Research Article

Antioxidant and Anti-inflammatory activity of *Plectranthus glandulosus* Leaf extracts

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Received: 05-04-2020; **Revised:** 24-06-2020; **Accepted:** 02-07-2020.

ABSTRACT

The hypothesis that *Plectranthus glandulosus* leaf extracts and fractions possess antioxidant and anti-inflammatory activities was tested. The antioxidant capacity was characterized by total phenolic content (TPC), total flavonoid content (TFC), 2.2-Diphenyl-1-picrylhydrazyl (DPPH) and 2.2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activities, and the reducing power. The ant-inflammatory activity was characterized by albumin denaturation, red blood cell (RBC) hemolysis and Proteinase inhibition. The EAF exhibited highest TPC, TFC (139.30±0.16 and 9.11± 0.01mgGAE/g) which resulted to higher DPPH, ABTS scavenging activities reducing power. The EAF showed highest ability in inhibiting albumin denaturation, RBC hemolysis and Proteinase activity.

Keywords: *Plecthrantus glandulosus*, antioxidant, anti-inflammatory, Phytochemical Screening.

INTRODUCTION

Two related patho physiological processes stimulated by abnormal production of free radicals, pro-inflammatory mediators, causing extreme cellular damage and involved in the pathogenesis of human chronic diseases are oxidative stress and inflammation[1,2]. It is also worth mentioning that oxidative stress and pro-inflammatory mediators can easily be induced by one another[1].

Oxidative stress is characterized by generation of reactive oxygen species, such as superoxide (O_2·), hydroxyl (·OH) and peroxyl (·OOH, ROO·) radicals. Reactive oxygen and nitrogen species can induce intracellular signaling cascade to stimulate expression of pro-inflammatory genes[3]. Inflammatory disorders are characterized by excessive activation of phagocytes, production of O_2·,·OH radicals as well as non-free radical species (H_2O_2). These can in turn harm surrounding tissue either by direct or indirect radicals formed from O_2 and provokes further inflammatory response characterized by chemo tactic factors [3].

Natural products are increasingly purported to exert potent beneficial actions to support health and may thus play a role in reducing the use of synthetic drug for treatment of metabolic complications[4]. A traditional health care system has been in use since prehistoric times and is still the most important health care system today for most of the world's population in low- and medium-income countries. With plants being the most frequently used ingredient in traditional recipes, many research activities have focused on the use of medicinal plants for the management of diseased conditions. In an earlier study [5] laid emphases on plants with antioxidant and anti-inflammatory potential that may treat various kinds of injuries or protect against diseases

Plecthrantus glandulosus Hook. F. (Lamiaceae) is one of such natural products with culinary applications as well as health benefits. It is a climbing herbaceous plant, three metres long that grows in mountainous and forest areas and widely distributed in West, Central and South of Africa[6]. *Plectranthus glandulosus* is used in traditional Cameroon medicine for the treatment of dermatitis, bellyache, venereal diseases, internal inflammation, lower abdominal and nerve ache. *Plectranthus glandulosus* is used as condiment in the Ewondo tribe under the name Ava[6].

Earlier studies reported on the antinociceptive and anti-inflammatory effects of the aqueous leaves extract of *Plectranthus glandulosus*. Hook. F. (Lamiaceae) in mice and rats[7]. Also, the essential oils of *Plectranthus glandulosus* plants have shown antioxidant and insecticidal activity against stored grain insects in North Cameroon [8]. Other studies on efficacy of *Plectranthus glandulosus* (lamiaceae) leaf extract fractions against *Callosobruchus maculatus* (coleoptera: bruchidae)[9]; phytochemicals and larvicidal activity of *plectranthus glandulosus* (lamiaceae) leaf extracts against *Anopheles gambiae*, *Aedes aegypti* and *Culexquinque fasciatus* have been reported [10].

The present study was undertaken to evaluate the in vitro antioxidant and anti-inflammatory activities of aqueous,

ethanolhydro ethanol, hexane, ethyl acetate, n-butanol and residual fractions of *Plectranthus glandulosus* leaves.

MATERIALS AND METHODS

Chemical

Ethanol, ethyl acetate, hexane, n-butanol, anhydrous sulfate magnesium, HCl, Mayer's reagent (Potassium mercuric iodide solution), NaOH, hydrochloric acid, ferric chloride, potassium ferrocyanide, Fehling's solution, chloroform, Folin–Ciocalteu, Catechine, quercetin,2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azino-bis(3 ethylbenzothiazoline-6-sulfonic acide) (ABTS), sodium persulfate, phosphate buffer, potassium ferricyanide, trichloroacetic acid, bovine serum albumin (BSA); sodium diclofenac sodium, phosphate buffer saline (PBS), trypsin, Tris/HCl buffer, casein, perchloric acid. All these chemicals were purchased from Sigma Aldrich.

Plant material

The fresh leaves of *Plectranthus glandulosus* were collected in January 2017 (7:30 am -9:00 pm Cameroon time) in Ngaoundere, Adamawa Region (plateau) of Cameroon (latitude 7_ 220 N and longitude 13_ 340 E, altitude of 1.100 masl). The identification of the plant was done at the National Herbarium, Yaoundé-Cameroon, where voucher specimen was deposited (41168-HCN).

Extraction and Fractionation of Plant Materials

Preparation of aqueous extract

The fresh leaves collected from their natural environment were washed with distilled water three times, shade dried and powdered. The powdered material (1200g) was then extracted by maceration in 10 liters of distilled water at room temperature for 2 days. The aqueous crude extract was then collected and dried using ventilated drying oven (MANESTY- PETRIE) at 70°C for 24 hours and the residue obtained was 133.84 g. The yield of the extraction process was calculated according to the following formula:

Yield= MS/MP X 100

MS = mass of the dry matter obtained; MP = mass of the starting powder.

Preparation of ethanol extract

The powdered plant material (1200g) was extracted by cold maceration in 14 liters of ethanol for 2 days. The ethanol extract was then collected and concentrated using rotary evaporator (BUCHI). The concentrated extract was further dried using ventilated drying oven (MANESTY- PETRIE) at 70°C for 24 hours. 34.6g of dry powdered extract was obtained and the yield calculated.

Preparation of hydro ethanol extract

The hydro ethanol solvent was prepared by mixing water and ethanol in the ratios of 70:30 (v/v). After 48 hours of maceration of 2400g powdered plant material with the hydro ethanol solvent, the extract was concentrated using rotary evaporator (BUCHI). After which drying was carried out using ventilated drying oven (MANESTY-PETRIE) at 70°C to give 263.09g of dry extract.

All crude extracts were stored in refrigerator at 4°C until needed.

Fractionation

The ethanol crude extract was fractioned in hexane, ethyl acetate, n-butanol in increasing order of polarity. The hydro ethanol extract (190g) was initially mixed with 500mlof distilled water and 450ml of hexane. The mixture was transferred into a separating funnel and after shaking for 10 minutes two phases were obtained. The hexane fraction which represents the upper organic phase was transferred in to an Erlenmeyer containing anhydrous magnesium sulfate which is used as desiccant making it possible to eliminate the traces of water. The lower organic phase was extracted many times adding fresh solvent (hexane) until a clear phase was obtained. The hexane fractions were then pooled and stored as such. The lower phase was then mixed with subsequent solvents (ethyl acetate and then n-butanol) and they followed same treatment as hexane fraction. At the end of the fractionation the residual fraction was called residue. The same rotary evaporator and ventilation drying oven were used to concentrate and dry the fractions. The yield of each process was calculated and the fractions were stored in refrigerator at 4°C until required.

Preliminary Phytochemical screening

The presence of possible phytochemical constituents (alkaloids, flavonoids, saponins, tannins, phenols, terpenoids, glycosides) in extracts and fractions were evaluated qualitatively[11, 12]. These tests were based on color change generated by reaction of the bioactive components with principal reagents mixture.

Assessment of in vitro antioxidant activity of extracts and fractions

Total phenolic content

Total phenolic content was analyzed using the Folin–Ciocalteu colorimetric method[13,14] with some modifications. 200 µl of extract, fraction of *Plectranthus glandulosus* leaf was added to 1ml Folin–Ciocalteu phenol reagent (10 times diluted). After 4 min, 800 µl of a sodium carbonate solution (75 mg/ml) was added in the reaction medium and incubated for 2 hours at room temperature. The absorbance was then measured at 765nm. Standard curve for gallic acid (GA) in the range 10-100 µm/ml was prepared in the same manner and results were expressed as mg gallic acid equivalent per gram of extract.

Total flavonoid content

Total flavonoid content was determined using the aluminum colorimetric method [15]using quercetin as the standard. A calibration curve of quercetin was prepared in the range of 10-100 µm/ml. Briefly 500 µl of extracts, and fractions were placed in different test tubes and to

each 1500µl of distilled water and 150µl de sodium nitrite ($NaNo_2$) (5%) were added and incubated for 5 minutes at room temperature. After which 150µl of $AlCl_3$ (10%) was added to the mixture and 6 minutes latter 500µl NaOH (4%) was added in the reaction medium. The mixture was homogenized and the absorbance of the solution (pink color) was read at 510 nm.

2, 2-Diphenyl-1-picrylhydrazyl radical(DPPH˙)-scavenging assay

Scavenging activities of extracts and fractions on DPPH˙ was measured according to the method developed by [16] with some modifications. The extracts, fractions and the standard (catechine) were prepared with concentration range of 300, 100, 30, 10, 3 and 1µg/ml. The DPPH was prepared at concentration of 0.1mM.Briefly in 2ml of DPPH, 500µl of extract, fraction or standard was added. After agitation the samples were incubated in dark room for 30 minutes. The optical density was read at 517nm.

The percentage inhibition was calculated according to the formula:

%DPPH = [(DPPH control - DPPH sample)/DPPH control] X 100.

Where DPPH sample is the absorbance of the sample solution in a steady state and DPPH control is the absorbance of DPPH˙ solution before adding the extract.

The values of the IC_{50} (The concentration causing 50% of inhibition) were graphically given by the linear regression.

Scavenging activity on 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS˙)+ assay

$ABTS^{\cdot+}$-scavenging activity was assessed according to the method described by Pellegrini [17] with some modifications. A mixture of ABTS (7.4 mmol/L) and sodium per sulfate (2.6 mmol/L) in water was prepared and stored at room temperature for 24 h in a dark room to produce $ABTS^{\cdot+}$. 8ml of $ABTS^{\cdot+}$ solution was diluted with 72 ml of distilled water and the absorbance adjusted to1.50 at 734nm. Different concentrations (300, 100, 30, 10.3 and 1µg/ml) of extracts, fractions and standard (catechine) solution (200µl) were added to the diluted $ABTS^{\cdot+}$solution (1800µl). After incubation at room temperature for 15 minutes, the absorbance was read at 734nm. The radical-scavenging activity was calculated as the percentage of inhibition according to the formula:

Percentage inhibition = [(ABTS control - ABTS sample)/ABTS control] X 100.

The $IC_{50}$was obtained graphically from the linear regression curve.

Ferric reducing antioxidant power (FRAP) assay

The reducing power was measured by the browning reaction method [18]. Varying concentrations (300, 100, 30, 10, 3 and 1µg/ml) of extracts, fraction and standard (rutin) solutions (1mL) were mixed with phosphate buffer (pH 6.6, 2.5 mL, 0.2 mol/L) and 1% aqueous potassium ferricyanide (2.5 mL). The mixture was incubated for 20 min at 50°C in water bath. An aliquot (2.5 mL) of 10% aqueous trichloroacetic acid was added to the mixture, which was subsequently centrifuged for 10 min at 3000 rpm. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% aqueous $FeCl_3$(2.5 mL), and the absorbance was measured at 700nm.

The values of effective concentration EC_{50} (concentration of the extract or fraction corresponding to an absorbance of 0.5 obtained by the interpretation of the linear regression curve) were graphically determined.

Assessment of in vitro anti-inflammatory activity of extracts and fractions

Inhibition of bovine serum albumin (BSA) denaturation

Inhibition of protein denaturation was evaluated using the method of Sakat [19]. Five hundred micro liters of 1% bovine serum albumin (BSA) was added to 100 µL of extracts, fractions of varying concentration (800, 400,200, 100, 50µg/ml). This was then allowed to stand at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The reaction mixture was then allowed to cool to room temperature and absorbance was recorded at 660 nm. Diclofenac sodium was taken as a positive control. All analyses were carried out in triplicates and percentage of inhibition for protein denaturation was calculated using the formula bellow:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Where Abs control is the absorbance of solution without extract Abs sample is the absorbance of extract, fraction, or standard solution.

The IC_{50} (The concentration causing 50% of inhibition) was graphically given by the linear regression.

Membrane stabilization

Preparation of red blood cell (RBC) suspension

Fresh cow blood (10 ml) obtained from the Yaoundé central abattoir was centrifuged in heparinized tubes at 3000 rpm for 10 min and the supernatant discarded. The residual RBC was then washed three times with equal volume of normal saline. The volume of the RBC after discarding the supernatant was measured and then reconstituted as 10% v/v suspension with phosphate buffer saline (PBS)[19].

Heat induced hemolysis

A volume of 100 µL of 10% RBC was added to 100 µL of the extract. The resulting solution was heated at 56°C for 30 minutes followed by centrifugation at 2500 rpm for 10 minutes at room temperature. Supernatant was collected, and absorbance was read at 560 nm. Diclofenac sodium was used as a positive control. Percentage of membrane stabilization was calculated applying the formula mentioned in section 1.6.1 above[20]. The IC_{50} (The

concentration causing 50% of inhibition) was graphically obtained from the linear regression.

Proteinase inhibitory action

The test was conducted in accordance with the method of Oyedepo[21]. The reaction mixture (2 ml) contained: 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml extracts at different concentrations (800, 400, 200, 100, 50µg/ml). The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of Proteinase activity was calculated as earlier mentioned in section 1.6.1 above. The values of the IC_{50} (The concentration causing 50% of inhibition) were graphically obtained from the linear regression.

Statistical analysis

The results are expressed as the mean ± SD for triplicates using Microsoft Excel 2013, IC_{50} and EC_{50} were calculated using non-linear regression with Graphpad prism 5 software Significance of difference between groups was evaluated by using analysis of variance ANOVA, $P<0.05$ was considered as statistically significant.

Extraction and phytochemicals analysis

The extraction yield for the crude extracts and fractions are presented in Table 1. Aqueous, ethanol and hydro ethanol extraction presented a yield of 11.15%, 2.88% and 10.96% respectively. Hexane fraction, ethyl acetate fraction, n butanol and residual fraction presented a yield of 34.11%, 10.34%, 3.15% and 42.26% respectively compared to the hydro ethanol crude extract. The total yield of the four fractions was 89.86 % instead of 100 %. Hence, we considered that approximately 10.14 % lost which may be due to the several washing procedures separation of fractions.

The phytochemical screening of crude aqueous, ethanol, hydro ethanol extracts and hexane, ethyl acetate, n-butanol, residual fraction revealed the presence of some secondary metabolites such as alkaloids, phenolic compounds, flavonoids, saponins, terpenoids, glycosides and tannins as shown in Table 2. Saponin was more in the aqueous, ethanol and hydro ethanol extracts and absent in the fractions. The phenolic compounds were abundant in the hydro ethanol extract, ethyl acetate and n-butanol fractions. Flavonoid was tested positive in all the extracts and fractions meanwhile terpenoids and glucosides were sparingly positive only in the fractions. Tannin was present in hydro ethanol extracts and the fractions. The ethyl acetate fraction was contained all bioactive components tested except saponin while the n-butaol fraction did not test positive for saponin and alkaloid.

RESULTS

Table 1: Yield of different crude extracts and fractions

Extracts/Fractions	Yield compared to the starting powder	Yield compared to hydro ethanolic crude extract
Aqueous extract	11.15 %	
Ethanolic extract	2.88 %	
Hydro ethanolic extract	10.96 %	
Hexane fraction	2.66 %	34.11 %
Ethyl acetate fraction	0.819 %	10.34 %
n- butanol fraction	0.25 %	3.15 %
Residual fraction	3.34 %	42.26 %

Table 2: phytochemical screening of crude extracts and fractions

Phytochemical compound tested	Aqueous extract	Ethanolic extract	Hydro Ethanolic extract	Hexane fraction	Ethyl acetate fraction	n-butanol fraction	Residual fraction
Saponins	+++	+++	++	-	-	-	+
Phenolic compound	-	-	+++	-	+++	+++	+
Flavonoids	+	++	++	T	++	++	+
Terpenoids	-	-	-	++	++	+	-
Glucosides	T	T	-	-	+	+	+
Tannins	-	-	++	-	+++	++	+
Alkaloids	T	T	T	-	++	-	+

Legend: + = Present at low concentration; **++** = present at moderate concentration; **+++** = present at high concentration; **T**= Present in trace; -= no present.

In vitro antioxidant activity of extracts and fractions

Total phenolic and flavonoid contents

Table 3 presents the TPC and TFC of extracts and fractions of *P glandulous* leaf. The ethyl acetate fraction presented the highest TPC and TFC of 139.3±0.16 mg GAE/g and 9.11± 0.01mgGAE/g respectively. This was closely followed by the n-butanol fraction while among the crude extract it was the hydro ethanol extract that presented the best TPC and TFC. The hexane fraction presented an insignificant amount of TPC and TFC.

Table 3: Total phenolic and flavonoids content of different crude extracts and fractions

Extracts/Fraction	Total phenolic content mgGAE/g of extract	Total flavonoids content mgGAE/g of extract
Aqueous extract	36.80±0.038	2.43±0.033
Ethanolic extract	40.50±0.25**	6.49±0.00***
Hydro ethanolic extract	64.28±0.2407***	11.84±0.03***
Hexane fraction	0.001±0.2344***	0.0006±0.01***
Ethyl acetate fraction	139.3±0.1680***	29.10±0.01***
n-butanol fraction	92.95±0.1156***	17.92±0.02***
Residual fraction	40.27±0.1389**	3.24±0.003**

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates; ***p≤0.01;***p≤0.001* significant difference compared to the aqueous extract.

DPPH scavenging activity

Figure1a and 1b shows the dose-response curve of DPPH radical scavenging activity of the different extracts and fractions of *Plectranthus glandulosus* compared to gallic acid. Among the extracts, hydro ethanol extract had a DPPH scavenging activity of 78.33% at the concentration of 300µg/ml. At very high concentration the plant extract showed high radical scavenging activity better that gallic acid. Meanwhile a concentration ranges of 3 – 30 µg/ml the gallic acid was a better scavenger than the extracts. At concentration 1µg/ml the extracts showed a higher percentage radical scavenging activity than gallic acid (30.76% and 16.43% respectively).

At concentration of 300µg/ml it was observed that the ethyl acetate fraction had the highest scavenging activity of 88.82% followed by n-butanol (85.26%) while at the same concentration gallic acid exhibited a DPPH scavenging activity of 74.82% (Fig 1b). But at the concentration of 10µg/ml gallic acid had significantly higher scavenging activity that ethyl acetate fraction (62.65±0.31% and 40.00±0.00% respectively).

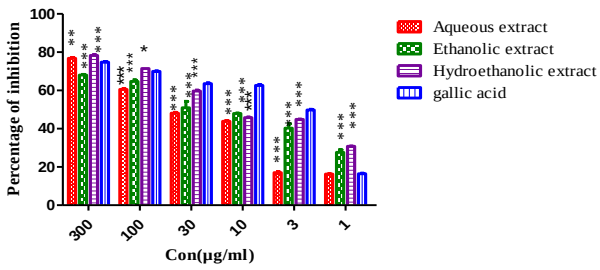

Figure 1a: DPPH scavenging activity of extracts compared to gallic acid

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates **p ≤ 0.05;**p≤0.01; ***p≤0.001* significant difference compared to gallic acid.

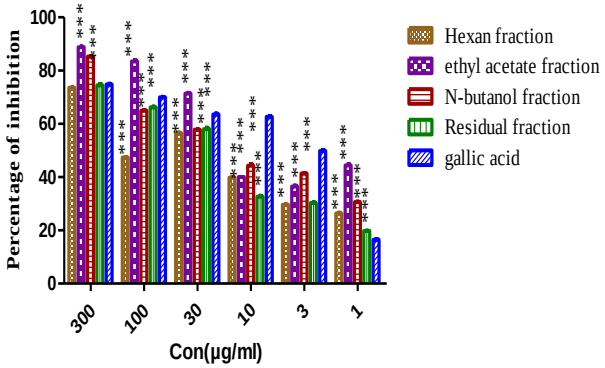

Figure 1b: DPPH scavenging activity of fractions compared to gallic acid

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates ***p≤0.01; ***p≤0.001* significant difference compared to gallic acid.

ABTS scavenging activity

The ABTS scavenging activity increased with concentration of the extracts and fractions. Furthermore among the extracts the ABTS scavenging activity of the hydro ethanol extracts was higher than that of aqueous and ethanol extract (Fig 2a). At the concentration of 300µg/ml the percentage of inhibition of hydro ethanol extract was 82.93 ±0.28%. Among the fractions, ethyl acetate fraction (89. 26 ±0.28%) exhibited an ABTS scavenging activity higher than other fractions (hexane, n-butanol and residual fraction) (Fig 2b). The ABTS scavenging activity of the extracts and fractions were lower than that of gallic acid (reference drug) that had a percentage scavenging activity of 95.12±0.00% at the

same concentration. Table 4 shows the value of $IC_{50}$of different extracts, fractions and gallic acid.

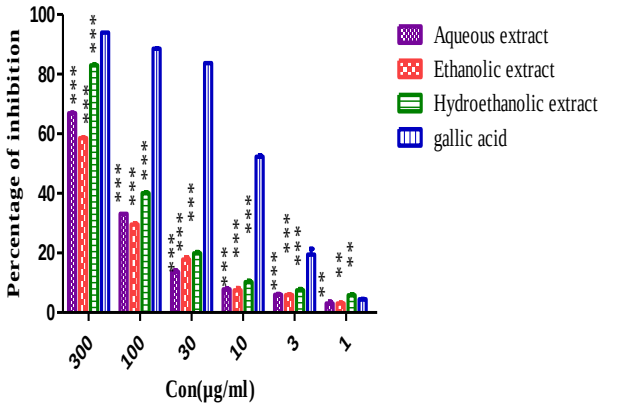

Figure 2a :ABTS scavenging activity of Extact copared to gallic acid

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates*; **p≤0.01;***p≤0,001* significant difference compared to gallic acid.

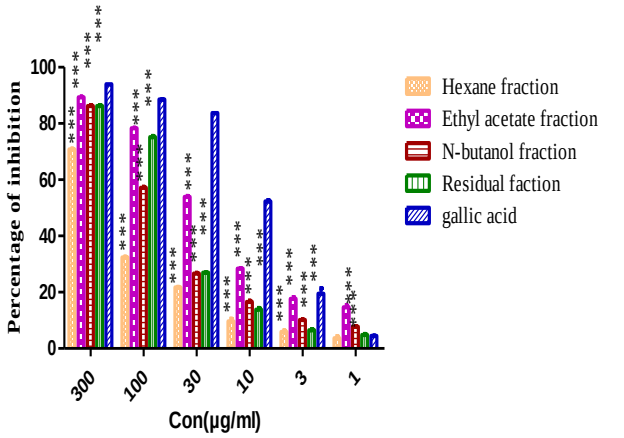

Figure 2b:ABTS scavenging activity of fractions compared to gallic acid

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates, ****p≤0.001* significant difference compared to gallic acid.

Ferric reducing power assay

Antioxidant activity has been associated with reducing potential and serves as an expression of sample antioxidant activity. The ferric reducing power assay was carried out by measuring absorbance. The absorbance of the plant extracts and fractions are presented in Figure 3a & b. Hydro ethanol extract showed the best EC_{50} (concentration of the extract or fraction corresponding to an absorbance of 0.5 obtained by the interpretation of the linear regression curve) of 40.97µg/ml and similar data were obtained for aqueous extract. For the fractions it was the ethyl acetate fraction which had 50.22µg/ml. The EC_{50} of rutin was 77.87µg/ml (Table 4). According to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species.

Table 4 presents the value of IC_{50} of extracts, fractions, gallic acid/rutin and their correlation coefficient with their respective percentage inhibition. It was observed that gallic acid with the lowest IC_{50} in DPPH and ABTS had the best scavenging activity hence a stronger antioxidant than the extracts and fractions studied. This was closely followed by the hydro ethanol and ethanol extracts in DPPH scavenging activity while in ABTS scavenging activity it was the ethyl acetate fraction that was best amongst the extracts and fractions. The aqueous extract showed a strong FRAP activity with the lowest EC_{50} value better than the reference standard (rutin) used. Overall the correlation values obtained were positive. For the DPPH the correlation coefficient was highest for the aqueous extract, residual fractions and gallic acid and similar for the ABTS. For the FRAP it was the ethyl acetate and ethanol extracts that had the highest correlation coefficient.

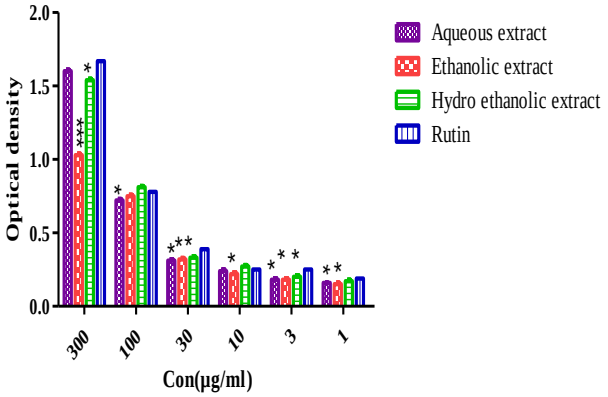

Figure 3a: Ferric reducing power assay of extract compared to rutin.

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates **p ≤ 0.05; ***p≤0.001* significant difference compared to rutin.

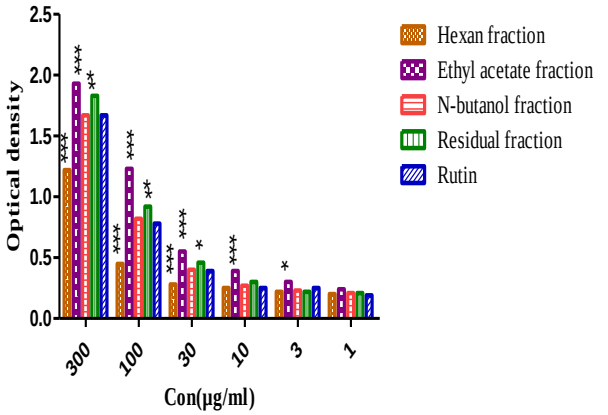

Figure 3b :ferric reducing power of fractions compared to rutin

Values represented: each bar represents the mean ± standard deviation; Analyses were done in triplicates **p ≤ 0.05;**p≤0.01; ***p≤0.001* significant difference compared to rutin.

Table 4: IC50 and Correlation coefficient (r) of different extracts and fractions in DPPH, ABTS essay and EC50 in Frap essay

Antioxidant activity IC50 (µg/ml) and Correlation coefficient (r)						
Essay	**DPPH**		**BTS**		**FRAP**	
Extracts/Fractions	**IC_{50}**	**Correlation coefficient (r)**	**IC50**	**Correlation coefficient (r)**	**EC_{50}**	**Correlation coefficient (r)**
Aqueous extract	11.56	0.9404	81.75	0.9522	8.43	0.9133
Ethanolic extract	4.524	0.8319	77.77	0.9425	71.11	0.9304
Hydro ethanolic extract	3.585	0.8387	66.36	0.9397	40.97	0.9173
Hexan fraction	8.100	0.8093	70.98	0.9277	82.71	0.7880
Ethyl acetate fraction	5.925	0.7898	17.68	0.9723	50.22	0.9310
n-butanol fraction	7.508	0.8380	48.31	0.9601	72.18	0.9017
Residual fraction	8.152	0.9226	40.67	0.9806	69.58	0.9160
Gallic acid	2.431	0.9247	8.237	0.9961	ND	
Rutine	ND	ND	ND	ND	77.87	0.8956

In vitro anti-inflammatory activity of extracts and fractions

Inhibition of bovine serum albumin denaturation

The plant extracts were effective in inhibiting heat induced albumin denaturation at different concentrations as shown in Figure 4a & b. Among the extract it was the hydro ethanol extract that had the highest percentage inhibition activity. However this was lower than the inhibition activity of diclofenac sodium in all dose level tested (Fig 4a). Amongst the fractions, maximum percentage inhibition, 91.48±0.98% was observed at 800µg/ml with n-butanol fraction followed by ethyl acetate fraction (91.36±0.93%) (Fig 4b).It was noted that there were no significant difference between n-butanol fraction, hexane fraction, diclofenac and hydro ethanol extract at 800µg/ml. However, these were not comparable to the diclofenac sodium (reference drug) at 600µg/ml. Between concentration of 400µg/ml to 50µg/ ethyl acetate fraction had the best percentages inhibition (88.89 ± 00 %, 88.77 ± 0.43%, 84.07 ± 0. 37%, 76.67 ± 0.37% at the concentration 400, 200, 100 and 50µg/mg respectively).Diclofenac Sodium percentage inhibition was 91.98±0.57% at the concentration 800µg/ml.

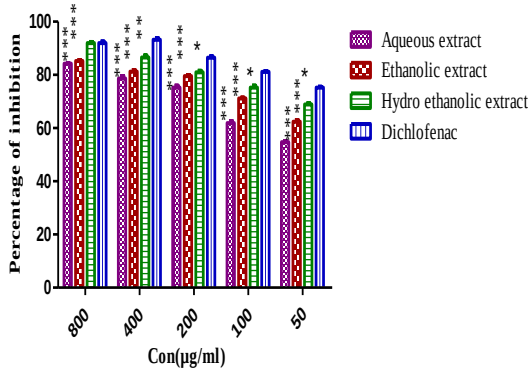

Figure 4a : extracts inhibition percentage of BSA denaturation compared to dichlofenac

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates **p ≤ 0.05; p≤0.001* significant difference compared to diclofenac.

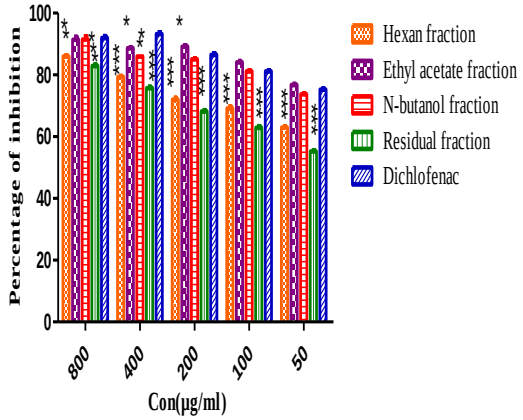

Figure 4b : fractions inhibition percentageof BSA denaturation compared to dichlofenac

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates **p ≤ 0.05; **p≤0.01;***p≤0.001* significant difference compared to diclofenac.

Membrane stabilization test

Tested extracts and fractions (800 to 50µg/ml) of the plant studied inhibited the heat induced hemolysis of RBCs to varying degree as shown in Figure 5a & b. The ethanol extract was the best inhibitor of membrane hemolysis and comparable to diclofenac (Fig 5a). Among the fractions it was ethyl acetate fraction that showed the maximum percentage inhibition 64.92±0.12% at 800µg/ml. The IC_{50} was calculated as 18.84µg/ml at correlation coefficient value (r) of 0.9561.The hydro ethanol extract which had percentage inhibition activity of60.07±0.12 % and IC_{50}of21.06µg/ml at same dose level was second. Diclofenac the reference drug showed the maximum percentage inhibition activity of 80.00.92 ± 0.20% at 800µg/ml but had a poor IC_{50} value of 52.45µg/ml.

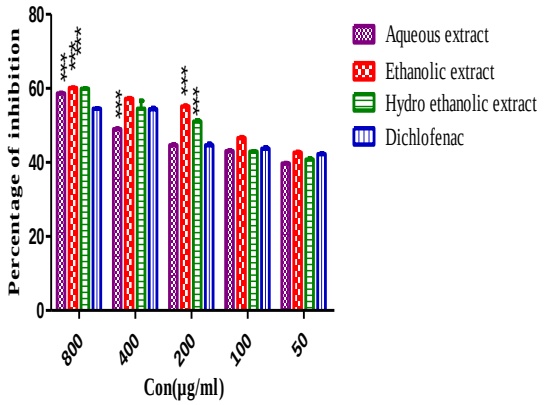

Figure 5a : extracts inhibition percentage of heat induced hemolysis of RBCs compared to dichlofenac

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates ****p≤0.001* significant difference compared to diclofenac.

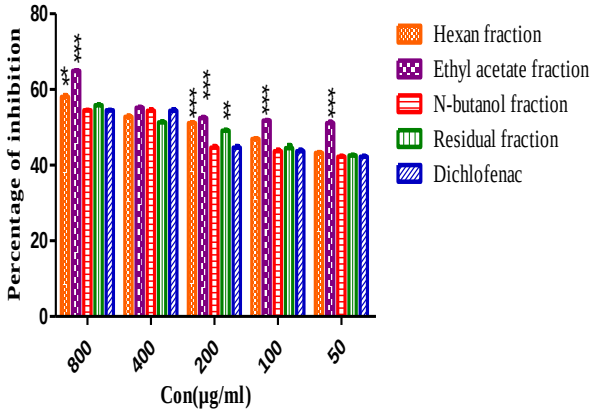

Figure 5b :fractions inhibition percentage of heat induced hemolysis of RBCs compared to dichlofenac

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates ***p≤0.01; ***p≤0,001* significant difference compared to diclofenac.

Proteinase Inhibitory Action test

Plectranthus glandulosus extracts and fractions exhibited significant Proteinase inhibitory activity at different concentrations (Figure 6a & b). The diclofenac sodium out smarted all extracts on Proteinase inhibition at all concentration levels. Among the extract it was the hydro ethanol extract that had the best activity. The ethyl acetate fraction showed maximum inhibition at 800µg/ml (78.98±00.27%) with IC_{50} value of 16.87µg/ml at correlation coefficient value (r) of 0.9978. Diclofenac sodium showed the maximum proteinase inhibition 80.05±0.27% at the same concentration and IC_{50} was 14.63µg/ml (Table 5).

Table 5 presents the IC_{50} and correlation coefficient between IC_{50} and percentage inhibition of plant extracts and fraction in relation of diclofenac sodium. Generally very strong correlation coefficients were obtained between IC_{50} and corresponding percentage inhibition activity. The IC_{50} value for albumin denaturation was found to be 17.03µg/ml for ethyl acetate fraction at correlation coefficient value (r) of 0.9987 followed by 17.26µg/ml for n-butanol fraction. Diclofenac sodium, a standard anti- inflammatory drug showed an IC_{50} of 17. 92 µg/ml. The aqueous extract had the highest IC_{50} values in albumin denaturation, membrane stabilization and proteinase denaturation inhibition while the ethyl acetate fraction had the lowest IC_{50} better than diclofenac sodium.

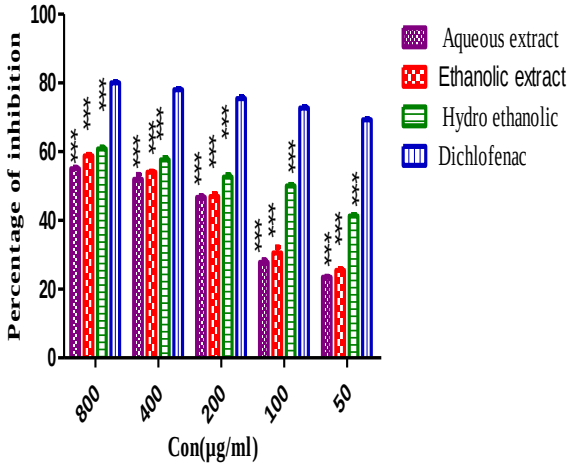

Figure 6a :extracts inhibition percentage of proteinase activity compared to dichlofenac

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates, ****p≤0.001* significant difference compared to diclofenac.

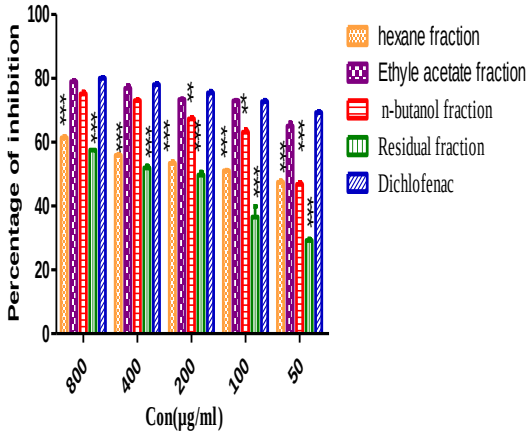

Figure 6b :fractions inhibition percentage of proteinase activity compared to dichlofenac

Values represented: each bar represents the average ± standard deviation; Analyses were done in triplicates **p ≤ 0.05;**p≤0.01;***p≤0.001* significant difference compared to diclofenac.

Table 5: IC50 and Correlation coefficient (r) of different extracts and fractions in BSA denaturation, membrane stabilisation and proteinase inhibition essay

Anti -inflammatory activity, IC50 (µg/ml) and Correlation coefficient (r)						
Essay	**Albumin denaturation**		**Membrane stabilization**		**Proteinase inhibition**	
Extracts/Fractions	**IC50**	**Correlation coefficient (r)**	**IC50**	**Correlation coefficient (r)**	**IC50**	**Correlation coefficient (r)**
Aqueous extract	31.55	0.9936	28.31	0.9659	73.75	0.9750
Ethanolic extract	23.11	0.9977	28.39	0.9853	73.24	0.9822
Hydro ethanolic extract	20.95	0.9924	25.33	0.9939	26.52	0.9955
Hexan fraction	21.95	0.9865	21.06	0.9894	18.90	0.9860
Ethyl acetate fraction	17.03	0.9987	18.84	0.9561	16.87	0.9969
n-butanol fraction	17.26	0.9949	19.90	0.9745	34.59	0.9978
Residual fraction	28.28	0.9899	20.23	0.9871	52.92	0.9880
Diclofenac	17.92	0.9957	52.45	0.9097	14.63	0.9961

DISCUSSION

Phytochemical screenings are often carried out to determine the presence of bioactive components present in the plant material so as to link them up to their biological activities. In the present study only a trace amount of alkaloid was detected in the extracts and fractions. Alkaloids have earlier been associated with anti-inflammatory anti malarial, antimicrobial cytotoxicity and antispasmodic while tannin has been associated with antibacterial, antitumor and antiviral activities due to their ability in precipitating microbial protein [12]. Just like alkaloids the glycoside was detected in trace amount in the present study. Meanwhile glycosides have been associated with management of congestive heart failure and cardiac arrhythmia by inhibiting Na+/K+ pump resulting to increased calcium ion necessary for contraction of heart muscles[12]. Generally, phenolic compounds including flavonoids have been studied mainly for their properties against oxidative damage leading to various degenerative diseases, such as cardiovascular diseases, inflammation and cancer [22]. Thus their presence in the plant extract in this present study is an indication of their antioxidant potential. Saponins have been associated with hypoglycemic activity, accelerating metabolism of cholesterol in the liver, antifungal, antimicrobial, veridical and anti-inflammatory activities[23]. Terpenoids fight against cancer, malaria, inflammation, and various infectious diseases [24].

Different methods were employed to evaluate the antioxidant capacity. This is because as earlier stated no one method is sufficient to determine antioxidant capacity of a plant extract or fraction [25] due to the complex nature of the plant material and their respective mechanisms of action [26].Among the crude extracts, the hydro ethanol extract presented the highest TPC and TFC while the ethyl acetate fraction was the best among the fraction. The phytochemical data showed these extracts to contain most of the bioactive components than the others. This is suggestive that the bioactive components present in the extracts and fractions influenced their TPC and TFC and hence makes these solvents best for extraction of flavonoids and polyphenols. Similar report has earlier been presented on the extractability of ethyl acetate [27]. Comparing the TPC of *Plecthrantus glandulosus* obtained in the present study to those of same genus earlier reported, it was observed that ethyl acetate leaf faction of *Plectranthus stocksii* had higher TPC (355.41 ± 8.48 mg GAE/g of extract)[28]. However, the TPC of ethanol and aqueous extracts of *Plectranthus glandulosus* leaf was higher compared to data on ethanol and aqueous leaf extracts of *Plectranthus amboinicus* (11.6µg/48 mg GAE/g and 9.4µg/ mg GAE/g of extract for respectively)[29]. In the same way *Plectranthus glandulosus* hydroethanol leaf extract had a higher value compared to *Plectranthus amboinicus* (8.4mg GAE/g of extract [30]. Flavonoids are important sub-branch of the polyphenol family and are known as antioxidants because they possess the ability to reduce oxidative damage in the cell. They can directly trap free radicals or scavenge them through a series of coupled reactions and also stabilization of lipid peroxidation hence preventing cellular damage[31].

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is often used in evaluating antioxidant activity because it can accommodate many samples within a short time and is sensitive even at low substance concentration [31]. This test operates the hydrogen atom donation antioxidant action characterized by a decrease in absorbance of the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical solution in the presence of an antioxidant substance. The IC_{50} (5.92µg/ml) value of *P. glandulosus* ethyl acetate leaf fraction was higher than that of *P. stocksii* ethyl acetate leaf fraction earlier published IC_{50} (3.46µg/ml)[28]. This makes *P. stocksii* a better DPPH radical scavenger than *P. glandulosus*. The obtained antioxidant capacities of the extracts and fractions may be related to their constituent metabolites and are closely dependent on the contents of the phenolic compounds and their molecular structure that play a role in the stabilization of resulting phenoxyl radicals via hydrogen

bonding or by expanded electron delocalization [31]. The ABTS assay depends on the antioxidant compound ability to scavenge ABTS radical. This assay measures the antioxidant capacity of lipophilic and hydrophilic compounds in the same sample[12]. Thus it is the appropriate radical scavenging assay for aqueous extracts. It has earlier been reported that ABTS˙+-scavenging activities correlates significantly with the abundance of phenolic compounds[32]. Thus the TPC observed this this study may be responsible for the ABTS˙+- scavenging activities. The Fe (III) reduction is an indicator of electron donating ability which is an important mechanism in phenolics as antioxidants. The electron-donating property indicates that the antioxidant compounds can act as primary and secondary antioxidants donors and can reduce the oxidized intermediates in lipid peroxidation process[28]. Just like plants in the same genus such as *Plectranthus stocksii* [28], *Plectranthus amboinicus* [29, 30, 33], *Plectranthus barbatus*[34] on which antioxidant activities were already carried out, the present study portrays *Plectranthus glandulosus*as a potential antioxidant source. The antioxidant property of *Plectranthus glandulosus* essential oil has earlier been reported [8] and these essential oils may contribute to its antioxidant property. The availability of exogenous antioxidant molecules found in plants may play a very important role in taking care of the excesses of intoxication, hence reducing the stress on endogenous antioxidant system[35]. Reason why a glass of fruit juice, a cup of tea, coffee etc is often advised in a stressful day. For all three methods used to ascertain the antioxidant capacity of *Plectranthus glandulosus* the hydro ethanolic extract portrayed the best activity comparable to the reference standards used in this study. This antioxidant capacity could stem from the high TPC and TFC of the extract as shown in this study. The phytochemical screening confirms the presents of TPC and TFC and reveals the presence of saponins and tannins which may contribute to the antioxidant capacity. Meanwhile for the fractions the ethyl acetate fraction had the best antioxidant capacity in all three methods used. Hence, we may suggest that the bioactive components contributing to the antioxidant capacity may be found in the ethyl acetate fraction.

Protein denaturation is a process in which protein loses its secondary and tertiary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of protein is a well-documented cause of inflammation [36]. Anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation [36]. In the present study we presented the anti-inflammatory activity of plant extracts and fractions to inhibit protein denaturation. The mechanism of protein denaturation certainly involves the bond breaking of the electrostatic, hydrogen, hydrophobic and disulfide bridges. The ability of *P glandulosus* extracts and fraction to effectively inhibit thermal protein denaturation means the stabilizing of these bridges either by single electron transfer of hydrogen atom transfer related to their antioxidant capacity determined in this study. Stabilization of the RBCs (red blood cells) membrane was studied to further establish the anti-inflammatory action of *Plectranthus glandulosus* because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extracts and fractions may well stabilize lysosomal membranes [37]. The extract and fractions were effective in inhibiting the heat induced hemolysis at different concentrations. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release [3]. The lysosomal enzymes released during inflammation produce various disorders and the extra cellular activity of these enzymes are said to be related to acute or chronic inflammation [3]. Non-Steroidal Anti-inflammatory drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [3]. Hence the ability of extracts and fractions of *Plectranthus glandulosus* leaf to stabilize the RBC which mimics the lysosomal membrane is a great stride towards the anti-inflammatory activity.

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases [36]. It was previously reported that leukocytes Proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors [36]. Plants in the same genus such as *Plectranthus amboinicus* [38] have earlier been reported to possess anti-inflammatory properties. *Plectranthus barbatus*[39] has been reported on antioxidant and anti-inflammatory activities. Flavonoids and related polyphenols from plants have been associated with antioxidant and anti-inflammatory activity which may translate to *Plectranthus glandulosus* leaf extracts and fractions activities.

The present revealed that the leaf extracts and fractions of *Plectranthus glandulosus* contained bioactive molecules that possess antioxidant and anti-inflammatory activities. Hence support its use in traditional medicine for the management of inflammations.

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Source of Support: None declared.

Conflict of Interest: None declared.