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In-vitro assays on *Mangifera indica* and *Embelia ribes* against *Ascaridia galli* of poultry

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Abstract

The anthelmintic effect of indigenous medicinal plants namely *Embelia ribes* (seed) and *Mangifera indica* (bark) was evaluated against the exogenous stages of the poultry nematode *Ascaridia galli. In vitro* egg embryonation assay showed that the methanolic extract of *Embelia ribes* showed better inhibitory effect (61.23%) on the embryonation of eggs of *Ascaridia galli* than its aqueous extract (58.20 %). Both *M. indica and E. ribes* had low inhibitory effect on the activity of second larval stage (L_2) inside egg shell. The *in vitro* assay for larvicidal activity on the deshelled larvae of *A. galli* revealed that the aqueous extract of *Embelia ribes* at a concentration of 20 mg/ml exhibited moderate inhibitory effect of 77.66±1.85%. Adult motility assay revealed that the methanolic extract of *E. ribes* had a significant anthelmintic efficacy of 88.33%. The extracts from *M. indica* were found to have very low to moderate anthelmintic properties against various developmental stages of *A. galli*.

Key words: *Ascaridia galli, Embelia ribes, Mangifera indica,* poultry.

Poultry is one of the fastest growing segments of the agricultural sector in India. The production of agricultural crops has been rising at a rate of 1.5-2 % per annum while that of eggs and broilers has been rising at a rate of 8-10 % per annum (Mehta *et al.* 2003). Huge losses in poultry have been linked to disease causing agents such as viruses, bacteria and parasites. *Ascaridia galli* has been incriminated as the most common, important and prevalent parasite of poultry (Pam *et al.* 2006; Luka and Ndams 2007). *Ascaridia galli* infection continue to be the most debilitating factor impeding poultry productivity resulting in retarded growth, weight loss, diarrhoea, poor absorption of nutrients, death and even the spread of fatal bacterial infections, consequently responsible for economic losses to the poultry industry (Gauly *et al.* 2007). Most of the parasite control programs are based upon a combination of hygienic measures and chemotherapeutic control. Various problems have evolved with chemotherapeutic control practices as parasites are developing resistance to several families of chemical antihelmintics rendering helminth infections rampant as ever (Chartier *et al.* 2001; Stear *et al*. 2007), chemical residues and toxicity problems (Muhammad *et al.* 2004). Therefore, possible alternatives such as the use of plant products that function by mechanisms other than those of chemotherapeutics, with the additional advantage of a natural origin have been recommended (Naidoo *et al*. 2008). The dried fruits of *Embelia ribes* (False black pepper/Vidhang) have been described in Ayurveda as a powerful anthelmintic, antifertility and antihyperlipidemic agent (Kekuda *et al*. 2009). *Mangifera indica* (Mango) has been reported to possess antiallergic and anthelmintic properties (Garcia *et al.* 2003). Hence, the present study was planned to evaluate and validate the *in vitro* effects of crude extracts of bark of *M. indica* and seeds of *E. ribes*, which are indigenous medicinal plants and are a part of ethno-veterinary practices of this region, against exogenous stages of *A. galli.*

Materials and Methods

Preparation of aqueous and methanolic extracts

The bark of *Mangifera indica* and seeds of *Embelia ribes* were collected from their natural habitats, shade dried and grinded to obtain the fine powder. The

aqueous and methanolic extracts were prepared as per technique described by Hussain *et al.* 2011. The per cent recovery of both aqueous and methanolic extracts was recorded. After getting the dried filtrate from each plant, it was lyophilized (lyophilizer- Alpha 1-2 LD Plus, Martin Christ Germany). The lyophilized aqueous and methanolic extracts were stored at 4 °C until use and dissolved in PBS on the day of the experiments to prepare stock solution and different dilutions for the purpose of evaluating anthelmintic activity.

Preparation of testing solutions

Different testing solutions of varying concentrations were prepared from the crude extracts using PBS as the solvent on the day of experiment. A positive control with albendazole (for evaluating activity against egg embryonation and L_2 inside the egg shell and piperazine (for evaluating activity against larvae and adult worms) was set corresponding to each of the doses in each of the *in vitro* assay. A negative control consisting of the diluents PBS (for evaluating activity against egg embryonation and L_2 inside the egg shell), Earls medium (for evaluating activity against larvae) and Ringer locke (for evaluating activity adult worms) was set corresponding to each of the doses in each of the *in vitro* assay. Three replications were done for each of the concentrations for both aqueous and methanolic extracts of all plant material and different plates were set for studying the effects at different time intervals.

The faeces of the infected birds were used for the collection of eggs. Also the adult *Ascaridia galli* female worms were collected from the intestines of infected birds from the slaughter houses. The eggs were collected from the uteri of the gravid female of *A. galli*. The uteri containing fertile eggs were squeezed out from the gravid females and the eggs were liberated by manipulation of the uteri. The eggs were separated by dropper and were washed 3-4 times in distilled water before placing them in clean petridishes at 30 ± 1^0 C for development for 12 days. The suspension of infective dose (500 eggs) was given to each of the five birds at 10 days of age to make the donor birds. Prior to giving infection to the donor birds their faeces were screened for presence of any parasitic stage and the birds were treated accordingly. Thereafter, the faeces of the birds were checked regularly for the presence of *Ascaridia galli* eggs.

***In vitro* egg embryonation assay**

It was conducted by the method described by Coles *et al.*1992 with some modifications that allowed the testing of the natural compounds (Alawa *et al.*2003). Suspension of *A. galli* eggs (0.15 ml; 100 eggs) was distributed in each of 96 Well U bottom micro titre plates (Tarsons) and mixed with the equal volume of different concentrations (20, 40, 60 mg/ml) of plant extract dissolved in PBS. The positive control wells received a single concentration (10mg/ml) of Albendazole (Virbac, 25mg/ml) in place of plant extracts plus the egg suspension while negative control wells contained the diluents(PBS) and the egg suspension. The eggs were incubated in this mixture at 30°±1C for 12 days. There were three replicates for each dose (for both aqueous and methanolic plant extracts) and positive and negative controls. The dead eggs, eggs at different stages of embryonation (2, 4, 8, 16 cell stage, morula etc.) and fully embryonated eggs (with L2) were counted in each set after 12 days of incubation. Data was expressed as percentage of unembryonated eggs.

***In vitro* assay for larvicidal activity against L_2 inside the egg shell**

The infective larvated eggs (second larval stage inside eggs) were prepared by collecting the eggs from the faecal samples by Flotation concentration technique as described above. The eggs were then suspended in distilled water kept in petri dishes (2.5 inches diameter). Later, few drops of 2% formalin as preservatives was added and incubated at 30°C ± 1°C for 12 days. The embryonated infective eggs thus obtained were stored at 4°C until used. The test was conducted by the method described by Coles *et al.* 1992 with some modifications. Egg suspension containing eggs with L2 stage (0.15 ml; 100 eggs) was distributed in each of 96 Well U bottom micro titre plates (Tarsons) and mixed with the same volume of different concentrations (20, 40, 60mg/ml) of plant extract .The positive control wells received different concentrations of Albendazole (10mg/ml) (Virbac, 25 mg/ml) in place of plant extracts while negative control wells contained the diluents PBS and the infective egg solution. The eggs were kept in this mixture at room temperature ($25\text{-}30^0$ C). The level of the wells was maintained by adding distilled water to them regularly. There were three replicates for each treatment and both positive and negative control. The

observations were recorded for each set after 6, 12, 24 and 48hrs. The eggs with the dead larvae (non motile) and live larvae inside the egg shell were counted after 6, 12, 24 and 48hrs.

***In vitro* assay for larvicidal activity**

This assay was carried to check the anthelmintic effect of the extracts against the deshelled L_2 stage larvae. The larvae were released from the larvated eggs by the procedure described by Hansen *et al*. (1956) and taken in the Earl's medium, in petridishes, with the help of coverslips (*Garadaghil et al. 2008).* Larval paralysis assay (Martin and Le Jambre 1979) was performed to evaluate larvicidal activity. The final concentration of L_2 larvae was kept around 100 L_2/100 µl of nutritive solution. Petri dishes containing different concentrations (5, 10, 20 mg/ml) of extract dissolved in PBS were incubated with larval suspension at room temperature (25-30^0 C).Larval suspension with nutritive medium and PBS at the same concentration as in test set served as negative control. Positive control wells contained same concentration of larvae with Piperazine citrate (10mg/ml). Each concentration of extract treatment had three replicates. The number of surviving (motile) and paralyzed (no observable motion during 5 sec) larvae were counted after 6, 12, 24 and 48 hours of incubation.

Adult motility assay

The *in vitro* trials of the aqueous and methanolic extracts were conducted on active and motile mature *A. galli* worms by petridish method. The worms were collected in Ringer Locke (RL) solution from intestines of freshly slaughtered infected birds. A minimum of 6 worms were exposed in three replicates to each of the plant extracts in separate petridishes at room temperature (25-30^{o}C).The efficacy of the extracts was evaluated at different concentrations (5, 10, 20 mg/ml) at room temperature. The positive control contained worms with piperazine citrate (10 mg/ml) (Kosalge and Fursule 2009) in RL and negative control had worms in RL. The inhibition of motility and/or mortality of the worms kept in the above treatments were used as the criterion for anthelmintic activity. The petridishes were incubated for sixteen hours at room temperature and the efficacy was observed by counting the dead parasites and expressed in percentages (%).The motility was observed after 2, 4, 8 and 16 hour intervals.

Statistical analysis. Comparison of the mean values of the treatments was made using unpaired Student's t test and the level of probability was considered significant when $P<0.05$.

Results and Discussion

The maximum percent recovery of *Embelia ribes* was obtained by making methanolic extract whereas the maximum percent recovery of *Mangifera indica* was through the aqueous extract (Table 1). *In vitro* egg embryonation assay revealed a relatively fair efficacy by *E. ribes* against the development of *A. galli* eggs. The results revealed that methanolic extract of *E.ribes* at the concentration of 60 mg/ml showed better inhibitory effect on the embryonation of eggs of *A. galli* than their aqueous extract at the same concentration. However *M. indica* (both the aqueous and methanolic extracts) showed very low inhibitory effect on the development of *A.galli* eggs (Table 2).

The *in vitro* assay for larvicidal activity against L_2 inside the egg shell revealed that both aqueous and methanolic extracts of *E.ribes* exhibited moderate anthelmintic activity (Fig.1). Both methanolic and aqueous extract showed inhibitory effect of 38.67±4.10 and 38.67±1.86%, respectively, at a concentration of 60 mg/ml at 48hrs post incubation. Inhibitory action of methanolic extract of *M. indica* against larvae inside shell was maximum (50.33±3.71%) with at a concentration of 60 mg/ml. However, the aqueous extract showed low inhibitory effect (30.33±2.91) at the same concentration.

In vitro assay for larvicidal activity revealed that both the aqueous and methanolic extracts of *E. ribes* exhibited moderate anthelmintic effect of 77.66±1.85 and 73.66±2.33 per cent, respectively, at a concentration of 20 mg/ml at 48 hrs post incubation on the deshelled larvae of *A. galli* (Table 3). The aqueous extract of *M. indica* showed highest efficacy of 63.33± 2.18 percent at 20 mg/ml 48 h post incubation. Almost, similar effect of methanolic extract was seen (62.33±1.45 per cent) at 20 mg/ml at 48 h post incubation.

Adult motility assay revealed a significant inhibitory effect of 88.33% of *E. ribes* (methanolic extract) at 20 mg/ml concentration 16 h post exposure (Table 4). Thus, indicating that the plant could be used as anthelmintic in poultry targeting the mature stages of *A. galli*. *M. indica* extracts did not produce any significant anthelmintic activity against the mature *A.galli* worms. The maximum efficacy of 38.33% was

seen at 20 mg/ml (aqueous extract) 16 h post exposure.

There are reports of anthelminthic activity of *E. ribes* (Garg and Mehta 1960; Guru and Mishra 1964) but no previous study on *A. galli*. *Embelia ribes* seed oil has been reported to be efficacious against *Pheritima posthuma* at concentrations 10, 50 and 100 mg/ml (Jalalpure *et al*. 2007). *Embelia ribes* was found to be very potent against soilborne non-parasitic infected with the nematode, *Trichinella spiralis*, it led to a significant decline in the number of parasite larvae encysted in the musculature (Garcia *et al*.2003). The crude ethyl acetate and ethanol extracts of *M.indica* L. was evaluated for anthelmintic activity using adult earthworms, in which *M. indica* root extracts exhibited a dose dependent inhibition of spontaneous motility (paralysis) and evoked responses to pin-prick. With higher doses (30 mg/ml

Table 1. Per cent recovery of the methanolic and aqueous extracts of plant parts

Name of the plant	*Embelia ribes*	*Mangifera indica*
Plant parts	Seeds	Bark
Initial weight (g)	50	50
Methanolic (g)	6.80	4.47
% Recovery	13.6%	8.94%
Aqueous (g)	4.5	4.7
% Recovery	9%	9.4%

Table 2. *In vitro* efficacy of the aqueous and methanolic extracts of *Embelia ribes* and *Mangifera indica* on the inhibition of development of *Ascaridia galli* eggs

Plants	Extract	Conc. mg/ml	No of eggs counted (Mean)	Developed eggs (Mean)	Undeveloped eggs (Mean)	Effect on development of eggs (% of undeveloped eggs)
Embelia ribes	Aqueous	20	100	49.02±2.65	50.98±2.65	50.98
		40	100	45.88±1.87	54.12±1.87	54.12
		60	100	41.8±2.34	58.20±2.34	58.20
	Methanol	20	100	43.71±2.40	56.29±2.40	56.29
		40	100	40.66±1.45	59.34±1.45	59.34
		60	100	38.77±2.19	61.23±2.19	61.23
Mangifera indica	Aqueous	20	100	74.66±2.33	25.34±2.33	25.34
		40	100	74±1.87	26±1.87	26
		60	100	68.66±1.53	31.34±1.535	31.34
	Methanol	20	100	82.33±1.76	17.67±1.76	17.67
		40	100	75.33±3.28	24.67±3.28	24.67
		60	100	72.67±2.91	27.33±2.91	27.33
Untreated			100	89.6	10.4	10.4
Albendazole			100	2	98	98

Fig 1 Effect of aqueous and methanolic extract of powdered seeds of *Embelia ribes* on the activity of L2 inside egg shell

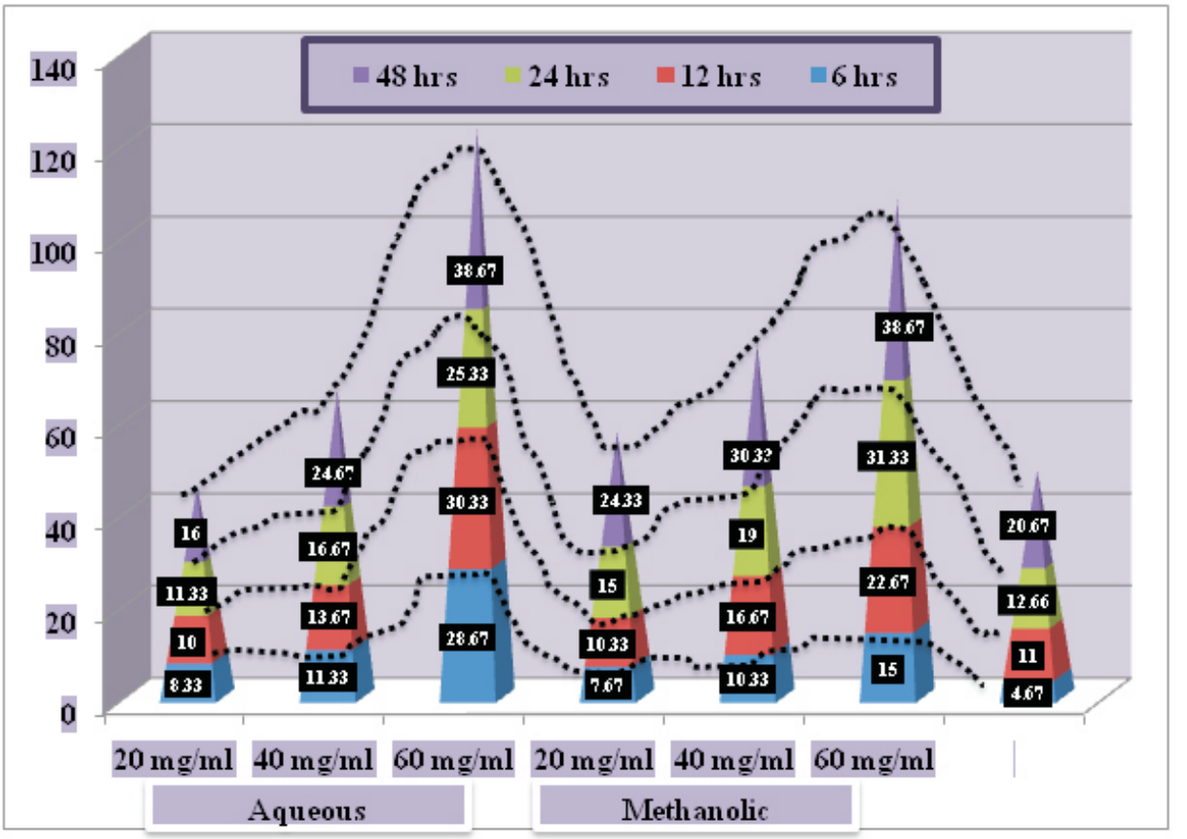

Table 3. ***In vitro*** **efficacy of the aqueous and methanolic extracts of** ***Embelia ribes*** **and** ***Mangifera indica*** **against deshelled larvae of** ***Ascaridia galli***

Treatments		% Mortality (MEAN±SE) at different concentration			
		Exposure time (hrs)			
Group	Conc.	6h	12h	24h	48h
Mangifera	5 mg/ml	17.00±1.15	23.33±2.40	25.00±2.88	26.00±1.53
indica(Aqueous)	10mg/ml	26.33±2.73	27.33±2.33	37.33±2.33	43.33±0.88
	20 mg/ml	40.66±0.88	47.00±2.31	51.33±2.33	63.33±2.18
Mangifera	5 mg/ml	11.00±2.65	11.00±1.15	16.00±2.52	22.33±1.85
indica(Methanolic)	10mg/ml	22.00±2.08	28.33±3.71	28.67±2.03	44.67±2.60
	20 mg/ml	32.67±1.45	37.00±3.51	48.00±1.53	62.33±1.45
Embelia ribes	5 mg/ml	16.33±2.02^{c}	22.66±1.45^{d}	32.66±1.76d^{e}	36.33±2.60^{d}
(aqueous)	10mg/ml	29.00±1.15^{b}	36.33±2.73^{bc}	45.33±2.02^{c}	55.66±1.76^{b}
	20 mg/ml	38.66±2.02^{a}	38.66±1.20^{b}	54.66±2.02^{b}	77.66±1.85^{a}
Embelia ribes	5 mg/ml	27.66±1.45^{b}	31.00±2.08^{c}	38.66±2.40^{d}	48.66±1.85^{c}
(methanolic)	10mg/ml	25.33±2.90^{b}	29.66±4.37^{cd}	29.66±2.02^{e}	52.33±1.76^{bc}
	20 mg/ml	41.66±2.18^{a}	58.33±1.76^{a}	64.00±2.64^{a}	73.66±2.33^{a}
Piperazine	10 mg/ml	100±0.00	100±0.00	100±0.00	100±0.00
Untreated		7.00±1.73	9.00±2.31	14.00±1.53	22±1.53

$^{a, b, c}$ Values with different superscripts within the same column are significantly different

Table 4. *In vitro* efficacy of the aqueous and methanolic extracts of *Embelia ribes* and *Mangifera indica* against adults of *Ascaridia galli* (Adult motility assay)

Plant	Extract	Conc (mg/ml)	2hrs			4hrs			8hrs			16hrs		
			Dead	live	%	Dead	live	%	Dead	live	%	Dead	live	%
Mangifera indica	Aqueous	5	0.00±0.00	6.00±0.00	0	0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	1.00±0.57	5.00±0.57	16.66
		10	0.33±0.33	5.66±0.33	5.5	1.00±0.57	5.00±0.57	16.66	1.00±0.57	5.00±0.57	16.66	1.66±0.66	4.33±0.66	27.66
		20	0.66±0.66	5.33±0.66	11	1.00±0.57	5.00±0.57	16.66	1.66±0.33	4.33±0.33	27.66	2.33±0.33	3.66±0.33	38.83
	Methanolic	5	0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	0.33±0.33	5.66±0.33	5.5	0.66±0.66	5.33±0.66	11
		10	0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	0.66±0.66	5.33±0.66	11	1.00±0.57	5.00±0.57	16.66
		20	0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	0.66±0.66	5.33±0.66	11	1.33±0.33	4.66±0.33	22.16
Embelia ribes	Aqueous	5	0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	0.33±0.33	5.66±0.33	5.5	1.33±0.33	4.66±0.33	22.16
		10	0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	0.66±0.33	5.33±0.33	11	1.66±0.66	4.33±0.66	27.66
		20	0.33±0.33	5.66±0.33	5.5	0.33±0.33	5.66±0.33	5.5	0.66±0.66	5.33±0.66	11	2.00±0.57	4.00±0.57	33.33
	Methanolic	5	0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	0.33±0.33	5.66±0.33	5.5	0.66±0.66	5.33±0.66	11
		10	0.00±0.00	6.00±0.00	0	0.66±0.33	5.33±0.33	11	0.66±0.33	5.33±0.33	11	1.33±0.33	4.66±0.33	22.16
		20	0.33±0.33	5.66±0.33	5.5	1.66±0.66	4.33±0.66	27.7	2.66±0.88	3.33±0.88	44.33	5.33±0.33	0.66±0.33	88.83
	Untreated		0.00±0.00	6.00±0.00	0	0.33±0.33	5.66±0.33	5.5	0.33±0.33	5.66±0.33	5.5	1.33±0.33	4.66±0.33	22.16
	Piperazine	10	4.00±0.57	2.00±0.57	66.66	6.00±0.00	0.00±0.00	100	6.00±0.00	0.00±0.00	100	6.00±0.00	0.00±0.00	100

of ethyl acetate extract), the effects were comparable with that of 3% piperazine citrate (Latha *et al.* 2010).

Hence, this was the first study to evaluate the efficacy of the indigenous medicinal plants *Embelia ribes* and *Mangifera indica* against *Ascaridia galli*. Although both the plant extracts were not as potent as synthetic anthelmintics, yet, *E. ribes* showed a moderate to good efficacy against exogenous stages of *A.galli*. Thus, could be utilized as an easily accessible source of natural anthelminthic. Further studies are required to assess the *in vivo* efficacy and toxicity. Nonetheless, the study can be used as a guide in continuing search for new natural products with potential medicinal and anthelmintic properties.

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