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Full Length Research Paper

Antioxidant, anti-cholinesterase and antibacterial activities of the bark extracts of *Garcinia hombroniana*

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This study reports an antioxidant, anti-cholinesterase, antibacterial activities and total flavonoids content of different extracts (water, methanol, ethyl acetate, dichloromethane and *n*-hexane) of the bark of *Garcinia hombroniana*. The antioxidant activity was evaluated using radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP) and Folin Ciocalteu assay (FCA). Ethyl acetate extract showed the highest antioxidant activity (DPPH = 8667.7 ±166.6 µmol TE/g (trolox equivalent), ABTS (IC50 = 3.03 µg/ml), FRAP (5579.8 ± 117.7.7 µmol TE/g) and FCA = 3320.4 ± 98.3 µmol GAE/g (gallic acid equivalent)). The flavonoids content of ethyl acetate extract is 2385.7 ± 87.3 RE/g (rutin equivalent) and 3317.6 ± 131.0 QE/g (quercetin equivalent) while the methanol extract is 2234.0 ± 90.0 RE/g and 3090.0 ± 135.1 QE/g). Both extracts are statistically close to each other. In anti-cholinesterase study, ethyl acetate extract showed the highest activity against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes with IC_{50} of 13.73 ± 1.56 and 32.17 ± 0.36 µg/ml, respectively. Dichloromethane and *n*-hexane extracts showed good antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*. The results of this study showed that the bark extracts of *G. hombroniana* have valuable pharmacological activities.

Key words: *Garcinia hombroniana*, bark extracts, antioxidant, anticholinesterase, antibacterial.

INTRODUCTION

Garcinia is a genus of plant belonging to the family Clusiaceae. These plants grow in the form of medium height trees or shrubs and are native to the regions of Asia, Northeast Australia, Madagascar, West Polynesia, Southern Africa and Tropical America (Patil, 2005; Xiwen et al., 2007). Many parts of the plant species of *Garcinia* such as the fruits, leaves, flowers, stem and bark have been used traditionally to treat various diseases including abdominal pain, dysentery, diarrhea, suppuration, infected wound, leucorrhoea, chronic ulcer and gonorrhea gonorrhea (Morton, 1987; Balemba et al., 2010; Moongkarndi et al., 2004a; Pierce, 2003; Braide, 1993; Garnett and Sturton, 1932). The fruit of many *Garcinia* species are edible and are commonly referred to as mangosteens.

Various biological and pharmacological activities have been reported on the species of *Garcinia*. These include anti-HIV (Chen et al., 1996), anticancer (Liu et al., 2010), antioxidant (Chiang et al., 2003), anti-tuberculosis (Lin et al., 2001), antifungal (Selvi et al., 2003), antibacterial (Negi et al., 2008) and the ability to prevent the formation of acute ulcer (Yamaguchi et al., 2000). The bioactive compounds reported from the plants are xanthones (Bennett and Lee, 1989), flavonoids (Kaikabo et al., 2009) and benzophenones (Williams et al., 2003). Among

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the many species of *Garcinia*, *Garcinia hombroniana* appeared to be the one which is rarely studied. The local name of the plant is “manggis hutan”, translated as “jungle mangosteen”. The fruits are edible, while the roots and leaves are used to relieve itching (Gimlette and Burkill, 1930). There has been an account on the low-density lipoprotein (LDL) antioxidation and antiplatelet aggregation activities of the compounds isolated from the twigs of *G. hombroniana* (Saputri and Jantan, 2012). Other reports on the randomly isolated compounds from the plant did not show any significant biological activities (Rukachaisirikul et al., 2000, 2005). In view of the limited amount of data concerning the biological and pharmacological potential of the plant, the present study is carried out to evaluate the antioxidant, anti-cholinesterase and antibacterial activities of the bark extracts of *G. hombroniana*.

EXPERIMENTALS

Chemicals

All the chemicals used in the antioxidant and antibacterial assays were purchased from Sigma Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) companies. Acetylthiocholine iodide (ATCl), acetylcholinesterase from electric eel (AChE), 5,5’-dithiobis[2-nitrobenzoic acid] (DTNB), butyrylcholinesterase from equine serum (BChE), *S*-butyrylthiocholine chloride and physostigmine for anti-Alzheimer’s activity were purchased from Sigma Chemicals (St. Louis, MO, USA). All the chemicals were of analytical grade.

Plant collection and extraction

Plant was collected from the Botanical Garden, Penang where a voucher specimen (PBGK12) was deposited. The air dried bark of *G. hombroniana* was extracted using Soxhlet with solvents of increasing polarity in a successive manner. The solvents used were *n*-hexane, dichloromethane, ethyl acetate and methanol. The extracts obtained were concentrated *in vacuo* at 40°C prior to drying with gaseous nitrogen.

Antioxidant assays

DPPH radical scavenging capacity assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay was carried out according to the method described by Brand-Williams et al., (1995) and modified by Thaipong et al., (2006). In brief, DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol. Prior to conducting the assay, a working solution with an absorbance of approximately 1.1 at 515 nm was prepared by diluting 10 ml stock solution with 45 ml methanol. A series of Trolox standard solutions in the concentration range of 50 to 800 µM was prepared. For the assay of plant extracts, samples were prepared at a concentration of 100 µg/ml. An aliquot of 150 µl of each standard solution (trolox) and samples (extracts) was added to 2850 µl of the DPPH solution and the mixture was allowed to incubate in the dark at room temperature for 24 h. The absorbance was then taken at 515 nm. A vehicle blank was prepared using methanol to serve as the control. The inhibition percentage was calculated according to the following formula:

$$\% \text{ inhibition} = \text{Acontrol} - \frac{\text{Asample}}{\text{Acontrol}} * 100$$

The capacity of the extracts to reduce DPPH was obtained from the standard curve and the results are expressed as µmol trolox equivalence (TE)/g of extract.

ABTS radical scavenging assay

ABTS radical scavenging assay was carried out in accordance to the method described by Thaipong et al., (2006) with some modifications. Stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulfate were prepared. Prior to the assay, both solutions were combined at equal volume and the mixture was allowed to react in the dark for 16 h to produce ABTS radical cation. Then, 1 ml of the solution was diluted with deionized water until an absorbance reading of approximately 0.73 was attained at 734 nm. A 150 µl aliquot of each sample with the concentration of 0.25, 1.5, 2.5, 5, 10, 15 and 25 µg/ml were added to 2850 µl of the diluted ABTS solution and the absorbance was measured at 734 nm after 6 min. Standard solutions of trolox and gallic acid were also prepared for comparison. A dose-response curve was constructed by plotting percentage inhibition against the concentration of the samples or standard. The concentration required to scavenge 50% of the free radicals (IC_{50}) was calculated using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, USA).

Ferric ion reducing antioxidant power (FRAP) assay

FRAP assay was done according to the method described by Benzie and Strain (1996) with some modifications. Working solution of FRAP was prepared by mixing 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3.6H_2O$. The freshly prepared mixture was warmed to 37°C prior to use. Standard solution of trolox was prepared in the concentration range of 50 to 800 µM. The samples were prepared at 100 µg/ml. 150 µl aliquot of each standard solution and samples was then added to 2850 µl of the FRAP reagent and the mixture was allowed to react in the dark at room temperature for 2 h. The absorbance of the product was then measured at 593 nm. Results were expressed in µmol TE/g of extract.

Folin-Ciocalteu assay (Total phenols assay)

The total phenols assay was performed according to the method described by Swains and Hillis (1959) and modified by Thaipong et al., (2006). Gallic acid standard solution in the concentration range of 50 to 1000 µM was used to construct the calibration curve. The samples were prepared at 100 µg/ml. A volume of 150 µl of each standard solution and samples was combined with 2400 µl of deionized water and 150 µl of 0.25 N Folin-Ciocalteu reagent and the mixture was allowed to react for 3 min. Then, 300 µl of 1 N Na_2CO_3 solution was added and the mixture was allowed to incubate for 2 h at room temperature (25°C) in the dark. Absorbance was then measured at 725 nm. The results were represented as µmol gallic acid equivalent (GAE)/g of extract.

Total flavonoids content analysis

The flavonoids content of the extracts of *G. hombroniana* was determined using the colorimetric assay described by Zhishen et al., (1999). In brief, a series of rutin and quercetin standards were prepared in the concentration range of 50 to 1000 µM. The extracts

have a concentration of 500 µg/ml. A volume of 0.5 ml of each standard and extracts was added to 4.5 ml of distilled water in a 10ml vial. 0.3 ml of 5% (w/v) $NaNO_2$ was added to the vial and mixed well. After 5 min, 0.6 ml of 10% (w/v) $AlCl_3$ was added followed by a waiting time of another 6 min. 2 ml of 1 M NaOH was then added and the solution was made up to the volume of 10 ml with distilled water. The absorbance was measured at 510 nm. The total flavonoids content of the extracts was expressed as µmol RE/g and µmol QE/g extract.

Cholinesterase enzymes inhibitory assay

Cholinesterase enzymes inhibitory potential of the test samples were determined by Ellman's microplate assay (Ellman et al., 1961) with some modification. Physostigmine was used as positive control. The test samples and physostigmine were prepared in DMSO at the initial concentration of 1 mg/ml. The concentration of DMSO in final reaction mixture was 1%. At this concentration, DMSO has no inhibitory effect on both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes.

AChE inhibitory assay

In this assay, 140 µl of 0.1 M sodium phosphate buffer (pH 8) was added to 96 wells microplate followed by 20 µl of test samples and 20 µl of 0.09 units/ml AChE enzyme. Then, 10 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was added into each well followed by 10 µl of 14 mM of acetylthiocholine iodide. Absorbance of the coloured end product was measured using Tecan Infinite 200 Pro Microplate spectrometer at 414 nm, 30 min after initiation of the enzymatic reaction.

Butyrylcholinesterase (BChE) inhibitory assay

BChE inhibitory assay adopt the same procedures as described earlier with BChE from equine serum as enzyme and *S*-butyrylthiocholine chloride as substrate. Absorbance of the test samples was corrected by subtracting the absorbance of their respective blank. A set of five concentrations was used to estimate the 50% inhibitory concentration (IC_{50}).

Antibacterial assay

Bacteria culture and preparation

The antibacterial activities of *G. hombroniana* extracts were evaluated on four types of bacteria, namely, *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 19659), *Pseudomonas aeruginosa* (ATCC 17588) and *Escherichia coli* (ATCC 25922). Prior to the assay, an inoculum of bacteria was prepared in Müller-Hinton broth from the bacteria culture and the inoculum was diluted to the turbidity of 0.5 McFarland Standard. The standardized bacteria inoculum was then used for both disc diffusion and minimum inhibitory concentration (MIC) assays.

Disc diffusion method

Approximately 400 µl of the bacteria inocula was pipetted and spread onto the agar prepared in a Petri dish. Separately, an aliquot of 10 µl extract with a concentration of 3 mg/ml was added to filter paper discs of 6 mm diameter. The discs were then placed on the inoculated agar. The Petri dish was incubated at 37°C for 24 h. Diameters of the clear inhibition zones were then measured. Vancomycin and streptomycin were used as positive control while the carrier solvent was used as the negative control.

MIC assay

Plant extracts prepared in DMSO were diluted in the culture broth prior to transferring into a 96-well plate. The final concentration of the samples were 62.5, 125, 250, 500, 1000 and 2000 µg/ml. The 96-well plate was then allowed to incubate at 37°C for 24 h. At the end of the incubation period, 50 µl of a freshly prepared solution of *para*-iodonitrotetrazolium (INT) with the concentration of 200 µg/ml was added to all wells and the plate was returned to incubation for another 30 min. Bacteria inhibition was evaluated by observing the colour changes in each well. MIC of the plant extract was regarded as the lowest concentration that inhibits bacteria growth. The inhibition results were compared with Vancomycin and Streptomycin.

Statistical analysis

All data were analyzed and expressed as means ± standard deviation of three replicates (n = 3). The differences between the assayed values of the various extracts were analyzed using one-way analysis of variance (ANOVA) which is a parametric test, followed by Tukey's honestly significant difference (HSD) Test at 95 and 99% confidence interval. Results with $p < 0.05$ were considered significant, while those with $p < 0.01$ were regarded as very significant. This analysis was carried out using Statistical Package for Social Sciences (SPSS) software, version 18.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Antioxidant activities and total flavonoids content

Antioxidants are gaining an increasing amount of attention in the recent decades as clinical trials and epidemiological studies have found a close correlation between the intake of fruits and vegetables rich in antioxidants to the reduction of risk in several chronic diseases, such as cancer, inflammation and atherosclerosis (Kris-Etherton et al., 2002; Gosslau and Chen, 2004; Podsędek, 2007; Asgarpanah and Ramezanloo, 2012). Although, several synthetic antioxidants are available to date such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), their applications are limited due to controversies over their tumour promoting properties (Hocman, 1988). As such, recent efforts have been concentrating on searching for safer antioxidants, particularly those of natural product origin.

In the present study, the bark extracts of *G. hombroniana* were evaluated for their antioxidant potential *in vitro*. Three assays, namely the DPPH, FRAP and Folin-Ciocalteu assays indicated that all polar extracts possessed antioxidative properties. The order of the antioxidative capacity of the extracts was found to be: ethyl acetate extract > methanol extract > water extract (Table 1). The non-polar extracts, such as the *n*-hexane and dichloromethane extracts, were found to have poor antioxidative effect. The ability of the plant extracts to

Table 1. Antioxidant activities of the various extracts of *G. hombroniana*.

Extract	DPPH assay (µmol TE/g)	FRAP assay (µmol TE/g)	Total phenols assay (FCA) (µmol GAE/g)	Total flavonoids assay	
				µmol RE/g	µmol QE/g
Water	7220.1±19.8*	4127.5±74.7	2312.9±94.0	1716.1±27.3*	2313.1±40.9
Methanol	7859.2±69.6*	4709.6±88.0	3320.0±88.8	2234.0± 90.0	3090.0±135.1
Ethyl acetate	8667.7±166.6*	5579.8±117.7*	3320.4±98.3	2385.7 ± 87.3	3317.6±131.0
Dichloromethane	<500	<2000	<600	-	-
n-Hexane	<500	<2000	<600	-	-

Results are mean values of 3 replicates ± SD. *The mean difference is statistically significant ($p < 0.05$).

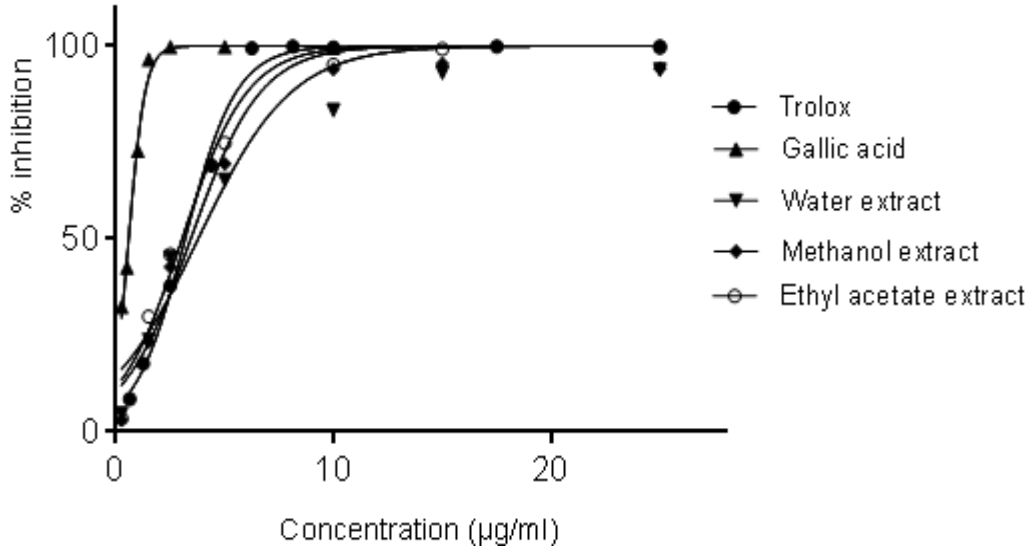


Figure 1. ABTS inhibition profile of the bark extracts of *G. hombroniana*.

inhibit $ABTS^{\cdot+}$ free radicals were further evaluated at seven different concentrations (Figure 1). The ethyl acetate, methanol and water extracts were found to have a comparable inhibition profile with IC_{50} of 3.03, 3.43 and 3.77 µg/ml, respectively as compared to 3.17 µg/ml for trolox. Complete inhibition of the $ABTS^{\cdot+}$ free radical was attained with 10 µg/ml of ethyl acetate and methanol extracts, and 15 µg/ml of water extract, while a slightly lower concentration of trolox 6.25 µg/ml was needed to inhibit all free radicals. On the other hand, all three extracts were found to have much lower antioxidative capacities as compared to gallic acid (IC_{50} = 0.58 µg/ml).

The polar extracts which showed strong antioxidative capacities were further evaluated for total flavonoids content (TFC). The methanol and ethyl acetate extracts were found to have almost equal content of flavonoids (Table 1). Statistically, the differences between the results observed for these two extracts were not significant ($p > 0.05$). As such, the strong antioxidative effect observed in all these three extracts may be correlated to their total flavonoids content.

Cholinesterase enzymes inhibitory assay

Alzheimer Disease (AD) is a chronic and progressive neurodegenerative disorder of the brain which is characterized clinically by deterioration in activities of daily living, behaviours and cognition (Grossberg, 2003). According to the cholinergic hypothesis, memory impairment in patients suffering from AD resulted from decreased level of neurotransmitter acetylcholine. Thus, attempts of researchers are to discover cholinesterase inhibitors in order to sustain the concentration of acetylcholine in the synaptic cleft and prolong its effect (Francis et al., 1999). Table 2 summarizes the IC_{50} and the selectivity index of examined bark extracts and standard drug, physostigmine. Among all the examined extracts, ethyl acetate extract demonstrated the strongest inhibitory activity against AChE and BChE (13.73 ± 1.56, 32.17 ± 0.36 µg/ml), in which AChE is approximately three times more potent than BChE. The dichloromethane extract showed a reasonable inhibition against BChE only (22.35 ± 1.4 µg/ml), while methanol and water extracts

Table 2. Anticholinesterase activity of bark extracts of *G. hombroniana*[1-2].

Extract	% inhibition at 50 µg/ml		IC_{50} (µg/ml)		Selectivity[a,b]	
	AChE	BChE	AChE	BChE	AChE	BChE
Dichloromethane	56.71[c]	66.71[d]	44.93[d]±5.76	22.35[b]±1.41	0.49[a]	2.01[c]
Methanol	54.87[b]	50.16[b]	43.04[c]±2.07	48.93[d]±7.09	1.13[b]	0.87[b]
Ethyl acetate	96.57[d]	53.83[c]	13.73[b]±1.56	32.17[c]±0.36	2.34[c]	0.43[a]
Water	53.46[a]	24.92[a]	50.84[e]±0.28	ND	-	-
Physostigmine**	-	-	0.037[a]±0.004	0.091[a]±0.003	2.40[c]	0.41[a]

[1]Values are mean ± standard deviation of three replicates (n = 3), ND = not detected. [2]Values in the same column with different alphabet superscripts are significantly different ($P < 0.05$) from each other, [a]selectivity for AChE is defined as IC_{50} (BChE)/IC_{50} (AChE), [b]Selectivity for BChE is defined as IC_{50} (AChE)/IC_{50} (BChE), "**" = positive control.

Table 3. Antibacterial activity of the bark extracts of *G. hombroniana.*

Extract	Disk diffusion (mm)				Minimum inhibitory concentration (MIC) (µg/ml)			
	S. aureus	*B. subtilis*	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*E. coli*
n-hexane	8	7.5	9.5	-	500	250	>2000	>2000
Dichloromethane	9	9.5	8	8	250	62.5	62.5	2000
Ethyl acetate	7	-	-	8.5	1000	500	>2000	>2000
Methanol	-	-	-	-	>2000	>2000	>2000	>2000
Water	-	-	-	-	>2000	>2000	>2000	>2000
Vancomycin*	16	26	-	12	>2000	400	400.0	400
Streptomycin*	16	23	-	15	>2000	>2000	>2000	>2000

Results are mean values of triplicate assays,- = no inhibition observed (6 mm), * = positive control.

showed very weak inhibition.

Antibacterial activity

The various extracts of *G. hombroniana* were tested for antibacterial activities *in vitro* using the disc diffusion technique and MIC assay. Results in Table 3 show that *n*-hexane extract (3 mg) was able to inhibit the growth of *S. aureus*, *B. subtilis* and *P. aeruginosa* on agar plates with an inhibition zone of 7.5 to 9.5 mm. Further evaluation of the *n*-hexane extract showed MIC values of > 100 µg/ml, indicating weak antibacterial activity. The dichloromethane extract (3 mg) was found to be active against all the four types of bacteria by disc diffusion, with inhibition zones between 8 and 9.5 mm. Further evaluation revealed that the dichloromethane extract was able to inhibit the growth of *B. subtilis* and *P. aeruginosa*, with a MIC value of 62.5 µg/ml. The ethyl acetate extract was found to inhibit the growth of *S. aureus* and *E. coli* with an inhibition zone of 7 and 8.5 mm in disk diffusion method. However, in MIC method the antibacterial effect was found to be very weak. The water and methanol extracts were inactive against all the four bacteria strains.

Conclusion

The various extracts of the bark of *G. hombroniana* were evaluated for antioxidant, anti-cholinesterase and antibacterial activities. The study showed that ethyl acetate extract has good antioxidant and anti-cholinesterase activities, while the dichloromethane extract has good antibacterial activity. However, the methanol and water extracts showed lower antioxidative capacities and they were weak cholinesterase inhibitors. The *n*-hexane extract was found to be inactive in all three biological studies. This study pointed out the potential of *G. hombroniana* as a new source of phytomedicine for treatment of Alzheimer's disease, bacterial infection and other diseases which occur from oxidative stress. Further work to isolate and characterize the constituents responsible for the biological activities of the plant is currently ongoing in our laboratory.

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