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In Vivo And *In Vitro* Toxicity of Binary Combination of Column Purified Fraction of *Asparagus Racemosus* and *Glycyrrhiza Glabra* Against the Sporocyst, Redia and Cercaria Larva of *Fasciola Gigantica*

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Abstract
In binary combination (1:1) of the activity of *A. racemosus* and *G. glabra* against *Fasciola gigantica* larva in *Lymnaea acuminate* observe *in vivo* and *in vitro* experiments, which is based on time and concentration-dependent toxicity. *In vivo* treatment represent the highest toxicity against cercaria larva, with 2Hr LC_{50} and 8Hr LC_{50} of *A. racemosus* and *G. glabra* at 64.85 mg/L and 39.78 mg/L, respectively while in *in vitro* treatment the toxicity against sporocyst larva, with 2Hr and 8Hr LC_{50} values of 64.59 mg/ml and 46.27 mg/ml, respectively. Negative regression between exposure period and LC_{50} is significant ($p<0.05$). Steep slope values, t-ratios >1.96, and g values <0.5 shows reliability of the data. This study suggests the potential of *A. racemosus* and *G. glabra* as larvicidal plant products against *F. gigantica*, offering a novel approach to control fascioliasis without harming intermediate host snails.
Key words: *Fasciola gigantica*, Fascioliasis, *Asparagus racemosus, Glycyrrhiza glabra*.

Introduction

Two major parasitic flukes *Fasciola hepatica* and *F. gigantica* causes a zoonotic disease fascioliasis in mammals. The infected cattle like Buffaloes, sheep, pigs, donkeys, goats, horses, camels and other herbivore and including human. (Fentie, *et al*., 2013). Fascioliasis causes damage in liver and other associated organ of ruminants due to which reducing body weight, infertility, growth and also increasing the mortality that affect the production of meat, milk, and wool (Kumar and Singh, 2006; Ramadan, *et al*., 2019). In previous some decades, infestation of fascioliasis in human has notably increases in worldwide mainly in Asia, Africa, and South America, where most common flukes species *F. hepatica* and *F. gigantica* exist (Mas-Coma *et al*., 2005; Ramadan, *et al.,* 2019). Nearly about 17 million human population infected by *Fasciola* infection globally and many more at the risk (Mas-Coma *et al.,* 2018; Kumar, *et al.,* 2020; Vishwakarma and Kumar, 2021a). Infection of *Fasciola* in human can transmitted by food, water, and sometimes combination of both, where ingestion of lentic freshwater wild plants is chief source, with waterweeds and other vegetables involved (Mas-Coma *et al.,* 2018). High infestation of fascioliasis is related with socio-economic activities, global changes, human and animal migration and disturbances in environment (Mas-Coma et al. 2009).

The adult *F. gigantica* is able to reproduce by both selfing and crossing, due to bisexuality. In infected cattle, buffaloes, human or other mammals *Fasciola* lives in bile ducts that reach in intestine with duct in unembryonated form of eggs and ejected outside the body through feces. (Sunita *et al.,* 2013a). The first stage ciliated larva of *Fasciola* (miracidium) hatches from egg in to fresh water bodies. The miracidium larva inter intermediate host fresh water snail *Lymnaea acuminata* by making pore in their body wall and develops into a sporocyst, then redia and cercaria. Cercaria

larva may infect the primary host mammals, including humans passively when they drinks contaminated water or eaten as encysted larva on leaves containing metacercaria larva (Hotez, *et al.,* 2012; Nyindo and Lukambagire, 2015).

The eaten fifth stage larva of *Fasciola* (metacercaria) reach in liver through blood and metamorphose into adult and causes hyperplasia of the bile ducts. In chronic phase hyperplasia of the gall bladder and biliary epithelium occurs and this leads to biliary tract obstruction (Vishwakarma and Kumar, 2021b; Sunita *et al.,* 2013). For the control of infestation of fascioliasis we can easily break the larval development of *Fasciola* in snail body (sporocyst, redia and cercaria). This freshwater host snail is one of the most important part of the aquatic ecosystem. If these larva will be destroyed by biodegradable phytochemical molluscicides at sub lethal concentration in the snail body, the rate of infection can be reduced without killing the snail. Binary combinations of *Asparagus racemosus* and *Glycyrrhiza glabra* (1:1) were tested against *Fasciola* larva in *in vivo* and *in vitro* condition. It is a new approach to reduce incidence of the fascioliasis without killing the intermediate host snail.

A. racemosus root extract was contain many chemical constituents like flavonoids, saponins, tannins, sterols, alkaloids, polyphenols and vitamin C, which were found to exhibit the greatest antioxidant activity, phytoestrogenic effect as well as antidiarrhoeal, antidyspepsia, adaptogenic, cardioprotective, antibacterial, immune adjuvant and antitussive effects (Taepongsorat and Rattana, 2018). The methanolic extracts of *A. racemosus* shows highest Ovicidal and larvicidal activity against *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* (Govindarajan and Sivakumar, 2014).

The primary phytochemical constituents of *G. glabra* root extract are glycyrrhizin, liquiritin, glabridin, triterpene, isoliquiritin, glycyrrhetic acid, saponins, flavonoids (Sangam and Sheela, 2017; Karkanis *et al.* 2018; Batiha *et al.* 2020; Murck, 2020). It shows antimicrobial activity against *Staphylococcus aurens*, *Candida albicans*, *Bacillus subtilis* (Demizu *et al.,* 1988; Mitscher *et al.,* 1980), and also against *Agrobacterium tumefaciens*, *Bacillus cereus*, *Bacillus subtilus* and *Pseudomonas syringae* (Ercisli, *et al.,* 2008). In traditionally the root of *G. glabra* are uses as anti-inflammatory, anti-cariogenic, antiulcer, antibacterial, antifungal, anti-viral, anti- allergic, antioxidant, immune stimulatory, anti-protozoan, anti-tumor (Martins, *et al.,* 2015, Karkanis, *et al.,* 2018), hepatoprotective, immunomodulatory, neuro-protective, memory enhancement, anti-diabetic, anti-asthmatic, haematinic, cerebro-protective, anti-tussive, hair growth promoting (Anagha, *et al.,* 2013; Zang, 2020). Other activities such as anticancer, anti-inflammatory, antimicrobial, antiviral, antioxidative, hepatoprotective, antidiabetic, and antiobesity activities.

Plant products are easily available, biodegradable and eco-friendly. The plant products have sufficient anthelmintic larvicidal activity in *in vivo* and *in vitro* (Sunita and Singh, 2011).

Materials and Methods

Animals

Infected adult *L. acuminata* (2.6±0.20 cm in length) were collected from ponds of local areas. The snails were allowed to acclimatize for 24Hr in laboratory condition. Each infected snail was dissected in a glass petri-dish containing 10ml of dechlorinated water at 22°C-24°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.

After dissection sporocyst, redia, and cercaria were separated in different Petri-dish containing 10 ml of dechlorinated water. These larva were kept in dechlorinated tap water where they survive up to 48Hr in laboratory condition.

Toxicity determination

In vivo

In *in vivo* toxicity of binary combination of *A. racemosus* and *G. glabra* (1:1) where determine against larva of *F. gigantica* in infected snail *L. acuminata*. After 2Hr, 4Hr, 6Hr and 8Hr of treatment of infected snails where dissected, then live and dead sporocyst, redia and cercaria larva were counted. Death of larva was stabilized by immediate arrest of locomotion movement. It was continuously monitor up to 48Hr in all treatments to ensure death. Present mortality of larva at each concentration for 2Hr, 4Hr, 6Hr and 8Hr was used for the determination of LC_{50}.

In vitro

In vitro toxicity of binary combinations of *A. racemosus* and *G. glabra* (1:1 ratio) were performed in the Petri dish by the method of Sunita and Singh (2011). Ten sporocyst, redia, and cercaria larva of *F. gigantica* were separated in different Petri dish containing 10 ml dechlorinated tap water. Treatments of *A. racemosus* and *G. glabra* in binary combinations (1:1 ratio) were made directly in the Petri dish containing 10 sporocyst/redia/cercaria. Mortality of sporocyst, redia and cercaria were observed after 2Hr, 4Hr, 6Hr and 8Hr of treatment. Counting of larva was performed with help of light microscope.

Lethal value (LC_{50}), low and upper confidence limits (LCL and UCL), Slop-values, t-ratio, g value and heterogeneity factor were measured 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).

Result

In vivo and in vitro larvicidal activity of different binary combinations (1:1 ratio) of *A. racemosus* and *G. glabra* against the sporocyst, redia and cercaria larva of *F. gigantica* is time and concentration dependent (Table 1-2). In *in vivo* treatments, binary combination of *A. racemosus* and *G. glabra* caused highest toxicity against cercaria larva. The 2Hr LC_{50} and 8Hr LC_{50} of *A. racemosus* and *G. glabra* against cercaria larva in *in vivo* treatment was 64.85 mg/L and 39.78 mg/L, respectively (Table 1). In *in vitro* treatments, binary combination of *A. racemosus* and *G. glabra* toxicity against sporocyst larva of 2Hr and 8Hr are 64.59mg/ml and 46.27 mg/ml, respectively. The 2Hr LC_{50} and 8Hr LC_{50} of *A. racemosus* and *G. glabra* against cercaria larva in *in vivo* treatment was 64.85 mg/L and 39.78 mg/L, respectively. 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_{50} 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).

Table:1 *In vivo* toxicity of binary combination (1:1) of column purified fraction in mg/L of *Asparagus racemosus* and *Glycyrrhiza glabra* against the sporocyst, redia and cercaria larva of *F. gigantica.*

Exposure	Larva	LC_{50}	LCL	UCL	Slope - value	t-ratio	g-value	Heterogeneity
2Hr	Sporocyst	74.02	67.83	87.32	0.57±0.32	3.48	0.29	0.34
	Redia	69.37	58.49	77.91	0.67±0.29	3.17	0.52	0.40
	Cercaria	64.85	52.37	73.15	0.44±0.72	2.58	0.37	0.21
4Hr	Sporocyst	66.91	58.82	79.54	0.89±0.63	2.20	0.63	0.42
	Redia	60.11	51.26	74.89	0.37±0.20	3.77	0.49	0.15
	Cercaria	58.20	50.46	67.42	0.25±0.48	3.05	0.38	0.19
6Hr	Sporocyst	59.50	52.30	76.44	0.65±0.37	2.91	0.21	0.27
	Redia	52.92	46.84	68.12	0.82±0.55	2.82	0.76	0.33
	Cercaria	48.43	41.10	53.76	0.31±0.14	3.61	0.68	0.40
8Hr	Sporocyst	51.22	43.89	72.05	0.49±0.57	3.38	0.55	0.25
	Redia	48.37	39.79	61.50	0.67±0.36	2.44	0.71	0.38
	Cercaria	39.78	31.44	58.18	0.56±0.30	3.12	0.20	0.14

Six batches of 10 infected snails were kept in binary combination (1:1) of column purified fraction solution of *A. racemosus* and *G. glabra*. In *in vivo* mortality of sporocyst/ redia/ cercaria larva were recorded every 2Hr up to 8Hr. Concentration given are the final concentration (w/v) in dechlorinated tape water. Significant negative regression ($p < 0.05$) was observed between exposure time and LC_{50} of treatments. TS- testing significant of the regression coefficient. LCL- lower confidence limits, UCL-upper confidence limits**.**

Table: 2 *In vitro* toxicity of binary combination (1:1) of column purified fraction in mg/ml of *Asparagus racemosus* and *Glycyrrhiza glabra* against the sporocyst, redia and cercaria larva of *F. gigantica.*

Exposure	Larva	LC_{50}	LCL	UCL	Slope - value	t-ratio	g-value	Heterogeneity
2Hr	Sporocyst	64.59	51.27	72.91	0.48±0.19	2.98	0.32	0.27
	Redia	60.22	52.69	73.28	0.63±0.41	3.13	0.44	0.14
	Cercaria	55.10	43.81	63.39	0.51±0.38	2.64	0.30	0.18
4Hr	Sporocyst	56.37	49.98	65.04	0.26±0.42	2.18	0.49	0.24
	Redia	52.89	43.76	61.12	0.76±0.55	3.22	0.21	0.37
	Cercaria	47.42	40.32	59.44	0.46±0.21	2.92	0.35	0.42
6Hr	Sporocyst	52.91	42.22	63.39	0.34±0.57	2.67	0.40	0.27
	Redia	49.76	39.19	58.82	0.29±0.65	2.50	0.26	0.49
	Cercaria	38.85	31.50	59.73	0.58±0.36	2.73	0.59	0.31
8Hr	Sporocyst	46.27	35.93	55.20	0.72±0.28	2.59	0.76	0.23
	Redia	40.39	31.22	48.62	0.39±0.12	2.44	0.22	0.43
	Cercaria	29.05	22.99	37.38	0.42±0.63	2.87	0.39	0.29

Six batches of 10 sporocyst/ redia/cercaria larva were kept in binary combination (1:1) of purified fraction solution of *A. racemosus* and *G. glabra*. In *in vitro* mortality of sporocyst/redia/cercaria larva were recorded every 2Hr up to 8Hr. Concentration given are the final concentration (w/v) in dechlorinated tape water. Significant negative regression ($p < 0.05$) was observed between exposure time and LC_{50} of treatments. TS- testing significant of the regression coefficient. LCL- lower confidence limits, UCL-upper confidence limits.

Discussion

The experiment represents the potential of the larvicidal activity of binary combinations of *A. racemosus* and *G. glabra* against *F. gigantica* larva in both *in vivo* and *in vitro* conditions. Fascioliasis is a zoonotic disease caused by *F. hepatica* and *F. gigantica*, which impart significant adverse effects on both animals and human health worldwide mainly low economic countries. The worldwide increase of fascioliasis in human to attract the needs of effective and ecofriendly control measures.

The results represent that the binary combination of *A. racemosus* and *G. glabra* has potent larvicidal activity against *F. gigantica* larva, and shows it is effective method to control the fascioliasis. The time and concentration-dependent toxicity express the importance of dosage and exposure duration to effective larval eradication.

The *in vivo* treatments show high toxicity against cercaria larva, representing the efficacy of the binary combination. The calculated LC_{50} values further support the efficacy of the treatment, with concentrations of *A. racemosus* and *G. glabra* resulting in significant larval mortality within specified time duration. The negative regression observed between exposure period and LC_{50} support the concentration-dependent nature of the larvicidal activity.

Furthermore, the slope values, along with the reliability of data represented by t-ratios >1.96 and g values <0.5, provide confidence in the accuracy of the results obtained. These findings suggest that *A. racemosus* and *G. glabra* could serve as effective and ecofriendly alternatives to the controlling fascioliasis without using to harmful larvicidal chemical.

However, the eco-friendly nature of plant-derived products makes them using for globally, providing a sustainable and biodegradable options to the control of parasitic infections. The phytochemical composition of *A. racemosus* and *G. glabra*, along with their anti-inflammatory, antimicrobial and medicinal properties, help to support their effectiveness as larvicidal agents against *F. gigantica*.

Thus, the present study help suggested the larvicidal activity of *A. racemosus* and *G. glabra* against *F. gigantica* larva, expose the new opportunity for further research into their application as eco-friendly and effective control measures for fascioliasis.

Conclusion

In *in vivo* and *in vitro* time and concentration dependent treatment of the larva of *F. gigantica* through binary combination (1:1) of the *A. racemosus* and *G. glabra* represent the potential to control its population. The findings indicates the potential of these plant-derived products as effective and eco-friendly agents for controlling fascioliasis. Specially, the observed time and concentration-dependent toxicity shows the importance of dosage and exposure duration to obtain maximum larval mortality. However, the calculated LC_{50} values shows the efficacy of *A. racemosus* and *G. glabra* combinations in larval mortality within specified time duration. The validity and accuracy of the results is further supported by slope values and the confidence limits. Thus the eco-friendly nature of plant-based larvicidal activity of plant-products shows the effective approach to control the incidence of fascioliasis without harming intermediate host snails and providing a sustainable solution to the worldwide parasitic infection control.

Conflict of Interest:

The authors have no conflict of interest.

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