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In vitro PHYTOTHERAPY OF VECTOR SNAILS BY BINARY COMBINATIONS OF LARVICIDAL ACTIVE COMPONENTS IN EFFECTIVE CONTROL OF FASCIOLIASIS

Kumari SUNITA, Pradeep KUMAR, Vinay Kumar SINGH & Dinesh Kumar SINGH

SUMMARY

A food-borne trematode infection fascioliasis is one among common public health problems worldwide. It caused a great economic loss for the human race. Control of snail population below a certain threshold level is one of the important methods in the campaign to reduce the incidence of fascioliasis. The life cycle of the parasite can be interrupted by killing the snail or *Fasciola* larva redia and cercaria inside of the snail *Lymnaea acuminata*. *In vitro* toxicity of different binary combinations (1:1 ratio) of plant-derived larvicidal active components such as citral, ferulic acid, umbelliferone, azadirachtin and allicin against *Fasciola* redia and cercaria were tested. The mortality of larvae was observed at 2h, 4h, 6h and 8h of treatment. In *in vitro* condition azadirachtin + allicin (1:1 ratio) was highly toxic against redia and cercaria (8h LC_{50} 0.006 and 0.005 mg/L). Toxicity of citral + ferulic acid was lowest against redia and cercaria larvae.

KEYWORDS: *Lymnaea acuminata*; Fascioliasis; Redia; Cercaria; Active larvicides.

INTRODUCTION

Fasciola spp is the causative agent of endemic fascioliasis in different parts of the world[16]. *F. hepatica* has worldwide distribution but predominates in temperate zones, while *F. gigantica* is found primarily in tropical regions[2,14,15]. This disease belongs to the plant-borne trematode zoonoses. The definite host is very broad and includes many herbivorous mammals, including humans. Bovine fascioliasis is very common in the eastern region of Uttar Pradesh, India[22]. SINGH & AGARWAL[25] reported that 94% of buffaloes slaughtered in local slaughtered house in Gorakhpur district are infected with *F. gigantica.* In northern India *Lymnaea acuminata* is the intermediate host of *F. gigantica*[2]. Although control of snail population below a threshold level is one of the important methods for effective control of fascioliasis[9,11,13], yet snails are one of the important components in the aquatic ecosystem. Release of molluscicides in aquatic system for snail control also affects the other non-target organism. The *Fasciola* larval stages sporocyst, redia and cercaria are in division phases of *F. gigantica* in the snail body. If these larvae will be destroyed by plant molluscicides at sublethal concentration in the snail body, the rate of infection can be reduced without killing the snail. Binary combinations of different plants derived active larvicides such as citral (*Zingiber officinale*), ferulic acid, umbelliferone (*Ferula asafoetida*), azadirachtin (*Azadirachta indica*), and allicin (*Allium sativum*)[30] were tested against *Fasciola* larvae in *in vitro* condition. It is a new approach to reduce incidence of the fascioliasis without killing the intermediate host snail.

MATERIAL AND METHODS

Animals: Infected adult *Lymnaea acuminata* (2.6 ± 0.20 cm in length) were collected locally. The snails were allowed to acclimatize for 24h in laboratory condition. Each infected snail was dissected in a glass Petri dish containing 10 mL l of dechlorinated water at 22 ºC-24 ºC. The pH of the water was 7.1-7.3 and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.5-7.2 mg/L , 5.2- 6.3mg/L and 102.0-105.0 mg/L, respectively.

After dissection redia and cercaria were separated in different Petri dish containing 10 mL l of dechlorinated water. These larvae were kept in dechlorinated tap water where they survived for up to 48h in laboratory conditions. Snail *L. acuminata* and *Fasciola gigantica* were identified by Zoological Survey of India (ZSI), Kolkata.

Active larvicidal components: Citral, ferulic acid, umbelliferone, azadirachtin and diallyl disulfide were purchased from Sigma chemical Co. (U.S.A). Allicin was prepared by the method of SINGH & SINGH[27].

Efficacy determination

In vitro: *In vitro* efficacy of active larvicidal components in binary combinations (1:1 ratio) were performed in petri dish by the method of SUNITA & SINGH[30]. Ten redia and cercaria of *F. gigantica* were separated into different Petri dishes containing 10 mL l dechlorinated

Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur 273009 (U.P), India.
Correspondence to: Prof. D. K. Singh, Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur 273009 (U.P), India. Tel +91-551 222187 (Off), Mobile: +91-9454211574. E-mail: dksingh_gpu@yahoo.co.in

tap water. Mortality of redia and cercaria were observed after 2h, 4h, 6h and 8h of treatment. In control, no treatments were given in Petri dish. Usually, in *in vitro* conditions (control) survival of 72h in dechlorinated water. Counting of larvae in control and treated groups was performed with the help of a microscope. Each experiment was replicated six times.

Effective concentrations to kill 50% (EC_{50}), low and upper confidence limits (LCL and UCL), Slop-values, t-ratio, g value and heterogeneity factor were calculated with the help of the POLO computer program of ROBERTSON *et al.*[19]. One way ANOVA and product moment correlation coefficient was applied by the method of SOKAL & ROHLF[28].

RESULTS

In vitro larvicidal activity of different binary combinations (1:1 ratio) of active components against the redia and cercaria larva of *F. gigantica* was time and concentration dependent (Table 1 and 2). In *in vitro* treatments, binary combinations of azadirachtin + allicin were more efficient against redia and cercaria. The 8h EC_{50} of azadirachtin + allicin against redia/cercaria larvae in *in vitro* treatment was 0.006mg/L / 0.005mg/L, respectively (Table 1 and 2). Citral + ferulic acid efficacy against both larval stages was lowest (Table 1 and 2). Significant ($p < 0.05$) negative regression was observed in between exposure period and EC_{50} of different plant products. There was no mortality of *F. gigantica* larvae in control group.

The slope values were steep and separate estimation of LC based on each six replicate were found within the 95% confidence limit of EC_{50}. The t-ratio was greater than 1.96 and the heterogeneity less than 1.0. The g-value was less than 0.5 at all probability levels (90, 95 and 99 respectively) (Table 1-2).

DISCUSSION

The present study clearly indicates that the active components of *Zingiber officinale* (citral), *Ferula asafoetida* (ferulic acid, umbelliferone), *Azadirachta indica* oil (azadirachtin) and *Allium sativum* (allicin) and their binary combinations have sufficient larvicidal activity against different larva of *F. gigantica* in *in vitro* treatments. The alcoholic extract of bulbs of *A. sativum* has also shown moderate *in vitro* antihelminitic activity against human *Ascaris lumbricoides*[18]. *A. sativum* has been reported to be effective in dysentery and also act as vermifuge[17,21]. Oil of *A. sativum* has also been reported to possess antihelmintic activity[8,10,29] and discards all injurious parasites in the intestine[17]. *In vitro* and *in vivo* efficacy of single treatments of the active components citral, ferulic acid, umbelliferone, azadirachtin and allicin against redia and cercaria larva of *F. gigantica* have potent larvicidal activity[30].

Z. officinale is a perennial plant and is considered to be the universal medicine in Ayurveda. The antihelminthic activity of ethanolic extracts of rhizomes of *Z. officinale* against human *Ascaris lumbricoides* is appreciable[18]. GOTO *et al.* [7] reported the lethal effect of *Z. officinale* on *Anisakis* larvae *in vitro*. The anti-filarial effect of *Z. officinale* against *Dirofilaria immitis* has been reported by DATTA & SUKUL[4]. ADEWUNMI *et al.*[1], SINGH *et al.*[26] have reported the molluscicidal activity of *Z. officinale.*

Ferulic acid and umbelliferone extracted from *Ferula asafoetida* root latex are potent molluscicides against *L. acuminata*[11,12,13]. The antioxidant, anticarcinogenic, antispasmodic, antihelminthic activity of *F. asafoetida* extract and ferulic acid were reported by various workers[5,6,20]. *Spigelia anthelmia* inhibits the motility of *Haemonchus contortus* larva[3], SINGH *et al.*[24] observed that *A. indica* have molluscicidal activity against *L. acuminata. In vitro* treatment of citral, ferulic acid, umbelliferone, azadirachtin and allicin caused toxicity against redia (8h EC_{50} 4.14, 0.45, 0.63, 0.07 and 0.01 mg/L, respectively) and cercaria (8h EC_{50} 6.08, 0.44, 0.27, 0.08 and 0.009 mg/L, respectively) larva of *F. gigantica*[30]. Binary combinations of ferulic acid + azadirachtin against redia larva is 64.28 times more effective than single treatment of ferulic acid, where as in case of cercaria citral + allicin was 53.80 times more effective than single treatment of citral (Table 3). Earlier it was reported that citral (24h EC_{50} - 68.95 mg/L), ferulic acid (24h EC_{50} - 2.21 mg/L), umbelliferone (24h EC_{50} - 3.43mg/L), azadirachtin (24h EC_{50} - 0.35mg/L), and allicin (24h EC_{50}- 6.34 mg/L) are potent molluscicides against *L. acuminata*[11,23,26,27]. 8h EC_{50} of binary combinations of these larvicidal components against redia/cercaria larva is 1.25 (allicin + umbelliferone)/ 1.23 (citral + umbelliferone) times low that kill the intermediate host *L. acuminata.* Different concentrations of binary combinations used to kill redia and cercaria are not toxic to the snail, even in a 24h exposure period. Use of these plants and their active component in killing the redia and cercaria of *F. gigantica* without killing the host snail is important. The snail is a crucial component of aquatic ecosystem. The present studies clearly demonstrate that direct treatment of the redia and cercaria will be a more efficient method to control fascioliasis.

The steep slope value indicates that a small increase in the concentration of different larvicide caused higher larval mortality. A t-ratio value greater than 1.96 indicates that the regression is significant. The heterogeneity factor value of less than 1.0 denotes that in the replicate test of random samples, the concentration response is limited and, thus, the model fits the data adequately. The index of significance of the potency estimation *g* indicates that the value of the mean is within the limit at all probability levels (90, 95, and 99, respectively) since it is less than 0.5[19].

It can be concluded from the present study that binary combination of these natural products will be more helpful in controlling the redia/ cercaria than their single components, because their larvicidal effect is 1.25 to 64.28/ 1.23 to 53.80 more effective in killing the larva. The effective toxic concentration in the binary combinations of each larvicidal component is lower and would be safer in aquatic environment.

RESUMO

Fitoterapia *in vitro* de caramujos vetores por combinações binárias de componentes ativos larvicidas como controle efetivo de fasciolíase

A infecção alimentar pelo trematóide da fasciolíase é uma dentre os mais comuns problemas de saúde pública mundiais, causando grande prejuízo econômico para a humanidade. Controle da população de caramujos abaixo de determinado nível é um dos métodos no campo mais importantes para a redução da incidência da fasciolíase. O ciclo de vida do parasita pode ser interrompido pela morte do caramujo ou da larva redia e cercária da *Fasciola* dentro da *Lymnaea acuminata*. Foi testada a toxicidade *in vitro* das diferentes combinações binárias (relação 1:1) entre os vários componentes larvicidas ativos da planta tais como citral,

Table 1
In vitro toxicity of different binary combinations (1:1 ratio) of active larvicidal components against the redia larva of *F. gigantica*

Exposure period	Treatment	EC_{50} mg/L (w/v)	Limits LCL	Limits UCL	Slope value	t-ratio	g-value	Heterogeneity
2h	Ci+Fe	29.500	17.18	13.84	1.050±0.306	3.439	0.325	0.13
	Ci+Um	18.532	12.28	51.62	1.709±0.408	4.190	0.219	0.29
	Ci+Az	5.700	3.806	21.58	1.338±0.401	3.334	0.346	0.15
	Ci+Al	0.547	0.364	2.246	1.266±0.394	3.210	0.373	0.11
	Fe+Um	2.401	1.573	10.07	1.350±0.407	3.313	0.350	0.14
	Fe+Az	0.041	0.030	0.081	1.091±0.330	3.302	0.390	0.16
	Fe+Al	0.062	0.044	0.096	1.284±0.429	2.995	0.428	0.09
	Az+Um	0.433	0.358	0.659	1.986±0.568	3.493	0.315	0.13
	Az+Al	0.015	0.010	0.021	1.308±0.382	3.420	0.382	0.15
	Al+Um	0.040	0.027	0.152	1.073±0.374	2.869	0.466	0.10
4h	Ci+Fe	15.330	10.31	40.30	0.998±0.286	3.491	0.315	0.13
	Ci+Um	15.704	9.762	68.67	1.140±0.330	3.454	0.322	0.17
	Ci+Az	3.720	2.662	9.86	1.167±0.375	3.115	0.396	0.13
	Ci+Al	0.346	0.247	0.892	1.124±0.372	3.023	0.420	0.10
	Fe+Um	1.553	1.111	4.450	1.214±0.392	3.094	0.401	0.11
	Fe+Az	0.024	0.016	0.038	1.046±0.324	3.230	0.368	0.13
	Fe+Al	0.039	0.019	0.050	1.413±0.433	3.267	0.360	0.12
	Az+Um	0.319	0.247	0.397	1.916±0.557	3.437	0.325	0.13
	Az+Al	0.010	0.005	0.013	1.370±0.391	3.501	0.313	0.15
	Al+Um	0.022	0.014	0.033	1.129±0.365	3.091	0.402	0.13
6h	Ci+Fe	8.107	5.239	12.78	0.996±0.280	3.561	0.303	0.14
	Ci+Um	9.500	6.392	28.46	0.995±0.309	3.223	0.370	0.15
	Ci+Az	2.218	1.435	3.41	1.112±0.365	3.045	0.414	0.13
	Ci+Al	0.202	0.119	0.301	1.084±0.364	2.975	0.434	0.10
	Fe+Um	0.987	0.687	1.66	1.156±0.390	2.965	0.437	0.11
	Fe+Az	0.014	0.005	0.020	1.076±0.327	3.290	0.355	0.14
	Fe+Al	0.026	0.008	0.038	1.410±0.446	3.174	0.381	0.20
	Az+Um	0.234	0.143	0.287	1.985±0.569	3.437	0.310	0.15
	Az+Al	0.007	0.003	0.009	1.566±0.418	3.750	0.273	0.18
	Al+Um	0.013	0.005	0.018	1.157±0.367	3.152	0.387	0.15
8h	Ci+Fe	4.463	1.968	6.50	1.023±0.282	3.624	0.292	0.15
	Ci+Um	5.172	3.187	8.63	0.948±0.301	3.148	0.387	0.15
	Ci+Az	1.304	0.470	1.84	1.136±0.366	3.100	0.400	0.14
	Ci+Al	0.118	0.035	0.171	1.119±0.367	3.048	0.413	0.11
	Fe+Um	0.607	0.256	0.831	1.186±0.398	2.977	0.433	0.14
	Fe+Az	0.007	0.002	0.013	1.145±0.340	3.367	0.378	0.28
	Fe+Al	0.022	0.001	0.012	1.092±0.342	3.342	0.243	0.29
	Az+Um	0.183	0.103	0.230	2.366±0.597	3.966	0.244	0.29
	Az+Al	0.006	0.003	0.008	2.324±0.516	4.401	0.190	0.33
	Al+Um	0.008	0.002	0.013	1.355±0.355	3.537	0.307	0.27

Ci = citral, Fe = ferulic acid, Um = umbelliferone, Az = azadirachtin, Al = allicin, LCL - lower confidence limits, UCL - upper confidence limits. Six batches of ten redia larva were exposed to different concentration of binary combinations of the above larvicides treatments. Mortality was recorded every 2h. Concentrations given are the final concentration (W/V) in the glass aquarium water. Significant negative regression ($p < 0.05$) was observed between exposure time and EC_{50} of treatments. Ts - testing significant of the regression coefficient. Ci+Fe:-7.76^{++}, Ci+Um:-10.50^{+}, Ci+Az:-8.70^{+}, Ci+Al:-7.73^{+}, Fe+Um:-8.00^{+}, Fe+Az:-6.97^{+}, Fe+Al:-12.34^{+}, Az+Um:-8.67^{++}, Az+Al:-16.24^{++}, Al+Um:-1.59^{+}. +: linear regression between x and y; ++: non – linear regression between log x and log y.

Table 2
In vitro toxicity of different binary combination of active larvicidal components against the cercaria larva of *F. gigantica.*

Exposure period	Treatment	EC_{50} mg/l (w/v)	Limits LCL	Limits UCL	Slope value	t-ratio	g-value	Heterogeneity
2h	Ci+Fe	34.962	20.928	140.729	1.330±0.340	3.917	0.250	0.18
	Ci+Um	19.579	12.392	67.064	1.525±0.386	3.947	0.246	0.23
	Ci+Az	5.364	3.618	19.816	1.297±0.395	3.284	0.356	0.12
	Ci+Al	0.567	0.369	2.851	1.213±0.392	3.096	0.401	0.12
	Fe+Um	2.457	1.608	10.004	1.379±0.409	3.370	0.338	0.11
	Fe+Az	0.044	0.031	0.109	1.165±0.334	3.486	0.316	0.10
	Fe+Al	0.079	0.062	0.129	1.575±0.438	3.592	0.298	0.11
	Az+Um	0.441	0.371	0.627	2.247±0.577	3.892	0.253	0.13
	Az+Al	0.018	0.012	0.032	1.115±0.380	2.933	0.446	0.10
	Al+Um	0.035	0.027	0.065	1.398±0.381	3.671	0.285	0.13
4h	Ci+Fe	24.654	15.072	107.980	1.036±0.298	3.477	0.318	0.13
	Ci+Um	10.942	7.931	21.679	1.415±0.330	4.290	0.209	0.27
	Ci+Az	3.577	2.529	10.369	1.098±0.372	2.951	0.441	0.10
	Ci+Al	0.352	0.248	1.026	1.082±0.371	2.919	0.451	0.12
	Fe+Um	1.713	1.181	6.953	1.148±0.392	2.925	0.449	0.09
	Fe+Az	0.028	0.019	0.050	1.028±0.324	3.168	0.383	0.15
	Fe+Al	0.054	0.038	0.072	1.454±0.430	3.379	0.336	0.11
	Az+Um	0.355	0.283	0.477	1.826±0.558	3.274	0.358	0.13
	Az+Al	0.011	0.005	0.014	1.213±0.386	3.143	0.389	0.16
	Al+Um	0.022	0.015	0.033	1.178±0.366	3.216	0.371	0.11
6h	Ci+Fe	13.986	9.277	38.423	0.926±0.283	3.276	0.358	0.12
	Ci+Um	8.974	6.111	24.322	1.005±0.308	3.262	0.361	0.14
	Ci+Az	2.067	1.204	3.157	1.056±0.364	2.899	0.457	0.10
	Ci+Al	0.202	0.113	0.306	1.046±0.364	2.875	0.464	0.12
	Fe+Um	0.939	0.688	1.454	1.232±0.371	3.059	0.346	0.09
	Fe+Az	0.016	0.007	0.022	1.037±0.325	3.192	0.377	0.16
	Fe+Al	0.036	0.019	0.047	1.510±0.436	3.462	0.320	0.13
	Az+Um	0.255	0.167	0.310	1.939±0.559	3.467	0.320	0.12
	Az+Al	0.008	0.123	0.320	1.950±0.651	3.321	0.421	0.18
	Al+Um	0.015	0.007	0.020	1.219±0.367	3.325	0.347	0.17
8h	Ci+Fe	7.092	4.030	11.147	0.920±0.278	3.304	0.352	0.11
	Ci+Um	4.911	2.969	7.862	0.963±0.301	3.196	0.376	0.14
	Ci+Az	1.189	0.323	1.729	1.088±0.367	2.966	0.436	0.11
	Ci+Al	0.113	0.027	0.166	1.076±0.367	2.920	0.448	0.12
	Fe+Um	0.595	0.273	0.803	1.257±0.400	3.143	0.389	0.10
	Fe+Az	0.009	0.002	0.014	1.128±0.336	3.356	0.341	0.19
	Fe+Al	0.026	0.011	0.036	1.736±0.461	3.765	0.271	0.48
	Az+Um	0.193	0.096	0.246	2.023±0.575	3.516	0.311	0.12
	Az+Al	0.005	0.002	0.008	1.589±0.454	3.718	0.278	0.29
	Al+Um	0.010	0.004	0.014	1.468±0.381	3.848	0.259	0.32

Ci = citral, Fe = ferulic acid, Um = umbelliferone, Az = azadirachtin, Al = allicin, LCL - lower confidence limits, UCL - upper confidence limits. Six batches of ten cercaria larva were exposed to different concentration of binary combinations of the above larvicides treatments. Mortality was recorded every 2h. Concentrations given are the final concentration (W/V) in the glass aquarium water. Significant negative regression ($p < 0.05$) was observed between exposure time and EC_{50} of treatments. Ts - testing significant of the regression coefficient. Ci+Fe:-15.33^{+}, Ci+Um:-5.53^{++}, Ci+Az:-9.61^{+}, Ci+Al:-7.58^{+}, Fe+Um:-8.94^{+}, Fe+Az:-8.21^{+}, Fe+Al:-7.97^{++}, Az+Um:-15.97^{+}, Az+Al:-7.41^{+}, Al+Um:-8.51^{++}. +: linear regression between *x* and *y*; ++: non – linear regression between log *x* and log *y*.

Table 3
Synergism in the *in vitro* larvicidal activity of different binary combinations against redia and cercaria larva of *F. gigantica* at 8h exposure period

Treatment	Redia		Cercaria	
	8h EC_{50} mg/L	Synergistic ratio	8h EC_{50} mg/L	Synergistic ratio
Ci	4.14	-	6.08	-
Fe	0.45	-	0.44	-
Um	0.63	-	0.27	-
Az	0.07	-	0.08	-
Al	0.01	-	0.009	-
Ci+Fe	4.463	-	7.092	-
Ci+Um	5.172	-	4.911	1.23
Ci+Az	1.304	3.18	1.189	5.15
Ci+Al	0.118	37.37	0.113	53.80
Fe+Um	0.607	-	0.595	-
Fe+Az	0.007	64.28	0.009	48.88
Fe+Al	0.022	20.45	0.026	16.92
Az+Um	0.183	-	0.193	-
Az+Al	0.006	11.66	0.005	16.00
Al+Um	0.008	1.25	0.010	-

Ci = Citral, Fe = Ferulic acid, Um = Umbelliferone, Az = Azadirachtin, Al = Allicin. Single treatments of active larvicides were reported by SUNITA and SINGH (2011).

ácido ferúlico, umbeliferone, azadiractina, e alicina contra a *Fasciola* redia e a cercária. A mortalidade das larvas foi observada após duas, quatro, seis e oito horas de tratamento. A condição *in vitro* azadiractina + alicina (relação 1:1) foi altamente tóxica contra redia e cercária (8h LC_{50} 0,006 e 0,005 mg/L). Toxicidade do citral + ácido ferúlico foi a mais baixa contra redia e larvas de cercária.

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