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In vivo Phytotherapy of Fresh Water Host Snail *Lymnaea acuminata* for the Control of *Fasciola* Larva by the use of *Glycyrrhiza glabra*

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A B S T R A C T

Fascioliasis is a worldwide zoonotic disease, caused by trematodes species of *Fasciola* among cattle and humans populations. *Fasciola hepatica* and *F. gigantica* are major two species of liver fluke, which causes economic loss of livestock keeper. The fluke species of *F. gigantica* is very common in the eastern part of Uttar Pradesh (India) which completes their life cycle in primary host cattle and intermediate host snail *Lymnaea acuminata*. Fascioliasis can be controlled by destroying the larval stages (sporocyst, redia, and cercaria) in intermediate host snail. The present study aims to evaluate *in vivo* toxicity of different preparation of plant *Glycyrrhiza glabra* against sporocyst, redia, and cercaria larva of *F. gigantica*. Each experimental setup contains 10 infected snails were kept in one-liter dechlorinated tap water with 6 replicates for different preparations and the same experimental setup was performed parallel without treatment for the control group. The dried root powder of *G. glabra* and their organic extracts (ether, chloroform, methanol, acetone, and ethanol) and column purified fractions were exposed in 1 liter dechlorinated water with 10 infected snails for *in vivo* larvicidal activity against sporocyst, redia, and cercaria within 2h, 4h, 6h, and 8h separately along with control group. The column purified fraction was shown after 2h LC_{50} 58.90, 59.27, and 64.91 mg/l and 8h LC_{50} 52.82, 50.05 and 54.20 mg/l efficacy against sporocyst, redia, and cercaria, respectively. The larvicidal activity of column purified fraction of *G. glabra* in *in vivo* exposure was observed redia>sporocyst>cercaria.

***Key words*:** Fascioliasis, *Glycyrrhiza glabra*, *Lymnaea acuminata*, Sporocyst, Redia, Cercaria

The trematode flukes species of *Fasciola hepatica* and *F. gigantica* are causative agents of fascioliasis in cattle including other vertebrates buffaloes, sheep, pigs, donkeys, goats, horses, camels, and human [1-2]. The infections of fascioliasis damage the liver of the animals and it cause liver cirrhosis which affects ruminants by decreasing, their development, growth, body weight, infertility, high mortality, lower production of wool, milk, and meat [3-5]. In the last few decades, human fascioliasis has considerably increased in different parts of the world especially in Asia, Africa, and South America, where common flukes *F. hepatica* and *F. gigantica* coexist [6-7]. In human, the main sources of fascioliasis are contaminated aquatic vegetables, food, and water that contain spores or larval stages of *Fasciola* [8]. The worldwide spreading of fascioliasis is concerned with global exchanges and movements of animals and humans, [9-12]. *Fasciola* is a hermaphrodite fluke that can reproduce by both cross and self-fertilization. The miracidium stages of *Fasciola* are ciliated which hatches from an egg in freshwater. It bores the body of the host snail *Lymnaea acuminata* and develops into a sporocyst, redia, and cercaria [13]. Cercaria larva can infect the definitive host mammals, including humans passively when the host drinks contaminated water or eaten contaminated aquatic vegetation as encysted metacercaria larva on leaves [14-15].

Larval stages of *Fasciola* can be killed in host snail by *in vivo* phytotherapy technique which can check the transmission of the fascioliasis infections in the primary host. Many synthetic larvicides are uses for the control of trematodes larva, but due to the development of resistance and adverse effect on the ecosystem and other non-target organisms, in phytotherapy study of plant-derived active components in *in vivo* are uses to break the life cycle of *F. gigantica* [16-17]. Phytochemicals are easily available, biodegradable, and eco-friendly. The medicinal plant *Glycyrrhiza glabra* commonly known as Mulethi is worldwide [18] and native herbs of the Southwest and Central regions of Asia and the Mediterranean basin [19-20]. *Glycyrrhiza glabra is also* known as "Liquorice" and "Sweetwood" [21]. This medicinal herb is found at high or low altitudes, up to 1200 meters above sea level [22]. The ethanolic extract of *Glycyrrhiza glabra* is responsible for powerful antioxidant activity through significant free radical scavenging, hydrogen-donating, metal ion chelating, anti-lipid peroxidative reducing abilities [23], antimicrobial, antiulcer,

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expectorant, and anxiolytic activities [24-25]. Traditionally, this plant is used as anti-protozoan, antimicrobial, anti-inflammatory, anti-ulcer, laxative, antibacterial, anti-tumor, anti-Hepato-protective, and malarial [26-27]. The present study aims to evaluate *in vivo* phytotherapy of host snails by the use of different formulations of the *G. glabra* for killing different larval stages of *F. gigantica*.

MATERIALS AND METHODS

Collection of snails

Adult snails *Lymnaea acuminata* (2.7±0.21 cm in length) were collected locally from the submerged field and different ponds of Muhammadabad Gohna, Mau, UP, (India). Snails were allowed to acclimatize for 48 hours in tap water (pH 7.3-7.5) at room temperature (26±0.10°C) before the experiment.

Identification of infected snails

Infected adult snails *L. acuminata* often shown more resources to growth than uninfected snails, which can grow larger than uninfected [28-30], slack locomotion than uninfected ones, appeared yellowish colour, feet are more swollen and shedding cercaria have appeared at the mouth of snails and shell morphology is changed in infected snails [31-33].

Preparation of root powder and extract of plant

The freshly dried root of *G. glabra* was purchased from the local market of Muhammadabad Gohna Mau, U.P. (India) and was authenticated by Dr. A. K. Singh, Department of Botany, S.G.N. Govt. P.G. College Muhammadabad Gohna Mau, U.P. (India). Dried roots were washed with tap water and dried under shade after complete drying, the root materials were cut into small pieces and then grind in an electric grinder and obtained crude powder, were then sieved with the help of fine mesh cloth. Finally, the powder was stored sterilized container at laboratory conditions in the incubator.

Ten-gram root powder of *G. glabra* was extracted with 200 ml of organic solvents (98.5% ether, 98.6% chloroform, 97.5% methanol, 98.2% acetone, and 95.1% ethanol) at room temperature for 24 hours. Each preparation was filtered through sterilized filter paper (Whatman No. 1) separately and the filtered extracts were subsequently evaporated under vacuum. The solid residues, thus obtained, were used for the determination of *in vivo* larvicidal activity. The root powder of *G. glabra* yielded 325 mg ether, 227 mg chloroform, 350 mg methanol, 365 mg acetone, and 240 mg ethanol extracts.

Preparation of column purified

Five-liter ethanol extract fraction of dried root powder of *G. glabra* was subjected to chromatography through a 5×45 cm column, silica gel (60-120 mesh). Two-milliliter fractions eluted with ethanol (95.1%) were collected. Ethanol was evaporated under a vacuum machine and the remaining solids column extract obtained was used for the determination of larvicidal activity of each collected fractions.

In vivo experimental essay

In *in vivo* toxicity experiment of root powder of *G. glabra* was formulated in organic extract (ether, chloroform, methanol, acetone, ethanol, and column purified) were determine at different concentrations against *F. gigantica* larva in infected snail *L. acuminata*. Ten infected snails which kept in one liter of dechlorinated tap water in an aquarium with six replicates for treatment and some experimental setup was parallel was repeated without treatment for the control group. After 2h, 4h, 6h, and 8h of the treatment infected snails were dissected, then live and dead sporocyst, redia, and cercaria larva were counted with help of a binocular light microscope. The death of larvae was stabilized by the observation of movement and no response within few minutes. It was continuously monitored for up to 48h in all the treated and control groups to ensure the death of the larva. No mortality was observed in the control group. Mortality of larvae at each concentration for 2h, 4h, 6h, and 8h was used for the determination of LC_{50} values. Lethal value (LC_{50}), lower and upper confidence limit (LCL and UCL), t-ratio, Slop-values, g value, and heterogeneity factor were calculated with the help of POLO computer programmed [34].

RESULTS AND DISCUSSION

In vivo toxicity of dried root powder, organic extract (ether, chloroform, methanol, acetone, and ethanol) and column purified of *G. glabra were* exposed against sporocyst, redia, and cercaria larva of *F. gigantica* were time and concentration-dependent (Table 1-3). The exposure of root powder of *G. glabra* was more effective against cercaria after 2h, 4h, 6h, and 8h the LC_{50} values were 77.53, 73.47, 70.25, and 68.05 mg/l, respectively (Table 3). The exposure of ethanolic extract against sporocyst, redia, and cercaria after 2h the LC_{50} was 62.49, 61.19, and 67.22 mg/l, respectively (Table 1-3). Whereas, 8h LC_{50} of ethanol extract against sporocyst, redia, and cercaria the LC_{50} values were 54.69, 52.69, and 58.44 mg/l, respectively. Likewise, the column purified fractions of dried root powder of *G. glabra* against sporocyst, redia, and cercaria in 2h exposure the LC_{50} values was 58.90, 59.27, and 64.91 mg/l and 8h exposure LC_{50} was 52.32, 50.05, and 54.20 mg/l, respectively (Table 1-3). The column purified fractions were more effective against sporocyst, redia, and cercaria. Maximum effects of column purified of *G. glabra* were observed against redia larva (8h LC_{50} 50.05 mg/l) (Table-2). Likewise, the toxicity of ethanolic extract, column purified fraction of dried root powder of *G. glabra* were observed redia>sporocyst>cercaria *in vivo* exposure.

In vivo anthelmintic larvicidal activity of dried root powder *Glycyrrhiza glabra* and their different organic extracts, column purified fractions were observed against *F. gigantica* larva (sporocyst, redia, and cercaria) at different concentration with different exposure period, in all exposure more effect was observed against redia larva. [35] has been investigated that ethanolic extract of root powder of *G. glabra* has saponins, tannin, alkaloid, flavonoids, phenolic compounds, and glycosides, and its shows antimicrobial activity against *Staphylococcus aurens*, *Candida albicans*, *Bacillus subtilis* [36-37], while methanolic extract of *G. glabra* has antibacterial property against *Agrobacterium tumefaciens*, *Bacillus cereus*, *Bacillus subtilus* and *Pseudomonas syringae* [38]. The methanolic extract of *G. glabra* in *in vivo* inhibits tyrosinase enzyme, with 21.2 µg/ml inducing 50% inhibition [39]. The primary phytochemical constituents of the *G. glabra* are glycyrrhizin, liquiritin, glabridin, triterpene, glycyrrhetic acid, saponins, isoliquiritin, and flavonoids [40-41]. *In vivo* studies of *G. glabra* revealed antiviral activity against HIV-1, SARS-related Coronavirus, respiratory syncytial virus, vaccinia virus, arboviruses, and vesicular stomatitis virus [42]. The chemical constituents of *G. glabra* are uses as antibacterial, anti-viral, antifungal, anti-

protozoan, anti-inflammatory, antiulcer, anti-allergic, anti-cariogenic, antioxidant, immune-stimulatory, anti-tumor [43], immunomodulatory, hepatoprotective, neuroprotective, memory enhancement, anti-diabetic, anti-asthmatic, haematinic, Cerebro-protective, anti-tussive, hair growth-promoting [42]. The exposure of different preparation of *Glycyrrhiza glabra* in *in vivo* against sporocyst, redia, and cercaria larva of *Fasciola gigantica* is more effective. In *in vivo larvicidal* activity of *Glycyrrhiza glabra* against *Fasciola* larva may be due to the presence of different bioactive chemical components that are easily diffused in the host snail body fluid and gradually diffuses in *Fasciola* larva and progressively bioaccumulation in sporocyst, redia, and cercaria along with exposure period and cause mortality. Whereas, ethanolic extract of *Glycyrrhiza glabra* in *in vivo* against *Fasciola gigantica* larva was observed high, which indicates that the phytochemicals of this plant easily dissolved in ethanolic solvents.

Table 1 *In vivo* larvicidal activity of dried root powder of *G. glabra* and their different organic extract, column purified fractions against sporocyst larva of *F. gigantica*

Exposure periods	Values	Larvicides (mg/l)						
		Glycyrrhiza glabra dried root powder	Ether extract	Chloroform extract	Methanol extract	Acetone extract	Ethanol extract	Column purified
2h	LC_{50}	83.10	78.06	76.22	80.94	74.02	62.49	58.90
	LCL	71.92	69.22	65.04	71.19	66.37	54.50	49.22
	UCL	95.64	89.76	83.48	89.32	81.29	70.92	65.47
	Slop-value	0.84±0.36	0.79±0.49	0.34±0.44	0.24±0.55	0.78±0.40	0.21±0.07	0.18±0.20
	t-ratio	3.19	2.97	2.64	2.87	2.91	2.56	3.14
	g-value	0.32	0.46	0.28	0.35	0.30	0.42	0.28
	Heterogeneity	0.14	0.19	0.17	0.20	0.15	0.18	0.14
4h	LC_{50}	80.75	74.18	72.67	77.58	70.34	59.99	56.72
	LCL	73.39	67.56	63.70	69.00	62.15	50.25	49.78
	UCL	87.61	82.40	80.33	86.91	82.35	71.54	65.99
	Slope-value	0.37±0.14	0.56±0.39	0.97±0.68	0.35±0.18	0.14±0.05	0.39±0.31	0.56±0.22
	t-ratio	3.39	2.78	3.05	2.99	2.72	2.22	2.29
	g-value	0.20	0.31	0.42	0.36	0.39	0.22	0.49
	Heterogeneity	0.21	0.13	0.16	0.14	0.17	0.21	0.19
6h	LC_{50}	78.34	73.18	70.84	74.22	68.29	57.02	54.49
	LCL	69.02	65.79	62.18	66.00	59.10	49.85	45.29
	UCL	86.23	80.05	78.39	82.21	76.55	65.12	62.34
	Slope-value	0.22±0.10	0.51±0.64	0.69±0.29	0.76±0.55	0.38±0.19	0.89±0.54	0.14±0.27
	t-ratio	2.45	2.63	2.79	3.15	3.20	3.07	3.01
	g-value	0.27	0.40	0.38	0.25	0.37	0.46	0.63
	Heterogeneity	0.11	0.17	0.34	0.22	0.15	0.19	0.20
8h	LC_{50}	75.66	71.20	68.57	72.10	65.91	54.69	52.32
	LCL	67.05	63.44	59.30	68.81	59.01	48.37	49.05
	UCL	82.69	80.56	74.49	80.19	72.31	61.05	60.92
	Slope-value	0.25±0.10	0.39±0.21	0.22±0.19	0.30±0.06	0.74±0.24	0.68±0.30	0.70±0.67
	t-ratio	2.97	2.76	2.54	3.16	2.19	3.33	2.79
	g-value	0.54	0.24	0.39	0.28	0.45	0.52	0.44
	Heterogeneity	0.14	0.26	0.12	0.19	0.13	0.16	0.15

In six batches 10 infected snails were kept in different concentrations of the above preparations. In *in vivo* mortality of sporocyst was recorded every 2h up to 8h. The concentration given is the final concentration (W/V) in the aquarium water
LCL- Lower Confidence Limits, UCL- Upper Confidence Limits

Table 2 *In vivo* larvicidal activity of dried root powder of *G. glabra* and their different organic extract, column purified fractions against redia larva of *F. gigantica*

Exposure periods	Values	Larvicides (mg/l)						
		Glycyrrhiza glabra dried root powder	Ether extract	Chloroform extract	Methanol extract	Acetone extract	Ethanol extract	Column purified
2h	LC_{50}	81.32	74.67	71.18	76.63	70.55	61.19	59.27
	LCL	73.49	65.51	63.82	69.42	62.71	52.99	46.00
	UCL	92.64	81.92	78.43	89.14	79.53	69.46	64.72
	Slop-value	0.85±0.62	0.72±0.22	0.44±0.31	0.59±0.27	0.63±0.41	0.69±0.12	0.91±0.62
	t-ratio	2.31	2.99	3.14	2.76	2.42	2.55	2.40
	g-value	0.43	0.24	0.28	0.37	0.42	0.26	0.44
	Heterogeneity	0.18	0.12	0.15	0.19	0.20	0.14	0.14

4h	LC_{50}	78.93	72.01	69.14	73.49	67.58	57.92	55.20
	LCL	69.22	64.31	60.50	66.23	59.48	49.06	48.77
	UCL	85.37	79.29	74.12	81.92	76.19	62.32	62.95
	Slope-value	0.30±0.11	0.55±0.29	0.67±0.49	0.29±0.07	0.43±0.22	0.68±0.45	0.82±0.37
	t-ratio	2.66	2.71	2.83	2.91	3.19	2.92	3.24
	g-value	0.48	0.32	0.56	0.29	0.35	0.42	0.31
	Heterogeneity	0.15	0.27	0.20	0.11	0.18	0.13	0.17
6h	LC_{50}	75.14	69.99	67.32	71.21	64.29	55.32	53.76
	LCL	68.49	61.99	59.18	64.22	55.07	41.87	40.08
	UCL	83.76	77.02	75.29	79.38	71.42	59.01	61.19
	Slope-value	0.75±0.59	0.30±0.12	0.57±0.36	0.72±0.43	0.91±0.62	0.70±0.36	0.54±0.19
	t-ratio	2.64	2.86	2.32	2.45	2.50	2.23	2.40
	g-value	0.40	0.63	0.29	0.38	0.30	0.27	0.18
	Heterogeneity	0.18	0.12	0.33	0.20	0.16	0.14	0.30
8h	LC_{50}	73.56	67.38	64.22	69.00	62.47	52.69	50.05
	LCL	65.91	58.78	58.37	60.13	57.81	40.28	37.21
	UCL	80.05	75.14	72.81	76.55	69.17	59.30	58.79
	Slope-value	0.86±0.23	0.66±0.49	0.39±0.20	0.79±0.09	0.36±0.55	0.44±0.62	0.29±0.05
	t-ratio	2.69	2.94	3.15	3.42	2.83	2.95	2.48
	g-value	0.76	0.52	0.40	0.33	0.46	0.26	0.41
	Heterogeneity	0.15	0.22	0.16	0.18	0.21	0.14	0.19

In six batches 10 infected snails were kept in different concentrations of the above preparations. In *in vivo* mortality of sporocyst was recorded every 2h up to 8h. The concentration given is the final concentration (W/V) in the aquarium water
LCL- Lower Confidence Limits, UCL- Upper Confidence Limits

Table 3 *In vivo* larvicidal activity of dried root powder of *G. glabra* and their different organic extract, column purified fractions against cercaria larva of *F. gigantica*

Exposure periods	Values	Larvicides (mg/l)						
		Glycyrrhiza glabra dried root powder	Ether extract	Chloroform extract	Methanol extract	Acetone extract	Ethanol extract	Column purified
2h	LC_{50}	77.53	79.14	76.37	78.59	75.44	67.22	64.91
	LCL	64.89	68.28	68.11	70.43	68.59	52.73	51.35
	UCL	83.22	84.74	80.39	87.21	82.49	73.94	70.50
	Slop-value	0.18±0.37	0.22±0.42	0.49±0.31	0.76±0.42	0.91±0.59	0.37±0.14	0.24±0.55
	t-ratio	2.29	2.74	2.59	2.91	3.14	2.22	3.41
	g-value	0.56	0.36	0.47	0.63	0.32	0.29	0.44
	Heterogeneity	0.13	0.17	0.20	0.22	0.17	0.16	0.14
4h	LC_{50}	73.47	75.39	73.08	74.57	71.94	63.67	60.76
	LCL	66.21	64.30	60.46	69.39	64.92	54.32	48.57
	UCL	85.39	86.02	81.90	86.78	79.56	70.99	68.09
	Slope-value	0.76±0.33	0.45±0.19	0.66±0.37	0.84±0.49	0.78±0.30	0.38±0.15	0.24±0.47
	t-ratio	3.07	3.29	2.83	2.67	2.51	3.12	2.89
	g-value	0.62	0.55	0.38	0.32	0.21	0.29	0.47
	Heterogeneity	0.19	0.13	0.11	0.18	0.15	0.13	0.15
6h	LC_{50}	70.25	72.23	70.94	72.09	68.37	61.84	57.92
	LCL	61.40	63.79	64.29	64.33	60.54	50.07	49.11
	UCL	79.06	81.91	79.14	80.65	75.39	68.49	63.79
	Slope-value	0.55±0.63	0.12±0.59	0.29±0.47	0.80±0.63	0.57±0.41	0.32±0.54	0.29±0.73
	t-ratio	3.37	3.09	2.084	2.67	2.44	2.79	2.37
	g-value	0.57	0.28	0.48	0.27	0.39	0.51	0.35
	Heterogeneity	0.19	0.16	0.12	0.18	0.20	0.17	0.36
8h	LC_{50}	68.05	70.37	68.28	70.83	66.08	58.44	54.20
	LCL	59.93	65.21	6.73	63.50	59.42	46.89	41.33
	UCL	75.46	79.99	79.06	78.91	74.33	65.76	60.92
	Slope-value	0.44±0.19	0.15±0.68	0.32±0.15	0.13±0.77	0.58±0.19	0.79±0.22	0.15±0.47
	t-ratio	2.85	2.93	2.14	3.29	3.14	2.90	2.54
	g-value	0.50	0.30	0.48	0.33	0.51	0.22	0.30
	Heterogeneity	0.14	0.20	0.16	0.21	0.19	0.16	0.18

In six batches 10 infected snails were kept in different concentrations of the above preparations. In *in vivo* mortality of sporocyst was recorded every 2h up to 8h. The concentration given is the final concentration (W/V) in the aquarium water
LCL- Lower Confidence Limits, UCL- Upper Confidence Limits

In result sections (Table 1-3) the value of heterogeneity is less than 1.0 which denote that replicate tests of the random sample and concentration-response lines would fall within the 95% confidence limits and thus the model fits adequately. Whereas, the steep slope values of this study indicate that a small increase in concentration of various *iv vivo* treatments caused mortality of sporocyst, redia, and cercaria larva (Table 1-3). The index of the significance of the potency estimating values indicates that the value of the mean is within the limit at all probability levels (90, 95, and 92 limits) since it is less than 0.5.

CONCLUSION

Anthelmintic larvicidal studies *in vivo* medicinal plant *Glycyrrhiza glabra* in different formulations (dried root powder, organic extracts of ether, chloroform, methanol, acetone, ethanol, and column purified fractions) against *F. gigantica* larva (sporocyst, redia, and cercaria) was effective in the different exposure period. All preparations and their treatment of *G. glabra* were analyzed that the phytochemicals of this plant are time and concentration dependents. Whereas, ethanolic extract of dried root powder of *G. glabra* shows higher anthelmintic larvicidal activity among all extract. It may be possible that phytochemicals of *G. glabra* are easily dissolved in the ethanol which might be responsible for the larval mortality. *In vivo exposure* of the *G. glabra* against sporocyst, redia and cercaria were revealed towards the phytochemicals are, easily enter in the larval body through body fluid of the host snail and cause toxic effects. The phytochemicals of *G. glabra* are may be useful in the control of fascioliasis after formulations and at suitable concentrations. Therefore, this study revealed more identification of the phytochemicals and their mode of action that how it's are effective at the molecular level in the *Fasciola* larval.

Conflict of Interest: The authors have no conflict of interest.

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