Bangladesh J. Bot. **49**(1): 171-177, 2020 (March) **- Short communication**

BIOLOGICAL ACTIVITY OF INFUSION AND DECOCTION EXTRACTS OF *HIBISCUS SABDARIFFA* L. CALYCES

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Keywords: *Hibiscus sabdariffa*, Anti-inflammatory, Cytotoxicity

Abstract

To compare the phytochemical and biological profiles, infusion and decoction extracts of dried *Hibiscus sabdariffa* calyces were prepared. Infusion extract had the highest phenolic content (292.42 ± 0.62 mg GAE/g extract) and antioxidant activity (TEAC = 3.09 ± 0.28). It also potently inhibited the activities of α-amylase (58.57 ± 1.4%), α-glucosidase (66.92 ± 2.5%) and 5-lipoxygenase (58.97 ± 1.17 µg/ml). All extracts showed weak cytotoxicity as tested on Vere monkey kidney cells.

Hibiscus sabdariffa L., a member of Malvaceae, is commonly known as Roselle. It is a perennial plant with a height of about 25 m, characterized by a smooth, cylindrical red stem and veins, and long green serrate leaves (Mohamed *et al.* 2007). The calyx is red and consists of five valves, each containing 3 - 4 kidney-shaped brownish seeds (Mohamed *et al.* 2007). Across cultures such as Egypt, Sudan, Trinidad and Tobago, Mexico, Nigeria, Malaysia, Indonesia, India, and South America, the calyx is used as food and medicine (Patel 2014, Ghosh *et al.* 2014). Brewing an infusion or a decoction of the calyces is still the popular extraction method for preparing the beverage (Rasheed *et al.* 2018). This method of preparation is believed to impart a strong effect on the therapeutic properties of the *H. sabdariffa* tea (Rasheed *et al.* 2018, Villani *et al.* 2013).

In folk medicine, it is used for the treatment of constipation, heart ailments, high blood pressure, urinary tract infections, cancer, diabetes and nerve disorders (Patel 2014, Ghosh *et al.* 2014). Extracts of *H. sabdariffa* calyces were reported to show antioxidant, anti-inflammatory, antihypertensive, antimicrobial, anticonvulsant and hypoglycaemic activities (Al-Mamun *et al.* 2011). They contain polyphenols, alkaloids, L-ascorbic acid, anthocyanins, β-carotene, β-sitosterol, citric acid, gossypetin, hibiscetin, mucopolysaccharide and polysaccharide (Hirunpanich *et al.* 2005, Rasheed *et al.* 2018) which may be responsible for the therapeutic properties.

Plant polyphenols (phenolic and flavonoids) are natural antioxidants (Yao *et al.* 2010) that modulate metabolic activities (Hanhineva *et al.* 2010) and ameliorate oxidative stress related diseases such as inflammatory conditions and diabetic complications (Yang *et al.* 2012). There are some reports on the inhibition of α-amylase and α-glucosidase activity by the extracts of *H. sabdariffa* (Ademiluyi and Oboh 2013, Rasheed *et al.* 2018), but limited on the inhibition of 5-lipoxygenase activity and on Vero monkey kidney cell lines. Inhibition of 5-lipoxygenase activity is proposed as a possible target for preventing inflammation induced diseases (Rådmark *et al.* 2007). While the cytotoxic effect of extracts has been evaluated using the Vero monkey kidney cells (McGaw *et al.* 2007). This is important more especially when traditional use of some plants extracts have been reported to cause deaths due to toxic effects (Winslow and Kroll 1998). Therefore, the aim of this study was to evaluate infusion and decoction extracts of the calyces of *H. sabdariffa* for total phenolic and total flavonoid content, antioxidant, cytotoxicity, and inhibition of α-amylase, α-glucosidase and 5-lipoxygenase activities.

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H. sabdariffa calyces were purchased from a local market in Lagos, Southwest of Nigeria (6°27´55.5192´´N and 3°24´23.2128´´E). Mr J. Mordi of the Federal Institute of Industrial Research Oshodi, Nigeria assisted in the identification and authentication of the plant. The voucher specimen (voucher number OLA005) was deposited and conserved in the Herbarium of the Department of Biology, Sefako Makgatho Health Science University, South Africa. Trypan blue (Fluka), doxorubicin (Pfizer), dimethyl sulfoxide (DMSO), methanol, and Whatman No. 1 filter paper were purchased from Merck (South Africa). All the other chemicals were purchased from Sigma (South Africa). Absorbance was read using an Epoch microplate reader (BioTek).

An infusion was prepared by addition of freshly boiled hot water (30 ml) to powdered calyces of *H. sabdariffa* (3 g) in a conical flask (Martins *et al.* 2014). After incubation for 10 min at room temperature, the mixture was centrifuged at 40,000 g for 5 min and filtered through a filter paper (No. 1). For the decoction, 30 ml of freshly boiled hot water was added to 3 g of powdered *H. sabdariffa* calyces each in three separate conical flasks. These flasks were heated at 60°C for 10, 20 and 40 min, respectively. All flasks were cooled to room temperature and filtered through a filter paper (No. 1) after centrifugation at 40,000 g for 5 min. The freeze dried extracts were stored in brown bottles at 4°C. The extracts (0.1 mg/ml) in distilled water were used as the working solutions for all assays in this study unless otherwise specified.

Total phenolic content of extracts was determined using the 96-well plate by method previously described (Olaokun *et al.,* 2017). To extracts (20 µl) in a 96-well plate was added 100 µl of 20% Folin-Ciocalteu reagent and 80 µl of 7.5% sodium bicarbonate solution. The mixture was shaken for 1 min, incubated for 60 min in the dark at room temperature and absorbance was measured at 760 nm. Total phenolic content was calculated from linear regression curve of gallic acid and results were expressed as mg gallic acid equivalent (GAE) per g of extract.

Total flavonoid content of extracts was determined using the 96-well plate method (Olaokun *et al.* 2017). To extracts (100 µl) in a 96-well plate was added 100 µl of 2% aluminium chloride. The mixture was shaken for 1 min, incubated for 15 min and the absorbance was read at 430 nm. Total flavonoid content was calculated from linear regression curve of quercetin and results were expressed as mg quercetin equivalent (QE) per g of extract.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of extracts was determined in a 96-well plate using a modified method (Olaokun *et al.* 2017). To extracts (50 µl) and positive control in serial dilution (8 - 500 µg/ml), 200 µl of DPPH (25 µg/ml) was added and change in absorbance was measured after 30 min incubation in the dark at 517 nm. Ascorbic acid and Trolox (1 mg/ml) were used as positive controls and extracts without DPPH as blank. Results were expressed as percentage reduction of the initial DPPH absorbance. Concentration of extract leading to 50% reduction of DPPH (IC_{50}) was determined.

Trolox equivalent antioxidant capacity (TEAC) of extracts was estimated as described by Olaokun *et al* (2017). Prior to assay, the preformed $ABTS^+$ (7 mM 2,2-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid)) radical was diluted in methanol (about 1:89 v/v) and equilibrated to give an initial absorbance (Ai) of 0.700 ± 0.02 at 734 nm. To extracts (20 µl) and trolox (1 mg/ml) (positive control) in serial dilution (8 - 500 µg/ml) was added 180 µl of the $ABTS^+$ radical and the mixture was allowed to react for 6 min. Extracts without $ABTS^+$ was used as blank. Absorbance (A) was read at 734 nm. Percentage change in absorbance was calculated for each concentration of extracts and trolox. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance versus concentration plot for the extracts and positive controls was divided by the gradient of the plot for trolox. TEAC of the extract is a ratio value and has no unit.

Inhibition of 5-lipoxygenase activity by extracts was determined as described by Olaokun *et al* (2017). To 20 µl of the extracts (4 mg/ml) or quercetin (1 mg/ml) (positive control) in five serial dilutions in 96-well plate was added 50 µl enzyme solution (400 units/ml cold 2 M borate buffer, pH 9). After incubation at 25°C for 5 min, 50 µl substrate solutions (10 µl linoleic acid dissolved in 30 µl ethanol, made up to 120 ml with 2 M borate buffer, pH 9.0) was added to start the reaction and incubated for another 5 min at 25°C. Thereafter, the absorbance was taken at 234 nm. For control sample (100% enzyme activity) distilled water replaced extracts while enzyme solution was replaced with buffer for blanks. Percentage enzyme inhibition of each extract compared with control sample was calculated and the results were expressed as IC_{50}.

The α-amylase inhibition assay described by Olaokun *et al* (2013) was utilised. Ice cold porcine pancreatic α-amylase solution (200 µl) at 4 unit/ml (type VI) was pre-incubated with 40 µl of extracts (20 mg/ml DMSO) and 160 µl of distilled water, and mixed in a screw-top plastic tube. Addition of 400 µl of potato starch (0.5%, w/v) in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride initiated the reaction, and thereafter incubated at 25ºC for 3 min. An aliquot of the mixture (200 µl) was removed and placed into a new tube containing 100 µl DNS colour reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31M sodium potassium tartrate in 2M sodium hydroxide) and placed into an 85°C water bath. After 15 min, this mixture was removed from the water bath, cooled and diluted with 900 µl distilled water. Absorbance was taken at 540 nm. For control sample (100% enzyme activity) DMSO replaced extracts while distilled water replaced enzyme solution for blanks. Acarbose was used as positive control. The percentage enzyme inhibition of each extract compared with control sample was calculated.

The α-glucosidase inhibition assay was determined using the method described by Olaokun *et al* (2013). Sucrose (200 µl of a 56 mM solution) in 0.1 M potassium phosphate buffer (pH 7) was mixed with 100 µl of extracts (2.5 mg/ml) in 50% aqueous DMSO in test tubes. After pre-incubation at 37°C for 5 min, 200 µl of rat intestinal α-glucosidase solution (25 mg/ml in ice-cold 50 mM phosphate buffer) was added. A 100 µl DMSO (50%) was used in place of extract for the control sample, acarbose (0.1 mg/ml) was used as the positive control. After thoroughly mixing, all tubes were incubated at 37°C for 20 min and then the reaction was stopped by adding 750 µl of 2 M tris-hydrochloric acid buffer (pH 6.9). The amount of liberated glucose was determined by the glucose oxidase method. Percentage enzyme inhibition of each extract compared with control sample was calculated.

The cytotoxic activity of extracts against Vero kidney cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was as described by McGaw *et al.* (2007). Cells at a density of 5.0×10^4 cells/ml in (minimal essential medium) MEM supplemented with 0.1% gentamicin and 5% foetal bovine serum was seeded (200 µl) into 96-well plate for the toxicity assays. Following overnight incubation at 37ºC in a 5% CO_2 incubator, the media of cells were removed and replaced in serial dilutions with 200 µl of fresh media containing either extracts (3.125 - 100 µg/ml) or doxorubicin (0.01 - 100 µM). After 48 hrs incubation, the media were removed and fresh media containing 30 µl of MTT (5 mg/ml in phosphate-buffered saline) were added into each well. After a further incubation period (4 hrs), the media were replaced with 50 µl of undiluted DMSO to dissolve the MTT crystals at the bottom of the wells. Absorbance was measured at 570 nm after gentle shaking. The LC_{50} values were calculated from a plot of log of concentration versus average absorbance of extract that resulted in a 50% reduction of absorbance compared to the untreated cells. The percentage cell viability was calculated as the absorbance of the treated cells divided by the absorbance of the untreated control cells.

Statistical analyses were done by one-way ANOVA and considered to be significantly different at $p < 0.05$. When significance was found, location of significance was determined by Bonferroni and Tukey HSD multiple comparison *post hoc* tests.

Extracts of *H. sabdariffa* calyces have been reported to be rich in polyphenols (Yang *et al.* 2012). Polyphenols are a large group of phytochemicals that consist of the flavonoids and phenolic acids (Hanhineva *et al.* 2010). The phenols of the calyces of *H. sabdariffa* were extracted by infusion and by decoction with additional heat treatment at constant temperature for 10, 20 and 40 min. The total phenolic content of the infusion extract (233.63 ± 0.57 mg GAE/g extract) was the highest (Table 1). Significant difference ($p > 0.05$) was found between phenolic content of infusion and decoction for 10 min, as well as between decoction for 10 min and those for 20 min and 40 min. The total flavonoid content of the decoction extract with 40 min heat was the highest (43.54 ± 0.05 mg QE/g extract) while the flavonoid content of decoction for 10 min was significantly different ($p > 0.05$) from those for 20 min. A trend of decrease in total phenolic content with increase in heat treatment time was observed similar to those of Rasheed *et al.* (2018) whose infusion extract contained more polyphenols than decoction extracts.

Table 1. Total phenolic content, total flavonoid content antioxidant activity of infusion and decoction extracts of *H. sabdariffa* calyces.

Test samples	Phenolic content mg GAE/g extract	Flavonoid content mg QE/g extract	TEAC	DPPH IC_{50} µg/ml
Infusion (0 min)	292.42 ± 0.62^{a}	43.06 ± 0.05	3.09 ± 0.28	13.22 ± 2.00^{de}
Decoction (10 min)	246.48 ± 0.22^{ab}	42.22 ± 0.09^{c}	2.86 ± 0.31	12.16 ± 1.51^{de}
Decoction (20 min)	244.12 ± 0.47^{b}	39.70 ± 0.03^{c}	2.91 ± 0.46	15.31 ± 1.15^{de}
Decoction (40 min)	233.63 ± 0.57^{b}	43.54 ± 0.05	2.44 ± 0.24	13.66 ± 2.11^{de}
Ascorbic acid	na	na	na	2.15 ± 0.44^{d}
Trolox	na	na	na	2.92 ± 0.54^{e}

Values are means of triplicate determinations done three times (n = 9) ± standard error. TEAC (Trolox equivalent antioxidant capacity) of the extract is a ratio value and has no unit; na: Not applicable. ^{a,b,c,d,e} Significant difference ($p < 0.05$) between values with same letter.

The TEAC of the infusion extract was the strongest (2.44 ± 0.24) while the DPPH radical scavenging ability of decoction with 10 min heat treatment was the strongest (IC_{50} of 12.16 ± 1.51 µg/ml) (Table 1). While no significant difference was found between the TEAC of extracts, significant difference ($p < 0.05$) was found between the DPPH activity of extracts and the positive control. No correlation was found between antioxidant activity by TEAC and DPPH which was in conformity with Floegel *et al.* (2011).

The extracts of *H. sabdariffa* inhibited the activity of 5-lipoxygenase with IC_{50} value ranging from 58.97 ± 1.17 to 78.77 ± 3.47 µg/ml (Table 2) with significant difference ($p < 0.05$) among them. The infusion extract showed higher inhibition in comparison to the decoction samples. Irrespective of the method of extraction, the extracts of *H. sabdariffa* calyces possessed anti-inflammatory activity by the inhibition of 5-lipoxygenase activity. To the best of the authors' knowledge, this was the first study to demonstrate the anti-inflammatory activity of *H. sabdariffa* by the inhibition of 5-lipoxygenase activity.

The extracts rich in polyphenols from a number of plants were effective for the inhibition of intestinal α-glucosidase and α-amylase, indicating their potential therapeutic effect on post-prandial glucose levels (McDougall *et al.* 2005). The infusion extract of *H. sabdariffa* calyces strongly inhibited the activity of α-amylase (58.57 ± 1.4%) and α-glucosidase (66.92 ± 2.5%) than the decoction samples (Table 2) with no significant difference among them. It might be due to the

higher levels of polyphenols in the infusion samples. The extracts generally demonstrated a stronger inhibitory activity on α-glucosidase than α-amylase.

Table 2. Cytotoxic, 5-lipoxygenase, α-amylase and α-glucosidase inhibitory activities of infusion and decoction extracts of *H. sabdariffa* calyces.

Test samples	Cytotoxicity LD_{50} µg/ml	Anti-inflammatory (LOX) activity IC_{50} µg/ml	α-amylase inhibitory activity (%)	α-glucosidase inhibitory activity (%)
Infusion (0 min)	263.50 ± 2.79^{a}	58.97 ± 1.17^{c}	58.57 ± 1.2	66.92 ± 2.5
Decoction (10 min)	257.77 ± 1.73	68.60 ± 2.26^{c}	55.77 ± 2.5	59.60 ± 3.0
Decoction (20 min)	262.43 ± 2.61^{b}	78.77 ± 3.47^{c}	56.46 ± 3.1	64.79 ± 1.9
Decoction (40 min)	280.37 ± 1.52	77.97 ± 2.20^{c}	52.92 ± 0.2	62.46 ± 1.6
Doxorubicin (µM)	15.69 ± 1.20^{ab}	nd	nd	nd
Quercetin	nd	7.43 ± 1.37^{c}	nd	nd
Acarbose	nd	nd	79.21 ± 0.1	94.45 ± 0.4

Values are means of triplicate determinations done three times (n = 9) ± standard error of mean;
LOX: 5-lipoxygenase, nd: not determined, ^{a,b,c}Significant difference ($p < 0.05$) between values with same letter.

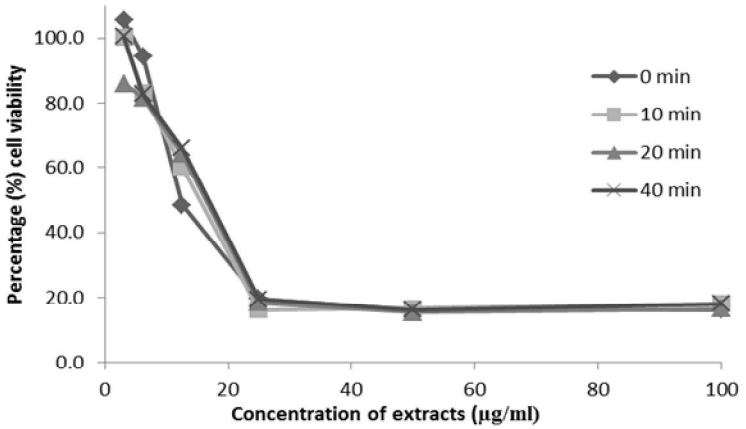

Fig. 1. Percentage viability of Vero monkey kidney cells treated with *H. sabdariffa* extracts (Infusion (0 min) and decoction (10, 20 and 40 min).

The extracts of *H. sabdariffa* were relatively and significantly ($p < 0.05$) less cytotoxic against the growth of Vero monkey kidney cells than doxorubicin. The cytotoxicity value of the extracts (LD_{50}) ranged from 257.77 ± 1.73 to 280.37 ± 1.52 µg/ml (Table 2). However, all the extracts in a concentration-dependent manner reduced the cell viability of the Vero kidney cells (Fig. 1).

In general all the aqueous extracts of *H. sabdariffa* are rich in polyphenols with several potential therapeutic benefits but the infusion extract had the highest phenolic content as well as stronger antioxidant and enzyme inhibitory activities. Although there have been no reports of the suspected toxicity of this plant since its long traditional use in folk medicine, comprehensive studies should be done to confirm its safety profile.

Acknowledgments

Sefako Makgatho Health Sciences University provided the DHET Research Development Grant (RDG) for this study, while the facility of the Phytomedicine Programme laboratory at the University of Pretoria was used for part of the study.

References

Adebayo SA, Dzoyem JP, Shai LJ and Eloff JN 2015. The anti-inflammatory and antioxidant activity of 25 plant species used traditionally to treat pain in southern African. BMC Complement Altern. Med. **15**(1): p.159.

Ademiluyi AO and Oboh G 2013. Aqueous extracts of Roselle (*Hibiscus sabdariffa* Linn.) varieties inhibit α-amylase and α-glucosidase activities *in vitro*. J. Med. Food. **16(1)**: 88-93.

Al-Mamun KH, Nesa L, Islam R and Munira S 2011. *In vitro* evaluation of the antibacterial, cytotoxic and insecticidal activities of *Hibiscus sabdariffa* fruits. Libyan Agric. Res. Center J. Int. **2**(3): 144-149.

Floegel A, Kim DO, Chung SJ, Koo SI and Chun OK 2011. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. J. Food Compost Anal. **24**(7): 1043-1048.

Ghosh I, Poddar S and Mukherjee A 2014. Evaluation of the protective effect of *Hibiscus sabdariffa* L. Calyx (Malvaceae) extract on arsenic induced genotoxicity in mice and analysis of its antioxidant properties. Biol. Med. **6**: 218.

Hanhineva K, Torronen R, Bondia-Pons I, Pekkinen J and Kolehmainen M 2010. Impact of dietary polyphenols on carbohydrate metabolism. Int. J. Mol. Sci. **11**(4): 1365-1402.

Hirunpanich V, Utaipat A, Morales NP, Bunyapraphatsara N and Sato H 2005. Antioxidant effects of aqueous extracts from dried calyx of *Hibiscus sabdariffa* Linn. (Roselle) *in vitro* using rat low-density lipoprotein (LDL). Biol. Pharm. Bull **28(3)**: 481-484.

Martins N, Barros L, Santos-Buelga C, Henriques M, Silva S and Ferreira IC 2014. Decoction, infusion and hydroalcoholic extract of *Origanum vulgare* L.: Different performances regarding bioactivity and phenolic compounds. Food Chem. **158**: 73-80.

McDougall GJ, Shpiro F, Dobson P, Smith P, Blake A and Stewart D. 2005. Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. J. Agri. Food Chem. **53**: 2760-2766.

McGaw LJ, Steenkamp V and Eloff JN 2007. Evaluation of Athrixia bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. J. Ethnopharmacol. **110**: 16-22.

Mohamed R, Fernandez J, Pineda M and Aguilar M 2007. Roselle (*Hibiscus sabdariffa*) seed oil is a rich source of gamma-tocopherol. J. Food Sci. **72**(3): S207-S211.

Olaokun OO, Mkolo NM, Mogale MA and King PH 2017. Phytochemical screening, antioxidant, anti-inflammatory and glucose utilization activities of three South African plants used traditionally to treat diseases. Biol. Med. (Aligarh) **9**: 412.

Olaokun OO, McGaw LJ, Eloff JN and Naidoo V 2013. Evaluation of the inhibition of carbohydrate hydrolysing enzymes, antioxidant activity and polyphenolic content of extracts of ten African *Ficus* species (Moraceae) used traditionally to treat diabetes. BMC Complement Altern Med. **13**(1): 94.

Patel S 2014. *Hibiscus sabdariffa*: an ideal yet under-exploited candidate for nutraceutical applications. Biomed. Prev. Nutr. **4**(1): 23-27.

Rådmark O, Werz O, Steinhilber D and Samuelsson B 2007. 5-Lipoxygenase: regulation of expression and enzyme activity. Trends Biochem. Sci. **32**(7): 332-341.

Rasheed DM, Porzel A, Frolov A, El Seedi HR, Wessjohann LA and Farag MA 2018. Comparative analysis of *Hibiscus sabdariffa* (Roselle) hot and cold extracts in respect to their potential for α-glucosidase inhibition. Food Chem. **250**: 236-244.

Ribeiro D, Freitas M, Tomé SM, Silva AM, Porto G, Cabrita EJ, Marques MMB and Fernandes E 2014. Inhibition of LOX by flavonoids: a structure–activity relationship study. Euro J. Med. Chem. **72**: 137-145.

Villani T, Juliani R, Simon EJ and Wu QL 2013. *Hibiscus sabdariffa*: Phytochemistry, quality control and health properties. ACS Symposium Series 1127(Chapter 14), 209-230

Winslow LC and Kroll DJ 1998. Herbs as medicines, Archives Intern. Med. **158**(20): 2192.

Yang L, Gou Y, Zhao T, Zhao J and Li F 2012. Antioxidant capacity of extracts from calyx fruits of roselle (*Hibiscus sabdariffa* L.). Afr. J. Biotechnol. **11**(17): 4063-4068.

Yao Y, Sang W, Zhou M and Ren G 2009. Antioxidant and α-glucosidase inhibitory activity of colored grains in China. J. Agric. Food Chem. **58**(2): 770-774.

(Manuscript received on 14 November, 2018; revised on 24 April, 2019)