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Research Paper

Efficacy of *Croton oblongifolius* fresh leaves in Goat helminthes: An in vitro study

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ARTICLE DETAILS

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ABSTRACT

The present investigation deals with the efficacy of Croton oblongifolius fresh leaves in goat helminths: An vitro study during 2020- 2021. Croton oblongifolius leaves samples were used for the purpose of phytochemical analysis and anthelmintic activity. Faecal samples of goats were collected from the slaughter house/goat farms in and around Shivamogga and Bhadravati areas. Crude methanolic extract of leaves of Croton oblongifolius was subjected to phytochemical tests, later antibacterial activity of this extract was done by disc diffusion assay..The anthelminthic activity of extract of Croton oblongifolius is found effective against the helminths that is infectious to goat .i e effective in the treatment of nematodes, cestodes and treamatodes. The anthelmintic inhibition increases as the concentration increases. After performing phyto-chemical analysis it has been concluded that a good amount alkaloids and a moderate amount of diterpenoide present which helps in inhibition of helminths in goats, a trace amount of other proteins and amino acids are also play a role in inhibition of helminthes.

1. Introduction

Croton oblongifolius Roxb, an important component of many Ayurvedic preparations, was described by Roxburgh from India, West Bengal and is typified by Hoxburgh s n. (BMD) and well-illustrated in Roxb., FL. Indica Iean. No. 1925 (-2212) (CAL). This binomial is a later homonym of two species described by two different authors for two different taxa. In 1814, Delile described and published the name *Croton oblongifolius* (as oblongifolium) in Florue Augyptiacne Illustratio p. 283, t. 51. 1. 1814 from Egypt. In 1828, Sprengel published the same binomial in Systema Vegetabilium 3: 850. 1826. Euphorbiaceae there is this entry, *Croton Roxburghii* Wall (C. polyandrum R. non Spr.r. It is a new name for Roxburgh's Croton polyandrum and not of Sprengel. In Botany and history of Hortus Malabaricus ed. by KS Manilal (1960) Mabberley presents an article "A reexamination of the Indian Catalogues" where he gives a list a list of names validly published in Wallich's second list (15401 There on page 90 he shows *Croton roxburghii* Wall as a new name based on *C. polyandrum* Rash., non Spr. *Baliospermum montanum* (Willd.) Muell-Arg. This rare document, though referred to by Veigt in his Hortus and Wallich in his Catalogue, was not taken seriously by later workers until Mabberley studied it carefully and found that there are several names validly published in it. There is now more interest in using herbal remedies due to the issue of anthelmintic drug resistance, their toxicity, and growing concerns about drug residues in animal products. The anthelmintic activity of novel plant compounds can be assessed in vitro using free-living stages of parasitic nematodes (Asase et al., 2005 ; Mares Mohammed et al.,2023). The aim of the present investigation is to know the efficacy of *Croton oblongifolius* fresh leaves in goat helminthes.

1.1 Description

This tree has leaves are either coarsely dentate, serrate or crenate, prominently-lobed subglobose fruit some 10 x 8–12 mm in size, and peltate/shield-like indumentum (hair covering on the plant), with rays of scales radiating in 1 plane (at

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least 80% webbing, rays free of such for only some 20% of total length). This species is native to Southeast Asia, Southern Yunnan and the Indian subcontinent. Countries and regions in which it occurs include: Thailand; Cambodia; Vietnam; Zhōngguó/China (southern Yunnan); Laos, Myanmar; India (including Assam); Bangladesh; East Himalaya; Sri Lanka

1.2 Habitat and ecology

The tree has a short lifespan. The tree is a pollen source for stingless bee species in the Lepidotrigona, Tetragonilla and Tetragonula genera at Nam Nao National Park, Phetchabun Province, northern Thailand. The tree occurs in deciduous and deciduous dipterocarp-oak forest. In the utilized edge of montane evergreen forest of Doi Suthep–Pui National Park, Chiang Mai Province, northern Thailand, the dominant species were *Glochidion lanceolarium, Litsea beusekomii, Schima wallichii, Erythrina stricta, Macaranga indica, Staphylea cochinchinensis, C. persimilis, Pinus kesiya, Litsea martabanica,* and *Clausena excavata*. The plant is the most abundant tree in the peripheral zone of Kuldiha Wildlife Sanctuary, Odisha, India, it was pervasive in the buffer zone, but of far lesser presence in the core zone. These zones reflected human interference in the landscape. Other abundant trees in the peripheral zone were *Shorea robusta, Glochidion lanceolarium, Caesalpinia digyna, Ziziphus oenoplia, Syzygium cumini* and *Stereospermum tetragonum.* In the buffer zone, apart from *C. persimilis*, other abundant trees were *Holarrhena pubescens, Macaranga peltata, S. robusta, Terminalia alata*, and *Pongamia pinnata*.

2. Materials and methods

The present investigation "Efficacy of *Croton oblongifolius* fresh leaves in goat helminths: An vitro study with plant constituents" was carried out during 2020 – 2021 in the Department of Veterinary Pharmacology and Toxicology, Veterinary college, Shivamogga, Shivamogga, Karnataka state. Leaves sample of *Croton oblongifolius* for the purpose of phytochemical analysis and anthelmintic activity were collected. Faecal sample of goats were collected from the slaughter house/goat forms in and around Shivamogga and Bhadravati.

2.1 Experimental details

The present investigation was carried using crude Methanolic extract of leaves of Croton oblongifolius, then it was subjected to phytochemical tests, later antibacterial activity of this extract was done by Disc diffusion assay.

2.2 Collection of Leaves of *Croton oblongifolius*

2 – 3 kgs of the Leaves of Croton oblongifolius plant were collected from vender of Bangalore.

2.3 Identification of collected plant

The collected plant was identified as '*Croton oblongifolius'* by Botanist Dr. Rajeshwari, Professor and Head of the department ,Department of Botany, Sahyadri science college, Shivamogga.

2.4 Preparation of crude Methanolic extract of Croton oblongifolius

Drying of leaves: Collected leaves were washed thoroughly by Distilled water, then shade dried and completely dried by using drier.

Grinding of leaves: The dried leaves were thinly powdered by using Grinder. Dissolve in Methanol for the release of phytocompounds: Then the powder of leaves was added in Methanol in the ratio of 1 : 5 ratio, like this we added 100 grams of leaf powder in 500 ml of Methanol in sterile plastic container. Then the container contained powder and methanol was kept about a week followed by constant mixing twice a day.

Separation of plant extract from the Methanol: After a week, for the purpose of obtaining pure plant extract, leaf powder in methanol was firstly filtered using sterile muslin cloth. Then the filtrate was subjected to Rotary evaporator for the separation of Plant extract and methanol, then the plant extract and Methanol were separated.

Drying of separated Plant extract: The plant extract separated from Methanol by the help rotary evaporator was in the form of thick paste, because it contained small amount of methanol in it, so we kept it in incubator orbital shaker for the purpose of drying. After extract was dried, the powder was obtained that is called as Crude Methanolic extract.

Fig 1:Incubator orbital shaker

Fig 2: Methanolic extract

Phytochemical analysis of Methanolic extract of Croton oblongifolius: Phytochemical analysis of Methanolic extract of Croton oblongifolius was done by using different Biochemical test for different Phytocompounds such as, primary and secondary metabolites present in extract.

2.5 Qualitative analysis of primary metabolites

2.5.1 Test for Carbohydrates

The plant extract (100mg) was dissolved in 5ml of distilled water and filtered. The filtrate was subjected to the following tests.

Benedict's test: About 0.5 ml of the filtrate was taken to which 0.5ml of Benedict's reagent was added. This mixture was heated for about 2 minutes in a boiling water bath. *(Red precipitate)*

Molisch's test : To 2ml of filtrate, 2 drops of Molisch's reagent was added, the mixture was shaken well and 1ml of conc. H2SO4 was added slowly along the sides of the test tube and allowed to stand. *(Violet ring at the junction of two liquids).*

Fehling's reduction test: 1ml of filtrate was boiled on water bath with 1 ml of Fehling's solution. *(Brick red colour)*

Barfoed's test : To 1ml of extract filtrate, 1ml of Barfoed's reagent was added, and heated on water bath for 2 minutes and observed**.** *(Red precipitate)*

Seliwanoff's test: 3ml of Seliwanoff's reagent was added to 1ml of extract solution and heated on a water bath for 1min and observed. (Rose red colour).

2.5.2 Test for proteins

*Biuret test:*A few mg of extract was taken in distilled water and filtered. To this 1 ml of 4% NAOH solution was added and a drop of 1% solution of CUSO4 was added. *(Pink/violet colour)*

Millon's test : To 2 ml of the above filtrate is added with a drops of Millon's reagent and observed*. (White precipitate)*

Test for amino acids: The extract (100mg) is dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate is subjected to test for amino acids.

*Ninhydrin test:*To 1ml of either of the extract filtrate, few drops of Ninhydrin reagent (10mg of Ninhydrin in 200ml of acetone) was added*. (Colour change)*

*Nitric acid test:*To 2 ml of extract, few drops of nitric acid was added along the sides of the tube *(yellow colour)*

2.5.3 Test for alkaloids

Qualitative analysis of secondary metabolites

A small portion of the extract was shaken with about 5-6 ml of 1.5% HCL and filtered. The filtrate was tested with the alkaloid reagents.

Mayer's test

Mayer's reagent was added in a test tube containing 2 to 3 ml of filtrate**.** *(Cream colour precipitate)*

Wagner's test

To a few ml of filtrate, few drops of Wagner's reagent were added by the sides of the test tube. *(Reddish brown).*

Hager's test

To a few ml of filtrate, 1 or 2 ml of Hager's reagent was added. *(Precipitate)*

Dragondroff's test

2.5.4 Test for Flavonoids.

To a few ml of filtrate, 1 to 2 ml of Dragondroff's reagent was added and observed. *(Reddish brown precipitate)*

Sulphuric acid test

A fraction of the extract was treated with few drops of conc. H2SO4. *(Orange colour)*

Lead acetate test

10% of lead acetate was added to 1 ml of extract and observed*. **(Yellow precipitate)***

Alkaline reagent test

To the aqueous solution, 10% ammonia solution is added and is heated. *(Yellow colour).*

Test for Glycosides

The MEAC of about 0.5 g was hydrolysed with 20ml of 0.1 ml of 0.1 N HCL and filtered using Whatmann No.1 filter paper. The filtrate was used to test presence of glycosides.

Keller-killiani test

1ml of MEAC filtrate is taken in a test tube to which 1.5 ml of glacial acetic acid is added along the sides of the tube, one drop of 5% FeCl3 and few drops of conc. H2S04 were added. *(Reddish brown colour)*

2.5.5 Test for phenolic compounds

Concentrated extract was made alkaline with few drops of 10% NAOH solution and then freshly prepared sodium nitroprusside solution was added to the solution. *(Blue colour)*

Lead acetate test : A small amount of MEAC is dissolved in 5 ml of distilled water to which 2 to 3 ml of 10% lead acetate solution was added. *(White precipitate).*

Gelatin test : A small amount of MEAC is dissolved in 5ml of distilled water to which 1% gelatin solution and 10% NaCl were added. *(White precipitation)*

Ferric chloride test andTest for Tannins : 50 mg of the MEAC was dissolved in 5 ml of distilled water to which few drops of neural 5% ferric chloride solution were added and observed**.** *(Green/ bluish black colour)*

Gelatin test : 50 mg of the MEAC was dissolved in 5 ml of distilled water to which 2 ml of 1% solution of Gelatin containing 10% NaCl was added to it*. (white precipitate)*

*Bromine water test ;*10 ml of bromine water was taken in test tube to which 500mg of the plant extract was added and observed. (Decolouration of bromine water).

2.5.6 Test for Saponins
The extract of about 50mg was diluted to 20ml with distilled water and slowly shaken in a graduated cylinder for 15 minutes. *(Froth formation)*

2.5.7 Test for phytosterols and Triterpenoids
The extract (0.5g) was treated with 10ml chloroform and filtered. The filtrate was used to test the presence of phytosterols and triterpenoids.
Lieberman – Bucharat test : The extract is dissolved in 2ml of acetic acid and to which 1 or 2 drops of conc. H_2SO_4 is added along the sides. *(Deep red ring at the junction)*
Salkowaski test : To the filtrate, few drops of conc. H_2SO_4 were added, shaken and allowed to stand. *(Change in colour)*
Test for Chalcones : To 0.5g of either of the extract 2 ml of ammonium hydroxide was added and observed. *(red colour)*
*Test for Coumarins :*10% aqueous solution of sodium hydroxide was added to 2ml of extract filtrate. *(Yellow colour.*

2.6 The in-vitro anthelmintic activity of Croton oblongifolius against goat helminths
The *in vitro*. trials for anthelmintic activity of crude methanolic extract were conducted on mature live *round worms* of goat. The mature worms were collected from the abomasums of freshly slaughtered goat in the local abattoir. The worms were washed in phosphate-buffered saline (PBS). 10 worms were exposed in triplicate to the following treatments at room temperature (25–30°C). The inhibition of motility and mortality of the worms subjected to the above treatments were used as the criteria for anthelmintic activity. The motility was recorded after 0, 1, 2, 3, and 6 hr intervals. Finally, the treated worms were kept for 30 min in the lukewarm fresh PBS to observe the revival of motility. Zafar lqbal et al (2008).

2.6.1 Chemicals and other materials:
- Analytical chemicals will be used for all other assay.
- Plastic ware like 24 well plates, Mc Master counting slide.
- Glass wares like petri dishes, watch glasses, Beakers, slides, cover slips will be used in the commercial suppliers.

Fig 3: The fecal sample suspension will be homogenized with pestle and morter

Fig 4: The homogenized mixture is collected in a beaker

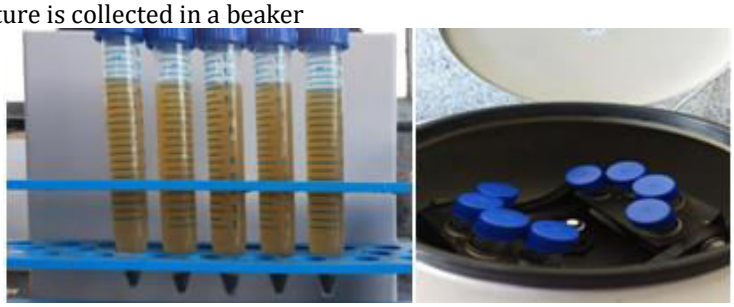

Fig 5: Centrifuge tube are placed in centrifuge machine and sample is collected in centrifuge tube

2.7 Experimental procedure
2.7.1 Collection and screening of faecal samples for detection of nematodes egg:
Faecal samples will be collected from goat at slaughter house in and around Shivamogga. Collected sample will be screened for isolation of eggs by salt flotation technique (soul sby 1982) .Positive samples will be processed by modification Mc master technique for EPG of round worms. Fecal sample with EPG more than 150 will be selected for EHA and LDA.

Fig 6: In-vitro antihelmintic assays

2.7.2 Collection of eggs for EHA

2.7.2.1 Procedure for collection of eggs was followed as described by Coles et al. (1992)

The fecal suspension will be homogenized with a laboratory stirrer .The suspension will be poured through a sieve (pore size of 0.15) and filtered the fecal sample solution. Then transfer to the centrifuge tube and centrifuged for 8 min at 1500-2000 rpm. After centrifugation remove the supernatant and add the saturated Sodium chloride to the tube and mix it well. Again centrifuged for 2 minutes in the 2000 rpm and in the top of the tube eggs was collected because the egg have low dencity compare with the Nacl, so that add exces of Nacl place the cover slip in the top of the tube. Collected the eggs from the tube and count the egg place it in the cover slip under the microscope. Make it upto the 200 µl of water contain 100 eggs approximate using the distilled water make the concentration of egg.

2.7.2.2 Egg hatch inhibition assay (EHIA)

Approximately, 100 eggs in 200 µL of water will be pipetted in to each well of a 24-well microtiter plate. To each of the test wells, 200µL of each plant extract will be added to a final volume of 400 µL per well. The plant extracts will be tested at concentrations of 2,4,6 and 8mg/mL. Similarly 200 µL of albendazole (standard drug) at 0.25mg/mL concentration and distilled water will be used as a positive control and non treated control respectively. Each test will be done in three replicates. The plate will be incubated in a BOD incubator at 37ºC for 48h. Thereafter, a drop of Lugol's solution will be added to stop further hatching. All unhatched eggs and L1 larvae in each well be counted under microscope. Percentage of inhibition of eggs is calculated using formula.

2.7.2.3 Larval development assay

Eggs are collected as described in the egg hatch assay.50µl amphoerinin will be added to 5ml of the egg suspension(100eggs/100µ) .100µl eggs suspension and 20µl of nutritive media and 20µl of lyophilized E-coli (100mg/1mL) to each well in 26 well plates.Incubation it in 22ºc for 48hrs duration of time. 10µl of different concentration of C*roton oblongifolius* added. Incubate it in 25ºc for 6 days duration. Two drops of lugol's iodine was added to the wells in the plates and observe the third stage of larvae counted under the microscope

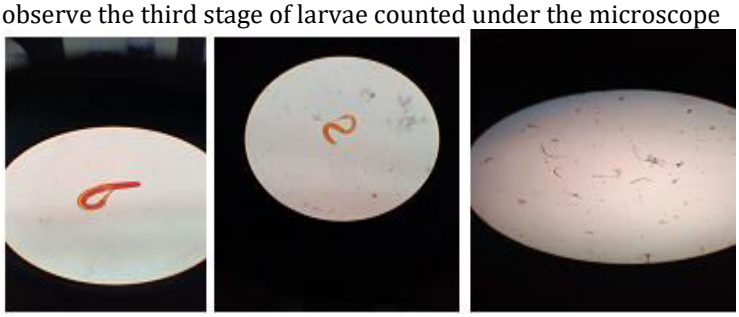

Fig 7: Third stage of larvae under the microscope

3. Result and discussion

3.1 Phytochemical analysis of Methanolic extract of Croton oblongifolius: showed following results,

Table. 1 List of Phytoconstituents in Methanolic extract of Croton oblongifolius

Phyto constituents	**Present or absent**
Carbohydrates	Absent
Proteins	Present
Amino acids	Absent
Alkaloids	Present
Flavonoids	Absent
Glycosides	Present
Phenolic compounds	Present
Tannins	Present
Saponins	Present
Triterpenoids	Present
Coumarins	Present

3.2 In vitro anthelmintic activity of croton oblongifolius against goat helminths:

In vitro results indicates the moderate anthelmintic activity of a croton oblongifolius activity of crude methanolic extract, it is good to study because it will longer to survival and longer survival a greater number of observations recorded for in vitro study.

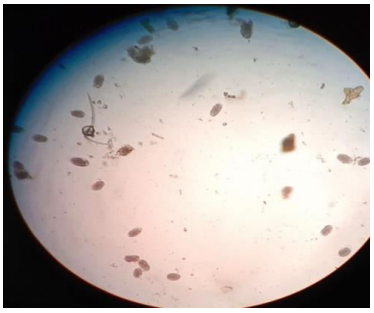

Fig 8: Microscopic view of round worms

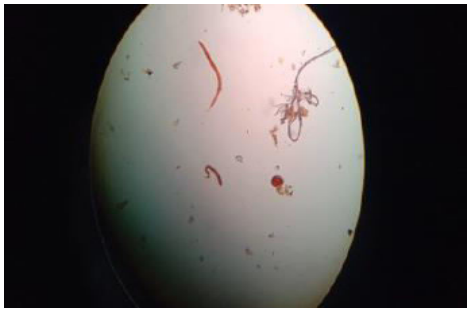

Fig 9: Egg and larva

After 5 to 6 trial and error, We came to an conclusion on increasing the dose, the anthelmintic activity or inhibition also increases so as shown in the below Table 2 in 2mg/mL it shows 43%, 4mg/mL it shows 62%, 6mg/mL it shows 72% and 8mg/mL it shows 82% the high inhibition was in 8mg/mL compare with others.

Table 2 : Concentration of Plant extract and anthelmintic activity

Concentration of Plant extract	Number of eggs	Number of larva	Percentage of inhibition
2mg/Ml	**43**	**58**	**43%**
4mg/Ml	**60**	**38**	**62%**
6mg/Ml	**70**	**28**	**72%**
8mg/Ml	**91**	**17**	**82%**

4. Conclusion

Natural products, especially the universal role of plants in the treatment of disease is exemplified by their employment in all the major system of medicine. The anthelmintic activity of extract of *Croton oblongifolius* is found effective against the helminths that is infectious to goat .i e effective in the treatment of nematodes, cestodes and treamatodes. The anthelmintic inhibition increases as the concentration increases. After performing phytochemical analysis it has been concluded that a good amount alkaloids and a moderate amount of diterpenoide is present which helps in inhibition of helminths in goats; a trace amount of other proteins and amino acids are also present.

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