Antioxidant, Anti-inflammatory, Cytotoxicity and Cytoprotection Activities of *Crassocephalum crepidioides* (Benth.) S. Moore. Extracts and Its Phytochemical Composition

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Abstract

The hexane, ethyl acetate, ethanol and water extracts of *Crassocephalum crepidioides* (Benth.) S. Moore. were assessed for their antioxidant, anti-inflammatory, cytoxicity and cytoprotective activities. The ethanol extract displayed profound antioxidant activities and inhibited the enzymatic activity of 5-lipoxygenase (5-LOX). The ethanol extract of *Crassocephalum crepidioides* (Benth.) S. Moore. at a concentration of 100 µg/ml, was non-toxic against MRC-5 and HepG2 cell lines. At the concentration of 25 µg/ml it gave a percentage of cytoprotection with the value of 69.47%. Phytochemical analysis of the ethanol extract revealed the presence of phenols and flavonoids with 422.22 gallic acid equivalent (mg/g) and 3.46 quercetin equivalent (mg/g). Phenolic compounds and flavonoids impart to *Crassocephalum crepidioides* (Benth.) S. Moore. its biological properties.

1. Introduction

Nature has been a continuous source of pharmacologically active molecules and medicinal herbs have been used by countless human generations [1]. The Asteraceae is one of the two largest families of flowering plants, which consist of more than 25000 species and 1000 genera of flowering plants [2-4]. Many species have been used as sources of rubber, medicines, edible oils, vegetables, pesticides and so on. The species in this family are commonly featured in the literature because of their anti-inflammatory, antioxidant, antimicrobial, analgesic and antipyretic activities [5].

Crassocephalum crepidioides (Benth.) S. Moore. (Asteraceae), known as thickhead or fireweed, is native to South Africa [6]. In Nigeria, the leaves are used to treat gonorrhoea, stomach upset and epilepsy [7]. It is also known in folk medicine in Japan for the treatment of acute hepatitis, fever and edema [8]. In Vietnam, Taiwan, Japan China and Malaysia, this plant is used for food [7, 9]. The plant is known to elaborate pyrrolizidine alkaloid (jacobine) and flavonoids (kaempferol and quercetin) [10-12]. Extracts of this plant elicited hepatoprotective activity and showed moderate antimutagenic activity in *Salmonella typhimurium* TA98 and TA 100 [13-14].

2. Materials and Methods

2.1. Materials

Gallic acid, β-carotene, 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), linoleic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu reagent, tween 80, linoleic acid, neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride), trypsin–EDTA (0.05% wt/vol trypsin, 0.02% wt/vol EDTA), RPMI-1640 medium and tert-butyl hydroperoxide 70% were purchased from Sigma Co. (St. Louis, MO, USA). Human recombinant 5-lipoxygenase enzyme and nordihydroguaiaretic acid (NDGA) were purchased from Calbiochem, Sandiego. FBS (Fetal Bovine Serum) was purchased from GIBCO, USA (cat. no. 10108-165) and pen-strep was purchased from BioWhittaker, USA (cat. no. BE13-114E). Dimethyl sulfoxide (DMSO), potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4), acetic acid, ethanol 97%, chloroform and phosphate buffer saline (PBS) were analytical grade.

2.2. Plant collection and extraction

Crassocephalum crepidioides (Benth.) S. Moore. was collected from Broga, Selangor, Malaysia. Voucher specimen (UNMC47W) was deposited in the herbarium of School of Pharmacy, Faculty of Science, University of Nottingham Malaysia Campus. Air-dried and finely milled samples (300 g) were sequentially extracted by hexane, ethyl acetate, ethanol and water. The extracts were concentrated using rotary evaporator (Buchi, USA) under reduced pressure at 40°C. Dried extracts were kept at -20°C until further tests were carried out. For stock solutions, 100 mg/ml of each extract was dissolved in DMSO (dimethyl sulfoxide).

2.3. Total phenolic contents

The total phenolic contents of the plant extracts were determined using the Folin-Ciocalteu assay, as described by Slinkard & Singleton [15]. Briefly, 20 µl of diluted extracts were mixed with 1.58 ml of water and 100 µl of Folin-Ciocalteu reagent. After standing for 5 minutes at room temperature, 300 µl of sodium carbonate (20% w/v) were added. The solutions were mixed and allowed to stand for 30 minutes at 40°C. Changes in absorbance were determined at λ: 765 nm against a blank using a UV-Vis Spectrophotometer (Biochrom Libra S12, USA). Gallic acid (50, 100, 150, 250 mg/ml) was used for a calibration standard curve. Results were expressed on fresh weight basis of mg gallic acid equivalents/g of sample.

2.4. Total flavonoid contents

Estimation of total flavonoid contents in extracts were carried out using the method described by Froehlicher *et al.* [16]. The flavonoid contents were expressed in mg of quercetin/g of plant extracts.

2.5. Antioxidant assay

2.5.1. Ferric reducing power antioxidant (FRAP) assay.

The antioxidant activity of the extracts was estimated by the FRAP method based on Benzie & Strain [17] with slight modifications. The working FRAP reagent was prepared freshly by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio. Briefly, 180 µl of the FRAP reagent was mixed with 20 µl of the test sample to obtain a final concentration of 1/10. Readings were taken after 90 minutes (λ: 593

nm) using a spectrophotometer (Dynex MRX-Revelation, USA). Ferrous sulphate concentrations in the range 1 µM – 125 µM ($FeSO_4.7H_2O$) were used for calibration. Trolox and quercetin were used as positive controls. The FRAP values were calculated as Ferrous Equivalents: the concentration of trolox/quercetin or extracts which produced an absorbance value equal to 1 mM of $FeSO_4$.

2.5.2. β-Carotene bleaching assay.

The β-carotene bleaching assay was conducted according to Miller [18] with some modifications. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml chloroform. Two ml of this solution was pipetted into a 100 ml round-bottom flask. After removal of chloroform in vacuo, 40 mg of linoleic acid, 400 mg of tween 80, and 100 ml of distilled water were added to the flask with vigorous shaking. The zero time absorbance was measured at λ: 490 nm using a spectrophotometer (Dynex MRX-Revelation, USA). Absorbance readings were recorded at 20 min intervals for 240 minutes. A blank, devoid of β-carotene, was prepared for background subtraction. Percentage of antioxidant activity (AA) was calculated using the following equation (25): % AA = ((DR control-DR sample)/DR control) x 100, where DR is degradation rate of sample (DR = ln (initial absorbance at time zero)/(absorbance at 240 minutes)/t (time in minutes)). EC_{50} (the concentration exhibiting 50% of the percentage antioxidant activity) of samples were calculated from the graph of antioxidant activity percentage against concentration of the extracts.

2.5.3. DPPH assay.

The DPPH assay was conducted according to the method described by Juan-Badaturuge *et al.* [19]. Twenty µl of sample (1 mg/ml to 0.003 mg/ml) were pipetted into each well. One hundred and eighty µl of DPPH (0.1 mM) were added. The plates were kept in the dark at room temperature for 30 minutes. The percentage of decolourisation was measured spectrophotometrically at λ: 550 nm. The DPPH scavenging effect (%) was calculated using the following equation: % Scavenging effect = ((A control- A sample)/A control) x 100.

2.6. Anti-inflammatory assay

The anti-inflammatory activity of *Crassocephalum crepidioides* (Benth.) S. Moore. crude extracts was determined using the method of Baylac & Racine [20] with linoleic acid as the substrate. Crude plant extracts (50 mg/ml) were prepared. Five µl of extract was mixed with 970 µl of phosphate buffer (pH 9) and 17 µl of linoleic acid in a 1 ml cuvette maintained at 25 °C. The mixture was shaken and 4 µl of the aliquoted enzyme and 4 µl of the phosphate buffer (4 °C) were pippeted to initiate enzyme reaction. Absorbance was measured at λ: 234 nm over a period of 10 minutes using spectrophotometer (Libra, USA). Absorbance was plotted graphically against the different concentrations used. Nordihydroguaiaretic acid (NDGA) was used as the positive control. The slopes of the straight-line portions of the sample and the control curves were used to determine the percentage activity of the enzyme [21].

2.7. Cytotoxicity and cytoprotection assays

2.7.1. Cell culture

The human hepatoma cell line (HepG2) and normal fibroblast lung cell line (MRC-5) were used for the cytoprotection assay. The cells were cultivated at 37°C in a humidified incubator

containing 95% air and 5% CO2 , in RPMI-1640 medium (Sigma, Germany) containing 10% inactivated fetal bovine serum (FBS) (Gibco, Tokyo, Japan) with the addition of 1% antibiotic solution (pen-strep).

2.7.2. Cytotoxicity assay

Cytotoxicity assay was conducted against MRC-5 and HepG2 cell lines. One and half ml of a 10^5 HepG2 cells/ml was inoculated into each well of a 24-well flat-bottom microplate, the 24-well microplate was cultivated for 24 h at 37°C in an incubator containing 95% air and 5% CO_2. The medium (1.5 ml) was removed from each well and replaced with 1.5 ml of fresh medium containing the plant extracts/pure compounds/standard and incubated for 24 h at 37°C. Neutral red solution (400 µl) was added after the medium was removed and the plate was kept at 37°C for 1 h. Next, fixative solution (400 µl) was added to stop the reaction and absorbance (540 nm) was measured using a microplate reader.

2.7.3. Cytoprotection assay

One and half ml of a 10^5 HepG2 cells/ml was inoculated into each well of a 24-well flat-bottom microplate, the 24-well microplate was cultivated for 24 h at 37°C in an incubator containing 95% air and 5% CO_2. The medium (1.5 ml) was removed from each well and replaced with 1.5 ml of fresh medium containing the plant extracts/pure compounds/standard. The concentration of the sample used was the concentration which not toxic for the cells, which is obtained from the cytotoxicity assay. Removal of the medium was repeated after 24 hours, 1.5 ml of fresh medium containing 1 mM t-BHP, was added and kept at 37°C under 5% CO2 for 5 h. Five hours later, the medium was discarded and neutral red uptake assay was used to measure the vialibility of the cells. Quercetin was used as the positive control that showed antioxidative effect on cells.

2.8. Statistical analysis

All data were expressed as mean ± standard deviation. Data were analyzed using one way anova followed by Tukey test using GraphPad Prism5 software. A significant difference was considered at the level of $P < 0.01$.

3. Results and Discussion

3.1. Extraction yield, total phenolic and flavonoid contents

Table 1. presents the yield and the total phenolic and flavonoid contents of the extracts. The water extract had the highest yield amongst the four extracts. In the Folin-Ciocalteu assay, gallic acid was used as a standard ($y = 0.0011x + 0.0018$, $R^2 = 0.9996$) and the results were expressed in Gallic Acid Equivalent (mg/g). The total phenolic contents of the four extracts varied from 62 to 422.22 mg/g extract. The total flavonoid content was evaluated using quercetin as a standard ($y = 0.0207x – 0.0202$; $R^2 = 0.9993$, with x as the concentration of quercetin in mg/ml). Our study revealed the presence of high amount of phenolic compounds and flavonoids in the ethanol extracts with value of 422.22 mg/g GAE and 3.46 mg/g quercetin equivalent, respectively.

Table 1.
Extraction yield and the total phenolic and flavonoid contents of the four extracts of *Crassocephalum crepidioides* (Benth.) S. Moore.

Extract	Extraction yield	Total phenolic contents	Total flavonoid contents
	% yield (w/w)	[Gallic Acid Equivalent (mg/g)]	[Quercetin Equivalent (mg/g)]
Hexane	2.50	62.00 ± 0.00A	0.98 ± 0.00A
Ethyl acetate	1.33	307.78 ± 0.00B	0.98 ± 0.00A
Ethanol	4.16	422.22 ± 0.05C	3.46 ± 0.00B
Water	9.07	324.67 ± 0.00D	1.28 ± 0.00C

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same capital letter are not significantly different ($P<0.01$) according to Tukey multiple comparison test.

3.2. Antioxidant activities

Antioxidants play an essential role in the prevention of cardiovascular diseases, cancers and neurodegenerative diseases, as well as inflammation and aging [22]. The antioxidant properties of *Crassocephalum crepidioides* (Benth.) S. Moore. were appreciated using FRAP, DPPH and β-carotene assays. The FRAP assay measures the total antioxidant activity of a sample by the reduction of ferric-tripyridyltriazine to an intense blue colouration ferrous complex, which is measured at λ: 593 nm [23]. The results are defined as FRAP values: the concentrations of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/l $FeSO_4.7H_2O$. Low FRAP values indicate high antioxidant activities. The extracts tested in our study displayed reducing abilities in the range of 1.16 µg/ml to 91.64 µg/ml (Table 2). The ethanol extract elicited remarkable reducing abilities, with FRAP values inferior to the values obtained with the standards trolox and superior from quercetin. The DPPH assay gave the similar result where ethanol extract gave IC_{50} values equal to 0.30 µg/ml.

The β-carotene bleaching method is widely used to measure the antioxidant activity of plant extracts (43). This method is based on the fact that linoleic acid produce a free radical which is reduced by β-carotene. The presence of an antioxidant prevents the reduction of β-carotene which remains yellowish-orange in colour [24]. The EC_{50} values represent the concentration at which 50% of β-carotene is reduced. A low EC_{50} value indicates a potent antioxidant activity. The rank order of EC_{50} values for *Crassocephalum crepidioides* (Benth.) S. Moore. were trolox > quercetin > ethanol > ethyl acetate > hexane > water. Overall, in the three experiments, the ethanol extract displayed profound antioxidant capacities. The present study demonstrates that *Crassocephalum crepidioides* (Benth.) S. Moore. is potent source of antioxidants.

Phenolic compounds and flavonoids can act as an antioxidant because of their ability to scavenge free radicals and to prevent the decomposition of hydroxyperoxides into free radicals [25]. Several reports emphasized on the fact that there is a positive relationship between total phenolic contents and antioxidant activity [26-29]. In agreement with this, our study demonstrated the existence of a positive correlation between the total phenolic contents and the FRAP values ($y = 388.66x + 135.36$, $r^2 = 0.8567$) and total phenolic contents and β-carotene bleaching values ($y = -1.3793x + 378.17$, $r^2 = 0.8980$), respectively. Therefore, one could draw an inference that phenolic compounds and flavonoids impart to *Crassocephalum crepidioides* (Benth.) S. Moore. its antioxidant properties.

Table 2.
Antioxidant activities of the four extracts of *Crassocephalum crepidioides* (Benth.) S. Moore.

Extract	**FRAP assay**	**DPPH assay**	**β-carotene bleaching assay**
	FRAP value (µg/ml)	**IC_{50} (µg/ml)**	**Ec_{50} (µg/ml)**
Hexane	91.64 ± 0.02A	0.75 ± 0.01A	8.6 ± 0.44A
Ethyl acetate	4.11 ± 0.01B	5.34 ± 0.01B	3.22 ± 0.41B
Ethanol	1.16 ± 0.13C	0.30 ± 0.01C	0.24 ± 0.24C
Water	1.60 ± 0.01D	0.48 ± 0.01D	18.81 ± 0.15D
Quercetin	1.31 ± 0.03C	11×10^3 ± 0.00F	0.13 ± 0.17C
Trolox	1.14 ± 0.02C	9×10^3 ± 0.00F	0.05 ± 0.17C

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

3.3. Anti-inflammatory activities

The mechanisms of inflammation involve a series of events in which the metabolism of arachidonic acid plays an important role [30]. Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures [20]. The conversion of linoleic acid to 1,3- hydroperoxy linoleic acid was followed spectrophotometrically by the appearance of a conjugate diene at λ: 234 nm on a UV spectrophotometer. A low IC_{50} value indicates a potent anti-inflammatory activity. The ethanol extract of *Crassocephalum crepidioides* (Benth.) S. Moore. inhibited the best enzymatic activity of 5-lipoxygenase (5-LOX) with an IC_{50} 5-LOX equal to 55.01 µg/ml (Table 3). Antioxidants and free radical scavengers have the potential to reduce radicals and to terminate the synthesis of leukotrienes. Therefore, inhibition of the 5-lipoxygenase enzyme can indirectly reduce free radical production [31]. Studies have implicated oxygen free radicals in the process of inflammation as blocking agents in the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase activity [32-33]. A combination of anti-inflammatory and antioxidant assays [34] constitutes a good indication on potential anti-inflammatory activity of a drug [35], as inhibition of the lipoxygenases is due to reaction of the inhibitor with free radicals generated at the active site of the enzyme [36].

Table 3.
Anti-inflammatory of the four extracts of *Crassocephalum crepidioides* (Benth.) S. Moore.

Extract	**Anti-inflammatory assay**
	IC_{50} 5-LOX (µg/ml)
Hexane	413.09 ± 0.03A
Ethyl acetate	526.62 ± 0.01B
Ethanol	55.01 ± 0.04C
Water	213.63 ± 0.01D
Nordihydroguaiaretic acid (NDGA)	5.33 ± 0.05E

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

3.4. Cytotoxicity and cytoprotective activities

This assay has been conducted to determine the safety of the plant extracts in an effort to verify the efficacy of plant studied. It is known, Asteraceae are cytotoxic [37] because of pyrrolizidine alkaloids. The cytotoxicity assay conducted in the ethanol extract, which displayed good antioxidant and anti-inflammatory properties. The ethanol extract of *Crassocephalum crepidioides* (Benth.) S. Moore. was non-toxic against MRC-5 and HepG2 cell lines at a concentration value of 100 µg/ml (Table 4).

Table 4.
Cytotoxicity of the ethanol extract of *Crassocephalum crepidioides* (Benth.) S. Moore on MRC-5 and HepG2 cell lines

Concentration (µg/ml)	**Cytotoxicity assay**	
	% Viability	
	MRC-5	**HepG2**
100	123.67	109.06
50	126.9	111.94
25	183.17	158.92
12.5	187.89	122.19
6.25	192.75	194.16
3.125	173.59	116.62
1.5624	277.43	183.84

The cytoprotective effect of *Crassocephalum crepidioides* (Benth.) S. Moore. ethanol extract was measured using *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damages in HepG2 cells. The concentration of ethanol extract and quercetin (standard) used in this assay were 100 µg/ml and 25 µg/ml, respectively. The ethanol extract of *Crassocephalum crepidioides* (Benth.) S. Moore., at a concentration of 25 µg/ml gave a percentage of cytoprotection which was higher than that of quercetin (Table 5).

2.6. Conclusions

This study is the first report on the phytochemical compositions, antioxidant, anti-inflammation, cytotoxicity and cytoprotective properties of hexane, ethyl acetate, ethanol and water extracts of the aerial parts of *Crassocephalum crepidioides* (Benth.) S. Moore. The ethanol extract of *Crassocephalum crepidioides* (Benth.) S. Moore., which abounds with phenolic compounds and flavonoids, displayed strong antioxidant, anti-inflammatory and cytoprotective activities. The present study warrants further phytochemical analysis, which may lead to the discovery of anti-inflammatory and/or cytoprotective agents of therapeutic value.

Table 5.

Cytoprotection of the ethanol extract of *Crassocephalum crepidioides* (Benth.) S. Moore. and standard on HepG2 cell lines

Concentration (µg/ml)	**% Vialibility**	
	Ethanol extract	**Quercetin (Standard)**
100	69.47	-
50	66.94	-
25	55.83	47.87
12.5	43.77	39.80
6.25	41.02	20.91
3.125	39.64	16.91
1.5624	35.73	13.77
0.7812	-	6.30
0.3906	-	2.21

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