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Total Phenolic and Flavonoid Contents, Antioxidant, Antibacterial and Anti-Inflammatory Activities and Toxicities of Ethanol Extracts from *Curcuma Mangga*, *Zingiber Officinale* and *Zingiber Montanum*

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Abstract

The objectives of this study were to determine and to compare total phenolic and flavonoid contents (TPC and TFC), antioxidant, antibacterial and anti-inflammatory activities and toxicities of ethanol extracts from *Curcuma mangga*, *Zingiber officinale* and *Zingiber montanum*. The study was performed by determinations of 1) TPC using Folin-Ciocalteu reagent method and TFC using colorimetric method of aluminum chloride, 2) antioxidant activities using DPPH, ABTS and FRAP assay, 3) antibacterial activities through evaluation inhibition of bacterial growth, MIC and MBC against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli,* and *Staphylococcus aureus*, 4) anti-inflammatory activity using Griess reaction to measure nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells and 5) toxicities using lethality assay in brine shrimps and MTT assay in the RAW 264.7 cells. The results revealed that all the extracts possess TPC and TFC, antioxidant, antibacterial and anti-inflammatory activities which the highest TPC and TFC and strongest activities were found in the *Z. montanum* extract. However, the extracts showed mildly to moderately toxic to brine shrimps, consider to *Artemia salina* nauplii, and nontoxic to the RAW 264.7 cells. And also, the results confirm the utilization of these extracts for the treatment and/or management of diseases that related to antioxidant, antibacterial and anti-inflammatory activities.

Keywords: *Curcuma mangga*, *Zingiber officinale*, *Zingiber montanum*, Antioxidant, Antibacterial, Anti-inflammation, Toxicities

Introduction

Spices in the family Zingiberaceae, including *Curcuma mangga*, *Zingiber officinale* and *Zingiber montanum* have been utilized not only as food spices, food preservatives and also as traditional medicine in many Asian countries for the treatment of various health problems and minor ailments. *C. mangga* has been reported to have anti-inflammation, antidiabetic, antioxidant [1,2,4] and antimicrobial activity [5]. *Z. officinale* is traditionally used for the prevention, treatment and management of various diseases. It possesses antioxidant, anti-inflammatory, antimicrobial, antibacterial and antioxidant [6,7,9] activities. Its active compounds can ameliorate inflammation [8]. *Z. montanum* is traditionally used for relieving

dysentery, constipation, dyspepsia, gastritis [10] and muscular and joint pain [12,13]. It displays antioxidant, antibacterial [11,14] and anti-inflammatory activities [13].

Despite a long traditional utilization of *C. mangga*, *Z. officinale* and *Z. montanum*, information on their chemical components and pharmacological activities still needs to be further determined. Therefore, this study was carried out to determine and compare TPC, TFC and antioxidant, anti-inflammatory and antibacterial activities of the extracts from *C. mangga*, *Z. officinale* and *Z. montanum*. To see whether it is safe for utilization, the toxicities of the extracts were investigated as well.

Materials and methods

Plant materials and plant extracts

The plant rhizomes used in this study were collected from the consistent region of Loei Province in the northeast of Thailand, namely *Curcuma mangga* Valeton (MSUT No. 8351), Zijp, *Zingiber officinale* Roscoe (MSUT No. 8352) and *Zingiber montanum* (J. Koenig) Link ex A (MSUT No. 8353).

Fresh rhizomes of the plants were washed, cut into small pieces, shade-dried, powdered and extracted [15]. Briefly, the plant powder was macerated separately by mixing 100 g of powder with 600 mL of 95 % ethanol, left for 7 days with occasional stirring and filtered. The filtrates were collected and evaporated to obtained extracts. The extracts were weighed for calculation of extract yield and stored at 4 °C before being used.

Determination of total phenolic content (TPC) and total flavonoid content (TFC)

Determination of TPC was carried out using Folin-Ciocalteu reagent method [15]. Briefly, 10 µL of each extract was separately mixed with 181.80 µL of 2 % sodium carbonate solution (Na_2CO_3) in a 96-well plate and left at room temperature. After 30 min, 10 µL of Folin-Ciocalteu reagent was added and mixed. The reaction mixture was allowed to stand in the dark at room temperature for a further 30 min. The absorbance was measured at 750 nm. The TPC was calculated and expressed as mg GAE/g.

Determination of TPC was performed using the colorimetric method of aluminum chloride [17]. Briefly, a volume of 20 µL of extract and different dilutions of a standard solution of Quercetin (QE) was added to a 96-well plate and then 7.50 µL $NaNO_2$ was added. After 5 min, 15 µL $AlCl_3$ followed by 50 µL NaOH and 107.5 µL distilled water were added and incubated at room temperature in darkness for 30 min. Absorbance was read at 430 nm. The TFC was calculated and expressed as mg QE/g.

Determination of antioxidant activities

The determination of antioxidant activities of the extracts was carried out using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay, ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-acid) cation radical scavenging assay and FRAP (ferric reducing antioxidant power) assay.

DPPH assay

The assay was performed according to Yongkhamcha *et al.* [16]. Briefly, 2 mL of 0.10 mM DPPH in ethanol was added to 200 µL of the different extracts (25, 50, 75 and 100 µg/mL). The mixture was incubated at room temperature for 30 min. After an incubation period, the absorbance measurements were taken at 517 nm. The activity was calculated and expressed as IC_{50} value.

ABTS assay

The assay was conducted according to Zheleva-Dimitrova [18]. Briefly, the stock solutions, including 7 mM ABTS and 2.40 mM potassium persulphate were prepared. The solution was then diluted by mixing 2 mL ABTS with 50 mL methanol. Different concentrations (1 mL) of the extracts were allowed to react with 2 mL of ABTS for 5 min. The absorbance was taken at 734 nm. The activity was calculated and expressed as IC_{50} value.

FRAP assay

The assay was performed according to Youn *et al*. [19]. Briefly, 20 µL of sample solutions was added to 150 µL of FRAP reagent. The mixture was incubated at 37 °C for 30 min. The absorbance was measured at 593 nm. The FRAP value was calculated and expressed as mgTE/mL.

To verify correlation between TPC or TFC and antioxidant activity, Pearson's correlation coefficients (r) was employed.

Determination of antibacterial activity

The bacterial strains used in this study were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus subtilis* obtained from the Department of Biology, Mahasarakham University.

Antibacterial activity of the extracts was carried out by determination inhibition of bacterial growth, minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC). The experiments were conducted according to Chaiyong *et al.* [20].

Inhibition of bacterial growth

Agar disc diffusion assay was utilized. Briefly, each bacterial strain (1.5×10^8 cells/mL) was inoculated on the surface of the nutrient agar plate. A volume of 10 mL of each extract (25 mg/mL) was dropped onto sterilized filter paper discs (5 mm in diameter). The discs were allowed to dry at room temperature before being placed on the test plates inoculated with the tested bacterial strains. The plates were incubated at 37 °C for 24 h. Inhibition of bacterial growth surrounding the discs was measured after 24 h and compared to Ciprofloxacin and 10 % Tween 20.

MIC

Broth dilution method was used for the determination of MIC (the lowest concentration that led to growth inhibition). A volume of 100 µL of each tested extract was added to the well of a 96-well plate containing 100 µL of Mueller Hinton Broth. Then 10 µL of bacteria (1.5×10^8 cells/mL) was added to each plate and incubated at 37 °C for 18 h. MIC was interpreted from the lowest concentration of the extract at which the bacteria did not demonstrate visible growth.

MBC

The MBC was determined by using the content from wells designated without visible turbidity (25, 50, 100 and 200 mg/mL) streaking on the agar plates. MBC (the lowest plant extract concentration that produced no cultivation on the nutrient agar plates) was investigated after the plates were incubated at 37 °C for 24 h.

Determination of anti-inflammatory activity

The anti-inflammatory activity of the tested extracts was determined by measuring nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells.

Prior to the determination of NO production, the determination of cell viability using MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reduction assay [21] was carried out to ensure that the RAW 264.7 cells were healthy and the extracts were nontoxic (% cell viability > 80 %).

Due to NO in the biological matrix is unstable and rapidly oxidizing to nitrite (NO^{2-}), thus in this study, NO production was carried out by measuring nitrite concentrations in the cultured medium using the Griess reaction [22]. Briefly, the RAW 264.7 cells were cultured in DMEM medium with PBS in a 96-well plate. LPS (1 µg/mL) was added to each well prior to the addition of the extracts. The plate was incubated at 37 °C and 5 % CO_2 for 24 h. Thereafter, the cultured medium was collected and transferred to a new plate followed by the addition of Griess reagent. The absorbance was measured at 570 nm. The percentage of NO production is calculated and compared to Diclofenac.

Determination of toxicities

Toxicities of the extracts were determined both to brine shrimp and to RAW 264.7 cells.

Toxicity to brine shrimp

Brine shrimp lethality assay [23] was used for toxicity testing of the extracts. Briefly, 10 nauplii of brine shrimp (*Artemia salina* L.) were cultured in each petri dish containing different concentrations of the extracts. The number of deaths in each petri dish was examined and counted after every 12 h for 24 h. Tween 20 (%) was used as a control. The percentage mortality was calculated and expressed as LC_{50} value.

Toxicity to RAW 264.7 cells

MTT assay [21] was used to determine toxicity to the RAW 264.7 cells after exposure to extracts. The toxicity to the RAW 264.7 cells was interpreted from % cell viability after exposure to the extracts, as % cell viability + % mortality = 100 %.

Statistical analysis

The results from the determination of TPC and TFC, antioxidant, antibacterial and anti-inflammatory activities and toxicities of ethanol extracts were analyzed using one-way analysis of variance (ANOVA), the differences among the tested groups were determined using Duncan's Multiple Range Test and the criterion for statistical significance was $p < 0.05$. Correlation of TPC or TFC and antioxidant activity was performed using Pearson's correlation coefficients. All results were expressed as mean ± standard deviation of 3 replications.

Results and discussion

Percentage yields

The percentage yields of the extracts from *C. mangga*, *Z. officinale* and *Z. montanum* were found to be significantly different ($p < 0.05$). They were 0.76, 0.75 and 0.46 % for the extracts from *Z. montanum*, *Z. officinale* and *C. mangga*, respectively as shown in **Table 1**.

TPC and TFC

The TPC and TFC of the extracts from *C. mangga*, *Z. officinale* and *Z. montanum* were different significantly ($p < 0.05$). The highest TPC (194.64 ± 1.27 mg GAE/g) was found in *Z. montanum* extract, followed by *Z. officinale* extract (170.47 ± 2.40 mg GAE/g) and *C. mangga* extract (126.67 ± 2.28 mg GAE/g), respectively. Meanwhile, the TFC was found to be highest in the *Z. montanum* extract (27.63 ±

0.60 mg QE/g) followed by *Z. officinale* extract (24.38 ± 0.24 mg QE/g) and *C. mangga* extract (20.26 ± 0.13 mg QE/g), respectively, as presented in **Table 1**.

Antioxidant activities

The results of the antioxidant activities of the extracts are shown in **Table 1**.

DPPH assay

The extracts from *C. mangga*, *Z. officinale* and *Z. montanum* possessed different DPPH scavenging activities significantly ($p < 0.05$). As IC_{50} values are inversely proportional to the antioxidant activity; the lowest IC_{50} value shows higher antioxidant activity, therefore the highest activity was found to be in the *Z. montanum* extact (IC_{50} of 0.24 ± 0.01 mg/mL), followed by *Z. officinale* extract (IC_{50} of 0.35 ± 0.01 mg/mL) and the *C. mangga* extract (IC_{50} of 0.39 ± 0.01 mg/mL), respectively.

ABTS assay

The *Z. montanum* extract showed the strongest ABTS activity with IC_{50} of 0.37 ± 0.01 mg/mL. followed by the *Z. officinale* extract with IC_{50} of 0.58 ± 0.02 mg/mL. The *C. mangga* extract showed the lowest activity with IC_{50} of 1.48 ± 0.05 mg/mL.

FRAP assay

The extracts displayed the different FRAP values significantly ($p < 0.05$). Due to the higher FRAP value is proportional to the higher antioxidant activity, therefore the *Z. montanum* extract displayed the highest antioxidant activity with FRAP value of 580.83 ± 8.61 mgTE/mL, followed by the *Z. officinale* extact (455.26 ± 7.27 mg TE/mL). The lowest activity was in the *C. mangga* extract with FRAP value of 109.99 ± 1.65 mg TE/mL.

Table 1 Percentage yield, TPC, TFC and antioxidant activities by DPPH, ABTS and FRAP assay of the extracts from *C. mangga*, *Z. officinale* and *Z. montanum*.

Extracts	% Yield	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH IC_{50} (mg/mL)	ABTS IC_{50} (mg/mL)	FRAP (mg TE/mL)
C. mangga	0.46^{a}	126.67 ± 1.28^{a}	20.26 ± 0.13^{a}	0.39 ± 0.01^{c}	1.48 ± 0.05^{c}	109.99 ± 1.65^{a}
Z. officinale	0.75^{b}	170.47 ± 0.40^{b}	24.38 ± 0.24^{b}	0.35 ± 0.01^{b}	0.58 ± 0.02^{b}	455.26 ± 7.27^{b}
Z. montanum	0.76^{b}	194.64 ± 1.27^{c}	27.63 ± 0.60^{c}	0.24 ± 0.01^{a}	0.37 ± 0.01^{a}	580.83 ± 8.61^{c}

Values are expressed as mean ± standard deviation of triplicates. The values followed by different superscript letters in the same column are significantly different at $p < 0.05$ among the tested groups.

Correlation between TPC or TFC and antioxidant activity

Pearson's correlation coefficients (r) demonstrated that there were significant correlations among TPC, TFC, DPPH, ABTS, and FRAP assay. TPC exhibied negative correlation to DPPH (r = –0.67) and ABTS (r = –0.83) and positive correlation to FRAP assay (r = 0.72). In line with TPC, TFC exhibited negative correlation to DPPH (r = –0.83) and ATBS (r = –0.57) and positive correation and FRAP assay (r = 0.78), as presented in **Table 2**.

Table 2 Correlation between TPC or TFC and antioxidant activity by DPPH, ABTS and FRAP assay of the ethanol extracts from *C. mangga*, *Z. officinale* and *Z. montanum*, by Pearson's correlation coefficients.

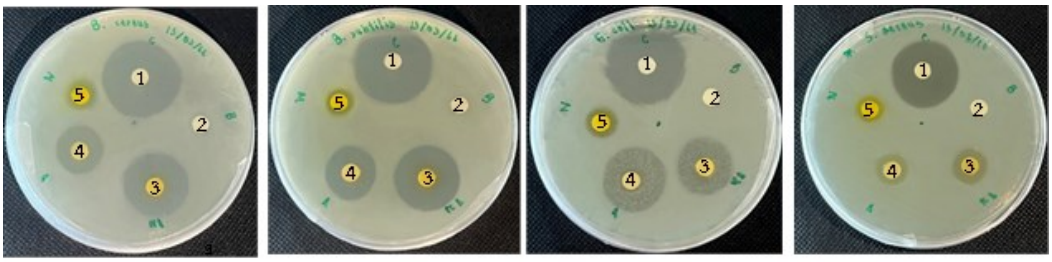
Active compound	Antioxidant activity		
	DPPH assay	ABTS assay	FRAP assay
TPC	–0.67*	–0.83**	0.72**
TFC	–0.83**	–0.57**	0.78**

* is significantly different at $p < 0.05$ and ** is significantly different at $p < 0.01$.

Antibacterial activities

Inhibition of bacterial growth

Disc diffusion assay demonstrated that the extracts from *C. mangga, Z. officinale and Z. montanum* inhibited the growth of bacterial strains, *B. cereus, B. subtilis, E. coli*, and *S. aureus*, as presented in **Figure 1**.



(A) (B) (C) (D)

Figure 1 Inhibition zones of bacterial growth; *B. cereus* (A)*, B. subtilis* (B)*, E. coli* (C), and *S. aureus* (D)*,* 1 = Ciprofloxacin; positive control, 2 = 10 % Tween 20; negative control, 3 = *C. mangga* extract*,* 4 = *Z. officinale* extract *and* 5 = *Z. montanum* extract.

The inhibition zones varied among the bacterial strains, from 4.67 ± 0.57 to 15.67 ± 0.57 mm. The greatest inhibition zone (15.67 ± 0.57 mm) was found in *B. cereus* that inhibited by *C. mangga* extract. The smallest zone was found in *S. aureus* (4.67 ± 0.57 mm) that inhibited by *Z. montanum* extract. The inhibition by the extracts was less than by Ciprofloxacin (23.33 ± 0.57 - 24.67 ± 0.57mm) (**Table 3**).

Table 3 Inhibition zones of the extracts from *C. mangga, Z. officinale and Z. montanum* against *B. cereus, B. subtilis, E. coli* and *S. aureus*, compared to Ciprofloxacin and 10 % Tween 20, by disc diffusion assay.

Sample	Inhibition zone (mm)			
	B. cereus	***B. subtilis***	***E. coli***	***S. aureus***
C. mangga	13.33 ± 0.57^{d}	15.67 ± 0.57^{d}	8.33 ± 0.57^{c}	8.00 ± 0.00^{d}
Z. officinale	9.67 ± 0.57^{c}	11.67 ± 0.57^{c}	12.67 ± 0.57^{d}	6.67 ± 0.57^{c}
Z. montanum	5.67 ± 0.57^{b}	6.67 ± 0.57^{b}	5.33 ± 0.57^{b}	4.67 ± 0.57^{b}
Ciprofloxacin	24.33 ± 0.57^{e}	24.33 ± 0.57^{e}	24.67 ± 0.57^{e}	23.33 ± 0.57^{e}
10 % Tween 20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are expressed as mean ± standard deviation of triplicates. Values followed by different superscript letters in the same column are significantly different at $p < 0.05$ among the tested groups.

MIC

At concentrations of 3.125, 6.25 and 12.50 mg/mL, the extracts from *C. mangga, Z. officinale and Z. montanum* could not inhibit the growth of the bacterial strains. However, the concentrations 25 mg/mL to 200 mg/mL could inhibit all of the tested bacterial strains and inhibited with the same MIC value (25 mg/mL), as shown in **Table 4**.

Table 4 Inhibitory and bactericidal activities of the extracts from *C. mangga, Z. officinale and Z. montanum* against bacterial strains; *B. cereus, B. subtilis, E. coli*, and *S. aureus.*

Concentration (mg/mL)	*C. manga*				*Z. officinale*				*Z. montanum*			
	B. cereus	***B. subtilis***	***S. aureus***	***E. coli***	***B. cereus***	***B. subtilis***	***S. aureus***	***E. coli***	***B. cereus***	***B. subtilis***	***S. aureus***	***E. coli***
Inhibitory activity												
3.125	+	+	+	+	+	+	+	+	+	+	+	+
12.50	+	+	+	+	+	+	+	+	+	+	+	+
25	-	-	-	-	-	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-	-	-	-	-
MIC (mg/mL)	25	25	25	25	25	25	25	25	25	25	25	25
Bactericidal activity												
25	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	-	+	+	+	-	+	+	+	+	-
100	+	+	-	+	+	+	-	-	+	+	+	-
200	-	-	-	-	-	-	-	-	+	+	+	-
MBC (mg/mL)	> 200	> 200	50	200	> 200	100	50	100	> 200	> 200	> 200	50

+ = Showing visible growth of bacterial strains and **-** = no visible growth of bacterial strains.

MBC

Bactericidal activities of the extracts from *C. mangga*, *Z. officinale* and *Z. montanum* against bacterial strains; *B. cereus*, *B. subtilis*, *S. aureus*, and *E. coli* are depicted in **Figure 2**.

At a concentration of 25 mg/mL, all the extracts could not exhibit bactericidal activity (showing the growth of bacterial strains). However, at the concentrations 50 - 200 mg/mL the extracts showed bactericidal activity. For a concentration of 50 mg/mL, the extracts from *C. mangga* and the *Z. officinale* exhibited bactericidal activity on *S. aureus*. In addition, at 100 mg/mL, the *C. mangga* extract exhibited bactericidal activity on *S. aureus* and *E. coli*, whilst *Z. officinale* extract exhibited bactericidal activity on *B. subtilis, S. aureus* and *E. coli*. Regarding to a concentration of 200 mg/mL, all the extracts exhibited the bactericidal activity on *E. coli*.

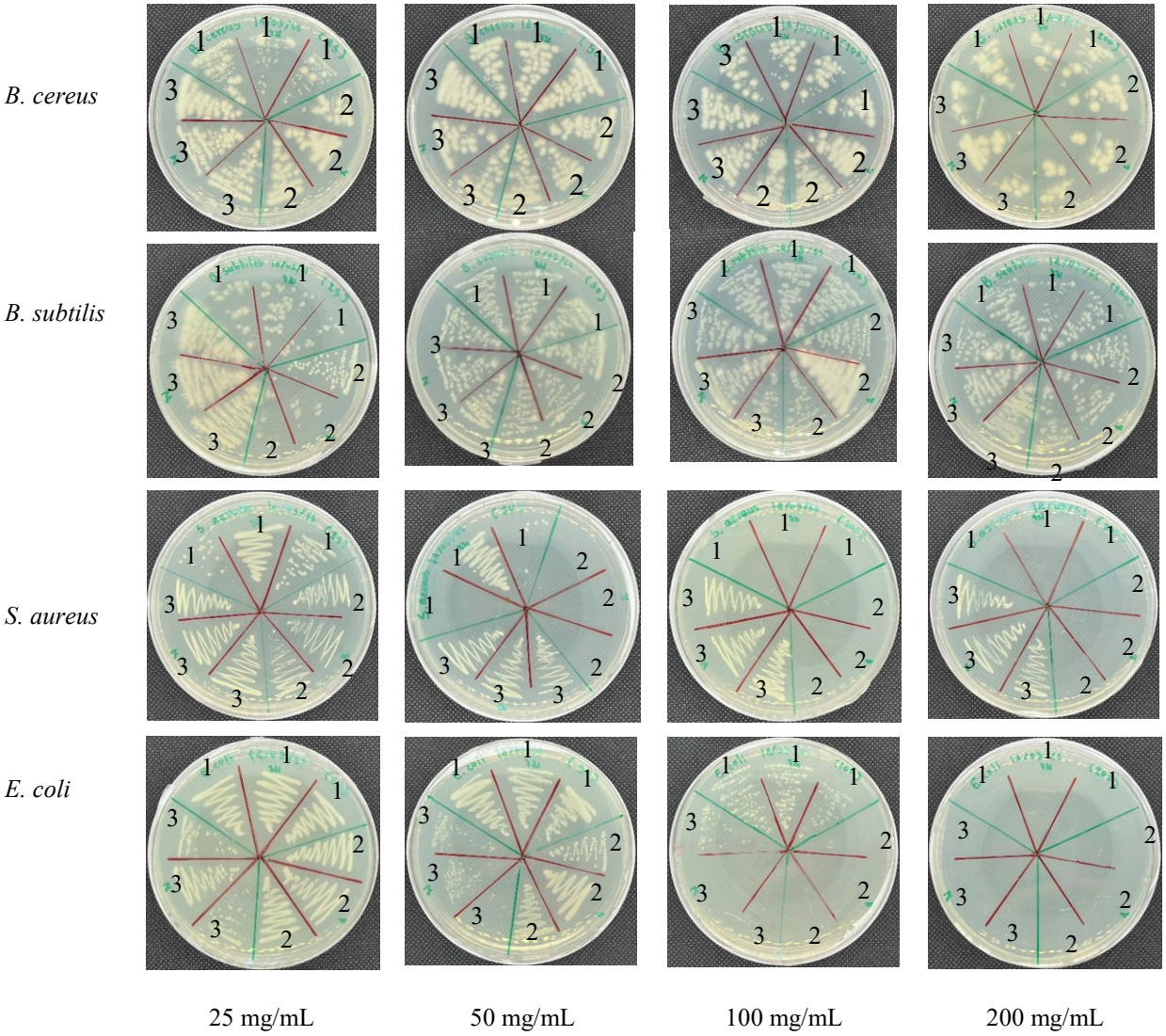


Figure 2 Bactericidal activities of the extracts from *C. mangga* (1), *Z. officinale* (2) and *Z. montanum* (3) with various concentrations against bacterial strains; *B. cereus, B. subtilis, S. aureus*, and *E. coli.*

The MBC values of the extracts against bacterial strains ranged from 50 to > 200 mg/mL (**Table 4)**. Regarding to the *C. mangga* extract, the MBC value for *S. aureus* was 50 mg/mL, for *B. cereus* and *B. subtilis* was > 200 mg/mL and for *E. coli* was 200 mg/mL. Focusing on the *Z. officinale* extract, the MBC value for *S. aureus* was 50 mg/mL, for *B. subtilis* and *E. coli* 100 mg/mL and for *B. cereus* was > 200 mg/mL. In case of the *Z. montanum* extract, the MBC value for *B. cereus, B. subtilis* and *S. aureus* was > 200 mg/mL, but for *E. coli* was 50 mg/mL.

Anti-inflammatory activity

The anti-inflammatory activity by measuring NO production using Griess reaction demonstrated that the extracts from *C. mangga, Z. officinale and Z. montanum* inhibited NO production in the LPS-stimulated RAW 264.7 cells concentration dependently. At the highest concentration (250 µg/mL), the extracts from *C. mangga, Z. officinale and Z. montanum* inhibited NO production in the LPS-stimulated RAW 264.7 cells by 30.54 ± 1.33, 31.96 ± 0.61 and 30.74 ± 1.14 %, respectively. At a concentration of 50 µg/mL, the extracts

from *C. mangga, Z. officinale and Z. montanum* inhibited the NO production in LPS-stimulated RAW 264.7 cells by 16.36 ± 0.94, 17.54 ± 0.91 and 17.11 ± 1.71 %, respectively, which were less than Diclofinac (27.25 ± 1.87 %) significantly ($p < 0.05$) (**Table 5**).

Table 5 Inhibition of NO production (%) in LPS-stimulated RAW 264.7 cells after exposure to the extracts from *C. mangga, Z. officinale and Z. montanum*, in comparing to Diclofenac.

Concentration (µg/mL)	Inhibition of NO production (%)			
	C. mangga	***Z. officinale***	***Z. montanum***	**Diclofenac**
25	8.87 ± 1.22[a]	10.91 ± 1.39[a]	8.94 ± 0.83[a]	ND
50	16.36 ± 0.94[a]	17.54 ± 0.91[a]	17.11 ± 1.71[a]	27.25 ± 1.87
100	19.26 ± 1.21[a]	21.65 ± 1.08[a]	19.97 ± 1.15[a]	ND
150	23.15 ± 1.57[a]	24.32 ± 1.51[a]	23.64 ± 0.23[a]	ND
200	27.26 ± 0.06[a]	28.22 ± 1.84[a]	27.81 ± 0.08[a]	ND
250	30.54 ± 1.33[a]	31.96 ± 0.61[a]	30.74 ± 1.14[a]	ND

Values are expressed as mean ± S.D of triplicates. The values followed by different superscript letters in the same row are significantly different at $p < 0.05$ among the tested groups. ND = no determination.

Toxicities

Toxicity to brine shrimps

The extracts from *C. mangga, Z. officinale and Z. montanum* exhibited mortality to the brine shrimps in concentration dependent manner with IC_{50} of 271.90 ± 0.61, 36.10 ± 0.70 and 40.23 ± 0.66 µg/mL, respectively, as shown in **Table 6**. At concentrations of 16, 32 and 64 µg/mL, the extract from *C. mangga* could not cause the mortality to the brine shrimp, whilst the extracts from *Z. officinale* and *Z. montanum* exhibited the mortality by 3.33 ± 3.33, 46.67 ± 6.67 and 83.33 ± 3.33 % and by 3.33 ± 3.33, 33.66 ± 3.33 and 76.66 ± 3.33 %, respectively. At concentrations of 128, 256 and 512 µg/mL, the *C. mangga* extract exhibited mortality by 20.00 ± 0.00, 26.66 ± 4.22 and 93.33 ± 4.22 %, respectively, meanwhile the extracts from *Z. officinale* and *Z. montanum* exhibited the mortality by 100.00 ± 0.00, 100.00 ± 0.00 and 100.00 ± 0.00 %.

Table 6 Mortality (%) of brine shrimp, *A. salina* after exposure to the extracts from *C. mangga, Z. officinale* and *Z. montanum*.

Concentration (µg/mL)	% Mortality of brine shrimp, *A. salina*		
	C. mangga	***Z. officinale***	***Z. montanum***
16	0.00 ± 0.00[a]	3.33 ± 3.33[b]	3.33 ± 3.33[b]
32	0.00 ± 0.00[a]	46.67 ± 6.67[b]	33.66 ± 3.33[b]
64	0.00 ± 0.00[a]	83.33 ± 3.33[b]	76.66 ± 3.33[b]
128	20.00 ± 0.00[a]	100.00 ± 0.00[b]	100.00 ± 0.00[b]
256	26.66 ± 4.22[a]	100.00 ± 0.00[b]	100.00 ± 0.00[b]
512	93.33 ± 4.22[a]	100.00 ± 0.00[b]	100.00 ± 0.00[b]
LC_{50} (µg/mL)	271.90 ± 0.61[b]	36.10 ± 0.70[a]	40.23 ± 0.66[a]

Values are expressed as mean ± standard deviation of triplicates. The values followed by different superscript letters in the same row are significantly different at $p < 0.05$ among the tested groups.

Toxicity to the RAW 246.7 cells

The extracts from *C. mangga, Z. officinale* and *Z. montanum* significantly ($p < 0.05$) exhibited various % cell viability of the RAW 264.7 cells. Decrease in % cell viability with an increase in the concentration of the extracts was occurred. The % cell viability was < 80 % when the extracts 500 to 1,000 µg/mL were administered and the *Z. officinale* extract provided the % cell viability of the cells less than the extracts from *C. mangga* and *Z. montanum*. At the concentration of 1,000 µg/mL, the extracts from *C. mangga, Z. officinale and Z. montanum* exhibited similar % cell viability (69.56 ± 1.72, 65.32 ± 2.51 and 67.19 ± 1.64 %, respectively) as depicted in **Figure 3**.

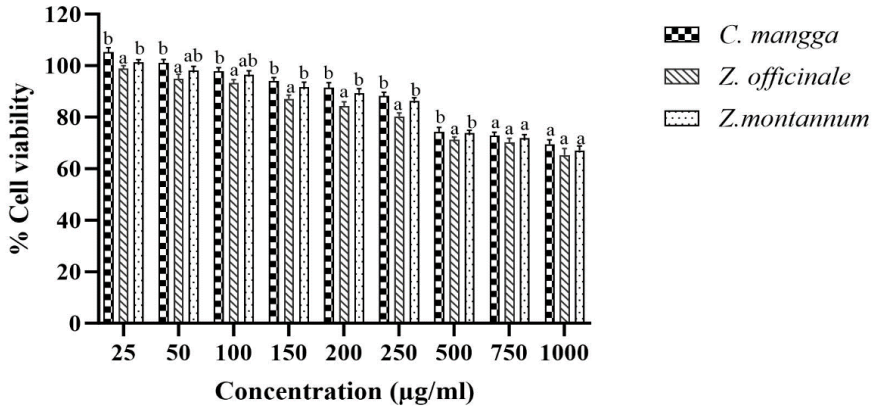


Figure 3 Percentage viability of the LPS-stimulated RAW 264.7 cells after exposure to the extracts from *C. mangga, Z. officinale* and *Z. montanum* Note; a, b and ab indicate the significant difference at $p < 0.05$ among the tested groups.

Results and discussion

According to the % yields of the extracts from *Z. montanum* and *Z. officinale* were not different, but higher than that from the *C. mangga* extract. The % yield of *Z. montanum* extract from this study which was extracted with 95 % ethanol using maceration process was less than the % yield of *Z. montanum* extract which was extracted with 50 % ethanol using an orbital shaker at 150 rpm for 24 h and also less than that was extracted with 50 and 75 % ethanol [11]. These imply that the differences in the extraction yields are influenced by the plant species, extracting solvents and process of extraction.

The determination of TPC revealed that the 95 % ethanol extracts from *C. mangga, Z. officinale* and *Z. montanum* possessed different TPC values and the highest TPC value was found in *Z. montanum* extract (194.64 ± 1.27 mg GAE/g) which was higher than 50 % ethanol extract (49.52 ± 0.32 mg GAE/g) [11]. In case of the *Z. officinale*, water extract shows higher TPC than ethanol extract [9]. This indicates that extraction processes and plant species are influenced the TPC values.

The determination of TFC demonstrated that the *Z. montanum* extract possessed the highest TFC value followed by the extracts from *Z. officinale* and *C. mangga*. Regarding to *Z. officinale* extract, the TFC value in this study (24.38 ± 0.24 mg QE/g) was less than flavonoids detected in chloroform: methanol extract (40.25 ± 0.21 mg QE/g) [24]. The different TFC values are likely resulted from the plant species and extracting solvents.

DPPH assay revealed that the *Z. montanum* extact exhibtied the highest activity, meanwhile the *C. mangga* extract exhibited the lowest activity. Regarding to *Z. montanum*, the methanol extract exhibited the activity (IC_{50} of 36.89 ± 2.53 μg/mL) less than ethanol extract (IC_{50} of 38.89 ± 0.27 μg/mL) [11]. The extract obtained from the rhizomes collecting from the different parts of Thailand exhibited different activities [12]. For the *C. mangga*, water extract possessed antioxidant activity with IC_{50} value of 212.70 mg/L, while the ethanol extract exhibited activity with IC_{50} of 1,073 μg/mL [1]. In the case of *Z. officinale*, petroleum ether extract exhibited antioxidant activity with IC_{50} of 8.29 ± 1.73 μg/mL [24], meanwhile, water extract exhibited antioxidant activity with IC_{50} of 43.90 mg/mL [9]. Indicating, DPPH scavenging activity is influenced by extracting solvents, plant species and plant distributions.

ABTS activity demonstrated that the extract from *Z. montanum* exhibited the strongest activity followed by the extracts from *Z. officinale* extract and the *C. mangga*. A study using *Z. officinale* showed that dried process displayed strong activity in comparison to fresh, stir-frying and carbonized processes [25]. These indicate that plant species and processes are associated with ABTS activity.

FRAP assay revealed that the *Z. montanum* extract exhibited the highest FRAP value followed by the extracts from *Z. officinale* and *C. mangga*. Using the same assay, the ethanol extract of *Z. montanum* had slightly higher FRAP value than the methanol extract [11]. This indicates that the plant species and extractions show variations in antioxidant properties.

According to TPC and TFC exhibied negative correlation to DPPH and ABTS, indicating an increase in TPC and TFC correspond to a decrease IC50 of DPPH and ABTS which contribute to increase antioxidant activity of the extracts, as IC_{50} values are inversely proportional to the antioxidant activity. In addition, TPC and TFC showed positive correlation to FRAP, suggesting an increase in TPC and TFC correspond to an increase antioxidant activity of the extracts, as increase in FRAP value leading to increase antioxidant activity. This suggestes that the active compounds (TPC and TFC). the is associated with antioxidant activity of the extracts.

Antibacterial activity demonstrated that the different tested extracts exhibited different antibacterial activities, as *C. mangga* extract showed the strongest inhibitory activity against *B. cereus* and the *Z. montanum* extract exhibited the lowest activity against *S. aureus*. Due to at 3.125, 6.25 and 12.50 mg/mL the extracts could not inhibit the bacterial growth, suggesting the ineffective activities of the extracts, which was in contrast to the effective activities at 25 to 200 mg/mL.

Based on the extracts inhibited the growth of *B. cereus B. subtilis* and *S. aureus* (gram-positive) and *E. coli* (gram-negative), indicating the extracts can inhibit both Gram-positive and Gram-negative bacteria.

According to the MIC of the extracts against *B. cereus, B. subtilis, E. coli*, and *S. aureus* was not different and was 25 mg/mL, it can be interpreted that the different extracts possess comparable activity. Nevertheless, the different antibacterial activity of the plant extracts has been reported, such as the n-hexane, chloroform and ethanol extracts from *Z. montanum* exhibit antibacterial activity higher than the ethyl acetate, n-butanol and water extracts [9].

The MBC values of the extracts from *C. mangga*, *Z. officinale* and *Z. montanum* against bacterial strains; *B. cereus* and *B. subtilis* were not different and was > 200 mg/mL, indicating the comparable bactericidal activities of the extracts. According to the MBC values of the extracts from *C. mangga*, *Z. officinale* and *Z. montanum* on *E. coli* were 200, 100 and 100 mg/mL, respectively, indicating the extracts from *Z. officinale* and *Z. montanum* possess the comparable bactericidal activity and higher than that of *C. mangga* extract. Due to the *Z. officinale* extract exhibited bactericidal activity with MBC value of 50 mg/mL on *S. aureus*, meanwhile the extract from *C. mangga* exhibited the activity with MBC of 100 mg/mL and *Z. montanum* with MBC of > 200 mg/mL, indicating the extract from *Z. officinale* possesses the strongest bactericidal activity.

Due to at the concentrations of 25 - 250 µg/mL the extracts from *C. mangga, Z. officinale* and *Z. montanum* could inhibit the NO production in LPS-stimulated RAW 264.7 cells, suggesting the extracts have anti-inflammatory activity as measured by inhibition of NO production. This result is in line with ethanol extract from *S. aromaticum* [21]. Base on at the highest concentration of 250 µg/mL the extracts from *C. mangga, Z. officinale and Z. montanum* inhibited NO production not exceeding to 50 %, suggesting the IC_{50} of the extracts for inhibition of NO production is higher than 250 µg/mL.

According to LC_{50} values lower than 12 µg/mL were considered highly toxic, between 12 and 100 µg/mL were moderately toxic, between 100 and 500 µg/mL were mildly toxic and above 500 µg/mL were nontoxic [26]. The extracts from *Z. officinale*, *Z. montanum* and *C. mangga* exhibited mortality to *A. salina* nauplii with LC_{50} of 38.17 ± 2.59, 39 ± 5.29 and 275.67 ± 17.25 µg/mL, respectively. Therefore, the extracts from *Z. officinale* and *Z. montanum* exhibit moderately toxic, meanwhile the *C. mangga* extract exhibits mildly toxic. At the concentration of 1,000 µg/mL, the extracts from *C. mangga*, *Z. officinale* and *Z. montanum* exhibited toxicity to the RAW 264.7 cells with the % cytotoxicity less than 50 %. This indicates that the LC_{50} for cytotoxicity is higher than 1,000 µg/mL and is nontoxic. Nevertheless, crude methanol extract from *C. mangga* has cytotoxicity against human cancer cell lines, whilst the water extract did not show any cytotoxicity [3]. These indicate that toxicities of the plant extracts is associated with the plant species, tested cells and extraction process.

Conclusions

The ethanol extract from *Z. montanum* possesses higher antioxidant, anti-inflammatory, antibacterial activities and toxicities than those from *C. mangga* and *Z. officinale*. Antioxidant activity of the extracts is attributed to radical scavenging by DPPH and ABTS assay, and reducing mechanisms by FRAP assays when anti-inflammatory activity is to inhibition of NO production, and antibacterial activity is to inhibition of bacterial growth and bactericidal activity. The extracts can be developed and applied for antioxidant, anti-inflammatory and antibacterial agents potentially. Further work is required to identify the bioactive compounds corresponding to antioxidant, anti-inflammatory and antibacterial activities.

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