Antibacterial, anti-inflammatory, and antioxidant effects of the leaves and stem bark of *Glyphaea brevis* (Spreng) Monachino (Tiliaceae): A comparative study

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ABSTRACT

Background: *Glyphaea brevis* (Spreng) Monachino (Tiliaceae) have traditional uses in the management of conditions characterized by infections, inflammatory disorders and oxidative stress. The paper aims to report the comparative data on the leaves and stem bark of *Glyphaea brevis* with respect to their antibacterial, anti-inflammatory and antioxidant effects. **Materials and Methods:** The antibacterial effects of the 70% ethanol extracts of the leaves and stem bark were determined using the agar well diffusion and micro dilution assays. The anti-inflammatory activity was assessed using the carrageenan-induced oedema model in 7-day old cockerels. Using the DPPH free radical scavenging, total antioxidant and total phenol content assays, the antioxidant potential of the extract was assessed. **Results:** The bark extract had the higher antibacterial effect against 6 of the 8 microorganisms used. Noteworthy are its activity against *Bacillus subtilis* and *Enterococcus faecalis* with lowest MIC value of 500 μg/mL respectively. In doses of 30, 100 and 300 mg/kg, both extracts reduced the carrageenan-induced oedema in 7-day old cockerels. Based on the ED_{50} values, both extracts demonstrated similar potencies (ED_{50} = 21.00 mg/kg). The stem bark extract exhibited higher free radical scavenging activity (IC_{50} = 1.392 mg/mL) compared to the leaf extract (IC_{50} = 9.509 mg/mL). In the total phenol content, the bark extract showed higher content (15.91 mg/g of dry mass) compared to the leaf extract (2.68 mg/g dry mass). Both extracts demonstrated equal potencies in the total antioxidant capacity determinations (0.60 mg/g dry weight of extract). **Conclusions:** The results of this work provide scientific evidence for the traditional uses of *Glyphaea brevis*.

Key words: Antimicrobial, Antioxidant, Carrageenan-induced oedema, Free radical, *Glyphaea brevis*.

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INTRODUCTION

The development of resistance of microorganisms to antimicrobial drugs in current use is on the increase. Antimicrobial resistance is one of the major challenges facing global public health.[1] The emergence of multidrug-resistant isolates in tuberculosis, acute respiratory infections, and diarrhea is the major worry. This coupled with the epidemic of HIV/AIDS has increased the need for new antimicrobial agents.[2]

Furthermore, the treatment of chronic inflammatory diseases is still problematic.[3] Medical research over the past years has yielded important anti-inflammatory drugs; steroidal and nonsteroidal. However, these drugs have numerous and severe side effects including gastric ulcers and redistribution of body fat. Therefore, agents of natural origin with relatively infrequent side effects are required. Natural products from the plant origin represent an unparalleled reservoir of molecular diversity to drug discovery and development.

Glyphaea brevis (Spreng) Monachino (Tiliaceae) is a tree mainly present in forest regrowths, swampy places, rocky

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savanna, forest galleries, and fallow land. The leaf decoction facilitates childbirth and hastens delayed labor. The leaves are also used for dyspepsia and ulcers. The root decoction is used as an aphrodisiac, appetizer, laxative, and as a remedy for chest pains, diarrhea, dysentery, and sleeping sickness. Again, the root decoction together with guinea grains is used as a beverage and bath for paralysis.[4,5] Apart from the investigation of the antimicrobial[6] and antioxidant effects[7] of the leaves, not much is reported on the other plant parts.

In this work, we report a comparative study of the antibacterial, anti-inflammatory, and antioxidant properties of the leaves and bark of *G. brevis.*

MATERIALS AND METHODS

Collection of plant material and preparation of extracts

The plant materials used in the studies were obtained from Kente, Amansie Central District in the Ashanti Region of Ghana in June 2009. Botanical identity was confirmed and voucher specimens, KNUST/HM1/2010/L31 and KNUST/HM1/2010/S004 for leaves and stem bark, respectively, were deposited at the Department of Pharmacognosy, College of Health Sciences, Kwame Nkrumah University of Science and Technology herbarium. The dried and ground leaves and stem bark each of 50 g were Soxhlet-extracted with 500 mL 70% ethanol.

Microorganisms used

Eight bacterial species including both Gram positive and Gram negative bacteria obtained from the University of Ghana Medical School were used. The Gram positive bacteria were *Enterococcus faecalis* ATCC 29212, *Bacillus thurigiensis* ATCC 13838, *Staphylococcus aureus* ATCC 25923, and *Bacillus subtilis* NCTC 10073. Gram negative bacteria used were *Salmonella typhi* NCTC 6017, *Escherichia coli* NCTC 9002, *Proteus vulgaris* NCTC 4175, and *Pseudomonas aeruginosa* ATCC 27853.

Animals

The animals used in this study were cockerels (*Gallus gallus*; strain shaver 579). They were obtained one-day post-hatch and maintained in stainless steel cages (34 × 57 × 40 cm^3) at 12–13 chicks per cage. They were fed on Chick Mash (GAFCO, Ghana) and allowed access to water *ad libitum*. An overhead incandescent illumination was maintained on a 12-h light–dark cycle chicks and room temperature was 29 ± 1 °C. Cockerels weighing between 40 and 55 g on day 7 were randomized into treatment groups and tested. Group sample sizes of 5 were used throughout the study. All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication No. 85-23, revised 1985) and were approved by the Ethics Committee of the Department of Pharmacology, KNUST.

Chemicals

All chemicals used were of analytical grade and purchased from Sigma Aldrich Co. Ltd., Irvine, UK. Organic solvents were also of analytical grade and purchased from BDH Laboratory Supplies (England). TLC was carried out on silica gel F_{254} aluminum sheets, product code OB 315394, purchased from Merck KGaA, Germany. Diclofenac and dexamethasone were purchased from Troge, Germany and Pharm-Intas, Belgium, respectively.

Antimicrobial assays

Agar well diffusion method

The agar diffusion method[8] was used to determine the antibacterial activity. Wells of 9 mm diameter were made in 20 mL nutrient agar (Oxoid) seeded with two loopfuls of a suspension of test organisms (10^5 CFU/mL) aseptically. The wells were filled with 100 μL of the crude extracts at a concentration of 5 mg/mL in methanol. The plates were allowed to diffuse for 1 h at room temperature, incubated at 37 °C for 24 h, after which zones of inhibition were measured. The test results are the mean of three replicates. Amoxycillin was included as a positive control.

Microdilution assay

Minimal inhibitory concentration (MIC) values were determined based on a micro-well dilution method.[9] Inocula of the microorganisms were prepared from the 12-h broth cultures and suspensions were adjusted to 10^5 CFU/mL.

The 96 well sterile plates were prepared by dispensing into each well 100 μL of double strength nutrient broth and 100 μL aliquot of the plant extract (7.8–1000 μg/mL) together with 20 μL of the inoculums. The microplates were incubated for 24 h at 37 °C. Bacterial growth was determined after addition of a 20 μL of a 5% solution of *p*-iodonitrotetrazolium salt (MTT) and incubating for further 30 min. Dark wells indicated the presence of microorganisms. Amoxycillin was included as a positive control. All experiments were carried out in three replicates.

Anti-inflammatory assay

Carrageenan-induced edema

The anti-inflammatory activity was evaluated in cockerels in groups of five animals for each dose, using the carrageenan-induced edema,[10] modified by Woode *et al.*[11] Foot volumes were measured by water displacement plethysmography[12] at 0, 1, 2, 3, 4, and 5 h after subplantar injection of

carrageenan (10 μL of a 2% w/v solution in saline) into the right foot pad of the chicks. The test groups received the leaf extracts. Three groups of cockerels containing five chicks in each group received the plant extract (30, 100, and 300 mg/kg, *p.o.*), bark extract (30, 100, and 300 mg/kg, *p.o.*), the standard groups received diclofenac (5, 15, and 50 mg/kg, i.p.) and dexamethasone (1, 3 and 10 mg/kg, i.p.) and the control animals received the vehicle only. All treatments were administered 30 min for i.p. route and 1 h for p.o. before carrageenan injection.

Statistical analysis

Raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0 then averaged for each treatment group. The time-course curves for foot volume was subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni's *post hoc t*-test. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). The inhibition percentage of edema was calculated for each animal group in comparison with its vehicle-treated group.

Differences in AUCs were analyzed by ANOVA followed by Newman–Keul's *post hoc t*-test. ED_{50} (dose responsible for 50% of the maximal effect) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

$$Y = \frac{a + (b - a)}{\left[1 + 10^{(\mathrm{Log}ED_{50} - X)}\right]}$$

where X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

GraphPad Prism for Windows version 5.00 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis and ED_{50} determinations. $P < 0.05$ was considered statistically significant.

Antioxidant assays

Rapid screening for antioxidants

Methanolic solutions of extracts were spotted on silica gel sheets and developed in chloroform–methanol (9:1 v/v). The plates were sprayed with 20 mg/L of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol, antioxidant compounds in the extracts gave clear zones against a purple background.[13]

Total phenol content determination

The Folin–Ciocalteu reagent was used for phenol content determination.[14] Each plant extract (1 mL of 0.125–2.5 mg/mL) in methanol was separately mixed with 1 mL Folin–Ciocalteu's reagent and 1 mL of aqueous Na_2CO_3 (2%). The mixtures were incubated at 25°C for 2 h, and then centrifuged at 10,000*g* for 10 min. The absorbance of the supernatant was determined at 760 nm. 1 mL methanol was processed in the same way as the test drugs and used as a blank. The standard curve was prepared by preparing tannic acid solutions (0.03–0.1 mg/mL) in methanol. Total phenol values are expressed in terms of tannaic acid equivalents (mg/g of dry mass).

Total antioxidant capacity

Total antioxidant capacities of the extracts were determined as described by Prieto *et al*.[15] Different concentrations were tested for both ascorbic acid and the plant extracts: ascorbic acid (0.03–1 mg/mL); extracts (0.125–2.5 mg/mL). One milliliter of each plant extract or ascorbic acid was separately mixed with 3 mL of reagent solution (0.6 M H_2SO_4, 28 mM Na_2HPO_4, and 4 mM ammonium molybdate), served as a positive control. The mixtures were incubated at 95 C for 90 min, and then centrifuged at 10,000*g* for 10 min and the absorbance of the supernatant determined at 695 nm. Methanol was processed the same way as test drugs and used as a blank. The standard curve was prepared using solutions of ascorbic acid (0.03–1 mg/mL) in methanol. Total antioxidant values are expressed in terms of ascorbic acid equivalent (mg/g of dry mass).

Free radical scavenging activity of the ethanolic extracts

Assay was performed by the DPPH method described by Blois.[16] The absorbance of the stable free radical solution (20 mg/L in methanol) was determined as A_0. Afterwards, 3 mL of free radical solution was added to 1 mL of the methanolic test extracts (3, 1.5, 0.75, and 0.375 mg/mL). The mixtures were allowed to stand for 30 min and absorbance was measured at 517 nm. The percentage reduction of DPPH was calculated by the following equation:

DPPH scavenging activity (%) = $100(A_0 – A_1)/A_0$.

With A_0 being the absorbance of the control and A_1 is the absorbance in the presence of the test sample. Data were presented as %DPPH scavenging effect against concentration and the IC_{50} determined. All data were computed using GraphPad Prism for Windows Version 5.00 (GraphPad Software, San Diego, CA, USA).

Phytochemical screening

The preliminary phytochemical screening was performed by the standard methods.[17]

RESULTS

Antimicrobial effects

Agar well diffusion

The highest amount of inhibition was observed from the stem bark extract against *B. thurigiensis* ATCC 13838. The leaf extract inhibited some Gram positive organisms but none of the tested Gram negatives. However, the bark extract inhibited the growth of all the Gram positive organisms tested, *Proteus vulgaris* NCTC 4175, and *Escherichia coli* NCTC 9002 [Table 1].

Micro-dilution assay

MICs were observed for the extracts that showed activity in the agar well diffusion assay. The extracts showed activity with MICs from 500 μg/mL to more than 1000 μg/mL [Table 2].

Anti-inflammatory activity

Carrageenan-induced edema

The results of the 70% ethanolic extract of the leaves and stem bark of *G. brevis* in carrageenan-induced foot edema were presented in Figure 1 and Table 3. When compared with the control, the leaf and bark extracts, diclofenac and dexamethasone significantly reduced the foot pad edema.

Based on the ED_{50} values [Table 3] obtained from the dose–response curves [Figure 1], the leaf and bark extracts showed similar potencies. Both extracts were, however, found to be about four- to sixfold less potent than diclofenac and dexamethasone.

Table 1: Zones of inhibition of the leaves and stem bark extracts of *Glyphaea brevis*

Microorganisms	Zones of inhibition (mm) ± SEM	
Gram positive	GBL	GBB
Bacillus subtilis NCTC 10073	–	15.33 ± 0.33
Bacillus thurigiensis ATCC13838	13.00 ± 0.58	16.00 ± 0.58
Staphylococcus aureus ATCC 25923	11.33 ± 0.67	15.67 ± 0.33
Enterococcus faecalis ATCC 29212	11.67 ± 0.33	14.33 ± 0.67
Gram negative		
Proteus vulgaris NCTC 4175	–	15.67 ± 1.33
Pseudomonas aeruginosa ATCC 27853	–	–
Salmonella typhi NCTC 6017	–	–
Escherichia coli NCTC 9002	–	14.33 ± 1.15

Each value in the table was obtained by calculating the average of three replicates ± standard error of the mean. Extract: GBL, *Glyphaea brevis* leaves; GBB, *Glyphaea brevis* bark.

DISCUSSION

The leaves and stem bark extracts of *G. brevis* showed considerable antibacterial activities (MIC = 500 to >1000 μg/mL) against the tested microorganisms. Between the extracts, the stem bark was more active. It exhibited antibacterial activity against six of the eight tested bacteria strains. The leaf extract showed activity against three of the four tested Gram positive organisms with no Gram negative activity. The most prominent activities of the stem bark extract were against *B. subtilis* and *E. faecalis* with an MIC value of 500 μg/mL.

Plant secondary metabolites including: alkaloids, glycosides, triterpenoids, tannins, etc. have demonstrated antibacterial activities.[18] The presence of any one or more of these phytoconstituents [Table 5] may be responsible for the observed antibacterial activities.

The cyclohexane, ethyl acetate, and methanol extracts of the leaves of *G. brevis* have been reported to possess antimicrobial activities.[6] In the recent study, the 70% ethanol extract of the bark of *G. brevis* showed wider spectrum of activity compared to the leaf extract.

Carrageenan-induced edema is biphasic and characterized by the release of serotonin and histamine in the first phase.

Table 2: Mean minimum inhibitory concentration (MIC; μg/mL) of the leaf and stem bark extract of *Glyphaea brevis* on various microorganisms. *n* =3

Microorganisms	Zones of inhibition (mm) ± SEM	
Gram positive	GBL	GBB
Bacillus subtilis NCTC 10073	NT	500
Bacillus thurigiensis ATCC13838	>1000	1000
Staphylococcus aureus ATCC 25923	1000	>1000
Enterococcus faecalis ATCC 29212	1000	500
Gram negative		
Proteus vulgaris NCTC 4175	NT	1000
Pseudomonas aeruginosa ATCC 27853	NT	NT
Salmonella typhi NCTC 6017	NT	NT
Escherichia coli NCTC 9002	NT	>1000

All experiments were carried out in triplicates. 200 μg/mL of amoxycillin served as a positive control. MIC readings for all wells were the same. Extract: GBL, *Glyphaea brevis* leaves; GBB, *Glyphaea brevis* bark. NT = not tested.

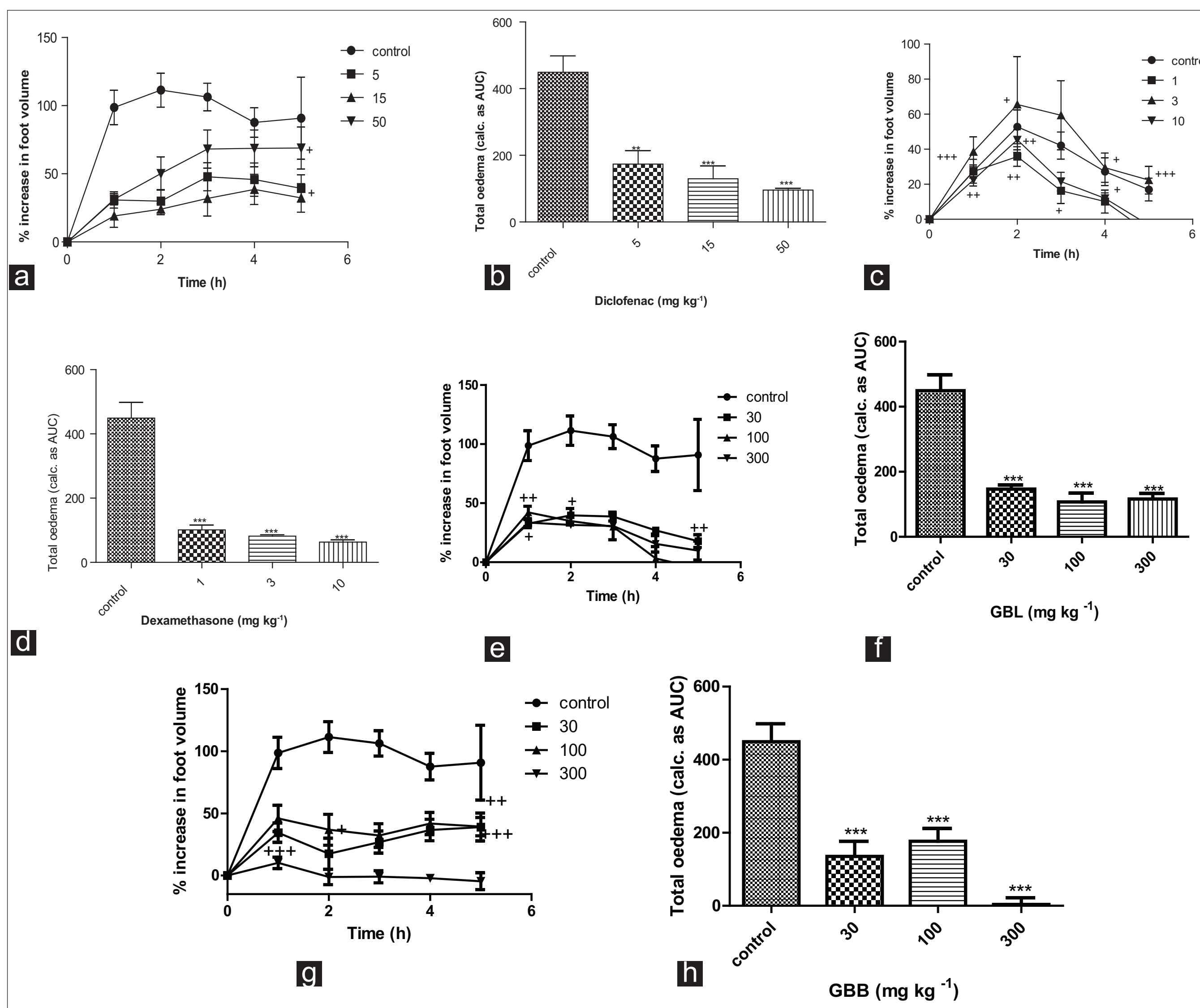

Figure 1: Effect of diclofenac (10 – 100 mg/kg; i.p.), dexamethasone (1 – 10 mg/kg; i.p.) and extract (30 – 300 mg/kg; p.o) on time course curve (a, c, e, g) and the total oedema response (b, d , f, h respectively) in carrageenan-induced oedema in chicks. Values are means ± SEM. (n =5). +++*P* < 0.0001; ++*P* < 0.001; +*P* < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). ****P* < 0.0001; ***P* < 0.001; **P* < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test.

Table 3: ED50 values for the effect of GLB, diclofenac, and dexamethasone in carrageenan-induced edema in chicks

Drug	ED50 (mg/kg)
GLB	21.16
GBB	21.30
Dexamethasone	3.43
Diclofenac	4.42

Extracts: GBL, *Glyphaea brevis* leaves; GBB, *Glyphaea brevis* bark

The second phase involves the release of prostaglandins. Kinins maintain the edema between the two phases.[19] This study clearly showed that the 70% ethanol extracts of the leaves and stem bark of *G. brevis* have anti-inflammatory effects. Both extracts significantly and equally reduced the edema produced by the subplantar injection of carrageenan. The results observed suggest an inhibitory effect, of both extracts, on the release and/or action of the mediators of inflammation. To the best of our knowledge, this is the first report of the anti-inflammatory effects of *G. brevis*.

Both extracts exhibited antioxidant properties in the various assays employed. The bark extract was higher in its content of phenols [Figure 2] and radical scavenging effects [Table 4]. Both extracts showed similar capacities in the total antioxidant assay [Figure 3].

Free radicals are often generated as by products of

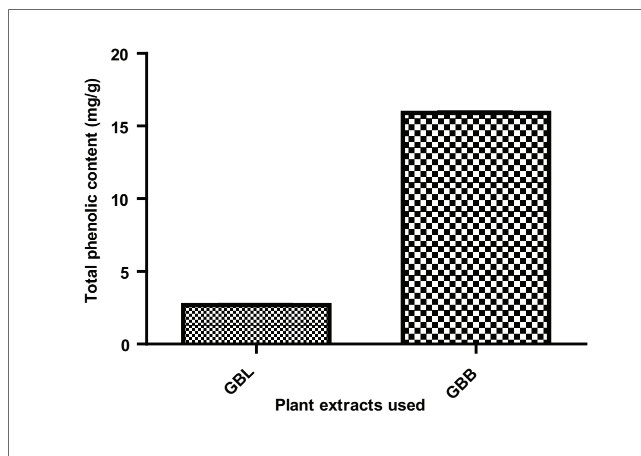

Figure 2: Total phenolic content (mg/g) in the 70% ethanolic extract of *Glyphaea brevis*. Extracts: GBL = *Glyphaea brevis* leaves, GBB = *Glyphaea brevis* bark.

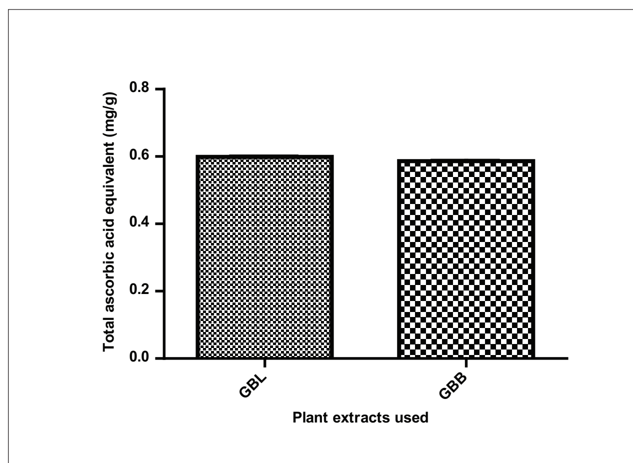

Figure 3: Total ascorbic acid equivalent (mg/g) in the 70% ethanolic extracts of *Glyphaea brevis*. Extracts: GBL = *Glyphaea brevis* leaves; GBB = *Glyphaea brevis* bark.

Table 4: IC_{50} values (mg/mL) for free radical scavenging activity by extracts

Extract	IC_{50} DPPH
GBL	9.509
GBB	1.392
Propyl gallate	0.0039

Extracts: GBL, *Glyphaea brevis* leaves; GBB, *Glyphaea brevis* bark

Table 5: Results of the phytochemical screening for all powdered plant materials

	Powdered plant materials	
	GBL	GBB
Phenolic	+	+
Reducing sugar	+	+
Alkaloids	+	+
Phytosterols	+	−
Triterpenoids	+	+
Saponins	−	+
Flavonoids	−	+

− = Absent, + = present. Extracts: GBL, *Glyphaea brevis* leaves; GBB, *Glyphaea brevis* bark

biological reactions or from exogenous factors and are implicated in neurodegenerative diseases, chronic ulcers, inflammatory diseases of the airway, and many more diseases of man.[20,21] The results noted above in the antioxidant assays validate their use in traditional medicine.

The antioxidant effects of the leaves of *G. brevis* using different assays have been reported before by Dakam *et al.*[7] However, the total antioxidant capacity, free radical scavenging, and total phenol content in the stem bark are being investigated for the first time.

The extracts of the leaves and stem barks of *G. brevis* in this work have exhibited different extents of antibacterial, anti-inflammatory, and antioxidant effects. These effects observed may be due to the presence of one or more of the plant secondary metabolites [Table 5]. The variations in the activities of the different plant parts could be due to unequal distribution of these secondary metabolites in the different plant parts, or up to different amounts of the same metabolites.

In conclusion, the 70% ethanol extracts of the leaves and bark of *G. brevis* possess antibacterial, anti-inflammatory, and antioxidant effects. In terms of antibacterial and antioxidant activities observed, the stem bark extract was the more potent. The anti-inflammatory activities were similar. These results indicate that the traditional use of *G. brevis* seems quite useful and reasonable. Further isolation of the various constituents responsible for these activities is in progress in our laboratories.

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