

EVALUATION OF ACUTE, SUBACUTE AND SUBCHRONIC TOXICITY OF *POLYGONUM GLABRUM*

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ABSTRACT

The aim of present study was to evaluate the safety dose range of methanol extract of *Polygonum glabrum* by acute, subacute and subchronic oral toxicity studies on animal model. In acute toxicity study, the animals were administered with an oral dose (2000 mg/kg) of methanol extract of *Polygonum glabrum* daily for a period of 15 days. In subacute and subchronic toxicity studies three doses of methanol extract of *Polygonum glabrum* (250, 500, and 1000 mg/kg) was administered to animals for 28 days and 90 days orally. Behavioral changes, adverse effects and mortality were recorded during the period of investigation. Body weight, organ weight, hematological, biochemical and histopathological studies were performed and compared with control group. In acute toxicity study, the animals did not show any changes in general behavior or any signs of toxicity. Also, no mortality was recorded. Methanol extract of *Polygonum glabrum* did not cause any significant difference ($p > 0.05$) in the body weight, relative organ weight, hematological, biochemical parameters and gross abnormalities during the 28 days study. However, in the 90-day study a significant increase in white blood cell and platelets were observed. Biochemical parameters such as total protein showed significant increase while creatinine and uric acid levels showed significant decrease. The results suggest that methanol extract of *Polygonum glabrum* is not likely to produce any significant toxic effect in animal models on oral administration.

Keywords: *Polygonum glabrum*, Acute, Subacute, Subchronic toxicity, Biochemical analysis, Hematological parameters.

INTRODUCTION

Traditional healing and use of traditional medicine is an important part in Indian culture. It was practiced by ancients long before the beginning of modern medicine. The book "Sushruta Samhita" compiled in 1000 B.C. comprises a complete chapter on herbal therapeutics and contains outstanding information about the use of medicinal plants¹. Such books have greater impact on phytochemists and researchers to do a meticulous study and study oriented research in medicinal plants. There is an upsurge in studies that are published to explain the role of natural products in drug discovery and therapeutics. However, efficacy of phytoconstituents should be carefully evaluated since pharmacology is inter-related to toxicology. Hence screening of plant extracts and isolated compounds for toxicity study is highly recommended by international conference on harmonization (ICH)². Assessment of plant extracts by toxicity studies are emphasized since the no observed adverse effect level (NOAEL) could be confirmed

through toxicological evaluation³. According to toxic substances control act (TSCA), a toxic effect is defined as structural or functional change in an experimental animal due to its exposure to chemical substance. These changes can be effected through acute, sub chronic or chronic exposure studies⁴.

In India, plants of *Polygonaceae* family are known to be rich source of bioactive compounds. Among them flavonoids are the most common components found in *Polygonum spp.* and have been used as chemotaxonomic markers of the genus⁵. *Polygonum glabrum* commonly called as dense flower knotweed is a perennial plant. It is an amphibious shrub with pink flowers and glossy brown seeds found in most parts of India. Various parts of the plants such as leaves, stem, flowers and roots are used in traditional system of medicine. Decoctions prepared from the plant parts are used for the treatment of rheumatism, jaundice, piles etc⁶. The leaves are used as an antimalarial agent in Sudan⁷. Leaves of *Polygonum glabrum* are used for the treatment of dysentery in south India. A decoction of the leaves and seeds are used as cardi tonic, astringent and anthelmintic⁸. The whole plant decoction is used as a remedy for colic pain, pneumonia and the boiled paste is applied in cuts and wounds⁹. Pharmacologically, the plant has been effectively screened for antihepatotoxic, antinephrotoxic, antimicrobial, anticancer, antidepressant, analgesic and antioxidant activities^{10,11}. The plant contains many flavonoids including diosmetin, rutin, hyperin and quercitrin. The leaves are rich in essential oil which contains many compounds like 6, 6-dimethyl-1, 3-heptadien-5-ol, oleic acid, 9-octadecenamide, isodecyl octyl ester and 1,2,3-benzenetriol¹². Compounds like aliphatic, alicyclic compounds, sesquiterpenes, sterols, phenolic acids as well as other trace elements were identified and some of them were isolated from the plant¹³. In spite of the broad spectrum of literature, less information on *Polygonum glabrum* toxicity studies are currently available. The purpose of present study was to investigate acute, subacute and subchronic oral toxicities of methanol extract of *Polygonum glabrum* in rats.

MATERIALS AND METHODS

Collection and extraction of the plant

Polygonum glabrum was collected from Tirupati (Andhra Pradesh) and further identified, confirmed & authenticated by Dr. Madavchetty, professor, Botany department, Sri Venkateswara University, Tirupati. The plant was preserved in the herbarium of GITAM Institute of Pharmacy, GITAM University, for future reference (Voucher specimen No -1916). The whole plant was washed, air-dried, homogenized to fine powder and stored under ambient conditions. About 500 gm of plant powder was extracted with methanol in a Soxhlet apparatus. The extraction was done continuously till a clear solvent was observed in the thimble. The excess solvent was removed from methanol extract using a rotary vacuum evaporator

under reduced pressure. The extracts were collected and percentage yield was calculated. Dried extract was preserved in desiccator for toxicity study.

Acute toxicity study

Acute oral toxicity study was conducted as per the guidelines recommended by OECD (No 425)¹⁴. The LD₅₀ of the extract was determined by using rats of either sex. The rats had free access to water followed by overnight fasting before starting the experiment. Animals were divided into two groups, each consisting of 5 animals. The control group animals received normal saline orally. The experimental rats received a single test dose of 2000 mg/kg of methanol extract of *Polygonum glabrum* using intra gastric tube for short term (48 hr) and long term (15 days) study¹⁵. After dosing, the animals were continuously observed for first one hour and then hourly for 4 hours and finally after every 24 hours up to 15 days. Changes in physical appearance (eye and skin color), behavioural response (tremors, convulsion, sleep, salivation, diarrhoea, lethargy) and other parameters such as body weight, respiration and temperature were recorded. The animals were also observed for any mortality during the course of the study and at the end of study period.

Subacute toxicity study

The oral subacute study was conducted according to OECD guidelines¹⁶. Twenty four rats (either sex) were totally used for the study. Rats were divided into four groups of 6 animals (3 males and 3 females) in each group. Group I was treated as control and received distilled water (2ml/kg, p.o). Group II, III and IV were considered as tested groups and received the plant extract (250, 500 and 1000 mg/kg body weight respectively. p.o) for 28 consecutive days. Body weight, organ weight, hematological and biochemical parameters were analyzed at the end of study period.

Subchronic toxicity study

The subchronic toxicity study was conducted according to OECD guidelines¹⁶. A total of twenty four rats (either sex) were used for the study. Rats were divided into four groups of 6 animals (3 males and 3 females) in each group. Group I served as control and received distilled water (2ml/kg, p.o). Group II, III and IV were treated as tested groups and received the plant extract (250, 500 and 1000 mg/kg body weight respectively. p.o) for 90 consecutive days. Body weight and organ weight along with other parameters were analyzed at the end of experiment.

Analysis of hematological and biochemical parameters

At the end of 28 and 90 days of treatment period all the animals were given anesthesia (chloroform) and sacrificed. Blood samples were collected from rats through cardiac puncture into tubes with EDTA for measuring hematological parameters. Blood was also collected in centrifuge tubes without an anticoagulant for estimation of biochemical parameters.

Body weight and organ weight

Body weight of each rat was recorded weekly throughout the study period and the % weight change for each rat at the end of each study was calculated.

% Weight change = (Difference between interval body weight and initial body weight ÷ initial bodyweight) x100.

After blood collection rats were euthanized and the internal organs (heart, lungs, liver, spleen, kidney, brain, testes and ovary) were removed and weighed to calculate the relative organ weights (ROW) followed by observation for any gross lesions.

$$\text{ROW} = (\text{organ weight}) / (\text{body weight}) \times 100$$

Hematological parameters

Blood samples were collected in centrifuge tubes with EDTA and used for the evaluation of hematological parameters¹⁷. Hemoglobin (Hb) content was estimated using hemoglobinometer. Total white blood count (WBC), red blood cell (RBC) and platelet count were estimated by hemocytometer. Mean cell volume (MCV) is estimated in relation to the volume of the average red cell in a sample. The Mean cell hemoglobin (MCH) is calculated from Hb and RBC. Mean corpuscular hemoglobin concentration (MCHC) is calculated based on average Hb concentration in the RBC. The hematocrit is calculated from RBC count and MCV.

Biochemical parameters

Blood collected in tubes without anticoagulant was centrifuged at 3000 r/min for 10 min. The separated serum was analysed for various clinical parameters such as alkaline phosphatase (ALP), aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT) were estimated based on standard techniques. Sodium, potassium and chloride were estimated using an electrolyte analyzer. Creatinine, urea, uric acid and total bilirubin were analysed by known methods. Total protein and albumin were determined by standard method.

Estimation of SGOT & SGPT assay

Serum glutamate oxaloacetate transaminase (SGOT) and Serum glutamate pyruvate transaminase (SGPT) were determined by the method of Reitman and Frankel¹⁸. 0.5ml of L-alanine (200mM) and 0.5ml of L-aspartate (200mM) were taken separately and pre incubated with 2mM of α ketoglutarate for 5 min at 37°C. To this 0.1ml of serum was added and the volume was adjusted to 1.0ml with sodium phosphate buffer (pH 7.4; 0.1M). The reaction mixture was incubated for 30 and 60 min for SGPT and SGOT respectively. A 0.5ml of 2, 4-dinitrophenyl hydrazine (1mM) was added to the reaction mixture and left for 30 min at room temperature. Finally, the color was developed by the addition of 5ml NaOH (0.4 N) and the product formed was read at 505nm.

Estimation of alkaline phosphatase

Alkaline phosphatase (ALP) was assayed by the method of Kind and King¹⁹. To 1ml of substrate 1.5 ml of buffer (carbonate-bicarbonate buffer, 0.1M, pH 10.0) was added and incubated at 37°C for 15minutes. The reaction was brought down by the addition of 1.0 ml of Folin's phenol reagent. After centrifuging the content, supernatant was removed. To the supernatant, 1.0 ml of 15% sodium carbonate solution and 0.1ml of magnesium chloride (0.1M), was added and mixture was incubated for 10 minutes at 37°C. The colour was read out 640 nm against the blank.

Estimation of creatinine

The serum creatinine was estimated by standard enzymatic method by Fabiny and Ertinghausen²⁰. To test tubes, each 1 ml of picric acid and sodium hydroxide and 0.1 ml of test serum was added and mixed gently and aspirated in Trace- 30 semi auto analyser. Result obtained on display was recorded.

Estimation of urea

Serum urea was determined by standard enzymatic method as described by Tiffany et al²¹. To 0.1ml of serum sample, 1 ml of ready to use blood urea agent in test tubes was added mixed gently and kept in semi auto analyser. After completion of assay, the readings on the monitor were recorded.

Estimation of uric acid

The serum uric acid was estimated by standard enzymatic method²². To test tubes, each 1 ml urizyme buffer (Polyhalogenated benzoic acid in Tris buffer at pH 7.5 + 0.05), 0.1ml of urizyme reagent (4-aminoantipyrine, peroxidase, uricase) and 0.025 ml of serum was added and incubated for 5 min at 37°C. Absorbance was measured at 510 nm.

Estimation of total bilirubin

Estimation of total bilirubin content was done by method of Malloy and Evelyn²³. 0.2ml of serum sample and 1.8 ml of distilled water was added to test tube. 0.5 ml of diazo reagent and 0.5 ml of 1.5% hydrochloric acid was added. Finally 2.5 ml of methanol was added and then allowed to stand for 30 minutes in ice and absorbance was read at 540nm.

Estimation of total protein

This assay was carried out by the method of Lowry et al²⁴. To 0.3 ml of serum solution, 2 ml of alkaline copper sulphate reagent was added. This solution is incubated at room temperature for 10 mins. Then 0.2 ml of Folin ciocalteau solution was added to each tube and incubated for 30 min. Absorbance was measured at 660 nm. Bovine serum albumin was used as the standard.

Estimation of albumin

This assay was carried out by dye method²⁵. To 5µl of serum, 1 ml of bromo cresol green reagent was added. The solution was incubated at room temperature for 10 min. Absorbance was measured at 630 nm.

Histopathology Study

The internal organs were preserved in a fixation medium of 10% buffered formalin for histopathological study. The organ paraffin sections were prepared, stained with haematoxylin and eosin, and processed for light microscope study.

Statistical Analysis

All values were expressed as the mean $\pm$ SEM. The results were analysed statistically by one-way Analysis of Variance (ANOVA) followed by Dunnet's test. P value < 0.05 was considered to be statistically significant.

Results

Acute toxicity study

Oral administration of methanol extract of *Polygonum glabrum* at a dose of 2000 mg/kg did not induce any drug related changes in behavior. Consumption of food and water was observed to be normal. There was no colour change in skin, eyes and faeces. However, sedation and lethargy was seen in rats. Above all, no mortality was recorded during acute toxicity study. The parameters observed for acute toxicity study after the administration of methanol extract of *Polygonum glabrum* are presented in (Table 1).

Table1: Effect of methanol extract of *Polygonum glabrum* on general appearance and behavioural changes in acute oral toxicity study.

Parameters	Dose (2000mg/kg)
Temperature	Normal
Urination	Normal
Rate of respiration	Normal
Skin colour	Normal
Eye colour	Normal
Diarrhoea	Not observed
Drowsiness	observed
Sedation	observed
Mortality	No

Subacute toxicity study**Effect of methanol extract of *Polygonum glabrum* on body weight of rats**

Differences in body weight of control and test animals were compared and percentage body weight was calculated (Table 2). Methanol extract of *Polygonum glabrum* caused a non-significant increase in percentage body weight in a non dose dependent manner. The highest weight gain was observed at a dose of 1000 mg/kg in the 28 days study.

Table 2: Effect of methanol extract of *Polygonum glabrum* on body weight of rats in 28 days

Study	Dose (mg/kg)	Percentage weight change (%)
Subacute (28 days)	Control	22.22± 3.54
	250	23.67± 2.39
	500	21.33± 4.57
	1000	31.01± 9.77

Values are expressed as mean ± SEM. n = 6 animals in each group; Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of methanol extract of *Polygonum glabrum* on relative organ weight of rats

The relative weight of each organ of treatment group animals was compared with control group (Table 3). The result indicates that, the vital organs (liver, kidney, heart, pancreas, brain, lungs, uterus, ovary, testis and spleen) were not adversely affected throughout the treatment by extract. A slight increase in the weight of lungs at a dose of 1000mg/kg was observed in the 28 days treatment. This increase may be dose dependent.

Table 3: Effect of methanol extract of *Polygonum glabrum* on relative organ weight of rats in 28 days.

Study	Organs	Control	Dose (mg/kg)		
			Methanol extract of PG (250mg/kg)	Methanol extract of PG (500mg/kg)	Methanol extract of PG (1000mg/kg)
Subacute (28 days)	Heart	0.47± 0.12	0.38± 0.06	0.42± 0.04	0.43± 0.02
	Lungs	0.72± 0.12	0.81± 0.14	0.83 ±0.18	0.92± 0.17*
	Kidneys	0.60± 0.04	0.63±0.05	0.70± 0.06	0.61± 0.01
	Spleen	0.44± 0.12	0.48 ±0.15	0.43 ±0.16	0.47± 0.13
	Liver	3.02± 0.17	3.05± 0.30	3.12± 0.15	3.17± 0.12
	Brain	0.95± 0.01	0.94± 0.06	0.91± 0.05	0.90± 0.04
	Testes	0.98± 0.05	0.96± 0.02	0.99± 0.04	0.93± 0.02
	Ovary	0.07± 0.00	0.08± 0.01	0.06± 0.02	0.09± 0.02

PG-*Polygonum glabrum* Values are expressed as mean ± SEM n = 6 animal in each group;

**P < 0.01, *P < 0.05 when compared to control. Data were analyzed by one-way ANOVA followed by Dunnet's test.

Effect of methanol extract of *Polygonum glabrum* on haematological parameters of rats

Oral treatment of rats with methanol extract of *Polygonum glabrum* for a period of 28 days revealed a non significant effect on hemoglobin, MCV, MCH, MCHC, hematocrit, platelet count, and total white blood cell (WBC) of treated animals. The plant extract at a dose level of 1000 mg/kg exhibited slight change (P < 0.05) in RBC level in treated rats. Similarly the plant extract at a dose level of 1000mg/kg exhibited slight increase in MCV level in treated rats (Table 4).

Table 4: Effect of methanol extract of *Polygonum glabrum* on haematological parameters of rats in 28 days.

Study	Haematological parameters	Control	Dose (mg/kg)		
			Methanol extract of PG (250mg/kg)	Methanol extract of PG (500mg/kg)	Methanol extract of PG (1000mg/kg)
Subacute (28 days)	Haemoglobin (g/L)	12.11±0.01	12.33±0.09	12.53±0.08	12.93±0.54
	Total RBC(x10 ¹² /L)	7.72 ±0.41	7.94± 0.93	7.97± 0.63	9.94 ±0.61*
	MCV (fL)	55.25± 1.31	55.45 ±1.41	56.35± 1.10	59.23± 1.23*
	MCH(pg)	17.39± 0.03	17.40± 0.09	17.45 ±0.07	17.47± 0.04
	MCHC(g/dl)	32.02 ±0.23	33.04± 0.34	34.05± 0.21	33.56 ±0.31
	Haematocrit (%)	36.30± 0.86	37.30± 0.67	38.90± 0.01	38.90± 0.64
	Platelet (x10 ⁹ /L)	308.01±0.45	320.01± 0.03	320.02± 0.05	321.02± 0.04
	Total WBC(x10 ⁹ /L)	6.40± 1.02	6.45 ±1.02	6.34± 1.26	6.50 ±1.01

PG-*Polygonum glabrum* Values are expressed as mean ± SD, n = 6 animal in each group;

***P < 0.001 **P < 0.01, *P < 0.05, when compared to control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of methanol extract of *Polygonum glabrum* on biochemical parameters of rats

Daily administration of methanol extract of *Polygonum glabrum* for a period of 28 days induced a significant change (p<0.05) in the total protein concentration at a dose of 1000 mg/kg respectively. Other serum biochemical parameters such as ALP, AST, ALT, creatinine, urea and albumin did not show any significant changes during the study period. Levels of serum electrolytes such as sodium, potassium and chloride were not affected by the administration of plant extract at all test doses. Rats receiving the plant extract at a dose of 1000 mg/kg showed a significant decrease in uric acid level when compared to control group (Table 5).

Table 5: Effect of methanol extract of *Polygonum glabrum* on biochemical parameters of rats in 28 days.

Study	Biochemical parameters	Control	Dose (mg/kg)		
			Methanol extract of PG(250mg/kg)	Methanol extract of PG(500mg/kg)	Methanol extract of PG(1000mg/kg)
Subacute (28 days)	ALP (U/L)	76.23± 9.04	75.23± 0.71	76.67± 2.08	77.89± 1.47
	AST (U/L)	53.5± 1.40	53.89± 6.70	52.34 ±0.45	52.33± 4.89
	ALT (U/L)	18.9± 2.30	19.45± 4.89	18.70± 8.97	18.64± 4.34
	Sodium meq/L	137.00± 0.44	138.12± 0.09	139.89± 0.07	136.45± 0.03
	Potassium meq/L	6.89± 0.04	6.77 ±0.03	6.54 ±0.05	6.72 ±0.01
	Chloride meq/L	107.34± 0.04	106.56± 0.01	108.34± 0.05	106.56± 0.09
	Creatinine mg/dL	1.45± 0.09	1.00± 0.03	0.85 ±0.06	0.83±0.04
	urea mg/dL	26.34± 0.00	25.43 ±0.09	24.45± 0.06	25.33± 0.00
	Uric acid mg/dL	3.76± 0.01	3.72 ±0.02	3.70 ±0.05	3.39 ±0.05*
	Total bilirubin (mg/dL)	0.65± 0.04	0.62 ±0.09	0.54± 0.04	0.51± 0.18
	Total protein (g/L)	7.72± 0.01	8.43 ±0.09	8.47 ±0.02*	8.96± 0.08*
	Albumin mg/dL	3.68 ±0.07	4.24± 0.08	3.85± 0.03	3.89 ±0.02

PG-*Polygonum glabrum* Values are expressed as mean ± SD, n = 6 animal in each group;

***P < 0.001 **P < 0.01, *P < 0.05, when compared to control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

Subchronic toxicity study

Effect of methanol extract of *Polygonum glabrum* on body weight of rats

The body weight of both control and plant extract treated groups over a period of 90 days did not show any significant changes (Table 6). A slight increase in body weight was observed in rats which received methanol extract of *Polygonum glabrum* at a dose of 1000mg/kg.

Table 6: Effect of methanol extract of *Polygonum glabrum* on body weight of rats in 90 days.

Study	Dose (mg/kg)	Percentage weight change (%)
Subchronic (90 days)	Control	67.57± 5.71
	250	67.91 ±6.76
	500	68.20± 8.69
	1000	71.65 ±4.24

Values are expressed as mean ± SEM. n = 6 animals in each group; Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of methanol extract of *Polygonum glabrum* on relative organ weight of rats

Long term administration of methanol extracts of plant did not cause any significant differences in calculated organ weights of test animals and control group (Table 7). However a significant ($p < 0.05$) increase in weight of spleen and testes was recorded at a dose of 1000mg/kg in the 90 days study period.

Table 7: Effect of methanol extract of *Polygonum glabrum* on relative organ weight of rats in 90 days.

Study	Organs	Control	Dose (mg/kg)		
			Methanol extract of PG (250mg/kg)	Methanol extract of PG (500mg/kg)	Methanol extract of PG (1000mg/kg)
Subchronic (90 days)	Heart	0.45± 0.12	0.37± 0.07	0.40± 0.05	0.42± 0.01
	Lungs	0.80± 0.13	0.85± 0.14	0.87 ±0.18	0.82± 0.17
	Kidneys	0.61± 0.03	0.62±0.05	0.59± 0.06	0.61± 0.07
	Spleen	0.64± 0.12	0.65 ±0.15	0.67± 0.13	0.74 ±0.16*
	Liver	3.12± 0.17	3.10± 0.30	3.32± 0.15	3.23± 0.12
	Brain	0.92± 0.01	0.83± 0.06	0.88± 0.05	0.90± 0.05
	Testes	0.78± 0.05	0.79± 0.09	0.81± 0.02	0.88± 0.04*
	Ovary	0.06± 0.00	0.06± 0.01	0.06± 0.05	0.07± 0.02

PG-*Polygonum glabrum* Values are expressed as mean ± SEM n = 6 animal in each group;

**P < 0.01, *P < 0.05 when compared to control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of methanol extract of *Polygonum glabrum* on haematological parameters of rats

During the 90 days treatment of rats with methanol extract of *Polygonum glabrum* a significant dose dependent increase in WBC and platelet count was observed (Table 8). Other parameters such as hemoglobin, RBC, MCV, MCH, MCHC and hematocrit of treated animals did not exhibit significant change when compared to control groups.

Table 8. Effect of methanol extract of *Polygonum glabrum* on haematological parameters of rats in 90 days.

Study	Haematological parameters	Control	Dose (mg/kg)		
			Methanol extract of PG (250mg/kg)	Methanol extract of PG (500mg/kg)	Methanol extract of PG (1000mg/kg)
Subchronic (90 days)	Haemoglobin (g/L)	11.11±0.01	11.33±0.09	12.23±0.08	12.43±0.54
	Total RBC($10^{12}/L$)	7.02 ±0.41	7.04± 0.93	7.09± 0.63	7.10±0.10
	MCV (fL)	54.25± 1.31	54.27 ±1.41	54.35± 1.09	55.24± 1.33
	MCH(pg)	17.78± 0.03	17.93± 0.09	18.24 ±0.07	18.56± 0.04
	MCHC(g/L)	34.02 ±0.23	33.01± 0.34	34.05± 0.21	34.66 ±0.31
	Haematocrit (%)	41.30± 0.80	43.02± 0.67	46.08± 0.01	47.08± 0.64
	Platelet count($10^9/L$)	378.01±0.45	467.12± 0.03	486.03± 0.56	509.02± 0.05*
	Total WBC($\times 10^9/L$)	10.40± 1.02	11.45 ±1.23	11.64± 1.26	13.60 ±1.10*

PG-*Polygonum glabrum* Values are expressed as mean $\pm$ SD, n = 6 animal in each group;

***P < 0.001 **P < 0.01, *P < 0.05, when compared to control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of methanol extract of *Polygonum glabrum* on biochemical parameters of rats

Repeated oral treatment with methanol extract of *Polygonum glabrum* for a period of 90 days did not exhibit any significant change in the serum levels of aspartate transaminase, alanine transaminase and alkaline phosphatase (Table 9). There was a slight decrease in the serum creatinine and uric acid levels in animals receiving plant extract at a dose of 1000mg/kg.

Table 9: Effect of methanol extract of *Polygonum glabrum* on biochemical parameters of rats in 90 days.

Study	Biochemical parameters	Control	Dose (mg/kg)		
			Methanol extract of PG (250mg/kg)	Methanol extract of PG (500mg/kg)	Methanol extract of PG (1000mg/kg)
Subchronic (90 days)	ALP (U/L)	66.33± 9.04	66.43± 0.71	65.77± 2.08	66.29± 1.47
	AST (U/L)	47.05± 11.40	47.29± 5.70	47.34 ±0.45	47.63± 4.89
	ALT (U/L)	17.99± 2.30	18.15± 1.89	18.05± 8.97	18.54± 2.34
	Sodium meq/L	137.18± 0.43	136.10± 0.09	138.9± 0.07	138.25± 0.03
	Potassium meq/L	5.89± 0.04	5.77 ±0.03	5.64 ±0.05	5.72 ±0.01
	Chloride meq/L	106.34± 0.04	107.56± 0.01	107.34± 0.05	107.96± 0.09
	Creatinine mg/Dl	1.05± 0.11	0.97± 0.13	0.90 ±0.05	0.80±0.09*
	urea mg/dL	23.04± 0.01	23.43 ±0.12	22.45± 0.22	23.30± 0.05
	Uric acid mg/dL	6.76± 0.01	6.73 ±0.02	6.70 ±0.01	5.15 ±0.05*
	Total bilirubin(mg/dL)	0.62± 0.03	0.60 ±0.02	0.59± 0.14	0.57± 0.11
	Total protein (g/L)	8.02± 0.01	8.13 ±0.09	8.45 ±0.02	8.76± 0.08
	Albumin mg/dL	4.98 ±0.01	5.01± 0.0	5.06± 0.11	5.10 ±0.07

PG-*Polygonum glabrum* Values are expressed as mean ± SD, n = 6 animal in each group;

***P < 0.001 **P < 0.01, *P < 0.05, when compared to control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of methanol extract of *Polygonum glabrum* on Histological studies

The harvested organs did not indicate any treatment related histological changes in the treated and control rats. The cellular architecture was maintained in the tissues of treatment and control group animals of both subacute and subchronic studies. Photomicrographs of liver (Fig A-D) and kidney (Fig E-H) histopathology are shown in Figure 1.

Discussion

Mainstreaming of traditional medicine, particularly herbal medicine has become important as it is used by people globally. Pharmacovigilance in herbal sector is essential to find the toxicological data and adverse effect of these treatments. Therefore, the present research was aimed to evaluate methanol extract of *Polygonum glabrum* for acute, subacute and subchronic toxicity studies and to detect the range of dose that could be used for further studies. The oral acute toxicity study was carried out on Wistar rats with single dose (2000 mg/kg) body weight. The rats were monitored continuously for a period of 15 days. The limit dose of 2000 mg/kg did not result in any mortality or pharmacotoxic symptoms in animals during the observatory period. Nevertheless, depression, lethargy and tiredness were apparent in rats which were treated with the dose. The above results suggests that LD₅₀ could be >2000 mg/kg as methanol extract of *Polygonum glabrum* seems to be safe at a dose level of 2000 mg/kg.

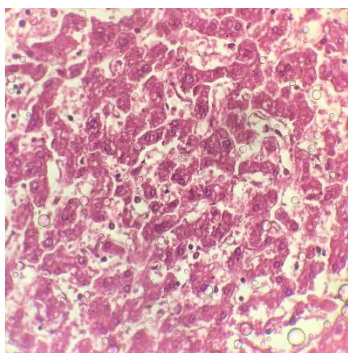


Fig 1 (A) Section of rat liver showing normal architecture for control group

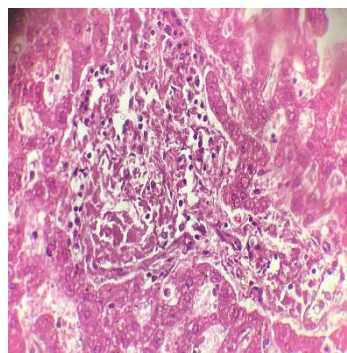


Fig (B) Section of rat liver treated with PG (250 mg/kg)

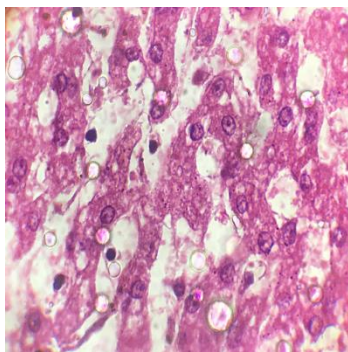


Fig (C) Section of rat liver treated with PG (500 mg/kg)

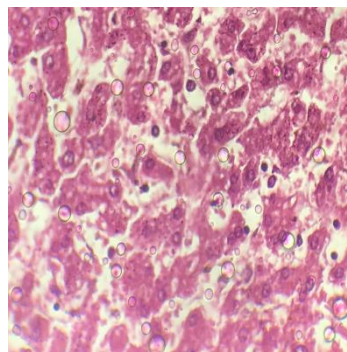


Fig (D) Section of rat liver treated with PG (1000 mg/kg)

Similar data was found in a study which evaluated acute toxicity of methanol extract of *Polygonum glabrum* leaves in rats²⁶. Also, in a study carried out by Banjiet al. the oral LD₅₀ of methanol-aqueous extract of *Polygonum glabrum* whole plant in rats was estimated to be more than 2000 mg/kg²⁷. The

present study is in accordance with previous results suggesting that methanol extract of *Polygonum glabrum* tested at the limit dose is essentially nontoxic. However, acute toxicity data does not validate the cumulative toxic effects that happen over a period of time even at small doses²⁸.

Hence a subacute (28 days) and subchronic (90 days) toxicity study (as per OECD guidelines) of *Polygonum glabrum* methanol extract was carried out by examining its effect on organ weight, haematological and biochemical parameters with doses of 250mg/kg, 500mg/kg and 1000mg/kg. In the study, an increase in body weight was observed in rats that received methanol extract of *Polygonum glabrum*. However, this gradual gain in body weight in all treatment groups was not statistically significant when compared to the control group. The comparative weight gain over time was a regular dose dependent increase. These results suggested that oral administration of methanol extract of *Polygonum glabrum* daily over a period of 28 and 90 days does not affect the normal growth of animals or their stability of appetite at any particular dose.

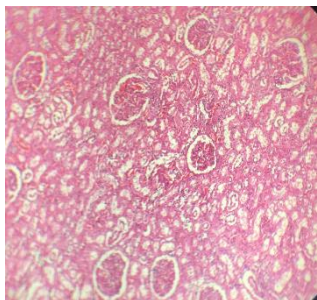


Fig (E) Section of rat kidney for control group

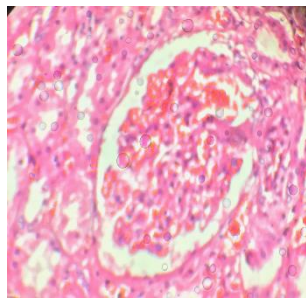


Fig (F) Section of rat kidney treated with PG (250 mg/kg)

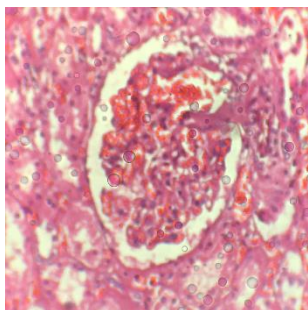
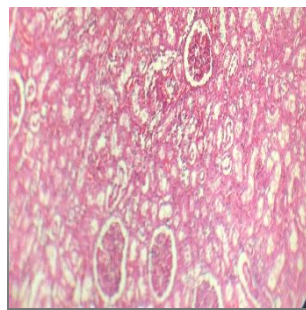


Fig (G) Section of rat kidney treated with PG (500 mg/kg)



(Fig H) Section of rat kidney treated with PG (1000 mg/kg)

Subacute and Subchronic toxicity studies provide time period for eliciting any toxicity on long term feeding. Crucial data about persistent or cumulative toxic effects on target organ systems are generally

acquired from these studies. Exposure to chemicals or any toxic drugs may result in weight reduction of internal organs²⁹. Correspondingly hypertrophy of organs is also an indication of the influence of toxic substance on our body. During the 28 days treatment period, methanol extract of *Polygonum glabrum* did not cause enlargement of any vital organs relative to control group. Macroscopic analysis of harvested organs from treatment animals did not show significant changes in color, volume and texture when compared with the control group. A slight increase in weight of lungs in rats which received a dose (1000mg/kg) of plant extract was noticed. This increase may be due to the heavy dose of plant extract. Similarly during the ninety days study, an increase in the weight of testes and spleen was observed at a dose of 1000mg/kg. However no treatment related pathological changes in tissues of these organs were revealed during histology analysis. Absence of any significant differences in the weight of overall vital organs provides support for the safety of *Polygonum glabrum*.

Several phytoconstituents present in the plant may penetrate and affect the bone marrow leading to impairment in the synthesis of blood cells. This can be followed by cell lysis causing abnormal changes in blood parameters³⁰. Methanol extract of *Polygonum glabrum* to experimental animals for twenty eight days at dose (1000mg/kg) caused slight increase in RBC and an increase in MCV (1000mg/kg) suggesting the influence of plant extract on erythropoietic system. Repeated oral treatment for 90 days led to an elevation of WBC and platelets. The significant increase in WBC implied that the plant extract plays a key role in boosting the immune system. The methanol extract did not show any abnormal change in haematological parameters ruling out the possibility of interference in blood cell synthesis. The above results also justify the non-toxicity of *Polygonum glabrum* in rats making it safe at all doses.

Assessment of serum biochemical parameters was carried out to find any potential alterations in kidney and hepatic functions by plant extract. Abnormal levels of serum creatinine, uric acid and urea are biomarkers of possible damage of kidneys³¹. Drugs or bioactive active compounds are generally metabolized in liver making it a vulnerable site for attack. Alteration in total protein, albumin and total bilirubin level indicates injury in hepatocellular and secretory functions of liver³². Administration of methanol extract of *Polygonum glabrum* to rats did not alter the biochemical parameters. The significant increase in parameters such as albumin and total protein observed in this study may suggest potential hepatoprotective effect of methanol extract of *Polygonum glabrum*. The lack of significant changes in biochemical values indicates that the plant extract does not affect hepatic or renal metabolism of rats.

Based on the above observation it can be said that methanol extract of *Polygonum glabrum* is essentially non toxic. These observations were further confirmed by the histological assessment of the organs.

Conclusion

Treatment with oral dose of 2000 mg/kg of methanol extract of *Polygonum glabrum* did not inflict any clinical toxicity symptoms or mortality in the acute toxicity study. Oral administration of methanol extract of *Polygonum glabrum* at dose of 250, 500 and 1000 mg/kg did not elicit any considerable change in body weights, organ weights, biochemical parameters, haematological parameters and histological analysis. The treatment doses also exhibited organ protective potential along with boosting immune system. It can be concluded that methanol extract of *Polygonum glabrum* was well tolerated in acute, subacute and subchronic toxicity studies.

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Conflict of interest

Authors declare no conflict of interest.

Reference

1. Singh VK and Abrar M. Medicinal Plants and Folklore, Today and Tomorrow. Printers and Publishers, 1990, New Delhi, 1-5.
2. Parasuraman S. Toxicological screening. Journal of Pharmacology & Pharmacotherapeutics. 2011; 2(2):74-79.
3. Setzer RW and Kimmel CA. Use of NOAEL, benchmark dose, and other models for human risk assessment of hormonally active substances. Pure Applied Chemistry, 2003; 75: 2151–2158.
4. Butterweck V and Nahrstedt A. What is the best strategy for preclinical testing of Botanicals? A critical perspective. Planta Medica, 2012; 78(8): 747–754.
5. Dzoyem JP, Nkuete AH, Kuete V, Tala MF, Wabo HK, Guru SK, Rajput VS et al. Cytotoxicity and antimicrobial activity of the methanol extract and compounds from *Polygonum limbatum*. Planta medica, 2012; 78(8):787-792.
6. Soudahmini E, Ganesh M, Senthil PL, Madhu C and Divakar. Herbal remedies of Madugga tribes of Siruvani forest, South India. Natural Product Radiance, 2005; 4(6): 492-499.

7. El Tahir A, Satti GM and Khalid SA. Antiplasmodial activity of selected Sudanese medicinal plants with emphasis on maytenus senegalemsis. Journal of Ethnopharmacology, 1999; 64(3): 227-233.
8. Shankar LH and Mishra PK. Study of aquatic medicinal plants of Hazaribagh district of Jharkhand, India. International research journal of pharmacy. 2012; 3(4): 405-409.
9. Koche DK, Shirsat RP, Syed Imran, Mohd. Nafees, Zingare AK and Donode KA. Ethnomedicinal Survey of nagzira wild life sanctuary, District Gondia (M.S.) India-Part II. Ethnomedicinal Leaflets, 2008; 1(8): 532-537.
10. Khan MF, Islam Rabbi SN, Fahima Aktar MD and Kawsar H. In vitro Cytotoxic, membrane stabilizing and thrombolytic activities of *Polygonum glabrum* Willd. Bangladesh Pharmaceutical Journal. 2014; 17(2): 202-204.
11. Babitha S, David B and Otilia JFB. Investigation on antioxidant and hepatoprotective activity of ethanolic leaf extract of *Polygonum glabrum* Wild on carbon tetrachloride- induced hepatotoxicity in rats. Spatula DD, 2012; 2(4): 199-205.
12. Adinarayana D, Ramachandraiah P and Syamasundar KV. Polyphenolic constituents of *Polygonum glabrum* seeds admixed with flowers and stem. Leather Science (Madras). 1980; 27: 268–270.
13. Jacobsson U and Muddathir AK. Four biologically active Sesquiterpenes of the drimane type isolated from *Polygonum glabrum*. Phytochemistry, 1992; 31(12):4207–4211.
14. The Organization of Economic Co-Operation and Development (OECD), The OECD guideline for testing of chemicals: 420-acute oral toxicity, OECD, Paris (1001) pp. 1–14.
15. Ghadirkhomi A, Safaeian L, Zolfaghari B, Aghaye-Ghazvini MR, Rezaei P. Evaluation of acute and sub-acute toxicity of *Pinus eldarica* bark extract in Wistar rats. Avicenna Journal of Phytomedicine, 2016; 6 (5): 558-566.
16. Ibrahim MB et al. Sub-acute and chronic toxicity profiles of *Markhamia tomentosa* ethanolic leaf extract in rats Journal of Ethnopharmacology, 2016; 193: 68–75.
17. Narhari D, Durajan G, Sharif H, Sheikh ZR. Evaluation of acute and subacute toxicity induced by methanol extract of *Terminalia citrina* leaves in Sprague Dawley rats. Journal of Acute Disease, 2015; 4(4): 316–321.
18. Reitman S, Frankel SA. Colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transminases. American Journal Clinical Pathology. 1957; 28(1): 56-63.

19. Kind P and King E. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *Journal of Clinical Pathology*, 1954; 7(4): 322.
20. Fabiny DL and Ertingshausen G. Automated reaction-rate method for determination of serum creatinine with the centrifichem. *Clinical Chemistry*. 1971; 17(8): 696-700.
21. Talke H and Schubert GE. Enzymatic urea determination in the blood and serum in the warburg optical test. *Klin Wochenschr.* 1965; 1(43): 174-175.
22. Buchanan MJ, Isdale IC and Rose BS. Serum Uric Acid Estimation: Chemical and Enzymatic Methods Compared. *Annals of the Rheumatic Diseases*. 1965; 24(3): 285-288.
23. Malloy HT and Evelyn KA. The determination of bilirubin with the photoelectric colorimeter. *The Journal of Biochemistry*, 1937; 119: 481-490.
24. Kingsley GR. The determination of serum total protein, albumin, and globulin by the biuret reaction. *Journal of Biological Chemistry*, 1939; 131: 197-200.
25. Suzuki Y. Determination method of serum albumin using a bromocresol green solution and a bromocresol purple solution containing no detergent as a color reagent. 2003; 52(4): 269-273.
26. Faheemuddin MD, Ahmed A and Shirisha SK. Antidiabetic activity of methanolic extract of *Polygonum glabrum* Wild Leaves in diabetic rats. *International Journal for Pharmaceutical Research Scholars*, 2016; 5(1).
27. Sreenivasamurthy B, Banji D and Banji O. Investigation on antioxidant and hepatoprotective activity of ethanolic leaf extract of *Polygonum glabrum* Willd. on carbon tetrachloride-induced hepatotoxicity in rats. *Spatula DD*. 2012; 2(4): 199-205.
28. Abotsi WKM, Ainooson GK and Gyasi EB. Acute and sub-acute toxicity studies of the ethanolic extract of the aerial parts of *Hillieria latifolia* (Lam.) (Phytolaccaceae) in rodents. *West Afr. J .Pharm*, 2011; 22: 27–35.
29. Wang et al. Acute and subchronic oral toxicity profiles of the aqueous extract of *cortex dictamni* in mice and rats. *Journal of Ethnopharmacology*. 2014; 158: 207–215.
30. Agbaje EO, Adeneye AA and Daramola AO. Biochemical and toxicological studies of aqueous extract of *Syzgium aromaticum* (L.) Merr & Perr in rodents. *African Journal of Traditional and Complementary Alternative Medicine*. 2009; 6: 241–254.
31. Newman DJ and Price CP. Renal function and nitrogen metabolites. In: Burtis CA, Ashwood ER. (Eds.), *Tietz Textbook of Clinical Chemistry*. W.B Saunders Company, Philadelphia, 1999; 1204–1270.

32. Tolman KG and Rej R. Liver function. In: Burtis, CA, Ashwood, ER. (Eds.), Tietz Text Book of Clinical Chemistry, 3rd edi. W.B. Saunders Co, Philadelphia PA, USA, 1999; 1125–1177.