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RESEARCH PAPER

Antinematicidal Potency of *Arum maculatum* L. (*Araceae*) to Control Macrocyclic Lactone Derivative-Resistant Gastrointestinal Roundworms in Ovine

Kareem Khoshnow Hamad*

*****Department of Biology, College of Science, Salahaddin University- Erbil, Kurdistan Region of Iraq

A B S T R A C T:

Antinematicidal resistance has been emerging between the gastrointestinal (GI) nematode populations in ovine to the popular broad-spectrum artificial drugs [say macrocyclic lactone (ML) derivatives] in most countries of the world including Kurdistan region of Iraq. Hence, the present study was designed to evaluate the efficacy of crude aqueous ethanol extract (CAEE) of native *Arum (A.) maculatum* L. (common name: snakehead) in eliminating ML derivative-resistant GI roundworms in ovine. The study was conducted on private sheep farms in Qushtapa district, Erbil governorate from October 2018 to March 2019. After confirmation of infestation with various GI nematodes (*Nematodirus* 37%, *Marshallagia* 42% and *Trichuris* 21%), the tentative animals were divided into six groups (n=15). One group was allotted for diagnosis of resistance against ML and one group for control. The faecal egg count reduction test (FECRT) has revealed the rampancy of resistance to the aforementioned antiparasitic. According to the RESO Computer Program, the calculated FECR% and lower confidence interval 95% were 69.83% and 48.4% respectively. For antiparasitic evaluation of the above phytomedicine, four groups (n=15) were exploited. The FECR% results post-therapy with 25, 50, 75, and 100 mg kg-^{1} BW of the *A. maculatum* CAEE were 21.13, 54.91, 73.28 and 96.27 respectively. According to these data and related references, the dose; 100 mg kg-^{1} BW was deemed effective, whilst other doses were ineffective. The ovicidal activity of the medicinal herb was also tested *in vitro* via executing egg hatch assay. The calculated LC_{50} value was 2.551µg ml^{-1} (range 2.454-2.647) after conduction of the assay. Having said, the same control group was employed because statistically, all the groups were belonged to the same population.

KEY WORDS: Medicinal herbs, antinematicidal resistance, ivermectin, alimentary tract nematodes, sheep
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1. INTRODUCTION

Antinematicidal resistance (AR) has become an international phenomenon threatening sheep and goat resources since the last century (Kaplan and Vidyashankar, 2012; Karrow *et al.*, 2014; Kalkal *et al.*, 2019). Relatively, among the renowned broad-spectrum synthetic antinematicidals, macrocyclic lactone (ML) derivatives such as ivermectin is vulnerable to evolve resistance against it by the gastrointestinal (GI) nematodes of small ruminants, particularly in under-developed nations (Hamad, 2018).

It is noteworthy to mention that in some regions where small ruminants are being reared intensively, the resistance has reached a considerable level (Hamad, 2012) due to mutations (Beech and Silvestre, 2010). To overcome this dilemma, parasitologists and experts in the domain of livestock wellbeing have promoted some substitute policies such as pasture management, nutritional supplementations, biological control, immunization, and genetic approaches. Pragmatically, the aforementioned substitutions have not accomplished significant results in the field (Stear *et al.*, 2007) especially in under-developed states where livestock owners

* **Corresponding Author:**
Kareem Khoshnow Hamad
E-mail: kk_hamad@yahoo.com
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are not well-educated and unaware about the problem of AR (Hamad, 2014).

Alternatively, phytomedicines have been advocated in African, Asian, and South Latin American countries to be a sharp sward to control rampancy of antinematicidal-resistant GI roundworms of livestock (Jabbar *et al*., 2006). Having said, medicinal plant crude extracts comprise several active constituents which, in turn, preclude the emergence of AR by alimentary tract parasitic roundworms owing to targeting different dewormer receptors (Athanasiadou *et al*., 2007). In this regard, antinematicidal activity of *Arum* (*A.*) *maculatum* L. leaf extract, which has been mentioned in folk medicine (Adams *et al.*, 2011), was trialed *in vitro* and *in vivo* to assess its ovicidal and adulticidal efficacy utilizing reliable parasitological assays. Having said, this growing plant in spring season contains some agro-chemical secondary metabolites say cyanogenic glycosides, saponins, monoterpenes and alkaloids (Avato *et al.,* 2006).

2. Material and Methods

2.1. Allotment of experimental lambs

Animals (n=90) having 3-6 months age, which had not been deparasited for the last two months, were comprised in the research work. To choose tentative lambs, the five point check, lower eyelid paleness (anemia) assessed by FAMACHA (score 1-5) and bottle jaw (submandibular edema) were applied for haematophagous worm infestations, whilst for all GI nematode infections, back score condition (1-5) and coat condition were utilized. On the other hand, for scour worms, fecal soiling (dag score 0-5) was exploited. Moreover, determination of egg per gram of feces (EPG) was performed. The animals, selected for the trial, were marked and arbitrarily allotted into six groups:

Group 1: ML derivative (ivermectin) resistance detection group (n=15)

Group 2: *A. maculatum* dose (25 mg kg-^{1} BW) group (n=15)

Group 3: *A. maculatum* dose (50 mg kg-^{1} BW) group (n=15)

Group 4: *A. maculatum* dose (75 mg kg-^{1} BW) group (n=15)

Group 5: *A. maculatum* dose (100 mg kg-^{1} BW) group (n=15)

Group 6: infected non-treated group (n=15)

(Coles *et al.,* 1992; Macedo *et al.*, 2010)

The trialed lambs did not get any other remedy during the experiment period and routine clinical inspections were carried out as well (Kahn, 2005). The experimental animals were chosen from private sheep ranches in Qushtapa district, Erbil governorate, whilst the laboratory techniques were executed at the Department of Biology, College of Science, Salahaddin University-Erbil.

2.2. Preliminary assays to detect natural infestations with GI nematodes

●***Faecal examination***

Qualitative and quantitative parasitological tests were performed to determine natural infections with different alimentary tract parasitic roundworms and during conducting other steps of the study (Soulsby, 1982; Coles *et al*., 1992; Iqbal *et al*., 2006a).

●***Coprological method***

Coprocultures were also applied to detect the involvement of various digestive system helminths in whole natural worm infestations following MAAF (1986). Faecal samples of each group of tentative animals were pooled and cultured in plastic containers. Amphotericin B (5 µg g^{-1}) was added to prevent fungal contaminations. The cultures were incubated for seven days at 27±1ºC. After this time, the nematode larvae (L_3) were collected using Baermann apparatus.

●***Baermann technique***

This assay was performed to collect the parasite larvae (L_3) from the coproculture method. Approximately 15g of the incubated faeces were wrapped up in medical gauze and put in the Baermann apparatus funnel. Lukewarm water was added to stimulate larval motility to the end of collecting tube. The "Baermann" was left overnight and a small volume of water was collected and poured in a plastic container. Then the water sample transferred to a petridish, Lugol's iodine was added to the culture (Iqbal *et al*., 2006a) larvae were identified following MAAF (1986).

2.3. Studies on antinematicidal resistance

●***Fecal egg count reduction test (in vivo assay)***

Ivermectin 1% belongs to the macrocyclic lactone family and produced by Cherished Pharmac. (pvt) Ltd., was obtained from a reliable veterinary clinic. Lambs (n=15) in group 1 were injected

ivermectin subcutaneously at the regular dose (0.2 mg kg-^{1} BW); whilst group 6 served as infected non-dewormed control. Coproculture of animals (group 1 and control) were conducted at day 0 (pre-deworming) and day 14 (post-deworming) as pointed out formerly. Eggs per gram of faeces (EPG) were counted employing Whitlock Universal Egg Counting Slide (provided by JA Whitlock & Company, PO Box 51, EASTWOOD NSW 2122 AUSTRALIA). Post- therapy EPG and involvement of all parasitic roundworms in the natural infections were determined. EPG were computed by Whitlock slide using the formula below:

EPG= Total eggs in chamber 1, 2 and 3/3 x 50 (dilution factor)

Faecal egg count reduction percentage (FECR %) was estimated utilizing the following formula (Coles *et al.*, 1992):

FECR % = [1- (mean EPG treatment/mean EPG control)] x100

RESO computer program (CSIRO Animal Health Research Laboratory, Private Bag 1, Parkville, Vic. 3052, Australia) was exploited to calculate the arithmetic mean, variance of counts, FECR% and 95% confidence interval. According to Coles *et al*. (1992), resistance is rooted if (**i**) the FECR% is less than 95% (**ii**) the lower limit of 95% confidence interval is less than 90% (**iii**) If just one of the two norms is met, resistance is suspected. Moreover, Gill (1996) has suggested that any negative values obtained from FECR% and lower limit of confidence interval should be equal to zero, interpreting that the resistance is broadly prevalent and has reached the catastrophic level.

2. 4. Assessment of plant extract for using against resistant GI nematodes

●***Extraction* of *Arum maculatum* leaves**

Arum (A.) maculatum leaves were harvested naturally from South of Erbil governorate. Leaves were dried in shade at room temperature. After dryness, materials were powdered utilizing an electric grinding machine. The powder was solved in 70% aqueous ethanol by cold maceration at 25-30 Cº and the materials were mixed 2-3 times daily by a stirrer. After three days, the materials were filtrated through a portion of leaky textile and the filtrate was collected in a container. The aforementioned process was repeated thrice. The extract was evaporated to dryness at room temperature. The crude aqueous-ethanol extracts (CAEE) was kept at 4°C until used against the pathogenic nematodes (Gilani *et al*., 2004).

●***Fecal egg count reduction test (in vivo assay)***

The lambs in group 2, 3, 4 and 5 were drenched *A. maculatum* leaf CAEE at doses; 25, 50, 75 and 100 mg kg-^{1} BW respectively, whilst, group 6 utilized as control (infested untreated). Fecal examinations and coprocultures of the experimental lambs were conducted at day 0 (pre-therapy) and on day 14 (post-therapy) as mentioned previously. Post- therapy EPG and parasite infestations were recorded. FECR % was calculated using the undermentioned formula:

FECR% = [1-(mean EPG treatment/mean EPG control)] x100

●***Egg hatch assay (in vitro assay)***

This assay was performed to assess the inhibitory activity of various doses of the CAEE on egg hatching of the parasitic nematodes. The technique was done according to the protocol recommended by Coles *et al*. (1992) with slight changes by some parasitologists to be proper for evaluation of ethnobotanicals (Macedo *et al*., 2010). One gram of CAEE was dissolved in 10 ml of 70% acetone and this was reckoned as mother solution (100 mg ml^{-1}) which was serially diluted in a 24 multiwell plate. The egg samples were treated with 12 concentrations (100-0.048 mg ml^{-1}) of the extract. For positive control, 0.025 mg ml^{-1} of albendazole5% was dissolved in 0.3% Dimethylsulfoxide (DMSO). The well of negative control got just 1ml of 70% acetone. Plate was incubated at 27°C ±1 for 48 hours and 70% relative humidity. After incubation, two drops of Lugol's iodine was added.

At least 100 of the unhatched eggs (dead and embryonated) and hatched larvae were counted to calculate the hatching inhibition percentage. The subsequent formula was exploited to assess hatching inhibition (%):

Hatching inhibition (%) = P test/ P total x100

P test: number of unhatched or embryonated eggs.

P total: number of unhatched or embryonated eggs + Larvae (L_1).

LC_{50} values were calculated for the eggs by probit analysis.

2. 5. *Statistical analysis*

The RESO computer program was exploited to analyze the mean EPG for determination of resistance in treatment and control groups (14 days post-injection with ivermectin). The emergence of resistance was evaluated by this

program through calculating FECR% and lower limit of confidence interval 95%. For analysis of the data recovered from FECRT and assessment the influence of different doses of *A. maculatum* CAEE on reduction of EPG, one-way ANOVA was applied, followed by application of Tukey test for comparison between doses.

The data obtained from EHA for different concentrations of *A. maculatum* CAEE to evaluate their ovicidal efficacy against nematode eggs; one-way ANOVA was applied utilizing Graphpad Prism (version 7). Tukey as multiple comparison tests was used to compare among doses. All procured data were expressed as Mean±SE. For calculation of LC_{50} (µg ml^{-1}) at 95% confidence interval for preventing 50% of egg hatching, probit analysis of LC_{50} value on the EHA was applied.

3. Results

3.1. Contributing GI nematode species in infections

The larvae (L_3) of *Nematodirus* (37%), *Marshallagia* (42%) and *Trichuris* (21%) were identified following Soulsby (1982) and MAAF (1986) after execution of pre and post-deworming coproculture and Baermann technique (table 1).

3. 2. Trials on ivermectin resistance

●Faecal egg count reduction test

In accordance with the statistical analysis and RESO computer program, the post-treatment EPG mean, FECR% and lower confidence interval 95%, on day 14 post-deworming with ivermectin, were 22.47 (control mean EPG= 730.80), 69.83 and 48.4, respectively. The aforesaid data had verified that the resistance was emerged towards the previous synthetic dewormer.

3. 3. Antinematicidal influence of *A. maculatum* leaf CAEE against resistant GI nematodes

●Faecal egg count reduction test

Pre-treatment proportions (0 day) of nematode larvae (L_3), depending on the coprological assay, were displayed in table 2. The antinematicidal activity of *A. maculatum* leaf CAEE (four various dosages) against ivermectin-resistant worms in the experimental lambs naturally infested with GI nematodes in addition to comparison between influences of different doses of the plant extract on the egg reduction mean were analyzed statistically and exhibited in table 3 and 4. As evident from the data, obtained after performing the coprological assay (Table 3), group 5 was exposed to an efficacious dose (100 mg kg^{-1} BW) of *A. maculatum* leaf extract, so no parasite larvae (L_3) were recovered. On the other hand, the results of FECRT (Table 4) demonstrated the effectiveness of *A. maculatum* leaf extract in reducing faecal egg count (FECR% =96.27) in group 5 when exposed to100 mg kg^{-1} BW. Having said, other doses were not effective (FECR% <80). There was a significant difference ($P<0.05$) between all doses.

●Egg hatch assay

The analysis of variance (ANOVA) regarding the data collected from EHA in evaluating the ovicidal activity of different concentrations of *A. maculatum* leaf CAEE via hatching inhibition (%) calculation had revealed various impacts of different concentrations (dose-dependent ovicidal potency) (figure 1). The computed LC_{50} was 2.551 µg ml^{-1} (range 2.454-2,647) at the level of 95% confidence interval.

Table 1 Pre-deworming (0 day) and post-deworming (after 14 days) percentage of nematode larvae (L_3) in the tentative lambs selected for detection of resistance against ivermectin based on pooled faecal samples of groups 1 and 6 (control)

Groups	Pre-deworming L_3 (%) of nematodes		
	Nematodirus	*Marshallagia*	*Trichuris*
Group 1 (Ivermectin group)	46	37	17
Group 6 Control (untreated)	43	35	22
	Post-deworming L_3 (%) of nematodes		
Group 1			

(Ivermectin group)	43	33	24
Group 6			
Control (untreated)	41	38	21

Table 2 Pre-treatment proportions (0 day) of nematode larvae ($L_{3)}$ in the lambs chosen for evaluation of different doses of *Arum maculatum* CAEE based on pooled faecal specimens of groups 2, 3, 4, 5 and 6 (control)

Groups	*Nematodirus* %	*Marshallagia* %	*Trichuris* %
Group 2			
(25mg kg-^{1} BW)	44	36	20
Group 3			
(50 mg kg-^{1} BW)	42	33	25
Group 4			
(75 mg kg-^{1} BW)	48	34	18
Group 5			
(100 mg kg-^{1} BW)	45	32	23
Group 6			
Control (untreated)	44	37	19

Table 3 Post-treatment proportions (on day 14) of nematode larvae ($L_{3)}$ in the lambs chosen for evaluation of different doses of *arum maculatum* CAEE based on pooled faecal specimens of groups 2, 3, 4, 5 and 6 (control)

Groups	*Nematodirus* %	*Marshallagia* %	*Trichuris* %
Group 2			
(25mg kg-^{1} BW)	47	34	19
Group 3			
(50 mg kg-^{1} BW)	44	33	23
Group 4			
(75 mg kg-^{1} BW)	49	30	21
Group 5			
(100 mg kg-^{1} BW)	not recovered	not recovered	not recovered
Group 6 (control)	45	38	17

Table 4 Mean egg per gram of faeces and percentages of faecal egg count reduction in lambs on day 14 post-treatment with different doses of *Arum maculatum* CAEE

Groups	Mean EPG±SE	Mean FECR%
Group 2 (25 mg kg-^{1} BW)	647.8±15.11**C**	21.13
Group 3 (50 mg kg-^{1} BW)	370.3±12.42 **BC**	54.91
Group 4 (75 mg kg-^{1} BW)	219.4±7.34 **AB**	73.28
Group 5 (100 mg kg-^{1} BW)	36.6 ± 3.38 **A**	96.27
Group 6 (control)	821.4±71.99 **D**	-

Means sharing similar letters are statistically non-significant (P>0.05)

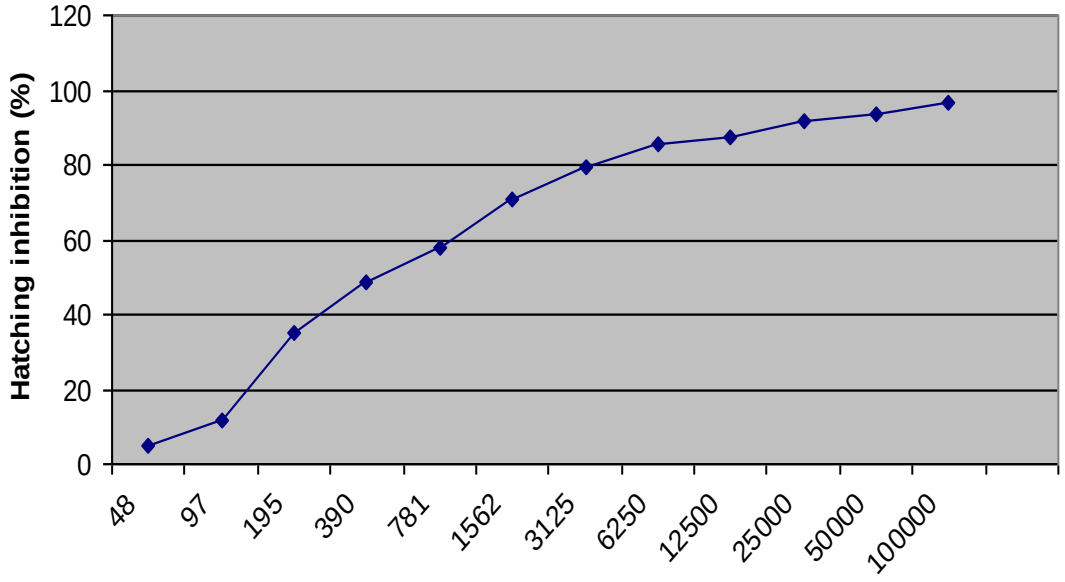

Figure 1 Correlation between the effects of various concentrations of *Arum maculatum* leaf extract and hatching inhibition (%)

4. Discussion

Definitely, recurrent annual use of ML derivative members has conduced to evolution and spread of AR between GI nematodes of sheep and goats every where (Waller, 2006; Hamad *et al*., 2017; Hamad *et al*., 2018). This dilemma has also become a menace to human beings (Beach and Silvestre, 2010). Certainly, GI nematodes of ovine and caprine have developed resistance dangerously against ML derivative members particularly in under-developed nations (Kaplan, 2004; Vercruysse *et al*., 2011). Therefore, this study, conducted in our region, had paid attention to the rampancy of resistance to ivermectin among GI nematode populations in naturally infected sheep using RESO computer program. The computed FECR% was (69.83), whilst the lower confidence interval was (48.4) which justify the emergence of resistance (Coles *et al*., 1992). It can be concluded that the resistance percentage between the GI nematode individuals of ovine in the study area was more than 25% and the worms were resistant to ivermectin. It is apparent that the AR could not be determined by these traditional

approaches if the resistance percentage is less than 25% between the GI nematodes (Martin *et al*., 1989).

The small ruminant owners are adapted to deparasite their livestock 2-3 times with ML derivative members yearly in the study zone (Veterinarian file in the research site). In this connection, Barnes *et al*. (1995); Waller *et al.* (1995); Kalkal *et al.* (2019) have stated that repeated annual use of an anthelmintic are closely related to the emergence and rampancy of resistance between alimentary tract roundworms. The study of Blackhall *et al.* (1998) has revealed that one allele of the putative ά-subunit gene is related to resistance against the dewormer. Nevertheless, Gill and Lacey (1998) have proposed that the mechanism of resistance to ivermectin might be unlike between various genera of parasitic nematodes. Having said, some investigators have also incriminated injecting low doses of the dewormer by shepherds, bad quality of the drug and storage conditions in under-developed countries as major predisposing factors enhancing emergence of resistance (Coles *et al*., 1995).

In contrast, absence of dependable commercialized substitutes to allopathic drugs at the present time, agrochemicals could be apposite alternatives to control GI nematodes in small ruminants particularly in sub-developed nations (Jabbar *et al*., 2006; Sindhu *et al*., 2014). Having said, medicinal plants contain many secondary metabolites which possess antinematicidal activity and their efficacy has been validated and documented in tropical and sub-tropical regions where nematodiasis is common (Hamad *et al.*, 2018). In continuation of the rare studies on phytomedicines, ivermectin-resistant GI nematodes were exposed to the native medicinal herb; *A. maculatum* leaf CAEE using *in vivo* and *in vitro* approaches. Obviously, several bioactive agrochemical substances have been extracted from *A. maculatum* leaves such as glycosides, flavonoids, alkaloids, fatty acids, and essential oils (Rahuman *et al*., 2008; Hussain *et al*., 2014). It is noteworthy mentioning that the efficacy of *A. maculatum* leaf CAEE has not been elaborated *in vitro* and *in vivo* against antinematicidal-resistant GI nematodes of small ruminants elsewhere, thus, it could be mentioned that this research work is a novel study in this domain. The FECR% results post-therapy with 25, 50, 75, and 100 mg kg-^{1} BW of the plant CAEE were 21.13, 54.91, 73.28 and 96.27 respectively. In this concern, it could be point out to the recommendations of W.A.A.V.P (second edition) edited by Wood *et al*. (1995) suggesting that any antiparasitic drug with FECR% (98) is counted highly effective; FECR% (80) and above is effective; whilst FECR% less than (80) is not encouraged to use in fighting pathogenic worms. Consequently, the dose; 100 mg kg-^{1} BW of the extract is effective (FECR% was 96.27). The study had also revealed that the highest doses were more efficacious as compared to the lowest doses. This therapeutic activity of ethnobotanicals was confirmed by several workers in the domain of herbology (Iqbal *et al*., 2006b; Hamad *et al*., 2013; Hamad, 2018; Ahmed *et al*., 2019).

5. Conclusions

Pursuant to the outcome of this research work, it may be concluded that the resistance proportion is above 25% between GI nematode individuals to ivermectin in ovine in Qushtapa district, Hawler governorate, where the study carried out. The likely cause beyond the rampancy of ivermectin resistance in the study zone is recurrent annual employment of ML derivatives. On the other hand, the highly effective dose of *A. maculatum* CAEE was 100 mg kg-^{1} BW which led to reduce mean EPG with percentage 96.27. This dose could be used in deparasiting sheep harboring ivermectin-resistant GI nematodes. Moreover, the EHA has demonstrated that the CAEE of *A. maculatum* can preclude egg hatching but not embryonations.

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Conflict of interest

The author attests that there is no conflict of interest regarding contents of the current research article.

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