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Antioxidant and prebiotic gut-microbiota effects of dietary phenolic compounds in *Etlingera elatior* extracts

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Abstract

Etlingera elatior, commonly found in South East Asia, is known to have high antioxidant activities. The purposes of the present work were to investigate the effects of different solvents on the extraction of total phenolic (TPC) and flavonoid compounds (FC) of *E. elatior* inflorescences, and to test their antioxidant and prebiotic effects. The highest yield (14.12%) of *E. elatior* extract was obtained from a 50% ethanol extract with the highest TPC and flavonoids contents being observed in 70 and 95% ethanol extracts. The ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and iron chelating activities were also investigated. The results showed that the 70% ethanol extract had high values for DPPH, FRAP, and ABTS, whereas the 95% ethanol extract had higher iron chelating activities. The *E. elatior* TPC showed a prebiotic-like effect by significantly decreasing the populations of harmful bacteria through the production of short chain fatty acids. The present work demonstrated that *E. elatior* has the potentials for applications as a natural functional ingredient for the modulation of gut microbiota.

Introduction

There has been a global rise in human death (71% of all deaths) caused by non-communicable diseases (NCDs) and metabolic syndromes. These NCD include cardiovascular diseases (17.9 million), cancers (9.0 million), respiratory diseases (3.9 million), and diabetes (1.6 million) (WHO, 2018). These NCDs are avoidable by dietary consumption. Behind of this is the action of gut microbiota utilising dietary nutrients to metabolites or sensing to other organs. It is known that some of NCDs or metabolic syndromes are strongly related to gut microbiome such as obesity, diabetes mellitus, chronic kidney disease, and colon cancer (Voreades *et al*., 2014; Singh *et al*., 2017). And recent studies show evidence of gut microbiota involves in Parkinson's and Alzheimer's diseases (Miraglia and Colla, 2019; Kowalski and Mulak, 2019).

Bioactive compounds in foods play an important role in providing health benefits to consumers (Jeevani Osadee Wijekoon *et al*., 2011). *Etlingera elatior*, known to have high antioxidant activities, belongs to the family Zingiberaceae, and is commonly found in South East Asia, particularly in the southern part of Thailand (Jackie *et al*., 2011). Previous studies have reported that the extract of *E. elatior* from Kelantan, Malaysia contained important amounts of phenolic and flavonoid compounds which contributed to their high antioxidant properties (Ghasemzadeh *et al*., 2015). Phenolic compounds or polyphenols are important phytochemicals, and previous studies have shown that polyphenols from blueberry are indicated as potential prebiotics and have anti-obesity effects through the improvement of the gut microbiota (Jiao *et al*., 2019). Polyphenol consumption has several beneficial functions such as improving gut health and lowering the risk of coronary heart disease (Kemperman *et al*., 2010). Dietary polyphenols have also been reported to play a role in the alteration and activity of colonic microbiota (Cardona *et al*., 2013); and Parkar *et al*. (2013) posited that dietary polyphenols have the potential to restore the gut microbiota balance.

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Acidified solvents such as water, ethanol, methanol, acetone, ethyl acetate, and their mixtures have been widely used to improve the phytochemical yield extracted from plant materials. However, the yield of phytochemicals obtained depends on the method of extraction employed (Haminiuk *et al*., 2014). The extraction of phenolics and flavonoids depends on the polarity of the extracting solvent (Lapornik *et al*., 2005; Turkmen *et al*., 2006). Dietary phenolics have shown antimicrobial effects on the gastrointestinal (GI) microbiota population and this ability affects the health of the GI tract and the metabolism of dietary phenolics (Selma *et al*., 2009). Polyphenols extracted from grape and red wine have been reported to enhance human health by modulating gut microbiota. Those results show that there is a potential relationship between gut microbiota and dietary polyphenols in relation to human health (Nash *et al*., 2018).

The purpose of the present work was to investigate the effects of different extraction solvents on the final yields of total phenolic and flavonoid compounds from *E. elatior* inflorescences, and to test their antioxidant activity and prebiotic properties by action of gut microbiota.

Materials and methods

Materials

The materials were 14 day-old fresh pink *E. elatior* inflorescences obtained from the local Phikunthong Development Study Centre, Narathiwat, Thailand. The chemicals used for HPLC analysis were purchased from Sigma-Aldrich (Darmstadt, Germany) and Carlo Erba (Cornared MI, Italy). The DNA probes (Bif164, Lab158, Chis150, Bac303, and Eub338) used for fluorescent *in situ* hybridisation (FISH) were purchased from Sigma-Aldrich (Darmstadt, Germany). All other chemicals were of analytical grade and purchased from Ajax Finechem Pty Limited (Taren Point, Australia) and Merck (Darmstadt, Germany).

Sample preparation

E. elatior inflorescences were washed with water, cut using a knife into 0.5 × 0.5 cm pieces, dried using a freeze dryer (Martin Christ, Germany) for 24 h, and ground using a commercial kitchen blender (Tefal, France). The powder was passed through a 35-mesh sieve to obtain a powder with particle size < 0.5 mm and then stored at 4°C.

Extraction of E. elatior

E. elatior powder (10 g) was added to 30 mL of four different solvents (distilled water and ethanol with concentrations of 50, 70, and 95% v/v) then mixed overnight at room temperature using an orbital shaker (Daihan, Korea) with a speed of 125 rpm. The extract was suction-filtered with Whatman No. 1 filter paper. The supernatant was pooled and evaporated using a rotary evaporator (Eyela, USA) (175 mbar, 45°C) and then freeze-dried. The crude extract was kept at -20°C in an aluminium-foil-covered bottle to protect it from light. The percentage crude extract yield was calculated using Eq. 1:

$$\text{Yield (\%)} = \frac{\text{weight of the crude extract (g)}}{\text{weight of the sample (g)}} \times 100\% \quad \text{(Eq. 1)}$$

Determination of total phenolic content of E. elatior extract

The total phenolic contents of the extracts were determined using Folin-Ciocalteu assay following the method of Jeevani Osadee Wijekoon *et al*. (2011) with modification. The sample extract (200 µL) was added into 1 mL of Folin-Ciocalteu reagent (10%, v/v) and incubated for 6 min, then 1 mL of sodium carbonate solution (10%, w/v) was added, adjusted with water up to 5 mL and incubated for 30 min at room temperature. Following incubation, the absorbance was measured using a UV-vis spectrophotometer (Biotek, USA) at 765 nm. A standard of gallic acid was prepared in the range of 50-300 µg/mL. The total phenolic contents were expressed as mg gallic acid equivalents (GAE) per gram of sample using Eq. 2:

$$y = 0.0028x, R^2 = 0.9965 \quad \text{(Eq. 2)}$$

where, y = absorbance, and x = GAE (mg/g).

Determination of total flavonoid content of E. elatior extract

The total flavonoid contents in the extracts were determined following the aluminium trichloride method of Jeevani Osadee Wijekoon *et al*. (2011) with modification. The sample extract (250 µL) was added to 1.25 mL of distilled water, followed by the addition of 75 µL of sodium nitrite solution (5%, w/v) and incubated for 5 min. To this mixture was then added 150 µL of aluminium chloride (10%, w/v) and it was again incubated for 6 min, then 0.5 mL sodium hydroxide (1 M) was added. The mixture was then

mixed vigorously using a vortex mixer (Vortex-Genie 2, Scientific Industries Inc., USA). The absorbance was measured using a UV-vis spectrophotometer at 510 nm. A standard of quercetin was prepared in the range of 300 - 900 µg/mL. The total flavonoid contents were expressed as mg quercetin equivalents (QE) per gram of sample using Eq. 3:

$$y = 0.0006x, R^2 = 0.9821 \quad \text{(Eq. 3)}$$

where, y = absorbance, and x = QE (mg/g).

Determination of antioxidant activities of E. elatior extract

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH radical scavenging assay technique was adapted from the method of Chan *et al*. (2007). A DPPH solution was prepared by adding 5.9 mg of DPPH to 100 mL of methanol. The mixture was mixed vigorously and incubated in the dark at room temperature for 3 h. The extracts (1 mL) were added to 2 mL of DPPH and incubated for 30 min at room temperature before measuring the absorbance at 517 nm. The DPPH radical scavenging activity was expressed as µg/mL of GAE per gram of the sample using Eq. 4:

$$y = -0.0608x + 0.6167, R^2 = 0.9963 \quad \text{(Eq. 4)}$$

where, y = absorbance, and x = GAE (µg/mL).

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed following the method of Maizura *et al*. (2011). Acetate buffer (300 mM, pH 3.6) was prepared by adding 16 mL of glacial acetic acid to 3.1 g of sodium acetate trihydrate. A 2,4,6-tripyridyltriazine (TPTZ) solution was prepared by adding 0.031 g of TPTZ to 40 mM of HCl. The FRAP reagent was prepared by mixing 100 mL of acetate buffer, 10 mL of TPTZ solution, 10 mL of iron (III) chloride hexahydrate and 12 mL of distilled water. The solution was incubated in a water bath at 37°C for 30 min. A sample of 30 µL of the plant extract was added to 1 mL of FRAP reagent and incubated in a dark room for 30 min. The absorbance was measured at 593 nm. The FRAP of the sample was expressed as mg GAE/g extract using Eq. 5:

$$y = 0.0146x + 0.1614, R^2 = 0.9915 \quad \text{(Eq. 5)}$$

where, y = absorbance, and x = GAE (mg/g).

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity

The ABTS radical scavenging activity was measured following the method of Thaipong *et al*. (2006). ABTS was dissolved in distilled water to obtain a final concentration of 7 mM. The ABTS solution was incubated at room temperature for 12 - 16 h then mixed with 2.45 mM potassium persulfate (1:0.5). A 150 µL sample of the plant extract was added to 2 mL of ABTS solution and mixed. The mixture was incubated in a dark room for 6 min and the absorbance was measured at 734 nm. The ABTS activity of the sample was expressed as mg GAE/g extract using Eq. 6:

$$y = -0.0227x + 0.8899, R^2 = 0.9926 \quad \text{(Eq. 6)}$$

where, y = absorbance, and x = GAE (mg/g).

Iron chelating activity

The iron chelating activity was measured following the method of Boonpeng *et al*. (2014). A 200 µL sample of the plant extract at different concentrations was mixed with 20 mL of iron (II) chloride solution (1.2 mmol/L in H_2O). Ferrozine solution (20 µL) with a concentration of 2.4 mmol/L was added to the mixture and incubated for 10 min. The absorbance was measured at 562 nm. The iron chelating activity of the sample was expressed as gram of EDTA equivalent per gram of sample using Eq. 7:

$$y = -0.0333x + 0.7490, R^2 = 0.9908 \quad \text{(Eq. 7)}$$

where, y = absorbance, and x = equivalent of EDTA (g/g).

Identification of phenolic compounds by high performance liquid chromatography

The phenolic compounds were identified by high performance liquid chromatography (HPLC) (Agilent model 1200 series, CA, USA) with a quaternary pump using a Zorbax Eclipse C18 column (Agilent, CA, USA) with a 5 µm diameter and a 4.6 × 250 mm length at 30°C with a 5 µL volume of injection. A diode array detector (DAD) was used in the HPLC system while the UV absorbance was set at 280 and 300 nm. The mobile phase was purified water with 1% (v/v) of formic acid (solvent A) and methanol (solvent B) with a flow rate of 1 mL/min. The elution

was achieved with the following gradient solvents; solvent A to solvent B: 90% to 10% for 10 min, 75% to 25% for 20 min, 40% to 60% for 30 min, 30% to 70% for 40 min and finally, returned to solvent B for 5 min. The phenolic compounds were identified by comparing the unknown samples with authentic samples. External calibration curves of the phenolic standard were used to analyse the concentration of phenolic acid in each of the samples. The phenolic acids were expressed as the mean ± standard deviation of three experiments (Del Pozo-Insfran *et al*., 2006).

In vitro faecal fermentation (batch culture) of E. elatior extract

The prebiotic properties of *E. elatior* extract were evaluated by batch culture faecal fermentation and compared to quercetin and gallic acid standards. An additional experimental sample for a negative control containing no carbon source was also tested. Fresh faecal samples were prepared from four volunteers who had not received antibiotic treatment for the past three months and mixed with 0.1 M of pre-reduced phosphate buffer (pH 7.0) in a stomacher for 2 min to give a final faecal slurry concentration of 10% (w/v). Water jacket glass vessels were filled with 90 mL of pre-reduced basal culture medium and stirred magnetically. The basal culture medium contained, per litre: 0.9 g peptone water, 0.9 g yeast extract, 0.045 g NaCl, 0.018 g K_2HPO_4, 0.018 g KH_2PO_4, 0.0045 g $MgSO_4.7H_2O$, 0.0045 g $CaCl_2.6H_2O$, 0.9 g $NaHCO_3$, 0.0225 g hemin, 0.225g L-cysteine HCl, 0.225 g bile salts, 0.9 mL Tween 80, 4.5 µL vitamin K, and resazurin solution (0.45 mL of 0.025% w/v). The pH and temperature were controlled (pH 6.8 ± 0.2, 37°C) throughout the experiment using a pH controller and a circulated-water bath, respectively. An anaerobic environment was maintained by constantly pumping nitrogen gas into the vessel. The basal culture medium was inoculated with 10 mL of faecal slurry and the samples were added and dissolved to give a final concentration of 1% (w/v). Samples (5 mL) were taken from each vessel at 0, 6, 12, 24, 48, and 72 h of incubation for short chain fatty acid (SCFA) analysis by HPLC and faecal bacteria enumeration by the FISH technique (Chaikliang *et al*., 2015). All the samples were kept at -20°C.

Enumeration of faecal bacteria by the FISH technique

Oligonucleotide probes were designed to target specific regions of 16S rRNA for bacterial populations using the FISH technique. The bacterial groups enumerated were *Bifidobacterium* spp. (Bif164), *Lactobacillus* / *Enterococcus* spp. (Lab158), *Bacteroides* spp. (Bac303), *Clostridium* / *Enterobacter* spp. (Chis150), and eubacteria (Eub338). Samples (375 µL) from the faecal batch cultures were fixed using 1,125 µL of cold 4% paraformaldehyde (pH 7.2) at 4°C overnight to immobilise the bacterial cells. The samples were then centrifuged (13,000 *G*, 15 min at 4°C) and washed twice in filtered 0.1 M PBS solution (pH 7.0). The pellets were resuspended in 150 µL of PBS, mixed with 96% ethanol (150 µL) then stored at -20°C for further use. The samples were dropped (20 µL) into each well of Teflon- and poly-L-lysine-coated six-well slides (Tekdon Inc., FL, USA). The samples on the slides were then dried for 10 min using a slide warmer (Digicon MD-700A, Thailand) at 46 - 50°C, dehydrated using alcohol series (50, 80, and 96%) for 3 min in each solution and were then returned to the slide warmer to evaporate the ethanol, before adding 50 µL of probe / hybidisation mixture (1:9; v/v) into each well. The procedure for the cells targeted with the Lab158 probe was slightly modified by applying 20 µL of lysozyme into each well for 15 min then washing for 2 - 3 s in water before being dehydrated in alcohol series. Hybridisation was performed for 4 h in a hybridisation chamber (Boekel Scientific, In Slide Out Slide Hybridiser 241000, Pennsylvania, US) at appropriate temperatures for the probe. Following hybridisation, the slides were washed with wash buffer (50 mL) for 15 min then briefly washed for 2 - 3 s in cold water and dried with a stream of compressed air. Antifade reagent (5 µL; Invitrogen, USA) was added into each well and covered with a cover slip. The slides were stored in the dark and the bacterial cells were counted under a fluorescent microscope (Nikon E400 Eclipse, Japan). For each well, 30 randomised views were counted in the range of 20 - 200 cells (Wichienchot *et al*., 2016).

The gut microbial balance effect of *E. elatior* extract was measured by the gut microbial balance index (GMBI) equation, modified from Rueangwatcharin and Wichienchot (2015):

$$\text{Gut microbial balance index (GMBI)} = \alpha + \beta - \gamma - \delta \quad \text{(Eq. 8)}$$

$$\alpha = (Bif_t / Bif_0) / Total \quad \text{(Eq. 9)}$$

$$\beta = (Lac_t / Lac_0) / Total \quad \text{(Eq. 10)}$$

$$\gamma = (Bac_t / Bac_0) / Total \quad \text{(Eq. 11)}$$

$$\delta = (Clos_t / Clos_0) / Total \quad \text{(Eq. 12)}$$

where,

Total = total numbers of eubacteria at 6, 12, 24, 48, and 72 h / total number at 0 h
α = change in numbers of bifidobacteria at 0, 6, 12, 24, 48, and 72 h
β = change in numbers of lactobacilli at 0, 6, 12, 24, 48, and 72 h
γ = change in numbers of bacteroides at 0, 6, 12, 24, 48, and 72 h
δ = change in numbers of clostridia / enterobacter at 0, 6, 12, 24, 48, and 72 h

SCFA analysis by HPLC

Samples from the batch cultures were centrifuged (13,000 g, 15 min at 4°C) and the supernatant was passed through a 0.22 µm membrane nylon filter. Samples were analysed for the concentrations of acetic, propionic, isobutyric, and butyric acids by HPLC (Agilent Technologies, USA) using an ion-exclusion Aminex HPX-87H (7.8 × 300 mm; Bio-Rad, USA) column at 50°C. The mobile phase was 0.005 M H_2SO_4 with a flow rate of 0.6 mL/min and data were acquired using Chemstation software (Agilent Technologies, USA) with the UV absorbance set at 210 nm. Quantification of the SCFAs was calculated from the calibration curves of acetic, propionic, isobutyric, and butyric acids (Chaikliang *et al*., 2015).

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 for Windows (IBM Corp. New York, USA). Data were expressed as means ± standard deviations from triplicate experiments (n = 3). The variation within the samples was analysed using one-way analysis of variance (ANOVA) and Duncan's multiple range test, and the level of statistical significance was set to $p < 0.05$.

Results and discussion

Extraction of phytochemicals from E. elatior

Phytochemicals from *E. elatior* were obtained by extraction from the inflorescences using water and ethanol as solvents with three different concentrations (50, 70, and 95%). The results showed that 50% ethanol gave the highest yield (14.12%) when compared with the other solvents. Extraction with 95% ethanol showed the lowest yield (5.33%) of *E. elatior* extract. These results suggest that extraction yields could be increased by increasing the polarity of the extraction solvent. The use of a high polarity solvent may cause other unwanted bioactive compounds (i.e. glycosides) to be extracted together with the target compound (phenolics), contributing to a high yield of the extract.

Total phenolic and flavonoid contents of E. elatior extract

Phenolic compounds from plants have been shown to have antioxidant properties for the scavenging of free radicals (Jeevani Osadee Wijekoon *et al*., 2011). The highest value of total phenolics (304.68 mg GAE/g extract) was obtained from the 70% ethanol extract and the lowest value (112.14 mg GAE/g extract) was found in the water extract. Jeevani Osadee Wijekoon *et al*. (2011) reported that a water extract of *E. elatior* (Jack) contained the lowest amount of phenolic compounds when compared with methanol and acetone extracts, and the result in the present work agrees with that finding. The phenolic compounds identified in the present work were gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, and ferulic acid. The concentrations of the phenolic compounds identified are shown in Table 1. The extract from 95% ethanol contained the highest concentration (89.89 ± 0.41 mg/g) of total phenolics identified. Chlorogenic acid was the dominant phenolic compound identified

Table 1. Concentration of phenolic compounds identified in *E. elatior* extracts.

Phenolic compound	Phenolic concentration (mg/g)			
	Water	50% ethanol	70% ethanol	95% ethanol
Gallic acid	ND	0.59 ± 0.02^{b}	0.75 ± 0.01^{a}	0.61 ± 0.03^{b}
Protocatechuic acid	ND	1.49 ± 0.00^{b}	2.12 ± 0.14^{a}	2.11 ± 0.25^{a}
Chlorogenic acid	3.60 ± 1.18^{d}	10.28.87 ± 0.85^{c}	40.14 ± 0.03^{b}	70.05 ± 1.59^{a}
Caffeic acid	1.24 ± 0.10^{d}	2.20 ± 0.04^{c}	3.18 ± 0.02^{b}	5.88 ± 0.02^{a}
Syringic acid	20.10 ± 0.06^{a}	4.34 ± 0.56^{c}	6.69 ± 0.09^{c}	9.70 ± 1.69^{b}
p-coumaric acid	0.30 ± 0.02^{c}	0.33 ± 0.03^{c}	0.47 ± 0.00^{b}	0.95 ± 0.02^{a}
Ferulic acid	ND	ND	0.21 ± 0.01^{b}	0.58 ± 0.02^{a}
Total	25.23 ± 1.32^{c}	19.22 ± 0.25^{d}	53.55 ± 0.05^{b}	89.89 ± 0.41^{a}

ND = not detected. Means with different superscript letters in the same column indicate significant difference ($p < 0.05$).

Table 2. Antioxidant activity of *E. elatior* extracts.

Solvent	DPPH (mg GAE/g extract)	FRAP (mg GAE/g extract)	ABTS (mg GAE/g extract)	Iron chelating activity (mg EDTA/g extract)
Water	98.79 ± 6.22[d]	18.58 ± 0.00[d]	15.85 ± 0.09[d]	8.27 ± 0.23[d]
50% ethanol	165.42 ± 7.20[b]	36.70 ± 0.48[b]	47.45 ± 1.39[b]	13.81 ± 0.32[b]
70% ethanol	234.34 ± 3.61	66.25 ± 0.55	57.52 ± 0.70	10.49 ± 0.43[c]
95% ethanol	111.69 ± 0.70[c]	32.41 ± 0.14[c]	33.79 ± 0.13[c]	24.10 ± 0.23

Means with different superscript letters in the same column indicate significant difference ($p < 0.05$).

from the ethanol extracts. A previous study reported that high concentrations of ethanol produced higher contents of phenolic compounds when compared with water extracts (Jung *et al*., 2006) as evidenced in the present work.

Flavonoids are the most common and widely distributed group of phenolic compounds in plants (Jeevani Osadee Wijekoon *et al*., 2011). In the present work, 95% ethanol extract produced the highest amount of flavonoids (400.95 mg GAE/g extract). Water extraction yielded the lowest amount of flavonoids, which agrees with Jeevani Osadee Wijekoon *et al*. (2011) and Do *et al*. (2014). The extraction solvent plays an important role and affects the yield of flavonoids during extraction (Mazandarani *et al*., 2012). Previous studies have reported that methanol has been widely used to extract flavonoids as it produces high yields (Sulaiman *et al*., 2011). However, the use of methanol for flavonoid extraction may not be feasible in the food industry due to the high risk of toxicity (Chan *et al*., 2011). Thus, water and ethanol are more suitable solvents for flavonoid extraction in the food industry with safety and feasible cost.

It has been reported that Thai black rice contains phenolic compounds including protocatechuic acid, syringic acid, caffeic acid, and *p*-coumaric acid (Pengkumsri *et al*., 2015). Brown rice contains protocatechuic acid, syringic acid, and chlorogenic acid (Sumczynski *et al*., 2016). Nine phenolic compounds were identified in the soaked Thai purple rice. These included gallic acid, protocatechuic acid, catechin, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid, rutin trihydrate, and *trans*-cinnamic acid (Owolabi *et al*., 2018). Phenolic compounds could be found in various plants which may vary in their types and concentrations. Most pigmented rice varieties were high in protocatechuic acid but *E. elatior* was high in chlorogenic acid. The type and concentration of phenolic compound could effect on the modulation of certain groups of gut microbiota.

Antioxidant activities of E. elatior extract

The antioxidant activity (AOA) of the inflorescence extract of *E. elatior* was evaluated using DPPH, FRAP, ABTS, and iron chelating assays. The AOA results showed that different solvents produced different AOAs. Table 2 shows that the extraction with 70% ethanol yielded the highest values for DPPH (234.34 ± 3.61 mg GAE/g extract), FRAP (66.25 ± 0.55 mg GAE/g extract) and ABTS (57.52 ± 0.70 mg GAE/g extract) activity. The highest value for iron chelating activity was found in the extract from 95% ethanol for which the value was 24.10 ± 0.23 mg EDTA/g extract. Water extraction showed the lowest AOA values, which was probably related to the polarity of the solvent, which plays a critical role in the extraction process, thereby affecting the solubility of the antioxidant compounds (Addai *et al*., 2013).

Methanol, acetone, ethanol, ethyl acetate, and propanol are the most common solvents used for the extraction of antioxidant compounds from fresh fruits and vegetables. The recovery of antioxidants is affected by both the solubility and the extraction time. The solubility of antioxidant compounds is affected by the polarity of the solvent. Thus, a screening process is important to identify the optimal solvents for specific extraction from specific sample (Wojdyło *et al*., 2007). Jeevani Osadee Wijekoon *et al*. (2011) reported that antioxidant compounds from *E. elatior* inflorescences can be a potential source of natural antioxidants for food and nutraceuticals.

Prebiotic effects of E. elatior extract

Faecal batch culture was used to evaluate the prebiotic effects of *E. elatior* extract. The bacterial groups counted were bifidobacteria, lactobacilli, bacteroides, clostridia / enterobacter, and eubacteria. The substrates were *E. elatior* extracts from the different extraction solvents (water, 50, 70, and 95% ethanol), with gallic acid and quercetin used as comparison and an additional control experiment without a carbon source.

Figure 1a shows the populations of bifidobacteria. The numbers of bifidobacteria populations in all of the substrates were lower as compared to the control during the faecal fermentation. The populations of lactobacilli are shown in Figure 1b. It was observed that not all the substrates supported the growth of

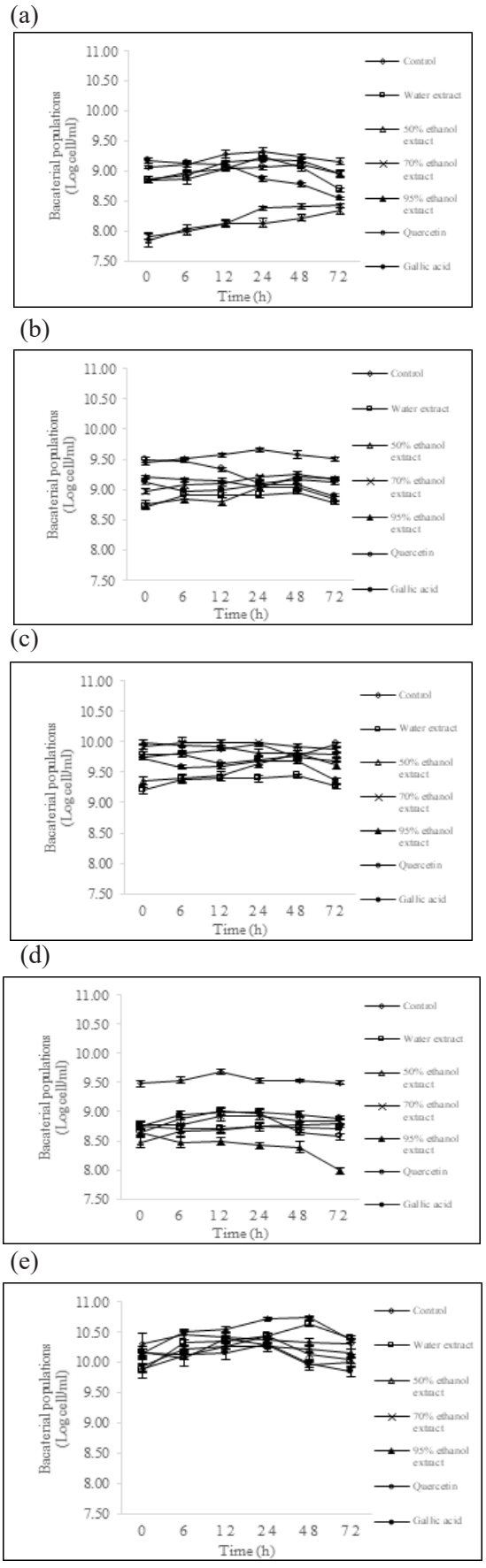

Figure 1. Faecal bacterial populations of (a) bifidobacteria, (b) lactobacilli, (c) bacteroides, (d) clostridia/enterobacter, and (e) total bacteria in faecal batch culture fermentation.

lactobacilli and that the population was lower than the control. The bacteroides populations (Figure 1c) decreased significantly ($p < 0.05$) after 6 h of fermentation with the *E. elatior* extracted with 95% ethanol. A significant decrease of clostridia populations (Figure 1d) was also observed in the fermentation of *E. elatior* extracted with 95% ethanol after 6 h. Figure 1e shows the total bacterial populations for all of the substrates, in which it was found that the populations of total bacteria for the fermentation of *E. elatior* extract with 95% ethanol increased significantly ($p < 0.05$) and were higher than the control after 6 h. The results indicated that no substrate promoted the growth of beneficial bacteria (bifidobacteria and lactobacilli). On the other hand, the bacteroides and clostridia / enterobacter populations were reduced by *E. elatior* extracted with ethanol during the faecal batch culture fermentation. Parkar *et al*. (2013), who studied the faecal microbial metabolism of polyphenols, showed that polyphenol fermentation stimulated the growth of Bifidobacterium spp. and decreased the ratio of Firmicutes to Bacteroidetes, as relative to control. Comparing to that study, the results obtained in the present work showed that the extracts of *E. elatior* did not support the growth of beneficial bacteria (bifidobacteria and lactobacilli) and that the populations of pathogenic bacteria decreased significantly ($p < 0.05$) during faecal fermentation. This could be due to the fact that the extract of *E. elatior* contains phenolic compounds which have antibacterial property.

The gut microbial balance index (GMBI) reflects the relationship between the growth of beneficial bacteria (bifidobacteria and lactobacilli) and pathogenic bacteria (clostridia / enterobacter and bacteroides) in relation to the changes of total bacteria (eubacteria). The GMBI values for each substrate are shown in Figure 2 which shows that the highest GMBI value (0.08) was found at 72 h in the fermentation of *E. elatior* extracted with 95% ethanol, followed by 70%, and 50% ethanol extraction (0.06 and 0.04, respectively). Phenolic compounds from *E. elatior* extracts showed higher GMBI values as compared to commercial gallic acid and quercetin. This is probably because of differences in the biodiversity of gut microbiota in the faecal inoculum (Parkar *et al*., 2013) and the chemical composition and molecular weight distribution of the substrates tested. Phenolic compounds from the ethanol extracts of *E. elatior*, therefore, showed the potential to improve gut balance by suppression of harmful or pathogenic bacteria.

Previous studies have focused on gut microbiota metabolism derived from non-digestible but

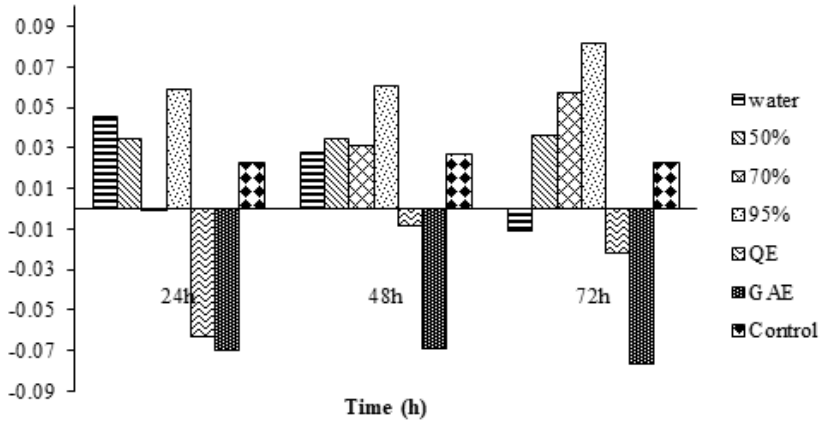

Figure 2. Gut microbial balance index (GMBI) of *E. elatior* extracts, Quercetin and gallic acid in faecal batch culture at 24, 48 and 72 h of fermentation.

fermentable carbohydrates and oligosaccharides (Healey *et al*., 2017; Ariestanti *et al*., 2019; Wang *et al*., 2019a). Recently, however, there has been increased attention paid to the gut fermentation of phenolic compounds due to the role these may play in reshaping the gut microbiota and the benefits they may provide to the host (Espín *et al*., 2017). Phenolic compounds have been reported to have beneficial effects related to their ability to modulate the gut microbiota (Serra *et al*., 2018) with many studies indicating the potential of polyphenols as a candidate prebiotic. However, few studies have been carried out to confirm this in vivo (Etxeberria *et al*., 2013). The present work shows that the extracts of *E. elatior* inflorescence from ethanol, which mostly contained chlorogenic acid, have a prebiotic-like effect. And it has a potential to improve the gut balance based on the in vitro study by faecal batch culture. These results are comparable with the previous results reported by Parkar *et al*. (2013). Ayimbila and Keawsompong (2018) evaluated the colonic fermentation of riceberry rice hydrolysates and found that Lactobacillus and Bacteroides growth were enhanced while the pathogenic bacteria were neither enhanced nor suppressed. The soaked rice polyphenols showed similar results to those of the present work in that it could suppress the growth of clostridia / enterobacter in faecal batch culture (Owolabi, 2019).

Short-chained fatty acids production

SCFAs are the metabolites produced by the gut microbiota metabolism which include acetate, propionate, butyrate, and some branch-chained fatty acids (BCFAs) such as isobutyrate. Table 3 shows the concentrations of SCFAs produced in the *E. elatior* extracts; gallic acid, quercetin, and the control experiment during the faecal fermentation batch culture. The results showed that acetate was produced in the fermentation of all the substrates. The highest concentrations of acetate (40.62 ± 1.37 mM) and propionate (38.21 ± 0.13 mM) were both found in the quercetin sample at 48 h. The *E. elatior* sample extracted with 50% ethanol and the quercetin sample yielded the highest concentration of isobutyrate (19.92 ± 0.04 mM) after 12 h of fermentation. All of the samples produced only small amounts of lactate during faecal fermentation and the highest concentration (8.58 ± 0.02 mM) was produced at 48 h from 50% ethanol *E. elatior* extract. The propionate produced from quercetin in the present work is higher than the amount reported by Parkar *et al*. (2013). This might be due to the use of different methods of fermentation and different faecal inoculum. The SCFAs produced during faecal batch culture were in the order: acetate and propionate followed by BCFA-isobutyrate and medium-chained fatty acid (MCFA)-lactate. The high amount of SCFAs produced in the present work suggest that phenolic compounds from *E. elatior* extract were used by intestinal bacteria to activate the production of SCFAs. It has been reported that concentration of each SCFA produced by gut fermentation depends on type of dietary substrate. Dietary fibres produced acetate and propionate while resistant starch (RS) produced butyrate (Wang *et al*., 2019b). Recently, gut fermentation of dietary polyphenols has raised interest due to it having the potentials to promote memory, stress, and neurodegeneration. Gut microbiota could transform dietary polyphenols to phenolic metabolites which in turn could affect human health (Frolinger *et al*., 2019).

Table 3. Concentration (mM) of short chain fatty acid in batch culture fermentation of *E. Elatior* extracts.

Substrate	Time (h)	Lactate	Acetate	Propionate	Isobutyrate	Butyrate	Total SCFA
Control	0	0.44 ± 0.00	1.56 ± 0.00^{f}	1.87 ± 0.13	9.75 ± 0.10^{a}	ND	13.62 ± 0.05^{a}
	6	ND	6.52 ± 0.03^{e}	ND	7.43 ± 0.03^{b}	ND	13.95 ± 0.03^{b}
	12	ND	7.35 ± 0.01^{d}	ND	ND	ND	7.35 ± 0.01^{e}
	24	ND	7.73 ± 0.31^{c}	ND	ND	ND	7.73 ± 0.31^{de}
	48	ND	8.07 ± 0.11^{b}	ND	ND	ND	8.07 ± 0.11^{cd}
	72	ND	8.45 ± 0.02^{a}	ND	ND	ND	8.45 ± 0.02^{c}
Water extract	0	ND	4.34 ± 0.23^{d}	4.11 ± 0.37^{a}	ND	ND	8.45 ± 0.3^{f}
	6	2.65 ± 0.00^{b}	8.11 ± 0.04^{c}	3.30 ± 0.04^{b}	ND	ND	14.06 ± 0.04^{e}
	12	1.75 ± 0.00^{c}	10.89 ± 0.11^{b}	3.37 ± 0.25^{b}	ND	ND	16.01 ± 0.13^{d}
	24	ND	10.84 ± 0.03^{b}	3.73 ± 0.05^{ab}	5.24 ± 0.04^{a}	ND	19.82 ± 0.03^{b}
	48	2.94 ± 0.00^{a}	11.65 ± 0.08^{a}	2.40 ± 0.02^{c}	4.50 ± 0.12^{b}	ND	21.49 ± 0.06^{a}
	72	ND	11.11 ± 0.04^{b}	2.47 ± 0.13^{c}	5.21 ± 0.20^{a}	ND	18.79 ± 0.12^{c}
50% ethanol extract	0	ND	5.93 ± 0.01^{c}	5.07 ± 0.02^{a}	17.20 ± 0.07^{c}	ND	28.20 ± 0.03^{c}
	6	2.69 ± 0.01^{b}	5.92 ± 0.16^{c}	4.56 ± 0.03^{b}	18.32 ± 0.00^{b}	ND	31.49 ± 0.05^{b}
	12	2.59 ± 0.00^{c}	9.39 ± 0.05^{b}	3.84 ± 0.02^{d}	19.92 ± 0.04^{a}	ND	35.74 ± 0.03^{a}
	24	ND	9.92 ± 0.00^{a}	3.91 ± 0.01^{c}	14.68 ± 0.03^{d}	ND	28.51 ± 0.01^{e}
	48	8.58 ± 0.02^{a}	9.91 ± 0.03^{a}	2.85 ± 0.02^{e}	5.35 ± 0.04^{e}	ND	26.69 ± 0.03^{d}
	72	1.28 ± 0.00^{d}	9.76 ± 0.01^{a}	2.01 ± 0.01^{f}	ND	ND	13.06 ± 0.00^{f}
70% ethanol extract	0	ND	5.68 ± 0.08^{d}	2.01 ± 0.01^{c}	ND	ND	7.69 ± 0.05^{d}
	6	1.14 ± 0.11	6.23 ± 0.49^{c}	6.07 ± 0.01^{a}	ND	ND	13.44 ± 0.2^{c}
	12	ND	12.51 ± 0.05^{a}	3.85 ± 0.55^{b}	8.12 ± 0.60^{a}	ND	24.48 ± 0.4^{a}
	24	ND	7.06 ± 0.06^{b}	4.07 ± 0.14^{b}	8.59 ± 0.27^{a}	ND	19.72 ± 0.16^{b}
	48	ND	7.12 ± 0.03^{b}	ND	ND	ND	7.12 ± 0.03^{d}
	72	ND	6.93 ± 0.13^{b}	ND	ND	ND	6.93 ± 0.13^{d}
95% ethanol extract	0	ND	9.86 ± 0.80^{c}	11.97 ± 0.38^{a}	5.96 ± 0.47^{b}	ND	27.78 ± 0.55^{b}
	6	3.59 ± 0.02^{a}	9.61 ± 0.03^{c}	6.62 ± 0.13^{d}	9.91 ± 0.19^{a}	ND	29.74 ±0 .09^{a}
	12	3.27 ± 0.45^{a}	12.09 ± 0.03^{b}	10.70 ± 0.03^{b}	ND	ND	26.07 ± 0.17^{c}
	24	2.13 ± 0.48^{b}	14.83 ± 0.35^{a}	10.20 ± 0.10^{c}	6.65 ± 0.75^{b}	ND	33.81 ± 0.42^{a}
	48	ND	15.45 ± 0.28^{a}	ND	ND	ND	15.45 ± 0.28^{e}
	72	ND	8.60 ± 0.10^{d}	ND	9.83 ± 0.14^{a}	ND	18.43 ± 0.12^{d}
Gallic acid	0	ND	ND	2.22 ± 0.51	9.86 ± 0.37^{b}	ND	12.09 ± 0.44^{d}
	6	ND	6.10 ± 0.21^{d}	2.15 ± 0.07	10.72 ± 0.56^{b}	ND	18.97 ± 0.28^{c}
	12	3.11 ± 0.01	17.19 ± 1.50^{bc}	2.18 ± 0.03	11.99 ± 0.09^{a}	ND	34.46 ± 0.41^{a}
	24	ND	13.70 ± 0.11^{c}	1.85 ± 0.10	ND	ND	15.55 ± 0.11^{d}
	48	ND	18.04 ± 3.34^{b}	ND	ND	ND	18.04 ± 3.34^{d}
	72	ND	22.00 ± 0.27^{a}	ND	7.00 ± 0.61^{c}	ND	29.00 ± 0.44^{b}
Quercetin	0	ND	1.50 ± 2.12^{e}	3.02 ± 0.05^{d}	14.02 ± 0.34^{a}	ND	19.85 ± 0.84^{cd}
	6	ND	7.45 ± 0.17^{d}	1.06 ± 0.00^{e}	11.91 ± 0.12^{b}	ND	21.16 ± 0.97^{bc}
	12	ND	12.08 ± 0.27^{c}	17.71 ± 0.84^{c}	19.92 ± 0.04^{c}	ND	34.59 ± 0.38^{b}
	24	ND	14.65 ± 0.03^{b}	33.98 ± 0.01^{b}	ND	ND	48.63 ± 0.02^{b}
	48	0.74 ± 0.00	40.62 ± 1.37^{a}	38.21 ± 0.13^{a}	ND	ND	78.83 ± 0.5^{a}
	72	ND	ND	ND	ND	ND	ND

ND = not detected. Means with different superscript letters in the same column indicate significant difference ($p < 0.05$).

Conclusion

The phenolic compounds in the ethanol extracts of *E. elatior* inflorescence were mainly phenolic acids, particular chlorogenic acid. These phenolic compounds showed not only antioxidant activity but also a modulatory effect on the gut microbiota. The phenolic compounds in the *E. elatior* extract showed a prebiotic-like effect by significantly ($p < 0.05$) decreasing the populations of harmful bacteria through the production of SCFAs. Thus, phenolic compounds from ethanol extracts of *E. elatior* have the potential as a functional ingredient for the promotion of gut health and gut-brain axis related diseases. The functional ingredient containing phenolic compound might be used for prevention or treatment of memory impairment in elderly population.

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