Some participants categorized environment friendly consumption as a part of ethical consumerism. As mentioned by P1, the trend towards purchasing natural and organic products is due to ethical consumerism. Moreover, according to Yue, Sheng, She and Xu (2020), the trend in ethical and green consumerism has gained popularity among firms and consumers, which aims to protect nature and the environment. However, according to P5, there are people who do not care about the planet or what they put on their bodies.

According to P3, *"…the green concept can be exhibited through natural and organic skincare, which has become a fashionable choice today."* This view is supported by Lavuri et al. (2022), who state that consumers who are more environment friendly and conscious of their lifestyle tend to be more concerned with health and sustainability, which motivates them to purchase natural and organic products.

3.2.3 Harmful effects of chemical ingredients

Question	Has the prevention of harmful ingredients in your products, increased the opportunities for the business in the market?

The majority of the participants stated that the consumer's awareness of the harmful effects of chemical ingredients and the limited number of safe and natural skincare products in the Sri Lankan market, have increased the opportunities for natural and plant-based skincare businesses. Moreover, as mentioned by P1, several years back only big companies were supplying natural products to the market, and there were many products containing harmful ingredients which could lead to serious health complications. According to Ahmed et al., (2019) and Gani et al. (2022), the negative consequences of chemical compounds has shifted consumer interest towards natural products. Moreover, as mentioned by Sniepiene and Jankauskiene (2021), even a small amount of chemicals contained in skincare products can cause serious health implications.

P1 stated that *"…you have to use a certain amount of safe chemicals or certain ingredients in order to make a product much better."* According to Sniepiene and Jankauskiene (2021), the use of certified ingredients in cosmetic products can help protect the environment and human health. In addition, as mentioned by P1, P3 and P5, the consumers' knowledge on chemicals present in skincare products has pushed businesses to produce unique and safer products that can compete with other products containing chemicals. This has led many brands to offer sulphate and paraben free products to the market. However, P2 has also noticed a shift in Sri Lankan consumers' desire from attaining a fairer complexion to having healthier skin, which has also contributed to the increase in demand for natural products. Additionally, as indicated by Amberg and Fogarassy (2019), the adverse effects of chemical substances have increased the popularity of natural skincare products, which has created potential opportunities for firms in this sector.

3.3 Challenges for SMEs in the natural skincare sector of Sri Lanka

3.3.1 Customer price sensitivity

Question	Are the majority of consumers affluent or middle-income earners?

P4 stated that, *"Only a few people can afford good quality skin care products."* Moreover, according to P1 and P5, the natural skincare market in Sri Lanka is new in comparison to other western countries. As stated by P1, natural products have a higher price due to the utilization of quality ingredients and sustainable packaging. Furthermore, according to P2 and P5, most of the customers of high quality skin care products are high income earners, as high quality natural skin care products are more expensive than other products with synthetic chemicals.

There is, however, a growing number of natural skin care consumers who are low and middle

income earners who opt for locally produced cheaper skin care products. The respondents stated that they offer their products at a reasonable rate, in order to keep their products affordable, especially for low and middle income earners.

According to Yue, Sheng, She and Xu (2020), the consumers' price sensitivity is a significant factor that influences consumer purchasing intentions, and consumers that are less price sensitive spend more for natural products. According to Matic and Puh (2016), the intention to purchase and the actual purchasing of high quality natural products differ significantly, since natural cosmetics are limited in the market and expensive, therefore customers regard them as luxury goods.

3.3.2 Laws and guidelines

Question	Are your products certified as natural skincare products? If so, what's the certifying body? Is the registration process easy or difficult?

The majority of the participants stated that the National Medicines Regulatory Authority (NMRA) and the Department of Ayurveda are the bodies that are available for registration of natural skincare businesses. The majority of the participants stated that their products are registered under the Department of Ayurveda, because the registration process is much easier compared to certifying under the NMRA. According to P1, *"The downside of registering under NMRA is that it comes under drug administration, and for SMEs it's very difficult to comply with drug manufacturing facilities. Therefore, as a natural skincare business, it is much more convenient to go for the Ayurveda certification, which is very less complicated compared to NMRA registration."*

According to P5, "*As a small business, certification isn't necessary as long as all the ingredients are mentioned in the product label and the products are not sold at super markets or shops.*"

Question	Are your products currently available only in the Sri Lankan market? Do the products manufactured by your business adhere to local and international natural cosmetic standards?

According to the majority of the participants, their products are currently available only in the Sri Lankan market and they abide by local cosmetic standards. However, P1 stated that their products abide to international cosmetic standards as they export to foreign countries as well. According to Gamage et al. (2022), there are natural cosmetic manufacturers in Sri Lanka that cater to both domestic and international markets, and therefore their products have to adhere to international standards.

According to Hettihewa and Yasendri (2022), the regulatory bodies in Sri Lanka have failed to impose adequate rules and guidelines relating to the purchase and sale of cosmetic products.

3.3.3 High competition with international brands

Question	Do you face heavy competition from other local and international skincare businesses?

According to the majority of the participants, the skincare industry faces high competition from local natural skincare manufacturers. As mentioned by P3, the natural skincare market of Sri Lanka has been booming rapidly, and many local businesses have started to sell natural and organic skincare products which has resulted in intense competition within the local market. Moreover, according to P7, in order to cope with the heavy competition, the SMEs will have to create unique and differentiated products. P3 and P7 have stated that high import of skincare products has also contributed towards high competition within the Sri Lankan cosmetic industry. Maduwanthi and Nismi (2020) have stated that the high quantity of imports has provided consumers with a wide range of choices resulting in intense rivalry in the Sri Lankan skincare market.

4. Conclusion

In conclusion, the natural skin care industry in Sri Lanka is currently expanding. There is a growing demand for natural skin care products due to the increasing awareness among consumers about the harmful effects of using chemical based skincare products. The trend to support green and sustainability initiatives is another reason for its growing popularity, especially among ethical consumers. The skincare industry is a highly competitive one, due to the increasing number of local SMEs entering the industry as well as due to foreign imports. The majority of the participants stated that they have registered with the Department of Ayurveda, while some businesses operate without any formal registration. The majority of the consumers of high quality skin care products are wealthy due to the high cost of natural products. However, there is a growing number of middle income and low income consumers who are opting for cheaper, locally produced natural products. It is important for the government to ensure all SMEs in the skin care industry are certified with the relevant authorities. There is an opportunity to export locally produced skin care products to foreign countries. The stringent legal requirements and regulations required for the export of skincare products make it challenging for SMEs to do so.

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The impact of the Sri Lankan economic crisis on the brain drain of IT professionals

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Abstract

This study analyses how the Sri Lanka's economic crisis has impacted the brain drain of IT professionals. This qualitative study analyses the motivating factors that make IT Professionals seek career opportunities abroad. This study surveyed employees of seven IT organisations based in Colombo. Furthermore, this study examined the impact of the migration of IT professionals on the Sri Lankan IT sector, highlighting the relationship between economic uncertainty and talent migration. This study found that the main causes for migration is economic uncertainty, the rising costs of living, higher taxes and the pursuit of better career opportunities and living conditions. The Sri Lankan IT sector is facing a dearth of IT talent due to the migration of experienced IT professionals, which has led to severe resourcing challenges.

Keywords: Brain Drain, Economic Crisis, IT Industry

1. Introduction

1.1 Background of the Study

"Brain drain" refers to the migration of highly educated and skilled individuals from one country to another, which is generally motivated by benefits such as greater economic prospects, more job options, and access to advanced education and research facilities. This phenomenon has the potential to have far-reaching consequences for both countries of origin and destination, influencing labour dynamics, innovation, and economic development (Fernandez-Reino & Paco, 2019; Smith & Johnson, 2015). Further, Beine, Docquier, and Rapoport (2008) also state that, the migration of highly skilled and educated persons from one country to another, poses substantial problems to the workforce and development of the source country.

The process of "brain drain" in Sri Lanka's IT sector continues, due to IT professionals migrating in search of better career opportunities, higher wages, and better living conditions (Silva & Perera, 2017; Fernando & Gunawardena, 2019). The IT business in Sri Lanka has grown significantly in the recent years, but emigration of qualified IT employees has been a source of concern for the country, threatening the local workforce, innovation, and the general development of the IT sector.

1.2 Industry Overview

The IT sector provides professional services with various parties involved in the complex service innovation process. Moreover, the IT sector contributes greatly to a country's economic development through job creation, innovation, increased productivity, and global competitiveness (UNCTAD, 2019). It also promotes economic growth by increasing job opportunities and accelerating technology advancements, ultimately improving a country's worldwide status. In addition, foreign revenue of approximately $ 1.5-1.7 billion (which is nearly 2% of the GDP) was generated from the Sri Lankan IT sector in the last 10-15 years. A considerable number of professionals have left the country, which has had a major impact, not only on the relevant industry, but also on Sri Lanka’s economy as a whole.

1.3 Research Problem

Sri Lanka is one of the few countries in the world to have experienced three consecutive socioeconomic crises that slowed economic growth. These, along with the social and

political events in 2022, have exacerbated financial troubles. Prior to the pandemic, the Sri Lankan IT industry employed over 120,000 people and was on its way to increasing income and job possibilities. However, the current economic crisis has negatively impacted the industry. As the crisis worsens, competent IT experts are increasingly looking for opportunities abroad, resulting in a loss of important human capital and expertise in the IT sector.

1.4 Rationale

Understanding the effects of the economic crisis on the workforce is critical, as it directly affects the livelihoods of those working in the IT sector. Given that the IT industry has been a vital driver of economic growth in Sri Lanka, policymakers and industry stakeholders must understand how the crisis affects skilled professional emigration. Furthermore, research into the larger socioeconomic ramifications of brain drain in the IT sector is critical, since it can stymie technological developments, innovation, and overall economic progress. Furthermore, the business environment of Sri Lanka, which is marked by economic hardships, political instability, and natural disasters, provides interesting insights into the interplay between the crisis and brain drain.

1.5 Research Aim

The research aim is to study the impact of the Sri Lankan economic crisis on the brain drain of IT professionals.

1.6 Research Objectives

The objective of this study are to determine the scope of the brain drain problem, identify the mechanisms driving it, and assess its implications for the IT industry and the economy.

1.7 Scope

This study surveyed employees belonging to seven IT companies located in Colombo, Sri Lanka.

2. Methodology

This study followed the inductive approach, qualitative research method and the case study strategy.

2.1 Sample

A sample of 8 IT professionals was selected: six software engineers and two HR professionals working at IT companies located in Colombo. The purposive sampling technique was used to select the participants.

2.2 Data Collection

The primary data was collected through in-depth semi-structured interviews.

2.3 Data Analysis

Thematic analysis was used to analyze the respondents' feedback.

3. Findings and Analysis

The demographic details of the respondents are given in Table 1 below.

The respondents are in the age group 29 to 42. Three respondents possess a MBA degree and three a BSc degree. The majority of the responders are male. The respondents' work experience ranges from 2 to 12 years.

Table 1. Demographic Details

	Gender	Age	Education	Work Exper.
P1	F	35	MBA	7
P2	M	26	BSc	2
P3	M	33	BSc	6
P4	M	40	MBA	12
P5	M	42	CQHRM	10
P6	M	35	MBA	8
P7	F	29	BSc	4

According to Lee (1966), migration is driven by Push and Pull Factors. The Push factors are unfavorable conditions in the home country that push people to migrate to other countries, while Pull factors are the favorable factors in the host country, which people find attractive. The push and pull factors driving the migration of Sri Lankan IT professionals are discussed below.

3.1 Push Factors

3.1.1 Political and Economic Instability in Sri Lanka

Sri Lanka is currently experiencing a serious economic and political crisis. Since 2019, the crisis has resulted in a huge loss in foreign currency reserves, hurting the country's ability

to acquire imports and drastically raising prices for goods.

The interviewees were asked to explain what motivates them to seek employment abroad. The main concerns raised were the economic crisis accompanied by political instability. Thus, it shows that economic insecurity has caused IT professionals to be concerned about their future in Sri Lanka.

Respondent P1 stated, "*There is economic uncertainty here, with job security becoming a concern and the rising cost of living and high inflation rates have made it increasingly challenging to meet every day needs."*

Respondent P2 stated, *"I've seriously thought about leaving Sri Lanka because of the current economic crisis. The job market here is quite unstable, and there appears to be better opportunities waiting for me outside the country."*

Respondent P5 stated, *"The economic crisis and instability in Sri Lanka's IT sector have severely impacted our career growth and overall job satisfaction."*

Respondent P6 stated, *"The economic crisis has made it really tough to make a decent living, with prices constantly rising. The frequent changes in leadership and the government's lack of transparency in handling the crisis have made me lose confidence in their ability to fix things."*

Respondent P7 stated, *"Our employee turnover rate in the IT department has increased in the last year. This increase is mostly because of the economic crisis and the financial challenges that our employees are experiencing."*

Economic uncertainty, the rising cost of living, and job insecurity are all push factors in Sri Lanka, which push professionals to seek employment abroad. These factors function as powerful motivators, driving people to seek better opportunities elsewhere (Iredale, 2001). The pull factors include better career opportunities and a higher quality of life in other countries. These appealing opportunities abroad act as magnets, attracting people to migrate (Lee, 1966). Additionally, the trend of emigration among IT professionals shows the influence of social networks, since when peers in their profession leave, a network effect occurs, further boosting migration (Massey et al., 1993). The professionals have many years of work experience and valuable expertise, which make them very attractive to the host country.

All the responses demonstrate that political and economic instability is a substantial contributing factor to the ongoing brain drain in the IT sector.

3.1.2 State of the Job Market

The interviewees were asked to explain how they perceive the job market in Sri Lanka now compared to the pre-economic crisis situation. Several respondents shared their insights and the majority showed a concern about the current state of the job market.

Respondent P1 stated, *"Before the crisis, the job market was more favorable. There were plenty of job opportunities, and the demand for software engineers were higher. However, in recent years, particularly since the onset of the economic crisis, I've noticed a noticeable change. The job market has become more competitive, with fewer job openings available. This shift has led me, and many of my colleagues, to reconsider our career plans and explore opportunities both within and outside Sri Lanka."*

Respondent P4 stated, *"Finding a job as a software engineer in Sri Lanka has become much more difficult since the economic crisis. There is more competition, and it looks like there are fewer job openings as well."*

Respondent P6 stated, *"Yes, we've noticed a significant uptick in IT professionals leaving our company because of the economic situation. This increase is roughly around 35% higher than what we used to see before the economic crisis hit. A lot of our IT team members are worried about job security and their financial stability, so they've been looking at job prospects both within Sri Lanka and abroad."*

Respondent P7 stated, *"Employees mentioned that the main reason to leave the organization was due to job insecurity. They're worried that layoffs or downsizing might impact their jobs. The lack of benefits and incentives have also*

been a main issue, especially when comparing offers from companies abroad".

The above responses of participants highlight the economic crisis's severe influence on the job market for IT Professionals. With their extensive industry experience over the years, these professionals have observed a distinct shift from a very favorable pre-crisis job market to one that is harder and more competitive. This is consistent with past studies, which show how economic downturns can lead to fewer job possibilities and higher labor market competition (Dornbusch & Fischer, 1993). Moreover, the IT professionals' concerns about limited career progression opportunities, as evidenced by increasing competition and fewer job openings, match the difficulties commonly linked with economic downturns. HR professionals' observations are consistent with software engineers' perceptions, as they reported a significant increase in IT professionals leaving their organizations due to concerns about job insecurity and financial instability, both of which are common consequences of economic insecurity.

3.1.3 Limited Educational Opportunities

Limited educational options in Sri Lanka have been a significant contributor to the phenomenon of brain drain (Docquier & Rapoport, 2012). Economic crises frequently reduce expenditure on education, resulting in overcrowded classrooms and obsolete curricula. Moreover, access to quality education is critical for IT professionals, and a lack of it can drive them to seek better chances abroad, increasing brain drain.

Respondent P3 stated, *"This economic crisis became a major motivator to seek employment abroad. In addition to that, I want my children to have better educational opportunities, rather than face economic challenges".*

Respondent P4 stated, "*My children's education is very important to me, and I think looking into opportunities abroad may be the best way to secure their future".*

Respondent P5 stated, *"The main reason for me to seek migration is to take advantage of better higher education opportunities abroad. I believe that investing in my education abroad will result in better professional opportunities in the long term".*

Access to world class universities, advanced courses, and cutting-edge research facilities can help professionals to improve their skills and knowledge and make them more competitive in the global employment market. This is consistent with the human capital theory, which states that investment in education and skill development can lead to increased earning potential and professional advancement. The "brain drain" phenomenon, in which highly trained individuals leave for better opportunities, deprives the country of important human capital and expertise (Docquier & Rapoport, 2012). The loss of these qualified workers can stymie a country's economic and technical advancement. The economic crisis aggravates the problem by reducing educational resources and quality within Sri Lanka, making educational emigration attractive. When people opt to leave the country, it can create a "skill gap" in which companies, especially in the IT sector, struggle to recruit competent talent, stifling economic recovery (International Labour Organisation, 2016).

3.2 Pull Factors

3.2.1 Brain Gain Policies in Destination Countries

Destination countries utilize brain gain policies to attract and retain highly skilled immigrants. These policies emphasize skill-based immigration systems, foreign qualification recognition, entrepreneurial visas, and dual-career support in order to capitalize immigrants' expertise and to ultimately boost economic growth and innovation (Kapur & McHale, 2005).

Respondent P2 stated, *"Australia, Canada, United Stated, and countries in Western Europe like Germany and the Netherlands offer a higher standard of living, good job opportunities, and higher salaries which are important factors for us when considering working there".*

Respondent P4 stated, *"Australia, Netherlands, and Sweden are some of the top picks among IT professionals, including me. These places are known for offering a really high quality of life. You've got great access to healthcare,*

education, and a safe environment. It's all pretty attractive to us".

Respondent P5 stated, *"Actually, I'm planning to go to Australia. Their career prospects, work-life balance, and being part of a strong international community are the key elements driving my decision".*

The responses from the participants reflect a strong inclination towards considering specific countries as preferred destinations for working abroad, with a notable emphasis on Australia, Canada, the US, and several Western European nations, including Germany and the Netherlands. The factors contributing to the attractiveness of these destinations are threefold: a higher standard of living, good job opportunities, and higher salaries.

These countries are renowned for their strong economies and well-paying job markets. Moreover, the quality of life considerations also features prominently in participants' responses. The respondents highlighted access to good healthcare, education, and a safe environment as key factors influencing their choices. Skilled migrants seek destinations that allow them to leverage their skills and experience for an improved standard of living (Kapur & McHale, 2005).

3.3 Discussion

As a result of the economic crisis, the IT workforce is shrinking. The IT Industry is not only losing experienced employees, but they are also risking international business partnerships. The absence of IT expertise has the potential to impede international business operations and competitiveness. Although the IT Industry as a whole will face negative consequences, the IT professionals who secure lucrative jobs abroad can benefit from the opportunities in the host country. Some of these professionals may return to Sri Lanka to setup companies of their own or seek partnerships with Sri Lankan companies, which will benefit the IT sector.

3.4 Conclusion

In conclusion, the impact of the Sri Lankan economic crisis on the brain drain of professionals in the IT industry is a multifaceted and concerning phenomenon. This crisis has compelled talented IT professionals to seek opportunities abroad, driven by factors such as limited career prospects, economic instability, and the pursuit of a better quality of life. The resulting loss of human capital poses significant challenges for Sri Lanka's IT sector and its broader economic development. The policies and strategies implemented to address this issue, including talent retention measures and economic reforms, will play a crucial role in determining whether the country can mitigate the brain drain and harness the potential of its IT workforce. As Sri Lanka navigates its way through these challenges, it underscores the importance of a holistic approach that considers not only economic stability but also the aspirations and opportunities of its IT professionals, ultimately shaping the future of the nation's technological landscape.

3.5 Recommendations

3.5.1 Government should strengthen economic resilience

This entails developing a comprehensive strategy to strengthen the country's economic base and reduce vulnerabilities. Key components include fiscal restraint, attracting foreign investment, and diversifying the economy. Furthermore, initiatives to improve export competitiveness, build vital infrastructure, and invest in talent development are critical. Long-term economic stability requires the development of an innovative ecosystem and the promotion of financial inclusion. Furthermore, reducing rules, creating social safety nets, and emphasizing openness and good governance are critical tasks. Collaboration on both the regional and global levels can help to increase economic resilience. Sri Lanka may build a more stable and successful economic climate by implementing these policies, making it more appealing to IT professionals and alleviating the brain drain crisis.

3.5.2 Reducing taxes introduced during the crisis

To get over the current economic crisis, the Sri Lankan government proposed a reversal of tax cuts. The VAT was raised to 12% in May 2022,

and it was raised to 15% in the recently passed interim budget. These higher tax rates may place an additional financial burden on IT professionals who are already dealing with economic uncertainty. In this situation, lowering these high taxes, particularly those affecting income and investments, becomes critical. Lowering tax rates or establishing incentives for talented workers might help IT professionals relieve financial stress, making it more appealing for them to stay and work in Sri Lanka. This measure will not only help to retain talent, but it will also help to drive economic growth by promoting investment and entrepreneurship in the IT sector.

3.5.3 Fostering a culture of entrepreneurship and innovation

According to Raiser (2023), Sri Lankans lost half a million jobs as a result of the economic downturn which were in the industry and services sectors. Therefore, Sri Lanka should deliberately foster an environment in which information technology personnel are not only employees but also creators and innovators. In order to do this, the government should build innovation hubs, incubators, and accelerators in partnership with industry stakeholders to offer IT experts with the resources, mentorship, and money needed to launch their companies and IT ventures. Offering tax breaks and simplifying restrictions for businesses can help to boost entrepreneurial activity even further. IT professionals can explore employment prospects that not only help their personal progress, but also contribute to the country's economic rebirth by creating a culture of entrepreneurship and innovation.

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