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Phytochemical analysis, anti-oxidant and analgesic activity investigation of methanolic extract of *Azadirachta indica* Leaves

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Abstract
Azadirachta indica plants have been used for centuries in Indian folk medicine. This research was performed to determine the presence of major phytochemicals and to evaluate the analgesic and anti-oxidant activity of methanolic extract of *Azadirachta indica* leaves. Methanolic extract from the leaves of the plant was prepared by using drying, grinding, filtration and solvent evaporation methods. Phytochemical analysis of the leaves indicated the presence of Glycosides, Alkaloids, Flavonoids, Tannins, Saponins and Terpenoids. The analgesic activity of the samples was studied using acetic acid induced writhing model in mice. Significant analgesic effect was monitored ($p<0.05$) as the oral extract dose of 500 mg/kg showed 47.48% inhibition of writhing movements compared to standard drug Diclofenac-Na which inhibited 90.65% writhing movement. Anti-oxidant activity of the leaves was evaluated using DPPH free radical scavenging activity method. The methanolic extract of the leaves showed mild free radical scavenging activity. The IC_{50} of the plant extract was 310.259 µg/ ml, whereas IC_{50} of reference agent Ascorbic acid was found to be 17.33 µg/ ml.

Keywords: *Azadirachta indica*, anti-oxidant, analgesic, phytochemical analysis

1. Introduction
The purpose of this study was to investigate the presence of phytochemicals in the methanolic extract of *Azadirachta indica* leaves and also to determine if the leaves contain any kinds of anti-oxidant and analgesic properties. The main reason for focusing on anti-oxidant properties is because application of external source of antioxidants can assist against oxidative stress. It is known that free radicals are one of the main factors which are required for mutation of DNA. Their involvement in the initiation stage of carcinogenesis is also a known fact. Free radicals, reactive oxygen species and reactive nitrogen species are generated by our body by various endogenous systems and exposure to different physiochemical conditions or pathological states [1, 2]. That is why it is necessary to find proper balance between free radicals and antioxidants for proper physiological function. If free radicals overwhelm the body's ability to regulate them, a condition known as oxidative stress occur. [3, 4] Free radicals thus adversely alter lipids, proteins, and DNA and trigger a number of human diseases. [5] The main reasons for focusing on analgesic properties are because Painkillers and anti-inflammatory drugs which are used these days for pain management are very effective in case of pain relief however, in most cases cause more or less adverse effects such as renal failure, allergic reactions, hearing loss or affecting platelet function. On the contrary many plant derived medicines has been used from centuries without any serious adverse effects for pain management. [6-8] Due to these reasons it is necessary to conduct researches to develop medicines from plant based origins to reduce the risk of adverse effects. *Azadirachta indica* also known as Neem is a tree in the mahogany family of Meliaceae. It is known as one of the most beneficial plants for human health for centuries. It is native to India, Burma, Bangladesh, Sri Lanka, Malaysia and Pakistan. It grows in tropical and semi-tropical regions. Neem is a fast-growing tree that can reach a height of 15–20 m, rarely to 35–40 m. It is evergreen, but in severe drought it may shed most or nearly all of its leaves [9, 10]. All parts of Neem tree are used for treatment of various kinds of conditions. Its parts are used as anthelmintic, anti-fungal, anti-diabetic, antibacterial, antiviral, contraceptive and sedative. Neem tree is used in many medicinal cases like treatment of skin diseases, to improve liver function, to detoxify the blood, to reduce fever, to treat dental conditions, in treatment of cough, asthma, ulcers, piles, intestinal worms, urinary diseases etc. [11, 12]. Neem leaf paste is applied to the skin to treat acne, and in a similar way is used for measles and chicken pox sufferers.

Practitioners of traditional Indian medicine recommend that patients suffering from chicken pox sleep on Neem leaves. A decoction prepared from Neem roots are ingested to relieve fever in traditional Indian medicine [13]. Due to the numerous health benefits of *Azadirachta indica*, it was chosen for conducting this study.

2. Materials and Methods

2.1. Plant Materials

Azadirachta indica leaves were collected from Mohammadpur, Dhaka and authentically confirmed from the Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh.

2.2. Preparation of Plant Extract

The collected plant leaves were dried at room temperature (30 ± 3° C), for 10 days to ensure the active constituents free from decomposition. The dried leaves were powdered in an electrical grinder after overnight drying in an oven below 50° C. The powder was extracted with methanol at room temperature. The bottle was kept at room temperature and allowed to stand for 10 days with occasional shaking. When the solvent became concentrated, the liquid alcohol contents were filtered through cotton & then through filter paper. Then, the solvent was allowed to evaporate using rotary evaporator at temperature 40-45° C. Finally, a highly concentrated methanol crude extract was obtained.

2.3. Phytochemical Screening Methods

2.3.1. Test for Glycosides

2 ml solution of the extract was taken into a test tube. 1 ml mixture of Fehling solution was added into the test tube. The tube was placed in a water-bath at 60° C. If brick red color forms that shows the presence of glycosides [14]

2.3.2. Test for Alkaloids

In testing for Alkaloids, about 0.5 g of extract will be stirred with 5 ml of 1 percent aqueous hydrochloric acid on a water bath; 1 ml of the filtrate is to be treated with a few drops of Mayer's reagent and a second 1 ml portion is to be treated the same way with Dragendorff's reagent. Presence of orange-red color indicates the presence of alkaloid [14].

2.3.3. Test for Flavonoids

A small quantity of test residue was dissolved in 5 ml of ethanol and treated with few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal. If the pink, crimson or magenta color is developed within a minute or two, that means presence of flavonoids [14].

2.3.4. Test for Tannins

About 5 mg of of plant extract will be stirred with 10 ml distilled water, filtered, and ferric chloride reagent will then be added to the filtrate. If dark green or deep blue color is obtained, it means tannins are present [14].

2.3.5. Test for Saponins

A few mg of the test residue was taken in a test tube and shaken vigorously with small amount of sodium bicarbonate and water. If stable, characteristic honeycomb like froth is obtained, it means saponins are present [14].

2.3.6. Test for Terpenoids

2 mg of the extract was dissolved in 2 ml of $CHCl_3$ and evaporated. 2ml of conc. H_2SO_4 was then added and heated for about 2 minutes. Development of a grayish color indicates the presence of terpenoids [15].

2.4. Analgesic property test

2.4.1. Drugs and Chemicals

Acetic acid was collected from laboratory of Bangladesh University. The standard drug Diclofenac-Na was purchased from Square Pharmaceuticals Limited of Bangladesh.

2.4.2. Experimental Animals

Eight week-old Swiss albino mice were (50-52 g) purchased from Jahangirnagar University, Dhaka, Bangladesh and were housed in animals cages under standard environmental conditions (22-25° C, humidity 60-70%, 12 hours light: 12 hours dark cycle). The mice were feed with standard pellet diet taken from, Jahangirnagar University Dhaka. The animals used in this study were cared in accordance with the guidelines on animal experimentation of our institute.

2.4.3. Experimental Protocols

For Analgesic test 9 mice were divided into three groups-
1. Control Group 2. Standard Group. 3. Extract Group
Each group comprises of 3 mice. Control group received 1% Tween 80 in water. Standard Group received Diclofenac sodium 10 mg/kg and Extract Group received 500 mg/kg extract because in various studies it has been observed that a higher dose such as 500 mg/kg displays better analgesic activity [16, 17].

2.4.4. Acetic acid-induced writhing test for Analgesic activity

The analgesic activity of the samples was studied using acetic acid-induced writhing model in mice. Acetic acid was administered intraperitoneally. Test samples and vehicle were administered orally 30 min before administration of acetic acid. Diclofenac-Na was administered 30 min before administration of acetic acid to standard group. The mice were observed for specific contraction of body referred to as "writhing" for the next 30 min. [18, 19].

2.4.5 Statistical Analysis

All values were expressed as mean ± Standard Deviation (SD). Statistical comparison were performed by One-way analysis of variance (ANOVA), followed by using Dunnet test. Results were considered as significant when p values less than 0.05 ($p<0.05$).

2.5 Free radical scavenging activity test

2.5.1 Reagents

1. Methanol
2. DPPH (1, 1 - diphenyl - 2 – picrylhydrazyl - hydrate)
The reagents were collected from the laboratory of Bangladesh University.

2.5.2. Procedure

Stock solution of the plant extract was prepared in methanol from which a serial dilution was carried out to obtain concentration of 5 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml. Diluted solutions (2 ml) were added to 2 ml solution of DPPH then mixed and allowed to stand for 30 minutes for reaction to occur. The absorbance was determined at 517 nm and from these values corresponding percentage of inhibitions were calculated. Then percent inhibitions were plotted against log concentration and from the graph IC_{50} was calculated. The experiment was performed 3 times and average absorption was noted for each concentration. [20, 21, 22]

3. Results

3.1: Results of Phytochemical screening

The results of the phytochemical screening of the methanolic extract of *Azadirachta indica* leaves are displayed in (table-1).

Table 1: Results of Phytochemical screening

Tested Groups	Methanolic extract of *Azadirachta indica* leaves
Glycosides	+
Alkaloids	+
Flavonoids	+
Tannins	+
Saponins	+
Terpenoids	+

Note: (+) indicates the presence of tested groups and (–) indicates the absence of tested groups.

3.2 Result of Analgesic test

Table-2 shows the analgesic effects of methanolic extract of *Azadirachta indica* leaves on acetic acid induced writhing model in mice.

Table 2: Analgesic effects of methanolic extract of *Azadirachta indica* leaves.

Animal Group	Writhing counts (Mean ± SD)	Percentage of writhing inhibition
Control group	46.33 (± .577)	
Standard group	4.33 (±1.52)	90.65
Extract group	24.33 (± 2.08)	47.48

Note: Values were expressed in mean ± Standard Deviation (SD). Each group was comprised of 3 animals (n=3). Control Group animal received vehicle (1% Tween 80 in water), Standard Group received Diclofenac Na 10 mg/ kg body weight, Extract Group was treated with 500 mg/kg crude extract of. *Azadirachta indica* leaves.

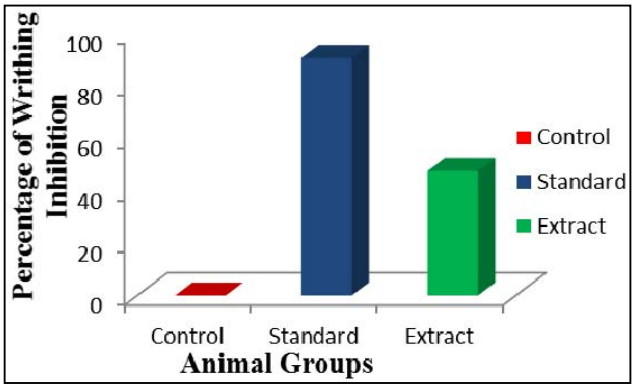

Fig 1: Analgesic activity of tested groups.

3.3 Result of Free radical scavenging activity test

Table 3: Free radical scavenging activity of ascorbic acid and methanol extract of *Azadirachta indica* leaves.

Conc. (µg/ml)	Percentage of Inhibition (ascorbic acid)	Percentage of Inhibition (*A. indica* leaves extract)
5	33.50	10.23
10	46.80	13.50
50	57.25	18.40
100	74.65	32.90
500	91.50	63.70

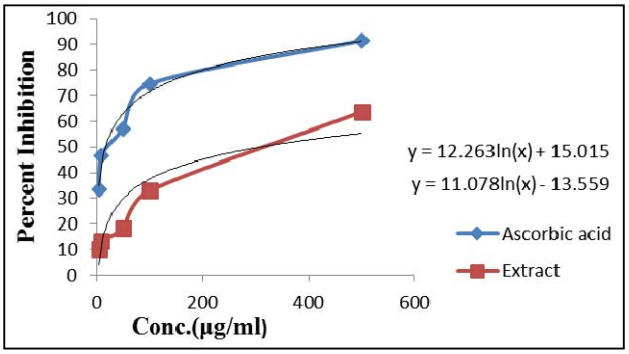

Fig 2: Free radical scavenging activity of ascorbic acid and *A. indica* leaves extract

Table 4: IC $_{50}$ values of ascorbic acid and methanolic extract of *Azadirachta indica* leaves

Sample	Equation	IC $_{50}$ (µg/ml)
Ascorbic acid	Y= 12.263 ln(x) + 15.015	17.33
A. indica leaves extract	Y= 11.078 ln(x) – 13.559	310.259

The anti-oxidant activity of the methanolic extract of *Azadirachta indica* leaves is evaluated using DPPH free radical scavenging activity method. The methanolic extract of *Azadirachta indica* has minor free radical scavenging activity. The IC $_{50}$ of the extraction was found to be 310.259 µg/ ml, whereas IC $_{50}$ of Ascorbic Acid was found to be 17.33 µg/ ml.

4. Discussion

Phytochemical screening of methanolic extract of *Azadirachta indica* leaves displayed that it contained Glycosides, Flavonoids, Tannins, Terpenoids, Alkaloids and Saponins

The analgesic tests displayed that it has good analgesic properties because the standard analgesic drug Diclofenac- Na inhibited 90.65% acetic acid induced writhing while the dose prepared by plant extract inhibited 47.48% acetic acid induced writhing. The analgesic effect is due to inhibition of the activity of cyclooxygenase-2 (cox-2) which results in the inhibition of prostaglandins synthesis. The extract may also have interfered with G-protein mediated signal transduction, an analgesic mechanism unrelated to inhibition of prostaglandin synthesis. It also may have augmented the peripheral mechanism through interference with the formation of prostaglandins in the central nervous system. These mechanisms have been implicated in the forms of analgesia induced by non-steroidal anti-inflammatory drugs [7, 8].

Phytochemicals like alkaloids, glycosides, saponins and terpenoids are known to induce analgesic mechanisms. All of these were present in the plant extract and were probably responsible for the good analgesic property.

The free radical scavenging activity test showed that the plant extract has mild free radical scavenging activity because the standard agent ascorbic acid was found to have IC $_{50}$ of 17.33 µg/ml while the IC $_{50}$ of the plant extract was found to be 310.259 µg/ ml. Tannins and flavonoids have the ability to donate electrons that results in converting highly reactive free radicals to non-reactive stable molecules. Flavonoids and tannins in the crude extract converted reactive non stable DPPH free radical into stable non-reactive DPPH-H form by donating electron or hydrogen radical. The scavenging activity of plant crude extract was comparable to the standard antioxidant ascorbic acid.

5. Conclusion

Our investigation displayed that the methanolic extract of *Azadirachta indica* leaves contains phytochemicals such as Glycosides, Alkaloids, Flavonoids, Tannins, Saponins and Terpenoids. The plant extract has minor anti-oxidant activity and good analgesic activity. From this study we have come to conclusion that there is a possibility of developing cheap analgesic drugs from this plant however, further research should be carried out on this field.

6. Acknowledgement

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7. Conflict Of Interest

The authors declare that there is no conflict of interest among them.

8. References

1. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010; 4(8):118-126.
2. Goldstein BD, Witz G. Free radicals and carcinogenesis. Free Radic Res Commun. 1990; 11(1-3):3-10.
3. Rahman K. Studies on free radicals, antioxidants, and co-factors. Clin Interv Aging. 2007; 2(2):219-236.
4. Clemens MR. Free radicals in chemical carcinogenesis. Klin Wochenschr. 1991, 15; 69(21-23):1123-34.
5. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. Free Radic Biol Med. 1990; 8(6):583-99.
6. Joseph CM, Jeffrey WB and Adara M. Natural anti-inflammatory agents for pain relief. Surg Neurol Int. 2010; 1:80.
7. Slater D, Kunnathil S, McBride J and Koppala R, Pharmacology of Nonsteroidal Antiinflammatory Drugs and Opioids. Semin Intervent Radiol. 2010; 27(4):400-411.
8. Hertel J. The Role of Nonsteroidal Anti-Inflammatory Drugs in the Treatment of Acute Soft Tissue Injuries. J Athl Train. 1997; 32(4):350-358.
9. Kumar VS and Navaratnam V. Neem (Azadirachta indica): Prehistory to contemporary medicinal uses to humankind. Asian Pac J Trop Biomed. 2013; 3(7):505-514.
10. Alzohairy MA. Therapeutics Role of Azadirachta indica (Neem) and Their Active Constituents in Diseases Prevention and Treatment. Evid Based Complement Alternat Med. 2016; 2016:7382506.
11. Lakshmi T, Krishnan V, Rajendran R and Madhusudhanan N. Azadirachta indica: A herbal panacea in dentistry – An update. Pharmacogn Rev. 2015; 9(17):41-44.
12. Raghavendra M, Maiti R, Kumar S, Acharya S. Role of aqueous extract of Azadirachta indica leaves in an experimental model of Alzheimer's disease in rats. Int J Appl Basic Med Res. 2013; 3(1):37-47.
13. Anyaehie UB. Medicinal properties of fractionated acetone/water neem [Azadirachta indica] leaf extract from Nigeria: a review. Niger J Physiol Sci. 2009; 24(2):157-9.
14. Nayak BS, Pinto PLM. Catharanthus roseus flowers extract has wound-heating activity in Sprague Dawley rats. BMC Complement Altern Med. 2006; 6(41):1-6.
15. Bargah RK. Preliminary test of phytochemical screening of crude ethanolic and aqueous extract of Moringa pterygosperma Gaertn. J Pharmacogn Phytochem. 2015; 4(1):07-09.
16. Ali K, Ashraf A, Biswas NN. Analgesic, anti-inflammatory and anti-diarrheal activities of ethanolic leaf extract of *Typhonium trilobatum* L. Asian Pac J Trop Biomed. 2012; 2(9):722-726.
17. Kaushik D, Kumar A, Kaushik P, Rana AC. Analgesic and Anti-Inflammatory Activity of *Pinus roxburghii* Sarg. Adv Pharmacol Sci. 2012; 2012:245431. doi: 10.1155/2012/245431.
18. Siddiqua SA, Majumder MM, Hossain MM, Alam MA, Haque S, Ghani A. Evaluation of analgesic and antioxidant potential of the leaves of Curcuma alismatifolia Gagnep. SJPS. 2008; 1(2):3-9.
19. Sheikh NW, Patel RD, Upwar NI, Mahobia NK, Seth MV, Panchal UR. Analgesic study of methyl alcohol extract of Cassia fistula Pod, J Pharm Res. 2010; 3(9):2218-2219.
20. Raquibul SM, Jamila M, Majumder MM, Akter R, Hossain MM, Ehsanul HM Analgesic and Antioxidant Activity of the Hydroethanolic Extract of *Mikania scandens* (L.) Wild Leaves Am J Pharmacol Toxicol. 2009; 4(1):1-7.
21. Ilavarasan R, Mallika M, Venkataraman S. Antiinflammatory and antioxidant activities of Cassia fistula Linn Bark extracts. Afr J Tradit Complement Altern Med. 2005; 2(1):70-85.
22. Benzie FF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 1999; 299:15-23.