



E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(6): 793-797
Received: 16-09-2019
Accepted: 18-10-2019

Senthilnathan M
Department of Veterinary
Pharmacology & Toxicology,
NTR College of Veterinary Science,
Gannavaram, Andhra Pradesh,
India

Afroz Jahan
Department of Veterinary
Pharmacology & Toxicology,
NTR College of Veterinary Science,
Gannavaram, Andhra Pradesh,
India

K Srinivas
Department of Veterinary Public
Health & Epidemiology,
NTR College of Veterinary
Science, Gannavaram, Andhra
Pradesh, India

P Ravi Kumar
Department of Veterinary
Pharmacology & Toxicology,
NTR College of Veterinary Science,
Gannavaram, Andhra Pradesh,
India

G Srinivasa Rao
Department of Veterinary
Pharmacology & Toxicology,
NTR College of Veterinary Science,
Gannavaram, Andhra Pradesh,
India

B Avinash
Department of Veterinary
Parasitology, NTR College of
Veterinary Science,
Gannavaram, Andhra Pradesh,
India

Corresponding Author:
Senthilnathan M
Department of Veterinary
Pharmacology & Toxicology,
NTR College of Veterinary Science,
Gannavaram, Andhra Pradesh,
India

In vitro anthelmintic activity of *Muntingia calabura* leaf extract against *Haemonchus contortus*

Senthilnathan M, Afroz Jahan, K Srinivas, P Ravi Kumar, G Srinivasa Rao and B Avinash

Abstract

Helminthiasis is a serious problem in the tropical regions including the Asian countries which cause substantial morbidity and mortality in livestock. Haemonchosis is one of the most significant parasitic diseases of livestock worldwide, affecting millions of small ruminants like sheep and goats. Medicinal plants are becoming increasingly popular in modern society as natural alternatives to synthetic drugs. With limitations of conventional treatments and development of drug resistance, research on herbal medicines is gaining momentum. In this scenario the present study was carried out to screen the acetone extract of *Muntingia calabura* (Leaves) for anthelmintic activity. *In vitro* anthelmintic activity was evaluated against *Haemonchus contortus* of sheep using adult motility assay (AMA) and egg hatch test (EHT) after preliminary phytochemical evaluation, and antioxidant assay. The phytochemical analysis revealed mainly the presence of flavonoids, tannins and saponins in *Muntingia* sp. The antioxidant activity of each compound was expressed as ascorbic acid equivalents. Antioxidant activity of *Muntingia calabura* exhibits 2.15696 mg/ml of ascorbic acid equivalent which is a well known antioxidant. The potency of extracts of *Muntingia calabura* is as par with albendazole. In AMA, a time-concentration dependent response was observed in all the treatment groups.

Keywords: Helminthiasis, *Muntingia calabura*, adult motility assay, egg hatch test, phytochemical analysis

Introduction

In developing countries like India, agriculture and its subsectors plays a vital role in its rapid economical growth. As all farmers cannot afford to rear cattle, they mainly prefer small ruminants for their additional daily income. Total number of sheep in the country as per 2012 census is 65 million numbers and contributes around 12.71% of the total livestock population. As state wise, Andhra Pradesh holds first place with 40.57% of total sheep population in India, followed by Karnataka and Rajasthan in second and third place with 14.73% and 13.95% respectively.

The serious problem in Asian countries which cause substantial morbidity and mortality in small ruminants like sheep and goat is haemonchosis, a parasitic condition caused by a gastrointestinal nematode called *Haemonchus contortus*. Currently, synthetic anthelmintics are widely used to control the disease, but having disadvantages of being costly, risk of environmental pollution and development of resistant population becomes serious issue (16). To encounter these problems natural plant extracts as dewormers for livestock has been used, however scientific validation of these practices and identification of the active compounds has been lacking. Keeping all these facts in view, the present study was carried out to screen the anthelmintic activity of *Muntingia calabura*.

Muntingia calabura, belongs to the family Muntingiaceae, is commonly called as Jamaican cherry. Kaneda *et al.*, 1991^[9] reported on the isolation of 12 flavonoids from the methanol extracts of the *M. calabura* roots. Many studies and researches revealed that it has antiproliferative activity^[21], Quinone reductase activity^[18], antiplatelet aggregation activity^[3], antibacterial activity^[19], antioxidant activity^[23], insecticidal activity^[2], antinociceptive activity^[22], anti-inflammatory activity^[20], antipyretic activity^[22], antiulcer activity^[8], antidiabetic activity^[17], anti-hypertensive activity^[15] and Cardioprotective activity^[11].

Material and Methods

Plant material collection

The young leaves of *Muntingia calabura* were collected from the NTR College of Veterinary

Science Hostel campus during the month of April, 2016. Plant was identified by faculty of Department of Botany, Acharya Nagarjuna University, Guntur. The collected plant samples were shade dried, powdered and stored in air tight containers separately.

Crude Extraction ^[7]

The freshly prepared powder (10g) was mixed in acetone solution (1:10) in a flask closed tightly with cotton plug and was kept at room temperature for 48 hours at 120 rpm in an orbital shaker. The contents of the flask were filtered through Whatman's Filter Paper (640md). The residue left in the flask was rinsed with little quantity of acetone and filtered through the same. Filtrate obtained was transferred to already weigh beaker and was kept for evaporation of solvent at room temperature. After complete evaporation of the solvent, the beaker was once again weighed to know the amount of extract. The per cent extractability was determined. The stock solution (100 mg/mL) was prepared in DMSO and was stored in cool and dry place until further used in this study.

Phytochemical screening

Muntingia calabura leaves and fruits extracts were analyzed for the phytochemical composition by qualitative methods using the standard protocols described by *Rosenthalr* (1930).

Test for Alkaloids

Dragendorff's Test - In a test tube containing 1 mL of extract, few drops of dragendorff's reagent were added and the colour developed was noticed. Appearance of orange colour indicates the test was positive for alkaloids.

Test for Saponins

About 1 mL of extract was diluted separately with 5 mL of distilled water and shaken in a test tube for 15 minutes. A 1 cm layer of foam indicates the presence of saponin.

Test for Carbohydrates

Benedict's test: 2 mL of Benedict's solution to extract was added and boiled on water bath. Red yellow or green precipitation indicates the presence of reducing sugars.

Molish's test: 2 mL of extract was placed in a test tube and 2 drops of molish's reagent was added. The solution was then poured slowly into a tube containing 2 mL of mL of concentrated sulphuric acid so that two layers form. The formation of a purple product at the interface of the two layers.

Test for Flavonoids

Ferric chloride test: To 1 mL of extract, 1 mL of ferric chloride solution was added. The formation of brown colour confirms the presence of flavonoids.

Test for proteins and amino acids

Biuret test: 1mL of 40% sodium hydroxide solution was and 2 drops of 1% copper sulphate solution were added to the extract. Formation of violet colour indicates the presence of proteins.

Xanthoproteic test: Add 1 mL of concentrated nitric acid to 1 mL of the extract. Heat the mixture and cool it. Slowly add sodium hydroxide (40% w/v in water) solution until the mixture becomes alkaline and a colour change is noted. If the

colour changes from yellow to orange, indicates the presence of an aromatic amino acid.

Ninhydrin test: 2 drops of freshly prepared 0.2 percent ninhydrin reagent was added to the extract solution and heated. Development of blue colour indicates the presence of proteins, peptides and amino acids.

Test for Tannins and phenolic compounds

Lead acetate test: A few drops of lead acetate solution were added to the acetone extract. The formation of white precipitate indicated the presence of tannins.

Ferric chloride Test: A few drops of ferric chloride solution were added to the little of the above filtrate. Green colouration in the filtrate of acetone extract indicated the presence of tannins.

Test for Terpenoids

Salkowski's test: 2 mL of chloroform was added to 0.5 g of the extract. Concentrated sulphuric acid (3 mL) was added carefully to form a layer. A reddish – brown colouration at the interface indicates the presence of terpenoids

Test for Resins

Foam test: 1 mL of extract was treated with few drops of acetic anhydride solution followed by 1 mL of concentrated sulphuric acid. Resins gives colouration ranging from orange to yellow

Total anti-oxidant activity by Phosphomolybdate assay

Assay was performed as described by Prieto *et al.* (1999).

Principle

This method is based on the reduction of Phosphomolybdic acid to Phosphomolybdenum blue complex by sodium sulphide. The obtained phosphomolybdenum blue complex is oxidized by the addition of acid and this causes a reduction in intensity of the blue colour.

Test protocol

In a 96 well microtitre ELISA plate, 200 µl of phosphomolybdate reagent (28mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M sulphuric acid) was added in all the wells. To this 20 µl of different concentrations of test compound was added and mixed gently. Then the plate was incubated at 90°C for 90 min in water bath. After incubation the samples were cooled to room temperature and the absorbance was measured at 695 nm against a blank sample. The antioxidant activity of each compound was expressed as ascorbic acid equivalents as calculated from the linear regression equation of the standard calibration curve for ascorbic acid.

In vitro assays for anthelmintic activity

Egg hatch assay

Pooled abomasal content samples were obtained by mixing several samples collected at the time of slaughter from a number of infected sheep. Eggs were isolated from the feces as per the method described by Coles *et al.* (4) and used in the assay.

Adult female worms of *H. contortus* were washed thoroughly with phosphate buffered saline (PBS). The worms were triturate in PBS and the suspension was centrifuged for 30 minutes at 2000 revolution per minute (rpm) and the sediment

was retained. The sediment was re-suspended in saturated solution of sodium nitrate and the suspension was again subjected to centrifugation as mentioned above. The supernatant fluid containing eggs were collected. Eggs were washed thrice with distilled water and adjusted to a concentration of 200 eggs/mL, using Mc Master's technique. Suspension containing approximately 200 eggs/mL was taken in 24 well titration plates. Acetone extract of *M. calabura* was added to plates at concentration of 10; 5.0; 2.5; 1.0; 0.5; 0.25 mg/mL. Same concentration of albendazole sulphoxide used as positive control, while PBS and egg suspension were taken as negative control. After incubation at 27°C for 48 hours, 2 drops of lugol's iodine solution was added into each well to prevent further hatching, and the number of eggs in each well were counted. The percentage of eggs, which failed to hatch at each drug concentration were calculated by using the formula.

$$\% \text{ of hatching} = (\text{number of hatched eggs} / \text{total number of eggs}) \times 100$$

The number of hatched eggs is the sum of hatched and embryonated eggs. The total number of eggs is the sum of hatched, embryonated, and unembryonated eggs.

The experiment was performed in triplicate

Adult Motility Test

The adult motility test (AMT) was performed by slightly modifying to Sharma *et al.*, 1971. The mature *H. contortus* worms were collected as above were washed thoroughly with PBS solution and finally suspended in the same. These worms were used for screening the anthelmintic activity of extract by petridish method at room temperature. Ten worms were exposed in triplicate to acetone extracts of *M. calabura* at six different concentrations (10; 5.0; 2.5; 1.0; 0.5; 0.25 mg/mL). As positive control, ten worms in triplicate were taken in piperazine at the dose rate of 12 mg/mL, while PBS along with worms were served as negative control. Inhibition of motility was taken as indication of worm mortality / paralysis. The observations were taken at regular intervals, until the worms in the negative control completely lost their motility.

Results and Discussion

Total anti-oxidant activity

Natural antioxidants like ascorbic acid, tocopherols and glutathione are very important against free radical damage caused by enzymes such as superoxide dismutase, catalase, etc. (6). Antioxidants in the food also acts as health protecting factor with nutritional and therapeutic effects (10, 12).

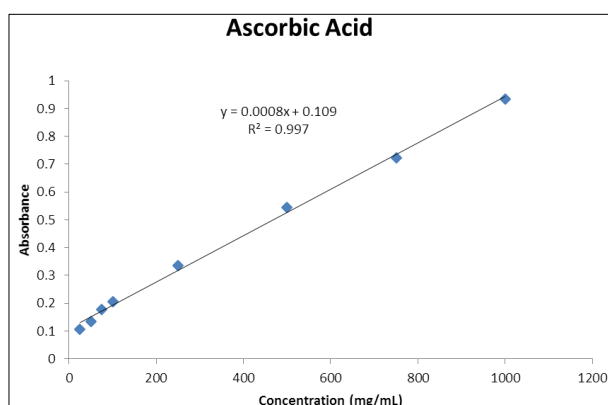


Fig 1: Anti-Oxidant Activity of Ascorbic acid using Phosphomolybdenum Assay

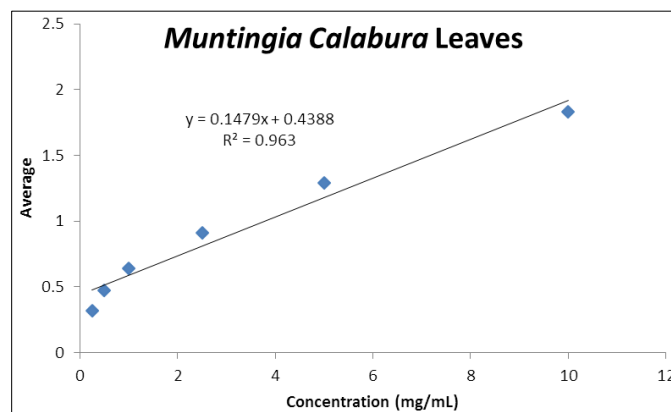


Fig 2: Anti-Oxidant Activity of *Muntingia Calabura* Leaves using Phosphomolybdenum Assay

Table 1: Anti-Oxidant Activity of *Muntingia Calabura* Leaves using Phosphomolybdenum Assay

Conc (mg/ml)	Absorbance	Calculated Concentration	Ascorbic acid equivalent (µg/ml)
10	1.834667	9.437908	2156.958
5	1.293	5.775524	1479.875
2.5	0.913333	3.208474	1005.292
1	0.643333	1.382916	667.7917
0.5	0.474667	0.242506	456.9583
0.25	0.321333	-0.79423	265.2917

In this study total antioxidant activity was estimated by Phosphomolybdenum assay. The calculated concentration of 10 mg/mL of *M. calabura* extract contains 2.15696 mg/mL of ascorbic acid equivalent, which is a well known antioxidant.

Adult motility test

The acetone extract of *M. calabura* exhibited the dose dependent anthelmintic action, as increase in the concentration, increased incidence of immobilization in treatment groups. But the anthelmintic activity of highest concentration (10mg/mL) of *M. calabura* was not par with the anthelmintic activity of piperazine (12 mg/mL). Piperazine paralyzed within 0.1 hour whereas highest concentration of *M. calabura* takes 0.48 hours to paralyze the worms. At lowest concentration *M. calabura* took 6.08 hours to paralyze and 7.02 hours to kill the worms. In PBS, which served as negative control, took 8 hours to produce 100% mortality. Piperazine (12 mg/mL) served as positive control, took 0.13 hours to cause mortality of the *H. contortus*.

Table 2: Anthelmintic activity of acetone extract of *Muntingia calabura*

Concentration (mg/mL)	Time (hour) for paralysis	Time (hour) for death
10.00	0.48±0.04	1.13±0.07
5.00	1.00±0.10	2.38±0.16
2.50	1.94±0.16	3.51±0.14
1.00	3.22±0.14	4.13±0.04
0.50	4.94±0.05	5.25±0.09
0.25	6.08±0.17	7.02±0.09
Piperazine (12 mg/mL)	0.1±0.33	0.13±0.33

Egg hatch assay

In Egg hatch assay, concentration estimates of acetone extract of *M. calabura* and albendazole sulphoxide are presented in table 2. According to IC50 (mg/mL) *M. calabura* extract where found to be equally as effective as albendazole

sulphoxide in order to inhibit the hatching of eggs; though it was not statistically significant ($p \geq 0.05$).

Table 3: IC50 values of screened compounds for inhibition of hatching of eggs

Compound	IC50 (mg/mL)	95% CI (Lower – Upper)
<i>M. calabura</i> leaves	1.435±0.189	0.611-3.389
Albendazole sulphoxide	1.039±0.110	0.634-1.704

Plants were intended for medicinal purpose for over the centuries. Plants have been basis for medical and veterinary treatments through much of history, and such traditional medicine is still widely used till date. In this study, wormicidal activity of *M. calabura* was evaluated using Adult motility test and Egg hatch assay. Obtained results show that *M. calabura* was found to be effective in case of inhibiting the hatching of eggs, whereas efficacy is not par with albendazole sulphoxide in adult motility assay. Dhawan & Gupta (2017) [5] compared different solvents for phytochemical extractions and were found that acetone yields 62.48% with moderate extraction of flavonoids, phenols and showed moderate antioxidant activity also. According to Avinash *et al.*, (2017) [1] acetone is very effective in breaking down the plant cell walls, semi polar in nature and highly volatile organic solvent, making it easy to isolate any dissolved substance simply by evaporation.

In adult motility test, *H. contortus* worms were exposed to different concentrations of plant extracts and their ability to paralyze/kill the worms was screened. Based on IC50 values, Acetone extracts of *M. calabura* was found to be effective as albendazole sulphoxide. Bandeira G N *et al.*, (2012) [2] reported that hexane and ethanolic extracts of *M. calabura* fruits and flowers prolonged larval duration by ~2 days in *Plutella xylostella* (Diamondback Moth), as compared with positive control (Cordycepin) 7.2 days. Phytochemical analysis revealed that presence of reducing sugars, resins, flavonoids, tannins, phenolics and saponins.

Conclusion

The present study showed that acetone extract of *M. calabura* leaves can be a potent *H. contortus* egg hatching inhibitor. This kind of herbal formulations are very safe to environment and never harms ecology. Phytochemical analysis reveals that presence of reducing sugars, resins, flavonoids, tannins, phenolics and saponins. However, the results were not statistically significant; and the search for anthelmintic activity from *M. calabura* remains unsettled. Hence, it is suggested that researchers should carry on further studies, mainly in *in-vivo* condition to prove the effectiveness against helminthes.

References

- Avinash B, Santi priya Ch, Kondaiah PM. In vitro evaluation of anthelmintic activity of *Nicotiana tobacum* extracts against *Haemonchus contortus*. International journal of science, Environment and Technology. 2017; 6(1):458-465.
- Bandeira GN, C da Camara AG, de Moraes MM, *et al.* Insecticidal activity of *M. calabura* extracts against larvae and pupae of diamondback, *Plutella xylostella* (Lepidoptera, Plutellidae). J King Saud Univ Sci. 2013; 25:83–9.
- Chen JJ, Lee HH, Shih CD, *et al.* New dihydrochalcones and anti-platelet aggregation constituents from the leaves of *Muntingia calabura*. Planta Med. 2007; 73:572-7.

- Coles GC, Bauer C, Borgsteede FHM, Geerts S, Taylor MA, Waller PJ. World Association for the Advancement of Veterinary Parasitology: methods for the detection of anthelmintic resistance in nematodes of Veterinary importance. Vet. Parasitol. 1992; 44:34-44.
- Dhawan D, Gupta J. Comparison of different solvents for phytochemical extraction potential from *Datura metel* plant leaves. International Journal of Biological Chemistry. 2017; 11(1):17-22.
- Goldie Uppal, Vijay Nigam. *In Vitro* antioxidant activity of ethanolic extract of *Euphorbia hirta* Linn. International Journal of Current Research. 2012; 4(10):155-159.
- Handa SS, Khanuja SPS, Longo G, Rakesh DD. Extraction Technologies for Medicinal and Aromatic Plants, (1stedn), no. 66. Italy: United Nations Industrial Development Organization and the International Centre for Science and High Technology, 2008.
- Ibrahim IAA, Abdulla MA, Abdelwahab SI, *et al.* Leaves extract of *Muntingia calabura* protects against gastric ulcer induced by ethanol in Sprague–Dawley rats. Clin Exp Pharmacol. 2012; S5:004.
- Kaneda N, Pezzuto JM, Soejarto DD, *et al.* Plant anticancer agents, XLVIII. New cytotoxic flavanoids from *Muntingia calabura* roots. J Nat Prod. 1991; 54:196–206.
- Morton JF. “Jamaica cherry,” in Fruits of Warm Climates, JF Morton, Ed., JFMorton, Miami, Fla, USA, 1987, 65–69.
- Nivethetha M, Jayasari J, Brindha P. Effects of *Muntingia calabura* L. on isoproterenol-induced myocardial infarction. J Med Singapore. 2009; 50:300–2.
- Ram Prakash Prajapat, Varun Gupta, Balram Soni, Deepak Choudhary, Veerma Ram, Anil Bhandari. Extraction and Isolation of Marmelosin from *Aegle marmelos*, synthesis and evaluation of their derivative as antidiabetic agent. Der Pharmacia Lettre. 2012; 4(4):1085-1092.
- Rosenthaler L. The chemical investigation of plants. Journal of Chemical Technology and Biotechnology. 1931; 50(18):356.
- Sharma Parashar B, Vatsa E, Chandel S, Sharma S. Phytochemical screening and anthelmintic activity of leaves of *Cedrus deodara* (Roxb). World journal of pharmacy and pharmaceutical sciences. 2016; 5(8):1618-1628.
- Shih CD, Chen JJ, Lee HH. Activation of nitric oxide signaling pathway mediates hypotensive effect of *Muntingia calabura* L. (Tiliaceae) leaf extract. Am J Chin Med. 2006; 34:857-72.
- Singh D, Swarnkar CP, Khan FA. Anthelmintic resistance in gastrointestinal nematodes of livestock in India. Journal of Veterinary Parasitology. 2002; 16:115-130.
- Sridhar M, Thirupathi K, Chaitanya G, *et al.* Antidiabetic effect of leaves of *Muntingia calabura* L., in normal and alloxan-induced diabetic rats. J Pharmacol. 2011; 2:626–32.
- Su BN, Park EJ, Vigo JS, *et al.* Activity-guided isolation of the chemical constituents of *Muntingia calabura* using a quinine reductase induction assay. J Phytochem. 2003; 63:335–41.
- Yasunaka K, Abe F, Nagayama A, *et al.* Antibacterial activity of crude extracts from Mexican medicinal plants

- and purified coumarins and xanthenes. J Ethnopharmacol. 2005; 97:293-9.
20. Zakaria ZA, Kumar GH, Zaid SNH, *et al.* Analgesic and antipyretic actions of *Muntingia calabura* leaves chloroform extract in animal models. Orient Pharm Exp Med. 2007a; 7:34-40.
 21. Zakaria ZA, Mohamed AM, Jamil NSM, *et al.* *In vitro* antiproliferative and antioxidant activities of the extracts of *Muntingia calabura* leaves. Am J Chin Med. 2011; 39:1-18.
 22. Zakaria ZA, Sulaiman MR, Jais AMM, *et al.* The antinociceptive activity of *Muntingia calabura* aqueous extract and the involvement of L-arginine/nitric oxide/cyclic guanosine monophosphate pathway in its observed activity in mice. Fundam Clin Pharmacol. 2006a; 20:365-72.
 23. Zakaria ZA. Free radical scavenging activity of some plants available in Malaysia. J Pharmacol Ther. 2007b; 6:87-91.