Chemical composition of *Neem* Leaves extract using Phytochemical Screening and GC-mass: antibacterial activity

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ABSTRACT

Medicinal plants such as leaves of *Neem* have very unique therapeutics properties which made them used widely to treat a lot of human diseases. The present study were conducted characterize the crude extract of Neem and evaluate their biological activities. Neem leaves were extracted using hot-extraction method, and evaluated for their chemical group compounds using phytochemical screening for the chemical group compounds. Meanwhile, Gas chromatography (GC), Energy-dispersive X-ray spectroscopy (EDX) and Fourier-transform infrared spectroscopy(FTIR) were used to conform to the fictional groups in the leave of *Neem*. The antibacterial activities of the cured extract of *Neem*were observed against two bacteria pathogens. Phytochemical screening analysis was done using common procedures and had shown the presence of alkaloids, carbohydrates, tannins, steroids, and flavonoids. Twenty-two compounds were identified in the GC-Mass spectrometry. The highest compounds were Pyridine,2,3,4,5-tetrahydro-3-methyl (17.03 %), 1-azabicyclo(3.1.0) hexane (12.16 %), and 2-Undecanol (7.63 %), while the lowest compounds were Heptafluorobutyric acid, n-tetradecyl ester (0.79 %) and 4-Methyl-3-pentenal (0.79 %). The EDX analysis presented two elements, which were carbon (53 %) and oxygen (46 %). The *Neem* band at 1668 cm−1 refers to the amide I C=O stretching, and the peak at 2140 cm−1 is associated to the alkyne group that exists in the phytoconstituents of *Neem* extract. Meanwhile, the peak that was monitored at 3301cm−1 corresponds to the amide A (N-H). The observed peaks are mainly discovered as flavanoids and terpenoids that exist significantly in the plant extract. The antibacterial activities of *Neem* leaves were investigated against gram-negative bacteria, such as *Escherichia coli*(*E.coli*) and gram-positive bacteria, such as *Staphylococcus aureus*. (S. *aureus*). The results had presented the factional activity for *Neem*against both pathogens.

Keywords:*Neem,* GC-mass, Phytochemical screening, Antibacterial

INTRODUCTION

The use of medicinal plants in the development of a drug is crucial to the human as they are being used to treat various kinds of diseases. Traditional treatment from the wild plants had been always referred to guide the researcher to discover the best medications to create a healthy life forhumans and animals [1]. However, there are still a few more medicinal plants that are still hidden and undiscovered, which requires further scientific evaluation [2, 16-19].

*Neem*is one of the Melia genus that belongs to the Meliaceae family. It is significantly distributed in India, Iran, Pakistan, Argentina, Brazil, Bermuda [14], China, Australia, and Malaysia [15]. Traditionally, *Neem*leaves were used as a medicine for various kinds of treatment such as insect pests, wound healing [7]. Different parts of *Neem*have been extracted and utilized for any kinds of skin infections such as the microbial and gastrointestinal tract[13, 20-24], hypoglycaemic, and antidiabetic [16, 28-33].

*Neem*planthas excellent biological activates such as Antifeedant activity, Hepatoprotective activity, Antidiabetic activity (anti-larvicidalactivity, and Anti-bacterial activity [16, 7, 6, 4].

*Neem*has many properties that have been investigated in the previous studies [11, 25-28], however, there is no study about a phytochemical screening of *Neem*leaves to identify the chemical components of the extract. In the present study phytochemical screening of *Neem*leaves have been done via using standard procedures along with investigated the biological properties of *Neem* leaves via identified its antibacterial activity against gram-negative bacteria, such as *Escherichia coli* and gram-positive bacteria, such as *Staphylococcus aureus*

The results of the phytochemical screening of *Neem*leaves have shown the presence of alkaloids, flavonoids, tannins, steroids, and carbohydrates. GC-mass spectroscopy discovered 22 compounds in the crude of *Neem* extract. EDX analysis has shown a high presence of carbon and oxygen. FTIR analysis graph showed the availability of factional groups that belonged to two of these active compounds, which are present in the leaves of *Neem* The extract of leaves had demonstrated the activity against *E.coli* and *S. aureus*.

Fig 1: Fresh and dried leaves of *Neem*

EXPERIMENTAL SECTION

Materials

Neem leaves were taken from Perak, Malaysia. Acetic acid, nutrient agar, and nutrient broth were bought from the Merck brand. Ascorbic acid,gallic acid, chloroform ($CHCI_3$), quercetin, and α-tocopherol (from Sigma-Aldrich Chemical Co. St. Louis, USA) were used as the standard in the process. On the other hand, methanol, sulphuric acid (H_2SO_4), acetone, ethanolic potassium hydroxide, ethanolic potassium hydroxide, phenolphthalein from (R&M/ Malaysia), hydrochloric acid (HCl), Hanus reagents.

Instrumentation

The leaves of *Neem*were characterized by using several equipment such as Gas chromatography – Mass spectrometry (GC-MS, Shimadzu GC-14B) analyzer, FTIR (Fourier-transform infrared spectroscopy) by Termo Scientific Perkin Elmer Model: Spectrum 100 Spectrometers, and EDX (Energy-dispersive X-ray spectroscopy) using FESEM instrument used was Hitachi SU8020.

Procedures

Drying and Extracting of *Neem* leaves

*Neem*leaves were cleaned many times using distilled water to eradicate the dust and fungus. The sun-dry method was used to the dried leaves of *Neem*for 7 days. The dried leaves were cut down to small pieces and kept for future work.

25 g of the leaves were extracted with 200 ml of distilled water in 250 ml conical flask. The leaves were boiled for 20 minutes and filtered to get the crude extract of *Neem*leaves. The extract of the leaves was stored at 4 ^{o}C for future work [18].

Phytochemical screening of *Neem*leaves

The screening of phytochemical was conducted for *Neem*leaves. Any inconsistencies in types or the presence of precipitate in solution were used as the demonstrator of positive reaction to these tests.

Alkaloids test

*M.dubia*extract was dissolved separately in a dilute HCL acid and later, was filtered. Mayer's, Wagner's, and Dragendroff's Reagent were used to treat the filtrates individually to identify the alkaloids.

A. Mayer's test: A drop or two of Mayer's reagent was added to the side of the test tube in a few ml of filtrate. The test is considered to be positive once there is an existence of white or creamy precipitate.

B. Wagner's test: A few drops of Wagner's reagent was mixed to a few ml of the filtrate at the side of the test tube. The test is positive when a reddish-brown precipitate appears.

C. Dragendorff's test: 1 or 2 ml of Dragendorff's reagent was mixed to a few ml of filtrate. The test is positive once a noticeable yellow precipitate appears.

Saponins Test

0.5 ml of *Neem*extract was added with 2.5 ml of distilled water and was shaken to mix the liquids. The mixture was then left for a few minutes. Based on the observation, the existence of saponins was confirmed because of the advancement of foam on the surface of the mixture.

Carbohydrates Test

*Neem*extract was added to 5 ml of distilled water to be dissolved and later, being filtered. Subsequently, Molash's Reagent was used to treat the filtrates. This was done to test for the presence of sugars. Based on the observation, a formation of violet ring indicated that the test is positive.

Cardiac glycosides Test:

A mixture of 0.5 ml of *Neem*extract, 1ml of Iron (III) chloride reagent, and few drops of concentrated H_2SO_4, was done to examine for the existence of cardiac glycosides. Based on the observation, the existence of cardiac glycosides is confirmed when a greenish-blue colour precipitate appears. Thus, it indicated that the test is positive.

Tannins Test

0.5 ml of *Neem* was added to 2 ml portion of the 0.1% Ferric chloride to examine for the existence of Tannins. The test is positive when a precipitate appears in brownish-green or blue-black colour.

Steroids Test

5ml of chloroform was added to 0.5 ml of *Neem.* Subsequently, 5 ml of Sulphuric acid was added to the sides of the test tube to examine the presence of steroids. Based on the observation, the test is considered positive once the upper layer changes to red and the layer of the sulphuric acid turn to yellow with green fluorescent.

Terpenoids Test

0.5 ml of *Neem*extract was added to 1ml of Chloroform and a condensed sulphuric acid was carefully added to a precisely a couple of drops to test for the presence of terpenoids. The test is positive once a reddish-brown coloration appears.

Flavonoids Test

2ml of 1% aluminum solution was added to 0.5 ml of *Neem*to test for the presence of flavonoid. The presence of flavonoid was confirmed one the mixture turns to yellow. This indicated that the test is positive.

Coumarins Test

0.5 ml of *Neem*was added to 3ml of NaOH (10%) to examine the presence of Coumarins test. The test is declared positive when a yellow coloration appears.

Antibacterial activities of *Neem*leaves

Agar Preparation

A combination of 8g of nutrient broth and 20g of agar powder was dissolved in 1000 mL of distilled water. The mixture was then sterilized by an autoclave at 121 ^{o}C for 20 minutes. Subsequently, the mixture was left to cool to 55 ^{o}C. 25 ml of cooled media was mixed to the plate and was left for it to solidify. Then, it was kept in the dark at 4 ^{o}C for further experimentation.

Antimicrobial Test

The bacterial strain test was shifted from the stock cultures as a streaked on the plate of nutrient agar (NA) and was left to be incubated for 24 hours. The bacterial colonies that had been segregated were then being used as the inoculums. A bacteriological loop was used to transfer the bacteria to autoclave the nutrient agar that was left to cool at 450C in a water bath and was mixed by gently swirling the flasks. The medium was then poured to sterile Petri plates for solidification and to be used for biotest [5]. A fresh culture of inoculums from each culture was marked on the nutrient agar media in a petri dish. Different concentrations of crude extract were used to observe the antibacterial activity. A 6mm diameter of filter paper discs, which contain the test compound at the desired concentrations, were positioned on the surface of the agar. The Petri dishes were incubated in a condition with 37°C. Generally, the diffusion of the antibacterial agent into the agar causes the inhibition of germs. The growth of the microorganism was examined and measured to determine the diameters of the inhibition growth areas.

RESULTS AND DISCUSSION

Phytochemicals are antibacterial that occurs naturally and known to be among the decent promising materials that are used in different forms. The crude of the *Neem*leaves undergoes phytochemical screening test. The test is confirmed to be positive when there is a change in colour or appearance of any precipitation during the test. The common chemical compounds that often undergo phytochemical screening tests are alkaloids, carbohydrates, cardiac glycosides, flavonoid, saponins, steroids, and tannins [12].

Based on the phytochemical screening test that was conducted onto the leaves *of Neem,* alkaloids, carbohydrates, flavonoid, tannins, and steroids were discovered to be present. These chemical group compounds can act as a reducing agent to convert the metal from its salt by changing the charge from +1, +2 to zero. In this study, the leaves of *Neem*had exhibited the availability of the major chemical group compounds to show their capability to function as reducing agents. Table 1 tabulates the results of the phytochemical screening test of the *Neem*leaves.

Table 1: Phytochemical screening of *Neem*leaves

	Phytochemical test	**Indicator**	**Result**
1	Alkaloid test		
	Mayer's	Creamy precipitate has been an appearance	**Positive**
	Wagner's,	The reddish-brown precipitate was observed	**Positive**
	Dragendroff's	The yellow precipitate has been identified	**Positive**
2	Carbohydrates	The observed to violet ring that identified	**Positive**
3	Coumarins test	Not noticing the yellow colour in the solution	Negative
4	Cardiac glycosides	Greenish-blue colour did not observe in the solution	Negative
5	Flavonoids test	The yellow colour for the solution was identified	**Positive**
6	Saponins Test	The foam has not observed to appear on the surface of the mixture	Negative
7	Steroids test	Green fluorescent has been observed	**Positive**
8	Tannins test	The solution colour change into Blue-black	**Positive**
9	Terpenoids test	The colure of solution didn't change to reddish-brown	Negative

GC-MS was used to identify the compound qualitatively and to measurement the compound quantitatively. According to [2], the compounds may be volatile or semi-volatile organic compounds. *M.dubia*leaves underwent the GC-MS analysis to identify the exact

composition of the leaves and the content of the compound, which can be determined based on the area of the peak, the retention time, and the molecular formula. Based on the analysis, 22 compounds were discovered in the leaves' composition. Three of these compounds exhibited high percentage in the leaves, which are 1-azabicyclo(3.1.0)hexane with the composition of 12.16%, Pyridine,2,3,4,5-tetrahydro-3-methyl with 17.03%, and 2-Undecanol with 7.63%. The molecular formulas of these compounds are; $C_{18}H_{29}F_7O_2$ and $C_6H_{10}O$ respectively. Generally, the overall composition of these compounds was 0.79%. Table 2 tabulates the percentage of the main component of *Neem*leaves.

Table 2: Main Components (%) of *Neem*leaves

No	R. Time	Composition (%)	Compound	Molecular formula
1	9.887	3.29	Acetyl cyanide	C_3H_3NO
2	9.887	3.29	2-Propynenitrile, 3-fluoro-	C_3FN
3	9.887	3.29	Ethyl isocyanide	C_3H_5N
4	10.154	12.16	1-azabicyclo(3.1.0)hexane	C_5H_9N
5	10.154	17.03	Pyridine, 2,3,4,5-tetrahydro-3-methyl-	$C_6H_{11}N$
6	10.264	1.45	1,2,3,6-Tetrahydropyridine	C_5H_9N
7	10.319	1.96	1-Methoxy-2-propyl acetate	$C_6H_{12}O_3$
8	12.079	1.02	Tans-1-Propenylcyclopropane	C_6H_{10}
9	12.361	3.88	1,6-Heptadiene	C_7H_{12}
10	13.037	7.62	2-Undecanol	$C_{11}H_{24}O$
11	13.131	1.49	2-Furanmethanamine	C_5H_7NO
12	13.171	1.35	Acetamide, N-2-propynyl-	C_5H_7NO
13	13.288	2.59	Acetonitrile, 2,2'-iminobis-	$C_4H_5N_3$
14	13.343	2.42	Cyclobutanone, 2-methyl-2-oxiranyl-	$C_7H_{10}O_2$
15	13.587	2.73	2-Hexenal	$C_6H_{10}O$
16	13.343	2.42	1-Butene, 2-ethyl-3-methyl-	C_7H_{14}
17	13.367	1.32	2-Pentanone, 3-methylene-	$C_6H_{10}O$
18	13.516	1.78	2(5H)-Furanone, 5-methyl-	$C_5H_6O_2$
19	13.642	0.79	Heptafluorobutyric acid, n-tetradecyl ester	$C_{18}H_{29}F_7O_2$
20	13.642	0.79	4-Methyl-3-pentenal	$C_6H_{10}O$
21	13.673	1.95	1,1,2,3-Tetramethylcyclopropane	C_7H_{14}
22	13.775	1.32	1,1,3-Trimethylcyclopentane	C_8H_{16}

The EDX technique detects the X-ray beams emitted from the sample during the process of the bombardment to characterize the composition of the element from the sample. The outcome of the technique highlighted the elements with the atomic number, which ranges from beryllium to uranium, to indicate that the relative x-ray counts at a certain energy level of the sample's constituent can assist in obtaining the quantitative result [17]. Besides that, EDX also helps to identify the percentage of the elements that exist in the crude of *Neem*leaves. The two elements that had been discovered in the crude were oxygen (O) and carbon (C). These elements were associated with the organic compounds that exist naturally in the plant. Figure 2 displays the mapping and graph of EDX.

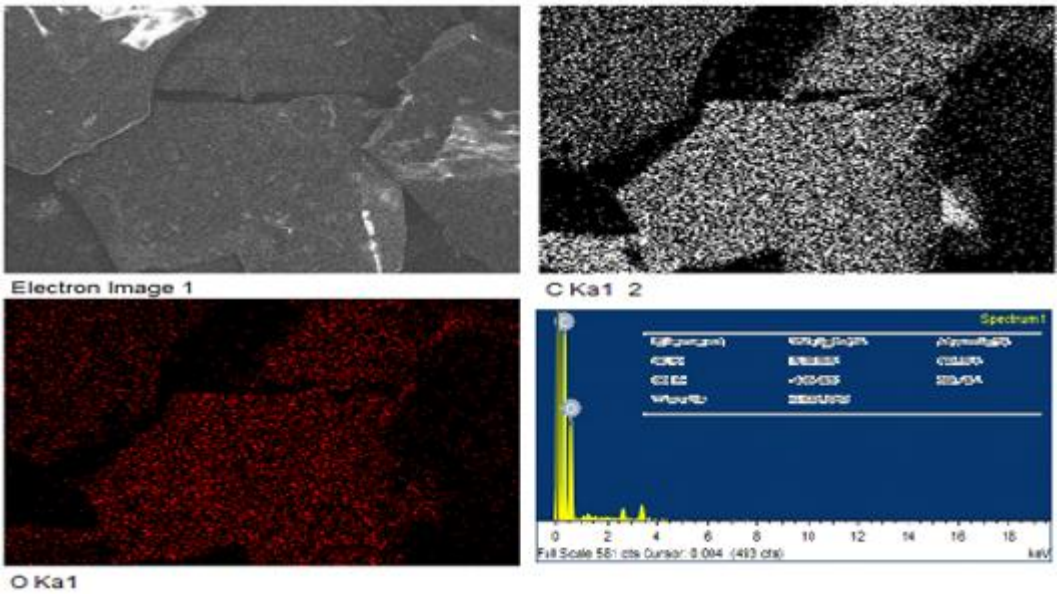

Fig 2: EDX mapping of leaves *Neem*

Besides EDX, the Fourier Transform Infrared Spectroscopy (FTIR) was also used as it was known to be a substantial analytical method as it can detect a few functional groups in the compounds. It was discovered that there were significant impacts to the chemical bond in a liquid once it interacted with the infrared light. The chemical bond will elongate, contract, and absorb the radiation when other molecules were present at a particular wavelength. Therefore, the main functional groups in the compound were recorded. Based on the record, the FTIR spectra noted the *Neem*band at 1668 cm-1 that referred to amide I C=O stretching [9]. The peak of 2140 cm-1 was associated with the alkaline group that exists in the phytoconstituents of *Neem*[8]. At 3301 cm-1, the peak was assigned to the amide A (N-H). Generally, the detected peaks are known to be as flavanoids and terpenoids that exist significantly in the extract of plants [10].

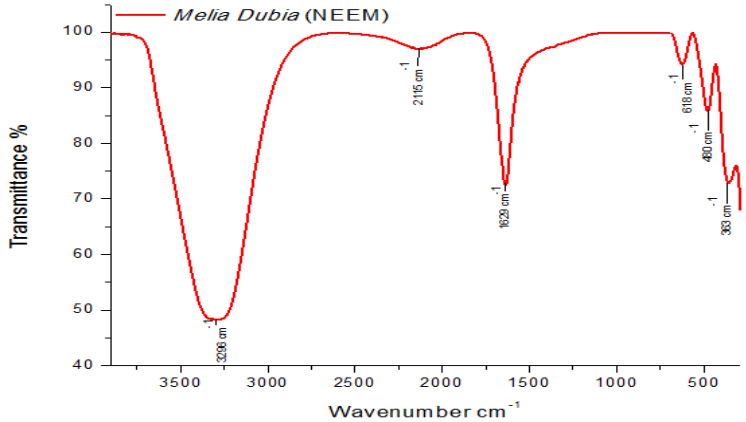

Fig 3: FTIR spectroscopy of *Neem*leaves

The antibacterial activity of *Neem*crude was investigated using two pathogens against gram-negative bacteria, such as *Escherichiacoli* and gram-positive bacteria, such as *Staphylococcusaureus*. The compounds of the chemical groups were identified and concluded that they were the cause of the antibacterial activity of the plants against bacteria. In this test, the distilled water was used as a negative control, while the ampicillin was used as a positive control. 5, 10, 20, 30 and 50 µg/ml concentrations of *Neem*crude were mixed with the distilled water to dissolve. Then, the mixtures were poured onto a filter paper of 6 mm and placed in the petri dish. As mentioned previously, the antibacterial activities of the*Neem*crude was affirmed based on the method of disk diffusion. A ruler was used to measure the ability of *Neem*crude to prevent bacterial growth against both bacteria. It was reported that the distilled water inhibition was zero in both bacteria. Meanwhile, the inhibition zones of ampicillin were 28.6 against *E.coli* and 22.1 against *S. aureus.* Overall, the inhibition bacteria growth of the *Neem*was 10, 11, 13, 15 and 16 mm against *E. coli* and 8, 10, 12, 13, 15 mm against *S. aureus*.

Table 3: Antibacterial activities of plant extract and control samples against *E. coli* and *S. aureus*

Bacteria	Ampicillin	Distilled water
E. coli	28.6 mm	0
S. aureus	22.1 mm	0
M. dubia (Neem)	*E. coli*	*S. aureus*
5 µg/ml	10 mm	8 mm
10 µg/ml	11 mm	10 mm
20 µg/ml	13 mm	12 mm
30 µg/ml	15 mm	13 mm
50 µg/ml	16 mm	15 mm

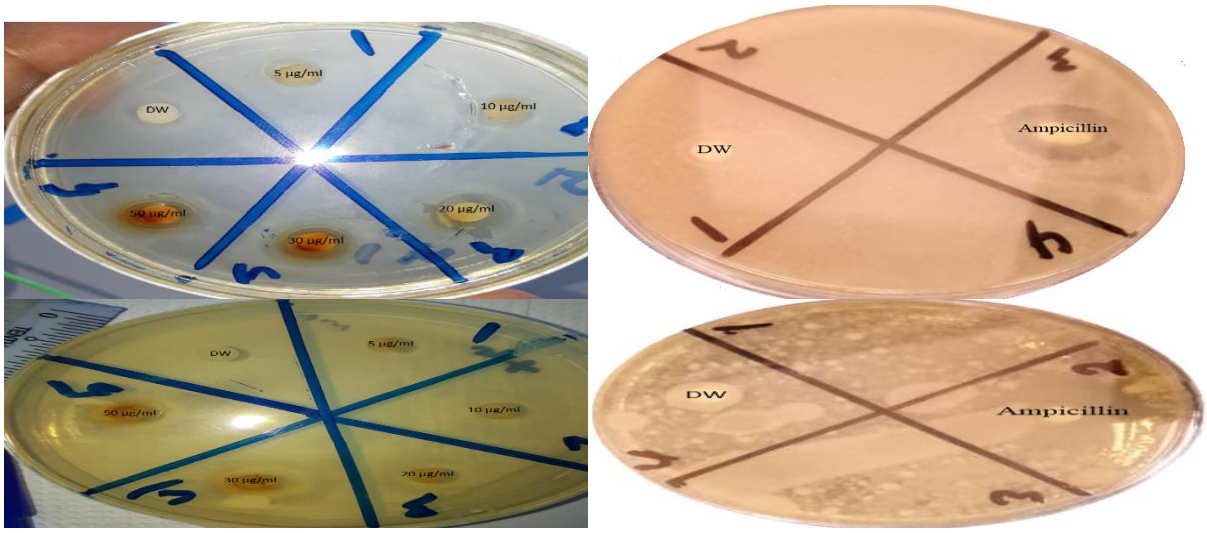

Fig 3: Antibacterial activities of extract of plant and control samples against *E. coli* and *S. aureus*

CONCLUSION

In this study, the *Neem*leaves were extracted via the hot-extraction method using distilled water as a solvent. The first procedure was to conduct the phytochemical screening of the leaves using common procedures. The compound of the chemical groups had been investigated, and it discovered the presence of alkaloids, carbohydrates, tannins, steroids, and flavonoids, which proven the ability of the plant to be utilized in the medical field. The aqueous solutions of *Neem*had exhibited 22 compounds using the GC-mass spectroscopy, and three of these compounds had shown high availability. Meanwhile, the FTIR was used to identify the factional groups that belonged to the phyto-constituents in the extract of *Neem.* The antibacterial activity of *Neem*extract was further examined to confirm its suitability to be used as an antibacterial agent in the medical Andbiological fields.

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