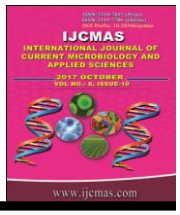
*Int.J.Curr.Microbiol.App.Sci* (2017) *6*(10): 780-789

*International Journal of Current Microbiology and Applied Sciences*
*ISSN: 2319-7706* **Volume 6 Number 10 (2017) pp. 780-789**
Journal homepage: http://www.ijcmas.com



**Original Research Article** https://doi.org/10.20546/ijcmas.2017.610.094

# Evaluation of Anthelmintic Efficacy of *Nicotiana tabacum* against Gastrointestinal Nematodes of Goats

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**Keywords**

*Nicotiana tabacum*, Anthelmintic activity, GI nematodosis, Goats.

**Article Info**

***Accepted:***
07 September 2017
***Available Online:***
10 October 2017

**A B S T R A C T**

The present study was planned to evaluate the anthelmintic efficacy of crude powder (CP), crude aqueous (CAE) and crude methanolic (CME) extracts of *Nicotiana tabacum* leaves against common gastrointestinal nematodes of goats using three *in-vitro* tests *viz.* adult motility test (AMT), egg hatch assay (EHA) and larval paralysis test (LPT) and one in-vivo test using faecal egg count reduction test (FECRT). AMT was conducted against freshly collected adult *Haemonchus contortus*. However, EHA and LPT tests were conducted against eggs and larvae of gastrointestinal nematodes, respectively of goats or AMT at different concentrations *viz.*0.156%, 0.312%, 0.625%, 1.25%, 2.5%, 5% and 10%. *In-vivo* trial was also conducted in goats naturally infected with mixed infection of GI nematodes using FECRT. In AMT, an average highest corrected motility of 100% was recorded from 0.625–10% concentration followed by 66.66% at 0.156 and 0.312% concentrations. Overall CP and CAE showed maximum (100%) adulticidal activity against *H. contortus* and minimum (71.43%) with CME. In EHA, CME of *N. tabacum* showed better $ED_{50}$=0.522 and $ED_{99}$= 21.32mg/ml in comparison to CAE ($ED_{50}$=0.40 and $ED_{99}$= 36.22mg/ml). The highest (100%) inhibition of egg hatching was observed with CAE @ 50 and 100mg/ml and CME @ 25, 50 and 100 mg/ml concentration. In case of LPT, crude methanolic extract has better ($ED_{50}$= 0.69 and $ED_{99}$= 9.58 mg/ml) values than crude aqueous extract ($ED_{50}$= 0.759 and $ED_{99}$= 14.9349 mg/ml). The highest (100%) paralysis of third stage larvae was observed with CAE @ 25, 50 and 100mg/ml and CME @12.5, 25, 50 and 100mg/ml concentration. In *in-vivo* trial, the highest (100%) reduction in faecal egg count was recorded against CAE @ both 100 and 200mg/kg b. wt. 14 days post-treatment. However, CME and CP both showed maximum effect of 33.33% and 15.79%, respectively @200mg/kg b. wt. on 14 days post-treatment.

## Introduction

Sheep and goats serve as major source of income for poor and landless farmers of Indian subcontinent, but the production performance of these animals is drastically reduced due to parasitic infections. Sheep and goats are highly susceptible to various helminth infections specially goats due to its unique grazing behaviour. Among nematodes, *Haemonchus contortus* is the most pathogenic gastrointestinal (GI) nematode distributed in different agro-climatic regions throughout the year. It causes heavy economic losses in terms of retarded growth, weight loss, lowered productivity and even mortality (Tyasi and Tyasi, 2015). Several methods have been employed for controlling the

infection that includes anthelmintic treatment (Singh and Yadav, 1997), nutritional management (Swarnkar and Singh, 2005), pasture management, biological control (Sanyal, 1998) and immunization (Smith, 2014). Use of chemical drugs remains the only option to control GI nematodosis under field condition because of unavailability and unfeasibility of the other methods of controlling the infection. Unfortunately, the excessive or haphazard use of chemical drugs had lead to emergence of resistance among the GI parasite in most of the countries including India (Rialch *et al.,* 2013). Use of chemical drugs and emergence of resistance are the factors which are forcing the researchers to find out an alternative control methods. The exploitation of herbal product is a better option for most of small scale farmers as herbs having medicinal property are distributed in different regions of the country. Several plants available in India have been identified having anthelmintic activity (Bhardwaj *et al.,* 2015; Kumar *et al.,* 2014 and 2016). Major benefits of herbs based dewormers include broad spectrum activity, non-toxic with wide margin of safety, cheaper and supplementary effects on animal health (Kuamr *et al.,* 2015).

*Nicotiana tabacum* having narcotic properties was originally native to America but now is cultivated in other countries including India and is locally known as tambacco. The ethno medical uses include use of the decoction of leaves as antispasmodics, expectorants, emetics, sedatives, and in rheumatic swellings, anesthetics, anticonvulsants, antibacterial, diuretics, antimicrobial, and for anti-fungal activities and anthelmintic activities (Nouri *et al.,* 2014). Keeping in mind, the use of the plant in traditional medicine, the present study was planned to evaluate the anthelmintic activity of *Nicotiana tabacum* against different stages of GI nematodes.

## Materials and Methods

### Selection of plant

Leaves of *Nicotiana tabacum* were purchased from local market of Pantnagar. *N. tabacum* was selected, on the basis of their documented anthelmintic properties or literature survey on traditional uses in India and other parts of the world.

The authentication and identification of the plant was confirmed by Department of Biological Sciences, College of Basic Science and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar.

### Preparation of extract

The leaves were air-dried under shade, coarsely grinded with the electric mixer grinder and stored in airtight container. Two extracts of the plant were prepared *viz.* Crude aqueous extract (CAE) and crude methanol extract (CME) using distilled water and methanol, respectively. About 50 gm of powdered sample was soaked separately in 400 ml of each solvent in 1 lit glass flasks, covered with aluminium foil and stirred every one hour interval initially for 2-3 times and left undisturbed for 8 hrs. At room temperature and then filtered by separating funnels using Whatman paper No.1. The filtrate was concentrated by using rotatory vacuum evaporator at 50-55$^0$C (Singh, 2001).

The extract residues were individually marked, kept in airtight glass petridishes in refrigerator at 4$^0$C till further use. Seven dilutions of each extract residues were prepared *viz.* 0.156%, 0.312%, 0.625%, 1.25%, 2.5%, 5% and 10% in locks solution. Thiabendazole was dissolved in Dimethyl Sulfoxide (DMSO) and distilled water at same concentrations and used as positive control and.

## *In-vitro* evaluation of enthelmintic activity of *N. tabacum*

### Adult motility test

### Collection of worms

Adult *Haemonchus contortus* worms were procured from the abomasii of goats freshly slaughtered at Lalkuan slaughter house. The worms were washed with distilled water and then transferred in wide mouth glass container having lukewarm normal saline. Then after, the collected worms were brought to the laboratory, Department of Veterinary Parasitology, College of Veterinary & Animal Sciences, Pantnagar. Several washings of the worms were made with normal saline solution and finally the worms were transferred in beaker containing Lock's solution and kept at $37^0$C in incubator to acclimatize before beginning the test **(**Bhatnagar *et al.,* 1961**)**

For AMT, ten freshly collected adult *H. contortus* were taken in each small petridishes having 15 ml of different dilutions of extracts and crude powder. Lock's solution was used as positive control. It was then incubated at $37^0$C±$1^0$C for 24 hrs and number of live and dead adult worms were counted at 1, 2, 4, 6, 12, 18 and 24 hrs interval. The viability of the worms was determined by pinch technique (the absence of motility for an observation period of 5-6 second) as described by Neogi *et al.,* (1964). The corrected mortality was calculated as per the formula given by Sangwan and Sangwan (1998).

$$\% \text{ corrected mortality} = \frac{\text{Total mortality- Control mortality}}{\text{Total mortality}} \times 100$$

### Egg hatch assay

Gastrointestinal (GI) nematodes eggs were collected by using standardized protocol adopted by the World Association for Advancement of Veterinary Parasitology (Coles *et al.,* 1992). The egg hatch test was performed as per Le Jambre (1976). Faecal samples were collected from local goats naturally infected with GI nematodes in a screw top plastic bottle and stored an aerobically. The bottles were almost fully filled with water and were shaken vigorously. Faecal samples were broken in mortar-pestle with water to make homogenised suspension. It was sieved through tea strainer of 0.15mm aperture, 20 cm diameter and then filtrate was transferred into centrifuge tube (15ml). Then after, it was centrifuged for 2 min. at 300×g and then supernatant was gently sucked off. Tubes were agitated to loosen the sediment, then saturated salt solution was added into the tube up to 2/3 rd and re-centrifuge for 3 min at 130×g. The tubes were filled with saturated salt solution until a meniscus was formed above the tube. A cover slip was gently placed above the meniscus by avoiding bubbles and left for few minutes. The cover slip was plucked off and the eggs were washed with deionised water into a conical centrifuge tube. These tubes were filled and the eggs were re-suspended in deionised water, the number of eggs were estimated per ml and diluted to 100-150/100µl. 100µl of fresh egg suspension containing about 100-150 fresh eggs were placed in each well of 96-multiwell plate containing 1 ml extract in multiwell plate, while control well received water instead of extract. Total volume of each well was made up to 2ml by adding 900µl of distilled water. Plates were incubated at 27°C for 48 hours. Two drops of Lugol's iodine were added to each well. A total of hundred eggs and hatched larvae were counted under compound microscope.

### Larval paralysis test

Faecal samples were collected directly from rectum of the animals and transferred in

faecal bags. The faecal sample collected from each group was pooled. Pooled sample was broken in a tray with spatula and mixed with adequate quantity of activated charcoal to avoid fermentation. The faeces were then packed loosely in petridish. The petridish was then incubated at $27^0$C in BOD incubator over a period of one week. The samples were kept moist by sprinkling water on every alternate day. The infective third stage larvae (L3 stages) were harvested by using Bearmann's apparatus (MAFF 1986). 100µl containing 100-150 live larvae were exposed to different concentrations of extracts ranging from 0.156% - 10% in 96 multiwell plates. After 24 hrs, live (motile) and dead larvae were counted.

## *In-vivo* evaluation of anthelmintic activity of *N. tabacum*

### Location of study area

Evaluation of anthelmintic efficacy of plant extracts residue was carried out in goats from Department of Livestock Production and Management, College of Veterinary and Animal Sciences, Pantnagar and also in local areas, Sanjay colony and Jha colony of Pantnagar.

### Grouping and treatment of animals

Animals having faecal egg per gram of faeces more than 200 and also having the history of not using any kind of anthelmintic treatment since last three months were selected. Forty two goats naturally infected with mixed infections of gastrointestinal nematodes of either sex, aged between 1-3 years were selected, numbered and weighed during each trial.

These animals were randomly divided into seven group *viz.* GI, GII, GIII, GIV, GV, GVI and GVII comprising of 6 animals each.

The GI, GII and GIII groups were treated with crude powder (CP), crude aqueous (CAE) and crude methanolic (CME) extracts of *Nicotiana* leaves, resprctively @100mg/kg b.wt. orally, while GIV, GV and GVI were treated @200mg/kg b.wt. orally with CP, CAE and CME, respectively. However, animals of GVII kept as untreated infected control.

### Faecal Egg Count Reduction Test (FECRT)

Faecal egg count from individual animals of each treated and control groups were performed on 0 day (before) of treatment, 7 and 14 day post-treatment (7 and 14 DPT) using modified Mc Master egg counting technique.

Faecal suspension was prepared using one gram of faecal sample in 14 ml saturated salt solution. Sample was sieved through tea strainer and transferred into plastic /glass test tube. Mc Master egg counting chamber of was charged and eggs of gastrointestinal nematodes were counted under 10X magnification of light microscope.

The intensity of egg per gram of faeces (EPG) was determined by modified Mc Master Technique (MAFF, 1971). The egg per gram of faeces was calculated as follows:

Egg per gram (EPG) = Number of eggs in the chamber x 50

### Determination of anthelmintic efficacy

The anthelmintic efficacy of the each extract residues was calculated using the following formula given by Dash *et al.,* (1988)

$$\% \text{ efficacy} = \frac{\text{EPG before treatment} - \text{EPG after treatment}}{\text{EPG before treatment}} \times 100$$

## Coproculture

Pooled faecal samples of each group were cultured to harvest the third stage larvae according to MAFF (1971) using Baermanns apparatus. GI nematode larvae were identified on the basis of their morphological characters as described by Soulsby (1965).

## Phytochemical analysis of extracts

Extract residues obtained from various solvents were tested for the presence of their phytoconstituents such as alkaloid, anthraquinones, tannins, flavonoids, saponins, glycoside, resins, triterpencs, reucing sugars, proteins and coumarins by standard procedures (Sofowara, 1982).

## Statistical analysis

The data of *in-vitro* trials *viz.* Egg Hatch Assay and Larval Paralysis Test were analyzed statistically using probit analysis for calculation of $ED_{50}$ and $ED_{99}$ by SPSS version 16. However, multivariat two ways ANOVA was used to analyze the data for calculation of F-value obtained from *in-vivo* trials and difference between the means were considered significant at $p>0.05$.

## Results and Discussion

In the present study, anthelmintic activity of *Nicotiana tabacum* was assessed against adult *Haemonchus contortus*, eggs and larvae of common gastrointestinal nematodes using three tests *viz*. adult motility test (AMT), egg hatch assay (EHA) and larval paralysis test (LPT), respectively and faecal egg count reduction test (FECRT).

## Adult motility test

CP and CAE extracts causes100% mortality at all tested concentration *viz.* 0.156%, 0.312%, 0.625%, 1.25%, 2.5%, 5% and 10% whereas CME showed 0, 0, 100, 100, 100, 100 and 100% mortality, respectively at 0.156%, 0.312%, 0.625%, 1.25%, 2.5%, 5% and 10% concentrations. Overall CP and CAE of *N. tabacum* showed 100% average corrected mortality while CME showed 71.43% only (Table 1). However, TBZ showed 100% mortality of the worms at 1.25%, 2.5%, 5% and 10% against all concentrations (Table 1).

An average highest corrected motility of 100% was observed from 0.625–10% followed by 66.66% at 0.312 and 0.156%. Overall *N. tabacum* showed overall 94.47% corrected mortality (Table 2).

## Egg hatch assay

CAE and CME extracts of *N. tabacum* caused significant inhibition of egg hatching with increasing dose (Table 3). CAE ($ED_{50}$=0.40 and $ED_{99}$= 36.22mg/ml) @ 50 and 100mg/ml concentration showed 100% inhibition. However, at 25.12.5, 6.25, 3.12 and 1.56 mg/ml concentration, inhibition of egg hatching were 99, 95, 90, 84 and 79%, respectively. CME ($ED_{50}$= 0.522 and $ED_{99}$= 21.320mg/ml) showed highest (100%) inhibition of egg hatching at 25, 50 and 100mg/ml concentration followed by 97, 93, 85 and 78% at 12.5, 6.25 3.125 and 1.56mg/ml concentration, respectively (Tables 3 and 4). In EHA, TBZ showed 100% inhibition in egg hatching at all concentrations (Table 2).

## Larval paralysis test

CAE and CME extracts of *N. tabacum* causes significant dose dependent paralysis of third stage ($L_3$) larvae of GI nematodes. The CAE ($ED_{50}$= 0.75 and $ED_{99}$= 14.34 mg/ml) caused 100% paralysis of third stage ($L_3$) larvae at 25, 50 and 100 mg/ml concentration. However, it was 99, 94, 86 and 73% at 12.5, 6.25, 3.125 and 1.56mg/ml, respectively.

Crude methanolic ($ED_{50}$= 0.69 and $ED_{99}$= 9.58 mg/ml) extract also caused 100% paralysis of $L_3$ larvae at 100, 50, 25 and 12.5 mg/ml concentration, while at 6.25, 3.125 and 1.56mg/ml concentration, it was 97, 89 and 78%, respectively (Tables 2 and 3). In LPT, TBZ caused 100% paralysis of third ($L_3$) stage larvae of gastrointestinal nematodes in all tested concentrations (Table 2).

## *In-vivo* anthelmintic efficacy of *N. Tabacum* using FECRT

Anthelmintic efficacy of the plant extracts was conducted in goats found positive with mixed infection of GI nematodes. Coproculture examination revealed the presence of *Haemonchus contortus* as predominant GI nematode followed by *Oesophagostomum columbianum*, *Trichostrongylus colubriformis* and *Strongyloides* spp. The highest (85.71%) reduction in faecal egg count was observed against CAE followed by CME (14.28%) and CP (-2.9%) on 7 DPT, however, it was 100, 28.57 and 5.88% on 14 DPT following treatment @ 100mg/kg b.wt. orally.

CAE showed highest (90%) reduction in faecal egg count followed by CME (25%) and lowest (10.53%) against CP on 7 day post-treatment while it was 100, 33.33and 15.79%, respectively on 14 day post-treatment @ 200mg/kg b.wt. orally (Table 4). Non-significant difference was observed by CP and CME.

## Phytochemical analysis

On phytochemical analysis, alkaloids, reducing sugar, saponin and tannins were found in CAE of *N. tabacum*. However, CME revealed the presence of alkaloids, coumarins, reducing sugar, saponin and tannins.

**Table.1** Average % corrected mortality of CP, CAE and CME of *Nicotiana tabacum* and TBZ against adult *H. contortus*

| Extracts | Average % mortality at different % concentration | | | | | | | Average % CM |
|---|---|---|---|---|---|---|---|---|
| | 0.156 | 0.312 | 0.625 | 1.25 | 2.5 | 5 | 10 | |
| CP | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| CAE | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| CME | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 71.43 |
| TBZ | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 57.14 |

CP-crude powder , CAE- crude aqueous extract, CME- crude methanol extract

**Table.2** % inhibition of egg hatching and larval paralysis at different concentrations of CAE and CME of *N. tabacum* and TBZ

| Concentration (mg/ml) | % inhibition of egg hatching | | | % of Larval paralysis | | |
|---|---|---|---|---|---|---|
| | CAE | CME | TBZ | CAE | CME | TBZ |
| 1.56 | 79 | 78 | 100 | 73 | 78 | 100 |
| 3.12 | 84 | 85 | 100 | 86 | 89 | 100 |
| 6.25 | 90 | 93 | 100 | 94 | 97 | 100 |
| 12.5 | 95 | 97 | 100 | 99 | 100 | 100 |
| 25 | 99 | 100 | 100 | 100 | 100 | 100 |
| 50 | 100 | 100 | 100 | 100 | 100 | 100 |
| 100 | 100 | 100 | 100 | 100 | 100 | 100 |

**Table.3** ED $_{50}$ and ED $_{99}$ (mg/ml) of CAE and CME of *N. tabacum* in EHA and LPT

| *N. tabacum* extracts | Tests | $ED_{50}$ mg/ml | | | $ED_{99}$ mg/ml | | |
|---|---|---|---|---|---|---|---|
| | | ED $_{50}$ | Lower limit | Upper limit | $ED_{99}$ | Lower limit | Upper limit |
| CAE | EHA | 0.40 | 0.14 | 0.74 | 36.22 | 20.80 | 95.33 |
| | LPT | 0.75 | 0.40 | 1.09 | 14.939 | 9.57 | 29.00 |
| CME | EHA | 0.52 | 0.21 | 0.864 | 21.32 | 13.22 | 49.41 |
| | LPT | 0.69 | 0.32 | 1.026 | 9.58 | 6.53 | 19.98 |

**Table.4** *In-vivo* anthelmintic efficacy of *N. tabacum* against mixed infection of GI nematodes in goats

| Groups | | GI | GII | GIII | GIV | GV | GVI |
|---|---|---|---|---|---|---|---|
| Treatment & dose rate (per kg b.wt) | | CP @100mg | CAE @100mg | CME @ 100mg | CP @ 200mg | CAE @ 200mg | CME @ 200mg |
| % FECRT | (7DPT) | -2.9 | 85.71 | 14.28 | 10.53 | 90 | 25 |
| | (14DPT) | 5.88 | 100 | 28.57 | 15.79 | 100 | 33.33 |

AMT of *N. tabacum* against *H. contortus* revealed that both CP and CAE showed maximum (100%) adulticidal activity against *H. contortus* followed by (71.43%) CME. Adulticidal activity of the decoction of *N. tabacum* against *H. contortus* has also been observed by Raje and Jangde (2003). However, Sindhu *et al.,* (2014) observed 100% mortality against aqueous extract slightly at higher concentration. 100% mortality of *H. contortus* against aqueous and methanolic extracts @25, 50 and 100mg/ml has been also observed by Iqbal *et al.,* (2006).

EHA trial of *N. tabacum* revealed that CME showed better $ED_{99}$ value (21.320mg/ml) in comparison to CAE (36.225mg/ml). CME also showed maximum inhibition in egg hatching at lower concentration than CAE. The dose-dependent ovicidal activity of the plant has also been reported by Hamad *et al.,* (2012).

The findings of LPT revealed that CME has better $ED_{50}$ and $ED_{99}$ ($ED_{50}$= 0.696 and $ED_{99}$= 9.585 mg/ml) values than CAE ($ED_{50}$= 0.759 and $ED_{99}$= 14.349 mg/ml) and also showed dose-dependent activity of larval paralysis. Larvicidal activity of the aqueous extract of *Nicotiana* has also been reported by Molef *et al.,* (2013).

*In-vivo* trials revealed that CAE showed 100% efficacy in goats naturally infected with mixed infections of GI nematodes at both 100 and 200mg/kg body wt. orally. Iqbal *et al.,* (2006) recorded 49.4% and 73.6% reduction in faecal egg counts in sheep naturally parasitized with gastrointestinal nematodes following treatment with crude aqueous and methanolic extracts @ 3mg/kg b. wt. on 5 days post-treatment However, Hamad *et al.,* (2012) observed 87.5% and 88.6% reduction in faecal egg count against crude aqueous methanolic extracts @2mg and 4mg/kg b.wt., respectively.

Anthelmintic effectiveness of *N. tabacum* occurs due to the presence of excitatory neuromuscular junctions containing ganglion type nicotinic receptors with acetylcholine as their neurotransmitter present in muscles of nematode parasites (Neal, 2002). The ganglion stimulant nicotine is known to found

in *N. tabacum* leaves and it activates these neuromuscular junctions causing spastic paralysis in the worm results into death and expulsion of the worms from the host (Bowman and Rand, 1980). Kambou and Guissou (2011) observed that aqueous extract contains alkaloids, steroids, saponins, tannins and triterpins. However, Kaushik *et al.*, (2011) found the presence of carbohydrate, flavonoids, phenols and tannins. Alkaloids, saponins and tannins as phytoconstituents have also been observed by Silva *et al.*, (2013). The presence of alkaloids, reducing sugar, saponins, flavonoids, steroids, terpenoids and tannins has been observed by Suleiman (2011). *Nicotiana tabacum* showed anthelmintic activity against mixed infection of gastrointestinal nematodes of goats might be due to presence of tannins, alkaloids, saponins, reducing sugars, coumarins, flavonoids and triterpenes. Tannins interact with proteins in nematode cuticle which possibly changes the chemical and physical properties (Athanasiadou *et al.,* 2007). Alkaloid may improve tonicity of gastrointestinal tracts and thus expell the worms or may have effect on nervous system (Lateef *et al.,* 2003; Ademola *et al.,* 2008). Saponins are naturally occurring chemical compounds found in wide variety of plants used as traditional ethanomedicinal at various concentrations.

Anthelmintic activity of saponins has been observed by Makkar *et al.,* (2007). Conjugated unsaturated system of selected saponins is involved in the formation of free radicals, which induces membrane damage through peroxidation of membranes in helmiths (Nandi *et al.,* 2004). Saponins enhance the cell membrane lipid peroxidation (Babu *et al.,* 1997). About 60% of plant materials are composed of liquid, containing mostly sapogenins, the non-glycosidic portion of saponins having significant anthelmintic activity (Silveira *et al.,* 2012).

The results of the present study clearly indicated that *N. tabacum* showed strong anthelmintic activity against different stages of gastrointestinal nematodes of goats. Therefore, it can be used as an alternative of chemical drugs in controlling gastrointestinal nematodosis in goats especially against anthelmintic resistant gastrointestinal nematodes under natural conditions.

## Acknowledgements

The authors wish to express gratitude to the Director, Experiment Station and Dean, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture and Technology, Pantnagar for giving financial assistance and all the necessary facilities during the entire course of the study.

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**How to cite this article:**

Sushmita Sastya, Rajeev Ranajan Kumar and Stuti Vatsya. 2017. Evaluation of Anthelmintic Efficacy of *Nicotiana tabacum* against Gastrointestinal Nematodes of Goats. *Int.J.Curr.Microbiol.App.Sci.* 6(10): 780-789. doi: https://doi.org/10.20546/ijcmas.2017.610.094