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# DETECTION AND DETERMINATION OF EFFECT OF ANTIBIOTIC ACTIVITY OF NYMPHAEA NOUCHALI (WATER LILY) AGAINST ESCHERICHIA COLI AND STAPHYLOCOCCUS AUREUS BACTERIA

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## *ABSTRACT*

With the emergence of antibiotic resistance within pathogenic bacteria, treating bacterial illnesses has become challenging. Therefore, a need of natural antimicrobial compounds to work alongside with the conventional antibiotics has arisen. Based on the past evidences on the presence of secondary metabolites responsible for antibacterial properties, the aquatic herb, Nymphaea nouchali (Blue water lily) was tested against pathogenic bacteria Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923). The ethanolic extract of the plant sample was assessed for the inhibitory activity both quantitatively and qualitatively using the laboratory techniques including antimicrobial susceptibility testing (ABST), minimum inhibitory concentration determination (MIC) and minimum bactericidal concentration determination (MBC) assays. At 50 mg/ml and 100 mg/ml concentrations, N. nouchali exhibited the highest inhibitory activity against S. aureus resulting zones of length 24.667± 0.471 mm and 26.334± 0.471 mm respectively. A significant difference in the inhibitory activity was expressed by the plant against the two bacterial strains at both the concentrations. In determination of MIC, a lower concentration of 12.5 mg/ml, expressed bacteriostatic properties against E. coli while, against S. aureus 25 mg/ml concentration expressed bacteriostatic properties. When determined the MBC, Nymphaea nouchali, expressed bactericidal properties at 50 mg/ml and 100 mg/ml concentrations against E.coli and S.aureus respectively. The results revealed the presence of phytochemical compounds that contained antimicrobial properties and the potent antibacterial activity of the plant, against pathogenic bacteria. Further analysis will be carried out using different solvent extraction methods.

Key words: Antibiotic resistance, Natural antimicrobial compounds, Inhibitory activity, Bacteriostatic, Bactericidal

## *INTRODUCTION*

Discovery of antibiotics is an immensely successful achievement of the 20th century that led the path to eliminate a wide range of diseases caused by a broad spectrum of bacterial strains. But the effectiveness of antibiotics remained for a limited period of time, due to the development of antibiotic resistance. Many physiological and biochemical processes are among the causes of the development of resistance which induces genetic alterations in the bacterial genome (Davies and Davies, 2010). This arises the requirement of discovery and synthesis of new antibiotics which is a costly and time consuming process. As a result, the use of natural antimicrobial plant extracts has gained much attention. Given the circumstances, studies that are conducted

in order to assess the sensitivity of selected human pathogenic bacteria against, phytochemical extracts of plant species seems appropriate.

Pathogenic bacteria are a group of microorganisms with the ability to cause a disease. Such diseases are usually treated with conventional antibiotics. Over time, development of antibiotic resistance has given rise to bacterial strains which does not show sensitivity towards the antibiotic treatments that were originally effective (Barbieri et al., 2017). Antibiotic resistance causes the sensitive bacteria to be eliminated leaving the resistant strains gain prominence. Also resistance can be acquired spontaneously through mutagenesis (Ventola, 2015). Misuse of antibiotics also inclined the antibiotic resistance. It has been revealed that, in 30% to 50% of the situations, the indication of treatments, the choice of antibiotic and the duration of the therapy is inappropriate (Ventola, 2015). Extensive usage of antibiotics in the treatment of livestock to avoid infections and to promote growth also could potentially aid in developing the resistant strains. The unavailability of novel antibiotics that can combat resistant bacteria also promotes the resistance (Ventola, 2015; De Zoysa et al., 2019). Development of antimicrobial resistance occurs through several processes including, modification of the bacterial proteins targeted by the antibiotics, inactivation of the antibiotics through enzymatic degradation, alterations of the permeability of membranes towards antibiotics and efflux pump (Bintsis, 2017). Acquired resistance can be inherited through genomic islands associated with horizontal gene transfer (HGT). To overcome this issue, various scientific studies have been conducted to assess the effectiveness of natural antimicrobial substances against human pathogenic bacteria (Bhavsar and Krilov, 2015; Foster, 2017).

**Human pathogenic bacteria**

The two bacterial strains Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) were utilized in this study based on their relevance to the phenomenon of antimicrobial resistance. Escherichia coli colonizes as normal flora in the gut of homoeothermic organisms. Though Escherichia coli is generally considered as non-pathogenic, some strains have been developed into pathogenic strains by acquiring certain virulence factors (Sarowska et al., 2019). Those virulent strains are responsible for many infections associated with the skin, urinary tract and central nervous system (Bhavsar and Krilov, 2015). Treating for such infections caused by Escherichia coli has become challenging due to the emergence of antibiotic resistance. Escherichia coli produces extended spectrum β – lactamases, an enzyme causing resistance against β – lactam antibiotics. Therefore Escherichia coli has developed multi drug resistance (MDR) over time (Pormohammad, Nasiri and Azimi, 2019; Rasheed et al., 2014). A study conducted by Dessie Regional Health Research Laboratory has proven that Escherichia coli has developed higher resistance against antibiotics like amoxicillin, erythromycin, and tetracycline (Kibret and Abera, 2011). Another study tested for the sensitivity of Escherichia coli isolated from patients with urinary tract infection has expressed a greater resistance towards antibiotics that are commonly in use (Olorunmola, Kolawole and Lamikanra, 2013). Staphylococcus aureus is a gram positive, human bacterial pathogen which is fast growing and round in shape. It consists of various virulence factors which enables the entry, establishment and evasion of the immune response. With the discovery and extensive use of the antibiotic penicillin, penicillin resistant strains of Staphylococcus aureus emerged which

were capable of synthesizing penicillinase, leading to penicillin resistance. To counter this methicillin was introduced as a treatment for infections caused by penicillin resistant Staphylococcus aureus. Currently, methicillin resistant Staphylococcus aureus (MRSA) strains have also been identified across the world (Appelbaum, 2007; Garoy et al., 2019). Multi drug resistant Staphylococcus aureus is responsible for many disease conditions including, pleuropulmonary, pneumonia, infective endocarditis and infections associated with skin and soft tissues (Tong et al., 2015; Gnanamani, Hariharan and Paul-Satyaseela, 2017). Recent studies have revealed, Staphylococcus aureus has developed resistance against vancomycin, which is used in treating methicillin resistant Staphylococcus aureus (MRSA) known as Vancomycin resistant Staphylococcus aureus (VRSA) (McGuinness, Malachowa and DeLeo, 2017).

**Antimicrobial properties of phytochemical extracts**

In order to counter the issue of the development of antimicrobial resistance, several studies have been conducted since the past few years in determining the applicability of treatments incorporated with plant phytochemicals in treating infectious diseases. According to Barbieri et al., (2017), through a series of studies, it has been revealed that secondary metabolites of plants are capable of altering the resistant mechanisms within the bacterial cells which can elevate the effectiveness of antibiotics in treating disease conditions. It has been evidenced that plants contain an array of phytochemicals with various important properties and antimicrobial property is also a prominent aspect. Secondary metabolites are naturally occurring chemical compounds which are responsible for the characteristic aroma, colour and texture, synthesised by various parts of the plants. Depending on the chemical formulation, secondary metabolites can be identified as several types including, polyphenols, terpenoids and alkaloids.

Secondary metabolites of the plant material can also be responsible for the inhibitory effect on both gram positive and gram negative pathogenic bacterial growth. Among those, polyphenols have found to be capable of passing through the gastrointestinal tract unabsorbed while affecting only the intestinal microbiota. This leads to the inhibition of the growth of pathogenic bacteria in the intestinal microbiota (Othman, Sleiman and Abdel-Massih, 2019). Flavonoids and tannins have been identified as polyphenols which commonly exhibit antimicrobial properties by suppressing the bacterial virulence factors. Terpenoids, a group of secondary metabolites comprise of an extensive amount of plant metabolites which are well known to be capable of inhibiting the growth of gram negative and gram positive bacteria (Barbieri et al., 2017). The antimicrobial compounds are capable of inhibiting the microbial growth either by chemically interfering with the synthesis of bacterial cell wall or by evading the bacterial resistant mechanisms. Therefore, in order to inhibit the bacterial growth, antimicrobial substance should be targeted on the biosynthesis of cell wall and proteins, disruption of the bacterial cell membrane, inhibition of metabolic pathways or replication of deoxyribonucleic acid (DNA) (Khameneh et al., 2019).

**Nymphaea nouchali**

Among the species which are potential sources of important antimicrobial compounds, Nymphaea nouchali has gained attention as a promising candidate. Therefore, the study aims to demonstrate the microbial inhibitory activity of ethanolic plant extracts of the Sri Lankan

plant species, Nymphaea nouchali. Nymphaea nouchali (Burm.f), also known as water lily, which belongs to the family of Nymphaeaceae, is an aquatic herb, grows in natural waterbodies. Water lily has been used in treating disease conditions including diabetes, menstrual disorders and inflammation since ancient times (Raja, Sethiya and Mishra, 2010). Nymphaea nouchali which is the national flower of Bangladesh, is considered as an herb which, each part of the plant has a medical significance in treating numerous conditions. The flower of the plant is considered to be consisting of healing properties towards conditions such as piles, renal disorders and cardiovascular disorders. The whole plant is used in Ayurveda medicine in treating sores and ulcers (Anitha and Balaji, 2012). Through previous studies it has been revealed that, the seed of the plant to be containing antifungal, antioxidant, antidiabetic and antibacterial properties. It has been reported, the seeds of the plant are rich in phytochemical compounds including, tannins, flavonoids, phenols and alkaloids (Sarwar et al., 2016). A study conducted to determine the sensitivity of Escherichia coli against methanol, ethyl acetate and acetone extracts of water lily, has discovered that all the extracts have displayed certain degree of inhibitory activity against the pathogens, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia and Sarcina lutea. Also, Nymphaea nouchali has been in use as an important ingredient in Indian Ayurveda medical formulations from a long time (Raja, Sethiya and Mishra, 2010) According to Hossain et al. (2014), the flower extract of Nymphaea nouchali has found to be rich in cytotoxic properties while the ethyl acetate extract of the plant leaf has been found to be rich in antimicrobial properties against a broad spectrum of bacteria due to the confirmed presence of phytochemicals. Therefore, ethanol extract of Nymphaea nouchali could potentially have some inhibitory activity against pathogenic bacteria, due to the presence of secondary metabolites including saponins, flavonoids and tannins (Dash et al., 2013).

Based on the past evidences, the current study will be targeted on determining the capacity of the ethanolic extract of the flower of Nymphaea nouchali in inhibiting the growth of gram positive and gram negative bacteria in depth. Moreover, the presence of phytochemicals responsible for the inhibitory activity will be determined. The potential antimicrobial properties will be assessed using microbiology laboratory techniques including antibiotic susceptibility testing (ABST), minimum inhibitory concentration determination (MIC) using broth dilution method and minimum bactericidal concentration determination (MBC).

## *METHODOLOGY*

**Sample selection**

The flower of the plant Nymphaea nouchali, commonly known as Blue water lily, was chosen as the sample to be assessed. The selected samples were obtained from a local market in Colombo, Sri Lanka. The procedure was adopted and modified from the protocols by Dash et al., (2013).

Disinfection, drying and pulverization

The plant samples were cleaned with normal tap water and distilled water, then was disinfected using 70% Ethanol. Next, the samples were dried under the shade for two weeks with applied pressure alternatively throughout the time period. Completely dried samples were then pulverized using a clean grinder.

**Preparation of the Ethanolic plant extracts**

From the pulverized sample of Nymphaea nouchali, 20 g was weighed using an analytical balance and mixed with 50 ml of 70% ethanol in sterile falcon tubes. The tubes with the respective mixtures were sealed using parafilm and kept in the roller mixer (KJMR-II) for 48 hours continuously. The extracted mixtures were filtered using a clean muslin cloth. The filtrate was transferred to fresh sterile petri plates and kept open inside a fume hood (BIOBASE, FH1000) for one week. The dried, crude plant extracts were scraped using a glass rod and weighed using an analytical balance. The extracts were reconstituted with Dimethyl sulfoxide (DMSO) in order to prepare three separate stock concentrations of 50mg/ml, 100mg/ml and 200 mg/ml (w/v).

**Preparation of the 0.5 McFarland standard**

Using an analytical balance, 0.1g of BaCl2 (HiMedia) (w/v) was weighed and added to a measuring cylinder. By adding distilled water, the solution was topped up to the level of 10 ml. The solution was mixed well and transferred to a fresh test tube. To a separate measuring cylinder 9.9 ml of distilled water was measured and transferred to a fresh test tube. To the test tube, 0.1 ml of absolute H2SO4 was added. For the preparation of 0.5 McFarland standard, to a separate fresh test tube, 0.05 ml from the BaCl2 solution and 9.95 ml of the H2SO4 solution which were prepared previously, was added. The resulting solution was used as the reference for the preparation of bacterial inoculums.

**Preparation of the sub cultures and the diluted bacterial suspensions**

Lysogeny broth (LB) was prepared by adding 3.29 g of lysogeny broth powder (HiMedia) into 90 ml of distilled water. The solution was mixed well and autoclaved at 15 lbs pressure (121℃) for 15 minutes. Two 15ml falcon tubes were taken to which, 5ml of the lysogeny broth was added. To each tube, three loops full of the bacteria Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923) taken from the respective stock cultures and were inoculated separately. The sub cultures were incubated overnight at 37℃. 0.5 McFarland standard was prepared. From the sub cultures of each bacteria, bacterial suspensions were prepared by dissolving the bacteria in 10 ml of distilled water and standardized to 0.5 McFarland standard by comparing the turbidity.

**Antibiotic susceptibility testing (ABST) using well diffusion method**

From Muller Hinton agar powder (HiMedia), 14.06g was weighed using an analytical balance and added to a conical flask containing 370 ml of distilled water. The solution was mixed well and autoclaved for 15 minutes at 15 lbs pressure (121℃). Autoclaved media was poured in to sterile petri plates and allowed to solidify at room temperature.

The prepared bacterial suspensions for Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923) were swabbed using sterile cotton swabs on solidified agar. On the seeded agar plates, wells were created using a sterile pipette tip disinfected with 70% Ethanol, as shown in figure 01. Each well was loaded with 50µl of the respective plant extracts of concentrations 50 mg/ml and 100 mg/ml. Distilled water was used as the negative control while, 1mg/ml concentrated Gentamycin solution was used as the positive control. The plates were sealed using parafilm and incubated overnight at 37 ℃. The inhibition zones were observed next day and were measured to the nearest millimeter using a ruler (Dahiya and Purkayastha, 2012).

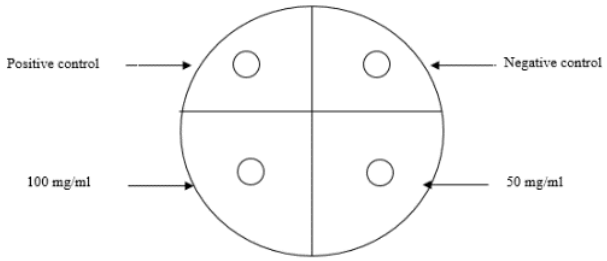



Figure 01 Layout of the petri plate for well diffusion

**Minimum Inhibitory Concentration (MIC) Determination**

Using a digital balance, 5.67 g of Mueller Hinton broth powder (HiMedia) was weighed and added to 77 ml of distilled water. The solution was mixed well and autoclaved for 15 minutes at 15 lbs pressure (121℃). The Ethanolic plant extract of the samples were reconstituted in order to obtain a series of concentrations with the highest concentration being 200 mg/ml followed by 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml. 1 ml from the respective reconstituted dilution of the extracts were added to each test tube containing 900 µl of Mueller Hinton broth. To end with, 100 µl of the prepared bacterial suspensions for Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923) were added accordingly. The final plant extract concentrations were 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml respectively. The tubes were incubated for 18-24 hours at 37 ℃. Positive controls were prepared by adding 1 mg/ml Gentamycin solution to 1 ml of broth seeded with the two bacterial strains. Negative controls were prepared using 1 ml of seeded broth. A sterility control to check quality of media was kept containing only 1 ml media.

**Minimum Bactericidal Concentration (MBC) determination**

Fresh plates of Tryptone soy agar were prepared by adding 33.2g of Tryptone soy agar powder (HiMedia) to 830 ml of distilled water. The mixture was autoclaved at 15lbs pressure for 15 minutes at 121℃. After cooling down to the room temperature, the mixture was poured in to sterile petri plates. The tubes with no visible growth in minimum bactericidal concentration determination were selected and streaked using a sterile inoculation loop on Tryptone soy agar plate surface. The plates were sealed using the parafilm and incubated for 18-24 hours at 37 ℃. The corresponding lowest concentration after minimum inhibitory concentration that had killed 99.9% of the bacteria or complete growth inhibition was determined as the minimum bactericidal concentration.

**Statistical analysis**

The raw data was obtained for three replicates for each experiment conducted and were analysed statistically. Graphs for the antibiotic susceptibility testing were generated using Graph Pad Prism Version 8.4.3 software. The concentrations were used as the independent variables and inhibition zone lengths were the dependent variables. The two – way ANOVA statistical analysis was carried out using IBM SPSS statistic data editor Version 21.0 software for the determination of the interaction between the concentrations of the test antibiotic with the two bacterial strains. Bacterial strains and the concentrations were used as the fixed variables while the inhibition zone lengths were used as the dependent variables. The analysis was conducted with a 95% confidence interval and the significance of the results were determined using the P – value ($P < 0.05$). The data represent, mean ± standard deviation.

## *RESULTS*

Results for antibiotic susceptibility testing (ABST) for Nymphaea nouchali

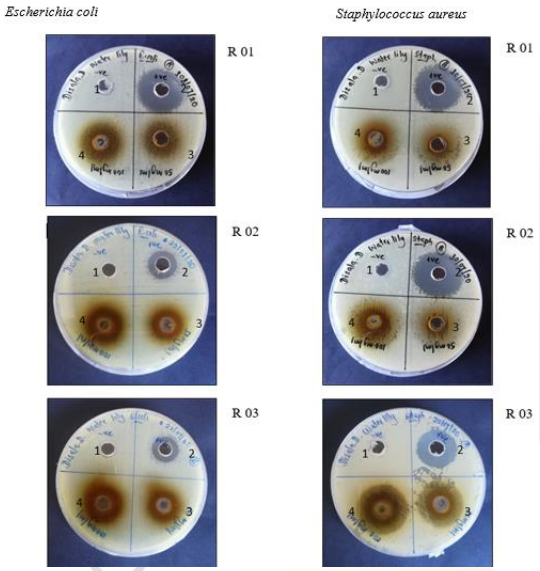



Figure 02 Inhibition zones produced by Nymphaea nouchali against Escherichia coli and Staphylococcus aureus. The wells 1, 2, 3 and 4 represents negative control, positive control, 50mg/ml, 100mg/ml concentrations of the plant extract respectively. (R = Replicate).

**Inhibition zone lengths from antibiotic susceptibility testing analysis**

Table 1.0 Zones of inhibition produced by Nymphaea nouchali (Mean ± SD)

| Bacteria | Concentrations (mg/ml) | | | |
|---|---|---|---|---|
| | 50 mg/ml | 100 mg/ml | Negative control | Positive control |
| *Escherichia coli* Zone length (mm) | 18.667± 1.247 | 21.667± 2.054 | 00.00 | 19.667± 3.091 |
| *Staphylococcus aureus* Zone length (mm) | 24.667± 0.471 | 26.334± 0.471 | 00.00 | 26.334± 2.494 |

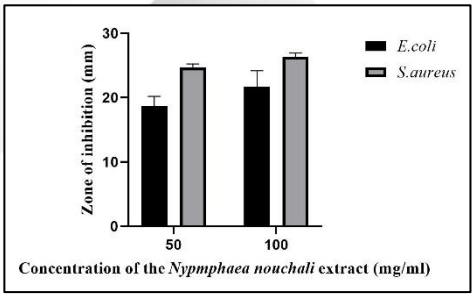



*Figure 03* Antibacterial activity of Nymphaea nouchali against Escherichia coli and Staphylococcus aureus at 50mg/ml and 100mg/ml concentrations. The grey and white bars each represents the mean ± SD of the inhibition zones expressed against Escherichia coli and Staphylococcus aureus.

Table 2.0 Two – way ANOVA test for Nymphaea nouchali

| Source | Type III Sum of Squares | df | Mean square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 103.000[a] | 3 | 34.333 | 14.714 | .001 |
| Intercept | 6256.333 | 1 | 6256.333 | 2681.286 | .000 |
| Bacteria | 85.333 | 1 | 85.333 | 36.571 | .000 |
| Concentration | 16.333 | 1 | 16.333 | 7.000 | .029 |
| Bacteria*Concentration | 1.333 | 1 | 1.333 | .571 | .471 |
| Error | 18.667 | 8 | 2.333 | | |
| Total | 6378.000 | 12 | | | |
| Corrected Total | 121.667 | 11 | | | |

R Squared = .847 (Adjusted R Squared = .789)

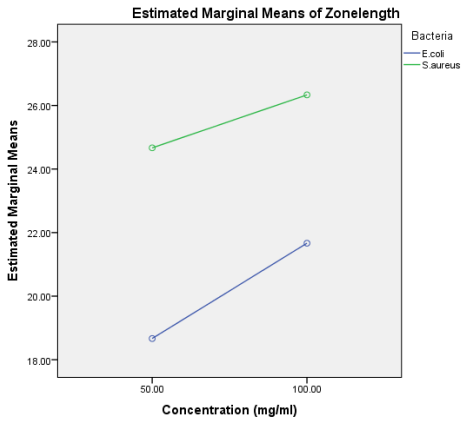



*Figure 04* The profile plot of the two way ANOVA analysis for Nymphaea nouchali.

The two-way ANOVA test resulted a significant difference between the zone lengths for two bacterial strains and a significant difference was observed for the inhibitory activity between the two concentrations by giving a $P < 0.05$ (Table 2.0). This was further proven by the ANOVA plot in Figure 04. The increase in the concentration of the plant extracts seems to have a main effect on both bacterial strains. However, there is no interaction effect observed between the bacterial strains and plant extracts since $P > 0.05$.

**Results for Minimum Inhibitory Concentration (MIC) determination of Nymphaea nouchali**

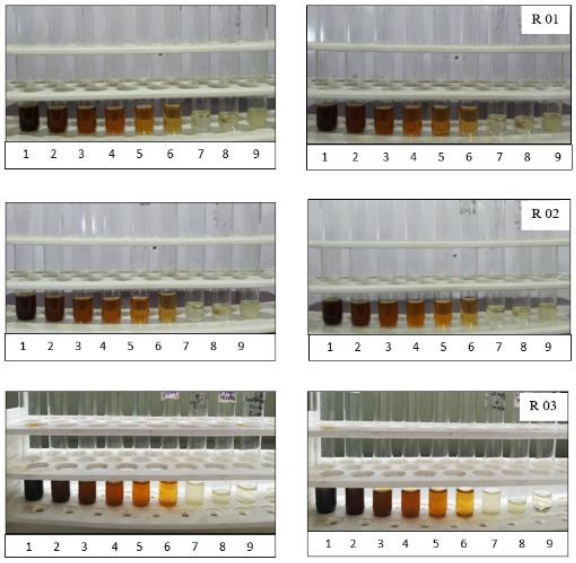



*Figure 05* Serial dilution of Nymphaea nouchali for the minimum inhibitory concentration determination against Escherichia coli and Staphylococcus aureus. The dilutions labeled from 1-6 are

100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml respectively. Labeled from 7-9 are positive control, sterility control and media seeded with bacteria respectively. (Replicate = R)

Table 3.0 Growth pattern of Escherichia coli and Staphylococcus aureus using different concentrations of Nymphaea nouchali extracts.

| Name of bacteria | Replicate No. | Concentrations of the plant extracts (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3.125 | 6.25 | 12.5 | 25.0 | 50.0 | 100.0 |
| *E. coli* | R1 | + | + | _ | _ | _ | _ |
| | R2 | + | + | _ | _ | _ | _ |
| | R3 | + | + | _ | _ | _ | _ |
| *S.aureus* | R1 | + | + | + | _ | _ | _ |
| | R2 | + | + | + | _ | _ | _ |
| | R3 | + | + | + | _ | _ | _ |

Key

\- No visible growth

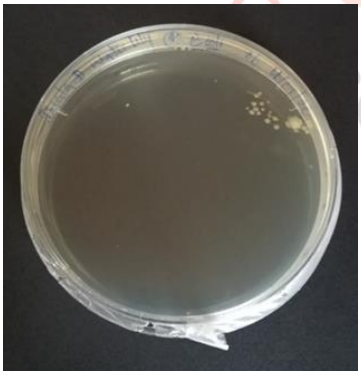
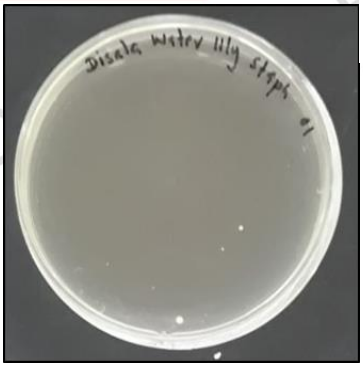
\+ Visible growth

R Replicate

Against Escherichia coli, Nymphaea nouchali expressed bacteriostatic properties at a lower concentration of 12.5 mg/ml, whereas against Staphylococcus aureus, bacteriostatic properties were observed at a concentration of 25 mg/ml.

**Results for Minimum Bactericidal Concentration (MBC) determination**

Table 4.0 Results for the minimum bactericidal concentration determination of Nymphaea nouchali against Escherichia coli and Staphylococcus aureus.

| ***Nymphaea nouchali*** | |
|---|---|
| *Escherichia coli* | *Staphylococcus aureus* |
|  50 mg/ml |  100 |

Nymphaea nouchali extract exhibited bactericidal properties against Escherichia coli at a concentration of 50 mg/ml while, against Staphylococcus aureus, bactericidal properties were exhibited at a concentration of 100 mg/ml. Therefore, when compared the two bacteria, overall greater inhibitory activity was observed against Escherichia coli.

## *DISCUSSION*

Due to the inclined growth of antimicrobial resistances, several efforts have been taken in the identification of novel antimicrobial compounds. Since ancient times, the suitability of phytochemical components which are synthesized by the plants in the application of treating pathogenic illnesses have been well determined. Therefore, the incorporation of natural plant components in the process of the antimicrobial drug discovery has currently gained much attention (Rossiter, Fletcher and Wuest, 2017). Hence, Nymphaea nouchali, an herb frequently used in indigenous medicine has gained attraction for its higher antimicrobial properties currently.

Antibiotic susceptibility testing, minimum inhibitory concentration and minimum bactericidal concentration was determined in plant extracts for the determination of antimicrobial properties exhibited by the plant against the two bacterial strains Escherichia coli and Staphylococcus aureus. For the extraction of phytochemicals, the solvent was selected according to the polarity of the solute (Altemimi et al., 2017). A qualitative analysis of the antimicrobial properties were carried out using antibiotic susceptibility testing. The antibacterial activities were quantitatively determined through minimum inhibitory concentration and minimum bactericidal concentration testing. The minimum concentration of the test antibiotic which inhibits the visible microbial growth showing bacteriostatic properties was determined using minimum inhibitory concentration determination (Owuama, 2017). Using minimum bactericidal concentration determination test, the minimum concentration of the test antibiotic required to kill 99.9% of the bacterial growth, showing bactericidal properties was determined (Debalke et al., 2018).

Through antimicrobial susceptibility testing, both the bacterial strains Escherichia coli and Staphylococcus aureus were found to be sensitive towards the phytochemical extract of Nymphaea nouchali. As shown in figure 02 and table 1.0, a greater inhibitory activity was expressed against Staphylococcus aureus than Escherichia coli at both 50mg/ml and 100mg/ml concentrations. The overall inhibitory activity against each of the bacterial strains increased noticeably by doubling the concentration, proving the presence of phytochemicals responsible for the inhibitory properties (Dash et al., 2013). When compared with the positive control, Nymphaea nouchali exhibited a greater level of inhibitory activity which was closer to that of Gentamycin. According to Dash et al., (2013) a similar inhibitory property of 20.00 ± 0.60 mm has been expressed at 4090 µg/ml concentration, by the methanol extract of the plant against Escherichia coli. According to a study conducted by Parimala and Shoba (2014) the ethanol extract of Nymphaea nouchali seeds has shown susceptibility against both Escherichia coli and Staphylococcus aureus. At the highest concentration of 1000 µg/ml, the greatest inhibitory activity has shown against Staphylococcus aureus resulting an inhibition zone of length 20 mm whereas, against Escherichia coli, the plant extract has expressed a zone of inhibition of 15 mm. When comparing the results with the current study, it is observable that a similar inhibitory pattern was expressed by the plant extract at 100

mg/ml concentration against both bacteria. In addition, a slightly lesser inhibitory activity has expressed against Escherichia coli than that of Staphylococcus aureus in both the instances. No contaminations were observed in the negative control. When tested for the minimum inhibitory concentration 12.5 mg/ml and 50 mg/ml showed inhibition of bacterial growth when visibly observed for Escherichia coli and Staphylococcus aureus respectively. The determination of minimum bactericidal concentration, Nymphaea nouchali exhibited bactericidal properties against Escherichia coli and Staphylococcus aureus at 50 mg/ml and 100 mg/ml concentrations respectively showing a greater sensitivity towards gram negative bacteria. Therefore, the plant extract was found to be susceptible against both gram negative and gram positive bacteria, which revealed the presence of phytochemicals responsible for the antimicrobial properties.

According to Parimala and Shoba (2014), it has been revealed the presence of phytochemical compounds such as phenols and polyphenols which are secondary metabolites of the plant, responsible for the antimicrobial properties in the extract of Nymphaea nouchali. Among polyphenolic compounds, tannins and flavonoids are considered to have a broad spectrum of antibacterial activity due to the ability to suppress the virulence factors in microbial genome of pathogens. Phenolic compounds including Gallic acid, catechin and quercetin has been found to be highly bioactive due to its ability in inhibition of pathogenic bacterial activity (Takó et al., 2020). Against both gram negative and gram positive bacteria, Gallic acid has been found to be showing a greater sensitivity by making irreversible alterations in the hydrophobicity of the bacterial cell wall.

According to ANOVA statistical analysis shown in figure 04 and table 2.0, the plant extract of Nymphaea nouchali expressed a significant difference between the inhibitory activity between the two bacterial strains and between the two concentrations by showing a P value lesser than 0.05. The difference in the susceptibility pattern against antimicrobial compounds occur due to the structural deviation of the cell wall among gram negative and gram positive bacteria. Gram positive bacteria, surrounded by multiple layers of peptidoglycan forming a thick outer covering provides protection to antimicrobial compounds which are active upon cell wall. On the other hand, gram negative bacteria with outer lipopolysaccharide membrane which lacks thick peptidoglycan layer, allows selective penetration of antibiotics depending on the polarity and molecular weight, to the targeted site (Silhavy, Kahne and Walker, 2010) Therefore, as observed in minimum bactericidal concentration determination (table 4.0) unlike in gram positive bacteria, a greater sensitivity can be observed in gram negative bacteria towards the antimicrobial substances, due to lower resistance provided by the lack of peptidoglycan layer (Guimarães et al., 2019).

It was observed that the inhibitory activity shown by the freshly extracted samples were observed to be high in antibacterial activity. Therefore, by using samples with a lesser storage time could improve the results significantly. However, further steps are required for the quantitative assessment since, the antibiotic susceptibility testing and minimum inhibitory concentration determination can be affected by the rate of diffusion in agar and the degree of solubility in media. Since the sample expressed promising inhibitory activity through further steps of purification, the active components of the plant can be isolated and used in further analysis for drug development.

For a better understanding of the potent inhibitory activity of the plant sample, phytochemical extraction can be carried out using different solvents such as methanol, acetone and chloroform. Increasing the concentrations of the plant sample also could be effective in achieving efficient pharmacokinetic and pharmacodynamics profiles. The isolated compounds could also be tested on different bacterial strains and on clinical isolates. By optimizing the protocols to isolate the active compounds responsible for the inhibitory properties, further analysis can be conducted to determine the suitability of compounds to be developed into a consumable drug. To develop the natural antimicrobial components into drugs, several steps including in vitro assays, in vivo assays to test on animal models and clinical trials can be proceeded. As for in vitro studies, the synergic effect of the test antibiotic in combination with another antibiotic can be determined using the fractional inhibitory concentration (FIC) index (Hughes et al., 2011; Andrade et al., 2016). The metabolic viability of cells could be determined using the 3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a high throughput screening method (Rai et al., 2018). The inhibition of biofilm production of the bacterial strains could also be analyzed. An in silico approach for drug designing could be utilized which can target other bacterial strains and model the compounds using several computational approaches. As the final stage, the drugs can be studied on humans during clinical trials in four phases I, II, III and IV for the determination of the effectiveness in treating a particular condition and using different computational approaches and bioinformatics tools, the toxicity of the test antibiotic and its lethal dose can be determined through Lethal Dose 50 (LD50) and Lethal Concentration 50 (LC50) assays (Parasuraman, 2011; Yang et al., 2018).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