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In-vitro Antioxidant Activities, Phytoconsituent and Toxicity Evaluation of Local *Bougainvillea glabra* Bract (Bunga Kertas)

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Abstract : The ethanolic extract of *B. glabra* bract (EEBGB) was evaluated for antioxidant properties using 4 different antioxidant pathways, quantification of phenolics, flavanoids and betalains. The EEBGB showed high antioxidant activity (IC_{50} <1.0 mg/mL) in DPPH free radical scavenging activity and superoxide radical scavenging. The EEBGB possessed good reducing power in FRAP (105.37 ± 5.3 mg TE/100 g of extract) and antioxidant capacity with ORAC score of 166,920 ± 27,962 µM TE/100g. Preliminary phytochemical screening on the bract indicated presence of flavanoid, tannins, steroids and triterpenes. The EEBGB also exhibited high phenolic content and flavonoid content of 76.74 ± 2.38 GAE mg/100 g and 250.10 ± 22.59 QE mg /100 g respectively. The total betalain content in EEBGB was found to be low. HPLC-PDA analysis showed the presence of quercetin as primary flavonoid constituent which confered antioxidant activity to the extract. Further evaluation of the cytotoxicity showed that the EEBGB is free from toxicity against WRL-68 human liver and monkey kidney vero cell with IC_{50} > 1000 ug/mL. Moreover, *B. glabra* bract had minimal aerobic microbe count and no heavy metal contamination. Thus, natural antioxidant substances in *B. glabra* bract scavenge excess free radicals and prevent oxidative damage, slows down the onset of premature aging symptoms and prevent degenerative chronic diseases. Therefore, the local B. glabra bracts can serve as a natural source of antioxidant in food and nutraceutical product development.

Key words: Antioxidant Activities, Phytoconsituent, Toxicity Evaluation of Local *Bougainvillea glabra*, Bunga Kertas.

Introduction

Natural antioxidants are gaining popularity due to the unwanted side-effects of synthetic antioxidants which cause adverse health conditions. Safe and effective natural polyphenolic antioxidants are found

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abundantly in plants. Clinical and epidemiology studies reported that there is an inverse co-relation between degenerative diseases and intake of natural antioxidant from plant foods[1].

Many reports and papers have highlighted the advantages of dietary intake of antioxidants from plant sources such as fruits, vegetables, cereals, nuts and grains. However, the antioxidant properties of an ornamental and flowering plant has been poorly studied. Natural antioxidants are primarily plant phenolic compounds occurring in all plant parts (wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen, and seeds)[2]. Flower is an important part of plant which contains a great variety of natural antioxidants, such as phenolic acids, flavonoids, anthocyanin and many other phenolic compounds[3]. Flower of plants have traditionally been used in many types of cooking to promote health. Flowers used in Traditional Chinese Medicine (TCM) has been documented in ancient books such as "Ben Cao Gang Mu" and "Yang Sheng Lu"[4]. It is important to identify and evaluate antioxidant potential of new sources as a beneficial contribution towards human health.

Figure 1. *Bougainvillea glabra* (flower and bract)

Bougainvillea glabra from the family Nyctaginanceae, is a popular flowering ornamental and edible plant[5] found growing throughout Malaysia. It is cultivated all over Malaysia for ornamental purposes. *B. glabra* has thin papery petaloid bracts with varying vibrant colours locally known as 'bunga kertas'. There is great interest in *B. glabra* bracts, a rich source of polyphenolic compounds, which act as natural antioxidants with health promoting properties[6]. Thus, the objectives of this study were to evaluate the antioxidant activities, quantification of phytoconsituent and toxicity of *B. glabra* bract.

Materials and Methods

Plant Material and Sample preparation

400 g fresh flowers of *B. glabra* were collected from a home garden at Taman Bersatu, Rawang. The collected flowers were processed to separate the bracts. All bracts were oven dried at 30 ^{0}C ± 1 ^{0}C for 72 hours. The dried material was then pulverized and passed through a sieve to obtained fine particles and was stored in air tight container 4 °C until further uses.

Extraction of polyphenolics

Ten grams of powdered *B. glabra* bracts was extracted with 1000 mL absolute ethanol analytical grade was purchased from Fisher Scientific. The mixture was placed on shaker for 72 hour at room temperature. Successive extractions were carried out to achieve maximum yield. The extract were then filtered and concentrated under reduced pressure using a rotary evaporator. The ethanolic extract of *B. glabra* bract (EEBGB) was then stored at 4°C until further analysis.

Antioxidant Evaluation

1, 2-Diphenyl-2-picrylhydrazl (DPPH) free radical scavenging system

The anti-free radical assay was carried out based on the method of Markandan et al [7]. The DPPH free radical scavenging activities of *B. glabra* compared in terms of their IC_{50} (mg/mL) values. All measurements were done in triplicate.

Xanthine/Xanthine oxidase superoxide scavenging activity (XOD)

The assay system was performed to evaluate scavenging activity of the EEBGB against superoxide free radical anions was determined by spectrophotometric method and previously described in detail by Vimala et al[8].

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was carried out based on the method of Musa et al[9].

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed as described by Huang et al.[10], with some modifications. AAPH (0.65 g) was dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 240 mM (made fresh daily). A fluorescein stock solution (1 mM) was made in 75 mM phosphate buffer (pH 7.4) and was diluted to ratio of 1:100 000. To the experimental wells, 150 µL of working sodium fluorescein solution was added. The blank wells received 25 µL of PBS. The sample wells received 25 uL of EEBGB. The plate was then allowed to equilibrate by incubating for 10 minutes at 37^0C. BMG Omega FLUOstar spectrophotometer with injectors was used with an excitation filter of 485±20 nm bandpass and emission filter of 528±20 nm bandpass. Reactions were initiated by the addition of 25 µL of AAPH solution using the microplate reader's injector for a final reaction volume of 200 µL. The fluorescence was then monitored kinetically with data taken every minute. ORAC values were calculated using MARS Data Analysis Reduction Software.

Content of Antioxidant Compounds

Preliminary Phytochemical Screening (Qualitative Analysis)

B. glabra bract was screened for alkaloids, flavonoids, saponions, tanins, steroids and triterpenoids. The preliminary phytochemical screening was carried out in FRIM phytochemical laboratory according to in-house standard procedure.

Determination Total Phenol Content (TPC) and Total Flavonoid Content (TFC)

Antioxidant activity was determined using the method described by Musa et al[9]. The calibration curve of gallic acid (GA) was used for the estimation of EEBGB activity capacity. The TFC was determined by the colorimetric method as described by Eghdami and Sadeghi[11]. A total of 100 µL EEBGB was transferred into microplate wells, followed by the addition of 0.03 ml of 10% (w/v) $NaNO_2$ solution. After 5 min of incubation, 0.3 mL of 10% $AlCl_3 \cdot 6H_2O$ solution was added, and the reaction was allowed to stand for another 5 min before 0.2 ml of 1% NaOH was added. The mixture was mixed well by vortexing, and the absorbance was measured immediately at 510 nm using FLUOstar Omega spectrophotometer.

Determination of Betalain Content

The content of betaxanthins and betacyanins in the EEBGB was determined according to the methods of Ravichandran et al.[12].The EEBGB (100 µL) was dissolved in 3.9 ml distilled water and measured at 538 nm and 480 nm respectively using UV–Vis spectrometer. The betalain content was calculated from the equation (mg/L) = [(A×DF×MW×1000) / (e)], where A is the absorption, DF the dilution factor and l the pathlength (1 cm) of the cuvette. For quantification of betacyanins and betaxanthins, the molecular weights (MW) and molar extinction coefficients (e) respectively are (MW=550g/mol; e= 60,000 L/mol cm in H2O) and (MW=308 g/mol; e=48,000 L/mol cm in H2O) were applied.

High performance Liquid Chromatography (HPLC) analysis

Antioxidant testing and HPLC profiling based on retention time were carried out on 6 polyphenolic compound standards. 10.0 mg of EEBGB was weighed in a glass tube followed by addition of 10.0 mL diluent (50% aqueous methanol) and the solution was homogenized. The EEBGB was then filtered through a 0.45 µm nylon membrane filter (Whatman, USA) prior to injection to HPLC. Standard addition technique was used for compound confirmation by spiking 5 µg/mL of pure standard into positive sample.

The EEBGB was analysed by a HPLC system (Waters Delta 600 with 600 Controller) with photodiode array detector (Waters 996). A Phenomenex-Luna (5 µm) column was used (4.6 mm i.d. x 250 mm)from Waters (USA) and for elution of the constituents, a gradient of two solvents denoted as A and B was employed. A was 0.1 % aqueous formic acid, whereas B was 0.1 % formic acid in acetonitrile. Initial conditions were 85 % A and 15 % B, with a linear gradient reaching 25 % B at t=12 min. This was followed by an isocratic elution until t=22 min, after which the programme returned to the initial solvent composition at t=25 min and continued until t=35 min. The flow rate used was 1.0 ml/min and the injection volume was 10 µl. Detection was carried out at 300 nm.

Toxicity Evaluation

Cytotoxicity Evaluation

WRL-68 and Vero cells, which are liver- and kidney-like cells, respectively, was used in this study. Cells were seeded in a 96-well plate at the density of 6×10^3 cells/well in 100 µL/well of complete culture medium. Following an overnight incubation to allow cell attachment, the culture medium was removed and the cells were washed with 150 µL/well of phosphate buffered saline (PBS). The cells were then be treated with the EEBGB at the desired concentrations in a total volume of 200 µL/well of phenol red-free medium containing 2% foetal bovine serum (FBS) (unless otherwise stated). The cells were exposed to the test samples for 72 hours at 37°C. After the treatment period, cell viability was measured by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay as described by Mosmann[13]. MTT (approximately 0.2 mg/mL, 40 µL/well) was added to each well and incubated at 37°C for 150 minutes. After the incubation time, medium containing MTT was removed and the formazan crystals formed was dissolved with 100 µL/well of DMSO. Absorbance was measured at 570 nm with a spectrophotometer. Dose-response curve for EEBGB was plotted and the median inhibition concentration (IC_{50}) was determined by non-linear regression using a variable slope model (GraphPad Prism 5, GraphPad Software Inc., California, U.S.A).

Heavy Metal Analysis and Microbial load

Microbial load count and heavy metal analysis for *B. glabra* bract was carried out by the Natural Products Quality Control Laboratory using in-house method, at the Forest Research Institute of Malaysia (FRIM).

Results and Discussion

Antioxidant Activity

Table 1: Antioxidant activity of EEBGB

Plant sample	DPPH IC_{50} mg/mL	XOD IC_{50} µg/mL	FRAP mg TE/100 g	ORAC µM TE/100 g
EEBGB	0.075±0.24	0.83±0.19	105.37±5.3	166,920±27,962

Values are means ± SD from three independent experiments.

The EEBGB (1mg/mL) was evaluated for antioxidant properties using 4 bioassay systems representing as shown in Table 1. The EEBGB exhibited high activities in all 4 antioxidant pathways. It showed DPPH free radical scavenging activity IC_{50} (0.075 mg/mL), superoxide radical scavenging activity IC_{50} (0.83 mg/mL), FRAP (127.47±10.0 mg TE/100 g of extract), and ORAC (194,540±1,669 µM TE/100 g).

The antioxidant evaluation shows great natural antioxidant potential of *B. glabra* bracts from plant sources. DPPH is a stable free radical, the free radical reducing activity of antioxidants based on one electron reduction. DPPH free radical scavenging activity determines the overall antioxidant potential of the *B. glabra* bracts, which shows its effectiveness, prevention, interception and repair mechanism against injury in a biological system [14,15].

Superoxide anions are by-products produce during proteins and cells metabolism and biochemical functions. Production of superoxide anions in excess are known to cause damage in cells which leads to many

diseases[16] and aging[17]. The superoxide anion scavenging property of *B. glabra* bracts possessed highest activity may be attributed to both neutralization of superoxide anion radicals via hydrogen donation and inhibition of xanthine oxidase by various phenolic present in the extract.

FRAP shows the second line of antioxidant defense where, reductant antioxidants scavenge active free radicals before they can attack target molecules[18]. In this study, the EEBGB were found to have potent FRAP which may be helpful in the prevention of oxidative stress related tissue damage. Individual members of the second line antioxidant defense team are deployed to prevent generation of ROS, to destroy potential oxidants and to scavenge ROS. Thus, oxidative stress induced tissue damage is minimized.

ORAC assay has been broadly in the food and dietary supplement industries as a method of choice to quantify antioxidant capacity[19]. The ORAC value has been used to suggest daily doses of vegetables products in order to meet parallel health protection[20,21].The dose recommended is between 3500 to 5000 ORAC units per day[19]. The current study showed that the *B. glabra* bracts contain potent antioxidant compounds.

Phytoconsituent Analysis

Phytochemical screening

Phytochemical screening is one of the methods that have been used to explore antioxidant compounds in plants. Phytochemicals are the individual chemicals from which plants are made and plants are the key sources of raw material for both pharmaceutical and aromatic industries. The bracts was observed to contain flavonoids, tannins, steroid and triterpenes with no traces of alkaloids and saponins (Table). A previous study by Sheeja et al.[22], the leaves of *B. glabra* are possess tannin flavonoids phenolic compound as major phytochemicals, but found no reports on the bract.

Table 2 Preliminary phytochemical screening of *B. glabra* bract

Phytoconstituents	**Alkaloids**	**Saponins**	**Tanins**	**Flavanoids**	**Steroid**	**Triterpenes**
Observation	No precipitate	No fronth	Brownish green	Light yellow	Brown ring	formation of green precipitate
Presence	-	-	+	++	+	+

(+++) highly presence, (++) moderately presence (+) slightly presence and (-) absence

Determination Total Phenol Content (TPC) and Total Flavonoid Content (TFC)

The EEBGB was further tested for chemical analysis to determine the total phenolic, flavonoid and betalain contents. As shown in Table 2, the EEBGB showed high total phenolic and flavonoid content (76.74± 2.38 GAE mg/100 g and 250.10 ± 22.59 QE mg /100 g respectively. Plant phenolics are known as powerful antioxidants that scavenge free radicals. Phenolics have the ability to neutralize free radicals due their hydroxyl group[23]. TFC was observed to be high, thus indicating that *B. glabra* bracts consist of hydrophobic flavanoids. The least polar quercetin and aglycones of flavanoid group (mythoxylated isoflavones, flavanones and flavonols) may be present in *B. glabra* bracts contributing to its antioxidant activity. Quercetin may be a potential active compound in flowers that scavenge harmful free radical and exhibits anti-oxidative properties[24].

Determination of Betalain Content

The total Betacyanins (red-ultra violet) and Betaxanthins (yellowish) obtained for *B. glabra* bract were measured low at 12.87±0.15 mg/L and 5.98±0.74 mg/L, respectively. This finding may be due to the fact that betalains are water-soluble nitrogen-containing pigments[25]. One of the most important physicochemical properties of betalain plant pigments are their significant polarity and ionization (dissociation, zwitter-ionic behavior) in aqueous solutions. The increased polar character of betacyanins and betaxanthins results in insolubilities in any of the popular organic polar or semi-polar solvents except of water and the mixtures with low-molecular alcohols[26,27].

Table 3 Phytoconsituent profile of *B. glabra* bract

Phyto-constituents		***B. glabra* bracts ethanol extract**
Total Content	Phenolics (GAE mg/100g)	76.74± 2.38
	Flavanoids (QE mg /100 g)	250.10± 22.59
Total Betalains Content	Betacyanins (mg/L)	12.87±0.15
	Betaxanthins (mg/L)	5.98±0.74

Values are means ± SD from three independent experiments.

High performance Liquid Chromatography (HPLC) analysis

The EEBGB was subjected HPLC analysis. The retention times (Rt) of the major peaks in the chromatogram were compared with those of standards phenolic standard compounds (p-hydrobenzoic acid, coumaric acid, quercetin, myceretin, kaemperol and ferulic acid) as shown in Figure 2a. The HPLC profile on EEBGB (1.0 mg/mL) in Figure 2b showed the presence of quercetin at 300 nm which are major antioxidant compounds in plants which have been proposed to have beneficial effects on health human health. Peak 3 in the chromatogram (Rt = 16.853 min) corresponded to quercetin (Rt = 16.930 min) (Figure 2a). The EEBGB was spiked with quercetin (5.0 µL), an increased from 0.1384 au to 0.2872 au in the peak height was observed and the results confirmed the presence of quercetin (Figure 2c). The Rt of other peaks in the chromatogram of *B. glabra* bract did not correspond to the Rt of other standards used. This showed that the antioxidant activity found in *B. glabra* bract is contributed by hundreds of different phenolic compounds, found as antioxidant potent plant polyphenols. Based on the standard curve, quercetin was quantified ($y = 2733.5x - 33014$, $r^2 = 0.997$). The concentration of quercetin in EEBGB was 12.07 µg/mL (0.1207%). Phenolic compounds are of different origins and functions, most of them belong to principal biologically highly active components of plant origin and play vital role of protecting organisms against harmful effects[28].

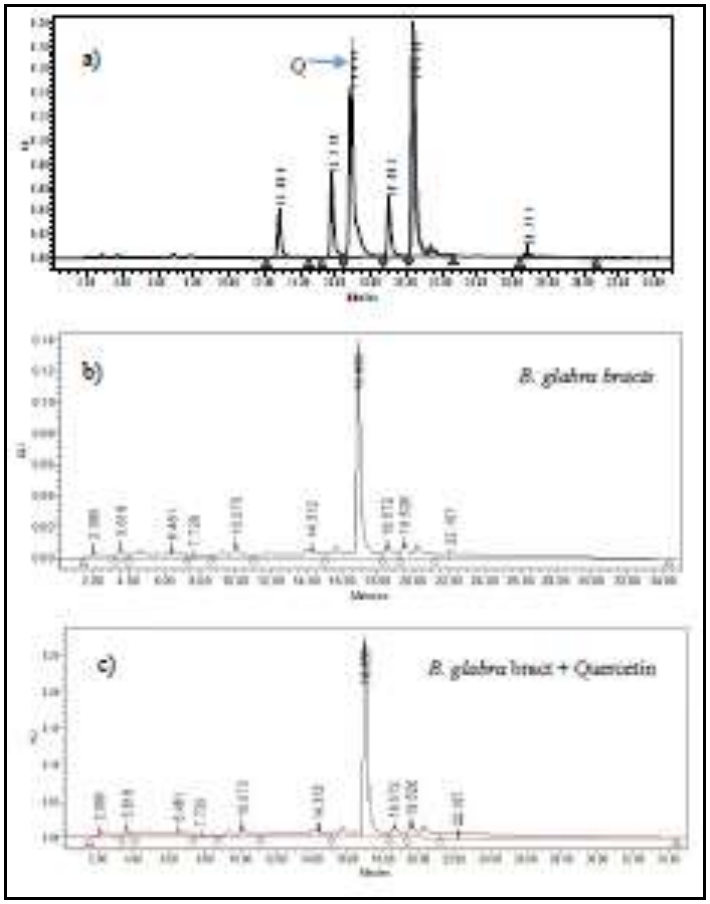

Figure 2 : HPLC Chromatogram of a) Quercetin standard b) *B.glabra* bractc) *B. glabra* bract + quercetin

Toxicity evaluation (Cytotoxicity, Heavy Metal and Microbial Load)

The EEBGB was also tested for cytotoxicity, microbial load count and heavy metals. As shown in Table 3, in the cytotoxicity analysis, the IC_{50} of the extract was found to be high in WRL-68 Human Liver cell lines (135.46 ± 20.43 µg/mL) and in vero monkey kidney cell line (269.10 ± 70.16 µg/mL) respectively, showing that extract has non-toxic effect against normal cell lines. Cell death not in excess of 50% of the negative control is considered free from toxic[29]. Thus, the results demonstrate that the *B. glabra* bract in absolute ethanol without cytotoxicity effect on the WRL-68 human liver and vero monkey kidney cells.

Table 3.Toxicity evaluation profile of *B. glabra* bract

Safety Profile		***B. glabra* bracts**	
		EEBGB	**Raw material**
Cytotoxicity LC_{50} (µg/ml)	WRL-68 Human liver cell	135.46 ± 20.43	NT
	Vero monkey kidney cell	269.10 ± 70.16	
Total Microbial Count cfu/mg	Aerobic microbe	NT	1.8×10^7
	Mould/Yeast		3.1×10^6
Heavy metal mg/kg	Lead (Pb)	NT	0.24
	Mercury (Hg)		0.25
	Cadmium (Cd)		0.01
	Arsenic (As)		0.21

NT= not tested

The heavy metal analysis in raw material of the *B. glabra* bracts (lead, mercury, cadmium and arsenic) showed free from contamination according to heavy metal poison act 1952. In the microbial load count analysis, total microbial and mould/yeast was found to be below permissible level. The *B. glabra* bract found to be safe without apparent contamination of microbial and heavy metal. The cytotoxicity and safety evaluation in the present study showed that, the *B. glabra* bracts is safe for consumption. Thus, the *B. glabra* bracts has great potential to be exploited as new source of natural antioxidants from plant with high content of polyphenolics which help to prevent chronic, degenerative, age-dependent diseases and aging symptoms.

Conclusion

The above investigaton showed that the *B. glabra* bract has potential source of natural antioxidant which neutralizes free radicals which could cause oxidative damage of cell membranes and DNA. This study of *B. glabra* bract indicated the presence of biocompound (quercetin) with possible commercial applications which can be added as ingredient of antioxidant and anti-aging food supplements that has beneficial effects for human health.

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