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IN VITRO ANTHELMINTIC ACTIVITY OF *AZADIRACHTA INDICA* (NEEM) AND *MELIA AZEDARACH* (BAKAIN) ESSENTIAL OILS AND THEIR SILVER NANOPARTICLES AGAINST *HAEMONCHUS CONTORTUS*

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ABSTRACT

Synthetic drugs are mostly used for the control and prevention of parasitic ailments in ruminants. However, the resource-poor people of developing countries use herbal medicines to treat their animals. Ethnoveterinary medicine along with its huge beneficial effects also prevents the emergence of drug resistance. Therefore, it is necessary to validate the efficacy of these medicinal plants scientifically for their future use to control endoparasitic infections. Thus, this study aims to evaluate the *in vitro* anthelmintic effects of two indigenous plant extracts namely *Azadirachta* (*A.*) *indica* and *Melia* (*M.*) *azedarach*. The essential oils (EOs) and silver nanoparticles (AgNPs) of these two plants were obtained from their seeds and leaves by hydro-distillation and centrifugation techniques. Their effects were studied by performing the egg hatch assay (EHA) and adult motility assay (AMA) against *Haemonchus contortus*. The results of their efficacy were analyzed using Probit analysis. In our study, EO and NP of *M. azedarach* resulted in a 50% reduction of egg hatching in EHA at 0.209 and 0.204µL/L, and for *A. indica* at 0.456 and 0.184µL/L respectively. Essential oils and AgNPs of *A. indica* and *M. azedarach* were found to be effective in AMA at 0.036, 0.362, 0.305, and 0.032µL/L respectively. Further studies are still required to know more about their effectiveness against different parasitic stages.

***Keywords*:** Ethnoveterinary Medicine, Bioassay, Ruminant, Nanotechnology, Herbal Plants

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1. INTRODUCTION

Livestock farming is the major source of income for the people of rural areas of the subcontinent region. For the prevention, control and treatment of animal diseases, resource-poor people all over the world mostly depend on traditional knowledge of medication. Such remedial practices are known as ethnoveterinary medicine (Farooq et al. 2008). Like Pakistan, the major sector of agriculture is livestock, which has a share of 60.07% in agriculture and 11.53% in the gross domestic product with a growth rate of 3.06% (Pakistan Economic Survey 2020-21). In Pakistan, mostly small farmers have 5-6 animals per family that contribute ≤60% of livestock share. To meet their daily needs, the maximum production of animals is necessary (Babar et al. 2011). Parasitism is a major problem faced by animals worldwide that decreases the productivity of animals, feed conversion ratio and growth rate (Sindhu et al. 2012). *Haemonchus* (*H.*) *contortus* is responsible for severe economic losses in livestock (Chaudary et al. 2007; Sajid et al. 2009; Wajiha and Qureshi 2020). In small ruminants, this is highly pathogenic and could cause acute infection (Kamaraj et al. 2010). Haemonchosis is associated with a decrease or loss of appetite, disruption of the normal functions of the gastrointestinal tract (GIT), variation in the absorption of proteins from the GIT leading to decreased protein content in the body, and alterations in the metabolism of energy and minerals (Maciel et al. 2006).

Pasture management and synthetic drug use are common methods for the control of helminths. The use of synthetic drugs for the control of helminths leads to drug resistance, which ultimately results in treatment failure. Drug residues in animal products are another major concern. Moreover, smallholders could not afford the expensive chemotherapies, and the unavailability of veterinary facilities in rural areas is another major issue (Štrbac et al. 2021). The major disadvantage of synthetic drugs used against parasites has revived the interest of researchers to find alternate control methods. The use of medicinal plants could offer a substitute for synthetic antiparasitic drugs (Sindhu et al. 2014). Many studies have indicated the use of various medicinal plants in animals as well as humans for the treatment of helminths. Some of these plants include *Azadirachta* (*A.*) *indica*,

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Eruca vesicaria, *Aloe vera*, and *Nicotiana tabacum* (Sindhu et al. 2012). However, there are still many plants that must be investigated for their anti-parasitic activity. *Azadirachta indica* and *Melia* (*M.*) *azedarach* are known for their therapeutic properties and are used as ethnobotanical antiparasitic (Costa et al. 2006). The farmers use different parts and forms of these plants to control helminths such as leaves, seeds, stems, roots, EOs, and aqueous extracts (Iqbal et al. 2010). Different studies conducted on these plants showed their effectiveness against many parasites including protozoa, helminths, and ectoparasites such as *Trichomonas vaginalis*, *H. contortus*, *Rhipicephalus microplus*, and *Triatoma infestans* etc. (Maciel et al. 2006). Besides their antiparasitic properties, these plants could also be used to combat certain diseases such as asthma, allergy, rashes, eczema, leprosy, and rheumatism and proved effective as antipyretic, antibacterial, and fungicidal (Qureshi et al. 2016). These medicinal plants are rich in compounds that have antiparasitic, anti-inflammatory, and antimicrobial activities such as azadirachtin, salanin, terpenoids, meliantriol, nimbin, saponins, alkaloids, tannins, acids, and steroids (Singh et al. 2020; Malar et al. 2020). Silver nanoparticles have unique biological, physical, chemical, properties and small size that make them more useful and effective. Studies indicated that AgNPs have larvicidal and vermicidal activities against the larvae and adult worms of *H. contortus*. Nanoparticle-mediated plant extracts showed more efficacy than their simple extracts (Rashid et al. 2016; Preet and Tomar 2017; Rehman et al. 2019). This study aims to select previously mentioned plants for the evaluation of in vitro anthelmintic activity of essential oils and nanoparticles of two important plants namely *A. indica* and *M. azedarach* leaves and seeds individually and in combination.

2. MATERIALS AND METHODS

The present study was carried out at the Department of Parasitology, University of Agriculture, Faisalabad-Pakistan (UAF), and all the experiments were repeated thrice.

2.1. Preparation of Plant Materials

Seeds of *A. indica* and *M. azedarach* were procured from the local market of Faisalabad while leaves were collected from the trees of the UAF botanical garden and got authenticated by a botanist at UAF. After washing, all the plant materials were shade dried and ground into a fine powder in an electric mill separately. Essential oils of seeds and leaves were obtained using the Clevenger apparatus by the hydro-distillation method as previously described by Fagbemi et al. (2021).

2.2. Preparation of nanoparticles

Silver nanoparticles were synthesized from seeds and leaves dried in a hot air oven at 60°C after soaking and washing with water. Five grams of powder of ground seeds and leaves previously stored at -20°C were mixed with hydrochloric acid in a flask to form a solution. The flask was stirred at 1000 rpm (revolution per minute) at room temperature and centrifuged at 9000rpm for 15min to isolate the nanoparticles. Nanoparticles were filtered through a millipore filter having a pore size of 220nm. Prepared EOs and NPs were refrigerated till further use.

2.3. *In vitro* anthelmintic activity

For the evaluation of *in vitro* anthelmintic activity, different bioassays were performed. The anthelmintic activity was checked by performing an EHA and AMA.

2.3.1. Egg hatch assay (EHA)

Egg hatch assay was performed by following the steps instructed by Coles et al. (1992). Adult females of *H. contortus* collected from the infected abomasum of sheep obtained from an abattoir in a local area were triturated in phosphate buffer saline (PBS) by using a mortar and pestle for the recovery of eggs and the suspension was strained through a tea strainer for the removal of debris as illustrated by Jambre (1976). The total volume was then adjusted to 100-200 eggs/mL using the McMaster technique (Soulsby 1982). Six 2-fold serial dilutions of EOs and NPs with distilled water were made having a concentration of 0.5, 0.25, 0.125, 0.075, 0.037, and 0.0185mL/mL. A flat-bottomed 96-well microtitration plate was filled with 100 eggs in each well containing 1.5 mL distilled water. One mL of diluted EOs and NPs were added to each well while maintaining positive and negative controls. The positive control well received oxfendazole at a concentration of 0.025mg/mL whereas the negative control well received only PBS and egg solution. These microtitration plates were incubated in an incubator at 27°C for 48 hours. At the end of incubation, a solution of Lugol's iodine was added to each well to stop further hatching of eggs and first-stage larvae (L_1) and eggs were counted. Inhibition of egg hatching was the criteria to evaluate the efficacy of selected plants' EOs and NPs.

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2.3.2. Adult motility assay (AMA)

Adult male and female *H. contortus* were collected from the abomasum of freshly slaughtered infected sheep for conducting AMA as illustrated by Singh et al. (1985). Six 2-fold serial dilutions of EOs and NPs in different concentrations (same as above) with PBS were prepared. For confirmation of worms, identification keys and worm size were used as described by Soulsby (1982). The right and left spicules had a length having variation of 39.8±2.7 and 21.0±1.7µm respectively whereas the body length had a variation ranging from 13.36±1.7mm (González-garduño et al. 2013). Initially, worms were placed in PBS and then transferred in Petri dishes separately in triplicate containing different concentrations of EOs and NPs at room temperature (25-30ºC). Levamisole was used in the positive control whereas PBS was used in the negative control. Inhibition of worm motility was used as an indicator to assess the mortality or paralysis of worms. The motility of worms was checked after every 2hrs of intervals till 8hrs post-treatment. On every observation, the number of motile and expired worms was counted, and non-motile worms were shifted and kept in lukewarm PBS for 10min. If worms revive their motility, they were considered alive otherwise dead.

2.3. Statistical analysis

The data obtained from the EHA was analyzed by probit test using "PoloPlus" (LeOra software, 2002) and lethal concentration LC_{50} was calculated. While the anthelmintic activity (AMA) was analyzed with one-way ANOVA and Turkey HSD using Statistica version 6 (Stat Soft, Inc., 2001) to detect significance among the group.

3. RESULTS

In EHA, a dose-dependent response of all the plant materials used as a candidate for anthelmintic activity was observed (refer to Fig. 1). Lethal concentrations (LC) estimate of EOs, NPs, and oxfendazole have been presented in Table 1. Essential oil and NPs of *M. azedarach* resulted in a 50% reduction of *H. contortus* eggs hatching at 0.370 and 0.204µL/L obtained from seeds, and 0.352, and 0.209µL/L obtained from leaves respectively. Essential oil and NPs of *A. indica* resulted in a 50% reduction of *H. contortus* eggs hatching at 0.456 and 0.184µL/L obtained from seeds, and 0.662 and 0.169µL/L obtained from leaves, respectively. The most effective treatments based on LC_{50} (LC_{50} in µL/L) of these two plants in combination or separately was the NPs (0.148). Results indicated that NPs of *A. indica* were more effective than simple EOs. The EOs of *M. azedarach* was more effective than all the other EOs. Silver nanoparticles of all other plant parts were found to be equally effective in EHA. Moreover, the NPs of all plants were found to be more effective as compared to their respective simple EOs.

In AMA, a time-dependent dose response with varying concentrations was observed (refer to Fig. 2). Lethal concentration estimates of EOs, NPs, and levamisole have been presented in Table 2. The essential oils and NPs of *A. indica* were observed against adult worm motility (50% dead worms at 8 hours post-treatment) obtained from leaves at

Table 1: Comparison of LC_{50} and LC_{99} (expressed in terms of µL/L) estimates of different plant essential oils and their silver nanoparticles evaluated for *in vitro* anthelmintic efficacy against *Haemonchus contortus* eggs.

Plant	Slope (SE)	χ2	$LC_{50\% v/v}$ (95% CI)	$LC_{90\% v/v}$ (95% CI)	$LC_{99\% v/v}$ (95% CI)
A. indica L (EO)	0.053 (0.069)	0.984	0.662 (0.417-1.317)	173.724 (38.905-2125.112)	16282.888 (1463.24-9407.93)
A. indica L (NPs)	0.646 (0.066)	0.447	0.169 (0.130-0.226)	16.247 (6.873-58.182)	673.268 (154.99-657.629)
A. indica S (EO)	0.529 (0.068)	0.652	0.456 (0.303-0.814)	120.028 (29.396-1226.12)	11284.491 (117.771-519.06)
A. indica S (NPs)	0.606 (0.065)	0.430	0.184 (0.142-0.250)	23.925 (9.144-102.540)	1268 (246.88-15336.65)
M. azedarach L (EO)	0.585 (0.068)	1.1916	0.352 (0.235-0.615)	54.690 (14.552-528.045)	3347.639 (378.67-144320.2)
M. azedarach L (NPs)	0.623 (0.066)	0.449	0.209 (0.158-0.291)	23.804 (9.202-99.968)	1131.230 (227.476-12994.4)
M. azedarach S (EO)	0.581 (0.068)	1.0171	0.370 (0.253-0.624)	517 (16.772-479.837)	3744.733 (467.48-118561.5)
M. azedarach S (NPs)	0.617 (0.066)	0.586	0.204 (0.154-0.284)	24.279 (9.296-103.837)	1195.061 (235.84-14209.85)
A+B+C+D (EOs)	0.604 (0.069)	1.3766	0.414 (0.267-0.781)	54.812 (13.833-628.503)	2945.226 (312.56-162502.5)
A+B+C+D (NPs)	0.780 (0.068)	1.1613	0.148 (0.11-0.194)	6.522 (3.235-18.236)	142.460 (43.150-839.235)

A = Azadirachta indica leaf B = Azadirachta indica seed C = Melia azedarach leaf D = Melia azedarach seed L = Leaf S = Seed EO = Essential oil NPs = Nanoparticles LC = Lethal concentration CI = Confidence interval.

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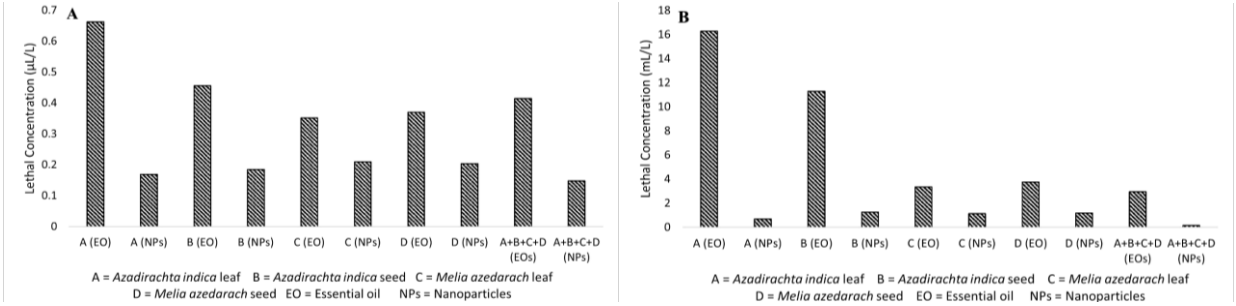

Fig. 1: 48 hours post treatment: eggs of *H. contortus* submitted to egg hatch test with EOs and NPs of leaves and seeds of *A. indica* and *M. azedarach* separately and in combination has shown LC_{50} (A) and LC_{99} (B).

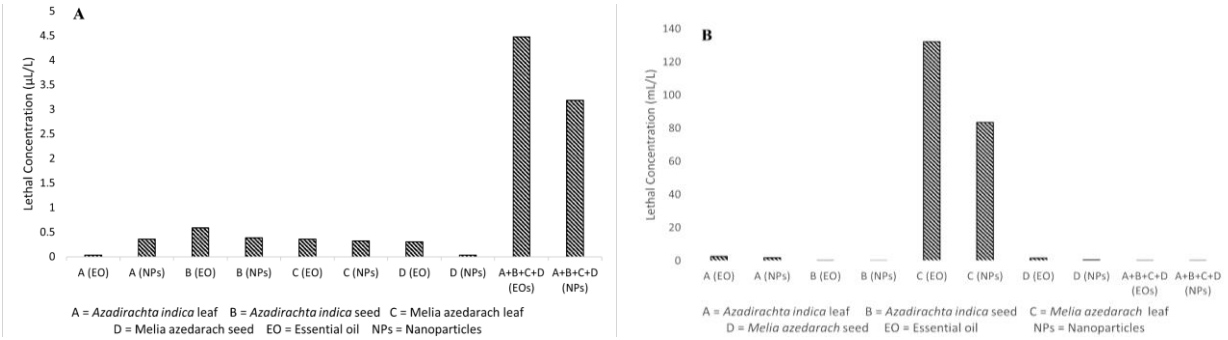

Fig. 2: Eight hours post treatment: mature live *H. contortus* submitted to adult motility assay with EOs and NPs of leaves and seeds of *A. indica* and *M. azedarach* separately and in combination has shown the LC_{50} (A) and LC_{99} (B).

Table 2: Comparison of LC_{50} and LC_{99} (expressed in terms of µL/L) estimates of different plant essential oils and their silver nanoparticles in combination as well as individually, eight hours post-treatment, evaluated for *in vitro* anthelmintic efficacy against adult *Haemonchus contortus* using adult motility assay.

Plant	Slope (SE)	X^2	$LC_{50\% v/v}$ (95% CI)	$LC_{90\% v/v}$ (95% CI)	$LC_{99\% v/v}$ (95% CI)
A. indica L (EO)	1.247 (0.141)	10.679	0.036 (0.027-0.0465)	36.386 (6.469-522.743)	2628.576 (346.05-135897.7)
A. indica L (NPs)	6.714 (0.752)	20.189	0.362 (0.186-0.963)	4.968 (3.397-548.493)	1843.921 (836.753-73133.2)
A. indica S (EO)	1.621 (0.187)	18.591	0.585 (0.485-0.785)	2.256 (2.017-13.265)	138.833 (37.844-2884.245)
A. indica S (NPs)	3.636 (0.349)	26.921	0.386 (0.172-2.761)	3.383 (2.495-25.534)	285.326 (12.143-2850.149)
M. azedarach L (EO)	5.992 (0.672)	24.573	0.36 (0.11-0.342)	12.487 (11.810-5223.858)	132184.178 (2130.2-131067.9)
M. azedarach L (NPs)	0.360 (0.063)	23.442	0.32 (0.334-0.053)	42.631 (5.896-2365.339)	83616.968 (1350.5-589653.4)
M. azedarach S (EO)	1.268 (0.147)	20.998	0.305 (0.017-0.495)	13.543 (3.439-601.037)	1643.324 (116.7-1322801.7)
M. azedarach S (NPs)	2.026 (0.417)	28.524	0.032 (0.021-0.096)	163.823 (16.135-37660.016)	584.315 (625.690-954.630)
A+B+C+D (EOs)	0.316 (0.063)	15.149	4.477 (2.978-6.731)	2.773 (1.286-5.980)	1.876 (0.617-5.706)
A+B+C+D (NPs)	11.950 (2.430)	46.628	3.187 (2.128-4.774)	6.456 (3.149-13.238)	11.478 (4.236-31.102)

A = *Azadirachta indica* leaf B = *Azadirachta indica* seed C = *Melia azedarach* leaf D = *Melia azedarach* seed L = Leaf S = Seed EO = Essential oil NPs = Nanoparticles LC = Lethal concentration CI = Confidence interval

0.036, and 0.362µL/L, and those obtained from seeds at 0.585 and 0.386µL/L respectively. The EOs and NPs of *M. azedarach* obtained from leaves resulted in 50% adult worm mortality at 0.36 and 0.32µL/L and those obtained from the seeds at 0.305 and 0.032µL/L respectively. A combination of NPs and EOs of two plant parts was found to be more effective among all the tested groups. However, the EOs of leaves of *A. indica* and *M. azedarach* showed higher efficacy than their respective testing groups.

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4. DISCUSSION

Ethnoveterinary medicine is defined as the indigenous knowledge and practices of people for caring for, treating the diseases of animals, and managing and healing livestock by using all resources other than synthetic allopathic drugs (Wanzala et al. 2005). People adopt different ways for the diagnosis, classification, and therapy of diseases in animals in rural areas or developing countries. Two plants included in the present study are known for their use against the arachnid, helminths, and protozoan (Sindhu et al. 2014). In this study, EHA, and AMA have been employed to validate the anthelmintic potential of these plants scientifically.

Melia azedarach and *A. indica* are the members of family Meliaceae. These are native to Asia, tropical and subtropical countries. Due to its vast applications, *A. indica* is referred to as "Tree of the 21st century" (Kumar and Navaratnam 2013). While *M. azedarach* is referred to as "A paradise Tree". It also has been extensively used in ethnoveterinary practices for centuries (Sharma and Paul 2013). Both *M. azedarach* and *A. indica* have a well-developed medicinal reputation as several studies have proved their antiparasitic (Mackinnon et al. 1997), antibacterial (Singh and Sastry 1997), anticancer, antifungal, anti-inflammatory, and anti-gastric ulcer activity (Bandyopadhyay et al. 2004).

Cala et al. (2012) documented the anthelminthic activity of *M. azedarach*. Fruit extract of *M. azedarach* results in inhibition of egg hatching and larval development and they also calculated the LC_{99} for egg hatch and larval development. The Eos of seeds of *A. indica* showed ideal activity against egg hatching and resulted in inhibition of hatching. All these findings support our results. Costa et al. (2008) also evaluated the *in vitro* anthelmintic activity of *A. indica*. They reported a reduction in egg hatching and larval development of EOs of *A. indica*. Another study was conducted by Maciel et al. (2006) for the assessment of the anthelmintic potential of *M. azedarach* EOs. The EOs of seeds and leaves of *M. azedarach* were evaluated. In our study, egg hatching and development of larvae were also prevented by the application of EOs obtained from *M. azedarach*. In this study, the results of the EHA and AMA agree with the results obtained by Nawaz et al. (2014). The silver nanoparticles biologically synthesized from the plant used against *H. contortus* also support the current findings (Tomar and Preet 2016). But certain variations in the efficacy values could be due to different plant compositions at different geographical locations. The anthelmintic activity of these plants is due to the presence of some compounds that exert their lethal effects against the *H. contortus* as the result findings indicated in a previous study (Malar et al. 2019).

5. Conclusion

Based on these results, it is possible to conclude that the EOs and NPs have a stronger and broader spectrum of anthelmintic activity. The use of nanotechnology in medicine is a new and interesting field in Pakistan so there is a great need for research on making NPs from easily available plants using different solvents that can be useful in various ways by providing ease of treatment. The antiparasitic activity of A. indica and M. azedarach was associated with the alkaloids and other ingredients; hence, there are a need for further studies to determine the active components as well as ascertain which parasite species or developmental stages are most susceptible to the effect of their EOs as well as NPs to further enhance their anthelmintic usefulness.

Author's Contribution

Zia ud Din Sindhu, Bilal Aslam, and Muhammad Amir Aslam conceived the idea and designed the study. Saira Batool, Mansoor Ahmad, and Muaz Khalid Chaudhary conducted the experiments. Muhammad Kasib Khan, and Muhammad Imran performed statistical analysis. Furqan Munir, Zia ud Din Sindhu and Rao Zahid Abbas were involved in the Writeup and proofreading of the manuscript.

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