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Phytochemical analysis and *in vitro* anti-nemathelminthic activity of selected ethnoveterinary herbal preparations used by local healers in small ruminants of Tirunelveli district of Tamil Nadu

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ABSTRACT

Small ruminants are of great significance to the Indian economy and provide living for two-thirds of the rural population. Gastrointestinal helminths of small ruminants pose a significant impact on small ruminant production. Inappropriate use of synthetic anthelminthics and very limited quantity of synthetic therapeutic agents favours the risk. Natural plant products are far more diverse than synthetic therapeutic agents and many of them have historically shown to be potential in traditional medicine in the treatment of a variety of gastrointestinal helminths. The present study was conducted in view of validating the anthelminthic activity of herbal preparations used by local healers and farmers of Tirunelveli district of Tamil Nadu. The herbal preparations used by local healers and farmers that were previously documented, was assessed for its anthelminthic activity in the current study. In vitro egg hatch assay was conducted. The study revealed a dose-dependent inhibition of egg hatching. The extracts were subjected to phytochemical analysis to evaluate the phytochemicals responsible for the activity. It was found that flavonoids, sterols, terpenoids, tannins and saponins might be accountable for the activity.

Keywords: *Aloe vera*, Egg hatch assay, Garlic, Neem, Turmeric.

INTRODUCTION

Small ruminants are significant to the Indian economy and provide living for two-thirds of the rural population. Goats and sheep play a significant part in the livelihood of a substantial number of small and marginal farmers as well as landless workers, especially in regions of the rainfed agro-ecosystem [1]. Helminths, which are a variety of parasites that pose a serious health risk to animals and significantly add to the workload of farmers, were extensively managed using pharmaceutical anthelmintics. Sadly, the over use and improper use of anthelmintic medications has resulted in an extremely high level of resistance [2]. Natural plant products are far more diverse than synthetic therapeutic agents and many of them have historically shown to be potential in traditional medicine in the treatment of a variety of gastrointestinal helminths [3]. Thus, the present study aims at *in vitro* assessment of anti-nemathelminthic activity of selected ethnoveterinary herbal preparations used by local healers in small ruminants of Tirunelveli district of Tamil Nadu and qualitative phytochemical analysis of the preparations to know the class(es) of phytochemical(s) responsible for the activity.

MATERIAL AND METHODS

Herbal Plant materials

The herbal plants to be evaluated were chosen according to documentation from local healers [4] and input from the EVM herbal Research and Training Centre, Veterinary University Training and Research Centre, Thanjavur. In this experiment, 13 herbal plants (three groups) were selected for evaluation of their anthelmintic activity against gastrointestinal nematodes of sheep. Plant materials were obtained from local market. The collected plant materials were dried in shade at ambient temperature and ground to powder. The powdered materials were separately stored in dark tightly closed glass bottles for the phytochemical analysis and extract preparations.

Experimental design

The experimental design of the current study has been depicted in the table 1.

Extraction of plant material

Plant extraction methods on selected plants were carried out according to the techniques described by Trese and Evans [5].

Procedure

The air-dried plant materials (5 grams) were roughly ground, macerated in a closed flask for 24 hours with 100 ml of different solvents (distilled water and alcohol), constantly stirred for 6 hours and then left to stand for 18 hours. After that, quickly filtered while taking precautions against solvent loss. Twenty-five millilitres of the filtrate were then dried to dryness at 105°C on a thin, flat-bottomed dish and weighed. The percentage of extractable material was then determined.

Identification of Phytochemical Constituents

Preliminary phytochemical analysis on selected plants was carried out according to the techniques described by Trese and Evans [5] and Kokate *et al.* [6].

***In vitro* egg hatch assay (EHA)**

EHA was conducted according to the techniques and recommendations of the World Association for Advancement of Veterinary Parasitology (WAAVP) for the detection of anthelmintic resistance in nematodes of Veterinary importance [7-9] with slight modifications. In this assay, the ability of the plant extract to inhibit embryonation and hatching of nematode eggs was evaluated. This assay was first described by Le Jambre [10].

Isolation of nematode eggs from faeces

Pooled fecal samples were obtained by mixing several samples collected per rectum from number of sheep. Eggs were isolated by a slight modification of the method described by Jackson *et al.* [11]. Forty milliliters of water were added to the fecal sample and kneaded thoroughly. The macerated faeces were then suspended in 1 liter of tap water and passed through a succession of sieves with progressively smaller mesh sizes (500, 75 and 35 µm).

The nematode eggs-containing retentate from the 35 µm sieve was cleaned, collected in a poly-allomer tube and centrifuged at 1,000 rpm for one to two minutes. The sediment was re-suspended in 10 to 12 ml of saturated sodium chloride solution after the supernatant was removed. The suspension was thoroughly and gently mixed before being centrifuged once more for one to two minutes at 1,000 rpm. The poly-allomer tube was pinched immediately below the meniscus with artery forceps and the material above the clamp was transferred into a 15 ml polystyrene tube and twice cleaned with distilled water. Several isolated eggs were combined to create a 10 ml volume. From this suspension, 100 microliters (µl) were pipetted; eggs counted and re-suspended in such a manner that 100 microliters (µl) of the suspension contained approximately 100 eggs.

Test protocol

Egg hatch assay was performed in 24 well plates as per the method described by Jackson *et al.* [11]. A 100 µl egg suspension (with approximately 100 eggs) was added to the wells in the test plate. To this 10 µl of plant extracts at concentrations of 10, 20, 40 and 80 mg/ml were added to 4 wells followed by the addition of 1890 µl of distilled water to make a volume of 2 ml in each well. Fenbendazole 0.1 µg/ml in the fifth well and distilled water (10 µl) in the sixth well were used as positive and negative controls, respectively. The plate was then incubated at 26 °C for 48 hours. An inverted tissue culture microscope was used to count the larvae and unhatched eggs after each well had received a drop of helminthological iodine. The mean number of eggs and larvae at each concentration was calculated and percentage of hatch was derived using the following formula:

$$\text{Percentage of hatch} = \frac{\text{Number of larvae}}{\text{Number of eggs + Number of larvae}} \times 100$$

Statistical Analysis

The means of different treatment groups were compared with control by one way Analysis of Variance. Critical values are estimated by using Duncan multiple range test using SPSS statistical package, version 17.0.

RESULTS

Extraction of plant material

The extraction yield of different plant groups in both aqueous and ethanol were presented in the figure 1. Variation in extraction yield was observed. The lowest yield was recorded for the ethanolic extract of fruits of Group T3 (1.120 per cent) and the highest yield was for the aqueous extract of Group T4 (19.82 per cent).

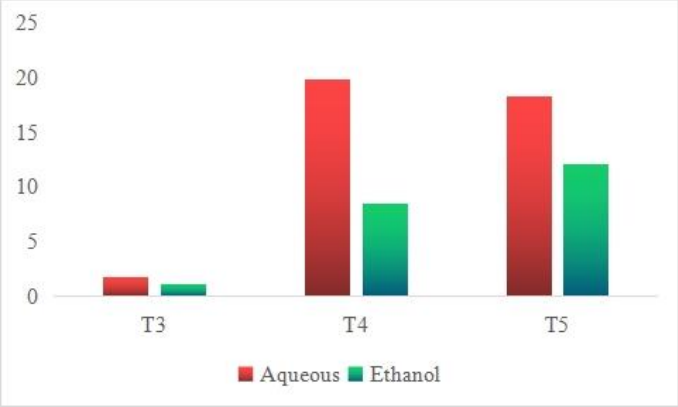

Figure 1: Plant material extraction yield

Phytochemical Analysis of plant preparations

The results of preliminary phytochemical analysis of T3, T4 and T5 were presented in the table 2. Phytochemical analysis of T3 revealed the presence of Flavonoids, Terpenoids, Carotenoids, Tannins, Red Sugar Anthraquinone, Glycoside, Protein and phenol.

***In vitro* evaluation of anthelminthic activity of the extracts**

The *in vitro* study was conducted to evaluate the anthelmintic activity of herb and herbal combination against gastrointestinal nematodes of sheep.

Egg hatch inhibition assay

In the present study, aqueous extracts of *Aloe vera* alone (T3) and combination of herbs with *Aloe vera* (T5) induced significant egg hatch inhibition in a dose dependent manner. Aqueous extracts of T3, T4 and T5 induced 97.75, 78.75 and 94 percent inhibition at 80 mg/ml, respectively. Ethanolic extracts of combination of herbs with

Aloe vera (T5) induced significant egg hatch inhibition of 94.75 percent at 80 mg/ml when compared with positive control (95.50 percent) (figure 2).

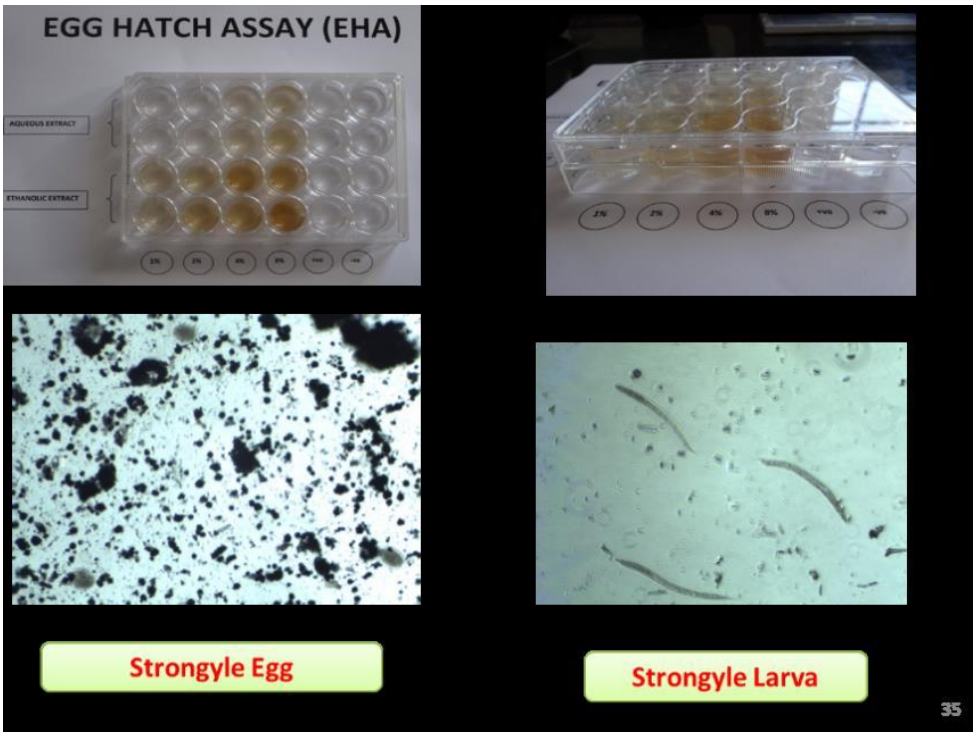

Figure 2: Egg hatch assay

Table 1: Experimental design

T1	Control group (Negative control)			
T2	Fenbendazole group (Positive control)			
T3	*Aloe vera* (Katrallai) whole leaf			
T4				
	1.	*Cuminum cyminum*	Cumins	50g
	2.	*Brassica juncea*	Mustard	50g
	3.	*Curcuma longa*	Turmeric	50g
	4.	*Piper nigrum*	Pepper	50g
	5.	*Allium sativum*	Garlic	50g
	6.	*Trigonella foenum graecum*	Fenugreek	50g
	7.	*Zingiber officinale*	Ginger	50g
	8.	*Leucas aspera*	Thumbai leaves	100g
	9.	*Azardirachta indica*	Neem leaves	100g
	10.	*Musa paradisica*	Banana stem	100g
	11.	*Momordica charantia*	Bitter gourd	100g
	12.	*Andrographis paniculata*	Nila vembu	100g
T5	*Aloe vera* whole leaf with herbal mixture (T3+T4)			

Table 2: Phytochemical analysis of herbal extracts

S. No.	Phytochemical	T3		T4		T5	
		Aqu	Eth	Aqu	Eth	Aqu	Eth
1.	Flavonoids	+	+	+	+	+	+
2.	Alkaloids	-	-	-	+	-	+
3.	Terpenoids	+	+	-	+	+	+
4.	Sterol	-	+	+	+	+	+
5.	Carotenoids	+	+	-	+	+	+
6.	Tannins	+	+	+	+	+	+
7.	Saponin	-	+	+	+	+	+
8.	Red. Sugar	+	+	+	+	+	+
9.	Anthroquinone Glycoside	+	+	+	+	+	+
10.	Protein	+	-	+	+	+	+
11.	Phenol	+	+	+	+	+	+

T3 - *Aloe vera* Whole Plant; T4 - Cumins, Mustard, Turmeric, Pepper, Fenugreek, Garlic, Ginger (each 50 grams), Thumbai leaves, Neem leaves, Banana stem, Bitter gourd, Nila vembu (each 100 grams); T5 - T3+T4; Aqu - Aqueous extract; Eth - Ethanolic extract.

DISCUSSION

Extraction of plant material

The variations in extraction yield may be due to the appropriate solvent-to-solid matrix ratio, optimal extraction temperature and pertinent particle size of the plant material and adequate duration of extraction [12]. The variation might also be a result of other variables including the climate, location, kind of soil, stressors, weather and seasonal variations where the plants are grown [13]. According to Gonfa *et al.* [14], methanol, ethanol and water have similar solubility properties because they contain hydroxyl group which is hydrophilic, however high percentage yield extract were obtained from extraction employing polar organic solvent.

Phytochemical Analysis of plant preparations

Out of the two extracts, the Ethanolic extract had the largest number of phytochemicals. Alkaloids were absent in the aqueous extract but present in all the ethanolic extracts. This can be attributed to the relative insolubility of alkaloids in water as compared to organic solvents. The result of present study is in agreement with Padmanabhan and Jangle [15] for phytochemical evaluation of herbal preparation (A combination of four medicinal plants). The collective or individual presence of phytochemicals in the extracts may possibly constitute the basis for the profound anthelmintic activity exhibited by the plant extracts as opined by Ruben *et al*. [16].

In vitro evaluation of anthelminthic activity of the extracts

Egg hatch inhibition assay

One of the main causes of wastage and decreased productivity in livestock rearing is helminths infection, which manifests itself through mortality, illness, slower growth, weight loss in young ones and late maturity of slaughter animals. Additionally, it impoverishes animals and damages their guts, resulting in anaemia, diarrhoea, anorexia, gastroenteritis, abdominal distension, emaciation, decreased feed intake and nutrient absorption, decreased milk and meat output, and decreased working ability, mostly in underdeveloped nations [17]. Anthelmintic have been the mainstay of sheep nematode parasite control for many years. However, the parasitic worms that infect animals have continuously and significantly contributed to the development of resistance over time [2]. Consequently, the discovery and development of new chemical substances for helminths control is greatly needed and has promoted studies of traditionally used anthelmintic plants, which are generally considered to be very important sources of bioactive substances [18].

Meenakshisundaram *et al.* [19] reported the anthelmintic activity of aqueous and ethanolic extract of *Aloe vera* with an ED_{50} of 0.5 mg/ml. Similarly, Ahmed *et al.* [20] evaluated *in vitro* anthelmintic activity of several plant extracts including *Allium sativum* and *Zingiber officinale* and reported a dose-dependent inhibition of egg hatching. Lavanya *et al.* [21] investigated and reported the *in vitro* anthelmintic activity of *Brassica juncea* and *Brassica oleracea.* Singh *et al.* [22] reported the anthelmintic activity of *Curcuma longa* and its synergistic activity with *Zingiber officinale* in a study with *Pheretima posthuma.* Kayesh *et al.* [23] reported the anthelmintic activity of aqueous and ethanolic extracts of leaves of *Leucas aspera* using *Pheretima posthuma,* which was found to be less than albendazole. Rabiu and Subhasish [24] reported the anthelmintic activity of *Azardirachta indica* using *Pheretima posthuma, Ascaridia galli* and *Raillietina spiralis,* with highest activity (36 % inhibition) at 40 mg/ml. Venkatesh *et al.* [25] reported the anthelmintic activity of *Musa paradisiaca* using *Pheretima posthuma* as experimental model and found that 100 mg/ml showed significant activity. Vinav *et al.* [26] reported the *in vitro* anthelmintic activity of *Momordica charantia* using *Eisenia foetida* as experimental model. They reported that the presence of phytochemicals like alkaloids, steroids and triterpenoids might be responsible for the activity. Raju *et al.* [27] reported the anthelmintic activity of *Andrographis paniculata*. Thus, these studies support the anthelminthic activity of the individual herbal plants. Kumar *et al.* [28] reported that anthelmintic activity of combination of *Amaranthus spinosus, Amaranthus caudatus* and *Amaranthus viridis* was more than the individual components.

CONCLUSION

Thus, from the present study ethanolic extract of herbal combinations (T5) showed better activity than T3 and T4, though aqueous extract showed a non-significant difference in activity. However, the activity of the herbal preparations was less than the control drug, fenbendazole. This may be presumably due to small concentrations of the active ingredient in the plant extract.

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Conflicts of Interest

The authors declare no conflict of interest.

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