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DETECTION AND DETERMINATION OF EFFECT OF ANTIBACTERIAL ACTIVITY OF AZADIRACHTA INDICA (NEEM) AGAINST ESCHERICHIA COLI AND STAPHYLOCOCCUS AUREUS BACTERIA

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ABSTRACT

Antibiotic resistance is a global crisis threatening the human population. In the past few decades, the number of bacteria that are resistant to multiple antibiotics have increased, making the treatment complicated, costly and leading to increased mortality. Therefore, it is fundamental to discover new drugs that plays an important role in treating bacterial infection. Medicinal plants contain active compounds to treat bacterial infection with novel mechanism of action. This study was aimed to evaluate the antibacterial activity of ethanolic extract of Azadirachta indica (Neem) against Escherichia coli and Staphylococcus aureus. Neem belongs to Meliaceae family and the active ingredients in neem play an important role in disease prevention and treatment. The antibacterial activity of neem was determined by well diffusion method against the selected pathogens. Two different concentrations of Azadirachta indica (Neem) were used in this study. The results were statistically analyzed by Student's T test. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also recorded. Neem extract at 50 mg/ml inhibited the growth of Escherichia coli and Staphylococcus aureus with zone of inhibition respectively 11.67±0.94 mm and 12.33±0.94 mm. Neem extract at 100 mg/ml inhibited the growth of Escherichia coli and Staphylococcus aureus with inhibition zones respectively of 12.33±0.94 mm and 12.67±1.25 mm.The MIC and MBC value of neem extract against Escherichia coli and Staphylococcus aureus was 25 mg/ml and 100 mg/ml. Further studies can be conducted in order to identify the novel compounds that was extracted from this plant sample which could be used to formulate potent antibacterial compounds against bacterial pathogens.

Keywords: Azadirachta indica (Neem), Antibacterial activity, Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), Antibiotic resistance

INTRODUCTION

Antibiotic resistance

Antibiotic resistance is a global problem affecting the human population associated with high morbidity and mortality. Antibiotic resistance has increased due to excessive usage of antibiotics and lack of new drug development (Bhalodia and Shukla, 2011). The era of antibiotics originated since Sir Alexander Fleming's discovery of penicillin (Ventola, 2015). The period from 1930 to 1960 was known as the Golden era of antibiotics. In this era pharmacological industries had produced numerous antibiotics and it was frequently prescribed to treat infectious diseases and this period ended due the emergence of antibiotic resistance (Ventola, 2015). Antibiotic resistance has reduced the efficacy of the antibiotics. This results the

treatment to be complicated, costly and difficult to execute (Lushniak, 2014). Individuals who consistently use antibiotics causes the spread of resistant bacteria in the community. Most of the antibiotics belong to the same class of drugs, therefore resistant to one antibiotic may result in resistant to the whole class of drugs. Antibiotic resistant microbes can affect human health, animal health and agriculture (WHO, 2020). According to Research and Development Corporation of US, the analysts have reported that in the future the world will experience a lack of potent antimicrobial agents to treat infectious diseases (Aslam et al., 2018).

Staphylococcus aureus and Escherichia coli

Staphylococcus aureus and Escherichia coli are opportunistic pathogen that leads to several infections such as urinary tract infection, skin infection and respiratory infection (Wimmerstedt and Kahlmeter, 2008). The disease severity various depending on the virulence factor of the pathogen. To cause an infection Escherichia coli and Staphylococcus aureus expresses the virulence factors such as enymes, toxins, surface proteins and adhesins (Frieri, Kumar and Boutin, 2016). The production of enzymes and toxins were controlled by numerous antibiotics. However, antibiotic resistance had limited the therapeutic options to treat bacterial infection (Frieri, Kumar and Boutin, 2016). To overcome this crisis drugs produced from natural sources such as medicinal plants, herbs and spices can be utilized to treat microbial diseases.

Role of medicinal plants

The connection between man and plants have evolved throughout history. There are almost 300,000 plants around the world but only 15% of the plants have been investigated to determine the pharmacological properties (Palhares et al., 2015). Medicinal plants are rich source of secondary metabolites which has been used as an alternative medicine from ancient times (Subramani, Narayanasamy and Feussner, 2017). In the developing countries 60 to 80% of the population use medicinal plants as a remedy to treat diseases (Palhares et al., 2015). According to World Health Organization, medicinal plants are one of the best source to produce antimicrobial drugs (Manandhar, Luitel and Dahal, 2019). The secondary metabolites present in medicinal plants are alkaloids, flavonoids, tannins and phenolic acids. These secondary metabolites possess a wide range of health benefits such as antimicrobial, anti-carcinogenic and anti-mutagenic (Subramani, Narayanasamy and Feussner, 2017). The secondary metabolites are present in almost all the cells but in different concentrations. The concentration varies depending on the plant part, climate change and growth phase. Generally, leaf is accumulated with high concentration of secondary metabolite than other parts of the plants (Subramani, Narayanasamy and Feussner, 2017). Recently, several studies have carried out to discover plant derived antibacterial compounds against multi-drug resistant human pathogens.

Azadirachta indica (Neem)

Neem is a fast growing tree which belongs to Meliaceae family. Neem is a plant that has been used in Ayurvedha, Homeopathic medicine and Unani (Alzohairy, 2016). United Nations has declared neem as the "Tree of 21st Century" (Gupta et al., 2017). The parts of neem have been used in traditional medicine to treat various diseases. For example, in India neem has been used to treat smallpox, gastrointestinal problems, diabetes, leprosy, hair problems and ulcers. In Indonesia it has been used to treat diuretics, headache, diabetes and heartburn (Gupta et al., 2017). Neem is considered as the storehouse of phytochemicals. Up to now more than 300 phytochemicals have been discovered and they are chemically diverse and

structurally complex (Gupta et al., 2017). The active compound in neem is azadirachtin (Alzohairy, 2016). Other compounds in neem are nimbidol, salannin, nimbidin, nimbanene and ascorbic acid. The active compounds in neem are responsible for the therapeutic properties such as anti-oxidant, antibacterial, anti-fungal and anti-inflammatory activity (Alzohairy, 2016). A study indicated that the crude extraction of neem consist of antibacterial compounds that can be used to treat infection in eyes and ears (Herrera-Calderon et al., 2019). The active compound in neem play a vital role in damaging the cell wall of the bacteria. Therefore, it inhibits the growth of bacteria. Moreover, azadirchtin has the ability to inhibit the DNA topoisomerase II (Shaila, Sumaiya and Laisa, 2016). A study indicated that the active compounds in neem has antibacterial property against gram positive and gram negative bacteria. However, it is less effective against gram positive bacteria than gram negative bacteria (Adamu et al., 2019). Another study showed that aqueous extract of neem leaf indicated high antibacterial activity than stem and bark. This is due to presence of azadirachtins, β-sitosterol and quercetin in neem leaves (Raja et al., 2013).

Significance of the project

Antibiotic resistance is a global threat to human health, animal, and environment. This is due to the spread of multi drug resistant bacteria throughout the world. Multi drug resistant strains have led to economic loss and loss of life (Michael, Dominey-Howes and Labbate, 2014). Around the world many organizations have recognized this burden and have recommended solutions to the global health problem (Michael, Dominey-Howes and Labbate, 2014). Antibiotic resistance is a consistent and a prevailing issue. Therefore, it is essential to discover new strategies to combat bacterial infections. Drugs derived from medicinal plants have been used as a remedy to treat various disease. Medicinal plants consist of various phytochemicals that can be used as an alternative to treat bacterial infections with improved safety and efficacy (Khan et al., 2013). In this study, Azadirachta india (Neem) has been used to investigate the antibacterial properties. The study will be conducted under the hypothesis that the ethanolic extract of neem can yield phytochemicals with antibacterial property that Staphylococcus aureus and Escherichia coli bacterial strains will be sensitive against. The purpose of this study is to detect the antibacterial activity of neem against Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923).

METHODOLOGY

Sample collection

The plant sample used in this study were leaves of Azadirachta indica (Neem) collected from home gardens in Colombo. Freshly collected leaves from neem were washed with tap water and distilled water and it was disinfected with 70% Ethanol. It was allowed to dry in shade for 4 days with exerted pressure. The dried neem leaves were pulverized into a fine powder and 50 g of powdered neem was measured and it was mixed with 50 ml of 95% Ethanol. The neem extract mixture in falcon tube was kept on rotary mixer for 48 hours. The extract was filtered through muslin cloth and it was evaporated under fume hood till all the ethanol was evaporated. The extract obtained was stored at 4°C.

Preparation of bacteria

Bacterial cultures of Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923) were obtained from Medical research institute Sri Lanka. The stock culture was maintained at 4°C and subcultures were prepared freshly from

stock cultures for further experiments prior to each procedure.

Preparation of neem extract stock solution for antibiotic susceptibility testing and minimal inhibitory concentration determination To prepare the neem extract 5.04g of the evaporated sample was measured and 25.2 ml of DMSO was added. The reconstituted solution concentration 200 mg/ml neem extract was obtained and was stored at 4°C. The 200 mg/ml plant extract was subjected to a serial dilution using DMSO to obtain different concentrations of plant extract such as 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml. Preparation of 0.5 McFarland Standard

To prepare 1% BaCl2, 0.1 g of BaCl2 was measured and it was mixed with 9.9 ml of distilled water. To prepare 1% H2SO4, 1 ml of Conc. H2SO4 was mixed with 9 ml of distilled water. To prepare 0.5 McFarland standard, 0.05 ml of 1% BaCl2 was mixed with 9.95 ml of 1% H2SO4.

Preparation of bacterial cultures

To prepare subculture of Escherichia coli and Staphylococcus aureus, 15 ml of Muller Hinton Broth (HiMedia) was prepared according to manufacturer's instruction. 0.315 g of Muller Hinton Broth was suspended in 15 ml of distilled water. It was mixed well and was autoclaved at 121°C for 15 minutes. Then, a loopful of stock cultures of Escherichia coli and Staphylococcus aureus was transferred to broth and it was incubated at 37°C for 24 hours. To dilute overnight cultures of Escherichia coli and Staphylococcus aureus required amount was added to distilled water corresponding to the turbidity of 0.5 McFarland Standard.

Antibiotic susceptibility testing using well diffusion method

Muller Hinton agar (HiMedia) was prepared according to manufacturer's instruction. To prepare Muller Hinton Agar, 11.4 g of Muller Hinton Agar was suspended in 300 ml of distilled water. It was mixed well and it was autoclaved at 121°C for 15 minutes. The Muller Hinton agar was poured into petri plates and it was let to solidify. Fresh overnight cultures of Escherichia coli and Staphylococcus aureus was used to prepare bacterial dilution and was swabbed uniformly on Muller Hinton agar plates using a sterile cotton swab. It was allowed to dry for 5 minutes. 1000 µl tip was sterilized and it was used to make four wells of 6 mm in diameter on the agar plate. Then 50 µl of 100 mg/ml neem extract and 50 µl of 50 mg/ml neem extract, 50 µl of 1 mg/ml gentamycin solution and 50 µl of distilled water was added to each of the four wells. The plates were sealed and it was incubated at 37°C for 24 hours. Results were recorded the following day. This procedure was modified by Abiy and Berhe, 2016.

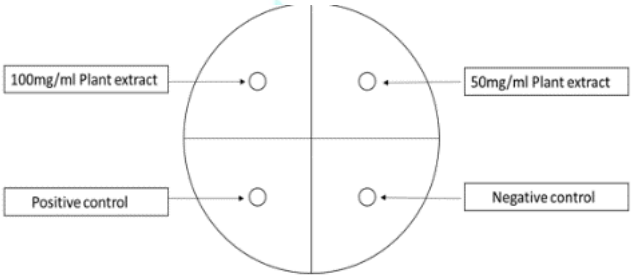

Figure 1 Petri dish layout used for well diffusion

Determination of Minimum inhibitory concentration (MIC)

For minimum inhibitory concentration (MIC) determination, 1 ml of Muller Hinton Broth and 1ml of each diluted neem extract concentrations were added to each of the six tubes. The plant extract of neem sample was reconstituted in order to obtain a series of concentrations with the highest concentration 200 mg/ml followed by 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml. Diluted bacterial suspension of Escherichia coli and Staphylococcus aureus was prepared by comparison with the 0.5 McFarland

Standard turbidity. Then 100 µl of this was added to tubes and it was incubated at 37°C for 24 hours. Positive control was prepared using 2 ml of Muller Hinton Broth with 200 µl of gentamycin solution (1mg/ml) and 100 µl of bacterial dilution was added. 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. To prepare negative control, 2 ml of seeded Broth was made by adding 200 µl of bacterial dilution. A media control was prepared to check sterility with 2 ml broth only. This procedure was modified from Stanley et al., 2014.

Minimum bactericidal concentration (MBC)

Fresh Tryptone Soy Agar (HiMedia) was prepared according to manufacturer's instruction. To prepare Tryptone Soy Agar, 18 g of Tryptone Soy Agar was suspended in 450 ml of distilled water. It was mixed well and it was autoclaved at 121°C for 15 minutes. The Tryptone Soy Agar was cooled down and the medium was poured to sterile petri plates. From the six dilutions used in minimum inhibitory concentration (MIC) test, the clear tubes with no visible bacterial growth were selected for minimum bactericidal concentration (MBC) since other tubes had more turbidity which indicates bacterial growth. The clear tubes were streaked on Tryptone Soy Agar plates using a sterile inoculation loop and it was incubated at 37°C for 24 hours. The plates were observed the following day and the lowest concentration corresponding to the plate that had 99% of the bacteria killed was presumed as the minimum bactericidal concentration (MBC).

Analysis of the results

The results of the experiments were recorded for triplicates and were analyzed by using GraphPad Prism version 8.4.3 software. The data represent mean and ± standard deviation. The significant difference was determined by using Student's t-test for a 95% confidence interval for a P-value less than 0.05.

RESULTS

Neem extract yield

The extraction yield of Azadirachta indica (Neem) was calculated using the following equation % yield of extract = (weight of extract/weight of sample) x 100%. The yield of extract from plants had a potential effect on the overall efficacy on antibacterial activity. In this study, neem had a yield of 3.34%.

Yield of extract = (1.67/50) X 100% =3.34%

Antibacterial activity of Azadirachta indica (Neem) against Escherichia coli and Staphylococcus aureus bacteria

Neem extract exhibited inhibitory effect against both Escherichia coli and Staphylococcus aureus. Neem extract against Staphylococcus aureus had the maximum zone of inhibition (12.67±1.25 mm) at 100 mg/ml and the minimum zone of inhibition at 50 mg/ml. Neem extract against Escherichia coli showed the highest activity (12.33±0.94 mm) at 100 mg/ml, followed by the lowest activity (1167±0.94 mm) at 50 mg/ml.

Microorganisms	Concentration	Zone of inhibition (mean±SD)
Escherichia coli	50 mg/ml	11.67±0.94
	100 mg/ml	12.33±0.94
Staphylococcus aureus	50 mg/ml	12.33±0.94
	100 mg/ml	12.67±1.25

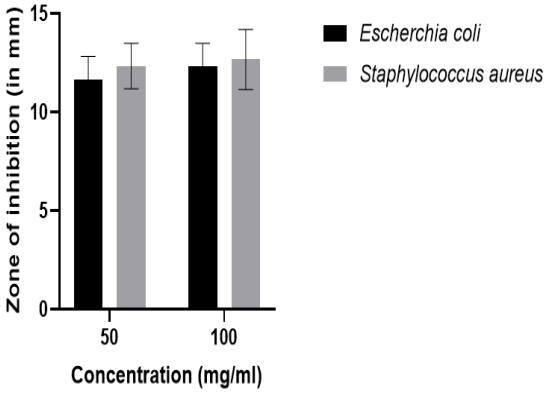

Figure 2 Results of antibiotic susceptibility testing of neem extract against Escherichia coli (black bars) and Staphylococcus aureus (grey bars). The data represent mean ± SD for three replicates

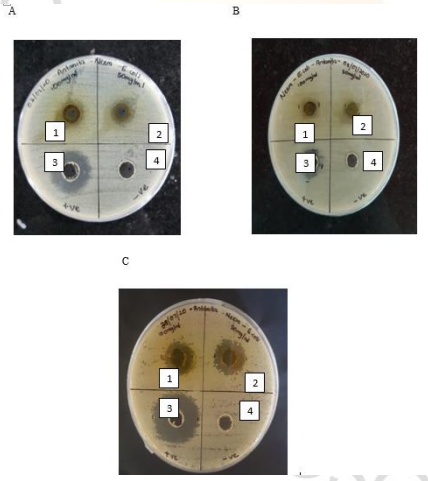

Figure 3 a, b and c shows the three replicates of the well diffusion of neem extract against Escherichia coli. 1-100 mg/ml extract 2-50 mg/ml extract 3-Positive control (Gentamycin) 4-Negative control (Distilled water)

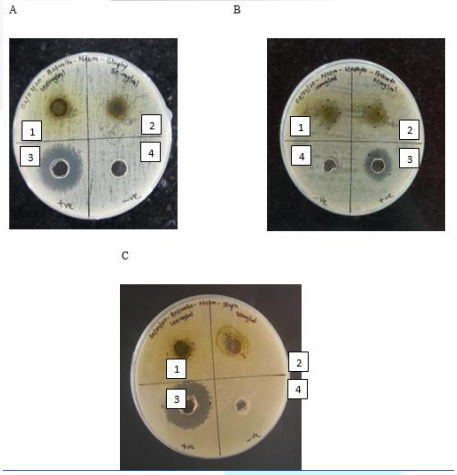

Figure 4 a, b and c shows the three replicates of the well diffusion of neem extract against Staphylococcus aureus. 1-100 mg/ml extract 2-50 mg/ml extract 3-Positive control (Gentamycin) 4-Negative control (Distilled water)

Unpaired T test									
	P value	Significantly different (P<0.05) ?	t	df	Difference between mean	Std. Error Mean	95% Confidence interval		R squared value
							Lower	Upper	
Escherichia coli	0.5185	No	0.7071	4	0.6667	0.9428	-1.951	3.284	0.1111
Staphylococcus aureus	0.7780	No	0.3015	4	0.3333	1.106	-2.736	3.403	0.02222

Table 2 Results of unpaired T test analysis for neem extract against Escherichia coli and Staphylococcus aureus

The two different concentrations of neem extract were analyzed by students T test and the P value was determined for a 95% confidence interval. Students T test result of neem extract showed that there is no significant difference among the two different concentrations against both Escherichia coli and Staphylococcus aureus. The p value of Escherichia coli was 0.5185 and the p value of Staphylococcus aureus was 0.7780.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is the lowest concentration that inhibits the growth of microorganisms. In this study the minimum inhibitory concentration of neem extract was determined by broth dilution method. Neem extract exhibited bacteriostatic effect ranging from 100mg/ml to 25mg/ml against Escherichia coli and Staphylococcus aureus.

Table 3 Growth inhibition observed Escherichia coli and Staphylococcus aureus treated with neem extract.

Plant extract	**Concentration (mg/ml)**	**Test Organisms**	
		Escherichia coli	***Staphylococcus aureus***
Azadirachta indica (Neem)	100	-	-
	50	-	-
	25	-	-
	12.5	+	+
	6.25	+	+
	3.125	+	+

Key: + growth, - no growth

The MIC value of neem extract was 25mg/ml against both Escherichia coli and Staphylococcus aureus. In figure 5, the two-fold serial dilution images of neem is included.

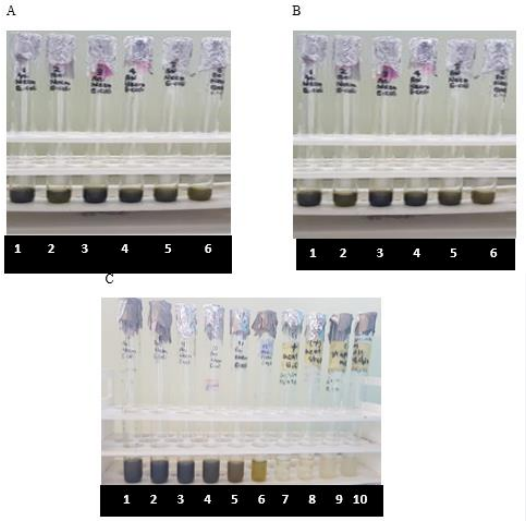

Figure 5 Serial dilution of neem extract to detect MIC against Escherichia coli. Figure 5a, 5b and 5c shows the three replicates of serial dilution to detect MIC of neem extract against Escherichia coli.

1-100 mg/ml extract 2-50 mg/ml extract 3-25 mg/ml extract 4-12.5 mg/ml extract

5-6.25 mg/ml extract 6-3.125 mg/ml extract 7-Gentamycin with Escherichia coli

8-Gentamycin with Staphylococcus aureus 9-Media with Staphylococcus aureus

10-Media with Escherichia coli

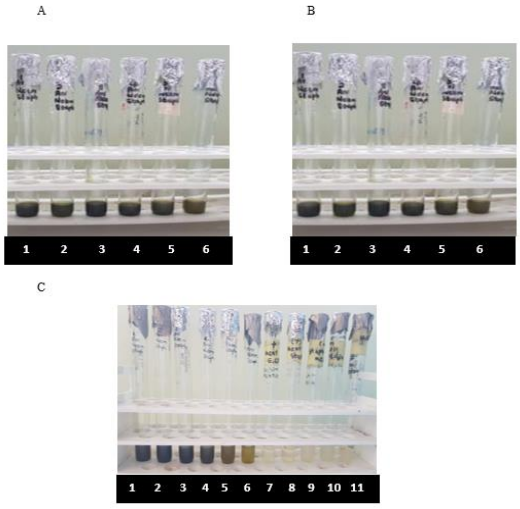

Figure 6 Serial dilution of neem extract to detect MIC against Staphylococcus aureus Figure 6a, 6b and 6c shows the three replicates of serial dilution to detect MIC of neem extract against Staphylococcus aureus

1-100 mg/ml extract 2-50 mg/ml extract 3- 25 mg/ml extract 4-12.5 mg/ml extract

5-6.25 mg/ml extract 6-3.125 mg/ml extract 7-Gentamycin with Escherichia coli

8-Gentamycin with Staphylococcus aureus 9-Media control

10-Media with Escherichia coli 11-Media with Staphylococcus aureus

Minimum bactericidal concentration (MBC)

Table 4 Growth pattern observed in agar plates of Escherichia coli and Staphylococcus aureus treated with the neem extracts used in this study

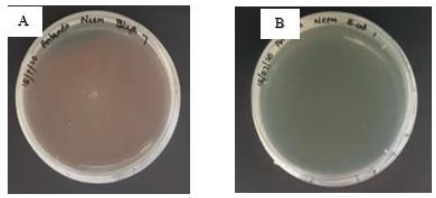

Figure 7 MBC test of neem against Escherichia coli and Staphylococcus aureus. a- MBC of neem extract against Staphylococcus aureus, b- MBC of neem extract against Escherichia coli

Plant extract	Concentration (mg/ml)	Microorganisms	
		Escherichia coli	*Staphylococcus aureus*
Azadirachta indica (Neem)	100	-	-
	50	+	+
	25	+	+
	12.5	+	+
	6.25	+	+
	3.125	+	+

Key: + growth, - no growth

The MBC value of neem extract was 100 mg/ml against both Escherichia coli and Staphylococcus aureus. Neem extract exhibited bactericidal effect against both bacterial strains.

DISCUSSION

In this study, ethanolic extraction procedure was carried out to obtain 3.34% of Azadirachta indica (Neem) extract. To obtain desirable yield of extract, solvent and sample were kept in contact for a longer time. Moreover, the particle size also has an impact on the yield of extract. When the particle size is finer, it will increase the surface area and that had an impact on the yield of extract (Masyitah and Bin, 2014). To extract the neem, 95% ethanol was used. This is because polar organic solvent can increase efficiency of the extraction (Masyitah and Bin, 2014). DMSO was used to prepare different concentrations of neem extract. DMSO is a polar aprotic solvent that has a unique ability of penetrating into the tissue without damaging the neem extract (Masyitah and Bin, 2014).

In this study antibacterial activity of the ethanolic extract of Azadirachta indica (Neem) against gram positive bacteria (Staphylococcus aureus) and gram negative bacteria (Escherichia coli) was evaluated on the basis of zone of inhibition qualitatively. The results of neem indicated that it had antibacterial property against both Escherichia coli and Staphylococcus aureus. Higher concentration of neem extract was more effective than the low concentration against both Escherichia coli and Staphylococcus aureus. Large clear zones were visible at high concentration and zones were smaller in comparison at low concentration. Moreover, neem was highly effective against Staphylococcus aureus compared to Escherichia coli. A study reported that neem extract has antibacterial property against gram positive but not on gram negative (Francine and Jannette, 2015). This might be due to the cell wall structure of gram negative bacteria. Gram negative bacteria consists of an outer membrane envelope that show resistance to antibiotics (Francine and Jannette, 2015). However, our study indicated that high concentration

and low concentration of neem was effective against both gram positive and gram negative bacteria. The antimicrobial property of neem extract was due to presence of varying degree of phytochemicals such as glycosides, steroids, terpenoids and alkaloids (Mistry, Sanghvi, Parmar and Shah, 2014). In this study, high concentration of neem was more effective than low concentration of neem. This is due to the high quantity of active compounds in high concentration of neem extract.

The minimum inhibitory concentration (MIC) values of Azadirachta indica (Neem) against Staphylococcus aureus and Escherichia coli was detected by observation of turbidity for visible bacterial growth and lower MIC values depict the effectiveness of the plant extract to inhibit the growth of the microorganisms used (Mummed et al., 2018). In this study 100 mg/ml, 50 mg/ml and 25 mg/ml of neem extract had a significant effect in reducing the growth of Escherichia coli and Staphylococcus aureus. The minimum concentration of neem that inhibits the growth of both Escherichia coli and Staphylococcus aureus was 25 mg/ml. While the minimum bactericidal concentration (MBC) value of neem extract against both Escherichia coli and Staphylococcus aureus was 100 mg/ml. A study by Abalaka, Oyewole and Kolawole, (2012), reported that the MIC and MBC values of neem extract were 5 mg/ml and 50 mg/ml against the tested pathogens such as Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Klebsiella ozanae, which is comparable to the results obtained in the present study. The difference in MIC value could be due to the assay variation, biological variation and variations in the laboratory (Mouton et al., 2017). In this study, antimicrobial activities of neem was assessed by ethanol extraction method. The results showed potential antibacterial effect of neem against both Escherichia coli and Staphylococcus aureus.

In future different solvents such as methanol, water and acetone can be used to extract the antibacterial compound in neem and can compare the antibacterial activity of neem with different solvent extraction procedures. Isolation and purification of phytochemicals is suggested to evaluate the phytochemical that could be used to treat infectious disease. Moreover, other plant parts of the sample should be studied to detect the potential antibacterial agent in neem extract and to assess the full spectrum of properties. The antibacterial efficacy of each of the neem plant part could be compared to detect the highest effective part against bacterial infections. Apart from the bacterial strains used in present study other strains and clinical isolates also could be evaluated similarly. Fungal infections are relatively less harmful than bacterial infection. However, immunocompromised patients are more prone for fungal infection. Therefore, these extracts could be tested for antifungal efficacy and the active compounds in neem that is effective against fungal infection could be discovered. The extracts could be evaluated in vivo to determine the pharmacology profiles and toxicity tests to derive a more comprehensive evaluation into the novel compounds.

CONCLUSION

In this study, the antibacterial activity of neem extract towards Escherichia coli and Staphylococcus aureus was investigated. Ethanolic extract of neem inhibited the growth of both the tested bacteria. High concentration of neem extract was more effective than the lower concentration of neem extract. Moreover, Staphylococcus aureus was susceptible to ethanolic extract of neem compared to Escherichia coli. The minimum concentration of neem that

inhibits the growth of both Escherichia coli and Staphylococcus aureus was 25 mg/ml and the minimum concentration that kills the growth of both Escherichia coli and Staphylococcus aureus was 100 mg/ml.

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