In vitro Anti-Inflammation and Selective Cytotoxicity of Vero and HepG2 Cells by Phenolic Extract From Roots of Hermannia Geniculata Eckl and Zehl

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Abstract
Anti-inflammatory and in vitro cytotoxic effect of phenols of Hermannia geniculata (PoHG) on Vero and HepG2 cells was carried out using Soybean lipooxygenase and MTT assays. PoHG extract exhibited a commendable inhibition of 5-lipoxygenase enzyme with IC50 value of (150 ± 0.03) µg/mL which is similar to the IC50: (110± 0.01) µg/mL of the standard (indomethacin). However, the extract was non-toxic to Vero cells with LC50 value >1.00 mg/mL but highly toxic to HepG2 cells (LC50: 0.05 mg/mL). A decrease viabilit of HepG2 cells was observed with increase in the concentration of the extract. There was less than 5% viable HepG2 cells at PoHG concentration of 750 µg/mL. The selectivity index of (20.00 and 33.33) was recorded for PoHG extract and doxorubicin respectively. The anti-inflammatory activities of PoHG suggested that the phenols extract may be useful in the management of inflammatory diseases like artherosclerosis, diabetes mellitus, rheumatoid arthritis and asthma. It is also safe for use while its antiproliferative activities can be exploited in search for anticancer agents.

Keywords: Phenols; Antiinflammatory; Anticancer; Hermannia geniculata; 5-lipoxygenase enzyme.

1. Introduction
Inflammation is simply defined as the body response to injury. Injury may result from exogenous sources including invasion of ectoparasites, viruses, bacteria and fungi while endogenous injury may result from endoparasites, defective immune system, hypersensitivity and anaphylactic reactions [1, 2]. The body inflammatory response involve secretion from neutrophils, monocytes and macrophages which produces several mediators primarily to resolve the cause of injury in a series of processes which regulate and cause resolution of the acute state of inflammation. It is possible that if resolution of inflammation is prolong it may move from an acute to a chronic stage. The development of chronic inflammation play key role in different pathological conditions like insulin resistance, ulcer, diabetes, arthritis and asthma [3].

The 5-lipoxygenase (5-LOX) enzyme is expressed in leucocytes and play a key role in leukotriene biosynthesis especially leukotriene B4 (LTB4) which help to increase vascular permeability during inflammation. Also, 5-LOX is a catalytic product of 5(s)-hydroperoxy-6-Trans-8, 11, 14-Cis-eicosatetraenoic acid (5-HPETE) which is further metabolized to 5- HETE a lipid mediator. Anti-inflammatory mediators play key role in the process of inflammation resolution [4]. Leukotriene has been established as mediator of asthma and inhibiting the biosynthesis of leukotriene have a therapeutic role to play in atherosclerosis and other inflammatory diseases [5-7]. Various steroidal and non-steroidal anti-inflammatory drugs has been developed which target inhibition of 5-LOX enzymes but severe side effect accompanied prolong use of these drugs which necessitate the search for a potent and safe natural products.

Africa is blessed with several medicinal plants that has anti-inflammatory activity. H. geniculata is among the medicinal plant species frequently used in South Africa for the management of different diseases [8-10]. It belongs to the genus of flowering plant from the family Malnaceae. It is a creeping shrubs with sub-oricbular broad crenate leaves, the length of the leaf is about 15mm and the texture may be viscid or sticky. Hermannia geniculata is readily identified by the hanging flowers, a typically green calyx encloses the base of free petals with five petals which are contorted with transversely expanded filament [11]. The plant is endemic in all provinces of South Africa. H. geniculata can also be found in Madagascar, Kenya, Lesotho, Saudi Arabia and other tropic countries [8, 12].

Traditionally, H. geniculata has been used in the treatment of several diseases like colic and diabetes mellitus. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders, management of diarrhoea, heartburn, stomach disorder and flatuency called “leletha” in pregnant Sotho women [9, 10].
The presence of different phytochemicals in plants account for their biologic activities. Among these phytochemical of pharmacological importance are phenols, flavonoids, saponins and alkaloids. Several phytochemicals from different plants may have some level of cells toxicity, due to the prolong use of anti-inflammatory agent it must be non-toxic to cells and safe for prolong use.

This study was design to test the anti-inflammatory properties of PoHG and determine its cytotoxic effect on Vero and HepG2 cells in order to determine its safety and possible antiproliferative activities.

2. Materials and Methods

2.1. Collection of Plants, Preparation and Extraction

The roots of *H. geniculata* was bought in Puthaditjhaba market, Qwaqwa Northern Free State, South Africa. Confirmation of the plant species was carried out by comparisms with an earlier voucher specimen (Ash/med/05/2013/QwHB) in the herbarium of the Department of Botany, University of Free State, Qwaqwa Campus, South Africa.

2.2. Extraction of Phenols

The roots was properly rinse with water, dried and chopped into pieces. It was air dried at 25°C, pulverized into fine powder with a laboratory blender (Labcon, South Africa) and kept at 4°C prior to extraction.

Phenol extraction was done using shake extraction procedure [13]. In brief, addition of 3:17 (v/v) mixture of 1 M HCl and 95% C₂H₅OH to 5000 mg of the root powder of *H. geniculata*. The mixture was placed on an orbital (rotary) shaker set at 30°C for a duration of 1hr. Centrifugation of the mixture was carried out at 3000 rpm for 10mins, the mixture was then sieved with Millex-HV syringe (0.45 µM) which is followed by evaporation of the filtrate under reduced pressure. The extract was stored at 4°C before using them for *in vitro* assays.

2.3. Soybean 5-Lipooxygenase Inhibition Assay

The assay was performed according to previously described procedure [14]. The assay is based on the formation of the complex Fe³⁺/xylmol orange with absorption at 560 nm. 5-lipooxygenase from *Glycine max* was incubated with different concentration of the extract or standard (0.00078-0.1 mg/mL) at 25°C for 5 min. Then linoelic acid (final concentration, 140 µM) in Tris-HCl buffer (50 mM, pH 7.4) was added and the mixture was incubated at 25°C for 20 min in the dark. The assay was terminated by the addition of 100 µL of FOX reagent [sulfuric acid (30 mM), xylmol orange (100 µM), iron (II) sulfate (100 µM), methanol/water (9:1)]. The lipooxygenase inhibitory activity was evaluated by calculating the percentage of the inhibition of hydroperoxide production from the changes in absorbance values at 560 nm after 30 min at 25°C. % inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance control] x100. The 50% inhibition of enzyme activity (IC₅₀) were determined graphically using the linear regression equation y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC₅₀ value.

2.4. Cell Culture of Vero and HepG2

The Vero cell and HepG2 cells were maintained at Department of Paraclinical Sciences, cell line laboratories, Onderstepoort campus, University of Pretoria, South Africa. The culture medium used for the cell culture was DMEM supplemented with 10% FBS. 100 µg/mL streptomycin and 100 units/mL penicillin was added to the culture medium. The cell culture environmental condition was 5% CO₂ humidified atmospheric condition at 37°C.

2.5. Cytotoxicity of Cell Lines

Viable cells growth after incubation of African green monkey (Vero) cells and Human hepatocarcinoma (HepG2) with PoHG extract was determined using the tetrazolium-based colorimetric (MTT) assay [15]. Briefly, cells that had reached sub-confluent in their culture medium were harvested, centrifuged and re-suspended in the growth medium at 5 x 10⁴ cells/mL. MEM was used as the growth medium and it was supplemented with FCS (5%) and 0.1% gentamcin (Vibrae). MEM (200 µL) was added to wells of columns 1 and 12 to maximize the “edge effect” and maintain the relative humidity. The 96 well plates were incubated at 37°C until the exponential growth phase of the cells were reached. MEM in the plates was removed carefully without disturbing the cells and washed in 150 µL PBS. It is important to minimize the disturbance of the cells during the aspiration of MEM. The serial dilutions of the PoHG extract at differing concentrations of 0.05 – 1.0 mg/mL were made in quadruplicate. The serial dilutions of the test extract were all prepared in MEM and the mixture were added to the wells. The microtitre plates were incubated at 37°C in 5% CO₂ for 48h. The cells that were not treated and doxorubicin chloride were used as negative and positive control respectively.

After 48 h of incubation of the plates, 30 µL MTT (stock of 5 mg/mL in PBS) was put into all the wells and the plates were subjected to 4 h of further incubation at optimum temperature of 37°C. After incubation, the MTT in the culture medium in each of the cell was removed gently without distorting the MTT crystals. The formazan crystals formed by MTT were dissolved by the addition of DMSO in each of the well. The plates were gently shaken to facilitate better dissolution of the MTT crystals. The MTT reduction by the cells was measured by taking their absorbance using a microplate reader (Synergy Multi-Mode Reader, BioTek) at 570nm and a reference wavelength of 630nm. The reader was blanked using the column 1 well which contains only MTT and medium. The LC₅₀ values was determined as the concentration of *Hermannia geniculata* extracts resulting in a 50% reduction of absorbance compared to untreated cells.
2.6. Selectivity Index (SI)

The degree of selectivity of PoHG extract was expressed by its SI value as suggested by. 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 which can also cause cytotoxicity in normal cells [16, 17]. Each SI value was calculated using the formula: SI = \( \frac{LC_{50} \text{normal}}{LC_{50} \text{cancer cell}} \times 100 \).

2.7. Statistical Analysis

This was carried out using Graph Pad 5 statistical package (Graph pad software, USA). The n=3; Mean ±SD for all in vitro assays. In vitro assays were subjected to a two way ANOVA and Bonferroni (Post Hock Test) which compares all pair of column. (P< 0.05) was considered as statistically significant.

3. Result

The result of 5-lipoxygenase inhibition of PoHG extract is shown in Table 1. PoHG has commendable inhibition of 5-lipoxygenase enzyme with IC\(_{50}\) (150 ±0.03) µg/mL which is similar to the reference compound indomethacin with (IC\(_{50}\): 110 ±0.01) µg/mL. Similar percentage inhibition of the enzymes is seen for PoHG and indomethacin at concentrations (3.13, 12.50 and 50.00) µg/mL. PoHG extract inhibit 5-lipoxygenase enzyme in a concentration dependent manner with the highest inhibition of the enzyme occurring at 100.00 µg/mL (Figure 1). The cytotoxicity of PoHG on Vero and HepG2 cells was evaluated using MTT assay. The LC\(_{50}\) of PoHG extract was >1 which is similar to the LC of >1 also recorded for the standard (Table 2). The observed responses of HepG2 cells toward increasing concentration of the extract was exponential decrease in viability of HepG2 cells. At concentration of 93.5 µg/mL, marked cancer cells fatality was observed and less than 5% of the cells were viable at the maximum concentration of 750 µg/mL (Figure 3). This is similar to the result recorded for doxorubicin. The selectivity index was (SI: 20 and 33.33) for phenols and doxorubicin respectively (Table 2).

Table-1. Showing IC\(_{50}\) values of the inhibitory capabilities of different extracts of Hermannia geniculata phenols on 5-lipoxygenase enzymes (µg/mL)

| Phenols    | Indomethacin |
|------------|--------------|
| 150 ± 0.03 | 110 ± 0.01   |

The values were expressed as Mean ± SEM of triplicate determination. Indomethacin is the standard anti-inflammatory agent used in this assay.

Table-2. Showing cytotoxic activity of the phenols of Hermannia geniculata on Vero and HepG2 cells expressed as LC\(_{50}\) (mg/mL) of plant extracts

| LC\(_{50}\) | Vero | HepG2 | SI   |
|-----------|------|-------|------|
| Phenols   | >1.00 ± 0.02 | 0.05  | 20.00|
| Doxorubicin| >1.00 ± 0.01 | 0.03  | 33.33|

Data are the means ± SD (standard deviation) n=8. Selectivity Index (SI) = IC\(_{50}\) Vero cell/ IC\(_{50}\) HEPG2 cell. SI value >2 indicating high selectivity.

Figure-1. Inhibition of 5-lipoxygenase enzyme activity by phenols of Hermannia geniculata root extract and indomethacin. Results represent three triplicate value, mean± standard deviation (SD)
Figure-2. Percentage viability of Vero cells at different concentrations of the Phenols of Hermannia geniculata roots extract and doxorubicin. Data represent the mean ± SE (standard deviation) of three independent experiments.

Figure-3. Percentage viability of HepG2 cell viability at different concentrations of the Phenols of Hermannia geniculata roots extract and doxorubicin. Data represent the mean ± SD (standard deviation) of three independent experiments.

4. Discussion
4.1. Anti-Inflammatory Activities of PoHG

Use of conventional drug like corticosteroid, salphasalazine which inhibit leukotriene biosynthesis through 5-lipoxygenase (5-LOX) inhibition has been in use for the management of inflammatory bowel diseases, acute colitis, rheumatoid arthritis and asthma [18, 19]. However, these anti-inflammatory drugs currently in use may effectively manage acute inflammatory conditions but prolong use as observed in chronic inflammation causes a lot of side effects which necessitate the need to search for natural products with inhibitory effect on 5-LOX enzyme. There are several reports of the inhibition of 5-lipoxygenase by phenolic extracts [20-23]. The result obtained from this study shows commendable inhibition of 5-lipoxygenase enzyme by PoHG extracts. The IC$_{50}$ values of 150±0.03 µg/mL recorded for phenols in this study is similar to the value of standard (indomethacin) (IC$_{50}$: 110±0.01) µg/mL. The enzyme 5-lipoxygenase is involved in the metabolic pathway of the conversion of arachidonic acid to form (5-hydroxyecosateteraenic acid) HETE which is a substrate for leukotriene production. The downstream metabolic products of leukotriene B$_3$ is important in the pathophysiology of asthma and acute colitis [3, 24, 25]. This suggested that down regulation of 5-LOX activity is a therapeutic option in managing many inflammatory related diseases. Several studies are in agreement with this suggestion [22, 26-28].
4.2. Phenols Cytotoxicity

The observed LC$_{50}$ value of PoHG on Vero monkey kidney cell lines is $>$ 1, this shows that the extract is safe at the highest concentration of 1 mg/mL while it showed selective toxicity of the human hepatocellular carcinoma cells. The viability of HepG2 cancer cell at 0.75 mg/mL is less than 5%. The selectivity index (SI: 20 and 33.33) was recorded for the phenols and the standard doxorubicin respectively. Extract with SI higher than 10 has been considered to have anticancer properties.

Our findings in this study shows that PoHG extract is safe, possess anti-inflammatory and anticancer properties. It may be a potent agent needed to manage asthma, arthritis, cancer and other inflammation induced illnesses.

5. Conclusion

The *in vitro* activities of the PoHG showed that the extract can be used in the management of asthma, diabetes and colitis. *In vivo* studies of the extract is ongoing in the laboratory. Also, the isolation and elucidation of the bioactive constituents of the extract is at an advance stage.

This results validate the traditional use of the plant in the treatment of inflammatory diseases.

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