



## Evaluation of potent hydro-alcoholic extract of leaves of *Azadirachta Indica* for isolation and identification of anti-helminthic compound

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### ABSTRACT

The traditions of collecting, processing and applying plant and plant-based medications have been handed down from generation to generation. In many African countries, traditional medicines, with medicinal plants as their most important components, are sold in marketplaces or prescribed by traditional healers (without accurate dose value) in their homes. The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine because the search for new potential medicinal plants is frequently based on an ethno-medicinal basis. The experiment was carried out to isolate and determine the anti-helminthic activity of the molecule (s) isolated from hydro-alcoholic extract of leaves of *Azadirachta indica* (Neem). The isolation and characterization of the potent molecule was undertaken by chromatographic and spectroscopic techniques. The compound was obtained as a greenish-brown powder after drying and was found to be a tetranor-triterpenoid limonoid. The compound showed *R<sub>f</sub>* value of 0.56 when compared to standard (0.57). The isolated compound showed the retention time of 3.8 minutes very much similar to that of the standard. The compound demonstrated paralysis as well as death of worms in a lesser time at 10 and 20 µg/ml compared to piperazine citrate and albendazole (15 mg/ml). It was observed that anti-helminthic activity was significant as the concentration of the compound varies. The results showed the significant potency of azadirachtin as a significant anti-helminthic.

**Keywords:** *Azadirachta indica*, azadirachtin, anti-helminthic compound, hydro-alcoholic extract, terpenoid.

### INTRODUCTION

The traditions of collecting, processing and applying plant and plant-based medications have been handed down from generation to generation. In many Afro-Asian countries, traditional medicines, with medicinal plants as their most important components, are sold in marketplaces or prescribed by traditional healers (without accurate dose value) in their homes. Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy.<sup>[1]</sup> The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine because the search for new potential medicinal plants is frequently based on an ethno-medicinal basis.<sup>[2-4]</sup> At least 12,000 phytochemicals (secondary metabolites) have been isolated from plants, a number estimated to be less than 10 % of the total. Translation of ethnobotanical information for isolation and identification of phytochemicals is required for revealing the pharmacological status of the important phytochemicals.<sup>[5]</sup> These secondary metabolites functions in defense against predators and pathogens as allelopathic agents or attractants in pollination and seed dispersion. Plants with anti-helminthic activity have been reviewed.<sup>[6]</sup> Anti-helminthic activity of some plants has also been reported akin to that of sorghum *Aliium sativum*, *Zingiber officinale*, *Cucurbita mexicana* and *Ficus religiosa*, *Artemisia brevifolia*, *Calotropis procera* *Nicotiana tabacum*,

*Butea monosperma* and *Swertia chirayta*<sup>[7-11]</sup> Previous study already revealed the anti-helminthic potency of some indigenous botanicals including *Azadirachta indica*.<sup>[12]</sup> Different researchers revealed the pharmacological, insecticidal, pesticidal and anti-helminthic properties of *Azadirachta indica* (Neem).<sup>[13-15]</sup> The present study was thus carried out to isolate the potent anti-helminthic compound from hydro-alcoholic extract of *Azadirachta indica*.

## MATERIALS AND METHODS

### Chemicals and Reagents required

The chemicals and reagents used were of analytical grade and were procured from Ranchem and CDH. Standard anti-helminthic drugs (positive controls), viz. albendazole and piperazine citrate were used for comparative evaluation.

### Collection of Plant material

The leaves of *Azadirachta indica* (Neem) belongs to Meliaceae family was selected for the study. The herbarium of plant material was taxonomically identified. The leaves were dried under shade and ground to form the fine powder.

### Preparation of Solvent Extracts

The powdered material was soaked in approximately 200 ml of hydro-alcoholic solvent (50 % v/v), on an electrical shaker for three hours at room temperature and then left to stand overnight. The solvent extract preparation was filtered in conical flasks using Whatmann filter paper No. 1. The filtrate was concentrated on a rotary evaporator at 50°C to yield semi-solid masses. The extracts were stored in a refrigerator at 4°C for further use.

### Phytochemical screening of the extract

The portion of the dry extract was subjected to the phytochemical screening using the method adopted.<sup>[16, 17]</sup> Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars, cardiac glycosides and terpenoids<sup>[18]</sup>

### Test for alkaloids

The 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl and further kept in water bath for about 2 minutes. 1ml of the filtrate was treated with Dragendroff's reagent. Turbidity or precipitation was taken as indicator for the presence of alkaloids.

### Test for tannins

About 0.5 g of the sample was dissolved in 10 ml of boiling water and will be filtered. Few ml of 6% FeCl<sub>3</sub> was added to the filtrate. Deep green colour appeared confirmed the presence of tannins.

### Test for flavanoids

About 0.2 gm of the extract was dissolved in methanol and heated for some time. A chip of Magnesium metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color confirmed the presence of flavanoids.

### Test for saponin

About 0.5 g of the plant extract was stirred with water in the test tube. Frothing persists on warming was taken as an evidence for the presence of saponin.

### Test for steroids

Salkowaski method was adopted for the detection of steroids. About 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. To the filtrate, conc. H<sub>2</sub>SO<sub>4</sub> was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids ring.<sup>[19]</sup>

### Test for cardiac glycoside

About 0.5 g of the extract was dissolved in 2ml of glacial acetic acid containing 1 drop of 1% FeCl<sub>3</sub>. This was under laid with conc. H<sub>2</sub>SO<sub>4</sub>. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

**Test for reducing Sugars**

1ml each of Fehling's solutions, I and II was added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2-5 minutes. The production of a brick red precipitate indicated the presence of reducing sugars.

**Test for terpenoids**

Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly. The production of red violet color was taken as an indicative for terpenoids.

**Isolation and identification of compound via chromatographic and spectroscopic techniques****Column chromatography**

The hydro-alcoholic extract was subjected to silica gel column chromatography for the isolation of the phytoconstituents. An appropriate column sized 5 cm diameter and 50 cm length was used. The column was packed with Silica gel G 70 -325. It was washed with water and rinsed with acetone and then dried completely. Little of pure cotton was placed at the bottom of column with the help of a big glass rod. Solvent hexane was poured into the column upto  $\frac{3}{4}$ <sup>th</sup> level. Hydro-alcoholic extract was mixed with equal amount of graded silica gel until it became free flowing powder. When it reached a defined state it was slowly poured into the column containing hexane solvent with slight movement of stirring by glass rod to avoid clogging. Little cotton was placed on top of silica gel-extract mixture pack to get neat column pack. The knob at the bottom was slowly opened to release the solvent. The elution was done using hexane, ethyl acetate and methanol in different ratios like ethyl acetate: methanol (50:50 – broad fraction). The fraction was collected separately and subjected to TLC. The solvents were evaporated by rotary vacuum evaporator.

**Conventional preparative TLC**

Silica gel G was used as an adsorbent for thin layer chromatography (TLC). The plates were activated in hot air oven at 110°C for one hour. A quantity of the finely divided absorbing agent silica gel G was prepared by the absorbent with twice the weight of distilled water and the mixture was made homogeneous by vigorous shaking for 5 minutes, then it was applied to the glass plate in a thin and uniform layer by using a stahl-type applicator or by means of a spreading device. The thickness of the applied layer was maintained at 2 mm to 4 mm and the plates were activated by being dried in a hot air oven, usually for 24 hours at 60°C. The plates were developed in the solvent, ethyl acetate: methanol: water (78:14:8 v/v/v). The selected fractions as resulted from column chromatography were spotted along with some probable standards as a fine and tiny droplet on the TLC plate occupied with silica slurry coating. All the TLC separations were performed at room temperature and detection was carried out by UV light at 354 nm. The various samples showed different intensities of the respective compounds inferring the presence of these compounds in varying amounts in those samples. The visualized compounds from TLC were quantified by the GelQuant-NET software provided by Biochemlab Solutions Co. using reference compounds.<sup>[20]</sup> First calibration curves were prepared from standard marker solutions and peak areas in terms of pixels were plotted against the corresponding concentration. All seven crude methanol extracts were visualized for their respective marker compounds from TLC plates and pixel ratio for each band was calculated using GelQuant-NET software. The active fractions/ pure compounds were further scraped from the silica gel plate and eluted from the silica gel with ethanol. The active compounds were filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica gel and this yielded more of compound(s) fraction.

**High-performance liquid chromatography (HPLC)**

HPLC analysis was performed using a Shimadzo LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermo-stated flow cell and a selectable wavelength of 340 nm. The detector signal was recorded on a Shimadzo LC2010 integrator. The column used was C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. Mobile phase used was 0.5% v/v formic acid and acetonitrile at a flow rate of 1.2 ml/minute and at column temperature 25°C. Injection volume was 20 µl of the diluted compound sample and detection of the chromatogram was carried in UV range. The complete procedure was performed both for isolated compound and standard. Both the chromatograms were interpreted by comparing the retention time (RT).

**Fourier Transform Infrared (FTIR) studies**

The IR spectrum of isolated compound was recorded in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a computerized FT-IR spectrometer (Perkin Co., Germany) in the range of 4400-400  $\text{cm}^{-1}$  by the KBr pellet technique. The molecular structure and its homology with any reported compound (if any) was determined by probable structural units (PSUs) as determined by IR- spectra.

**Anti-helminthic bioassay**

Healthy adult Indian earthworms, *Pheretima posthuma*, (Annelida, Megescolecidae) due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings were used in the study. <sup>[21]</sup>

**Assessment of Anti-helminthic activity**

Anti-helminthic activity was assessed using earthworms by the reported methods with slight modifications. <sup>[22]</sup> Samples of the compound were diluted in 1 % N-saline (0.85 % Sodium chloride) solution for anti-helminthic activity. To obtain a stock solution, different working solutions of the isolated compound was prepared to get a concentration range of 05, 10 and 20  $\mu\text{g/ml}$ . Six earthworms were soaked separately within the petri-dishes in the different sets within 6 groups at 0 time. The time (in minutes) of paralysis and death of earthworms were recorded after placing in the compound dilutions in reference to the positive controls. Paralysis is meant by the motility of the earthworms after their placement in the extracts and controls. The death time shows the non-motility/death of the worms.

**Groups subjected for the study:**

**I Group:** Vehicle/Negative Control: 1 % N-saline (0.85 % Sodium chloride)

**II Group:** Positive Control: Piperazine citrate (15 mg/ml)

**III Group:** Positive Control: Albendazole (15 mg/ml)

**IV Group:** Test: Isolated compound (azadirachtin) of *Azadirachta indica* (Neem) - 5  $\mu\text{g/ml}$

**V Group:** Test: Isolated compound (azadirachtin) of *Azadirachta indica* (Neem) - 10  $\mu\text{g/ml}$

**VI Group:** Test: Isolated compound (azadirachtin) of *Azadirachta indica* (Neem) - 20  $\mu\text{g/ml}$

**Statistical Analysis**

All the experiments were performed in triplicates and the data are reported as mean  $\pm$  SD.

**RESULTS****Phytochemical screening**

The hydro-alcoholic extracts of leaves of *Azadirachta indica* after screening for anti-helminthic activity were qualitatively detected for phytochemical screening. The results confirmed the presence of tannins, flavanoids, saponin, reducing sugars and terpenoids in the hydro-alcoholic extract while alkaloids, steroids and glycosides were found absent. The results are shown in **Table 1**.

**Table 1: Phytochemical screening of the hydro-alcoholic extract of *Azadirachta indica***

Phytochemicals	Hydro-alcoholic extract of <i>Azadirachta indica</i>
Alkaloids	-
Tannins	+
Flavanoids	+
Saponin	+
Steroids	-
Cardiac glycoside	-
Reducing sugars	+
Terpenoids	+

\*+, present; -, absent

**Column and Thin layer chromatography**

The pure compound was isolated from the hydro-alcoholic solvent by gradient column chromatography and further identified under UV light on TLC plate. The compound showed similar Rf value (0.56) very much similar to that of standard compound, azadirachtin (0.57). The results of TLC are shown in **Figure 1**.

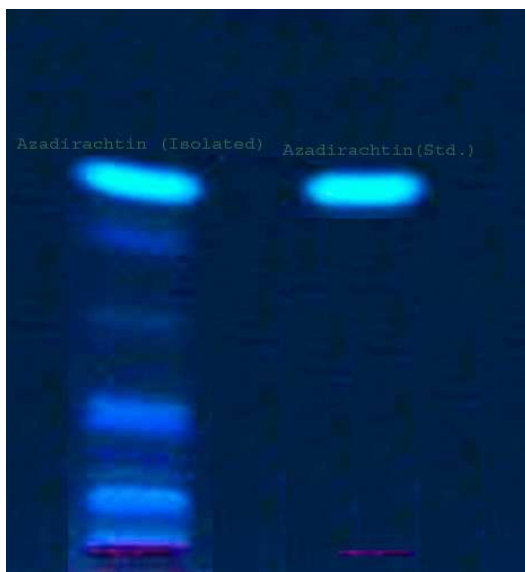


Figure 1: TLC of the isolated compound (azadirachtin) of *Azadirachta indica* after visualization under UV light

#### HPLC assay

Further, the isolated compound was identified by HPLC purification studies after interpreting with that of standard compound. The isolated compound showed almost similar retention time (3.853 minutes) as that of the standard, azadirachtin (3.842 minutes). The results of HPLC are shown in **Figure 2 (a)** and **(b)**.

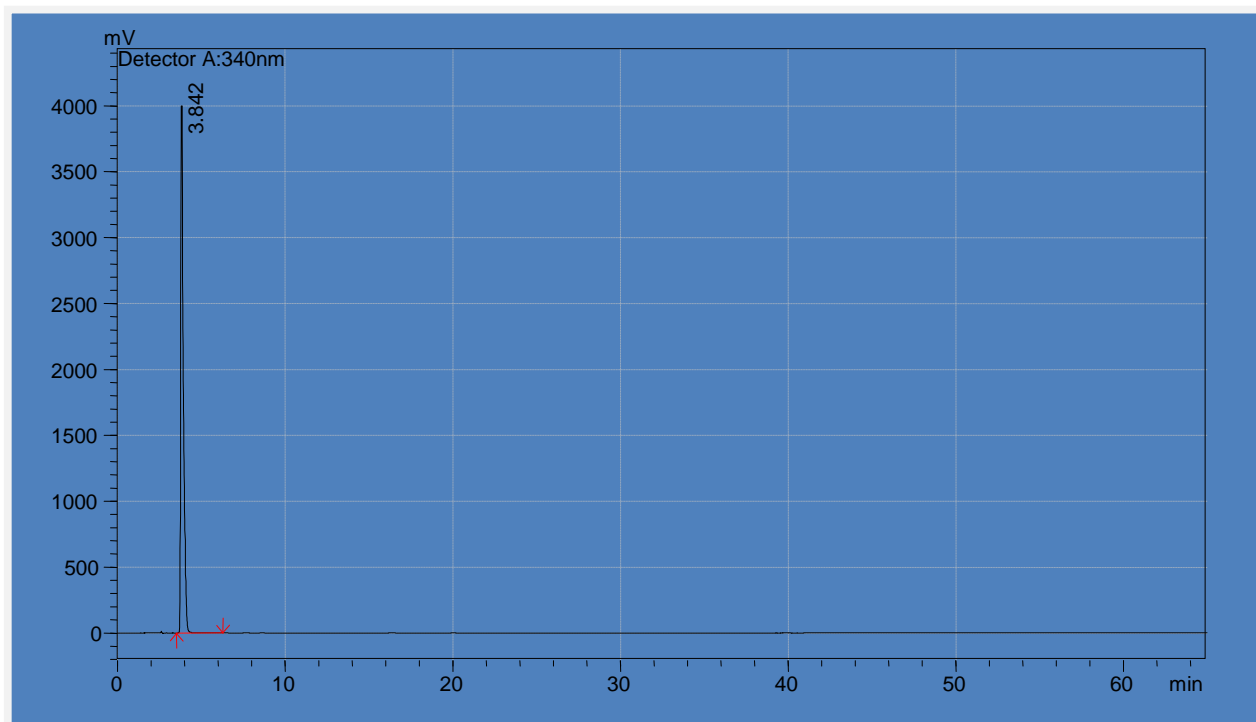


Figure 2 (a): HPLC chromatogram of the standard (azadirachtin)

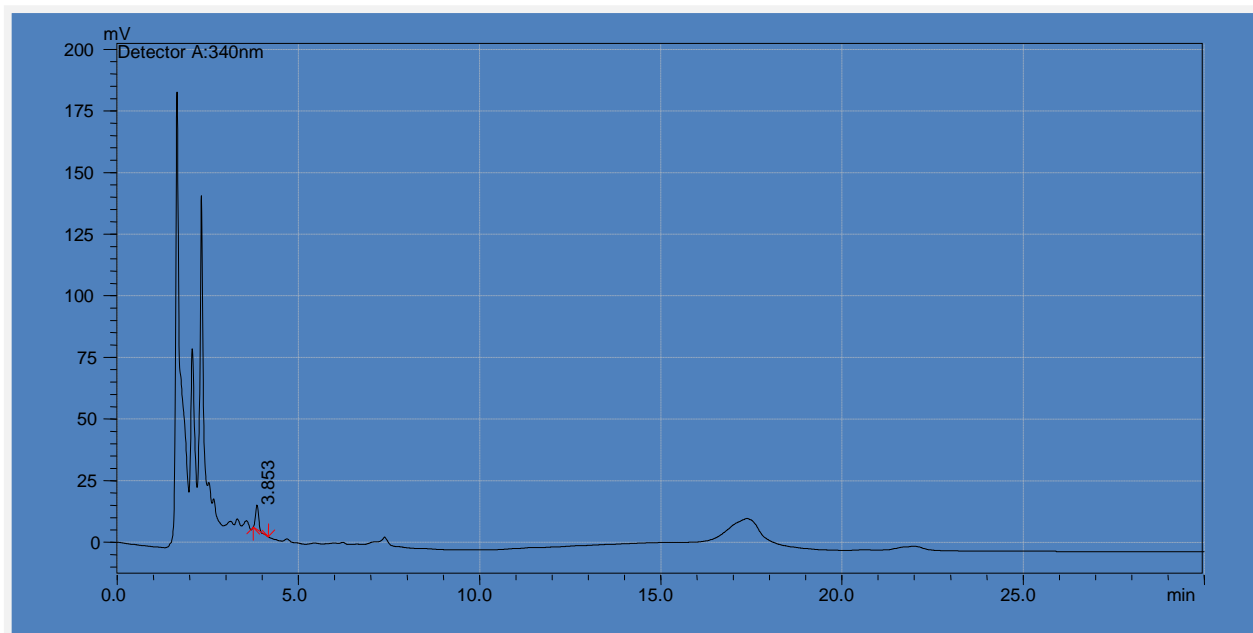


Figure 2 (b): HPLC chromatogram of the isolated compound from the hydro-alcoholic extract of *Azadirachta indica*

### IR spectroscopy

The FT-IR spectra of the isolated compound, azadirachtin was isolated from hydro-alcoholic extract of *Azadirachta indica* at  $4000\text{--}400\text{ cm}^{-1}$ . The determinations of probable structural units (PSUs)/functional groups of the isolated compound were interpreted with that of standard. The results of FT-IR spectra of the isolated compound and standard are shown in **Figure 3** and **Table 2**.

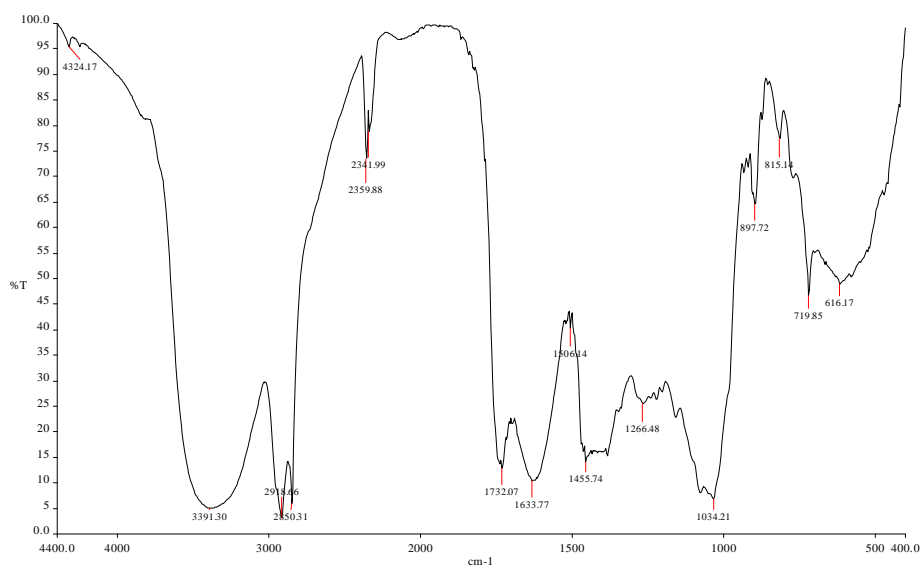


Figure 3: IR- spectra of isolated compound (azadirachtin) from *Azadirachta indica*

Table 2: Functional groups of the isolated compound (azadirachtin) as recorded by FT-IR spectra

Wave number (cm <sup>-1</sup> )	Functional group predicted
616.06	Aliphatic cyclic ether
933.70	Aromatic C-H out of plane bend
1079.37	C=O stretching
1403.46	Carboxylic acid ester

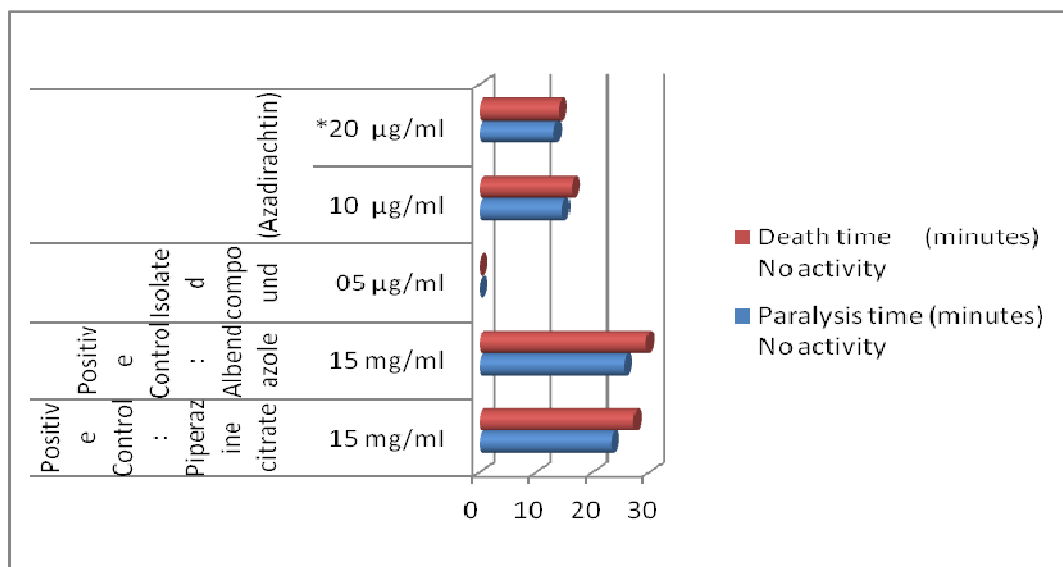
### Anti-helminthic assay

In the present investigation, azadirachtin as an anti-helminthic molecule is isolated from *Azadirachta indica*. The compound, azadirachtin was scrapped off from silica gel coated TLC plate and diluted in N-saline. The compound demonstrated paralysis as well as death of worms in a less time as compared to piperazine citrate and albendazole (15 mg/ml). The results of anti-helminthic activity are recorded in Table 3 and Figure 4. The results were found to be significant at  $p < 0.5$ .

Table 3: Anti-helminthic activity of the isolated compound (azadirachtin) and standard drugs

Group	Treatment	Concentration (mg/ml)	Paralysis time (minutes)	Death time (minutes)
1.	Vehicle: 1 % N-Saline	--	No activity	No activity
2.	Positive Control: Piperazine citrate	15 mg/ml	23.3±0.6	27.3±0.5
3.	Positive Control: Albendazole	15 mg/ml	25.6±0.6	29.5±0.5
4.	Isolated compound (Azadirachtin)	05 µg/ml	No activity	No activity
		*10 µg/ml	14.54±0.2	16.34±0.2
		*20 µg/ml	13.14±0.2	14.04±0.2

\*Level of significance  $p < 0.5$

Figure 4: Anti-helminthic activity of compound, azadirachtin isolated from *Azadirachta indica*

## DISCUSSION AND CONCLUSION

The present study suggests that, *Azadirachta indica* (Neem) is a source of valuable anti-helminthic agents apart from other pharmacological constituents. The current study leads to the isolation and identification of novel anti-helminthic molecule (Azadirachtin) from Neem. The present study stresses on the exploration of diverse novel molecules from different plants which can be utilized for different pharmacological activities. The present study thus concluded that azadirachtin can be utilized as one of the constituent in preparation of anti-helminthic drug or can be independently utilized as potent anti-helminthics.

## REFERENCES

- [1] P.A. Cox. Will tribal knowledge survive the millennium. *Sci.* 2000; 287, 44–45.
- [2] Muhammad A., Khan M.Z. and Saqib M. Use of neostigmine in massive ivermectin toxicity in cats. *Vet. Hum. Toxicol.* 2004; 46: 28–29.
- [3] Akhtar M.S., Iqbal Z., Khan M.N. and Lateef M. Anthelmintic activity of medicinal plants with particular reference to their use in animals in Indo-Pakistan subcontinent. *Small Rumin. Res.* 2000; 38: 99–107.
- [4] Iqbal Z., Munir M.A, Khan M.N, Akhtar M.S. and Hassan I.J. In vitro inhibitory effects of *Sorghum bicolor* on hatching and moulting of *Haemonchus contortus* eggs. *Int. J. Agri. Biol.* 2001a; 3: 451–453.
- [5] Iqbal Z., Nadeem Q.K., Khan M.N., Akhtar M.S. and Waraich F.N. In vitro anti-helminthic activity of *Allium sativum*, *Zingiber officinale*, *Curcubita mexicana* and *Ficus religiosa*. *Int. J. Agri. Biol.* 2001b; 3: 454–457.
- [6] Iqbal Z., Lateef M., Ashraf M., and Jabbar A. Anthelmintic activity of *Artemisia brevifolia* in sheep. *J. Ethnopharmacol.* 2004; 93: 265–268.
- [7] Iqbal Z., Lateef M., Jabbar A., Muhammad G. and Khan M.N. Anthelmintic activity of *Calotropis procera* (Ait.) Ait. F. flowers in sheep. *J. Ethnopharmacol.* 2005; 102: 256– 261.
- [8] Iqbal Z., Lateef M., Jabbar A., Ghayur M.N and Gilani A.H. In vitro and in vivo anthelmintic activity of *Nicotiana tabacum* L. leaves against gastrointestinal nematodes of sheep. *Phytoh. Res.* 2006a; 20: 46–48.
- [9] Iqbal Z., Lateef M., Jabbar A., Ghayur M.N. and Gilani A.H. In vivo anthelmintic activity of *Butea monosperma* against Trichostrongylid nematodes in sheep. *Fitoterapia* 2006b; 77: 137–140.
- [10] Iqbal Z., Lateef M., Akhtar M.S., Ghayur M.N. and Gilani A.H. In vivo anthelmintic activity of ginger against gastrointestinal nematodes of sheep. *J. Ethnopharmacol.* 2006c; 106: 285–287.
- [11] Shubham, Bhardwaj U., Mathur A. Isolation and identification of amarogentin as an anti-helminthic compound in *Swertia chirayta*. *J. Chem. Pharm. Res.* 2016; 8(4): 1374-1381.
- [12] Akhtar M.S., Iqbal Z., Khan M.N. and Lateef M. Anthelmintic activity of medicinal plants with particular reference to their use in animals in Indo-Pakistan subcontinent. *Small Rumin. Res.* 2000; 38: 99–107.
- [13] GC Coles, C Bauer, FHM Borgsteede and S. Geerts. World Association for the Advancement of Veterinary Parasitology methods for the detection of anthelmintic resistance in nematodes of Veterinary importance. 1992; *Vet. Parasitol.*, 44: 35–44.
- [14] Al-Qarawi, A.A., Malunoud O.M, Sobaih M.A, Haroun E.M. and Adam S.E.I. A preliminary study on the anthelmintic activity of *Calotropis procera* latex against *Haemonchus contortus* infection in Najdi sheep. *Vet. Res. Commun.* 2001; 25: 61–70.
- [15] Jain R., Shubham, Ansari S., Aswal D., Mathur A. Anti-helminthic potential of solvent extracts of indigenous botanicals. *Int. J. App. Bio. Pharm. Tech.* 2005; 6 (4).
- [16] Mathur A., Verma S.K., Singh S.K., Prasad G.B.K.S., Dua V.K. Investigation of the antimicrobial, antioxidant and anti-inflammatory activity of the compound isolated from *Murraya koenigii*. *It. J. Appl. Bio. Pharm. Tech.* 2011; 2(1).
- [17] AO Sofowora. *Traditional Medicine and Medicinal Plants in Africa*. 1993; 2nd Ed., University of Life Press, 320.
- [18] GE Trease, WC Evans. *Pharmacognosy*. 1989; 14th Edition, Brown Publication.
- [19] Agarwal A., Singhvi I.J., Bele D., Sharma K., Gupta S.K., Karwani G., Kumawat M. Evaluation of steroids in face creams of different marketed brands. *Int. J. pharm. Tech.* 2011; 3(2): 2480-2486.
- [20] Saito T., Hayamizu K., Yanagisawa M., Yamamoto O., Wasada N., Kinugasa S., Tanabe K., Tamura T. *Integrated Spectral Data Base System for Organic Compounds*. 2004.
- [21] Iqbal Z., Lateef M., Khan M.N. Anthelmintic activity of *Swertia chirayta* against gastrointestinal nematodes of sheep. *Fitoterapia*. 2006; 77: 463-465.
- [22] Ghosh T., Maity T.K., Bose A. and Dash G.K. Anthelmintic activity of *Bacopa monierri*, Indi. *J. Nat Pro.* 2005; 21:16-19.