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Phytochemical Screening and Study of Antioxidant and Antimicrobial Activities of Leaf Extracts of *Azadirachta indica*

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The present study was conducted to identify the bioactive compounds, antimicrobial and antioxidant activities of *Azadirachta indica* (neem) leaves. The leaf extract of *A. indica* was prepared using different polarities of solvents: methanol, chloroform and hexane. The methanol extract showed the highest percentage in the yield of the crude extract of *A. indica* leaves (4.52%) followed by the chloroform extract (2.93%) and the hexane extract (0.4%). The phytochemical screening of the leaf extracts showed the positive results for alkaloids, flavonoids, terpenoids, tannins, saponins and steroids which were responsible for the observed antioxidant and antimicrobial activities. For the antimicrobial activity, an experiment by using the disc diffusion method indicated that the methanol crude extract exhibited the strong antimicrobial effect towards Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Esherichia coli* and *Samonella thypi*). The methanol crude extract of *A. indica* showed the maximum zone of inhibition (15 mm) against *B. subtilis*, followed by *S. aureus* (12mm), *E. coli* (9 mm) and *S. thypi* (9 mm). The antioxidant activity by using DPPH assay of methanol, chloroform and hexane crude extracts was also determined. The maximum inhibition of free radicals obtained were 93.28%, 82.53% and 65.45% respectively when the 1000 µg/mL crude extract was used. The highest antioxidant activity was found in the methanol crude extract followed by the chloroform extract, and the lowest antioxidant activity was in the hexane extract. Therefore, the findings suggest that the *A. indica* leaves have a great potential as a plant of phytopharmaceutical importance, and it is crucial to conduct further research on extracts from leaves of *A. indica* for identification, purification and isolation of compounds having antibacterial and antioxidant activities.

Keywords: *Azadirachta indica*; bioactive compounds; phytochemical; antimicrobial; antioxidant

I. INTRODUCTION

Azadirachta indica (neem) is a tree in the Meliaceae family. Neem is called 'arista' in Sanskrit, a word that means 'perfect, complete and perishable' (Girish and Shankara, 2008). It is an attractive evergreen tree that is native to the Indian subcontinent. It is also planted throughout the Southeast Asia, Australia, East and sub-Saharan Africa, Fiji, Mauritius, and many countries in Latin America (Puvan *et al.*, 2015). It has been used for a long time in solving global, agricultural, and environmental and health problems (Natarajan *et al.*, 2003, Girish and Shankara, 2008 and Bassey *et al,* 2016). Different parts of Neem such as leaf, bark and seed oils have been shown to exhibit wide pharmacological activities including; antioxidant, antimalarial, antimutagenic, anticarcinogenic, anti-inflammatory, antihyperglycemic, antiulcer and anti-diabetic properties (Reddy *et al.*, 2013). According to Bassey (2016), both the leaf and stem bark of neem extracts contain bioactive compounds such as alkaloid, flavonoids, tannin, saponin, polyphenol and reducing sugar that exhibited the antimicrobial activity. In other work, Maria and Romilly (2017), also reported both fresh and sun-dried neem leaves contained alkaloids, flavonoids, tannins, saponins, steroids, resins, bitter, cardiac glycosides, reducing sugar and triterpenes and volatile oils that have great potential for an

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antimicrobial activity. In Malaysia, neem leaves are commonly used as an alternative treatment for chicken pox, a highly contagious disease caused by the primary infection of varicella zoster virus. There have been very limited studies on the bioactive compound for antioxidant, antimicrobial and antifungal activities of Malaysian neem leaves. Puvan *et al.*, (2015), did the only study on the antifungal activity of neem leaves in Malaysia. Thus, the aim of the present study was to identify bioactive compounds for antioxidant and antimicrobial activities of neem leaves in the different polarity of solvent extracts.

II. MATERIALS AND METHOD

The plant of *A. indica* was selected for the study. The leaves of this plant were collected from Sepang, Selangor. About 1 kg of fresh maturated leaves of the plant was properly washed in tap water and then rinsed in distilled water. Then, the leaves were air- dried at room temperature (27^{o}C) for one week (Pandey *et al.,* 2014). After that, the leaves were cut into small pieces then ground to uniform powder by using mixture grinder.

A. Extraction Method

Approximately 1000 g of leaf powder was weighted. Then, each 1000 g of leaf powder was soaked into three different polarities of solvents namely methanol, chloroform and hexane for 72 hours. After the extraction, the extracts obtained were filtered and concentrated (Arunkumar and Muthuselvam, 2009). Then, the extract was used for the phytochemical screening. The yields of extracts were calculated by using the following formula:

$$\text{Percentages yield} = \frac{\text{Weight of dry crude extract}}{\text{Weight of dry leaves}} \times 100$$

B. Phytochemical Screening

The extracts were analysed by the following procedures to test for the presence of the alkaloids, flavonoids, saponins, terpenoids, tannins and steroids.

1. Test for alkaloids (Wagner's Test)

2 ml of crude extracts were treated with a few drops of the Wagner's reagent (Iodine in Potassium Iodide). The formation of brown/reddish precipitate indicates the presence of alkaloids (Olabinri *et al.,* 2014).

2. Test for flavonoids (Lead Acetate Test)

2 ml of crude extracts were treated with a few drops of the lead acetate solution. The formation of yellow colour precipitate indicates the presence of flavonoids (Raphael, 2012).

3. Test for saponins (Frothing Test)

2 ml of crude extracts were diluted with 1 ml distilled water. Then, the mixture was shaken vigorously, and the persistent froth formation was observed after 10 minutes (Edeoga *et al.,* 2005).

4. Test for terpenoids (Salkowski Test)

2 ml of crude extracts were dissolved in 1 ml of chloroform. Then, a few drops of concentrated sulfuric acid were added to the mixture. The reddish-brown colour indicates the presence of terpenoids (Edeoga *et al.,* 2005).

5. Test for tannins (Ferric Chloride Test)

About 2 ml of the extract was added with a few drops of $FeCl_3$ solution. The formation of blue black or dark green colour indicates the presence of tannins (Bassey *et al.,* 2016).

6. Test for steroids (Liebermann-burchard test)

About 2 ml of crude extracts were mixed with 1 ml of acetic acid in the test tubes. Then, a few drops of concentrated sulfuric acid are added to the mixture. The green colour indicates the presence of steroids (Bassey *et al.,* 2016).

C. Antioxidant Activity

2,2-diphenyl-1-picrylhydrazyl radical (DPPH) Scavenging Activity Analysis: The antioxidant activity of *A. indica* leaf extract was determined based on a slightly modified Dhakal *et al.* (2016), and Paini *et al.* (2014) method. The neem leaf extract with various concentrations (7.81 to 1000 μg/ml) were prepared. After that, 0.2 ml of the extracts were placed in the test tube and added with 3.8 ml of DPPH in methanol. Ascorbic acid was used as a reference standard and a control solution was prepared with the same amount of methanol and DPPH. Meanwhile, the pure methanol acts as a blank

solution. When the DPPH free radical reacted with the antioxidant compound, the capacity of compounds donating the hydrogen atom was reduced. The decrease in DPPH scavenging activity could be identified based on the absorbance of the solution that was measured at 517 nm (Ayoola *et al.,* 2008) after the mixture was homogenized wisely and left in the dark at the room temperature for 30 minutes. The DPPH free radical scavenging activity was stated as % inhibition = $[(A_0-A_t) / A_0] \times 100\%$, where A_0 was the control absorbance at t = 0 seconds and A_t was the antioxidant absorbance at t seconds.

D. Antimicrobial Activity

1. Media Preparation of Nutrient Agar (NA)

The agar was prepared by using NA powder (38 g) dissolved in 1 L distilled water until the mixture was completely dissolved. The media was autoclaved at 121°C for about 20 minutes. After autoclaving, the media was left to cool to 40°C and poured into sterile petri dishes.

2.Culturing Microbe of Nutrient Broth (NB)

The broth was prepared by using NB powder (38 g) dissolved in 1 L distilled water until the mixture was completely dissolved. The medium was autoclaved at 121°C for about 20 minutes. After autoclaving, 5 ml of the medium was poured into small bottles (10 ml).

3. Sample Preparation

2 mg of the crude extract was weighed and dissolved in methanol (1 ml) for the antimicrobial activity.

4. Disc Diffusion Method

The antimicrobial assay on cultures of microbes was tested using the disc diffusion method. The disc (6 mm) was prepared by impregnating it in the methanol solution for each sample. Each culture was prepared for turbidity and spread on the test plate. The paper disc containing 2 mg of the fractionated plant extract was placed on the agar surface that was previously inoculated with the suspension of each microbe to be tested. All determination was made in triplicate.

5. Control test

In the antimicrobial test, amoxycillin was used as the positive control while the DMSO solvent was used as the negative control. The inhibition diameter of the bacteria was determined after an incubation at 37°C for 24 hours (1 day). The presence of the clear inhibition zone around each disc indicated the antimicrobial activity. The four bacteria used in this study included positive (*Bacillius subtilis* and *Staphylococcus aureus)* and negative (*Esherichia coli* and *Samonella thypi*) gram stain bacteria that were obtained from the microbiology laboratory of UiTM Kuala Pilah, Negeri Sembilan.

III. RESULT AND DISCUSSION

The bioactive compounds presented in the crude extract of *A. indica* were extracted by using the solvent extraction method with different polarities of solvents: methanol (polar), chloroform (semi-polar) and hexane (non-polar) are shown in Table 1. The differences in the polarity of solvents and method of extraction have attributed to the presence of the bioactive compounds (Mondali *et al.,* 2009). The highest percentage of the crude extract yield of *A. indica* was by using methanol (4.52%) compared to chloroform (2.93%) and hexane (0.40%). A similar finding in which alcohol extracts presented the highest yield compared to other type of solvent was also reported (Klebson *et al.,* 2018, Ogbonna *et al.,* 2016; Vasantharaj, 2013; Prashanth and Krishnaiah, 2014).

Table 1. Percentage yield of total crude extract

Solvents	Weight of powder sample (g)	Weight of crude extract	Yield (%)
Hexane	1046.69	4.03	0.40
Chloroform	1017.72	29.82	2.93
Methanol	987.94	44.61	4.52

The preliminary investigation of bioactive compounds in *A. indica* leaf crude extract contained six phytochemical components as shown in Table 2. The results show that majority of the phytochemicals in *A. indica* extracted by methanol had polar properties, followed by chloroform (semi-polar) and lastly by hexane (non-polar). It was observed that alkaloids, flavonoids, terpenoids, saponins, tannins and steroids were present in the methanol extract.

Flavonoids, terpenoids, and saponins were absent in the chloroform extract. While for the hexane crude extract, only tannin was tested positive.

The research conducted by Zainab *and Hossein* (2016) and Susmitha *et al.,* (2013) also showed a positive result for alkaloids, flavonoids, tannins, saponins and steroids in the methanol crude of *A. indica*. However, only limited journals reported on the chemical compounds in the leaves of *A. indica*. The presence of terpenoids in the leaves of *A. indica was reported by Alase et al., (*2015), Vinoth *et al., (*2012) and Daniel and Danish *(*2011). In addition, the studies by Asif (2013); Girish and Shankara (2008) reported the isolation of 135 additional chemical compounds from the diverse fragments of the leaves.

Table 2. Phytochemical components of *Azadirachta indica* leaf crude extract

Components	CH_3OH	$CHCl_3$	C_6H_{14}	Observation
Alkaloids	+	+	-	Reddish brown precipitate
Flavonoids	+	-	-	Yellow precipitate
Saponins	+	-	-	Froth formation
Terpenoids	+	-	-	Reddish brown colour at the interfaces
Tannins	+	+	+	Blue-black or dark green colour
Steroids	+	+	-	Green colour

Note:Meth=methanol, Chloro=chloroform, Hex=hexane
:Presence(+); Absence (-)

Table 2 indicates that all crude extracts contained tannins. This is because tannins can be present in the two groups which are hydrosable tannins (gallic tannin) and condense tannins. The phytochemical test of tannins using the ferric chloride test can differentiate the types of tannins.: the blue colour is specified for gallic tannins whereas the green colour is specified for catecholic tannins (Vinoth *et al.,* 2012; Vasantharaj *et al.*, 2013). The test of tannins for the hexane crude extract of *A. indica* changed to blue-black colour which indicated the presence of gallic tannins while the methanol and chloroform extracts changed to dark-green colour which indicated the presence of cathecholic tannins.

The phytochemical content was correlated with the antioxidant and antimicrobial activities. The antioxidant activities were determined by measuring the scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) by using ascorbic acid as the standard. DPPH is a very stable organic free radical with deep violet colour which gives the maximum absorption within the 515-528 nm. When antioxidant activities are present, the DPPH radical scavenging activity increases as the concentration of phenolic compounds or the degree of hydroxylation of the phenolic compounds increases (Sanchez-Moreno *et al.*, 1999).

The methanol crude extract showed the best antioxidant activity with 93.28 % inhibition of free radicals compared to chloroform (82.53%) and hexane (65.45%) at the concentration of 1000 µg/mL as displayed in Table 3. The methanol crude extract showed a higher antioxidant activity than chloroform and hexane due to the presence of polar compounds such as phenol and flavonoids. *A. indica* has polar compounds that are valuable effectively in several radicals related to the pathological condition since the compounds can donate hydrogen to a free radical in order to eliminate the odd electron (Dhakal *et al.*, 2016).

Table 3. The percentages radical scavenging activity with various concentrations.

	Concentration of sample, µg/mL	1000	500	250	125	62.5	31.25	15.63	7.81
% of radical scavenging	Ascorbic acid	97.50	97.31	97.12	96.74	96.35	95.97	95.39	95.38
	Methanol	93.28	91.36	81.11	70.25	65.43	55.87	48.37	43.76
	Chloroform	82.53	71.59	63.72	61.81	59.88	47.41	45.11	36.47
	Hexane	65.45	63.92	57.39	55.85	53.55	28.02	19.96	13.82

The bioactive compound in the extract of A. *indica* inhibited the growth of microbes as shown in Table 4. Four bacteria were used in the antimicrobial studies including positive (*B. subtilis* and *S. aureus)* and negative (*E. coli* and *S. thypi*) Gram stain bacteria. The methanol crude extract of *A. indica* showed the maximum zone of inhibition (15 mm) against *B. subtilis,* followed by *S. aureus* (12 mm), *E. coli* (9 mm), and *S. thypi* (9 mm). Amoxycillin (positive control) inhibited 14 to 26 mm whereas DMSO (negative control) showed no inhibition zone as approved by Lin *et al.,* (2011). Amoxycillin (positive control) showed the largest inhibition zone compared to the *A. indica* crude extracts (Sarmiento *et al.,* 2011). Gram-positive bacteria are mostly susceptible whereas the Gram-negative bacteria are mostly resistant because of their cell wall (Francine *et al.,* 2015). However, our present study indicated that Gram-negative bacteria formed a minimum inhibition zone. A similar finding was reported by Mohammed and Omer (2015) whereby *E. coli* was spotted the lowest zone of inhibition (7.5 mm). Moreover, the alkaloids, flavonoids and saponins are the

sources of antibiotics for the protective mechanism against pathogens (Panchal *et al.,* 2013).

Table 4. The antimicrobial activities of *A. indica* crude extract

Inhibition zone (mm)	*S. aureus*	*B. subtilis*	*E. coli*	*S. thypi*
Hexane	7	12	7	7
Chloroform	8	13	8	7.5
Methanol	12	15	9	9
Amoxycillin	25	26	26	14
DMSO	6	6	6	6

IV. CONCLUSION

Thus, in our present study, the phytochemical screening of neem leaves in the different polarity of solvent extracts revealed the present of many phytochemical components. This study may be useful to further explore the pharmacological and biosynthetic activities of the neem leaves.

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