

EVALUATION OF ANTHELMINTIC ACTIVITY OF *CITRUS RETICULATA*: *IN VITRO* AND ITS PHYTOCHEMICAL INVESTIGATION

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ABSTRACT

Objective: The prevalence of worm infestation is high in underdeveloped and developing countries due to poor sanitation and lack of health education. *Citrus reticulata*, fruit belonging to citrus family *Rutaceae* is a common fruit native to Asia. Peels of the fruit are rich in limonene, a potent phytoconstituent having anthelmintic activity. However, proper utilization of peels has not been done as it is not consumed and is therefore discarded. The main objective of the study was to explore various phytoconstituents present in *C. reticulata* and its anthelmintic effect.

Methods: Various concentration of methanolic extract and volatile oil of *C. reticulata* were subjected for assessment of anthelmintic activity in earthworms. Time of paralysis and time of death were used as an evaluation parameter. Albendazole (25 mg/ml) was used as a standard drug.

Results: Phytochemical test revealed the presence of alkaloids, carbohydrates, tannins, flavonoids, terpenoids, and glycosides. Concentration-dependent anthelmintic effect was observed with the extract were 150 mg/ml concentration of methanolic extract showed paralysis of test worm (earthworm) at 5.76 minutes and death at 19.16 minutes, respectively.

Conclusion: *C. reticulata* peel has shown substantial anthelmintic activity using *in vitro* model on earthworms. Hence, further research is required to understand its mechanism of action using *in vivo* models to confirm its anthelmintic potential.

Keyword: *Citrus reticulata*, Anthelmintic activity, Phytochemical constituents, *Pheretima phostuma*.

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INTRODUCTION

Helminthiasis is one of the most widespread infectious disease affecting mostly children and pregnant women. Around 2 billion people are infected by helminths (*Ascaris lumbricoides*, hookworm, *Trichuris trichiura*, and *Hymenolepis nana*) especially in the developing countries having poor sanitation [1]. *Citrus reticulata* commonly referred to as Mandarin orange or Tangerine is a sweet, juicy, easy to peel citrus fruit belonging to family *Rutaceae*. In Sanskrit, it is called Narangi and is originated in South East Asia. It is the second most widely cultivated citrus fruit after sweet orange (*Camellia sinensis*) and is cultivated well in countries such as Vietnam, Japan, Southern China, and Nepal.

They are orange colored small fruit bearing easily divided sections with loose skin. Fruit is rich in vitamin C, Vitamin A, flavonoids such as narirutin and hesperidin [2], phenolic acids, volatile oil, minerals such as calcium and potassium [2]. Dry fruit rind of the plant is edible, used as flavoring agent having sweet, spicy flavor. Fruits are consumed as juice or eaten fresh, used in beverages and preparation of jams and candies. Apart from its high nutritional value, it also has high medicinal value. Fruits are laxative, anthelmintic and aphrodisiac [3] as it possesses chemical constituents such as limonoid glycoside [4], flavonoid glycoside like - Kaempfer ride trimethyl ether; quercetin 3, 7, 3', 4'-tetramethyl ether, steroids, λ terpinene, and few flavones [5]. Oil obtained from fruit pulp has been traditionally used as sedative, diuretic, antispasmodic, antiseptic and in improving blood circulation [6]. In Ayurveda, dried peel has been used in the regulation of abdominal distension to enhance digestion and decrease phlegm. D-Limonene is a major constituent along with α - pinene and β - pinene in the oil extract of the fruit peel and is responsible for its antimicrobial property [7]. It has skin sensitizing effect in the presence of sunlight which is due to the presence of a coumarin; bergapten [8]. It also has anti-proliferative

effect on various cancer cell lines such as human gastric cancer cells (SNU-668) and colon cancer cells (SNU-C4) [9], used to treat dyspepsia, hiccups, and vomiting. It possesses various pharmacological activities such as antibacterial [10], antifungal, anti-inflammatory, and anticancer activities.

Therefore, due to its high nutritional and medicinal value, the anthelmintic potential of this particular species of *Citrus* family has been chosen to explore its preliminary phytochemical constituents and *in vitro* anthelmintic effect.

METHODS

Plant

Fruit of *C. reticulata* (mandarin) was purchased from local market of Bengaluru, South India. The plant and the plant material were identified and authenticated by Dr. Shivananda T.N, Principal Scientist, Department of Medicinal and Aromatic Plant, Indian Institute of Horticultural Research, Bengaluru, India and thereafter voucher herbarium specimen No: CR-311/KCP was preserved in the Department of Pharmacognosy in Krupanidhi College of Pharmacy, Bengaluru, India.

Preparation of extract

Fruits were washed thoroughly using tap water and were peeled off manually. All the peels were segregated into two halves where one-half were shade dried at room temperature for 10-12 days. The dried peels were further made to smaller size and its methanolic extract was prepared by soxhlation technique.

Volatile oil extraction

Volatile oil was obtained from the remaining half of fresh peels (750 g) by Clevenger's apparatus in 3 batches using distilled water as solvent

and temperature was maintained at 45°C. Slight yellow colored volatile oil was obtained with a yield of 0.11%.

Experimental animal

The ethical clearance was obtained from the Institutional Animal Ethics Committee (Approval No: 2015/PCOL/05). The experiment was conducted using Indian Adult earthworm (*Pheretima posthuma*) which was purchased from University of Agricultural Sciences, GKVK, Bengaluru - 65. The earthworms of 6-8 cm in lengths and 0.2-0.4 cm in width were used for all the experimental protocol. Before initiating the experiment, earthworms were in normal saline to remove the waste/unwanted matter. Since earthworms aid in increasing the fertility of the soil and are easily available in fields; also they anatomically and physiologically resemble the human intestinal roundworm parasite *A. lumbricoides*. Therefore, earthworms have been selected as an experimental animal for the preliminary evaluation of anthelmintic activity, *in vitro*.

Drugs and chemicals

Methanolic extract and volatile oil of the peel of *C. reticulata* fruit, albendazole (Intas Pharmaceuticals LTD, Ahmedabad), dimethyl sulfoxide, 0.9% NaCl, anhydrous formic acid of analytical grade were used during the experimental protocol.

Phytochemical screening

The methanolic extract of the dried peels was screened for the presence of various phytoconstituents such as alkaloids, glycosides, carbohydrates, tannins, flavonoids, and steroids qualitatively as per the standard protocol [11].

Anthelmintic assay

Anthelmintic activity was assessed in such a way that two earthworms were placed on each Petri dish consisting of 20 ml of solution. Methanolic extract of orange peel at four different concentrations (25, 50, 100 and 150 mg/ml), orange peel oil at (0.5%, 1%, 1.5% and 2%) concentrations was used as test while albendazole at the concentration of 25 mg/ml was used as reference standard and control was normal saline. Observation for the time of paralysis and death of worms were used as an evaluation parameter in which worm was considered to be paralyzed if it shows no movement except when shaken vigorously. Time of death was recorded if the worm does not show movement even after shaking externally or when placed on 50°C water or if the colour of worm fades away [12]. All the results shown in Table 1 are expressed as a mean \pm standard error of mean of six worms in each group [13].

RESULT

Preliminary phytochemical screening

The preliminary phytochemical screening revealed the presence of various phytoconstituents in the extract. It showed the presence of carbohydrates, flavonoids, alkaloids, terpenoids, phytosterols, tannins, and steroids and the results are compiled in Table 2.

Anthelmintic assay

The *in vitro* anthelmintic activity of peels of *Citrus reticulata* was evaluated in earthworms using parameters; Time of paralysis and Time of death which is shown in Table 2.

DISCUSSION

Preliminary phytochemical investigation showed the presence of alkaloids, glycosides flavonoids, carbohydrates, tannins, steroids, and terpenoids in the methanolic extract which might be responsible for its potent anthelmintic activity. Limonene has been reported to be a major active principle in citrus peels and it has shown anthelmintic activity in ruminants [14]. Orange being a citrus fruit, volatile oil from its peel also consists of high amount of limonene. Anthelmintic assay when performed *in vitro* on earthworm, both the extract as well as volatile oil exhibited anthelmintic effect in a dose-dependent manner (Table 1). From the above result, all the concentrations of the extract of *C. reticulata* and its volatile oil were found to exhibit strong

Table 1: Anthelmintic activity of *C. reticulata*

Extract	Concentration (mg/ml)	Mean \pm SEM	
		Time of paralysis in minutes	Time of death in minutes
Normal saline	-	-	-
Methanolic extract	25	38.28 \pm 0.32	57.71 \pm 0.58
	50	26.52 \pm 0.31	37.89 \pm 0.51
	100	8.42 \pm 0.19	28.18 \pm 0.64
	150	5.76 \pm 0.19	19.16 \pm 0.60
Albendazole (standard)	25	1.44 \pm 0.04	4.63 \pm 0.36
Volatile orange peel oil	0.5	18.21 \pm 0.11	80.48 \pm 0.26
	1	16.66 \pm 0.25	56.43 \pm 0.44
	1.5	12.60 \pm 0.28	46.88 \pm 0.98
	2	10.66 \pm 0.37	32.22 \pm 0.61

C. reticulata: *Citrus reticulata* SEM: Standard error mean, each value represents mean \pm SEM (n=6)

Table 2: Preliminary phytochemical tests on methanolic extract of *C. reticulata*

Test	Methanolic extract of <i>C. reticulata</i>
Test for carbohydrates	
Fehling's test	++
Benedict's test	++
Test for alkaloids	
Mayer's test	+
Wagner's test	+
Test for glycosides	
Legal's test	++
Baljet test	+
Test for steroids	
Salkowski test	++
Liebermann-Burchard test	+
Test for saponins	
Foam test	-
Test for flavonoids	
Shinoda test	++
Lead acetate test	+
Test for protein	
Biuret test	-
Test for tannins and phenolic compounds	
FeCl ₃ test	+
Gelatin solution test	+
Test for phytosterols	++
Test for terpenoids	+

More prominent color observed (++) , Less prominent color observed (+), absent (-), *C. reticulata*: *Citrus reticulata*

anthelmintic effect when compared to standard drug. At concentration of 150 mg/ml, methanolic extract of *C. reticulata* showed paralysis at 5.76 \pm 0.19 minutes and death at 19.16 \pm 0.60 minutes while 2% concentration of volatile oil showed paralysis at 10.66 \pm 0.37 minutes and death at 32.22 \pm 0.61 minutes. These two concentrations showed better anthelmintic effect when compared to others. Albendazole showed paralysis of earthworm at 1.44 \pm 0.04 minutes and death after 4.63 \pm 0.36 minutes. Limonene is a major constituent of citrus fruits but its exact mechanism of anthelmintic effect is not identified, but several former research on limonene suggests its inhibitory effect on the plasma membrane pumps, growth of nematodes, enzymes of parasite and it certainly disturbs the metabolic pathway of the worms.

CONCLUSION

From the results obtained through our studies, it can be concluded that *C. reticulata* has a potent anthelmintic effect. The peel of the fruit

is generally not utilized but has high value. Therefore with the help of this knowledge, further research has to be conducted to find out the exact mechanism and isolate the active principle responsible for its anthelmintic effect which can aid in exploring a novel anthelmintic drug.

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