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Antioxidant and antibacterial activity of leaves of *Etlingera* species (Zingiberaceae) in Peninsular Malaysia

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Abstract

Methanolic extracts from fresh leaves of five *Etlingera* species were screened for total phenolic content (TPC), antioxidant activity (AOA), and antibacterial activity. Analysis of TPC was done using the Folin–Ciocalteu method. Evaluations of AOA included 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability, ferric-reducing antioxidant power (FRAP), ferrous-ion chelating (FIC) ability, and β-carotene bleaching (BCB) activity. Antibacterial activity was screened using the disc-diffusion method. Highest TPC, ascorbic acid equivalent antioxidant capacity (AEAC), and FRAP were found in leaves of *E. elatior* and *E. rubrostriata*. Leaves of *E. maingayi*, with the lowest TPC, AEAC, and FRAP, had the highest FIC ability and BCB activity. Ranking of TPC and AOA of different plant parts of *E. elatior* was in the order: leaves > inflorescences > rhizomes. Leaves of highland populations of *Etlingera* species displayed higher values of TPC and AEAC than those of lowland counterparts. Leaves of *Etlingera* species exhibited antibacterial activity against Gram-positive but not Gram-negative bacteria.

Keywords: Etlingera; Leaves; Highland; Lowland; Total phenolic content; Antioxidant activity; Antibacterial activity

1. Introduction

Etlingera Giseke of the family Zingiberaceae are tall forest plants, with larger species reaching 6 m in height (Khaw, 2001). In the *Phaeomeria* group, inflorescences are borne on erect stalks protruding from the ground and, in the *Achasma* group, inflorescences are subterranean with flowers appearing at soil level (Lim, 2000, 2001). The varying shades of pink and red colours of bracts and flowers make *Etlingera* species very attractive plants. A total of 15 *Etlingera* species has been recorded in Peninsular Malaysia (Lim, 2001).

Plants of *Etlingera* have various traditional and commercial uses. In Sabah, Malaysia, the hearts of young shoots, flower buds, and fruits of *E. elatior*, *E. rubrolutea*, and *E. littoralis* are consumed by indigenous communities as condiment, eaten raw or cooked (Noweg, Abdullah, & Nidang, 2003). In Thailand, fruits and cores of young stems of *E. littoralis* are edible, and flowers of *E. maingayi* are eaten as vegetables (Sirirugsa, 1999). There are no reports on the use of rhizomes of *Etlingera* species.

Inflorescences of *E. elatior* are widely cultivated throughout the tropics as spices for food flavouring and as ornamentals. They are commonly used as ingredients of dishes such as *laksa asam*, *nasi kerabu*, and *nasi ulam* in Peninsular Malaysia (Larsen, Ibrahim, Khaw, & Saw, 1999). Farms in Australia and Costa Rica are cultivating the species and selling its inflorescences as cut flowers (Larsen et al., 1999). In Malaysia, fruits of *E. elatior* are used traditionally to treat earache, while leaves are applied for cleaning wounds (Ibrahim & Setyowati, 1999). Leaves of *E. elatior*, mixed with other aromatic herbs in water, are used by *post-partum* women for bathing to remove body odour.

Flavonoids in the leaves of *E. elatior* have been identified as kaempferol 3-glucuronide, quercetin 3-glucuronide,

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quercetin 3-glucoside, and quercetin 3-rhamnoside (Williams & Harborne, 1977). Flavonoid content of inflorescences of *E. elatior* has been estimated to be 286 and 21 mg of kaempferol and quercetin (per kg dry weight), respectively (Miean & Mohamed, 2001).

Phytochemical studies on rhizomes of *E. elatior* led to the isolation of two new and six known compounds of diarylheptanoids, labdane diterpenoids, and steroids (Habsah et al., 2005). Using the ferric thiocyanate method, lipid peroxidation inhibitory activity of the isolated diarylheptanoids was greater than that of α-tocopherol. Ethanolic extracts from the flower shoots of *E. elatior* have antimicrobial activity and are cytotoxic to HeLa cells (Mackeen et al., 1997).

Past studies on the antioxidant activity (AOA) of ginger species were confined to rhizomes (Jitoe et al., 1992; Habsah et al., 2000; Zaeoung, Plubrukarn, & Keawpradub, 2005). Their rhizomes have been reported to contain antioxidants comparable to α-tocopherol. Although leaves of ginger species have been used for food flavouring and as traditional medicine, hardly any research has been done on their AOA.

In our previous study (Chan, Lim, & Lim, in press), total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves and rhizomes of five wild and six cultivated ginger species, belonging to seven genera, were screened. The seven genera were *Alpinia*, *Boesenbergia*, *Curcuma*, *Elettariopsis*, *Etlingera*, *Scaphochlamys*, and *Zingiber*. Results showed that leaves of *Etlingera* had the highest TPC and AEAC. Values were significantly higher than those of rhizomes.

In this study, TPC, AOA, and antibacterial activity of leaves of five *Etlingera* species were analysed. In *E. elatior*, TPC and AOA of the different plant parts were compared. Altitudinal variation in leaf TPC and AEAC of four *Etlingera* species was also studied. This study represents the first systematic analysis of TPC, AOA, and antibacterial activity of leaves of *Etlingera* species.

2. Materials and methods

2.1. Plant materials

Five species of *Etlingera* studied were *E. elatior* (Jack) R.M. Smith, *E. fulgens* (Ridl.) C.K. Lim, and *E. maingayi* (Bak.) R.M. Smith of the *Phaeomeria* group, and *E. littoralis* (Koenig) Giseke and *E. rubrostriata* (Holtt.) C.K. Lim of the *Achasma* group. Their identification in the field was based on taxonomic descriptions and photographic illustrations of Khaw (2001) and Lim (2000, 2001). Characteristic scent of leaves when crushed was another useful cue for species identification. Voucher specimens were deposited at the herbarium of Forest Research Institute Malaysia (FRIM).

Leaves of highland populations of *Etlingera* species were sampled from Janda Baik and Genting Highlands in Pahang, and from Ulu Gombak in Selangor, while leaves of lowland populations were sampled from FRIM. For each species, mature leaves were sampled from three different clumps. Altitude of locations where populations were sampled was measured using a Casio altimeter (Model PRG-70-1VDR).

Rhizomes of *E. elatior* were collected from FRIM while its inflorescences were purchased from the supermarket. For comparison as positive controls, young leaves of the tea plant, *Camellia sinensis* (L.) Kuntze, were collected from a tea plantation in Cameron Highlands, Pahang, and rhizomes of *Curcuma longa* L. and *Zingiber officinale* Roscoe were purchased from the supermarket.

2.2. Chemicals and reagents

For TPC analysis, Folin–Ciocalteu's phenol reagent (Fluka, 2N), gallic acid (Fluka, 98%), and anhydrous sodium carbonate (Fluka, 99%) were used; for DPPH assay, 1,1-diphenyl-2-picrylhydrazyl (Sigma, 90%) was used; for FRAP assay, ferric chloride hexa-hydrate (Fisher Scientific, 100%), potassium ferricyanide (Unilab, 99%), trichloroacetic acid (HmbG Chemicals, 99.8%), potassium dihydrogen orthophosphate (Fisher Scientific, 99.5%), and dipotassium hydrogen phosphate (Merck, 99%) were used; for FIC assay, ferrozine (Acros Organics, 98%) and ferrous sulphate hepta-hydrate (HmbG Chemicals) were used; for BCB assay, β-carotene (Sigma, Type 1: synthetic), chloroform (Fisher Scientific, 100%), linoleic acid (Fluka), and Tween 40 (Fluka) were used. For disc-diffusion assay, paper discs (Oxoid, 6 mm), Muller–Hinton agar (Oxoid), nutrient broth (Oxoid), and streptomycin susceptibility discs (Oxoid, 10 μg) were used.

2.3. Preparation of extracts

For the analysis of TPC and AOA, fresh leaves (1 g) were powdered with liquid nitrogen in a mortar and extracted using 50 ml of methanol, with continuous swirling for 1 h at room temperature. Extracts were filtered and stored at −20 °C for further use. Rhizomes and inflorescences were extracted in a similar manner.

For the screening of antibacterial activity, leaves of each species were cut into small pieces and 100 g were weighed and freeze-dried. Dried samples were then crushed in a mortar with liquid nitrogen and extracted with 250 ml of methanol three times for 1 h each time. Samples were filtered and the solvent was removed using a rotary evaporator. Dried extracts were kept at −20 °C for analysis.

2.4. Methanol extraction efficiency

To test the efficiency of methanol extraction, second and third extractions were conducted. After filtration, residues, along with the filter paper, were transferred back into the extraction vessel and extracted again each time with 50 ml methanol. Measurement of extraction efficiency was based on TPC.

2.5. Determination of total phenolic content

Total phenolic content (TPC) of plant extracts was determined using the Folin–Ciocalteu assay reported by Kahkonen et al. (1999). Folin–Ciocalteu reagent (1.5 ml; diluted 10 times) and sodium carbonate (1.2 ml; 7.5% w/v) were added to the extracts (300 μl; triplicate). After 30 min, absorbance was measured at 765 nm. TPC was expressed as gallic acid equivalents (GAE) in mg per 100 g. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$).

2.6. Determination of antioxidant activity

2.6.1. DPPH assay

The DPPH free radical-scavenging (FRS) assay used by Miliauskas, Venskutonis, and van Beek (2004) was adopted with modifications. Different dilutions of the extract (1 ml; triplicate) were added to 2 ml of DPPH (5.9 mg/100 ml methanol). Absorbance was measured at 517 nm after 30 min. FRS ability was calculated as IC_{50} and expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg AA/100 g (Leong & Shui, 2002) as follows:

$$\text{AEAC (mg AA/100g)} = \text{IC}_{50(\text{ascorbate})}/\text{IC}_{50(\text{extract})} \times 100,000$$

The IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

2.6.2. FRAP assay

The ferric-reducing antioxidant power (FRAP) assay reported by Chu, Chang, and Hsu (2000) was adopted with modifications. Different dilutions of the extract (1 ml) were added to 2.5 ml phosphate buffer (0.2 M; pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid solution (2.5 ml; 10% w/v) was added to stop the reaction. The mixture was then separated into aliquots of 2.5 ml and diluted with 2.5 ml of water. To each diluted aliquot, 500 μl of ferric chloride solution (0.1% w/v) were added. After 30 min, absorbance was measured at 700 nm. FRAP of extracts was expressed as mg GAE/g. The calibration equation for gallic acid was $y = 16.767x$ ($R^2 = 0.9974$).

2.6.3. FIC assay

The ferrous-ion chelating (FIC) assay used by Singh and Rajini (2004) was adopted. Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were diluted 20 times. $FeSO_4$ (1 ml) was mixed with different dilutions of extract (1 ml), followed by ferrozine (1 ml). Absorbance was measured at 562 nm after 10 min. The ability of extracts to chelate ferrous ions was calculated as follows:

$$\text{Chelating effect } \% = (1 - A_{\text{extract}}/A_{\text{control}}) \times 100$$

where A_{extract} and A_{control} are absorbance of the extract and negative control, respectively.

2.6.4. BCB assay

The β-carotene bleaching (BCB) assay reported by Kumazawa et al. (2002) was adopted. β-Carotene/linoleic acid emulsion was prepared by adding 3 ml of β-carotene (5 mg/50 ml chloroform) to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was evaporated under vacuum and oxygenated ultra-pure water (100 ml) was added and mixed well. Initial absorbance of the emulsion was measured at 470 nm. Aliquots of the emulsion (3 ml) were mixed with 10, 50, and 100 μl of extract and incubated in a water bath at 50 °C for 1 h. Bleaching rate of β-carotene was measured at 470 nm and 700 nm. Measurement at 700 nm is needed to correct for the presence of haze. Bleaching rate was expressed as AOA (%) and calculated as follows:

$$\text{Bleaching rate (BR)of β-carotene} = \ln(A_{\text{initial}}/A_{\text{extract}})/60$$

$$\text{AOA } (\%) = 1 - (\text{BR}_{\text{extract}}/\text{BR}_{\text{control}}) \times 100$$

where A_{initial} and A_{extract} are absorbances of the emulsion before and 1 h after incubation, and $\text{BR}_{\text{extract}}$ and $\text{BR}_{\text{control}}$ are bleaching rates of the extract and negative control, respectively.

2.7. Screening for antibacterial activity

The disc-diffusion method described by Chung, Chung, Ngeow, Goh, and Imiyabir (2004) was used to screen for antibacterial activity. Agar cultures of Gram-positive bacteria (*Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella cholerasuis*) were prepared. Suspensions of bacteria (100 μl) were spread evenly onto 20 ml Mueller–Hinton agar preset in 90 mm Petri dishes. Paper discs (6 mm diameter) were impregnated with 1 mg of plant extract dissolved in 100 μl solvent, and transferred onto the inoculated agar.

Streptomycin susceptibility discs (10 μg) and methanol-impregnated disc were used as positive and negative controls, respectively. After incubation overnight at 37 °C, inhibition zones were measured and recorded as mean diameter (mm). Antibacterial activity was also expressed as inhibition percentage of streptomycin and arbitrarily classified as strong for inhibition of $\geqslant 70\%$, moderate for inhibition $50 < 70\%$, and weak for inhibition $<50\%$.

3. Results and discussion

3.1. Descriptions of plant specimens

Photographs of plants of the five species of *Etlingera* studied are shown in Fig. 1. Leaves of *E. elatior* are entirely green, sometimes flushed pink when young, and emit a pleasant sour scent when crushed. Leaves of *E. fulgens* are dark green, shiny, undulated, and their underside is bright red when young. They emit a pleasant sour scent similar to those of *E. elatior*. Leaves of *E. maingayi* are red-

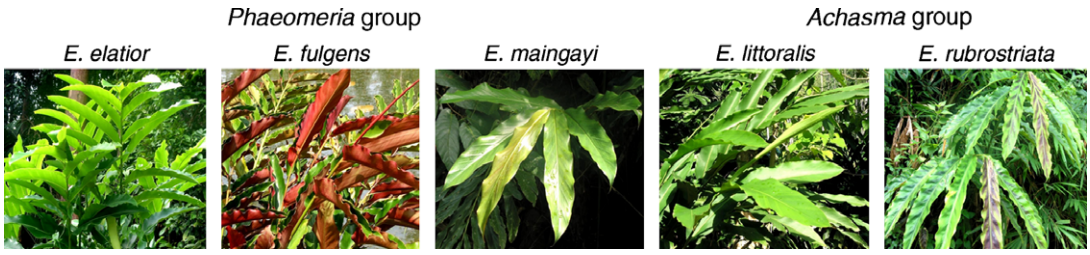

Fig. 1. Photographs of plants of five species of *Etlingera* studied.

dish below, translucent when young, and emit an unpleasant sour scent. Leaves of *E. littoralis* are entirely green, sometimes flushed pink when young, and do not have any scent. Leaves of *E. rubrostriata* are green with distinctive purplish-brown bars on upper surface and do not have any scent.

3.2. Methanol extraction efficiency of leaves

Methanol showed high extraction efficiency of leaves of *Etlingera* species. Yields of the first extraction ranged from 82.7% in *E. rubrostriata* to 88.2% in *E. maingayi*. A second extraction yielded 12.9% and 7.8%, and a third yielded 4.4% and 4.0%, respectively.

Methanol has been recommended for the extraction of phenolic compounds from fresh plant tissues. It is a suitable solvent due to its ability to inhibit polyphenol oxidase, which could alter antioxidant activity (Yao et al., 2004). High methanol extraction efficiency has been reported for leaves and flowers of *Alpinia* species (Wong, 2006), and for young leaves of *C. sinensis* (Chan, Lim, & Chew, 2007).

3.3. Total phenolic content of leaf extracts

Total phenolic content (TPC) of leaf extracts was determined using the Folin–Ciocalteu method and expressed in mg GAE/100 g. Of the *Etlingera* species analysed, leaves of *E. elatior* and *E. rubrostriata* had the highest TPC (Table 1). Values were 3550 ± 304 and 3480 ± 390 mg GAE/100 g, respectively. Leaves of *E. maingayi* and *E. fulgens* had the lowest TPC of 1110 ± 93 and 2540 ± 91 mg GAE/100 g, respectively.

Table 1
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing antioxidant power (FRAP) of leaves of *Etlingera* species (fresh weight)

Etlingera sp.	TPC (mg GAE/100 g)	Antioxidant activity (AOA)	
		AEAC (mg AA/100 g)	FRAP (mg GAE/g)
E. elatior	3550 ± 304a	3750 ± 555a	19.6 ± 2.1a
E. rubrostriata	3480 ± 390ab	3540 ± 401a	16.6 ± 2.4a
E. littoralis	2810 ± 242bc	2930 ± 220b	11.6 ± 1.0b
E. fulgens	2540 ± 91c	2030 ± 126c	9.4 ± 0.4c
E. maingayi	1110 ± 93d	963 ± 169d	4.9 ± 0.8d

Values of TPC, AEAC, and FRAP are means ± SD ($n = 3$). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$, as measured by the Tukey HSD test.

3.4. Antioxidant activity of leaf extracts

Antioxidant activity (AOA) of leaf extracts from *Etlingera* species was evaluated using the DPPH, FRAP, FIC, and BCB assays. Activity was expressed in mg AA/100 g, mg GAE/g, chelating ability (%) and AOA (%), respectively.

Results showed that leaves of *E. elatior* and *E. rubrostriata* had high AEAC and FRAP (Table 1). Values were 3750 ± 555 mg AA/100 g and 19.6 ± 2.1 mg GAE/g for *E. elatior*, and 3540 ± 401 mg AA/100 g and 16.6 ± 2.4 mg GAE/g for *E. rubrostriata*, respectively. Moderately high AEAC and FRAP were found in the leaves of *E. littoralis* and *E. fulgens*. Values were 2930 ± 220 mg AA/100 g and 11.6 ± 1.0 mg GAE/g for *E. maingayi*, and 2030 ± 126 mg AA/100 g and 9.4 ± 0.4 mg GAE/g for *E. fulgens*, respectively. Lowest values of 963 ± 169 mg AA/100 g and 4.9 ± 0.8 mg GAE/g were found in the leaves of *E. maingayi*.

Among the species of *Etlingera* studied, leaf AEAC and FRAP shared the same order of ranking as leaf TPC i.e., *E. elatior* > *E. rubrostriata* > *E. littoralis* > *E. fulgens* > *E. maingayi*. It is evident that *Etlingera* species with high leaf TPC also have high AEAC and FRAP.

In terms of FIC ability, the trend was reversed with leaves of *E. maingayi* and *E. fulgens* having the highest values (Fig. 2). Leaves of *E. maingayi* and *E. fulgens* were superior, and leaves of *E. elatior* and *E. littoralis* were comparable to the FIC ability of young leaves of *C. sinensis* (positive control). Lowest values were found in the leaves of *E. rubrostriata*.

It can therefore be seen that leaves of *Etlingera* species with high TPC, AEAC, and FRAP have low FIC ability and vice versa. This would mean that phenolic compounds in extracts responsible for antioxidant activities of scavenging free radicals and reducing ferric ions might not be directly involved in ferrous ion chelation. The compounds responsible could be nitrogen-containing compounds, which are generally better chelators than are phenols. Similar observations were made with leaves of *Alpinia*. Of four species studied, leaves of *Alpinia galanga*, with the lowest TPC, AEAC, and FRAP, exhibited the highest FIC ability (Wong, 2006).

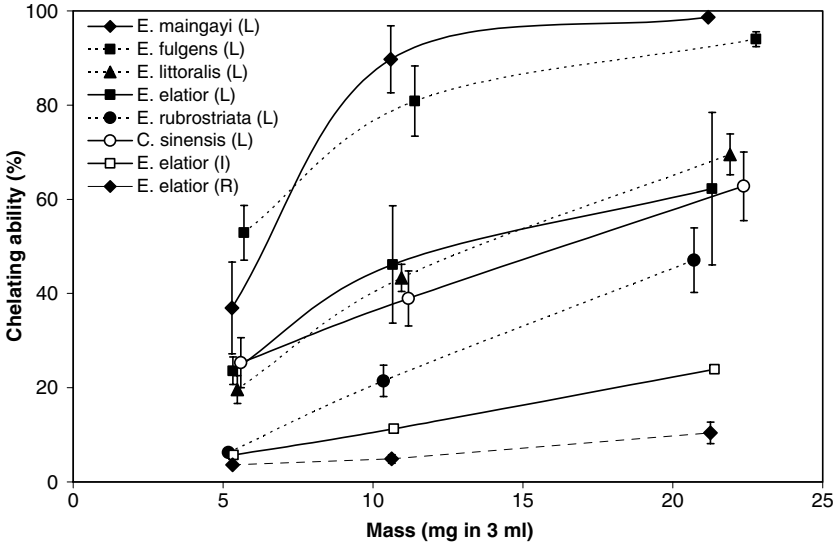

Fig. 2. Ferrous-ion chelating (FIC) ability of leaves of *Etlingera* species and different plant parts of *Etlingera elatior* (fresh weight). Young leaves of *Camellia sinensis* were used as positive control. Results are means ± SD ($n = 3$). Abbreviations: E., *Etlingera*; C., *Camellia*; R, rhizomes; L, leaves; I, inflorescences.

In terms of BCB activity, leaves of *E. maingayi* had the highest values. Its leaf BCB activity was better than that of rhizomes of *C. longa* with leaves of *E. rubrostriata*, *E. littoralis*, and *E. elatior* having slightly lower values (Fig. 3a). Although leaves of *E. fulgens* showed the lowest BCB activity, values were higher than that of young leaves of *C. sinensis* but lower than that of *Z. officinale* rhizomes (Fig. 3b).

With the exception of *E. fulgens*, leaves of all *Etlingera* species studied showed high BCB activity, comparable with that of rhizomes of *C. longa* and superior to that of young tea leaves and rhizomes of *Z. officinale*. High BCB activity of leaves of *Etlingera* species reflects their ability to strongly inhibit lipid peroxidation. There appears to be no correlation between BCB activity and AOA, as measured by the other assays. This is supported by findings of Lim and Quah (2007) that methanolic extracts of six cultivars of *Portulaca oleracea* showed that TPC correlated well with AEAC and FRAP but not with BCB activity.

3.5. TPC and AOA of extracts from different plant parts

Analyses of different plant parts of *E. elatior* showed that leaves had significantly higher TPC, AEAC, and FRAP than had inflorescences and rhizomes at $P < 0.05$ (Table 2). Values were 3550 ± 304 mg GAE/100 g, 3750 ± 555 mg AA/100 g, and 19.6 ± 2.1 mg GAE/g for leaves, 295 ± 24 mg GAE/100 g, 268 ± 45 mg AA/100 g, and 1.5 ± 0.2 mg GAE/g for inflorescences, and 187 ± 46 mg GAE/100 g, 185 ± 59 mg AA/100 g, and 0.9 ± 0.2 mg GAE/g for rhizomes, respectively.

Similarly, leaves of *E. elatior* showed superiority over inflorescences and rhizomes in terms of FIC ability (Fig. 2). FIC ability of leaves was comparable to that of young leaves of *C. sinensis*. BCB activity of leaves was much higher than that of rhizomes but slightly lower than that of inflorescences. BCB activities of inflorescences and rhizomes were comparable to those of rhizomes of *C. longa* (Fig. 3a) and young leaves of tea (Fig. 3b), respectively. Ranking of TPC and AOA (AEAC and FRAP) was in the order: leaves > inflorescences > rhizomes.

In Zingiberaceae, it is generally believed that antioxidants and other secondary metabolites are transported to the rhizomes where they are accumulated. This implies that rhizomes would have higher AOA than would other plant parts. Rhizomes of cultivated species have been reported to possess radical-scavenging compounds comparable to commercial antioxidants on a weight per weight basis. Jitoe et al. (1992) reported that AOA of extracts of *Alpinia*, *Amomum*, *Curcuma*, and *Zingiber* rhizomes were comparable to α-tocopherol. Extracts of *Z. officinale* rhizomes had better radical-scavenging ability than had butylated hydroxytoluene and quercetin (Stoilova, Krastanov, Stoyanova, Denev, & Gargova, 2007).

In our previous study (Chan et al., in press), screening of five wild and six cultivated ginger species showed that leaf TPC and AEAC were generally higher than those of rhizomes. Out of the 11 species screened, eight species had significantly higher leaf TPC and/or AEAC. Outstanding leaf TPC and AEAC of both *E. elatior* and *E. maingayi* were seven and eight times higher than those of rhizomes, respectively.

Results of this study on the different plant parts of *E. elatior* reaffirmed that TPC and AOA of leaves were significantly higher than those of rhizomes at $P < 0.05$. TPC, AEAC and FRAP were 19, 20, and 22 times higher in leaves than in rhizomes, respectively. Leaves of wild and cultivated *Etlingera* species therefore contain more antioxidants than do other plant parts.

Recently, Elzaawely, Xuan, and Tawata (2007) reported that ethyl acetate extracts from leaves of *Alpinia zerumbet*

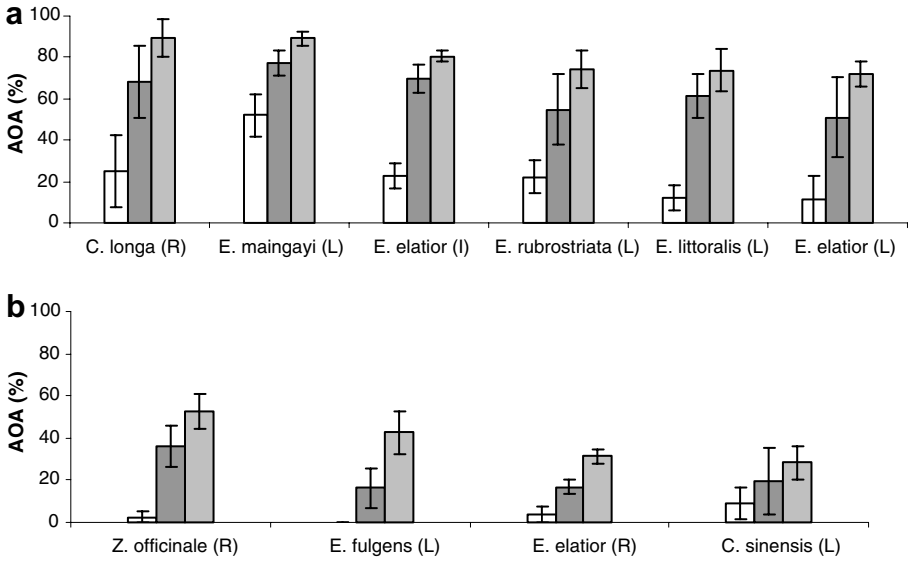

Fig. 3. β-Carotene bleaching (BCB) activity of leaves of *Etlingera* species (fresh weight). Rhizomes of *Curcuma longa* and *Zingiber officinale*, and young leaves of *Camellia sinensis* were used as positive controls. Results are means ± SD ($n = 3$). For each species, left, middle, and right bars represent extract concentrations of 0.2, 1.0, and 2.0 μg in 3 ml, respectively. Abbreviations for Fig. 3a: C., *Curcuma*; E., *Etlingera*; R, rhizomes; L, leaves; I, inflorescences. Abbreviations for Fig. 3b: Z., *Zingiber*; E., *Etlingera*; C., *Camellia*; R, rhizomes; L, leaves.

Table 2
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing antioxidant power (FRAP) of different plant parts of *Etlingera elatior* (fresh weight)

Plant part of *E. elatior*	TPC (mg GAE/100 g)	Antioxidant activity (AOA)	
		AEAC (mg AA/100 g)	FRAP (mg GAE/g)
Leaves	3550 ± 304a	3750 ± 555a	19.6 ± 2.1a
Inflorescences	295 ± 24b	268 ± 45b	1.5 ± 0.2b
Rhizomes	187 ± 46c	185 ± 59c	0.9 ± 0.2c

Values of TPC, AEAC, and FRAP are means ± SD ($n = 3$). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$, as measured by the Tukey HSD test.

showed higher inhibition of β-carotene oxidation and scavenging activity of free radicals than did rhizomes. This further supports our result that leaves have free-radical scavengers that are more effective than those found in rhizomes.

3.6. Altitudinal variation in TPC and AEAC of leaf extracts

Leaves of all *Etlingera* species sampled from highland populations were found to have higher TPC and AEAC than those of lowland counterparts. Leaves of *E. rubrostriata*, *E. elatior*, and *E. fulgens* showed significantly higher values with greater altitude at $P < 0.05$, while *E. littoralis* was marginally higher (Table 3). Highest leaf TPC and AEAC were found in highland populations of *E. elatior*, with values of 3550 ± 304 mg GAE/100 g and 3750 ± 555 mg AA/100 g, and of *E. rubrostriata*, with values of 3480 ± 390 mg GAE/100 g and 3540 ± 401 mg AA/100 g, respectively. Lowland populations of *E. fulgens* had the lowest values of 1280 ± 143 mg GAE/100 g and 845 ± 159 mg AA/100 g, respectively.

Higher altitudes seem to trigger an adaptive response in *Etlingera* species. Higher leaf TPC and AEAC of highland populations over those of lowland counterparts might be due to environmental factors, such as higher UV-B radiation and lower air temperature. There is increasing evidence that enhanced UV-B radiation induces production of phenolic compounds in plants (Bassman, 2004). Enzymes associated with the synthesis of phenolics are produced in greater quantities or show increased activity (Chalker-Scott & Scott, 2004). Phenylalanine ammonia lyase (PAL) is up-regulated, resulting in the accumulation of flavonoids and anthocyanins, which have antioxidant ability (Jansen, Gaba, & Greenberg, 1998). Low temperatures have also been shown to enhance PAL synthesis in a variety of plants, leading to increased production of flavonoids and other phenolics (Chalker-Scott & Scott, 2004).

3.7. Antibacterial activity of leaf extracts

Using the disc-diffusion method, leaves of all five *Etlingera* species were found to inhibit Gram-positive *B. cereus*, *M. luteus*, and *S. aureus* (Table 4). Leaves of *E. elatior*, *E. fulgens*, and *E. maingayi* exhibited moderate inhibition of the three bacteria. Moderate inhibition was shown by the leaves of *E. rubrostriata* on *B. cereus* and *S. aureus*, and by the leaves of *E. littoralis* on *S. aureus*.

Mean diameter of the zone of inhibition of streptomycin was 23 mm for *M. luteus*, and 17 mm for *B. cereus* and *S. aureus* (Table 4). Methanol showed no inhibitory effect on the three bacteria. Streptomycin was used as positive

Table 3
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves of *Etlingera* from highland and lowland locations (fresh weight)

Etlingera sp.	Location	Altitude (m asl)	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
E. elatior	Janda Baik	400	3550 ± 304a	3750 ± 555a
	FRIM	100	2390 ± 329b	2280 ± 778b
E. rubrostriata	Ulu Gombak	300	3480 ± 390a	3540 ± 401a
	FRIM	100	2430 ± 316b	2640 ± 508a
E. littoralis	Genting Highlands	800	2810 ± 243a	2930 ± 220a
	FRIM	100	2340 ± 386a	2220 ± 913a
E. fulgens	Janda Baik	400	2270 ± 31a	2030 ± 126a
	FRIM	100	1280 ± 143b	845 ± 158b

Values of TPC and AEAC are means ± SD ($n = 3$). For columns of each species, values followed by the same letter (a–b) are not significantly different at $P < 0.05$ measured by the Tukey HSD test. ANOVA does not apply between species.

control because it has been used as the antibiotic for Gram-positive and Gram-negative bacteria.

Among the Gram-positive bacteria, *S. aureus* appeared to be more sensitive. Screening for antibacterial activity of 191 plant extracts belonging to 30 families of plants from Sabah, Malaysia, showed similar results (Chung et al., 2004). About 52% of the extracts inhibited *S. aureus*. For all five *Etlingera* species, leaves showed stronger antibacterial activity than did rhizomes.

Leaves of *Etlingera* showed no antibacterial activity on Gram-negative bacteria of *E. coli*, *P. aeruginosa*, and *S. cholerasuis*. Antibacterial studies of extracts from various ginger species also showed no inhibition of Gram-negative bacteria (Chandarana, Baluja, & Chanda, 2005; Wong, 2006).

Gram-negative bacteria have an outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to the underlying structures (Chopra & Greenwood, 2001). This renders the Gram-negative bacteria generally less susceptible to plant extracts than the Gram-positive bacteria.

Preliminary investigation on the use of ethylenediamine tetraacetic acid (EDTA) to improve the efficacy of leaf extracts of *Etlingera* species against Gram-negative bacteria was carried out. Adding 2 mM EDTA to the agar caused *P. aeruginosa* to be susceptible to all leaf extracts of *Etlingera* species but inhibited the growth of *E. coli* and *S. cholerasuis*.

Table 4
Antibacterial activity of leaves of *Etlingera* species against Gram-positive bacteria using the disc-diffusion method

Etlingera sp.	Zone of inhibition in mm (inhibition %)		
	B. cereus	*M. luteus*	*S. aureus*
E. elatior	10(59)++	13(57)++	11(65)++
E. fulgens	11(65)++	12(52)++	11(65)++
E. littoralis	8(47)+	9(39)+	9(53)++
E. maingayi	9(53)++	14(61)++	11(65)++
E. rubrostriata	9(53)++	11(48)+	10(59)++
Streptomycin	17	23	17
Methanol	–	–	–

Mean diameter of the zone of inhibition is in millimetres. Figures in parentheses are inhibition percentages compared to streptomycin. Antibacterial activity is categorized as strong +++ for inhibition ⩾ 70%, moderate ++ for inhibition 50 < 70%, or weak + for inhibition < 50%. Abbreviations: *B.*, *Bacillus*; *M.*, *Micrococcus*; *S.*, *Staphylococcus*.

EDTA has been reported to permeabilise the outer membrane of *P. aeruginosa*, making it susceptible to antibiotics and certain antiseptic agents (Haque & Russell, 1974). Bacteria can be either exposed to the permeabiliser, together with the antibiotic, or pre-treated with the permeabiliser prior to introduction of the antibiotic (Ayres, Furr, & Russell, 1999).

In this study, the pre-treatment method was not effective. This suggests a different mode of action for EDTA, possibly synergistic with plant extracts. It is the first time the method has been used for testing antibacterial activity of plant extracts. Initial findings warrant further investigations.

4. Conclusion

Results showed that methanolic extracts from fresh leaves of *Etlingera* species had high values of TPC, AEAC, and FRAP. Species with the highest leaf TPC, AEAC, and FRAP possessed the lowest leaf FIC ability and vice versa. The FIC ability of leaves of *E. maingayi* and *E. fulgens* was superior to that of young leaves of *C. sinensis*. Leaves of *Etlingera* species exhibited high BCB activity, matching that of rhizomes of *C. longa* and superior to that of young tea leaves. Ranking of TPC and AOA of different plant parts of *E. elatior* was in the order: leaves > inflorescences > rhizomes. Leaves of highland populations of *Etlingera* species had higher values of TPC and AEAC than had those of lowland counterparts. Leaves of *Etlingera* species inhibited Gram-positive but not Gram-negative bacteria. With promising antioxidant and antibacterial properties, leaves of *Etlingera* species have great potential to be developed into natural preservatives and herbal products, applicable to the food and nutraceutical industries. Unlike the commercial use of rhizomes, the harvesting of leaves does not result in destructive sampling of plants.

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