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In vivo phytotherapy of snail by plant derived active components in control of fascioliasis

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ABSTRACT

Fascioliasis is worldwide zoonotic disease, which caused great economic loss to human being. *In vivo* killing of *Fasciola* larva (redia and cercaria) is one of the effective methods to control of fascioliasis. *In vivo* toxicity of different binary combinations (1:1ratio) of plant derived active components such as citral, ferulic acid, umbelliferone; azadirachtin and allicin were tested against *Fasciola* larva (redia and cercaria) in snail *Lymnaea acuminata*. Mortality of larvae were observed at 2h, 4h, 6h and 8h of treatment. *In vivo* exposure of azadirachtin + allicin (1:1 ratio) was highly toxic against redia and cercaria (8h LC_{50} 0.007and 0.006 mg/L). Combinations of citral + umbelliferone, cirtal + ferulic acid were less toxicity against redia and cercaria larva than azadirachtin + allicin. *In vivo* use of these larvicidal active components in the binary combinations at sublethal does will be more helpful in controlling the redia/cercaria than their individual components.

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1. Introduction

Fasciolosis is an economically important disease caused by *Fasciola hepatica* and *F. gigantica* (Singh and Agarwal, 1981; Mas-Coma *et al.,* 2009). *F. hepatica* has worldwide distribution but *F. gigantica* is the most common *Fasciola* species infecting ruminant in the tropical regions of Asia (Agarwal and Singh, 1988; Mas-Coma *et* *al.,* 2005; Sunita and Sing, 2011). The definite host is very broad and includes many herbivorous mammals, including humans. Singh and Agarwal, (1981) reported that 94% of buffaloes slaughtered in local slaughtered house in Gorakhpur district are infected with *F. gigantica.* Control of snail population below a threshold level is one of the important methods for effective control of fasciolosis (Sunita and Singh, 2011; Kumar and Singh, 2006; Kumar *et al.,* 2009; Jaiswal and Singh, 2009; Srivastava *et al.,* 2013). Snails are the important components of the aquatic ecosystem. If the larvae of *Fasciola* i.e. redia, cercaria will be destroyed by active components of plants in binary combination at sublethel concentration within the snail body, the rate of infection in livestock as well as human can be reduced without killing the snail. Different plants derived component which are active against snail *Lymnaea acuminata* as molluscicides (Kumar and Singh, 2006; Singh *et al.,* 1995; Singh *et al.,* 1997) such as citral, ferulic acid, umbelliferone, azadirachtin, and allicin were tested in binary combinations (1:1 ratio) against *Fasciola* larva in *in vivo* (Sunita and Singh, 2011). It is a new therapy to reduce incidence of the fascioliasis without killing the intermediate host snail *L. acuminata,* which is one of the important component of aquatic ecosystem. In this way we will achieve the goal that is without killing the snail we can reduce the incidence of fasciolosis.

2. Materials and methods

2.1 Animals

Adult infected *Lymnaea acuminata* (2.6±0.20 cm in length) were collected locally. Cercaria shedding infected and uninfected snails was separated in two groups. Snails were allowed to acclimatize for 24h in laboratory condition. After treatment each infected snail was dissected in a glass petridish containing 10 ml of dechlorinated water at 22^{o}C-24^{o}C.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.

2.3 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 and Singh, (1995).

2.4 Toxicity determination

2.4.1 In vivo

In vivo toxicity of active larvicidal components in binary combinations (1:1 ratio) were performed in the glass aquaria. Infected snails were exposed to different concentrations of cirtal, ferulic acid, umbelliferone, azadirachtin and allicin by the methods of Sunita and Singh, (2011). After 2h, 4h, 6h and 8h of treatment, infected snails were dissected. Than live and dead redia and cercaria larva were counted with the help of microscope. Mortality of redia and cercaria larva was established by immediate arrest of locomotion/ movement. It was continuously monitored up to 48h in all treatment to ensure death. Percent mortality of larva at each concentration for 2h, 4h, 6h and 8h was used for determination of $LC_{50.}$

Lethal value (LC_{50}), low and upper confidence limits (LCL and UCL), Slop-values, t-ratio, g value and heterogeneity factor were calculated with the help of POLO computer programmed of Robertson, et al., (2007). One way ANOVA and product moment correlation coefficient was applied by the method of Sokal and Rohlf, (1996).

3. Results

In *in vivo* larvicidal activity of different binary combinations (1:1 ratio) of active components against the redia and cercaria larva of *F. gigantica* is time and concentration dependent (Table 1-2). In *in vivo* treatments binary combination of azadirachtin + allicin caused highest toxicity against redia and cercaria larva. The 8h LC_{50} of azadirachtin + allicin against redia/cercaria larva in *in vivo* treatment was 0.007 mg/L / 0.006 mg/L, respectively (Table 1-2). Toxicity of citral + umbelliferone, citral + ferulic acid against redia and cercaria larval stage were lower than azadirachtin + allicin (Table 1-2). Significant ($p<0.05$) negative regression was observed in between exposure period and LC_{50} of different plant products.

The slope values were steep and separate estimation of LC based on each six replicate were found within the 95% confidence limit of LC_{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).

4. Discussion

It is evident from the result section that active larvicidal component of *Zingiber officinal* (citral), *Ferula asafoetida* (ferulic acid, umbelliferone), *Azadirachta indica* oil (azadirachtin) and *Allium sativum* (allicin) and their binary combinations have sufficient larvicidal activity against redia and cercaria larva of *Fasciola gigantica* in *in vivo* treatments. The alcoholic extract of bulbs of *A. sativum* has also shown moderate *in vitro* anthelminthic activity against human *Ascaris lumbricoldes* (Kalesaraj, 1975). *A. sativum* has been reported to be effective in dysentery and also act as vermifuge (Nadkarni, 1976). Oil of *A. sativum* has also been reported to possess anthelmintic activity (Steenis-Kruseman, 1953; Hoppe, 1975; Kirtikar and Basu, 1981) and discards all injurious parasites in the intestine (Nadkarni, 1976). *In vitro* and *in vivo* 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 (Sunita and Singh, 2011).

Zingiber officinal is perennial plant and is considered to be the universal medicine in Ayurveda. The anthelminthic activity of ethanol extracts of rhizomes of *Zingiber officinale* against human *Ascaris lumbricoldes* is appreciable (Kalesaraj, 1975; Kalesaraj, 1974). Goto et al., (1990) reported the lethal effect of *Zingiber officinale* on *Anisakis* larvae *in vitro.* The anti filarial effect of *Z. officinale* against *Driofilaria immitis* has been reported by Datta and Sukul (1987). Adewunmi *et al.,* (1990), Singh *et al.,* (1997) have reported the molluscicidal activity of *Z. officinale.*

Ferulic acid, umbelliferone is (*Ferula asafoetida*) one of the potent molluscicides Aagainst *L. acuminata* (Kumar and Singh, 2006; Kumar *et al.,* 2009; Kumar *et al.,* 2011). Antioxidant, ant carcinogenic, antispasmodic, anthelminthic activity of *F. asafoetida* extract and ferulic acid were reported by various workers (Eigner and Scholz, 1999; Saleem *et al.,* 2001; Fatehi *et al.,* 2004). Azadirachtin, an active component of *Azadirachta indica* inhibits the motility of *Haemonchus contortus* larva (Assis *et al.,* 2003), observed that *A. Indica* have sufficient molluscicides activity against *L. acuminata. In vitro* treatment of citral, ferulic acid, umbelliferone, azadirachtin and allicin caused toxicity against redia (8h LC_{50} 4.14, 0.45, 0.63, 0.07 and 0.01 mg/L, respectively) and cercaria (8h LC_{50} 6.08, 0.44, 0.27, 0.08 and 0.009 mg/L, respectively) (Sunita and Singh, 2011), whereas in *in vivo* the binary combinations of these larvicides are more effective than singly treatment. Synergistic ratio in *in vivo* larvicidal activity of different binary combinations (1:1) was observed in 2h, 4h, 6h and 8h of exposure period. The binary combination of ferulic acid + azadirachtin 8h $LC_{50,}$ 26.47, 24.44 times more effective than the single treatment against redia and cercaria larvae, respectively (Table-3). Present study clearly demonstrates that different larval stages inside the snail body can be killed without killing the snails. Earlier it has been reported that citral (24h LC_{50} - 68.95 mg/L), ferulic acid (24h LC_{50} -2.21 mg/L), umbelliferone (24h LC_{50} -3.43mg/L), azadirachtin (24h LC_{50} -0.35mg/L), and allicin (24h LC_{50}-6.34 mg/L) are active molluscicides against *L. acuminata* (Kumar and Singh, 2006; Singh *et al.,* 1995; Singh and Singh, 1995; Singh *et al.,* 1997). Lethal concentration (LC_{50}) of binary combinations of these larvicidal components against redia and cercaria larva is many time low to kill the intermediate host *L. acuminata.* The different concentration of binary combinations in *in vivo* treatment to kill redia and cercaria in not toxic to *L. acuminata*, even in 24h exposure period. So that, use of these plants and their active component in killing the redia and cercaria of *F. gigantica* inside snail body directly, without killing the host snail is important, as snail is crucial component of aquatic ecosystem. In *in vivo* killing of redia and cercaria of *F. gigantica* is beneficial as it kills directly target larva of *F. gigantica.*

The steep slope value indicates that a small increase in the concentration of different larvicides caused higher larval mortality. A t-ratio value greater than 1.96 indicates that the regression is significant. Heterogeneity factor value less than 1.0 denote that in the replicate test of random sample the concentration response limits and thus the model fits the data adequately. The index of significance of the potency estimation g indicates that the value of mean is within the limit at all probability level (90, 95, and 99, respectively) since it is less than 0.5.

Table1

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

Exposure period	Treatment	LC_{50} mg/l (w/v)	Limits LCL	Limits UCL	Slope value	t-ratio	g-value	Heterogeneity
2h	Ci+Fe	30.509	19.349	131.804	1.252±0.366	3.416	0.329	0.18
	Ci+Um	25.122	17.554	60.624	1.957±0.461	4.136	0.213	0.28
	Ci+Az	7.254	5.288	20.106	1.707±0.506	3.375	0.337	0.14
	Ci+Al	0.855	0.602	2.489	1.458±0.423	3.449	0.323	0.13
	Fe+Um	4.254	2.693	18.360	1.212±0.356	3.409	0.330	0.13
	Fe+Az	0.061	0.045	0.130	1.348±0.404	3.476	0.345	0.12
	Fe+Al	0.091	2.693	18.36	1.212±0.356	3.409	0.330	0.13
	Az+Um	0.574	0.484	0.821	2.239±0.623	3.593	0.297	0.10
	Az+Al	0.023	0.015	0.043	1.134±0.325	3.489	0.316	0.12
	Al+Um	0.043	0.036	0.066	1.986±0.568	3.493	0.316	0.20
4h	Ci+Fe	17.539	12.610	39.439	1.202±0.346	3.476	0.318	0.14
	Ci+Um	21.078	14.164	69.979	1.357±0.346	3.510	0.312	0.06
	Ci+Az	5.156	3.986	10.688	1.519±0.481	3.160	0.382	0.14
	Ci+Al	0.573	0.421	1.331	1.22±0.401	3.065	0.409	0.09
	Fe+Um	2.753	1.886	8.493	1.082±0.338	3.197	0.376	0.10
	Fe+Az	0.039	0.027	0.056	1.297±0.397	3.265	0.360	0.12
	Fe+Al	0.056	0.025	0.077	1.297±0.383	3.404	0.378	0.14
	Az+Um	0.443	0.363	0.531	2.583±0.620	3.714	0.278	0.10
	Az+Al	0.014	0.006	0.019	1.170±0.329	3.561	0.303	0.13
	Al+Um	0.33	0.025	0.043	1.737±0.555	3.128	0.393	0.12
6h	Ci+Fe	10.334	7.211	15.066	1.208±0.340	3.551	0.305	0.14
	Ci+Um	13.750	9.892	33.242	1.97±0.365	3.279	0.346	0.14
	Ci+Az	3.464	2.502	4.796	1.467±0.474	3.097	0.400	0.11
	Ci+Al	0.351	0.218	0.501	1.196±0.396	3.022	0.421	0.08
	Fe+Um	1.540	0.996	2.637	1.008±0.332	3.332	0.417	0.11
	Fe+Az	0.025	0.013	0.033	1.414±0.404	3.500	0.314	0.14
	Fe+Al	0.040	0.013	0.057	1.363±0.407	3.571	0.343	0.14
	Az+Um	0.340	0.234	0.403	2.336±0.634	3.687	0.283	0.11
	Az+Al	0.009	0.003	0.013	1.312±0.345	3.803	0.265	0.16
	Al+Um	0.023	0.012	0.029	1.787±0.56	3.192	0.399	0.13
8h	Ci+Fe	6.335	3.236	8.626	1.248±0.345	3.618	0.293	0.15
	Ci+Um	8.288	5.811	12.605	1.148±0.381	3.207	0.373	0.14
	Ci+Az	2.327	1.123	3.011	1.519±0.481	3.160	0.385	0.12
	Ci+Al	0.216	0.076	0.301	1.249±0.403	3.102	0.399	0.09
	Fe+Um	0.861	0.310	1.243	1.037±0.336	3.082	0.404	0.11
	Fe+Az	0.017	0.005	0.024	1.412±0.419	3.366	0.339	0.12
	Fe+Al	0.032	0.012	0.045	1.917±0.497	3.858	0.258	0.26
	Az+Um	0.275	0.177	0.331	2.850±0.689	3.698	0.225	0.21
	Az+Al	0.007	0.003	0.010	1.830±0.401	3.234	0.184	0.33
	Al+Um	0.018	0.008	0.023	2.113±0.588	3.594	0.297	0.26

Abbreviation: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 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 LC_{50} of treatments. Ts - testing significant of the regression coefficient. Ci+Fe:-8.08^{++}, Ci+Um:-11.00^{+}, Ci+Az:-0.11^{+}, Ci+Al:-1.08^{+}, Fe+Um:-2.32^{++}, Fe+Az:-1.71^{+}, Fe+Al:-3.77^{++}, Az+Um:-1.69^{++}, Az+Al:-1.58^{+}, Al+Um:-0.52^{+}.

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

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

Exposure period	Treatment	LC_{50} mg/l (w/v)	Limits LCL	Limits UCL	Slope value	t-ratio	g-value	Heterogeneity
2h	Ci+Fa	35.355	22.907	114.671	1.578±0.402	3.922	0.250	0.19
	Ci+Um	26.197	17.623	75.290	1.758±0.441	3.981	0.242	0.24
	Ci+Az	6.099	5.075	18.663	1.662±0.500	3.325	0.347	0.12
	Ci+Al	0.889	0.606	3.325	1.347±0.418	3.223	0.370	0.09
	Fe+Um	4.398	2.769	0.696	1.230±0.357	3.440	0.325	0.12
	Fe+Az	0.064	0.048	0.135	1.418±0.408	3.476	0.318	0.11
	Fe+Al	0.155	0.100	0.276	1.258±0.373	3.369	0.338	0.14
	Az+Um	0.624	0.520	0.977	2.222±0.628	3.539	0.307	0.12
	Az+Al	0.028	0.019	0.053	0.990±0.324	3.734	0.411	0.07
	Al+Um	0.045	0.037	0.067	2.128±0.505	3.700	0.281	0.12
4h	Ci+Fa	25.994	17.273	88.472	1.267±0.347	3.481	0.288	0.14
	Ci+Um	21.514	14.175	83.358	1.294±0.385	3.362	0.340	0.18
	Ci+Az	5.016	3.835	11.338	1.418±0.282	2.968	0.436	0.10
	Ci+Al	0.574	0.421	1.363	1.218±0.400	3.044	0.414	0.09
	Fe+Um	2.920	1.944	11.639	1.025±0.338	3.033	0.418	0.08
	Fe+Az	0.043	0.031	0.065	1.316±0.398	3.265	0.352	0.14
	Fe+Al	0.103	0.073	0.175	1.163±0.372	3.126	0.393	0.16
	Az+Um	0.468	0.389	0.578	2.243±0.618	3.714	0.292	0.11
	Az+Al	0.015	0.007	0.022	1.057±0.325	3.623	0.364	0.13
	Al+Um	0.034	0.027	0.042	2.022±0.560	3.613	0.294	0.12
6h	Ci+Fa	16.191	11.547	37.147	1.125±0.342	3.282	0.356	0.12
	Ci+Um	13.162	9.536	29.982	1.199±0.364	3.291	0.354	0.13
	Ci+Az	3.283	2.183	4.537	1.381±0.473	2.922	0.450	0.10
	Ci+Al	0.344	0.214	0.483	1.189±0.396	3.004	0.405	0.08
	Fe+Um	1.532	1.037	2.434	1.093±0.333	3.281	0.357	0.11
	Fe+Az	0.027	0.014	0.027	1.265±0.399	3.168	0.383	0.17
	Fe+Al	0.053	0.025	0.072	1.335±0.389	3.240	0.327	0.13
	Az+Um	0.359	0.248	0.427	2.184±0.625	3.495	0.166	0.11
	Az+Al	0.009	0.002	0.014	1.153±0.337	3.423	0.328	0.16
	Al+Um	0.025	0.017	0.31	1.973±0.561	3.519	0.310	0.17
8h	Ci+Fa	9.264	5.861	13.395	1.128±0.339	3.325	0.347	0.11
	Ci+Um	7.940	5.240	11.734	1.156±0.358	3.229	0.368	0.13
	Ci+Az	2.163	0.833	2.868	1.441±0.481	2.996	0.428	0.10
	Ci+Al	2.209	0.065	0.294	1.221±0.403	2.677	0.419	0.09
	Fe+Um	0.880	0.322	1.270	1.029±0.336	3.066	0.408	0.13
	Fe+Az	0.018	0.006	0.025	1.381±0.414	3.366	0.334	0.20
	Fe+Al	0.042	0.022	0.056	1.799±0.439	3.564	0.228	0.33
	Az+Um	0.281	0.163	0.344	2.432±0.657	3.701	0.280	0.13
	Az+Al	0.006	0.002	0.010	1.407±0.368	3.820	0.263	0.26
	Al+Um	0.019	0.011	0.024	2.287±0.581	3.906	0.252	0.30

Abbreviation: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 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 LC_{50} of treatments. Ts - testing significant of the regression coefficient. Ci+Fe:-1.45^{++}, Ci+Um:-4.13^{++}, Ci+Az:-0.36^{+}, Ci+Al:-0.98^{+}, Fe+Um:-2.32^{++}, Fe+Az:-1.60^{+}, Fe+Al:-3.30^{++}, Az+Um:-1.77^{++}, Az+Al:-0.31^{++}, Al+Um:-2.11^{++}.
+: linear regression between *x* and *y*; ++: non – linear regression between log *x* and log *y*.

Table 3
Synergism in *in vivo* larvicidal activity of different binary combinations (1:1) against redia and cercaria larva of *F. gigantica* at 8h exposure.

Treatment	Redia		Cercaria	
	8h LC_{50} mg/l	Synergistic ratio	8h LC_{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	6.335	-	9.264	-
Ci+Um	8.288	-	7.940	-
Ci+Az	2.327	1.77	2.163	2.81
Ci+Al	0.216	19.16	2.209	2.75
Fe+Um	0.861	-	0.880	-
Fe+Az	0.017	26.47	0.018	24.44
Fe+Al	0.032	14.06	0.042	10.47
Az+Um	0.275	-	0.281	-
Az+Al	0.007	10.00	0.006	13.33
Al+Um	0.018	-	0.019	-

Abbreviations: Ci= Citral, Fe=Ferulic acid, Um= Umbelliferone, Az= Azadirachtin, Al= Allicin
(*) Sunita and Singh, (2011).

5. Conclusion

It can be concluded from the above study the *in vivo* use of these larvicidal active components in the binary combinations at sublethal does will be more helpful in controlling the redia/cercaria than their individual components. The effective toxic concentration in the binary combinations of each larvicidal component is lower and would be safer in aquatic environment and non target organisms.

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