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Research Article

IN VITRO EVALUATION OF ANTHELMINTIC EFFECTS OF THE UNRIPE FRUITS OF *AEGLE MARMELOS* (INDIAN BAEL)

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ABSTRACT: Resistance to commonly used anthelmintics stipulate alternative control methods. The use of natural herbal products with anthelmintic properties can address the issue. In this regard, the present study was conducted to determine the *in vitro* antiparasitic activities of aqueous and methanolic extracts of *Aegle marmelos* by using different *in vitro* assays at various concentrations of extracts. The evaluation was done by egg hatch assay (EHA) and larval development assay (LDA) on *Strongyle-type* eggs and larval stages of *Haemonchus contortus* isolated from the goat fecal samples respectively. In EHA, 74.34% and 79.71% were the mean inhibition percent against *Strongyle* type eggs from hatching at the highest concentration of 50 mg/ml tested and the change was 7.2 and 8.24 fold higher than negative PBS control while 77.45% and 85.78% was mean inhibition percentage in terms of *Haemonchus contortus* L3 larvae development at the highest concentration 1 mg/ml tested and the change was 14.5 and 18.3 fold higher than negative control by aqueous and methanolic extract of *A. marmelos,* respectively. Both extracts showed dose-dependent inhibition at all the concentrations tested and were similar to positive control-like treatment with 0.125 mg/ml albendazole for EHA and LDA. The study concluded that the aqueous and methanolic extracts of *A. marmelos* have antiparasitic potential and can be used to control parasitic infection.

Key words: Anthelmintic activity, *Haemonchus contortus*, egg hatch assay, larval developmental assay, *Aegle marmelos*.

INTRODUCTION

According to the 20th livestock census, India has 535.78 million livestock population out of which 192.49 million are cows and about 20.5 million people depend upon livestock for their livelihood (DAHD 2019). Domestic ruminants have a significant contributory role in the upliftment of the socio-economic condition of farmers all over the world. In India, 2/3rd of rural community's livelihood depends on livestock (DAHDF 2019). Moreover, it also contributes 4.11% to GDP (gross domestic product) and 5.1% to total GVA (gross value added) (NDRI 2019). Parasitism harms livestock health and productivity in terms of considerable economic loss in terms of production, reproduction performance, growth, lower weight gain, reduced work capacity, condemnation of affected organs, and even death of severely affected animals (Raza *et al.* 2014, Swarnakar *et al.* 2015, Rashid *et al.* 2019) as well as enhanced susceptibility to other diseases. However, subclinical GI parasitism is more frequent especially in Rajasthan (Choudhary *et al.* 2018, Panwar *et al.* 2019).

Plants are used in the healthcare of animals and man for a long back (Pattanayak *et al.* 2013, Pattanayak 2021) and study for validation of the reported efficacies of such plants as some effective medicines is a very important aspect of modern-day research (Saif *et al.* 2021, Patel *et al.* 2022, Paul and Sujatha 2022).

The plant medicines are effective against varieties of ecto and endo parasites (Yadav *et al.* 2021), Bacteria

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(Pattanayak 2018, Hassan *et al.* 2022), fungi (Singh *et al.* 2020), etc. For control of endoparasites of animals, the administration of anthelmintics is believed to be the only easy and effective approach and indiscriminate use of anthelmintics has resulted in the development of resistance to one or more of the widely used anthelmintic in many countries (Verma *et al.* 2018, Kalkal *et al.* 2020). Awareness of food safety concerning drug residues in animal products and lack of any novel drug constrained development costs (Knox *et al.* 2003) shifting the focus to the herbal plants with medicinal properties (Sunita *et al.* 2017). *Aegle marmelos*, (Indian Bael) is known for its ethnomedicinal uses as antiviral, antibacterial, anti-ulcerative (Maity *et al.* 2009, Balakumar *et al.* 2011), hepato-protective, cardio-protective, anti-diabetic, anti-diarrhoeal, demulcent, antipyretic, anti-inflammatory, anti-fungal, anti-tumor, digestive stimulator, tonic and astringent activities (Baliga *et al.* 2011, Pattanayak *et al.* 2015, Devi and Vanitha 2016) with the effective use of unripe Bael fruit in the diarrhoea management (Mir *et al.* 2010). As the effect of *A. marmelos* extract of nematode intermediate stage is still lacking so current study shows the potential of *A. marmelos* extracts on controlling nematodes eggs as well as larval stages and can be used along with anthelmintic drugs to reduce the pasture load of parasite and anthelmintic resistance management.

MATERIAL AND METHODS

Collection of plant materials

The fresh plant materials were collected from the local market and identified by the Botanical Survey of India, Jodhpur (No.: BSI/AZRC/I.12012/Tech./2019-2020 (PI.Id)/712 Date 17.03.2020 serial no. 37). The unripe fruits of *A. marmelos* were used for the preparation of aqueous and methanolic extracts.

Processing and extraction

The fresh unripe fruits of *A. marmelos* were washed with clean water, and air dried under shade for two weeks, close observation was made to avoid the growth of fungus and molds. The plant material was finally dried in a hot air oven and grounded mechanically by a mixer grinder into fine particles and dried powders were stored in a clean, air-tight container at a dark place till further use. Finally, grounded crude powders of unripe fruits of *A. marmelos* were used for the formation of aqueous and methanolic extracts by Soxhlet extraction (continuous hot extraction) as per Mahire and Patel (2020) method. The extracts thus obtained were packed in an amber color bottle and stored in a dark place till further use.

Test used to access *in vitro* anthelmintic activity

To evaluate the *in vitro* anthelmintic activity of aqueous and methanolic extracts of *A. marmelos* on *Strongyle* type eggs and *Haemonchus contortus* larvae, egg hatch assay (EHA) and larval development assay (LDA) respectively, were performed. Doses were selected based on previous trials and available reports (Har *et al.* 2012, Sridhar *et al.* 2014, Wagh *et al.* 2017).

Egg hatch assay (EHA)

Strongyle-type eggs isolated from the feces of naturally infected goats were used in the present study. The fecal samples were collected from the rectum of infected goats and eggs were isolated by using the centrifugal floatation technique described by Soulsby (1986). EHA was performed based on the method described by Coles *et al.* (2006) with slight modifications. In the assay, 100 µl of egg suspension containing about 100-150 fresh *Strongyle*-type eggs was dispensed into individual wells of a flat-bottom 96-well culture plate (Costar, Corning, New York, USA) and the volume was made up to 200 µl with distilled water PBS (pH 7.2). The egg suspension in each treatment well was mixed with an equal volume of the aqueous and methanolic plant extracts to obtain the following final concentrations: 50, 25, 12.5, and 6.25 mg/ml. Albendazole at 0.125 mg/ml was used as the positive control, as per the report of Rakesh *et al.* (2016) who reported 100% inhibition of larval development at this concentration, whereas untreated eggs in PBS (pH 7.2) served as negative control. The plates were incubated under humidified conditions at 27°C for 48 hrs and later a drop of Lugol's iodine solution was added to each well to stop further hatching. A total of hundred eggs either hatched (egg with larvae) or unhatched eggs were counted under a compound microscope using the 40X objective. The test was carried out with three replicates for each concentration along with control wells. The efficacy of percent inhibition of egg hatching is determined by following the formula given by Coles *et al.* (1992).

$$\text{Inhibition percent (\%)} = (1 - \text{P test}/\text{P control}) \times 100$$

Where P test is the number of hatched eggs or larval form L1 and P control is the respective numbers in PBS control.

Larval development assay (LDA)

The *H. contortus* larvae were isolated from the *H. contortus* eggs recovered from the feces of naturally infected goats in the present study. Sufficient L1 larvae were recovered from the eggs after incubation for 24 hours at 27°C by following the methods of Hubert and Kerboeuf (1992).

Larval development assay was also performed as per Hubert and Kerboeuf (1992) with slight modifications. For LDA, 50 µl of eggs suspension containing 50-100 eggs of *H. contortus* were dispensed into individual wells of a flat-bottom surface 96 wells culture plate (Costar, Corning, New York, USA) as same in EHA with 20 µl of yeast extract prepared in Earle's salt solution (Hubert and Kerboeuf 1984) and 10 µl of amphotericin-B (0.3 mg/ml) were added. The final volume was made up to 200 µl with PBS (pH 7.2). Outer rows of the wells were filled with distilled water. The plates were incubated under humidified conditions for 24 hrs at 27°C to enable the eggs to hatch into first-stage larvae (L1). After this period, the treatment wells were added with equal volume, 200 µl of the aqueous and methanolic extracts of *A. marmelos* at different concentrations of 1 mg/ml, 0.5 mg/ml, 0.250 mg/ml, and 0.125 mg/ml in accordance with the findings of (Wagh *et al.* 2017, Rakesh *et al.* 2020). Appropriate positive (0.125 mg/ml albendazole) and negative (PBS) controls were kept the same as in EHA. The plates were incubated for another 6 days, to permit the larval development from the first stage (L1) to the infective third stage (L3) at 27°C in humidified conditions. One drop of Lugol's iodine was then added, and fifty larvae from each well were counted by separating the larvae into two classes, third-stage larvae (L3) and other developmental stages larvae (L1 and L2) with the aid of a compound microscope using a 40X objective.

The experiment was carried out with three replicates for each concentration along with control wells. The inhibition percentage was calculated according to the equation:

Percent inhibition of development to L3 larvae will be calculated by following the formula according to the method of Coles *et al.* (1992)

$$\text{Inhibition percent (\%)} = (1 - \text{P test}/\text{P control}) \times 100$$

Where P test is the number of larvae that developed into infective larvae (L3) in treatment wells and P control is the respective numbers in PBS control.

Statistical analysis

Graph Pad Prism 7 software (Graph Pad Software, La Jolla, CA) was used for statistical calculations and presentations of graphs in figures and tables. One-way ANOVA was performed, followed by Tukey's multiple comparisons test. The level of significance for experiments was revealed as follows: ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$. Results have been expressed as means ± standard errors of the means (SEM).

RESULTS

***In vitro* anthelmintic activity**

Assessment of *in vitro* anthelmintic activities of aqueous and methanolic extracts of unripe fruit of *A. marmelos* was determined by egg hatch assay and larval development assay and the percent inhibition of hatching of *Strongyle* type eggs and development of *H. contortus* larvae respectively. Tested concentrations of aqueous and methanolic extracts of *A. marmelos, i.e.* 50, 25, 12.5, and 6.25 mg/ml showed dose-dependent enhancement ($p \leq 0.0001$) in ovicidal activities when compared to negative control with complete details in Table 1.

The highest concentration of aqueous and methanolic extracts of *A. marmelos, i.e.* 50 mg/ml showed 7.20 and 8.24 fold ($p \leq 0.0001$) enhancement in ovicidal activity as compared to negative (PBS) control and positive (Albendazole) control depicted only 1.27 and 1.18 fold change ($p \leq 0.0001$) as compared to aqueous and methanolic extract respectively (Fig. 1a and 1b).

The present report has shown dose-dependent egg-hatching inhibition at tested concentrations for both aqueous and methanolic extracts of *A. marmelos* with minor differences which may be attributed to the variation in solubility of the active constituents in the two solvent systems (Eloff 1998). Also, their uniform activities could probably be due to the presence of similar or related chemicals having ovicidal properties in both extract types and needs further exploration (Fig. 2a and 2b).

Egg inhibition in the current study is comparable to similar studies conducted earlier for other plants (Eguale *et al.* 2011, Rakesh *et al.* 2016). Eguale *et al.* (2011) evaluated the anthelmintic potential of different herbal plants at different concentrations using egg hatch assay and concluded that aqueous and hydro-alcoholic extracts of *Leonotis ocymifolia* and *Leucas*

martinicensis showed 100% egg hatching inhibition at the highest concentration of 1 mg/ml plant extracts tested while plant-derived compound bromelain at different concentrations and resulted that 43.25% mean efficacy at 1 mg/ml of highest concentration which was statistically less from the positive control albendazole (Rakesh *et al.* 2016).

Aqueous and methanolic extracts of *A. marmelos* inhibited the hatching of *Strongyle* eggs significantly at 50 mg/ml concentration with many fold changes in the present study (Fig. 1c). Most of the eggs remain unhatched after treatment suggesting that the bioactive compounds were lethal to the eggs. Previous studies indicated that plants used in the current study contain several phytochemical compounds like tannins, alkaloids, coumarins, steroids, polysaccharides, carotenoids, 2-furocoumarin psoralen, riboflavin, mamelon, phenolics, pectins, flavonoids, and terpenoids (Singh *et al.* 2009, Keowkase *et al.* 2020) with possible pharmacological activities. Bioactive compounds such as tannins or saponins are known to be water-soluble polyphenols (Chung *et al.* 1998) and other phytochemicals possibly penetrated the different layers of the egg and inhibited the formation of larva and enzymes responsible for the hatching of *H. contortus* eggs reported by Molan *et al.* (2000) and Marie-Magdeleine *et al.* (2009). The hatching of nematode eggs is initiated by environmental stimuli which lead to the release of so-called hatching enzymes proteases, lipases, chitinases, beta-glycosidases, and leucine aminopeptidases (Sommerville and Rogers 1987) and inhibition of these enzymes are known to reduce the rate of egg hatch or inhibit the process completely Arnold *et al.* (1993).

Larval development assay

In a similar trend, tested concentrations of aqueous and methanolic extracts *i.e.* 1, 0.50, 0.25, and 0.125 mg/ml showed dose-dependent enhancement ($p \leq 0.0001$) inhibition of L1 or L2 larvae of *H. contortus* as compared to negative control (Table 2) (Fig 1d-1f). The highest concentration of aqueous and methanolic extracts of *A. marmelos, i.e.* 1 mg/ml showed 14.53 and 18.36 fold ($p \leq 0.0001$) enhancement in inhibition of L1 or L2 larvae as compared to negative (PBS) control and positive (Albendazole) control depicted 1.27 ($p \leq 0.0001$) and 1.13 ($p \leq 0.01$) fold change as compared to aqueous and methanolic extracts respectively with complete details in Table 2 (Fig. 2c and 2d). Larval inhibition reported in the present study following the reports of Molan *et al.* (2003) may be due to the condensed tannins which are known to affect the biological processes of nematode (Hoste *et al.* 2006) as well as larval development in a dose-dependent manner (Molan *et al.* 2003). Leaf extract of *A. marmelos* has been reported earlier to inhibit the motility of *Brugia malayi* microfilaria completely @ 100 ng/ml concentration (Sahara *et al.* 2008) and cause reduction in diarrhea and parasitic ova of *Ancylostoma caninum* in dogs after oral ingestion (Har *et al.* 2012). Ethanol solvent allows the extraction of more natural bioactive compounds than aqueous extracts so the efficacy of ethanolic extract might be due to the amount of polar products coming from their traction that these extracts have weakened oocyst walls more rapidly and inhibited the sporulation process more readily (Arlette *et al.* 2019).

Interaction of water-soluble tannin with bacterial cells and yeast in a culture medium with eggs will protein complexes which will be either precipitated or not swallowed by developing larvae and cause damage to the mucosal lining of the gastrointestinal tract of larvae Molan and Faraj (2010). Another possible mechanism was that condensed tannin might cause damage to the pharyngeal muscles and prevent the feeding of the larvae, this leads to inhibition of the development of L1 to L3 larvae, indicating their *in vitro* and *in vivo* anti-parasitic potential. *A. marmelos* fruits have tannins as biologically active compounds (Bhardwaj and Nandal 2015) which may be responsible to reduce the motility of the L3 larvae with possible interference to neuromuscular coordination of the larvae and may induce paralysis (Molan *et al.* 2009).

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Table 1. Mean inhibition percent of aqueous and methanolic extracts of unripe fruit of *A. marmelos* to inhibiting *Strongyle* type eggs.

Concentration (mg/ml)	Mean inhibition (percentage ± SEM)	
	Aqueous extract	Methanolic extract
50 mg/ml	74.34^{a}±0.72	79.71^{a}±0.87
25 mg/ml	64.31^{b}±1.29	70.47^{b}±0.96
12.5 mg/ml	52.05^{c}±1.03	59.41^{c}±0.64
6.25 mg/ml	39.76^{d}±1.49	48.34^{d}±0.63
Albendazole (0.125 mg/ml)	94.42^{e}±0.64	94.10^{e}±0.35
PBS	10.33^{f}±0.33	9.67^{f}±0.67

[All data were analyzed by One-way ANOVA analysis, followed by Tukey's multiple comparisons test. a, b, c, d and f are the level of significance for with $p \leq 0.0001$ or $p \leq 0.001$. All results are expressed as means ± standard errors of the means (SEM)].

Table 2. Mean inhibition percent of aqueous and methanolic extract of unripe fruit of *A. marmelos* to inhibition of development of L1 or L2 larvae into L3 larvae of *H. contortus*.

Concentration (mg/ml)	Larval Inhibition (percentage ± SEM)	
	Aqueous extract	Methanolic extract
1 mg/ml	77.45^{a}±0.91	85.78^{a}±1.97
0.50 mg/ml	67.57^{b}±1.18	76.57^{b}±1.41
0.25 mg/ml	56.31^{c}±1.15	66.63^{c}±1.80
0.125 mg/ml	45.05^{d}±1.44	55.34^{d}±0.68
Albendazole (0.125 mg/ml)	96.50^{e}±0.66	97.18^{e}±0.67
PBS	5.33^{f}±1.33	4.67^{f}±0.67

[All data were analyzed by One-way ANOVA analysis, followed by Tukey's multiple comparisons test. a, b, c, d and f are the level of significance for with $p \leq 0.0001$ or $p \leq 0.01$. All results are expressed as means ± standard errors of the means (SEM)].

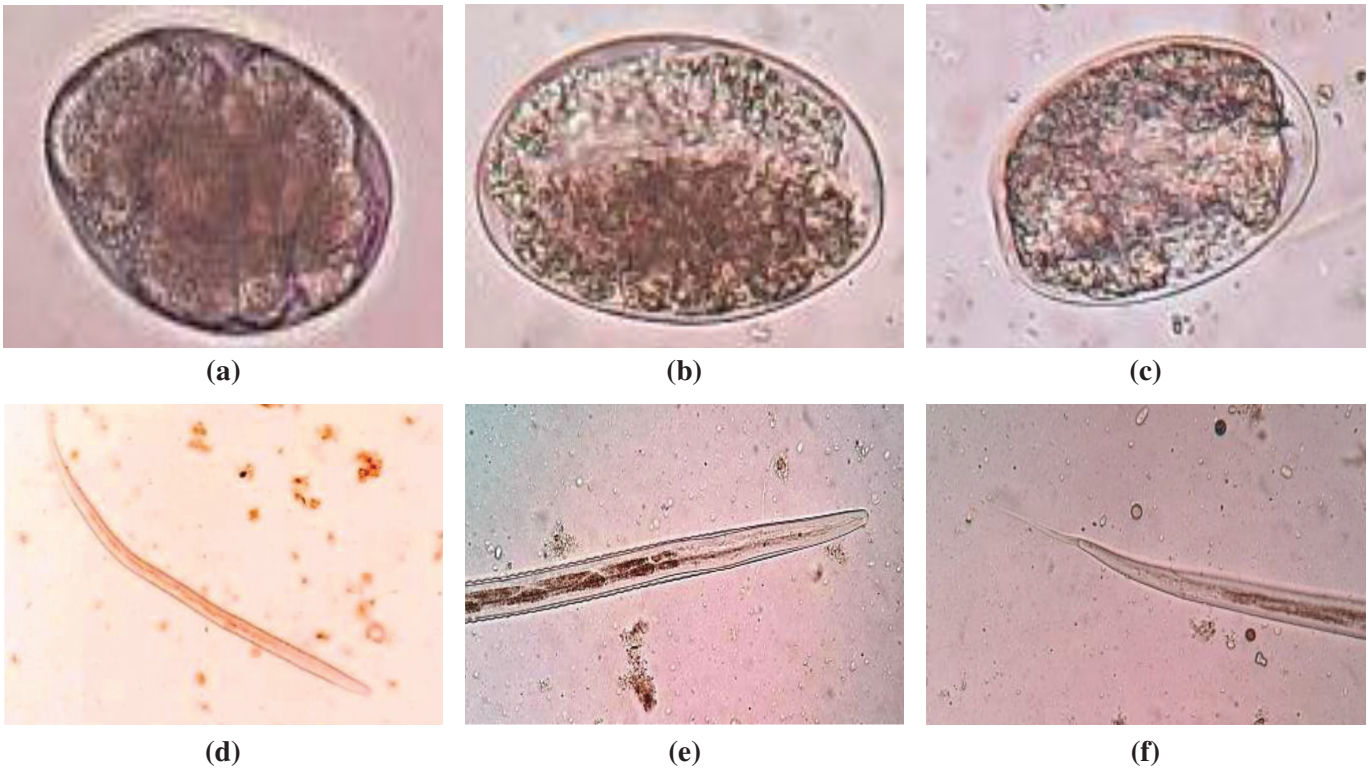

Fig. 1. Photomicrographs of nematode.
[(From left to right, a-f): 1a-1c: depicts different stages of *Stronglye* egg; 1d-1f indicate the typical L1 larvae of *H. contortus*, the mouth part of L3 larval stage of *H. contortus* and the characteristics kinking of tail of *H. contortus,* respectively].

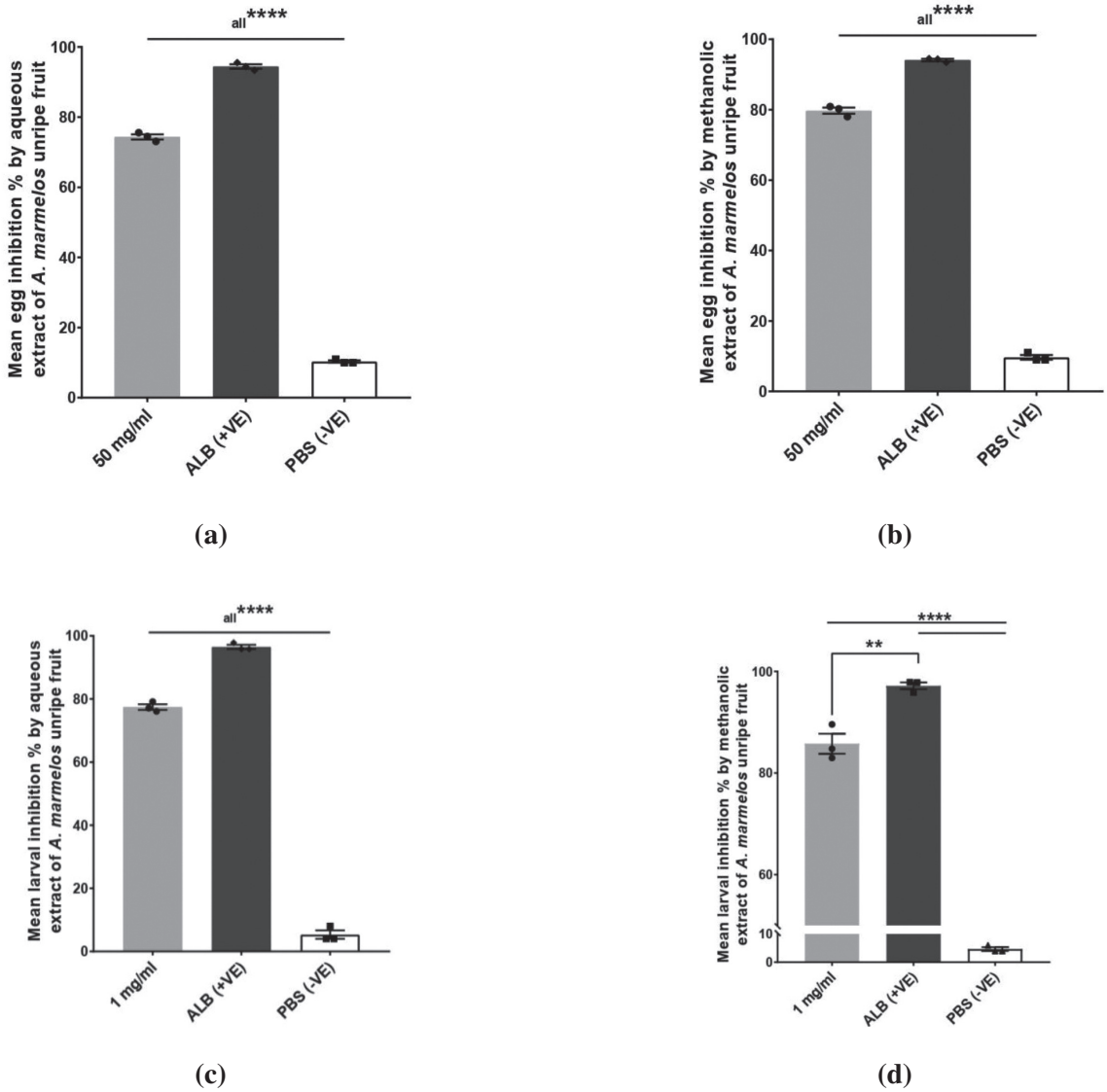

Fig. 2. *In-vitro* ovicidal (on *Stronglye* egg) activity and L1 or L2 larval (on *H. contortus*) inhibition activity by aqueous and methanolic extract of *A. marmelos* (From left to right, a-d).

[Histograms represent the percent ovicidal activity of aqueous (Fig 2a) and methanolic extract (Fig 2b) of *A. marmelos* on Stronglye egg. Histograms represent the percent L1 or L2 *H. contortus* larval inhibition by aqueous and methanolic extract in 2c and 2d respectively. All data were analyzed by One-way ANOVA analysis, followed by Tukey's multiple comparisons test. The level of significance for experiments was revealed as follows: ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$. All results are expressed as means ± standard errors of the means (SEM)].

CONCLUSION

Plant derivatives are much-needed alternatives to the currently available chemotherapeutic antiparasitic drugs. Considerable inhibition indicates that *A. marmelos* have anthelmintic potential against *Strongyle*-type egg and larvae of *H. contortus*. The methanolic extract has shown slightly better inhibition. Extraction and characterization of exact bioactive compounds from *A. marmelos* and *in vivo* evaluation in animal models also need to be accomplished in the near future.

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