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Proceeding Paper

Sub-Chronic Toxicological Evaluation of the Sesquiterpene Lactone-Enriched Fraction of *Tithonia diversifolia* (Hemsley) A. Gray in Experimental Rats †

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Abstract: The growing interest in herbal and alternative medicines demands information on the toxicity risk assessment of the various plant extracts used in traditional medicines. The rich presence of sesquiterpene lactone, a potentially toxic phytochemical, in *Tithonia diversifolia* necessitates the toxicological evaluation of its biologically active constituents. This study evaluated the in vivo sub-chronic toxicity of the moderately polar fractions of *T. diversifolia* in a rat model. The ethyl acetate soluble portion from the methanol extract was separated using the vacuum liquid chromatographic method. Three dose levels—an observed adverse effect level (OAEL) of 2000 mg/kg, a no-observed adverse effect level (NOAEL) of 80 mg/kg, and an intermediate dose of 500 mg per kg body weight of rats per day—were selected for a 28-day period of repeated dosing for the sub-chronic toxicological evaluation. The LC-MS dereplication of the active fractions showed the presence of sesquiterpene lactones such as diversifolin, diversifolin methylether, tagitinin A, tagitinin C–F, woodhousin, and orizatin, as well as many unidentified peaks. There was a significant reduction ($p < 0.05$) in the weights of the rats dosed with OAEL and their food consumption of the fraction during week 1, which normalized during the subsequent weeks of the study. The histopathological examination showed mild necrosis and degeneration of hepatocytes in the centrilobular areas of the rats treated with OAEL of the active VLC fraction. There were no *T. diversifolia*-related adverse toxicological events in rats receiving 2000 mg/kg/day when dosed orally for 28 days.

Keywords: sesquiterpene lactones; phytotoxicity; adverse effects; herbal preparation

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1. Introduction

Toxicological evaluations of phytomedicine are vital due to the high burden of drug toxicity arising from willful use, side effects, or chronic abuse of herbal medicines [1]. The incidences of drug toxicity are more common in herbal products due to unmetered or poor monitoring of their usage or unknown toxicity potential. Despite the continued reliance on phytomedicine, there are still major gaps in our understanding of the mechanism of action, incompatibility potential with orthodox medicines, adverse herbal reactions, and contraindications in their usage [2]. These issues have continued unabated in herbal medicines, including *Tithonia diversifolia* [3].

Tithonia diversifolia is an important tropical medicinal plant of the Asteraceae family, known for its richness in sesquiterpene lactones (STLs) [3]. This has increased its potentiality for toxicity due to non-selective off-targets binding and the interaction of the nucleophilic α-methylene-γ-lactone of STLs with the thiol group of proteins [3,4]. They also possess diverse pharmacological activities resulting from the structure–activity relationship, pharmacokinetics, and other known properties of the STLs.

Several important biological activities of *T. diversifolia* have been reported [5]. However, the potential to cause serious deleterious effects when formulated or used in folklore medicines has elicited interest in the investigation of its toxicological profile. This study, therefore, evaluated the in vivo sub-chronic toxicity of the moderately polar STL-enriched fraction of *T. diversifolia* in a rat model.

2. Experimental

2.1. Collection and Extraction of Plant Material

The leaves of *T. diversifolia* were collected in Enugu, Nigeria, in January 2021 and authenticated by a taxonomist, Mr. Felix Nwafor of the Department of Pharmacognosy and Environmental Medicine, University of Nigeria. A voucher specimen (ID: PCG/UN/2021/Atd) of the collection was deposited at the herbarium. The plant was dried under shade for 14 days, reduced to a coarse powder, and macerated in 95% methanol for 48 h. The filtrate was concentrated to dryness under a vacuum.

2.2. Fractionation and Chromatographic Separation

The MeOH extract (50 g) was fractionated in hexane, ethyl acetate, and butanol using a separating funnel. The EtOAc fraction containing STLs was subjected to vacuum liquid chromatographic separation using a gradient mixture of EtOAc and dichloromethane. The fraction of the separation containing STLs was used for the toxicity evaluation.

2.3. LC-MS Dereplication of the STL Fraction

The dereplication was performed using UHPLC/ESI-QTOF MS/MS with the following properties. Chromatographic separations: Dionex Ultimate 3000 RS LS System with a Dionex Acclaim RSLC 120 and C18 column; mobile phase: binary gradient (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid); flow rate: 0.8 mL/min; injection volume: 5 µL; and detection: Dionex Ultimate DAD-3000 RS over 200–400 nm wavelength.

2.4. Bioassay Dosing Schedule

The experimental rats were divided into four groups (n = 5) and treated as follows: Group 1 received 2000 mg/kg of STL fraction (observed adverse effect level), group 3 received 80 mg/kg of STL fraction (no-observed adverse effect dose), group 2 received 500 mg/kg (25% of OAEL), and group 4 was the untreated control.

2.5. Toxicological Evaluation

A general toxicological evaluation, clinical pathological examination, and histopathological studies were conducted, following the reported protocols [6–9].

2.6. Data Analysis

Data are presented as mean ± SEM, (n = 5). Variation among groups was assessed by ANOVA followed by a post hoc two-sided Dunnett's test. In all cases, $p < 0.05$ was accepted as statistically significant.

3. Results and Discussion

3.1. Phytochemical Analysis

The LC-MS chromatogram (Figure 1) of STL fraction identified prominent peaks that matched the fragmentation patterns, retention time, and/or UV spectra of some of the known STLs hypothesized in a previous study, such as diversifolin, diversifolin methylether, tagitinin A, tagitinin C–F, woodhousin, and orizatin [10].

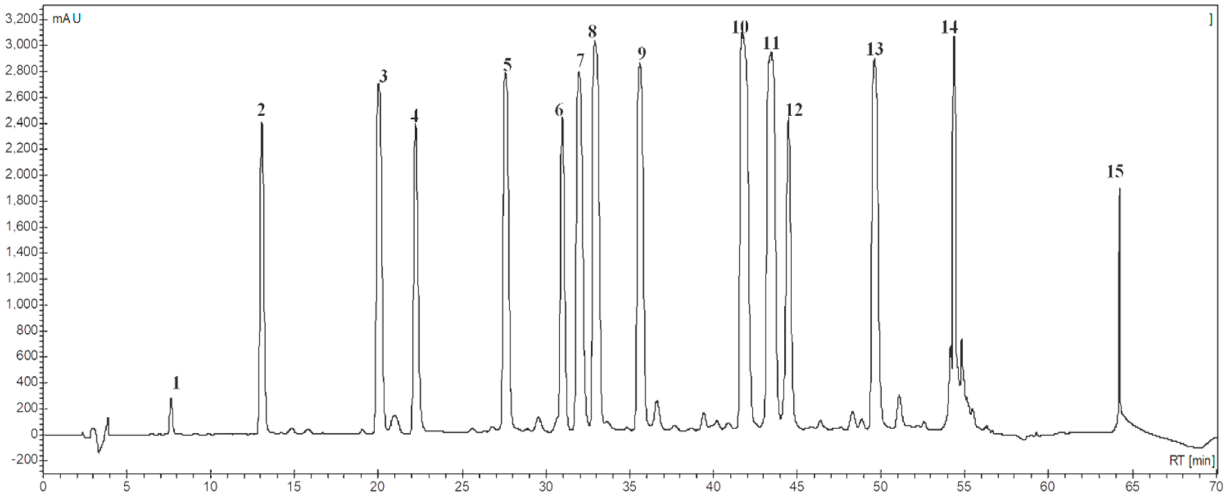

Figure 1. UHPLC–MS of VLC–STLs showing possible STLs. Peaks represent base peak chromatograms; c = 10 mg/mL; *m*/*z* 50–1500 Da. Orizatin (**3**), tagitinin A (**5**), tagitinin E (**6**), tagitinin C (**7**), diversifolin (**8**), tagitinin F (**10**), tagitinin D (**11**), woodhousin (**12**), anddiversifolin methylether (**13**).

3.2. Toxicity of STL Fraction

Apart from the group 1 rats, which exhibited delayed fecal evacuation within the first week of the study, there was no treatment-related weight loss, food intake, or fecal excretion. All the vital signs were stable throughout the study. The alcoholic extract and saponins-rich extract of the plant have demonstrated high safety profiles in 21-day toxicological studies [11].

3.3. Clinical Pathological Examination

The liver is an important detoxification point due to the presence of various metabolizing enzymes. In the hepatic enzyme parameters, there were no significant differences between the ALP, AST, and ALT of the highest-dosed rats compared to the untreated group of rats (Table 1). These enzymes are located in the hepatic cells and are usually released into the blood plasma when the liver is compromised.

Table 1. Clinical pathological effects of VLC-STLs on experimental rats.

Parameter/Groups	1	2	3	4
AST (i/uL)	11.85 ± 0.05^{a}	11.47 ± 0.08^{a}	10.90 ± 0.46^{b}	11.93 ± 0.06^{a}
ALT (i/uL)	11.77 ± 0.17^{a}	11.49 ± 0.02^{b}	10.92 ± 0.16^{c}	11.97 ± 0.09^{a}
ALP (iu/L)	51.79 ± 1.81^{a}	47.22 ± 2.41^{b}	42.76 ± 3.64^{c}	53.33 ± 3.19^{a}
PCV (%)	40.67 ± 1.15^{a}	39.33 ± 1.15^{a}	38.67 ± 1.53^{a}	40.67 ± 1.15^{a}
RBC ($\times 10^{6}/\mu L$)	7.33 ± 0.58^{a}	7.43 ± 0.60^{a}	6.67 ± 0.42^{b}	7.50 ± 0.50^{a}
Hb (g/dL)	10.24 ± 0.35^{a}	9.91 ± 0.22^{a}	9.05 ± 0.89^{b}	10.47 ± 0.11^{a}
WBC ($\times 10^{6}/\mu L$)	9.00 ± 0.87^{a}	7.73 ± 0.50^{b}	7.27 ± 0.64^{b}	9.40 ± 0.53^{a}

Data are expressed as mean ± SEM (n = 5). ^{a,b,c} Values across rows with the same superscripts are not statistically different. $p > 0.05$ when compared with the untreated group.

3.4. Histopathological Examination of Liver

The liver sections in groups 2–4 presented normal histo-architecture of the liver, with mild necrosis and/or degeneration of the hepatocytes in the centrilobular areas of the rats in groups 2 and 3 (Figure 2). The affected hepatocytes appeared to be swollen, with clear vacuolated cytoplasm and pyknotic nuclei. The livers of rats in group 1 showed marked vacuolar degeneration and necrosis of the hepatocytes in the centrilobular and mid-zonal areas of the hepatic lobules (arrow).

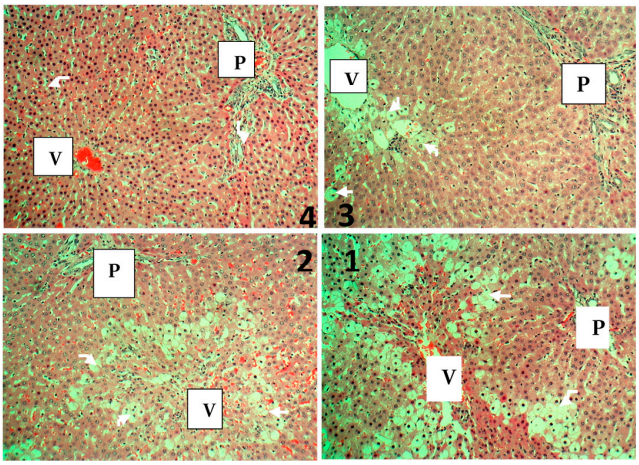

Figure 2. Liver sections of rats in groups 4 (normal), 3 (mild degeneration), 2 (mild necrosis), and 1 (marked degeneration). P represents the portal triads of the hepatic vein, hepatic artery, and bile ducts. V is the central vein. Arrows point to the hepatic lobules.

4. Conclusions

There were no *T. diversifolia*-related adverse toxicological events in rats. An observed-adverse-effect level (OAEL) of 2000 mg/kg/day was observed when dosed orally for 28 days.

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Institutional Review Board Statement: The permission to use the animals for this study was reviewed and granted by the University of Nigeria ethical committee (Reference No.: FPSRE/UNN/2021/00015) for research. The study was conducted per the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or the US guidelines (NIH publication #85-23, revised in 1985).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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