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Original Article

Evaluation of Anthelmintic Potential of Leaves and Fruits of *Zanthoxylum rhetsa*

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ABSTRACT

Background: *Zanthoxylum rhetsa* of family Rutaceae is traditionally employed for treatment of intestinal worms, urinary tract infection, tooth ache, asthma, bronchitis and rheumatism. Thus, the objective of the study was to screen *in vitro* anthelmintic activity of leaves and fruits of *Zanthoxylum rhetsa* on *Eisenia fetida* and *Tubifex tubifex*. **Material and Methods**: Total methanol extract and solvent fractions of methanol extract were prepared by maceration and solvent solvent extraction process respectively. The chromatographic fingerprints of total methanol extracts of leaves and fruits were developed using HPTLC. **Results**: The leaf and fruit extracts showed the presence of alkaloids, flavonoids, terpenoids, coumarins, essential oils and saponins. The extracts exhibited significant anthelmintic activity as evidenced by decrease in paralysis death time in the treatment groups when compared to standard. **Conclusion**: The results suggest that fruits and leaves of *Z rhetsa* have promising anthelmintic activity and further studies are required to identify the phytochemicals responsible for anthelmintic activity.

Key words: Anthelmintic, Fruit, Leaf, *Zanthoxylum rhetsa*, HPTLC, Phytochemicals.

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INTRODUCTION

Soil-transmitted helminth infections are generally prevalent in tropical and subtropical countries and are caused by infestation with roundworms, whipworms and hookworms. It affects around 24% of population worldwide and can cause substantial morbidity including weakness, malnutrition, gastrointestinal distress, impaired growth and physical development in human beings.[1] The anthelmintic drugs, albendazole, metronidazole and pyrantel currently used for treatment and prevention of infection generally produce side effects such as G.I distress, dizziness, headache, allergic reactions and increase of serum aminotransferase activity.[2] Also the indiscriminate use of these drugs have led to widespread resistance in humans resulting in treatment failure.[3] Thus, there is a need to find newer drugs for effective treatment and control of the infection.

Medicinal plants have been used as therapies in traditional health care systems since prehistoric times and even today more than 80% of the world's population relies on traditional herbal medicine to meet their primary health care needs. Plant secondary metabolites exhibit antiparasitic activity by various mechanisms by damaging DNA (intercalation and alkylation), altering membrane integrity, affecting microtubules and neuronal signal transduction.[4] Hence, medicinal plants have received considerable attention as alternative source of compounds for the treatment of parasitic diseases.

The genus *Zanthoxylum* (Rutaceae) comprises over 200 species distributed worldwide in tropical and temperate regions of Eastern and Southeast Asia, America and Africa. These have been traditionally used for the treatment of parasitic infections. Many *in vitro* and *in vivo* studies have been conducted that have validated antiparasitic activity of plants of genus *Zanthoxylum*.[5,6]

Zanthoxylum rhetsa (Syn: *Z. budrunga*, *Z. limonella*) family Rutaceae, is found in India, Bangladesh, Burma and Himalayan region. In India, it is found in the north eastern states and in eastern and western ghats of peninsular India. The plant is routinely used as food and medicine.[7] Traditionally, plant is employed for treatment of intestinal worms, urinary infection, heart troubles, tooth ache, asthma, bronchitis and rheumatism. The Naga tribe in the north-eastern region of India employ the leaf decoction in the treatment of intestinal worm infections and as insecticide. Volatile oil of fruit known as "Mullilam oil" has been used as antiseptic, anti-inflammatory, mosquito repellent, hypocholesterolemic and soothing agent for dental caries.[8] A study conducted by Yadav and Tangpu showed good therapeutic activity of *Z. rhetsa* leaf extract against larval stage of *Hymenolepis diminuta* and moderate efficacy against immature and adult stages of parasite when compared to standard drug praziquantal.[9] In this study, the focus in on screening the anthelmintic activity of

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leaves and fruits of *Zanthoxylum rhetsa* and to conduct preliminary phytochemical analysis of the methanol extracts.

MATERIALS AND METHODS

Collection and authentication of plant parts

Leaves and fruits of were collected from Karkala, Udupi district, Karnataka, India (13° 12'54".36"N latitude; 74° 59'46".25"E longitude; 87.79m altitude). The samples were authenticated by Dr. Rajendra D. Shinde, Blatters Hebarium, St. Xaviers College, Mumbai. The fresh leaf and fruits were shade dried, powdered and stored in well closed containers until use.

Preparation of methanol extract and its solvent fractions

The methanol extract was prepared by simple maceration process wherein 100 grams of dried powdered plant material was soaked in methanol 1 L at room temperature for 7 days with occasional shaking. It was filtered and filtrate concentrated using distillation apparatus and evaporated on electric water bath at 40°C to remove solvent. 50 g of crude methanol extract was suspended in distilled water (500 ml) and partitioned with solvents (500 ml x 3 times) in order of increasing polarity i.e petroleum ether, ethyl acetate and methanol. The fractions were individually subjected to dryness on electric water bath below 40°C. The total methanol extract, petroleum ether fraction, ethyl acetate fraction and methanol fraction of fruit was labelled as TMF, PEF, EAF and MEF respectively and the total methanol extract, petroleum ether fraction, ethyl acetate fraction and methanol fraction of leaf was labelled as TML, PEL, EAL and MEL respectively

Chromatographic fingerprint of secondary metabolites in total methanol extract[10,11]

Total Methanol extract of fruit and leaf was dissolved by sonication in 1 ml HPTLC grade solvent. HPTLC fingerprint profile for various secondary metabolites such as alkaloids, saponins, terpenoids, flavonoids, coumarins, essential oils and coumarins were developed individually using standard solvent systems and detection was done by use of respective derivatising agents (Table 1). Analysis was performed on 10 cm × 10 cm Merck, TLC plates silica gel 60 F 254. Samples were applied by Linomat 5 as 8 mm wide bands and 11.4 mm apart from middle of bands by spray-on technique along with nitrogen gas supply for simultaneous drying of bands. The extracts were applied at a concentration of 15 µl and 20 µl for leaf and fruit respectively. Plates were developed to a distance of 70 mm at room temperature (28 ± 2°C) with mobile phase in a CAMAG glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were derivatised with corresponding derivatising reagents and scanned at 540 nm by CAMAG TLC scanner run on visioncats-server, version 2.3.16286.1 software.

Table 1: HPTLC fingerprinting of phytochemicals.

Phytochcmicals	Solvent System	Detection/Derivatising agent
Flavonoid	Ethyl acetate: Formic acid: Glacial acetic acid: Water (10:0,5:0.5:1.3)	Natural products reagent at UV366mn
Essential oils	Toluene: Ethyl acetate(9.3:0.7 v/v)	Vanillin Sulphuric Acid Reagent at 540mn
Saponins	Chloroform: Acetic acid: Methanol: water (6.4:3.2:1.2:0.8)	Anisaldehyde Sulphuric Acid Reagent at 540 nm
Terpenoids	n-Hexane: Ethyl acetate (5:5)	Anisaldehyde Sulphuric Acid Reagent at 540 nm
Coumarins	Chloroform (100%)	UV254nmandUV366nm
Alkaloids	Toluene: ethylacetate: diethylamine (7:2:1)	Dragendroff's reagent at 540nm

Anthelmintic activity

Anthelmintic activity was conducted on Indian earthworm *Eisenia fetida* (Annelida) and aquarium worms *Tubifex tubifex* (Annelida) due to their anatomical and physiological resemblance with the human intestinal worm parasites. The anthelmintic assay was carried out as per reported protocols with minor modifications.[12,13] The earthworms of average size 8-10 cm and aquarium worms of average sizes of 1-1.5 cm were procured form local supplier. The assay was performed for screening of anthelmintic activity of total methanol extract and the prepared petroleum ether, ethyl acetate and methanol fractions. Three concentrations (50, 75 and 100 mg/ml) of extracts were prepared as a suspension in aqueous solution of 1% w/v Carboxy Methyl Cellulose (CMC). Six earthworms (2 in a petridish) and approximately 5 g of tubifex (n=3) were placed in 20 ml of extract solution and the time of paralysis and death of the worms were recorded. The time of paralysis was noted when no movement was observed even after the worms were shaken vigorously. The time for death for earth worm was recorded after when worms neither moved when shaken vigorously nor when dipped in warm water at 50°C. The death of tubifex worms was confirmed when the worms lost motility and by discoloration of the body. The observations were done for a maximum time period of 120 min. The anthelmintic activity of the extracts were compared albendazole (40 mg/ml) as a standard and 1% w/v carboxy methyl cellulose as a control.

Statistical analysis

The time of paralysis and death was summarized as mean and standard deviation. the data was analyzed statistically by one-way ANOVA followed by Tukey's post hoc test, $P<0.05$ being considered as statistically significant.

RESULTS

Results of phytochemical investigations and chromatrographic fingerprint of the compounds via HPTLC is depicted in Figure 1. As per the results obtained, the fruits and leaf showed presence of alkaloids, essential oils, triterpenoids, coumarin, saponin and flavonoids. The methanol extract of fruits and leaves exhibited almost equal number of bands for alkaloids, essential oils and triterpenoids. The number of bands detected for coumarins, flavonoids and saponins were higher in fruits when compared to leaf. Thus, fruits extract was found to be rich in coumarins, flavonoids and saponins in comparision to leaf extract.

The anthelmintic activity of methanol extracts and its fractions on the earthworms and tubifex worms are depicted in Figure 2 and Figure 3 respectively. The activity of extract treated groups were compared with the standard (Albendazole 40mg/ml) treated group and were recorded as the paralysis and death time of the worms. The time was recorded for a maximum of 120 min as per the protocol. From the results obtained, a dose dependent effect was shown by all treatments groups. In both the models, TML showed significant anthelmintic activity ($P<0.05$) at all three doses when compared to the standard. PEL exhibited negligible or no activity at the tested concentrations whereas the EAL showed potent activity at 100 mg/ml dose and moderate to poor activity at lower concentrations. MEL showed very potent activity and paralysis and death time significantly reduced ($P<0.05$) when compared to the standard. Anthelmintic activity of leaf fractions ranged from MEL > EAL > PEL. Fruit extracts showed potent activity ($P<0.05$) only higher dose (100mg/ml). No activity was observed for all the treated groups at 50 mg/ml and

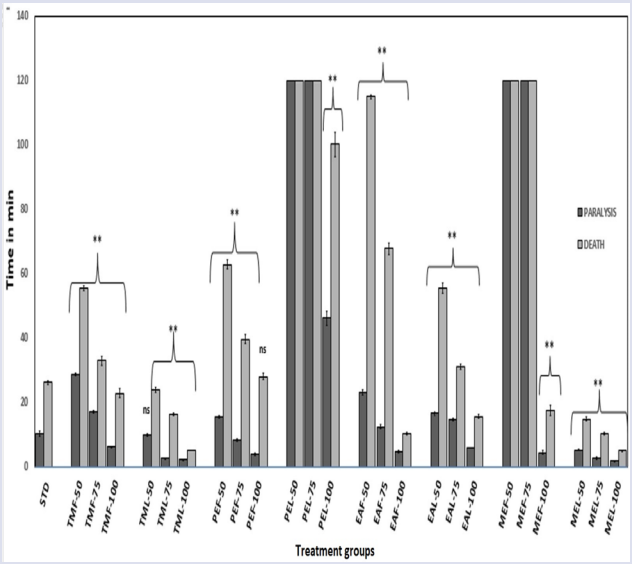

Figure 1: Anthelmintic activity on tubifex worms.
Results are expressed as mean ± SEM, n=3, ** P and t;0.05, as compared to standard, ns= not significant

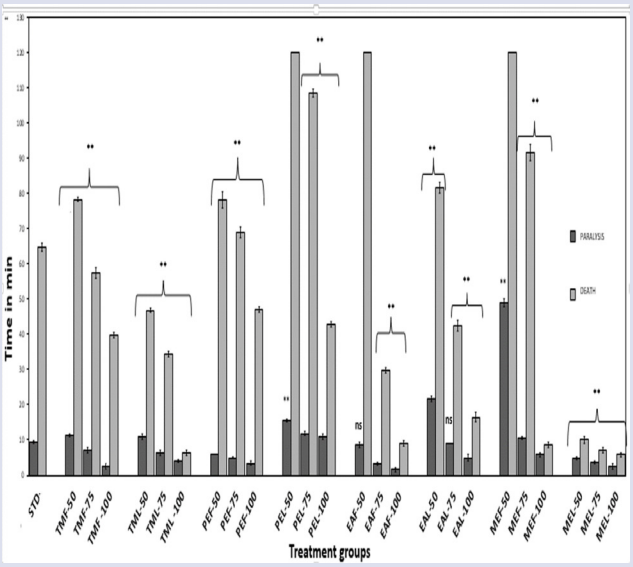

Figure 2: Anthelmintic activity on earthworms.
Results are expressed as mean ± SEM, n=6, ** *P*<0.05, as compared to standard, ns= not significant.

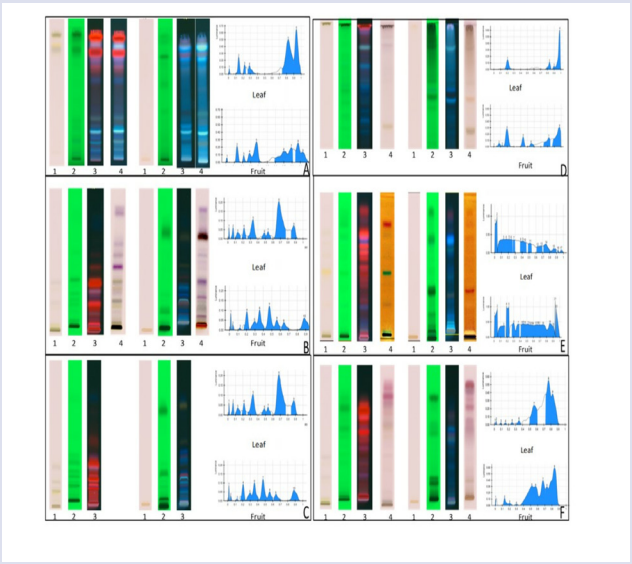

Figure 3: Chromatographic fingerprint secondary metabites in total methanol extracts of leaves and fruits.
A-Flavonoids, B- Essential oils, C- Coumarins, D- Saponins, E- Alkaloids, F- Terpenoids
Track 1- Plate before derivatisation in normal light, Track 2- Plate before derivatisation UV-254, Track 3- Plate before derivatisation UV 365, Track 4- Plate after derivatisation in normal or UV light.

moderate to poor activity was observed at 75 mg/ml treated groups when compared to standard. From the comparison of death time of extracts at 100mg/ml, EAF and MEF exhibited most potent activity in comparison to TMF and PEF.

DISCUSSION

From the results of our investigations, we propose that the leaf and fruits show potent anthelmintic activity. Whereas, in leaves, anthelmintic activity of leaf fractions ranged from MEL > EAL > PEL showing that polar phytoconstituents exhibited potent activity in compared to the non-polar phytoconstituents. The activity of the fruits too could be attributed to polar constituents since the ethyl acetate and methanol fraction yielded better activity when compared to the petroleum ether fraction. Many classes of phytoconstituents have reported to be good anthelmintic with various mechanism of action.[14] Alkaloids show anthelmintic action due to their neurotoxic property or DNA damaging property. Terpenoids, essential oils and phenyl propanoids show antiparasitic property since they disrupt fluidity and permeability of membrane of the parasite. Saponins are known to destabilize the parasite membrane by increasing the content of pro-apoptotic calcium dependent proteins and decrease the absorption and transport of sugar in the gut of helminths. The anthelmintic activity of coumarins can be attributed to the choline esterase inhibitory activity exerted by compounds. Thus, the phytochemicals alone or in combination is responsible for anthelmintic activity shown by the samples.

CONCLUSION

Natural products have been used since ancient times as anthelmintics. To overcome the side effects and growing wide spread resistance of synthetic anthelmintic drugs, newer natural product based drugs are the need of the hour.[2,3] Traditionally *Z. rhetsa* has been used to treat intestinal worms.[5,6] In this study methanol extract of leaves and fruits have shown to have potent anthelmintic activity. The polar constituents of methanol extract of the leaf and fruits have been found to have better anthelmintic potential when compared to non-polar phytoconstituents. Based on this study, it can be concluded that leaf and fruits of *Z. rhetsa* can be potential sources of lead compounds for anthelmintic therapy. Further studies to identify and characterise lead compounds from leaves and fruits as anthelmintic are essential.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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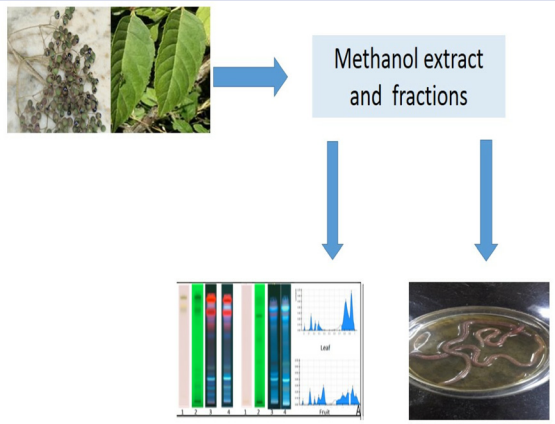
GRAPHICAL ABSTRACT



SUMMARY

- Methanol extract of fruits and leaf of *Z. rhetsa* prepared by maceration
- Fractions of methanol extract produced by solvent solvent extraction process
- Anthelmintic activity tested on *Eisenia fetida* and *Tubifex tubifex*
- Total methanol extract and polar constituents of fruits and leaves have shown to have potent anthelmintic activity.
- Leaf and fruits of *Z. rhetsa* can be potential sources of lead compounds for anthelmintic therapy.

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