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Research Article

Assessment of median lethal dose and anti-mutagenic effects of *Glycyrrhiza glabra* root extract against chemically induced micronucleus formation in *Swiss albino* mice

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ABSTRACT

Background: To assess the median lethal dose and evaluate the anti-chemotherapeutic effects of hydro-methanolic root extract of *Glycyrrhiza glabra* on the cyclophosphamide (CP) induced mutagenicity in bone marrow cells of *Swiss albino* mice.
Methods: For the assessments of $LD_{50,}$ hydro-methanolic root extract of *Glycyrrhiza glabra* were intra-peritoneally administered at doses of 200, 400, 600, 800, 1000, and 1200 mg/kg body weight. For the mutagenicity study, bone marrow micronucleus test was used and the single i.p. of *Glycyrrhiza glabra* extract given at the dose of 300, 450, and 600 mg/kg body weight, 24 hrs prior the administration of CP (at the dose of 50 mg/kg body weight).
Results: The present investigations revealed that, the median lethal dose/LD_{50} was observed at the dose of 833.3 mg/kg body weight. The results suggest that, the doses of 450 and 600 mg/kg body weight expressed significant preventive potential against CP induced Micronucleus formation in student 't' test at dose dependent manner in the bone marrow cells of *Swiss albino* mice. *Glycyrrhiza glabra* root extract alone has not induced micronucleus formation.
Conclusion: Based on this study, it may be concluded that *Glycyrrhiza glabra* root extract possess anti-mutagenic behavior and this hydro-methanolic crude extract may be safe as per the LD_{50} was observed.

Keywords: Bone marrow, Cyclophosphamide, *Glycyrrhiza glabra,* Micronucleus assay, Mutagenicity, Toxicity

INTRODUCTION

Plants have been used for medicinal purposes since before recorded history. Since the origin of human's life, plants continue to play a curative and therapeutic role in preserving human health against disease. An LD_{50} is a standard measurement of acute toxicity that is stated in milligrams (mg) of extract material per kilogram (kg) of body weight. A process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation is known as mutagenesis or mutagenicity. A micronucleus is the erratic (third) nucleus that is formed during the anaphase of mitosis or meiosis and also referred to Howell-Jolly bodies. Micronuclei are characterized as newly divided daughter cell, which can contain a whole chromosome or part of a chromatid in the cells. This includes damage caused by radiation, harmful chemicals, and random mutations that occur throughout the genome.

Glycyrrhiza glabra (*G. glabra* L.; Family: *Fabaceae*) is a traditional medicinal herb, which grows in various parts of the world. This herb is known as 'mulaithi' in northern India and roots are called as Licorice commonly. The yellow color of licorice is due to the flavonoid content of the plant, which includes liquiritin, isoliquiritin (a chalcone) , and other compounds.[1] The roots and rhizomes of *Glycyrrhiza glabra* have been widely used in medicines for its unique and diverse pharmacological properties viz., antiviral, anticancer, anti-ulcer, anti-diabetic, anti-inflammatory, immunostimulant, anti-allergenic, etc.[2,3] In addition, the roots are also used as a flavoring and sweetening agents with tobacco chewing, chewing gums,

candies, toothpaste, and beverages.[4] Phytochemical investigations have demonstrated that the major bioactive components of *Glycyrrhiza glabra* roots are flavonoids and pentacyclic triterpene saponin, Alkaloids, Glycosides, etc. The hydromethanolic root extract possess anti-bacterial and antioxidant potential because the presence of these secondary metabolites or phytoconstituents.[5] Liquorice is effective in the reducing of pain and inflammation of stomatitis mouth ulcers by the reduction in ulcer size and speed healing.[6]

METHODS

Plant collection and identification

The root of *Glycyrrhiza glabra* were procured from Bhopal (Madhya Pradesh), India and authenticated by Botanist, Dr. Zia Ul Hasan (Voucher Specimen No: 441/BOT/Safia/13) Prof. & Head, Dept. of Botany, Safia Science College, Bhopal, Madhya Pradesh (India).

Chemicals

Cyclophosphamide (CP) was purchased from Sigma chemical Co., U.S.A. HBSS, albumin, EDTA, saline, methanol, May-Grunwald, Giemsa, and other chemicals were reagents grade.

Preparation of Glycyrrhiza glabra root extract

The collected root were dried in the shade and grinded with mechanical grinder. About 30 g powder was filled in a separating funnel with 50% methanol for 48 hrs. The collected residues kept at 55-60°C in boiling water bath to concentrate it and finally transfer into the hot air oven to dry it. About 5.8 g powder of crude extract was obtained (yield = 19%) and used for the further studies.

Experimental animals

Random bred of male *Swiss albino* mice (7-8 weeks old), weighing 23±2 g body weight obtained from the animal colony of our Research Center were used for the experiments. Experimental animals were handled according to the Institutional Legislation, regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. These animals were housed in polypropylene cages in the animal house at temperatures of 22±1.5°C and 12 hrs light and dark cycle. The animals were provided with standard pallet diet (from Golden feed Ltd., New Delhi, India) and water *ad libitum*.

Experimental design

Acute toxicity study (median lethal dose/LD_{50})

Acute intra-peritoneal toxicity test was performed as per OECD-423 guidelines. All the animals were randomly distributed into one control and six experimental groups, containing six animals per group. All groups were intraperitoneally administered 200, 400, 600, 800, 1000 and 1200 mg/kg body weight hydro-methanolic extract of *Glycyrrhiza glabra* and the control group received vehicle alone (double distilled water [DDW]) by oral route using intragastric syringe following the method of Lorke.[7] The animals were observed continuously for first 72 hrs, 7 days and after those 14 days for any signs of behavioral changes, toxicity, mortality, and body weight.

The median lethal dose (LD_{50}) calculated as, the dose at which 50% mortality was observed during 2 weeks. The animals were observed and the studies were terminated after 2 weeks. The LD_{50} value was calculated with "Arithmetic method of Karber."[8,9]

The sum of the product was divided by the number of animals in a group and the resulting quotient was subtracted from the least lethal dose in order to obtain LD_{50} value.

LD_{50} = the apparent least dose lethal to all in a group − $(a.b)/N$

Where N = number of animals in each group.

a = dose difference.

b = mean mortality.

Anti-mutagenic activity (micronucleus assay)

Micronucleus assay test was performed as per the method reported by Schmid[10] and modified by Aron *et al.*[11] and standardized by Agrawal *et al.*, 1998.[12] The animals were randomly divided into six groups and each group having four mice. The required dose was dissolved in an appropriate solvent (DDW) and administered intraperitoneally before 24 hrs of CP treatment. Positive control group was received only CP injection. The animals were sacrificed after 24 hrs of CP administration and slides of Bone marrow were prepared. After staining with May-Grunewald and Giemsa, total of 1000 cells were scored at magnification of ×1000 (100 × 10) for each group.

Experimental groups

Group I (vehicle alone): Double distilled water (DDW).

Group II (positive control): Single i.p. of CP (50 mg/kg body weight) administered.

Group III (*Glycyrrhiza glabra* extract alone): Single i.p. of 300 mg/kg body weight of *Glycyrrhiza glabra* hydromethanolic root extract.

Group IV (*Glycyrrhiza glabra* extract + CP): 300 mg/kg body weight of *Glycyrrhiza glabra* hydromethanolic root extract before 24 hrs of CP 50 mg/kg body weight.

Group V (*Glycyrrhiza glabra* extract + CP): 450 mg/kg body weight of *Glycyrrhiza glabra* hydromethanolic root extract before 24 hrs of CP 50 mg/kg body weight.

Group VI (*Glycyrrhiza glabra* extract + CP): 600 mg/kg body weight of *Glycyrrhiza glabra* hydromethanolic root extract before 24 hrs of CP 50 mg/kg body weight.

Statistical analysis

The experimental results were expressed as mean ± standard error mean. Data were assessed followed by student 't' test. $p<0.05$ was considered as statistically significant.

RESULTS

Toxicological study

The results of the toxicity study revealed that different groups treated with hydromethanolic *Glycyrrhiza glabra* root extract showed no specific signs and mortality was compared to the control group i.e. Vehicle alone (DDW). The all mice moved well and fed normally and no significant change was found on the body weight of mice up to the dose of 600 mg/kg body wt when compared with the untreated control group. The behavior of mice has changed at the dose of 800 mg/kg body wt and loss of body weight of mice was observed. Some mice showed signs and symptoms such as; general weakness, loss of appetite, restlessness, and sleepy behavior after the increasing of dose as dose depending manner. All mice were died at the dose of 1200 mg/kg body weight. As per the calculation by "Arithmetic method of Karber," the LD_{50} was observed at the dose of 833.3 mg/kg body weight. Results are summarized in Table 1a & 1b.

Anti-mutagenic activity

The results revealed that, when the CP given at a single dose i.e. 50 mg/kg body weight cause a high incidence of micronucleus formation in bone marrow cells of *Swiss albino* mice (positive control group i.e. Group I) and caused bone marrow toxicity as evidenced by a decrease in the polychromatic erythrocyte/normochromatic erythrocyte ratio. The frequency of micronucleated polychromatic erythrocyte (MNPCEs) was 4.33±0.61 for the CP (50 mg/kg body weight) treated group which was significantly higher in student 't' test at $p<0.05$. The frequency of MNPCEs in the Group III, i.e. *Glycyrrhiza glabra* extract alone, at the concentration of 300 mg/kg body weight was found 0.34±0.21, when compared with the untreated control group, it was revealed that the extract was not genotoxic to the bone marrow cells of mice. The mice which received single application of *Glycyrrhiza glabra* root extract at doses of 300, 450, 600 mg/kg body weight 24 hrs before the single administration of CP at the dose of 50 mg/kg body weight exhibited significant reduction in the micronucleus formation when compared to the positive control group. Group V (*Glycyrrhiza glabra* 450 mg/kg + CP 50 mg/kg body weight) & Group VI (*Glycyrrhiza glabra* 600mg/kg + CP 50mg/kg

Table 1a: The effect of *Glycyrrhiza glabra* hydromethanolic root extract on mortality and body weight of *Swiss albino* mice over a period of 2 weeks.

Sr. No.	No. of mice	Treatment doses (mg/kg body weight)	Rout of administration	Percentage of mice died (%)	Observation of body weight in days mean±SEM		
					0 day	7 days	14 days
1.	06	Vehicle alone/positive control (DDW)	PO	-	27.20±0.20	27.90±0.20	28.50±1.20
2.	06	*Glycyrrhiza glabra* root extract (200 mg/kg body weight)	IP	-	22.20±0.34	23.50±0.50	24.50±0.22
3.	06	*Glycyrrhiza glabra* root extract (400 mg/kg body weight)	IP	-	24.33±0.42	25.20±0.32	26.30±0.80
4.	06	*Glycyrrhiza glabra* root extract (600 mg/kg body weight)	IP	-	24.70±0.21	25.60±0.68	26.8±1.2
5.	06	*Glycyrrhiza glabra* root extract (800 mg/kg body weight)	IP	50	27.18±0.34	20.74±1.2	17.4±0.3
6.	06	*Glycyrrhiza glabra* root extract (1000 mg/kg body weight)	IP	83.33	29.50±0.45	25.34	24.86
7.	06	*Glycyrrhiza glabra* root extract (1200 mg/kg body weight)	IP	100	29.15±1.22	Death	Death

Data (body weight) presented as the mean and standard error mean among mice (n=6). IP: Intra-peritoneal, PO: *Per os* (oral)

body weight) expressed significant in the student 't' test at $p<0.05$. The results suggest that, the percentage of protection in the frequency of CP-induced MN formation was significantly increased in a dose dependent manner. Therefore, it appears that *Glycyrrhiza glabra* extract showed anti-mutagenic potential. Results are summarized in Table 2. Figure 1 showing MNPCE and graphical representation of anti-mutagenic effects in Figure 2, Figure 3, Figure 4.

DISCUSSION

Acute toxicity test gives clues on the range of doses that could be toxic to the animal; it could also be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics.[13] The supplementation of *Glycyrrhiza glabra* extract elevated blood pressure. This is thought to be due to the effect of extract on the rennin-angiotensin-aldosterone system. Phytochemicals are thought to have positive or negative effects on animals. In addition to hypertension, extract showed hypokalemia (potassium loss) and sodium retention, resulting in edema.[14] Tannins and Flavonoids are thought to have both pro-oxidant and antioxidant effects on the body. While the antioxidant protects the tissues and organs, the pro-oxidant damages the tissues and organs. The weight changes of the animals during the period of observation which was more visible

Table 1b: Arithmetic calculation of LD_{50} by Karber method.

Sr. No.	No. of mice in groups	Treatment doses (mg/kg body weight)	Dose difference	No. of death	Mean death	Dose difference X mean death
1.	06	*Glycyrrhiza glabra* root extract (200 mg/kg body weight)		-		
			200		-	-
2.	06	*Glycyrrhiza glabra* root extract (400 mg/kg body weight)		-		
			200		-	-
3.	06	*Glycyrrhiza glabra* root extract (600 mg/kg body weight)		-		
			200		1.5	300
4.	06	*Glycyrrhiza glabra* root extract (800 mg/kg body weight)		3		
			200		4	800
5.	06	*Glycyrrhiza glabra* root extract (1000 mg/kg body weight)		5		
			200		5.5	1100
6.	06	*Glycyrrhiza glabra* root extract (1200 mg/kg body weight)		6		

LD_{50}: Lowest dose that killed 100% − Σ dose difference×mean death/N, LD_{50}: 833.3 mg/kg body weight

Table 2: The effects of *Glycyrrhiza glabra* hydro-methanolic root extract against micronucleus formation by cyclophosphamide in bone marrow cells of *Swiss albino* mice.

Sr. No.	Groups	Treatment doses (mg/kg body weight)	MNPCE±SEM	PCE/NCE ratio±SEM	Protection (%)
1.	I (n=4)	Cyclophosphamide alone (50 mg/kg body weight)	4.33±0.61	1.13±0.18	-
2.	II (n=4)	*Glycyrrhiza glabra* (300 mg/kg body weight) + CP (50 mg/kg body weight)	2.34±0.49	0.99±0.02	45.95
3.	III (n=4)	*Glycyrrhiza glabra* (450 mg/kg body weight) + CP (50 mg/kg body weight)	1.5±0.93*	1.06±0.05	65.35
4.	IV (n=4)	*Glycyrrhiza glabra* (600 mg/kg body weight) + CP (50 mg/kg body weight)	0.67±0.34*	1.02±0.03	84.52
5.	V (n=4)	*Glycyrrhiza glabra* alone (300 mg/kg body weight)	0.34±0.21	1.02±0.02	-
6.	VI (n=4)	Vehicle alone (DDW)	0.12±0.08	0.43±0.08	-

*Statistical significance as compared to cyclophosphamide at $P<0.05$ followed in student 't' test. PCE: Polychromatic erythrocyte, NCE: Normochromatic erythrocyte, MNPCE: Micronucleated polychromatic erythrocyte, SEM: Standard error mean

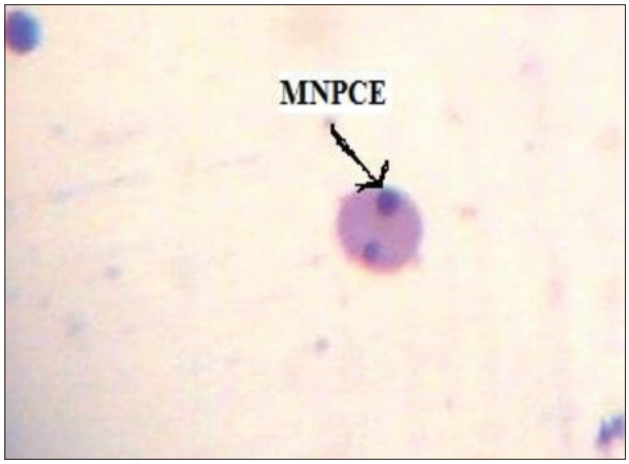

Figure 1: Micronuleated polychromatic erythrocyte.

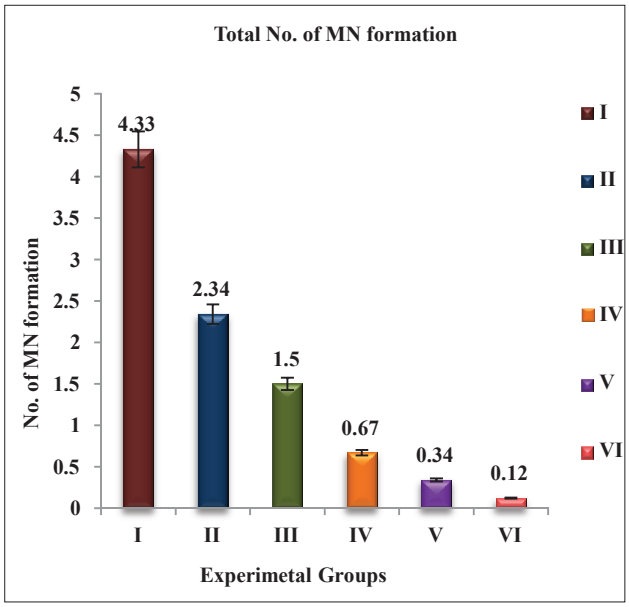

Figure 2: Effect of *G. glabra* root extract on MN formation.

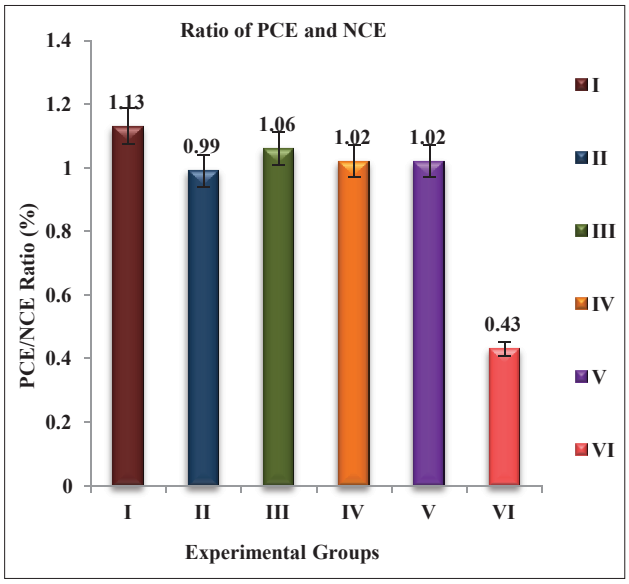

Figure 3: Effect of *G. glabra* root extract on PCE/NCE Ratio.

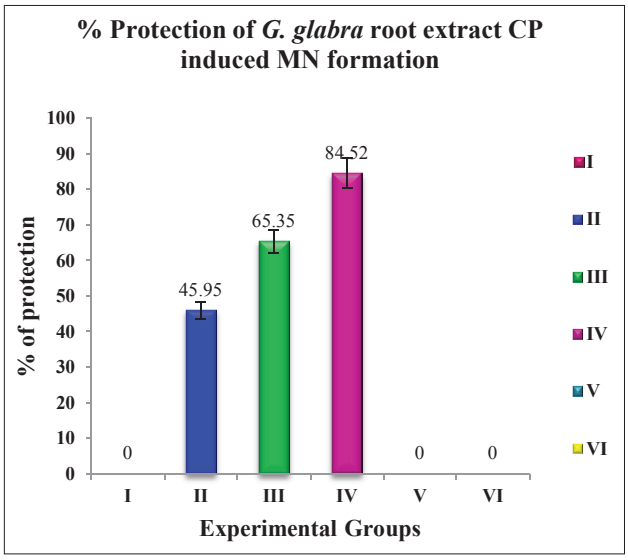

Figure 4: Effect of *G. glabra* root extract on % of protection against CP induced MN formation.

at higher doses, suggest the presence of tannins which are thought to interfere with the absorption of nutrients making them unavailable and thereby reducing feed intake.[15]

Traditionally, the plant has been recommended as a prophylaxis for gastric and duodenal ulcers and dyspepsia as an anti-inflammatory agent during allergenic reactions.[16] The aqueous extract, or *Glycyrrhiza glabra* inhibits *in vivo* and *in vitro* proliferation of *Ehrlich ascites* tumor cells and inhibits angiogenesis in *in vivo* assay, peritoneal and chorioallantoic membrane assay.[17]

CP is belongs a class of drugs known as alkylating agent, which have been used to treat some kind of cancer. After studying the mode of action of CP, it demonstrated that, this induces inhibition of deoxyribonucleic acid (DNA) replication and leading cell death. CP exerts its cytotoxic effects on both resting and dividing lymphocytes. In that case, it is working as immunosuppressant. It is a potent anticancer drug, but it also affects normal cell division by decreasing the immune responses. The MN formation with CP administration showed that it has mutagenic behavior.

In present investigation, the root extract of *Glycyrrhiza glabra* exhibited anti-chemotherapeutic potential by decreasing the MN formation at the given doses. It may be due to the reducing immunosuppressant effects of CP and increasing the immune responses in the experimental animals. The presence of Glycyrrhizin, which is a triterpenoid saponin glycoside, stimulates medicinal properties of *Glycyrhiza glabra* Linn. From the above findings, it seems that, the hydromethanolic crude extract of *Glycyrrhiza glabra* possesses the anti-mutagenic

potential. It may be because of the Glycyrrhizin and presence of many other phytoconstituents such as; tannins flavonoids, alkaloids, etc. The anti-mutagenic activity of the extract was found to be dose dependent. The significant anti-mutagenic activity in the above three different doses of *Glycyrrhiza glabra* extract against direct acting mutagens suggest that this extract may directly protect DNA damage from mutagens. The underlying mechanism behind the anti-mutagenic action of *Glycyrrhiza glabra* is still unknown.

Thus, there is an immense need to study the natural *Glycyrrhiza* constituents to reduce their toxic effects for generating the advanced versions of the bioactive compounds to be used as anti-mutagenic drugs from nature in future. Glycyrrhizin, glycyrrhetinic acid, glabridin and isoliquiritigenin hold a strong promise in designing future drugs.

CONCLUSION

It is concluded on the basis of results of present studies that acute administration of hydromethanolic root extract of *Glycyrrhiza glabra* may be safe as the LD_{50} determined. The present findings thus suggest that root extract of *Glycyrrhiza glabra* may be utilized to formulate anti-mutagenic drugs and in future it may be used as an alternative drug because most of the anticancer drugs used in present days showed mutagenic responses in animal models. Further studies are however needed to isolate and characterize the active principles responsible for anti-mutagenic activity. After that, this herbal formulation may include with the chemotherapy because it may be reduce the side-effects of chemotherapeutic agents and may increase the life span of patients who are suffering from cancer.

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