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RESEARCH ARTICLE

In vitro anthelmintic activity and phytochemical analysis of for tropical plants against *Haemonchus contortus*

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Abstract

The present study was conducted to test anthelmintic activity of various plants against *Haemonchus contortus*. The experimental study included the *in vitro* anthelmintic trials against adult parasites, larval stages and eggs of *Haemonchus contortus.* The adulticidal assay results revealed that adulticidal activity was maximum for *Annona squamosa* leaves MeOH (methanolic) extract (LC_{50} 48.6 mg/ml at 1 hour post exposure) followed by *Eucalyptus globulus* leaves MeOH extract (LC_{50} 50.81 mg/ml at 1 hour post exposure). The maximum larvicidal activity was shown by *Catharanthus roseus* leaves MeOH extract (LC_{50} 11.30 mg/ml at 2 hour post exposure). Egg hatch assay revealed that the maximum ovicidal activity was attributed to *Eucalyptus globulus* leaves MeOH extract (LC_{50} 7.06 mg/ml) and *Annona squamosa* leaves MeOH extract (LC_{50} 5.8 mg/ml). *Syzigium cumini* was having minimum anthelmintic activity among selected plants. Anthelmintic activity of these plant extracts may be attributed to presence of alkaloids, tannins and essential oils in the crude extracts. The qualitative phytochemical analysis of the methnolic extracts of *Eucalyptus globulus* and *Annona squamosa* leaves showed the presence of these phytochemical constituents. TLC analysis showed the presence of various constituents at different Rf values.

Introduction

Anthelmintics alone cannot combat ever-changing strains of *H. contortus*. The current state of crisis regarding widespread anthelmintic resistance calls for alternative control strategies. A multifaceted approach that includes pasture management, careful planning and observation, and individualized treatment is required to control gastrointestinal helminthes. To circumvent the problem of drug resistance, the only realistic strategy would be to develop novel non-chemical approaches that decrease the need of treatment and to use the anthelmintics that remain effective in a more intelligent manner (Sanyal, 2005). The goal of alternative control strategy is to develop methods or products that can be used in place of commercial dewormers to reduce dependency on them. One of the effective methods is to use herbal products which are having anthelmintic property. Major benefits of herbal dewormers can include broad spectrum activity, non-toxic with huge margin of safety to the animals, cost effectiveness, cultural acceptability and supplementary effects on animal health. For centuries, plants provided mankind with useful sometimes lifesaving drugs. The importance of herbal medicine can be realized from their prominence in

prescription and drug markets. In the last century the sources of different drugs expanding including a range of different types of plants as well as animals. Medicinal plants have formed the basis of traditional medicine system. In Indian subcontinent, Ayurvedic and Yunani medicine system are very popular and are in use for centuries.

Materials and Methods

Selection of plants: Four plants were selected for present study on the basis of basic ayurvedic knowledge and local availability of plants in the area.

Plant material collection and extraction: Leaves of *Annona squamosa* (Custard apple, Sarifa), *Eucalyptus globulus* (Safeda, Eucalyptus), *Syzygium cumini* (Jamun) and *Catharanthus roseus* (Sadabahar) were collected form herbal garden maintained in CIRG, Makhdoom. Leaves were dried in shade. After drying, leaves were powdered in a grinder and stored at room temperature for further processing. Crude extract was prepared by using Methanol (SRL lmtd.) as solvent. About 100 gm of the coarsely grounded plant material was taken in a porous cellulose thimble and placed in soxhlet extractor (ASGI, India) with 3000 ml flask containing about 2500 ml solvent at a temperature 60±5˚C. The extraction was allowed to continue for 20-22 cycles. Solvent was evaporated using rotary evaporator (Heidolph, Germany) and extract were stored in refrigerator (LG, India) at -20˚C for further use.

Collection of parasites: The abomasum and its content of goats were collected from Municipal Corporation Slaughter House situated in Nalman Chauraha Mandi, Agra (U.P.) and postmortem room CIRG, Makhdoom for gross examination and recovery of the alimentary nematodes according to the techniques described by Urquhart *et al*. (1996). Adult worms were washed and collected in Ringer's Lactate solution and stored at 370 C for further tests.

***In vitro* Studies:** Three replicates of each concentration were used in the following procedures and average values were used for final calculations. The viability of parasitic stages in ringer's lactate solution were recorded and taken as negative control in the experiments. Following *in vitro* methods were conducted in the experiment.

Egg Hatch Assay: The egg hatch test was described by Le Jambre (1976) and a standardized protocol was adopted by the World Association for Advancement of Veterinary Parasitology (Coles et al., 1992).

Larvicidal Activity: In this study larval development test was performed by the method described by Hubert and Kerboeuf, (1992). L3 stages were obtained from faecal culture of eggs obtained from adult parasites and incubated for 7 days. 50 live larvae were exposed to different concentration of ectracts. The percentage dead count and LC_{50} concentrations were calculated.

Adulticidal Assay: Adulticidal assay was conducted on mature live *Haemonchus contortus* following procedure described by Sharma *et al.* (1971). The LC_{50} values were calculated by probit analysis using SPSS 17 software.

Phytochemical analysis: Qualitative analysis was done as described by Debela, 2002 and thin layer chromatography was done to determine Rf (Retention Factor) values of various constituents. Rf values are used to quantify the movement of various compounds present in crude extract according to their polarity along the plate and it is calculated as distance travelled by the substance/ distance travelled by solvent (solvent front) along the plate.

Results

Yield of Extracted Plant Materials: The percentage yield of *Eucalyptus globulus* (leaves) was highest (27%), while it is lowest for *Syzygium cumini* leaves (18.4%). The details of all extracts about percent yield and physical characteristic are given in table 1.

S. No.	Common names	Botanical names	Physical characteristics and per cent yield			
			Nature	Color	Odour	% Yield
1	Jamun	*Syzygium cumini*	Sticky	Black	Peculiar	18.4
2	Safeda	*Eucalyptus globulus*	Oily, Sticky	Blackish green	Aromatic	27
3	Sarifa	*Annona squamosa*	Oily, sticky	Greenish Black	Aromatic	19.6
4	Sadabahar	*Catharanthus roseus*	Oily , sticky	Blackish green	peculiar	19

Table 1: Percentage yield of different plants using alcoholic extraction methods and physical property of extract.

Anthelmintic activity

Ovicidal activity: The effective doses required to induce 50% inhibition of hatching are calculated by probit analysis and given in table 2. The percentage inhibition of hatching of eggs exposed to *Annona squamosa* leaves MeOH extract recorded was 87.95, 93.46, 94.97, 96.96, 98 and 100 at 1.56, 3.125, 6.25, 12.5, 25 and 50 mg/ml respectively and LC_{50} value calculated was 5.8 mg/ml. The results of percentage mortality are portrayed in table 3. The comparison of ovicidal activity revealed that *Annona squamosa* and *Eucalyptus globulus* has maximum anthelmintic activity on the basis of egg hatch assay.

S. No.	Plant Extract	LC 50(mg/ml)
1	***Eucalyptus globulus* leaves MeOH**	46.6
2	***Annona squamosa* Leaves MeOH extract**	6.4
3	***Syzigium cumini* Leaves MeOHextraxt**	51.2
4	***Catharanthus roseus* Leaves MeOH**	82.7

Table 2: In vitro anthelmintic activity of plant extracts and expressed in LC_{50} on the egg of *Haemonchus contortus* exposed for 48 hours

	negative control	1.56	3.125	6.25	12.5	25	50
Annona Squamosa	38.48485	87.95181	93.46939	94.97487	96.9697	98	100
Eucalyptus globulus	41.66667	69.35484	75	80.26316	84	87.5	100
Syzigium cumini	42.91262	73.86935	87.80488	85.3211	97.08738	98.85057	100
Catharanthus roseus	25.70776	66.87898	84.84848	88.4058	97.32143	99.32432	100

Table 3: The percentage inhibition of egg hatch of Haemonchuscontortus after 48 hours exposure to different concentration of plant extracts (mg/ml)

Larvicidal assay: The effective LC_{50} doses for larvae exposed to herbal extract are portrayed in table 4. The maximum larvicidal activity was of *Catharanthus roseus* leaves with LC_{50} concentration 11.30 mg/ml after 2 hours post exposure. The minimum activity was shown by *Syzygium cumini* herbal extract.

Plant	Time(hour)	LC50(mg/ml)
Annona squamosa	2	31.39
	3	20.97
	4	13.14
Eucalyptus globulus	2	33.53
	3	17.59
	4	11.89
Catharanthus roseus	2	11.30
	3	7.95
	4	3.44
Syzigium cumini	2	65.31
	3	47.86
	4	34.25

Table 4: LC_{50} of selected plants against larval stages (larvicidal activity) of *H. contortus* at different time intervals.

Adulticidal activity: The effective LC_{50} dose required to kill 50 % of adult parasites exposed to herbal extract are portrayed in table 5. The *Annona squamosa* and *Eucalyptus globulus* are having most potent adulticidal activity with LC_{50} concentration 48.62 and 50.81 mg/ml at 1 hour exposure respectively.

Plant	Time	LC50
Annona squamosa	1 hour	48.62
	2 hour	22.71
	3 hour	15.32
Eucalyptus globulus	1 hour	50.81
	2 hour	39.68
	3 hour	29.15
	6 hour	21.76
Catharanthus roseus	1 hour	105.61
	2 hour	35.90
	3 hour	28.95
	6 hour	27.06
Syzigium cumini	3 hour	61.61
	4 hour	45.25
	5 hour	39.45
	6 hour	30.44

Table 5: Adulticidal assay LC_{50} of various plant extracts against *H. contortus*adult parasites

Phytochemical analysis

Phytochemical analysis of different extracts were conducted by different tests to know the presence of (active constituents) alkaloids, flavonoids, saponins, steroids, carbohydrates, glycosides, tannins, phenolic compounds, protein, amino acids and triterpenoids. Phytochemical analysis of methanolic extract of *S. comuni* (leaves) revealed that alkaloids, flavonoids, tannins and fixed oils were present in it. The methanolic extract of *A. squamosa* (leaves) showed presence of flavonoids, tannins and alkaloids. Methanolic extract of *E. globulus* (leaves) showed the presence of alkaloids, flavonoids, fixed oils and tannins during analysis. The methanolic extract of *Catharanthus roseus* was positive for alkaloids, flavonoids and tannins. The detailed results are portrayed in table 6.

The TLC plate was developed for the plant extracts namely *A. squamosa*, *E. globulus*, *S. cumini* and *C. roseus* using Merck HPTLC plate on mobile phase n Hexane: acetone (75:30). The plate was run for 15 minutes to get a solvent front of 7.1 cm. The TLC plate developed for *A. squamosa* (leaves) showed four components with Rf values of 0.10, 0.53 0.74 and 0.89 under UV light at 366 nm wave length, while in case of *E. globulus* (leaves) four components were detected in TLC plate with Rf values of 0.09 0.70 0.77 and 0.86. TLC plate developed for *S. cumini* showed four components with Rf values 0.029, 0.57, 0.77 and 0.86. Similarly *C. roseus* showed presence of five components with Rf values 0.16, 0.50, 0.61, 0.77 and 0.86. The results are portrayed in table 7 and figure 1.

S.No.	Plant extract	Chemical constituents							
		Alkaloids	Flavonoids	Saponins	Carbohydrates	Tannins	Glycosides	Fixed oils	Protein & A.Acid
1	***Syzigium comuni* leaves MeOH extract**	+	+	-	-	-	-	+	- -
2	***Annona squamosa* Leaves MeOH extract**	+	+	-	-	+	-	-	-
3	***Eucalyptusglobulus* leaves MeOH extract**	+	-	-	+	+	-	+	-
4	***Catharanthus roseus* Leaves MeOH**	+	+	-	-	+	-	+	-

Table 6: Phytochemical analysis of different herbal extracts for presence of various active constituents

***Eucalyptus globulus* leaves MeOH extract**	***Syzigium comuni* leaves MeOH extract**	***Annona squamosa* Leaves MeOH extract**	***Catharanthus roseus* Leaves MeOH**
0.09	0.029	0.10	0.16
0.70	0.51	0.53	0.50
0.77	0.77	0.74	0.61
0.86	0.89	0.89	0.77
			0.86

Table 7: Rf values of various spots detected under 366 nm on TLC plate (Sovent front= 71 mm). Sovent system used was n-Hexane:Acetone(75:35).

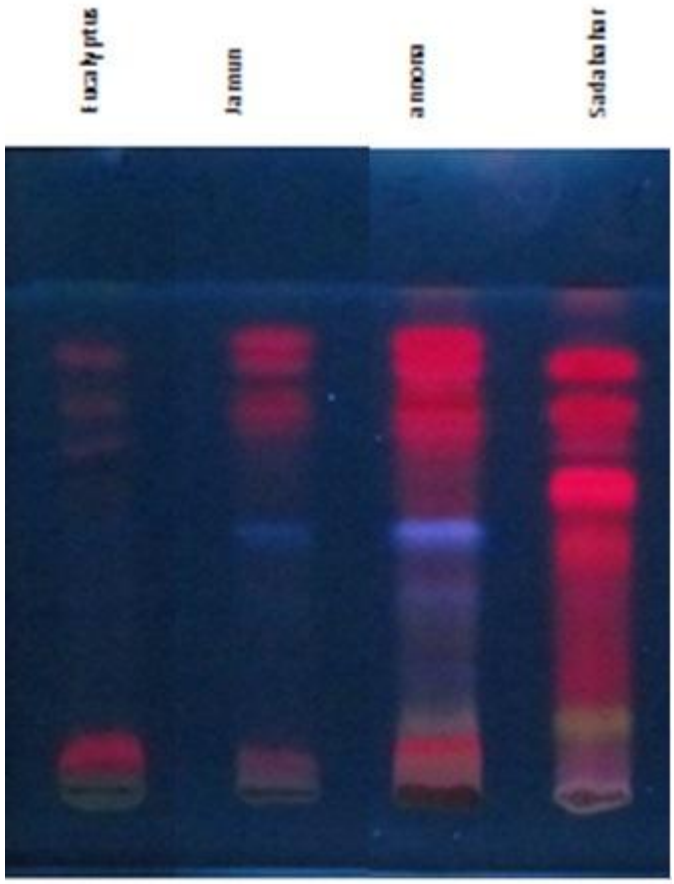

Figure 1: TLC plate developed (Mobile phase: n-Hexane: Acetone) for various extract under study visualised under 366 nm.

Discussion

The botanical extracts from the plant leaves, roots, seeds, flowers and bark in their crude form have been used as conventional anthelmintics for centuries and are still used for this purpose in many parts of the world (Anthony *et al*., 2005). Much effort has been focused on plant extracts or phytochemicals as potential sources of gastrointestinal nematodes-control agents (Hordegen, 2006; Eguale *et al*., 2011). The adulticidal activity assay of various plant extracts revealed that the methanolic extracts of *A. squamosa* and *E. globulus* leaves had lowest LC_{50} concentration at one hour exposure i.e. 48.63 and 50.81 mg/ml respectively. Other plants of the study i.e. *C. roseus also* showed fair amount of adulticidal activity with LC_{50} value 105.61 mg/ml 1 hour post exposure. The adulticidal activity may be attributed to presence of condensed tannins and fixed oil present in these plant extracts. The anthelmintic activity of herbal extracts was reported by various workers in India and abroad whose results comply with present study. Some of them are Nwude and Ibrahim, 1980; Minja, 1989; Akhtar *et al*., 1999; Iqbal *et al.*, 2001 and Iqbal *et al*., 2002. The anthelmintic activity of herbal extracts has been reviewed by Akhtar*et al.*, 2000.

The larvicidal activity test and Egg hatch assay results showed that the methanolic extracts of *A. squamosa* and *E. globulus* leaves have potential anthelmintic efficacy while other plant extracts used in study showed moderate to low activity. Hordegen *et al.* (2006) screened extracts or ingredients of six different plant species and tested

against exsheathed infective larvae of *H. contortus* and showed an anthelmintic efficacy of up to 93 percent, relative to pyrantel tartrate. Costa *et al.* (2008) tested ethyl acetate and ethanol extracts of *A. indica* on *H. contortus* eggs and larvae and ethanol extract was found more effective, inhibiting egg hatching by 99.77 percent at 3.12 mg/ml and larval development by 87.11percent at 50 mg/ml. These variations in *in vitro* activity may due to difference in method and type of extraction and variation in collection time, maturity ofseed or leaves which ever was taken, composition of soil and different climate or geographical area in which plant is grown (Paech and Tracey 1955). Efficacy of plants can vary with the growth stage, season, soil and climate etc. In consideration to above factors proper time of collection and processing of plant materials should be standardised.

Phytochemical analysis of selected crude plant extracts reveals the presence of Alkaloids, flavonoids, tannins and fixed oils in *E. globulus, A. squamosa* and *C. roseus* leaves extract. The presence of these constituents contributes to anthelmintic activity of plants. Foo *et al*., 1996 reported that there is a negative relationship between dietary condensed tannins concentration and abomasal nematode numbers suggest that the concentration of condensed tannins may be responsible, but the possibility remains that the structure (or plant origin) of condensed tannins may also exert an effect. Feeding forages containing condensed tannins, such as Sulla and *L. pedunculatus*, significantly increased the growth rate of parasitized lambs (Niezen *et al.*, 1995)

Thin layer chromatography of selected plants showed 4 to 5 constituent in each plant extracts. The separation of constituents was based on the polarity of compounds. Compounds with less polarity travels farther on the TLC plate. Standardization and characterization of herbal drugs is a topic of continuous scientific interest in the herbal drug industry. With the advent of modern chromatographic systems there is an ever increasing intent to produce and develop easy, rapid, convenient and cost effective methods for standardization of plant constituents (Selvamani *et al.*, 2009).

Conclusion

Ancient literatures described the use of various plant extracts and components for the treatment of endoparasitism and other diseases. Present study indicates that the plant constituents can give promising results if included in control strategies against parasitic infections. To conclude, in future studies, there is need of phytochemical and clinical trials to determine efficacy of these extracts. Molecular mechanisms of action can be explored in near future. Efforts should be made to standardise the plant extracts with good anthelmintic activity and formulate best herbal preparations to replace or complement the synthetic drugs which are currently in use.

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