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Biological and Pharmacological Properties of *Zanthoxylum ovalifolium* Weight, Leaves (Rutaceae) from Different Solvents Extracts



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ABSTRACT

The present study explores the phytochemical, antibacterial and anthelmintic activity of leaf extracts of *Zanthoxylum ovalifolium* Wight. The phytochemical analysis revealed that the major phytoconstituents of the plants are alkaloids, terpenoides, phenols, tannins, flavonoids, steroids and glycosides. In view of this petroleum ether, chloroform and ethanol *Z. ovalifolium* leaf extracts were evaluated separately for the activity on adult Indian earthworms, *Pheretima posthuma*, using albendazole as reference standards. Different concentrations (20, 40 and 60 mg/ml) of the extracts were tested for anthelmintic capacity by the determination of time of paralysis and death of Indian earthworms, *P. posthuma*. The antibacterial activity of the extracts was screened against five bacterial strains viz., *E. coli*, *P. syringae*, *X. compestris*, *S. aureus* and *P. aeruginosa*. Antibacterial activity was conducted by the agar well diffusion method. The extract showed varies levels of antibacterial activity on different test bacteria. The zone of inhibition was determined against the microorganisms. The effects of these extracts were compared to standard drug amoxicillin. Future studies are in process to isolate the active principles responsible for the activity.



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INTRODUCTION

Herbal medicines are in great demand in the developed as well as in developing countries for primary health care because of their wide biological and medicinal activities, higher safety margin and lower costs. Since the time immemorial, our traditional system of medicine and folklore claiming that medicinal plants as a whole or their parts are being used in all types of diseases successfully including antibacterial and anthelmintic, antiinflammatory etc. The medicine practiced by our forefathers has been the precursor of modern pharmaceutical medicine. This includes herbal medicine and phytotherapy, which is prevalent in Chinese, Ayurvedic (Indian), and Greek medicine [2].

India has richest plant based medicinal source because of its rich biodiversity compare to other developing countries. Herbal plants constitute very important national resources of health sector. There herbal medicines are mainly used for health care due to their cost value, effectiveness and less side effects on human body [14].

The potential for developing antimicrobials from higher plant, as it will lead to the development of phytomedicine to act against microbes. Natural products either as pure compounds or as standardized plant extract unlimited opportunities for new drug. Therefore, researchers are increasingly turning their attention to folk medicine to develop better drugs against microbial infection [8].

Plants have the ability to synthesize a wide range of chemical compounds that are used to perform importance of biological function, and to defend against attack from predators, such as, pathogenic microbes, insects and herbivorous mammals [5].

The secondary metabolites of plants were found to be source of various phytochemicals, that could be directly used as more acceptable to the human body, when compare to modern synthetic drug. Thus, the most important factor needed is to derive the maximum benefit from the traditional system of medicine phytochemical and pharmacological approaches, which lead to drug discovery referred as natural product screening. Plants have some important secondary metabolites, such as, phenolics, carotenoids, flavonoids, ascorbic acid etc., these chemical properties viz., antioxidant, antimicrobial, antiinflammatory, anticancer etc. [1].

The vast potentiality of plant have not been adequately evaluated considering the vast potentiality of plants as sources of antimicrobial drug, with reference to antibacterial and anthelmintic agents a systematic investigation was undertaken to screen, the local plant like *Zanthoxylum ovalifolium* for its antibacterial and anthelmintic activities.

Helminthiasis is the condition resulting from worm infestation, and is one of the major prevalent diseases in the world particularly in tropical countries. Lack of adequate sanitary facilities and supply of pure water coupled with poverty and illiteracy are some of the factors responsible for wide spread nature of this disease in the developing countries [18]. Helminthiasis is a common health problem in global level, but is more common in developing countries, with poorer personal and environmental hygiene. Anthelmintics are drugs, which expel parasitic worm (helminthes) from the body, by either stunning or killing them. The gastro-intestinal helminthes becomes resistant to currently available anthelmintic drug; therefore, there is a foremost problem in treatment of helminthes diseases. Moreover, these drugs are unaffordable because of their high cost. These factors paved the way for herbal remedies as alternative anthelmintics [13].

The survey of literature revealed that, no considerable work is carried out on *Z. ovalifolium*. *Zanthoxylum* sp is a prickly and deciduous aromatic shrub and tree belongs to the family Rutaceae, comprises 250 species native to warm temperate and subtropical region of the world. Traditionally leaves, fruits and barks are also used fragrances and flavorings agents for foods and beverage and are also recommended as an alternative source for constituting numerous bioactive photochemical, which can be potentially used for insect control. The main components of *Zanthoxylum* oil are oleic acid, palmitic acid, linoleic acid, and 2-undecanone. There are 12 species of this genus and some of them are used to cure toothache, snake bite, enteritis, diarrhea, arthritis stomachic, rheumatism, bronchitis, are interms of phytochemistry more than 90 species have been studied and among secondary metabolites, that appear most frequently are alkaloids, triterpenoids and lignins [17].

Zanthoxylum is represented by around 12 species from India. Chemical studies have revealed the occurrence of many secondary metabolites, one of species reported from India is *Z. ovalifolium* [9].

Z. ovalifolium stem brownish, smooth leaflet 8-12; an elliptic-abovate, apex abruptly acuminate, glabrous nerves 15-18 pairs with an irregular intra-marginal vein; petiole 5-10 cm long, paniculate flower, few male flowers 2-3 long, sepals 4, triangular petals, white stamens 4, female flower 3-4 mm long and dark brown seed. The plant *Z. ovalifolium* plays a very important role in traditional medicine to cure number of diseases. The plant has been reported to possess antioxidant, antiinflammatory, natural pesticide, antimicrobial, anthelmintic, hepatoprotective, antiproliferative and antifungal activity [15].

However, so far no study has been reported of anthelmintic and antibacterial activity of leaf materials. Hence, the present study was therefore undertaken to evaluate the *in-vitro* antibacterial and anthelmintic activity of different solvent leaf extracts of *Z. ovalifolium*.

MATERIALS AND METHODS

The methodology which was adopted to evaluate the phytochemical, antibacterial and anthelmintic activities of the leaf *Z. ovalifolium* is as follows:

Collection of plant material

The leaves of *Z. ovalifolium* (Fig.1) were collected from different forest regions of Sringeri (T), Chikamagalore (D), Karnataka during the month of December 2014. The plant was identified by using standard floras [10, 22]. The herbarium specimen has been deposited in the Dept. of P. G. Studies and Research in Applied Botany, Jnana Sahyadri, Kuvempu University, Shankaraghata. The collected materials were washed thoroughly 2-3 times with running water and once with sterile distilled water and dried in shade at room temperature, then stored in a plastic zip bag, an air sealed polyethylene bag at room temperature before extraction.

Phytochemical analysis

The main groups of secondary metabolites (alkaloids, flavonoids, glycosides, saponins, steroids, tannins, phenols and terpenoids) are the presence and absences of these phytoconstituents were tested during the present study.

Chemicals

All the solvents viz., petroleum ether, chloroform and ethanol used in this study were purchased from Hi-Media Laboratories Pvt. Ltd, of analytical grade.

Preparation of crude extract

The extracts were prepared according to the methodology [4]. The powdered material was subjected to Soxhlet extraction with various solvents ranging from non-polar to polar. The solvents used were petroleum ether, chloroform and ethanol. Each time before extraction with next solvents the marc was air dried. All the extracts were concentrated by rotary flash evaporator. Each extraction was carried out for 48 h at suitable temperature and preserved at 4°C for further experiments.

Qualitative tests

Qualitative phytochemical analysis was done to determine the presence of active secondary metabolites using standard procedure [12].

Antibacterial activity

The antibacterial activities of crude extracts were studied comparatively with that of control DMSO and standard drug (Amoxicillin) by agar well diffusion method against some selected plant and human pathogenic bacteria.

Source of microorganisms

The following bacterial strains were used to study the antibacterial activity of leaf extracts of *Z. ovalifolium* viz., *Escherichia coli* (MTCC-1698), *Pseudomonas syringae* (MTCC-1604), *Xanthomonas compestris* (MTCC-3386), *Staphylococcus aureus* (MTCC-902) and *Pseudomonas aeruginosa* (MTCC-431). These organisms were received and authenticated from MTCC, Chandigarh, India and the cultures were maintained at 4°C for further use.

Agar well diffusion method

The agar well diffusion assay technique was used [21]. 20 ml of the sterilized nutrient agar was poured uniformly in a petriplate and allowed to solidify and then 100 µl of suspension of the test organisms was spread evenly on medium with sterilized L- shaped glass spreader to get a uniform smear of bacteria. After that the wells were prepared with the help of a clean and sterilized cork borer of 6 mm diameter. Different solvent extract of leaves at various concentrations (25%, 50% and 100%) respectively. The tests were carried out by triplicates for each solvent extract for each test organisms. All the plates were incubated at 35°C for 24 h in

BOD incubator to favor the complete growth of the test organism. The bacterial activity was determined by recording zone of inhibition around the well and compared with that produced by amoxicillin which was the standard antibiotic used for the test.

Anthelmintic activity

Worm collection and authentication

Indian adult earthworms (*Pheritima posthuma*) were used to study anthelmintic activity. The earthworms were procured from the Department of Horticulture Shivamogga (D), Karnataka were identified in the Department of Applied Zoology, Kuvempu University, Jnana Sahyadri, Shankaraghatta-577451, Shivamogga (D), Karnataka.

Standard drug

For present study albendazole had taken as standard drug. The concentration of standard drug was prepared in 1% gum *Acacia* in normal saline to give 10 mg/ml concentration.

Experimental design

The anthelmintic assay was carried as per the method of minor modifications [3]. The assay was performed on adult Indian earthworm, *P. posthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings [7, 16, 19, 20]. Because of easy availability, earthworms have been used widely for the initial *in-vitro* evaluation of anthelmintic compounds [6]. Three sets (petroleum ether, chloroform and methanol extract treatment) in five groups were taken each containing six earthworms of approximately equal size (6.0 ± 1.0 cm). Albendazole (10 mg/ml) was taken as standard drug. The plant extracts of *Z. ovalifolium*, different concentrations were prepared by dissolving in minimum quantity of DMSO and making upto the final volume with normal saline to obtain 20 mg/ml, 40 mg/ml and 60 mg/ml concentrations. One of the groups is taken as control group, which was treated with normal saline. Time for paralysis was noted when no movement of any sort could be observed except the worms were shaken vigorously. Time for death of worms were recorded after as pertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 50°C. All the test solution and standard drug solution were prepared freshly before starting the experiments [11].

RESULTS



Figure 1. Natural habitat of *Z. ovalifolium*

Table 1. Yield of leaf extracts of *Z. ovalifolium* obtained from different solvents (250 gm in 800 ml)

Solvents	Yield of extract (gm)
Petroleum ether	4.02
Chloroform	3.26
Ethanol	3.01

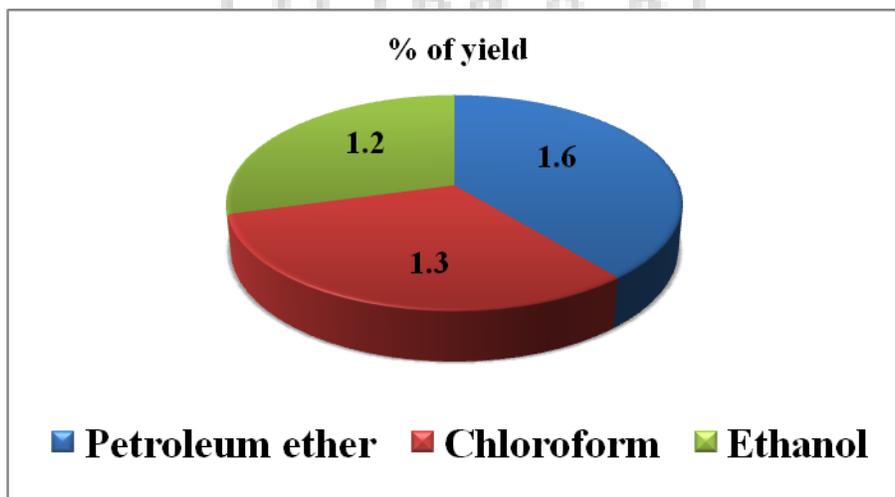


Figure 2. Percent of yield of leaf extracts of *Z. ovalifolium* obtained from different solvents

Phytochemical analysis

Table 2. Results of qualitative phytochemical analysis of secondary metabolites of *Z. ovalifolium*

Sl. No	Secondary metabolites	Name of the test	<i>Z. ovalifolium</i>		
			Petroleum ether	Chloroform	Ethanol
1	Alkaloids	Mayer's test	-	-	-
		Wagner's test	+	+	+
2	Tannins	Ferric chloride test	-	-	-
		Gelatin test	+	-	+
3	Glycosides	Keller-killiani's test	-	-	+
		Legal's test	-	-	-
4	Phenols	Ferric chloride test	-	+	+
		Ellagic test	+	-	+
5	Flavonoids	Ferric chloride test	-	-	+
		Shinoda test	-	-	-
		Alkaline reagent test	-	-	+
		Lead acetate test	-	-	-
6	Steroids	Salkowski's test	+	+	+
7	Saponins	Foam test	-	-	-
8	Triterpenoids	Salkowski's test	+	-	+

Note: '+' = presence and '-' = absence.

Antibacterial activity

Table 3. Antibacterial activity of *Z. ovalifolium* leaf extracts against plant and human pathogenic bacteria

Test organism	Diameter of zone of inhibition (mm) / conc. mg/ml										
	Petroleum ether extract			Chloroform extract			Ethanol extract			Control	Standard drug
	25	50	100	25	50	100	25	50	100	DMSO	Amoxicillin
<i>P. syringae</i>	11.5	12.3	14.0	15.0	16.0	20.4	4.0	10.0	12.3	0.0	24.0
<i>E. coli</i>	5.5	8.7	15.0	6.0	10.0	15.0	5.5	7.4	12.0	0.0	22.5
<i>P. aeruginosa</i>	6.5	10.2	13.5	4.0	16.5	19.4	9.4	10.5	16.6	0.0	22.5
<i>S. aureus</i>	4.5	11.7	19.8	9.4	10.0	19.0	5.5	6.0	12.0	0.0	21.5
<i>X. campestris</i>	9.5	10.0	10.5	13.8	14.8	18.0	6.0	8.5	18.0	0.0	20.3

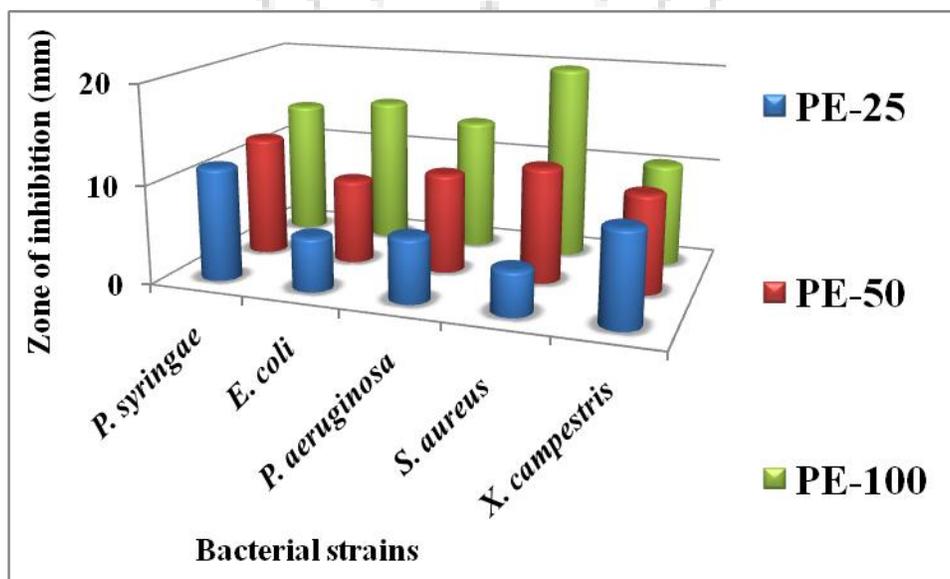


Figure 3. Antibacterial activity of petroleum ether extracts of *Z. ovalifolium* against various strains

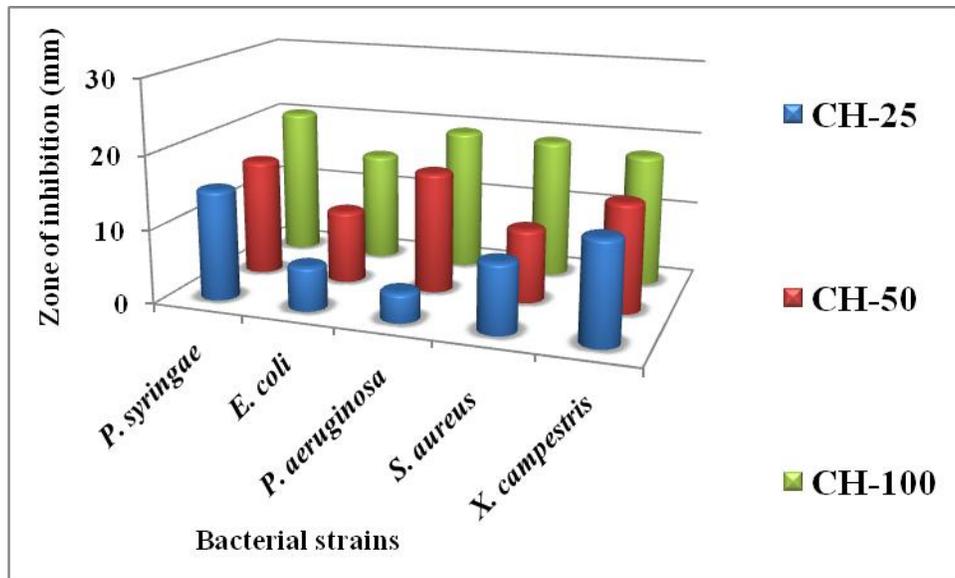


Figure 4. Antibacterial activity of chloroform extracts of *Z. ovalifolium* against various strains

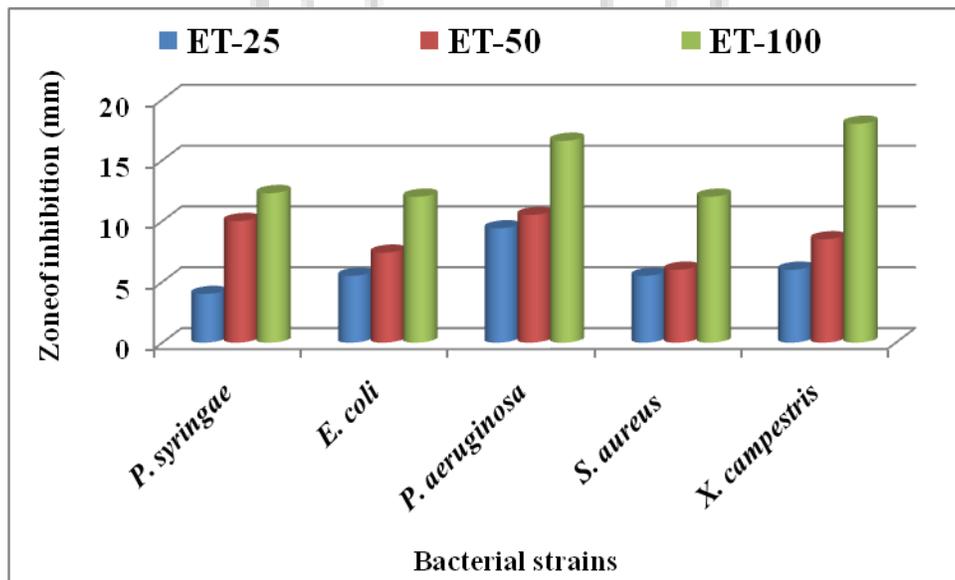


Figure 5. Antibacterial activity of ethanol extracts of *Z. ovalifolium* against various strains

Anthelmintic activity

Table 4. Anthelmintic activity of *Z. ovalifolium* leaf extracts against *Pheritima posthuma*

Treatments	Concentrations (mg/ml)	Paralysis time (min)	Death time (min)
Control	-	-	-
Standard drug (Albendazole)	10	21.98	50.62
Chloroform extract	20	56.74	90.82
	40	50.13	86.22
	60	46.22	84.16
Petroleum ether extract	20	54.15	82.11
	40	48.84	80.14
	60	43.11	79.16
Ethanol extract	20	41.18	75.83
	40	38.18	60.42
	60	35.92	65.11

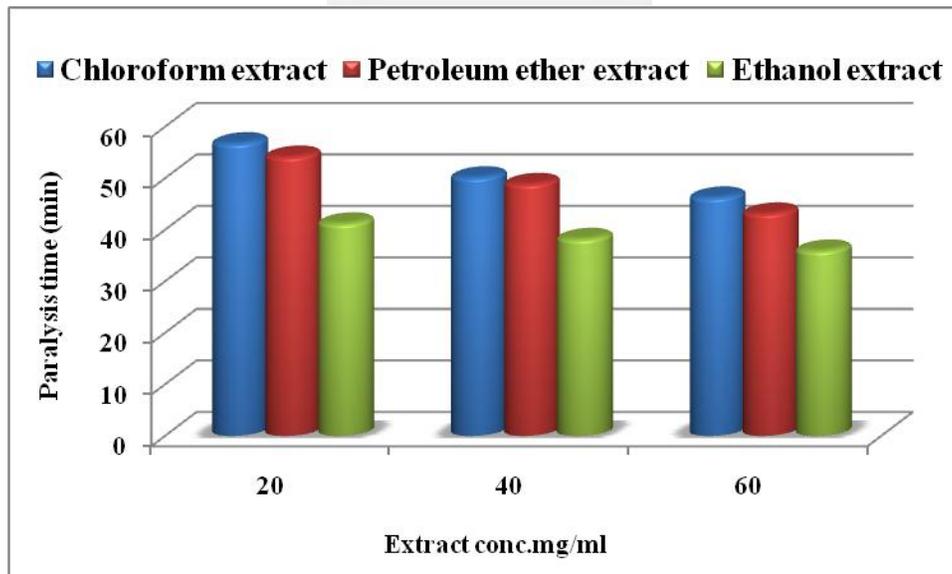


Figure.6. Comparative data of paralysis time at different concentrations against *P. posthuma*

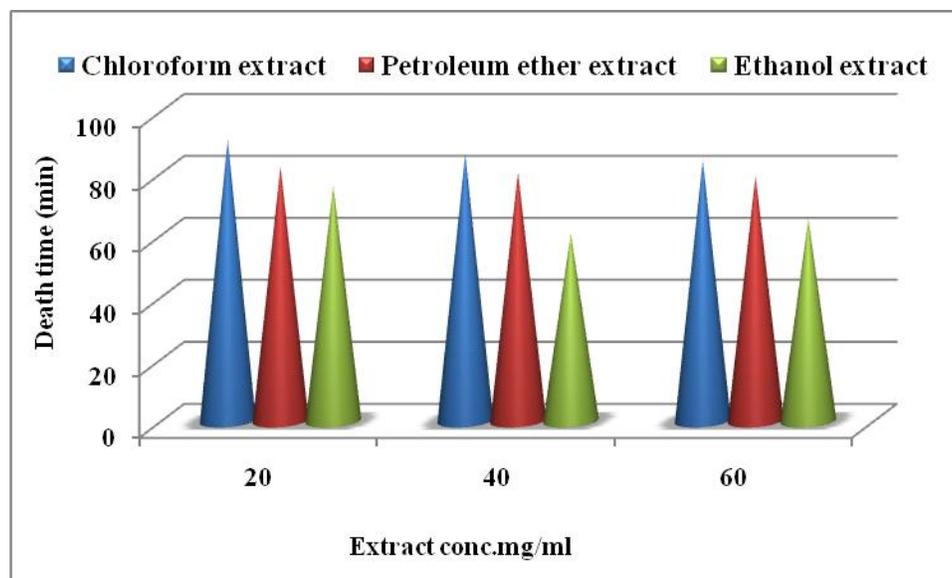


Figure.7. Comparative data of death time at different concentrations against *P. posthuma*

DISCUSSION

In the present study, Soxhlet extract method is used to obtain crude extract leaf extraction of *Z. ovalifolium*, by using the successive solvents like petroleum ether, chloroform and ethanol. The result of leaf extract shows that, the higher percentage yield was obtained in the petroleum ether followed by chloroform and ethanol (Table.1 and Fig.2).

Phytochemical analysis

The data of preliminary screening of phytochemicals i.e., secondary metabolites of all the three extracts of leaf sample of *Z. ovalifolium* revealed the presence of alkaloids, saponins, tannins, phenols, glycosides, steroids, terpenoids and flavonoids. The petroleum ether leaf extract showed the presence of alkaloids, tannins, phenols, steroids, terpenoids. The chloroform leaf extract showed the presence of alkaloids, phenols, steroids. The ethanol leaf extract shows the presence of alkaloids, tannins, glycosides, phenols, flavonoids, steroids, and terpenoids (Table.2).

Antibacterial activity

The results of antibacterial activity (Table.3) of *Z. ovalifolium* leaf extracts assayed by agar well diffusion method. A total of five pathogenic bacteria were selected, out of them, four bacterial species were belongs to human pathogenic, which include *P. syringae*, *E. coli*, *P. aeruginosa* and *S. aureus* and one species belongs to plant pathogenic i.e., *X. campestris*. The agar well

diffusion method was followed to assess the antibacterial activity of different leaf extracts. The rate of antibacterial activity of leaf extracts was determined by considering the zone of inhibition formed around the well, which was loaded with different concentrations. The zone of inhibition was measured in millimeter (mm).

The rate of antibacterial assay revealed that, the different solvent extracts obtained from the leaf material like, petroleum ether, chloroform and ethanol extracts at different concentration of 10 mg/ml, 20 mg/ml and 100 mg/ml showed, varied degree of antibacterial activity, against all selected human pathogenic as well as plant pathogenic bacteria. In case of petroleum ether (Fig.3) leaf extract, at higher concentration (100 mg/ml) showed maximum zone of inhibition against *Staphylococcus aureus* (19.5 mm), followed by *Escherichia coli* (15.0 mm), *Pseudomonas syringae* (14.0 mm), *Pseudomonas aeruginosa* (13.5 mm) and *Xanthomonas campestris* (10.5 mm).

Similarly, chloroform (Fig.4) leaf extract showed varied degree of zone of inhibition against *Pseudomonas syringae* (20.4 mm), *Pseudomonas aeruginosa* (19.4 mm), *Staphylococcus aureus* (19.0 mm), *Xanthomonas campestris* (18.0 mm) and *Escherichia coli* (15.0 mm).

In case of ethanol (Fig.5) leaf extract has, also showed, different zone of inhibition against pathogenic bacteria viz., *Escherichia coli* (12.0 mm), *Staphylococcus aureus* (12.0 mm), *Pseudomonas syringae* (12.3 mm), *Pseudomonas aeruginosa* (16.6 mm), and *Xanthomonas campestris* (18.0 mm). The standard drug amoxicillin showed varied degree of inhibition zone at 10 mg/ml against *Pseudomonas syringae* (24.0 mm), *Escherichia coli* (22.5 mm), *Pseudomonas aeruginosa* (22.5 mm), *Staphylococcus aureus* (21.50 mm) and *Xanthomonas campestris* (20.3 mm). The DMSO was used as a control in the experiment.

The overall study of antibacterial activity of different solvent extracts of *Z. ovalifolium* leaf showed, the inhibition zone against the selected pathogenic bacteria, whereas, chloroform leaf extracts shows more antibacterial activity, when compare to the ethanol and petroleum ether extracts at higher concentration i.e., 100 mg/ml. Whereas, the standard drug amoxicillin treated against the pathogenic bacteria at the concentration of 10 mg/ml showed higher degree of inhibition zone against tested pathogenic bacteria. Hence, it is also showed significant antibacterial activity.

Anthelmintic activity

The result of anthelmintic activity of different solvent extracts of *Z. ovalifolium* leaves is given in Table.4. The crude leaf extracts were evaluated for anthelmintic activity on the basis of time taken for paralysis and death of earthworms. Paralysis was said to occur when worms were not able to move even in normal saline. Death time was concluded; when they lost their motility it was also confirmed by dipping the worm in warm water.

Time taken by chloroform extract to paralyze earthworms at the concentration of 20, 40 and 60 mg/ml are 56.74, 50.13 and 46.22 min respectively. Time taken by chloroform leaf extract to cause death in earthworms at the concentrations of 20, 40 and 60 mg/ml are 90.82, 86.22 and 84.16 min respectively (Fig.6 and 7).

Time taken by petroleum ether extract to paralyze at different concentrations of 20, 40 and 60 mg/ml are 54.15, 48.84 and 41.8 min respectively and time taken to cause death at the same concentrations are 82.11, 80.14 and 79.16 min respectively (Fig.6 and 7).

Time taken by ethanol extract to paralyze at different concentrations of 20, 40 and 60 mg/ml are 41.18, 35.18 and 30.92 min respectively and time taken to cause death at the same concentrations are 75.83, 65.42 and 60.1.min respectively (Fig.6 and 7).

However in case of standard drug, albendazole treated worms showed varied results. The time taken by albendazole to paralyze earthworms at the concentration of 10 mg/ml was 21.98 and time taken to cause death at same concentration was 59.92 (Table 4).

The overall results of anthelmintic activity of different solvent extracts of leaf material of *Z. ovalifolium* reveals that, the ethanol leaf extract was found to have significant anthelmintic activity, when compared to chloroform and petroleum ether extracts at higher concentrations i.e., at 60 mg/ml, whereas, the standard albendazole treated worms at the concentration of 10 mg/ml were also showed significant anthelmintic activity. Overall, the three solvent extracts of leaf materials of *Z. ovalifolium* have proved of having considerable anthelmintic activity.

CONCLUSION

The *Zanthoxylum ovalifolium* is an important angiosperm plant. The information about phytochemical, antibacterial and anthelmintic properties of this plant is scanty. Hence, through

this study, the aspects of phytochemical, antibacterial and anthelmintic activities were considered for the present investigation.

In the present study, the different solvent extracts of *Z. ovalifolium* leaves showed considerable antibacterial activity against *P. syringae*, *X. compestris*, *E. coli*, *P. aeruginosa* and *S. aureus*. The phytochemical analysis of leaf materials of the tested plant showed the presence of secondary metabolites like alkaloids, steroids, tannins, glycosides, terpenoids, flavonoids and phenols. Among three solvents leaf extracts, the significant antibacterial activity has observed in chloroform extract, whereas, moderate level of antibacterial activity has obtained in ethanol and petroleum ether extracts.

Similarly, the crude extracts of leaf material of the test plants obtained in chloroform, petroleum ether and ethanol, showed varied degree of anthelmintic activity. The ethanol leaf extract showed significant anthelmintic activity when compare to petroleum ether and chloroform extracts.

From the present study, it is confirmed that the leaves of *Z. ovalifolium* possess considerable biological and pharmacological properties.

Further, in future it is necessary to identify and isolate the possible active phytoconstituents responsible for the pharmacological activity and also to evaluate the mechanism of action at the cellular and molecular level.

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