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Chromatographic Analysis and *In Vitro* Cytotoxic Properties of Different Root Extracts of *Hermannia geniculata* Eckl. & Zeyh on Vero, HepG2 And RAW 264.7 Macrophage

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SUMMARY

Hermannia geniculata is widely used in the management of several illnesses in South African traditional medicine. Chromatographic analysis, in vitro cytotoxicity, and biological activities of secondary metabolites present in *Hermannia geniculata* root extracts were investigated. Vero monkey kidney cells, human hepatocellular carcinoma (HepG2) cells, and RAW 264.7 macrophage cell lines were used to determine the cytotoxicity of the extracts using MTT assay. The capabilities of the plant extracts to inhibit 5-lipoxygenase enzyme activities, the overproduction of nitric oxide (NO) following lipopolysaccharide (LPS)-activated RAW 264.7 macrophages by the ethanol extract was evaluated. Results showed selective toxicity of the extracts with LC_{50} values of Vero cells ranging from (0.40-0.57 mg/mL) while the LC_{50} value of HepG2 cells varies between (0.016-0.136 mg/mL). The selectivity indexes (SI) were recorded (31.87, 18.87, 33.33, and 13.52) for ethanol, hydro-ethanol, decoction, and aqueous extracts respectively. The ethanol extract inhibited NO production in a concentration-dependent manner showing a decrease of 82% at a concentration of 0.1 mg/mL. Its LC_{50} value (3.64 mg/mL) is lower and significantly different ($p<0.05$) compared to quercetin (standard) with an LC_{50} (8.28 mg/mL). Similarly, the ethanol extract is a potent inhibitor of 5-lipoxygenase enzyme with the lowest IC_{50} value of 0.14 mg/mL which is significantly different ($p<0.05$) from other extracts and indomethacin (standard). The GC-MS chromatograms of the ethanol extract revealed five principal compounds that have been reported to have antioxidant, anti-inflammatory, and antifungal properties. This result indicated that *Hermannia geniculata* root extracts is not toxic to Vero and RAW 264.7 macrophage cell lines and toxic to HepG2 cell lines used in this experiment, it may also possesses antiiflammatory and antiploriferative activities which could be exploited in the development of new, safer, and efficacious drugs.

Keywords: Cytotoxicity; Nitric oxide; 5-lipoxygenase; *Hermannia geniculata,* Antioxidant GC-MS.

INTRODUCTION

Phytotoxicology describes the clinical and experimental aspects of plant poisoning which may lead to different pathological conditions in humans and animals (Mecina *et al*., 2016). Medicinal plants contain secondary metabolites that have variable pharmacological properties used in the treatment of diseases. It is acceptable worldwide due to its effectiveness, affordability, and availability in managing several ailments (Ogundajo *et al*., 2018). However, herbal remedies are generally accepted to be safe and totally free from any adverse side effects but studies have shown safety concerns on the use of medicinal plants (Kooti *et al*., 2017; Tariq *et al*., 2018). An early record of sweet clover poisoning in cattle which led to hypothrombinemia has demonstrated the toxicological effect of plant metabolites on heamatological indices in animals (Botha and Penrith, 2008). Moreover, aqueous extracts of mistletoe from kola administered for 14 days causes anaemia, hypoglobinemia, and platelet count reduction in tested animals (Ladokun *et al*., 2015). *In vivo* toxicological studies conducted on some plant phenolic compounds induce break in the DNA double-strand, mutation of DNA adduct, and chromosomal disorder, therefore, causing cancer, papilloma and acts as carcinogens (Aljabr *et al*., 2017). Re-evaluation of daouri and juniper tar used in the management of skin diseases occurs due to the results of experimental studies that revealed its nephrotoxicity (Ghorani-Azam *et al*., 2018). Medicinal plants that can be considered safe for use should have low toxicities and show no adverse effect on the consumers. Thus, long and short term effect of biologically active phytochemicals on the genome, cells, tissue, organs, and the body system is required in order to increase confidence in the safety to human and also in the development of pharmaceutical. The use of *in vitro* cytotoxicity assays is important in evaluating the toxicity of biologically active components of plants' secondary metabolites which may be of pharmaceutical interest (Mcgaw *et al*., 2014).

Many Plants' phytochemicals have proven to be effective in inhibiting overproduction of nitric oxide (NO) a signaling molecule which is useful to the body at a normal concentration but becomes pathological when its production is unregulated which may cause cell death, neurodegeneration and contribute to ischemic reperfusion injury. Also, 5- lipoxygenase enzyme has been implicated in the pathogenesis and progression of inflammatory diseases, diabetes and cancer (Fukumura *et al*., 2006; Sharma *et al*., 2007; Xu *et al*., 2012).

Hermannia geniculata is a flowering plant in the family *Malvaceae*. It is a tropical plant endemic in South Africa and found worldwide (Gwynne-Evans, 2015). It is commonly used in South Africa to treat diabetes, diarrhea in pregnant women, ulcer and skin diseases (Kazeem and Ashafa, 2015). This study was carried out to evaluate

the cytotoxic effect of *Hermannia geniculata* root extracts to determine its safety and other pharmacological activities.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, and sodium-pyruvate (Hyclone Laboratory Inc, United Kingdom), fetal bovine serum (Capricorn Scientific Gmbh, South Africa). Penicillin, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), streptomycin, ethanol, dimethyl sulfoxide (DMSO), and doxorubicin hydrochloride were obtained from (Pfizer, South Africa). Griess reagent containing N-(1-naphthyl)ethylenediamine dihydrochloride, and 2% sulfanilamide in 5% phosphoric acid was purchased from (Thermo Fisher, Leiden, Netherland)

Plant Collection

Hermannia geniculata roots were purchased from a registered herbal vendor in Puthaditjhaba, Sesting market, Qwaqwa, Northern Free State Province, South Africa. Confirmation of the species identity was carried out with the herbarium specimen with voucher specimen file number (5056.000-10700) (Moffett, 1993) at the University of Free State, Qwaqwa Campus, South Africa. It was also compared with our earlier Voucher specimen (Ash/med/05/2013/QwHB) (Kazeem and Ashafa, 2015) at the herbarium.

Preparation and Extraction

The roots were washed with water to remove all debris and chopped into small pieces. It was further air dried to a constant weight. The dried root material was pulverized into fine powdered using waring laboratory blender (Labon, Durban, South Africa).

The dried powdered material (30 g each) was extracted in 300 mL of distilled water (aqueous), hot water at 40ºC (decoction), ethanol and hydro-ethanol (1/1, v/v) hydroethanolic) , with constant shaking on Labcon platform shaker (Laboratory Consumables, PTY, Durban, South Africa) for 72 hours and the mixture was filtered using Whatman No. 1 filter paper (Sigma-Aldrich, Germany). The extracts were concentrated to dryness *in vacuo* at 40º C using a rotary evaporator (Cole-Palmer, South Africa).

GC–MS analysis of the ethanol extract

The GC-MS analysis of the chemical constituents in the ethanol extract of *Hermannia geniculata* roots was carried out on Agilent Technologies 6890 Series gas chromatography together with an Agilent 5973 Mass Selective detector which is operated by Agilent Chemstation software using a described method (Adam, 2007). The column was an Agilent eHP-5MS capillary column (30m× 0.25mm internal diameter, 0.25 µm film thickness). Ultra-pure helium was used as the carrier gas at a flow rate and linear velocity of 0.57 mL/min and a 27.5 cm/sec respectively. The injector temperature was fixed at 250 °C. The initial oven temperature (50 °C), was set to rise to 250 °C at the rate of 15 °C/min and hold

time of 4 min at each increment. Injections of 1 μL were made in the splitless mode with a split ratio of 20:1. The mass spectrometer was run in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating factors were set appropriately. Identification of the components was accomplished by matching the mass spectral data, retention times and fragmentation pattern of the unidentified components of the sample analyzed with those from Wiley libraries and National Institute of Standards and Technology (NIST).

Cell culture of the Vero and HepG2

The African green monkey kidney (Vero) cell lines, and Human Hepatocellular carcinoma ((HepG2 cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 units/ mL penicillin and 100 µg/ mL streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2 for 24 h.

Cytotoxicity of Vero and HepG2 cells

Viable cells growth after incubation of Vero and HepG2 cells incubated with *Hermannia geniculata* extracts were determined using the tetrazolium-based colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay (Mosmann, 1983). Briefly, The Vero and HepG2 cells were plated at a density of 10^4 cells per well in 96-well microtitre plates. After seeding it was ensured that the cells got 80% confluency within 24- 48 h then the cells were treated with increasing concentrations of the extracts dissolved in dimethyl sulfoxide (DMSO) and diluted in the fresh culture medium. During each experiment, the maximal concentration of DMSO in the medium did not exceed 1%. Doxorubicin hydrochloride was used as a positive control and negative controls were included. After incubation for 48 h at 37 °C with 5% CO2, the culture medium was discarded and replaced by a fresh medium with 30 μL of thiazolyl blue tetrazolium bromide (5 mg/mL) dissolved in phosphate-buffered saline. After incubation for 4 h, the medium was aspirated and the formazan crystals were dissolved in 50 μL of DMSO for 15 min. The absorbance was measured spectrophotometrically at 570 nm in a Biotek Synergy microplate reader. The viability rate of treated cells was calculated for each concentration and the 50% inhibitory concentrations (IC_{50}) for cancer cells lines and the 50% lethal concentrations (LC_{50}) for normal Vero cells were determined by plotting the graph of viability rate versus the concentrations.

Selectivity index (SI)

Selectivity index was used to determine the selective toxicity of the extracts on normal and cancerous cells.

The degree of selectivity of the compounds was expressed by its SI value as suggested by (Mcgaw *et al*., 2014). High SI value (≥10) of an extract suggests selective toxicity against cancer cells, while a compound with SI value (<10) is considered to give general toxicity even to normal cells (Mcgaw *et al*., 2014). Each SI value was calculated using the formula: SI = IC_{50}

normal cell/IC_{50} cancer cell (Mcgaw *et al*., 2014).

Assay of nitric oxide production and viability of LPS- activated RAW 264.7 macrophages

Cell culture of RAW 264.7 Macrophages

The RAW 264.7 macrophage cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in a plastic culture flask in DMEM containing L-glutamine supplemented with 10 % FCS and 1 % PSF solution under 5 % CO_2 at 37 °C. NO inhibitory activity and viability of LPS-activated RAW 264.7 macrophages were determined. The RAW 264.7 macrophages cells were seeded in 96 well-microtitre plates and was activated by incubation in medium containing 1 µg/ mL LPS for 24 h alone (control) or lipopolysaccharide with different concentrations of ethanol extract of *H. geniculata* dissolved in DMSO. Quercetin served as a positive control (NO inhibitor) for the reduction of NO overproduction (Mu *et al*., 2001).

Measurement of nitrite

Nitric oxide released from macrophages was determined as described by (Elisha *et al*. 2016) by measuring the nitrite concentration in the culture supernatant using the Griess reagent (Sigma Aldrich, Germany).

Cell viability

To determine whether the observed nitric oxide inhibition was not due to cytotoxic effects of the extract, cell viability assay was carried out on the RAW 264.7 macrophage cells as described (Mosmann, 1983).

Soybean 5-Lipoxygenase Inhibition Assay

The assay was performed according to a previously described procedure (Pinto *et al*. 2007).

Statistical Analysis

Statistical analysis was performed using a Graph Pad Prism 5 statistical package (Graph Pad Software, San Diego, MA, USA). Data were expressed as means of replicate determinations ± SD, for *in vitro* assays and was subjected to Dunnet tests were used to determine P-value for differences between tested samples and positive controls.Statistical significance were considered when $P < 0.05$.

RESULTS

The GCMS result of the ethanolic extract of *H. geniculata* (Table I, Figure 6) indicated the presence of five compounds 2-keto-butyric-acid, 2, 2-Bis (4-nitrobenzyl)-1-phenylbutane-1,3-dione, n-Undecane, 1,4,5,8-tetrathiadelin and imidazo-1,5-pyrimidine.

The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT reduction assay was conducted on both Vero and HepG2 cells to measure the effect of the extracts on the cells during a 48 hours exposure and also detect changes in the mitochondrial functionality. The result of the cell viability of Vero cells was displayed (Table II and Figure 1). It showed that the viability of the cells decreases with increasing concentration of extracts. The lowest extracts concentration of 0.05 mg/mL

has the highest cells viability which ranges from 75-97 % and the LC_{50} value of the extracts decreases as follows: ethanol (0.57 ± 0.05) > hydroethanolic (0.50 ± 0.00) > aqueous (0.48 ± 0.02) > decoction (0.40 ± 0.02). The percentage of cell viability observed in this study showed that the extract is non-toxic to Vero cells.

The effect of the plant extract on HepG2 (Table II and Figure 2) showed that at a concentration of 0.75 mg/mL after 48 hours exposure, the viability of the cancer cell was reduced to about 7-9% for all the tested extracts. Their respective LC_{50} values were (0.012± 0.000, 0.016± 0.001, 0.027± 0.003, and 0.039± 0.001) which are similar with the value for doxorubicin LC_{50} (0.032± 0.002). The selectivity index (Table 1), the SI values (12.03, 18.51, 31.87, and 33.33) for aqueous, hydro-ethanol, ethanol and decoction.

The IC_{50} values of NO production (Table III, Figures 3 and 4) showed that ethanolic extract has lower and significantly different ($p<0.05$) IC_{50} value (3.64 ± 0.123 mg/mL) compared to the reference compound quercetin with (IC_{50}: 8.28 ± 0.045). The highest concentration of the extract inhibited NO production by 82% and inhibition of NO production by the extract is in a concentration dependent manner (Figure III) while the RAW 264.7 macrophage cells exposed to the extract did not show any significant difference ($p<0.05$) in the percentage viability of the cells at all concentration of the extracts tested (Figure 5).

Hermannia geniculata extracts exhibited a pronounced inhibition of 5-LOX enzyme compared to the standard indomethacin, their respective IC_{50} value decreased in the following order ethanol< hydro-ethanol< decoction< aqueous with their respective IC_{50} values are (0.14± 0.06, 2.06± 0.00, 3.85± 0.02, 7.15± 0.13 and indomethacin IC_{50}: 3.24±0.12) (Table IV and Figure 5). The comparisons of the means on a two way ANOVA showed that IC_{50} value for ethanol is lower and significantly different ($p<0.05$) from all the other extracts and indomethacin.

TABLE I: Showing cytotoxic activity expressed as LC_{50} (mg/mL) of *Hermannia geniculata* root extracts

LC_{50}: Values: (mg/mL)	Vero cell	HepG2 cell	SI
Ethanol	0.57± 0.052	0.016± 0.001	31.87
Hydro- ethanol	0.50± 0.001	0.027± 0.003	18.51
Decoction	0.40± 0.022	0.012± 0.000	33.33
Aqueous	0.48± 0.021	0.039± 0.001	12.03
Doxorubicin	>1.00± .125	0.032± 0.002	31.25

Data are the means ± SD (standard deviation) n=3. Selectivity Index (SI) = IC50 Vero cell/ IC50 HepG2 cell. SI value ≥10 indicating high selectivity

TABLE II: Showing effect of the *Hermannia geniculata* roots extracts on 5-lipoxygenase enzyme expressed as IC_{50} (mg/mL)

Ethanol	Hydro-ethanol	Decoction	Aqueous	Doxorubicin
0.14±0.06^{a}	2.06± 0.00^{b}	3.85± 0.02^{c}	7.15± 0.13^{d}	3.24 ± 0.12^{d}

Data are the means ± SD (standard deviation) n=3. ^{abcd}Values in the row with different alphabet are significantly different ($p<0.05$).

TABLE III: Showing nitric oxide (NO) inhibition capabilities of the *Hermannia geniculata* root extracts expressed as IC50 values in mg/mL

	Ethanol	Quercetin
IC_{50} (mg/mL)	3.64 ± 0.123^{a}	8.28 ± 0.045^{b}

Data are the means ± SD (standard deviation) n=3; ^{ab}Values in a row with different alphabet are significantly different (p<0.05).

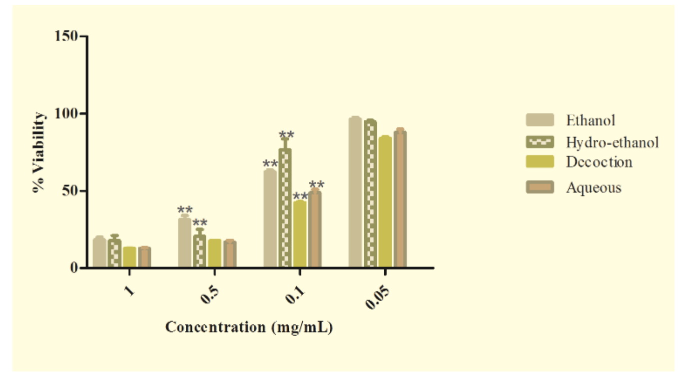


Fig 1 The percentage cell viability of the Vero cells at different concentrations of *Hermannia geniculata* roots extracts. Data represents mean± SEM (standard deviation) of three independent experiments. *Mean significantly different at (p< 0.01) and **Mean significantly different at (p<0.05)

TABLE IV: Gas Chromatography- Mass Spectrometry (GC-MS) chromatogram of Compounds present in ethanolic extract of *Hermannia geniculata* roots

S/N	Constituents	Retention time (Min)	Area %	Molecular formular	Molecular weight (g/mol)
1	2-keto-butyric-acid	11.058	1.32	$C_4H_6O_3$	102
2	2,2-Bis(4-nitrobenzyl)-1-phenylbutane-1,3-dione	14.143	1.36	$C_{24}H_{20}N_2O_6$	432
3	n-Undecane	15.529	1.71	$C_{11}H_{24}$	156
4	1,4,5,8-tetrathiadelin	26.961	3.41	$C_6H_{10}S_4$	210
5	Imidazo(1,5-a)pyrimidine	29.865	92.20	$C_6H_5N_3$	119

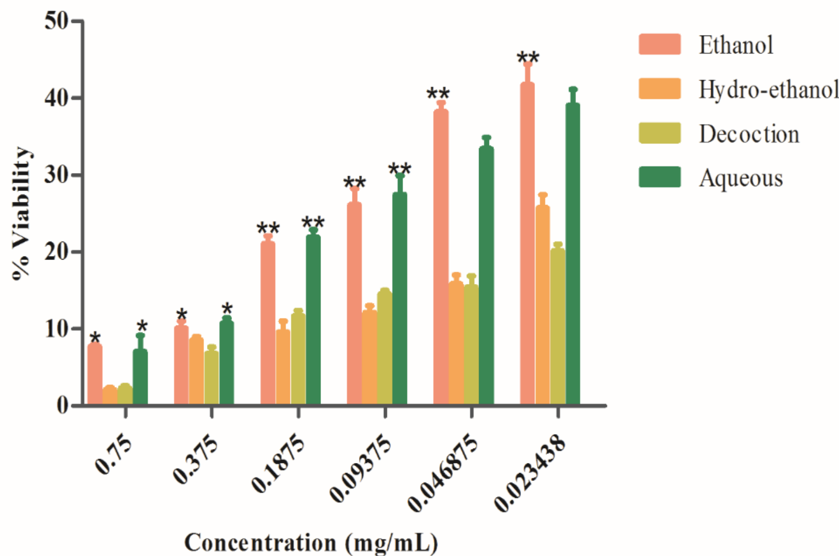


Fig 2 The percentage cell viability of the HepG2 cells at different concentrations of *Hermannia geniculata* roots extracts. Data represents mean± SEM (standard deviation) of three independent experiments. *Mean significantly different at (p< 0.05) and **Mean significantly different at (p<0.001)

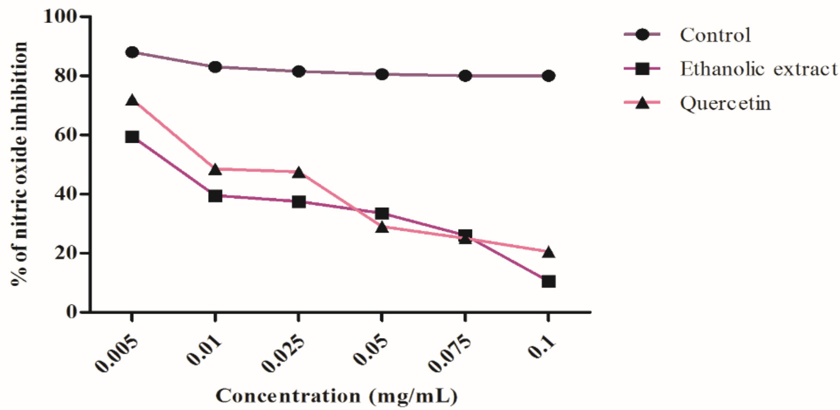


Fig 3 The percentage inhibition of nitric oxide production at different concentrations of the *Hermannia geniculata* roots extracts. Data represents mean± SD (standard deviation) of three independent experiments

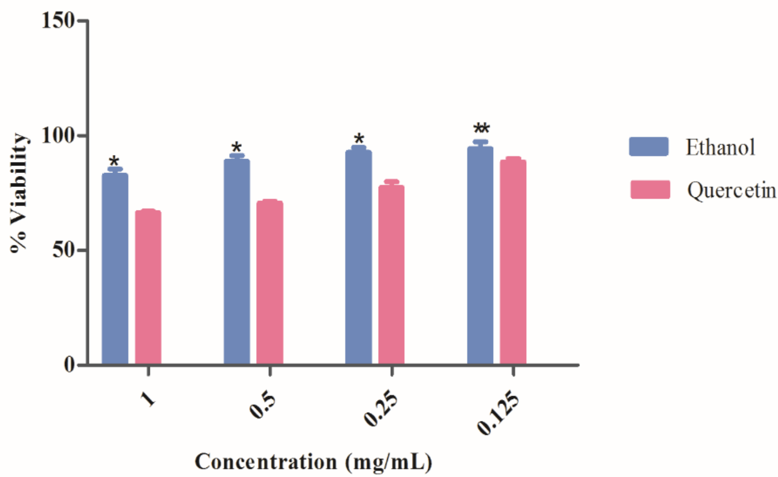


Fig 4 The percentage cell viability of the RAW 264.7 macrophage cells at different concentrations of *Hermannia geniculata* roots extracts. Data represents mean± SD (standard deviation) of three independent experiments. *Mean significantly different at ($p< 0.01$) and **Mean significantly different at ($p<0.05$)

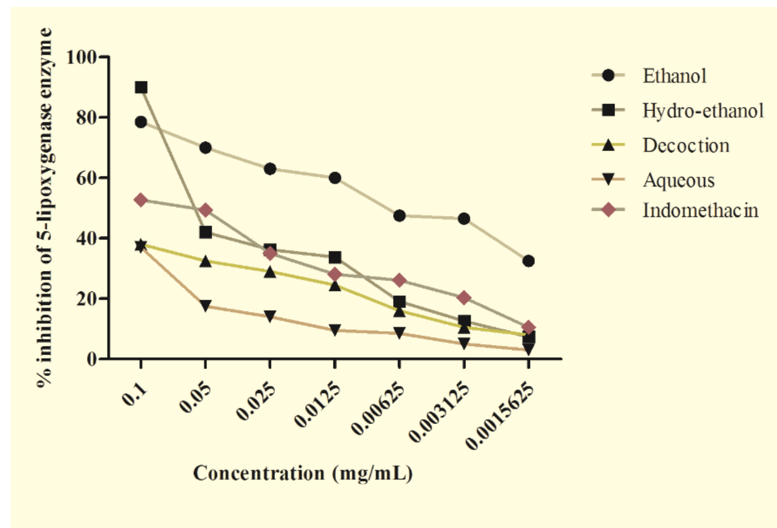
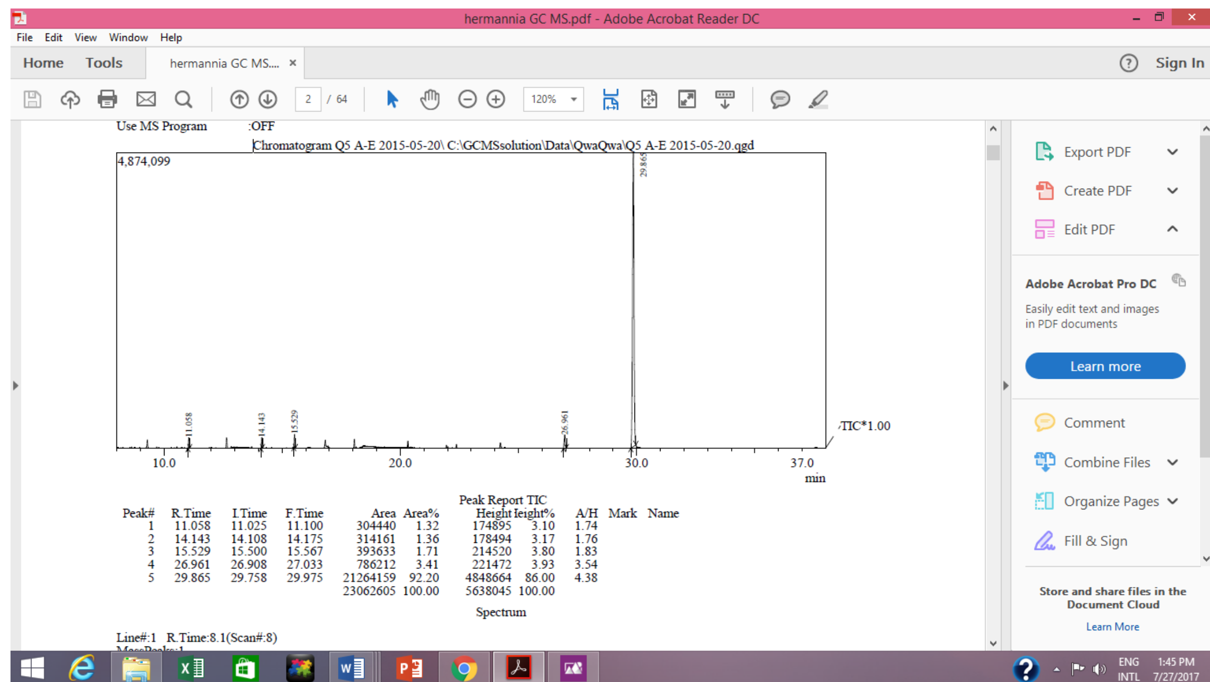


Fig 5 The percentage inhibition of 5-lipoxygenase enzyme at different concentrations of *Hermannia geniculata* roots extracts. Data represents mean± SD (standard deviation) of three independent experiments.



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	11.058	11.025	11.100	304440	1.32	174895	3.10	1.74		
2	14.143	14.108	14.175	314161	1.36	178494	3.17	1.76		
3	15.529	15.500	15.567	393633	1.71	214520	3.80	1.83		
4	26.961	26.908	27.033	786212	3.41	221472	3.93	3.54		
5	29.865	29.758	29.975	21264159	92.20	4848664	86.00	4.38		
				23062605	100.00	5638045	100.00			

Fig 6 GC-MS chromatogram of ethanolic extract of *Hermannia geniculata* roots extract.

DISCUSSION

The GC-MS result of the ethanolic extract of *H. geniculata* indicated the presence of five compounds. The 2-keto-butyric-acid reported in this study has been documented as a substance that is capable of stimulating

the biosynthesis of fibronectin by fibroblast, endothelial cells, astrological cells and hepatocyte as a sequela to inflammation and tissue repair (Tanaka and Nishida, 1985). Also, 2, 2-Bis (4-nitrobenzyl)-1-phenylbutane-1,3-dione and n-Undecane are excellent reducing equivalents and have antioxidant properties (Azzena *et al*., 1996; Mzé-Ahmed *et al*., 2012). The antifungal activities of 1,4,5,8-tetrathiadelin have been documented (Adeogun *et al*., 2016). Imidazo-1,5-pyrimidine pharmacological importance includes; antiulcer, antihypertensive, and antitrypanosomal (Novinson *et al*., 1976; Rovnyak *et al*., 1992; Patil *et al*., 2008). Furthermore, it has been reported to have activity against NF-Kb and AP-1 protein expression (Rovnyak *et al*., 1992).

Cytotoxicity effects of plant extracts have been previously reported and *in vitro* cytotoxicity testing essentially helped to determine the effect of phytochemicals on cells (Mcgaw *et al*., 2014). The result of our extract on hepatocytes, and macrophage cells showed all the extract has an $LC_{50} \geq$ 0.40 mg/mL which showed that the extract is safe and not toxic to the cells. The variation recorded in the LC_{50} values in the extracts may be due to different phytochemical like the phenols, alkaloids, saponins, tannins, triterpenes and glycosides extracted by the different solvent used. This is in agreement with other previous reports which documented toxicity of certain phytochemicals like tannins, saponins, and phenolic (Mbatchou and Dawda, 2012; Pawlowski *et al*., 2012; Harun *et al*., 2014; Sulaiman *et al*., 2014; Mecina *et al*., 2016).

Selective cytotoxicity of plant extracts on cancer cells rather than normal, and non-transformed cells have been documented (Du *et al*., 2010). Selective cytotoxicity of extract ≥10 signifies that the agent is non-toxic to normal cells but has potential anticancer activity (Mcgaw *et al*., 2014). The average selective index recorded for all the tested extracts is ≥20 suggested the anticancer potential of the plant extracts.

The ethanolic extract has a lower inhibitory effect than the standard quercetin on the production of nitric oxide (NO) by the LPS stimulated RAW 264.7 macrophages cells. In this study, the highest concentration of the extract inhibited production by 82% NO, and inhibition of NO production by the extract occurs in a concentration-dependent manner.

NO is synthesized from L-arginine by three isoforms of NO synthase (NOS) two are constitutive and the third is induced nitric oxide synthase (iNOS) is activated during inflammation and produces a high level of NO for a long period (Komers *et al*., 1994; Cui *et al*., 2005; Villalobo, 2006). NO diffuses very rapidly through water and cell membranes and easily combine with superoxide anion ($.O_2^-$) to form peroxynitrite ($ONOO^-$) a potent mutagenic oxidant. NO production may contribute to DNA damage, renal hyperfiltration and hyperperfusion observed in the pathogenesis of diabetes a complication (Komers *et al*., 1994). NO inhibitors are a useful agents in preventing cell death and inflammatory diseases which is a sequela to cell apoptosis. Plant extracts have been used in suppressing NO production in LPS- stimulated RAW 264.7 murine macrophages cell line (Elisha *et al*.,

2016; Mfotie *et al*., 2017). The extent of NO production can be determined by measuring the concentration of nitrite, a stable oxidized product (Mfotie *et al*., 2017). The result of RAW 264.7 macrophage cell viability showed that there was no significant difference ($p<0.05$) in the percentage viability of the cells at all concentrations of the extracts tested. Therefore, the observed reduction of NO production seen at higher concentrations of the extracts may be due to the inhibitory effect of the extract on iNOS enzymes. This result confirms the NO inhibitory activities of *Hermannia geniculata* root extracts.

Inhibition of 5-lipoxygenase (LOX) enzyme block the development of several diseases including cancer, diabetes, and several inflammatory diseases. Ethanol extracts have potent inhibitory effect on 5-LOX enzyme activity. The extract concentration of 0.1 mg/mL exhibited 40-89% inhibition of the enzyme activities. Inhibition of 5-LOX enzyme blocked the biosynthesis of 5-HETE which is an essential factor in the survival of several cancer cells (Ghosh and Myer, 1998) thus one of the an effective mechanism of action of *H. geniculata* root extracts on Human hepatocellular carcinoma cell may be through the inhibition of 5-LOX which is the enzyme that catalyzes the biosynthesis of 5-HETE an essential factor in the survival and proliferation of the liver cancer cells. Also, observed inhibition of 5-lipoxygenase enzyme suggested that extracts of *H. geniculata* roots may be used as an inflammatory agent.

CONCLUSION

The selective toxicity, anti-lipoxygenase and inhibitory potentials of *H. geniculata* confirmed that the root extracts have a toxic effect on HepG2 cells but not toxic to Vero cells, and it also inhibits the production of NO in LPS activated RAW 264.7 cells. The *in vitro* cytotoxicity test of the root extracts from *Hermannia geniculata* on different cell lines showed that it is safe to normal cell lines, the chromatographic analysis of ethanol extract indicated the presence of compounds of pharmacological importance which may be exploited for their anti-inflammatory, antiproliferative, and anticancer properties.

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