An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

ASSESSMENT OF ANALGESIC ACTIVITY AND PHYTOCHEMICAL SCREENING OF *ZANTHOXYLUM ARMATUM* SEEDS – AN *IN VIVO* DESIGN

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ARTICLE INFO

Key Words

Zanthoxylum armatum, Diclofenac sodium, ethanol, seeds, analgesic activity, Hot Plate Method, Tail Clip Method.

ABSTRACT

Background: Medicinal plants which act as therapeutic agents are also a good source of information for a wide variety of phytochemical constituents which can be developed as drugs with precise and good selectivity. The scientific evaluation of medicinal plants used in the preparation of folk remedies has provided modern medicine with effective pharmaceuticals for the treatment of diseases. **Objective:** In the present research, we evaluated the analgesic effect and phytochemical investigation of *Zanthoxylum armatum* (Rutaceae) was studied on animal models. **Methods:** The analgesic effect of ethanolic seed extract of *Zanthoxylum armatum* was studied in Wistar Rats using Hot Plate Method and Tail Clip Method at a doses of 200mg/kg and 400mg/kg. Diclofenac Sodium is used as a standard drug at a dose of 25mg/kg. **Results:** The results showed dose dependent and significant ($p<0.001$) increase in pain at 60 minutes. The effects of the extract were significantly lower than those produced by Diclofenac Sodium. **Conclusion:** The obtained results showed significant analgesic activity in both mechanisms i.e. centrally and peripherally comparatively control and standard. Furthermore, a detailed and systematic approach can be done in exploiting and identifying the phytopharmacology to explore in knowing the maximum potentiality of the plant which will be useful to mankind.

1. INTRODUCTION:

India has richest plant based medicinal traditional system because of its rich biodiversity. Like India, in other developing countries, herbal plants constitute very important national resources of health sector. These herbal medicines are mainly used for health care due to their cost value, effectiveness and lesser side effects on human body. So, the pharmaceutical industries are directly or indirectly dependent upon the plant material. The Indian Himalayan Region (IHR) recognized amongst 34 biodiversity, hot spots in the world. It contains about 1,748 different species of medicinal plants. Drugs presently used for the management of pain and inflammatory conditions are narcotics (e.g. opioids), non-narcotics (e.g. salicylates) or

corticosteroids (e.g. hydrocortisone). All of these drugs cause well known side and toxic effects. Moreover, synthetic drugs are very expensive to develop, as the successful introduction of a new product calls for approximately 3000-4000 compounds to be synthesized, tested and screened, the cost of development of which ranges from 0.5 to 5 million dollars. On the other hand, many medicines of herbal origin have been used since long ago without any adverse effects. It is therefore essential that efforts be made to develop cheaper drugs. Medicinal plants and herbal medicine are one of the current areas of investigation that possess all the hallmarks of modern biomedical science. This necessitates efforts in order to identify plants that have potential for medical cure.

Plant profile: *Zanthoxylums* are deciduous and evergreen shrubs and trees from the family Rutaceae. They are native to warm temperate and subtropical region of the world. The genus is a rich source of various chemicals such as alkaloids, amides, flavonoids, lignans, sterols and terpenes etc. Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject of very intense pharmacological studies. The secondary metabolites especially the benzophenanthridine alkaloids are considered to be very important in world of medicine. The genus is occurring in Eastern and Southeast Asia (India, Bangladesh, Bhutan, China, Myanmar, Cambodia, Vietnam, Thailand, and Malaysia etc.), America (Mexico, Northern South America, Puerto Rico, Brazil, Argentina, Paraguay, Uruguay etc.) and Africa (Ethiopia, Somalia south to eastern Botswana, Kenya, Tanzania and Rwanda, Zimbabwe etc.) *Z. armatum* is a deciduous shrub or small tree which grows in well drained alluvial, black soil. In India, it has been reported from the warmer valleys of the Himalaya from Jammu and Kashmir to Assam and Khasi, in the Eastern Ghats in Orissa and Andhra Pradesh (1,200 m) and the lesser Himalayan regions in the northeastern part of India for example, Naga Hills, Meghalaya, Mizoram, and Manipur. The English name of the plant is 'Winged prickly ash' and commonly known as 'Timur' or 'Toothache tree'.

Other common names

Hindi-Darmar, Nepali dhaniya, Tejpat, Tumuru. **Bengali**-Gaira, Ttambul. **Oriya**-Tundopoda. **Sanskrit**-Tumburu, Dhiva, Gandhalu, Tejovati. **Manipuri**-Mukthrubi. **Nepali**-Timur, Nepali peeper. **Urdu**-Dambrary, Tamu. In its natural habitat, it grows up to 6 m in height with dense foliage and armed branched flattened prickles. Leaves are compound, 4 to 20 cm long, imparipinnate, rachis winged, serrate with gland dots and aromatic, containing a flavor like lime and mint. The ripe fruit follicles are usually reddish in colour and 4 to 5 mm in diameter. The dried fruit also contain an aroma that is present in brown fruit wall (pericarp-shell). It may be able to develop numbing or anesthetic feeling on the tongue. Seeds are solitary, globose, shining and have bitter taste. Flowering occurs in the months of March and April. The green or yellow flowers are present in dense terminal and axillary sparse panicles. Seed - best sown in a greenhouse as soon as it is ripe in the autumn. Stored seed may require up to 3 months cold stratification, though scarification may also help. Sow stored seed in a cold frame as early in the year as possible. Germination should take place in late spring, though it might take another 12 months. Prick out the seedlings into individual pots when they are large enough to handle and grow them on in a cold frame for their first winter. Plant them out in early summer.

Taxonomy

Domain: Eukaryota
Kingdom: Plantae
Subkingdom: Viridaeplantae

Phylum: Tracheophyta
Subphylum: Euphyllophytina
Infraphylum: Radiatopses
Class: Magnoliopsida
Subclass: Rosidae
Superorder: Rutanae
Order: Rutales
Suborder: Rutineae
Family: Rutaceae
Genus: *Zanthoxylum*
Species: Z. *armatum*

The objective of this study was to screen the petrochemical constituents and evaluate the analgesic activity of *Zanthoxylum armatum* on pain induced rats.

MATERIALS AND METHODS

Plant Material: The seeds of the plant *Zanthoxylum armatum* were collected from Raj and Company, Neemuch, Madhya Pradesh, India in the month December 2015. The plant material was identified and authenticated by Mr. G. Baba Shankar Rao, Department of Pharmacognosy and Phytochemistry, School of Pharmacy, Anurag Group of Institutions, Venkatapur (Voucher no. 0668).

Extraction: The seeds of *Zanthoxylum armatum* were dried in shady place and then powdered using mechanical grinder and stored in air tight container. About 150gms of the powder was extracted using soxhlet apparatus for 12hours using ethanol as solvent. The extract was made free from solvent by keeping it on water bath at 50-60 ^{0}C for about 6 hours.

Qualitative Preliminary Phytochemical Screening: The qualitative phytochemical screening is performed for plant extract to find out the primary and secondary metabolites present in it and chemical composition by using standard procedures. The phytochemical screening is studied under two headings

1. Screening for primary metabolites and

2. Screening for secondary metabolites.

Screening for Primary Metabolites– The phytochemical screening for primary metabolites is done for detection of carbohydrates, proteins, amino acids, fats and fixed oils.

Detection of Carbohydrates – About 50mg of the extract is dissolved in 5ml water and filtered. The filtrate is tested for the presence of carbohydrates.

1. Molish Test: To 2ml of filtrate, 2 drops of alcoholic solution of α – napthol was added. The mixture was shaken well and 1ml of concentrated sulphuric acid was added slowly along the sides of the test tube and observed for colour. The formation of violet ring at the junction of two liquids indicates the presence of carbohydrates.

2. Fehling's Test: About 50mg of extract was hydrolysed with 10ml of dilute hydrochloric acid and neutralized with alkali. The mixture was heated with 1ml of Fehling's A and Fehling's B each and observed for precipitate. Formation of red precipitate indicates the presence of reducing sugar.

3. Barfoed's Test: To 1ml of filtrate, 1ml of Barfoed's reagent was added and heated on boiling water bath for 2minutes and observed for precipitate. Formation of red precipitate indicates the presence of reducing sugar.

4. Benedict's Test: To 0.5ml of filtrate, 0.5ml of Benedict's reagent was added. The mixture was heated on boiling water bath for 2minutes and observed for precipitate. Formation of orange red precipitate indicates the presence of reducing sugar.

Detection of Proteins and Amino acids – About 100mg of extract was dissolved in

10ml of distilled water and filtered through Whatmann's filter paper and the filtrate was subjected to test for amino acids and proteins.

1. Millon's Test: To 2ml of filtrate, 2ml of Millon's reagent was added, heated to boil and observed for precipitate. The formation of white precipitate which turns to red upon heating indicates the presence of amino acids and proteins.

2. Biuret Test: To 1ml of filtrate, 1ml of 10% Sodium Hydroxide solution was added and heated to boil. To this a drop of copper sulphate solution was added and observed for colour. The formation of purple violet colour indicates the presence of amino acids and proteins.

3. Ninhydrin Test: To 2ml of test solution, few drops of 0.5% Ninhydrin reagent was added and boiled for few minutes and observed for colour. The formation of violet or blue colour indicates the presence of amino acids and proteins.

Detection of fixed oils and Fats

1. Saponification Test: Treat the extract with few drops of 0.5N alcoholic potassium Hydroxide and a drop of phenolphthalein solution. The resultant is heated on a water bath for about 1 to 2 hours. Formation of soap due to neutralization of alkali indicates the presence of fatty material.

Screening for Secondary Metabolites – The phytochemical screening for secondary metabolites is done for the detection of alkaloids, glycosides, steroids, terpenoids, flavonoids, phenolic compounds, tannins and saponins.

Detection of Alkaloids – About 50mg of solvent free extract was dissolves in the same solvent used for extraction and filtered. The filtrate was tested for the presence of alkaloids.

1. Mayer's Test: To 0.5ml of filtrate, 2 drops of Mayer's reagent (solution of potassium mercuric iodide) was added along the sides of the test tube and observed for precipitate. The formation of creamy precipitate indicates the presence of alkaloids.

2. Wagner's Test: To 0.5ml of filtrate, 2 drops of Wagner's reagent (solution of iodine in potassium iodide) was added along the sides of the test tube and observed for precipitate. Formation of reddish brown precipitate indicates the presence of alkaloids.

3. Dragendorff's Test: To 0.5ml of filtrate, 2 drops of Dragendorff's reagent (solution of potassium bismuth iodide) was added and observed for precipitate. Formation of a prominent reddish brown colour precipitate indicates the presence of alkaloids

4. Hager's Test: To 0.5ml of filtrate, 1ml of Hagner's reagent (saturated picric acid solution) was added and observed for precipitate. Formation of a prominent yellow colour precipitate indicates the presence of alkaloids.

Detection of Glycosides – For the detection of glycosides, about 50mg of extract was hydrolyzed with concentrated Hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate was subjected to the following tests.

1. Borntrager's Test: To 2ml of hydrolysate, 3ml of chloroform was added and shaken. To this separated chloroform layer, 1ml of 10% ammonia solution was added and observed for colour. Formation of pink colour indicates the presence of anthraquinone glycosides.

2. Keller – Killani Test: About 50mg of the extract was dissolved in 2ml of glacial acetic acid and 2 drops of 5% ferric chloride solution was added and mixed. Then 1ml of Sulphuric acid was added. Reddish brown colour appears at the junction of the two liquid layers and upper layer appears bluish green colour indicates the presence of steroidal glycosides.

Detection of Steroids and Terpenoids

1. Leibermann – Burchard's Test: To 50mg of extract in 2ml of chloroform was treated with 2 drops of acetic anhydride , 2 drops of concentrated sulphuric acid was then added along the sides of the test tube and observed for colour. Red, Pink or violet colour appears at the junction of the liquids indicates the presence of steroids/triterpenoids and their glycosides.

2. Salkowski's Test: 50mg of extract in 2ml of chloroform was treated with 2 drops of concentrated sulphuric acid, shaken well and allowed to stand and observed for colour. The formation of yellow coloured layer indicates the presence of triterpenes and formation of reddish brown coloured layer indicates the presence of steroids.

Detection of Phenolic compounds and Tannins

1. Ferric Chloride Test: About 50mg of extract was dissolved in 2ml of distilled water and then 2 drops of neutral 5% ferric chloride solution was added and observed for colour. Formation of blue, green or black colour indicates the presence of phenolic compounds.

2. Lead Acetate Test: About 50mg of extract was dissolved in 2ml of distilled water and to this 3ml of 10% lead acetate solution was added and observed for the precipitate. The formation of white precipitate indicates the presence of phenolic compounds.

3. Bromine Water Test: About 50mg of extract was dissolved in 2ml of distilled water; 1ml of bromine water was added and observed for the decolouration of bromine water. Decolouration of bromine water indicates the presence of phenolic compounds.

Detection of Flavonoids

1. Shinoda Test: 10mg of extract was dissolved in 2ml of alcohol. To this, two fragments of Magnesium Turnings and 0.5ml of concentrated Hydrochloric Acid was added and observed for the colour. Formation of Magenta or Crimson Red colour indicates the presence of flavonoids.

2. Alkaline Reagent Test: 10mg of extract was dissolved in 2ml of water and treated with 1ml of 10% ammonium hydroxide solution and observed for the colouration. Two drops of dilute Hydrochloric Acid was added and again observed for the discolouration. The formation of an intense yellow colour which turns to colourless on addition of dilute acid indicates the presence of flavonoids.

Detection of Saponins

1. Foam Test: 10mg of extract was dissolved in 3ml of water, shaken well and allowed to stand and observe for the formation of foam. Formation of foam indicates the presence of saponins.

Selection of dose: The LD50 value of the *Zanthoxylum armatum* extract has been reported to be 2000mg/kg for the assessment of analgesic activity, doses of 200mg/kg and 400mg/kg were selected.

Chemicals and Drugs: Diclofenac Sodium, ethanol, olive oil were from institutional laboratory.

Pharmacological Investigation

Experimental Animals: Wistar Rats weighing 200-250gms were procured from institutional animal house. The animals were housed in solid bottom polypropylene cages with a stainless steel grill on top with a clean paddy husk, at an ambient temperature and humidity, with 12-12h light and dark cycle. The experimental protocol was cleared from the Institutional Animal Ethics Committee (IAEC) before conducting the experiments. All the protocols and the experiments were conducted in strict compliance with the ethical principles and guidance provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (I/IAEC/LCP/013/2015WR♀ + ♂).

Test for Central Analgesic Activity

Hot Plate Method in Wistar Rats: The paws of mice and rats are very sensitive to heat at temperature which is not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The Hot plate, which is commercially available, consists of an electrically heated surface. The temperature is controlled to 55 ^{0}C to 56 ^{0}C. This can be a copper until either licking or jumping occurs is recorded by a stop watch. Wistar Rats were kept. Diclofenac Sodium 25mg/kg was prepared freshly. Wistar Rats of either sex were divided into four different groups each containing six animals, the animals were marked individually. Food was withdrawn 12 hours prior to drug administration till completion of experiment. The animals were weighed and numbered appropriately. The test drugs and standard drugs were given orally. After minutes, the animals are placed on the hot plate and the observations were recorded and at the time interval of 60, 90, 120 minutes. The results of Hot Plate method in Wistar Rats were tabulated.

Test for Peripheral Analgesic Activity

Tail Clip Method in Wistar Rats: The analgesic activity of the samples was evaluated using tail clip method in Wistar Rats. An artery clip is applied to the root of the tail of the rat with a weight between 18 and 25gms are used. The test drugs were administered orally to fasted animals. The test groups and control group consists of 18 Wistar rats. The drug administered 15, 30 and 60minutes prior testing. An artery clip is applied to the root of the tail to induce pain. The animal quickly responds to these noxious stimuli by biting the clip or the tail near the location of the clip. The time between stimulation onset and response is measure by a stop watch in1/10 seconds increments.

RESULTS:

Phytochemical investigation of ethanolic extract of *Zanthoxylum armatum* seeds: The ethanolic extract of *Zanthoxylum armatum* seeds were subjected to qualitative chemical tests to determine the chemical constituents present in the extract like alkaloids, terpenoids, phenolic compounds, volatile oils and flavonoids etc. were illustrated in Table 2.

Fig. 1: *Zanthoxylum armatum* plant

Fig. 2: *Zanthoxylum armatum* dried seeds

Table 1: Animal grouping

Groups	Name	Treatment
I	Control	Olive oil
II	Standard	Diclofenac sodium 25mg/kg
III	Low dose	Extract 200mg/kg
IV	High dose	Extract 400mg/kg

Table 2: Preliminary phytochemical analysis of *Zanthoxylum armatum* seeds

Constituents	Ethanol extract
Terpenoids	+
Saponins	+
Steroids	+
Carbohydrates	-
Flavonoids	+
Alkaloids	+
Tannins	-
Fixed oils and fats	-
Phenols	+
Glycosides	-
Volatile oils	+
Amino acids	+
Fatty acids	+
Anthraquinones	-

Analgesic Activity:

Test for central analgesic activity

Table 3: Effect of ethanolic extract of *Zanthoxylum armatum* on paw withdrawal time of rat using Eddy's Hot Plate

Groups	Response latency in seconds			
	0 min	**30 min**	**60 min**	**90 min**
Control	2.2±0.2	2.3±0.8	2.16±0.1	2.5±0.2
Standard	2.3±0.8	7.9±0.6	7.9±0.6	11.6±1.0
Low dose	2.2±0.7	5.1±0.7	5.1±0.7	5.1±0.6
High dose	2.0±0.2	6.3±0.5	6.3±0.5	5.9±0.4

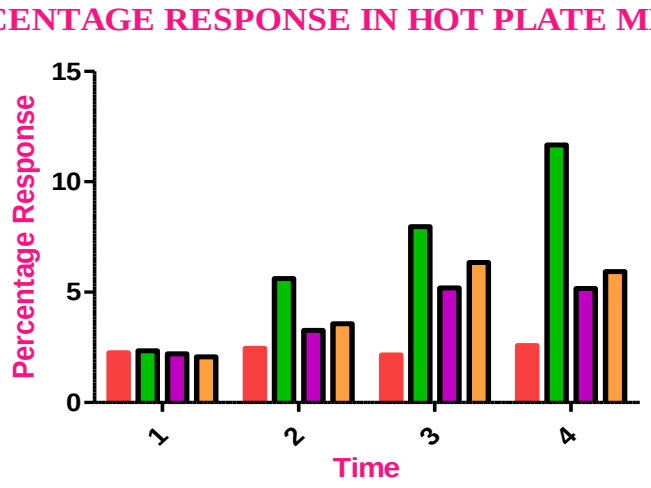

Fig. 3: Parentage response in hot plate method

Test for peripheral analgesic activity

Table 4: Effect of ethanolic extract of *Zanthoxylum armatum* using Tail Clip Method

Groups	Response latency in seconds		
	15 min	**30 min**	**60 min**
Control	4.00±0.36	3.50±0.42	3.83±0.30
Standard	8.66±0.21	9.66±0.33	10.33±0.33
Low dose	5.00±0.36	5.16±0.30	5.33±0.21
High dose	7.16±0.37	7.50±0.34	8.33±0.42

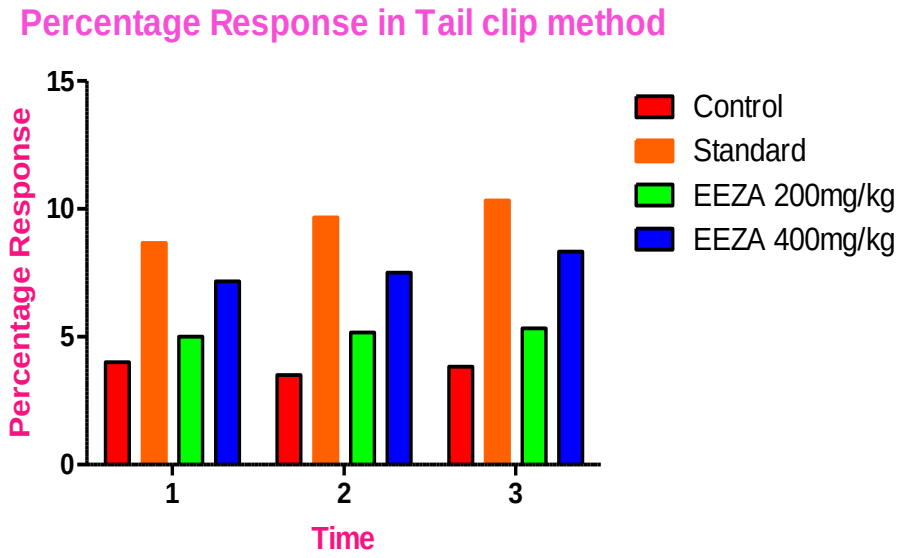

Fig. 4: Parentage response in tail clip method

DISCUSSION

Pain is 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage'. Treatment with ethanolic extract of *Zanthoxylum armatum* at doses of 200 mg/kg and 400 mg/kg body weight shows dose dependent analgesic activity was found to be most significant at the dose of 400 mg/kg ethanolic extract of *Zanthoxylum armatum* when compared with the control animals. The results of the present study demonstrated that the extract at the doses tested was shown to possess analgesic activity evident in all the models, signifying that it possesses both central and peripherally mediated activities. The treatment with ethanolic extract of *Zanthoxylum armatum* at doses of 200 mg/kg and 400 mg/kg body weight shows significant analgesic activity in the Hot plate Model. Ethanolic extract of *Zanthoxylum armatum* at the above mentioned doses produced a gradual dose dependent increase in the response latency, which started at 30min at oral administration of ethanolic extract of *Zanthoxylum armatum*, and was significant ($p<0.01$) in comparison to the control group from 30 min onwards. When compared to the standard drug Diclofenac Sodium the increase in response latency produced by ethanolic extract of *Zanthoxylum armatum* 200 mg/kg and 400 mg/kg was significant ($p<0.01$) at 90 min. The hot plate test is considered to be

selective for opioid like compounds which are centrally acting analgesics in several animal species. Treatment with ethanolic extract of *Zanthoxylum armatum* shown significant analgesic activity in the Hot plate testy may in part be mediated by opioid receptors. These findings indicate that ethanolic extract of *Zanthoxylum armatum* may contain opioid like compounds which are responsible for the analgesic activity of the plant. Tail Clip test is known to be a sensitive analgesic method. Oral administration of the extract shows its activity by the prolongation of time. Treatment with ethanolic extract of *Zanthoxylum armatum* at 200mg/kg and 400 mg/kg body weight, significantly ($p<0.001$), standard drug Diclofenac significantly ($p<0.001$) increased the time latency period for removing the clip from tail. Also it is known that chemically induced nociception can be due to an acute inflammation in the peritoneal area. The plant extract exhibited significant Analgesic Activity in central as well as peripheral analgesic models and possibly mediated its effects through diverse mechanisms that may involve both central and peripheral pathways. *Zanthoxylum armatum* seeds are potential remedy for the treatment of various pain states.

CONCLUSION

The purpose of this study was to evaluate the analgesic effect of *Zanthoxylum armatum* seed ethanolic extract in Wistar Rats using Hot plate method and Tail clip method. Ethanolic extract of *Zanthoxylum armatum* at doses 200mg/kg and 400mg/kg body weight produced a dose dependent increase in the latency period of time. In both models, ethanolic extract of *Zanthoxylum armatum* showed significant increase in the reaction time at 90 mins compared to standard drug Diclofenac sodium. Ethanolic seeds extract of *Zanthoxylum armatum* at 200mg/kg and 400mg/kg significantly suppressed the Tail Clip method and Hot Plate Method in rats and the effect was statistically significant even when compared to standard drug Diclofenac sodium. To summarize the present data indicates that the administration of ethanolic extract of *Zanthoxylum armatum* extract showed significant analgesic activity mediated both centrally and peripherally. In conclusion, the plant *Zanthoxylum armatum* has been proven for their rich anti oxidant, neuroprotective, hepatoprotective, anti-inflammatory, anti-tumor, anti-bacterial, piscicide and larvicidal activity, further the analgesic role of the seed ethanolic extract was proved by this study, indicates the potential of *Zanthoxylum armatum* seed extract. There is a need for further evaluation of different fractions of ethanolic extracts of *Zanthoxylum armatum* seed as a potential remedy for the treatment of various pain states. This knowledge about the medicinal plants usage can also be extended to other fields like field of pharmacology. This study is among the early research in order to justify potential pharmacological properties of *Zanthoxylum armatum* seeds for its analgesic activity. It is hoped that the finding of this research would be beneficial and contribute to the development of better analgesic medicines from phytochemicals.

ACKNOWLEDGEMENT: The authors wish to thank the management of School of Pharmacy, Anurag Group of Institutions, Venkatapur, Ghatkesar, Telangana, India for providing necessary equipment for research, constant encouragement, praiseworthy inspiration, facilities and support.

Conflict of interest

Author declares that there is no conflict of interest to disclose.

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