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SHORT COMMUNICATION

In vitro Experiments Revealed the Anthelmintic Potential of Herbal Complex against *Haemonchus contortus*

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ABSTRACT

In an era of drug resistance, new medicinal plants are being evaluated for control of parasites. In the present study, the anthelmintic activity of the extract of herbal complex (HC) composed of four herbs (*Origanum vulgare*, *Cinnamomum verum*, *Rosmarinus officinalis* and *Capsicum annuum*) was assessed against eggs and adult *Haemonchus* (*H.*) *contortus*. Egg hatch test (EHT) and adult motility assay (AMA) was performed to evaluate the anthelmintic potential of HC. The reference drug used in EHT was oxfendazole and for AMA two drugs i.e. oxfendazole (2.265 mg ml^{-1}) and levamisole (3mg ml^{-1}) were used. In EHT, LC_{50} values of HC and oxfendazole were 498 and 1.6 ppm, respectively. In AMA, 100% mortality of *H. contortus* was observed after 6 hr of treatment with HC (100 mg ml^{-1}) where as two positive control groups could not kill all worms after this exposure time. These results indicated the anthelmintic potential of HC.

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INTRODUCTION

Small ruminant production is a major economic contributor in the livelihood of rural communities in developing countries like Pakistan. But, production and profit in sheep and goat farming are significantly affected by infection with gastrointestinal nematodes (GINs). Among different types of GINs, *Haemonchus* (*H.*) *contortus* is a highly pathogenic blood-feeding nematode of small ruminants, which is a significant cause of mortalities in small ruminants worldwide (Fayaz *et al.*, 2019). Infection with this nematode not only results in decreased milk and meat yield but it also causes anaemia in animals. Commercially available anthelmintics, used for control of this parasite, are less effective now due to development of drug resistance. In addition to the development of resistance, drug residues in animal products is another reason for the search of alternative control strategies and replacement of allopathic drugs.

Research has been conducted on different medicinal plants as an alternate anthelmintics in order to manage GINs in small ruminants (Fayaz *et al.*, 2019). Plants have a broad spectrum of pharmacological effects, with antibacterial, antiviral and anti-parasitic properties (Khater *et al.*, 2018). A number of individual plants have been reported for their anthelmintic activity (Zaman *et al.*, 2017). Anti-parasitic activity of *Origanum* (*O.*) *vulgare*, *Cinnamomum* (*C.*) *verum*, *Rosmarinus* (*R.*) *officinalis* and *Capsicum* (*Ca.*) *annuum* have been reported in the literature. However, it is also reported in the literature that activity of medicinal plants my increase due to synergistic effect when used in a combination (Zaman *et al.*, 2012). So, we used these four plants in a combination (herbal complex; HC) to check their in vitro anthelmintic activity by performing egg hatch test (EHT) and adult motility assay (AMA).

MATERIALS AND METHODS

Plant Material and preparation of extract: Whole plants of Oregano (*O. vulgare*), Cinnamon (*C. verum*), Rosemary (*R. officinalis*) and Chili (*Ca. annuum*) were procured from a local market of Faisalabad, Pakistan and identified by a botanist. Dried plants were ground finely to a powder in an electric mill and mixed properly in equal quantity. The crude aqueous extract (CAE) of this powdered HC was prepared by boiling 100 g material in 1000 mL of water for 2 h. After cooling to room temperature, it was filtered using Whatman # 1 filter paper. The filtrate was then evaporated in a rotary evaporator and the extract was stored at 4ºC until used.

***In vitro* anthelmintic activity:** Methods described by Sindhu *et al*. (2014) were adopted for evaluation of *in vitro* anthelmintic activity and all the experiments were performed in triplicate. The brief description along with minor modifications are as follows.

Egg Hatch Assay: adult female *H. contortus* worms, obtained from abomasum of sheep, were triturated in Phosphate buffer saline (PBS) to liberate eggs. After cleaning, the concentration of eggs was adjusted to approximately 100 eggs per 0.1 mL. A stock solution of HC and oxfendazole was prepared in tap water and then different two-fold serial dilutions were prepared. The maximum concentration of HC was 50mg/mL and it was serially diluted to 0.78mg/ml. similarly, Oxfendazole was used as a positive control with the maximum concentration of 2.831µg/ml and the last concentration of 0.044238 µg/mL. The test was performed in 24-well flat-bottom titration plats. Each well was filled with 1 mL of the test solution and 100 eggs were added in that while using water as a negative control.

Adult motility assay: Mature live *H. contortus* were suspended in PBS and seven worms were exposed to test solution in a Petri plate. The number of dead and live worms were counted at different time intervals after treatment. Following were the treatments:

1. The HC @ 100, 50 and 25 mg ml^{-1} (candidate compound)
2. Oxfendazole@ 2.265 mg ml^{-1} (positive control)
3. Levamisole @ 3mg ml^{-1} (positive control)
4. PBS (negative control)

Statistical analysis: Per cent mortality calculated in AMA was analyzed by ANOVA and Tukey HSD to check difference among treatment groups. Data Obtained from EHT were subjected to probit analysis for estimation of lethal concentration (LC).

RESULTS AND DISCUSSION

Exposure of *H. contortus* eggs to HC and oxfendazole resulted in a dose-dependent inhibition of egg hatching. There was, however, a statistically significant ($P<0.05$) difference between the efficacy of oxfendazole and HC (Table 1). The LC_{50} value of HC and oxfendazole were 498 and 1.6 ppm, respectively. The slope and standardized residual values indicated a good fit of data to log probit model (Fig. 1). In contrast to EHT, AMA indicated better results with HC as compared commercial drugs used as a positive control. A dose-dependent response was observed and 100% mortality was observed in the group treated with 100 mg/mL of HC after six hours of post-exposure. Two synthetic drugs used in this experiment did not kill the 100% worms after 6 hours. Detailed results are presented in Fig. 2.

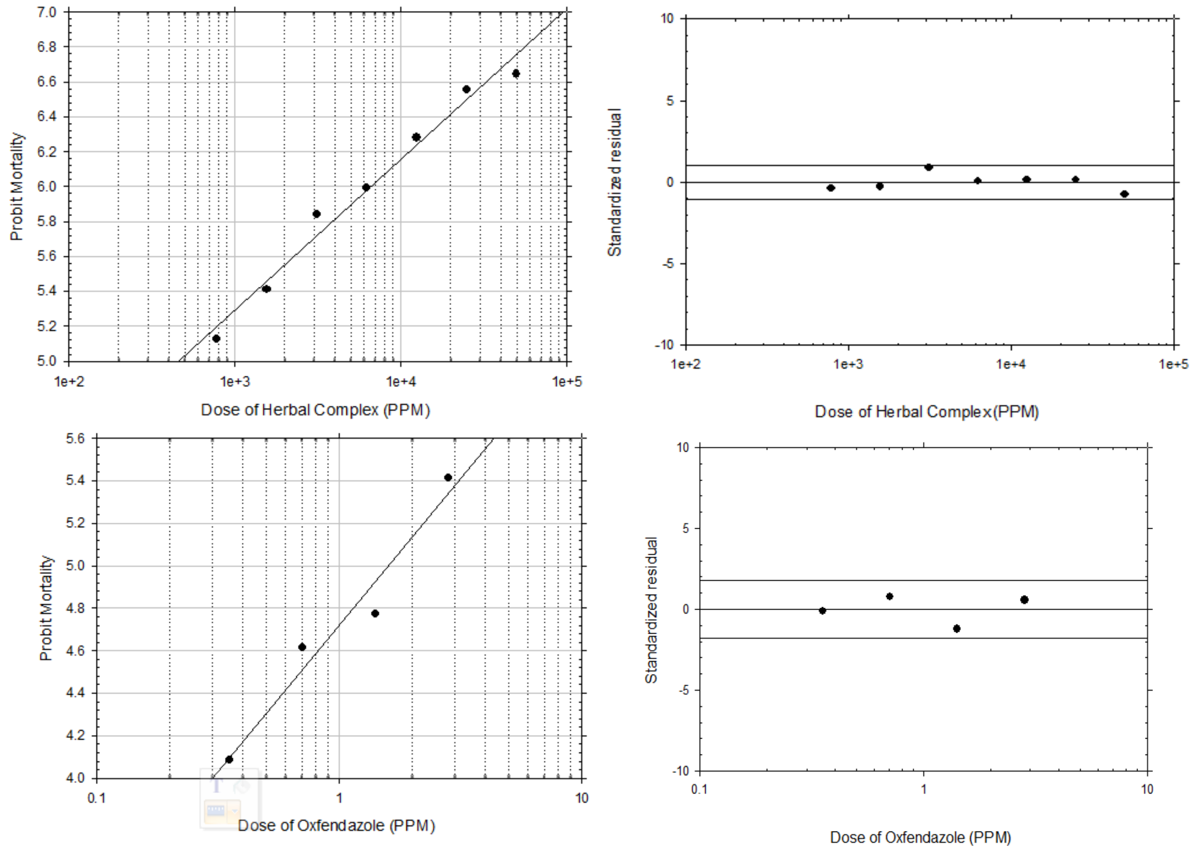

Fig. 1: Probit un-hatched x log concentration (left) and standardized residual x log concentration (right) plot of herbal complex (top) and oxfendazole (bottom) subjected to egg hatch test.

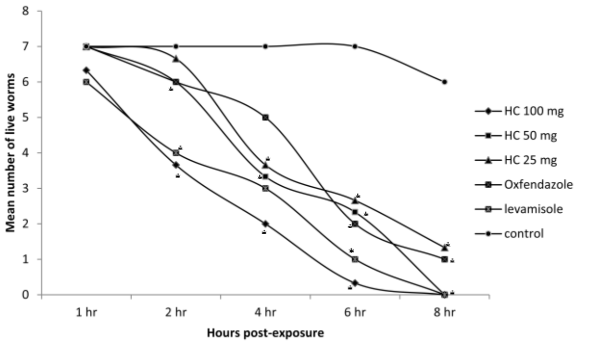

Fig. 2: *In vitro* time- and dose-dependent anthelmintic effect of a herbal complex [HC (*Origanum vulgare*: *Cinnamomum verum*: *Rosmarinus officinalis*: *Capsicum annuum*)] against adult *Haemonchus contortus* worms. PBS was used as control. *Means significantly different (P<0.05) from previous value.

Table 1: Different lethal concentration (LC) values in parts per million (PPM) along with slope and chi-square obtained after *in vitro* evaluation of treatment effects on eggs of *Haemonchus contortus*

	Herbal *complex*	Oxfendazole
Slope ± Standard *error*	0.898±0.104	1.370±0.202
χ^2	1.57	2.43
LC_{30} (95% CI)	129.69 (45.4-256.0)	0.7 (0.2-1.1)
LC_{50} (95% CI)	498 (251-790)	1.6 (0.9-5.9)
LC_{90} (95% CI)	13337 (9335-21885)	13.8 (4.5-5752)

Results of herbal complex, used in the present study, revealed the positive anthelmintic effect in both *in-vitro* tests (EHT and AMA). Before the *in-vivo* study, these *in-vitro* tests are performed to save the time and cost, which allows the assessment of the efficacy of various anthelmintic compounds for the duration of the existence cycle of the parasite (Demeler *et al.*, 2013). The plants used in the Herbal complex have already been reported for their anthelmintic, antimicrobial and other biological effects individually in numerous studies. For example, finely chopped *Ca. annuum* (100 g) given orally with jaggery have shown the anthelmintic activity in sheep naturally parasitized with mixed species of gastrointestinal nematodes (GINs) (Babar *et al*., 2012). A number of active metabolites have been reported in *R. officinalis*, including camphor, α-bisabolol, 1, 8-cineole, terpineol-4-ol, α-terpineol, limonene, gallic acid, rutin, diosmin, hesperidin, quercetin and kaempfero, which have antibacterial and anthelmintic activity (Váradyová *et al*., 2018). In addition to aiming at the treatment of animals with specific plants, farmers have been using mixed plant prescriptions regularly based on *Ca. annuum* and other plants for stomachics and galactagogues (Babar *et al*., 2012). This is well known fact with the support of previous studies that plants when used in combinations, show very good effects because of their synergistic effects (Sreeja and Pooja, 2017). For example, *O. vulgare* essential oil when used in combination with nanoemulsion containing anthelmintic drug against human parasites, showed better results as compared to their effect when used individually, therefore, this is suggestive to use such plants as an integrated approach to enhance the action potential of anthelmintics. Likewise, in the present study, the herbal complex showed the 100% efficacy after the 6 hours exposure in terms of the effect on adult motility.

The main constituent responsible for the anthelmintic activity is still unknown for most of the plants but the literature suggests that plants containing tannin can be considered as potential strategic alternatives for the control of nematode infection in small ruminants. Plants included in this study i.e. *O. vulgare, C. verum, R. officinale and Ca. annum* is found to be rich in tannins and that might be the cause of their anthelmintic activity against *H. contortus* (Rasool *et al*., 2019). Moreover, all the plants used in this HC act as an immunomodulator and have other biological activities too. So, the plants in combination not only act as anthelmintic but also have some other nutritional effect which may enhance the milk and meat quality.

Findings of present research indicate that HC appears to be viable against *H. contortus* and HC could be a cheap alternative of synthetic drugs. Use of HC should be preferred over individual compounds to utilize the synergistic impacts among the phytochemicals of the plants. The HC evaluated in this investigation is accessible and viable against GIN of sheep. However, compounds that are active *in vitro* may not show same activity *in vivo*. This is because of variables identified with the bioavailability and pharmacology of the compound tried. For example, possible destruction or insolubility of biologically active compound in the rumen, as well as protection of the worm in the stomach, could be possible reasons for it (Peneluc *et al*., 2009). This limitation of the experiment should be considered while planning the use of HC without any *in vivo* trials.

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