OPEN ACCESS

Phytopharmacological Communications

DOI: doi.org/10.55627/ppc.004.002.0793

Research Article

Evaluation of *in-vitro* Cytotoxicity and *in-vivo* Toxicity of Aqueous Fraction of *Ribes orientale*

Ambreen Malik Uttra[1*], Alamgeer[2], Muhammad Talha Raheem[3], Sumera Qasim[4]

[1]Department of Pharmacology, College of Pharmacy, University of Sargodha, Sargodha 40100, Pakistan
[2]Department of Pharmacology, Punjab University College of Pharmacy, University of the Punjab, Lahore 54000, Pakistan
[3]Department of Electrical and Computer Engineering, COMSATS University Islamabad, Lahore Campus 54000, Pakistan
[4]Department of Pharmacology, College of Pharmacy, Jouf University, 72341 Aljouf, Saudi Arabia
***Correspondence**: ambreen.malik@uos.edu.pk

Abstract

The present research investigated the probability of toxicity of the aqueous fraction of *Ribes orientale* (ROAF) after acute, subacute, and sub-chronic dose administration. Mice were given varying doses between 10–5000 mg/kg during the acute trial. Rats were administered 50, 100, and 200 mg/kg doses for 30 days, whereas 300, 600, and 900 mg/kg doses were given for 14 days. The rats' weight as a whole, organ weight, hematology and biochemical indices, and histology of liver, kidney, and heart tissue were assessed.None of the tested dose incited notable behavioral alterations or mortality in acute poisoning, the lethal dose 50 (LD50) was higher than 5000 mg/kg. For sub-acute toxicities, ROAF did not affect body or organ weight, while only slight changes in both serum and blood measurements were identified. Only slight changes in lymphocytes and neutrophils, total protein, ALP, and glucose at some doses were noted but these changes were not allied with histopathological alterations. In sub-chronic toxicity, ROAF did not modify weights (body/organ), and non-significant variances in hematology and biochemistry were observed, affirmed by histopathology. Therefore, the results above indicate that ROAF is rather safe to take orally for therapeutic usage at lower dosages because it did not cause death in animals or create any notable side effects related to hematological, biochemical, or structural processes.

Keywords: *Ribes orientale,* Acute, Sub-acute, Sub-chronic, Hematology, Biochemistry.

1. Introduction

Plant-based medicines have been used since prehistoric times (Hardy 2021). In the present era, herbs have enticed the attention of man as basic resources for drug development. This upsurge in the utilization of plant-based remedies is owing to the credence that these are natural, safe, and innocuous. Yet, the natural source of these herbal preparations does not assure their safety, because there is now increasing proof that many herbal medications do result in potential toxicity to their consumers (Arsad et al. 2013). Consequently, additional scientific consideration is now being given to evaluate the probable toxicity of plant-based remedies and to foresee the consequences of plant therapies adopted by humans/animals (Mensah et al. 2019).

About 200 species of currants and gooseberries can be found in Europe, North America, South America, and Asia under the genus Ribes (Grossulariaceae) (Kendir and Köroğlu 2015). *Ribes orientale* is a deciduous shrub that flowers from April to May and grows to 1.8 m (6ft). The flowers are dioecious and pollinated by insects. It is found in stony slopes and rocks up to 4000 m in the Himalayas (Polunin and Stainton, 1984). In Gilgit Baltistan, Pakistan, natives use the roots of *Ribes orientale* Desf. (local name: Ghonashatooh) to

treat fever, headaches, joint discomfort, and rheumatism (Khan and Khatoon 2007). In Turkey, leaves of *Ribes orientale* have also been employed for diaphoretic and diuretic potential (Kendir and Köroğlu 2015). Blood is not only a good indicator to ascertain an organism's health but also a good pathological mirror of the entire body. The cellular constituent of blood is valuable in immunotoxicology to appraise the immunotoxic prospective of a substance (Vlata et al. 2021). Previously, the anti-arthritic activity of *Ribes orientale* aqueous-ethanolic extract and fractions was examined and results explored a highly significant activity of *Ribes orientale* in reducing chronic inflammation. Similarly, another investigation provided evidence of the wound-healing potential of *Ribes orientale*, supporting its diverse pharmacological applications (Kendir et al. 2019). As *Ribes orientale* has been utilized traditionally for various medicinal purposes, thus the current study aimed to explore its acute, sub-acute, and sub-chronic toxicity. Building upon past research, the investigation specifically examined the aqueous fraction of *Ribes orientale* aqueous-ethanolic extract, demonstrating a pronounced anti-arthritic effect of aqueous fraction compared to other fractions (Uttra et al. 2019). Hence, scientific information regarding the safety of *Ribes orientale* is critical before it can be developed into an alternative therapy for rheumatoid arthritis or other inflammatory diseases. A battery of tests including no observed adverse effect level (NOAEL) and reference dose (RfD) were performed. The combination of its promising pharmacological activities and the need for further safety evaluations makes *Ribes orientale* a strong candidate for future clinical research and potential therapeutic applications.

2. Materials and Methods

2.1. Collection and Extraction of Plant

The Assistant Professor of Botany, Dr. Shair Wali Khan at Karakoram International University in Gilgit Baltistan, Pakistan, acknowledged and validated the *Ribes orientale* Desf roots that were obtained from that region during the months of April-May. A voucher specimen (number RO-15-19) was turned in to the University of Sargodha's College of Pharmacy herbarium for future use. We used the same extraction process described in our earlier paper (Uttra et al. 2019).

2.2. Experimental Animals

Standard diet and water were provided to the Sprague Dawley rats (170-190 g) and Swiss albino mice (20-25 g), with a 12-hour light/dark cycle maintained at 23 to 25°C. Both male and female rats and mice were used. This work was conducted in accordance with the Declaration of Helsinki (1964). The University of Sargodha's College of Pharmacy's Animal Ethics Committee approved the experimental protocols (Approval No. 51A46 IEC UOS).

2.3. Acute Toxicity Study (LD50)

Lorke's method (Lorke 1983) was used to carry out acute toxicity in two phases. In the first phase, 3 groups of mice (n=3) were administered 10, 100, and 1000 mg/kg/ p.o. ROAF. In the next phase, 3 groups of fresh mice (n=3) were executed 1600, 2900, and 5000 mg/kg/ p.o. of ROAF. In both phases, mice were noticed for toxicological signs/mortality for the first 4 h, subsequently the first 24 h, and thus regularly for a week. The LD50 was examined centered on the final observed mortality and the safety margin was calculated by the following formula (Erhirhie et al. 2018):

Equation 1:

$$\text{Safety margin} = \text{LD50}/\text{Highest safe dose}$$

2.4. Sub-acute Toxicity Study

The OECD guidelines 407 with minor adaptations were followed to perform a sub-acute toxicity study. Three doses (300, 600, and 900 mg/kg/po) were given daily for 2 weeks to 3 groups of rats (n = 6). In the 4^{th} group (n = 6), vehicle control was given only distilled water for a similar period. After treatment, body weights were determined on the first study day and then checked once a week after that. At the culmination of 14 days of examination time, hematologic, biochemical, and histopathologic examinations were done (Nath and Yadav 2015).

Table 1: The impact of ROAF on body weights of rats in a sub-acute toxicity study

Treatments	Body weight (g)		
	Day 0	Day 7	Day 14
Normal Control	177.167 ±2.330	180.833 ±2.301	183.167 ±2.197
Aqueous Fraction (300 mg/kg)	176.167 ±1.905^{ns}	178.333 ±2.319^{ns}	181.167 ±2.725^{ns}
Aqueous Fraction (600 mg/kg)	178.500 ±2.172^{ns}	183.000 ±2.671^{ns}	185.333 ±3.403^{ns}
Aqueous Fraction (900 mg/kg)	174.167 ±1.138^{ns}	177.167 ±2.167^{ns}	180.000 ±2.671^{ns}

The findings are shown as mean ± SEM (n=6) via Two-way ANOVA with Bonferroni posttest, which showed no statistically significant differences between doses within each parameter.

2.5. Sub-chronic Toxicity Study

OECD guideline 407 with minor variations was pursued and rats were distributed into 4 groups (n=10). Three doses of ROAF (50, 100, and 200 mg/kg) were given daily for 1 month to 3 groups of rats. Rats' body weight was measured every week after the first day of the trial. Rats were sacrificed on the 31st day of the study. On the 31st day of the study, hematologic, biochemical parameters, and histopathologic modifications were noted (Singh and Kumar 2011).

2.6. NOAEL and RfD Calculation

NOAEL was defined as the maximum exposure level at which the frequency or severity of adverse effects did not increase in a way that was statistically or physiologically significant in comparison to the control group of rats. Moreover, RfD was calculated by following the formula (Lewis et al. 2002).

Equation 2:

$$RfD = NOAEL/ UF_1 X UF_2 X UF_3$$
$$= NOAEL/10x10x10$$
$$= NOAEL/100$$

UF= Uncertainty factor

UF_1= A 10-fold factor for human variability

UF_2= A 10-fold factor for extrapolation from animals to humans

UF_3= A 10-fold factor for less than chronic exposure

2.7. Assessment of cytotoxicity using resazurin test

The *in-vitro* effect of the aqueous fraction of *Ribes orientale* on cell viability was evaluated by resazurin assay. For this purpose, Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC, Health Protection Agency, Porton Down, Salisbury, Wiltshire, United Kingdom). Cells were seeded in a 24-well plate at a density of 2.5×10^4 cells/well for 10 days in a final volume of 500 µL of Modified Eagle Media (MEM) cell culture medium with Earle's balanced salts supplemented with 10% fetal bovine serum (FBS), 2.0 mM L-glutamine and 1% penicillin-streptomycin at 37°C in 5% CO_2 environment. The culture medium was refreshed every other day. When the cells were about 80 % confluent (80% of the surface of the flask covered by cell monolayer after 8-10 days), they were washed twice with phosphate buffer saline (PBS) pre-warmed at 37°C. Test solution (0.5% m/v), positive control prepared in white MEM, and negative control (0.5% v/v Triton X-100) were added in 500 µL volume in triplicate to the cell culture. Then, treated cells were incubated in a 5% CO_2 environment at 37°C for 3 and 24 h. Next, test solutions were removed and cells were washed twice with pre-warmed PBS. A 2.2 µM diluted resazurin solution in 500 µL volume was supplemented to every well and cells were

Table 2: The impact of ROAF on hematological and biochemical parameters of rats in 14 days.

Parameters	Normal control	Aqueous (300 mg/kg)	Aqueous (600 mg/kg)	Aqueous (900 mg/kg)
Hematological parameters				
TLC (×10³/μL)	6.767±0.362	6.167±0.657^{ns}	7.233±0.501^{ns}	7.033±0.264^{ns}
Neutrophils (%)	22.500±1.118	28.667±2.66^{ns}	27.667±1.606^{ns}	30.000±2.352*
Lymphocytes (%)	74.000±0.730	68.333±2.929^{ns}	69.500±2.349^{ns}	66.167±1.600*
Monocytes (%)	2.33±0.333	2.000±0.365^{ns}	1.667±0.333^{ns}	2.333±0.422^{ns}
Eosinophils (%)	1.000±0.258	1.000±0.258^{ns}	1.000±0.365^{ns}	1.500±0.322^{ns}
RBCs (×10⁶/μL)	7.135±0.075	7.227±0.086^{ns}	7.137±0.045^{ns}	6.947±0.140^{ns}
Hb (g/dL)	12.717±0.111	12.867±0.167^{ns}	12.700±0.121^{ns}	12.767±0.275^{ns}
HCT (%)	41.850±0.249	41.933±0.206^{ns}	41.367±0.274^{ns}	41.300±0.258^{ns}
MCV (fl)	56.150±0.239	56.933±0.653^{ns}	57.133±0.667^{ns}	56.567±0.440^{ns}
MCH (pg)	17.500±0.278	17.633±0.246^{ns}	17.933±0.184^{ns}	17.667±0.308^{ns}
MCHC (g/dL)	30.700±0.151	30.767±0.217^{ns}	31.067±0.206^{ns}	30.633±0.184^{ns}
Platelets (×10³/μL)	734.000±10.076	706.333±38.489^{ns}	811.333±41.046^{ns}	750.000±43.626^{ns}
Biochemical parameters				
ALT (U/L)	30.000±1.732	23.667±0.667^{ns}	33.667±1.726^{ns}	29.667±3.584^{ns}
AST (U/L)	188.500±4.780	174.333±1.726^{ns}	177.167±4.028^{ns}	180.000±4.712^{ns}
ALP (U/L)	236.000±4.091	202.500±12.460*	198.833±4.722*	200.500±9.472*
Total protein (g/dL)	6.417±0.070	6.300±0.086^{ns}	6.133±0.049*	5.800±0.058***
Albumin (g/dL)	2.050±0.022	2.133±0.049^{ns}	2.000±0.037^{ns}	2.200±0.073^{ns}
Urea (mg/dL)	28.000±2.477	24.333±2.348^{ns}	28.333±1.838^{ns}	23.667±1.978^{ns}
Creatinine (mg/dL)	0.800±0.058	0.667±0.042^{ns}	0.833±0.102^{ns}	0.933±0.076^{ns}
LDH (U/L)	244.333±1.453	242.833±1.579^{ns}	246.833±1.424^{ns}	249.000±1.571^{ns}
CK (U/L)	88.000±1.807	85.167±1.662^{ns}	87.500±1.565^{ns}	88.667±1.909^{ns}
Total Cholesterol (mg/dL)	77.000±4.782	78.667±1.667^{ns}	76.667±2.951^{ns}	87.333±2.390^{ns}
Triglycerides (mg/dL)	62.500±2.446	53.833±2.725^{ns}	56.000±2.595^{ns}	57.333±2.591^{ns}
Glucose (mg/dL)	183.000±0.577	220.000±11.547**	223.000±5.550**	276.333±4.566***

Values recorded are the mean ± SEM (n=6). Analysis was done with one-way ANOVA followed by Dunnett's multiple comparison test. ***= $p<0.001$, **= $p<0.01$, *= $p<0.05$, ns= non-significant compared with vehicle control. TLC=Total leukocyte count, RBC=Red blood cells, Hb=Hemoglobin, HCT=Hematocrit, MCV=Mean corpuscular volume, MCH=Mean corpuscular hemoglobin, MCHC=Mean corpuscular hemoglobin concentration, ALT=Alanine aminotransferase, AST=Aspartate aminotransferase, ALP=Alkaline phosphatase, LDH=Lactate dehydrogenase, CK=Creatine Kinase.

incubated for 3 h. The 100 μL of supernatant was subsequently transferred to a black 96-well plate and fluorescence intensity was measured at 540 nm using a microplate reader with background subtraction at 590 nm (Vetter *et al.* 2010). The percentage of viable cells was calculated using the following equation:

Equation 3:

$$\text{Cell viability} = \frac{\text{Experimental values-Negative contr}}{\text{Positive control-Negative control}}$$

2.8. Statistical Analysis

Using Graph Pad Prism 5.0, statistical analysis was performed using one-way ANOVA followed by Dunnett's test and two-way ANOVA followed by Bonferroni post-test. The data were expressed as mean ± SEM, and $p<0.05$ was deemed statistically significant.

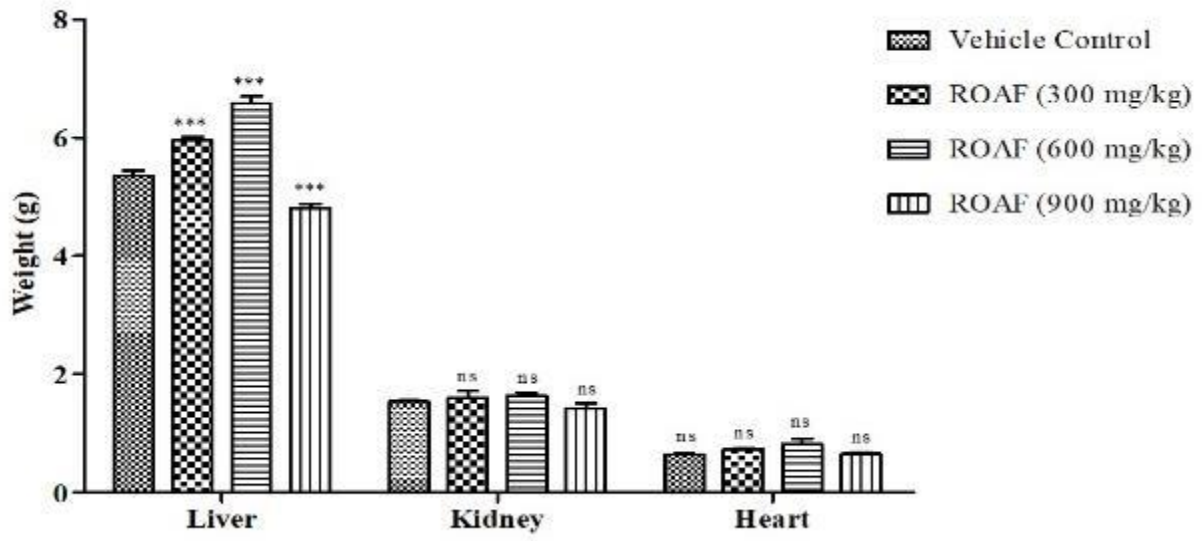

Figure 1: The effect of ROAF on Sprague Dawley rats' organ weights after 14 days of treatment. The findings are shown as mean ± SEM (n=6), using two-way ANOVA. ***= p<0.001, ns=non-significant, compared to vehicle control.

3. Results

3.1 Acute Toxicity

Behavioral changes due to ROAF toxicity detected in mice administered ≥1000 mg/kg dose showed comprised sleep, weakness, and reduced activity for the first 24 h. However during the remainder of the trial period, no alterations in behavior were noticed, and at all doses used, no deaths were reported seven days following therapy. Thus, LD50 was appraised to be >5000 mg/kg b.w. Hence, a safety margin of ROAF is calculated as:

$$\text{Safety margin} = \text{LD50/Highest safe dose} = \frac{5000 \text{ mg/kg}}{200 \text{ mg/kg}} = 25$$

3.2 Sub-Acute Toxicity

Over the course of 14 days, body weights were gradually increased, and non-significant differences were seen between the control and treatment groups at any point (Table 1). Furthermore, there were no significant variations were noted in the weights of the kidney and heart with any of the tested doses during the necropsy. However, animals that were given 300 and 600 mg/kg of ROAF had liver weights that were considerably ($p<0.001$) higher compared to vehicle control, while animals given 900 mg/kg dose had liver weights that were significantly lower than the vehicle control (Figure 1). Additionally, all hematological parameters showed no discernible alterations (Table 2). However, for rats treated with the highest ROAF dose (900 mg/kg),showed a significant increase ($p<0.01$) in neutrophils and eosinophils while a significant decrease ($p<0.01$) in lymphocytes was seen. Likewise, changes in biochemical parameters of treated animals were statistically non-significant. However, rats treated with the lowest and medium doses showed a significant decrease ($p<0.05$) in alanine phosphate (ALP) and those administered with the highest dose showed a significant increase ($p<0.01$) in ALP values. Similarly, total protein values for rats treated with the lowest and median doses were significantly reduced with $p<0.05$ and $p<0.001$, correspondingly, whereas with the highest dose non-significant changes were observed. Moreover, glucose values were significantly increased at all the doses but a highly significant ($p<0.001$) increase was observed with the median dose. Furthermore, histopathologic examination of the liver, kidney, and heart sections showed no abnormalities (Figure 3-5).

Table 3: The impact of ROAF on rats' body weights in sub-chronic toxicity

Treatments	Body weight (g)				
	Day 0	Day 7	Day 14	Day 21	Day 30
Vehicle Control	175.900 ± 0.924	177.400 ± 1.655	180.600 ± 1.628	183.200 ± 0.879	185.200 ± 0.772
Aqueous Fraction (50 mg/kg)	173.200 ± 0.879 ns	175.700 ± 1.086 ns	176.900 ± 1.260 ns	182.800 ± 1.618 ns	184.400 ± 1.543 ns
Aqueous Fraction (100 mg/kg)	176.300 ± 1.438 ns	178.600 ± 1.522 ns	180.600 ± 1.392 ns	185.300 ± 1.106 ns	186.600 ± 0.945 ns
Aqueous Fraction (200 mg/kg)	175.400 ± 1.185 ns	176.300 ± 1.212 ns	178.600 ± 1.628 ns	183.300 ± 1.106 ns	187.200 ± 0.757 ns

The findings are shown as mean ± SEM (n=10) via Two-way ANOVA using Bonferroni posttest, which showed no statistically significant differences between doses within each parameter.

3.3 Sub-Chronic Toxicity

Oral administration of 50, 100, and 200 mg/kg doses of ROAF to rats for 30 days showed that ROAF did not display mortality and toxicity symptoms. Moreover, body weight gradually increased in all the groups (Table 3). The 30 days administration of 50, 100, and 200 mg/kg ROAF, individually demonstrated no significant impact ($p>0.05$) on kidney, liver, and heart weight as paralleled with vehicle control (Figure 2). Moreover, Table 4 describes that ROAF administration at sub-chronic doses did not significantly modify ($p>0.05$) hematologic and biochemical factors at all doses employed. Also, kidney, liver, and heart were excised after 30 days from all treatment groups and discovered no abnormalities upon gross examination (Figure 3-5).

3.4 NOAEL and RfD

A dose of 200 mg/kg of ROAF was found NOAEL since it did not result in any statistically significant changes in the frequency/severity of adverse effects between treated rats and their respective vehicle control rats. Instead, it did produce considerable changes in the total protein at 600 and 900 mg/kg, ALP and glucose at 300, 600, and 900 mg/kg, or in hematopoietic cells at 900 mg/kg. This NOAEL suggests that the RfD with a safety factor of 100 would be 2 mg/kg body weight/day.

3.5 Resazurin-Cytotoxicity Assay

According to the resazurin test results (Figure 6), Caco-2 cells treated with a 0.5% solution of ROAF showed only a 10% drop in viability and integrity at the third hour and an 11% reduction at the twenty-four-hour mark. Here, Caco-2 cells exposed to negative control showed a 100% protective effect, while positive control cultured in white MEM caused 94% cytotoxicity at 3 h and 24 h, 95.33%.

4. Discussion

The safety of using medicinal plants over an extended period is becoming more and more significant. In experimental animal models, toxicological evaluation is done to establish a safe dose for human ingestion (Ismail et al. 2014). *Ribes orientale* is used worldwide to treat numerous disorders but, scientific records on its adverse effects and toxicity are lacking, so additional studies concerning its safe consumption are vital. In acute toxicity, no adverse effect was discerned in mice up to 5 g/kg ROAF dose. As reported earlier, those substances are considered safe that demonstrate an oral LD50 >5 g/kg (Atsamo et al. 2011). Henceforth, it can be anticipated that ROAF lacks acute oral toxicity.

The results illustrate that 14 and 30 days of treatment of rats with ROAF at high and low

Table 4: The impact of ROAF on hematological and biochemical parameters of rodents in 30 days toxicity.

Parameters	Normal control	Aqueous Fraction (50 mg/kg)	Aqueous Fraction (100 mg/kg)	Aqueous Fraction (200 mg/kg)
Hematological parameters				
TLC (×10³/µL)	6.720±0.133	6.530±0.185^{ns}	6.650±0.186^{ns}	6.820±0.136^{ns}
Neutrophils %	42.800±1.750	40.000±1.468^{ns}	41.700±1.334^{ns}	40.800±1.497^{ns}
Lymphocytes %	74.200±1.750	68.900±1.804^{ns}	75.900±1.567^{ns}	78.600±1.384^{ns}
Monocytes %	2.500±0.167	2.500±0.167^{ns}	2.200±0.133^{ns}	2.400±0.163^{ns}
Eosinophils %	1.000±0.000	1.300±0.153^{ns}	1.400±0.163^{ns}	1.400±0.163^{ns}
RBCs (×10⁶/µL)	7.198±0.017	7.201±0.017^{ns}	7.153±0.019^{ns}	7.258±0.021^{ns}
Hb (g/dL)	13.740±0.107	13.460±0.099^{ns}	14.000±0.086^{ns}	13.870±0.124^{ns}
HCT (%)	44.480±0.159	41.020±0.998^{ns}	42.870±1.890^{ns}	44.800±1.134^{ns}
MCV (fl)	56.290±0.191	56.410±0.405^{ns}	55.260±0.351^{ns}	56.320±0.335^{ns}
MCH (pg)	17.530±0.116	17.700±0.159^{ns}	17.570±0.166^{ns}	17.330±0.187^{ns}
MCHC (g/dL)	32.610±0.204	33.040±0.148^{ns}	33.180±0.195^{ns}	33.260±0.234^{ns}
Platelets ×10³/µL)	838.900±18.747	820.500±4.954^{ns}	810.800±4.565^{ns}	804.700±3.836^{ns}
Biochemistry parameters				
ALT (U/L)	34.400±1.514	33.000±1.626^{ns}	30.500±1.778^{ns}	34.300±1.647^{ns}
AST (U/L)	188.500±3.138	195.800±1.679^{ns}	184.300±1.758^{ns}	190.400±1.815^{ns}
ALP (U/L)	268.100±2.554	273.500±2.115^{ns}	272.200±2.356^{ns}	275.700±2.196^{ns}
Total protein (g/dL)	6.120±0.158	5.970±0.237^{ns}	6.150±0.151^{ns}	5.820±0.206^{ns}
Albumin (g/dL)	3.340±0.115	2.930±0.153^{ns}	3.010±0.176^{ns}	3.440±0.110^{ns}
Urea (mg/dL)	28.300±1.033	29.000±1.585^{ns}	25.300±2.011^{ns}	26.900±1.906^{ns}
Creatinine (mg/dL)	0.880±0.061	0.7100±0.038^{ns}	0.870±0.042^{ns}	0.890±0.053^{ns}
LDH (U/L)	246.000±2.011	253.800±2.453^{ns}	244.600±2.428^{ns}	255.200±3.791^{ns}
CK (U/L)	87.200±1.806	87.300±1.513^{ns}	82.600±1.833^{ns}	89.600±2.034^{ns}
Total Cholesterol (mg/dL)	87.300±1.647	81.100±1.810^{ns}	84.600±2.146^{ns}	86.000±1.713^{ns}
Triglycerides (mg/dL)	81.500±1.641	82.300±1.838^{ns}	79.700±1.283^{ns}	84.900±1.941^{ns}
Serum Glucose (mg/dL)	186.400±1.956	182.800±1.971^{ns}	185.400±1.634^{ns}	192.800±2.086^{ns}

The findings are shown as mean ± SEM (n=10) via One-way ANOVA using Dunnett's multiple comparison test. ns= non-significant compared with normal control. RBC=Red blood cells, TLC=Total leukocyte count, Hb=Hemoglobin, HCT=Hematocrit, MCH=Mean corpuscular hemoglobin, MCV=Mean corpuscular volume, MCHC=Mean corpuscular hemoglobin concentration, AST=Aspartate aminotransferase, ALT=Alanine aminotransferase, ALP=Alkaline phosphatase, CK=Creatine Kinase., LDH=Lactate dehydrogenase.

doses, respectively did not present any toxic signs/death. The non-significant differences in rats' body weight administered sub-acute and sub-chronic doses of ROAF describe no modification in the metabolic mechanisms of rats, which may accordingly disrupt body weight and hormones (Awodele et al. 2012). Furthermore, it has been stated that escalated organ weight directs inflammation, whereas decreased organ weight illustrates cellular constriction (Ashafa et al. 2009). In the current study, reduced liver weight at sub-acute doses suggests that ROAF did cause hepatic inflammation and non-significant changes in liver weight at sub-chronic doses suggest that ROAF is safe at low doses. Further, no changes in kidney and heart weight in treated groups depict no renal or cardiac inflammation at both low and high doses.

Valuation of hematologic parameters can be employed to govern the deleterious influence of test substances on blood and to explicate blood-involved properties of plant extracts (Yakubu et al. 2007). Administration of rats with high doses of ROAF produced minor hematologic variations while sub-chronic doses resulted in non-significant hematologic changes. It was noticed

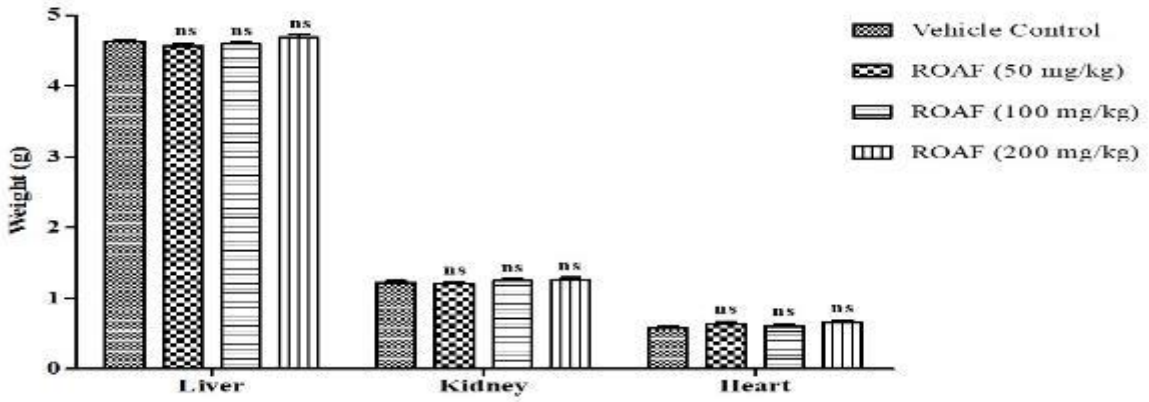

Figure 2: The effect of ROAF on Sprague Dawley rats' organ weights after 30 days of treatment. The findings are shown as mean ± SEM (n=10), using two-way ANOVA. ns=non-significant, compared to vehicle control

that levels of RBC, HCT, Hb, MCH, MCV, and MCHC in rats administered ROAF for 14 and 30 days, respectively were within normal range, which depicts that the plant is non-toxic and results justify that at all doses of ROAF doesn't induce anemia, which is often accompanied with bone marrow toxicity (Kifayatullah et al. 2015). Contrariwise, the significant increase in neutrophils and decrease in lymphocytes with 900 mg/kg of ROAF suggest some anti-lymphocytic and anti-tumor potential of a plant at maximal dose (Chanda et al. 2015). In addition, monocytes, TLC, and eosinophils remained within the normal range at low and high doses.

Serum biochemistry is employed to detect any changes in the liver, kidney and heart. AST and ALT were used as biomarkers for predicting possible hepatotoxicity (Umbaugh and Jaeschke 2021). The increased serum ALT and AST levels can be considered an initial sign of cell injury (Mukinda and Eagles 2010). The functioning of ALP is associated with the working of hepatocytes and an improvement in its activity could be due to amplified production/obstructed biliary tract (Manjunatha et al. 2005). In the present study, non-significant proliferation was ascertained in ALT and AST levels at both high and low doses of ROAF that staunchly submit that the plant did not alter hepatocytes and therefore, metabolism in rats. Besides, sub-acute doses of ROAF prompted the substantial decline in ALP while sub-chronic doses didn't modify this parameter. Further, histopathologic variations (centrilobular degenerative modifications, necrosis/steatosis) associated with raised liver enzymes were also absent in the current examination. Besides, serum total protein and albumin designate liver state and type of damage (Yakubu et al. 2005). Total protein was decreased at 600 and 900 mg/kg doses, which might be owing to poor nutrition/malabsorption (Agbaje et al. 2009). These outcomes propose that the secretory task of the liver was not compromised by ROAF.

Likewise, serum urea and creatinine concentrations provide an understanding of plant effect on glomerular/tubular portions of the kidney (Ashafa et al. 2009). A rise in creatinine and urea levels indicates kidney damage. Non-significant influence of ROAF on renal function markers at all the doses investigated may submit that the regular working of nephrons at the glomerular/tubular level remained unaffected. The blood chemistry findings validate histological outcomes of kidneys that indicate no impairment of renal cells (Ismail et al. 2014).

LDH is a useful index of cardiac and hepatocellular injury (Ismail et al. 2014). The enzyme CK performs an imperative role in furnishing energy for cardiac and skeletal muscle contraction. A raised CK level specifies

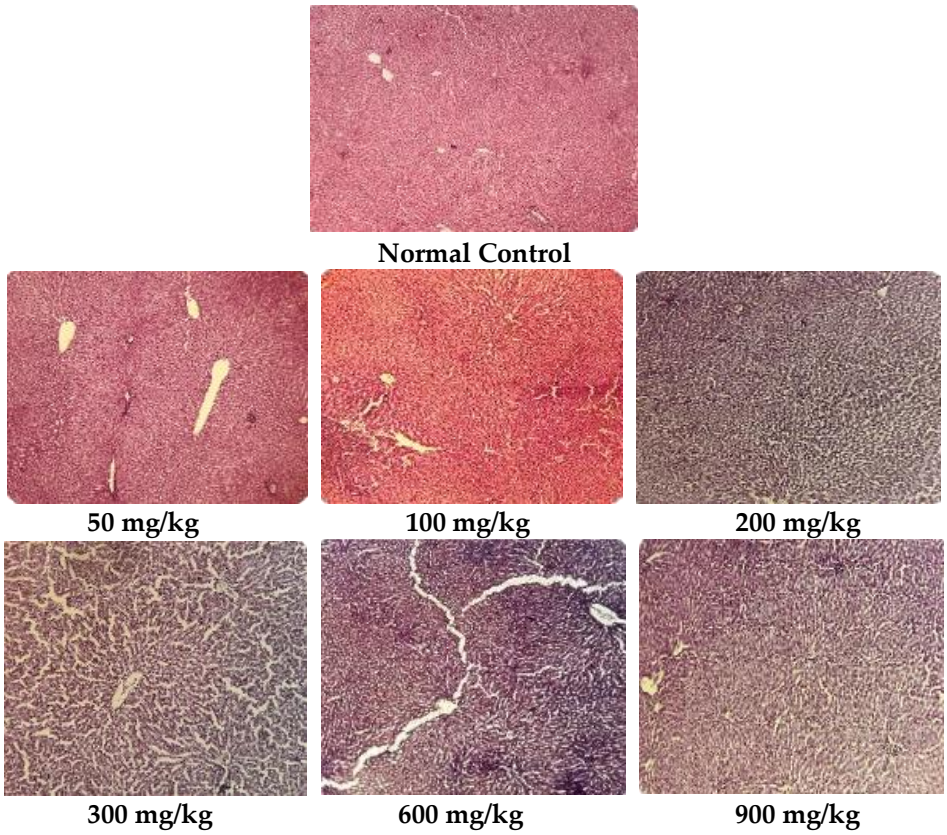

Figure 3: Histology of liver sections (H&E, 100x) of rats treated with sub-acute and sub-chronic doses of ROAF.

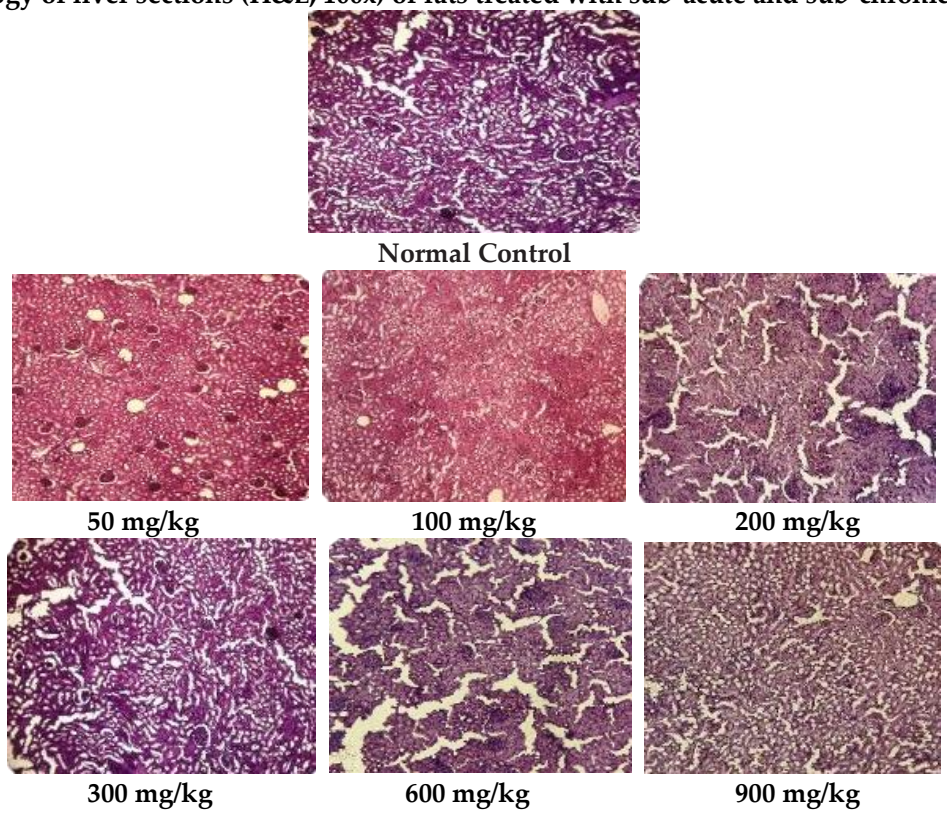

Figure 4: Histology of kidney sections (H&E, 100x) of rats treated with sub-acute and sub-chronic doses of ROAF.

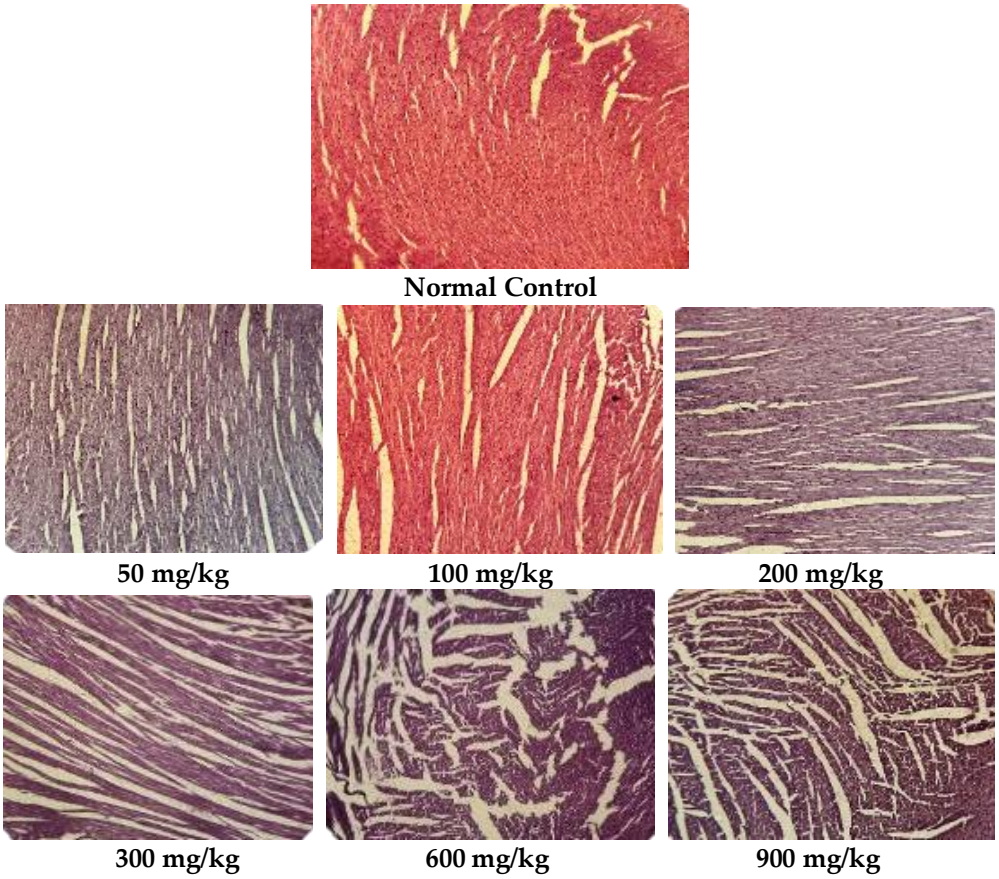

Figure 5: Histology of heart sections (H&E, 100x) of rats treated with sub-acute and sub-chronic doses of ROAF.

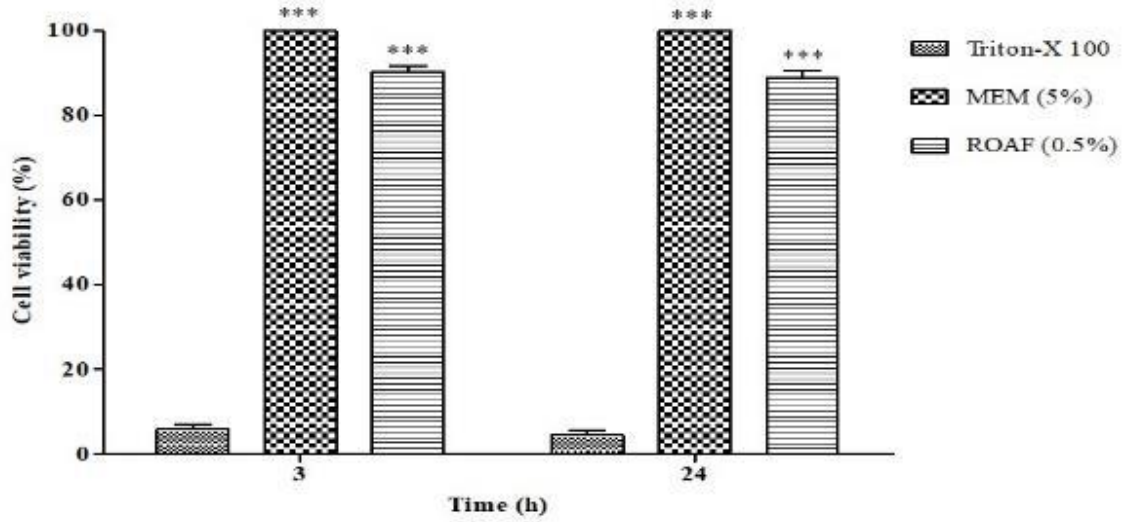

Figure 6: The impact of ROAF on the viability of Caco-2 cells in the resazurin test. The findings are shown as mean ± SEM (n=3), using two-way ANOVA followed by Bonferroni posttest. ***= $p<0.001$, compared to negative control. MEM= Modified Eagle Medium.

damage/stress to any of these organs like myocardial infarction (Nwose 2013). The findings recommend that cardiac performance remained unchanged with high and low doses of ROAF, as supported by histology results, which exhibited no lesions. Additionally, changes in lipid concentration (triacylglycerol and cholesterol) offer expedient evidence on lipid metabolism and coronary heart ailments (Caselli et al. 2001). The non-significant impact of ROAF on serum triglyceride and cholesterol could be explained by an improvement in cholesterol biosynthesis. Furthermore, apart from cholesterol synthesis, the liver is the major site for glucose synthesis (Anderson and Borlak 2008). At sub-acute doses of ROAF, hyperglycemia was noted. Yet, no considerable variabilities were distinguished in glucose concentration at sub-chronic doses, thus suggesting that ROAF at low doses does not affect carbohydrate metabolism in rats.

Furthermore, according to Rasmussen et al. 2011, the screening for acute toxicity can be done through a cytotoxicity test. The ability of Caco-2 cells to metabolize resazurin dye enabled researchers to assess the cell line's in vitro survival following three and twenty-four hours of exposure to ROAF (Costa et al. 2021). According to the findings of current research, ROAF increased the viability of Caco-2 cells at the tested dose (0.5%) after 3 and 24 hours of incubation and did not exhibit any appreciable toxicity.

5. Conclusion

In brief, aqueous fraction of *Ribes orientale* hydroalcoholic extract could be considered as devoid of potential toxicity as, it did not produce mortality and substantial alterations in hematology and biochemistry values, along with histopathology at doses tested. On the other hand, excessive use of this plant must be done so with caution because prolonged use may cause hyperglycemia and liver abnormalities.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

NA.

Study Approval

The study was approved by the biosafety and ethical review committee, College of Pharmacy, University of Sargodha.

Consent Forms

NA.

Acknowledgments

We thank the College of Pharmacy, University of Sargodha, for providing lab facilities.

Data Availability

All the data related to the manuscript is available with the authors.

Author Contributions

"AMU" performed experimental work, data collection and result compilation, literature search, and manuscript preparation. "A" supervised the research work. MTR statistically evaluated the results. SQ refined the manuscript for publication. The authors read and approved the final manuscript for publication.

References

Anderson, Nora, and Jürgen Borlak. 2008. "Molecular Mechanisms and Therapeutic Targets in Steatosis and Steatohepatitis." *Pharmacological Reviews* 60 (3): 311–57. https://doi.org/10.1124/pr.108.00001.

Siti Rosilah Arsad, Norhaizan Mohd Esa, Hazilawati Hamzah, and Fauziah Othman. 2013. "Evaluation of Acute, Subacute and Subchronic Oral Toxicity of Rhaphidophora Decursiva (Roxb.) Schott Extract in Male Sprague Dawley Rats" 7

(41): 3030–40. https://doi.org/10.5897/jmpr2013.2611.

A. O. T. Ashafa, M T Yakubu, D S Grierson, and A J Afolayan. 2009. "Toxicological Evaluation of the Aqueous Extract of Felicia Muricata Thunb. Leaves in Wistar Rats." 8 (6): 949–54. https://doi.org/10.5897/ajb2009.000-9156.

Atsamo, Albert D., Télesphore B. Nguelefack, Jacques Y. Datté, and Albert Kamanyi. 2011. "Acute and Subchronic Oral Toxicity Assessment of the Aqueous Extract from the Stem Bark of Erythrina Senegalensis DC (Fabaceae) in Rodents." *Journal of Ethnopharmacology* 134 (3): 697–702. https://doi.org/10.1016/j.jep.2011.01.023.

Awodele, Olufunsho, Ibrahim Adekunle Oreagba, Saidi Odoma, Jaime A. Teixeira da Silva, and Vincent Oluseye Osunkalu. 2012. "Toxicological Evaluation of the Aqueous Leaf Extract of Moringa Oleifera Lam. (Moringaceae)." *Journal of Ethnopharmacology* 139 (2): 330–36. https://doi.org/10.1016/j.jep.2011.10.008.

Caselli, Chiara, Raffaele De Caterina, Jeff M Smit, Jonica Campolo, Mohammed El Mahdiui, Rosetta Ragusa, Alberto Clemente, et al. 2021. "Triglycerides and Low HDL Cholesterol Predict Coronary Heart Disease Risk in Patients with Stable Angina." *Scientific Reports* 11 (1). https://doi.org/10.1038/s41598-021-00020-3.

Chanda S, Parekh JY, Vaghasiya RD, Baravalia Y, and Nair R. 2015. "Medicinal plants-from traditional use to toxicity assessment: a review." *International Journal of Pharmaceutical Science and Research* 6(7): 2652-2670.

Costa, Pedro, Ana T. P. C. Gomes, Márcia Braz, Carla Pereira, and Adelaide Almeida. 2021. "Application of the Resazurin Cell Viability Assay to Monitor Escherichia Coli and Salmonella Typhimurium Inactivation Mediated by Phages." *Antibiotics* 10 (8): 974. https://doi.org/10.3390/antibiotics10080974.

Erhirhie, Earnest Oghenesuvwe, Chibueze Peter Ihekwereme, and Emmanuel Emeka Ilodigwe. 2018. "Advances in Acute Toxicity Testing: Strengths, Weaknesses and Regulatory Acceptance." *Interdisciplinary Toxicology* 11 (1): 5–12. https://doi.org/10.2478/intox-2018-0001.

Hardy, Karen. 2020. "Paleomedicine and the Evolutionary Context of Medicinal Plant Use." *Revista Brasileira de Farmacognosia* 31 (1): 1–15. https://doi.org/10.1007/s43450-020-00107-4.

Ismail, Zakiah, Siti Zaleha Halim, Noor Rain Abdullah, Adlin Afzan, Badrul Amini Abdul Rashid, and Ibrahim Jantan. 2014. "Safety Evaluation of Oral Toxicity OfCarica PapayaLinn. Leaves: A Subchronic Toxicity Study in Sprague Dawley Rats." *Evidence-Based Complementary and Alternative Medicine* 2014: 1–10. https://doi.org/10.1155/2014/741470.

Kendir, Gülsen, Ipek Süntar, Ali Osman Çeribaşı, and Ayşegül Köroğlu. 2019. "Activity Evaluation on Ribes Species, Traditionally Used to Speed up Healing of Wounds: With Special Focus on Ribes Nigrum." *Journal of Ethnopharmacology* 237 (June): 141–48. https://doi.org/10.1016/j.jep.2019.03.038.

Gülsen Kendir, and Ayşegül Köroğlu. 2015. "In Vitro Antioxidant Effect of the Leaf and Branch Extracts of Ribes L. Species in Turkey." *International Journal of Pharmaceutical Sciences Research* 2 (1). https://doi.org/10.15344/2394-1502/2015/108.

Khan, Sher Wali, and Surayya Khatoon. 2007. "Ethnobotanical studies on useful trees and shrubs of Haramosh and Bugrote valleys, in Gilgit Northern Areas of

Pakistan." *Pakistan Journal of Botany* 39 (3): 699–710.

Kifayatullah, Muhammad, Mohd. Shahimi Mustafa, Pinaki Sengupta, Md. Moklesur Rahman Sarker, Arindam Das, and Sreemoy Kanti Das. 2015. "Evaluation of the Acute and Sub-Acute Toxicity of the Ethanolic Extract of Pericampylus Glaucus (Lam.) Merr. In BALB/c Mice." *Journal of Acute Disease* 4 (4): 309–15. https://doi.org/10.1016/j.joad.2015.06.010.

Lewis, Richard J, Richard A Billington, Eric Debryune, A.O Gamer, B W Lang, and Francis Carpanini. 2002. "Recognition of Adverse and Nonadverse Effects in Toxicity Studies" 30 (1): 66–74. https://doi.org/10.1080/01926230252824725 .

Lorke, Dietrich. 1983. "A New Approach to Practical Acute Toxicity Testing." *Archives of Toxicology* 54 (4): 275–87. https://doi.org/10.1007/bf01234480.

Manjunatha BK, Vidya SM, Dhiman P, Pallavi R, and Mankani KL. 2005. "Hepatoprotective activity of Leucas hirta against CCl 4 induced hepatic damage in rats." *International Journal of Experimental Biology* 43(8):722-727.

Mensah Merlin LK, Gustav Komlaga, Arnold D. Forkuo, Caleb Firempong, Alexander K. Anning, and Rita A. Dickson. 2019. "Toxicity and Safety Implications of Herbal Medicines Used in Africa." *Herbal Medicine*, January. https://doi.org/10.5772/intechopen.72437.

Mukinda, James. T., and Peter. F.K. Eagles. 2010. "Acute and Sub-Chronic Oral Toxicity Profiles of the Aqueous Extract of Polygala Fruticosa in Female Mice and Rats." *Journal of Ethnopharmacology* 128 (1): 236–40. https://doi.org/10.1016/j.jep.2010.01.022.

Nath, Purobi, and Arun Yadav. 2015. "Acute and Sub-Acute Oral Toxicity Assessment of the Methanolic Extract from Leaves of Hibiscus Rosa-Sinensis L. In Mice." *Journal of Intercultural Ethnopharmacology* 4 (1): 70. https://doi.org/10.5455/jice.20141028021746.

Chibueze Nwose. 2013. "Effect of Ethanolic Leaf Extract of Kalanchoe Pinnata on Serum Creatine Kinase in Albino Rats." *Journal of Pharmacognosy and Phytochemistry* 1 (5): 08-12.

Polunin, O., Stainton, A. (1984). Flowers of the Himalayas. Oxford University Press, New Delhi, India.

Rasmussen, R.R., P.H. Rasmussen, T.O. Larsen, T.T. Bladt, and M.L. Binderup. 2011. "In Vitro Cytotoxicity of Fungi Spoiling Maize Silage." *Food and Chemical Toxicology* 49 (1): 31–44. https://doi.org/10.1016/j.fct.2010.09.007.

Singh, Gireesh Kumar, and Vikas Kumar. 2011. "Acute and Sub-Chronic Toxicity Study of Standardized Extract of Fumaria Indica in Rodents." *Journal of Ethnopharmacology* 134 (3): 992–95. https://doi.org/10.1016/j.jep.2011.01.045.

Umbaugh, David S, and Jaeschke Hartmut. 2021. "Biomarkers of drug-induced liver injury: A mechanistic perspective through acetaminophen hepatotoxicity." *Expert Review of Gastroenterology & Hepatology* 15(4): 363-375. DOI: 10.1016/j.jep.2010.01.022

Uttra, Ambreen Malik, Alamgeer, Muhammad Shahzad, Arham Shabbir, Shah Jahan, Ishfaq Ali Bukhari, and Asaad Mohamed Assiri. 2019. "Ribes Orientale: A Novel Therapeutic Approach Targeting Rheumatoid Arthritis with Reference to Pro-Inflammatory Cytokines, Inflammatory Enzymes and Anti-Inflammatory Cytokines." *Journal of Ethnopharmacology* 237 (June): 92–107. https://doi.org/10.1016/j.jep.2019.03.019.

Vetter, A, G Perera, K Leithner, G Klima, and A. Bernkop-Schnürch. 2010. "Development and in Vivo Bioavailability Study of an

Oral Fondaparinux Delivery System." *European Journal of Pharmaceutical Sciences* 41 (3-4): 489–97. https://doi.org/10.1016/j.ejps.2010.08.001.

Zacharenia Vlata, Emanuela Corsini, and Aristidis M Tsatsakis. 2021. "Modern Aspects of Immunotoxicology." *Elsevier EBooks*, January, 233–45. https://doi.org/10.1016/b978-0-323-85215-9.00045-3.

Yakubu, Musa Toyin, Musbau Adewumi Akanji and Adenike T. Oladiji. 2005. "Aphrodisiac potentials of the aqueous extract of Fadogia agrestis (Schweinf. Ex Hiern) stem in male albino rats." *Asian Journal of Andrology* 7(4):399-404. DOI:10.1124/pr.108.00001

Yakubu, Musa Toyin, Musbau Adewumi Akanji and Adenike T. Oladiji. 2007. "Hematological evaluation in male albino rats following chronic administration of aqueous extract of Fadogia agrestis stem." *Pharmacognosy Magazine* 3: 34.