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Chemical Characterization and Biological Study of *Azadirachta indica* Extracts

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Abstract: The utilization of the plant parts (bark, leaves and roots) of *Azadirachta indica* from Malaysia for research purpose were limited and most of the previous studies focused only on the leaves. Thus, this study focused on the volatile compounds and the cytotoxic study of crude and fractions obtained from bark, leaves and roots of *A. indica*. The analysis of the volatiles of the crude and fractions was performed via Gas Chromatography-Mass Spectrometry (GC-MS). The volatiles present vary according to the plant parts and extraction solvents. The bark aqueous fraction yielded highest amount of volatiles (19 compounds) that corresponds towards various reported activities. The n-hexadecanoic acid was the most frequently occurring compound detected in all extracts, bark (40.14 and 20.1%), leaf (65.18 and 44.79%) and root (20.55 and 9.14%) for crudes and fractions, respectively. Meanwhile, the cytotoxic bioassay against brine shrimp (*Artemia salina*) resulted that; the fractions were more toxic compared to the crude extracts. Among all the extracts, leaf ethyl acetate fraction provides a lethal concentration value (1.35 ± 0.40 ppm) of the lowest, followed by bark ethyl acetate (1.38 ± 0.33) and leaf chloroform fraction (2.14 ± 0.35 ppm) and thus indicating the highest cytotoxic effect. The bioassay proved that fractions provide a better extraction technique for the extraction of toxic phytochemicals compared to the crude extracts.

Keywords: *Azadirachta indica*, Crude, Fractions, Volatile Compound, Cytotoxic Bioassay

1. Introduction

Neem (*Azadirachta indica*) (vernacular name in Malay: 'Pokok Mambu') is consumed widely as traditional medicine for the treatment of several diseases, including chicken pox, fever, skin infections, oral care, as a tonic for ulcers, high blood pressure and diabetes [1]. The literature reported that, *A. indica* possesses many medically beneficial bioactive phytochemicals such as hydrocarbon, terpenoids, phenolic, alkaloids, and their derivatives [2]. Some of these phytochemicals exhibits acaricidal [3], antibacterial [4], gastroprotective [5], immu-nostimulant [6] and insecticidal [7] properties. The occurrence and the concentration of secondary metabolites are restricted to the plant taxonomy. Basically, these metabolites are not related to primary metabolism, but exhibit several biological activities or defense. Certain secondary metabolites are classified to be volatile compounds [8].

The extraction of the *A. indica* extracts had been studied trough many different ways [2]-[3]. However, no previous cytotoxic work related to the comparison of crude and fractions of *A. indica* plant parts (bark, leaves and roots) in a

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single study. This comparison of the various parts with a variety of solvents scientifically proves the suitable extraction method of the toxic compounds according to best solvent and plant part. However, very limited study concerning the *A. indica* extracts from Malaysia is available in the literature. Almost, all the reported studies in Malaysia were on the leaf extracts [9] and none includes the root.

One of the effective methods to evaluate the cytotoxicity of a substance is via shrimp lethality assay [10]. The concept of this assay is to kill a laboratory-cultured invertebrate model, *Artemia salina*. This method, predicts the acute toxicity avoiding the usage of laboratory animals that are being demanded by certain organizations [11]. It is recognized to be simple, fast, effective and reproducible assessments of toxicity (e.g. detection of fungal and cyanobacteria toxins, natural products, pesticides and heavy metals) [12]. This assay is vital in determining the responses of human normal and cancer cells as a preclinical assessment for drugs. Therefore, this work aims to identify the volatile compounds of crude and fractions of *A. indica* extracts.

2. Materials and Methods

2.1 Plant Materials

Plant, *A. indica* was harvested directly from the tree from Teluk Intan, Perak, Malaysia. The taxonomy identification was conducted. The parts (bark, leaves and roots) were dried and the moisture content was analyzed via moisture content analyzer; and grounded into a coarse powder using a grinder (0.25 mm sieve). All the samples were sealed and kept in desiccators to avoid fungal activities.

2.2 Extraction of Plant Materials

The crude extracts of grounded parts (barks, leaves and roots; each 50 g) were extracted via maceration in acetone, chloroform, ethanol and reflux in ethanol. The maceration was conducted for 5 days, whereas the reflux method was conducted for minimum extraction of 6 h. The extracts were filtered, evaporated and dried under open air.

The fractionation conducted with some modification of previous study [2]. Grounded part (barks, leaves and roots; 200 g each) was soaked with 80% methanol solution. The mixture was left to settle for a day. The solution cakes were sonicated for about 30 min, filtered and evaporated. The concentrated extract was partitioned with solvents of different polarities, including; hexane (Fraction 1), chloroform (Fraction 2) and ethyl acetate (Fraction 3), respectively with increasing polarity. The aqueous and organic layer was separated using separation funnel. The leftover was the aqueous layer (Fraction 4). All collected fractions were filtered to obtain particle free extract, then dried and stored for further study.

2.3 Determination of Volatile Compounds

Volatiles of all extracts (crudes and fractions) were determined via Agilent Technologies 7890A Gas Chromatography (GC) System coupled with Mass Spectrometry (MS) detector. Each sample was prepared at 1000 ppm via dilution in respective solvents and was injected into the system; except for aqueous extracts that were placed in solid form. Blank analysis was also performed. The chromatography settings are; injection source: GC auto sampler and Thermal separation probe (TSP); injection volume: 1 µL (organic sample); injection mode: split less and split ratio 1:5 and oven temperature: initial 35 °C, increase to 180 °C (6 °C/min), held 5 min, increase to 230 °C (1 °C/min) and held 20 min; and initial 35 °C (2 min), increase to 180 °C (2 °C/min), held 5 min, increase to 230 °C (6 °C/min) and held 30 min; for organic and aqueous samples, respectively. Other settings; column: non-polar capillary DB-1 of 100% dimethyl-polysiloxane (30 m x 0.53 mm id, film thickness 0.25 µm); carrier gas: helium (1 mL/min); ionization energy: 70 eV; front inlet pressure: 6.78 psi, oven equilibrium time: 3 min; maximum oven, post run, front inlet, MS source and MS quad temperature: 350, 290, 250, 230 and 150 °C, respectively, for both organic and aqueous samples. The compounds were characterized with the National Institute of Standards and Technology (NIST) Library Chem Station software.

2.4 Cytotoxicity Bioassay

2.4.1 Preparation of samples

The appropriate amount of each extract was dissolved in artificial sea water with 0.5 ml of dimethyl sulfoxide (DMSO, Merck, Germany) to prepare 50 mL of extract solutions (50, 100, 500, and 1000 ppm). The positive control was 0.1% of potassium dichromate (Sigma-Aldrich, USA) (Control 1); whereas the negative control was 1% DMSO (Control 2).

2.4.2 Source of seawater and organisms

Artificial sea water (35 ppt) was prepared with commercial sea salt in 1 L of distilled water and filtered. The solution was transferred into a cylindroconical tube (hatching system) with 1.5 g/L of *A. salina* eggs (Super Eagle, China). The eggs were exposed to an optimal incubation period of 24 h (27-29 °C) with lateral illumination by a light tube (500-1000 Lx) and aeration. The solution was swirled once a while to maximize cysts hatching. After 24 h, the aeration was stopped to separate the nauplii from hatched shells and placed into containers containing the same concentration of artificial seawater, light source and aeration. The nauplii were fed with 0.06% yeast solution starting from the first day of hatching up to 48 h of bioassay study.

2.4.3 Determination of cytotoxicity

The lethality bioassay was performed with minor modification from previous study proposed by Nguta et al. (2013) [13]. Test was conducted using prepared test samples (50, 100, 500, and 1000 ppm) and controls. An amount of 3 mL of each test sample and controls was transferred into test tubes and a number of 10 nauplii were released into them. The tubes were stored at 27-29 °C. The lethality of the nauplii were observed within its activity range and evaluated after 24 h. The moribund nauplii within 10 s were counted as dead. The assay was performed in triplicate and values were recorded.

2.5 Statistical Analysis

Lethality data were calculated through percentage mortality, according to equation (1) and corrected lethality in relation to the negative control were calculated and corrected by applying Abbott's formula as in equation (2) [14]. The corrected percentage mortality was transferred into a graph through Probit analysis in the presence of linear functions [15]. Cytotoxicity was reported as LC50 that represents 50% of moribund or killed nauplii. The significant difference of the LC50 values was analyzed through ANOVA and Tukey's multiple range test ($P<0.05$) [16].

$$\% \text{ Mortality} = \frac{\text{Moribund or dead nauplii}}{\text{Total tested nauplii per replicate}} \times 100\% \quad (1)$$

$$\text{Corrected \% Mortality} = 1 - \frac{\text{Nauplii in treated sample}}{\text{Nauplii in control}} \times 100\% \quad (2)$$

3. Results and Discussions

3.1 Volatile Phytochemicals of *A. indica*

Major volatile compounds (>10%), found in the crude (Table 1) are 9-octadecenoic acid, (E)-, an unsaturated fatty acid, identified in bark maceration in ethanol extract (10.8%); eicosane, an alkane, from leaf chloroform extracts (15.6%); hexadecanoic acid, methyl ester, a fatty acid methyl ester, present in maceration in ethanol extract of leaf (20.6%); n-hexadecanoic acid, a fatty acid chain, detected in bark chloroform and reflux in ethanol, leaf chloroform, maceration and reflux in ethanol (10.8%, 15.6%, 17.7%, 16.6% and 25.8%, respectively) and octadecanoic acid, methyl ester identified in bark acetone and maceration in ethanol extracts (13.4 and 10.6%, respectively).

Whereas, the most abundant volatile compounds (>10%), identified in the fractions (Table 2a and b) were: 9,12,15-octadecatrien-1-ol, (Z,Z,Z)-, a fatty alcohol, detected in the bark and leaf hexane (17.04 and 23.35%, respectively); benzoic acid, 4-hydroxy-3,5-dimethoxy-, an ester, present in root aqueous (13.4%); n-hexadecanoic acid, found in bark and leaf hexane (11.7% and 19.1%) and leaf chloroform (14.9%); pentadecanoic acid, 14-methyl-, methyl ester identified in bark chloroform (16.4%); phytol, a diterpene alcohol from the leaf hexane (13.9%) and chloroform (62.25%) fractions, and p-xylene from leaf ethyl acetate fraction (16.1%). In similarity for both crude and fractions, n-hexadecanoic acid, is a compound that occurs more frequently in all the extracts. This compound found to be present in bark, leaves and roots of the plant, but in different amount according to the extraction solvent polarity. Most of the extracts differ, in terms of the components found in different solvent extract and the extraction method, in which most of them were detected in different percentage level and the nature of the compounds were also differing.

3.2 Cytotoxicity of *A. indica* Extracts

The brine shrimp, *A. salina* lethality bioassay was used to indicate the cytotoxicity that involves many pharmacological effects and diseases (e.g. pesticide, antitumor and anticancer) as they corresponds similarly as a mammalian system [38]. The cytotoxic effect of *A. salina* on the exposure towards the extracts was represented in Table 3. The result shows that, the degree of lethality is directly proportional to the concentration of the extracts, whereby the mortality rate increases with the sample concentration.

The probit graph was plotted to perform the empirical probit, calculate the slope and intercepts in the form of linear regression to give out the 50% lethal concentration (LC_{50}) values [39].

Theoretically, extract that exhibits smaller LC_{50} values are more toxic. From the overall results of the crude extracts, bark present to be the best part, according to the LC_{50} value and toxicity profile. The extracts of acetone (660.69 ± 0.87 ppm), maceration (660.69 ± 0.87 ppm) and reflux in ethanol (549.54 ± 0.79 ppm) present to provide LC_{50} values that lie between the toxicity level (≥500≤1000 ppm: weak toxicity). The second active part would be root as the acetone extracts present to have the lowest LC_{50} value (457.09 ± 0.88 ppm) (<500 ppm: toxic) and chloroform extract shows weak toxicity (LC_{50} 645.65 ± 0.94 ppm); whereas maceration and reflux in ethanol is non-toxic (>1000 ppm). The leaf extract are with a low toxicity level of acetone (645.65 ± 0.94 ppm) and chloroform (891.25 ± 0.71 ppm) while toxic factors were not detected in extracts of maceration and reflux in ethanol.

According to previous studies, it had been reported that, the *A. indica* leaves ethanol crude extract showed LC_{50} value of 23 ppm [40], 28 µg/mL [38], 36.81 mg/mL [41], and 37.15 mg/mL [42] against *A. salina*. Therefore, with the comparison of the obtained results in reference of past study, the current results are acceptable as the LC values achieved, lies between the reported results.

The increasing chronological order of the solvent in extracting the crude with high toxic phytochemicals, would be the maceration in ethanol < reflux in ethanol < chloroform < acetone. Acetone extract of the root is the only extract that presents to fall between the toxic levels of LC_{50}. The maceration and reflux in ethanol are mostly non-toxic for both leaf and root extracts. Chloroform extracts are listed to act better than the ethanol extracts as only one extract of the bark chloroform is non-toxic.

The two different extraction techniques, of crude alone and partitioning of crude methanol extract to its subsequent solvent fractions, resulted in toxicity activities that are variable in this study. The technique proves that the partitioning method present to exhibit more significant toxic effect, whereby the overall mortality range lies between the ranges of 25.71 to 97.50%. The fractions of two immiscible solvents obtain through partitioning are effective in separating the complex mixtures of the plant metabolites according to polarity that exhibits activities at different levels. The low concentration of the bioactive compounds in the crude might prejudice their activity.

Table 1: Volatile phytochemicals of *A. indica* crude extracts

Compounds	Percentage of compound in crude extract (%)												Reported activity
	Bark				Leaf				Root				
	AC	CH	ME	RE	AC	CH	ME	RE	AC	CH	ME	RE	
1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	-	-	-	-	-	-	-	-	2.0	-	-	-	Antimicrobial [17]
1-Heptadecene	2.4	-	-	-	-	-	-	-	-	-	-	-	NR
1-Hexadecene	-	-	-	-	-	-	-	-	0.4	-	-	-	NR
1-Nonadecene	2.0	-	-	-	-	-	-	-	-	-	-	-	NR
1-Octadecene	-	-	-	-	-	-	-	-	0.6	-	-	-	NR
2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	-	3.2	1.7	-	-	-	-	-	4.0	3.0	1.1	-	Antimicrobial [18]
2-Tetradecene, (E)-	-	-	-	-	-	-	-	-	0.2	-	-	-	Antifungal [19]
9-Octadecenoic acid, (E)-	-	4.7	10.8	-	-	-	-	-	-	-	-	-	Antiviral [20]
alpha.-Cadinol	-	-	-	-	-	-	-	-	-	-	0.3	-	Antibacterial, antifungal [21]
Caryophyllene oxide	-	1.2	-	-	-	-	-	-	-	-	-	-	NR
Cycloheptasiloxane, tetradecamethyl-	-	-	-	-	1.6	-	-	6.8	-	-	-	-	Antioxidant [22]
Dichloroacetic acid, heptadecyl ester	-	-	-	-	-	-	-	-	-	1.6	-	-	Antiviral [23]
Docosane	-	-	-	-	-	0.9	-	-	-	-	-	-	Antibacterial [17]
Eicosane	-	2.3	-	-	-	15.6	-	-	-	-	-	-	Antibacterial, antifungal [17]
Ethyl Oleate	-	-	6.4	-	-	-	-	-	-	-	-	-	Antiviral [20]
Ferruginol	-	-	-	-	-	-	-	-	-	6.0	-	-	Gastroprotective and ulcer healing effect [24]
Hentriacontane	-	-	-	-	-	-	-	-	-	0.3	-	-	Antibacterial, antifungal [17]
Heptacosane	-	-	-	-	-	2.1	-	-	-	0.5	-	-	Antioxidant [17]
Heptadecanoic acid, ethyl ester	-	-	5.9	-	-	2.3	-	-	-	-	-	-	NR
Hexadecanoic acid, butyl ester	-	-	-	-	-	-	-	-	-	5.6	-	-	Antioxidant [25]
Hexadecanoic acid, methyl ester	-	-	-	-	-	-	20.6	-	-	-	0.3	-	Antimicrobial [17]
n-Hexadecanoic acid	5.6	10.8	8.2	15.6	5.1	17.7	16.6	25.8	1.0	1.8	7.9	9.8	Antimicrobial [17]
Nimbiol	-	-	-	-	-	-	-	-	1.1	-	-	-	Anti-acne [26]
Octacosane	-	0.5	-	-	-	1.6	-	-	-	-	-	-	Antiviral [20]
Octacosyl acetate	-	-	-	-	-	3.1	-	-	-	-	-	-	Toxicity [27]
Pentadecane	1.9	0.6	-	-	-	-	-	-	-	-	-	-	Antiviral [20]
Octadecanoic acid, methyl ester	13.4	-	1.0	-	-	-	10.6	-	3.2	0.3	0.8	-	Antimicrobial [28]
Retinoic acid, methyl ester	-	6.6	-	-	-	-	-	-	-	-	-	-	NR
Tetratriacontane	-	-	-	-	-	1.9	-	-	-	-	-	-	Anti-acne [26]

Abbreviation: AC: Acetone; CH: Chloroform; ME: Maceration in ethanol; RE: Reflux in ethanol

Table 2a: Volatile phytochemicals of *A. indica* fractions

Compound	Percentage of compound in fraction (%)										Reported activity
	Bark*			Leaf				Root*			
	HE	CH	AQ	HE	CH	EA	AQ	HE	CH	AQ	
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	-	-	7.6	-	-	-	9.1	-	-	5.4	Antimicrobial [17]
1-Eicosene	-	-	2.1	-	-	-	1.3	-	-	-	Antioxidant, larvicidal [21]
1-Octadecene	-	-	1.5	-	-	-	-	-	-	-	NR
2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	-	0.4	-	-	-	-	-	-	-	-	Antimicrobial [18]

Table 2b: Volatile phytochemicals of *A. indica* fractions

Compound	Percentage of compound in fraction (%)										Reported activity
	Bark*			Leaf				Root*			
	HE	CH	AQ	HE	CH	EA	AQ	HE	CH	AQ	
2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl-, (all-E)-	-	-	1.3	-	-	-	-	-	-	-	NR
2-Methoxy-4-vinylphenol	-	-	-	-	-	-	-	-	-	3.1	Antimicrobial [29]
2-Propenal, 3-(4-hydroxy-3-methoxy phenyl)-	-	-	-	-	-	-	-	-	0.3		Uterotonic [30]
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	-	-	5.8	-	-	-	5.4	-	-	2.0	NR
3-Hydroxy-4-methoxybenzoic acid	-	-	3.6	-	-	1.2	-	-	-	0.2	Antidiabetic [31]
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	-	0.6	-	-	-	-	-	-	3.4	-	Analgesic, fungicide [29]
4,4,8-Trimethyltricyclododecane-2,9-diol	-	2.5	-	-	-	-	-	-	-	-	Antimicrobial [29]
5-Octadecene, (E)	-	-	-	-	-	1.8	1.3	-	-	-	Antioxidant [32]
7-Hydroxy-6-methoxy-2H-1-benzopyran-2-one	-	-	1.7	-	-	-	-	-	-	-	Anti-carcinogenic [17]
7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	2.3	-	-	-	-	-	-	-	4.0	-	NR
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	17.0	4.5	-	23.3	-	-	-	-	2.4	-	Antibacterial [33]
9,12-Octadecadienoic acid (Z,Z)	-	-	-	-	-	-	-	9.3	-	-	NR
9,17-Octadecadienal, (Z)-	0.8	-	-	-	-	-	-	-	-	-	NR
9-Octadecenoic acid (Z)-, methyl ester	-	-	-	-	-	-	-	-	1.6	-	Rodenticide [29]
alpha.-Cubebene	-	-	-	0.3	-	-	-	-	-	-	Wound healing [34]
Benzene, 1,2,3,4-tetramethyl-	-	-	-	0.7	-	-	-	-	-	-	NR
Benzene, 1,2,4,5-tetramethyl	-	-	0.3	-	-	-	-	-	-	-	NR
Benzoic acid	-	-	2.5	-	-	-	-	-	-	-	Antifungal [17]
Benzoic acid, 4-hydroxy-3,5-dimethoxy-	-	-	1.0	-	-	-	-	-		13.4	Antimicrobial, antioxidant [35]
beta.-Sitosterol	-	0.6		-	-	-	-	-	1.8	-	Antiviral [20]
Caryophyllene	0.4	-	1.2	-	-	-	-	-	-	-	Larvicidal [21]
Caryophyllene oxide	0.9	-	1.0	-	-	-	-	-	-	-	NR
Catechol	-	-	-	-	-	1.3	-	-	-	-	NR
Cyclohexane, 1-ethenyl-1-methyl-2, 4-bis(1-methylethenyl)-, [1S-(1.alpha,2.beta, 4.beta)]	-	-	-	0.2	-	-	-	-	-	-	Antimicrobial [36]
Cyclononasiloxane, octadecamethyl-	-	-	1.1	-	-	-	-	-	-	-	Anticancer [37]
Dodecanoic acid	0.2	0.2	-	-	-	-	-	-	-	-	NR
Eicosane	-	-	-	2.7	-	-	-	-	-	-	Antimicrobial [17]
gamma.-Elemene	-	-	-	7.7	-	-	-	-	-	-	Antifungal [17]
gamma.-Sitosterol	-	-	-	-	-	-	-	-	0.3	-	Antibacterial [17]
Hexacosane	-	-	-	1.6	-	-	-	-	-	-	Anti-acne [26]
Methyl stearate	-	1.9	-	-	-	-	-	-	3.1	-	Antiviral [20]
n-Hexadecanoic acid	11.7	4.0	4.4	19.1	14.9	7.0	3.9	2.8	3.0	3.5	Antimicrobial [17]
Octadecanoic acid	2.3	0.3	1.4	2.3	-	-	1.9	-	-	-	Antifungal, antibacterial [17]
Oleic Acid	8.1	2.3	-	7.3	-	-	-	5.7	1.6	-	Antiviral [20]
Pentadecanoic acid, 14-methyl-, methyl ester	-	16.4	-	-	-	-	-	-	1.1	-	Antifungal [17]
Phenol, 2,4-bis(1,1-dimethylethyl)	-	-	-	-	-	2.5	-	-	-	-	Antimicrobial [28]
Phenol, 2,6-dimethoxy	-	-	2.4	-	-	-	4.6	-	-	5.0	Antimicrobial [29]
Phenol, 2,6-dimethoxy-4-(2-propenyl)-	-	-	-	-	-	-	-	-	0.2	5.7	NR
Phenol, 2-methoxy	-	-	1.6	-	-	-	-	-	-	-	NR
Phytol	-	-	-	13.9	62.3	-	-	-	-	-	Anticancer [17]
p-Xylene	-	-	-	-	-	16.1	-	-	-	-	Antioxidant, antifungal [17]
Squalene	-	-	-	-	-	-	0.7	-	-	-	Antibacterial, pesticide [17]
Stigmasterol	-	0.5	-	-	-	-	-	-	4.5		Anticancer [20]
Tetradecanoic acid	0.4	0.3	0.6	-	-	-	0.7	-	-	2.7	Antiviral [20]
Tricosane	-	-	0.3	-	-	-	-	-	-	-	Anti-acne [26]

*****No compounds reported for EA fractions of bark and root. Abbreviation: HE: Hexane; CH: Chloroform; AQ: Aqueous; EA: Ethyl acetate

In this technique of fractionation, the plant part of the leaf, present to be the best, followed by the bark and finally root. Leaf fractions have the lowest LC_{50} values of 1.35 ± 0.40, 2.14 ± 0.35, 25.12 ± 0.35 and 45.71 ± 0.32 ppm (ethyl acetate, chloroform, hexane and aqueous, respectively). The *A. indica* was also analyzed for cytotoxicity, and had reported LC_{50} values of 1.3 µg/ml for hexane leaf extract [43], and the current study proves that the ethyl acetate solvent extracts are almost at the similar toxic level (1.35 ± 0.40 ppm). According to the other analysis, the aqueous and methanol extracts achieved higher LC_{50} values (101.26 ± 3.7 and 61.43 ± 2.9 µg/mL) [44] compared to the value obtained in this study (45.71 ± 0.32 ppm).

A researcher, reported that the aqueous root and bark extract of *A. indica* had achieved an LC_{50} of 285.8 mg/ml [13] that is far high compared to the current data (LC_{50} 3.24 ± 0.18 and 4.68 ± 0.15 ppm). Therefore, the LC values of the extract were found to be lower than the previous studies and thus indicate that the prepared extract was rich in bioactive compounds.

This data is supported by the similar extracting solvent itself, whereby the best solvent for all the three parts would be the ethyl acetate with lowest LC_{50} values (1.35 ± 0.40, 1.38 ± 0.33, and 2.29 ± 0.25 ppm for leaf, bark and root, respectively) followed by chloroform as the extracts present to achieve LC_{50} values closer to the ethyl acetate (LC_{50} in bark 2.29 ± 0.29 ppm and leaf 2.14 ± 0.35 ppm). The aqueous and hexane fractions present to be in the third and fourth ranking. The LC_{50} value of hexane in root fraction was very high (LC_{50} 281.84 ± 0.24 ppm) compared to the other fractions. The overall chronological order for the best solvent in fractionation of the plant material would be the ethyl acetate > chloroform > aqueous > hexane. A researcher had pointed out that the constituents of the bioactive may differ in each extract depending on its solubility range in solvent and thus extraction should be carried out in a wide range of solvents with variable polarities [45].

The American National Cancer Institute had set up criteria that a sample should possess LC_{50} limit of at least 30 ppm to prove that the sample is promising and suitable for further purification [46]. Therefore, the crude extracts with LC_{50} values ranges from the 457.09 ± 0.88 to 891.25 ± 0.71 ppm are not suitable to be further purified. On the other aspect, all the fractions with LC_{50} values of 1.35 ± 0.40 to 25.12 ± 0.35 of the *A. indica* are suitable to be purified to isolate and further identify the bioactive phytochemical except for the hexane fraction of bark (35.48 ± 0.21 ppm) and root (35.48 ± 0.21 ppm).

A group of researcher had listed out a research study that had achieved LC_{50} values of less than 500 ppm together with its biological activity and some of them are the flowers of *Calendula officinalis* with LC_{50} of 245 µg/mL for anti-inflammatory and wound healing activity, whereas the leaf of *Vinca rosea* with LC_{50} of 170 µg/mL shows anti-diabetic and anti-cancer effect [47]. Therefore, in comparison of the findings in this study, the root acetone crude extract and all the fractions of the extract have potential bioactive compounds.

Table 3: LC_{50} values of *A. indica* crudes and fractions against *A. salina*

Crude				**Fraction**			
LC_{50} (ppm)		**95% confidence interval**	**Toxicity level**	**LC_{50} (ppm)**		**95% confidence interval**	**Toxicity level**
Bark				***Bark***			
AC	660.69 ± 0.87 [a]	13.46 - 33140.81	Weak toxicity	HE	35.48 ± 0.21	13.49 - 91.20	Toxic
CH	>1000	NC	Non-toxic	CH	2.29 ± 0.29 [e]	0.78 - 10.72	Toxic
ME	660.69 ± 0.87 [a]	13.46 - 33140.81	Weak toxicity	EA	1.38 ± 0.33 [f]	0.31 - 6.17	Toxic
RE	549.54 ± 0.79	15.55 - 19384.13	Weak toxicity	AQ	3.24 ± 0.18	1.41 - 7.59	Toxic
Leaf				***Leaf***			
AC	645.65 ± 0.94 [b]	9.46 - 44632.74	Weak toxicity	HE	25.12 ± 0.35	5.13 - 125.89	Toxic
CH	891.25 ± 0.71	36.17 - 22111.72	Weak toxicity	CH	2.14 ± 0.35 [e]	0.45 - 10.23	Toxic
ME	>1000	NC	Non-toxic	EA	1.35 ± 0.40 [f]	0.22 - 8.13	Toxic
RE	>1000	NC	Non-toxic	AQ	45.71 ± 0.32	10.47 - 199.53	Toxic
Root				***Root***			
AC	457.09 ± 0.88	8.72 - 24127.07	Toxic	HE	35.48 ± 0.21	95.50 - 831.76	Toxic
CH	645.65 ± 0.94 [b]	9.46 - 44632.74	Weak toxicity	CH	23.44 ± 0.30	6.03 - 91.20	Toxic
ME	>1000	NC	Non-toxic	EA	2.29 ± 0.25 [e]	0.74 - 7.08	Toxic
RE	>1000	NC	Non-toxic	AQ	4.68 ± 0.15	2.40 - 9.12	Toxic

* The data represent the means ± standard deviation of three replicates. Means with the same letter are not significantly different at (Tukey's test, $p \leq 0.05$).
Toxicity level of extracts: LC_{50} values >1000 ppm (non-toxic), ≥500≤1000 ppm (weak toxicity) and <500 ppm (toxic) [48].
Abbreviation; AC: Acetone; CH: Chloroform; ME: Maceration in ethanol; RE: Reflux in ethanol; HE: Hexane; AQ: Aqueous; EA: Ethyl acetate; NC: Not calculate

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References

[1] Kumar, V.S. and Navaratnam, V. 2013. Neem (*Azadirachta indica*): Prehistory to Contemporary Medicinal Uses to Humankind. Asian Pac. J. Trop. Biomed., 3(7): 505-514.

[2] Hossain, M.A., Al-Toubi, W.A.S., Weli, A.M., Al-Riyami, Q.A. and Sabahi, J.N. 2013. Identification and Characterization of Chemical Compounds in Different Crude Extracts from Leaves of Omani Neem. J. Taibah Univ. Sci., 7: 181–188.

[3] Du, Y.H., Li, J.L., Jia, R.Y., Yin, Z.Q., Li, X.T., Lv, C., Ye, G., Zhang, L. and Zhang, Y.Q. 2009. Acaricidal Activity of Four Fractions and Octadecanoic Acid-tetrahydrofuran-3,4-diyl Ester Isolated from Chloroform Extracts of Neem (*Azadirachta indica*) Oil Against *Sarcoptes scabiei var. cuniculi* Larvae *In-vitro*. Vet. Parasitol., 163:175–178.

[4] Zhang, Y.Q., Xu, J., Yin, Z.Q., Jia, R.Y., Lu, Y., Yang, F., Du, Y.H., Zou, P., Lv, C., Hu, T.X., Liu, S.L., Shu, G. and Yi, G. 2010. Isolation and Identification of the Antibacterial Active Compound from Petroleum Ether Extract of Neem Oil. Fitoterapia. 81: 747–750.

[5] Bandyopadhyay, U., Biswas, K., Chatterjee, R., Bandyopadhyay, D., Chattopadhyay, I., Ganguly, C.K., Chakraborty, T., Bhattacharya, K. and Banerjee, R.K. 2002. Gastroprotective Effect of Neem (*Azadirachta indica*) Bark Extract: Possible Involvement of H^+-K^+-ATPase Inhibition and Scavenging of Sydroxyl Radical. Life Sci., 71: 2845-2865.

[6] Kumar, V.S. and Navaratnam, V. Rajasekaran, A. and Nair, N. 2012. Isolation and Characterization of Glucosamine from *Azadirachta indica* Leaves: An Evaluation of Immunostimulant Activity in Mice. Asian Pac. J. of Trop. Biomed., S1561-S1567.

[7] Siddiqui, B.S., Afshan, F., Ghiasuddin, Faizi, S., Naqvi, S.N.H., Tariq, R.M. 2000. Two Insecticidal Tetranortriterpenoids from *Azadirachta indica*. Phytochem., 53: 371-376.

[8] Holopainen, J.K. and Blande, J.D. 2012. Sensing in Nature. Advances in Experimental Medicine and Biology (eds L. Carlos) pp. 17-31. United States; Landes Bioscience and Springer.

[9] Othman, F., Motalleb, G., Peng, S.L.T., Rahmat, A., Basri, R. and Pei, C.P. 2012. Effect of Neem Leaf Extract (*Azadirachta indica*) on c-Myc Oncogene Expression in 4T1 Breast Cancer Cells of BALB/c Mice. Cell J. 14(1): 53-60.

[10] Michael, A.S., Thompson, C.G., Abramovitz, M. 1956. *Artemia salina*, A Test Organism for a Bioassay. Sci., 123: 464.

[11] Molina-Salinas, G.M. and Said-Fernandez, S. 2006. A Modified Microplate Cytotoxicity Assay with Brine Shrimp Larvae (*Artemia salina*). Pharmacol., 3: 633-638.

[12] Carballo, J.L., Hernandez-Inda, Z.L., Perez, P. and Garcia-Gravalos, M.D. 2002. A Comparison between Two Brine Shrimp Assays to Detect *In-vitro* Cytotoxicity in Marine Natural Products. BioMed. Central Biotechnol., 2: 1-5.

[13] Nguta, J.M. and Mbaria, J.M. 2013. Brine Shrimp Toxicity and Antimalarial Activity of Some Plants Traditionally Used in Treatment of Malaria in Msambweni District of Kenya. J. Ethnopharm., 148: 988–992.

[14] Abbott, W.S. 1987. A Method of Computing the Effectiveness of an Insecticide. J. American Mosq. Control Assoc., 3(2):302-303.

[15] Finney D.J. 1971. Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve. 3rd edn. Cambridge University Press.

[16] Nathan, S. S., Kalaivani, K. and Murugan, K. 2005. Effects of Neem Limonoids on the Malaria Vector *Anopheles stephens* Liston (Diptera: *Culicidae*). Acta Trop., 96(1): 47-55.

[17] Akpuaka, A., Ekwenchi, M.M., Dashak, D.A. and Dildar, A. 2013. Biological Activities of Characterized Isolates of n-Hexane Extract of *Azadirachta indica* A. Juss (Neem) Leaves. New York Sci. J., 6(6):119-124.

[18] Kalaiarasan, A. and John, S.A. 2011. GC-MS Analysis of *Bulbophyllum KaitenseRechib. Pseudobulbs* Estern Ghats of India. Int. J. of Chem. App., 3(3): 215-220.

[19] Yogeswari, S., Ramalakshmi, S., Neelavathy, R. and Muthumary, J. 2012. Identification and Comparative Studies of Different Volatile Fractions from *Monochaetia kansensis* by GCMS. Global J. of Pharmacol., 6(2): 65-71.

[20] Helmy, W.A., Abd-Alla, H.I., Amer, H. and El-Safty, M.M. 2007. Chemical Composition and '*In-vitro*' Antiviral Activity of *Azadirachta indica* A. Juss (Neem) Leaves and Fruits against Newcastle Disease Virus and Infectious Bursal Disease Virus. Australian J. Basic Appl. Sci., 1(4): 801-812.

[21] El-Hawary, S.S., El-Tantawy, M.E. Rabeh, M.A. and Badr, W.K. 2013. Chemical Composition and Biological Activities of Essential Oils of *Azadirachta indica* A. Juss. Int. J. Appl. Res. Nat. Prod., 6(4). 33-42.

[22] Rathinamala, J., Anjana, J.C., Sruthy, P.B. and Jayashree, S. 2013. Phytochemical Screening and GC-MS Analysis of Bioactive Compounds from *Mimusops elengi.* Ann. Pharma Res., 1(2): 32-34.

[23] Ara, I., Bukhari, N.A., Aref, N.M., Shinwari, M.M.A. and Bakir, M.A. 2012. Antiviral Activities of *streptomycetes* Against Tobacco Mosaic virus (TMV) in *Datura* Plant: Evaluation of Different Organic Compounds in Their Metabolites. African J. of Biotechnol., 11(8): 2130-2138.

[24] Rodriguez, J.A., Theoduloz, C., Yanez, T., Becerra, J. and Schmeda-Hirschmann, G. 2006. Gastroprotective and Ulcer Healing Effect of Ferruginol in Mice and Rats: Assessment of its Mechanism of Action using *In-vitro* Models. Life Sci., 78(21): 2503-9.

[25] Prakash, O., Gondwal, M. and Pant, A.K. 2011. Essential Oils Composition and Antioxidant Activity of Water Extract from Seeds and Fruit Pulp of *Skimmia anquetilia* N.P. Taylor & Airy Shaw. Indian J. Nat. Prod. Res., 2(4): 435-441.

[26] Nand, P., Drabu, S. and Gupta, R.K. 2012. Insignificant Anti-acne Activity of *Azadirachta indica*

Leaves and Bark. J. Pharm. Negative Results, 3(1): 29-33.
[27] Robertson, S., Narayanan, N. and Nargis, N.R.R. 2012. Toxicity Evaluation on Hydroalcoholic Extract of Leaf and Stem Bark of *Prosopis cineraria.* Int. J. Pharm. Pharm. Sci., 4(3): 113-118.
[28] Senthilkumar, N., Murugesan, S. and Vijayalakshmi, K.B. 2012. GC-MS-MS Analysis of *Trichilia connaroides* (Wight & Arn.) Bentv (Meliaceae): A tree of Ethnobotanical Records. Asian J. Plant Sci. Res., 2(2): 207-211.
[29] Moorthy, V. and Boominathan, M. 2011. The Antimicrobial Activities of Crude Extracts and Fraction of *Psidium guajava* and *Azadirachta indica* against *Staphylococcus aureus* in Chronic Disease Affected Patients. Int. J. Universal Pharm. Life Sci., 1(2):160-173.
[30] Sewram, V. 1997. Supercritical Fluid Extraction and Analysis of Indigenous Medicinal Plants for Uterotonic Activity. Ph.D. thesis. Department of Chemistry and Applied Chemistry, University of Natal, Durban, South Africa.
[31] Saravanamuttu, S. and Sudarsanam, D. 2012. Antidiabetic Plants and Their Active Ingredients: A Review. Int. J. Pharm. Sci. Res., 3(10): 3639-3650.
[32] Conforti, F., Statti, G.A. and Menichini, F. 2007. Chemical and Biological Variability of Hot Pepper Fruits (*Capsicum annuum var. acuminatum* L.) in Relation to Maturity Stage. Food Chem., 102: 1096–1104.
[33] Growther, L., Savitha, N. and Niren Andrew, S. 2012. Antibacterial Activity of *Punica granatum* Peel Extracts against Shiga Toxin Producing *E. coli.* Int. J. Life *Sci.* Biotech. Pharma Res., 1(4): 164-172.
[34] Pandey, I.P., Ahmed, S.F., Shhimwal, S. and Pandey, S. 2012. Chemical Composition and Wound Healing Activity of Volatile Oil of Leaves of *Azadirachta indica* A. Juss. Adv. Pure App. Chem., 1(3): 62-66.
[35] Liu, H., Wang, K., Zhao, J., Wang, M. and Zhou, L. 2012. Secondary Metabolites from *Halostachys caspica* and Their Antimicrobial and Antioxidant Activities. Rec of Nat. Prod., 6(1): 57-61.
[36] Arunkumark, V. and Paridhavi, M. 2013. Evaluation of the Components and Antimicrobial Activity of Volatile oil from *Zanthoxylumlimonella* Fruit. Int. J. Pharma Bio Sci., 4(2): 777-787.
[37] Chen, Y., Zhou, C., Ge, Z., Liu, Y., Liu, Y., Feng, W., Li, S., Chen, G. and Wei, T. 2013. Composition and Potential Anticancer Activities of Essential Oils Obtained from Myrrh and Frankincense. Oncol. Lett., 6: 1140-1146.
[38] Elhardallou, S.B. 2011. Cytotoxicity and Biological Activity of Selected Sudanese Medicinal Plants. Res. J. Med. Plant, 5: 201-229.
[39] Throne, J.E., Weaver, D.K., Chew, V. and Baker, J.E. 1995. Probit Analysis of Correlated Data: Multiple Observations over Time at One Concentration. J. Economic Entomol., 88(5): 1510-1512.
[40] Rahmani, M., Mohd Ismail, H., Ahmad, F., Manas, A.R. and Sukari, M.A. 1992. Screening of Tropical Plants for the Presence of Bioactive Compounds. Pertarnka. 15(2): 131-135.
[41] Chowdhury, N., Ghosh, A. and Chandra, G. 2008. Mosquito Larvicidal Activities of *Solanum villosum* Berry Extract against the Dengue Vector *Stegomyia aegypti.* BoiMed Central Complement Altern Med., 8:10.
[42] Al-Emran, A., Shahed, S.M., Farzana Ahmed, Saha, S.K., Das, S.C. and Bachar, S.C. 2011. Evaluation of Brine Shrimp Lethality and Antimicrobial Activity of *Azadirachta indica* Leaf Extract on Some Drug Resistance Bacteria in Bangladesh. Pharmacognosy J., 3(20): 66-71.
[43] Islam, M., Al-Amin, M., Ali Siddiqi, M.M., Akter, S., Haque, M.M., Sultana, N. and Chowdhury, A.M.S. 2012. Isolation of Quercetin-3-*O*-β-D-Glucopyranoside from the Leaves of *Azadirachta indica* and Antimicrobial and Cytotoxic Screening of the Crude Extracts. Dhaka Univ. J. Sci., 60(1): 11-14.
[44] Kirira, P.G., Rukunga, G.M., Wanyonyi, A.W., Muregi, F.M., Gathirwa, J.W., Muthaura, C.N., Omar, S.A., Tolo, F., Mungai, G.M. and Ndiege, I.O. 2006. Anti-plasmodial Activity and Toxicity of Extracts of Plants Used in Traditional Malaria Therapy in Meru and Kilifi Districts of Kenya. J. Ethnopharm., 106:403–407.
[45] Bulbul, I.J., Zulfiker, A.H.M., Hamid, K., Khatun, M.H. and Begum, Y. 2011. Comparative Study of *In-vitro* Antioxidant, Antibacterial and Cytotoxic Activity of Two Bangladeshi Medicinal Plants- *Luffa cylindrica* L. and *Luffa acutangula.* Pharmacognosy J., 3(23): 59-66.
[46] Rana, S., Bhatt, C., Kanaki, N. and Zaveri, M. 2012. Evaluation of cytotoxicity of some selected medicinal plants. Adv. Res. Pharm. Bio., 2(3): 290-295.
[47] Jimenez, C.D.C., Flores, C.S., He, J., Tian, Q., Schwartz, S.J. and Giusti, M.M. 2011. Characterisation and Preliminary Bioactivity Determination of *Berberis boliviana Lechler* Fruit Anthocyanins. Food Chem., 128: 717-724.
[48] Bastos, M.L.A., Lima, M.R.F., Conserva, L.M., Andrade, V.S., Rocha, E.M.M. and Lemos, R.P.L. 2009. Studies on the Antimicrobial Activity and Brine Shrimp Toxicity of *Zeyheria tuberculosa* (Vell.) Bur. (Bignoniaceae) Extracts and Their Main Constituents. Ann. Clin. Microbiol. Antimicrob., 8(16): 1-6.

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