SUPPLEMENTARY TABLES AND FIGURES ONLINE

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Tropical plants as alternative anthelmintics against *Cotylophoran cotylophorum* of sheep

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Cotylophoran cotylophorum is one of the most prevalent amphistomes in sheep in tropical and sub-tropical areas including India (Soulsby 1987). In the absence of vaccines, control of amphistomes relied largely on anthelmintics, which led to increased drug resistance, and forced use of medicinal plants as alternative control strategies (Waller 1999). The plants with anthelmintics activity have phytochemicals that impede energy generating pathways, resulting in paralysis and death of worms (Veerakumari and Munuswamy 2000).

Though, various plants have been tested for their anthelmintic efficacies against amphistomes (Rajesh *et al.* 2017), there is no scientific report on *Anacardium occidentale L* (Anacardiaceae), *Pistacia vera L* (Anacardiaceae), *Illicium verum Hook.f.* (Schisandraceae) and *Artocarpus heterophyllus Lam* (Moraceae) popularly known as cashew, pistachio, star anise, and jackfruit, respectively. Previous studies conducted on bacteria (Bisignano *et al*. 2013), and nematodes (Davuluri *et al.* 2020) involving extracts of these plants revealed their antimicrobial and anthelmintic activity. During extraction of antioxidants from selected Indian medicinal plants alcohol was able to extract more metabolites (Kaneria *et al*. 2012). Higher concentrations of the active metabolites in the extracts aid in transcuticular absorption of extracts into the parasite body and higher efficacy. A significant inhibition in the activity of lactate dehydrogenage (LDH) was also noticed in nematodes on *in vitro* exposure to hydro-alcoholic extracts of cashew, star anise and jackfruit (Davuluri *et al*. 2020). Therefore, the present study was designed to evaluate the anthelmintic activity of hydro-alcoholic extracts of these four tropical plants on adult *Cotylophoran cotylophorum* of sheep using worm motility inhibition assay. The effects of extracts on amphistome LDH activity, a key energy metabolism-related enzyme activity was evaluated, which could accentuate the assay results.

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Shell of pistachio, fruit of star anise and seed of jack fruit were procured from local markets, while the shell of cashew was collected from Srikakulam, its habitat. Hydro-alcoholic extraction, yield calculation and detection of active chemical constituents in each extract were carried out (Davuluri *et al*. 2020). Albendazole (99.8%; M/S PVS Laboratories Ltd., Vijayawada, India) was used for comparison. Albendazole and extracts (initially dissolved in DMSO) were diluted in Hedon-Fleig solution at the desired concentrations.

Worm motility inhibition assay: The assay was performed on adult live amphistomes (Sharma *et al*. 1971). Amphistomes collected from the rumen of naturally infected sheep and slaughtered at local abattoirs in Andhra Pradesh, India were washed in PBS (*p*H 7.2) and maintained in H-F solution (*p*H 7.2). Amphistomes were identified as *Cotylophoran cotylophorum* based on morphology (Soulsby 1987). Twenty-five actively moving amphistomes were incubated in 4.0, 1.4, 0.5, 0.2, and 0.05 mg/mL concentrations of each extracts in separate petri dishes at 37±1°C in triplicates. Amphistomes exposed to albendazole (30 µg/mL) and H-F solutions were served as positive and negative controls, respectively. Worms' motility was observed under a dissecting microscope (×20) at intervals of 15 min till the worms in negative control lost their motility, for 75 min of assay. The assay was replicated twice. The paralytic state and/ or mortality of the exposed worms were used as a sign for anthelmintic effect. The live and dead worms in triplicates of each concentration and control were counted. Percent worm mortality was calculated (Rabel *et al*. 1994).

Enzyme assay: LDH activity in amphistomes was analysed using Lactate Dehydrogenase Activity Assay Kit (MAK066, Sigma-Aldrich, USA). LDH reduces NAD (nicotinamide adenine dinucleotide) to NADH (reduced NAD+). The assay quantifies the LDH activity, i.e. the amount of NADH generated in biological samples and is detected by calorimetry (450 nm). Amphistomes were incubated for 75 min in 4.0, 1.4, 0.5, 0.2, and 0.05 mg/mL

Table 1. Mean per cent inhibition ± SEM of different extracts on *C. cotylophorum* worm's motility

Extract (mg/mL)	15 min	30 min	45 min	60 min	75 min
I. verum					
4	16.0 ± 0.0^{b5}	24.0 ± 0.0^{b4}	30.7 ± 1.3^{b3}	44.0 ± 0.0^{b2}	52.0 ± 0.0^{b1}
1.4	5.3 ± 1.3^{b5}	18.7 ± 1.3^{c4}	24.0 ± 0.0^{c3}	36.0 ± 0.0^{c2}	46.7 ± 1.3^{c1}
0.5	0.0 ± 0.0^{c5}	8.0 ± 0.0^{d4}	16.0 ± 0.0^{d3}	22.7 ± 1.3^{d2}	37.3 ± 1.3^{d1}
0.2	0.0 ± 0.0^{d4}	4.0 ± 0.0^{e3}	4.0 ± 0.0^{e3}	8.0 ± 0.0^{e2}	20.0 ± 0.0^{e1}
0.05	0.0 ± 0.0^{d3}	0.0 ± 0.0^{f3}	0.0 ± 0.0^{f3}	4.0 ± 0.0^{f2}	6.7 ± 1.3^{f1}
ABZ (30 ug/mL)	48 ± 0.0^{a5}	56 ± 0.0^{a4}	72 ± 0.0^{a3}	80 ± 0.0^{a2}	98.7 ± 1.3^{a1}
HFS	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{g}	1.3 ± 1.3^{g}
P.vera					
4	20.0 ± 0.0^{5}	30.7 ± 1.3^{b4}	34.7 ± 1.3^{b3}	46.7 ± 1.3^{b2}	54.7 ± 1.3^{b1}
1.4	8.0 ± 0.0^{c5}	22.7 ± 1.3^{c4}	25.3 ± 1.3^{c3}	38.7 ± 1.3^{c2}	49.3 ± 1.3^{c1}
0.5	4.0 ± 0.0^{d5}	12.0 ± 0.0^{d4}	20.0 ± 0.0^{d3}	29.3 ± 1.3^{d2}	40.0 ± 0.0^{d1}
0.2	1.3 ± 1.3^{e5}	6.7 ± 1.3^{e4}	4.0 ± 0.0^{e3}	9.3 ± 1.3^{e2}	24.0 ± 0.0^{e1}
0.05	0.0 ± 0.0^{e3}	0.0 ± 0.0^{f3}	0.0 ± 0.0^{f3}	4.0 ± 0.0^{f2}	10.7 ± 1.3^{f1}
ABZ(30 ug/mL)	48 ± 0.0^{a5}	56 ± 0.0^{a4}	72 ± 0.0^{a3}	80 ± 0.0^{a2}	98.7 ± 1.3^{a1}
HFS	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{g}	1.3 ± 1.3^{g}
A. occidentale					
4	22.7 ± 1.3^{b5}	34.7 ± 1.3^{b4}	42.7 ± 1.3^{b3}	56.0 ± 2.3^{b2}	64.0 ± 2.3^{b1}
1.4	10.7 ± 1.3^{c4}	26.7 ± 1.3^{c3}	30.7 ± 1.3^{c3}	46.7 ± 1.3^{c2}	60.0 ± 2.3^{b1}
0.5	8.0 ± 0.0^{c5}	16.0 ± 0.0^{d4}	24.0 ± 0.0^{d3}	40.0 ± 0.0^{d2}	48.0 ± 0.0^{c1}
0.2	1.3 ± 1.3^{d5}	6.7 ± 1.3^{e4}	10.7 ± 1.3^{e3}	20.0 ± 0.0^{e2}	32.0 ± 0.0^{d1}
0.05	0.0 ± 0.0^{d3}	0.0 ± 0.0^{f3}	1.3 ± 1.3^{f3}	10.7 ± 1.3^{f2}	18.7 ± 1.3^{e1}
ABZ (30 ug/mL)	48 ± 0.0^{a5}	56 ± 0.0^{a4}	72 ± 0.0^{a3}	80 ± 0.0^{a2}	98.7 ± 1.3^{a1}
HFS	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{g}	1.3 ± 1.3^{f}
A. heterophyllus					
4	58.7 ± 1.3^{a5}	74.7 ± 1.3^{a4}	82.7 ± 1.3^{a3}	92.0 ± 0.0^{a2}	100.0 ± 0.0^{a1}
1.4	49.3 ± 1.3^{b5}	56.0 ± 0.0^{b4}	68.0 ± 0.0^{c3}	80.0 ± 0.0^{b2}	88.0 ± 0.0^{b1}
0.5	34.7 ± 1.3^{c5}	42.7 ± 1.3^{c4}	56.0 ± 0.0^{d3}	64.0 ± 0.0^{c2}	72.0 ± 0.0^{c1}
0.2	24.0 ± 0.0^{d5}	32.0 ± 0.0^{d4}	44.0 ± 0.0^{e3}	50.7 ± 1.3^{d2}	66.7 ± 1.3^{d1}
0.05	16.0 ± 0.0^{e5}	24.0 ± 0.0^{e4}	32.0 ± 0.0^{f3}	36.0 ± 0.0^{e2}	52.0 ± 0.0^{e1}
ABZ (30 ug/mL)	48 ± 0.0^{b5}	56 ± 0.0^{b4}	72 ± 0.0^{b3}	80 ± 0.0^{b2}	98.7 ± 1.3^{a1}
HFS	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{g}	0.0 ± 0.0^{f}	1.3 ± 1.3^{f}

SEM, Standard error of mean; ABZ, Albendazole; HFS, Hedon-Fleig solution. Means with different superscripts (numerical) within a row differ significantly (P<0.01). Means with different superscripts (alphabet) within a column differ significantly (P<0.05) in particular plant extract.

concentrations of each extract, maintaining positive (worms in H-F solution) and negative (worms in albendazole) controls. Following incubation, sample for assay was prepared according to manufacturer's instructions. The assay was performed thrice on duplicated samples. Enzyme activity (nmole/min/mL=milliunit/mL) in extracts was ascertained and compared with controls. Data from assays was subjected to ANOVA and also subjected to probit analysis against the logarithm of extract concentration.

The per cent yield obtained were 9.53 for *A.heterophyllus* seed, 18.28 for *A. occidentale* shell, 20.27 for *I. verum* fruit and 3.53 for *P. vera* shell. Phytochemical screening of the extracts is presented in Supplementary Table 1 and Supplementary Fig. 1. Extracts induced dose- and time-dependant anthelmintic responses (P<0.01) (Table 1). Furthermore, onset of response was directly proportional to their dose rates. Complete (100%) mortality of worms was observed 75 min post exposure at higher (4 mg/mL) tested concentration with *A. heterophyllus* seed extracts. At the same time all the worms were still alive in negative control and were found dead in the albendazole treated group. However, the inhibition of worm's motility was at lower concentration than reported earlier (Hossain *et al*. 2012). The observed differences are possibly attributed to the extract phytochemical composition. Extracts of *A. heterophyllus* seed induced 50% mortality of amphistomes at lower concentration (LD_{50}=0.05 mg/mL) compared to that of *I. verum* fruit (LD_{50}=2.22 mg/mL), *P. vera* shell (LD_{50}=1.83 mg/mL) and *A. occidentale* seed (LD_{50}=0.82 mg/mL) extracts (supplementary Table 2; Fig. 2.).

The LDH activity in amphistomes exposed to extracts and albendazole was less (P<0.01) compared to negative control indicating inhibition of activity of LDH catalysing oxidation reaction (Table 2). Anthelmintics usually achieve their activity by altering the activity of glycolytic enzymes (Veerakumari and Munuswamy 2000). LDH is an important glycolytic enzyme of helminth parasites that catalyses oxidation of lactate and reduction of pyruvate to maintain the cytosolic redox potential.

Inhibition of LDH activity in amphistomes exposed to extracts indicated transtegumental diffusion of extracts phytochemicals, like anthelmintics, into amphistome to

Table 2. Effect of different hydroalcoholic extracts on lactate dehydrogenase activity of *C. cotylophorum*

Extract	ABZ (30 ug/mL)	Concentration of extracts (mg/mL)					HFS
		4	1.4	0.5	0.2	0.05	
I. verum	19.91 ± 0.15^{e}	22.96 ± 0.09^{d1}	24.65 ± 0.04^{c1}	25.14 ± 0.17^{c2}	25.82 ± 0.19^{c2}	29.52 ± 0.25^{b1}	31.56 ± 1.19^{a}
P. vera	19.91 ± 0.15^{f}	20.97 ± 0.16^{f2}	22.59 ± 0.05^{e2}	25.58 ± 0.08^{d1}	27.15 ± 0.02^{c1}	28.92 ± 0.11^{b2}	
A. occidentale	19.91 ± 0.15^{c}	14.93 ± 0.09^{e3}	16.28 ± 0.10^{de3}	17.25 ± 0.06^{d3}	19.68 ± 0.17^{c3}	27.11 ± 0.04^{b3}	
A. heterophyllus	19.91 ± 0.15^{c}	7.83 ± 0.05^{f4}	9.95 ± 0.07^{e4}	14.25 ± 0.06^{d4}	18.98 ± 0.07^{c4}	26.27 ± 0.05^{b4}	

SEM, Standard error of mean; ABZ, Albendazole; HFS, Hedon-Fleig solution. Means with different superscripts (alphabet) within a row differ significantly (P<0.01) in particular plant extract; Means with different superscripts (numerical) within a column differ significantly (P<0.05) between different plant extract.

target tegumental enzymes. This inhibition induces lactic acid accumulation and arrest the metabolic pathways. Consequently, there is reduced production of ATP causing starvation, paralysis and death of the parasite. Inhibition of LDH activity was dose-dependent (P<0.05). In comparison to albendazole (19.91±0.15 milliunit/mL), significant level of inhibition (18.98±0.07 milliunit/mL) was observed at a minimum concentration (0.2 mg/mL) following exposure with *A. heterophyllus* seed extract, which might be due to the amino acids that are exclusively present in it. High-protein diets shorten lifespan in in sterile ants because of certain amino acids that are key elements behind this process. Free amino acids shortened lifespan even more than proteins in sterile ants (Arganda *et al*. 2017). Though non-protein based low molecular weight compounds have dominated the study of natural products, proteins produced by plants play a crucial role in defence against pathogens (Soares *et al*. 2019).

In a previous study on nematodes (*Haemonchus contortus*), extracts of *A. heterophyllus* seed exhibited lower anthelmintic activity than *A. occidentale* shell (Davuluri *et al*. 2020). The difference in the activity of same plants against nematode in earlier study and trematode at present might be related to the morphological/functional properties of the parasite's external surfaces. The complex structure of the cuticle in nematode compared with the tegument in trematode could explain the differences observed in the trials. The nematode's cuticle is considered to be a barrier limiting entry of larger molecules into the parasite (Fetterer and Rhoads 1993). Proteins, which are larger molecules compared to other chemical constituents in the extracts, could not enter the *H. contortus* cuticle. While studying the drug transport mechanisms in helminths, it was evidenced that fenbendazole diffusion in nematode was markedly lower than those measured in the trematode (Mottier *et al*. 2006).

It is concluded that the *A. heterophyllus* seed extract exhibited potent inhibitory effect on motility and LDH activity of amphistomes (*C. cotylophorum*) and could be valuable alternative anthelmintic. Further, *in vivo* validation of *in vitro* results could endorse the seed extract of *A. heterophyllus* as a potential anthelmintic.

SUMMARY

Effect of hydroalcoholic extracts of *Pistacia vera* L. shell, *Anacardium occidentale* L. shell, *Illicium verum* Hook.f. fruit, and *Artocarpus heterophyllus* Lam seed was assessed *in vitro* against adult, and lactate dehydrogenase (LDH) of *Cotylophoran cotylophorum* of naturally infected sheep using worm motility assay and LDH activity assay kit, respectively. Extracts exhibited significant dose- and time-dependent anthelmintic responses by causing mortality of worms. *Artocarpus heterophyllus* seed exhibited maximum activity with 100% mortality of worms at a higher concentration of 4 mg/mL. Probit analysis revealed that the extracts of *A. heterophyllus* seed induced 50% worm mortality at a lower concentration (LD_{50}=0.05 mg/mL) compared with those of *I. verum* fruit (LD_{50}=2.22 mg/mL), *P. vera* shell (LD_{50}=1.83 mg/mL) and *A. occidentale* seed (LD_{50}=0.82 mg/mL) extracts. Evidently, *A. heterophyllus* seed extracts inhibited the LDH activity catalyzing the oxidation of lactate in adult *C. cotylophorum*. Qualitative screening of the extracts showed existence of amino acids, alkaloids, flavonoids, tannins, and saponins, which are accountable for the anthelmintic effects observed.

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