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. The 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 by 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 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 and many unidentified peaks. There was a significant reduction ($p < 0.05$) in the weights of and food consumption by the rats dosed with OAEL of the fraction on 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 with a 2000 mg/kg/day when dosed orally for 28 days.

Introduction

Toxicological evaluation of phytomedicine is 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 understanding 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 their potentiality for toxicity due to the 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. The study, therefore, evaluated the *in vivo* sub-chronic toxicity of the moderately polar STL-enriched fraction of *T. diversifolia* in a rat model

Experimental

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 the 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.

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.

LC-MS dereplication of the STL fraction

The dereplication was performed with UHPLC/ESI-QTOF MS/MS of 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; detection: Dionex Ultimate DAD-3000 RS over 200-400 nm wavelength.

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 represented untreated control.

Toxicological evaluation

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

Data analysis

Data were presented as mean ± SEM, (n=5), variation among groups by ANOVA followed by post-hoc using a 2-sided Dunnett's test. In all cases, a $p < 0.05$ was accepted as statistically significant.

Results and Discussion

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].

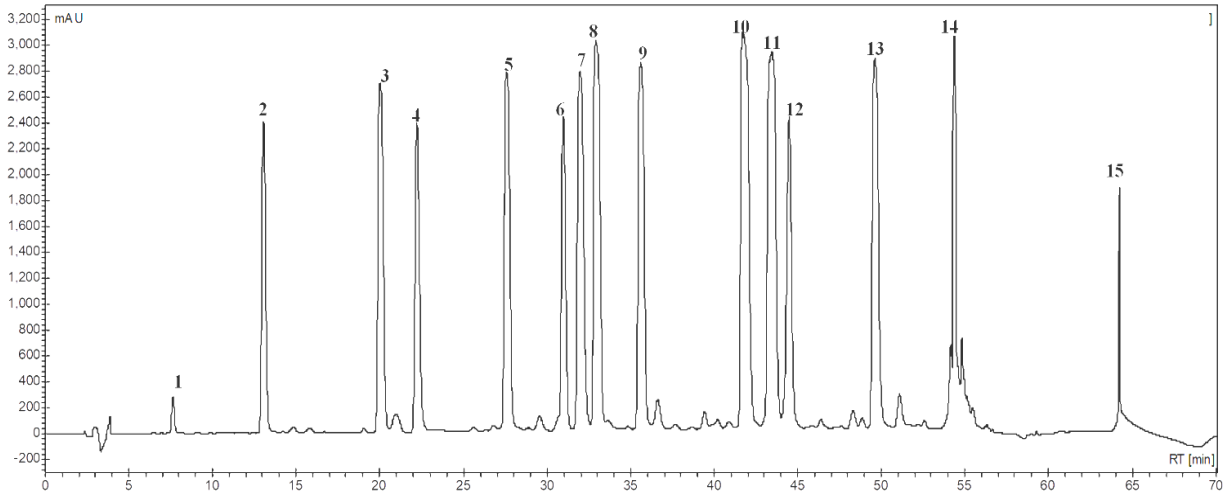

Figure 1. UHPLC-MS of VLC-STLs showing possible STLs; peaks represent base peak chromatograms; c= 10 mg/mL; m/z 50-1500 Da. Orizatin (**3**), tagitinin A (**5**), tagitinin E (**6**), tagitinin C (**7**), diversifolin (**8**), tagitinin F (**10**), tagitinin D (**11**), woodhousin (**12**), diversifolin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

Toxicity of STL fraction

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

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 with the untreated group of rats. 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 ($x10^{6}/\mu L$	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 ($x10^{6}$ /μL)	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 the row with the same superscript are not statistically different. ($p > 0.05$) when compared with the untreated group.

Histopathological examination of liver

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

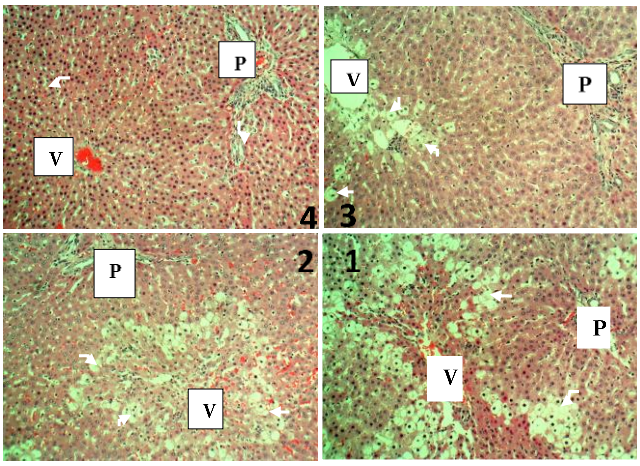

Figure 2. Liver section of rats in groups 4 (normal), 3 (mild degeneration), 2 (mild necrosis), and 1 (marked degeneration). P represents the portal triads of the hepatic vein; hepatic artery and bile ducts. V is the central vein. Arrows point to the hepatic lobules.

Conclusion

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

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