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Anthelmintic efficacy of *Glycyrrhiza glabra* on the nematode *Haemonchus contortus* – In vitro study

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***Abstract: Haemonchus contortus*, the twisted stomach worm is the most common and the most destructive of the gastrointestinal helminth. It is found in the abomasum of the sheep and goat. Chemical control of helminths coupled with improved management has important worm control strategy throughout the world. However, increasing problems of development of resistance in helminths against anthelmintics have led to the proposal of screening of medicinal plants for their anthelmintic activity. In the present investigation, the anthelmintic efficacy of *Glycyrrhiza glabra* was assessed based on its effect on the motility of *Haemonchus contortus*. In the present study, hexane, chloroform, ethyl acetate, ethanol and aqueous extracts of rhizome of *Glycyrrhiza glabra* were tested for their efficacy against the nematode, *H. contortus*. Adult live worms were collected from the abomasum of the sheep and were maintained *in vitro* in Hedon-Fleig solution (pH 7.0) at 37°C. The worms were exposed to various concentrations (1, 3 and 5 mg/ml) of the plant extracts for 24 h. Based on the gross visual observation on the motility of the worms in various concentrations of different extracts, ethanol extract of rhizome of *Glycyrrhiza glabra* were selected for motility test. Five different sub-lethal concentrations of ethanol extract of rhizome of *Glycyrrhiza glabra* were selected for further studies. Quantitative measure on the motility of the treated parasites using Electronic Micromotility Meter (EMM) confirmed the inhibitory effect of *Gg*EE on *H. contortus*. Motor activity of *H. contortus* was inhibited in *Gg*EE treated worms at 0.5 mg/ml after 8 h of exposure. The worms did not show any revival after 8 h and the worm death was confirmed by their pale white colour appearance. Active motility was observed in the untreated control worms throughout the period of incubation. The inhibition of the motility is directly proportional to the concentration of the extracts and period of incubation and maximum inhibition occurred in the highest concentration after 8 h of exposure. GgEE possesses a remarkable anthelmintic activity against *Haemonchus contortus*. It may serve as an alternative for anthelmintic chemotherapeutic agents and could be used in safe and eco friendly manner. However, this study warrants further *in vivo* studies for practical utility.**

***Keywords: Haemonchus contortus*, *Glycyrrhiza glabra* and Electronic Micromotility Meter (EMM).**

I. INTRODUCTION

Livestock play an important role in the socio-economic life of India. India has the largest livestock population in the world, which contributes nearly 7% towards its national income. The sheep, an important economic livestock species mostly reared for meat and wool. Sheep rearing provides livelihood supports in terms of income and employment to millions of land less, for the marginal and small pastoralist. Sheep manure is an important source of organic fertilizer and contributes to a part of earning of a farmer, particularly in India (Reddy and Rao, 2009). Intestinal parasites are one of the major problems in the world, especially in tropical and subtropical countries. The intestinal infection would be due to protozoan or helminth parasites (Tariq and Tantry, 2012). Many species of parasites are seen in sheep and goats and usually include *Haemonchus*, *Oesophagostomum*, *Ostertagia*, *Cchabertia*, *Nematodirus*, *Trichuris*, *Moniezia* and *Fasciola*. *Haemonchus contortus* it is considered to be the most important parasite of sheep and goats and is the single most important constraint to sheep production in India (Vatta *et al*., 2001).

Haemonchus contortus is a nematode belonging to the Phylum Nemathelminthes, Class Nematoda, Super Family Trichostrongyloidea and Family Trichostrongylidae (Dwight, 2003). Parasite management is the first line defense against worm infection. It is usually done with chemical anthelmintics. Chemotherapy is a major treatment modality used for the control of helminth infection in livestock. However, the indiscriminate use of these drugs without appropriate association with other methods to fight parasite infection has favoured the development of helminth resistance to various drugs in the gastrointestinal tract of small ruminants (Morales and Pino, 2001). Synthetic anthelmintic treatments are often impracticable in developing countries due to relatively high price of these anthelmintics (Tariq and Tantry, 2012). This danger has given impetus to the search for new drugs, with attention focusing on the search for plant products and the application of plant products as alternative methods of control. Naturally produced plant anthelmintics offer an alternative that can overcome some of these problems and is both sustainable and environmentally acceptable (Gasbarre *et al*., 2001). A large number of plant products are being used to combat gastro-intestinal parasites of livestock (Kozan *et al*., 2006; Lyndem *et al*., 2008; Al-Shaibani *et al*., 2009; Kosalge and Fursule, 2009; Manolaraki *et al*., 2010). In the present study, anthelmintic efficacy *Glycyrrhiza glabra* was investigated against the nematode *Haemonchus contortus.*

Glycyrrhiza glabra commonly known as Atimaduram in Tamil belongs to the family Leguminosae. It is one of the most widely used herbs from the ancient medical history of Ayurveda, both as a medicine and also as a flavoring herb. *Glycyrrhiza glabra* possesses biological activities such as anti-inflammatory, antiviral, anticancer, anti-ulcer, anti-diabetic, anti-oxidant, anti-thrombic, anti-malarial, anti-fungal, anti-bacterial, immune-stimulant, antithrombotic, anticonvulsant and anti-allergenic (Damle, 2014). *Glycyrrhiza glabra* found to contain important phytoconstituents such as glycyrrhizin, glycyrrhizinic acid, glabrin A and B and isoflavones.

All effective anthelmintics directly or indirectly affect the motility of the worms. Motility of helminths is a good indicator of worm's viability and can serve as a valuable parameter for evaluation of the spectrum of sensitivity of a specific helminth species to a variety of anthelmintics. Hence, motility is considered as an important parameter in evaluating the efficacy of drugs. Visual assessment of motility involved observation for presence of free spontaneous movement in medium (Bowen and Vitayavirasak, 2005). However, interpretation of anthelmintic property by visual observation is very time consuming, demanding close microscopical examinations and the results are also highly variable. Electronic micromotility meter provides a more dependable quantitative measure of the motor activity of drug-treated parasites and could ascertain the effects of the drug on the parasites (Veerakumari, 2003). Hence, the present *in vitro* investigation was designed with an aim to elucidate the anthelmintic potential of *Glycyrrhiza glabra* against the nematode, *Haemonchus contortus.*

II. MATERIALS AND METHODS

In the present investigations, anthelmintic potential of *Glycyrrhiza glabra* against the nematode, *Haemonchus contortus* was studied *in vitro*

In vitro maintenance of *Haemonchus contortus*

Adult live worms were collected from the abomasum of the sheep (Fig. 1) slaughtered at a local abattoir in Chennai (Figs.1 and 2) The worms were washed thoroughly in physiological saline and maintained in Hedon-Fleig solution (pH 7.0).

Preparation of Hedon-Fleig solution

Hedon-Fleig solution (pH 7.0) is the best medium for *in vitro* maintenance of *C.cotylophorum* (Veerakumari, 1996). It is prepared by dissolving 7 g of sodium chloride, 0.1 g of calcium chloride, 1.5 g of sodium bicarbonate, 0.5 g of disodium hydrogen phosphate, 0.3 g of potassium chloride, 0.3 g of magnesium sulphate and 1 g of glucose in 1000 ml of distilled water.

Collection of plant material

The roots of *Glycyrrhiza glabra* (Fig. 2) were purchased from Lakshmi stores, Chennai - 600 003. The plant materials obtained were identified and authenticated by a botanist in the Department of Botany, Pachaiyappa's College, Chennai. The vouchered specimens are deposited at Pachaiyappa's College, Chennai – 600 030.

Extraction of roots of *Glycyrrhiza glabra*

The rhizome of *Glycyrrhiza glabra* (Fig.3) was cleaned, shade dried and coarsely powdered. Successive solvent extraction was done by cold percolation method (Harborne, 1998) by soaking in hexane, chloroform, ethyl acetate and ethanol successively in an aspirator bottle for 48 h. Aqueous extracts of nuts of roots of *Glycyrrhiza glabra* were also prepared. After 48 h, the extracts were filtered by Whatman Filter paper No.1. The solvent was removed by distillation using Evator Rotary Evaporator (Fig.4) and the extracts were concentrated and dried in Lyodel Freeze Dryer (Fig.5).

Gross visual observation on the motility of the worms incubated in various plant extracts

Twenty five millilitres of Hedon-Fleig solution containing various concentrations (1, 3 and 5 mg/ml) of the aqueous and solvent extracts were individually distributed in 25 ml conical flasks and five worms were maintained in each flask. The activity of the worms was checked at various intervals (5, 15, 30 min, 1, 2, 4, 6, 8, 12, and 24 h) of exposure. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extracts. Based on the motility of the worms, the observations were categorised as very active (++++), moderately active (+++), slightly active (++), sluggish (+) and dead (-). The wormswith no movement were regarded as dead. Based on the motility of the parasite for 8 h of exposure, five different sub-lethal concentrations were selected. The parasites were incubated for 2, 4 and 8 h in the various sub-lethal concentrations of the plant extracts for further *in vitro* studies.

Quantitative measurement of motility of wormsusing Electronic Micromotility Meter (EMM)

EMM has been found to be highly sensitive, and suitable for *in vitro* quantitative assay of the motility of helminths. In the present study, the motility of the control and treated worms was measured. The percent inhibition of motility of drug-treated worms was calculated using the formula,

$$\% \text{ inhibition of motility} = \frac{C-T}{C} \times 100$$

where,

C - deviation of voltage signal in the control worms

T - deviation of voltage signal in the fluke treated with plant extracts

Statistical analysis

Statistical analyses were performed with the statistical program for the social sciences SPSS version 16.0. The significance of drug induced inhibition in the motility of the worms was assessed using analysis of variance (ANOVA) for different concentrations of ethanol extract of *Glycyrrhiza glabra* (*Ga*EE). Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation ($P < 0.05$) using Bonferroni test. Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants ($P < 0.01$) using Bonferroni tes

III. RESULTS AND DISCUSSION

The present study elucidated the anthelmintic potential of *Glycyrrhiza glabra* against the nematode, *Haemonchus contortus* was studied *in vitro*. Preliminary investigations on the efficacy of the aqueous, hexane, chloroform, ethyl acetate and ethanol extracts of *Glycyrrhiza glabra* (Table 1) on the flux of *Haemonchus contortus* was studied. The control worms were highly motile and active throughout the experimental period, whereas the movement of the drug–treated worms was severely curbed. It is evident from Table 7 that ethanol extract of *Glycyrrhiza glabra* were effective in decelerating the motility of *Haemonchus contortus*.

The effect of ethanol extract of *Glycyrrhiza glabra* on the motility of *Haemonchus contortus* was quantitatively measured using electronic micromotility meter. Ethanol extract of *Glycyrrhiza glabra* showed inhibitory effect on the motility of the worms. The inhibition of the motility is directly proportional to the concentration of the extracts and period of incubation (Table 2; Fig. 3) and maximum inhibition occurred in the highest concentration after 8 h of exposure. The inhibitory effects of ethanol extract of *Glycyrrhiza glabra* on the motility at different concentrations, for each period of duration are significant ($P < 0.05$). The inhibitory effects of the extracts between the different hours of incubation are significantly different for each concentration ($P < 0.01$) and it is clear that ethanol extract of *Glycyrrhiza glabra* exhibited an excellent parasiticidal activity against *Haemonchus contortus*.

In vitro assays provide cheaper, economical and rapid turnover in contrast to in vivo assays as for as anti-parasitic properties of plants and plant extracts are concerned. *In vitro* tests determine the effects of anthelmintic drugs on physiological processes like hatch, development, mortality and motility of the adult and larval helminth parasites. In this study, the effect of aqueous and various solvent extracts of *G. glabra* against *H. contortus* was assessed visually based on their effect on the motility of the worms. Depending on the concentration of the test medium, drug-treated worms showed a complete loss of movement at varying intervals. *Gg*EE inhibited the motor activity of *H. contortus* at 0.5mg/ml concentration. Similarly, aqueous and ethanol whole plant extracts of *Actinopteris radiata* exhibited more potential activity at higher concentration in *H. contortus* (Carvalho *et al*., 2012; Kumarasingha *et al*., 2016). Motor activity of *H. contortus* was inhibited in *Gg*EE treated worms at 0.5 mg/ml after 8 h of exposure. The worms did not show any revival after 8 h and the worm death was confirmed by their pale white colour appearance. Active motility was observed in the untreated control worms throughout the period of incubation.

Rainietina echinobothrida, a cestode parasite exposed to different concentrations of root peel extract of *Carex baccans* for varying time duration results in flaccid paralysis and death (Roy and Tandon 1996; Tandon *et al*., 1997; Dasgupta *et al*., 2010). Challam *et al*. (2010) reported that a similar kind of dose dependent motility was also recorded among trematode and cestode parasites, treated in vitro with the crude extract of several plants like *Flemingia vestita*, *Alpinia nigra*, *Millettia pachycarpa*, *Acacia oxyphylla* and *Lysimachia ramose*. Several investigators reported the inhibitory effect of plant extracts on the motility of *Haemonchus contortus*, *Fasciola gigantica*, *Gigantocotyle explanatum*, *Gastrothylax crumenifer*, *Fasciolopsis buski*, *Ascaris suum* and *Raillietina echinobothrida* (Iqbal *et al*., 2010; Kushwaha *et al*., 2004; Jeyathilakan *et al*., 2010; 2012). The interpretation of anthelmintic activity *in vitro* assays is mostly relied on the visual observation on the motility and viability of drug-treated parasites. However, to be accurate it is necessary that it should be substantiated by some quantitative measure. Hence, the motility of *H. contortus* was further quantitatively assessed using EMM developed by Veerakumari (2003) and hence the results were amicable to statistical analysis. *Gg*EE significantly inhibited the motility of *H. contortus*, the inhibitory effect being concentration and time-dependent.

Similar studies using the motility meter have shown an inhibitory effect on the motility of larval and adult helminth parasites treated with chemical anthelmintics (Veerakumari and Priya, 2006; Veerakumari and Lakshmi, 2006; Sathish kumar and Veerakumari, 2017 a, b). A characteristic feature of most of the GI adult parasites is their motility, which is an adaptation to survive in the host's gut, withstanding vigorous peristalsis and resist by evacuation. It may also help them in their feeding and reproduction. Motility is brought about by the contraction of the body musculature, which is well developed in nematodes. The normal functioning of the muscles requires a considerable amount of energy. Inhibition of motility might be largely due to muscular paralysis, which could also be brought about by the deleterious effects on the structure and / or physiological functions of the worms. *Ga*EE possesses a remarkable anthelmintic activity against *H. contortus*. It may serve as an alternative for anthelmintic chemotherapeutic agents to avoid their toxic side effects and development of resistance in a safe and eco friendly manner.

IV. CONCLUSION

The present study elucidated the anthelmintic effect and also the possible mode of action of *Gg*EE on *H. contortus*. *Gg*EE have multiple mode of action and are capable of causing a number of detrimental effects which altogether accounts for their effectiveness in combating the nematode, *H. contortus*. *Gg*EE might affect and disrupt the motility of the worms thus causing their death. Plants having multiple mode of action are very promising as they are eco-friendly, do not leave residues on animal products and development of resistance could be minimized. Hence, *Gg*EE could be used as an alternative drug to combat haemonchosis in livestock.

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APPENDIX - A

List of Tables:

Table 1: Chronological observations on the viability and motility of *Haemnchous contortus* exposed to various extracts of *Glycyrrhiza glabra*

Extracts	**Concentrations mg/ml**	**5min**	**15min**	**30min**	**1h**	**2h**	**4h**	**6h**	**8h**	**12h**	**24h**
Control		++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
*Gg*HE	1	++++	++++	++++	++++	+++	++	++	++	++	++
	3	++++	++++	++++	++++	+++	++	++	++	++	++
	5	++++	++++	++++	++++	++	++	++	+	+	-
*Gg*CE	1	++++	++++	++++	++++	+++	+++	+++	++	++	++
	3	++++	++++	++++	+++	++	++	++	++	++	+
	5	++++	++++	++++	++	++	++	+	+	+	-
*Gg*EaE	1	++++	++++	++++	++++	++++	+++	+++	++	+	+
	3	++++	++++	+++	+++	++	++	++	++	+	-
	5	++++	+++	+++	+++	++	++	++	+	-	-
*Gg*EE	1	++++	++++	+++	++	++	++	++	++	+	-
	3	+++	+++	++	++	+	+	+	-	-	-
	5	+++	++	+	+	-	-	-	-	-	-
*Gg*AE	1	++++	++++	+++	++	+++	+++	++	++	+	-
	3	++++	+++	++	++	++	+	+	-	-	-
	5	+++	+++	++	++	+	-	-	-	-	-

very active (++++); moderately active (+++); slightly active (++); sluggish (+); and dead (-).

Gg*HE- Hexane extract of *Glycyrrhiza glabra

Gg*CE- Chloroform extract of *Glycyrrhiza glabra

Gg*EaE- Ethyl acetate extract of *Glycyrrhiza glabra

Gg*EE- Ethanol extract of *Glycyrrhiza glabra

Gg*AE – Aqueous extract of *Glycyrrhiza glabra

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Table 2: Chronological observations on the viability and motility of *H. contortus* exposed to sub lethal concentrations of *Gg*EE

Extracts	Conc. mg/ml	5min	15min	30min	1h	2h	4h	6h	8h	12h	24h
Control		++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
*Gg*EE	0.005	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++
	0.01	++++	++++	++++	++++	++++	++++	++++	+++	++	++
	0.05	++++	++++	++++	++++	++++	++++	++	++	+	+
	0.1	++++	++++	++++	++++	++++	+++	++	++	+	+
	0.5	++++	++++	++++	++++	++++	++	++	+	-	-

Table 3: Quantitative measure on the motility of *H. contortus* treated with *GgEE*

Conc. mg/ml*	% inhibition (mean ± SD of n=5) at various periods of incubation**		
	2h	4h	8h
***Gg*EE**			
0.005	18.61 ± 0.40	34.21 ± 0.14	57.24 ± 0.17
0.01	24.52 ± 0.42	46.22 ± 0.20	63.17 ± 1.21
0.05	29.37 ± 0.36	52.12 ± 0.13	74.30 ± 0.23
0.1	38.24 ± 0.27	56.21 ± 0.18	79.3 ± 0.15
0.5	47.12 ± 0.13	68.03 ± 0.21	87.21 ± 0.21

Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (P < 0.05) using Bonferroni test

** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P < 0.01) using Bonferroni tes

List of Figures:

Fig. 1 *Haemonchus contortus*

Fig. 2 Rhizome of *Glycyrrhiza glabra*

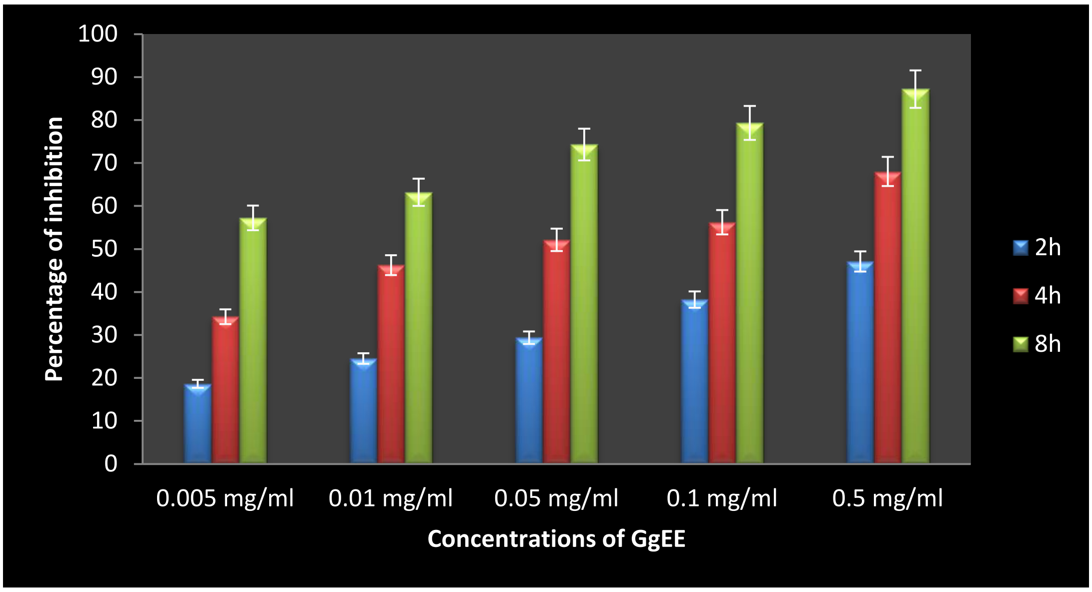

Fig. 3 Effect of *Gg*EE on motility of *H. contortus*