Antiparasitic Activity of Plants Extract against Gastrointestinal Nematodes and *Rhipicephalus microplus*

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**ABSTRACT**

Parasites are the significant factors influencing the health and production status of animals all over the globe. The high cost of currently available drugs and the emergence of parasitic-resistant strains has highlighted the importance of alternate control strategies. One of the effective ways to combat parasitism is to develop plant-based medicines. The current study aimed to develop a formulation based on the leaves of three traditional plants, *Calotropis procera*, *Syzygium cumini*, and *Ziziphus jujube*. Crude methanolic extract of leaves was evaluated for its antiparasitic activity utilizing egg hatch test (against nematode eggs), adult motility assay (against adult nematodes), and modified larval immersion test (against ticks). Meanwhile, sheep acquiring natural infections of Haemonchus contortus were used to determine in vivo anthelmintic activity. Results from the egg hatch test indicated that 41.05% of the extract inhibited 50% hatching of eggs (LC50): much higher than oxfendazole (4.11%; control group). More interestingly, it was observed that the effects of plant extract on adult worms increased with time. LC50 of plant extract reduced from 176.03 to 4.74% after 12 hours of treatment of adult nematodes. A similar response was observed against *Rhipicephalus microplus* ticks and reduced the exact dose required to induce LC50 after 24 hours of treatment (3641.11%) to 1576.55% after six days of treatment. On the other hand, the moderate activity of the extract was indicated in sheep acquiring natural infections of *H. contortus*. The results show that plants can induce antiparasitic activity; however, further research into plants' bioactive molecules is required to get a maximum reduction of parasites.

**Key words:** *Calotropis procera*, *Syzygium cumini*, *Ziziphus jujube*, *Rhipicephalus microplus*, *Haemonchus contortus*.

**INTRODUCTION**

Parasites are responsible for sustainable morbidity and mortality in animals globally and considerable productivity losses to animal owners (Mehmood et al. 2017; Rashid et al. 2018; Fentahun 2020). Control of parasites as well as parasitic diseases depend on antiparasitic drugs whose number is very limited. Meanwhile, strong resistance has been developed by parasites against the available antiparasitic drugs (Capela et al. 2019). Keeping this in mind, farmers as well as the veterinarians are looking at alternate measures due to the resistance developed against drugs. Plants have been considered as an alternate of antiparasitic drugs due to their pharmacological properties reported in recent past. Moreover, easily availability of plants and fewer side effects has increased their importance to be used as plant-based medicines. These plant-based medicines have been used for years to treat human and animal illness, including parasitic diseases (Pohl et al. 2018; Adhkari et al. 2021). Researchers believed that natural medicines may use different pathways to combat parasitic diseases that usually differ from targets of currently available antiparasitic drugs (Hrcikova and Velebny 2013; Roeber and Kahn 2014). Limited availability of data about systemic evaluation of efficacy of plants, mode of action and variety of active compounds in plants is not allowing plant-based antiparasitic drugs to available commercially (Eguale et al. 2011; Hoste et al. 2006; Nawaz et al. 2015).

Therefore, it is quite necessary to demonstrate the efficacy of plant-based medicines against parasites to develop effective control measures against parasites.

In recent past, extracts prepared from leaves, bark, flowers and stem of plants have been tested against parasites to evaluate their efficacy. Among the plants reported to have broad antiparasitic activity are Azadirachta indica, Dalbergia sissso, Diospyros antistandra, Punica granatum, Artemisia herba-alba, Achillea millefolium and Tanacetum vulgare (Flota-Burgos et al. 2020; Ahmed et al. 2020; Buza et al. 2020; Nawaz et al. 2015). Bark extract of D. antistandra was found to inhibit 98% hatching of Ancylostoma caninum (Flota-Burgos et al. 2020). Similarly, Ahmed et al. (2020) found that the highest concentration (10mg/mL) of crude methanolic extracts of Artemisia herba-alba and Punica granatum caused a significant nematocidal activity against Haemonchus contortus. Likewise, ethanolic and aqueous extracts prepared from flowers, leaves and fruits of Calotropis procera, Azadirachta indica and Punica granatum showed significant anthelmintic effects against Gastrolymphis indicus. Live adult worms of Gastrolymphis indicus died within 4 hours of treatment with ethanolic and aqueous extract of plants (Aggarwal et al. 2016). Therefore, the results of these studies suggest that plants can be potential sources for preparation of novel antiparasitic drugs (Esteveam et al. 2017; Azadbakht et al. 2020; Vecchi et al. 2021).

Although the antiparasitic activity of these plants has been evaluated individually, however, synergistic scientific validation of the use of these plants is an important research issue. Keeping this in mind, the current study was designed to determine synergistic effect of extract prepared from the leaves of Calotropis procera, Syzygium cumini and Ziziphus jujube. The crude methanolic extract prepared was tested against nematodes and ticks.

**MATERIALS AND METHODS**

**Collection of Plant Materials**

Leaves of Calotropis procera, Syzygium cumini and Ziziphus jujube were selected on the basis of bitter taste, aromatic smell and ethnomedical literature surveys. Fresh leaves of the plants were collected from Faisalabad, Pakistan. The taxonomic identification was made by a botanist from the Department of Botany, University of Agriculture, Faisalabad.

**Preparation of Extract**

Extract from leaves of plants was prepared according to the instructions provided by Kamaraj et al. (2011). Briefly, leaves were washed thoroughly and placed in shaded areas at room temperatures. Dried leaves were powdered mechanically by means of steel blender. 100gm of each plant material was taken and mixed thoroughly. Approximately 300gm of the powdered leaves was extracted with 2200mL of methanol in soxhlet apparatus (Qualigens) for 8h. The extract was concentrated at 45ºC under reduced pressure (22–26mmHg). The remainder acquired was subjected to 4ºC till further usage.

**In vitro Anthelmintic Activity**

Anthelmintic efficacy of the crude methanolic extract of plants was determined against adult worms as well as the eggs of *H. contortus* by egg hatch test and adult motility assay (Coles et al. 1992; Singh et al. 1985). For adult motility assay, live adult worms of *H. contortus* were collected directly from sheep abomasum. Worms washed with distil water were placed in petri dishes kept at room temperature. Three groups of worms in the petri dishes were made. Group 1 was treated with plant extract at dose concentrations of 100, 50, 25, 12.5, 6.25, and 3.125%. Group 2 was kept as positive control and treated with levamisole @5500, 2750, 1375, 687.5, and 343.75ppm. Meanwhile, group 3 was treated with phosphate buffered saline. Worms were examined regularly for the next 10h and their motility was recorded at 0, 2, 4, 6, 8, and 10h.

**Egg Hatch Test**

Feces of sheep infected with *H. contortus* were collected to isolate eggs. First the fecal sample of sheep was analyzed for the presence of eggs. The identification of eggs was carried out in the laboratory of Parasitology, University of Agriculture, Faisalabad. Following confirmation of presence of eggs of *H. contortus* in the faeces, 10-15gms of fecal sample was collected directly from the rectum of infected sheep. Number of eggs in 50µL was determined and adjusted to 100-150 eggs/mL. Approximately 100 eggs were added to each well of microtiter plate. It was followed by addition of same volume (1mL) of extract to each well. Wells containing only egg suspension and PBS were considered as control groups. After two days incubation of microtiter plate at 27ºC, hatched (larvae) as well as unhatched eggs were counted from each well. Three replicates were used for each concentration of extract and control group. Data were expressed as percentage of un-hatched eggs and PoloPlus was used to calculate LC values.

**In Vivo Anthelmintic Activity**

In vivo anthelmintic activity was evaluated by fecal egg count reduction test (FECRT). For that purpose, 50 sheep were selected at Livestock Production and Research Institute (LPRI), Bahadarnagar, Okara, Pakistan. Sheep were first examined for the presence of worms by fecal examination test. After confirmation, sheep were divided into five groups. Group A, B and C were treated with 2, 4, and 8mL/kg body weight (BW) of extract, respectively. Meanwhile, Group D was kept as untreated control whereas Group E was treated with levamisole (1mL/kg BW). Fecal examination of each treated and untreated sheep was performed at 0, 4, 8 and 12 days of treatment. Finally, FECR was estimated.

\[
\text{FECR} (%) = \frac{\text{post treatment EPG} - \text{pre treatment EPG}}{\text{pre treatment EPG}} \times 100
\]

**Acaricidal Activity of Leaf Extract of Plants**

Acaricidal activity of crude methanolic extract prepared form leaves of plants was evaluated against tick, *Rhipicephalus microplus*. First, *R. microplus* ticks, feeding on their respective hosts, were collected and their morphological identification was carried out to confirm the specie. Following confirmation, ticks were allowed to lay eggs by providing them ambient temperature (27ºC) and humidity (90%). Soon after laying, eggs were transferred (10gm) to 5mL syringes which were cut from the needle.

side to remain open. Finally, the open end was closed with nylon gauze and syringes containing eggs were kept in incubator at 27°C for hatching. After hatching, 12-14 days old larvae were used to check the efficacy of extract against them. Two groups of syringes were made; one treated with extract (100-3.125%) while the other was treated with ivermectin. Two mL of the solution (extract or levamisole) were administered into the respective syringes containing larvae. Syringes were shaken for 30s and the test solution was drawn from the syringes. Soon after the treatment, syringes were kept again in incubator (27°C and 90% humidity). Lastly, the number of live and dead ticks was recorded at the end of each observation.

Statistical Analysis
Data of syringe test, EHA and AMA was investigated by probit analysis using Polopus (LeOra, 2002) software. Meanwhile, FECRT data was analyzed by analysis of variance and Turkey’s test using SAS software (SAS 1998).

RESULTS

In vivo and In vitro Anthelmintic Activity
Time dependent response (in vivo) was observed in sheep treated with plant extract. Animals treated with 2mL/kg BW showed maximum reduction (16.67%) after 4 days of post treatment. Meanwhile, levamisole showed 18.75% reduction of EPG at the same day. However, 4mL/kg BW of extract was effective (23.80%) after 8 days of treatment; less effective than levamisole which showed 31.25% efficacy at the same day. Similarly, a dose of 8mL/kg BW reduced worm burden up to 37.03% after 12 days, which was about 54% in comparison with levamisole (68.45%) (Table 1). However, 155.02% reduction was observed in those sheep that were not treated with any extract or wormicidal drug.

In vitro efficacy of plant extract was evaluated by applying directly to the sheep and by evaluating its effect on eggs as well as adult worms isolated from the sheep.

Egg Hatch Test
LC50 (95% CI) of extract was calculated as 41.05% (30.5-39.8%) while LC50 of oxendazole was found to be 4.14µg/mL (3.75-4.57%). LC90 of extract was significantly different from LC99 (P<0.05) while non-significant difference was observed between LC90 and LC99 of oxendazole (Table 2).

Adult Motility Assay
Time dependent response of extract was observed in adult motility assay (Table 3). At 2h post treatment, LC50 (95% CI) of extract was found to be 176.03% (99.64-732.34%). Ninety five percent confidence interval for LC50 were very narrow indicating a good fit model. LC99 of extract and oxendazole after 12h of treatment were found to be 0.4264% (0.1014-0.9001%) and 2.1327% (0.1871-6.2249%). The difference observed between LC90 and LC99 of extract and levamisole after 10h post treatment was statistically non-significant (P>0.05).

Bioassay (syringe test)
LC50 (95% CI) calculated after 24h was 3641.10% (1251.44-21874.22%), statistically different (P<0.05) as compared to the LC50 after 6 days 10.669% (9.7474-11.6261%). This significant difference in values (concentration) after 24h and 6 days exhibited slow onset of the activity of extract. In addition to LC50, same response was observed in case of LC90 and LC99 of extract and ivermectin after 24h and 6 days of treatment (Table 4).

DISCUSSION
In order to get maximum production from livestock, it is required to maintain their good health status by following high quality managemental practices, providing balanced diet and protecting them from diseases. Alongside bacterial and viral diseases, protection from parasitic diseases is also required to gain efficient production as parasites are responsible for significant economic losses (Narladkar 2018; Charlier et al. 2020). Several factors such as high cost of antiparasitic drugs, widespread use of antiparasitic drugs and resistance developed by parasites have hindered the control against parasitism (Smout et al. 2010; Capela et al. 2019). Therefore, scientists are looking for alternate control strategies to combat parasitism. Formulations based on locally available plants will be a fine solution to such problems due to their cost effectiveness and easily availability (Liu et al. 2020; Jayawardene et al. 2021). This study was therefore, designed to check the efficacy of three locally available plants against internal and external parasites. In our study, it was observed that plants are capable of inducing moderate antiparasitic activity. Moreover, time dependent as well as dose dependent response was observed which was very much similar to previous studies. Iqbal et al. (2005) reported time dependent anthelmintic activity of extract prepared form Calotropis procera. Similarly, ethanolic and aqueous extract of C. procera induced time dependent anthelmintic activity against larvae of Gastrothylax indicus (Aggarwal et al. 2016).

Table 1: In vivo anthelmintic activity of Calotropis procera, Syzygium cumini and Ziziphus jujube

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12.67±6.8b</td>
<td>17.96±7.7b</td>
<td>21.61±9.5ab</td>
</tr>
<tr>
<td>B</td>
<td>7.81±3.4ab</td>
<td>14.34±4.4ab</td>
<td>19.12±6.11a</td>
</tr>
<tr>
<td>C</td>
<td>11.29±4.8ab</td>
<td>19.93±4.97b</td>
<td>28.66±5.29b</td>
</tr>
<tr>
<td>D</td>
<td>6.91±4.56a</td>
<td>13.72±7.29a</td>
<td>48.34±4.6ca</td>
</tr>
<tr>
<td>E</td>
<td>-30.69±8.45c</td>
<td>-67.55±15.83c</td>
<td>-117.68±23.76c</td>
</tr>
</tbody>
</table>

Group A=2mL/kg BW of extract; Group B=4mL/kg BW of extract; Group C=8mL/kg BW of extract; Group D=untreated control; Group E=treated with levamisole. Data shown in Mean±SE. a,b,c-Row means with different superscripts are either significant or highly significant.

Table 2: In vitro anthelmintic activity of Calotropis procera, Syzygium cumini and Ziziphus jujube against ovu of Haemonchus contortus

<table>
<thead>
<tr>
<th>NAME</th>
<th>Slope (SE)</th>
<th>X²</th>
<th>LC50 % v/v (95 % CL)</th>
<th>LC90 % v/v (95 % CL)</th>
<th>LC99 % v/v (95 % CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract %</td>
<td>0.86 (0.042)</td>
<td>7.01</td>
<td>34.51 (30.73-39.12)</td>
<td>437.70 (327.54-619.97)</td>
<td>3472.17 (2179.60-6098.44)</td>
</tr>
<tr>
<td>Oxfndz (µg/mL)</td>
<td>0.773 (0.346)</td>
<td>1.105</td>
<td>4.14 (3.75-4.56)</td>
<td>40.57 (32.92-51.83)</td>
<td>260.81 (183.84-394.81)</td>
</tr>
</tbody>
</table>

LC50, LC90 and LC99 were recorded, and results were compared with oxendazole.
The importance of *Syzygium cumini* and *Ziziphus jujube* as medicinal plants has been acknowledged in different countries (Shad et al. 2014; Chagas et al. 2015; Ishtiaq et al. 2021). Therefore, it has been believed that these plants possess certain antiparasitic properties. At the moment, *Calotropis procera* is known for its antiparasitic activity as literature is not available about antiparasitic properties of other two plants. As these plants are easily available in the field, therefore, antiparasitic activities associated with them will allow researchers to introduce them to animal feed to reduce parasitic burden within their animals. Further investigations are required to fractionate and identify the active components of plants used in this study.

### Conclusion

Development of resistance against parasitic drugs has led scientists to develop drugs based on medicinal plants. Keeping this in mind, the present study accessed the antitick and anthelmintic activity of methanol extract prepared from the leaves of *Calotropis procera*, *Syzygium cumini* and *Ziziphus jujube*. The extract showed moderate antiparasitic activity. However, further investigations are suggested to evaluate bioactive molecules mainly responsible for antiparasitic properties of these plants.

### Conflict of Interest Declaration

The authors have no financial or other association with persons or organizations that could have inappropriate influence on this paper or bias the contents of this research article.

### Author’s Contribution

Mohsin Nawaz: Methodology and Writing, Jinlin Zhou; Supervision and designed the experiment, Imran Khalid, Asim Shamim, Abid Hussain: Editing and Data curation, Zulfiquar Ahmed, Mohammad Waqas, Imran Ahmed; contributed reagents, materials and tools, Mohsin Nawaz, Muhammad Irfan Malik: Writing and follow up.

### REFERENCES


