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Fatty Acid Composition and Antibacterial Activity of Neem (*Azadirachta indica*) Seed Oil

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Abstract: Pneumonia and skin diseases are some types of severe infections occur worldwide due to pathogenic bacteria such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). These bacteria's do contribute towards other infections in human and thus lead towards unhealthy life. The aim of this study was to determine the fatty acid composition and to investigate the toxic effect of seed oil of Neem, genus *Azadirachta* of *Azadirachta indica* (Family: *Meliaceae*) against two multi-drug resistant bacteria, namely; *E. coli* and *S. aureus*. The oil was extracted for 6 h through soxhlet method using hexane as solvent. The fatty acid composition was analyzed via Gas Chromatography-Mass Spectrometry (GC-MS). The composition resulted in detection of 8 fatty acids, whereby the dominant compound is linoleic acid of 34.69% and followed by oleic-, stearic-, palmitic-, arachidic-, behenic-, lignoceric-, and palmiticoleic acid at the percentage of 20.46, 20.42, 18.66, 3.59, 0.80, 0.55, and 0.17%, respectively. The bioassays were conducted through in vitro activity via disc diffusion method with five (5, 20, 50, 80, and 100 mg/mL) different concentrations. Streptomycin and 1% Dimethyl sulfoxide (DMSO) were used as positive and negative controls respectively. The bioassay provided inhibition zone that lies between 8.7 to 11.7 mm and 8.7 to 13.0 mm for *E. coli* and *S. aureus* respectively at the concentration of 5 to 100 mg/mL. The antibacterial activity of Neem (*A indica*) seed oil against these bacteria depends on the correlation of its concentration and thus isolation of bioactive components should be conducted.

Keywords: Neem, *Azadirachta indica*, seed oil, fatty acid composition, antibacterial activity.

INTRODUCTION

For the past few years, it had been reported that pneumonia is the leading cause of death among the children worldwide as this dangerous disease had been killing an estimated amount of 1.2 million children under the age of five years every year. This amount contributes towards 19% of deaths among the children and the death rate is higher than the total deaths contributed by AIDS, malaria and tuberculosis. Whereas, the total number of new pneumonia cases reported every year were recorded to be around 156 million. From that total amount, around 8.7% of these cases were recorded to be severe and need serious hospitalization [1, 2]. Besides that, skin diseases that were caused by the bacterial infections do also needs major concern. Among many types of bacteria identified, *Staphylococcus aureus* (*S. aurues*) had been listed as one of the most common bacteria that contribute towards pneumonia and skin diseases. Other than *S. aurues, Escherichia coli* (*E. coli*) were also found out to contribute towards several infections such as abdominal cramps, bloody or watery diarrhea, fever and vomiting. Kidney failure that is also known as hemolytic uremic syndrome (HUS) is the severe level of *E. coli* infections and this had been reported mostly among the children's. Even though most of the *E.coli* had been listed as one of the harmless strains of bacteria as they live in human, but the infections were transferred through the intake of contaminated water, food, fruits and vegetables, under-cooked meat and unpasteurized milk [3].

Accordingly, the initiative and effort for the search of new alternatives are needed to provide vaccine or non-harmful biological products to stop the action of these pathogenic bacteria. These products could be a non-harmful substance towards both human being and environment. Flora contains many biologically active compounds which have potential for development as medicinal or curative agents [4]. The use of medicinal plants has now becoming one of the targets as therapeutic aids to fight against these ailments as they are from natural source and they contribute towards less environmental effects and other harmful diseases.

Among many well-known medicinal plants, Neem (*Azadirachta indica*) is one of the plants that have been identified in curing various infections. The plant Neem belongs to the big mahogany family of *Meliaceae* and the genus *Azadirachta* of *A indica.* This plant were origin from India but now it has wide distribution in huge numbers in

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tropical, subtropical, semi-arid and wet-tropical regions of the world [5]. The roots, stems, barks, leaves, seeds, flowers and fruits of *A. indica* have chemically bioactive substances such aspeptides, alkaloids, tannins, phenols, sterols, flavonoids and glycosides that contributes in fighting against the bacteria. Among all the parts of *A. indica*, the seeds are listed as one of the most popular source of medicaments in antibacterial activity as the oil contains extensive spectrum against antibacterial infections [4].

Therefore, the objective of this study was to determine the fatty acid composition and to investigate the toxic effect of seed fixed oil of *A. indica* against two multi-drug resistant bacteria, namely, *E. coli* and *S. aureus.* The comparative of the seed fixed oil with well-known and commercially used standard antibiotic, streptomycin were reported to support the obtained data.

MATERIALS AND METHODS

Source of Seeds and Extraction of Oil

A. indica seeds were collected in September 2012 from mature tree growing in Teluk Intan, Perak and the taxonomy identification of plant was done by a botanist of the School of Environmental Sciences and Natural Resources, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia. The seeds were cleaned and dried under room temperature in an open area of a room for about 1 month. The kernels were removed from the dried seeds. The seeds were crushed using mortar and pestle. Then, the seed fixed oil was extracted via solvent continuous method (Soxhlet) for 6 h by using n-hexane. After complete extraction, the solvent were evaporated via rotary evaporator and further drying under open air in a dark area at room temperature. The yield percentage (w/w %) of obtained seed fixed oil were calculated.

Transmethylation of Fatty Acid

A solution of 2M KOH (Methanolic potassium hydroxide) was prepared. An amount of 100 mg of oil sample was dissolved in 10 mL of hexane in a test tube. Then 1 mL of 2M KOH was added into the same test tube and was vortex. After 15 min, the hexane phase was collected and washed twice with 4 mL of water. The hexane phase was further dried over an anhydrous sodium sulfate.

Fatty Acid Composition Analysis

Crude oils were analyzed as methyl ester to determine the fatty acid composition. The analysis was performed on Agilent Technologies 7890 A Gas Chromatography (GC) Systems coupled with Mass Spectrometry (MS) detector. 1 µL/L of fatty acid methyl ester solution was injected into the system by using GC auto sampler. Helium was used as carrier gas at the flow rate of 1 mL/min. The separation was performed on nonpolar capillary DB-1 of 100% dimethyl-polysiloxane with 30 m lengths, 0.25 mm diameter and 0.25 µm thickness. The GC oven temperature was initially set to 60°C for 3 min and was increased to maximum of 240°C at the rate of 3°C/min. Once 240°C were reached, the temperature was maintained constant for 10 min. The inlet temperature was set at 250°C. The mass detector conditions were set as split less mode, injector temperature of 250°C and ion-source temperature of 230°C. The library search for the obtained peaks was carried out through National Institute of Standards and Technology (NIST) Library Chem Station software.

Preparation of Test Samples

Stock solution of 100 mg/mL was prepared by dissolving 5 g of oil with 0.5 mL of dimethyl sulfoxide (DMSO) and diluted up to 50 mL with distilled water. Serial dilution method from the prepared stock solution was used to prepare 10 mL of five different concentrations (5, 20, 50, 80, and 100 mg/mL) accordingly. Prepared samples were labeled and stored in freezer for further antibacterial bioassay. Antibiotic Streptomycin at different concentrations (5, 20, 50, 80 and 100 mg/mL) as per samples were used as positive control (Control 1), whereas 1% DMSO (Control 2) and distilled water (Control 3) were used as negative controls for comparison.

Source of Microorganisms

The organisms used for the antibacterial analysis were *E. coli* and *S. aureus*. The strains of microorganisms were identified, characterized and obtained from the bacterial stock of Microbiology Laboratory of Faculty of Industrial Sciences & Technology (FIST), Universiti Malaysia Pahang (UMP), Pahang, Malaysia. Subcultures of the organisms were done on separate plates by touching the colonies using a sterilized loop and transferring them into a new medium and were further incubated at 35±2°C. Subcultures were continued every 24 h until pure colonies were obtained. The obtained pure colonies were incubated and were used in 24 h of preparation and growth.

Preparation of Medium

An amount of 16 plates containing nutrient agar was prepared by dissolving 13.48 g of nutrient agar in 480 mL of distilled water. The mixture was mixed thoroughly and heated with frequent agitation and boiled for 1 min. The nutrient agar was autoclaved at 121°C for 15 min. The agar was then transferred into each plate at the depth of 4 mm, was allowed to solidify on a flat surface at room temperature and was kept inverted at 4 to 8°C for bioassay.

Determination of Antibacterial Activity

The antibacterial activity of the oil was determined via disc diffusion method according to method proposed in previous bacteria bioassay with some modification [6]. The inoculum were prepared using a sterile inoculating loop; whereby five isolated colonies were touched on the sub cultured organisms and were suspended in 2 mL of sterile saline. The saline tube was vortex to obtain a smooth suspension. A sterile swab was dipped into the inoculum tube and was streak over the surface of the agar in a back-and-forth motion and by moving across and down the plates. The plates were rotated 60° and streaking was repeated again twice to ensure uniformity. The plates were then allowed to stand at room temperature for 3 to 5 min. The blank sterile filter paper discs were placed on the agar surface by using sterile forceps and the disc were pressed against the agar surface. By using a micropipette, 10 µL/L of sample were placed on each disc, the sample were allowed to absorb into the disc and the plate lid were replaced and allowed to dry for 5 min. The plates were inverted and placed in an

Table 1. Fatty Acid Composition of Neem (*Azadirachtaindica*) Seed Fixed Oil

Fatty Acid	Systematic Name	Formula	Structure	Area (%)
Linoleic acid	9,12-octadecadienoic acid	$C_{18}H_{32}0_2$	C18:2	34.69
Oleic acid	9-octadecenoic acid	$C_{18}H_{34}O_2$	C18:1	20.46
Stearic acid	octadecanoic acid	$C_{18}H_{36}O_2$	C18:0	20.42
Palmitic acid	hexadecanoic acid	$C_{16}H_{32}O_2$	C16:0	18.66
Arachidic acid	eicosanoic acid	$C_{20}H_{40}O_2$	C20:0	3.59
Behenic acid	docosanoic acid	$C_{22}H_{44}O_2$	C22:0	0.80
Lignoceric acid	tetracosanoic acid	$C_{22}H_{48}O_2$	C24:0	0.55
Palmiticoleic acid	9- hexadecenoic acid	$C_{16}H_{30}O_2$	C16:1	0.17

incubator at 35±2°C. Each analysis was done in triplicate against each test bacterium and the antibacterial activity was evaluated by obtaining the average inhibition zone for the bacterial growth after 24 and 48 h of analysis. The diameter of the zones of growth inhibition was measured in mm on the under surface of the petri dish and were recorded.

RESULTS

Fatty Acid Composition of Neem (*A indica*) Fixed Oil

The total yield (% w/w) of oil obtained from seeds was calculated and was found out to be 37%. The extracted oil has physical properties of pale greenish yellow color with a pungent smell. In the past study, it had been reported that the same method of Soxhlet and hexane as solvent used for extraction of oil yielded 28.4%, which is lesser than the yield obtained in current study [4]. The difference in the percentage of yield may be contributed by the origin of the plant, time of harvesting of the fruit, maturity of the fruit and the drying process. The analysis of crude *A indica* seed fixed oil as methyl ester shows the presence of 8 different fatty acids that includes both saturated and unsaturated fatty acids as in Table **1**. The total percentage of fatty acids identified were approximately 99.34% whereby the results were represented as the relative percentage area from the sum of all identified peaks. In this study, the linoleic acid (C18:2), was found to be the major composition of fatty acid in *A indica* seed fixed oil at the percentage of 34.69%. Other fatty acids, that were the oleic- (C18:1), stearic- (C18:0), palmitic- (C16:0), arachidic- (C20:0), behenic- (C22:0), lignoceric- (C24:0), and palmiticoleic acid (C16:1) were present at the percentage of 20.46, 20.42, 18.66, 3.59, 0.80, 0.55, and 0.17%, respectively.

Antibacterial Activity of Neem (*A indica*) Fixed Oil

Through this study, the bioassay on the antibacterial activity exposed that the *A indica* seed fixed oil inhibited the growth of different pathogenic bacteria that is the Gram-negative bacteria, *E. coli* and Gram-positive bacteria, *S. aureus*. Both bioassay results were as represented in Fig. (**1** and **2**) for *E. coli* and *S. aureus* respectively. The maximum inhibition zone shown by the seed fixed oil towards *E. coli* was 11.7 mm at the concentration of 100 mg/mL after 48 h of exposure. Other concentrations, 5, 20, 50, and 80 mg/mL shows inhibition zone of 8.7, 9.7, 10.3, and 11.3 mm respectively upon 48 h. As for *S. aureus*, the highest inhibition zone was 13 mm at 100 mg/mL of seed fixed oil after 48 h. The other concentrations of the oil do also shows inhibition towards *S. aureus* where by, 5, 20,50 and 80 mg/mL had 8.7, 9.7, 10.7, and 11 mm of inhibition respectively.

The analysis does reveal that the commercially used antibiotic streptomycin does also contribute to hinder the growth of both pathogenic bacteria. Streptomycin shows good inhibition towards *E. coli* as compared to *S. aureus*. The results were 32 mm of inhibition for *E. coli* and 30 mm for *S. aureus* at 100 mg/mL at 48 h. The negative control, 1% of DMSO and distilled water do not show any antibacterial activity as zero inhibition was recorded.

DISCUSSION

Many studies have reported that the major fatty acid content of Neem (*A. indica*) seed fixed oil is oleic acid whereby the percentage lies between 25-61.9% [7-14]. But the obtained results were supported by other reported study, in reporting that the major content was linoleic acid at 38.26% and followed by oleic acid at 34.09% [15]. The lowest content of fatty acid is represented by palmiticoleic acid (0.17%) and it is agreeable with some of the identified studies, where by the percentage were 0.1 and 0.2% respectively [13, 14]. A researcher had conducted a review study on the Neem (*A. indica*) seed fixed oil and had summarized that palmitic-, stearic-, oleic-, arachidic- and behenic acid lies between 17.3-34.3, 6.6-24, 25.4-57.9, 1.24-1.3, and 0.23-1.73%, respectively [14]. Some of the obtained results in this study were acceptable and similar with previous studies. The difference in fatty acid and its composition may be attributed due to the origin, plant species and their growth conditions [13].

The bioassay provided inhibition zone that lies between 8.7 to 11.7 mm for *E. coli* and 8.7 to 13.0 mm for *S. aureus* at the concentration range of 5 to 100 mg/mL respectively. It had also been, reported that the seed fixed oil hexane extract of this plant shows inhibition zone of larger diameters between 13-14 mm against bacteria strains of *E. coli* [4]. Other than that, a study conducted on the comparison of Neem (*A indica*) oil and Neem (*A indica*) oil soap against *S. aureus* and both test shows positive results [16]. The mixture of oil (1:1 v/v) with control drugs such as the lamisil and

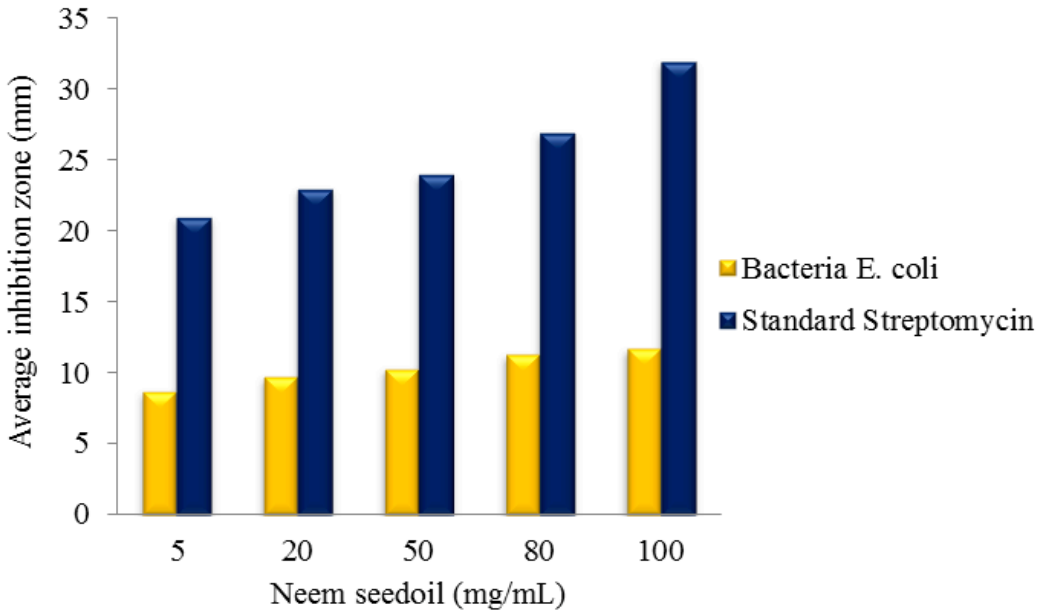

Fig. (1). Antibacterial activity of Neem (*A indica*) seed fixed oil against *E. coli* after 48 h.

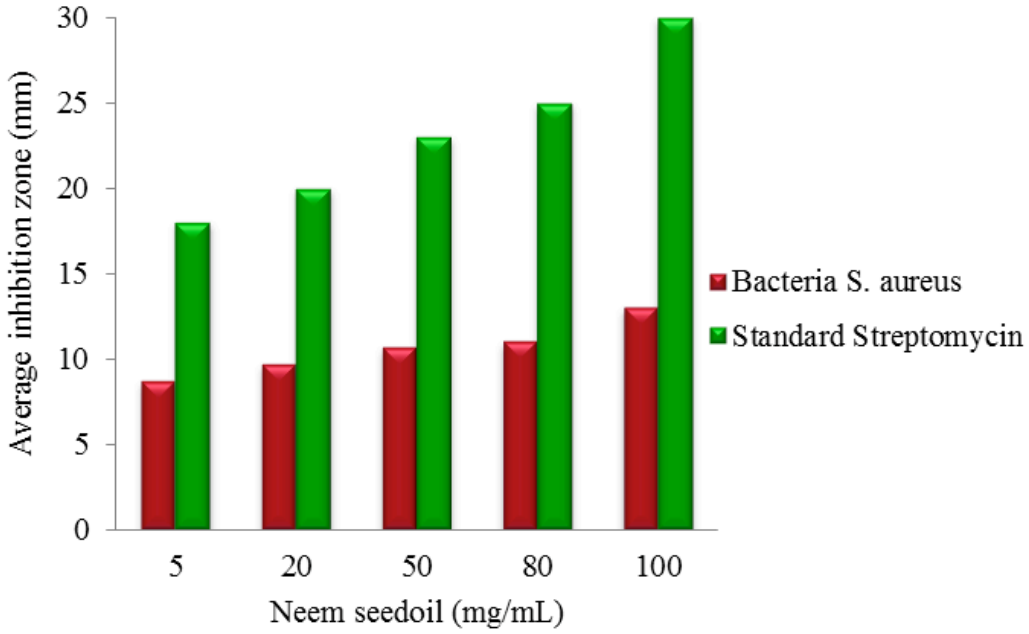

Fig. (2). Antibacterial activity of Neem (*A indica*) seed fixed oil against *S. aureus* after 48 h.

whitfield ointment resulted in higher inhibition zone compared to the pure Neem (*A indica*) seed fixed oil or soap. Neem (*A indica*) oil do shows average inhibiton of 17, 17.5, 19, and 19.5 mm on *Pseudomonas aeruginosa, Salmonella typhi, S. aureus* and *E.coli* respectively [17]. The tube dilution method that was adopted by past researcher, to test the antibacterial activity of this oil shows inhibition at different percentage according to its concentration and it was observed that the pathogens inhibited were 21.24, 71.42, and 7.14% at 500, 125, and 250 µL/ml, respectively. These results were also influenced by the temperature and energy whereby many bacteria could be killed at the temperature range of 4-37°C [18].

As in Fig. (**1** and **2**) of this study, the standard antibiotic streptomycin, revealed higher inhibition activity for both strains of bacteria than the extracted seed fixed oil of the plant. The activity of this antibiotic might be attributed by the condition of the antibiotic whereby it is in pure state or condition and has gone through various production process, thus been recognized as a standard antibiotic that could be commercially used. As comparison with crude seed fixed oil, the inhibition exerted were lesser than the standard antibiotic used and this might be because the oil contains some compounds that indirectly do not corresponds towards the bacteria. The compounds might have some other biological activities as verified by other studies. The seed fixed oil inhibited the growth of both *E. coli* and *S. aureus* together with positive control at different level of inhibition subjected on the concentration level.

Among *E. coli* and *S. aureus*; *E. coli* was more resistant towards the Neem (*A indica*) seed fixed oil as shown by the smaller size of growth inhibition zone. Therefore, many factors such as type of bacteria, type of solvent, extraction methods, temperature and pH of extracts were found out to be influencing the bacterial activity [4]. Therefore, the Neem (*A indica*) seed fixed oil used in this study to test the antibacterial activity shows results that are acceptable when compared with previous studies. Neem (*A indica*) seed fixed oil on its own might not able to behave as a strong antibacterial agent. Besides Neem (*A indica*), there are various plant been identified in behaving as therapeutic agent towards fighting the bacteria such as the *Swietenia mahagoni* oil, Aniseed oil, Calamus oil, Camphor oil, Cedarwood oil, Clove oil, Lavender oil, Lomongrass oil, Lime oil, Nutmeg oil, Basil oil, Peppermint oil and Rosemary oil [19-25]. Therefore, suggestions on the combination of the oil of one plant with oil of another plant and also a combination of plant oil with commercial drugs could be practiced as it proves that the synergistic combinations yield in better outcomes in fighting against these bacteria that ruin human's life [16].

CONCLUSION

The composition resulted in detection of 8 fatty acids, whereby the dominant compound is linoleic acid of 34.69% and followed by oleic, stearic, and palmitic acid at the percentage of 20.46, 20.42, and 18.66%. The hexane seed fixed oil of this study had demonstrated the antibacterial activity against *E.coli* and *S. aureus* that are promising. The antibacterial activity of Neem (*A indica*) seed fixed oil against these pathogenic bacteria depends on the correlation of its concentration. The problem associated with pneumonia and skin diseases contributed by pathogenic bacteria had increased resistance with commercial drugs and therefore could be solved by developing the Neem (*A indica*) seed fixed oil as one of the active drug to fight against these bacteria. This oil had been used for many other purposes in the traditional medicines and had been proven to be effective as medicinal agents. Thus, further isolation of bioactive components that corresponds towards this activity should be carried out.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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