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Evaluation of Antioxidant, Anti-tyrosinase and Antibacterial Activities of Selected *Hibiscus* Species

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Abstract

Leaves and flowers of selected *Hibiscus* species, used in traditional medicine, were evaluated for antioxidant, antityrosinase and antibacterial activities. Information on these species is meagre and this study would contribute new and additional knowledge on the bioactivities of the genus. Antioxidant properties (AOP) of six species assessed were total phenolic content (TPC), total anthocyanin content (TAC), ascorbic acid equivalent antioxidant capacity (AEAC), ferric reducing power (FRP), ferrous ion chelating (FIC) ability and lipid peroxidation inhibition (LPI) activity. Antityrosinase and antibacterial activities of four species were assessed using the modified dopachrome and disc diffusion methods, respectively. Leaves and flowers of *Hibiscus tiliaceus* showed outstanding AOP. Leaves of species with high TPC and AEAC had low FIC ability and *vice versa.* Red flowers which yielded the highest TAC also displayed high FIC ability and LPI activity. Leaves of *H. tiliaceus* had the strongest antityrosinase (AT) activity. With very strong AOP and AT activity, leaves of *H. tiliaceus* have potentials to be developed into functional food and skin care products. At 1 mg extract/disc, leaves of *Hibiscus sabdariffa* were found to inhibit Gram-positive bacteria of *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus aureus*. At 2 mg extract/disc, leaves of *H. sabdariffa* inhibited both Gram-positive and Gram-negative bacteria of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella choleraesuis*. This is the first report of leaf extracts of *H. sabdariffa* inhibiting Gram-negative bacteria. Adding 1 mM of ethylenediamine tetraacetic acid (EDTA) to the agar slightly enhanced the antibacterial activity of leaves of *H. sabdariffa* on Gram-negative bacteria. With a wide spectrum of inhibition against Gram-positive and Gram-negative bacteria, leaves of *H. sabdariffa* are worthy of further investigation as a natural wide spectrum antibacterial agent.

Keywords: *Hibiscus*, antioxidant, antityrosinase, antibacterial, leaves, flowers.

Introduction

The genus *Hibiscus* (Malvaceae) comprises about 275 species in the tropics and sub-tropics (Dasuki, 2001). Within the Malesian region, 43 species are found. Most *Hibiscus* species have a remarkable colour pattern with the base of corolla forming a deep-coloured heart (Lowry, 1976). Another feature is flower colour change among species of which the most spectacular is in flowers of *Hibiscus mutabilis* L. Leaves of *Hibiscus* are simple, lobed, alternate or spiral and have paired stipules (Ng, 2006). Flowers are radially symmetrical with cup-shaped calyx, five petals joined at the base, style bearing many stamens and stigma with five hairy lobes.

With attractive and colourful flowers, plants of *Hibiscus* are widely planted as ornamentals and are used in traditional medicine. Of the species studied, leaves and flowers of *H. mutabilis*, believed to have emollient and cooling effect, are used to relieve swellings and skin infections (Dasuki, 2001). Leaves and flowers of *Hibiscus rosa-sinensis* L. are used as an antiseptic for boils and ulcers. The sap from flowers is used as colouring agent. Leaves of *Hibiscus sabdariffa* L. are used as poultice for abscesses and ulcers. Young shoots and leaves are eaten raw or cooked as vegetable. The red fleshy calyces are widely used to make beverages and jams. Stems and roots of *Hibiscus taiwanensis* Hu have been used as anti-inflammatory, antifungal, antipyretic, and antihelminthic agents (Wu *et al*., 2005). Flowers of *Hibiscus tiliaceus* L. are widely used for birth control and for treating skin infections (Rosa *et al*., 2006).

Leaves and flowers of selected *Hibiscus* species are used in traditional medicine. Information on their antioxidant, antityrosinase and antibacterial activities is meagre. This study would contribute new and additional knowledge on the bioactivities of the genus.

Materials and Methods

Plant materials

Hibiscus species studied were *H. mutabilis*, *H. rosa-sinensis, H. sabdariffa*, *Hibiscus schizopetalus* (Dyer) Hook. f., *H. taiwanensis* and *H. tiliaceus* (Fig. 1). Voucher specimens of species studied were deposited in the herbarium of Monash University Sunway Campus in Malaysia. Leaves and flowers for screening were collected a day before extraction and kept fresh in sealed plastic bags in a refrigerator. From each location, three individual plants per species were sampled.

Fig. 1. *Hibiscus* species studied.

Extraction

For analysis of bioactivities, plant materials (1 g) of fresh leaves and petals were powdered with liquid nitrogen in a mortar and extracted using methanol (50 ml), with continuous swirling for 1 h at room temperature

with an orbital shaker. Extracts were filtered under suction and stored at -20°C for further use.

Antioxidant properties

Antioxidant properties assessed were total phenolic content, total anthocyanin content, radical scavenging activity, ferric reducing power, ferrous ion chelating ability and lipid peroxidation inhibition activity.

Total phenolic content (TPC) was determined using the Folin-Ciocalteu assay (Kähkönen *et al*., 1999). Extracts (300 µl; triplicate) were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before absorbance at 765 nm was measured. TPC was expressed as gallic acid equivalent (GAE) in mg per 100 g of material. The calibration equation for gallic acid was y = 0.0111x - 0.0148 (R^2 = 0.9998), where y is absorbance and x is concentration of gallic acid in mg/l.

Total anthocyanin content (TAC) was determined by the pH differential method (Teow *et al*., 2007). Potassium chloride solution (2 ml, 1 M and pH 1.0) was added to 1 ml of extract in triplicate. Measurements were blanked against sodium acetate buffer (2 ml, 1 M and pH 4.5) with the same amount of extract. Absorbance was measured at 520 nm and 700 nm. TAC was expressed as cyanidin-3-glucoside equivalent (CGE) in mg per 100 g of sample. The molar extinction coefficient of cyanidin-3-glucoside was 26 900.

Radical scavenging activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Miliauskas *et al*., 2004). Different dilutions of extract (1 ml) were added to 2 ml of DPPH (5.9 mg/100 ml methanol). Absorbance was measured at 517 nm after 30 min. Radical-scavenging was calculated as IC_{50} and expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg AA/100 g = $IC_{50(ascorbate)}/IC_{50(sample)}$ x 10^5. The IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

Ferric reducing power (FRP) was assessed using the potassium ferricyanide assay (Chu *et al*., 2000). Different dilutions of extract (1 ml) were added to 2.5 ml phosphate buffer (0.2 M and pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). The mixture was incubated at 50 ^oC for 20 min. Trichloroacetic acid solution (2.5 ml and 10% w/v) was added to stop the reaction. The mixture was then separated into aliquots of 2.5 ml and diluted with 2.5 ml of water. To each diluted aliquot, 0.5 ml of ferric chloride solution (0.1% w/v) was added. After 30 min, absorbance was measured at 700 nm. FRP was expressed as mg GAE/g. The calibration equation for gallic acid was y = 16.767x (R^2 = 0.9974), where y is absorbance and x is concentration of gallic acid in mg/ml.

Ferrous ion chelating (FIC) ability was assessed using the ferrous-ferrozine assay (Singh and Rajini, 2004). Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were diluted 20 times. $FeSO_4$ (1 ml) was mixed with different dilutions of extract (1 ml), followed by ferrozine (1 ml). Absorbance *(A)* was measured at 562 nm after 10 min. Chelating ability = $(1 - A_{sample}/A_{control})$ x 100%.

Lipid peroxidation inhibition (LPI) activity was determined using the β-carotene bleaching assay (Kumazawa *et al*., 2002). Emulsion of β-carotene and linoleic acid was prepared by adding 3 ml of β-carotene (5 mg in 50 ml chloroform) to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was evaporated under vacuum and oxygenated ultra-pure water (100 ml) was added and mixed well. Initial absorbance (*A*) of the emulsion was measured at 470 nm. Aliquots of the emulsion (3 ml) were mixed with 10, 50 and 100 ml of extracts and incubated in a water bath at 50 °C for 1 h. Bleaching rate of β-carotene was measured at 470 and 700 nm. Measurement at 700 nm was needed to correct for the presence of haze. LPI expressed as antioxidant activity (AOA) was calculated as bleaching rate (BR) of β-carotene = $\ln(A_{initial}/A_{sample})/60$ and AOA = $(1 - BR_{.sample}/BR_{control})$ x 100% where $A_{initial}$ and A_{sample} are absorbance of the emulsion before and 1 h after incubation, and BR_{sample} and $BR_{control}$ are bleaching

rates of the sample and negative control, respectively.

Antityrosinase activity

Antityrosinase (AT) activity was determined using the modified dopachrome method with L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate (Masuda *et al*., 2005). Assays were conducted in a 96-well microtitre plate and a plate reader was used to measure absorbance at 475 nm with 700 nm as reference. Samples were dissolved in 50% dimethylsulphoxide (DMSO). Each well contained 40 µl of sample with 80 µl of phosphate buffer (0.1 M, pH 6.8), 40 µl of tyrosinase (31 units/ml) and 40 µl of L-DOPA (2.5 mM). Each sample was accompanied by a blank that had all the components except L-DOPA. Results were compared with a control consisting of 50% DMSO in place of sample. AT activity was calculated as $(A_{control} - A_{sample})/A_{control} \times 100\%$. Leaves of *Psidium guajava* L. (Guava) were used as positive control as they have very high AT activity (Vimala *et al*., 2006).

Antibacterial activity

The disc diffusion method (Chung *et al*., 2004) was used to screen for antibacterial activity of leaf extracts. Agar cultures of Gram-positive bacteria of *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus aureus*, and Gram-negative bacteria of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella choleraesuis* were prepared. Suspensions of bacteria (100 µl) were spread evenly onto 20 ml Mueller-Hinton agar preset in 90 mm Petri dishes. Paper discs (6 mm diameter) were impregnated with 1 mg of extract dissolved in 100 µl solvent, and transferred onto the inoculated agar. Streptomycin susceptibility discs (10 µg) and methanol impregnated disc were used as positive and negative controls, respectively. After incubation overnight at 37 ^{o}C, inhibition zones were measured and recorded as mean diameter (mm). Antibacterial activity was also expressed as inhibition percentage of streptomycin and arbitrarily classified as strong for inhibition of ≥ 70%, moderate for inhibition 50 < 70%, and weak for inhibition < 50% (Chan *et al*., 2007).

Antibacterial activity was also determined using the minimum inhibitory dose (MID) method (Mackeen *et al*., 2000). For species with antibacterial activity, the disc diffusion method was repeated using diluted extracts and loaded onto paper discs. The MID is the minimum concentration of extract in mg/disc that showed positive inhibition of test bacteria. Two approaches were adopted to enhance the efficacy of extracts to inhibit Gram-negative bacteria (Wong, 2008). The first approach was to repeat the disc diffusion method by increasing the extract concentration from 1 to 2 mg extract/disc. The second approach was to add 1 mM of ethylenediamine tetraacetic acid (EDTA) to the agar before culturing the bacteria. The diffusion method was repeated using 1 and 2 mg extract/disc.

Results and Discussion

Description of species

Leaves of *H. mutabilis* (Confederate rose) are broadly ovate with mostly five triangular lobes. Flowers are white in the morning, turning pink in the afternoon, and red in the evening. Leaves *H. rosa-sinensis* (China rose) are ovate with serrated margins. Flowers are red with a long and slender style, anthers yellow and stigma red. Leaves of *H. sabdariffa* (Roselle) are broadly ovate with 3-5 lobes and stems are reddish. Flowers open pinkish in the morning, turning orange in the evening. Leaves of *H. schizopetalus* (Coral hibiscus) resemble those of *H. rosa-sinensis*. Flowers are red, pendulous with a long extended style and petals are finely dissected. Leaves of *H. taiwanensis* (Cream hibiscus) are finely toothed and flowers are yellow with a prominent deep brown eye. Leaves of *H. tiliaceus* (Sea hibiscus) are heart-shaped, and flowers are bell-shaped with maroon-coloured heart and

stigma. Flowers are yellow in the morning, turning orange-red in the evening.

Antioxidant properties

Leaves of *H. tiliaceus* showed outstanding antioxidant properties (AOP) with TPC and AEAC values of 2080 mg GAE/100 g and 2370 mg AA/100 g, respectively (Table 1). Values were 2.4 and 2.7 times higher than those of *H. mutabilis* which ranked second. Based on TPC and AEAC, ranking was: *H. tiliaceus* > *H. mutabilis* > *H. sabdariffa* > *H. taiwanensis* > *H. schizopetalus* ~ *H. rosa-sinensis.*

Table 1. Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves of *Hibiscus* species

Hibiscus species	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
H. tiliaceus	2080 ± 419^{a}	2370 ± 539^{a}
H. mutabilis	861 ± 92^{b}	877 ± 137^{b}
H. sabdariffa	523 ± 61^{c}	351 ± 40^{c}
H. taiwanensis	403 ± 3^{d}	233 ± 22^{d}
H. schizopetalus	336 ± 50^{e}	94 ± 26^{e}
H. rosa-sinensis	301 ± 21^{e}	96 ± 35^{e}

Values of TPC and AEAC are means ± SD ($n = 3$). For each column, values followed by the same letter (a-e) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. For each species, samples were collected from the same location. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

Leaves of *H. schizopetalus*, *H. sabdariffa* and *H. rosa-sinensis* had better FIC ability than those of *H. mutabilis*, *H. tiliaceus* and *H. taiwanensis* (Fig. 2). Leaves of species with higher TPC and AEAC had lower FIC ability for *H. tiliaceus* and *H. mutabilis*, and *vice versa* for *H. schizopetalus* and *H. rosa-sinensis*. This suggests the presence of compounds in leaves of *H. schizopetalus* and *H. rosa-sinensis* with relatively weak radical scavenging activity but good metal chelating ability that could prevent the generation of hydroxyl radicals *via* Fenton's reaction.

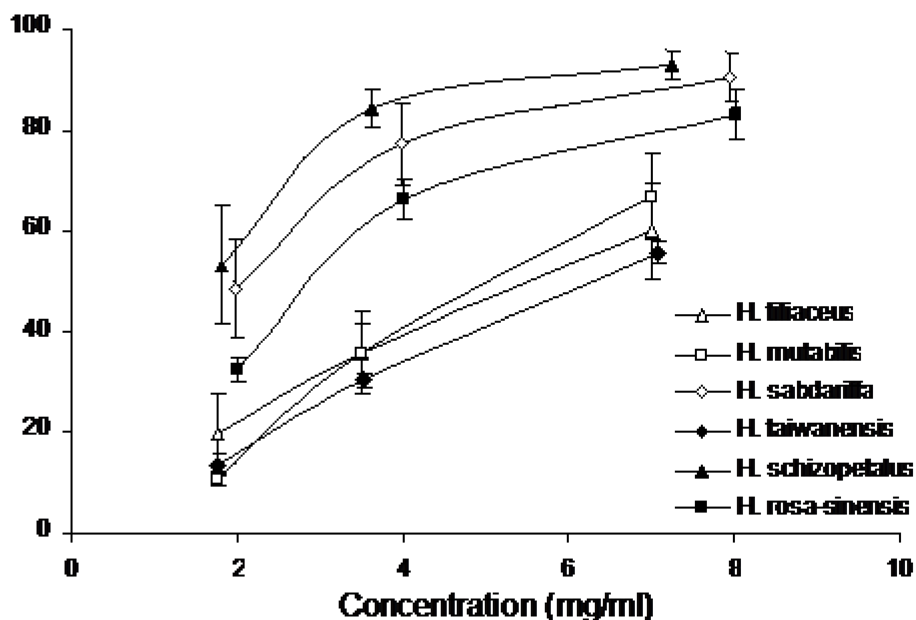


Fig. 2. Ferrous ion chelating ability of leaves of *Hibiscus* species

Flowers of *H. tiliaceus* with TPC, AEAC and FRP values of 2420 mg GAE/100 g, 3180 mg AA/100 g and 14 mg GAE/g, respectively, were significantly higher than all other species (Table 2). Based on TPC, ranking was: *H. tiliaceus* > *H. rosa-sinensis* > *H. taiwanensis* ~ *H. schizopetalus* ~ *H. mutabilis* > *H. sabdariffa*. The red flowers of *H. rosa-sinensis* and *H. schizopetalus*, which yielded the highest TAC, displayed high FIC ability and LPI activity (Fig. 3). Species with low TAC such as *H. mutabilis* and *H. sabdariffa* displayed low or no FIC ability and LPI activity. TAC appears to be positively correlated with FIC ability and LPI activity in flowers of *Hibiscus* species. Anthocyanins with potent metal-chelating activity have been reported in peels of the egg plant *Solanum melongena* L. (Noda *et al.*, 2000) and leaves of *Perilla pankinensis* Decne (Gülcin *et al.*, 2005).

Table 2. Total phenolic content (TPC), total anthocyanin content (TAC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) of flowers of *Hibiscus* species

Hibiscus species	TPC (mg GAE/100 g)	TAC (mg CGE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
H. tiliaceus	2420 ± 167[a]	64 ± 5[a]	3180 ± 678[a]	14 ± 1.3[a]
H. rosa-sinensis	735 ± 46[b]	284 ± 17[b]	640 ± 56[bc]	4.0 ± 0.3[b]
H. taiwanensis	580 ± 79[c]	92 ± 10[c]	761 ± 98[b]	3.6 ± 0.4[bc]
H. schizopetalus	516 ± 30[c]	192 ± 21[d]	520 ± 50[c]	3.0 ± 0.2[c]
H. mutabilis	495 ± 23[c]	16 ± 2[e]	562 ± 37[c]	2.4 ± 0.1[d]
H. sabdariffa	264 ± 61[d]	43 ± 12[f]	230 ± 60[d]	1.5 ± 0.3[e]

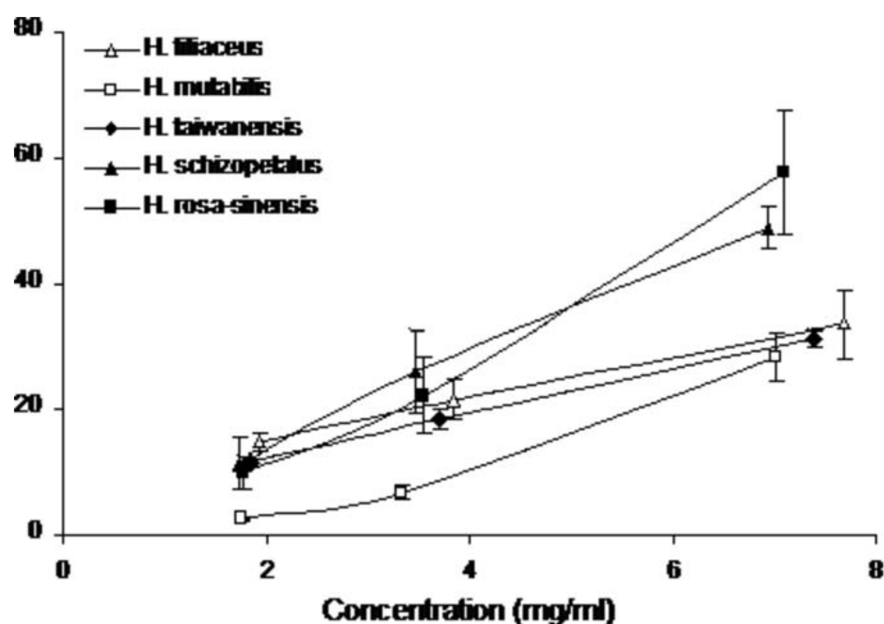
Values of TPC, TAC, AEAC and FRP are means ± SD (n = 3). For each column, values followed by the same letter (a-f) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. For each species, samples were collected from the same location. Abbreviations: GAE = gallic acid equivalent, AA = ascorbic acid and CGE = cyanidin-3-glucoside equivalent.

Of the six *Hibiscus* species, leaves and flowers of *H. tiliaceus* had the strongest AOP. A likely explanation is that *H. tiliaceus* is the only indigenous tree species while the other species are exotic shrubs and herbs. Being long-lived, trees have to produce a wide range of chemical defenses against herbivores and infections. Higher antioxidant activity of trees than shrubs and herbs has been reported (McCune and Johns, 2007).

Based on AOP of leaves and flowers, the six *Hibiscus* species can be divided into three categories (Wong *et al*., 2009). They are species with comparable values in leaves and flowers (*H. tiliaceus*), species with significantly higher values in leaves than flowers (*H. mutabilis* and *H. sabdariffa*), and species with significantly higher values in flowers than leaves (*H. taiwanensis*, *H. rosa-sinensis* and *H. schizopetalus*).

Antityrosinase activity

Of four species of *Hibiscus* tested, leaves of *H. tiliaceus* (42%) had the strongest AT activity (Table 3). Values were comparable to leaves of *P. guajava* (41%) as positive control. Ranking of AT activity was: *H. tiliaceus* > *H. mutabilis* > *H. rosa-sinensis* ~ *H. sabdariffa*. Leaves of *H. tiliaceus* displayed the highest tyrosinase inhibition among 39 tropical plant species screened by Masuda *et al.* (2005).



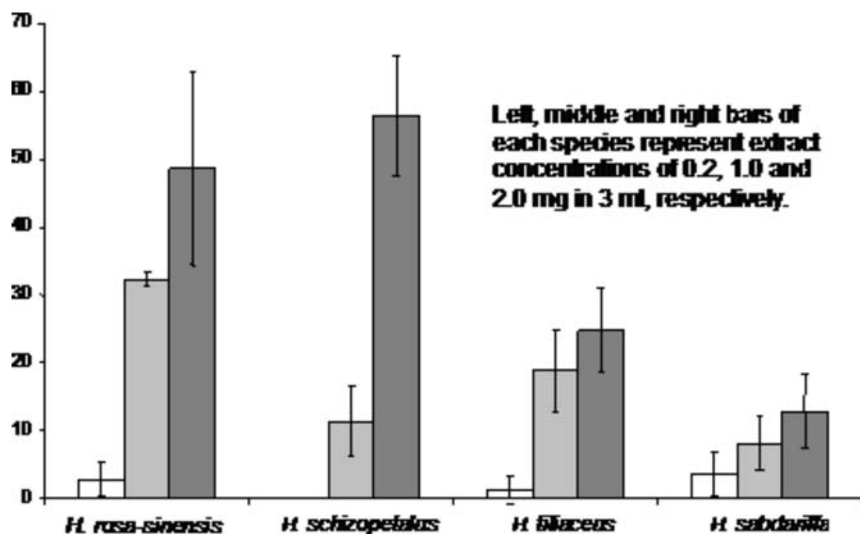


Fig. 3. Ferrous ion chelating (FIC) ability (a) and lipid peroxidation inhibition (LPI) activity (b) of flowers of *Hibiscus* species

Antibacterial activity

At 1 mg extract/disc, leaves of *H. tiliaceus* and *H. sabdariffa* were found to inhibit Gram-positive bacteria of *M. luteus*, *S. aureus* and *B. cereus* (Table 4). Leaves of *H. tiliaceus* exhibited moderate antibacterial activity against all three Gram-positive bacteria. Leaves of *H. sabdariffa* weakly inhibited *M. luteus* and *B. cereus.* Mean diameter zone of inhibition of streptomycin was 20 mm for *M. luteus*, 18 mm for *S. aureus* and 18 mm for *B. cereus*. Leaves of all four *Hibiscus* species showed no antibacterial activity on Gram-negative bacteria of *P. aeruginosa*, *S. choleraesuis* and *E. coli*.

Table 3. Antityrosinase (AT) activity of leaves of *Hibiscus* species

Hibiscus species	AT activity (%)
H. tiliaceus	42 ± 3^{a}
H. mutabilis	25 ± 4^{b}
H. rosa-sinensis	11 ± 3^{c}
H. sabdariffa	5 ± 4^{c}
Psidium guajava	41 ± 3^{a}

Values of AT activity are means ± SD (n = 3). Values followed by the same letter (a-c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Concentration of extracts used was 0.5 mg/ml. Leaves of *P. guajava* were used as positive control.

Table 4. Antibacterial activity of leaves of *Hibiscus* species at 1 mg extract/disc

Hibiscus species	Zone of inhibition in mm (percentage inhibition)					
	Gram-positive bacteria			Gram-negative bacteria		
	M. luteus	*S. aureus*	*B. cereus*	*P. aeruginosa*	*S. choleraesuis*	*E. coli*
H. mutabilis	–	–	–	–	–	–
H. rosa-sinensis	–	–	–	–	–	–
H. sabdariffa	9^{+}	–	8^{+}	–	–	–
H. tiliaceus	10^{++}	9^{++}	9^{++}	–	–	–
Streptomycin	20	18	18	14	14	14

Antibacterial activity is categorized as moderate $^{++}$ for inhibition 50 < 70% or weak $^{+}$ for inhibition < 50% based on inhibition percentage compared to streptomycin.

Chung *et al.* (2004) reported that none of 191 plant extracts from 30 families inhibited Gram-negative bacteria. Similarly, screening of extracts of 26 edible plant species by Alzoreky and Nakahara (2003) showed that Gram-negative bacteria were not susceptible to plant extracts. Screening of five different extracts from five ethnomedicinal plant species also showed that most of the antibacterial activity detected was against Gram-positive bacteria (Murugan *et al*., 2008). Gram-negative bacteria are generally less susceptible to plant extracts than Gram-positive bacteria due to their outer membrane of lipopolysaccharide and lipoprotein, which is resistant towards antibacterial substances (Chopra and Greenwood, 2001; Alzoreky and Nakahara, 2003).

For Gram-positive bacteria, MID of leaf extracts of *H. sabdariffa* was 1 mg/disc for *M. luteus* and *B. cereus*, and 2 mg/disc for *S. aureus* (Table 5). MID of leaf extracts of *H. tiliaceus* was 0.5 mg/disc for *M. luteus*, 0.25 mg/disc for *S. aureus* and 1 mg/disc for *B. cereus.*

Table 5. Minimum inhibitory dose (MID) of leaves of *Hibiscus sabdariffa* and *H. tiliaceus*

Extract (mg/disc)	Zone of inhibition in mm (percentage inhibition)					
	H. sabdariffa			*H. tiliaceus*		
	M. luteus	*S. aureus*	*B. cereus*	*M. luteus*	*S. aureus*	*B. cereus*
2	13^{++}	9^{++}	9^{++}	8^{+}	9^{++}	7^{+}
1	7^{+}	–	7^{+}	8^{+}	9^{++}	7^{+}
0.5	–	–	–	7^{+}	8^{+}	–
0.25	–	–	–	–	7^{+}	–
0.125	–	–	–	–	–	–
Streptomycin	20	18	18	21	17	17

Antibacterial activity is categorized as moderate $^{++}$ for inhibition 50 < 70% or weak $^{+}$ for inhibition < 50% based on inhibition percentage compared to streptomycin.

Screening for antibacterial activity of plant extracts is normally done using the disc diffusion method at concentration of 1 mg extract/disc. By increasing the concentration to 2 mg extract/disc, leaves of *H. sabdariffa* displayed moderate inhibition on all six Gram-positive and Gram-negative bacteria (Table 6). Gram-negative bacteria were not inhibited at 1 mg extract/disc. Results indicated that phenolic compounds in leaves of *H. sabdariffa* might be bactericidal and the mode of action might be dose dependent. At 2 mg extract/disc, antibacterial activity of leaves of *H. tiliaceus* remained the same as 1 mg extract/disc. Inhibition was moderate on *S. aureus*, and weak on *M. luteus* and *B. cereus* with no inhibition on the three Gram-negative bacteria. Results indicated that the inhibitive properties of phenolic compounds in leaves of *H. tiliaceus* might be non-dose dependent. This is the first report of leaf extracts of *H. sabdariffa* inhibiting Gram-negative bacteria of *P. aeruginosa*, *S. choleraesuis* and *E. coli.* None of plant extracts from 50 species reported by Wiart *et al.* (2004) inhibited *E. coli.* With a wide spectrum of inhibition against Gram-positive and Gram-negative bacteria, leaves of *H. sabdariffa* are worthy of further investigation as a natural wide spectrum antibacterial agent. Similar wide spectrum of antibacterial activity has been reported in extracts of *Andrographis paniculata* (Burm. F.) Nees (Sule *et al.*, 2010).

Table 6. Antibacterial activity of leaves of *Hibiscus sabdariffa* and *Hibiscus tiliaceus* at 2 mg extract/disc

Species and extract (mg/disc)	Zone of inhibition in mm (percentage inhibition)					
	Gram-positive bacteria			Gram-negative bacteria		
	M. luteus	*S. aureus*	*B. cereus*	*P. aeruginosa*	*S. choleraesuis*	*E. coli*
H. sabdariffa						
2	13^{++}	9^{++}	9^{++}	9^{++}	8^{++}	8^{++}
1	7^{+}	–	7^{+}	–	–	–
Streptomycin	20	16	18	14	13	14
H. tiliaceus						
2	8^{+}	9^{++}	7^{+}	–	–	–
1	8^{+}	9^{++}	7^{+}	–	–	–
Streptomycin	21	17	17	14	13	14

Antibacterial activity is categorized as moderate $^{++}$ for inhibition 50 < 70% or weak $^{+}$ for inhibition < 50% based on inhibition percentage compared to streptomycin.

Adding 1 mM of EDTA to the agar slightly enhanced the antibacterial activity of leaves of *H. sabdariffa* on Gram-negative bacteria (Table 7). At 2 mg/disc, extracts of *H. sabdariffa* moderately inhibited *E. coli* and

weakly inhibited *P. aeruginosa.* At 1 mg/disc, leaves of *H. sabdariffa* weakly inhibited *P. aeruginosa* which showed no inhibition without EDTA. Leaves of *H. tiliaceus* showed no activity on Gram-negative bacteria at extract concentrations of 1 and 2 mg/disc. This study also yielded other interesting results. EDTA adversely affected the bactericidal activity of streptomycin. With *S. choleraesuis*, the antibiotic showed no zones of inhibition. In the absence of EDTA, the antibiotic yielded zones of inhibition of 13 mm. With *P. aeruginosa*, EDTA enhanced the bactericidal effect of streptomycin as the zone of inhibition was enlarged to 22 mm compared to 14 mm without EDTA. Haque and Russell (1974) have reported that EDTA can permeabilise the outer membrane of *P. aeruginosa*, making the bacteria susceptible to antibiotics and certain antiseptic agents. Sensitivity of Gram-negative bacteria to antibacterial agents is mainly attributed to increasing permeability of the outer membrane and releasing endogenous phospholipases degrading membrane lipids (Alzoreky and Nakahara, 2003). In this study, adding 1 mM of EDTA to the agar rendered streptomycin ineffective against *S. choleraesuis* but enhanced the efficiency of the antibiotic against *P. aeruginosa*. This observation has not been reported before. Alzoreky and Nakahara (2003) had reported that EDTA inhibited the growth of *B. cereus* while *S. aureus* grew prolifically.

Table 7. Antibacterial activity of leaves of *Hibiscus sabdariffa* and *Hibiscus tiliaceus* on Gram-negative bacteria with 1 mM of EDTA added to the agar (fresh weight)

Species	Extract (mg/disc)	Zone of inhibition in mm (inhibition %)		
		Gram-negative bacteria		
		P. aeruginosa	*S. choleraesuis*	*E. coli*
H. sabdariffa	2	9^{+}	8	8^{++}
	1	7^{+}	–	–
H. tiliaceus	2	–	–	–
	1	–	–	–
Streptomycin		22	–	15

Based on inhibition percentages compared to streptomycin, antibacterial activity is categorized as moderate $^{++}$ for inhibition 50 < 70%, or weak $^{+}$ for inhibition < 50%.

Conclusion

Of six *Hibiscus* species screened, leaves and flowers of *H. tiliaceus* showed outstanding AOP. Leaves of species with high TPC, AEAC and FRP had low FIC ability. Red flowers with high TAC is positively correlated with FIC ability and LPI activity. Based on AOP of leaves and flowers, the six species screened can be categorized into species with comparable values in leaves and flowers, species with significantly higher values in leaves than flowers, and species with significantly higher values in flowers than leaves. Of four species of *Hibiscus* tested, leaves of *H. tiliaceus* had the strongest AT activity. With strong AOP and AT activity, leaves of *H. tiliaceus* have potentials to be developed into functional food and skin care products. At 1 mg extract/disc,

leaves of *H. sabdariffa* were found to inhibit Gram-positive bacteria of *M. luteus*, *S. aureus* and *B. cereus* but not Gram-negative bacteria of *P. aeruginosa*, *E. coli* and *S. choleraesuis.* At 2 mg extract/disc, leaves displayed inhibition on all six Gram-positive and Gram-negative bacteria. Adding 1 mM of EDTA to the agar slightly enhanced the antibacterial activity of leaves against Gram-negative bacteria. This is the first report of leaf extracts of *H. sabdariffa* inhibiting Gram-negative bacteria. With a wide spectrum of inhibition against both Gram-positive and Gram-negative bacteria, leaves of *H. sabdariffa* are worthy of further investigation as a natural wide spectrum antibacterial agent.

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