In vitro and In vivo Anthelmintic Activity of Leaves of Azadirachta indica,
Dalbergia sisso and Morus alba Against Haemonchus contortus

Mohsin Nawaz, Sajid Mahmood Sajid, Muhammad Zubair, Jibran Hussain, Zulfiqar Abbasi, Abrar Mohi-Ud-Din and Muhammad Waqas

Abstract: This study was conducted to evaluate the anthelmintic activity of water extract prepared from leaves of Azadirachta indica, Dalbergia sisso and Morus alba against ova and adult worms of Haemonchus contortus. Anthelmintic activity of water extract of Azadirachta indica, Dalbergia sisso and Morus alba was determined using fecal egg count reduction test, adult motility assay and egg hatch test. In fecal egg count reduction test, water extract of plants (Azadirachta indica, Dalbergia sisso and Morus alba) was administrated to the animals (sheep) at the dose rate of 02, 04 and 8 ml/kg b. wt. After 12 days of treatment, the plants extract (Azadirachta indica, Dalbergia sisso and Morus alba) induced 89%, 87% and 36% reduction in EPG, respectively. In adult motility assay, slow onset of the activity of extract was observed. LC50 of extract observed after 2 h of treatment was found to be 74% and it reduced to 5% after 10 h of treatment. The extract showed significant difference (P<0.05) between LC90 and LC99 in egg hatch test. LC (95% CL) of extract was found to be 97% (65-140%). Time and dose-dependent response of the plants extract was observed. Results indicated that extracts of Azadirachta indica, Dalbergia sisso and Morus alba are capable of inducing anthelmintic activity.

Key words: Haemonchus contortus - Azadirachta indica - Dalbergia sisso and Morus alba

INTRODUCTION

Dealing with the treatment against parasites, drug resistance is produced in the parasites [1]. The problem of resistance has been limited the use of drugs [2,3]. In addition to this problem, drugs are sometimes associated with adverse effects on host which include hypersensitivity, immunosuppressant and allergic reactions [4]. This leads to search for new drugs by pharmacological screening of medicinal plants [5]. Also, in developing countries, traditional medicine is accessible and affordable treatment [6]. Therefore, attempts are being made to identify new naturally occurring plants having antiparasitic activity [7-9]. The medicinal plants considered as a affluent resources of ingredients which can be used in drug development and synthesis [10].

Extracts of different medicinal plants have been tested for their action against the parasites in vitro and in vivo and have been found effective [11-13]. Various parts of neem tree (barks, seeds, leaves and stem) have been used by scientists to control both ecto- and endo-parasites. For example, Hordegen et al. [14] prepared the ethanolic extract from the seeds of Azadirachta indica and then evaluated its efficacy against Haemonchus (H.) contortus. Similarly, neem oil was used to check its activity against poultry red mite, Dermanyssus gallinae by Lundh et al. [15]. Likewise, Morus alba has been reported for its antiparasitic activity [16,17]. The water and methanolic extracts from the stem bark powder of Morus alba was used against the sheep infected with nematodes. Both extracts showed remarkable reduction in EPG [16]. The root extract of the plant Morus indica was examined against buffalo ascariasis (Neascus vitulorum) and was found to expel adult worms [18].

MATERIALS AND METHODS

Extraction of Plant Material: Fresh leaves of the plants were collected from the District Faisalabad after
identification and authentication of the plant by a botanist from the Department of Botany, University of Agriculture, Faisalabad. Leaves of each plant were chopped and soaked in ample quantity of distilled water in plastic buckets and were vigorously shaken after every 24 h. After 30 days, contents of buckets were heated at 25-30°C till 10% of the total volume was achieved. Material was then sieved and extract was stored at 4°C for further use [19].

**In vitro Anthelmintic Activity:** In vitro anthelmintic activity of the plant extract was evaluated against *H. contortus* using:

- Adult motility assay (AMA).
- Egg hatch test (EHT)

For EHT, procedure described by Coles *et al.* [20] was adopted while AMA methodology was designed according to Singh *et al.* [21].

**Adult Motility Assay:** Mature live male and female worms of *Haemonchus contortus* collected from abomasums of sheep were placed in separate petri dishes (n = 10) at room temperature. Petri dishes were divided into three groups:

**Group 1:** Treated with different concentrations (100, 50, 25, 12.5, 6.25, 3.125%) of extract

**Group 2:** Treated with different concentrations of levamisole {positive control (5500, 2750, 1375, 687.5, 343.75ppm) and PBS (sham treatment)}

**Group 3:** Phosphate Buffered Saline (sham treatment)

Motility of the worms was examined after 0, 2, 4, 6, 8 and 10 hours interval post treatment. Live and dead worms were recorded for each group.

**Egg Hatch Test:** The nematode eggs were isolated by using the technique described by Hubert and Kerboeuf [22]. Ten to fifteen gram of faeces was collected directly from the rectum of sheep naturally infected with *H. contortus*. The concentration of eggs was estimated in 50µl samples and adjusted to 100-150 eggs/ml. The egg suspension was diluted with filtrate from the first step of egg extraction that would have been centrifuged for 5 min at 1000 rpm.

Approximately 100 freshly collected eggs (1 ml egg suspension) of *H. contortus* were added per well of 24 well microtitre plate and mixed with the same volume of plant extract. Egg suspension and PBS was administrated to the control plates. The microtitre plate was incubated at 27 °C for 48 h. Unhatched eggs as well as first stage larvae in each well of the plate were counted. Three replicates were used for each concentration of extract and control group.

**In vivo Anthelmintic Activity:** Fecal egg count reduction test (FECRT) was performed to evaluate the *in vivo* anthelmintic activity of the herbal formulation. Fifty sheep naturally infected with *H. contortus* were selected at Livestock Production and Research Institute (LPRI), Bahadarnagar, Okara, Pakistan. Animals were randomly divided into five groups (n=10). The groups received the following treatments:

- **Group A:** Treated with 2 ml/kg b.w. of extract
- **Group B:** Treated with 4 ml/kg b.w. of extract
- **Group C:** Treated with 8 ml/kg b.w. of extract
- **Group D:** Untreated control (sham treatment)
- **Group E:** Treated control with levamisole @ 1ml/ 5kg b.w

Fecal examination of each animal was performed on 0, 4, 8 and 12 days post-treatment. FECR was calculated by using the following formula:

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

**Statistical Analysis:** Data was analyzed by probit analysis using poloplus [23] computer program. Lethal concentration values (LC<sub>50</sub>, LC<sub>90</sub> and LC<sub>99</sub>) were calculated.

**RESULTS**

**Egg Hatch Assay:** LC<sub>50</sub> (95% CL) of the plants extract inhibiting 50% hatching of eggs were found to be 97% (65-140%) while LC<sub>50</sub> of oxfendazole was calculated as 24 ppm (17-37%). Ninety-five percent confidence interval for LC<sub>50</sub> of both plants extract and oxfendazole were narrow indicating a high degree of repeatability. Similarly, significant difference (P < 0.05) was observed between LC<sub>50</sub>and LC<sub>90</sub>of extract whereas oxfendazole showed non significant difference (P > 0.05) between LC values (LC<sub>50</sub> and LC<sub>90</sub>). X<sup>2</sup> values and slopes of extract are presented in Table 1.

**Adult Motility Assay:** The plants extract exhibited time based response against adult worms of *H. contortus*. After 2 h of treatment, LC<sub>50</sub> (95% CL) of extract was found to be 74% (54-119%) while only 5% (3-6%) of
Table 1: Comparison of the Anthelmintic activity of plants extract and Oxfendazole against eggs of *Haemonchus contortus*, *In vitro*

<table>
<thead>
<tr>
<th>Name</th>
<th>Slope (SL)</th>
<th>$X^2$</th>
<th>LC$_{50}$ % v/v (95% CL)</th>
<th>LC$_{90}$ % v/v (95% CL)</th>
<th>LC$_{99}$ % v/v (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract (%)</td>
<td>0.902 (0.050)</td>
<td>103.51</td>
<td>97 (66-140)</td>
<td>2556 (1221-2829)</td>
<td>36574 (10237-334717)</td>
</tr>
<tr>
<td>Oxfndz (PPM)</td>
<td>0.969 (0.102)</td>
<td>34.460</td>
<td>24 (17-37)</td>
<td>507 (206-2841)</td>
<td>6073 (1369-111690)</td>
</tr>
</tbody>
</table>

Table 2: Comparison of the Anthelmintic activity of plants extract and Levamisole against adult *Haemonchus contortus*, *In vitro*

<table>
<thead>
<tr>
<th>Exposure time (Post treatment)</th>
<th>Slope (SE)</th>
<th>$X^2$</th>
<th>LC$_{50}$ % v/v (95% CL)</th>
<th>LC$_{90}$ % v/v (95% CL)</th>
<th>LC$_{99}$ % v/v (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2hr</td>
<td>Extract (%)</td>
<td>2.185 (0.119)</td>
<td>153.71</td>
<td>74 (54-119)</td>
<td>286 (162-895)</td>
</tr>
<tr>
<td></td>
<td>Levamisole (PPM)</td>
<td>2.441 (0.170)</td>
<td>29.360</td>
<td>15 (9-25)</td>
<td>77 (42-278)</td>
</tr>
<tr>
<td>4hr</td>
<td>Extract (%)</td>
<td>1.251 (0.071)</td>
<td>265.52</td>
<td>46(27-120)</td>
<td>493 (166-9604)</td>
</tr>
<tr>
<td></td>
<td>Levamisole (PPM)</td>
<td>2.098 (0.121)</td>
<td>45.089</td>
<td>4182 (3413-5485)</td>
<td>17073 (11311-32389)</td>
</tr>
<tr>
<td>6hr</td>
<td>Extract (%)</td>
<td>1.856 (0.077)</td>
<td>384.54</td>
<td>15 (9-25)</td>
<td>77 (42-278)</td>
</tr>
<tr>
<td></td>
<td>Levamisole (PPM)</td>
<td>1.481 (0.088)</td>
<td>37.168</td>
<td>4582 (3413-5485)</td>
<td>17142 (10779-34513)</td>
</tr>
<tr>
<td>8hr</td>
<td>Extract (%)</td>
<td>2.253 (0.098)</td>
<td>415.98</td>
<td>6 (3-10)</td>
<td>25 (15-72)</td>
</tr>
<tr>
<td></td>
<td>Levamisole (PPM)</td>
<td>1.573 (0.088)</td>
<td>97.874</td>
<td>1237 (891-1699)</td>
<td>8081 (4768-21483)</td>
</tr>
<tr>
<td>10hr</td>
<td>Extract (%)</td>
<td>3.445 (0.208)</td>
<td>125.64</td>
<td>5 (3-6)</td>
<td>12 (9-18)</td>
</tr>
<tr>
<td></td>
<td>Levamisole (PPM)</td>
<td>1.562 (0.095)</td>
<td>94.803</td>
<td>586 (356-812)</td>
<td>3874 (2495-8785)</td>
</tr>
</tbody>
</table>

Table 3: Effect of plants extract and levamisole on Eggs per gram of faeces (Mean ± ST DEV)

<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>20.0 ± 11.8</td>
<td>52.4 ± 8.3</td>
<td>89.5 ± 36.7</td>
</tr>
<tr>
<td>B</td>
<td>22.0 ± 6.5</td>
<td>52.5 ± 10.2</td>
<td>87.2 ± 33.8</td>
</tr>
<tr>
<td>C</td>
<td>6.7 ± 1.1</td>
<td>26.5 ± 1.6</td>
<td>36.2 ± 1.2</td>
</tr>
<tr>
<td>D</td>
<td>79.1 ± 18.2</td>
<td>75.9 ± 27.5</td>
<td>95.1 ± 5.4</td>
</tr>
<tr>
<td>E</td>
<td>-363.8 ± 70.0</td>
<td>-342.1 ± 48.8</td>
<td>-627.1 ± 92.6</td>
</tr>
</tbody>
</table>

In vivo Anthelmintic Activity: After 4 days of post treatment, 31.53% was the maximum reduction observed in extract treated animals and was found very low when compared with levamisole (95.55%). Likewise, 4 ml/kg b.wt. of extract induced 67.77% reduction of EPG after 8 days of treatment while levamisole exhibited 100% reduction at the same day. -400% reduction was observed in untreated animals. Animals of group A (2 ml/kg b.wt) and B (4 ml/kg b.wt) showed similar reduction of EPG but only 37.14% reduction was observed after 12 days of treatment in the animals treated with higher dose of extract (8 ml/kg b.wt). Again 100% reduction was observed in case of levamisole treated animals. -666.66% reduction was observed in case of untreated animals. Although the plants extract induced good reduction but its efficacy was very low as compared to levamisole as shown in Table 3. Average % reduction of plants extract and levamisole is shown in Figure 1.

**DISCUSSION**

All the three plants included in this study were selected on the basis of their cost, availability and reported anthelmintic activity. *Azadirachta indica* (Meliaceae) is commonly available medicinal plant all over the Pakistan. Extract prepared from leaves, seeds, bark and fruit of neem is known to possess anthelmintic [24], acaricidal [25] and anticoccidial activity [26]. Similarly, *Dalbergia sisso* is a folk remedy for anthelmintic, abortifacient, antipyretic, expectorant and treatment of skin diseases and various digestive disorders [27]. Oil of the plant has been used against some mosquito species including *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*. Anthelmintic [28], antimicrobial [29], and antiviral effects of *Morus alba*...
have been reported. Flavonoids isolated from root bark of Morus alba have shown antioxidant [31], and antidiabetic activity in different model systems [32].

Antiparasitic activity of neem plant has been evaluated for several times whereas other two plants (Morus alba and Dalbergia sisso) were tested for the first time. Findings of current work showed similarities with some previous studies. Results of EPG of this study showed similarity with the results of [33-35]. Dried leaves at the dose rate of 30 g per goat/day given for 5 days showed no anthelmintic activity [34]. Worku et al. [35] reported that aqueous extract containing water soluble proteins from neem were not effective anthelmintics in goats which is comparable with our findings. Feeding neem leaves up to 40 % level as blocks to calves did not show any difference in EPG count [33]. Wong et al. [36] and Khadijah et al. [37] found that the use of pelleted neem and fresh neem showed no significant difference in faecal egg counts, compared with controlled sheep. Chandrawathani et al. [38] reported that feeding neem leaves to the sheep naturally infested with H. contortus had no effect on the faecal egg counts.

Results of egg hatch test and adult motility assay of present study agree with the findings by Bray et al. [39] and Tipu et al [26] and Dakpogan et al. [40] and Hordegen et al. [14]. Seeds of Azadirachta indica were found to show good anthelmintic activity (93%) against larvae of H. contortus [14]. Crude methanolic extract prepared from the seeds of A. indica was highly effective against gastrointestinal nematodes of sheep [41]. Ferreira et al. [42] observed 84.91% efficacy of aqueous leaf extract of A. muricata in EHT while the extract induced 89.08% mortality. 2.5, 5.0 and 7.5 mg/mL of acetone and aqueous extracts of Mentha longifolia and Artemisia arfira inhibited egg hatching and larval development [43]. Likewise, Rahman et al. [44] observed 40% larval mortality of H. contortus at 24 h post treatment with 4mg/mL of methanolic extract of Azadirachta indica. Leaves of Cocos nucifera inhibited 100% hatching in EHT while induced 99.77% mortality of worms in larval developmental test [45]. Rahman et al. [46] observed 40% mortality of larvae of Haemonchus contortus against methanolic extract of Azadirachta indica.

CONCLUSIONS

Results of current study suggest that water extract of Azadirachta indica, Dalbergia sisso and Morus alba is capable of inducing antiparasitic activity. However, difference between the results of this study and previous studies may be due to the methodology applied as well as use of parts of plants. It is therefore necessary to further investigate the toxicity and efficacy of these plants on large scale with different extraction methods.

REFERENCES


