Indian Journal of Experimental Biology
Vol. 47, August 2009, pp. 643-648

Molluscicidal activity of *Piper cubeba* Linn., *Piper longum* Linn. and *Tribulus terrestris* Linn. and their combinations against snail *Indoplanorbis exustus* Desh.

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Received 6 January 2009; revised 3 March 2009

The toxic effect of dried berries powder of *P. cubeba*, dried fruit powder of *P. longum* and *T. terrestris* singly as well as in combination [binary(1:1) and tertiary (1:1:1)] were studied against snail *I. exustus*. Toxicity of these plant products were time and concentration dependent. Ethanol extracts of these plants were more effective than that of other organic solvents. 96 h LC_{50} value of column purified fraction of *T. terrestris* against *I. exustus* was 9.57 mg/l, where as 96 h LC_{50} values of column purified fractions of *P. longum* and *P. cubeba* were 11.57 mg/l and 10.93 mg/l, respectively. Binary (1:1) combination of *P. cubeba* (PC) + *P. longum* (PL) (41.78 mg/l) was more effective than *P. cubeba* (PC) + *T. terrestris* (TT) (42.17 mg/l) and *P. longum* (PL) + *T. terrestris* (TT) (55.84 mg/l) respectively; while tertiary (1:1:1) combinations of *P. cubeba* (PC) + *T. terrestris* (TT) + *T. foenum-graecum* (TF) (10.67 mg/l) was more effective than rest of the combinations. These plants can be used as potent source of molluscicides against the snail *I. exustus*.

Keywords: Active components, Fascioliasis, *Indoplanorbis exustus*, Molluscicides, *Piper*, *Tribulus*

Some of the fresh water snails are the vectors of digenean trematode (*Fasciola hepatica* and *Fasciola gigantica*) larvae, which causes endemic fascioliasis to man and his domestic cattle's[1-3]. Like the schistosomes, the life cycles of these trematodes are tightly knitted to water and snails[4,5]. *Indoplanorbis exustus* is acknowledged intermediate hosts of these liver flukes[2,6]. The best method to control trematode infection is to control the population of vector snail[2,7-9]. Several attempts have been made to reduce the incidence of fascioliasis by using synthetic pesticides and plant derived products against transmitting snails[2-3,6,10-11]. Uses of plant products as molluscicide are ecologically sound and culturally more acceptable than synthetic one. A large number of plant products have been identified as molluscicide against harmful snails[12]. In the present study common medicinal plants such as *Piper cubeba* (Piperaceae), *Piper longum* (Piperaceae) and *Tribulus terrestris* (Zygophyllaceae) were tested singly as well as in combinations against the snail *Indoplanorbis exustus* Desh., to explore their molluscicidal activity.

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Materials and Methods

Animals—Adult *Indoplanorbis exustus* (0.85 ± 0.036 cm in length) were collected locally from Ramgarh Lake and low-lying submerged fields. The animals were acclimatized for 72 h in laboratory condition. Experimental animals (10) were kept in glass aquaria containing 3 litre of dechlorinated tap water maintained at room temperature (22°-24°C). The *p*H of the water was 7.1-7.3 and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.5-7.1, 5.2-6.2 and 102-104 mg/l, respectively. Dead animals were removed immediately from the aquaria to avoid any contamination.

Plants—Berries of *Piper cubeba*, dried fruit of *Piper longum* and spiny fruit of *Tribulus terrestris* were collected locally and identified by Prof. S.K. Singh, Plant Taxonomist from the Department of Botany, D.D.U. Gorakhpur University, Gorakhpur, (UP) India.

Crude plant product—Stalked berries of *P. cubeba*, dried fruit of *P. longum* and dried spiny fruit of *T. terrestris* were pulverized separately with a grinder and the crude powder thus obtained were used for the toxicity experiments.

Organic solvent extracts—Stalked berries of *P. cubeba*, dried fruit of *P. longum* and dried spiny

fruit of *T. terrestris* (5 g each) were mixed with 100 ml each of 95% ethanol, 98% ether, 99.7% chloroform and 98% acetone at room temperature. After 24 h in each extracts solvent was removed under vacuum and remaining dried parts were used for determination of molluscicidal activity. *P. cubeba*-ethanol-580 mg, chloroform-630 mg, acetone-430 mg, ether-380 mg, *P. longum*-ethanol-630 mg, acetone-670 mg, chloroform-570 mg, ether-510 mg and *T. terrestris*- ethanol-710 mg, chloroform-520 mg, acetone-620 mg, ether-540 mg were obtained after extraction.

Column purification—Ethanol extract fraction (100 ml) of stalked berries of *P. cubeba*, dried fruit of *P. longum* and dried spiny fruit of *T. terrestris* were subjected to silica gel (60-120 mesh, Qualigens Glass, Precious Elctrochemindus private limited, Mumbai, India) chromatography through a 5×45 cm column. Fractions (5 ml) with ethanol (95%) were collected. Ethanol was evaporated under vacuum and remaining solids were used for determination of molluscicidal activity.

Pure compounds—Piperine (1-piperoylpiperidine), cubebene (α-cubebene), harmine (7-methoxy-1-methyl-9H-pyrido [3,4b] indole), harmane (Aribine, 1-methyl-9H-pyrido [3,4b] indole) were purchased from Sigma Chemical Co. USA.

Thin layer chromatography—Molluscicidal components present in berries of *P. cubeba*, dried fruit of *P. longum* and dried spiny fruit of *T. terrestris* were identified by TLC. TLC was done on 20×20 cm pre coated silica gel (Precious Electro Chemical Industry, Pvt. Limited, Mumbai). The solvent was benzyne ethyl acetate (90:10). Co-migration of column purified fraction of the plant with pure components cubebene, harmane, harmine and piperine were done on TLC plate. TLC plates were developed by iodine vapour.

Treatments of animal—Toxicity experiments were performed as per Singh and Agarwal[13]. Six aquaria were set for each concentration of plant derived molluscicides. Experimental animals (10) were kept in each glass aquarium containing 3 litre of dechlorinated tap water. Snails were exposed to different concentrations of single, binary (1:1) and tertiary (1:1:1) combinations of *P. cubeba, P. longum* and *T. terrestris* against *I. exustus* (Table 1). Mortality was recorded at 24 h intervals up to 96 h exposure periods. Control animals were kept in an equal volume of dechlorinated water under similar condition without treatment.

Table 1—Different concentrations of *P. cubeba*, *P. longum* and *T. terrestris* used singly as well as in combinations against the snail *I. exustus*

Materials used	Test concentration (mg/l)
Fruit powder of *Piper cubeba*	90,120, 150, 200
Chloroform extract	25, 35, 50, 70
Acetone extract	20, 25, 35, 50
Ether extract	50, 70, 90, 120
Ethanol extract	10, 12, 15, 20
Column extract	10, 12, 15, 20
Cubebene	2, 3, 5, 7
Fruit powder of *Piper longum*	90, 120, 150, 200
Chloroform extract	20, 25, 35, 50
Acetone extract	20, 25, 35, 50
Ether extract	50, 70, 90, 120
Ethanol extract	10, 15, 20, 25
Column extract	10, 12, 15, 20
Piperine	5, 7, 9, 12
Fruit powder of *Tribulus terrestris*	90, 120, 150, 200
Chloroform extract	35, 50, 70, 90
Acetone extract	30, 35, 50, 70
Ether extract	70, 90, 120, 150
Ethanol extract	25, 30, 40, 50
Column extract	10, 20, 40, 60
Harmane	5, 7, 9, 12
Harmine	1, 2, 3, 5
PC + PL	30, 50, 70, 90
PC + TT	30, 50, 70, 90
PC + PL + CT	50, 70, 90, 120
PC + TT + CT	30, 50, 70, 90
PC + PL + TF	20, 25, 35, 50
PC + TT + TF	10, 20, 30, 50
PL + TT	50, 70, 90, 110
PL + TT + CT	30, 35, 45, 55
PL + TT + TF	30, 50, 70, 90

PC= *P. cubeba*; PL= *P. longum*; TT= *T. terrestris*; TF= *Trigonella foenum-graecum*; CT= *Cinnamomum tamala*

Lethal concentration (LC_{50}), lower and upper confidence limits (LCL and UCL), slope values, t-ratio, g-values and heterogeneity factors were calculated by using the POLO computer programme[14]. The regression coefficient between exposure time and different values of LC_{50} was determined[15].

Table 2—Toxicity of fruit powder of *P. cubeba, P. longum and T. terrestris* and their different organic solvents and purified fraction against snail *I. exustus*

Exposure period (h)	Tested materials	*P. cubeba*			*P. longum*			*T. terrestris*		
		LC_{50} (mg / l)	Limits		LC_{50} (mg / l)	Limits		LC_{50} (mg / l)	Limits	
			LCL	UCL		LCL	UCL		LCL	UCL
24	Fruit powder	254.48	204.33	455.58	208.49	173.75	328.79	246.64	200.39	419.28
	Chloroform extract	76.88	64.01	109.53	55.87	46.23	82.53	103.11	87.38	142.56
	Ether extract	154.87	124.29	265.66	117.44	104.52	142.74	189.88	156.34	305.71
	Acetone extract	69.63	52.24	154.59	56.52	46.79	83.34	81.56	66.99	124.98
	Ethanol extract	19.13	17.27	22.76	27.13	23.38	36.04	51.11	43.73	73.72
	Column purified	23.36	18.97	43.66	21.20	18.49	27.93	73.97	50.92	167.81
	Cubebene	7.89	12.58	6.24	-	-	-	-	-	-
	Piperine	-	-	-	12.41	8.24	16.42	-	-	-
	Harmane	-	-	-	-	-	-	14.85	11.94	25.61
	Harmine	-	-	-	-	-	-	7.05	4.92	16.34
48	Fruit powder	200.79	169.87	295.42	159.49	139.62	198.26	221.84	179.46	412.51
	Chloroform extract	60.29	50.71	82.82	43.87	37.31	59.52	81.83	71.39	101.56
	Ether extract	110.64	93.01	162.61	103.97	90.48	132.83	160.91	133.18	267.03
	Acetone extract	53.69	42.43	101.17	47.62	39.16	74.43	57.71	50.38	72.38
	Ethanol extract	16.34	14.56	19.78	24.81	20.85	35.81	36.32	32.44	41.21
	Column purified	17.44	15.38	22.27	18.53	16.22	24.46	56.37	36.93	176.49
	Cubebene	5.24	4.35	6.97	-	-	-	-	-	-
	Piperine	-	-	-	6.21	4.68	9.38	-	-	-
	Harmane	-	-	-	-	-	-	10.57	8.91	15.27
	Harmine	-	-	-	-	-	-	4.28	3.25	7.30
72	Fruit powder	130.80	113.51	149.19	122.58	102.41	140.29	153.31	129.99	202.95
	Chloroform extract	41.82	34.99	49.98	34.31	29.78	41.26	67.23	58.58	81.11
	Ether extract	82.68	72.60	95.77	78.46	67.57	91.06	117.38	103.63	140.49
	Acetone extract	41.08	34.06	61.26	31.45	27.08	36.94	46.91	41.17	54.77
	Ethanol extract	13.37	11.95	14.85	19.94	16.81	26.68	30.21	26.14	33.40
	Column purified	14.45	12.41	17.46	14.81	12.87	17.99	19.76	6.96	32.67
	Cubebene	3.75	2.96	4.73	-	-	-	-	-	-
	Piperine	-	-	-	1.57	0.66	3.71	-	-	-
	Harmane	-	-	-	-	-	-	6.41	5.26	7.29
	Harmine	-	-	-	-	-	-	2.19	1.44	3.16
96	Fruit powder	108.58	92.30	120.86	103.16	87.51	114.58	108.81	90.97	121.95
	Chloroform extract	32.41	27.45	36.57	25.84	21.79	29.28	47.80	39.44	54.71
	Ether extract	62.14	51.93	69.94	62.22	47.59	72.32	101.49	88.45	115.67
	Acetone extract	26.88	21.69	31.41	23.72	20.18	26.54	37.81	32.70	42.29
	Ethanol extract	11.05	9.49	12.16	12.63	10.16	14.47	26.13	22.87	28.47
	Column purified	10.93	9.48	11.97	11.57	9.78	12.86	9.57	4.55	13.72
	Cubebene	2.40	1.76	2.90	-	-	-	-	-	-
	Piperine	-	-	-	0.82	0.12	1.87	-	-	-
	Harmane	-	-	-	-	-	-	5.31	4.12	6.10
	Harmine	-	-	-	-	-	-	1.30	0.82	1.69

Significant negative regression ($P<0.05$) was observed between exposure time and LC_{50} of treatments.

Ts- testing significant of the regression coefficient-

P. cubeba (fruit powder)-7.54^{+}; chloroform extract- 11.06^{+}; acetone extract-31.76^{+}; ether extract-8.74^{++}; ethanol extract-28.74^{+}; column extract-8.92^{+}; cubebene-8.24^{+}.

P. longum (fruit powder)-10.92^{++}; chloroform extract- 17.59^{+}; acetone extract- 10.17^{+}; ether extract- 12.18^{+}; ethanol extract- 6.13^{+}; column extract- 23.16^{+}; piperine- 5.30^{++} .

T. terrestris (fruit powder)-8.44^{+}; chloroform extract- 20.87^{+}; acetone extract- 16.90^{++}; ether extract- 8.50^{+}; ethanol extract- 86.50^{++}; column extract-6.72^{+}; harmane- 7.15^{+}; harmine- 6.46^{+}.

+: linear regression between x and y; ++: non-linear regression between log x and log y.

Table 3—Binary combinations (1:1) of crude powder of *P. cubeba*, *P. longum*, *T. terrestris* and tertiary combinations (1:1:1) with other known molluscicidal plants against the vector snail *I. exustus*

Exposure Period (h)	Tested material	LC_{50} (mg /l)	Limits		Slope value	t-ratio	g-value	Hetero-geneity
			LCL	UCL				
24	PC + PL	109.17	88.54	170.32	2.79±0.59	4.69	0.18	0.23
	PC + TT	104.08	86.27	151.59	2.99±0.61	4.91	0.16	0.25
	PC + PL + CT	133.75	111.03	201.52	2.99±0.67	4.43	0.20	0.13
	PC + TT + CT	95.70	78.41	144.01	2.44±0.53	4.58	0.18	0.12
	PC + PL + TF	37.95	32.87	47.49	2.67±0.57	4.71	0.17	0.18
	PC + TT + TF	53.77	40.93	91.66	1.89±0.39	4.92	0.16	0.13
	PL+ TT	119.21	104.80	152.30	4.30±0.83	5.18	0.14	0.32
	PL + TT + CT	64.63	55.58	91.72	4.39±0.99	4.43	0.20	0.27
	PL + TT + TF	91.14	75.05	134.55	2.36±0.52	4.54	0.19	0.18
48	PC + PL	81.18	68.34	110.06	2.42±0.51	4.77	0.17	0.17
	PC + TT	73.07	62.39	93.27	2.46±0.50	4.92	0.16	0.11
	PC + PL + CT	77.16	67.36	87.88	3.09±0.61	5.04	0.15	0.14
	PC + TT + CT	79.54	66.67	108.86	2.30±0.49	4.61	0.18	0.13
	PC + PL + TF	30.25	25.54	35.69	2.49±0.56	4.45	0.19	0.14
	PC + TT + TF	34.78	27.37	50.97	1.62±0.34	4.71	0.17	0.10
	PL + TT	106.93	93.64	137.63	3.37±0.71	4.71	0.17	0.18
	PL + TT + CT	54.46	48.74	67.51	4.34±0.89	4.84	0.16	0.18
	PL + TT + TF	63.60	55.75	74.39	2.93±0.50	5.83	0.11	0.12
72	PC + PL	60.53	50.39	75.28	2.12±0.48	4.46	0.19	0.13
	PC + TT	55.08	46.14	65.38	2.36±0.48	4.92	0.16	0.12
	PC + PL + CT	62.97	51.34	71.68	3.01±0.62	4.87	0.16	0.16
	PC + TT + CT	54.32	44.59	65.42	2.17±0.47	4.58	0.18	0.14
	PC + PL + TF	23.44	17.63	27.49	2.45±0.57	4.29	0.21	0.16
	PC + TT + TF	17.10	12.92	20.97	1.89±0.34	5.52	0.13	0.22
	PL + TT	85.51	74.99	102.39	2.88±0.67	4.34	0.20	0.17
	PL + TT + CT	41.99	38.69	46.11	4.72±0.85	5.53	0.13	0.13
	PL + TT + TF	49.06	42.13	55.73	3.04±0.49	6.15	0.10	0.14
96	PC + PL	41.78	33.83	48.26	2.78±0.49	5.65	0.12	0.19
	PC + TT	42.17	34.98	48.19	3.02±0.49	6.07	0.10	0.21
	PC + PL + CT	53.54	44.89	59.82	4.39±0.72	6.11	0.10	0.54
	PC +TT + CT	40.97	33.72	46.92	3.03±0.49	6.06	0.10	0.17
	PC +PL + TF	21.34	18.43	23.54	4.77±0.75	6.37	0.09	0.33
	PC +TT + TF	10.67	6.67	13.83	1.92±0.37	5.26	0.14	0.19
	PL + TT	55.84	46.29	62.58	3.98±0.71	5.61	0.12	0.24
	PL + TT + CT	35.22	32.19	37.74	5.74±0.91	6.31	0.10	0.16
	PL + TT + TF	40.49	33.48	46.25	3.13±0.50	6.21	0.10	0.27

PC = *Piper cubeba*; PL = *Piper longum*; TT = *Tribulus terrestris*; TF = *Trigonella foenum-graecum*; CT = *Cinnamomum tamala*; LCL = lower confidence limits; UCL = upper confidence limits. Significant negative regression ($P<0.05$) was observed between exposure time and LC_{50} of treatments.

Ts- testing significant of the regression coefficient-

PC+PL- 14.75^{+}; PC+TT- 10.60^{++}; PC+PL+CT- 12.96^{++}; PC+TT+CT- 12.26^{+}; PC+PL+TF- 10.11^{++}; PC+TT+TF- 6.98^{+}; PL+TT- 7.69^{+}; PL+TT+CT- 12.90^{+}; PL+TT+TF- 22.54^{++}.

+: linear regression between x and y; ++: non- linear regression between log x and log y.

Results

The toxicity of crude powder of berries of *P. cubeba*, fruit of *P. longum* and *T. terrestris*, their organic solvent extracts and combinations [binary (1:1) & tertiary (1:1:1)] against *I. exustus* were time and concentration dependent. There was a significant negative regression in between exposure period and LC_{50} values of all the treatments. The LC_{50} of stalked berries of *P. cubeba*, fruit of *P. longum* and *T. terrestris* powder at 24 h was 254.48, 208.49, 246.64 mg/l and at 96 h were 108.58, 103.16, 108.81 mg/l, respectively (Table 2). Among all organic solvent extracts, the ethanol extract of these plants were more toxic (Table 2). The 96 h LC_{50} values of ethanol extracts of berries of *P. cubeba*, fruit of *P. longum* and *T. terrestris* against *I. exustus* were 11.05, 12.63 and 26.13 mg/l, respectively. The 96 h LC_{50} values of column purified fractions of berries of *P. cubeba*, fruit of *P. longum* and *T. terrestris* were 10.93, 11.57 and 9.57 mg/l, respectively. Among binary combinations (1:1), PC+PL (96 h LC_{50}-41.78 mg/l) was more effective than PC+TT (96 h LC_{50}-42.17 mg/l) and PL+TT (96 hr LC_{50}-55.84 mg/l), respectively (Table 3). In tertiary combinations (1:1:1), PC+TT+TF (96 h LC_{50}-10.67 mg/l) was more effective than PC+PL+TF (96 h LC_{50}-21.34 mg/l), PL+TT+CT (96 h LC_{50}-35.22 mg/l), PL+TT+TF (96 h LC_{50}- 40.49 mg/l), PC+TT+CT (96 h LC_{50}- 40.97 mg/l) and PC+PL+CT (96 h LC_{50}- 53.54 mg/l), respectively (Table 3). TLC analysis demonstrated that the R_f values of harmane (0.98) and harmine (0.16) were equivalent to the R_f values of two spots in column purified fraction of *T. terrestris* (0.98 and 0.16). The R_f value of piperine (0.54) was equivalent to the R_f value of column-purified fraction in *P. longum* (0.54). The R_f value of cubebene is (0.49) equivalent to the R_f value of column-purified fraction (0.49) in *P. cubeba*. 96 h LC_{50} values of harmane, harmine, piperine and cubebene were 5.31, 1.30, 0.82 and 2.40 mg/l, respectively (Table 2).

Discussion

The present results clearly indicate that the berries of *P. cubeba*, dried fruit of *P. longum* and *T. terrestris* are potent molluscicides. Their toxic effects are time and concentration dependent. Higher toxicity of ethanol extract among other organic extract indicate that the molluscicidal component present in these plants are more soluble in ethanol. It is evident from the co-migration of column purified component and pure compounds on TLC plate that the molluscicidal activity of *P. cubeba* fruit powder may be due to the cubebene, whereas in *P. longum* and *T. terrestris* it may be due to the presence of piperine and harmane, harmine, respectively.

The main constituent of *P. longum* is an alkaloid, piperine[16]. *P. longum* has anti-inflammatory and antioxidant property[17]. The berries of *P. cubeba* have anti-inflammatory, antioxidant and antitumor properties[18-20]. If piperine is given at doses of 5-10 mg/kg body weight for 30 days, it resulted in significant reduction in weights of testis and accessory sex organs and damaged the seminiferous tubules of rats[21]. A comparison of molluscicidal activity of crude powder of all the three medicinal plants with other molluscicidal plants clearly indicates that these are more effective. Thus 96 h LC_{50} values of *P. cubeba*, *P. longum* and *T. terrestris* against *I. exustus* are 108.58, 103.16 and 108.81 mg/l, respectively. They are lower than *P. nigrum* (white; 121.97 mg/l), *Cinnamomum tamala* (705.42 mg/l) and *Carum carvi* (313.47 mg/l)[3,22].

Binary and tertiary combination of all three plants taken in the present study clearly show that their toxicity against *I. exustus* is more pronounced than their single treatment. It indicates that *I. exustus* can be eliminated more efficiently with even smaller quantities of these plants in different mixed combinations. The higher toxicity of binary and tertiary combinations than single components may be due to their action at different sites in the snail body or, it may be possible that detoxifying enzymes in the snail body are inhibited by one of the component present in binary and tertiary combination, which causes higher titer of active molluscicidal component at their target site in the snail body[23].

It is evident from steep slope value that a small increase in the concentration of different plant products might cause a marked mortality in snails. A *t*- ratio greater than 1.96 indicates that regression is significant. Heterogeneity factor values lower than 1.0 denote that in the replicate tests of random samples the concentration response curves would fall with in the 95% confidence limits and thus, the model fits over data adequately. The index of significance of the potency estimation g-value less than 0.5 indicates that the value of the mean is with in the limits of all the probability limits.

It can be concluded that the powder of berries of *P. cubeba*, dried fruit of *P. longum* and *T. terrestris*

singly or in combination, are effective molluscicides against the vector snail *I. exustus*.

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