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Phytochemical, Antimicrobial, Cytotoxic, Analgesic and Anti-Inflammatory Properties *of Azadirachta Indica*: A Therapeutic Study

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Abstract

In this study the ethanol extract, n-hexane extract and chloroform extract of *Azadirachta indica* were first evaluated for phytochemical study. The phytochemical screening of the three extracts of *Azadirachta indica* exhibited the presence of important secondary metabolites such as flavonoids, terpenoids, steroids and tannins. The extracts showed potential antimicrobial activities against thirteen different strains of microorganisms. Secondly, they were screened *in vitro* for cytotoxicity test by brine shrimp lethality bioassay and results illustrated significant ($p<0.05$) cytotoxicity against *Artemia salina.* To test the analgesic properties of ethanol extract of *Azadirachta indica,* hot plate and acetic acid induced writhing methods were used. At two different doses (250 and 500 mg/kg body weight), the analgesic tests were performed on Swiss Albino mice. Also, the anti-inflammatory tests were performed by carrageenan induced paw edema method on long Evans rats at the two different doses of 250 and 500 mg/kg body weight using ethanol extract. Our results indicated that *Azadirachta indica* possesses remarkable analgesic and anti-inflammatory activity.

Keywords: *Azadirachta indica;* Phytochemical; Cytotoxicity; Antimicrobial; Analgesic; Anti-inflammatory

Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals in current use have been plants used by indigenous people [1]. It has been reported that about 64% of the total global population remains dependent on traditional medicine and medicinal plants for provision of their health-care needs [2]. In order to promote the use of medicinal plants as potential sources of antimicrobial compounds, it is pertinent to thoroughly investigate their composition and activity and thus validate their use [3]. Some phytochemicals produced by plants have antimicrobial activity allowing these plants to be studied and used for the development of new antimicrobial drugs [4]. The effectiveness of phytochemicals in the treatment of various diseases may lie in their antioxidant effects [5]. Secondary plant metabolites are largely unexplored in 'conventional' animal production systems. In the past, plant metabolites were generally considered as sources of anti-nutritional factors. Recent bans and restrictions on the use of animal antibiotic growth promoters stimulated interest in bioactive secondary metabolites of plan source as alternative performance enhancers [6].

Azadirachta indica (Neem) belonging to Meliaceae family is one of the two species of the genus *Azadirachta*. Although it is native to Bangladesh, it is also available in other tropical and sub-tropical regions such as India, Burma, Sri Lanka, Malaysia and Pakistan [7-9]. Neem is an evergreen and fast-growing tree of reaching an average height of 15-20 meter which might be 35-40 meter in rare cases. Numbers of biologically active compounds have been isolated from the leaf, bark and seeds of this plant. A bitter principle Nimbidin found in Neem bark was found to be antipyretic, non-irritant and effective in skin diseases such as eczema, arsenical dermatitis, burn ulcers, furunculosis, herpes labialis, scabies and seborrhaeic dermatitis [10,11]. It is also useful for warts and dandruff. Nimbidin and sodium nimbidinate from the bark of Neem were reported to show spermicidal actions. Bark extracts are evident to show very promising diuretic and anti-inflammatory properties. Neem bark has shown anti-bacterial activity against various Gram-positive organisms [12]. The aims of the present investigation were the phytochemical properties of ethanol, n-hexane and chloroform extract of *A. indica*, the antimicrobial activity of three extracts in terms of the diameters of growth of inhibition of supplied test organisms, the cytotoxic effect of the bark extract, analgesic effects of ethanol extract and anti-inflammatory effects of ethanol extract.

Materials and Methods

Collection and identification of plants

The plant *A. indica* was collected from Jessore, Bangladesh during the month of November-December. The bark of the plant was collected, sun dried and pilled off. The plant was identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka (Accession No. 30950).

Extraction of the plant

The collected plant barks were aerated by fan aeration to be partially dried. Then they were heated through Oven to be fully dried at bellow 40°C for two days. The fully dried barks were then grinded to make them powder by the help of a suitable grinder. The whole powders were extracted by cold extraction with three solvent ethanol, n-hexane and chloroform and kept for a period of three (03) days accompanying

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occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then these were filtered through Whatman filter paper. The filtrate (ethanol, n-hexane and chloroform extract) obtained was evaporated by Rotary evaporator (Bibby RE-200, Sterilin Ltd., UK) at 5 to 6 rpm and at 68°C temperature. It rendered a gummy concentrate of greenish black color. The gummy concentrate was designated as crude extract. Then the crude extract was dried by freeze drier and preserved at 4°C [13].

Phytochemical analysis

All the extracts were subjected to qualitative phytochemical screening to identify the presence of alkaloids, flavonoids, carbohydrates, gum, reducing sugars, saponins, steroids, tannins and terpenoids using the established methods as described by Harborne and Sazada et al. [14,15]. Briefly, Alkaloids, flavonoids and tannins were respectively tested with Wagner reagent, concentrated HCl and 0.1% ferric chloride. Molish reagents & concentrated sulfuric acid for gum, sulfuric acid for steroids, α-napthol and sulfuric acid for reducing sugar and chloroform and concentrated HCl for terpenoids were used as reagents. Saponin was identified based on the ability to produce suds.

Antimicrobial assay

The antimicrobial activity for different extract was determined by the disc diffusion method [16]. Both Gram-positive and Gram-negative bacterial strains and fungi were used for the test. The bacterial strains used for the investigation are listed below (Table 2). Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Resulting discs were then placed on nutrient agar medium seeded with the microbial strains. A positive control disc (impregnated with kanamycin 30 μg/disc) and negative control disc (impregnated with extraction solvent) was used to compare antimicrobial effects of the extracts [17,18]. These plates were then left for 24 hrs at 4°C to allow maximum diffusion. There was a gradual change in concentration in the media surrounding discs. The plates were then incubated at 37°C for 24 hrs to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition visualized surrounding the medium was used to measure the antimicrobial effects of the extracts.

Cytotoxicity screening

Cytotoxicity of the extracts was assayed by brine shrimp lethality bioassay which was first introduced by Meyer et al. [19]. In this procedure, *Artemia salina* was used as a convenient monitor for the screening. Twenty hours aged young nauplii of brine shrimp hatched in artificial seawater (3.8% NaCl solution) were employed against the test extract. Extract solution (20 mg/ml) at a volume of 5, 10, 20, 40, 80 and 160 μl were taken in 6 vials which were supplemented with 4 ml of sea water containing 30-35 brine shrimp nauplii to make the final concentration of the vials as 25, 50, 100, 200, 400 and 800 μg/ml respectively. DMSO (50 μl diluted to 5 ml) as negative control and colcichine as positive control were used in this assay. The vials were inspected after 24 hrs using a magnifying glass to count the number of survived nauplii in each vial. The percentage (%) of mortality was calculated for each concentration through the following formula:

% mortality = (no. of dead nauplii / no. of live nauplli taken) × 100

Analgesic activity screening

Animals: Swiss albino mice (age 4-5 weeks, average weight 25-30 gm) of both sex and Wistar albino rats (6-7 weeks, average weight 180-200 gm) of either sex were used for this study. The animals were acclimatized in the animal house of the Department of Pharmacy, North South University, Bashundhara, Dhaka-1229, Bangladesh. They were housed in standard polypropylene cages and maintained at the temperature (23 ± 2)°C, humidity 55-60%) with a 12 hrs light-dark cycle. They were given standard pellet diet and tap water *ad libitum*.

In vitro Experimental Protocols

Hot plate test in mice

The hot-plate test was carried out with the method described by Lanhers et al. [20] and modified by Mahomed and Ojewole [21]. Mice were divided into four groups of each containing four animals. The mice were placed inside a beaker on the hot plate to record their response to electrical heat induced pain stimulus. Licking of the paws or jumping out of the beaker was taken as an indicator of the animal's response to heat-induced pain stimulus. The time for licking paws or jumping out of the beaker was taken as reaction time (s). Reaction time was measured once before treatment. The mean of this reaction time constituted initial reaction time before treatment. Each of the test mice was thereafter treated with negative control distilled water, positive control diclofenac sodium (2.5 mg/kg body weight) and ethanol extract at the doses of 250 mg/kg and 500 mg/kg body weight orally. Thirty minutes after treatment, the reaction time of each group was again counted five times individually in one hour interval. Percentage (%) of analgesic score was calculated as:

Percentage (%) of analgesic effect = Tb-Ta/Tb × 100

Where, Tb= Reaction time (in second) before drug administration

Ta = Reaction time (in seconds) after drug administration

Acetic acid induced writhing test in mice

Acetic acid is administered intra-peritoneally to the experimental animals to create pain sensation. As a positive control, any standard non-steroidal anti-inflammatory drugs (NSAID) can be used. In the present study Ketorolac was used to serve the purpose. The plant extract was administered orally in two different doses (250 and 500 mg/kg body weight) to the Swiss Albino mice after an overnight fast [22]. Test samples and vehicle were administered orally 30 minutes prior to intra-peritoneal administration of 0.7% v/v acetic acid solution (0.1 ml/10 gm). Animals were kept individually under glass jar for observation. Each mouse of all groups were observed individually for counting the number of writhing they made in 10 minutes commencing just 5 minutes after the intra-peritoneal administration of acetic acid solution. Full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while Ketorolac (10 mg/kg) was used as a reference substance (positive control).

Anti-inflammatory activity screening

Carrageenan induced rat paw edema method for anti-inflammatory activity: Anti-inflammatory effect of *A. indica* extract was carried out by carrageenan induced rat paw was edema model

described by Winter et al. [23] with minor modifications [24]. Rats were randomly divided into four groups each consisting of five animals. Group I was given drinking water only (negative control), Group II was given diclofenac as anti-inflammatory agent (positive control), Group III and group IV were given the test sample at the dose of 250 and 500 mg/kg body weight (test control) respectively. After 30 minutes of oral administration of the test materials, 1% carrageenan was injected to the left hind paw of each animal. The volume of paw edema was measured at 1/2, 1, 2, 3 and 8 hrs using plethysmometer (7150, UGO Basile, Italy) after administration of carrageenan. The right hind paw served as a reference of non-inflamed paw for comparison. The average increase in the percentage of paw volume was calculated and compared against the control group. Percent inhibition was calculated using the formula:

$$\% \text{ Inhibition of paw edema} = \frac{V_c - V_t}{V_c} \times 100$$

Where, V_c and V_t represent average paw volume of control and treated animal respectively.

Statistical analysis

The data are expressed as the mean ± SEM (Standard Error Mean) analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was used as the test of significance. $p<0.05$ were considered as the minimum level of significant. All statistical tests were carried out by using statistical software package (SPSS, version 18.0, IBM Corporation, NY, USA).

Results

Acute toxicity

Oral administration of graded doses (250 and 500 mg/kg) of the ethanol extract of *A. indica* to rats and mice did not produce any significant changes in behavior, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects during the observation period. No mortality was recorded in any group after 24 hrs of administering the extract to the animals.

Preliminary phytochemical screening of the extract of *A. indica* revealed the presence of various bioactive components of which alkaloid, cardiac glycosides, steriods, reducing sugars and gums, carbohydrates were the most prominent. The ethanol extract reveals various bioactive components were the most prominent than other extracts and the result of phytochemical test has been summarized in the Table 1. Our results indicate the presence of phytochemicals in the order of ethanol extract>n-hexane extract>chloroform extract. The above data shows the higher yield of phytochemicals in ethanol extract.

Antimicrobial screening

The ethanol, n-hexane and chloroform extracts (500 µg/disc) of the aerial part of *A. indica* were screened against thirteen human pathogenic bacteria and one fungus to check antibacterial activities by disc diffusion method. Chloroform extracts (500 µg/disc) showed antibacterial activity with the average zone of inhibition of 8-10 mm by disc diffusion method (Table 2), among the tested bacteria and fungi, the growth of *Shigella dysenriae* (12 mm) was highly inhibited. In case of ethanol extract the highest activity was also seen against the growth of *Escherichia coli* having the zone of inhibition of 14 mm. Besides this, the ethanol extracts showed good activity against the growth of *Bacillus sereus* (10 mm), *Bacillus subtilis* (10 mm), *Staphylococcus aureus* (11 mm), *Escherichia coli* (14 mm), *Salmonella paratyphi* (10 mm) and *Salmonella typhi* (10 mm), *Shigella dysenteriae* (10 mm), *Pseudomonas aureus* (10 mm) (Table 2).

Cytotoxicity screening

Following the procedure of Mayer et al. [19] the lethality of the crude ethanol, n-hexane and chloroform extract of *A. indica* to brine shrimp was determined on *A. salina* after 24 hrs of exposure the samples and the positive control, colcichine. The results of *A. indica* on different extracts (concentration of extract 25, 50, 100, 200, 400 and 800 µg/ml respectively) are shown in Table 3. The percentage mortality

A. indica extract	Tannins	Flavonoids	Saponins	Gums & Carbohydrate	Steroids	Alkaloids	Reducing sugar	Terpenoids
Ethanol	+++	++	+++	++	++	+++	++	_
n-Hexane	-	-	-	++	++	++	++	++
Chloroform	_	_	_	++	+++	+++	+++	+

Symbol (+) indicates presence and (-) indicates absence of phytochemicals.

Table 1: Result of phytochemical screening of plant fraction and extracts.

Test organisms	Ethanol extract (500 µg/disc)	n-hexane extract (500 µg/disc)	Chloroform extract (500 µg/disc)
Bacillus sereus	10	-	9
Bacillus subtilis	10	-	7
Bacillus megaterium	8	-	-
Staphylococcus aureus	11	-	9
Sarcina lutea	10	-	-
Salmonella paratyphi	10	-	6
Salmonella typhi	10	-	10
Escherichia coli	14	-	10
Vibrio mimicus	-	-	-
Vibrio parhaemolyticus	6	8	-
Shigella dysenteriae	10	-	8
Shigella boydii	8	-	-
Pseudomonas aureus	10	-	9

Values are expressed as zone of inhibition in millimeter (mm), (n=3).

Table 2: Results of zone of inhibition of microorganisms

increased with an increase in concentration. Ethanol, n-hexane extract and colchicine showed 100.0% mortality to brine shrimp at 800 μg/ml. The LC_{50} obtained from the best-fit line slope were found to be μg/ml, μg/ml and μg/ml for ethanol and n-hexane extract (Table 3).

Analgesic activity

Effect of plant extract on hot-plate test: The ethanolic extract of *A. indica* exhibited statistically significant (p<0.05) analgesic effect in hot plate test of white albino mice. The results presented in table 4 and 5. From these results we could concluded that the meal significantly increased the reaction time of mice in a dose-related manner. The dose dependent effect reached 42.80% and 61.90% at the doses of 250 and 500 mg/kg body weight respectively (Tables 4 and 5).

Acetic acid induced writhing test in mice: The results of acetic acid induced writhing responses in mice which indicate the analgesic activity of the ethanol extracts of were presented in Table 6. It was found that the extract of *A. indica* and Ketorolac at the assayed doses caused a significant (p<0.05) inhibition on the writhing responses induced by acetic acid when compared to the control (Table 6).

Anti-inflammatory activity screening

Carrageenan induced rat paw edema method for anti-inflammatory activity: The effective values, calculated for each group are presented in Tables 7 and 8. In control animals, the sub plantar injection of carrageenan produced a local edema that increased progressively to reach a maximal intensity three hour after the injection of the phlogistic agent. Ethanol extract of the *A. indica* showed a significant dose depended reduction at 500 mg/kg body weight. However, maximum inhibition of edema was found to be 22.22% at eight hour of study at a dose of 500 mg/kg body weight respectively (Tables 7 and 8).

Discussion

This research reported the phytochemical, antimicrobial, cytotoxic, analgesic and anti-inflammatory properties of *A. indica* both in *in vitro* and *in vivo* models. Phytochemical constituents play the major role guiding the pharmacological effects of individual plant source. Chemical screening is thus performed to allow localization and targeted isolation of new or useful phytochemical constituents with potential activities. This procedure recognizes occurrence of the known phytochemical metabolites in extracts either in preliminary stage or at the earliest stages of separation and this is very important in economic context.

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional medicinal plants are used primarily water as the solvent but in our studies we found that plant extracts in organic solvent (ethanol) provided more consistent antimicrobial activity compared to those extracted in n-hexane or chloroform extract. So we have decided to continue our experiments by using ethanol extract. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity [25]. Ethanolic extracts of plants generally possess terpines and phenolics, which are reported by different workers as antimicrobial compounds [26,27]. Medicinal plants containing different types of phytochemical have a long history of serving people throughout the world. Actually phytochemicals show a variety of pharmacological actions in human body [28].

The result of the phytochemical investigation of the ethanol extract of *A. indica* indicates the presence of alkaloids, flavonoids, steroids, gum and carbohydrates, reducing sugar, saponins and tannins. Results of n-hexane and chloroform extracts indicate the presence of steroids, gum and carbohydrates, reducing sugar, terpenoids and alkaloids. The presence of alkaloids represents the possibility of some biological activity of the extracts of *A. indica*; such as anti-arrhythmic, anti-cholinergic, anti-tumor, vasodilating, anti-hypertensive, cough expectorant, anesthetic, analgesic, muscle relaxant, anti-pyretics, anti-malarial, anti-protozoal agent [29-35]. The presence of flavonoids represent

Concentration	C1.398	C1.699	C2.000	C2.301	C2.602	C2.903	LC50 (μg/ml)
Control	6.18 ± 0.57	8.98 ± 0.57	10.65 ± 0.57	11.76 ± 0.57	11.76 ± 0.57	20.00 ± 0.57	-
Colcichine	45.00 ± 0.57	65.00 ± 0.57	75.00 ± 0.57	100.00 ± 0.57	100.00 ± 0.57	100 ± 0.57	6.92
Ethanol extract	4.00 ± 0.57	9.00 ± 0.57	19.00 ± 0.57	45.00 ± 0.57	77.00 ± 0.57	100 ± 0.57	30.20
n-hexane extract	5.00 ± 0.57	10.00 ± 0.57	13.00 ± 0.57	36.00 ± 0.57	71.00 ± 0.57	100 ± 0.57	32.36

Data were expressed as mean ± SEM and analyzed by one way ANOVA, Post hoc and Dunnett's test. All of the results were compared with the standard (colchicine); p<0.05.

Table 3: Results of brine shrimp lethality assay for *A. indica* extract.

Treatment	0 Hr	½ Hr	1 Hr	2 Hrs	3 Hrs	4 Hrs
Control	11.47 ± 0.33	9.45 ± 0.41	8.62 ± 0.64	7.9 ± 0.55	6.92 ± 0.48	5.55 ± 0.33
Positive control	9.40 ± 0.83	11.95 ± 0.47	13.35 ± 0.41	14.9 ± 0.43	16.97 ± 0.24	14.45 ± 0.37
A. indica (250 mg/kg)	8.32 ± 0.73	9.85 ± 1.04	10.67 ± 1.11	11.75 ± 1.56	13.47 ± 1.26*	10.80 ± 1.31
A. indica (500 mg/kg)	12.92 ± 1.68	14.70 ± 1.28	16.4 ± 0.86	17.47 ± 0.83	18.45 ± 0.63*	16.02 ± 0.85

Values in the results are expressed as mean ± SEM, significantly different in comparison with control at *p<0.05.

Table 4: Analgesic effect of the ethanol extract of A. indica using the hot-plate method.

Treatment	% Inhibition				
	½ Hr	1 Hr	2 Hrs	3 Hrs	4 Hrs
Positive control	27.13 ± 3.43	42.02 ± 0.24	58.51 ± 0.35	80.53 ± 0.95	53.62 ± 0.91
A. indica (250 mg/kg)	13.78 ± 0.57	26.93 ± 0.63	35.22 ± 0.97	42.80 ± 0.25*	23.99 ± 0.78
A. indica (500 mg/kg)	18.39 ± 1.37	28.25 ± 2.30	41.23 ± 1.39	61.90 ± 3.03*	29.81 ± 0.76

Values in the results are expressed as mean ± SEM, significantly different in comparison with control at *p<0.05.

Table 5: Percent inhibition of the standard (control) and two different concentrations (250 and 500 mg/kg of *A. indica*) of the extract compared with their respective means at 0 hr (Analgesic activity).

Treatment	Mean ± SEM	% Inhibition
Control	42.80 ± 3.10	-
Positive control (Ketorolac sodium)	20.80 ± 0.86	51.40%
A. indica 250 mg/kg	30.80 ± 2.47*	28.03%
A. indica 500 mg/kg	20.40 ± 2.83	52.33%

Values in the results are expressed as mean ± SEM, significantly different in comparison with control at *p<0.05.

Table 6: Analgesic effect of the ethanol extract of *A. indica* using the acetic acid induced writhing test in mice.

Treatment	0 Hr	½ Hr	1 Hr	2 Hrs	3 Hrs	4 Hrs	8 Hrs
Control	0.73 ± 0.03	0.91 ± 0.04	1.07 ± 0.04	1.31 ± 0.06	1.59 ± 0.08	1.57 ± 0.08	1.49 ± 0.07
Positive control	0.73 ± 0.01	0.91 ± 0.03	0.99 ± 0.86	1.12 ± 0.04	1.17 ± 0.04	1.11 ± 0.05	0.94 ± 0.04
A. indica (250 mg/kg)	0.87 ± 0.03	1.06 ± 0.05	1.09 ± 0.06	1.13 ± 0.01	1.15 ± 0.13*	0.94 ± 0.06	0.90 ± 0.04
A. indica (500 mg/kg)	0.72 ± 0.02	0.84 ± 0.02	0.90 ± 0.82	0.97 ± 0.05	1.11 ± 0.05*	1.03 ± 0.02*	0.88 ± 0.04

Values in the results are expressed as mean ± SEM, significantly different in comparison with control at *p<0.05.

Table 7: Anti-inflammatory effect of ethanolic extract of *A. indica* on carrageenan induced rat paw inflammation.

Treatment	% Inhibition					
	½ Hr	1 Hr	2 Hrs	3 Hrs	4 Hrs	8 Hrs
Positive control	24.66 ± 0.48	35.62 ± 0.44	53.42 ± 0.43	60.27 ± 0.43	52.05 ± 0.38	28.77 ± 0.16
A. indica (250 mg/kg)	21.84 ± 0.56	18.39 ± 0.38	16.09 ± 0.59	32.18 ± 0.30	8.05 ± 0.26	17.24 ± 0.29
A. indica (500 mg/kg)	16.67 ± 0.27	25.00 ± 0.16	34.72 ± 0.34	54.17 ± 0.24*	43.06 ± 0.15	22.22 ± 0.30*

Values in the results are expressed as mean ± SEM, significantly different in comparison with control at *p<0.05.

Table 8: Percent inhibition of the standard and two different concentrations of the extract compared with their respective means at 0 hr (Anti-inflammatory activity).

the possibility of some biological activity of the extracts of *A. indica*; such as it can modify allergens, viruses, and carcinogens indicating the potentiality flavonoids to be biological "response modifiers". It can also be used as an anti-allergic, anti-inflammatory [36] anti-microbial [37] and anti-cancer therapies [38]. Its dietary uses are also very widely known. The presence of tannins represent the possibility of some biological activity of the extracts of *A. indica*; such as anti-diarrheal, hemostatic, anti-hemorrhoidal, anti-inflammatory, astringent, anti-infective. It can be used for immediate relief of sore throats, diarrhea, dysentery, hemorrhaging, fatigue, skin ulcers and as a cicatrizing on gangrenous wounds. It may have anti-viral effect which tannins have. It can also be used against poisons. It also possesses antioxidant effects. All these effects may be due to the presence of tannins in the extract of *A. indica* because tannins are previously reported to show such effects [39]. The presence of saponins indicates that it can be used as dietary supplements as saponins have these properties.

The antimicrobial potential of plants was compared according to their zone of inhibition against the several pathogenic organisms. The plant extract from various extraction processes showed their potential activity against bacteria. None of the crude extracts of *A. indica* demonstrated significant inhibition of growth of the test microorganisms. This was probably due to the development of partial or complete resistance of the microorganisms against the test samples, which might be consequence of the indiscriminate use of antibacterial agents [37]. However, the results of this study showed that the extracts used can inhibit the growth of Gram-positive and Gram-negative bacteria [40]. Based on the statistical analysis, this inhibition is moderately significant than the standard broad spectrum antibiotic (30 μM of Kanamycin). From the result, regarding the antimicrobial activity, it can be concluded that the ethanol, n-hexane and chloroform extract of bark of *A. indica* possesses prospective broad spectrum antimicrobial potency against the given test organisms and the order the potency should be ethanol> chloroform>n-hexane.

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the extracts as well as isolated compounds to assess the toxicity towards brine shrimp which could also provide an indication of possible cytotoxic properties of the test materials [37]. Brine shrimp nauplii have been previously utilized in various bioassay systems. Among these applications, there have been the analyses of pesticidal residues, mycotoxins, stream pollutants, anesthetics, dinoflagelate toxins, morphine-like compounds, carcinogenicity of phorbol esters and toxicants in marine environment [41]. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay [42-44].

The variation in BSLA results may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids or terpenoids) present in the extracts. Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 100 ppm or μg/ml) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.

Effect of ethanol extract of *A. indica* was measured in hot plate method which is one of the most common tests for evaluating the analgesic efficacy of drugs/compounds. The paws of mice and rats are very sensitive to heat at temperature which is not damaging to the skin. The responses are shaking, jumping, withdrawal of the paws and licking of the paws. The time until this response is prolonged after administration of centrally acting analgesics [45]. *A. indica* extract at the dose of 250 and 500 mg/kg showed the significant (p<0.05) increase in latency time as compared to control. Positive control diclofenac Na showed significant (p<0.05) analgesic activity at the dose of 10 mg/kg.

The analgesic activity was expressed as mean increase in latency after drug administration. *A. indica* exhibited potent analgesic activity at the doses of 250 and 500 mg/kg. These extracts showed analgesic activity at low dose of 250 mg/kg even in first hour in test. These results indicate that ethanolic extract of *A. indica* can produce significant analgesic effect.

The acetic acid-induced writhing is a sensitive method to evaluate peripherally acting analgesics. Ethanol extract of *A. indica* possesses analgesic effects in the model of acetic acid-induced writhing test. Acetic acid induced writhing in mice finds much attention in the screening of analgesic drugs. Acetic acid-induced abdominal writhing and the visceral pain model release prostaglandin, via cyclooxygenase pathway, which plays a role in the nociceptive mechanism. This model of response is thought to be mediated by peritoneal mast cells acid sensing ion channels and the prostaglandin pathway. In other words, the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products [37]. The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability. Results of the present studies show that ethanol extract of *A. indica* produced significant analgesic effect which may be due to the inhibition of the synthesis of the arachidonic acid metabolite.

Carrageenan-induced edema involves the synthesis or release of mediators at the injured site. These mediators include prostaglandins, especially the E series, histamine, bradykinins, leucotrienes and serotonin all of which also cause pain and fever [46,47]. Inhibitions of these mediators from reaching the injured site or from bringing out their pharmacological effects normally ameliorate the inflammation and other symptoms [48,49]. In the present study, it has been shown that the ethanol extract of the *A. indica* possessed a significant anti-edematogenic effect on paw oedema induced by carrageenan.

Conclusion

The present study indicated that the ethanol extract of *A. indica* may have potential use in medicine. From the previous study and our investigation it may be concluded that, ethanol extract possesses more pharmacological properties than n-hexane and chloroform extracts. In our study, the ethanol extracts of *A. indica* showed significant dose dependent inhibition of analgesic effect. These current studies suggested ethanol extract of *A. indica* demonstrated significant result and possess analgesic properties. Now our next aim is to isolate the leading compounds and to establish their chemical structure.

References

1. Balick JM, Cox PA (1996) Plants, People and Culture: the Science of Ethnobotany. Scientific American Library, New York pp: 228.
2. Cotton CM (1996) Ethnobotany: Principle and Application. John Wiley and Sons, New York pp: 399.
3. Nair R, Chanda S (2006) Activity of some medicinal plants against certain pathogenic bacterial strains. Indian J Pharmacol 38: 142-144.
4. Nascimento GGF, Locatelli J, Freitas PC, Silva GL (2010) Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Braz J Microbiol 31: 247-256.
5. Akinmoladun AC, Ibukun EO, Obuotor EM and Farombi EO (2007) Phytochemical constituent and antioxidant activity of extract from the leaves of Ocimum gratissimum. Sci Res Essay 2: 163-166.
6. Greathead W (2003) Extraction and Antimicrobial Effect of Thai Herbs and Spices Extracts. J Agri Food Chem 43: 361-371.
7. Schumacher M Cerella C, Reuter S, Dicato M, Diederich M (2011) Anti-inflammatory, pro-apoptotic, and anti-proliferative effects of a methanolic neem (Azadirachta indica) leaf extract are mediated via modulation of the nuclear factor-Î°B pathway. Genes Nutr 6: 149-160.
8. Pandey V Agrawal V, Raghavendra K, Dash AP (2007) Strong larvicidal activity of three species of Spilanthes (Akarkara) against malaria (Anopheles stephensi Liston, Anopheles culicifacies, species C) and filaria vector (Culex quinquefasciatus Say). Parasitol Res 102: 171-174.
9. Prachayasittikul S, Suphapong S, Worachartcheewan A, Lawung R, Ruchirawat S, et al. (2009) Bioactive metabolites from Spilanthes acmella Murr. Molecules 14: 850-867.
10. Khan NM, Khan FA, Mu TH, Khan ZU, Khan M, et al. (2015) Potentiality of Neem (Azadirachta indica) Powder in Rheology Modification of oil-in-water emulsion. J Food Process Engineering 38: 190-196.
11. Sittie AA, Lemmich E, Olsen CE, Hviid L, Brøgger Christensen S (1998) Alkamides from Phyllanthus fraternus. Planta Med 64: 192-193.
12. Majumder M, Shukla AK, Arunachalam A (2008) Nutrient release and fungal succession during decomposition of weed residues in a shifting cultivation system. Communications Biometry and Crop Sci 3: 45-59.
13. Haque M, Haque ME, Rahman MM (2008) Antibacterial and cytotoxic activities of Capparis zeylanica Linn roots. Ars Pharmaceutica 49: 31-37.
14. Harborne JB (1998) Phytochemical Methods: A guide to modern techniques of plant analysis. (3rd Edition) Chapman and Hall, London pp: 302.
15. Sazada S, Verma A, Rather AA, Jabeen F, Meghvansi MK (2009) Preliminary phytochemicals analysis of some important medicinal and aromatic plants. Adv Biol Res 3: 188-195.
16. Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45: 493-496.
17. Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal Biochem 269: 337-41.
18. Chang CC, Yang MH, Wen HM, Chern JC (2002) Estimation of total flavonoids content in propolis by two complementary colorimetric methods. J Food Drug Anal 10: 178-182.
19. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen JB, Nicholsand DE, et al. (1982) Brine shrimp; a convenient general bioassay for active plant constituents. Planta Medica 45: 31-34.
20. Aguinaldo AM, Espeso EI, Guevara BQ, Nonato MG (2005) Phytochemistry. In: Guevara BQ (ed.) A Guidebook to Plant Screening: Phytochemical and Biological. University of Santo Tomas, Manila, Philippines.
21. Moon YJ, Wang X, Morris ME (2006) Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro 20: 187-210.
22. Veitch NC (2007) Isoflavonoids of the leguminosae. Nat Prod Rep 24: 417-464.
23. Jiang H, Zhan WQ, Liu X, Jiang SX (2008) Antioxidant activities of extracts and flavonoid compounds from Oxytropis falcate Bunge. Nat Prod Res 22: 1650-1656.
24. Kim HP Son KH, Chang HW, Kang SS (2004) Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci 96: 229-245.
25. Sankar KD, Debdulal B, Sourav C, Krishnendu BK (2010) Antimicrobial activities of some medicinal plants of West Bengal. Int Pharma and Bio Sci 1: 1-10.
26. Dwivedi S (2007) Terminalia arjuna Wight & Arn.--a useful drug for cardiovascular disorders. J Ethnopharmacol 114: 114-129.
27. Sanches NR, Cortez DAG, Schiavini MS, Nakamura CV, Dias Filho BP (2005) An Evaluation of Antibacterial Activities of Psidium guajava (L.). Braz Arch Biol Tech 48: 429-436.
28. Uddin SMN, Amin MN, Shahid-Ud-Daula AFM, Hossain H, Md. Haque MM, et al. (2014) Phytochemical screening and study of antioxidant and analgesic potentials of ethanolic extract of Stephania japonica Linn. J Medicinal Plant Res 8: 1127-1133.
29. Asif M (2013) A review on spermicidal activities of Azadirachta indica. J of Pharmacog and Phytochemistry 1: 61-79.
30. Abalaka M, Oyewole OA, Kolawole AR (2012) Antibacterial activity of Azadirachta indica against some bacterial pathogens. Adv in Life Sci 2: 5-8.
31. Chandrawathani P, Zary Shariman Y, Premaalatha B, Rahimah H, Norhafiza NH, et al. (2014) Evaluation of Neem leaf (Azadirachta indica) product for worm control on goats. Malaysian J Veterinary Res 4: 5-12.
32. Jafari S, Saeidnia S, Ardekani MRS, Hadjiakhoondi A, Khanavi M (2013) Micromorphological and preliminary phytochemical studies of Azadirachta indica and Melia azedarach. Turkish J of Botany 37: 690-697.

33. Khatkar S, Dhiman P, Sachin, Malik N, Khatkar A, Redhu N (2013) Biological and medicinal properties of Azadirachta Indica: A review. Int J of Pharma Professional Res 4: 979- 985.
34. Raja Ratna Reddy Y (2013) Antimicrobial activity of Azadirachta indica (neem) leaf, bark and seed extracts. Int J Res Pytochem Pharmacol 3: 1-4.
35. Manisha SY, Sachin AN, Amrita KA (2014) Review on neem plant. World J Pharmacy and Pharmaceutical Sci 3: 590-598.
36. Wu JH Tung YT, Chien SC, Wang SY, Kuo YH, et al. (2008) Effect of phytocompounds from the heartwood of Acacia confusa on inflammatory mediator production. J Agric Food Chem 56: 1567-1573.
37. Dzubak P Hajduch M, Vydra D, Hustova A, Kvasnica M, et al. (2006) Pharmacological activities of natural triterpenoids and their therapeutic implications. Nat Prod Rep 23: 394-411.
38. Chowdhury SA, Sohrab MH, Haque MR, Choudhury MH, Rashid MA (2009) Phytochemical and biological investigations of Polygonum lanatum. Orien Pharm Exp Med 8: 97-102.
39. Singh UP Maurya S, Singh DP (2005) Phenolic acids in neem (Azadirachta indica): a major pre-existing secondary metabolites. J Herb Pharmacother 5: 35-43.
40. Dudonné S Vitrac X, Coutière P, Woillez M, Mérillon JM (2009) Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. J Agric Food Chem 57: 1768-1774.
41. Cioffi G D'Auria M, Braca A, Mendez J, Castillo A, et al. (2002) Antioxidant and free-radical scavenging activity of constituents of the leaves of Tachigalia paniculata. J Nat Prod 65: 1526-1529.
42. Molyneux P (2003) The use of the stable free radical diphenylpicrylhydrazy (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol 26: 211-219.
43. Brand-Williams W, Cuevelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss u-Technol 28: 25-30.
44. Moon JK Shibamoto T (2009) Antioxidant assays for plant and food components. J Agric Food Chem 57: 1655-1666.
45. Chuanphongpanich S, Suttajit M, Phanichphant S, Buddhasukh D, Sirithunyalug B (2006) Antioxidant Capacity of Broccoli Seeds Grown in Thailand. Chiang Mai J Sci 33: 117-122.
46. Erlund I (2004) Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability and epidemiology. Nutr Res 24: 851-874.
47. McLaughlin J, Chang C, Smith D (1991) Proceedings of the 18th National Seminar and Unesco Workshop on Natural Products. Institute of Advance Studies, University of Malaysia, Malaysia.
48. El-Hawary SS, El-Tantawy ME, Rabeh MA, Badr WK (2013) Chemical composition and biological activities of essential oils of Azadirachta indica A. Juss. International J Appl Res Nat Pro 6: 33-42.
49. Srinivasa K, Jagadeesh K, Revankar SP (2014) Analgesic effect of Azadirachta indica (Neem) in albino rats. J Medical and Health Sci 3: 93-96.

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