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In vitro CONTROL OF FISH HELMINTH PARASITE, *Euclinostomum heterostomum* BY FLAVONOID-RICH EXTRACTS OF *Mangifera indica*

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author IK gave the concept, designed the study, did the analysis or interpretation and wrote the manuscript. Authors SR, YK and MMR did the data collection or processing and managed the literature search. All authors read and approved the final manuscript.

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ABSTRACT

The study was conducted to determine anthelminthic activity of mango plant (*Mangifera indica*) known widely for its medicinal value. Alcoholic extracts of leaves of mango plant was tested as a therapeutic agent for controlling *Euclinostomum heterostomum,* trematode parasite in fish by '*in vitro'* method. Qualitative and quantitative estimation of the ethyl acetate extract showed higher flavonoid content compared to ethanol and chloroform extracts. The ethyl acetate extract was found to be effective in killing parasites significantly (LC_{50} value 8.2016 mg/l) which clearly demonstrates the anthelminthic activity of mango leaves. Acute toxicity tests of ethyl acetate extracts on host animals *(Channa punctata)* showed the lethal concentration (LC_{50}) value of 194.98 mg/l, thus a considerable lower effective working solution can be used for application for control of parasites without causing damage to host animals. On the basis of above study, it can be concluded that the mango leaves can be used as an alternative to synthetic drugs applied indiscriminately to control helminth parasites which has resulted in serious health concern.

Keywords: Anthelminthic; *Channa punctata*; trematode; mango.

1. INTRODUCTION

In India intensification of aquaculture practices has resulted in wide spread outbreak of parasites and microorganisms causing a serious public health issues [1]. Indiscriminate use of synthetic drugs and chemicals has lead to multidrug resistance, bioaccumulation, bioconcentration and transmission of drug residues in the food chain. Hence search for alternative modes for control of parasites has lead to the use of plant based herbal drugs.

Plants are known to possess secondary metabolites like flavonoids, alkaloids, terpenoids, steroids, phenols, glycosides and tannins which are made of several active components accounting to the therapeutic effects of the plants [2]. Flavonoids are also known as nature's tender drugs as they possess various pharmacological activities making them a huge reservoir of medicines and drugs [3,4].

Medicinal plants like mango are known to treat various diseases. Mango tree (*Mangifera indica* L.),

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belonging to Anacardiaceae are widely distributed in India and are known to possess antimicrobial [5], antifungal [6], antitumor [7] activities.

Mangifera indica has been known for its ethnopharmacological value and has been used for a long time as an anthelminthic agent [8,9]. Patel et al. [10] has worked on anthelminthic activity of *Mangifera indica* leaves using *Pheretima posthma* as model in general however reports on efficacy of its secondary metabolites, antioxidant activity was not reported.

Hence the present study was carried to evaluate the efficiency of flavonoid-rich fractions of mango leaves in controlling helminthiasis in fish using *Euclinostomum heterostomum* [11] as a model (helminth) for *in vitro* evaluation to justify the traditional beliefs.

2. MATERIALS AND METHODS

2.1 Study Location for Host Sampling and Collection of Helminthes

The study was conducted on 240 specimens of *Channa punctata* (length 12-17 cm and weight 40-60 g) reared in fish farms under the tropical conditions of West Bengal. The trematodes collected from *Channa punctata* were kept in 0.67% saline solution in a petridish incubated at 37°C for use in *in vitro* studies. Few were fixed with AFA or 70% ethanol. Morphometric identification was performed using selected identification keys [12,13,14].

2.2 Qualitative Evaluation of Plant Extracts

Dried leaves of mango plant was prepared for qualitative evaluation [15]. Detection of flavonoids (Alkaline Reagent Test), alkaloids (Mayer's Test: and Dragendroff's Test) [16], and saponins (Froth Test) [17] was performed. Test for steroids and triterpinoids (Salkowski test), phenols (Ferric Chloride Test) [18] and tannins (Gelatin Test) was performed [19].

2.3 Quantitative Estimation of Secondary Metabolites

Dried leaves of mango plant was prepared according to [15] for quantitative evaluation. Total phenolic content of ethanol, chloroform, ethyl acetate extract of mango leaves was determined with Folin-Ciocalteu method [20] using Gallic acid was used as the reference compound.Total flavonoid content of the mango leaves were determined according to a modified colorimetric method [21] using Quercetin as standard. 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging activity was performed according to [22]. Half maximal inhibitory concentration (IC_{50}) values denote the concentration of sample which is required to scavenge 50% of DPPH free radicals.

2.4 Screening for *In-vitro* Anthelmintic Assay

In vitro bioassay was carried out using groups of trematodes (4-5 each) in which 25 ml of various concentration of ethyl acetate extracts and Albendazole as standard was prepared in a Petri dish [23]. Observations were made from the time taken for paralysis of trematodes to set-in was noted. Control worms were maintained in phosphate-buffered saline with 1% dimethylsulfoxide at (37±1)°C.

2.5 Acute Toxicity Test and Estimation of LC_{50} of Experimental Fishes

Five day bioassay was performed to determine the acute toxicity of ethyl acetate extracts using varying concentrations (10, 25, 50, 75 and 100 mg/l) on host animal [24]. The LC_{50} results were analyzed according to Probit analysis [25] and the working dose was determined. All treatments were done in three replicates.

2.6 Statistical Analysis

Data was represented as mean ± SD for each group (n=6). Statistical significance, SD and analysis of variance (ANOVA) was determined at 5% level. $P<0.005$ was considered significant.

3. RESULTS AND DISCUSSION

Helminthes are the most widespread parasitic group infecting both invertebrate and vertebrate. In the present study a total of 68 parasites representing different species and some unidentified species were collected from the host fish (*Channa punctata*).The parasite predominantly obtained from the liver of the infected fishes was identified as *Euclinostomum heterostomum,* a common trematode parasite [11].

Indiscriminate use of commercially available anthelmintic drugs like ampicillin, cefotaxime, streptomycin, kanamycin, gentamicin have resulted in resistance of parasites [26] thus there is an urgent need for alternative modes for controlling this problem. The phytochemicals present in plants can act as active constituent for controlling the rate of parasitic infestation. The preliminary phytochemical analysis of ethyl acetate extracts of mango leaf powder exhibited higher number of secondary metabolites like, triterpenoids, alkaloids, flavanoids, phenols, saponins and tannins in comparison to ethanolic and chloroform extract (Table 1).

Table 1. Phytochemical constituents of *Mangifera indica* extracts based on solvents used

S/No.	Phytochemical constituents	Various extract of plant material		
		Chloroform	Ethyl acetate	Ethanol
1.	Flavonoids	+	+ + +	++
2.	Alkaloids	-	++	+
3.	Steroids	+	-	-
4.	Tannins	+	+	+
5.	Saponins	-	+	-
6.	Triterpenoids	+	++	-
7.	Glycosides	-	-	+++
8.	Phenols	+	+	+

(Present: +; Absent: -; ++moderate presence; +++high presence)

The concentrations of total phenolic contents and of flavonoids (Quantitative assay) in the ethyl acetate extracts was found to be higher than in chloroform and ethanolic extracts (Tables 2 and 3). Flavonoids are known for its antiparasitic effects against a number of parasites and microbes [27,28,29]. The DPHH scavenging activity is shown in Fig 1. The radical scavenging effects of the extracts showed concentration-dependence. Ascorbic acid was used as a standard antioxidant as it has strong DPPH scavenging property [30].

Udem et al. [31] reported that the *Mangifera indica* ethanolic extract showed 79.09 ± 0.42 percentage inhibition at 100 mg/ml while 20 mg/ml showed 73.17 ± 1.81 percentage inhibition in DPPH scavenging activity while in the present study chloroform extract, ethyl acetate and ethanol at 100 µg/ml showed 73.37±1.25, 94.3± 2.34 and 81.7±3.25 percentage inhibition which is much higher than the earlier study.

The IC_{50} values in the present study were found to be 59.075 ± 2.21µg/ml, 49.169 ±0.11µg/ml, 24.366 ±1.88 µg/ml, 6.2341 ± 3.34 µg/ml for chloroform, ethanol, ethyl acetate and acorbic acid respectively. The lower the IC_{50} values the higher the free radical activity. Hence it can be concluded that ethyl acetate fractions have higher antioxidant activity than chloroform and ethanol extracts. Thus the role of flavonoids and antioxidant properties of *Mangifera indica* for control of helminth parasites in fishes have been studied by *in vitro* method.

Table 2. Concentrations of total phenolic content in the plant extracts expressed in terms of Gallic acid equivalent (GAE) (mg of QE/g of extract)

Extract	Milligram of GAE/g of plant extract
Chloroform Extract	108.12
Ethyl Acetate extract	360.10
Ethanolic extract	258.16

Table 3. Concentrations of flavonoids in the plant extracts expressed in terms of Quercetin equivalent (QE) (mg of QE/g of extract)

Extract	Milligram of GAE/gm of plant extract
Chloroform Extract	108.12
Ethyl Acetate extract	360.10
Ethanolic extract	258.16

In vitro tests to evaluate the worm motility for prospecting of novel anthelmintic agents was reported [32,33]. The advantage of these assays are that the compounds or extracts are tested with the different life-cycle stages of the parasite in direct contact. So far, *in vitro* screenings of potential anthelmintic agents prior to in vivo testing have been shown to be a rational and practical strategy since they save time and money and also minimize the number of animals necessary for the development of a new therapeutic agent [34].

Thus *in vitro* assay for evaluating effect of ethyl acetate extract of *Mangifera indica* against *Euclinostomum heterostomum* was performed on varying doses for twenty four hours [23]. The results (Table 4), showed a significant dose-response relationship, with higher doses causing greater mortality. After twenty four hr exposure ethyl acetate extract at concentrations of 10 mg/l or more resulted in 60% mortality. Anthelmintic activity of plant extracts on *E. heterostomum* reported in present study is in agreement with the findings of earlier workers on different helminth parasites [35-41].

The lethal concentration (LC_{50}) value of the different doses was calculated arithmetically using probit analysis [25]. The lethal concentration (LC_{50}) values of ethyl acetate extract of *Mangifera indica* was determined and found to be 8.2016 mg/l (Table 5 and Fig. 2). Tariq et al. [37] reported LC_{50} for aqueous and ethanolic extracts of *Achillea millifolium* on *Haemonchus contortus* to be 0.05 and 0.11 mg/ml

respectively whereas Aggarwal et al. [42] showed that LC_{50} values to be 12.05 mg/ml ± 3.24 and 23.52 mg/ml ± 6.4 for *C. procera*, 24.37 mg/ml ± 4.11 and 21.02 mg/ml ± 4.6 for *A. indica*, 18.92 mg/ml ± 4.54 and 24.43 mg/ml ± 6.96 for *P. granatum* for ethanolic and aqueous extracts respectively. Different ranges of concentration and efficacy were observed by various researchers working with different solvents, extraction procedures for isolation of active molecules which corroborates with the present study [43,44].

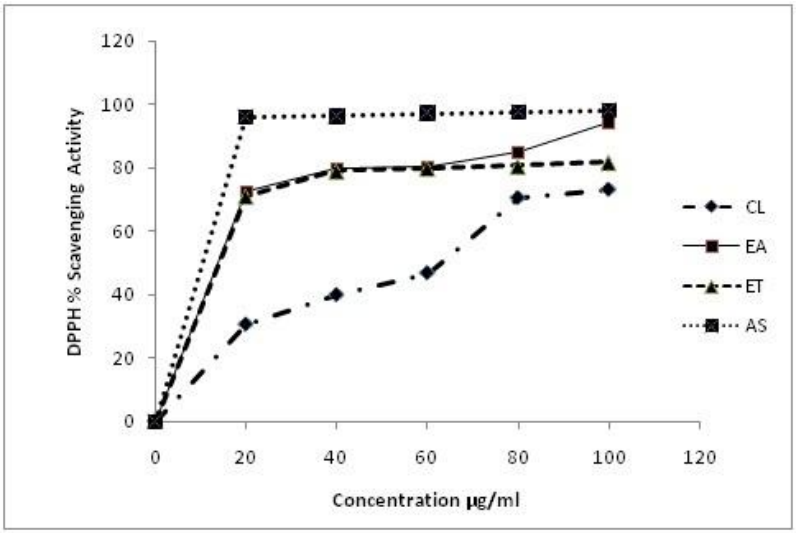

Fig. 1. DPPH scavenging activity assay of various extracts of *Mangifera indica*
CL-Chloroform, EA-Ethyl Acetate, ET-Ethanol, AS-Ascorbic acid

Table 4. Survivability of parasites in ethyl acetate extract of *Mangifera indica*

Dose (mg/l)	Time of paralysis (hour)	Time of death(hour)
1	15 ±2.1	28 ± 1.5
2	12 ± 0.32	22 ± 0.85
4	10 ± 2.36	18 ± 3.21
6	7 ± 1.98	16 ± 2.47
8	5 ± 0.25	14 ± 2.15
10	3 ±2.69	12 ± 1.28
12	2 ± 0.37	10 ± 1.32
14	1.5 ±1.57	9 ± 5.48
16	0.75 ± 8.23	7 ±027
Standard	3.5 ±0.65	13 ±1.2

Table 5. Acute toxicity of ethyl acetate extracts of *Mangifera indica* to trematode parasite

Concentration (mg/l)	Log10 concentration	Total number of animals	Number of animals dead	% Mortality	Corrected mortality (%)	Probit
0	0	5	0	0	0	-
1	0	5	0	0	0	-
2	0.3001	5	1	20	20	4.16
4	0.60206	5	1	20	20	4.16
6	0.778151	5	2	40	40	4.75
8	0.90309	5	2	40	40	4.16
10	1	5	3	60	60	5.25
12	1.0792	5	3	60	60	5.25
14	1.1461	5	4	80	80	5.84
16	1.2041	5	4	80	80	5.84

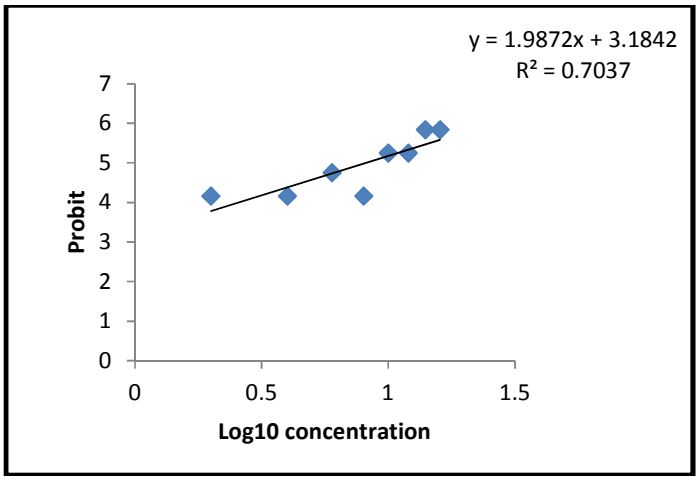

Fig. 2. Probit analysis for estimation of LC_{50}

The use of crude extracts as anthelminthic drugs is essential to study if the extract has any kind of toxic effects over healthy fish due to its application. A bioassay was hence performed for five days for determining the acute toxicity of ethyl acetate extracts of *Mangifera indica* to healthy fishes and lethal concentration (LC_{50}) was estimated (Table 6 and Fig. 3). The fish survived easily in the ethyl acetate extract at doses of 10–75 mg/l for five days (Fig. 3). However on increasing concentration reduced the survivability of the fishes which might be due to suspension of solids over gills thus reducing the oxygen supply to the gills. Hence increasing the concentration of the extracts up to 75 mg/l does not have any detrimental effect on the host and can be effectively used in aquaculture.

The LC_{50} value was found to be 194.98 mg/l which was much higher than the effective working solutions ie 10–75 mg/l used in the experiments (Table 6 and Fig. 4). Thus ethyl acetate extracts can effectively be used in controlling helminth parasites in aquaculture without causing any detrimental effect on fishes. LC_{50} values of the distilled water, 50% ethyl alcohol and absolute ethyl alcohol extracts of *A. zapota* leaf parts for *C. punctatus* were 279.35, 175.89, 125.69 ppms, respectively were reported by Nasiruddin et al. [45]. Similar results were observed by various workers in terms of the working with plant extracts [46-48].

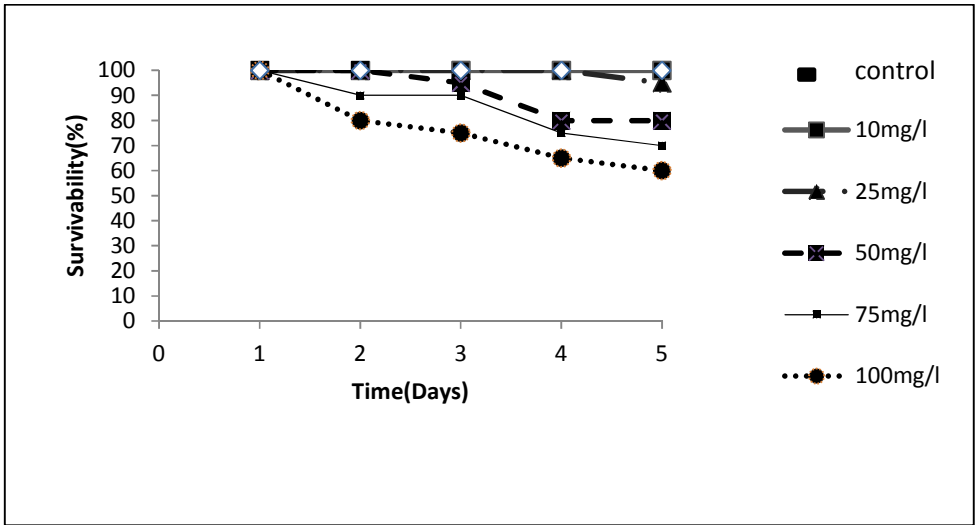

Fig. 3. Graphical representation of the survivability % of fish during acute toxicity of ethyl acetate extracts of mango to fish after 5 days of bioassay

Table 6. Acute toxicity of ethyl acetate extracts of mango to fish after 5 days of bioassay

Concentration (mg/l)	Log_{10} concentration	Total number of animals	Number of animals dead	% Mortality	Corrected mortality (%)	Probit
Control	0	15	1	6.67	0	-
10	1	15	0	0	0	-
25	1.39	15	4	26.67	20	4.16
50	1.69	15	5	33.33	26.68	4.39
75	1.85	15	6	40	33.35	4.56
100	2	15	7	46.67	40.02	4.75

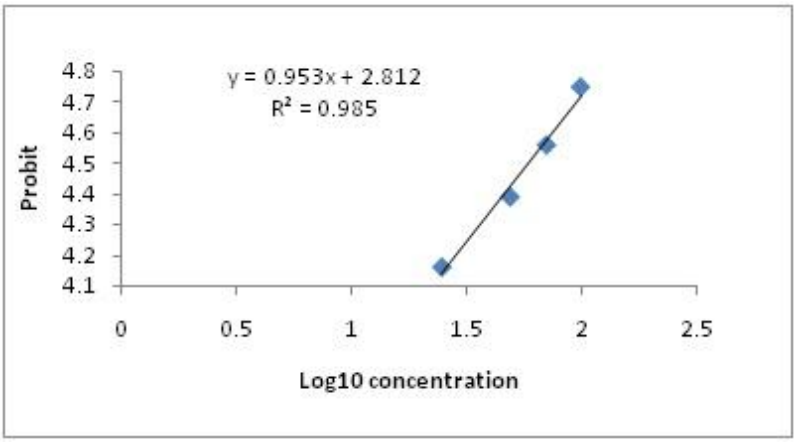

Fig. 4. Probit analysis for estimation of LC_{50} values

4. CONCLUSION

The present study using mango leaves extract inhibited motility of trematode parasites at low concentration, increasing the concentration of the plant extracts resulted in early paralysis, thus indicating dose-dependent efficacy of the test materials. Similar observation of using plant extract as anthelminthic drug has been made by some authors [49-52]. Moreover low toxic effect on host fishes as evaluated from LC_{50} values make if an effective for its use in aquaculture practices.

These findings indicated that the mango leaves extract contains active principles which can act as effective drugs against the trematode parasites affecting the fish. Hence it is of outmost necessity to identify bioactive compounds present in mango leaves which might be responsible for its anthelminthic activity.

ETHICS APPROVAL

As per CPCSEA instruction's protocol for experimentation on fishes, does not require approval from ethical committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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