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In-vitro Evaluation of Anthelmintic Activity of *Nicotiana tabacum* Extracts against *Haemonchus contortus*

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Abstract: Gastrointestinal parasite *Haemonchus contortus* is one of the most pathogenic and prevalent nematode parasite of sheep in India, causing huge economic losses. The present study was conducted to evaluate the anthelmintic activity of *Nicotiana tabacum*. Acetone extract was prepared by subjecting dried plant material to cold maceration method. *In-vitro* anthelmintic tests like adult motility test (AMT) and egg hatch assay (EHA) were performed at varying concentrations for evaluation of anthelmentic activity. In adult worm motility test, different dilutions (10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ ml 0.625 mg/ml, 0.31mg/ml and 0.156 mg/ml) of extracts were tested and piperazineadepate (12 mg/ml) was used as positive referral and PBS as negative control. At higher dose (10 mg/ml), worms were completely paralyzed within minutes of exposure to *Nicotiana tabacum.* As the concentration of the compounds decreased the degree of immobilization got delayed in all the treatment groups. The total time taken for mortality of worms with reference drug piperazineadepate (12mg/ml) was 5 min. In EHA revealed that the IC_{90}value for 3.581 mg/ml and 2.413 mg/ml aqueous extract and acetone extract respectively and it was 2.362 mg/ml for albendazole.
Keywords: *Nicotiana tabacum*, Anthelmintic activity, *Haemonchus contortus* Adult motility test, Egg hatch assay.

INTRODUCTION

In India, major proportion of farmers depends on agriculture for livelihood and prefers dairy or small ruminant rearing as an additional daily income. Sheep population in India is 74.0 millions contributing the 6.8 percent of total world population and 369 metric tons of meat production. Sheep farming in Andhra Pradesh occupies first place with 40.57 percent of total sheep population of India (GOI, 2012).

Haemonchus contortus, a predominant gastrointestinal nematode in the small ruminants throughout the world. At present the use of synthetic anthelmentics is the backbone to control the GI infections. However, improper use of anthelmentics, the helmenths are developing

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resistance to anthelmentics which has become a major problem worldwide and have many negative side effects including residues in food and environmental contamination if these products are improperly used (Singh *et al.,* 2002). To overcome this necessary to develop alternative new drug candidates with different mode of actions. Contrarily, no new anthelmintic class has reached the market for many years. This limitation might be due to high cost of drug development and approval, estimated to be as much as $100 million (Kaminsky *et al.,* 2008). These setbacks lead to exploitation of ethno pharmacology, which remained as an alternative and promising field in supplementing data about plant metabolites with have anthelmintic properties.

There is an urgent need to develop eco-friendly alternatives to chemicals in commercial use. Alternatively, natural bioactive phytochemicals are increasingly used for helmentic control as they have additional advantages such as low toxicity and more eco-friendly nature (Kamaraj et *al.,* 2011)

India has a rich culture of medicinal plants and spices, that include more than 2000 species with high potential abilities for Ayurvedic, Unani, Siddha traditional medicines but only very few have been studied pharmacologically for their potential medicinal value. Medicinal plants with anthelmintic activity have been reported for use in rural India (Sujith *et al.,* 2015). In the current study attempts were made to know the anthelminthic activity of *Nicotiana tabacum* .

***Nicotiana tabacum*/tobacco** (Family Solanaceae) popularly known as Pogaaku (Telugu)/ Hogesoppu (Kannada) is a stout, viscid annual herb growing upto 1-3 m in height. Traditionally it has been used to treat skin diseases, local infections, bronchitis, asthma and inflammation. In India, the leaves of tobacco plant have been used as sedative, antispasmodic, antiseptic, emectic and nacrotic. Tobacco leaves are rich in polyphenols besides containing vegetal oil, flavonoides, alkaloids and citric acid (Sharma *et al.,* 2016).

MATERIALS AND METHODS

Plant extracts preparation

The leaves of *Nicotiana tabacum* was collected from fields of different parts of Andhra Pradesh and identified by the Department of Botany, Sri Venkateswara University, Tirupati. The leaves were washed thrice in water to remove dust. Leaves were shade dried and ground into fine powder using blender. Acetone extract (AE) of each plant material under study was prepared by cold maceration method as previously described by Sindhu *et al.* (2012a). Prepared AEs were refrigerated till further use.

The aqueous leaf extract was prepared as described earlier (Shankar *et al*., 2004). Fifty grams of leaf powder was mixed with 500 mL distilled water and boiled for about 30 min. on a hot plate. The boiled solution was filtered using Whatman No.1 filter paper and clear aqueous extract was obtained. The extract was stored at 4°C until further use. Extracts were subjected for preliminary phytochemical studies.

Adult motility test

The adult motility test (AMT) was performed by slightly modifying to (Sharma *et al.,* 1971) Mature *H. contortus* worms were collected from abomasums of slaughtered infected sheep. Adult motility test (AMT) was conducted in Petri dishes at room temperature (27-30^{0}C). Ten worms were exposed in triplicate to acetone extracts of the *Nicotiana tabacum* at seven different concentrations (10mg/ml; 5mg/ml; 2.5mg/ml; 1.25mg/ml; 0.62mg/ml; 0.31mg/ml; 0.15mg/ml) prepared in PBS and seven different concentrations (6mg/ml; 3mg/ml; 1.5mg/ml; 0.75 mg/ml; 0.375 mg/ml; 0.1875mg/ml; 0.09375mg/ml and 0.046875 mg/ml) of aqueous extract. As positive control, ten worms in triplicate were taken in Piperazine @ 12mg/ml, while PBS alone with worms served as negative control. Inhibition of motility was taken as indication of worm mortality/ paralysis. The observations were taken at regular time intervals until worms in the negative control completely lost their motility.

Egg hatch assay

Adult female worms of *H. contortus* as collected above were washed thoroughly with phosphate buffer saline and triturated in the same. The suspension was centrifuged for 3 min. at 2000 rpm and sediment is retained. Saturated solution of Sodium nitrate was used to re-suspend the sediment. The suspension was subjected for centrifugation as mentioned above and the top most fluid containing eggs was collected. Eggs were washed thrice with distilled water and adjusted to a concentration of 200 eggs/ml, using the McMaster technique.

The egg hatch assay (EHA) was performed by slightly modifying Coles *et al.,* 1992 method. Egg suspension of 1 ml containing approximately 200 eggs was taken in 24 well titration plates. Acetone and aquoeus extracts of *Nicotiana tabacum* were added to plates at different concentrations. As positive control different concentrations of albendazole (2.5; 1.25; 0.62; 0.31mg/ml) were taken into wells, while PBS along with egg solution taken as negative control. The plates were incubated at 28^{0}C for 48 hrs. A drop of Lugol's iodine was added into each well to stop the reaction and number of eggs and L_1 larvae were counted. The experiment was performed in triplicate.

Statistical Analysis

The oviposition inhibition percentage from egg hatch assay was subjected to probit analysis to calculate lethal concentration ($IC_{50}$and IC_{90}) for respective compounds using Statistical Package for Social Sciences (SPSS 19.0 V IBM, Illinois, Chicago). The lethal concentrations were expressed by 95% fiducial limits. The 50 and 90 percent of the maximal oviposition inhibition index was calculated by Regression analysis using Microsoft Excel 2013. The level of significance was set at $p<0.05$. The results from AMT were analyzed with ANOVA using Statistical version.

RESULTS AND DISCUSSION

Adult motility test

The acetone and aquoeus extracts of *Nicotiana tabacum* showed dose dependent anthelminthic activity as the concentration of the compounds decreased the degree of immobilization got delayed in all the treatment groups. At higher dose acetone extract (10mg/ml) of *N. tabacum* exhibited more anthelminthic activity, as worms were completely paralyzed within one minute of exposure and for aqueous extract higher dose (6mg/ml) worms were completely paralyzed within four minute of exposure. At lowest dilution it took 16.33 min, 24.33 min for acetone and aqueous extracts respectively. Total time taken for mortality of worms for the above two extracts remained as 19.67 and 29.0 min (Table 1 & 2). In PBS which acted as negative control, time taken for 100% mortality was 8 hrs. The total time taken for mortality of worms with reference drug Piperazineadepate (12mg/ml) was 5.0 min.

Table 1: Anthelmintic activity of acetone extract of Nicotiana

Concentration (mg/mL)	**Time (min) for paralysis**	**Time (min) for mortality**
10.00	1.00 ± 0.00	1.00 ± 0.00
5.00	2.00 ± 0.00	4.67 ± 0.33
2.50	4.67 ± 0.33	5.67 ± 0.33
1.25	6.67 ± 0.33	9.67 ± 0.33
0.625	10.67 ± 0.33	13.00 ± 0.58
0.312	12.67 ± 0.33	15.00 ± 0.00
0.156	16.33 ± 0.33	19.67 ± 0.33
Piperine 12mg/mL	5.33 ± 0.33	7.67 ± 0.33

Table 2: Anthelmintic activity of aqueous extract of Nicotiana

Concentration (mg/mL)	Time (min) for paralysis	Time (min) for mortality
6.00	4.00 ± 0.58	5.00 ± 0.58
3.00	6.67 ± 0.67	8.00 ± 0.58
1.50	10.33 ± 0.33	12.67 ± 0.33
0.75	13.33 ± 0.67	15.67 ± 0.88
0.375	17.00 ± 0.58	19.33 ± 0.33
0.188	22.00 ± 0.58	25.67 ± 0.33
0.094	24.33 ± 0.33	29.00 ± 0.58
Piperine 12mg/mL	5.33 ± 0.33	7.67 ± 0.33

Egg hatch assay

In EHA, inhibitory concentration estimates of acetone and aquoeus extracts of *Nicotiana tabacum* and albendazole are represented in table 3. Based on IC_{50} and IC_{90}valuesmost effective plants in the order of significance were; acetone extract *N.tabacum* (IC_{50}:1.039&IC_{90}: 2.413mg/ml), *aqueous extract* (IC_{50}: 0.605&IC_{90}: 3.581mg/ml) Acetone extract of tobacco was found to be more lethal than *aqueous extract*. IC_{50} and IC_{90}values of for albendazole (1.050 & 2.362 mg/ml), though it was not statistically significant ($p \geq 0.05$) at IC_{90}.

Table 3: Hatchability inhibition by various plant extracts

Compound	IC_{30}	IC_{50}	IC_{80}	IC_{90}	Slope (SE)	Intercept (SE)	X^2 (df)
Albendazole	0.754 (0.592-0.913)	1.050 (0.865-1.299)	1.788 (1.426-2.552)	2.362 (1.801-3.736)	3.641 (0.121)	-0.078 (0.033)	173.362 (10)
Nicotiana Aqueous Extract	0.292 (0.250-0.336)	0.605 (0.535-0.683)	1.945 (1.665-2.328)	3.581 (2.940-4.523)	1.659 (0.044)	0.362 (0.024)	64.194 (19)
Nicotiana acetone extract	0.736 (0.570-0.899)	1.039 (0.849-1.298)	1.807 (1.426-2.638)	2.413 (1.814-3.939)	1.899 (0.055)	0.636 (0.027)	45.818 (19)

Values are inhibitory concentrations with 95% Fiducial Confidence Intervals in parenthesis
IC: Inhibitory concentration; SE = Standard error; df = degrees of freedom
Probit analysis using IBM SPSS 19.0 V

Over the centuries, plants were used as important source of medicines to treat different aliments of humans and animals. In India earliest records of curative properties of some herbs is documented in Rig-Veda. In this study, the wormicidal activity of the *Nicotiana tabacum*

was validated by conducting AMT and EHA, results demonstrates the wormicidal activity of crude acetone extract of *N.tabacum* was found it as effective as albendazole.

Acetone extraction was employed for separating plant metabolites as it breaks down cell walls, semi polar in nature and is miscible with the pigments. It is very volatile organic solvent, making it easy to isolate any dissolved substance simply by evaporation. In AMT, *H.contortus* worms were exposed to different concentrations of plant extracts and their ability to paralyze/kill the worms was screened. Based on IC_{90} values, Acetone extracts of *N.tabacum* was even found to be as effective as albendazole. Sindhu *et al.,* (2014) tested the wormicidal activity of crude extract of *N.tabacum* and found it as effective as levamisole. *In-vitro* and *In-vivo* tests performed by Iqbal *et al.,* (2006) on aqueous and methanol extracts of *N. tabacum* revealed dose dependent activity against gastrointestinal parasites of sheep.

Phytochemical analysis of tobacco revealed presence of phenols, alkaloids and saponins that have been known for various biological effects. The wormicidal activity of tobacco is mainly attributed to nicotine, a ganglionic stimulant that tends to activate the neuromuscular junctions causing spastic paralysis leading to death. Presence of phenols and saponins. Different parts of *N. tabacum* are widely used for their narcotic, anti-inflammatory, antirheumatic and antiparasitic properties (Nadkarni 1976). Any ganglion stimulant would tend to activate these neuromuscular junctions causing a spastic paralysis in the worms leading to their death and expulsion from the host. Tobacco leaf is known to contain nicotine, a ganglion stimulant (Bowman and Rand, 1980).

The wormicidal activity could be due to its strong corrosive action on cuticle and tegument of helminthes.

CONCLUSION

The present study demonstrated the plant extracts have the have potent anthelmentic activity. This herbal preparations also advisable due to its lower toxicity to environment. Phyto chemical analysis of tobacco revealed presence of phenols, alkaloids and saponins that have been known for various biological effects. The wormicidal activity of tobacco is mainly attributed to nicotine. However, further studies are needed to studies on the toxicity, evaluation of the effect. *In-vivo* condition and the establishment of the recomnded doses for animals are to be recommended.

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