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Modulation of the *in vitro* Oxidative Stress and Erythrocyte Cell Membrane Integrity Using Aqueous, Hydroethanolic and Ethanolic Stem-Barks Extracts of *Greenwayodendron suaveolens* (Engl. & Diels) Verdc

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Abstract

Pneumonia, a respiratory infection induces acute or chronic inflammation, characterized by increased activity of lymphocytes and neutrophils, thus generating oxygen-free radicals that decrease the endogenous antioxidants defence system. The aim of this experimental study focused on the capacity of nontoxic aqueous, hydroethanolic and ethanolic extracts of *Greenwayodendron suaveolens* (Engl. & Diels) Verdc. subsp. *suaveolens* to regulate free reactive species and protein inflammation generated by infectious disease. The phytochemical screenings of *G. suaveolens* extracts were carried out according to precipitation and colorimetric methods. The total antioxidant and flavonoid contents were determined by the Folin-Ciocalteu and Aluminium Chloride ethanolic methods. The efficiency of *G. suaveolens* extracts on free

radicals was evaluated using DPPH•, $ABTS^{+\bullet}$, and FRAP methods. The anti-inflammatory properties of extracts were evaluated according to *in vitro* protein (BSA) denaturation, Proteinase Inhibitory Action, and Red Blood Cell Membrane stabilization assays. The *G. suaveolens* aqueous, hydroethanolic and ethanolic extracts were used for the acute toxicity assessment according to the OECD protocol. The obtained results showed the presence of flavonoids, phenols, polyphenols, tannins, anthocyanins, alkaloids, terpenoids, and sterols as secondary metabolites families in *G. suaveolens* extracts. The highest contents of total antioxidants and flavonoids were highlighted in the hydroethanolic extract. However, it's the *G. suaveolens* aqueous extract that showed the best free radical DPPH• and $ABTS^{+\bullet}$ scavenging activities (SC_{50}) of 11.06 µg/mL and 15.16 µg/mL respectively. The highest ferric-reducing activity was found in *G. suaveolens* ethanolic extract with 866.23 µg EGA/mg of dry weight. The hydroethanolic extract has shown a high anti-inflammatory activity through BSA denaturation and erythrocyte membrane haemolysis with inhibitory concentrations 50 (IC_{50}) of 48.63 and 59.22 µg/mL respectively. In contrast, proteinase inhibitory activity revealed a better potential of IC_{50} (34.19 µg/mL) for the ethanolic extract. In oral acute toxicity, all treated groups revealed neither mortality nor any significant alteration in behaviour and locomotion. The lethal dose 50 (LD_{50}) of *G. suaveolens* extracts was >5000 mg/kg. These results suggest that *G. suaveolens* stem-barks extracts may serve as therapeutic sources to prevent inflammation induced by oxidative stress, an important feature of infectious diseases.

Keywords

Greenwayodendron suaveolens, Secondary Metabolites, Oxidative Stress, Antioxidant Activity, Anti-Inflammatory Properties and Oral Acute Toxicity

1. Introduction

Pneumonia is an infection of the lung tissue [1] [2]. The major causative agents are bacterial in origin [3] [4]. Bacterial pneumonia, however, remains a leading cause of morbidity and mortality around the world, despite significant improvements in health care [5] [6] [7]. It is characterized by acute or chronic inflammation of the lung alveoli. This inflammation, in response to bacterial infection, is essential for pathogen clearance, and, alveolar macrophages, lymphocytes and neutrophils play a crucial role in this process [8] [9] [10]. However, it is important to observe that during bacterial proliferation, the causative agents generate ROS, which is primarily responsible for the pro-inflammatory processes highlighted during the physiopathology of the disease [11]. A second source of ROS production has also been highlighted, namely superoxide released by neutrophils and involved in the worsening of the disease. Subsequently, the presence of ROS at the site of infection induces lipid peroxidation and loss of protein function, and leads to collateral cell and tissue damage, causing organ failure [8] [12]. On the other hand, many researchers have highlighted the capacity of nat-

ural products to scavenge the free ROS and resorb inflammatory damage [13] [14] [15]. These natural products are antioxidant compounds, including phenolic compounds and others that bear free hydroxyl groups on aromatic rings, which are the most reactive [8]. Antioxidants present in medicinal plant products help to stimulate the biological and cellular defence systems against oxidative stress [16].

Greenwayodendron suaveolens (Engl. & Diels) Verdc. subsp. *suaveolens* is a monophyletic rainforest tree, the genus being endemic to Tropical Africa [17]. Most taxonomic treatments recognize two species, namely, *G. oliveri* in Western Africa, and *G. suaveolens* in Central and Eastern Africa including Nigeria. The recent studies conducted by Lissambou *et al.*, presented the evidence for the existence and recognition of six distinct species of Greenwayodendron [18]. *G. suaveolens* is a deciduous medium-sized to large tree, up to 35 - 45 m tall. Some of its vernacular names in Cameroon include "Otungui" (Ewondo), "Otunga" (Fang), "Moabé noir" (Nzime), "Ntoulen" (Bassa'a), and "Botounga" (Baka) [18]. It is used by the population of Cameroon to treat gonorrhoea, infertility, malaria, stomach ache, headache, epilepsy, toothache psychosis and rheumatism. It's also considered as facilitating childbirth, diuretic, purgative and aphrodisiac. In Gabon and Cameroon, bark ash is rubbed in scarification, on the forehead to treat psychosis and bark paste is applied externally to treat headaches, epilepsy, rheumatism, toothache and malaria [18].

To date, to the best of our knowledge, very limited documented data are available on the preventive or curative effect of *G. suaveolens* species on oxidative stress and inflammatory damage. Therefore, the aim of the present study was to investigate the capacity of nontoxic aqueous, hydroethanolic and ethanolic extracts of *Greenwayodendron suaveolens* (Engl. & Diels) Verdc. subsp. *suaveolens* to regulate free reactive species and protein inflammation.

2. Material and Methods

2.1. Chemicals Reagents

Gallic acid and quercetin (Sigma-Aldrich, St. Louis, MO, United States) were used as standards for the antioxidant standard. Sodium diclofenac (Bayer Pharma AG, Germany) was also used as a standard reference to confirm the anti-inflammatory effects of plant extracts. Sodium nitrate, aluminum chloride, 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2'-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid (ABTS$^{+\bullet}$), potassium persulfate, and 2,4,6-Tris(2-pyridyl)-*s*-triazine (Sigma Aldrich, France) were used as chemical reagents for the *in vitro* antioxidant activity. Bovine Serum Albumin, perchloric acid, casein, and trypsin (Medibest, Cameroon) were used for the anti-inflammatory activity.

2.2. Plant Material

The *Greenwayodendron suaveolens* stem-barks were collected at Kala Mount

(48˚51'N; 2˚17'E) in Yaoundé (Cameroon) on March 25th, 2018. The plant was identified by the Cameroon National Herbarium under the identification number 45578-HNC.

2.3. Preparation of the Aqueous, Hydroethanolic and Ethanolic Extracts

The extracts were prepared by maceration, based on the method described by Moni *et al.* with slight adaptations [19]. The stem-barks of *G. suaveolens* were air-dried at room temperature and weighed. 200 g of each pulverized and dried plant material were extracted by maceration in an aqueous, hydroethanolic (30/70; v/v) mixture and ethanolic solution, twice for 48 hours each at room temperature (27˚C ± 2˚C) in enclosed flasks. The mixtures were filtered through a Whatman N˚ 1 filter paper and evaporated under reduced pressure using a rotatory evaporator to obtain aqueous, hydroethanolic and ethanolic extracts. The resulting extracts of aqueous, hydroethanolic and ethanolic were freeze-dried, sealed, and kept in a refrigerator at 4˚C for further use. Fresh stock solutions were prepared for the experiment whenever required.

2.4. Phytochemical Analysis of *G. suaveolens* Extracts

Preliminary qualitative phytochemical screening of secondary metabolites in aqueous, hydroethanolic and ethanolic extracts was performed according to the methods described by Trease and Evans, and Harborne [20] [21].

2.5. *In vitro* Antioxidant Testing

2.5.1. Preparation of Samples for the *in vitro* Experiments

For *in vitro* assays, the aqueous, hydroethanolic and ethanolic extract stock solutions were dissolved in 99% methanol (Sigma Aldrich, France) at the concentration of 1.5 mg/mL, after being mixed for 5 min using a vortex mixer (Remi Cyclo Mixture, CM 101, Nimboliadda, Kachiguda Hyderabad - 500027, Telangana, India). The concentration of the stock solution of gallic acid, quercetin and sodium diclofenac was 1.5 mg/mL. The concentrations of tested extracts and standards were selected based on preliminary studies by Betote *et al.* on the essential oil of *G. suaveolens* [8].

2.5.2. Determination of Total Antioxidant Content

The total antioxidant content was evaluated according to the spectrometric method using the Folin-Ciocalteu reagent as described by Vinson *et al.* [22]. 20 µL (1.5 mg/mL) of aqueous, hydroethanolic or ethanolic extracts were mixed with 980 µL of a 10-fold diluted Folin-Ciocalteu reagent. After 4 min of incubation, 1 mL of 7.5% Na_2CO_3 solution was added and the mixture was allowed to stand for 120 min at room temperature. The absorbance was then measured at 765 nm. The calibration curve ($y = 0.0001375x + 0.1359$; R^2: 0.96) was created using a freshly prepared aqueous solution of gallic acid. The results were expressed in µg EGA/mg of dry extract.

2.5.3. Determination of Total Flavonoid Content

Evaluation of the total flavonoids in plant extracts was performed using the method of Zhishen *et al.* [23]. To 0.5 mL of sample (1.5 mg/mL), 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After incubating for one hour at room temperature, the absorbance was measured at 420 nm. A yellow colour indicated the presence of flavonoids. Total flavonoid content was calculated as quercetin equivalent per milligrams of dry extract (µg/mg) using the following equation based on the calibration curve: $y = 0.0003743x + 0.01513$; R^2: 0.99, where x was the absorbance and y was the quercetin equivalent (µg EGA/mg of dry extract).

2.5.4. DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Free Radical Scavenging Assay

A standard solution of 2,2-diphenyl-1picrylhydrazyl ($DPPH^{\bullet}$) was prepared by dissolving 3 mg of DPPH was dissolved in 75 mL of methanol [24]. This solution was diluted 2-fold with methanol to obtain 225 mL of final solution. 2000 µL of the DPPH solution was added to test tubes and 500 µL of plant extracts and gallic acid at six concentrations (1500, 750, 375, 187.50, 93.75 and 46.87 µg/mL) were then added to each test tube to a final volume of 2.5 mL per tube. All tests were performed in triplicate in a dark room. The optical density was measured at a wavelength of 517 nm using a Thermo-Fisher-Scientific spectrophotometer (Evolution 300 UV−VIS), after 30 min of incubation. The results were calculated as scavenging percentage (%SC), scavenging concentration 50 (SC_{50}), efficacy concentration 50 (EC_{50}) and antiradical power (AP).

2.5.5. ABTS Radical Scavenging Activity

The cationic ABTS radical scavenging activity, which is the most widely used method for determining the antioxidant activity of plant extracts, involves following the kinetics of discoloration of the $ABTS^{+\bullet}$ ion, as described by Re *et al.* [25]. ABTS (2,2'-azinobis-(3-ethylbenzothiazolin-6sulfonic acid)) was prepared by mixing 0.0384 g of ABTS and 0.00662 g of potassium persulfate ($K_2S_2O_8$) with 10 mL of distilled water. The mixture was incubated for 16 hours at room temperature, and protected from light before use. For this assay, the ABTS solution was diluted with ethanol and the absorbance was adjusted to 0.700 (±0.02) at 734 nm with stability at 30˚C (initial optical density). 3.0 mL of this diluted ABTS solution was added to the 30 µL of extracts/gallic acid (1500, 750, 375, 187.50 and 93.75 µg/mL) in a test tube and the mixture was agitated to homogenize. Absorbance was immediately read at 734 nm after agitation. The scavenging percentage (%SC), scavenging concentration 50 (SC_{50}), efficacy concentration 50 (EC_{50}) and antiradical power (AP) were also calculated.

2.5.6. Ferric Reducing Antioxidant Power Assay

The Ferric-Reducing Antioxidant Power assay (FRAP) measures the ability of an antioxidant substance to reduce the tri-pyridyl-triazine ferric complex (Fe^{3+} - TPTZ) to the tri-pyridyl-triazine ferrous complex (Fe^{2+} - TPTZ). The FRAP solution was prepared as follows: 14.1 mg of TPTZ was diluted in 9 mL HCl at 40

mM then ferric chloride ($FeCl_2$ at 20 mM) and acetate buffer (300 mM; pH: 3.6) were added in the ratio of 1:1:10 respectively to form the FRAP solution. 1950 µL of FRAP solution was added into different test tubes, and then 50 µL of the extracts at 1500 µg/mL was added [8]. The tests were repeated in triplicate, and the mixture was incubated for 30 min. The optical density was measured at 593 nm using a Thermo-Fisher-Scientific: Evolution 300 (UV-VIS) spectrophotometer. A solution of standard antioxidant (Gallic acid), whose absorbance was measured under the same conditions as the samples, was used as the positive control. An increase in absorbance indicates an increase in the reducing power of the tested extract and the results are expressed in micrograms of Gallic acid equivalent per milligram of dry extract (µg/mg).

2.6. *In vitro* Anti-Inflammatory Assay

2.6.1. Bovine Serum Albumin (BSA) Denaturation Assay

Anti-denaturation assay was conducted as described by Juvekar *et al.* as follows with slight modifications by Betote *et al.* [8] [26]. The reaction mixture consisted of the aqueous, hydroethanolic and ethanolic extracts or sodium diclofenac at five concentrations (1500, 750, 375, 187.50 and 93.75 µg/mL) and 5 % aqueous solution of bovine serum albumin. The mixture was incubated at 37˚C for 20 min and then heated to 70˚C for 15 min. After cooling the samples, the turbidity was measured at 660 nm using a Thermo-Fisher-Scientific (Evolution 300 UV-VIS) spectrophotometer. The experiment was repeated in triplicate. The collected data was used to calculate the inhibition percentages (%I) and the inhibitory concentration 50 (IC_{50}) for each extract.

2.6.2. Proteinase Inhibitory Action

The test was performed according to the modified method of Oyedepo and Famurewa [27]. The reaction mixture (2 mL) consisted of 0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4) and 1mL of each test extract or sodium diclofenac at concentrations of 1500, 750, 375, 187.50 and 93.75 µg/mL. The reaction mixture was incubated at 37˚C for 5 minutes and then 1 mL of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min, after which 2 mL of 70% perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was repeated in triplicate and the inhibition percentages (%I) and inhibitory concentration 50 (IC_{50}) of proteinase inhibitory for each extract were calculated as follows:

$$\mathrm{I}(\%) = \frac{\mathrm{Abs}_{\mathrm{control}} - \mathrm{Abs}_{\mathrm{extracts/sodium\ diclofenac}}}{\mathrm{Abs}_{\mathrm{control}}} \times 100,$$

where, $Abs_{control}$ is the absorbance of control tube and $Abs_{extracts/sodium\ diclofenac}$ is the absorbance of sample tube.

2.6.3. Red Blood Cell Membrane Stabilization

Preparation of red blood cell (RBC) suspension: Sheep blood was collected in

heparinised tubes and centrifuged at 3000 rpm for 10 minutes. The obtained residual solution was washed three times with saline. The RBC layer was collected and diluted to make a (10%; v/v) using phosphate buffer saline [27] [28].

For heat-induced haemolysis: 1 mL of 10% RBC was added to 1 mL of extract solution (1500, 750, 375, 187.50 and 93.75 µg/mL). The mixture was heated at 56˚C for 30 minutes and then centrifuged at 2500 rpm for 10 minutes at room temperature. The supernatant was collected, and the absorbance was read at 560 nm [28]. Sodium diclofenac was used as a positive control. The results were calculated as RBC membrane destabilization inhibition percentage (%I).

2.7. Oral Acute Toxicity of *G. suaveolens* Extracts

The acute toxicity study was conducted according to the procedures outlined by the Organization for Economic Co-operation and Development guidelines 425 [29]. Forty-two Wistar female albino rats were used for this study. After one week of acclimatization, the rats (n = 6 per group) were feed with aqueous, hydroethanolic and ethanolic extracts by gavage at the doses of 2000 and 5000 mg/kg body weight. Distilled sterile water served as the control group. The general condition, clinical signs, and mortality of each animal were recorded for several hours after extract administration and once daily thereafter for 14 days, along with their body weight. All rats were sacrificed by 100% CO_2 inhalation and underwent gross necropsy examination at day 15 [30]. Mortality and LD_{50} were calculated using the following the OECD methods.

2.8. Statistical Analysis

The data were normalized and expressed as Mean ± SD (n = 3) for the *in vitro* assays and (n = 6) for oral acute toxicity testing. One-way ANOVA (Tukey's and Dunnett's tests) was used for analysis. A difference between standard molecules and *G. suaveolens* extracts concentrations was considered significant at $p < 0.05$. The data was graphically represented using the Graph Pad Prism 9.0.1 software (Microsoft, USA). All the parameters were determined by the SPSS Statistic Software version 23.0.

3. Results

3.1. Qualitative Phytochemical Analysis of *G. suaveolens* Extracts

The aqueous, hydroethanolic and ethanolic extracts of *Greenwayodendron suaveolens* were used to determine the presence of different types of secondary metabolites. The results shown in **Table 1** indicated the presence of eight (08) bioactive compounds families in the *G. suaveolens* extracts: alkaloids, phenols, polyphenols, tannins, flavonoids, anthocyanins, terpenoids, and sterols. While other classes of secondary metabolites were found in all three extracts, saponins and anthraquinones were found exclusively in the *G. suaveolens* aqueous, hydroethanolic and ethanolic extracts.

Table 1. Summary of phytochemical screening of *G. suaveolens* aqueous, hydroethanolic and ethanolic extracts.

Secondary metabolites	*G. suaveolens* extracts		
	Aqueous	Hydroethanolic	Ethanolic
Phenols	++	+	+++
Polyphenols	+++	++	+
Flavonoids	++	+++	++
Alkaloids	++	+++	+
Tannins	++	++	+++
Saponins	–	–	–
Terpenoids	++	++	++
Steroids	++	++	+
Anthocyanins	++	++	+++
Anthraquinones	–	–	–

Legend: (–): Absence of secondary metabolites; (+): Present in small concentration; (++): Present in moderately high concentration; (+++): Present in very high concentration.

3.2. *In vitro* Antioxidant Activities of *G. suaveolens* Extracts

3.2.1. Total Antioxidants Content

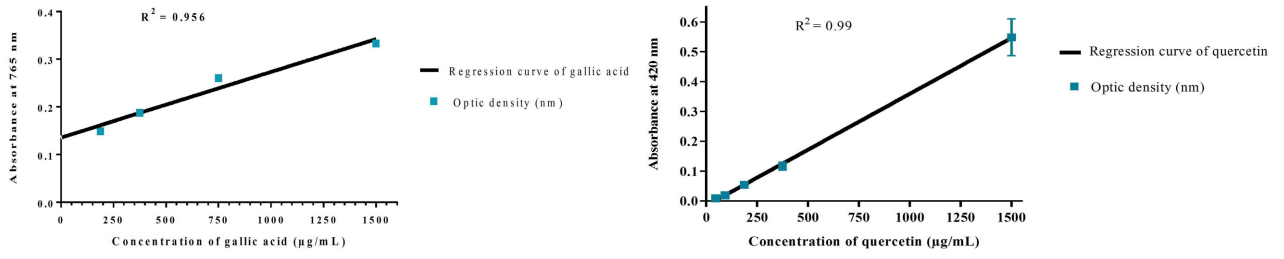
Total antioxidants content of *G. suaveolens* extracts was determined using the Folin-Ciocalteu method and expressed in µg gallic acid equivalents per mg of dry weight. The results were obtained from the gallic acid calibration curve ($p < 0.05$) shown in Figure 1(a). The aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks have a total antioxidants content of 3491.39 ± 17.48, 6340.84 ± 22.82 and 3084.12 ± 21.13 µg EGA/mg of dry weight respectively (Table 2). These *G. suaveolens* stem-barks extracts contain high quantities of secondary metabolites, which may be responsible for the plant's antioxidant properties.

3.2.2. Total Flavonoids Content

Total flavonoids content of *G. suaveolens* stem-barks extracts was expressed as µg quercetin equivalents per mg of dry weight (Figure 1(b)). Test samples were analysed in triplicate. Table 2 represents the analytical data for the total flavonoids content of the aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks.

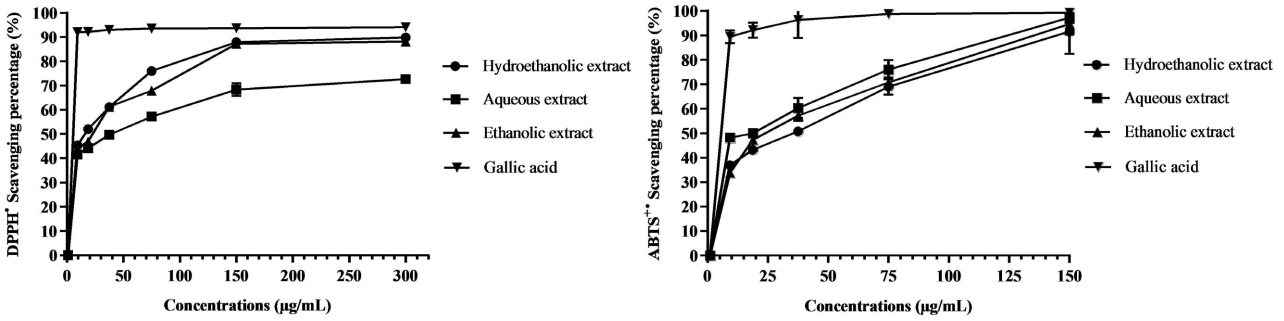
3.2.3. DPPH Free Radical Scavenging Capacity

Figure 2(a) shows the dose-response curve of free DPPH radical scavenging activity of *G. suaveolens* extracts, compared with gallic acid. The results indicate that the reference molecule had higher activity than the aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks ($p < 0.001$). The inhibition percentages were 72.66%, 89.89%, 88.12% and 94.09% respectively for aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens*, and gallic acid at 300 µg/mL.



(a) (b)

Figure 1. Standard calibration curves of gallic acid and quercetin for the determination of total antioxidants (a) and total flavonoids (b) contents respectively in the aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks.



(a) (b)

Figure 2. Variation curve of DPPH• (a) and ABTS+• (b) radicals scavenging percentage as a function of the concentration of aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks.

Table 2. Total antioxidants and flavonoids contents of aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks.

G. suaveolens extracts	Total antioxidant content (µg EGA/mg of dry weight)	Total flavonoid content (µg EQuerc/mg of dry weight)
Ethanolic	3084.12 ± 21.13^{c}	650.28 ± 2.52^{b}
Hydroethanolic	6340.84 ± 22.82^{a}	714.53± 3.10^{a}
Aqueous	3491.39 ± 17.48^{b}	561.40 ± 4.48^{c}

Legend: µg EGA/mg of dry weight: micrograms Gallic acid equivalents per milligrams of dry weight; µg EQuerc/mg of dry weight: micrograms Quercetin equivalents per milligrams of dry weight. Data are expressed as Mean ± SD. Means assigned to letters a, b and c (a > b > c) are significantly different at $p < 0.05$ (Tukey's test).

The scavenging capacity 50 (SC_{50}), effective capacity 50 (EC_{50}) and antiradical power (AP) are presented in Table 3. The obtained results showed that the higher antiradical power is found in gallic acid (3.38 ± 0.00) and the most active among the extracts was the aqueous extract (1.21 ± 0.00).

3.2.4. Cationic ABTS Radical Scavenging Ability

The aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks were effective scavengers of the cationic ABTS radical (Figure 2(b)), and the

Table 3. Summary of the *in vitro* antiradical capacity of aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks on DPPH• free radical.

G. suaveolens extracts	*In vitro* antiradical capacity		
	SC_{50} (µg/mL)	EC_{50} (µg Ex/mg of DPPH)	AP ($a \times 10^{-3}$)
Ethanolic	$13.78 \pm 0.66^{d***}$	$1033.38 \pm 0.27^{d***}$	$0.97 \pm 0.00^{d***}$
Hydroethanolic	$12.62 \pm 0.58^{c***}$	$946.58 \pm 0.11^{c***}$	$1.06 \pm 0.00^{c***}$
Aqueous	$11.06 \pm 1.76^{b***}$	$829.58 \pm 0.05^{b***}$	$1.21 \pm 0.00^{b***}$
Gallic acid	3.94 ± 0.22^{a}	295.87 ± 0.27^{a}	3.38 ± 0.00^{a}

Legend: µg/mL: micrograms of sample per millilitre of solution; µg Ex/mg of DPPH: micrograms of dry sample per milligrams of DPPH; SC_{50}: Scavenging concentration 50; EC_{50}: Efficacy concentration 50; AP: Antiradical power. Data are expressed as Mean ± SD. Means assigned to letters a, b, c and d (a > b > c > d) are significantly different at $p < 0.05$ (Tukey's test). Means assigned to "***" are significantly different at $p < 0.001$ (One-way ANOVA followed by Dunnett's test).

activity was lower than gallic acid. The SC_{50}, EC_{50} and AP of ABTS radical assay are presented in Table 4. The results showed that the ABTS radical scavenging ability of the aqueous extract (0.39 ± 0.00) was better than the hydroethanolic (0.29 ± 0.00) and ethanolic (0.30 ± 0.00) extracts of *G. suaveolens* stem-barks ($p < 0.05$).

The result analysis of antiradical activity of *G. suaveolens* stem-barks extracts has shown that, the biomolecules containing in aqueous, hydroethanolic and ethanolic extracts have more efficiency ($p < 0.001$) on the scavenging of free radical DPPH• compared to cationic radical $ABTS^{+\bullet}$. Concerning the scavenging power of each extract, the aqueous extract of *G. suaveolens* stem-barks presented a better antiradical capacity ($p < 0.05$) during DPPH• and $ABTS^{+\bullet}$ assays (Table 5).

3.2.5. FRAP Testing

The reducing ability of *G. suaveolens* stem-barks extracts result in the reduction of ferricyanide Fe^{3+} [TPRZ-Fe (III)] to ferrocyanide Fe^{2+} [TPTZ-Fe (II)] by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue colour at 593 nm. The results obtained were calculated from the calibration curve of gallic acid (Figure 3). The reducing power of stem-barks of *G. suaveolens* aqueous, hydroethanolic and ethanolic extracts was 564.41 ± 1.52, 656.71 ± 4.64 and 866.23 ± 2.36 µg EGA/mg of dry weight respectively. These results show that the ethanolic extract of *G. suaveolens* presented a better-reducing ability of heavy metals than aqueous and hydroethanolic extracts ($p < 0.001$).

3.3. *In vitro* Anti-Inflammatory Properties

3.3.1. Inhibition of Bovine Serum Albumin Denaturation

The aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks displayed significant inhibitory activity on albumin denaturation (Figure 4(a)).

Table 4. Summary of the *in vitro* antiradical capacity of aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks on cationic $ABTS^{\bullet+}$ radical.

G. suaveolens extracts	*In vitro* antiradical capacity		
	SC_{50} (µg/mL)	EC_{50} (µg Ex/mg of ABTS)	AP ($a \times 10^{-3}$)
Ethanolic	$19.72 \pm 0.09^{c***}$	$3286.65 \pm 1.65^{c***}$	$0.30 \pm 0.00^{c***}$
Hydroethanolic	$20.16 \pm 0.05^{d***}$	$3360.55 \pm 0.95^{d***}$	$0.29 \pm 0.00^{d***}$
Aqueous	$15.16 \pm 0.03^{b***}$	$2526.96 \pm 0.52^{b***}$	$0.39 \pm 0.00^{b***}$
Gallic acid	4.32 ± 0.00^{a}	719.35 ± 0.95^{a}	1.39 ± 0.00^{a}

Legend: µg/mL: micrograms of sample per millilitre of solution; µg Ex/mg of ABTS: micrograms of dry sample per milligrams of ABTS; SC_{50}: Scavenging concentration 50; EC_{50}: Efficacy concentration 50; AP: Antiradical power. Data are expressed as Mean ± SD. Means assigned to letters a, b, c and d (a > b > c > d) are significantly different at $p < 0.05$ (Tukey's test). Means assigned to "***" are significantly different at $p < 0.001$ (One-way ANOVA followed by Dunnett's test).

Table 5. Comparison of antiradical power (AP) between ABTS and DPPH assays.

G. suaveolens extracts	Antiradical power (AP)	
	AP_{DPPH}	AP_{ABTS}
Ethanolic	0.97 ± 0.00^{c}	$0.30 \pm 0.00^{b***}$
Hydroethanolic	1.06 ± 0.00^{b}	$0.29 \pm 0.00^{c***}$
Aqueous	1.21 ± 0.00^{a}	$0.39 \pm 0.00^{a***}$

Legend: Data are expressed as Mean ± SD. Means assigned to letters a, b and c (a > b > c) are significantly different at $p < 0.05$ (Tukey's test). Means assigned to "***" are significantly different at $p < 0.001$ (One-way ANOVA followed by Dunnett's test).

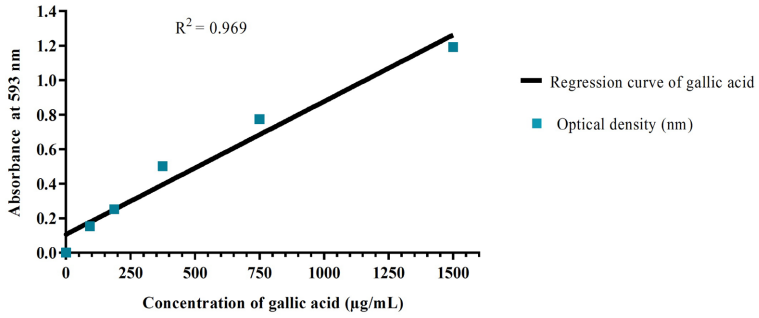


Figure 3. Standard calibration curve of gallic acid for the determination of the reducing power of aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks.

The results presented on Table 6 show a significant inhibitory activity on bovine serum albumin denaturation, with inhibitory concentrations 50 (IC_{50}) of 97.29 ± 0.91, 48.63 ± 0.00 and 120.02 ± 0.93 µg/mL respectively for aqueous, hydroethanolic and ethanolic extracts. The hydroethanolic extract has shown a better IC_{50} (48.63 µg/mL) compared to aqueous and ethanolic extracts ($p < 0.05$). The positive control Sodium diclofenac showed an IC_{50} of 36.83 µg/mL.

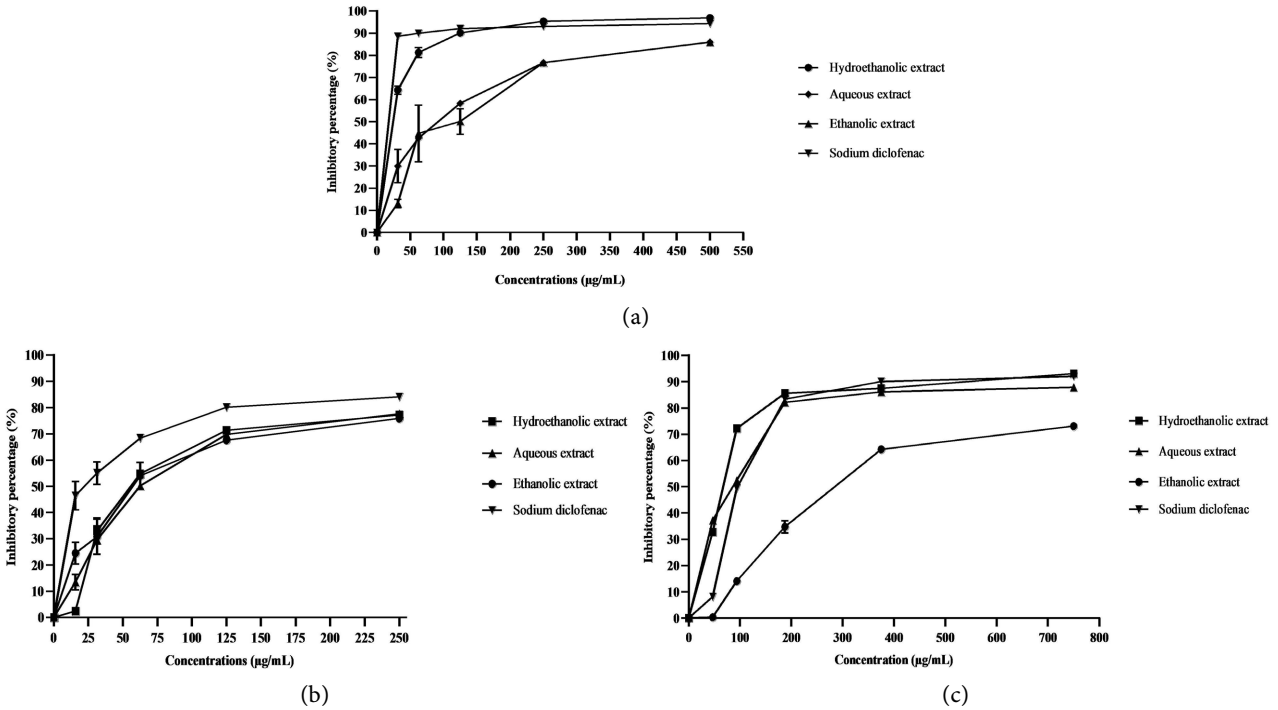


Figure 4. Effect of aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks on bovine serum albumin denaturation (a) proteinase inhibitory action (b) and red blood cell membrane haemolysis (c).

Table 6. Summary of IC_{50} of Sodium diclofenac and aqueous, hydroethanolic and, ethanolic extracts of *G. suaveolens* stem-barks on bovine serum albumin denaturation, proteinase inhibitory action and red blood cell membrane haemolysis.

G. suaveolens extracts	Inhibition of BSA denaturation	Proteinase inhibitory action	RBC membrane stabilization
	IC_{50} (µg/mL)		
Ethanolic	$120.02 \pm 0.93^{d***}$	$34.19 \pm 1.00^{b***}$	$185.42 \pm 2.00^{d***}$
Hydroethanolic	$48.63 \pm 0.00^{b***}$	$39.43 \pm 2.08^{c***}$	$59.22 \pm 2.00^{a***}$
Aqueous	$97.29 \pm 0.91^{c***}$	$41.12 \pm 0.95^{d***}$	$61.49 \pm 2.00^{b***}$
Sodium diclofenac	36.83 ± 0.74^{a}	19.87 ± 2.75^{a}	90.73 ± 1.00^{c}

Legend: IC_{50}: Inhibitory concentration 50; Data are expressed as Mean ± SD. Means assigned to letters a, b, c and d (a > b >c > d) are significantly different at $p < 0.05$ (Tukey's test). Means assigned to "***" are significantly different at $p < 0.001$ (One-way ANOVA followed by Dunnett's test).

3.3.2. Proteinase Inhibitory Activity

The aqueous, hydroethanolic and, ethanolic extracts of *G. suaveolens* stem-barks exhibited significant anti-proteinase activity (Figure 4(b)). The calculated inhibitory concentrations 50 were 34.19 µg/mL, 39.43 µg/mL and 41.12 µg/mL 19.87 µg/mL for ethanolic, hydroethanolic and aqueous extracts of *G. suaveolens* respectively. Statistically, Sodium diclofenac presented an IC_{50} value significantly lower compared to the *G. suaveolens* extracts ($p < 0.001$). The results are tabulated in Table 6.

3.3.3. Red Blood Cell Membrane Stabilizing Activity

The aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* exhibited a significant protective effect on erythrocyte membranes i.e. the extracts protected red blood cells from heat-induced lysis at all the concentrations used in a dose-dependent manner (**Figure 4(c)**). The IC_{50} values of haemolysis were 61.49 ± 2.00, 59.22 ± 2.00 and 185.42 ± 2.00 µg/mL for aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* respectively (**Table 6**). The aqueous and hydroethanolic extracts had higher efficacy in inhibiting heat-induced haemolysis of normal red blood cell membranes than the standard drug Sodium diclofenac which showed an IC_{50} of 90.73 ± 1.00 µg/mL ($p < 0.05$).

The comparative results of anti-inflammatory potential of the hydroethanolic, ethanolic and aqueous extracts of *G. suaveolens* stem-barks are shown in **Figure 5**. This representation shows that all extracts exhibited an anti-inflammatory activity with an $IC_{50} < 200$ µg/mL. The hydroethanolic extract was the most active, with considerable activity exhibited in all anti-inflammatory tests in this study. The anti-inflammatory activity of the reference molecule showed a better ability to inhibit the *in vitro* inflammatory properties of protein denaturation and proteinase activity stimulation than the *G. suaveolens* extracts ($p < 0.05$). Interestingly, the aqueous and hydroethanolic extracts exhibited better efficacy than Sodium diclofenac for RBC membrane stabilization ($p < 0.05$).

3.4. Acute Oral Toxicity Evaluation of *G. suaveolens* Stem-Barks Extracts

The limit test of acute oral toxicity study was under the OECD guideline N˚ 425. The tested animals were treated with a single administration of hydroethanolic, ethanolic and aqueous extracts of *G. suaveolens* stem-barks at two doses of 2000 and 5000 mg/kg b.w. respectively. The results show that, the average body weights of rats were normally increased within the 14-day observation period (**Figure 6**). In addition, there were no mortality, abnormal clinical sign, or significant gross lesions highlighted during and after the experimental study (**Table 7**). The LD_{50} of the extracts of *G. suaveolens* stem-barks were considered greater than 5000 mg/kg b.w.

4. Discussion

The present study was undertaken to investigate whether the nontoxic extracts of *Greenwayodendron suaveolens* (Engl. & Diels) Verdc. subsp. *suaveolens* could regulate free reactive oxygen species and inhibit proteins inflammation. Medicinal plants have always been considered to be the major sources of bioactive molecules worldwide [14]. Acute or chronic inflammatory diseases figure prominently among the multitude of ailments against which medicinal plants are used. In the management of oxidative stress-related diseases like infectious pneumonia, plants medicines represent an important therapeutic choice mainly in developing countries, and their efficiency are justified at least in part by their ability to thwart the deleterious effects of Reactive Oxygen Species (ROS) [8].

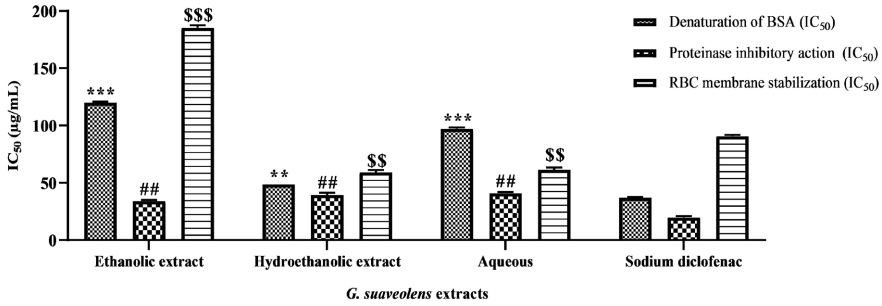


Figure 5. Status of the percentage of body weight of Wistar female rats during the oral acute toxicity testing ($p < 0.05$). Body weight evolution of normal control was analysed as statistically identical between the tested groups.

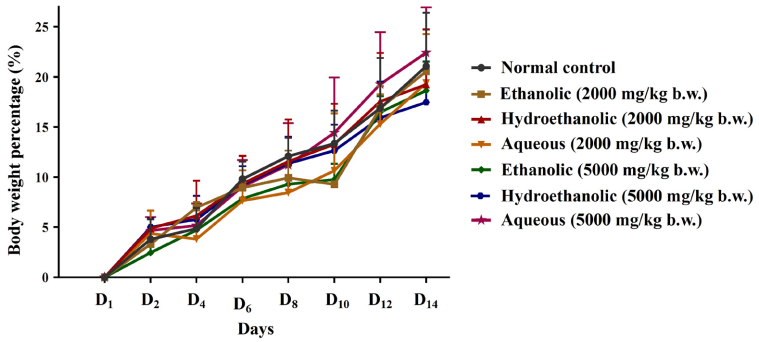


Figure 6. Comparison of anti-inflammatory efficacy of hydroethanolic, ethanolic and aqueousextracts of *G. suaveolens* stem-barks (One-way ANOVA followed by Dunnett's test) obtained by BSA denaturation inhibition test (***: $p < 0.001$; **: $p < 0.05$ vs. IC_{50} value of Standard drug), action inhibitory of proteinase test (###: $p < 0.001$; ##: $p < 0.05$ vs. IC_{50} value of Standard drug) and RBC membrane stabilization test ($$$: $p < 0.001$; $$: $p < 0.05$ vs. IC_{50} value of Standard drug).

Table 7. Administration effect of aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks on the status of relative weight percentage ($p < 0.05$) of kidney, lung, liver, spleen and heart of normal control and tested groups.

G. suaveolens extracts	Doses (mg/kg b.w.)	Relative weight percentage (%)				
		Kidneys	Lung	Liver	Spleen	Heart
Normal control		0.71 ± 0.05[a]	0.72 ± 0.12[a]	3.66 ± 0.33[a]	0.60 ± 0.02[a]	0.34 ± 0.03[a]
Ethanolic	2000	0.89 ± 0.00[a]	0.74 ± 0.03[a]	3.70 ± 0.49[a]	0.63 ± 0.45[a]	0.33 ± 0.08[a]
	5000	0.69 ± 0.07[a]	0.77 ± 0.31[a]	3.20 ± 0.43[a]	0.53 ± 0.13[a]	0.36 ± 0.06[a]
Hydroethanolic	2000	0.72 ± 0.04[a]	0.68 ± 0.12[a]	3.67 ± 0.20[a]	0.72 ± 0.51[a]	0.35 ± 0.06[a]
	5000	0.68 ± 0.09[a]	0.61 ± 0.05[a]	3.21 ± 0.40[a]	0.71 ± 0.23[a]	0.33 ± 0.02[a]
Aqueous	2000	0.69 ± 0.19[a]	0.75 ± 0.11[a]	3.62 ± 0.46[a]	0.63 ± 0.09[a]	0.34 ± 0.13[a]
	5000	0.73 ± 0.10[a]	0.72 ± 0.19[a]	3.22 ± 0.46[a]	0.73 ± 0.09[a]	0.35 ± 0.03[a]

Legend: (n = 6) represents the number of animals tested per group. Data are expressed as Mean ± SD. The means assigned "a" are significantly identical at $p < 0.05$ (Tukey's test).

In this study, the quantitative and qualitative phytochemical analysis has revealed the presence of various secondary metabolites that represent candidate

responsible for the biological and/or pharmacological effects of *G. suaveolens* extracts. These metabolites include phenols, polyphenols, flavonoids, alkaloids, catechic tannins, triterpenes, steroids and anthocyanins, in accordance with previous report from Ajayi *et al.* and Tsouh *et al.* who also showed the presence of phenols, polyphenols, flavonoids, glycosides, acetogenins, terpenoids, alkaloids and reducing sugars [31] [32] in *G. suaveolens* species. For instance, the phenolic compounds (phenols, polyphenols and flavonoids), and other compounds which have a free hydroxyl group on their chemical structures or aromatic rings like acetogenins, alkaloids, terpenoids, glycosides or reducing sugars are chemically proven antioxidants [33] [34]. These chemical groups are documented to confer free radicals scavenging ability to plant extracts via hydrogen or electron donations [33] [34] [35]. The presence of these compounds implies that *G. suaveolens* could have protective and therapeutic implications for humans [8].

The results of this study showed that the stem-barks extracts of *G. suaveolens* content an important amount of antioxidants. The quantification tests showed that the hydro-ethanolic, ethanolic and aqueous extracts of *G. suaveolens* stem-barks are rich in components that possess free hydroxyl functions (-OH) and aromatic rings. It appears from this study that the hydro-ethanolic extract of *G. suaveolens* contains the highest total antioxidants amount such as 2 times higher than the aqueous and ethanolic extracts.

Previous report showed that the antioxidants content of the essential oil of the same plant was 26 times lower than the hydroethanolic extract. The difference in antioxidants contents in *G. suaveolens* stem-barks extracts is probably due to the chemical nature of the compounds present in each extract [8]. The extraction of these compounds by polar solvents enhances the extraction of polar compounds rich in free hydroxyls like phenols, polyphenols, anthocyanins, tannins, alkaloids and flavonoids [36]. These bioactive components are major contributors of the antioxidant capacity of most plants [13] [15].

Determination of total flavonoids in the extracts also showed that the hydroethanolic extract of *G. suaveolens* contains the greatest amount of flavonoids. These results showed that polar mixed organic solvent (hydroethanolic mixture) provides better extraction and higher flavonoids content compared to distilled water, which is more polar [13]. Thus, the results showed that, the extraction of *G. suaveolens* stem-barks with distilled water, the highest polar solvent helped to limit or slow down the extraction process of polar antioxidant compounds present in the plant. These extracted secondary metabolites have been shown to possess various biological properties related to antioxidant mechanisms [14], including scavenging of free radicals and reactive oxygen species (ROS) and/or chelation and reduction of metal ions [14] [37]. Furthermore, the capacity of flavonoids to affect a wide range of pro-inflammatory proteins and enzymes has been reported [14] [38]. Because acute inflammation involves proteins denaturation and dysfunction of enzymes, *G. suaveolens* secondary metabolites might be useful in thwarting the deleterious effects of Oxidative species in the process of acute inflammation.

The antiradical activity of a secondary metabolite can be defined as its ability to scavenge hydrophilic DPPH or cationic ABTS free radicals [8]. The antiradical activity results of this study showed that *G. suaveolens* aqueous extract exhibits the best hydrophilic DPPH free radical scavenging capacity ($p < 0.05$) with antiradical powers of 1.21 ± 0.00, 1.06 ± 0.00 and 0.97 ± 0. In contrast, for the cationic radical ABTS, the hydroethanolic extract showed better reactivity (0.39 ± 0.00) compared to the aqueous (0.29 ± 0.00) and ethanolic (0.30 ± 0.00) *G. suaveolens* extracts ($p < 0.001$). These results suggest that, the aqueous extract of *G. suaveolens* has the greatest ability to scavenge hydrophilic free radicals while the hydroethanolic extract seems to have a better ability to scavenge the cationic radical ABTS. The results obtained showed that the polar secondary metabolites of *G. suaveolens* extracts have the ability to donate hydrogen ions (H^+) to free radicals, and contribute to the redox potential, electron-transferring capacity, and singlet antioxygen action. This would slow down or inhibit the propagation of lipoperoxidation [8] [13] [14], which is known to have a mechanism involving multiple consequences such as the inactivation of enzymes by oxidation of thiol groups, the reduction or disruption of the fluidity of cell membranes, tissues and organs during inflammatory infections [8] [19] [39].

Reducing power of Fe^{3+} to Fe^{2+} is another test to measure the antioxidant potential of the *G. suaveolens* extracts, the capacity to reduce heavy metal being the key process to highlighting the antioxidant potential. In our study, all the extracts exhibited high reducing antioxidant power as indicated by the significant Fe^{3+} reducing potential of *G. suaveolens* stem-barks extracts. These results are consistent with our previous study [8] showing that the essential oil of *G. suaveolens* stem-barks presented lower reducing power (94.75 ± 1.66 µg EGA/mg of dry weight) than the plant extracts, indicating that the essential oil contains a low amount of total antioxidants, and this could be elicited by the fact that compared the essential oil, *G. suaveolens* extracts are richer in phenolic compounds and others compounds bearing free hydroxyl groups on their aromatic rings, conferring a better heavy metal reducing and chelating ability [8]. However, it is worth noting that, as found in one report, no significant correlation could be found between the ferric-reducing ability of each *G. suaveolens* extract and, its total antioxidants content [40]. This result was corroborated by Nahak *et al.* who showed that an antioxidant that is effective in one test is not necessarily effective in another one [41].

In this study, it was verified that, because of their content in secondary metabolites, the extracts of *G. suaveolens* could act as primary and/or secondary antioxidants by scavenging free radicals, or through their ability to reduce heavy metals reducers [42]. The primary antioxidants (antiradicals or true antioxidants) are biomolecules capable of interrupting the autocatalytic chain by blocking lipid free radicals by a transfer of a hydrogen radical.

Meanwhile the secondary antioxidants (preventive antioxidants) act on other oxidation factors. They are able to delay the oxidation of lipids by indirect mechanisms such as oxygen reduction or complexation of metal ions [8]. **Table 8**

Table 8. Summary of total antioxidants content, primary and secondary antioxidants of *G. suaveolens* stem-barks extracts.

G. suaveolens extracts	Total antioxydants content	Primary/curative antioxydant	Secondary/preventive antioxydant	Pharmacological effect
		µg EGA/mg dry weight		
Ethanolic	3084.12 ± 21.13^{c}	4.59 ± 0.22^{a}	866.23 ± 2.36^{a***}	**Preventive**
Hydroethanolic	6340.84 ± 22.82^{a}	4.21 ± 0.19^{b}	656.71 ± 4.64^{b***}	
Aqueous	3491.39 ± 17.48^{b}	3.69 ± 0.57^{c}	564.41 ± 1.52^{c***}	

Legend: Data are expressed as Mean ± SD. Means assigned to letters a, b and c (a > b > c) are significantly different at $p < 0.05$ (Tukey's test). Means assigned to "***" are significantly different at $p < 0.001$ (One-way ANOVA followed by Dunnett's test).

summarizes the pharmacological effect of *G. suaveolens* organic extracts that had a highest amount of antioxidant chelator than the curative antioxidants. The values obtained, were statistically different with a significance level ($p < 0.001$). These results showed that *G. suaveolens* stem-barks can be used to prevent oxidative damages. The free radical scavenging capacity of *G. suaveolens* in this study corresponded to previous study that has already been done by Betote *et al.* [8]. For this purpose, *G. suaveolens* can be considered as antioxidant sources and has real therapeutic potential.

During bacterial infections, the infectious site environment is dominated by pro-inflammatory processes generated by the pathogen's virulence factors [19] [43]. In this environment, the processes of denaturation of proteins, destabilization of red blood cells membranes and catalytic overactivity of proteolytic enzymes are accentuated by the damages induced by ROS, leading to the loss of their biological functions [44]. It would be interesting to note that, the aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* extracts inhibit protein denaturation and regulate their activities in order to reduce inflammation. Inhibition of BSA denaturation, proteolytic enzyme overactivity and destabilization of cell membrane, are considered to be the best *in vitro* models for preventing or treating inflammation-associated bacterial infections. *G. suaveolens* extracts have slightly lower anti-inflammatory properties than Sodium diclofenac. However, the concentration-dependence of the biological efficacy of tested extracts was also observed [8] [19].

To further support the biological activities of the extracts this study showed the presence of bioactive constituents that have a strong anti-inflammatory activity with IC_{50} values lower than 200 µg/mL. This anti-inflammatory activity can be correlated with the antiradical and heavy metals reduction and/or chelation activities, conferred by the presence of active biomolecules present in *G. suaveolens* stem-barks extracts [8] [45]. Thus, the secondary metabolites of *G. suaveolens* extracts could act by trapping and complexing ROS, which result in the reconstitution of electrostatic, hydrogen, hydrophobic and disulphide bonds altered during inflammation [46]. These biomolecules, which can be considered to be anti-inflammatory mediators, would better modulate the activities of proteases and pro-inflammatory cells that play an important role in the develop-

ment of tissue damages during inflammatory reactions [8] [47]. It could be stated from the results of this present study that, aqueous, hydroethanolic and ethanolic extracts of *G. suaveolens* stem-barks are able to control protein denaturation, catalytic activity of proteolytic enzymes and stabilize cell membranes.

Herbal substances today symbolize safety in contrast to the synthetics that are regarded as unsafe to humans and the environment [48] [49]. With regard to the oral acute toxicity evaluation of *G. suaveolens* extracts, the lethal dose 50 was determined using Wistar female albino rats at the concentration of 2000 and 5000 mg/kg b.w. of *G. suaveolens* extracts. The results showed no major changes in behaviour and no mortality were observed in all groups. Thus, the *G. suaveolens* extracts stem-barks can be considered to be safe at a dose level of 5000 mg/kg b.w., and the LD_{50} is considered > 5000 mg/kg b.w. Any pharmaceutical drug or phytomedicine with an oral LD_{50} higher than 1000 mg/kg could be considered safe and low toxic [50].

5. Conclusion

Lower respiratory tract diseases, the main pathophysiological mechanism of which is inflammation is mainly induced by reactive oxygen species. These diseases are caused by microorganisms and the immune system response of the host. *In vitro* study of *G. suaveolens* stem-barks extracts revealed the antioxidant and anti-inflammatory activities of this plant. The *G. suaveolens* extracts have a high capacity to scavenge the hydrophilic DPPH and cationic ABTS radicals. For the reduction of Fe^{3+}, the highest activities were obtained with the three extracts tested. Concerning inflammation, the extracts of *G. suaveolens* stem-barks showed a high inhibitory potential of BSA denaturation, catalytic overactivity of proteolytic enzymes and destabilisation of erythrocyte cell membranes. Also, the results of the present study showed that *G. suaveolens* stem-barks extracts presented had a LD_{50} > 5000 mg/kg b.w., and are constituted by bioactive antioxidant and anti-inflammatory molecules that can be used traditionally for the treatment of inflammatory pathologies of infectious origin.

Abbreviations

EGA: Equivalent of Gallic acid; EQuerc: Equivalent of Quercetin; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); TPTZ: 2,4,6-Tris(2-pyridyl)-*s*-triazine; BSA: Bovine Serum Albumin.

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Authors' Contributions

P. H. D. B. Initiated the project, participated in laboratory research (phytochem-

ical analysis, antioxidative stress and anti-inflammatory activities, toxicological testing) and data analysis, and wrote and revised the manuscript article. M. G. A. and F. N. participated in the toxicological testing, data analysis, and revision of the manuscript article. E. D. F. N. M. Participated in the anti-inflammatory activity and revised the manuscript article. A. S. F. Y. participated in the statistical analysis of data and revision of the manuscript. G. A. A. Guided the investigation on the antioxidant efficacy of plant extracts and revised the manuscript article. N. N. Guided the research work and revised the manuscript. M.A. N. Initiated the project, guided the research work, and revised the manuscript. All authors read and approved the final version of the manuscript.

Availability of Data and Materials

All the results presented in this study were carried out by authors, and the data used as references were properly cited.

Ethics Approval and Consent to Participate

The current research protocol was duly approved by the Institutional Animal Ethical Committee of the Faculty of Medicine and Biomedical Sciences, University of Yaoundé I, Yaoundé, Cameroon (ethical approval N˚: 443/UYI/FMSB/VDRC/DAASR/CSD). The care of the animal was performed as per the Organization for Economic Cooperation and Development Guideline (OECD) guidelines.

Consent for Publication

Non-Applicable.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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