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Original article

Sub-acute toxicological studies 2α, 3β, 21β, 23, 28-penta hydroxyl 12-oleanene isolated from roots of *Laportea crenulata* Gaud

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Background: 2α,3β,21β,23,28-penta hydroxyl 12-oleanene was isolated from roots of *Laportea crenulata* Gaud (Urticaceae) as a new triterpenoid and its antifungal activities was evaluated against a number of fungi where moderate antifungal activities were reported. However, no toxicological study has yet been carried out.
Objective: The sub-acute toxicity of 2α,3β,21β,23,28-penta hydroxyl 12-oleanene was studied on albino mice.
Methods: The triterpenoid was administered on intraperitoneal route at 300 μg per mouse (20-27g) daily for 14 consecutive days. The studies included the determination of changes in body weight, hematological profiles (total count of red blood cell, white blood cell and platelet, differential count of white blood cell, erythrocyte sedimentation rate, and hemoglobin percentage), and biochemical parameters of blood (serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase, serum bilirubin, creatinine, and urea) as well as histopathology of the liver, kidney, heart, and lung.
Result: The changes in body weight, hematological, and biochemical parameters were statistically not significant when compared to control group mice. Histopathologically no abnormality was found on liver, kidney, heart, and lung of experimental group mice after treatment when compared to that of control group mice.
Conclusion: In sub-acute toxicity studies, the triterpenoid was found to be nontoxic. We suggest further studies such as chronic toxicological studies as well as route selection experiments.

Keywords: Biochemical parameters, body weight, hematological parameters, histopathological study, 2α,3β,21β,23,28-penta hydroxyl 12-oleanene

Toxicology is the part of pharmacology that deals with the adverse effect of bioactive substance on living organisms [1]. In order to establish the safety and efficiency of a new drug, toxicological studies are very essential in animals such as mice, rat, guinea pigs, dog, rabbit, and monkey under various condition of drug. No drug is used clinically without clinical trial as well as toxicity studies [1]. Toxicological studies help to make decision whether a new drug should be adopted for clinical use or not [2]. Depending on the duration of drug exposure to animals, toxicological studies may be of three types (acute, sub-acute, and chronic toxicological studies) [3]. In acute toxicity studies, single dose of drug is given in large quantity to determine immediate toxic effect. Acute toxicity studies are commonly used to determine LD_{50} of drug or chemicals. In sub-acute toxicity studies, repeated doses of drug are given in sub-lethal quantity for a period of 14 to 21 days. Sub-acute toxicity studies are used to determine effect of drug on biochemical and hematological parameters of blood as well as to determine histopathological changes. In chronic toxicity studies, drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of drug.

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Laportea crenulata Gaud (syn. *Urtica crenulata*, Fam. Urticaceae), locally known as Agnichutra, is an evergreen shrub that is widely distributed in Bangladesh, India, and Malay island [4]. The roots of the plant used traditionally for the treatment of bleeding from nose and/or mouth, excessive gas in the stomach, constipation, weakness, asthma, gout, mumps, whooping cough, and chronic fever [4, 5]. The roots of the plant also have stimulant, stomachic, and diuretic properties [5, 6]. Previously, we reported the isolation of 2α,3β,21β,23,28-penta hydroxyl 12-oleanene from roots of Laportea crenulata as a new compound (with two known compounds β-sitosterol 3-β-D-glucopyranoside and β-sitosterol) and its moderate antifungal activity [7]. No other compound has yet been reported from the plant. In continuation of our biocompatibility search, this study was designed to investigate sub-acute toxicity of 2α,3,β21β,23,28-penta hydroxyl 12-oleanene.

Materials and methods

Plant materials

The roots of *Laportea crenulata* was collected from various part of Rangpur district of Bangladesh and identified by Prof. A.T.M. Naderuzzaman, Department of Botany, University of Rajshahi, Bangladesh where its voucher specimen (No. 1239) was deposited. The roots were cut, air-dried, and ground into powder.

Plant materials extraction and fractionation

Powdered dried roots (900g) of the plant were extracted (cold) with ethanol (5L) in flat bottom glass containers, through occasional shaking and stirring for 10 days. The whole extract was filtered and the solvent were evaporated to dryness in vacuo with an rotary evaporator at 40-50°C to afford a blackish green mass (45g), which was further extracted with petroleum ether (3x50 mL), chloroform (3x50 mL), and methanol (3x50 mL) to afford petroleum ether (13g), chloroform (7g), and methanol (9g) fractions, respectively [8, 19]. The preliminary phytochemical screening of the different fractions was carried out by chemical tests and thin layer chromatographic methods [9, 20].

Isolation of the triterpenoid

The petroleum ether soluble fraction (3g) was subjected to column [10, 11] using n-hexane, chloroform, and methanol of increasing polarity. Column chromatography fractions eluting with 100% chloroform to 40% methanol in chloroform was subjected to preparative TLC (Silica gel PF_{254}) with solvent system ethyl acetate:cyclohexane (2:1) to afford compound 1 (53.30mg). Its structure 2α,3β,21β,23,28-penta hydroxyl 12-oleanene) was confirmed on the basis of various spectroscopic methods (IR, HR-EIMS, ^{1}H and ^{13}C NMR including JMOD, COSY, NOESY, HMBC, HSQC). In solubility test, the compound was sparingly soluble in water and soluble in methanol, ethanol, ethyl acetate, and chloroform.

Experimental animals

The experiment was carried out on Swiss albino male mice [12]. They were two to three months old, weighing between 20-27g (average weight is 22.13g). They were collected from the Animal Branch of International Center for Diarrheal Disease Research, Bangladesh. Mice were treated with guidelines and approval of animal research ethical council of International center for Diarrheal Disease Research, Bangladesh.

Maintenance of mice

The mice were housed in iron cages (considering group) under temperature and light controlled condition [13]. They were fed with a balanced diet [14] and tap water. The animals were maintained under this condition for 15 days before experiment to adjust with food and environment.

Acute Toxicity assay

Acute toxicity study was performed by grade doses of the triterpenoid in albino mice using intraperitoneal administration. They were observed continuously for the first two hours for toxic symptoms and up to 24 hours for mortality [15].

Grouping of mice

Individual weight of the mice was taken and they were grouped in two, randomly. The mice of group B were used for experiment while those of group A were used as control. Number of mice in each group was six [16, 17].

Administration of sample

The compound, 2α, 3β, 21β, 23,28-penta hydroxyl 12-oleanene was dissolved in distilled water with the help of tween-80 as co-solvent, so that each 0.2 ml solution contains 300 μg 2α,3β,21β,23,28-penta

hydroxyl 12-oleanene. Each mouse of experimental group (group B) was administered 0.2ml of sample solution (contain 300 μg compound) daily, for 14 consecutive days and each mouse of control group (group A) was administered 0.2ml of isotonic vehicle daily, for 14 consecutive days. Intraperitoneal route was used for these administrations.

Blood collection

For hematological study (total and differential blood cell count, ESR and percent hemoglobin determination), blood was drawn from the tail vein of both groups before drug administration, at 7th day of treatment and after completion of treatment. For biochemical study, blood was collected at 15th day after completion of treatment from the jugular veins of each mouse. Finally, all mice were sacrificed and liver, kidney, heart, and lung were removed for histological study.

Gross general observation

During the experimental period their behavior, central nervous system (CNS) excitation, CNS depression, muscular weakness, salivation, diarrhea, and food intake were observed. The body weight of each mouse of groups A and B were measured before drug administration and after treatment prior to sacrificing the animals.

Investigation of hematological profiles

The hematological parameters determination like total count (TC) of RBC, WBC and platelet, differential count (DC) of WBC, ESR, and hemoglobin percentage were performed just before drug intake, at seventh day of treatment, and after completion of treatment. Hemoglobin percentage was estimated by Sahli's acid hematin method using Sahli's hemometer [18]. Total count of RBC, WBC and platelet was carried out by automated cell counter (Beckman Coulter, China). Differential count (DC) of WBC was carried out by microscopy using Leishman' stain. ESR was determined by Westergren's method using Westergren tube and stand [18].

Investigation of biochemical parameters

Biochemical parameters (serum glutamate oxaloacete transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase, serum bilirubin, creatinine, and urea) were determined at 15th day after completion of treatment. Determination of biochemical parameters was carried out using automated biochemical analyzer (Vitros-250, Johnson and Johnson Co., USA).

Histopathological investigation

Histopathological investigation was carried out 15 days after completing treatment and collection of blood. Both experimental and control groups mice were sacrificed and liver, kidney, heart, and lung were isolated for investigation. The tissues were separately sliced in piece, fixed in 10% formaline for three days, processed, stained, mounted on glass slides, and observed under high power microscope at the Department of Pathology, Rajshahi Medical College, Bangladesh.

Statistical analysis

The results were presented as mean standard deviation where n=6. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range test. The analysis was performed using SAS statistical software.

Results

In acute toxicity study, the triterpenoid was found to be safe and no mortality was observed at a dose as high as 200 mg/kg. The test compound did not affect the change in body weight over the treatment period with mean weight±SD in treated group as 22.7±1.1 (before experiment) and 22.5±1.0g (after experiment) and in control group as 23.5±1.4 (before experiment) and 23.9±1.4g (after experiment) ($P > 0.1$, n=6 each). Other parameters of gross general observation of mice showed no noticeable change during treatment by the compound.

The hematological profiles of the experimental and control group mice were determined before treatment, at the seventh day of treatment and after completion of treatment and compared to check the hematological disorders. No significant changes in the values of RBC, WBC, platelet, and differential WBC count, ESR, and hemoglobin percentage of experimental group mice were observed when compared to that of control group mice as shown in **Table 1**. Biochemical parameters of blood (SGOT, SGPT, alkaline phosphatase, serum bilirubin, creatinine, and urea) were determined after treatment by 2α,3β,21β,23,28-penta hydroxyl 12-oleanene and compared to that of control group mice to check any change of these parameters. It was found that most of the parameters were slightly changed

with respect to control group mice but remained within the normal range (**Table 2**). Histopathologically, no abnormality was found on the liver, kidney, lung, spleen, and heart of both control and experimental animals.

Discussion

The acute toxicity study result reveals that the triterpenoid might be considered a broad nontoxic substance. The changes in body weight over treatment period were found trivial in both control (gained weight 1.7%) and experimental group (lost weight 0.9%). The insignificant changes in hematological profiles (total count of red blood cell, white blood cell, and platelet, differential count of white blood cell, erythrocyte sedimentation rate and hemoglobin percentage) of the experimental group indicated that 2α,3β,21β,23,28–penta hydroxyl 12-oleanene has no adverse effect on bone marrow of mice. No significant changes in biochemical parameters (serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase, serum bilirubin, creatinine, and urea) of the experimental group mice indicate that the triterpenoid is free from significant adverse effect on liver and kidney functions. Since in histopathological study the compound showed no abnormality in the organs (liver, kidney, lung, spleen, and heart) of the experimental mice, thus the compound has no significant adverse effect on cellular structures.

The isolation of 2α,3β,21β,23,28–penta hydroxyl 12-oleanene from roots of *Laportea crenulata* as a new compound and its moderate antifungal activity was reported previously [7] but sub-acute toxicity studies is the first report for the compound. The previous report of biological effect of the triterpenoid and its present toxicological studies suggest that the triterpenoid can be safely subjected to chronic toxicological studies, structure activity relationship studies, and route(s) selection experiments.

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Table 1. Effect of 2α,3β,21β,23,28–penta hydroxyl 12-oleanene on hematological parameters of mice

Haematological parameters	Control group			Experimental group		
	Normal mice	Treated with vehicle		Normal mice	Treated with compound 1	
	1st day n=6, M±SD	7th day n=6, M±SD	14th day n=6, M±SD	1st day n=6, M±SD	7th day n=6, M±SD	14th day n=6, M±SD
Total RBC count (million/cc)	5.1±0.2	5.1±0.1	5.2±0.1	5.1±0.2	5.4±0.3	5.3±0.3
Total WBC count (thousand/cc)	5.9±0.3	5.9±0.3	6.0±0.3	6.3±0.3	6.4±0.2	6.3±0.2
- Neutrophil	3637.7±198.7	3681.3±204.9	3746.3±176.5	3882.75±158.6	3937±133.3	3906±133.3
Differential - Lymphocyte	1760.3±96.1	1781.3± 99.1	1812.8±85.3	1878.8±76.8	1905±64.5	1890±64.5
count of WBC - Monocyte	310.8±16.9	314.3± 17.5	320.5±15.1	331.8±13.8	336.5±11.3	333.8±11.6
(no/cu. mm) - Eosinophil	134.5±7.5	136 ±7.6	139±6.7	143.5±5.9	145.8±5.1	144.8±5.1
Platelet count (thousand/cc)	273.8±9.9	276.5± 4.7	280.3±1.5	250.0±2.5	254.7±1.6	262.5±2.3
Haemoglobin (g/100 ml)	15.6±0.5	15.8±0.6	15.9±0.6	15.5±0.4	15.6±0.4	15.5±0.4
ESR (mm/1st hour)	20.3±1.1	21.5±1.1	22.2±1.0	21.8±1.5	24.3±0.7	25±3.2

Table 2. Effect of 2α,3β,21β,23,28–penta hydroxyl 12-oleanene on biochemical parameters of mice blood after intraperitoneal administration of 300 μg/mouse/day for 14 consecutive days

Biochemical parameters	Control group n=6, M±SD	Experimental group n=6, M±SD	% of change	Calculated t value
SGPT (IU/L)	11±1.22	12±0.70	+9.09	+1.42
SGOT (IU/L)	13.75±0.43	14.5±1.5	+5.45	+0.96
SALP (IU/L)	0.48±0.03	0.49±0.03	+2.08	+0.50
Serum bilirubin (mmol/L)	0.28±0.02	0.30±0.02	+2.90	+1.57
Creatinine (mg/dl)	0.54±0.02	0.55±0.02	+1.85	+0.64
Urea (mg/dl)	28.5±3.35	30.25±3.96	+6.14	+0.67

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