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Carica papaya L. (Caricacea) as herbal alternative to anthelmintics for the control of *Ascaridia galli* in poultry

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Abstract

The anthelmintic effect of *Carica papaya* was evaluated against the exogenous and endogenous stages of the poultry nematode *Ascaridia galli.* This study aimed at assessing whether *C.papaya* could emerge as an alternative to anthelmintics for the control of *A. galli* in poultry production. For this purpose, the anthelmintic effect of both aqueous and methanolic extracts of *C. papaya* (seeds) was evaluated. Aqueous extract of *C. papaya* was found to be 93.33% effective against the deshelled larvae of *A. galli* at 60 mg/ml concentration. Adult motility assay revealed that the *C. papaya* aqueous extract was 100% effective in killing the adult worms of *A. galli* @ 20mg/ml. The anthelmintic effect was concentration and time dependent. The *in vivo* trials revealed that the aqueous extract of *C. papaya* @ 40 mg/ kg was effective in reducing the egg counts by 90.47% and the total worm count by 83.66% at 15 day post treatment.

Key words: *Ascaridia galli, Carica papaya,* poultry.

In spite of the multidimensional benefits derivable from the poultry industry, the importance of diseases in the economics of a poultry farm needs no justification. Disease does indeed affect the profitability of a poultry breeding enterprise either directly or indirectly (Say, 1987). *Ascaridia galli* has been incriminated as the most common, important and prevalent endoparasite of poultry (Luka and Ndams, 2007; Katakam *et al*., 2010; Brar *et al*., 2016). *Ascaridia galli* infection impedes poultry productivity and is consequently responsible for economic losses to the poultry industry. The infection results in retarded growth, weight loss, diarrhoea, poor absorption of nutrients, death and even the spread of fatal bacterial infections (Katoch *et al*., 2012). 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 (Stear *et al.,* 2007) chemical residues and toxicity problems (Muhammad *et al.,* 2004).

Therefore, these constraints in the use of synthetic anthelmintics in less developed countries underline the advantages of herbal remedies as an alternative treatment.

Therefore, plant products as possible alternatives that function by mechanisms other than those of chemotherapeutics have been recommended (Naidoo *et al.,* 2008). Papaya (*Carica papaya*) fruit has the tremendous health benefits and the leaf and seeds are used against the various health disorders including liver cirrhosis, parasitic infections and digestive disorders. Its anticancer properties have also been reported (Aruoma *et al.*, 2014). Studies going back many decades have indicated that the cysteine proteinases (CPs) from papaya, collectively known as 'papain', have the ability to control gastrointestinal parasites in humans and animals, including soil transmitted helminths (Moraes *et al*., 2017). This study aimed at assessing whether *C. papaya* could emerge as an alternative to anthelmintics for the control of *A. galli* in poultry production. As there are very few reports from India on the efficacy of *Carica papaya L. (Caricacea)* seeds against *A. galli*, hence, the present study was planned to evaluate and validate the *in vitro* and *in vivo* effects of seeds of *C. papaya*, which are a part of ethno-veterinary practices of this region, against exogenous and endogenous stages of *A. galli.*

Materials and Methods

The seeds of *Carica papaya* were collected, shade dried and then grinded to obtain fine powder. The aqueous and methanolic extracts were prepared as per technique described by Hussain *et al.,* 2011. After getting the dried filtrate, 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. For preparation of donor birds, the faeces of the infected birds were used for the collection of eggs. Also, the adult *Ascardia 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 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 *A. galli* eggs.

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 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. There were three replicates for each dose (for both aqueous and methanolic plant extracts) and positive and negative controls. The eggs were incubated in this mixture at 30±1°C for 12 days. 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. 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. 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 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 mature *A. galli* 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°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.

Experimental trial: One day old, 24 female chicks (layers) were procured and kept in controlled conditions. The birds were maintained on standard ration and water *adlib* in parasite free conditions. The birds were divided into four groups of six birds each as given below.

Group 1: Uninfected Untreated control.
Group 2: Infected Untreated Control
Group 3: Crude extract (Aqueous extract of *Carica papaya*) - 40 mg/ kg Body Wt. for three days
Group 4: Piperazine citrate - 10 mg/ kg Body Weight as a single dose (Treated control)

The Groups 2, 3 and 4 were administered 500 infective eggs of *A. galli* each, *per os* on 10th day of life. After the administration of infective doses, faecal sample of experimentally infected birds was checked regularly till the faeces were found positive for the *A. galli* eggs.

Faecal egg count reduction test (FECRT)

Faecal egg counts per gram of faeces (EPG) was performed, for each infected bird when all the birds showed positivity for *A. galli* eggs, by Stoll's dilution method (Soulsby, 1982). EPG was calculated for all the birds before treatment (Day 0) and on days 5, 10, 15 post-treatment. Faecal Egg count percent reduction (FECR) was calculated using the following formula: FECR (%) = {(pre-treatment EPG – post-treatment EPG)/pre-treatment EPG} × 100

Critical worm count

The efficacy of treatment was also calculated on the basis of worm count in experimentally infected birds of each group. All the birds were slaughtered 15thday post treatment. Postmortem examination was performed for each bird as described by Alcorn (2001). After decapitation, the entire gastrointestinal tract including the oesophagus was collected from each bird. The gastrointestinal tract was opened in a longitudinal section and the intestinal contents, of each bird, were collected in separate petridishes and carefully washed through a sieve for collection of parasite.

The efficacy of the drug on the basis of post mortem worm count was evaluated as per the method of Soulsby (1982):

AE (%) = (A-B/A) 100; AE = Anthelmintic efficacy; A = Number of parasites in infected untreated (control) birds and B = Number of parasites in treated birds.

Statistical analysis: Statistical analysis of data was carried out by means of analysis of variance (ANOVA) using Students-Newman-Keuls test (intra-group comparison) and Dunnett's test (inter-group comparison) of Instat software (Graphpad) at 5 % and 1% level of significance.

Results and Discussion

Egg embryonation assay revealed that methanol & ethanol extracts of *Carica papaya* seeds had moderate efficacy against the development of *Ascaridia galli* eggs (Table1). The methanolic extract of *C. papaya* at 60 mg/ml exhibited maximum efficacy (68.33%) followed by its aqueous extract at same concentration (67.75%). These findings are in corroboration with the findings of Islam *et al.* (2008) that recorded similar anthelmintic effect with *C. papaya* leaves and reported that almost all preparations of papaya leaves showed strong efficacy against development of *A. galli* eggs. They further indicated higher efficacy of methanolic extract than aqueous extract against development of A. *galli* eggs. This could be explained on the basis that the active ingredients of papaya leaves are more soluble in alcoholic extract than water. Furthermore. as the dose of papaya seeds increases, more significant is the reduction in the development of *A. galli* eggs. This dose-dependent relationship has been affirmed by the study of Jacqeus *et al.* (2012). The anthelmintic efficacy of papaya might be due to presence of proteolytic enzymes such as papain, chymopapain and lysozymes.

In vitro assay on the activity against L2 inside egg shell revealed that methanolic extract of seeds of *C. papaya* exhibited inhibition of 38.67±4.10% at highest concentration of 60mg/ml at 48 hrs post incubation (Table 2). There was no significant difference between control and the aqueous concentrations of *C. papaya*. The results are in corroboration with the study conducted by Furtado *et al. (*2005) who showed a low anthelmintic activity of the mature seeds of *C. papaya* on the development of gastrointestinal nematode eggs in ovines.

The *in vitro* assay against the deshelled larvae revealed that the aqueous extract of *C. papaya* showed marked larvicidal activity against the larvae of *A. galli*.(Fig.1.). The highest efficacy of 93.33±1.20% was exhibited at the highest concentration of 60 mg/ml after 48hrs post incubation. Similarly the methanolic extract also showed considerable anthelmintic against the deshelled larvae (75.33±2.73) at a concentration of 20 mg/ml at 48hrs post hours incubation.

The adult motility assay revealed that aqueous extracts of *C. papaya* showed 100 % efficacy 8h post exposure at 20 mg/ml. However, majority of the worms exposed to control (RL) remained alive till 16 h post exposure (Table 3). The results, thus, indicated that *C. papaya* could be used as anthelmintic in poultry against the mature stages of *A. galli*. Our observation is in-agreement with the study of Ameen *et al.*(2012) who demonstrated successful control of helminths including *A. galli* through extraction of papaya seeds. Similarly, Alam *et al.*(2014) testified various medicinal plants against *A. galli* and demonstrated the best control with papaya seed extract followed by neem. Nagesh *et al.*(2002) identified the compound Benzyl isothiocyanate (BITC) responsible for the anthelmintic activity in *C. papaya*. In addition, cysteine proteases (CPs) in *C. papaya* have anthelmintic properties and are likely to have more than one target site on the cuticle, making rapid development of resistance very unlikely (Levecke *et al.,*2014). Thus, these are a serious contender for an alternative backup drug should anthelminthic resistance occur against the benzimidazoles.

Table 1. *In vitro* efficacy of the aqueous and methanolic extracts of *Carica papaya* on the inhibition of development of *Ascaridia galli* eggs

Plants	Extract	Conc. mg/ml	No of eggs counted (Mean)	Developed eggs (Mean ± SE)	Undeveloped eggs (Mean ± SE)	Effect on development of eggs (% of undeveloped eggs)
Carica papaya	Aqueous	20	100	38.97±0.72	61.02±0.72	61.02
		40	100	36.12±0.88	63.88±0.88	63.88
		60	100	34.25±1.45	65.75±1.45	65.75
	Methanol	20	100	36.12±1.33	63.88±1.33	63.24
		40	100	34.5±0.577	65.54±0.577	65.54
		60	100	31.7±0.67	68.33±0.67	68.33
Control	Albendazole		100	2	98	98

Table 2. Effect of aqueous and methanolic extract of powdered seeds of *Carica papaya* on the activity against L2 inside egg shell (% inhibition of motility of larvae ± SE)

Dose(mg/ml)	Extract	6hrs	12hrs	24hrs	48hrs
20		10.33 ± 2.91^{bc}	11.00 ± 1.53^{c}	21.00 ± 0.58^{abc}	23.67 ± 1.86^{bc}
40	Aqueous	14.67 ± 1.45^{b}	15.67 ± 2.40^{c}	14.33 ± 2.19^{d}	27.00 ± 1.15^{bc}
60		16.00 ± 2.08^{b}	28.00 ± 1.53^{a}	21.67 ± 1.20^{ab}	28.67 ± 1.86^{b}
20		11.67 ± 1.20^{b}	22.00 ± 1.53^{b}	17.00 ± 1.15^{bcd}	22.67 ± 1.20^{c}
40	Methanolic	11.33 ± 1.86^{b}	13.67 ± 1.45^{c}	16.67 ± 1.45^{cd}	24.67 ± 2.60^{bc}
60		28.67 ± 1.86^{a}	30.33 ± 2.40^{a}	25.33 ± 2.33^{a}	38.67 ± 4.10^{a}
Control		4.67 ± 1.20^{c}	11 ± 0.57^{c}	12.66 ± 1.45^{d}	20.67 ± 2.33^{bc}

[a, b, c] Values with different superscripts within the same column differ significantly

The *in vivo* test results showed that at Day 0 the average amount of EPG between groups did not vary significantly. However, following the last faecal sample analysis (day 15th post treatment) the percentage reduction of eggs was significant (P<0.05) with 90.47 % and 98.95% reduction in *C. papaya* and Piperazine group, respectively (Table 4). This confirmed 90.47% efficacy of the aqueous extract of seeds of *C. papaya* against *A.galli*. Adu *et al.*(2009), however, reported an overall efficacy of 77.7% and established the role of papain as anthelmintic by virtue of its ability in digesting parasitic cells.

The efficacy of treatment was also determined based on critical worm counts. In Group 2 (infected untreated) worm count was recorded to be 33.67±2.63. However a significant (P<0.05) reduction was seen in Group 3 (*C.papaya*) where only an average of 5.5± 1.34 worms was recorded. The results are comparable with the study conducted by Mursof and He (1991) where single dose of papaya latex completely freed the birds from *A. galli* infection one week after the treatment at single a dose of 1447.89 mg, while the untreated infected controls harboured a mean number of 50 worms adult worms. The best results were observed in Group 4, treated with piperazine, in which the worm count was reduced to Zero (0) establishing piperazine to be a potent drug against *A. galli* infection. These findings are in agreement with the findings of Shahadat *et al.,* 2008, who reported the number of *A.galli* to be higher in untreated chickens and were decreased in treated groups. They further observed that the total number of worms was lower in groups; treated with some conventional medication rather than any herbal medication. Similar efficacy of piperazine has been documented by Nilsson and Alderin (1988) where a 100 % efficacy both against mature and immature *A. galli* were observed in controlled anthelmintic tests.

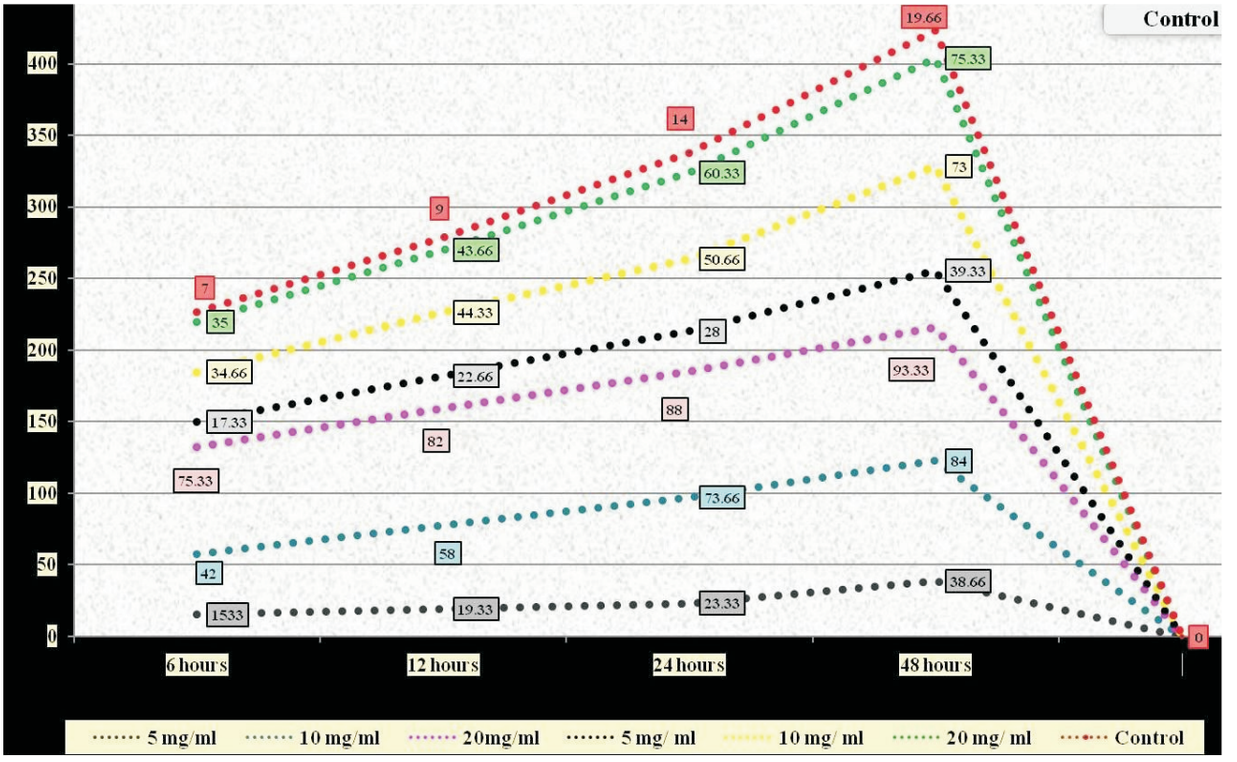

Fig.1. Effect of aqueous and methanolic extract of powdered bark of *Carica papaya* on the deshelled larvae of *Ascaridia galli*

Table 3. ***In vitro*** **efficacy of the aqueous and methanolic extracts of** ***Carica papaya*** **against mature** ***Ascaridia galli*** **worms (Adult motility assay)**

Extract	Dose	2hr			4 hrs			8hr			16hr		
	(mg/ml)	Dead	live	%	Dead	live	%	Dead	live	%	Dead	live	%
Aqueous	5	0.00±0.00	6.00±0.00	0	0.00±0.00	6.00±0.00	0	0.00±0.00	6.00±0.00	0	1.66±0.88	4.33±0.88	27.66
	10	0.00±0.00	6.00±0.00	0	1.33±0.33	4.66±0.33	22.1	2.33±0.33	3.66±0.33	38.83	3.66±0.33	2.33±0.33	61
	20	1.00±0.57	5.00±0.57	16.66	3.33±0.33	2.66±0.33	55.5	6.00±0.00	0.00±0.00	100	6.00±0.00	0.00±0.00	100
Methanolic	5	0.00±0.00	6.00±0.00	0	0.00±0.00	6.00±0.00	0	0.66±0.66	5.33±0.66	11	1.33±0.33	4.66±0.33	22.1
	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	0.66±0.33	5.33±0.33	11
	20	0.33±0.33	5.66±0.33	5.50	0.66±0.66	5.33±0.66	11	1.00±0.57	5.00±0.57	16.66	2.66±0.66	3.33±0.66	44.33
Negative control		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	0
Positive control	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

Table 4. Effect of *Carica papaya* and Piperazine on faecal egg count of chicken experimentally infected with *Ascaridia galli*

Group	Treatment	Dose	Pre treatment	Post Treatment		
			Day 0	Day 5	Day 10	Day 15
1	Uninfected untreated	---------	0.00	0.00	0.00	0.00
2	Infected untreated	---------	1038.88 ± 100.15[a]	1072.21 ± 96.76[a]	1099.99± 83.88[a]	1177.75± 78.25[a]
3	Infected treated with *Carica papaya*	40mg/kg bwt (aqueous extract)	1049.99 ± 115.39[a]	744.44± 100.25(29.10)[a]	338.88± 56.71[b](67.72)	99.99± 14.90[b](90.47)
4	Infected treated with Piperazine	10 mg/kg bwt	1061.10 ± 104.50[a]	511.11± 104.59(51.83)[ab]	183.33± 33.054[b] (82.72)	11.11± 7.03[b](98.95)

[a,b,c] values with different superscripts within the same column are significantly different. Values are compared with control group 2. Level of significance P<0.01

Conclusion

This was the first study to evaluate the efficacy of the *Carica papaya* seeds against endogenous and exogenous stages of *Ascardia galli*. Although, *C. papaya* plant extract was not as potent as synthetic anthelmintic, yet, it showed very good efficacy against all the stages of *A.galli*. Thus, it could be utilized as an easily accessible source of natural anthelminthic. It could also be considered a serious contender for an alternative backup drug should anthelminthic resistance occur against the benzimidazoles. Further studies are required to assess the mode of action, active ingredients and toxicity potential of this herbal compound. 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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