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 antimicrobial 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 (IC₅₀: 55, SI: 1.81) is more cytotoxic and more selective than GE (IC₅₀: 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.

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 (O₂⁻), hydrogen peroxide (H₂O₂), peroxy radical (ROO⁻) and reactive hydroxyl (OH⁻) radicals while RNs include: nitric oxide (NO) and peroxy nitrite anion.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 signaling pathways hence contribute to tumour development through regulation of cellular proliferation, angiogenesis and metastasis.⁴ 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.³ 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.⁴ 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

venereal diseases, fevers and sore throats, liver, kidney complaints and cancer especially.6 Gardenia aqualla (Hausa name: Gaude; G), a shrub plant of Rubieca 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.7 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 Hydroxytoluene (BHT), 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical, p-nitrophenyl-b-D-glucopyranoside (PNPG), 5,5′-dithiobis 2-nitrobenzoic acid (DTNB), napthylenediamine hydrochloride, sulphamidamide, 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 thepowdered 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 (NHRMC), 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-ciocalteau reagent method as described by Demirey et al.9 was employed to determine the total phenolic contents of the plant extracts. While Aluminium chloride colorimetric method was used for total flavonoids determination.

Assessment of antioxidant activities
The anti-oxidant activities of the plants root extracts were determined by DPPH Radical Scavenging Assay (1,1-diphenyl-2-picryl hydrazyl).10 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 Valencia.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 Nasir 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 cm² 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 colourless-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% CO₂.20,21

Statistical analysis
All data were expressed as mean ± standard deviation (SD).1 The differences were statistically significant at p<0.05. Statistical analyses were carried out using primers of Biostatistics program V, 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), vanillin 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 acid (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\textsubscript{50} of 100µg/ml and 55µg/ml respectively; given a selectivity index of 1.81. Whereas, GE revealed an IC\textsubscript{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.

ROS are generally generated from aerobic metabolism in the mitochondria and microsomes as well as metabolism of xenobiotic. Oxidative stress results from the imbalance between ROS/RNS and antioxidants.
mechanisms of the body. 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. These could explain also why BE had greater activity compared to GE; since it contains higher concentrations of polyphenols and flavonoids than GE.

**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.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Extracts (0.1mg/ml) and Zones of inhibitions(mm)</th>
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<tbody>
<tr>
<td></td>
<td>BE</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>20</td>
</tr>
<tr>
<td><em>Staph. aureus</em></td>
<td>26</td>
</tr>
<tr>
<td><em>Vibrio Sp.</em></td>
<td>18</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>29</td>
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<tr>
<td><em>E. coli</em></td>
<td>18</td>
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</tbody>
</table>

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.
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 and in vivo. On the other hands, BE and GE inhibited HRBC haemolysis (with IC$_{50}$ $117\mu g/ml$ and $137\mu g/ml$ respectively) and heat induced albumin denaturation (with IC$_{50}$ $151\mu g/ml$ and $206\mu g/ml$ 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. 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 pathologic conditions; especially fibrosis and cancer. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) impede inflammation by either inhibiting lysosomal enzymes or by stabilizing the lysosomal membrane. 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 $\mu g/ml$, hence considered cytotoxic. BE showed higher activity against HepG2 (IC$_{50}$: $55\mu g/ml$) than BHK-21 cell line (IC$_{50}$: $100\mu g/ml$) and selectivity index (SI) of 1.81. Similar result was reported in some members of this genus. 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$_{50}$: $478.60\mu g/ml$) and S.I. of 0.99 this agrees to the findings of Tagne et al. 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. 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.
CONCLUSION
This work establishes the potential of BE and GE as antioxidant, anti-inflammatory, anticancer as well as antimicrobial agents. Follow up studies, in vitro testing and isolation of active compounds are recommended.

ACKNOWLEDGEMENT
The authors wish to acknowledge the contribution of Dr. Shymaa Abdul-gaffar in the course of this research and Alexandria University for providing enabling atmosphere for the research.

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]-2,5-diphenyaltetrazolum bromide; DPPH: (1,1-diphenyl-2-picryl hydrazyl); BHK-21: Baby hamster kidney fibroblast cell line; HepG2: Human liver cancer cell line; NSAIDs: Nonsteroidal AntiInflammatory Drugs.

REFERENCES
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 *in vitro*. We conclude that *Combretum glutinosum* extract possessed robust and wide biological activities, especially antioxidant and anticancer activities.

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Cite this article: Muhammad BY, Shaban NZ, Elrashidy FH, Ghareeb AD. Anti-Oxidant, Anti-Inflammatory, Anti-Proliferative and Anti-Microbial Activities of *Combretum glutinosum* and *Gardenia aqualla* extracts *in vitro*. Free Radicals and Antioxidants. 2019;9(2):66-72.