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Research Article

Phytochemical Screening, Antioxidant, and Antimicrobial Activities of Leaves, Stems, and Rhizomes of *Etlingera coccinea* (Blume) S. Sakai & Nagam

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ABSTRACT
Etlingera coccinea (Blume) S. Sakai & Nagam has been used by various indigenous communities in parts of Asia as a traditional remedy for food poisoning, stomach ache, and gastric problems. The aim of this study was to evaluate the antimicrobial activity of methanolic extracts of leaves, stems, and rhizomes of *E. coccinea*, to support its use as a treatment for food poisoning. Another aim was to screen the major phytochemical constituents of these extracts and evaluate their antioxidant activity, which may be significant for other potential uses of this plant. The disc-diffusion method was used to assay antimicrobial activities. Phytochemicals including anthraquinones, cardiac glycosides, saponins, and steroids were detected using chemical analyses. The total phenolic content (TPC), total flavonoid content (TFC), and total flavonol content (TFlC) were estimated using spectrophotometric methods. The antioxidant activity (AOA) of extracts was evaluated using five methods: the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the 2,2-azino-bis-(3-ethylenebenzothiozoline-6-sulphonic acid) (ABTS) radical scavenging assay, the ferric-reducing antioxidant power (FRAP) assay, the reducing power activity (RPA) assay, and the total antioxidant capacity (TAC) assay. The disc-diffusion assay showed that none of the extracts from different plant parts of *E. coccinea* had antibacterial (Gram-positive and Gram-negative) or antifungal activities. The TPC, TFC, TFlC, and AOA of the extracts could be ranked, from strongest to weakest, as follows: leaves > stems > rhizomes. There were significant correlations between the AOA and TPC, TFC, and TFlC. The results of the antimicrobial activity assays do not validate the use of *E. coccinea* as a remedy for food poisoning. However, the high content of flavonoids and flavonols, and strong antioxidant activity of the leaf extract suggest that this plant has significant potential for use in food and pharmaceutical industries.

Keywords: *Etlingera coccinea*, phytochemical screening, phenolic compounds, antioxidant activity, antimicrobial activity.

INTRODUCTION

In recent years, there has been increasing interest in natural plant-derived products as sources of active compounds with antimicrobial and antioxidant properties, because of their relatively low toxicity and strong bioactivities. Natural active compounds can be used to prolong the shelf-life of food by inhibiting the growth of spoilage or pathogenic microorganisms and by protecting food from oxidative damage[1]. Phytochemicals with antioxidant properties play a vital role in maintaining human health and preventing oxidative damage caused by free radicals. Such phytochemicals can reduce the risk of several disorders including cancer, atherosclerosis, ageing, arthritis, central nervous system injury, and degenerative diseases[2]. The use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone in food products is strictly regulated because of uncertainties about their safety and side effects such as liver damage and carcinogenesis[3].

Infectious diseases caused by bacteria, fungi, viruses, and parasites are still significant threats to public health because of the dramatic increase in microbial resistance to existing preservatives or antimicrobial agents. Synthetic antibiotics have certain disadvantages and side effects. Therefore, natural compounds derived from plant tissues have received attention as novel antimicrobial and antioxidant compounds that are suitable for use in food and medicine.

Etlingera, a genus in the ginger family (Zingiberaceae), contains more than 100 different species. It is an Indo-Pacific terrestrial and perennial herb found predominantly close to the equator, between sea level and 2500 meters[4]. Borneo and New Guinea have the largest number of

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Table 1: Moisture content and extraction yield of leaves, stems, and rhizomes of *Etlingera coccinea.*

Plant Part	Moisture content[a] (%)		Extraction yield (mg/g)
	Oven Drying	Freeze Drying	
Leaves	63.91 ± 2.01 [be]	64.26 ± 1.93 [cg]	93.20 ± 3.65 [d]
Stems	81.40 ± 2.71 [e]	79.91 ± 2.61 [g]	44.60 ± 4.20 [d]
Rhizomes	82.48 ± 1.95 [b]	83.87 ± 2.07 [c]	61.13 ± 2.89 [d]

Values are mean ± SD (*n*=3). [a]Moisture content was calculated as a proportion of fresh weight. In each column, values with the same superscript letter are significantly different (P < 0.01; Tukey's HSD and LSD tests).

Table 2: Phytochemical components of methanolic extracts of *Etlingera coccinea* leaves, stems, and rhizomes.

Phytoconstituent	Test performed	Methanolic extract of		
		Leaves	Stems	Rhizomes
Alkaloids	Mayer's reagent	-	-	-
	Wagner's reagent	-	-	-
	Dragendoff's reagent	-	-	-
Cardiac glycosides	Keller–Killiani Test	+	+	+
Steroids	Salkowski's Test	+	+	+
	Liberman–Burchard test	+	+	+
Saponins	Frothing test	+	+	-
Anthraquinones	Borntrager's test	+	+	-

+ presence, - absence.

Table 3: Total phenolic, flavonoid, and flavonol contents of methanolic extracts of *Etlingera coccinea* leaves, stems, and rhizomes.

Plant extract	Total phenolic content (mg GAE/g DW)[a]	Total flavonoid content (mg QE/g DW)[b]	Total flavonol content (mg QE/g DW)[b]
Leaves	13.49 ± 0.258	5.33 ± 0.102	8.68 ± 0.166
Stems	7.94 ± 0.012	1.87 ± 0.003	3.8 ± 0.005
Rhizomes	2.64 ± 0.242	0.84 ± 0.077	1.14 ± 0.104

Values are means ± SD (*n*=3). Within columns and rows, mean values were significantly different (P < 0.001; Tukey's HSD and LSD test)

[a] Total phenolic content is expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

[b] Total flavonoid and flavonol contents are expressed as mg quercetin equivalents per g DW (mg QE/g DW).

Etlingera species.

The Malesian region harbors approximately 70 species of *Etlingera*. Various *Etlingera* species have traditional and commercial uses as foods, condiments, medicines, and as ornamental plants. The well-known species *Etlingera elatior* (torch ginger) is used as an ornamental plant and as a spice for culinary purposes. This species is cultivated throughout Borneo. The core of the young stem (*Etlingera littoralis*), fruit (*E. littoralis, Etlingera fimbriobracteata*) and flowers (*E. elatior, Etlingera maingayi*) are consumed as condiments, either raw or cooked, by indigenous communities in Malaysia, Brunei, and Thailand. The rhizome of *Etlingera punica* is used as a spice, which is a common ingredient in noodles and curries in the Chantaburi Province of Thailand. Leaves of *E. elatior* are used to clean wounds, and are mixed with other aromatic herbs and used in bathing to remove body odor.

Etlingera coccinea is native to Sumatra, Java, the Malay Peninsula, and Brunei, where it grows in various habitats. This species is known as 'tuhau' to the Dusuns and the Muruts in Sabah (Malaysia), as 'tubu nanung' to the Kelabits in Sarawak, and as 'tepus' to the Ibans in Brunei and Sarawak[5]. The inflorescence consists of several long, red flowers with a yellow border, and appears at ground level. The center of the leafy shoot is used as a condiment in Borneo and Java. The fruits are edible, and the seed oil has a characteristic aroma. Traditionally, *E. coccinea* has been used by various indigenous communities as a remedy for stomach ache, food poisoning, and gastric problems[5].

Several previous studies have evaluated the components and bioactivities of *Etlingera* species. Chan et al. (2008) screened 26 ginger species belonging to nine genera (*Alpinia, Boesenbergia, Curcuma, Elettariopsis, Etlingera, Hedychium, Kaempferia, Scaphochlamys,* and *Zingiber*), and found that leaves of several *Etlingera* species (*E. elatior, Etlingera rubrostriata, E. littoralis, Etlingera fulgens,* and *E. maingayi*) had high total phenolic content (TPC) and strong radical-scavenging activity[6]. Chan et al. (2007) reported on the antibacterial and high tyrosinase inhibition activities of *E. elatior, E. fulgens,* and *E. maingayi*. Stigmast-4-en-3-one and stigmast-4-en-6b-ol-3-one were isolated from *E. elatior*, and showed strong antitumor activities in *in vitro* tests[7]. To date, however, there have been no reports on the phytochemical characterization and biological activities of the methanolic extracts of leaves, stems, and rhizomes of

E. coccinea. The objectives of the present study, therefore, were to screen methanolic extracts of leaves, stems, and rhizomes of this plant species for phytochemicals, and to evaluate their antioxidant and antimicrobial activities.

MATERIALS AND METHODS

Chemicals

Ammonium molybdate, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid (AA), butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), potassium ferricyanide, and quercetin were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). All other solvents and chemicals were of analytical grade and were obtained from Merck & Co (Kenilworth, New Jersey, USA) and BDH Chemicals Ltd. (Poole, Dorset, UK).

Plant materials

The leaves, stems, and rhizomes of *E. coccinea* were collected from the riverine forest of Sg. Temburong, Temburong District, Brunei Darussalam in January 2013. The species was identified by Dr. Kamariah Abu Salim (Universiti Brunei Darussalam) and confirmed by Dr. Axel Poulsen (University of Oslo). Voucher specimens (No. SUD 1) and a spirit collection of the flowers (No. SUD 1) were prepared and deposited in the Universiti Brunei Darussalam Herbarium (UBDH) and the National Herbarium of Brunei Darussalam (BRUN).

Determination of moisture content

For each sample (leaves, stems, and rhizomes), 20 g fresh tissue was dried in an oven at 60°C, and another 20 g fresh tissue was dried in a freeze dryer under vacuum to constant weight. The moisture content in terms of percentage of fresh weight was determined. Dried samples were stored in desiccators at room temperature until further use.

Preparation of methanolic extracts

The plant materials (leaves, stems, and rhizomes) were rinsed, cut into small pieces with a sharp knife, and dried in a freeze dryer under vacuum for 2 days. The samples were ground to a fine powder using a laboratory mill. Then, a 30-g sample of each powdered plant part was mixed with 250 mL methanol and extracted in a Soxhlet apparatus for 6–8 h. The extracts were concentrated under reduced pressure at 40°C using a rotary evaporator. The crude extracts were dried in a freeze dryer and stored at 4°C in the dark until use. The yield of each methanolic extract was calculated based on mg extract per g freeze-dried plant material, as follows: percentage yield = weight of freeze dried crude (mg)/ freeze dried sample (g) × 100.

Phytochemical screening

Phytochemical screening was carried out to identify alkaloids, cardiac glycosides, steroids, and anthraquinones as described by Evans (2009)[8] and Sofowora (1996)[9]. These analyses were based on visual observations of a color change or the formation of precipitates after the addition of specific reagents.

Determination of phenolic compound contents

Total phenolic content

The TPC was determined using the Folin–Ciocalteu method[10] with minor modifications. Plant extract (0.5 mL of a 1 mg/mL solution) was mixed with 2.5 mL Folin–Ciocalteu reagent (10%) and 2 mL sodium carbonate (75% w/v). After vortexing for 15 s, the mixture was incubated at room temperature in the dark for 30 min and the absorbance was measured at 765 nm using a UV-visible spectrophotometer. The TPC was calculated using the regression equation from the calibration curve constructed using gallic acid standards (0 to 300 ppm) and expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW).

Total flavonoid content

The total flavonoid content (TFC) was determined as described by Lin and Tang (2007)[11]. Plant extract (500 µL) was mixed with methanol (1.5 mL), 10% aluminum chloride hexahydrate (0.1 mL), 1 M potassium acetate (0.1 mL), and distilled water (2.8 mL). The reaction mixture was incubated at room temperature for 40 min, and then the absorbance was measured at 415 nm using a UV-visible spectrophotometer. The results are expressed as mg quercetin equivalents per gram dry weight (mg QE/g DW). These values were calculated from a quercetin calibration curve (concentration range, 0–50 µg/mL).

Total flavonol content

The total flavonol content (TFlC) was determined using the method of Kumaran and Karunakaran (2007)[12]. Plant extract (1 mL of a 1 mg/mL solution) was mixed with 1 mL aluminum trichloride (20 mg/mL in methanol) and 3 mL 50 g/L sodium acetate solution. The mixture was incubated at 20°C for 2.5 h and then the absorption was measured at 440 nm. A standard calibration curve was prepared using quercetin (0–50 ppm) with the method described above. The results are expressed as mg quercetin equivalents per gram dry weight (mg QE/g DW).

Evaluation of antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl free radical-scavenging activity

The capacity of the methanolic extract to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured as described by Ćavar et al. (2012)[13] with minor modifications. A solution of DPPH (1 mM) was prepared in methanol, and 3 mL of this solution was added to 300 µL of various concentrations (5–500 µg/mL) of methanolic extract. The reaction mixture was left to react in the dark for 30 min at room temperature, and the absorbance was measured at 517 nm using a UV-visible spectrophotometer. Ascorbic acid and BHT (at the same concentration as the plant extracts) were also subjected to the same procedure for comparison. The inhibitory concentration (IC) was calculated as follows:

$IC\ (\%) = [(A_0 - A_t)/A_0] \times 100$

where A_0 is the absorbance value of blank sample and A_t is the absorbance value of the tested sample. The concentrations that reduced the absorption of DPPH solution by 50% (IC_{50}) were calculated. A lower IC_{50} value indicates stronger antioxidant activity.

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging activity

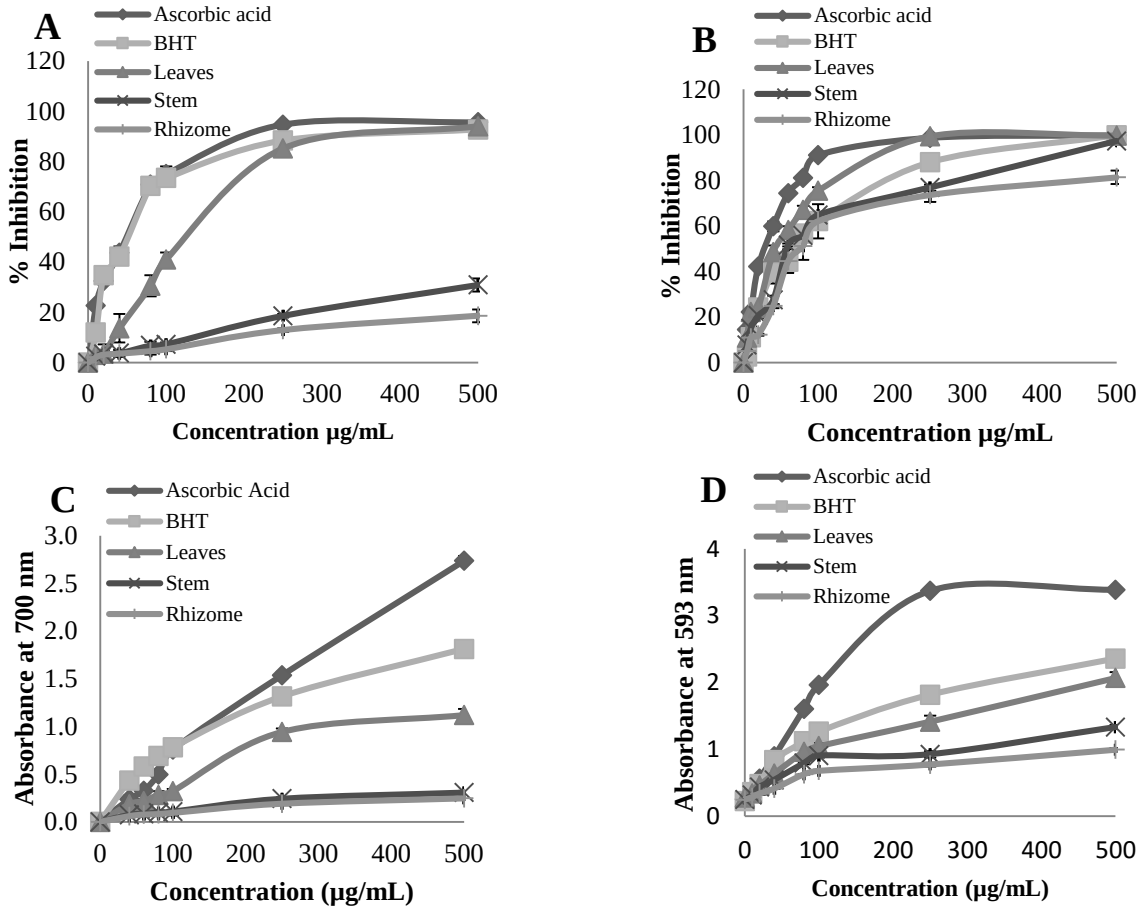

Figure 1: Antioxidant and reducing power activity of methanolic extracts of *E. coccinea* leaves, stems and rhizomes. (A) DPPH radical scavenging activity, (B) ABTS radical scavenging activity, (C) Reducing power activity, (D) FRAP

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging activity of the plant extracts and standards (ascorbic acid and BHT) was determined according to Shao et al. (2013)[14] with minor modifications. Briefly, equal volumes of 7 mM ABTS and 2.45 mM potassium persulfate were mixed together to prepare the ABTS solution, which was then incubated at room temperature in the dark for 16 h. Prior to the assay, the ABTS solution was diluted with methanol (1:50) to obtain an absorbance of 0.706 ± 0.01 at 734 nm. Then, 0.3 mL extract at various concentrations (5–500 µg/mL) or methanol (negative control) was added to 3 mL ABTS solution. The mixture was vortexed for about 1 min and allowed to stand at room temperature for 30 min before reading the absorbance at 734 nm. The radical scavenging activity was calculated using the following equation:

$$\% \text{ scavenging (IC \%)} = (1 - A_s/A_c) \times 100$$

where A_s is the absorbance of sample and A_c is the absorbance of the control. The IC_{50} values represent 50% inhibition of formation of the ABTS radical.

Reducing power activity assay

Plant extracts and standards (ascorbic acid and BHT) prepared at the same concentrations were analyzed using the reducing power activity (RPA) assay as described by Oyaizu (1986)[15]. The assay mixture consisted of 1 mL plant extract at various concentrations (40–500 µg/mL), 2.5 mL sodium phosphate buffer (200 mM, pH 6.6), and 2.5 mL 1% (w/v) potassium ferricyanide. The mixture was incubated at 50°C for 20 min and then 2.5 mL 10% (w/v) trichloroacetic acid was added to stop the reaction. The mixture was centrifuged at 3000 rpm (930 *g*) for 10 min, and the supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL freshly prepared ferric chloride solution 0.1% (w/v). The absorbance of the solution was measured at 700 nm. The concentration of the extract that produced an absorbance value of 0.5 (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration. Higher absorbance of the reaction mixture indicates stronger reducing power.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay was conducted as described by Benzie and Strain (1996)[16] with some modifications. The FRAP reagent was prepared fresh by mixing 100 mL acetate buffer (300 mM, pH 3.6), 10 mL TPTZ solution (10 mM TPTZ in 40 mM hydrochloric acid), and 10 mL ferric chloride heptahydrate (20 mM). The solution was warmed to 37°C before use. The FRAP reagent (3 mL) was added to 0.3 mL plant extract at various concentrations (5–500 µg/mL) or methanol (reagent blank), and the mixture was incubated for 30 min in the dark. The increase in absorbance due to the reduction

Table 4**:** Antioxidant activity of methanolic extracts of *Etlingera coccinea* in DPPH, ABTS, RPA, FRAP, and TAC (phosphomolybdate) assays.

Plant part	IC_{50} in DPPH radical scavenging assay (µg/mL)	IC_{50} in ABTS radical scavenging assay (µg/mL)	EC_{50} in reducing power assay (µg/mL)	EC_{50} in ferric reducing assay (µg/mL)	Total antioxidant capacity (mg AE g^{-1})
Leaves	$*196.72^{a} \pm 0.28$	$106.55^{a} \pm 2.59$	$*204.67^{a} \pm 7.69$	$28.22^{a} \pm 0.08$	$254.25^{a} \pm 0.05$
Stem	$**777.13^{b} \pm 7.15$	$*152.95^{b} \pm 1.56$	$**760.06^{b} \pm 0.43$	$38.61^{b} \pm 1.51$	$*116^{b} \pm 0.02$
Rhizome	$1380.28^{c} \pm 2.37$	$*173.14^{b} \pm 1.78$	$1000.12^{b} \pm 0.62$	$*64.75^{c} \pm 2.65$	$*107.25^{b} \pm 0.01$
Ascorbic Acid	$105.60^{a} \pm 4.39$	$*64.67^{c} \pm 0.24$	$*72.80^{a} \pm 5.44$	$16.87^{d} \pm 0.42$	-
BHT	$122.00^{a} \pm 6.64$	$147.02^{b} \pm 2.46$	$59.33^{a} \pm 6.08$	$22.43^{d} \pm 2.69$	-

Values are mean ± SD (*n*=3). Within each column and row, means followed by the same superscript letter and number of asterisks are not statistically different ($P < 0.05$; Tukey's HSD).
Total antioxidant capacity (TAC) is expressed as mg ascorbic acid equivalents per g dry weight (mg AE /g DW)

Table 5: Pearson's correlation coefficients between phenolic compound contents (TPC, TFC, and TFIC) and activity in antioxidant assays (DPPH, ABTS, RPA, FRAP, and TAC).

	TPC[a]	TFC[b]	TFIC[c]	DPPH[d]	ABTS[e]	RPA[f]	FRAP[g]	TAC[h]
TPC	1							
TFC	0.962^{**}	1						
TFlC	0.990^{**}	0.991^{**}	1					
DPPH	-0.982^{**}	-0.934^{**}	-0.967^{**}	1				
ABTS	-0.923^{**}	-0.939^{**}	-0.941^{**}	0.961^{**}	1			
RPA	-0.956^{**}	-0.973^{**}	-0.974^{**}	0.941^{**}	0.932^{**}	1		
FRAP	-0.960^{**}	-0.851^{**}	-0.913^{**}	0.954^{**}	0.836^{**}	0.883^{**}	1	
TAC	0.897^{**}	0.980^{**}	0.948^{**}	-0.850^{**}	-0.882^{**}	-0.925^{**}	-0.743^{*}	1

[a] Total phenolic content, [b]Total flavonoid content, [c]Total flavonol content, [d]IC_{50} in DPPH assay, [e] IC_{50} in ABTS assay, [f] EC_{50} in reducing power activity assay, [g] EC_{50} in FRAP assay, [h] Total antioxidant capacity
** Correlation significant at the 0.01 level (2-tailed).
* Correlation significant at the 0.05 level (2-tailed).

of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ (ferrous-tripyridyltriazine complex) was monitored at 593 nm. The EC_{50} was determined as the concentration of each sample required to give an absorbance of 0.5 at a wavelength of 593 nm. The antioxidant potential of a sample was compared with those of the standards, ascorbic acid and BHT, at the same concentration.

Total antioxidant capacity

The total antioxidant capacity (TAC) of the plant extracts was evaluated according to Kubola and Siriamornpun (2008)[17]. The phosphomolybdenum assay is based on the reduction of Mo(VI) to Mo(V). The assay mixture contained 0.3 mL sample (1 mg/mL) and 3 mL reagent solution [0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate (4:2:4)]. The reaction mixture was incubated at 95°C for 90 min in a water bath, and then cooled to room temperature. The absorbance was measured against a blank (methanol) at 695 nm. Ascorbic acid (5–500 µg/mL) was used as the standard. Antioxidant activity was expressed as mg ascorbic acid equivalents per g dry weight (mg AE/g DW).

Determination of antimicrobial activity

Microorganisms

Two species of Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and three species of Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Bacillus spizini* ATCC 6633, and *Staphylococcus aureus* ATCC 25923) were used as test microorganisms in the antibacterial activity assays. All of these bacteria were purchased from BioMérieux, Inc. (Durham, NC, USA). Four fungal species were used in the antifungal assay: *Candida albicans* ATCC 10231, *Aspergillus niger* 16404, *Trichophyton rubrum* 28188, and *Saccharomyces cerevisiae* ATCC 9763. The fungi strains were obtained from Oxoid Limited (Basingstoke, Hampshire, England).

Disc-diffusion assay

The disc-diffusion method was used to evaluate the antimicrobial activity of the methanolic extracts. Bacterial stock cultures were prepared by inoculating nutrient agar (NA) plates with a commercial bacteriological loop containing the test organism. Strains of *C. albicans and S. cerevisiae* were maintained on Sabouraud Dextrose Agar (SDA), and *A. niger* and *T. rubrum* were maintained on Potato Dextrose Agar (PDA).

All inocula were prepared in appropriate broth and standardized by adjusting the turbidity of the suspension to that of the McFarland Standard 0.5. The standardized cultures were inoculated onto plates by swabbing uniformly over the entire surface of the medium. After swabbing with pathogenic microorganisms, sterile paper discs (made from Whatman No. 1 filter, 6 mm diameter) impregnated with 10 µL plant extract or a negative control (methanol plus equivalent quantities of DMSO, which was used to dissolve the plant extract) were placed carefully on the surface of the inoculated medium. Standard

antimicrobial disks [penicillin G (10 µg/disc), tetracycline (30 µg/disc), chloramphenicol (75 µg/disc) and gentamycin (30 µg/disc) for bacteria and amphotericin (10µg/disc), fluconazole (10 µg/disc), itraconazole (10 µg/disc) for fungi, respectively] were used as positive controls. The plates were incubated at 37°C for 24 h for bacterial strains and at 25°C for 48–72 h for fungal strains. The zone of inhibition including the disc was measured in mm as an indication of antimicrobial activity.

Statistical analyses

All results are expressed as mean ± standard deviation of at least three replicates. Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS; IBM Corp., Armonk, NY, USA). Tukey's pair-wise comparison (HSD) and least significant difference (LSD) tests were performed to analyze variance and determine significant differences among mean values ($P < 0.05$). Pearson's correlation test was performed using SPSS to determine inter-relationships among TPC, TFC, TFlC, and antioxidant activity.

RESULTS AND DISCUSSION

Moisture content and percentage yield

The moisture contents of different parts of *E. coccinea* after oven- and freeze-drying are shown in Table 1. After oven-drying, the moisture contents of leaves, stems, and rhizomes were 63.91%, 81.74%, and 82.48%, respectively. After freeze-drying, the moisture contents of leaves, stems, and rhizomes were 64.24%, 79.91%, and 83.87%, respectively. For each plant part, there was no significant difference ($P > 0.05$) in moisture content between oven-drying and freeze-drying. However, the moisture content of leaves was significantly ($P < 0.001$) lower than those of stems and rhizomes. The moisture contents did not differ significantly ($P > 0.05$) between stems and rhizomes. The moisture content of *E. coccinea* leaves was comparable to those of *Alpinia zerumbet* leaves (60% ± 2%) and *E. elatior* leaves (68% ± 5%), but lower than that of *Curcuma longa* leaves (83% ± 2%)[18].

Methanol was chosen as the solvent because it is suitable for extracting phenolic compounds from plant tissues and it can prevent microbial growth. The extraction yields of the methanolic extracts differed significantly ($P < 0.05$) among leaves, stems, and rhizomes (93.20, 44.60, and 61.13 mg/g freeze-dried weight, respectively; Table 1). These values were comparable to the extraction yields from members of the genera *Alpinia*, *Curcuma*, *Vanoverberghia*, *Hedychium*, and *Zingiber* recorded by Chan et al. (2008)[6].

Phytochemical screening

The leaves, stems, and rhizomes of *E. coccinea* were screened for secondary metabolites (Table 2). We did not detect any alkaloids, but cardiac glycosides and steroids were detected in all plant parts. Saponins and anthraquinones were detected in leaves and stems, but not in rhizomes. Similar compositions of secondary metabolites have been reported for leaves *Alpinia nigra* and rhizomes of several species in the genus *Hedychium (H. spicatum, H. coronarium*, and *H. rubrum*)[19,20]. These secondary metabolites are used in various industrial and medicinal applications.

Cardiac glycosides are secondary metabolites that are produced by several plants. They are used to treat congestive heart failure and cardiac arrhythmia. They function by inhibiting the Na^+/K^+ pump, which increases the calcium concentration in the sarcoplasmic reticulum. This increases the force of contraction, and allows more time for ventricular filling.

Steroids have a wide variety of effects in animals. They affect the development, activity, and plasticity of the nervous system and modulate pain[21]. Plant steroids have cardiotonic activities, and so they are useful in the management of hypertension. Because of their structural similarities to various anabolic hormones including sex hormones, plant steroids have the potential to function as effective, natural, and safe alternatives to treat age- and disease-associated muscle loss, or to improve endurance and physical performance[22].

Saponins are natural detergents found in many plants. They bind to cholesterol to form insoluble complexes; this prevents cholesterol reabsorption and thus reduces blood cholesterol levels in humans. They have been shown to react with cholesterol in the protozoal cell membrane, causing cell lysis of *Giardia lamblia*, the causal agent of giardiasis. Saponins have several other biological functions such as anti-inflammatory, expectorant, immune stimulating, and antineoplastic effects[23].

Plant secondary metabolites such as anthraquinones (emodin, aloe-emodin, chrysophanol, rhein, physcion) and their respective glycoside derivatives have been shown to have various biological activities including antioxidant, cytotoxic, antimicrobial, antifungal, antitumor, antidiabetic, antiproliferative, and immunoenhancing activities[24,25].

Total phenolic, flavonoid, and flavonol contents

Phenolic compounds from plants show a range of bioactivities including antioxidant, anticarcinogenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities. They play an important role in the prevention of diabetes, osteoporosis, neurodegenerative diseases, and major cardiovascular diseases such as hypertension[26,27].

Flavonoids (flavones, flavonols, flavanols, chalcones, flavanones, isoflavonoids, neoflavonoids, biflavonoids, flavanonols, and anthocyanins) are the most widespread group of polyphenols. They are potent natural antioxidants and exhibit a wide range of biological activities including antibacterial, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory activities[27].

The antioxidant mechanism of flavonoids is related to several components of their chemical structure: the hydroxyl groups in the 3 and 5 positions; an *o*-diphenolic group (in ring B); and a 2-3 double bond conjugated with the 4-oxo function. Flavonoids hinder the diffusion of free radicals by stabilizing membrane fluidity, thereby restricting the peroxidation reaction[28]. Flavonols and procyanidins can prevent access of deleterious molecules

by interacting with membrane phospholipids; thus, they affect membrane rheology[29].

The leaves of *E. coccinea* had significantly ($P < 0.05$) higher TPC than those of stems and rhizomes (Table 3). The TPC in leaves, stems, and rhizomes were 13.49, 7.94, and 2.64 mg GAE/g DW, respectively. Based on these results, the TPC of *E. coccinea* was higher than those of *Etlinger belalongensis*, *Etlinger volutina*, and *Zingiber pseudopungens* (10.07, 5.30, and 8.10 mg GAE/g DW, respectively) but comparable to those of *E. maingayi* (11.10 mg GAE/g DW) and *E. fulgens* (12.10 mg GAE/g DW)[6].

Several authors have reported high concentrations of phenolic compounds in the methanolic extracts of leaves from species in the genera *Alpinia*, *Curcuma*, and *Etlingera*[6,30]. Chan et al. (2007) reported that in *Etlingera* species (*E. maingayi*, *E. elatior*, *E .littoralis*, and *E. fulgens*), the TPC was seven to eight times higher in the leaves than in the rhizomes. In other studies[4,31], rhizomes of *Kaempferia galangal*, *Curcuma manga*, and *C. longa* had low TPC (1.12, 0.57, and 1.72 mg GAE/g DW, respectively).

The leaves of *E. coccinea* had the highest flavonoid content (5.33 mg QE/g DW) followed by the stems (1.87 mg QE/g DW) and rhizomes (0.84 mg QE/g DW). The flavonoid content in the stem was similar to those reported for *E. belalongensis*, *E. volutina*, and *Z. pseudopungens* (2.57, 2.80, and 1.97 mg CE/g TFC, respectively)[32]. The leaves also had the highest flavonol content (8.68 mg QE/g DW), more than double that in the stems (3.8 mg QE/g dry weights) and more than six times that in the rhizomes (1.14 mg QE/g DW). To our knowledge, this is the first study to analyze the total flavonol content of *Etlingera* species.

As reported by Herrmann (1988)[33], flavonols and flavones are present in all types of plants and their formation depends on the light conditions. Consequently, they accumulate to higher concentrations in leaves than in other parts of the plant. Herrmann (1988) also reported that both elevated CO_2 and ultraviolet-B light levels increase the production of phenolic compounds, including flavonoids such as anthocyanins, isoflavonoids, flavonol glycosides, flavoproteins, phenolic acids, and coumarins. High constitutive levels of these secondary metabolites accumulate in the vacuoles of epidermal cells and protect the underlying tissues by absorbing ultraviolet wavelengths. This could explain why *Etlingera* species have higher contents of phenolic compounds and higher antioxidant activities in leaves than in stems and rhizomes.

Antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The assay based on the stable DPPH free radical is the most widely used method to determine the antioxidant capacity of plant extracts. The deep purple color of the DPPH radical changes after it accepts an electron from an antioxidant. Figure 1A shows the dose–response curve of DPPH radical scavenging activity of the methanolic extracts of *E. coccinea*, compared with two standards: ascorbic acid and BHT. Ascorbic acid showed the highest activity (96.74%), followed by BHT (93.54%), leaves (93.33%), stems (32.09%), and rhizomes (17.85%) at the same concentration (500 µg/mL). The activity of the leaf extract was higher than those of the stem and rhizome extracts.

The IC_{50} values of leaves, stems, and rhizomes are shown in Table 4. When these values were compared with those of the standards, ascorbic acid (IC_{50} = 105.60 µg/mL) and BHT (IC_{50} = 122 µg/mL), the leaf extract (IC_{50} = 196.72 µg/mL) showed strong activity but stems (IC_{50} = 777.13 µg/mL) and rhizomes (IC_{50} = 1380.28 µg/mL) exhibited very weak radical scavenging activities. A lower IC_{50} value indicates stronger DPPH scavenging activity, while a higher IC_{50} value indicates a lower scavenging activity.

Comparing our values with those reported for other plants, the leaf extract of *E. coccinea* showed stronger free radical scavenging activity than those of leaf methanolic extracts of *A. zerumbet*, *A. purpurata*, and *Adiantum philippense* (IC_{50} values for DPPH assay: 260, 350, and 210 µg/mL, respectively)[34,35].

The radical scavenging activity of rhizomes was weaker than those of leaves and stems, but stronger than those of *A. galangal* rhizomes (IC_{50} 2.3 mg/mL) and *Z. officinale* rhizomes (IC_{50} 4.30 mg/mL)[34]. However, the average DPPH-scavenging activity of methanolic extracts of rhizomes of various *Alpinia* species (*A. kawakamii*, *A. kusshakuensis*, *A. mesanthera*, *A. officinarum*, *A. pricei*, *A. shimadai*, *A. uraiensis*) and *Curcuma* species (*C. domestica*, *C. longa*, *C. viridiflora*, *C. zedoaria*) were reported as approximately 67% and 73%, respectively[30], higher than the values obtained in this study.

2,2-azino-bis-(3-ethylenebenzothiozoline-6-sulphonic acid) radical scavenging activity

The ABTS assay is another commonly used method to measure the relative radical scavenging activity of hydrogen-donating and chain-breaking antioxidants in plant extracts. As shown in Figure 1B, both plant extracts of *E. coccinea* and standards (ascorbic acid and BHT) significantly reduced the $ABTS^+$ radical as their concentrations increased. The $ABTS^+$ free radical scavenging activity of *E. coccinea* varied significantly among the different plant parts (Table 4). At a concentration of 500 µg/mL, the percentage inhibition of ABTS radical formation radicals by the leaf extract (99.68%) and stem extract (97.27%) were similar to those of the standards, ascorbic acid (99.76%) and BHT (99.86%), while the rhizome extract showed the lowest activity (81%). The extracts and standards could be ranked based on their IC_{50} values, from strongest $ABTS^+$-scavenging activity to weakest, as follows: ascorbic acid (64.67 µg/mL) > leaves (106.55 µg/mL) > BHT (147.02 µg/mL) > stems (152.95 µg/mL) > rhizomes (173.14 µg/mL). To our knowledge, there have been no other kinetic studies on the ABTS scavenging activity of methanolic extracts of *Etlingera* species.

Reducing power activity

In the RPA assay, Fe^{3+} reduction is an indicator of electron-donating activity. The solution changes from yellow to different shades of blue and green depending on the power of a compound to reduce the free radicals**.** The

basic principle of this assay is the conversion of the ferric–ferricyanide complex to the ferrous form depending on the presence of antioxidants. Figure 1C shows the dose–response curves for the reducing power of methanolic extracts of *E. coccinea* and standards. The plant extracts were able to reduce the ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in concentration-dependent manner, and the activities of the extracts differed significantly. As the concentration of extract in the reaction mixture increased, the reducing power also increased. The methanolic leaf extract showed stronger reducing power capacity than those of stems and rhizomes. The EC_{50} values of ascorbic acid, BHT, leaves, stems, and rhizomes were 72.80, 59.33, 204.67, 760.06, and 1000.12 µg/mL, respectively (Table 4). The reducing powers of standards at all concentrations were higher than those of leaves, stems, and rhizomes. In this assay, the activity of the leaf extract was almost three times higher than that of the stem extract, and five times higher than that of the rhizome extract. The standards and plant parts could be ranked based on their EC_{50} values, from strongest reducing power to weakest, as follows: ascorbic acid > BHT > leaves > stems > rhizomes.

The RPA has been used in several studies to determine the antioxidant activity of medicinal plants[13,14]. However, this assay has never been used to evaluate the antioxidant activity of *Etlingera* species. Kathirvel and Sujatha (2012) reported that *Dryopteris cochleata* extracts showed strong reducing power in the RPA (EC_{50} values of 243, 327, 378, 494 µg/mL for acetone, ethyl acetate, methanol, and chloroform extracts, respectively)[36]. These values are lower than the EC_{50} value of *E. coccinea* leaves (204.67 µg/mL) determined in this study. In some studies, the reducing power was evaluated based on changes in absorbance, rather than the EC_{50} value. Thus, based on the absorbance at 700 nm, the reducing activity of *E. coccinea* leaves (1.18 at 500 µg/mL) was comparatively higher than those of *Morus indica* (0.256 at 500 µg/mL), *Cistus ladaniferus* (1.47 at 1,000 µg/mL), *Centaurea polypodiifolia* (1.17 at 800 µg/mL), *Centaurea pyrrhoblephara* (0.87 at 800 µg/mL), *Centaurea antalyense* (0.85 at 800 µg/mL), black chokeberry (0.71 at 500 µg/mL), and an edible mushroom (0.587 at 2 mg/mL)[37,38].

Ferric reducing antioxidant power

The FRAP assay is a reliable method for assessing the antioxidant capacity of polyphenols. This assay is based on the ability of the extract to reduce the ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}–TPTZ). The Fe(II)–TPTZ complex turns blue in the presence of antioxidant compounds, which exert their action by donating a hydrogen atom to break the free radical chain[16]. The results of FRAP assay for methanolic extracts of leaves, stems, and rhizomes of *E. coccinea* and standards are shown in Figure 1D. The two standards, ascorbic acid and BHT, showed the highest activity in this assay. Among the plant extracts, the leaf extract showed the highest activity (EC_{50} = 28.22 µg/mL), followed by the stem extract (EC_{50} = 38.61 µg/mL) and the rhizome extract (EC_{50} = 64.75 µg/mL) (Table 4). The high polyphenol content in leaves may explain the high FRAP activities. The RPA and FRAP results followed a similar trend; that is, from strongest to weakest antioxidant activity: ascorbic acid > BHT > leaves > stems > rhizomes. Chan et al. (2007) reported high FRAP values for leaves of *E. elatior* (19 mg GAE/g DW) *E. rubrostriata* (16.6 mg GAE/g DW), and *E. littoralis* (11.6 mg GAE/g DW). Unfortunately, these values cannot be compared with our results because of the different standards used. However, compared with the EC_{50} values reported for other plant species, leaves of *E. coccinea* (EC_{50} 28.22 µg/mL) showed stronger antioxidant activity than leaves of *Piper betle* (EC_{50} 50.7 µg/mL), *Piper betleoides* (EC_{50} 69.7µg/mL), and *Piper wallichii* (EC_{50} 69.7 µg/mL), all of which were considered to show significant ferric-reduction power[39].

Total antioxidant capacity

The *E. coccinea* leaf extract showed the highest TAC (254.25 mg AE/g DW) and the rhizomes showed the lowest (107.25 mg AE/g DW) (Table 4). The plant parts could be ranked based on their TAC, from highest to lowest, as follows: leaves > stems > rhizomes. The differences in reducing capacity among the plant parts may be because of differences in their phenolic contents. To the best of our knowledge, this is the first report of the use of the TAC assay to determine the antioxidant capacity of *Etlingera* species. The TAC of the *E. coccinea* leaf extract was higher than those of methanolic extracts of aerial parts of *Centaurea pyrrhoblephara* (226.15 mg AE/g DW) and fruit pulp of *Annona squamosa* (207 µg α-tocopherol/g DW) but similar to that of a *Solanum surattense* leaf extract (257 mg AE/g DW)[40,41].

Correlation between antioxidant compound contents and antioxidant activity

It is important to evaluate the antioxidant activity of extracts of different plant parts using different antioxidant assays (DPPH, ABTS, FRAP, RPA and TAC) to cover the range of endogenous compounds. Antioxidants encompass a wide range of polyphenols, reducing agents, and nucleophiles that vary in their solubility and localization, redox potential, specificity, and mechanism of action. Some methods measure the activity of only hydrophilic antioxidants (e.g., Folin and FRAP), while the ABTS assay measures the activities of both hydrophilic and lipophilic antioxidants. The DPPH assay only detects the activity of antioxidants that are soluble in organic solvents, especially alcohols. Therefore, the evaluation of overall antioxidant capacity based on one chemical reaction is somewhat unrealistic. For this reason, we used multiple assays to evaluate the overall antioxidant capacity of these extracts. These assays allowed us to create an 'antioxidant profile', which included reactivity of both aqueous and lipid/organic radicals directly via radical quenching and radical reducing mechanisms, and indirectly via metal complexing. Thus, the differences among the results of the various antioxidant assays used in this study reflect differences in the types and amounts of antioxidant compounds in the various extracts.

Kubola et al. (2008) reported on the correlation between the phenolic compounds content and antioxidant activity

of plant extracts[17]. Recently, Farhat et al. (2013) reported significant correlations between antioxidant activities (detected by DPPH, ABTS, and FRAP assays) and caffeic, carnosic acid, carnosol, methyl carnosate, apigenin, and genkwanin, and polyphenol contents[42].

In this study, the contribution of polyphenolic compounds in the methanolic extracts of *E coccinea* to the total antioxidant activity was evaluated by Pearson's correlation coefficients (Table 5). For all of the *E. coccinea* extracts, there were significant correlations (r^2 values of 0.851–0.982) between *in vitro* antioxidant activities (DPPH, ABTS, RPA, FRAP, and TAC) and antioxidant compound contents (TPC, TFC, and TFIC). These results are consistent with those of some other reports[43]. The strong correlations between different tests (r^2 values ranging from 0.743 to 0.961) demonstrated that the antioxidant assays used in this study are effective and complementary to each other for evaluating the antioxidant activities of plant extracts. The results indicated that phenolic compounds (TPC, TFC, and TFlC) are the major contributors to the antioxidant activities of *E. coccinea* extracts.

Antimicrobial activities

The disc-diffusion method was used to evaluate the antimicrobial activity of the plant extracts against some common fungi and Gram-positive and Gram-negative bacteria. None of the extracts of *E. coccinea* showed antimicrobial activity against Gram-positive (*S. aureus*, *B. subtilis*, and *B. spizinium*), Gram-negative bacteria (*E. coli*, *P. aeruginosa)*, or fungi (*A. niger*, *T. rubrum*, *C. albicans*, and *S. cerevisiae*) (data not shown). In a previous study, leaf extracts of *Etlingera* species (*E. elatior*, *E. rubrostriata*, *E. fulgens*, *E. maingayi*, and *E. littoralis*) inhibited the growth of Gram-negative bacteria but only weakly inhibited Gram-positive bacteria[31]. So far, there have been no reports that *E. coccinea* stem and rhizome extracts inhibit the growth of bacteria and fungi.

CONCLUSION

The results of this study showed that extracts from different plant parts of *E. coccinea* have no antimicrobial activity. Thus, these results do not validate the traditional use of the plants to treat food poisoning. However, different parts of the plant have different antioxidant activities. The leaves contained high concentrations of phenolic compounds and leaf extracts showed strong antioxidant activity in all of the antioxidant activity assays. The plant parts could be ranked based on their phenolic, flavonoid, and flavonol contents, as follows: leaves > stems > rhizomes. The same rank order was observed in the antioxidant activity assays. Antioxidant activity measured with the DPPH radical, ABTS radical, RPA, FRAP, and TAC assays showed significant correlations with the phenolic compound contents. Therefore, these methods are appropriate techniques for assessing the antioxidant capacities of *Etlingera* species. The antioxidant properties of the leaf suggest that it could be considered as an alternative to synthetic compounds as an additive for foods and pharmaceuticals.

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