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Chemical Analysis, Antibacterial Activities and Uses of Leaves and Calyces of *Hibiscus sabdariffa* Grown in Dodoma, Tanzania

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Abstract

Preliminary phytochemical screening of aqueous and ethanolic extracts of *Hibiscus sabdariffa* grown in Tanzania revealed the presence of secondary metabolites like steroids, tannins, saponins, glycosides, terpenoids, flavonoids along with L-ascorbic acid (vitamin C) and iron(II). Furthermore, both leaves and calyces showed antibacterial activities (agar well diffusion method) against selected bacterial species (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Shigella sonnei*), but calyces possessed potent antibacterial activities compared to leaves. The results also supported the claimed traditional uses of this plant. When interrogated during the cross-sectional study in Dodoma region, 54% of the respondents claimed the plant is used to treat anaemia (supposedly as it increases haemoglobin levels), 23% claimed it is used in the preparation of local wine and the remaining respondents stated use in both areas. Furthermore, the intake of *H. sabdariffa* leaves and calyces on regular basis can boost the immunity system and helps in preventing bacterial and viral infections as the plant is loaded with flavonoids and vitamin C. Thus, the results observed for the plant *H. sabdariffa* that is grown in Dodoma in small scale for traditional uses, paves a way for consideration of future large scale production of pharmaceutical and neutraceutical products in Tanzania.

Keywords: Phytochemical screening, *Hibiscus sabdariffa*, antibacterial activity, L-ascorbic acid and iron(II).

Introduction

Hibiscus sabdariffa L. belonging to family Malvaceae is commonly known as "roselle" and locally called as "choya" in Tanzania. *H. sabdariffa* is an important ethnomedicinal plant where all the parts of the plant are used differently worldwide in food and medicine (Abu-Tarboush et al. 1997, Qi et al. 2005). This species is widely grown in Central and West Africa and South East Asia with different views on its origin (Crane 1949, Cobley 1976, Qi et al. 2005, Obouayeba et al. 2014). Many researchers worldwide have worked on nutritional and medicinal properties of the plant focusing mainly calyces (Mohagheghi et al. 2011, Mungole and Chaturvedi 2011, Okereke et al. 2015). Al Snafi (2018) reviewed the presence of alkaloids, anthocyanins, flavonoids, phenols, saponins, tannins, polyuronides,

cardiac glycosides, reducing sugars, carbohydrates, proteins, gums, essential minerals and volatile oils and also pharmacological activities like antibacterial, antifungal, antiviral, anticancer, apoptotic, immunological, antioxidant, hypolipidemic, antidiabetic, smooth muscle relaxant, gastrointestinal, anti-inflammatory, analgesic, antipyretic, protective effects, wound healing and wide range of cardiovascular and CNS effects. Additionally, Owoade et al. (2019) also reviewed the presence of chemical constituents and pharmacological properties of this plant.

H. sabdariffa is cultivated mainly in the central and coastal regions of Tanzania. It is locally cultivated for the production of calyces on small scale and sold in local markets in Dodoma region (Peter et al. 2014); whereby is commercially cultivated to produce hibiscus tea infusions by Kimango Farm Enterprises Ltd on the Mkata plains in Morogoro region, Tanzania. Few studies on dried calyces of this plant have been done in Tanzania regarding iron content (Maregesi et al. 2013), optimization of extraction conditions for iron and ascorbic acid (Peter et al. 2014) and levels of selected elements mainly iron and its possible effect on human blood pressure in Zanzibar (Mohamed et al. 2019). However, no research has been done concerning chemical constituents and *in vitro* anti-bacterial activity of *H. sabdariffa* (roselle) leaves grown in Dodoma, Tanzania though roselle leaves in African folk medicine are used as antimicrobials, emollients, antipyretics, diuretics, antihelmenthic, sedatives and also as a soothing cough remedy, whereas in India, leaves are used as vegetable and poultice on abscesses (Singh et al. 2017). Therefore, the aim of the study was to find out the traditional uses of the plant in Dodoma and screen aqueous and ethanolic extracts of *H. sabdariffa* leaves for the presence of chemical constituents like secondary metabolites along with iron II and ascorbic acid (vitamin C). Another objective was to assess whether the aqueous and ethanolic leaves and calyces extracts possess antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Shigella sonnei* and then to compare the results of antibacterial activities between leaves and calyces. Thus, the observed results may fill the information gap that existed on chemical constituents especially vitamin C which acts as an immune booster and *in vitro* anti-bacterial activity of *H. sabdariffa* grown in Dodoma and pave a way for future large scale production of pharmaceutical and neutraceutical products in Tanzania.

Materials and Methods

Plant collection

Fresh leaves and calyces of uninfected and healthy *H. sabdariffa* plant were collected in May 2019 from the Kikuyu area located at latitude 6°7'16" S and longitude 35°46'29" E, Dodoma, Tanzania. Carefully hand plucked samples were placed in ziploc bags and transported safely to the Pharmaceutical Laboratory at St John's University of Tanzania, Dodoma, Tanzania and then air dried for two weeks at room temperature (25–27 °C). The plant was identified by a botanist and authenticated and preserved at the University's herbarium.

Plant extraction

The under-shade air-dried, pulverized samples were soaked for 24 hours in water and ethanol. The filtered crude extracts were concentrated *in vacuo* using a rotary evaporator while maintaining water bath temperature below 40 °C to avoid thermal decomposition of labile compounds. The weight of the crude extracts was determined and about 20 mg each of the extracts was taken to carry out the antimicrobial activities.

Phytochemical screening

Phytochemical screenings of aqueous and ethanolic leaf and calyces extracts of *H. sabdariffa* were carried out using standard procedures (Begum et al. 2021) and were conducted at the Pharmaceutical Laboratory of St John's University of Tanzania.

***Test for alkaloids (Wagner's test)*:** Both the extracts were treated with few drops of Wagner's reagent (0.5 g of iodine and 1.5 g

of potassium iodide were dissolved in 5 mL of distilled water and the solution was diluted to 20 mL using water). The formation of reddish brown precipitate indicates the presence of alkaloids.

***Test for terpenoids (Salkowki's test)*:** Extracts were dissolved in 2 mL of chloroform and treated with 2 mL of concentrated sulphuric acid to form a layer. Reddish brown colouration at the interface indicates presence of terpenoids.

***Test for flavonoids (Alkaline reagent test)*:** Extracts were treated with few drops of 20% sodium hydroxide solution. Formation of an intense yellow colour, which turns colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

***Test for sterols (Liebermann-Burchard's test)*:** Extracts were diluted with chloroform and filtered. Few drops of acetic anhydride were added to the filtrates and boiled. The cooled filtrates were treated with few drops of concentrated sulphuric acid. Formation of a brown ring at the junction indicates the presence of phytosterols.

***Test forphenols (Ferric chloride test)*:** Extracts were treated with 3–4 drops of 5% ferric chloride solution. Formation of bluish-black colour indicates the presence of phenols.

***Test for saponins (Foam test)*:** The extracts were diluted with distilled water and warmed on water bath. Development of persevering forth affirms the vicinity of saponins. Furthermore, the frothing was mixed with three drops of olive oil and then shaken vigorously. Formation of emulsion indicates the presence of saponins.

***Test for tannins (Braymer's test)*:** Extracts were boiled with 20 mL water and then filtered. The filtrates were treated with 10% alcoholic ferric chloride solution and formation of brownish green to blue-black colour indicates presence of tannins.

***Test for cardiac glycosides (Keller Kelliani's test*):** The extracts were dissolved with 4 mL of distilled water and then treated with 2 mL of glacial acetic acid containing few drops of ferric chloride solution. Further, 2 mL of concentrated sulphuric acid was added carefully without mixing the solution. Formation of a brown ring at the interface indicates the presence of deoxysugars (characteristics of cardenolides).

***Test for carbohydrates (Benedict's test)*:** Extracts were treated with Benedict's reagent (cupric citrate complex) and heated gently. Orange-red/brick red/rusty brown precipitate indicates presence of reducing sugars.

***Test for proteins (Biuret test)*:** Extracts were dissolved in 4 mL of distilled water and then treated with an equal volume of 1% sodium hydroxide solution followed by 3 drops of aqueous copper (II) sulphate solution. A colour change from blue to purple/violet indicates presence of proteins.

***Test for quinones*:** Extracts were treated with concentrated HCl. Formation of yellow colouration or precipitate indicates the presence of quinones.

Qualitative and quantitative analysis of ascorbic acid

Qualitative analysis (Riddhu and Payal 2014) and quantitative analysis (Ciancaglini et al. 2001) of ascorbic acid were carried out by using standard procedures at Pharmaceutical Laboratory of St John's University of Tanzania.

***Qualitative analysis of ascorbic acid*:** Extracts were dissolved in 2 mL of distilled water. Added 0.1 g of sodium bicarbonate and 20 mg of ferrous sulphate and were shaken vigorously and allowed to stand. A deep violet colour was produced which disappeared by addition of 5 mL of 1 M sulphuric acid indicating presence of ascorbic acid (Riddhu and Payal 2014).

Quantitative analysis of L-ascorbic acid: L-ascorbic acid was estimated using direct iodometric titration reported by Ciancaglini et al. (2001) with slight modification. Twenty (20) fresh leaves and calyces were crushed and dissolved in 100 mL distilled water and then filtered. 20 mL of the filtrate was dissolved in 80 mL distilled water followed by 1 mL of HCl (15% v/v) and 1 mL starch indicator solution (1% w/v) and then titrated with 0.01 M iodine solution. The end-point was identified by appearance of dark blue-black colour which persisted for 60 seconds. Titrations were repeated thrice where the

average gave the experimental titre value. Then, the number of moles of iodine required on average were calculated as follows:

Number of moles = Molarity x Average titre volume in L

From the balanced chemical equation

$C_6H_8O_6 + I_2 \rightarrow C_6H_6O_6 + 2H^+ + 2I^-$

(Ascorbic acid) (Dehydroascorbic acid)

1 mole of iodine reacts with 1 mole of ascorbic acid. Therefore, number of moles of ascorbic acid present will be equal to the number of moles of iodine and later mass of ascorbic acid can be calculated as follows:

Mass of ascorbic acid = No. of moles of ascorbic acid (equivalent to moles of Iodine obtained above) x Molar mass of ascorbic acid.

Quantitative analysis of iron II

Quantification of iron was carried out in the College of Natural and Mathematical Sciences research laboratory at The University of Dodoma, Dodoma, Tanzania using spectrophotometric analysis (Sandell 1959).

***Sample preparation*:** 10 g of air-dried leaves and 5 g of air-dried calyces were weighed separately and ground into coarse powder and later soaked in 100 mL of distilled water and ethanol separately in four conical flasks and covered with aluminium foil and left for 12 hours. Then they were filtered and labelled 1, 2, 3 and 4, respectively. Furthermore, fresh leaves and fresh calyces weighing approximately 40 g each were also plucked from the plant and crushed in mortar and pestle and then divided into two equal portions. One portion was mixed with 50 mL of distilled water and to the other portion 50 ml of ethanol was added. Then they were filtered to make samples 5, 6, 7 and 8, respectively. All eight prepared samples were taken to the College of Natural and Mathematical Sciences research laboratory at the University of Dodoma to quantify iron(II).

***Preparation of stock solution for spectrophotometric analysis*:** The stock solution was prepared by dilution of 0.1 g of ammonium iron(II) sulphate ($Fe(NH_4)_2(SO_4)2.6H_2O$) in 1 litre of distilled water followed by addition of 1.6 mL of 0.3% o-phenanthroline and then 1.6 mL of 10% hydroxylamine hydrochloride making 100 ppm stock solution. Iron(II) reacts with o-phenanthroline to form a coloured complex ion. The intensity of the coloured species was measured using a UV-Vis spectrophotometer of Agilent technologies Mode (Cary 60.UV-Vis 5.0.0.999). A calibration curve (absorbance versus concentrations) was constructed for iron(II) and the concentration of the unknown iron sample was determined from the equation y = mx + c (deduced from the graph).

Antibacterial assay: Agar well diffusion method

Eight crude extracts obtained from fresh and dried *H. sabdariffa* leaves and calyces of aqueous and ethanolic extracts were tested by using agar well diffusion method (Begum et al. 2020) at Microbiology Laboratory, Dodoma Regional Referral Hospital, Dodoma, Tanzania. The gram-positive bacteria, namely *Staphylococcus aureus* and gram-negative bacteria, *Salmonella typhi*, *Escherichia coli* and *Shigella sonnei* clinical isolates were used for this study. Bacterial strains were isolated, cultured and inoculated into Mueller Hinton Agar (MHA) plates and incubated at 37 °C for 24 hrs for sensitivity test. MHA was used for clear and easy observation of the zones of inhibition. The dried plates containing MHA were then punched with sterile cork borer of 6 mm diameter to make open wells. Then, 0.1 mL of 24 hrs broth culture of each of the test microorganisms containing ca.log 7.0 CFU mL^{-1} was aseptically and evenly spread on the punched MHA plate.

One hundred microlitres of the test extracts were introduced into the wells that were effectively labelled. Standard antibiotic, ceftriaxone at a concentration of 30 µg served as positive antibacterial control before the inoculated plates were incubated at 37 °C for 24 hrs. After incubation time, the plates were taken out of the incubator, the results observed and the zones of inhibition measured in millimetres using a transparent ruler. The diameter of the zone of inhibition

was related to the susceptibility of the isolate. The antibacterial potentials of the experimental extracts were evaluated according to their zones of inhibition against various pathogens tested and the results (zones of inhibition) were compared with the activity of the standard drug, ceftriaxone.

Traditional uses of *Hibiscus sabdariffa* grown in Dodoma

A descriptive cross-sectional study design was applied to find out the traditional uses of the plant *Hibiscus sabdariffa* L. among the local people of Dodoma. As the number of people involved in cultivating *H. sabdariffa* in Dodoma urban by May 2019 was only 137 farmers, a sample size of 56 farmers were interviewed on the conventional uses.

Results

Phytochemical screening

The results of phytochemical screening of the aqueous and ethanolic extracts of *H. sabdariffa* leaves are tabulated in Table 1 for the presence of alkaloids, flavonoids, steroids, phenols, saponins, tannins, cardiac glycosides and ascorbic acid and absence of terpenoids, carbohydrates, proteins and quinones is shown.

Estimation of L-ascorbic acid

The concentration of L-ascorbic acid in water extract of *H. sabdariffa* fresh leaves and calyces was found to be 57.44 mg/100 g and 37.93 mg/100 g, respectively, when estimated by direct iodometric titration using 0.01 M iodine solution.

Spectrophotometric determination of iron(II) in *H. sabdariffa*

The absorbance of prepared samples were read at 405 nm, compared at 0.05 g/mL and converted to mg/100 g, and the results are tabulated in Table 2.

Table 1: Qualitative analysis of phytochemicals found in aqueous and ethanolic extracts of *Hibiscus sabdariffa* leaves grown in Dodoma, Tanzania

Chemical constituents	Aqueous extract	Ethanol extract
Alkaloids	+	+
Terpenoids	-	-
Flavonoids	+	+
Sterols	+	+
Phenols	+	+
Saponins	+	+
Tannins	+	+
Cardiac glycosides	+	+
Carbohydrates	-	-
Proteins	-	-
Quinones	-	-
Ascorbic acid (Vitamin C)	+	+

Key: + present, - absent

Table 2: Iron(II) readings for the samples of *H. sabdariffa*

S.N.	Sample conc. (g/mL)	Absorbance	Concentration of iron(II) (mg/100 g)
1	0.083	0.2556	1.332
2	0.083	0.3027	1.572
3	0.050	1.2396	17.84
4	0.050	0.4359	6.28
5	0.379	0.5812	1.103
6	0.379	0.3439	0.652
7	0.500	0.4523	0.65
8	0.500	1.0763	1.548

Key: 1: Dry leaves water extract, 2: Dry leaves ethanol extract, 3: Dry calyces water extract, 4: Dry calyces ethanol extract, 5: Fresh leaves water extract, 6: Fresh leaves ethanol extract, 7: Fresh calyces water extract and 8: Fresh calyces ethanol extract.

***In vitro* antibacterial activity**

The zones of inhibition for *in vitro* antibacterial activity are recorded in millimetres as shown in Table 3.

Table 3: Diameters of the zones of inhibition in millimetres for the samples of *H. sabdariffa*

Sample number	1	2	3	4	5	6	7	8	C
S. aureus	12	8	16	17	10	7	8	9	23
S. typhi	7	8	11	12	8	9	7	7	7
E. coli	7	8	10	9	7	8	9	7	32
S. sonnei	7	11	17	14	8	12	11	12	31

Key: 1 represents dry leaves water extract, 2 represents dry leaves ethanol extract, 3 represents dry calyces water extract, 4 represents dry calyces ethanol extract, 5 represents fresh leaves water extract, 6 represents fresh leaves ethanol extract, 7 represents fresh calyces water extract and 8 represents fresh calyces ethanol extract. The letter C represents ceftriaxone, the control used.

Traditional uses of *Hibiscus sabdariffa* in Dodoma

Among the respondents interrogated in Dodoma urban region for the uses of this plant, 54% claimed to use this plant to treat anaemia as it supposedly increases haemoglobin levels, 23% used it for leisure in preparation of local wine and the remaining respondents stated both the reasons. Additionally, 30% of respondents asserted to grow roselle for domestic uses, 20% for commercial purposes and 50% for both commercial and domestic uses. Moreover, 94% of respondents claimed calyces to be the most used part of the plant. Furthermore, 60% of respondents stated that the parts other than calyces are thrown away, 20% used as animal feed, 13% as manure and 7% burn the remaining parts. In order to ensure availability of the plants for the next growing season, 67% preserved the seeds appropriately from the previous season, 17% bought the seeds from the local market and for 16% seedlings were grown around their houses.

Discussion

The present study investigated the traditional uses, chemical analysis and antibacterial activities of leaves and calyces of *Hibiscus sabdariffa* growing in Dodoma. This study is in agreement with reports of Peter et al. (2014) for cultivating *H. sabdariffa* for its calyces in small scale basis in central and coastal regions of Tanzania for domestic and commercial use in treating anaemia and in wine production. The widespread uses of calyces in Dodoma may be due to lack of knowledge on nutritional and economic values of other parts of the plant. To fill the information gap, water and alcoholic extracts of *H. sabdariffa* leaves were screened for phytochemicals and the results revealed presence of phenolic compounds which are in agreement with Obouayeba et al. (2014). Also, presence of flavonoids and glycosides are in agreement with Alaga et al. (2014). Furthermore, presence of steroids, saponins and tannins and absence of quinones are in agreement with Adamu and Ngwu (2015), but the present study revealed presence of alkaloids though Adamu and Ngwu (2015) reported absence of alkaloids. Ismail et al. (2008) reported presence of proteins and carbohydrates in plant seeds, while the present study revealed absence of proteins and carbohydrates in leaves. Results also showed the presence of iron(II) in both water and ethanolic extracts of *H. sabdariffa* leaves and calyces where the iron concentration was highest in dry calyces water extract (17.84 mg/100 g) followed by dry calyces ethanolic extract (6.28 mg/100 g). Dried calyces showed better concentrations of iron compared to fresh calyces and dry and fresh leaves. Thus, presence of iron(II) ion in the dry calyces of the plant justifies its use for treatment of anaemia among the local community. However, the results of ascorbic acid and iron(II) varied slightly from that of Peter et al. (2014) and Maregesi et al. (2013) due to different extraction methods used and soil composition, time and season when the plant was collected for the study. The concentrations of L-ascorbic acid (vitamin C) in water extract of *H. sabdariffa* fresh leaves and calyces were found to be 57.44 mg/100 g and 37.93 mg/100 g, respectively.

Furthermore, results for the *in vitro* antibacterial activities of all the extracts of *H. sabdariffa* have shown activities against the selected bacterial species. The agar well diffusion method was used as it is known to allow better diffusion of the extracts into the medium, thus enhancing contact with the test organisms and ensuring more accurate results, and this observation agrees with the work of Omenka and Osuoha (2000). Though all the extracts of *H. sabdariffa* showed antibacterial activities to different extends ranging zones of inhibition (ZOI) of 7–17 mm, still potent activity was observed by ethanolic and water extracts of calyces with ZOI 12 and 11 mm, respectively, when compared to the positive control, ceftriaxone (ZOI 7 mm) followed by leaf extracts which showed similar effect to that of ceftriaxone (Table 3). As a matter of fact, the ethanol and water extracts of dry calyces have shown to be more effective in inhibiting growth of *S. typhi* compared to the 30 µg ceftriaxone which was used as control. The antibacterial activities of this plant were previously reported by Alian et al. (1983) and Sekar et al. (2015) though few tested species differed by strain and/or by activity. The antimicrobial activities demonstrated by the extracts of *H. sabdariffa* justify some of the ethno-pharmacological claims about this plant in the treatment of diseases caused by some of the tested pathogens.

Conclusion

This study concludes that both calyces and leaves of *Hibiscus sabdariffa* grown in Dodoma possess similar chemical constituents though vary in concentrations. Traditionally, calyces are most commonly used compared to leaves and also they exhibited more significant antibacterial activities compared to leaves against the selected bacterial species. The phytochemical constituents of the aqueous and ethanolic extracts of roselle leaves have shown similarity to that of calyces and to referenced data, with some differences that may be due to genetic variability, type of soil and its composition, time and season of sample collection and the solvent used for extraction. Further studies are required to investigate the nutritional contents of roselle leaves, fruits, seeds and other parts of the plant. Also, more research is required to determine the exact phytochemicals that are responsible for the antibacterial activities as well as the nature of the inhibition, whether bacterial static or bactericidal. This plant species is easily grown and can be a source of chemical constituents like phenols and flavonoids (free radical scavenging agents), iron (haemotonic) and vitamin C (immune booster). The study has helped in bridging the information gap that existed concerning the leaves of roselle grown in Dodoma and paved a way for consideration of future large scale production of pharmaceutical and neutraceutical products in Tanzania.

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