Antioxidant and antimicrobial activities of the extracts of the Calyx of *Hibiscus Sabdariffa* Linn

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**Capsule Summary:** The antioxidant and antimicrobial activities of the aqueous and ethanol (hydro-ethanol) of *Hibiscus sabdariffa* were assessed. Results showed that hydro-ethanol extracts showed higher antioxidant activity in terms of TAC, EC50 and DPPH as well as antimicrobial activity against set of microbes.

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**INTRODUCTION**

Oxidative stress and cellular metabolism generates reactive oxygen species (ROS) that contributes to the etiology, pathogenesis and progression of several diseases including inflammations, cancer, and cardiovascular diseases (Stanner et al., 2002). Dietary antioxidants can inactivate ROS and provide protection from oxidative damage and are therefore considered important therapeutic and prophylactic agents against disease development (Farrukh and Mukhtar, 2002). Additionally, escalating levels of antibiotic resistance strains of pathogenic microorganisms necessitates the search for new antibiotics with high therapeutic potential. Therefore, screening dietary sources of natural products for novel antimicrobial agents is a priority. *H. Sabdariffa* Linn is a multi-purpose plant used widely for nutritional and medicinal purposes throughout West Africa (Dokosi, 1998; Qi et al., 2000). The calyces are phytochemical rich and have been shown to contain bright red-pigmented flavonoids (Ali et al., 2005). Prior studies have confirmed the presence of glycosides, flavonoids, saponins, essential oils, saponosides, triterpenes, coumarins, tannins, sugars, steroids, triterpene glycosides, and polyphenolic compounds as phytoconstituents in the calyces of *H. sabdariffa* (Ali et al., 2005).

Aqueous decoction of the calyx of *H. Sabdariffa* is used for food coloring (Frimpong et al., 2014) and for the preparation of a soft drink widely consumed for varied health benefits (Fasoyiro et al., 2005; Fasoyiro et al., 2005). Prior studies have demonstrated that the calyx extracts of *H. sabdariffa* are anti-

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**A B S T R A C T**

The calyces of *Hibiscus sabdariffa* Linn are widely used for nutritional and medicinal purposes in Ghana. This study assessed the antioxidant and antimicrobial activities of the aqueous and the ethanol (hydro-ethanol) extracts (30%) of the calyces of *H. sabdariffa* Linn. The hydro-ethanol extract exhibited a more potent antioxidant potential versus aqueous extract as demonstrated by its higher Total Antioxidant Capacity (TAC) and its lower EC50 for both the DPPH and the Hydroxyl radical scavenging activities. Additionally, the hydro-ethanolic extract displayed more potent antimicrobial activities with larger zones of inhibition in the agar diffusion assay and lower MICs in the broth dilution assay against a set of microorganisms including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans*. The data supports the ethnomedicinal use of the aqueous infusion of *H. sabdariffa* Linn for the management of urinary tract infections and other symptoms that may be linked to oxidative stress.
hypertensive (Ajaya, 2007); anti-atherosclerosis in rabbits (Chang-Che, 2003); and a diuretic that decreases urinary concentrations of creatinine, uric acid, citrate, tartrate, calcium, sodium, potassium and phosphate in humans (Alarcón-Alonsoa, 2012). An additional earlier study has examined the antioxidant activities of the ethanolic extract of the calyx of H. sabdariffa (Yang, 2012) while an unrelated study has also assessed the antibacterial effects of the ethanolic extracts of the calyx of H. sabdariffa in ground beef and apple juice (Chao, 2008). Since the ethnomedical use of the calyx of H. sabdariffa in Ghana concentrates on aqueous decoctions, this study assesses the antimicrobial and antioxidant activities of the aqueous and the hydro-ethanolic (70% water/30% ethanol) extracts of the calyces of H. Sabdariffa.

The results show that both extract (aqueous and hydro-ethanolic) display substantial antioxidant activities as demonstrated by the relatively low EC50s in DPPH radical and hydroxyl radical assays. However, the Hydro-ethanol extract exhibited a more potent antioxidant potential than the aqueous extract as demonstrated by its higher Total Antioxidant Capacity (TAC), and its lower EC50s for the DPPH and Hydroxyl Radical scavenging activities. Additionally, the hydro-ethanolic extract displayed better antimicrobial activities with larger zones of inhibition in agar diffision assay and lower MICs in broth dilution assay against a panel of five microorganisms including E. coli, S. aureus, P. aeruginosa, B. subtilis and C. albicans. The data provides reasonable experimental supports for the widespread prophylactic use of H. sabdariffa calyx extract for the management of hypertension, urinary tract infection and diverse other symptoms in Ghana.

MATERIALS AND METHODS

Chemicals

DPPH (2,2-Diphenyl-2-picrylhydrazyl), potassium sodium phosphate, sodium carbonate, ascorbic acid and ammonium molybdate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Nutrient broth and nutrient agar were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ferric chloride, Ethanol and Methanol were obtained from Merck Chemical Supplies (Damstadt, Germany). All reagents were of analytical grade.

Collection and Preparation of the calyx

The calyces of H. sabdariffa was obtained from the Central market of Kumasi, Ghana. Purchased calyces were identified by a botanist at the Department of Applied and Theoretical Biology of the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. Voucher specimen was deposited at the School of Botany of the KNUST. Prior to extraction, the calyces were initially air dried, and pulverized and stored in an air tight container.

Phytochemical Extraction

Soxhlet apparatus was filled with 50 g of pulverized dried calyx. A 500 mL water (aqueous extract) or a 500 mL of the hydro-ethanol mixture of (350 mL water: 150 mL ethanol (98%)) was added to the calyces in the soxhlet. Soxhlet extraction was then performed for 3 h and extraction solvents removed at 45°C on a rotavap. Extracts were stored at -20°C until needed for analysis.

Basic Phytochemical Screening

To determine the broad classes of phytoconstituents present in the calyces of H. sabdariffa, portions of the aqueous and hydro-ethanolic extracts were assayed using protocols previously described (Trease and Evans, 1984)

Alkaloids: Acidified solution of alkaloid produce a white-yellowish precipitate upon the addition of a few drops of Mayer’s reagents. Extracts were initially heated on a boiling water bath with 2% hydrochloric acid. The mixture was allowed to cool and few drops of Mayer’s reagent added. A yellow precipitate indicated the presence of alkaloids.

Terpenoids and steroids: 250 µL of acetic anhydride and 250 µL of chloroform was added in small amount of the extract (2 mg). Concentrated sulphuric acid (1 mL) was added slowly and a red-violet color indicated the presence of terpenes while a green-bluish color was indicative of the presence of steroids.

Flavonoids: Small amount of the extract was mixed with 1.5 mL of 50% methanol and warmed. Metal magnesium was then added followed by 5-6 drops of concentrated hydrochloric acid. The formation of a red color indicated the presence of flavonoids while an orange color indicated the presence of flavones.

Tannins: A 0.5 mL of an aqueous solution of the extract was mixed with 1-2 drops of ferric chloride solution. A blue color indicated the presence of gallic tannins while a greenish-black color indicated the presence of catechol tannins.

Coumarins: 500 µL of an aqueous solution of the extract was mixed with 750 µL of 10 % NaOH. The formation of a yellow colour is indicative of the presence of coumarins.

Saponins: 500 µL of an aqueous solution of the extract was mixed with 1 mL of 1% sodium bicarbonate and the solution shaken. A persistent froth is indicative of the presence of saponins.

Glycosides: Few drops of acetic anhydride was added in small amount of the extract (2.0 mg) in a test tube followed by 2-3 drops of concentrated sulphuric acid. A blue-green color shows the presence of glycosides.

Antioxidants capacity determination assays

Total Antioxidant Capacity (TAC): The total antioxidant capacity was evaluated using a slight modification of the method described by Prieto et al. (1999). Briefly, a 3 mL each of the aqueous and the hydro-ethanolic extract of concentration (0.375, 0.75, 1.5 and 3.0 mg/mL) were placed in a test tube. A 0.3 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium
Table 1: Results of the phytochemical screening on the aqueous and hydro-ethanolic extracts of Hibiscus Sabdariffa Linn

<table>
<thead>
<tr>
<th>Tests</th>
<th>Aqueous</th>
<th>Hydro-ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins and Polyphenols</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Anthraquinone Glycosides</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Saponins</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>General Glycosides</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Steroids and Triterpenoids</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>

Table 2: Total Antioxidant Capacity (TAC) of the Hibiscus Sabdariffa Linn extracts expressed as ascorbic acid equivalent (AAE)

<table>
<thead>
<tr>
<th>Conc (mg/mL)</th>
<th>AAE of hydro-ethanol extract</th>
<th>AAE of aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375</td>
<td>0.189±0.002</td>
<td>0.122±0.003</td>
</tr>
<tr>
<td>0.75</td>
<td>0.488±0.003</td>
<td>0.425±0.005</td>
</tr>
<tr>
<td>1.5</td>
<td>1.311±0.005</td>
<td>1.243±0.003</td>
</tr>
<tr>
<td>3</td>
<td>2.758±0.002</td>
<td>2.595±0.004</td>
</tr>
</tbody>
</table>

Table 3: Estimated EC50 values for the DPPH and the Hydroxyl radical assays

<table>
<thead>
<tr>
<th>Extract</th>
<th>DPPH Assay</th>
<th>Hydroxyl radical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>0.2099±0.0011</td>
<td>0.6888±0.0009</td>
</tr>
<tr>
<td>Hydro-ethanol</td>
<td>0.2003±0.010</td>
<td>0.6879±0.0012</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.0361±0.0008</td>
<td>0.2094±0.0007</td>
</tr>
</tbody>
</table>

Phosphate and 4 mM ammonium molybdate) was added and the resulting mixture incubated at 95°C for 90 min. The mixture was allowed to cool to room temperature, and the absorbance of each solution was measured in triplicates on the UV-visible spectrophotometer (LKB Biochrom, Cambridge, Cambridge, England, Model 4050) at 695 nm. The blank contained all the reagents with no extract. Ascorbic acid was used as the standard antioxidant drug. The total antioxidant capacity was expressed as Ascorbic Acid Equivalents (AAE). All experiments were replicated three times and the data was expressed as Mean ± SD.

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging assay: The DPPH free radical scavenging activity was determined as previously described (Govindarajan et al., 2003; Govindarajan et al., 2005). Briefly, 1 mL solutions of different concentrations (0.375, 0.75, 1.5 and 3 mg/mL in methanol) of aqueous and hydro-ethanol extracts were placed in different test tubes. A 3 mL 0.1 mM methanolic solution of 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) (20 mg/L) was added (5). The tubes, shaken vigorously and kept on a waterbath at 25°C for 30 min. The control was prepared as above without any extract, and methanol was used for the baseline correction. Ascorbic acid (0.094-3.0 mg/mL) was used as a standard free radical scavenger. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: % radical scavenging activity (control OD-sample OD/control OD) 100. Dose response curve was plotted between % inhibition and concentrations. Linear regression analysis was carried out calculating the effective concentration of the sample required to scavenge DPPH radical by 50% (EC50). All tests were carried out in triplicate and the data was expressed as Mean ± SD.

Hydroxyl radical scavenging activity: A previously described method by Rajeshwar et al. (2005) was used with slight modification. Briefly, a 2.0 mL ethanolic solution of the sample extracts at various concentrations (0.375, 0.75, 1.5, 3.0 mg/mL) were placed in different test tubes. A 2.0 mL solution of a 6 mM FeSO4 solution was added followed by the addition of 2.0 mL solution of 6 mM of H2O2. The solution was shaken vigorously and allowed to incubate at room temperature for 10 min. A 2.0 mL solution of 6 mM Salicylic acid was added to all the tubes and left at room temperature for 30 min for color development solution. The intensity of the color formed was measured spectrophotometrically at 510 nm against reagent blank. The percentage hydroxyl radical scavenging is calculated by the following formula: % hydroxyl radical scavenging activity = 1 - (difference in absorbance of sample/difference in absorbance of blank) 100. All experiments were done in triplicates and the data was expressed as Mean ± SD. Dose response curve was plotted between % inhibition and concentrations. Linear regression analysis was used to calculate the effective concentration of the sample required to scavenge DPPH radical by 50% (EC50).

Antimicrobial assay

Agar Diffusion: The agar diffusion assay described by Smânia et al. (1995) was adopted with slight modifications. Briefly, Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Staphylococcus aureus (ATCC 25923), Bacillus subtilis (ATCC 6633) and Candida albicans (strain NCPF 113; National Collection of Type Cultures, London, England) were grown in Mueller-Hinton agar and broth (Difco Laboratories). The strains were incubated at 37°C for 18 h, and were diluted to a final concentration of approximately 0.1-4 CFU/mL. Each bacterial suspension (1 mL) was spread over the surface of Mueller-Hinton agar in a petri-dish containing five wells that were bored at 7 mm diameter. The wells were filled with extracts of different concentrations (Tables 4 and 5) dissolved in methanol (100 microliter). The negative control was methanol (100 microliter). Ciprofloxacin and fluconazole (at different concentration in Table 5) were used as positive controls. The petri-dish was incubated at 37°C for 24 h. Each test was carried out in triplicate and the data was expressed as Mean ± SD. The results were expressed in terms of the diameter of the inhibition zone.
Inhibition zone diameters were measured to the nearest whole millimeter from the center to the point where there was no visible growth (clear zones) after 24 h for all microorganisms.

**Broth dilution assay:** Broth dilution assay was performed using a modification of the protocol described by Murray et al. (1999). The microbial culture was inoculated in the nutrient broth (MH broth) and incubated at 37°C for 24 h. Then a 20 mL fresh broth was seeded with 0.25 mL of the 24-hour broth cultures and a two-fold serial dilution method was performed as described below. The dried *H. sabdariffa* extract was dissolved in 85% methanol to obtain a 20 mg/mL solution that was sterilized by filtration through a 0.45 μm membrane filter. A 0.2 mL solution of the material was added to 1.8 mL of the seeded broth to obtain the first dilution. A 1 mL of this dilution was further diluted with 1 mL of the seeded broth to produce the second dilution, and the

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**Table 4:** Zones of inhibitions for extracts (mm) in Agar Diffusion Assay

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Hydro-ethanol</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (mg/mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>26.33±0.35</td>
<td>21.00±0.24</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>25.00±0.23</td>
<td>19.00±0.53</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>23.00±0.54</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.87±0.15</td>
<td>-</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>37.01±0.65</td>
<td>29.25±0.75</td>
</tr>
</tbody>
</table>

**Table 5:** Zones of inhibitions (mm) for standard drugs (Ciprofloxacin and Fluconazole) in Agar Diffusion Assay

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Organisms</th>
<th><em>E. Coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. aureus</em></th>
<th><em>B. subtilis</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zone of inhibition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>43.46±0.54</td>
<td>57.58±0.24</td>
<td>45.29±0.93</td>
<td>51.87±0.33</td>
<td>40.35±0.56</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>41.75±0.55</td>
<td>53.45±0.54</td>
<td>39.18±0.82</td>
<td>43.37±0.65</td>
<td>31.57±0.15</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>39.85±0.25</td>
<td>47.35±0.73</td>
<td>33.73±0.29</td>
<td>39.45±0.44</td>
<td>28.81±0.37</td>
<td></td>
</tr>
<tr>
<td>0.125</td>
<td>25.65±0.53</td>
<td>43.76±0.89</td>
<td>31.64±0.48</td>
<td>33.94±0.15</td>
<td>22.27±0.84</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6:** Broth dilution for the Hydro-ethanol extract

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>-</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>-</td>
</tr>
</tbody>
</table>

+ indicates microbial growth; - indicates no microbial growth

**Table 7:** Broth dilution for the aqueous extract

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>-</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>-</td>
</tr>
</tbody>
</table>

+ indicates microbial growth; - indicates no microbial growth
process was repeated until seven dilutions were obtained (10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0.3125 mg/mL and 0.15625 mg/mL). Tubes containing only seeded broth and 85% methanol were kept as controls. All tubes were incubated for 24 h at 37°C. All determinations were made in triplicate and the data was expressed as Mean ± SD. MICs (expressed either in µg/mL or mg/mL) were taken as the lowest extract concentrations that showed complete growth inhibition and were represented by the last tube with no visible violet color from the addition of 0.1 mL of MTT. Ciprofloxacin and fluconazole (at different concentration in Table 8) were used as positive controls.

Statistical Analysis

All data were reported as the mean ± SD of the set of triplicates determinations as estimated using Microsoft Excel software. Student’s t test was used to compare the data, and all tests were considered statistically significant at p < 0.05.

RESULTS AND DISCUSSIONS

Phytochemical Screening

The phytochemical richness of the calyx of H. Sabdariffa is demonstrated by the phytochemical screen that revealed the presence of eight different phytochemicals in both the aqueous and the hydro-ethanolic extracts. Both extracts displayed the presence of the same type of phytochemical with the conspicuous absence of alkaloids (Table 1). The confirmed presence of flavonoids, carotenoids and other phytochemicals including anthraquinones that are broadly exhibit health beneficial bioactivities undergirds the use of H. Sabdariffa as a nutritional supplement and as an ethnomedicine in diverse herbal medicinal practices throughout Africa (Qi et al., 200). The results of the phytochemical screen also confirmed prior literature reports of the presence of glycosides, flavonoids, saponins and tannins in the calyx of H. Sabdariffa Linn (Ali et al., 2005).

Total antioxidant capacity

The presence of phytochemicals traditionally associated with antioxidant activities in the extracts of the calyx of H. Sabdariffa provided the foundational support for the observed substantial total antioxidant capacity (TAC) defined as the cumulative ability of the chemical constituents of the extracts to scavenge free radicals and expressed in ascorbic acid equivalents (AAE).

The hydro-ethanol extracts exhibited higher relative AAE values than the water extracts (Table 2). Since ethanol has a relative higher solvent strength than water, it could potentially extract a higher diverse pool of phytochemicals with antioxidant capacities than water could. TAC levels displayed a dose-dependent relationship with both extracts.

DPPH Radical Scavenging Activity: The display of total antioxidant capacity led to the examination of the DPPH radical scavenging activities of the extracts. The DPPH Radical Scavenging Activity of the hydro-ethanolic extract was higher than that of the aqueous extract at all examined concentrations (Fig. 1). Differences in the values of DPPH Radical Scavenging Activity were marginally lower at the lowest examined concentration (0.375 mg/mL) but became pronounced at higher concentrations (0.75, 1.5 and 3.0 mg/mL). All examined concentrations of both hydro-ethanolic and aqueous extracts had DPPH Radical Scavenging Activities that were substantially lower than that of the Ascorbic acid control. Scavenging of DPPH radical was found to rise with increasing concentration of the extracts. Antioxidant activities generally correlated with the
observed trend in the total antioxidant capacity with the hydro-
ethanol extract exhibiting higher antioxidant activities than the
aqueous extract. Ethanol displays good extraction efficiency than
water and could potentially extract a larger pool of anti-oxidant
compounds than water could.

**Hydroxyl Radical Scavenging Activity:** The pattern of hydroxyl
radical scavenging displayed by both extracts were similar to that
of the DPPH Radical Scavenging Assay. Just like the DPPH
assay, the hydro-ethanolic extracts showed a relatively higher
hydroxyl radical scavenging activity than the aqueous extract
(Table 5). Differences in hydroxyl radical scavenging activities
between the two extracts were more pronounced at higher
examined concentrations (1.5 and 3.0 mg/mL). A dose-dependent
scavenging of hydroxyl radicals was also observed for each
extract. Ascorbic acid displayed the highest hydroxyl radical
scavenging activity at all assayed concentrations.

**EC50 values for the DPPH and Hydroxyl radical scavenging:**
EC50 values correlated with radical scavenging activities of both
extracts in both the DPPH and the Hydroxyl radical scavenging.
Consequently, the EC50 values for the DPPH Radical and the
Hydroxyl radical scavenging activities of the hydro-ethanol
extracts were marginally lower than that of the water extract.
And in both DPPH Radical and the Hydroxyl radical scavenging
assays, Ascorbic acid displayed the lowest EC50 value (Table 3).
The decreasing order of potency of radical scavenging for the
DPPH and Hydroxyl radical, as provided by EC50 values, is
Ascorbic acid < hydro-ethanol extract < aqueous extract.

**Antimicrobial activities**

**Agar Diffusion Assays:** A pronounced dose-dependent zones of
inhibition was achieved with the use of the hydro-ethanolic
extract on *B. subtilis, E. coli* and *C. albicans* (Table 4). Inhibition
of growth of *S. aureus* and *P. aeruginosa* by the hydro-ethanolic
extract were minimal and occurred only at the highest tested
extract concentration (10 mg/mL): *S. aureus* (23 mm) and *P.
aeruginosa* (0.87 mm). *C. albicans* displayed the highest zones
of inhibition for each tested hydro-ethanolic extract concentration.

Compared to the hydro-ethanolic extract, the aqueous extract
did not display as effective anti-microbial effect on the test
organisms. Much higher concentrations of aqueous extracts were
required to inhibit the growth of *Bacillus subtilis, Escherichia
coli, Staphylococcus aureus* and *Candida albicans* than was
required for the hydro-ethanol extract (Table 4). Both hydro-
ethanolic and aqueous extracts displayed the least anti-microbial
effect on *Pseudomonas aeruginosa*. In fact no zone of inhibition
was observed for the aqueous extract on *Pseudomonas
eaeruginosa* at all tested concentrations. Clearly, *Pseudomonas
eaeruginosa* displayed the minimum anti-microbial effect of the
hydro-ethanolic extract.

Examined concentrations for the standard drugs (the anti-
bacteria Ciprofloxacin and the anti-fungi Fluconazole) were ten-
fold difference lower than the concentrations of tested extract
(Table 5). Even at a tenth of the concentration of the extracts, the
standard Ciprofloxacin displayed zones of inhibition that were at
least twice as high as that of the extracts for all examined
bacteria species. Fluconazole, at 10-fold lower concentrations,
also showed relatively higher zones of inhibition for *C. albicans*.

**Broth Dilution Assays:** Both extracts displayed anti-microbial
activity in the broth dilution assay at concentrations that are
indicative of potent bioactivity. Little variability in inhibition
potency on the panel of microorganisms was seen with both
extracts in the broth dilution assay. For all microbial species, the
hydro-ethanolic extract displayed a more potent anti-microbial
activities as demonstrated in the lower MICs (625 µg/mL) (Table
6) that were a 2-fold concentration factor lower than that of the
aqueous extract (1.25 mg/mL) (Table 7).

Present study showed that the qualitative patterns of the
MICs of the broth dilution method correlated with the patterns of
the zones of inhibition in the agar diffusion assay. Concordance
of results between agar diffusion and broth dilution assays were
much more reasonable for the hydro-ethanolic extracts. Both
agar diffusion and broth dilution assays are in agreement that the
hydro-ethanol extract is a more potent anti-microbial agent than
the aqueous extract. In both the agar diffusion and the broth
dilution assays, the hydro-ethanol extracts inhibited microbial
growth better than the aqueous extract as demonstrated larger
zones of inhibition and in the MICs for the hydro-ethanolic
extract that were lower by one tube dilution in all cases
compared to the aqueous extracts.

While the qualitative pattern of inhibition of microbial
species in the agar diffusion assay agrees with that of the broth
dilution assay, the extract concentration required for the
inhibition in the agar diffusion for both extracts were higher (by
at least 2-fold for the Hydro-ethanol extract and 5-fold for the
aqueous extract) than the broth dilution assay. The higher
concentrations needed for microbial growth inhibition in the agar
diffusion can be attributed to the low rate of diffusion of extract
form site of placement through the agar plate to the microbes and
at a tenth of the concentration of the extract, Ciprofloxacin
displayed broth dilution MICs against the four bacterial
specimens that were at least 10³ times higher than that of both
extracts (values range from 0.625 to 1.25 µg/mL): *E. coli* (0.625
µg/mL), *P. aeruginosa* (1.25 µg/mL) *B. subtilis* (0.625 µg/mL), *S.
aureus* (0.625 µg/mL) (Table 8). Similarly, fluconazole showed
an MIC against *C. albicans* (0.625 µg/mL) that was also at least
10³ times higher than that of the extracts.

Earlser study has concluded that the ethanol extracts of HS
inhibits the growth of food spoilage bacteria in beef and apple
juice with MICs that are lower by at least a10-fold when
compared with that of this study (Chao and Yin, 2008). Anti-
microbial activities was attributed primarily to the presence of
Protocatechuic acid, a known microbial growth inhibitor, in the
calyx extract and consequently, the ethanolic extract of HS calyx
was deduced to be a safe additive that can prevent bacterial contamination in food (Chao and Yin, 2008).
Anti-oxidant EC50 values differed and were at least 10-fold difference lower in previously published work that used ABTS anti-oxidant assay method (Yang et al., 2012). Nevertheless, the conclusions deduced from this study agree with that of earlier studies: that the hydro-ethanolic extract represents a safe and a better source of anti-oxidant due primarily to its higher radical scavenging activities.

CONCLUSION

_Hibiscus sabdariffa _calyx is a rich source of polyphenolic antioxidants and potential anti-microbial compounds. Anecdotal evidence from nutritional supplementation and ethnomedicinal practices suggested the possibility that the biologically active molecules in the aqueous extract of the calyces of _H. sabdariffa _have both anti-oxidant and anti-microbial activities. Because of the possibility that the pharmacological activities of the aqueous extracts of the calyces could be attributed to its anti-oxidative and anti-microbial activities, this study investigated the anti-oxidant and anti-microbial activities of the hydro-ethanol and aqueous extracts of calyx of _H. sabdariffa. _

Both extracts displayed remarkable phytochemical diversity with the notable presence of the same types of phytochemicals that includes tannins and polyphenols that accounts largely for their observed antioxidant activities. The higher TAC of the hydro-ethanolic extract suggested that the hydro-ethanolic extract of _H. sabdariffa _calyx contain either higher concentrations or different structural polyphenol phytochemicals.

Anti-oxidant assays using DPPH and hydroxyl radical scavenging methods displayed a pattern of results for both extracts that were: lower in potency compared to that of Ascorbic acid; concentration-dependent; and correlated directly with the TAC of the extracts. The hydro-ethanol extract, however, exhibited relatively lower EC50 for the DPPH and the Hydroxyl radical assays and suggested a more potent anti-oxidant activity than the aqueous extracts of _H. sabdariffa_. The EC50 values obtained with the reference standard Ascorbic acid tended to be lower than that of the hydro-ethanol and aqueous extracts. Consequently, tannin and related polyphenol constituents that contributed to the anti-oxidant activities might differ in concentration or in structural diversity between the hydro-ethanolic and aqueous extracts.

In addition to containing antioxidants that can directly inactivate free radicals, extracts of _H. sabdariffa _exhibited the ability, in vitro, to inhibit microbial growth when introduced to a panel of microbial specimens. Total inhibition of microbial growth in the agar diffusion assay and in the broth dilution assay occurred at a lower _H. sabdariffa _concentration when extracted with hydro-ethanol than when extracted with water. The extract constituents that contribute to the antimicrobial activities are unknown and establishing the mechanistic contribution of all the phytoconstituents to the overall antioxidant and anti-microbial activities of the extracts requires further investigation.

The therapeutic and prophylactic benefits of _H. sabdariffa _may be attributable to its substantial attenuation of free radical damage to cells and to its appreciable inhibition of microbial infections. Regular intake of _H. sabdariffa_, as happens in ethnomedicinal use, may have beneficial role in diseases prevention. This study provides valuable scientific information that supports the ethnomedicinal use and dietary intake of the aqueous extracts of the calyces of _H. sabdariffa _throughout West Africa.

REFERENCES


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