



Phytochemical screening and anthelmintic activity of root extract of *Hugonia mystax linn* against *Pheretima posthuma*: an *in vitro* Study

Naganagar Praneeth Reddy¹, Kadali Sai Priyanka², Rachana Jillella³, Jeppala Sripriya⁴, Vasudha Bakshi⁵, Narender Boggula^{6*}
¹⁻⁶ Department of Pharmaceutical Chemistry, School of Pharmacy, Anurag Group of Institutions, Venkatapur, Ghatkesar, Telangana, India

DOI: <https://doi.org/10.33545/26647222.2019.v1.i2a.11>

Abstract

Background: Helminth infections are among the most common infections in man in developing countries they pose a large threat to public. These infections can affect most population in endemic areas with major economic and social consequences.

Objective: In this paper, an ethnomedicinal plant, *Hugonia mystax Linn.* Was evaluated for its preliminary phytochemical screening and *in-vitro* anthelmintic activity.

Method: The earthworms resembled the intestinal roundworm parasites of human beings both anatomically and physiologically and hence were used to study the anthelmintic activity. The worms were acclimatized to the laboratory condition before experimentation.

Results: Preliminary phytochemical screening showed the presence of various classes of secondary metabolites such as flavonoids, phenols, saponins, steroids, tannins and terpenoids. Anthelmintic activity of ethanolic extract of root showed significant activity. The anthelmintic activity revealed the medicinal potential of *H. mystax* to develop a drug against various human ailments.

Conclusion: The present study brings detailed pharmacognostical profile of roots of *Hugonia mystax L.* The species, *H. mystax* has a restricted global distribution, occurring only in India and Sri Lanka. It is an unexplored medicinal plant in the Indian medicinal system. According to ethnobotanical information, the root powder of *H. mystax* is used as the best anti-dote, anthelmintic, febrifuge and for the treatment of peptic ulcers.

Keywords: *Hugonia mystax*, anthelmintic activity, piperazine citrate, paralysis, phytochemicals

Introduction

Ayurveda, the ancient healing system of India, grew luxuriantly from the Vedic period in India. In history, the classical texts of Ayurveda like Charaka samhita and Sushruta samhita were written around 1000BC. Medicinal plants like turmeric, ashwagandha, ginger, brahmi, manjistha and tulsi are integral part of ayurvedic medicines. All these plants have been used for the treatment of diseases, traditionally and their secondary metabolite constituents are the sources of important modern drugs such as atropine, codeine, digoxin, morphine, quinine and vincristine. According to one estimate nearly 70 % of the synthetic drugs have been derived from medicinal plants. Herbal medicine plays vital role in maintaining the health and wealth of mankind. Majority of world population use herbal medicines (1). The World Health Organization (WHO) reports that approximately 21,000 plants have been used for medicinal purposes. Herbs have stood the test of time for their safety, efficacy, cultural acceptability and minimal side effects. Therapeutic power of some plants is mainly due to the presences of some secondary metabolites, which collectively are referred to as phytochemicals. These phytochemicals have potential to be developed as herbal medicines or could serve as precursors for modern medicine. It is now widely understood that free radicals are involved in the pathogenesis of many diseases (2).

Helminthiasis is an infection disease caused by nematode worms such as *Ascaris lumbricoides*, *Trichuris trichiura*, *Nectator americanus* and *Ancylostoma duodenale*. Infection occurred when ingesting food contaminated eggs or larvae, hands or

utensils or through penetration of the skin by infective hookworm larvae in contaminated soil. Helminth infections are among the most common infections in man, affecting a large proportion of the world's population (3). In developing countries they pose a large threat to public health and contribute to the prevalence of malnutrition, anemia, eosinophilia, and pneumonia. Although the majority of infections due to worms are generally limited to tropical regions, they can occur to travellers who visited those areas and some of them can develop in temperate climate (4). Helminthiasis is a disease in which a part of the body is infested with worms such as pinworm, roundworm, or tapeworm. Typically the worms reside in the gastrointestinal tract but may also burrow into the liver and other organs; infected people excrete helminth eggs in their faeces, which then contaminate the soil in areas with inadequate sanitation. However, increasing problems of development of resistance in helminths against anthelmintics have led to the proposal of screening medicinal plants for their anthelmintic activity. The plants are known to provide a rich source of botanical anthelmintics (5). As we know very well, now a days the medicinal preparation available in the market from which most of them either not effective up to the mark or has to develop resistance resulting in reoccurrence again. Plant derived drug serve as a prototype to develop more effective and less toxic medicines. Helminth infection is a medical and public health problem of high magnitude, both in humans and domestic animals, causing considerable suffering and poor growth. In addition, helminths of livestock pose a serious

economic loss, in particular in areas where extensive grazing is practiced (6, 7).

Anthelmintics or anti helminthics are drugs that expel parasitic worms (helminths) from the body. They may also be called vermifuges (stunning) or vermicides (killing). An anthelmintic will destroy or expel worms from the digestive system. Helminthic infections are among the most common infection in human beings, affecting a large proportion of the world's populations. Diseases caused by helminth parasites in livestock continue to be a major productivity constraint, especially in small ruminants in the tropical and subtropical countries (8).

The genus *Hugonia* L. of family Linaceae comprise about 40 species in the world; of which *Hugonia mystax* L. was reported from India. This plant *Hugonia mystax* is locally known as Modirakanni. Ethnobotanically, the fruits are used by the tribals of Kalakad Mundanthurai for the treatment of Rheumatism. Roots were used as anthelmintic, astringent and also used for dysentery, snake bite, fever, inflammation and rheumatism. Biological activities such as analgesic, anti-inflammatory and ulcerogenic were also reported. Roots of *Hugonia mystax* were evaluated for preliminary phytochemical screening and anthelmintic activity. Preliminary phytochemical screening showed the presence of various classes of secondary metabolites such as flavonoids, phenols, saponins, steroids, tannins and terpenoids.

Taking into consideration of medicinal value and utility, the present study was planned to explore anthelmintic potential of the medicinal plant named *H. mystax* (9, 10).

The aim of present study is to investigate the anthelmintic activity of ethanolic extract of root of *Hugonia mystax* Linn against *Pheretima posthuma* (in vitro study).



Fig 1: *Hugonia mystax* plant



Fig 2: *Hugonia mystax* root



Fig 3: *Hugonia mystax* root powder

Materials and Methods

Plant material

Fresh roots of *Hugonia mystax* was collected from surroundings of Medchal dist., Telangana, India.

Preparation of extract

The root of *Hugonia mystax* was collected, washed with running tap water, shade-dried at room temperature and grounded in a manual mill to get a coarse powder of 60 mesh. Powdered plant materials of *Hugonia mystax* was extracted with 80% ethanol in a soxhlet apparatus at 40 °C. Extraction was done with solvent until the supernatant in the soxhlet apparatus became transparent (for 48 hours). The extracts were filtered through a Buchner funnel with whatman filter paper no. 1. The filtrate was evaporated to dryness under reduced pressure using rotary evaporator at 40 °C. The crude extract was stored at 4 °C in airtight bottles in refrigerator. The ethanolic extract of the root was used for anthelmintic activity.

Qualitative phytochemical evaluation

The different chemical tests were performed for establishing profile of the extract for its chemical composition; the following chemical tests for various phytoconstituents in the ethanol extract was carried out as described below.

(A) Test for alkaloids

1. **Dragendorff's Test:** In a test tube containing 1 ml of extract, few drops of Dragendorff's reagent was added and the colour developed was noticed. Appearance of orange colour indicates the presence of alkaloids.
2. **Wagner's Test:** To the extract, 2 ml of Wagner's reagent was added; the formation of a reddish brown precipitate indicates the presence of alkaloids.
3. **Mayer's Test:** To the extract, 2 ml of Mayer's reagent was added, a dull white precipitate revealed the presence of alkaloids.
4. **Hager's Test:** To the extract, 2 ml of Hager's reagent was added; the formation of yellow precipitate confirmed the presence of alkaloids.

(B) Test for terpenoids

1. **Salkowski test:** To 1 ml of extract, tin (one bit) and thionyl chloride were added. Appearance of pink colour indicates the presence of terpenoids.
2. **Hirshonn reaction:** When the substance was heated with trichloroacetic acid, red to purple colour was observed.

(C) Test for steroids

1. **Liebermann Burchard Test:** To 1ml of extract, 1ml of glacial acetic acid and 1ml of acetic anhydride and two drops of concentrated sulphuric acid were added. The solution become red, then blue and finally bluish green indicates the presence of steroids.

(D) Test for coumarins

1. To 1 ml of extract, 1 ml of 10% sodium hydroxide was added. The presence of coumarins is indicated by the formation of yellow colour.

(E) Test for tannins

1. To few mg of extract, ferric chloride was added, formation of a dark blue or greenish black colour showed the presence of tannins.
2. The extract was mixed with basic lead acetate solution; formation of white precipitate indicated the presence of tannins.

(F) Test for saponins

1. To 1 ml of the extract, 5 ml of water was added and the tube was shaken vigorously. Copious lather formation indicates the presence of saponins.

(G) Test for flavones

1. **Shinoda Test:** To the extract, a few magnesium turnings and 2 drops of concentrated hydrochloric acid were added, formation of red colour showed the presence of flavones.
2. To the extract, 10% sodium hydroxide or ammonia was added; dark yellow colour shows the presence of flavones.

(H) Test for quinones

1. To 1 ml of the extract 1 ml of concentrated sulphuric acid was added. Formation of red colour shows the presence of quinones.

(I) Test for flavanones

1. To the extract, 10% sodium hydroxide was added and the colour changes from yellow to orange, which indicates the presence of flavanones.
2. To the extract, conc. sulphuric acid was added, and the colour changes from orange to crimson red, which indicates the presence of flavanones.

(J) Test for anthocyanins

1. To the extract, 10% sodium hydroxide was added, and the blue colour shows the presence of anthocyanins.
2. To the extract, conc. sulphuric acid was added, and the yellowish orange colour confirms the presence of anthocyanins.

(K) Test for anthraquinones

1. **Borntrager's test:** The extract was macerated with ether and after filtration; aqueous ammonia or caustic soda was added. Pink red or violet colour in the aqueous layer after shaking indicates the presence of anthraquinones.

(L) Test for phenols

1. **Ferric chloride test:** To the extract, few drops of 10 % aqueous ferric chloride were added. Appearance of blue or green colour indicates the presence of phenols.

(M) Test for proteins

1. **Biuret Test:** To the extract, 1 ml of 40% sodium hydroxide solution and two drops of one percent copper sulphate solution were added. Formation of violet color indicates the presence of proteins.
2. **Xanthoprotein Test:** To the extract, 1 ml of concentrated nitric acid was added. A white precipitate was formed; it is then boiled and cooled. Then, 20% sodium hydroxide or ammonia was added. Orange colour indicates the presence of aromatic amino acids.
3. **Tannic Acid Test:** To the extract, 10% tannic acid was added. Formation of white precipitate indicates the presence of proteins.

(N) Test for carbohydrates

1. **Molisch's Test:** To the extract, 1 ml of alpha-naphthol solution, and concentrated sulphuric acid through the sides of test tube were added. Purple or reddish violet colour at the junction of the two liquids revealed the presence of carbohydrates.
2. **Fehling's Test:** To the extract, equal quantities of fehling's solution A and B were added and on heating, formation of a brick red precipitate indicates the presence of carbohydrates.
3. **Benedict's test:** To 5 ml of Benedict's reagent, extract was added and boiled for two minutes and cooled. Formation of red precipitate showed the presence of carbohydrates.

(O) Test for amino acids

1. **Ninhydrin test:** Two drops of ninhydrin solution were added to the extract, a characteristic purple colour indicates the presence of amino acids.

(P) Test for fixed oils and fats

1. **Spot Test:** A small quantity of extract was pressed between two filter papers. Oil stains on the paper indicates the presence of fixed oils and fats.

(Q) Test for volatile oils

1. To the section of drug, add alcoholic solution of Sudan III. Formation of red colour obtained by globules indicates the presence of volatile oils.
2. To the thin section of drug, add a drop of tincture alkaline. Formation of red colour indicates the presence of volatile oils.
3. To the test sample, add 1% Osmic acid. Formation of black colour indicates the presence of volatile oils.

***In vitro* anthelmintic activity**

The root extract of *Hugonia mystax* were evaluated for anthelmintic activity in *Pheretima posthuma* (Indian adult earth worm). Indian adult earth worm 4 -5 cm in length and 0.1 - 0.2 cm in width was used for the *in vitro* anthelmintic bio assay of

ethanolic extract. The earthworms resembled the intestinal roundworm parasites of human beings both anatomically and physiologically and hence were used to study the anthelmintic activity. The worms were acclimatized to the laboratory condition before experimentation. The earthworms were divided into five groups of six earth worms in each and placed in eight Petri dishes containing the extract solutions or the reference drugs as mentioned below-

Experimental grouping of earthworms

Group -1: Received normal saline which served as control.

Group-2: Received piperazine citrate suspension at a dose of 10mg/ml which served as the standard.

Group-3: Received ethanolic extract of stem bark of *Hugonia mystax* at a dose of 25mg/ml.

Group -4: Received ethanolic extract of stem bark of *Hugonia mystax* at a dose of 50mg/ml.

Group-5: Received ethanolic extract of stem bark of *Hugonia mystax* at a dose of 100mg/ml.

Paralysis was said to occur when the worms do not revive even in normal saline. Death was concluded when the worms lost their motility followed with fading away of their body colour. Observations were made for the time taken to paralysis and death of individual worms.

Results and Discussion

Preliminary Phytochemical analysis of ethanolic extract of root of *Hugonia mystax* was presented in Table 1. Phytochemical studies have revealed the presence of several phytochemicals including alkaloids, glycosides, flavonoids, steroids, proteins, phenolic compounds, anthraquinones and tannins.

Table 1: Preliminary phytochemical screening of *Hugonia mystax* root

Constituents	Ethanol extract
Terpenoids	+
Saponins	+
Steroids	+
Phenols	+
Flavonoids	+
Coumarins	-
Carbohydrates	+
Alkaloids	+
Quinones	-
Tannins	+
Proteins	+
Oils & fats	-
Anthraquinones	+
Anthocyanins	-
Amino acids	+
Volatile oils	-

*+' = Present, '-' = Absent

Table 2: Anthelmintic activity of ethanolic extract of *Hugonia mystax* root

Extract	Concentration (mg/ml)	Paralysis time (minutes)	Death time (minutes)
Control – Normal saline	-	-	-
Piperazine citrate	10	28.55±1.16	58±1.03
Ethanolic root extract of <i>Hugonia mystax</i>	25	55.15±1.17	126.2±1.21
	50	48.7±1.13	118.9±1.16
	100	35.1±1.05	66.4±0.82

All value represent Mean \pm SD, n=6 in each group. Comparisons made between standard versus treated groups, $p < 0.05$ was considered significant.

The main advantages of using *in vitro* assays to screen the anti-parasitic properties of the plants and plant extracts include low costs and rapid turnover which allow the screening of plants at large scale. In addition, these tests measured the effect of anthelmintic activity directly on the processes of hatching, development and motility of parasites without interfering the internal physiological functions of the host. Anthelmintic activity of root of *Hugonia mystax* was carried out on *Pheretima posthuma*. Different concentrations of the ethanolic extracts were used for the studies. The time taken for paralysis and death of earthworms were recorded in Table 2. The data reveals that the ethanolic extract at the concentration of 25, 50 and 100 mg/ml showed death time 126.2, 118.9 & 66.4 min respectively. The effect increased with concentration of extracts on dose dependent manner. The extract caused paralysis followed by death of the worms at all dose levels. The anthelmintic activity of ethanolic extract of root of *Hugonia mystax* was compared to the standard drug piperazine citrate (purchased from local medical store). Phytochemical analysis of the crude extract revealed the presence of tannins as one of the chemical constituents. Tannins were shown to produce anthelmintic activities. Chemically tannins are polyphenolic compounds. It is possible that tannins contained in the extracts of *Hugonia mystax* produced similar effects. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastro intestinal tract of host animal or glycoprotein on the cuticle of the parasite and cause death.

The preliminary phytochemical analysis of the extract has shown the presence of phenolics, like tannins and flavonoids as well as anthraquinones. The function of the anthelmintic drugs, like piperazine citrate, is known to cause paralysis of the worms so that they are expelled in the faeces of man and animals. Piperazine citrate by increasing chloride ion conductance of worm muscle membrane produces hyper polarization and reduced excitability that leads to muscle relaxation and flaccid paralysis. The extracts not only demonstrated this property, but they also caused death of the worms. Synthetic phenolic anthelmintics like niclosamide, interfere with the energy generation in the helminth parasites by uncoupling the oxidative phosphorylation. Another possible mechanism of action is that they bind to free proteins in the gastrointestinal tract of the host animal or to glycoprotein on the cuticle of the parasite and by this cause death. Tannins have also been shown to produce anthelmintic activities. There are reports for anthelmintic property of phenolics present in plant extract like *Hugonia mystax* root.

Conclusion

The traditional claim of root of *Hugonia mystax* as an anthelmintic has been confirmed as the extract shown activity against *Pheretima posthuma*. Further studies are necessary to isolate and reveal the active compound contained in the crude extract of *Hugonia mystax* responsible for activity and to establish the mechanism of action. 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 extract 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.

Acknowledgement

The authors wish to thank the management of School of Pharmacy, Anurag Group of Institutions, Venkatapur, Ghatkesar, Telangana, India for providing necessary equipment for research, praiseworthy inspiration, constant encouragement, facilities and support.

Conflict of interest

There is no conflict of interest.

Sponsorship

Nil.

References

- Vimalavady A, Kadavul K. Phytochemistry screening and antimicrobial activity of the leaves of the *Hugonia mystax* Linn. (Linaceae). *Indian Journal of Natural Products and Resources*. 2012; 3(2):161-165.
- Rajeswari G. GC-MS analysis of bioactive components of *Hugonia mystax* L. (Linaceae). *Research Journal of Pharmaceutical, Biological and chemical sciences*. 2012; 3(4):301-308.
- Harborne JB. A guide to Modern Techniques of plant analysis, 3rd ed., Kluwer academics publishers, USA, 1998.
- Evans WC. *Trees and Evans Pharmacognosy*, 15th ed., Elsevier India Pvt Ltd, New Delhi, 2006.
- Narender Boggula. *In vitro* evaluation of anti-oxidant activity of different extracts of *Aerva lanata* leaves. *World Journal of Pharmacy and Pharmaceutical Sciences*. 2016; 5(8):756-761.
- Mohankumar M, Lalitha. *In vitro* anthelmintic activity of *Hugonia mystax* leaves Linn in Indian Adult Earthworm. *Journal of Pharmacognosy and Phytochemistry*. 2015; 3(5):19-22.
- Pillai LS, Nair BR. A comparative study of the anthelmintic potential of *Cleome viscosa* L. and *Cleome burmanni* W. *Indian J Pharm Sci*. 2011; 73(1):98-100.
- Narender Boggula. Evaluation of *in vitro* anti-oxidant activity of dried roots of *Abrus precatorius*. *World Journal of Pharmaceutical Research*. 2017; 6(3):1619-1628.
- Sujani C, Radhika K, Rajasekhar KK, Shankarananth V. Synthesis, characterisation and anthelmintic activity of some novel 1,4-Disubstituted azetidinones. *International Journal of Novel Trends in Pharmaceutical Sciences*. 2015; 5(3):135-142.
- Martin RJ. "Γ-Aminobutyric acid and piperazine activated single channel currents from ascaris suum body muscle", *Br. J Pharmacol*. 1985; 84(2):445-461.
- Taur DJ, Kulkarni VB, Patil RY. Chromatographic evaluation and anthelmintic activity of *Eucalyptus globulus* oil. *Pharmacognosy Res*. 2010; 2(3):125-127.
- Narender Boggula, Swetha Reddy, Naram Reddy, Teja Sri Alla, Azmath Farhana, Jainendra Battineni. *et al.* Phytochemical evaluation and *in vitro* anti-bacterial activity of dried seeds of *Abrus precatorius*. *Int. J Pharm. Sci. Rev. Res*. 2017; 44(1):101-107.
- Salhan M, Kumar B, Sharma P, Sandhar HK, Gautam M. Comparative anthelmintic activity of aqueous and ethanolic leaf extract of *Clitoria ternatea*. *IJDDR*. 2011; 3(10):62-68.
- Acharyya S, Dash GK, Brahma DK, Chhetree RR. Preliminary phytochemical investigation and anthelmintic activity of *Acacia suma* (Roxb) barks. *IRJP*. 2011; 2(1):136-141.
- Mali RG, Hundiwale JC, Sonawane RS, Patil RN, Hatapakki BC. Evaluation of *Capparis deciduas* Linn for anthelmintic and antimicrobial activities, *Indian Journal of Natural Product*. 2004; 20(4):10-12.
- Sabhi M, Ramezani M, Jaffari G, Haravi G, Bahaeddini F, Aynehi Y. *et al.* Survey of Indian plants for saponins Alkaloids, Flavonoids and Tannins, the plant of Capparidaceae, *International Journal of Crude Drug Research*. 1985; 23(4):165-177.
- Nargund VLG. Anthelmintic activity of 8-Fluoro-9 substituted (1, 3) – Benzothiazolo (5, 1-b)-1, 3, 5-triazoles on *Pheretima posthuma*. *Indian Drugs*. 1999; 36(2):137-139.
- Nayak BS, Jena PK, Sahu NP, Nayak UK, Patro KB. Comparative study of anthelmintic activity between aqueous and ethanolic extract of *solanum surattense* Linn. *International Journal of Pharmacy and Pharmaceutical Sciences*. 2009; 1(1):103-107.
- Rubini D, Sudhahar D, Anandaragopal K. Phytochemical investigation and anthelmintic activity of *Celosia cristata* leaf extract. *Int Res J Pharm*. 2012; 3(5):335-336.
- Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: a review. *J Int Pharm Sci*. 2011; 1(1):98-106.
- Tuse TA, Bidkar AA, Bhale SA, Patanka RD. *In-vitro* anthelmintic activity of aerial roots of *Ficus benghalensis*. *International Journal of Pharmacological Research*. 2011; 1(1):10-13.
- Loukas A, Hotez PJ Goodman, Gilman's the *Pharmacological Basis of Therapeutics*, 2006, 1073-1093.
- Suleiman MM, Mamman M, Aliu YO, Ajanusi JO. Anthelmintic activity of the crude methanol extract of *Xylopi aethiopic a* against *Nippostrongylus brasiliensis* in rats. *Veterinarski Arhiv*. 2005; 75(6):487-495.
- Dabadi P, Koti BC, Swamy AV, Chandrakala. *In-vitro* anthelmintic activity of *Mimusops elengi* Bark. *Indian Journal of Novel Drug delivery*. 2011; 3(4):307-310.
- Dash GK, Suresh P, Sahu SK, Kar DM, Ganpaty S, Panda SB. Evaluation of *Evolvulus alsinoides* Linn. for anthelmintic and antimicrobial activities, *Journal of Natural Remedies*. 2002; 2(2):182-185.
- Deore SL. *In-vitro* anthelmintic activity of *Cassia tora*, *International Journal of Chem. Tech. Research*. 2009; 1(2):177-179.
- Gupta A. Pharmacognostic, Phytochemical and physiochemical studies of *Atrocarpus hetrophyllus* leaf, *Journal Natural Product & Plant Resource*. 2011; 1(4):20-26.
- Singhal KC. Evaluation of *Evolvulus alsinoides* Linn. For anthelmintic and antimicrobial activities, *Journal of Natural Remedies*. 2011; 15(2):119-122.
- Tripathi KD. *Essential of Medicinal Pharmacology*. Jaypee Brothers Medical Publishers, 2003, 808-809.

30. Williams LC, Wilkins. Remington, the science and practice of Pharmacy. Welter Klumer Health, New Delhi, 21st Ed, 2006; 2:773-774.
31. Santapau H, Henry AN. A Dictionary of flowering plants in India. Council of Scientific and Industrial Research, New Delhi, 1983, 103.
32. Srinivasu U, Amrutia Jay N, Katharotia Reena. J. International Research Journal of Pharmacy, 2011; 2(8):130-131.
33. Mulla WA, Thorat VS, Patil RV, Burade KB. Anthelmintic activity of leaves of *Alocasia indica* Linn. International Journal of PharmTech Research. 2010; 2(1):26-30.
34. Shaibani IRMA, Phulan MS, Arijoand A, Qureshi TA. Ovicidal and larvicidal properties of *Adhatoda vasica* (L.) Extracts against gastrointestinal nematodes of sheep *in vitro*. Pakistan vet. J. 2008; 28(2):79-83.
35. Vidyadhar S, Saidulu M, Gopal TK, Chamundeeswari D, Rao U, Banji D. *In vitro* anthelmintic activity of the whole plant of *Enicostemma littorale* by using various extracts. International journal of applied biology and pharmaceutical technology. 2010; 1(3):1119-1125.
36. Sharma US, Sharma UK, Singh A, Sutar N, Singh PJ. *In vitro* anthelmintic activity of *Murraya koenigii* linn. Leaves extracts. International journal of pharma and bio sciences. 2010; 1(3):1-4.
37. Shruthi A, Latha KP, Vagdevi HM, Vaidya VP, Pushpa B, Shwetha C. *et al.* *In vitro* Anthelmintic Activity of Leaves Extract of *Wrightia tinctoria*. International Journal of ChemTech Research. 2010; 2(4):2043-2045.
38. Mason PA, Sturman G. Some pharmacological properties of piperazine. Br. J Pharmac, 1972; 44:169-176.
39. Narender Boggula, Himabindu Peddapalli. Phytochemical analysis and evaluation of *in vitro* anti-oxidant activity of *punica granatum* leaves. International Journal of Pharmacognosy and Phytochemical Research. 2017; 9(8):1110-1118.
40. Laverack MS. The physiology of earthworms. Pergamon. New York, 1963, 179.