

## STUDY OF ANTHELMINTIC ACTIVITY OF *AEGLE MARMELOS* (LINN.) CORREA (RUTACEAE) ESSENTIAL OILS FROM LEAVES AND RIND

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### Abstract

In this study 'anthelmintic activity of essential oils (EOs), of leaves and rind of *Aegle marmelos* (Linn.) Correa ver. Bel were evaluated. 'Adult motility assay' was employed using *Haemonchus contortus* adult worms. EOs were used at three levels viz. 10, 35 and 50  $\mu$ L/10 ml in phosphate buffer solution (PBS) plus 10  $\mu$ L of tween 20 (as carrier/emulsifier). Levamisole was used as a positive control at 0.55 mg/ml concentration. Each concentration of essential oils obtained from different plant parts exhibited varied anthelmintic activity. The best dose dependant effect on adult mortality was by EOs from *Aegle* rind (50  $\mu$ L), with  $R^2$  value 0.981, followed by EOs from leaves with  $R^2$  value 0.86.  $LC_{50}$  values were 34.92 and 26.65 for rind and leaves respectively. Change in the color of the dead worms was an indicator parameter. This was of the fact that the EOs might have damaged the skin of the worms or after transcutaneous penetration into the body has disrupted the circulatory system by causing constriction of blood vessels. Hence it is concluded that EOs extracted from *A. marmelos* have anthelmintic properties and can be used as replacement of costly synthetic chemicals.

**Keywords:** Essential oils, *Aegle marmelos*, anthelmintic activity

### INTRODUCTION

Helminthiasis, a world over disease of grazing animals, is of especial concern in developing countries regarding inappropriate control activities (Lateef *et al.*, 2003). For this purpose synthetic anthelmintics have been used deliberately. The synthetic chemicals are costly as well as not accessible to the farmers in rural areas. It is a common practice of livestock producers to use local plants as dewormers. Besides chemical residual and toxicity problems, the use of synthetic chemicals also pose other hazards like resistance in helminths to various anthelmintic compounds (Adrian *et al.*, 2004). This has prompted the search for medicinally important plants for their 'anthelmintic activity' world-over (Lateef *et al.*, 2003). Therefore, the use of essential oils is thought to be more important and environment friendly. It is well reported that essential oils obtained from various plants like, *Croton zehntneri* and *Lippia sidoides* (Camurça *et al.*, 2007) and that from

*Eucalyptus staigeriana* (Lara *et al.*, 2010) inhibited larval development of *Haemonchus contortus*. Extracts from different parts of plants also had 'anthelmintic effects on the eggs and mature *Haemonchus contortus* (Egualé *et al.*, 2007; Gbolade and Adeyemi 2008; Tariq *et al.*, 2008; Fall *et al.*, 2008; Sujon *et al.*, 2008; Nery *et al.*, 2010; Ademola *et al.*, 2010). The family Rutaceae is of great economic importance for its numerous edible fruits of *Citrus* and *Aegle* genera. Other species like those of *Murraya*, *Zanthoxylum*, and *Skimmia* have medicinal properties. The presence of essential oils in members of family Rutaceae with diverse activities has an increasing demand for natural sources of anthelmintics. Thus, this study was conducted to explore the anthelmintic activity of essential oil obtained from different parts of *Aegle marmelos*. It is found in many countries of South-eastern Asia and used for the treatment of different diseases and is known for its hypoglycemic activities in humans and animal systems (Sabu and

Ramadasan 2004). The ripened fruit is useful for the treatment of diabetes (Kamalakkannan and Prince 2003), constipation, dyspepsia, and body temperature regulation problems (Kalaivani *et al.*, 2009). Essential oils extracted from *A. marmelos* exhibited promising activities against fungi like, *Phylospora tucumanensis*, *Ceratocystis paradoxa*, *Sclerotium rolfsii*, *Curvularia lunata*, *Helminthosporium sacchari*, *Fusarium moniliforme* and *Cephalosporium sacchari* (Mahilrajan *et al.*, 2014). *A. marmelos* EO has been recommended as a plant-based antimicrobial in food protection over synthetic preservatives (Singh *et al.*, 2009).

*Aegle marmelos* (Family - Rutaceae), usually known as Bel, a deciduous and evergreen tree is an indigenous species from Pakistan. Ripe fruits of this tree are used for curing diarrhea, gastric trouble and as heart tonic. However, there are not much biological studies on *Aegle marmelos* essential oils for the anthelmintic activity specifically in Pakistan. Therefore, we decided to perform a systematic study of EOs from this plant for anthelmintic activity. For this purpose Eos were extracted and their anthelmintic was studied using *Haemochus* as model organism. Aim was clear to search for cost effective and hazard free alternate to the synthetic chemicals, presently in use as anthelmintics.

## MATERIALS AND METHOD:

### i. Extraction of essential oils:

Different parts i.e., rind and leaves of *Aegle marmelos* were selected to analyze the 'anthelmintic activity' of essential oils. The above mentioned plant parts were chosen keeping in mind their ethno-botanical use in Pakistan and were obtained from their natural habitat, identified and authenticated by an expert, at the Department of Botany, G.C. University, Lahore. The respective plant parts were separated and subjected to hydro-distillation for

about four hours. The EOs obtained thus were dried with anhydrous sodium sulphate and put in brown colored glass bottles, at temperature of about 4° C.

### ii. Physical and Chemical analysis:

Physical characteristics were recorded and chemical composition was determined through GC-MS analysis.

### iii. *In vitro* anthelmintic activity (adult motility assay):

*In vitro* trials of the essential oils were carried out on adult '*Haemonchus contortus*' of sheep as by Sharma *et al.*, (1971). The adult worms of either sex were obtained from the 'abomasum' of freshly sacrificed sheep in the local slaughterhouse. These worms were given washing and then put in petri-dish having phosphate buffer (PBS) in it. Ten worms, *H. contortus* were given the following treatments, in triplicates, in petri dishes separately at room temperature (25-30° C). 10 µL of Tween 20 was added to each concentration of essential oils, as emulsifier, along with 10 ml PBS uniformly.

1. 10 ml PBS + 10 µL (oil) + 10 µL Tween-20
2. 10 ml PBS + 35 µL (oil) + 10 µL Tween-20
3. 10 ml PBS + 50 µL (oil) + 10 µL Tween-20
4. Levamisole at rate of 0.55 mg/ml was taken as a positive control.
5. 10 ml PBS + 10 µL Tween-20 (control)

On the basis of slight change in their body color and inhibition of movement, dead worms were recognized easily. The time taken during motility was noted. The dead worms were put in the lukewarm (37°C) fresh PBS for 30 minutes to see revival of motility in worms if any (Images 1, 2, and 3). Following criteria was used to interpret the results of adult motility assay in the present work were (a) time taken for mortality of worms (*H. contortus*) and (b) response of worms to different

concentrations of essential oils, i.e., dose dependant effect.

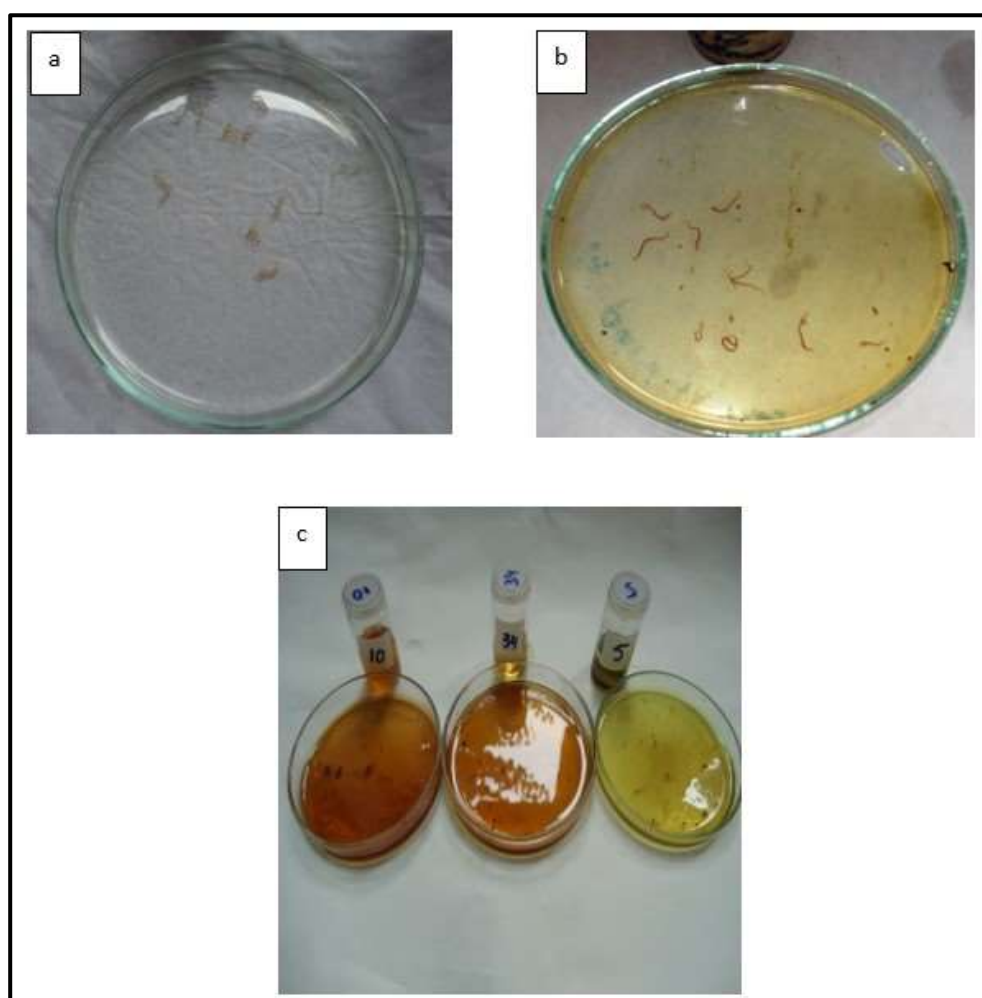
#### iv. STATISTICAL ANALYSIS:

One-way ANOVA, LSD and Probit regression tests were applied, using SPSS 13.0

(statistical software) on the data for statistical analysis to draw conclusions. Table 1. Shows that on hydro distillation percent yield of EOs was maximum from *Aegel* leaves 0.31% followed by 0.02 % from rind with clear yellowish colored oils.

**Table 1.** Yield and physical characteristics of essential oils obtained from different parts of *Aegel marmelos*

Plant	Part used	Place of collection	Weight in Kg	Weight of oil in g	Yield in %	Color of oil
<i>Aegel marmelos</i>	Rind	Lahore	2.700	0.60	0.02	Clear yellowish oil
<i>A. marmelos</i>	Leaves	Lahore	8.255	25.77	0.31	Clear yellowish oil



**Plate 1:** (a) *H. contortus* worms in PBS, (b) *H. contortus* worms in EO (enlarged view) and (c) *H. contortus* worms in different Eos.

## CHEMICAL COMPOSITION OF ESSENTIAL OILS:

Table 2 indicates that D-Limonene dominated in Eos from rind as well as leaves followed by 3-Carene from leaves and psi- Cumene in rind EO. Category wise, Monoterpene is of highest percentage compound in essential oils of *Aegel marmelos*, leaves and rind followed by Esters and Hydrocarbons. This has already been reported by Suvimol and Pranee (2008).

## LC<sub>50</sub> AND REGRESSION CO-EFFICIENT:

Essential oils from *A. marmelos* rind and leaves had LC<sub>50</sub> 34.92 and 26.65 respectively. R<sup>2</sup>

## DISCUSSION:

The use of herbal products such as essential oils in anthelmintic actions has many benefits. Firstly, EOs have a rich chemical composition of many bioactive compounds with vast pharmacological prospective (Ferreira *et al.*, 2016), which can result in high activity against nematodes, as shown in our *in vitro* study. Moreover, a large number of compounds in EOs that belong to different chemical classes may contribute to a reduced susceptibility to resistance (Ferreira *et al.*, 2016, Fonseca *et al.*, 2013). Some reports state that botanical anthelmintics may be regarded well tolerated by animals from the toxicology view and are related to the low amount of residues in meat and milk (Ferreira *et al.*, 2018). Although this is not proven and requires precise studies upon this, a large number of EOs, as well as their ingredients belonging to sundry chemical groups, offers a chance to find those who best meet these requirements, along with suitable efficacy. This also denotes varied price depending on the EO. Finally, their local distribution allows easy acquisition (Ferreira *et al.*, 2018) and, along with other stated factors, offers the opportunity of a maintainable possibility for nematode control in ruminants.

values for *A. marmelos* rind was 0.98, while leaves showed 0.86.

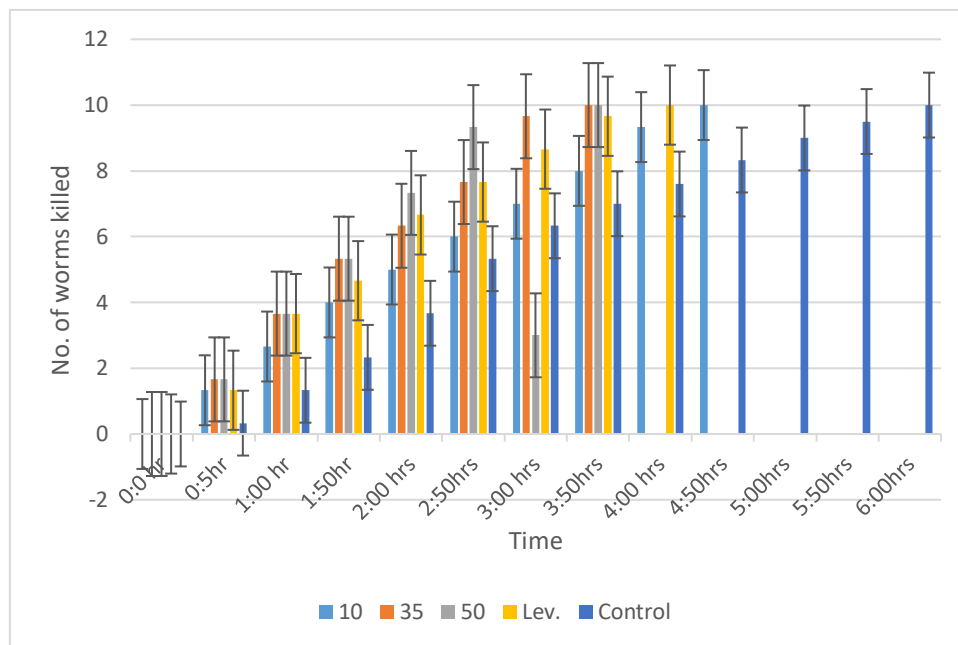
Results obtained after applying, One way ANOVA and LSD test, showed that anthelmintic activity of 10µL essential oils *Aegel marmelos* (leaves and rind), was less than anthelmintic activity of the positive control viz. Levamisole. As far as anthelmintic activity of 35µL of essential oils *Aegel marmelos* (leaves and rind), is concerned, it was similar to that of anthelmintic activity of the positive control viz. Levamisole. On the other hand, anthelmintic activity of 50µL of essential oils *Aegel marmelos* (leaves and rind), was greater to that of positive control.

In present study *In vitro* evaluation for anthelmintic activity of essential oils of different plant parts was tested using adult motility assay (AMA). Essential oils from every plant part incorporated in this study showed anthelmintic activity in opposition to *Haemonchus contortus* as clear from results of adult motility assay. A wide variation was observed in the anthelmintic effects of essential oils regarding intensity and dose dependant effects. **Figures 1 and 2**, show time dependent *in vitro* anthelmintic activity of essential oils from *Aegel marmelos* rind and leaves. Concentration of 50 µL oils proved more potent than Levamisole. Chemical analyses showed Monoterpenes to be the major phytochemicals in the EOs in essential oils followed by Esters and Hydrocarbons. This has already been reported by Suvimol and Pranee (2008). Monoterpenes have acaricidal activity (Cetin *et al.*, 2010). The anthelmintic efficacy of EOs may be due to single or a combined effect of the compounds or chemical groups. The speculated mechanism of action might be disruption of membranes by the lipophilic compounds (Suresh *et al.*, 1997; Amaral *et al.*, 1998). Variations in the 'anthelmintic activity' of the EOs tested in this experiment may be as a result of varied targets on

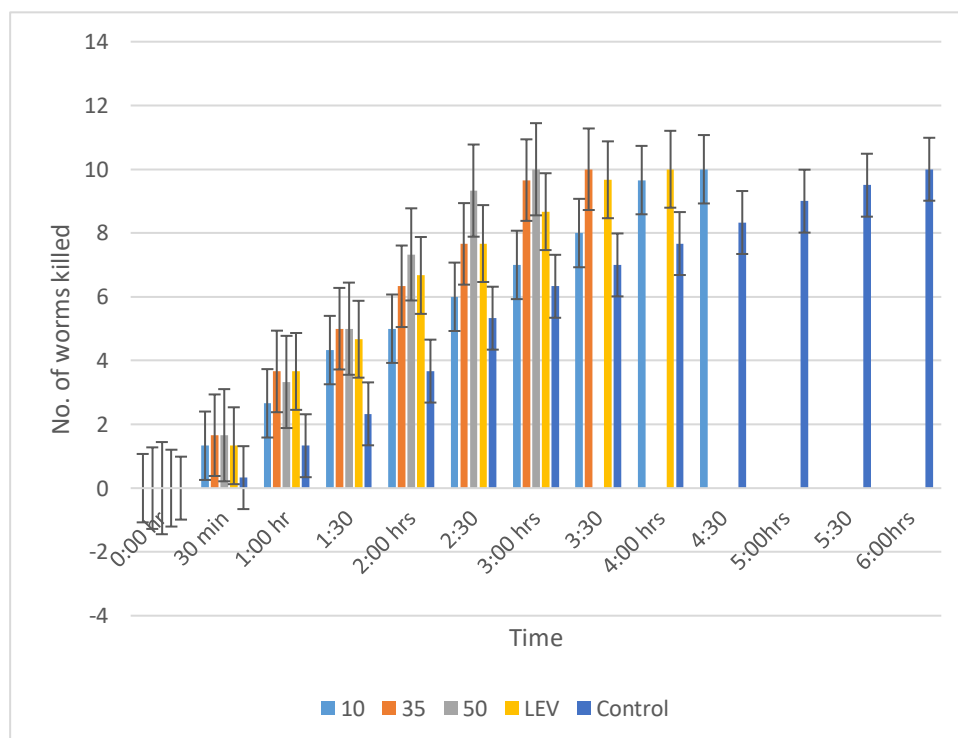
the parasites for action of the active chemicals present in EOs, with differences in quality and/or quantity. The dermal application of the EOs indicates that EOs might interfere with the osmotic balance of the worm resulting into death of worms. Higher the dose, the more pronounced was the rate of mortality. Different compounds/active principles of EOs may have different targets to exert anthelmintic effect on adults. The reported mechanisms are uncoupling of oxidative phosphorylation (Weinbach & Garbus, 1969). There are some similar targets among bacteria, fungi, protozoa, and helminths, which can also be hit by the compounds with 'anthelmintic activity'. These may be inhibition of enzymes, making complexes with proteins, polysaccharides, forming channels of ions, etc. These specific actions might disturb the usual processes of biochemical and physiological nature, thus depriving of nutrition, changes in structure, disturbing neuromuscular aspects, and other effects on 'helminths'. These are the most recognized targets for 'anthelmintics' in common use, (Kohler 2001; Mottier *et al.*, 2006).

An efficient alternative to commercial anthelmintics is immediately required due to the increase of resistance and the economic losses it involves. Therefore, testing of different EO samples may contribute to discovery of valuable anthelmintic agents. The present study demonstrated the high *in vitro*, anthelmintic potential of the tested

natural-based formulations against sheep gastrointestinal nematodes. From this perspective, the highest potential was shown by Eos from A. *marmelos* rind followed by Eos from leaves. EOs contained compounds such as psi-Cumene, D-Limonene, Myrtenyl acetate, Artemiseole, Myrcenol, Columbin in high percentages. Nevertheless, this study is another confirmation of the possible role of botanical anthelmintics in the control of small ruminant's GINs. Hence looking from a forthcoming viewpoint, the results of the present study may be of practical importance in combating anthelmintic resistance. In spite of encouraging results in present study, a lot of variation in conditions faced *in vivo*, like bio-transformations during metabolic pathways, interactions with 'feed materials' and uptake by the body, the outcome by the *in vitro* method could not be considered applicable to *in vivo* activity. Thus, these results should be confirmed by *in vivo* experimentation, so as to standardize doses and develop a drug. Dead worms from each petri dish were finally placed in luke warm PBS for 30 minutes, but no revival of motility was seen.



**Figure 1.** Time dependant mortality of adult *Haemonchus contortus* by essential oils (10 µL, 35 µL, 50 µL) from *Aegel marmelos* (Linn.) Correa. Bel leaves



**Figure 2.** Time dependant mortality of adult *Haemonchus contortus* by essential oils (10 µL, 35 µL, 50 µL) from *Aegel marmelos* (Linn.) Correa. Bel Rind

**Conflict of interest:**

There is no conflict of interest regarding research work in this publication.

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