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Original Article

Antioxidant, Anti-inflammatory, Antiproliferative and Antimicrobial Activities of *Combretum glutinosum* and *Gardenia aqualla* Extracts *in vitro*

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ABSTRACT

Objectives: Plants represent a diverse template that could be tapped as sources of novel drugs. This study highlights antioxidant, anti-inflammatory, anti-proliferative and as well anti-microbial activities of *Combretum glutinosum* (BE) and *Gardenia aqualla* (GE) roots ethanol extracts. **Methods:** The Biological activities were evaluated using the established standard methods. **Results:** Both extracts exhibited antioxidant and anti-inflammatory activities with BE being better than GE. Anti-proliferative (cytotoxicity) assay on HepG2 and BHK-21 cell lines similarly revealed that BE (IC_{50}: 55, SI: 1.81) is more cytotoxic and more selective than GE (IC_{50}: 478.60, SI: 0.99). For the antimicrobial study, BE inhibited the growth of pathogenic bacteria; *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio species* and *E. coli*, while GE extract showed activity against Candida albicans. **Conclusion:** We conclude that *Combretum glutinosum* extract (BE) possessed the most robust activity from this study. Hence, it could be a promising source of novel drug with wide biological activities, especially antioxidant and anticancer activities.

Key words: Antioxidant, Cytotoxicity, Cancer cell line, Anti-microbial, Drug discovery.

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INTRODUCTION

Medicinal plants contain secondary metabolites, some of which have the capacity to prevent or treat many pathological conditions. These secondary metabolites could inhibit or modulate inflammatory response and oxidative Stress (O.S.); which in turn, could prevent or treat pathological conditions.[1] Free Radicals (F.R.) are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism.[2] FRs include Reactive Oxygen Species (ROs) and Reactive Nitrogen species (RNs). The most common ROs include: Superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), peroxy radical (ROO^-) and reactive hydroxyl (OH^-) radicals while RNs include: nitric oxide (NO) and peroxy nitrite anion.[2,3] Under normal circumstances, homeostasis exists between FRs generated in the body and antioxidants available to scavenge them. However, a shift in this balance causes O.S. which could cause inflammation, tissue injury DNA-damage, increased mutation rate within cells and thus promoting oncogenic transformation.[1] In addition, O.S. can trigger signalling pathways hence contribute to tumour development through regulation of cellular proliferation, angiogenesis and metastasis.[3]Antioxidant offers resistance against O.S. by scavenging F. R, inhibiting Lipid Peroxidation (LPO) and prevent damage to proteins and nucleic acids thus, preventing disease progression.[4] Antioxidants include both enzymatic such as Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), catalase, glutathione reductase and non-enzymatic such as glutathione, Vitamins A, C and among others.[5] On the other hand, increasing incidence of drug-resistance, adverse effect and toxicity has stimulated the effort of scientists and pharmaceutical industries to search for drugs from natural sources.[6] Out of 109 new antibacterial drugs, approved in the period 1981–2006, 69% got their root from medicinal plants and other natural compounds and 21% of antifungal drugs were natural derivatives or compounds mimicking natural products.[7] Nigeria is a country that is blessed with vast arrays of flora most of which are yet to be discovered and utilized maximally in biomedical research, to arrive at a drug. Some of these flora used in Nigerian traditional medicine include; *Combretum Spp and Gardenia aqualla* among others.

The *Combretum glutinosum (Hausa name: Baushe; B)*, belongs to the family *Combretaceae* consisting of 20 genera with at least 600 species. In West Africa *C. glutinosum* is used as an important source of yellow to brownish dye for cotton textiles beside it medicinal purposes. Many species of Combretum (*Combretaceae*) have been used as traditional medicines for many applications, including abdominal disorders, bacterial infections, diarrhea, bilharzias, malaria, respiratory infections, pneumonia, skin and

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venereal diseases, fevers and sore throats, liver, kidney complains and cancer especially.[8] *Gardenia aqualla* (Hausa name: Gaude; G). a shrub plant of *Rubeacea* family, grows up to 3 metres high in the savannah; found in Senegal, Nigeria Sudan and other west African countries. Medicinally the leaf is used to treat leprosy, the root to treat oral infections, the fruit for ear infection and the stem bark is used to treat bowel disorders.[9]These plants are very important from the pharmaceutical point of view; therefore, present study was carried out to investigate; the anti-oxidant, anti-inflammatory, anti-proliferative (anticancer activity) as well as anti-microbial activities of their ethanol extract.

MATERIALS AND METHODS

Chemicals

Trichloro Acetic Acid (TCA), Thiobarbituric Acid (TBA), Butylated Hydroxytoluen (BHT), 1,1-diphenyl-2picryl hydrazyl (DPPH) radical, p-nitrophenyl-b-D-glucopyranoside (PNPG), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), napthylenediamine hydrochloride, sulphanilamide, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Organic solvents of HPLC-grade ethanol 95% were obtained from Merck (USA). All other chemicals and reagents were of analytical grade.

Collection and extraction of plant samples

Plants samples (which include the roots, leaves and fruits) were collected in January 2015 from Keffi, Nasarawa State, Nigeria. The roots were authenticated at the Department of plant science and Biotechnology, Nasarawa State University, Keffi and Voucher specimens were deposited at the herbarium. The roots were washed with water, cut into pieces, grinded with pestle and mortar then allowed to dry in the shade. A small mesh sieve was used to obtain small particles of about 100µm. The larger particles were discarded while the powdered kept in air tight plastic container until further use. The dried powdered root of each plant (100g) was exhaustively defatted with petroleum ether then soaked in 300ml ethanol (95%) and left for 48 hr. The extracts were obtained by filtration, then concentrated using rotary evaporator at 55°C and 100 rpm (BÜchi, Switzerland) then lyophilized (DISHI, DS-FD-SH10, Xian Heb Biotechnology Co, China) to obtain extracts in powdered form. The extracts were kept at -20°C until used.

Human Blood

Human participants and their specimen (blood) met the ethical standards for donor agreement, made mandatory by national regulatory bodies. Participants signed informed consent for the use of their blood in this study. Blood samples (2 ml each) were collected from five healthy individuals who did not take any medication two weeks prior to collection.

Animals

Experimental procedure was approved by Alexandria University Animal Ethics Committee (AEC) and animals received tender care as contained in the guide lines of National Health and Medical Research Council (NHMRC), Arab Republic of Egypt. Six male rats (150-200g body weight) were obtained from animal house of medical research institute, Alexandria University (Egypt). Animals were left to adapt to our laboratory for two weeks before the experiment. The livers were harvested from the animals under anaesthesia and washed in cold saline, then one gram of each liver was homogenized in 9 ml phosphate buffer saline. The homogenate was centrifuged at 3000 and metabolites containing supernatant was carefully decanted for further biochemical assessments.

Phytochemical composition

Dried powdered plants root extracts were spectrophotometrically screened for total phenolic and flavonoids. The Folin-ciocalteu reagent method as described by Demiray *et al.*[10] was employed to determine the total phenolic contents of the plant extracts. While Aluminium chloride colorimetric method was used for total flavonoids determination.[5]

Assessment of antioxidant activities

The anti-oxidant activities of the plants root extracts were determined by DPPH Radical Scavenging Assay (1,1-diphenyl-2picryl hydrazyl).[11] The method of Halliwel *et al.*[12] was used to assay HO. Nitric oxide scavenging activity was estimated using Griess reagent.[13] The lipid peroxidation assay was carried out by a method modified and used by Ghareeb *et al.*[14] Glutathione peroxidase (GPx) activity (EC NO:1.11.19) was determined by the method of Paglia and Valentia.[15] and Determination of superoxide dismutase (SOD) activity (EC NO:1.15.1.1) by the method of Markland and Marklund.[16]

Assessment of *in vitro* anti-inflammatory activity

Inhibition of albumin denaturation and Membrane stabilization test were used to test for anti-inflammatory activity.[17]

Antimicrobial assay

The indicator bacteria used in current investigation were *Pseudomonas aeruginosa* ATCC: 8739, *Staphylococcus aureus* ATCC: 6538, *Escherichia coli* ATCC 8739 and *Vibrio* sp. The assay was carried out as described by Nassir *et al.*[18] and anti-fungal activity of the samples was determined by disk diffusion method on Muller Hinton agar (MHA) medium.[19]

Cytotoxicity assay by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

Exponentially growing cells were trypsinized, counted and seeded at the appropriate densities (5000 cells/0.33 cm^2 well) into 96-well microtiter plates. Cells were incubated in a humidified atmosphere at 37°C for 24 hrs. Then cells were exposed to the two extracts, at the desired concentrations (0.1, 1, 10, 100 and 1000 µg/ml) for 72 hrs. At the end of the treatment period, media were removed, cells were incubated with 200 µl of 5% MTT solution/well (Sigma Aldrich, MO) and allowed to metabolize the dye into a coloured-insoluble formazan complex for 2 hrs. Medium was discarded from the wells and the formazan crystals were dissolved in 200 µl/well acidified isopropanol for 30 min, covered with aluminium foil and with continuous shaking using a MaxQ 2000 plate shaker (Thermo Fisher Scientific Inc, MI) at room temperature. Absorbance was measured at 570 nm using a SpectraMax plus Microplate Reader (Molecular Devices, CA). The cell viability was expressed relative to the untreated control cells. Human hepatocellular carcinoma (HepG-2) and baby hamster kidney cell line (BHK-21) were originally purchased from American type culture collection (ATCC, Wesel, Germany) and grown in the tissue culture lab of the Egyptian company for production of vaccines, sera and drugs (Vacsera, Giza, Egypt). The cells were transferred to our laboratory and maintained in Dulbecco Modified Eagle Medium (DMEM). Both were Supplemented with 1% of 100 mg/ml of streptomycin, 100 units/ml of penicillin and 10% of heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in humidified incubator containing 5% CO_2.[20,21]

Statistical analysis

All data were expressed as mean ± standard deviation (SD). The differences were statistically significant at $P< 0.05$. Statistical analyses were carried out using primers of Biostatistics program V_5 for analysis of unpaired student t- Test and one way (ANOVA).

RESULTS

Characterization of the plants extracts.

Total phenolic content

The result shows that the total phenolic content in BE and GE are 70.61mg and 40.76mg per gram dry extract, as Gallic acid equivalent respectively.

HPLC analysis of polyphenolic compounds: The result expressed in mg/g (Figure 1a and b) shows that, BE contains; catechins (24.12mg), vanillic acid (0.46mg), epigallocatechin gallate (0.28mg), kaempferol (0.0051mg), rutin (0.0018mg), quercetin (0.0084mg), apigenin (0.0073mg) while GE contains; 2,5-dihydroxy benzoic acid (25.52mg), vanillic acid (0.47mg), salicylic acid (0.0013mg), ferulic acid (0.069mg), naringenin (0.0082mg) and rosmarinic aid (0.0051mg).

Total flavonoids content

The result shows that the total flavonoid content in BE and GE are 100.44mg and 0.51mg per dry extract as quercetin equivalent respectively.

Free radical scavenging and antioxidant activity

DPPH

Figure 2a shows that BE and GE have DPPH scavenging activity and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE.

Hydroxyl radical scavenging activity

Figure 2b shows that BE and GE have HO· scavenging activity and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE.

Nitric oxide radical scavenging activity

Figure 2c shows that BE and GE have NO· scavenging activity and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE.

Inhibition of lipid peroxidation activity

Figure 2d shows that BE and GE have scavenging/antioxidant activity by impeding lipid peroxidation and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE.

Antioxidant activity

SOD activity

Figure 3a shows that BE and GE cause significant activation in SOD activity and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE

GPx activity

Figure 3b shows that BE and GE cause significant activation in SOD activity and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE.

Anti-inflammatory activity

Inhibition of RBC haemolysis

Figure 4a shows that BE and GE have activity against hypotonic solution induced RBC haemolysis and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE.

Inhibition of albumin denaturation

Figure 4b shows that BE and GE have activity against heat induced albumin denaturation and these activities increase in concentration dependent manner. It also shows that BE has better activity than GE

Cytotoxicity assay against normal and cancerous cells lines

Figure 5 shows the activity of BE against normal BHK-21 and HepG2 with an IC_{50} of 100µg/ml and 55µg/ml respectively; given a selectivity index of 1.81. Whereas, GE revealed an IC_{50} of 478.00µg/ml and 478.60 µg/ml respectively; given a selectivity index of 0.99

Antimicrobial activity

The Effect of BE and GE on selected pathogens is described in the (Table 1). BE has activity against all the pathogens while GE has activity against only two.

DISCUSSION

The result of phytochemical analysis showed that the total phenolic content in BE and GE are 70.61mg and 40.76mg per g dry extract, as Gallic acid equivalent respectively. While total flavonoids are 100.44mg and 0.51mg per dry extract as quercetin equivalent respectively**.** HPLC analysis revealed both extracts especially BE being very rich in flavonoids. Previous phytochemical studies showed that BE contains sterols, triterpenes, flavonoids, saponins, coumarins and tannins. While TLC revealed the presence of Apigenin, Genistein, Rutin and quercetin.[22] ROS are generally generated from aerobic metabolism in the mitochondria and microsomes as well as metabolism of xenobiotic.[23] Oxidative stress results from the imbalance between ROS/RNS and antioxidants

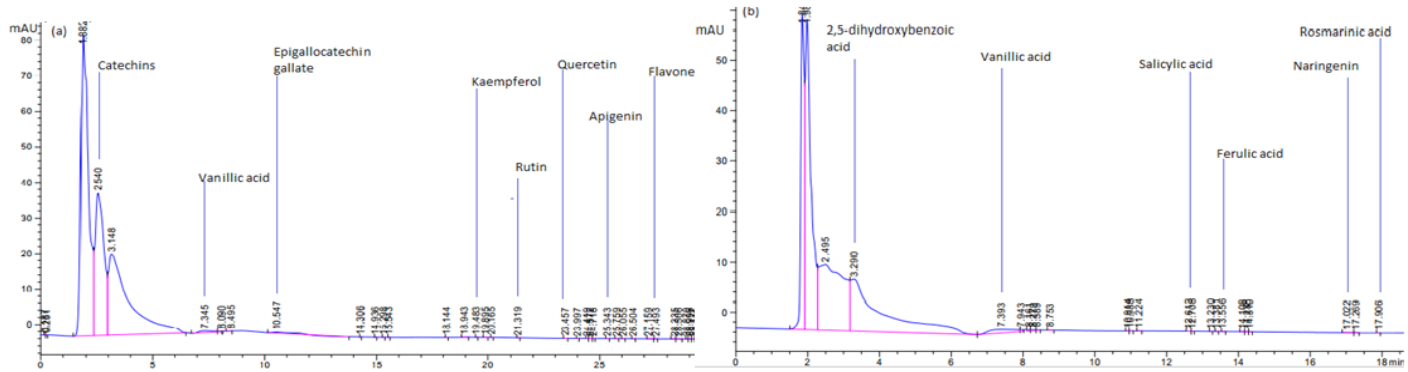

Figure 1: HPLC analysis of polyphenolic compounds present in studied plants. (A): *Combretum glutinosum* extract and (B): *Gardenia aqualla* extract.

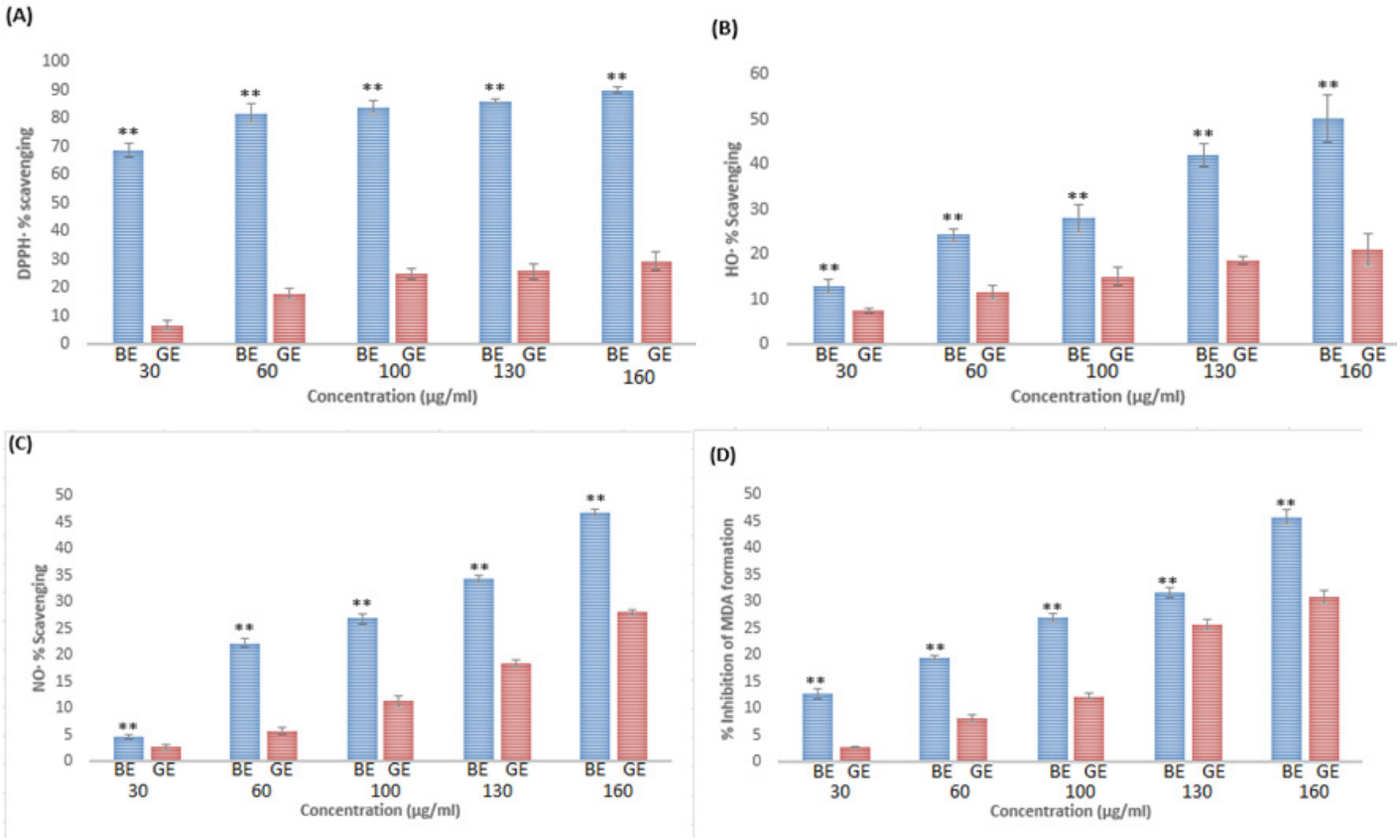

Figure 2: Radical scavenging effect of BE and GE. (A): DPPH (B) hydroxyl radical (C): Nitric oxide radical (D) Inhibition of MDA formation. Where BE: *Combretum glutinosum* and GE: *Gardenia aqualla*. Data are presented as Mean ± SD (*n*=3), values differ within column significantly at **P< 0.05.

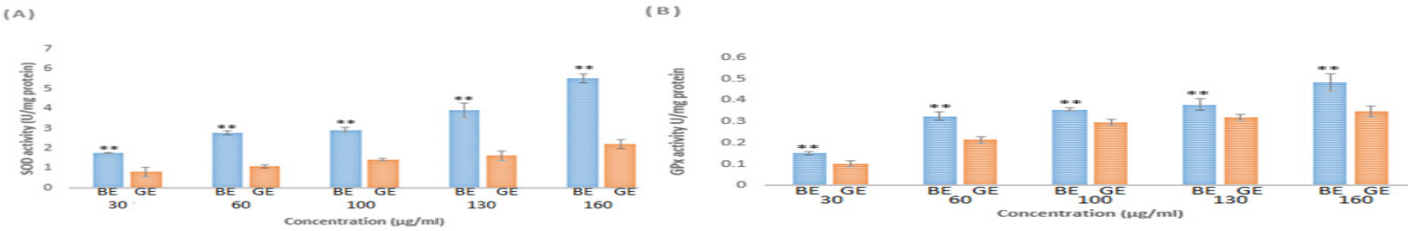

Figure 3: Effect of different concentrations of studied plants on some antioxidant enzymes activities in rat liver homogenate (A): Superoxide dismutase (SOD) and (B): Glutathione peroxidase (GPx). Where BE: *Combretum glutinosum* extract and GE: *Gardenia aqualla* extract Data are presented as Mean ± SD (*n*=3) values differ within column significantly at** P< 0.05.

mechanisms of the body.[24] Recently natural products are used as a source of pharmaceutical antioxidants. Therefore, in this study the antioxidant, anti-inflammatory, anticancer and antimicrobial activities of BE and GE were evaluated.

The result of this study showed that the IC_{50} of BE for DPPH, HO· NO· are 22 ± 1.3µg/ml, 160 ± 0.8µg/ml and 360 ± 2.8µg/ml respectively while GE are 271 ± 2.1 µg/ml, 235± 1.6µg/ml and 432 ± 3.8µg/ml respectively. This indicates that BE has better scavenging activities for the various radicals than GE. These activities increased with increase in concentrations of the extracts. Similarly, BE and GE inhibited TBA-MDA adduct formation *ex vivo*. These activities could be because of the polyphenols and flavonoids found in these extracts. Compounds such as quercetin, kaempferol, catechins and apigenin identified in BE has been found to possess free radical scavenging and antioxidant activity.[14] These could explain also why BE had greater activity compared to GE; since it contains higher concentrations of polyphenols and flavonoids than GE.

Table 1: The table describes the effect of BE and GE against selected microbial pathogens culture after 24 hrs.

Pathogens	Extracts (0.1mg/ml) and Zones of inhibitions(mm)	
	BE	GE
Pseudononas aeruginosa	20	-ve
Staph. aureus	26	-ve
Vibrio Sp.	18	-ve
Candida albicans	29	12
E. coli	18	14

BE has activity against all pathogens tested while GE has activity against two pathogens only. Where BE: *Combretum glutinosum* extract and GE: *Gardenia aqualla* extract.

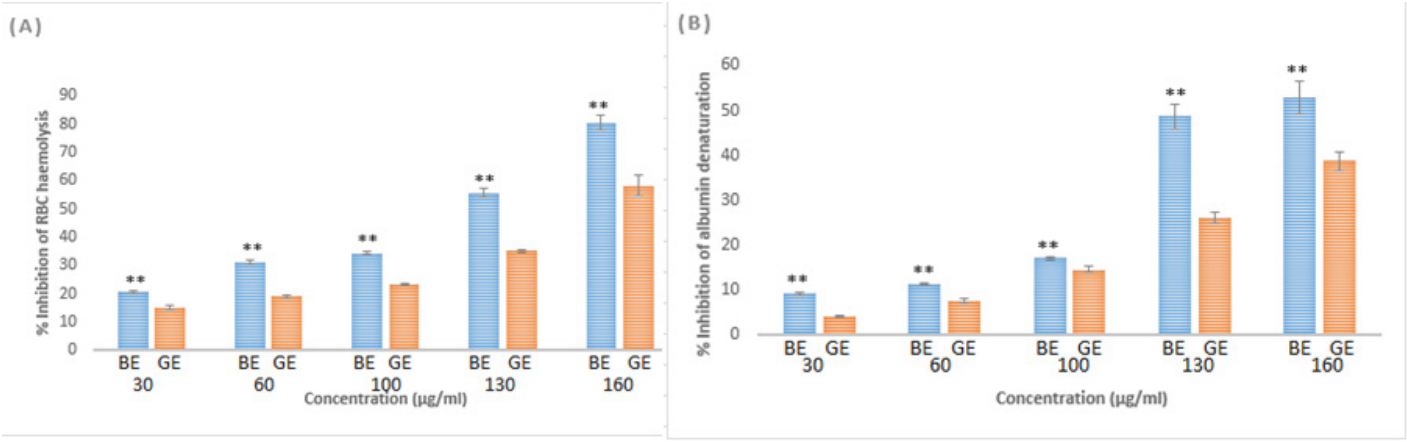

Figure 4: The anti-inflammatory activities of studied plants at different concentrations. (A): percentage inhibition of RBC haemolysis and (B): Percentage inhibition of albumin denaturation. Where BE: *Combretum glutinos*um extract and GE: *Gardenia aqualla* extract. Data are presented as Mean ± SD (*n*=3) values differ significant at *P***< 0.05.

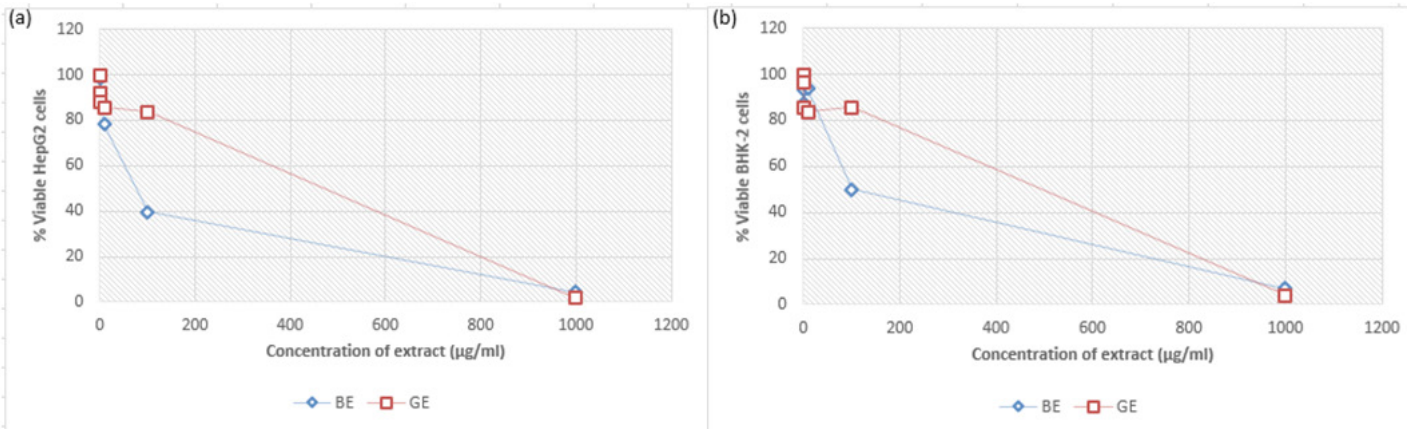

Figure 5: Effect of different concentrations of BE and GE on normal and cancer cells viability. (A): Activity HepG2 cells line and (B): Activity against BHK-21 cells line. Where BE: *Combretum glutinosum* extract and GE: *Gardenia aqualla* extract

In addition, the results showed that both BE and GE increased the activity of SOD and GPx in liver homogenate *ex vivo.* This activation was increased with increased concentrations of extracts. BE exhibited significantly (P<0.05) greater activation of the enzymes activity compared to GE. These results agree to the previous studies which found that Polyphenols such as Quercetin, Kaempferol and resveratrol increase SOD, GPx and Catalase activity *in vitro*[25] and *in vivo*.[26,27] On the other hands, BE and GE inhibited HRBC haemolysis (with IC_{50} 117µg/ml and 137µg/ml respectively) and heat induced albumin denaturation (with IC_{50} 151µg/ml and 206µg/ml respectively). Since cell membranes are similar in component and architecture, HRBC is therefore, like lysosomal membrane. For this reason, protection of HRBC membrane from lysis due to hypo tonicity and inhibition of heat induced albumin denaturation are considered as tests for anti-inflammatory activity.[14,28] An inflammatory process resulting from infection and/or damaged tissues accompanied by the release of lysosomal enzymes (such as glycosidases, proteases and sulphases) and inflammatory mediators, is considered a hallmark for many pathological conditions; especially fibrosis and cancer.[14,29] Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) impede inflammation by either inhibiting lysosomal enzymes or by stabilizing the lysosomal membrane.[14] For both RBC membrane stabilization and albumin denaturation assay, our extracts showed activity in concentration dependent manner. Likewise, NO· which could act as pro oxidant or an inflammatory mediator was found to be decreased by the extracts in concentration dependent manner (Figure 2) with BE having significantly (P<0.05) higher activity. This suggests the possibility of BE to serve as possible pharmaceutical lead compound to isolate antioxidant, anti-inflammatory /or anticancer drugs.

In vitro cytotoxicity (MTT) assay against BHK-21 and HepG2 cell lines, revealed that Both BE and GE have an IC_{50} of less than 500 µg/ml, hence considered cytotoxic.[30] BE showed higher activity against HepG2 (IC_{50}: 55 µg/ml) than BHK-21 cell line (IC_{50}: 100 µg/ml) and selectivity index (SI) of 1.81. Similar result was reported in some members of this genus.[31] However, to the best of our knowledge this is the first finding on the ethanol root extract of this species against cancer cell line. On the other hand, GE indicated lower cytotoxicity (IC_{50}: 478.60 µg/ml) and S.I. of 0.99 this agrees to the findings of Tagne *et al*.[32] The variation in the cytotoxicity of BE and GE could be related to differences in their phytochemical compositions.

The result in Table 1 shows that plant BE has activity against all the pathogenic organisms tested, which agrees with the findings of Wimaluk *et al*.[8] who found that BE has activity against all the pathogens tested including *S. typhimurium* and *K pneumoniae*. On the other hand, GE has activity against *Candida albicans* and *E. coli*. This agrees to the findings of Suvarnalatta *et al*.[33]

CONCLUSION

This work establishes the potential of BE and GE as antioxidant, anti-inflammatory, anticancer as well as antimicrobial agents. Follow up studies, *in vivo* testing and isolation of active compounds are recommended.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BE: *Combretum glutinosum* extract; **GE:** *Gardenia aqualla* extract; **MTT:** 3-[4,5-dimethylthiazole-2-yl]-2,5diphenyltetrazolium bromide; **DPPH:** (1,1-diphenyl-2picryl hydrazyl; **BHK-21:** Baby hamster kidney fibroblast cell line; **HepG2:** Human liver cancer cell line; **NSAIDs:** Nonsteroidal AntiInflammatory Drugs.

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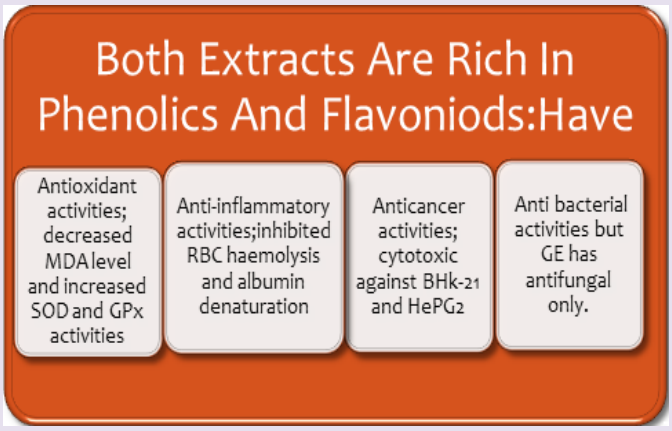
GRAPHICAL ABSTRACT



SUMMARY

This study highlights antioxidant, anti-inflammatory, anti-proliferative and as well antimicrobial activities of *Combretum glutinosum* and *Gardenia aqualla* roots ethanol extracts. Both extracts exhibited antioxidant and anti-inflammatory activities, Anti-proliferative as well as antimicrobial activities *invitro*. We conclude that *Combretum glutinosum* extract possessed robust and wide biological activities, especially antioxidant and anticancer activities

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