Available online at http://www.ajol.info/index.php/njbas/index
Nigerian Journal of Basic and Applied Sciences (June, 2022), 30(1):77-80
DOI: http://dx.doi.org/10.4314/njbas.v30i1.11
ISSN 0794-5698

Full Length Research Article

In-vitro Cytotoxicity Effects and Bioactive Constituents of Chloroform Extract of *Vernonia glaberrima* Welw. Ex O. Hoffm (Asteraceae)

[1]A. M. Alhassan, [1]A. Uba, [2]C. Muhammad, [2]H. L. Bako, [3]I. Malami, [4]Q. U. Ahmed, [5]Y. I. Alkali
[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria
[2]Department of Pure and Applied Chemistry, Faculty of Science, Usmanu Danfodiyo University, Sokoto, Nigeria.
[3]Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria
[4]Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Pahang DM, Malaysia
[5]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical sciences, Usmanu Danfodiyo university, Sokoto, Nigeria
[*Corresponding Author: E-mail: alhasanma@yahoo.com]

ABSTRACT
Vernonia glaberrima plant is used traditionally in the treatment of cancer, diabetes and malaria in North central Nigeria. This study was designed to evaluate the cytotoxic effect of chloroform extract of *Vernonia glaberrima* and isolate some bioactive constituents. Neutral red cytotoxicity assay was performed using breast cancer (MCF7), cervical cancer (HeLa) and Liver cancer (HepG2) cells upon exposure to predetermined concentrations of the chloroform extract. The cell viability was determined colorimetrically after 72 hours of incubation. The extract was fractionated using silica gel column chromatography and preparative TLC. Isolated compounds were characterized using FTIR and NMR spectroscopy. The extract inhibited 50% cell proliferation of MCF7, HeLa and HepG2 cells at an IC_{50} value of 14.02 ± 0.03, 15.78. ± 0.04, and 16.77± 0.1 µg/mL, respectively. Two compounds namely, lupeol (1) and betulinic acid (2) were isolated from the extract. The two isolated compounds have been previously reported in literature to possess anticancer activities. Hence, the findings from this study demonstrate the potential anticancer properties of chloroform extract of *Vernonia glaberrima*.
Keywords: Cytotoxicity, *Vernonia glaberrima*, Cancer, Natural product, Drug discovery

INTRODUCTON
Cancer is a devastating disorder and a major cause of morbidity and mortality. It is the second leading cause of death after cardiovascular disorders (Rumgay *et al*., 2021). In the year 2020, an estimated 19.3 million new cancer cases and 10.0 million cancer related mortality worldwide was reported (Sung *et al*., 2021). The prevalence of cancer in Nigeria is very high, with about 250,000 new cases and 10,000 cancer deaths being recorded every year (Baba and Hincal, 2018). Medicinal plants have been in use for centuries as source of a wide variety of biologically active compounds. Plant products in the form of powder, decoction or ointments are extensively used to treat diverse ailments by generations of traditional medicine practitioners (Alhassan and Ahmed, 2016). Indigenous plants that are used in the traditional medicinal practice represent an important yet underutilized source of bioactive natural products. Natural products provide unlimited opportunities for new drug discovery because of their availability and unmatched chemical diversity.

Vernonia glaberrima (Asteraceae)*;* Synonym: Shìwaakar-jan-gagari (Hausa language)*,* is a locally available plant found in North Central Nigeria. It is used traditionally in the treatment of several disorders including malaria, diabetes, menstrual pain, cancer and skin diseases (Burkill, 1985; Alhassan *et al*., 2018). Several scientific studies have been carried out on the extracts of this plant in an effort to verify these traditional claims. Previous researches have shown that *V. glaberrima* extract has significant activity against diabetes mellitus, malaria and inflammation (Abdullahi *et al*., 2015a; Abdullahi *et al*., 2015b; Abdullahi *et al*., 2015c). Methanol extract of *V. glaberrima* has been reported to exhibit significant cytotoxic effect against skin cancer cell lines (A375) while it was relatively less active against breast and colon cancer cell lines (Alhassan *et al*., 2018). Considering the clinical and economic importance of cancer, this study investigated the anticancer potential of chloroform extracts of *V. glaberrima* on a panel of cancer cell lines as well as isolation of some phytoconstituents.

MATERIALS AND METHODS
Cell lines and Reagents
The cell lines -breast cancer (MCF7), cervical cancer (HeLa) and Liver cancer (HepG2) cells were bought from American Tissue Culture Collection, United States of America (ATCC, USA). All solvents were of analytical grade and purchased from Merck (Merck KGaA, Darmstadt, Germany). Penicillin, streptomycin, EMEM media were purchased from Gibco (USA) while the Neutral red cell cytotoxicity assay kit was bought from BioVision, Inc., USA**.**

Collection of Plant Material and Authentication
Leaves of *V. glaberrima* were collected in September, 2019 in Nasarawa LGA of Nasarawa State. The leaves were authenticated by Dr Ibrahim Malami of Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto. The sample of the plant was deposited in the Herbarium with reference number, PCG/UDUS/ASTE/003. The leaves where dried, grinded to powder form and stored in plastic bags prior to use.

Extraction of Plant Material
Powdered plant material (400 g) was placed in an extraction flask and macerated with hexane (2 L) for 72

hours to remove the fatty material. It was then further extracted with chloroform (2 L) for 72 hours at room temperature. The supernatant was collected, filtered and the solvent was evaporated at 40°C using rotary evaporator to obtain the chloroform extract. The process was repeated to ensure exhaustive extraction was achieved while residual solvent was allowed to evaporate in an open beaker for three days.

Cell Culture

The MCF7 (Breast), HeLa (Cervical) and HepG2 (Liver) cell lines were sub-cultured in EMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were maintained at a temperature of 37 °C in a humidified incubator maintained on 5% CO_2. The cells were harvested by trypsinization at 70%–80% confluence for investigation.

Cytotoxicity Assay

Neutral red cytotoxicity assay was performed according to the protocol as described by Repetto *et al*. (2008) with slight modifications. The experiment was conducted in Centre for Advanced Medical Research and Training (CAMRET), UDUS. Briefly, MCF7, HeLa and HepG2 cells were seeded in 96-well plate at a concentration of 0.5×10^4 cells/well and incubated overnight in a humidified CO_2 incubator at 37°C. After overnight incubation, cells were treated with chloroform extract of *V. glaberrima* in a serially diluted concentrations starting with 200, then 100, 50, 25, 12.5 and 6.25 μg/mL and incubated at 37°C in a for another 72 h. DMSO (0.1%, v/v) was used as a negative control. After 72 h, all media was aspirated, well containing cells were washed with PBS and 100 µL of neutral red media was added into each well containing cells. The plates were again incubated at 37°C for 2 h. After 2 h, all neutral red media was aspirated, washed with PBS and 100 µL of destaining solution was added into each well. The plates were shaken for 10 min and the absorbance was read at 630 nm using a microplate reader. All experiments were conducted in triplicates. The percentage inhibition was expressed as the number of viable cells to the total cell population and the potency of the extract to inhibit cell proliferation by 50% was expressed as IC_{50}.

Isolation and Characterization of Phytoconstituents

The dried chloroform extract (10.5 g) was adsorbed in silica gel, dried and loaded unto an open chromatographic column already packed with silica gel (60-120 mesh size). The column was eluted in gradient mode using the following solvent systems; hexane/ethylacetate 100:0, 99:1, 95:5, 90:10 and 80:20. The fractionation process was monitored using TLC silica gel 60 F_{254} (solvent system: hexane/ethyl acetate 9:1). Sixty-four fractions were collected. The fractions were combined into 10 sub fractions based on similarity of their TLC profile. The sub fractions were labelled A – J. Sub-fraction I (combined fractions 38 – 50; solvent system: hexane/ethylacetate 95:5) was subjected to further fractionation. The sub-fraction (1.2 g) was subjected to another column chromatography for further purification. The column was eluted in a gradient mode using chloroform/hexane 10:90, 20:80, 30:70, 40:60, 50:50 and 60:40. Fifty fractions were collected. Fractions 9 – 36 were combined based on similarity of their TLC profile and dried to yield a 200 mg yellowish whitish isolate. The TLC analysis of the isolate reveal the presence of two major spots with traces of chlorophyll. The isolate was then subjected to preparative TLC (solvent system: hexane/ethyl acetate 9:1) to yield two compounds labeled compounds 1 and 2.

FT-IR analysis of the isolated compounds was done using the ATR mode (Perkin-Elmer IR spectrometer) while ^1H and ^{13}C-NMR spectra of the compounds dissolved deuterated chloroform were obtained on an Avance III Bruker Spectrometer at 600 and 150 MHz, respectively.

Statistical Analysis

The cytotoxicity experiment was conducted in triplicate. The potency of the extract to inhibit cell proliferation by 50% (expressed as IC_{50}) was determined using non-linear regression analysis. The GraphPad Prism software version 9.30 was used for the analysis.

RESULTS AND DISCUSSION

Cell Viability Studies

Figure 1 shows the effect of extract concentration on the viability of the three cancer cell lines. The percentage cell viability was determined in MCF7, HeLa and HepG2 cells using neutral red uptake assay. The cell viability was measured colorimetrically after 72 h of incubation in the presence of chloroform extract of *V. glaberrima*. The extract inhibited 50% cell proliferation of MCF7, HeLa and HepG2 cells at an IC_{50} value of 14.02 ± 0.03, 15.78 ± 0.04, and 16.77 ± 0.1 µg/mL respectively. The results indicate a dose dependent increase in inhibitory activity of the extract on all cell lines tested.

There have been limited studies on the anticancer properties of *V. glaberrima*. Although, research has shown that methanol extract of this plant inhibited the *in vitro* proliferation of skin cancer cell lines (A375) with IC_{50} of 26.23 µg/mL (Alhassan *et al*., 2018), the cytotoxic effect of chloroform extract of this plant on MCF7, HeLa and HepG2 cells is being reported for the first time. The IC_{50} values of the extract against all the three cancer cell lines are less than 20 µg/mL which is an indication of the potency of the plant extract against the cancer cells.

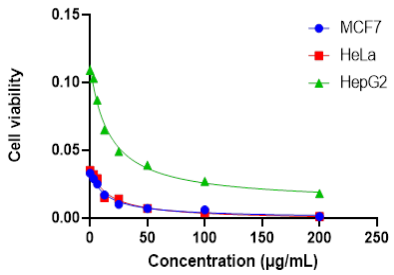

Figure 1. Cell viability of MCF7, HeLa and HepG2 cells treated with crude chloroform extract of *V. glaberrima* at different concentrations.

Isolation and Characterization

The separation of chloroform extract using open column chromatography afforded the isolation of two lupane triterpenoids namely, lupeol (**1**), and betulinic acid (**2**). The structures of these compounds are shown in Figure 2. The structures of the compounds were elucidated using FTIR and ^{1}H and ^{13}C NMR spectroscopy. The results obtained were also compared with the data earlier reported on the compounds.

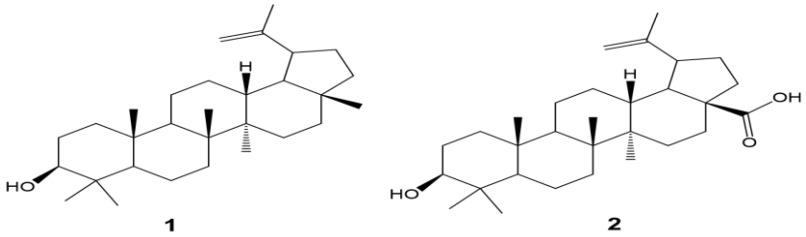

Figure 2: Structures of isolated compounds

Compound 1: White crystals (120 mg); FTIR : 3310.80 (OH-stretching), 2939.0 (CH-stretching), 1639.6 (C=C stretching), and 1387.0 and 1378.0 cm-1(OH-bending); ^{1}HNMR [600MHz, $CDCl_3$, δ (ppm)]: 3.21 (1H, dd, J = 11.4 Hz, 4.8Hz, H-3), 2.42 (1H, dt, J = 11.2 Hz, 5.4 Hz, H-19), 1.39 (1H, m, H-6), 0.99 (3H, s, H-23), 0.79 (3H, s, H-24); 0.86 (3H, s, H-25), 1.06 (3H, s, H-26), 0.97 (3H, s, H-27), 0.81(3H, s, H-28), 4.58 (1H, d, J = 2.4 Hz, H-29α), 4.71 (1H, d, J = 1.8 Hz, H-29β), 1.70 (3H, s, H-30), ^{13}C-NMR [$CDCl_3$, 150MHz, δ (ppm)]: δ 38.9 (C-1), 27.4 (C-2), 79.0 (C-3), 38.8 (C-4), 55.4 (C-5), 18.3 (C-6), 34.3 (C-7), 40.9 (C-8), 50.5 (C-9), 37.2 (C-10), 21.0 (C-11), 25.2 (C-12), 38.1 (C-13), 42.8 (C-14), 27.5 (C-15), 35.6 (C-16), 43.2 (C-17), 48.0 (C-18), 48.34(C-19), 151.0 (C-20), 29.9 (C-21), 40.0 (C-22), 28.0 (C-23), 15.4 (C-24), 16.0 (C-25), 16.1 (C-26), 14.6 (C-27), 18.0 (C-28), 109.3 (C-29), 19.3 (C-30) (Kang *et al*., 2013)

Compound 2: White powder (35 mg); FTIR: carboxyl OH stretching (broad) at 2939.29 (OH – stretch of COOH), 1683.69 (C=O stretching), 1233.60 (C-O stretch) and 881.75 cm^{-1} (CH_3 bending); ^{1}HNMR [600MHz, $CDCl_3$, δ (ppm)]: 3.21 (1H, dd, J = 11.4 Hz, 6.0 Hz, H-3), 3.02 (1H, td, J = 10.8, 4.8 Hz, H-18), 1.00 (3H, s, H-23), 0.85 (3H, s, H-24), 0.78 (3H, s, H-25), 0.97 (1H, s, H-26), 4.76 (1H, s, H-29α), 4.63 (1H, s, H-29β), 1.72 (3H, s, H-30), 13C-NMR [CDCl3, 150MHz, δ (ppm)]: δ 38.8 (C-1), 28.0 (C-2), 79.0 (C-3), 36.6 (C-4), 55.4 (C-5), 18.3 (C-6), 34.4 (C-7), 40.7 (C-8), 50.6 (C-9), 37.2 (C-10), 20.9 (C-11), 25.5 (C-12), 38.7 (C-13), 42.5 (C-14), 30.6 (C-15), 32.1 (C-16), 56.2 (C-17), 46.9 (C-18), 49.3(C-19), 150.3 (C-20), 30.5 (C-21), 37.0 (C-22), 28.7 (C-23), 16.0 (C-24), 16.1 (C-25), 16.5 (C-26), 15.3 (C-27), 179.4 (C-28), 109.6 (C-29), 19.4 (C-30) (Bisoli *et al*., 2008).

Lupeol (**1**) was obtained in relatively higher amounts (120 mg), suggesting the isolate is a major phytoconstituent present in the chloroform extract compared to betulinic acid (**2**) which was obtained in a relatively small amount (35 mg). Isolation of Betulinic acid from *V. glaberrima* is being reported for the first time while lupeol was previously reported (Alhassan *et al.,* 2018) to be present in the plant. Both compounds are lupane-type triterpenoids with similar structure. The only difference is that the methyl group at position 28 of lupeol is being replaced by a carboxiylic acid group in betulinic acid. Previous studies have shown that lupeol possess antiproliferative effects against breast cancer cells (MCF7) (Pitchai *et al*., 2014), hepatocellular carcinoma (HCC) cell lines (Liu *et al*., 2021) and human cervical carcinoma (HeLa) cells (Prasad *et al*., 2018). Betulinic acid has also been shown to inhibit the proliferation of hepatocellular carcinoma (Liu *et al*., 2019). Based on the reported effects of these compounds, it is likely that these compounds play major roles in the observed cytotoxic effects of the chloroform extract of *V. glaberrima*.

CONCLUSION

The cytotoxic effects of the chloroform extract of *V. glaberrima* on MCF7, HeLa and HepG2 cells demonstrate the anticancer potential of this plant. The presence of lupeol and betulinic acid may partly account for the observed bioactivity. Other yet to be identified constituent which are

present in the chloroform extract tested could have contributed to the observed cytotoxic effect. Further phytochemical investigations are needed to identify other constituents present in the extract that could be active against cancer cell lines. The finding of this study serves as a prelude for further investigations towards the discovery of new anticancer medication from *V. glaberrima* either as single chemical entity or standardized herbal preparation.

ACKNOWLEDGEMENTS

This research was funded by Tertiary Education Trust Fund (TETFund) through Institutional Based Research (IBR) Grant 2012-2016, 4th Batch Research Project Intervention Disbursement allocated to Usmanu Danfodiyo University, Sokoto, Nigeria.

CONFLICT OF INTEREST

The authors wish to state that there is no conflict of interest.

REFERENCES

Abdullahi, M. I., Uba, A., Umar, A., Alhasan, A. M., Yusuf, A.J., Yunusa, A., Bello, S.S., Nasir, I., Ibrahim, Z.Y.Y., Musa, K. M., Aminu, M. (2015a). Studies on phytochemical constituents, toxicity, analgesic and anti-inflammatory activities of *Vernonia glaberrima*. *Der Pharma Chemica*, **7**: 273–278.

Abdullahi, M. I., Uba, A., Yaro, A., Maxwell, O., Yusuf, A. J. and Kabir, S. (2015b). Phytochemicalscreening, acute toxicity study and evaluation of antidiabetic properties of the methanolicleaf extract of *Vernonia glaberrima* (Asteraceae). Journal of *Pharmaceutical, Chemical and Biological Sciences,* **3**: 169–177.

Abdullahi, M. I., Uba, A., Yunusa, A., Kaita, H. A., Yaro, A. H., Hassan, S., Alhassan, A. M., Umar, A., Bello, S. S., Nasir, I., Ibrahim, Z.Y.Y., Alebiosu, C. O. and Yusuf, A. J. (2015c). Evaluation of the anti-malarial effect of the methanolic leaf extract of Vernonia glaberrima (Asteraceae). *IOSR Journal of Pharmacy and Biological Sciences,* **10**:2319–7676.

Alhassan, A. M. and Ahmed, Q.U. (2016). *Averrhoa bilimbi* Linn.: A review of its ethnomedicinal uses, phytochemistry, and pharmacology. *Journal of Pharmacy and Bioallied Sciences*, **8**(4): 265-271.

Alhassan, A. M., Ahmed, Q. U., Latip, J., Shah, S. A. A., Khan, A. a. Y. F., Sarian, M. N., Wahab, R. A.; Taher, M.; Abdullahi, M. I.; Khatib, A. (2018). Phytoconstituents from Vernonia glaberrima Welw.Ex O. Hoffm.Leaves and their Cytotoxic Activities on a Panel of Human Cancer Cell Lines. *South African Journal of Botany*, **116**: 16-24.

Baba, I. A. and Hincal, E. (2018). Cancer incidence in Nigeria and border countries. *Malaysian Journal of Medical and Biological Research,* **5**(1): 7-12.

Bisoli, E., Garcez, W. S., Hamerski, L., Tieppo, C. and Garcez, F.R. (2008). Bioactive pentacyclic triterpenes from the stems of combretum laxum. *Molecules*, **13**(11): 2717–2728.

Burkill, H. M. (1985). Vernonia GlaberrimaWelw. ex O. Hoffm. Useful Plants West Tropical Africa. vol. 1.

Kang, S. C., Lim, S. Y. and Song, Y. J. (2013). Lupeol is one of active components in the extract of chrysanthemum indicumlinne that inhibits LMP1-induced NF-κB activation. *PLoS One,* **8**: e82688.

Liu, K., Zhang, X., Xie, L., Deng, M., Chen, H., Song, J. and Luo, J. (2021). Lupeol and its derivatives as anticancer and anti-inflammatory agents: Molecular mechanisms and therapeutic efficacy. *Pharmacological Research,***104**:105373.

Liu, W., Li, S., Qu, Z., Luo, Y., Chen, R., Wei, S. and Wang, Q. (2019). Betulinic acid induces autophagy-mediated apoptosis through suppression of the PI3K/AKT/mTOR signaling pathway and inhibits hepatocellular carcinoma. *American Journal of Translational Research*, **11**(11): 6952-6964.

Pitchai, D., Roy, A. and Ignatius, C. (2014). In vitro evaluation of anticancer potentials of lupeol isolated from Elephantopus scaber L. on MCF-7 cell line. *Journal of Advanced Pharmaceutical Technology & Research*, **5(**4): 179-184.

Prasad, N., Sabarwal, A., Yadav, U. C. and Singh, R. P. (2018). Lupeol induces S-phase arrest and mitochondria-mediated apoptosis in cervical cancer cells. *Journal of biosciences,* **43**(2): 249-261.

Repetto, G., Del Peso, A. and Zurita, J. L., 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature protocols, **3**(7):1125-1131.

Rumgay, H., Shield, K., Charvat, H., Ferrari, P., Sornpaisarn, B., Obot, I., Islami, F., Lemmens, V.E., Rehm, J. and Soerjomataram, I. (2021). Global burden of cancer in 2020 attributable to alcohol consumption: A population-based study. *The Lancet Oncology*, **22**(8): 1071-1080.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. and Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *A Cancer Journal for Clinicians*, **71**(3): 209-249.