Pharmacogn. Mag.
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ORIGINAL ARTICLE

In vitro Anti-Inflammation, Selective Cytotoxicity, and Inhibition of Induced Nitric Oxide from Lipopolysaccharide-Stimulated RAW 264.7 Macrophages Activities of Flavonoids from *Hermannia geniculata* Eckl and Zeyh Roots Extract

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Submitted: 12-Oct-2019 Revised: 31-Oct-2019 Accepted: 09-Jan-2020 Published: 28-Aug-2020

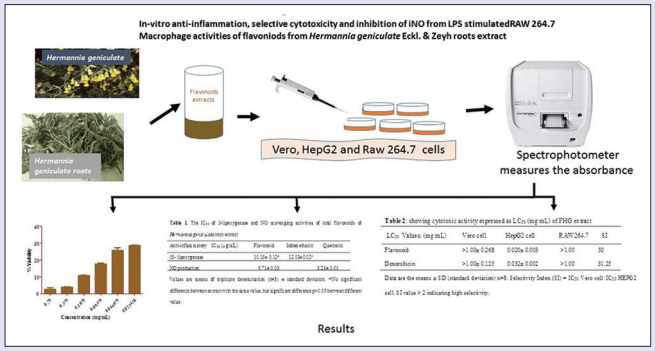
ABSTRACT

Aim: Evaluation of *in vitro* anti-inflammatory activities of flavonoids of *Hermannia geniculata* (FHG) roots extract, selective cytotoxicity of Vero and hepatocellular carcinoma (HepG2) cells, as well as its capabilities to inhibit the production of induced nitric oxide (iNO) in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage was investigated. **Materials and Methods:** Soybean 5-lipoxygenase (5-LOX) Inhibition and the tetrazolium-based colorimetric (MTT) assay methods were used. **Results:** The extract exhibited 73% inhibition of 5-LOX enzyme better than indomethacin (standard) with their respective IC_{50} (10.15 ± 0.02 and 12.03 ± 0.02). It was observed that 65% of the Vero cells were viable at the highest concentration of 1 mg/mL of FHG extract and the LC_{50} value is 1 mg/mL. Furthermore, the LC_{50} value of 0.02 mg/mL was recorded for HepG2 cells at the concentration of 0.75 mg/mL. Selectivity index (SI) for the FHG extract is 50 which is higher than the 31.2 SI value of the standard. Inhibition of iNO production was observed in LPS activated RAW 264.7 Macrophages with the highest concentration of 0.1 mg/mL decrease iNO production by 87%. **Conclusion:** This study indicated that FHG extract possesses anti-inflammatory and antiproliferation properties.

Key words: Anti-inflammatory, flavonoids, *Hermannia geniculata*, nitric oxide

SUMMARY

- *In vitro* anti-inflammatory evaluation of flavonoids of *Hermannia geniculata* roots extract and its selective cytotoxicity was carried out. The result justifies the conventional use of the plant in treating inflammatory diseases. The flavonoid extracts are not toxic to Vero cells and macrophages cells but highly toxic to cancer cells.



Abbreviations used: FHG: Flavonoids of *Hermannia geniculata*; iNO: Induced nitric oxide; LPS: Lipopolysaccharide; NO: Nitric oxide; iNOS: Induced nitric oxide synthase.

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DOI: 10.4103/pm.pm_447_19

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INTRODUCTION

Inflammation implies innate body response to injurious stimuli; this is done by mobilization of leukocytes and plasma to the site of injured tissue. There are accumulation and subsequent amplification of leukocyte during inflammation. Physiological response to inflammation entails the termination of pro-inflammatory signaling pathways and removal of inflammatory cells, thus restoring the normal function of the tissue.[1] Derangement of this mechanism may cause chronic inflammation which has been indicted in the pathogenesis of several diseases such as dementia, autoimmune diseases, arthritis, diabetes, inflammatory bowel diseases, and vascular diseases.[2]

Search for agents that can efficiently enhance the resolution of inflammation is of great pharmacological significance. Medicinal plants have served as an effective therapy in the management of several ailments. Most people in rural communities depend largely on the use of medicinal plants to manage different kinds of diseases.[3,4] Thus, there is an increasing demand for herbal based food supplements, health products, nutraceuticals, pharmaceuticals, and cosmetics worldwide.

Hermannia geniculata, also known by the Basotho tribe in South Africa as "kgwakgwa," is among the popular species frequently used for medicinal purpose in South Africa.[5,6] It is of the genus of flowering plant from the subfamily *Byttnerioideae* in the narrow family *Malnaceae*.[7] The plant is

Cite this article as: Adeniran LA, Ohanaka JN, McGAw LJ, Ashafa AOT. *In vitro* anti-inflammation, selective cytotoxicity, and inhibition of induced nitric oxide from lipopolysaccharide-stimulated RAW 264.7 macrophages activities of flavonoids from *Hermannia geniculata* Eckl and Zeyh roots extract. Phcog Mag 2020;16:S345-9.

seen across South Africa, Madagascar, East Africa, North to West Africa, and Saudi Arabia. Decoctions of *H. geniculata* are often used in the conventional Basotho medicine.[8,9] It is used in the management of diarrhea, heartburn, stomach disorder, and flatulency called "leletha" in pregnant Basotho women. Further, the dry root material is chopped, boiled in water, and taken three times daily to ameliorate blood sugar disorders.[5]

The role of 5-lipoxygenase (5-LOX) and NO being regulated by nuclear factor kappa (NF-κB) in causing inflammatory diseases has been documented; therefore, we seek to investigate the anti-inflammatory effect of the flavonoids of *H. geniculata* (FHG) using soybean 5-LOX and induced nitric oxides (iNOs) from RAW 264.7 macrophage cells, cytotoxic effect of FHG on Vero monkey kidney cells, human hepatocellular carcinoma cells (HepG2), and RAW 264.7 macrophage cells, to document the anti-inflammatory, anti-proliferative, and safety of FHG to kidney and immune cells.

MATERIALS AND METHODS

Plant collection, preparation, and extraction

H. geniculata roots were purchased from local market in Phuthaditjhaba, 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) at the herbarium.

Extraction of flavonoids

The roots of *H. geniculata* were thoroughly washed and rinsed with clean water. It was chopped into small pieces and dried in an open air in the laboratory. After drying, it was grinded into fine powder using warring laboratory blender (Labcon, Durban, South Africa) and kept at 4°C; the extraction of flavonoid was done through a described method.[10]

Determination of extraction yield

The yield of flavonoids from the extracts of *H. geniculata* roots (FHG) was determined through this simple calculation. Extraction yield % = PO/PI × 100.

- PO: Weight of the powder before extraction
- PI: Weight of the dry extract after extraction.

Chemical and reagents

Chemicals purchased from different suppliers include: ferric chloride, xylenol orange (Searle company, England), Sodium dodecyl sulfate, iron (II) sulfate, sodium nitrite and 5-lipoxygenase (Glycine max) (Sigma, Germany), linoleic acid (Merck, Darmstadt, Germany); other chemicals were of analytical grade; the water used was glass distilled.

Soybean 5 – lipoxygenase inhibition assay

The assay was carried out through a described procedure.[11] Different extracts or standard concentration (0.78, 1.6, 3.1, 6.3, 12.5, 25, 50, and 100) µg/mL was incubated with 5– lipoxygenase for 5 min at 25°C. linoleic acid (140 µM) in Tris–HCl buffer (50 mM, pH 7.4) was added and the resultant mixture was incubated in the dark for 25°C for 20 min. The assay was terminated by the addition of 100 µl of FOX reagent consisting of sulphuric acid (30 mM), xylenol orange (100 mM), iron (II) sulfate (100 mM) in methanol/ water (9:1). Determination of the inhibitory effect of extract/standard on the enzyme was done by calculating the inhibition of H_2O_2 production from change in absorbance values at 560 nm after 30 min exposure at 25°C.

Thereforem percentage inhibition of enzyme activity = (absorbance of control – absorbance of test sample)/absorbance of control × 100. The 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation Y = Mx + C, where Y is the percentage activity and equal 50, M is the slope, C is the intercept, and x is the IC_{50} value.

Cell culture of hepatocellular carcinoma and Vero

The HepG2 and Vero cells were obtained from the Cell Line Laboratory of Paraclinical Sciences Department, Onderstepoort Campus, University of Pretoria, South Africa. DMEM supplemented with 10% fetal bovine serum culture medium, penicillin 100 unit/ml, and streptomycin 100 µg/mL was added to the culture medium. The condition of the incubation was 37°C and 5% CO_2 humidified atmospheric condition.

Cytotoxicity of cell lines

Observed growth after incubating the cell with extract of FHG was evaluated using tetrazolium based calorimetric (MTT) assay.[12] Briefly, subconfluent cells were harvested, centrifuged, and re-suspended in the growth medium at 5 × 10^4 cells/mL. The growth medium was MEM supplemented with fetal calf serum (FCS) (5%) and 0.1% gentamicin (Vibrae). MEM (200 µL) was added to a well of columns 1 and 12 to minimize the edge effect and maintain the relative humidity. The incubation of the well plate was carried out at 37°C until when we get to the exponential growth phase of the cell. The growth medium (MEM) was gently aspirated from the plates without agitating the cells; the cells were further washed with 150 µL of PBS. In quadruplicate, FHG extracts' serial dilution was carried out in concentration ranging from 0.05 to 1.0 mg/mL. FHG extract serial dilutions made in MEM, and the mixture added to the wells. Incubation of the microtiter plates was done for 48 h at 37°C in 5% CO_2. A column in the microtiter plate contained untreated cells, while doxorubicin was also plated which serves as respective negatives and positive control. Doxorubicin was prepared at concentration varying from 750 µg/mL to 1 mg/mL. After incubating the plates for 48 h, 30 µL MTT (stock of 5 mg/mL in PBS) was added to all the wells in the plate. The plate was further incubated at 37°C for 4 h. Afterward, gentle aspiration of the MTT in the cultured medium was carried out making sure that it did not affect the formed MTT crystals. Dimethyl sulfoxide (DMSO) was added into the medium to dissolve the formed MTT crystals. To help dissolving the MTT crystal, the plates can be slightly shaken. Absorbance of the MTT reduction by the cells was measured using a microplate reader (Synergy Multi-Made Reader, BioTek) at 570 nm using a reference of 630 nm. The wells in the column 1 which contain MTT medium were used to blank the reader. Determination of FHG extracts that cause 50% reduction of the absorbance compared to untreated cells was used as the LC_{50} value.

Selectivity index

The degree of FHG extracts selectively was expressed as selectivity index (SI) as described.[13] Values >2 suggest selective toxicity while <2 value extracts general toxicity.[14] Calculation for the SI value was done using the formula

$SI = IC_{50}$ (normal cells)/IC_{50}

NO production in the lipopolysaccharide (LPS) activated RAW 264.7 macrophage viability assays.

Cell culture of RAW 264.7 macrophage

A procedure[15] was adopted. Briefly, macrophage cells (RAW 264.7) were procured from the American Type Culture Collection (Rockville, MD, USA). It was cultured in a DMEM containing L-glutamine and supplemented with FCS (10%), PSF (1%), and CO_2 (5%) at a temperature

of 37°C. The NO-scavenging capabilities of FHG from LPS-activated macrophages and the cytotoxicity potential of the extracts on RAW 264.7 murine macrophages cells were investigated. Incubation of bacterial LPS in a medium seeded with cells in a 96-well plate was carried out to activate production of NO. Different concentration of extracts (5, 10, 25, 50, 75, and 100) µg/mL was added to the wells. Water was used as the positive and negative control, respectively.

Measurements of nitrite

The NO released by the 264.7 RAW macrophage cells was evaluated.[15] Briefly, nitrite concentration in the cultured supernatant was aspirated from each well and put in a new well. Addition of 100 µL of Griess reagent was carried out in the new wells. The resultant solution absorbance was read on a microplate reader (Biotech Synergy) at 550 nm after 10 min. The concentration of nitrite was calculated using regression analysis of serial dilution of $NaNO_3$ which is the standard. Inhibitory capability of the nitrite produced was evaluated in relation to %inhibition of the negative control (0% inhibition of nitrite).

RAW 264.7 macrophage cell viability

To know whether the observed NO inhibition was not due to the toxic effect of the extracts on cells, MTT assay was carried out on the RAW 264.7 macrophages cells as described by previous study.[12] 30 µL MTT (stock of 5 mg/mL in PBS) was added to all the wells in the plate. The plate was further incubated at 37°C for 4 h. Afterward, gentle aspiration of the MTT in the cultured medium was carried out making sure that it did not affect the formed MTT crystals. DMSO was added into the medium to dissolve the formed MTT crystals. To help dissolving the MTT crystal, the plates can be slightly shaken. Absorbance of the MTT reduction by the cells was measured using a microplate reader (Synergy multi-made Reader, BioTek) at 570 nm using a reference of 630 nm. The wells in the column 1 which contain MTT medium were used to blank the reader. Determination of FHG extracts that cause 50% reduction of the absorbance compared to untreated cells was used as the LC_{50} value.

Statistical analysis

Statistical analysis was performed using a GraphPad Prism 5 Statistical Package (GraphPad Software, San Diego, MA, USA). All experiments were conducted in triplicate and values expressed as the mean ± standard deviation. Variations in mean were calculated using one-way Analysis of variance (ANOVA), and means were statistically significant if P <0.05. *Post hock* analyses were carried out using Bonferroni comparison tests. Statistical significance was considered at $P < 0.05$.

RESULTS

The yield of total flavonoids was 1.68 g from the 30 g extract of plant materials

In Table 1 and Figure 1, the effect of FHG extract on 5-LOX enzyme involved in arachidonic acid metabolism showed a concentration-dependent inhibition of the enzyme with the highest concentration of 0.1 mg/mL showing about 89% inhibition. The IC_{50} value of the extract is higher and significantly different ($P < 0.05$) from the reference compound (indomethacin). The respective IC_{50} value for FHG extract and indomethacin was 10.15 ± 0.12 and 12.03 ± 0.42, respectively.

The cytotoxicity of the extract was tested on two cell lines, the normal African green monkey kidney epithelium cells (Vero cells) and human HepG2 cells to determine the viability of the cells exposed to the extracts. The results are shown in Table 2 and Figures 2 and 3. Doxorubicin was used as the positive control. MTT assay was conducted to determine the cells viability after 48 h of exposure to the extract. It was observed that 75% of the Vero cells were viable at the highest concentration of 1 mg/mL of FHG extract and the LC_{50} value is 1 mg/mL. Furthermore, the percentage viability of the HepG2 cells was lowest at the highest concentration of 0.75 mg/mL with <5% of the cell viable. The LC_{50} value is 0.02 mg/mL.

The results of the SI are shown in Table 2; the SI for FHG extract is 50 which is higher than the SI of doxorubicin value of 31.25.

The inhibitory potentials of FHG extract on excessive and uncontrolled production of proinflammatory molecule NO was assayed. The results are shown in Figures 4 and 5. FHG extract significantly reduced the production of NO from LPS-activated RAW264.7 macrophages at the highest concentration of 100 µg/mL, and the inhibition is dose dependent. The IC_{50} of 3.61 mg/mL was lower and significantly different ($P < 0.05$) from reference compound (quercetin) with IC_{50} value of 6.72 mg/mL. They also determined the percentage viability of the extract to ascertain the viability of the cell using MTT assay. The LC_{50} value of the FHG extract on the cell is >1.00 mg/mL.

Table 1: 5-lipoxygenase and nitric oxide-scavenging activities of total flavonoids of *Hermannia geniculata* root extract

Anti-inflammatory	Flavonoid IC_{50} (µg/mL)	Indomethacin IC_{50} (µg/mL)	Quercetin IC_{50} (µg/mL)
05- lipoxygenase	10.15±0.12[a]	12.03±0.02[b]	
Nitric oxide	6.71±0.03		8.28±0.05

Values are expressed as mean of triplicate determination (n=3)±SD. [a,b]No significant difference between extract with the same value, but significant difference P>0.05 between different value. SD: Standard deviation

Table 2: Cytotoxic activity of flavonoids of *Hermannia geniculata* extract expressed as LC_{50} (mg/mL)

LC_{50} (mg/mL)	Vero cell	HepG2 cell	RAW 264.7	SI
Flavonoid	>1.00±0.268	0.020±0.003	>1.00	50
Doxorubicin	>1.00±0.125	0.032±0.002	>1.00	31.25

Data are expressed as mean±SD n=8. SI: IC_{50} vero cell/IC_{50} HEPG2 cell. SI value >2 indicating high selectivity. SD: Standard deviation; SI: Selectivity index

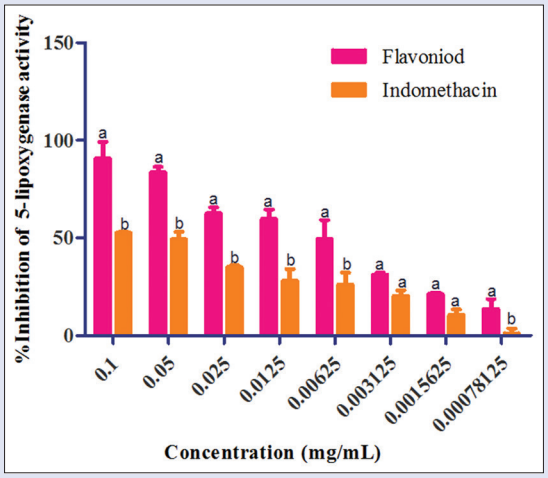

Figure 1: Antilipoxygenase activity of flavonoids of *Hermannia geniculata* root extract. Data represent the mean ± SE (standard deviation) of three independent experiments

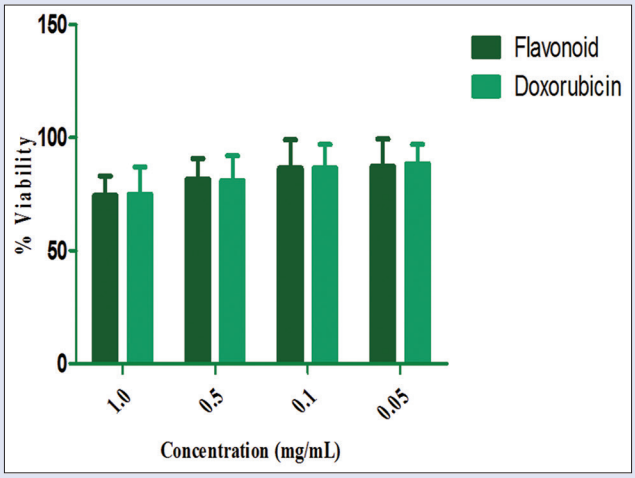

Figure 2: The percentage viability of Vero cell viability at different concentrations of the flavonoids of *Hermannia geniculata* extract. Data represent the mean ± SE (standard deviation) of three independent experiments

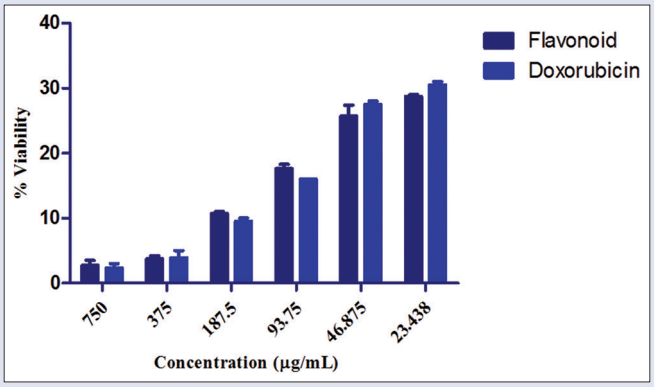

Figure 3: The percentage viability of HepG2 cell viability at different concentrations of the flavonoids of *Hermannia geniculata* extract. Data represent the mean ± standard deviation of three independent experiments

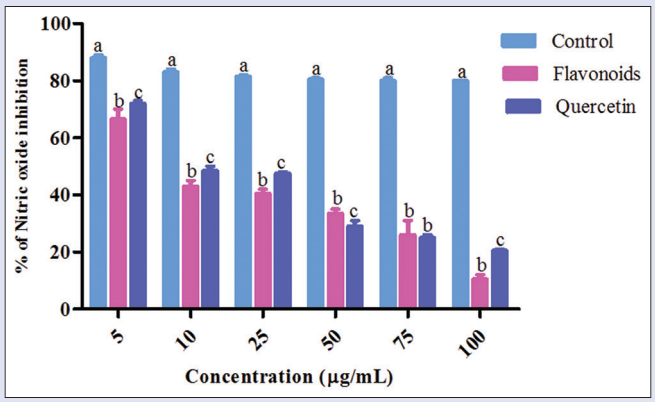

Figure 4: Nitric oxide inhibitory activity of the extract from flavonoids of *Hermannia geniculata* on RAW 264.7 macrophages. Data represent the mean ± SE (standard deviation) of three independent experiments

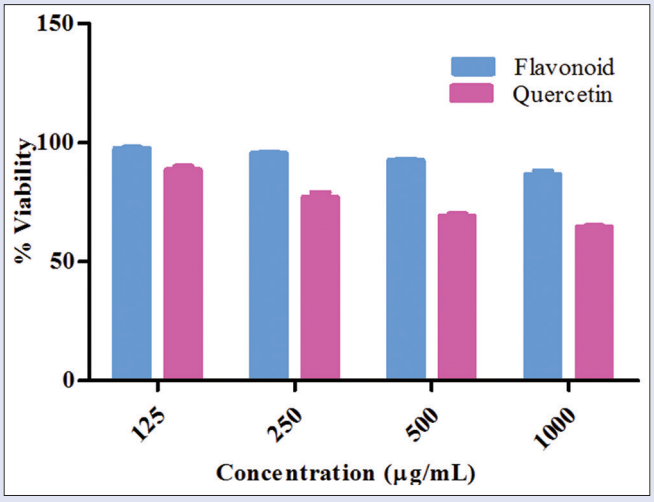

Figure 5: Nitric oxide inhibitory activity of the extract from flavonoids of *Hermannia geniculata* on RAW 264.7 macrophages with respect to their cell viability. Data represent the mean ± standard deviation of three independent experiments

DISCUSSION

In vitro anti-inflammatory assay

The result of the *in vitro* anti-inflammatory assay is presented in Table 1. FHG extract exhibited a varying degree of inhibitory effect on 5-LOX enzyme. The inhibition of lipoxygenase was dose dependent, with the highest concentration of the extract showing the highest degree of inhibition. This anti-inflammatory result supported the conventional use of *H. geniculata* roots in the management of different inflammatory diseases.[5,16] The IC_{50} value of FHG extract is 10.15 mg/mL which demonstrates also the good activity of FHG extract against the enzyme. The IC_{50} value is comparable to the positive control indomethacin which has an IC_{50} of 12.03 mg/mL. Flavonoids have been demonstrated to have antilipoxygenase activity.[17] Flavonoids are also known to affect a number of regulatory enzymes, which are essential to inflammation and immune response. Inhibition of 5-LOX enzyme by the FHG extract will inhibit the oxidation of arachidonic acid from membrane phospholipid to leukotrienes, which is a molecule required in the biosynthesis of several proinflammatory cytokines.[18]

We investigated the cytotoxicity effect of the FHG extract on HepG2 and Vero cell lines. Doxorubicin, a known anticancer drug, was used as the positive control. The respective LC_{50} values and their SI are presented in Table 1. For HepG2 assay, the LC_{50} value of FHG extract was 0.02 mg/mL, which is comparable to the value of doxorubicin. The percentage viability of the cells to different concentration of the extract is presented in Figure 2. It showed that antiproliferative activity of the extracts is dose dependent and the highest concentration of 0.75 mg/mL produces the highest cytotoxicity in the tested extract. The extracts are less toxic to Vero cells with LC_{50} value >1 mg/mL. The percentage viability is presented in Figure 2; it showed that the FHG extract was not toxic to the cells at all concentration. This result showed that all the extract may be safe at concentration of 1 mg/mL. The SI represents the overall activity of each extracts.[13] As seen in Table 2, the extract SI value is 50. This SI ndex greater >2 suggested that the extract may have anticancer properties. This is similar to work of Badisa *et al.*[13] where compounds having SI greater than 2 are being accepted to have promising anticancer activity.

Nitric oxide inhibitory activity

NO scavenging is important because it is a proinflammatory molecule, which can diffuse across the cell membranes. Low-grade NO production is beneficiary for maintaining the normal body functions, but unregulated production of NO reacts with superoxide anion generating peroxynitrite which causes oxidation of low-density lipoprotein, and this is a key process that leads to cell apoptosis, subsequent inflammation, and atherosclerosis.[19,20] Therefore, Nitric oxide inhibitors are useful agents that may prevent apoptosis observed in inflammatory diseases. Report of quercetin suppression of NO production in LPS stimulated RAW 264.7 murine macrophages cell line has been documented.[20] The extent of NO production can be determined by measuring the concentration of nitrite, a stable oxidized product.[11,17] In our present work, RAW 264.7 macrophages were treated with LPS and various concentration of FHG extract. Quercetin serves as the positive control. The NO production and the cell viability were measured.

The decrease in the NO produced from the percentage inhibition and the lower IC_{50} value of 6.71 mg/mL compared to the value of quercetin IC_{50} of 8.28 mg/mL in Table 1, and Figure 4 suggests that the FHG extract may be able to inhibit NO production from LPS-stimulated macrophages cells. This results was similar to the work of Elisha *et al.*[17] where flavonoids were described to be able to inhibit NO production. Further, Mfotie *et al.*[21] attributed the capacity of extracts to inhibit NO production to the phenolic content of the extracts, which regulate the synthesis of iNO synthase by inhibiting the nuclear transcription factor NF-κB. Furthermore, the hydroxyl group on position 3 of C-ring and two hydroxyl groups in B-ring are important site for NO scavenging.[22,23] The cytotoxicity of the extracts against RAW 264.7 cells was also tested by MTT assay, the result is presented in Figure 5. It showed that there was no significant cytotoxic effect of the extracts on the RAW 264.7 macrophages cells at all concentrations of the extract, which may account for the observed inhibition of NO production.

CONCLUSION

The anti-inflammatory and NO-scavenging capabilities of the FHG extracts indicated the pharmacological potentials of *H. geniculata* in the treatment of oxidative stress-related disorders. Further works on the *in vivo* biological activities are ongoing in the laboratory.

Acknowledgements

I wish to acknowledge Dr Njoya Mfotie, Dr Ogundajo Tayo for their technical support during the research work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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