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An *in-vitro* approach for evaluating anthelmintic activity of *Kandelia candel* and *Rhizophora apiculata*

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Abstract
Objective: Evaluating Anthelmintic activity of *Kandelia candel* and *Rhizophora apiculata* extracts by using *in vitro* assay.
Methods: The serial exhaustive extraction was carried out with a series of solvents: chloroform, ethyl acetate, methanol, ethanol and water with increasing polarity using Soxhlet apparatus. The concentrated and dried extracts were evaluated for anthelmintic activity by employing standard *in vitro* method (*Pheretima Posthuma* model).
Results: *In vitro* anthelmintic study shows that in case of both *Kandelia candel* and *Rhizophora apiculata* methanol extract showed higher anthelmintic activity when compared to other solvent extracts.
Conclusion: Results confirm that among tested extracts of both *Kandelia candel* and *Rhizophora apiculata* methanol extract of *Kandelia candel* exhibited highest anthelmintic activity by causing paralysis and death in the *Pheretima Posthuma* with good timing. This study provides scientific evidence that the leaves of *Kandelia candel* and *Rhizophora apiculata* have anthelmintic efficacy. Further study requires purification, characterization and structural elucidation of phytochemicals from these extracts that may help in the development of new drug formulations against various parasitic infections.

Keywords: *Kandelia candel, Rhizophora apiculata*, *Pheretima Posthuma* model, *in vitro* anthelmintic activity

1. Introduction
Helminthes infections are commonly found in community and being recognized as cause of much acute as well as chronic illness among the various human beings as well as cattle. More than half of the population of the world suffers from various types of infection and majority of cattle's suffers from worm infections [1]. Helminthes infections are also among the most common infections in humans, affecting a large proportion of the world's population in developing countries and produce global burden of disease and contribute to the prevalence of malnutrition, anemia, eosinophilia and pneumonia [2]. Inhabitants of tropical or subtropical, low income countries are at risk; children often get infected with one or more species as they born and remain infected throughout their lives. In some cases these infection results in discomforts and doest cause substantial ill health and results into serious morbidity [3, 4]. 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. In developing countries these parasite infections became threat to society by causing severe morbidity, including lymphatic filariasis, onchocerciasis and schistosomiasis [5]. It is proven that people with poor hygiene are most susceptible for parasitic infections. A person may infect with a worm either by eating contaminated food or drinking contaminated water [6]. In human body helminthes parasites are present in intestinal tract and also found in tissue [7]. As per World Health Organization (WHO), only few drugs are frequently used in the treatment of parasitic infections [8] but because of limited availability and affordability of modern medicines most of the world's population depends to greater extent on traditional medical remedies. The currently available drugs like Albendazole they are capable of a broad spectrum action against the intraluminal parasites and tissue parasites and have limitations for use in pregnancy and in children who are younger than 2 years of age [9]. The traditional medicines hold a great promise as a source of easily available effective anthelmintic agents to the people, particularly in tropical developing countries [10] and already many plants have been used to treat parasitic infections in humans and animals [11-14]. In the present study, *Kandelia candel* and *Rhizophora apiculata* plants were selected for *in vitro* anthelmintic study. *Kandelia candel* is the mangrove tree belonging to Rhizophoraceae family which is distributed along the western region of India. *K. candel* whole plant is reported to have antidiabetic activity [15, 16]. In fact rhizophoraceae species are known to have pharmacological activities. Methanolic extract of *K. candel* is used as antihyperglycemic agent in India [17] and bark, flowers and leaves were

reported to have antiviral and antimicrobial properties [18, 19]. *Rhizophora apiculata* is the tree species of mangrove tree belonging to rhizophoraceae family. In Malaysia, the leaves of *R. apiculata* are assayed as antibreast cancer [20]. Studies on HPLC investigation of *R. apiculata* have shown the presence of catechin monomer, antioxidant flavonoid [21]. This plant is reported to possess anti-inflammatory and anti-tumor properties and is also used to regulate the antioxidant enzymes in biological system [22]. Presence of tannins is reported in the bark of *R. apiculata,* which is known to possess antibacterial and antiviral properties [23-25]. Bark of *R. apiculata* is used as a traditional medicine in the treatment of diarrhoea and wounds [26, 27]. In Malaysia, pyroligneous acid from *R. apiculata* species have been used as sterilizing agent, deodorizer, fertilizer, antimicrobial agent and growth promoting agent [28]. Alkaline extract from leaf of *R. apiculata* reported to inhibit the HIV replication and HIV induced cytopathic effects. Some other studies have confirmed the antiviral property of *R. apiculata* extracts, which may be due to presence of anti polysaccharide in the extracts that acts as an antiviral agent [29]. Literature survey indicates that there are only few pharmacological studies are reported on these plants However these above mentioned plants have not been subjected for investigation for their anthelmintic activity. With this background, the present study was undertaken to evaluate anthelmintic activities of *Kandelia candel* and *Rhizophora apiculata*.

2. Materials and Methods

2.1 Plant collection

Leaves of *Kandelia candel* and *Rhizophora apiculata* were collected from Mangrove region, Sadashivgad, Karwar, Uttar Kannada District, Karnataka, India during the period of May, 2015. The leaves were identified and authenticated by Dr. Kotresha K, Dept of Botany, Karnatak Science College, Dharwad; Karnataka by referring to the voucher specimen deposited in the Dept of Botany, Karnatak Science College, Dharwad, Karnataka, India. Fresh plant leaves material was collected and washed under running tap water, shade dried and then homogenized to coarsely powder. The powder was stored in airtight containers at -20 °C for further use for crude solvent extraction.

2.2 Drugs and chemicals

All the solvents, chemicals and the standard drug Piperazine citrate (SD Fine Chemicals Ltd., Mumbai).

2.3 Crude Extraction

Coarsely powdered dried leaves of *Kandelia candel* and *Rhizophora apiculata* [100g each] were subjected to successive solvent extraction using Soxhlet apparatus separately. The extraction of each plant leaves material was done with different solvents in their increasing order of polarity which includes chloroform, ethyl acetate, methanol, ethanol and distilled water. Each time the plant material was dried and later extracted with next high polar solvents (following the strategy of extraction in series of increasing the solvent polarity). All extracts were concentrated in Buchi rotary evaporator, followed by removal of traces of solvent by using desiccator.

2.4 Test organism

Indian adult earthworms (*Pheretima posthuma*) collected from the University of Agriculture Sciences, Dharwad, India. The earthworms were maintained under normal vermicomposting medium with adequate supply of nourishment and water. Before the initiation of experiment the earthworms were washed with normal saline. Adult earthworms of approximately 4 cm in length and 0.2-0.3 cm in width were used for the experiment. This organism was selected model for anthelmintic activity due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings. [30, 31]

2.5 Extract preparation for experiment

The porously powdered plants material was used for extract preparation. After extraction, the crude extract was stored in desiccator until further use. Each solvent extracts and standard drug Piperazine citrate were dissolved in 0.5% DMSO in normal saline (v/v). Whereas, the crude aqueous extract of all plants was directly dissolved in normal saline and used for evaluation for anthelmintic activity.

2.6 Anthelmintic activity

The anthelmintic activity of *Kandelia candel* and *Rhizophora apiculata* extracts was evaluated by the following the method of Dash *et al* [32]. For each plant Twenty seven groups of animals with three earthworms in each groups, each earthworm were separate released into 20 ml of desired formulation in normal saline, Group 1 earthworm were released in 20 ml normal saline in a clean Petri plate. Group 2, 3, 4, 5, 6 earthworms were released in 20 ml normal saline containing 50, 100, 150, 200 and 250 mg/ml of chloroform extract respectively. Similarly, group 7, 8, 9, 10, 11 earthworms were released in 20 ml normal saline containing 50, 100, 150, 200 and 250 mg/ ml of ethyl acetate extract respectively. Same thing will be followed for methanol, ethanol and aqueous extracts for each plant. Last group of earthworms were released in 20 ml normal saline containing standard drug piperazine citrate (100 mg/ml). Earthworms were observed; the time taken for paralysis and the time taken for death was monitored and documented in minutes. Paralysis time was analyzed based on the behavior of the earthworm with no revival body state in normal saline medium. Death was concluded based on total loss of motility with faded body color. [33]

2.7 Statistical analysis

All experiments were performed in triplicates (n=3) and the data are presented as the mean ± standard error. Differences between the means of the individual groups were analyzed using the analysis of variance procedure of SPSS software 20 Version (IBM). The significance of differences was defined at the $p<0.05$ and $p<0.01$ level.

3. Results and Discussion

Parasitic helminthes are worm-like organisms that live and feed off living hosts, receiving nourishment and protection while disrupting their hosts' nutrient absorption, causing weakness and disease in human and animals inflicting heavy production losses. Anthelmintics are those agents that expel parasitic worms (helminthes) from the body, by either stunning or killing them [1] Various problems have been evolved with chemotherapeutic control practices such as parasites are developing resistance to several families of chemical anthelmintic [2]. Even the most common drugs like Piperazine salt have been shown to have side effects like nausea, intestinal disturbance and giddiness [34]. Ideal anthelmintic drug should have broad spectrum of action, high percentage of cure with single therapeutic dose, free from

toxicity and should be cost effective. Since many effective drugs has been isolated from traditionally reported plants. Phytochemicals or plant based agents are known to provide a rich source of botanical anthelmintics [35, 36]. Various medicinal plants have been reported on their application as anthelmintic drug in man and mammals [37, 38]. In the present study the concentrated and dried extracts of *Kandelia candel* and *Rhizophora apiculata* were evaluated for *in vitro* anthelmintic activity by varying the concentration (50-250mg/20ml) with using Indian earth worm (*Pheretima posthuma*) as animal model. In case of both *Kandelia candel* and *Rhizophora apiculata* among different solvent extracts methanol extract exhibited highest anthelmintic activity at 250mg/20ml concentration with paralysis time 46.66±1.20185 (min) and 60.66±0.88192 (min) respectively and in case of death time it was observed to be 67.33±1.76383 (min) and 84.33±1.45297 respectively. Results were compared with standard drug Piperazine citrate which shows paralysis time 31.33±1.76383 (min) and death time 36.00±1.15470 (min) at 100mg/20ml concentration. (Results are shown in Figure.1 and Figure.2). The anthelmintic activity of remaining extracts was depicted in Table.1 and Table.2. Several phytochemicals have potential to alter metabolic pathways of earth worms and there by produce mortality in the earth worms and they have ability to bind with the free proteins present in the gastrointestinal tract of earth worm and cause death [39, 40]. The results indicate that methanol extract of *Kandelia candel* and *Rhizophora apiculata* shown appreciable anthelmintic activity but in performed *in-vitro* assay methanol extract of *Kandelia candel* exhibited promising significant activity where as other tested extracts of both plants showed the least anthelmintic activity.

Table 1: *In vitro* Anthelmintic activity of different solvent extracts of *Kandelia candel* against *Pheretima posthuma*

Test Samples	Concentration in mg/20ml	Paralysis Time in minutes	Death time in minutes
Control (Normal Saline)	-	112.66±4.48454	204.33±3.17984
Chloroform Extract	50	100.00±2.30940**	129.33±1.76383*
	100	94.66±1.76383**	126.00±3.05505
	150	87.33±0.66667**	119.00±2.64575*
	200	85.33±1.76383**	119.00±2.08167*
	250	83.66±3.17980**	111.33±4.66667*
Ethyl acetate Extract	50	118.66±1.85592*	139.00±2.08167*
	100	124.66±2.90593*	141.00±2.08167*
	150	116.66±3.52767*	128.33±0.88192*
	200	109.66±3.17980*	127.00±2.51661*
	250	104.00±3.46410*	116.66±2.40370
Methanol Extract	50	68.66±2.40370**	60.00±0.55050**
	100	60.66±1.76383**	81.33±1.76383**
	150	56.66±2.90593**	75.33±1.76383**
	200	47.33±1.76383**	73.33±3.52767**
	250	46.66±2.66667**	67.33±1.76383**
Ethanol Extract	50	109.33±2.90593**	125.00±2.64875**
	100	101.33±1.76383**	121.33±1.76383**
	150	98.00±2.30940**	121.33±4.66667**
	200	92.66±1.76383**	110.33±2.60342**
	250	89.66±2.33333**	105.33±1.33333**
Aqueous Extract	50	119.66±2.60342**	140.33±2.72845**
	100	109.66±3.17980**	128.66±2.40370**
	150	125.33±2.90593**	142.00±1.15470**
	200	112.00±4.16333**	128.33±0.88192**
	250	104.00±3.46410**	110.66±1.76383**
Standard Drug Piperazine citrate	100	31.33±1.76383**	36.00±1.15470**

Results are expressed as Mean±SE (n=3); * to at the $p<0.01$.
Correlation is significant at the 0.01 level (2-tailed)**
Correlation is significant at the 0.05 level (2-tailed)*

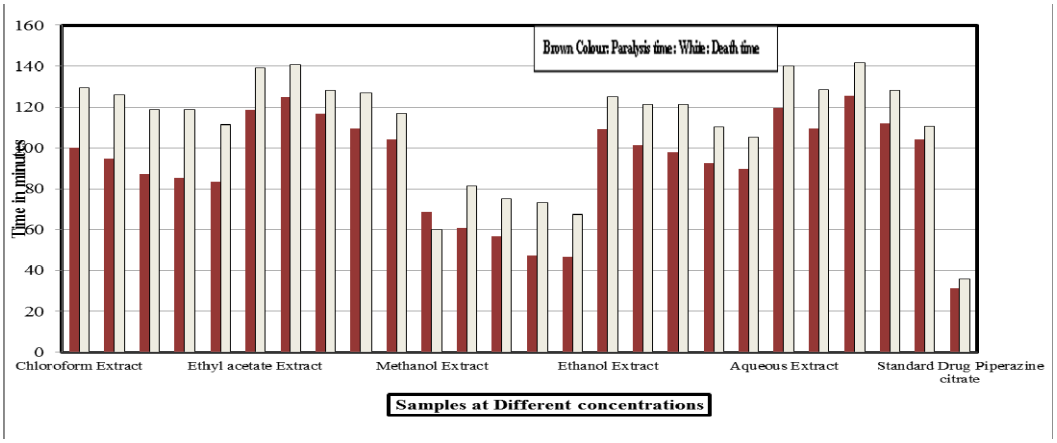

Fig 1: *In vitro* Anthelmintic activity of different solvent extracts of *Kandelia candel* against *Pheretima posthuma*

Table 2: *In vitro* Anthelmintic activity of different solvent extracts of *Rhizophora apiculata* against *Pheretima posthuma*

Test Samples	Concentration in mg/20ml	Paralysis Time in minutes	Death time in minutes
Control (Normal Saline)	-	112.66±4.48454	204.33±3.17984
Chloroform Extract	50	132.66±2.90593	153.33±3.52767
	100	127.00±2.98167	142.00±1.15470
	150	123.00±2.51661*	141.33±3.52767
	200	124.00±2.30940	138.00±2.30940
	250	116.33±1.85592**	130.00±1.15470*
Ethyl acetate Extract	50	115.33±2.40370*	138.66±2.40370**
	100	109.00±3.21455*	131.00±2.51661**
	150	117.33±3.71184*	131.33±1.33333**
	200	105.33±4.66667*	122.00±2.04375**
	250	99.00±1.73205*	115.66±2.33333**
Methanol Extract	50	82.66±1.76383**	110.00±2.30940**
	100	76.00±2.30940**	104.00±1.15470**
	150	76.66±2.40370**	103.33±2.90593**
	200	65.33±1.76383**	93.33±2.90593**
	250	60.66±1.76383**	84.33±1.45297**
Ethanol Extract	50	92.66±1.76383**	119.33±1.76383**
	100	91.00±1.52753**	116.00±3.05505**
	150	84.33±1.20185**	101.33±1.76383**
	200	78.33±1.20185**	94.66±1.76383**
	250	80.33±0.88192**	91.66±1.45297**
Aqueous Extract	50	115.33±2.40370**	138.66±2.40370**
	100	101.66±2.02759**	126.00±1.15470**
	150	107.66±1.45297**	129.00±2.08167**
	200	95.00±1.73205**	124.00±2.30940**
	250	94.00±2.30940**	116.00±3.78594**
Standard Drug Piperazine citrate	100	31.33±1.76383**	36.00±1.15470**

Results are expressed as Mean±SE (n=3); * significant at the $p<0.01$.
Correlation is significant at the 0.01 level (2-tailed)**
Correlation is significant at the 0.05 level (2-tailed)*

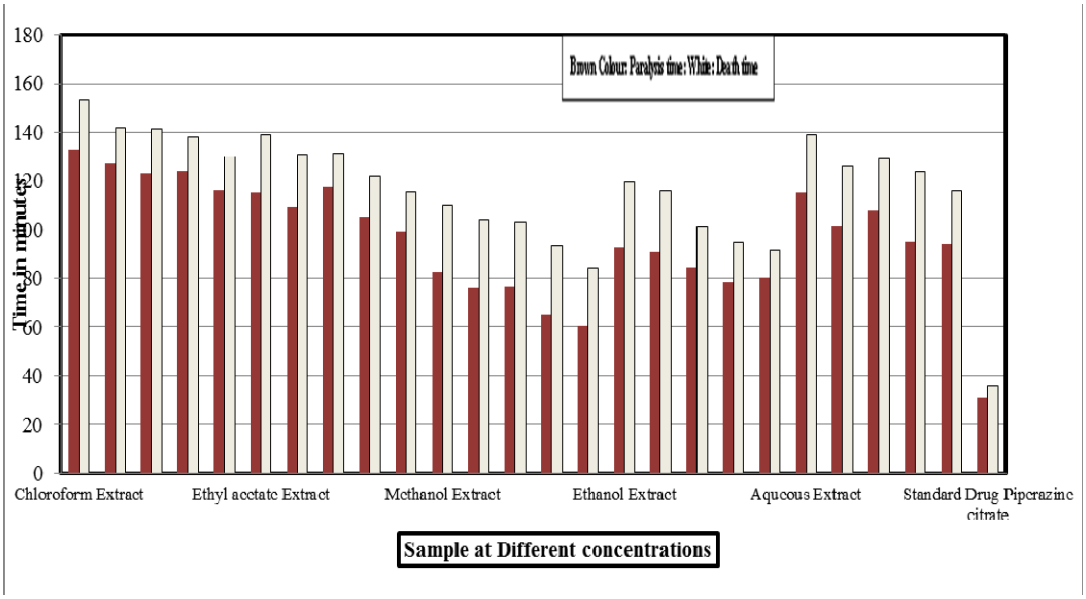

Fig 2: *In vitro* Anthelmintic activity of different solvent extracts of *Rhizophora apiculata* against *Pheretima posthuma*

4. Conclusion

In the present study in performed *in-vitro* method by using Indian earth worm (*Pheretima posthuma*) as a animal model for anthelmintic activity shows that all the tested extracts of *Kandelia candel* and *Rhizophora apiculata* showed anthelmintic activities but over viewing of the present study, it clearly shows that among tested extracts methanol extract of *Kandelia candel* exhibited high potential anthelmintic activity over all extracts with good timing for both paralysis and Death time. Based on the present study results it can be used for the development of new pharmaceutical drugs for treatment and curing of Helminthiasis and also this study shows that these extracts offer a safe method or supplement treatment strategy to control Helminthiasis. However, further detailed study is needed to isolate and purification of constituents from the plant for anthelmintic activity.

5. Conflict Of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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