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Chemical Antioxidant Activity of *Thunbergia Laurifolia* Linn. (Rang Chuet) Leaves and its Combined Extracts

Chattip Suntharak^{1,2} and Ratchadaporn Oonsrivilai^{1,2,*}

¹School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakorn Ratchasima, 30000, Thailand

²Health and Wellness Research Group, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand

*Corresponding author. E-mail: roonsivi@g.sut.ac.th

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Abstract

Plant extracts and various natural products may exhibit a synergistic interaction. *Thunbergia laurifolia* Lindl., also known as "Rang Chuet" in Thailand, is a plant with antioxidant properties widely used in traditional medicine. *Zingiber officinale* (ginger), a flavoring ingredient in foods, is well known for its numerous health benefits. There have been no studies on the interaction of antioxidant activity of Rang Chuet with other plant extracts up until now. Thus, this study aimed to investigate the interaction of Rang Chuet water extract (RWE) and ginger ethanol extract (GEE). First, the total phenolic content (TPC) and total flavonoid content (TFC) of RWE and GEE were determined. High-performance liquid chromatography spectrometry (HPLC) was used to examine the phytochemical profile of RWE. The RWE represented coumaric, caffeic, protocatechuic, rosmarinic, and gallic acid as phenolic compounds and apigenin as flavonoids. Chemical antioxidant assays were performed on single and combined extracts using the ABTS + radical cation scavenging capacity assay (ABTS), DPPH free radical scavenging capacity assay (DPPH), ferric reducing antioxidant power assay (FRAP), and ferric chelating assay (FIC). A substantial body of research demonstrated that the combined extracts of RWE and GEE had an additive interaction in various antioxidant assays. Furthermore, a 5:1 v/v ratio of RWE and GEE demonstrated a synergistic effect in DPPH and FRAP assays. The synergistic ratio could be used to create nutraceutical formulations or various food products with antioxidant activity to prevent oxidative diseases.

Keywords: Antioxidant capacity; phytochemical profile; synergistic; *Thunbergia laurifolia* Lindl.; *Zingiber officinale*

1. Introduction

Several chronic diseases, including aging, atherosclerosis, diabetes, hypertension, Alzheimer's disease, kidney disease, and cancer, are adversely affected by oxidative stress (Roberts & Sindhu, 2009). According to the findings of epidemiological studies, the consumption of plant-based foods may help reduce oxidative damage (Batool *et al.*, 2019). Polyphenols, a class of phytochemical compounds, are specialized plant metabolites that benefit human health by counteracting the pathological effects of oxidative stress (Zhang & Tsao, 2016). Therefore, numerous studies have attempted to assess the health benefits associated with antioxidant activity.

Thunbergia laurifolia Lindl. belongs to the Acanthaceae family and is widely distributed in India and Southeast Asia. It is commonly known in Thai as Rang Chuet. Rang Chuet leaves are widely used as an antidote for poisons. This plant has antioxidant, anti-inflammatory, antimicrobial, anti-diabetic, anti-pyretic, and anti-hyaluronidase activities (Chaiyana *et al.*, 2020; Chan *et al.*, 2012; Junsri *et al.*, 2017; Oonsivilai *et al.*, 2007; Preechasuk *et al.*, 2020). Rang Chuet has been used in Thai traditional medicine, so researchers are interested in studying its bioactive compounds and its unique phytochemical compounds. *Zingiber officinale* (ginger) well has many health benefits. Gingerol and zingerone, the main active compounds, have antioxidant effects by reducing free radical scavenging (Haniadka *et al.*, 2013). Furthermore, ginger has anti-inflammatory properties due to a combination of 1-dehydro-[6]-gingerdione, 6-shogaol, 6-dehydroshogaol, and hexahydrocurcumin (Li *et al.*, 2012).

Combining plant extracts and various natural products may result in a synergistic interaction between their various phytochemicals, hence enhancing the efficacy of plant extracts. (Karimi *et al.*, 2015). Structural phytochemicals may possess similar or overlapping, which may have complementary effects in their antioxidant activities. The interaction of phytochemicals in food, plant extracts, and natural antioxidants has been reported in various studies *in vitro* and *in vivo* (Hajimehdipoor *et al.*, 2014; Tian *et al.*, 2021; Wang *et al.*, 2011; Xu *et al.*, 2014).

This study aimed to determine the effect of the drying method on synergistic effect by identification of phytochemical compounds in Rang Chuet water extracts (RWE) using high-pressure liquid chromatography (HPLC) coupled with a DAD detector. Various chemical antioxidant assays were used to assess the antioxidant properties of RWE and ginger ethanol extract (GEE) and their combined extracts. Furthermore, specific combinations with synergistic effects and high antioxidant activity will be chosen.

2. Materials and Methods

2.1 Plant preparation

Plant materials

Thunbergia laurifolia Lindl. leaves were collected in March 2021 from the Non-toxic Agriculture Cooperative under the Royal Initiative of His Majesty the King (NACR) The people of a neighborhood's Development Study Center in Nakhon Ratchasima Province, Thailand. The herbarium voucher specimen (Ratchadaporn 001) was prepared and deposited at the School of Food Technology,

Suranaree University of Technology. The Rang Chuet fresh leaves were dried in an oven at 60°C for 3 h. In another way, the Rang Chuet fresh leaves were dried in a microwave vacuum dryer (Marchcool, Bangkok, Thailand) at 2,400 watts for 11 min. until moisture content was less than 13%. Dried Rang Chuet leaves were grounded in a blender (National, MX-T2GN, Taiwan). The powder was sieved through mesh size 0.2 mm and was kept at -20°C in a vacuum package until use. Commercial ginger extract was purchased from Now Foods (Illinois, USA) and stored at -20°C until further use.

Preparation of RWE, GEE, and CRGE

The Rang Chuet powder was extracted by the method described by Oonsivilai *et al.* (2006). The 100 mg powder was extracted with 12 ml of boiling water ($95 \pm 2^\circ\text{C}$) in a shaking water bath (Sheldon, WS27-2 SHEL LAB, Oregon, USA) at 37°C for 15 min. Centrifugation at 3,000 g (Thermo IEC, Waltham, MA) was applied for 3 min. between extraction and was repeated a total of 3 times. The filtrates were combined, and the volume was adjusted to 50 ml. Aliquots of 5 mL were transferred to 12 ml test tubes (VWR, Wilmington, NC) and sublimated by freeze dryer (CHRIST, Alpha 2-4 LSCplus Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The RWE was stored at -2°C until use. For the preparation of GEE, as Commercial ginger extract contains ginger extract and ginger power so it has to be re-extracted, extraction steps were the same as Rang Chuet extraction except using 95% ethanol for solvent extraction and drying with a rotary evaporator (Büchi®, R200, New Castle, United States) instead of using a freeze dryer. The GEE was stored frozen at -20°C until use. The combined extracts of Rang Chuet and ginger with the following ratios 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, and 1:5 v/v.

2.2 Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), 2,4,6-tri (2-pyridyl)-striaizine (TPTZ), Ferric chloride, chloride-6-hydrate, ferrous sulfate 7-hydrate, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulfate, gallic acid, Ascorbic acid, Butylated hydroxytoluene (BHT), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu phenol reagent, Sodium acetate ($\text{C}_2\text{H}_3\text{NaO}_2$), anhydrous sodium carbonate (Na_2CO_3), sodium nitrite (NaNO_2), aluminum chloride (AlCl_3) and sodium hydroxide (NaOH), ferrozine were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). Methanol, Glacial acetic acid, and Hydrochloric acid (37% w/w) were used for chemical analysis. For extraction, solvents including 95% ethanol were purchased from Mallinckrodt-Baker (Phillipsburg, NJ, USA). Acetic acid and acetonitrile solution (Mallinckrodt-Baker, Phillipsburg, NJ, USA) were certified HPLC and ACS grades for HPLC. The phenolic and flavonoid standard compounds (Sigma Aldrich, St. Louis, MO, USA).

2.3 Determination of Phytochemical profile of RWE

The RWE from both oven- and microwave-drying were determined by phenolic and flavonoid compounds by HPLC as described by Oonsivilai *et al.* (2007). The Agilent 1100 Series HPLC system equipped with a diode array detector was used for the analyte. The ZORBAX Eclipse® XDB-C18 Analytical 4.6×150 mm reversed-phase column (Agilent, US) with a guard column also packed with

ZORBAX Eclipse® XDB-C18 was applied. The solutions were separated by gradient elution with a 1.0 ml/min flow rate at 35°C using a binary mobile phase of water/acetic acid (98:2, v/v) in reservoir A and acetonitrile in reservoir B. The initial solvent proportion of 99:1 A/B with a linear gradient to 70:30 A/B 20 min was applied, followed by a 5 min with linear gradient back to 99:1 A/B and 5 min equilibration at the initial condition for a total chromatographic run time of 30 min. Detection and tentative identification of phenolic and flavonoid compounds were accomplished using in-line PDA data in the 270–340 nm wavelength range. The RWE were weighted and dissolved with DI water at a concentration of 25 mg/ml. The determination was carried out in triplicate. The solutions were filtered through a 0.22 µm PETP membrane before injection.

In addition, the reference phenolic acids standard consists of caffeic acid, p-coumaric acid, ferulic acid, gallic acid, protocatechuic acid, rosmarinic acid, and sinapic acid was used to standardize. Moreover, the flavonoid standard compounds were quercetin, and apigenin was also applied. Reference standards stock solutions of gallic, protocatechuic, caffeic, and p-coumaric acid were prepared by accurately weighing and dissolving with DI water. The ferulic acid, sinapic acid, quercetin, and apigenin were dissolved in 95% ethanol to obtain the final concentration of 500 µg/ml. Then, the stock solutions were diluted to a range of 1–500 µg/ml. Calibration curves were established to determine the quantification of phenolic acids and flavonoids. The RWE from oven-dried or microwave-dried leaves with high phenolic acids and flavonoid content were selected to further study.

2.4 Determination of total phenolic and flavonoid contents and the interaction of antioxidant activities of RWE and GEE

The total phenolic and total flavonoid contents of RWE and GEE were investigated. The chemical antioxidant activities of extracts were compared to commercial standards such as BHT, ascorbic acid, and Trolox using various assays.

Total phenolic content

The total phenolic content (TPC) was determined by the Folin-Ciocalteu procedure described by Oonsivilai *et al.* (2007), and gallic acid was used as a standard. Aliquots of 0.02 ml gallic acid standard/sample/blank were mixed with 1.58 ml of deionized water in a 1.5 ml cuvette. Folin-Ciocalteu reagent of 0.1 ml was added, and the solution was mixed and stood for 5 min. Then, 0.3 ml of Na₂CO₃ (20% w/v) was added to the mixture and stored in the absence of light for 120 minutes. Absorbance was measured at 765 nm using a spectrophotometer (GENESYS, 10S Vis, Wisconsin, USA). The gallic acid (0, 100, 200, 300, 400, and 500 mg/L) was used to set. The results were expressed as mg gallic acid equivalents (GAE)/g.

Total flavonoid content

Total flavonoid content (TFC) was determined using quercetin as a standard. The 0.1 ml of RWE and GEE were added to 0.3 ml of distilled water followed by 0.03 ml of 5% NaNO₂. After 5 min at 25°C, 0.03 ml of 10% AlCl₃ was added. After incubation for 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to volume (1 ml) with deionized water (DI water). The absorbance is measured at 510 nm using a

spectrophotometer. The total flavonoid content was expressed as mg quercetin equivalents.

ABTS+ cation radical scavenging assay

ABTS+ cation radical scavenging capacity (ABTS) of extracts was determined according to Ksouri *et al.* (2009) with modifications. The ABTS+ cation radical was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the absence of light temperature for 12–16 h. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. In a final volume of 2 ml, the reaction mixture comprised 900 μ l of ABTS+ solution and 100 μ l of the sample extracts at various concentrations, and distilled water was used as control. The reaction mixture was homogenized, and its absorbance was recorded at 734 nm at 6 min after mixing. ABTS + scavenging capacity was expressed as IC_{50} (mg/ml).

DPPH free radical scavenging assay

The scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined by the method described by Oonsivilai *et al.* (2008). Briefly, a 100 μ M violet solution of 0.1 mM DPPH in methanol was prepared. Aliquots of 0.1 ml of standard/sample/blank were transferred into a test tube. After adding 1.90 ml DPPH solution, the solution was mixed and then stand for 15 min. The absorbance of the solution at 515 nm was read.

Ferric-reducing antioxidant power assay

Ferric-reducing antioxidant power (FRAP) assay was carried out according to Oonsivilai *et al.* (2007) procedure with slight modification. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM ferric (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and warmed at 37°C in a water bath before use. 50 μ l of the sample were added, along with 150 μ l of DI water to 1.5 ml of the FRAP reagent. The reaction mixture absorbance was then recorded at 593 nm after incubation for 4 min. The standard curve was constructed using ferrous sulfate stock solution (100–1000 μ M), and the results were expressed as mM ferrous (II)/g dry weight of extract. All measurements were taken in triplicate, and the mean values were calculated.

Ferric chelating assay

The Ferric chelating (FIC) assay was carried out according to Junsri *et al.* (2017) with slight modification. Briefly, a 50 μ l of the sample, 1,000 μ l of a DI water, and 50 μ l of 1 mM $FeCl_2$ were mixed and incubated for 5 min in the dark, then 50 μ l of 5mM ferrozine was added and incubated for 20 min. The absorbance was measured at 562 nm. The results were expressed as μ g EDTA equivalents.

2.5 Determination of the interaction of antioxidant activities

From the previous determination of the antioxidant activities by DPPH, ABTS, FRAP, and FIC assays in section 2.4, the interaction effect between the RWE and GEE is shown by the combination index (CI) as the following formulation.

$$CI = \frac{C1}{C_{x,1}} + \frac{C2}{C_{x,2}}$$

where C1 and C2 are the concentrations of the single extracts and C_{x,1} and C_{x,2} are the concentrations of single extract required to achieve the same effect upon % single extract in formulation to produce the obtained effect x. From DPPH and ABTS assay results, the extracts that showed low IC₅₀ value with high antioxidant activity would show a CI value less than 0.90, which interprets a synergistic effect. On the other hand, the CI values of extracts determined by FRAP and FIC assays showed higher than 1.10, which interprets a synergistic effect. Additionally, the CI of antioxidant activity in a range of 0.91–1.10 means a nearly additive effect. Then, the expected value (E) is calculated as the average of individual observed amounts for each 1 and 2 combination extracts, and the observed value (O) came through the observed amounts for combined extracts (Liu *et al.*, 2016).

2.6 Statistical analyses

Statistical analyzes were performed using the SPSS statistics for windows, version 23.0, NY: IBM corp. The results were expressed as the mean ± standard error of three replications per sample. Comparisons between groups were performed using Student's t-test, and P<0.05 was selected as the level required for statistical significance. Statistical comparisons of multiple groups were assessed by one-way analysis of variance (ANOVA) according to Duncan's multiple range test at a significance level of 5%

3. Results and Discussion

3.1 Phytochemical profiling of RWE

The presence of phenolic and flavonoid compounds in RWE was determined using HPLC analysis. Table 1 shows the phytochemical profiles of RWE from both oven-dried and microwave-dried leaves extracts. RWE from microwave vacuum-dried leaves contained apigenin and several phenolic compounds, i.e., gallic acid, protocatechuic, caffeic acid, coumaric acid, and rosmarinic acid at 129 ± 13, 415 ± 31, 919 ± 39, 1,030 ± 88, and 378 ± 14 µg/g extract, respectively. Alternatively, the analysis of RWE from oven-dried leaves revealed only phenolic compounds: gallic acid, protocatechuic acid, caffeic acid, coumaric acid, and ferulic acid. Phytochemical profiling of RWE from other scientific reports was composed of phenolic compounds, including caffeic acid, protocatechuic acid, chlorogenic acid, rosmarinic acid, and vinixin. They also have reported finding such flavonoids as apigenin, catechin, isoquercetin, quercetin, and rutin in RWE (Chuthaputti, 2010; Junsu *et al.*, 2017; Nanthakarn *et al.*, 2020; Oonsivilai *et al.*, 2007; Preechasuk *et al.*, 2020). This study quantified the phenolic and flavonoid compounds in RWE. However, the quantification of phytochemicals varied from the previous finding due to differences in the growth location, leafage, type of extractant, and extraction preparations (Chaiyana *et al.*, 2020; Oonsivilai, 2006).

Besides, the choice of drying methods could have influenced the phenolic and flavonoid contents. Generally, the Rang Chuet leaves were dried in an oven or sun drying. E. Chan and Lim (2006) mentioned that the total phenolic compounds of oven-drying declined approximately 70-80% while microwave-drying declined approximately 38-41%. Wojdylo *et al.* (2014) explained that irreversible oxidative

occurred during drying and exposure to thermal degradation of phenolic compounds with increased heat intensity. Involving a study on phenolic compounds in Rang Chuet, E. W. C. Chan *et al.* (2012) reported that microwave-dried leaves tea of Rang Chuet had total phenolic compound values at $3,050 \pm 127$ mg GAE/100 g RM, which was higher than oven-dried leaves tea of Rang Chuet at $1,800 \pm 57$ GAE/100 g RM. Likewise, Phahom *et al.* (2017) who studied the effect of microwave on phenolic compounds in Rang Chuet leaves extract showed higher total phenolic content and caffeic acid when applied high-microwave power.

Therefore, this research selected microwave vacuum drying technology for drying Rang Chuet leaves because it might preserve phenolic compounds including other bioactive compounds that are heat sensitive. Furthermore, Therdthai and Zhou (2009) implicated that microwave drying took 85-90% shorter drying times in the drying process of mint leaves compared with hot air drying, which was a way to save energy and time in the food and pharmaceutical industries.

Table 1 The phytochemical profiles of Rang Chute extracts analyzed by HPLC

Photo chemical	$\mu\text{g/g}$ of extract									Total
	Gallic acid	Proto-catechuic acid	Caffeic acid	Coumaric acid	Ferulic acid	Sinapic acid	Rosmarinic acid	Quercetin	Apigenin	
Oven drying	64 $\pm$ 42 ^b	330 $\pm$ 7.8 ^b	1027 $\pm$ 34 ^a	162 $\pm$ 8.0 ^b	18 $\pm$ 0.8	ND	ND	ND	ND	1,640 $\pm$ 10 ^b
Microwave vacuum drying	128 $\pm$ 11 ^a	418 $\pm$ 14 ^a	919 $\pm$ 20 ^b	1,025 $\pm$ 49 ^a	ND	ND	374 $\pm$ 14	ND	107 $\pm$ 2.9	2,976 $\pm$ 19 ^a

Data are mean $\pm$ SD; Data in the same column with different superscripts are significantly different ($P < 0.05$). ND= not detected.

From table 1, the main phytochemical compounds in RWE from microwave-dried leaves are caffeic, coumaric, and rosmarinic acids. Caffeic acid (CA) was reported as the main chemical constituent found in Rang Chuet (Nanthakarn *et al.*, 2020; Ruangpayungsak *et al.*, 2018) and showed antidote activity that might have mechanisms related to detoxification, and the effects were attributed to excellent anti-inflammatory and antioxidant activity (Ahn *et al.*, 2017; da Cunha *et al.*, 2004; Gülçin *et al.*, 2016). Moreover, the results had discovered rosmarinic, which showed a variety of health benefits; antioxidant, anti-inflammatory, anti-diabetic, and anticancer (Gülçin *et al.*, 2016; Hossan *et al.*, 2014; Ngo *et al.*, 2018). However, this study did not discover high rosmarinic acid content same as the 50% methanol extraction method at ambient temperature by Nanthakarn *et al.* (2020). However, the amount of phytochemical compounds in RWE from microwave vacuum-dried leaves showed a higher quantity than RWE from oven-dried leaves which was a conventional method.

3.2 Chemical antioxidant activity of RWE and GEE

3.2.1 Total phenolic and flavonoid contents

The total phenolic content (TPC) and total flavonoid content (TFC) of RWE and GEE are presented in Figure 1, which showed the significant differences between them ($P < 0.05$). Especially, RWE showed high total phenolic contents at 130 ± 2.86 mg GAE/g extract. This result might have been affected by microwave vacuum drying which helped preserve phenolic compounds during the drying step. Therefore, the result showed an increase in TPC up to 20% in RWE from microwave-dried leaves. Their quantity was close to that reported by Phahom *et al.* (2017), who applied the microwave with blanching for drying Rang Chuet leaves.

Additionally, this study showed that microwave drying took a shorter drying time than oven drying which reduced drying times from 3 hrs. to 11 min. Furthermore, the TPC of GEE was 53 ± 1.37 mg GAE/g extract. This result agreed with a previous study that the TPC of the ginger methanol extract was 71.1 mg GAE/g dry weight (El-Ghorab *et al.*, 2010). However, the results showed that the TPC of the ginger extract was 136 mg GAE/g extract in the study of Bursal *et al.* (2012). In addition, the GEE showed total flavonoid content at a level of 225 mg Quercetin E/g extract, which was significantly higher than RWE ($P < 0.05$).

Furthermore, the various results from previously studied might be due to extracting methods with different solvents, which significantly affected the quantification of total flavonoid content. Notably, the ginger used in this study was from a commercial product and the preparation method was unknown. However, this work focused on the synergistic effect of both extracts.

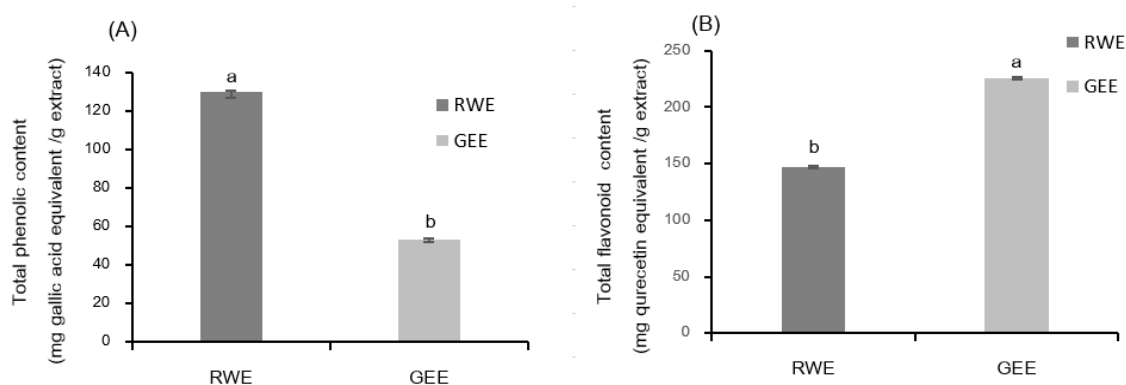


Fig 1 Total phenolic content (A) and total flavonoid content (B) of RWE and GEE. Data are mean $\pm$ SD (n=3)

^{a, b} Different letters over the error bars denote that the means differed significantly ($P < 0.05$) on extracts.

3.2.2 Chemical antioxidant activities of RWE and GEE

The antioxidant activities of RWE and GEE are shown in Table 2. The DPPH assay, RWE, showed the IC_{50} value at 1.10 ± 0.07 mg/mL, followed by ginger at 1.53 ± 0.05 mg/mL. DPPH free radical scavenging capacity of RWE is related to its phenolic contents. As a result, phenolic compounds are the major components responsible for the radical scavenging capacity of Rang Chuet extract (Chaiyana

et al., 2020). However, both extracts were less effective than commercial standards including Vit C, Trolox, and BHT in the case of DPPH free radical scavenging.

In contrast, ABTS + radical cation scavenging capacity of GEE showed IC_{50} value at 1.30 ± 0.0 mg/mL, which was higher than RWE, which showed IC_{50} value at 1.82 ± 0.01 mg/mL with significant differences ($P < 0.05$). As ABTS assay is effective in phenolics, and flavonoids solubilized in ethanol, some bioactive compounds in RWE may not be soluble in the ethanol solvent system. Therefore, it could reduce a potential ABTS + radical cation scavenging capacity in RWE. However, DPPH and ABTS assays are radical or radical oxygen species scavenging methods whose dominant mechanism is single electron transfer (SET). This may also have hydrogen atom transfer at the same time. Furthermore, the antioxidant activities also depend on antioxidant structure and characteristics, solubility and partition coefficient, as well as the solvent system, scavenging capacity with different assays may not be the same (Shahidi & Zhong, 2015).

Table 2 Antioxidant activities of the DPPH and ABTS assays

Samples	IC_{50} of DPPH · free radical scavenging capacity (mg extract/ml)	IC_{50} of ABTS + radical cation scavenging capacity (mg extract/ml)
Rang Chuet extract	1.10 ± 0.07^c	1.82 ± 0.01^e
Ginger extract	1.53 ± 0.05^d	1.30 ± 0.01^d
BHT	0.35 ± 0.02^b	0.08 ± 0.00^c
Vit C	0.08 ± 0.00^a	0.04 ± 0.00^a
Trolox	0.08 ± 0.10^a	0.05 ± 0.00^b

Data are mean $\pm$ SD; Data in the same row with different superscripts are significantly different ($P < 0.05$).

The ferric reducing power (FRAP) assay represents a non-radical redox potential-based method. The reducing power of GEE was shown at a level of 0.714 ± 0.01 mmol Fe (II) Equiv./g extract, which was higher than the reducing power of RWE, which was shown at a level conducted by Liu *et al.* (2016), which indicated 0.806 mmol of Fe (II) Equiv./g (consider using the same unit as shown in Table 3) extract.

Ferrous ions chelating activity (FIC) assay measures metal chelation capacity. The results showed that RWE showed FIC at 2.857 ± 0.08 mg EDTA equip./g extract, which was a significant difference from GEE ($P < 0.05$). Thus, plant extracts could chelate the transition metal ions (Shahidi and Zhong, 2015). Furthermore, caffeic and protocatechuic acids were presented in RWE, and previous reports explained that they could form complexes with ions (Genaro-Mattos *et al.*, 2015; Kakkar and Bais, 2014).

Table 3 Antioxidant activities of FRAP and FIC assays.

Samples	Reducing power (mmol Fe (II) Equiv./g extract)	Ferric ion cheating (mg EDTA Equiv./g extract)
Rang Chuet extract	0.593 ± 0.03 ^d	2.857 ± 0.08 ^b
Ginger extract	0.714 ± 0.01 ^e	1.380 ± 0.03 ^c
BHT	2.42 ± 0.02 ^c	-
Vit C	8.68 ± 0.10 ^a	-
Trolox	5.76 ± 0.02 ^b	-
EDTA	-	16.80 ± 0.01 ^a

Note: Data are meant ± SD; Data in the same column with different superscripts are significantly different (P < 0.05). - Do not measure.

This study implicated that both RWE and GEE had competencies in chemical antioxidant activities, hydrogen atom transfer (HAT), single electron transfer (SET), non-radical redox potential, and metal chelation capacity. However, RWE was not as potent as those commonly used as commercial antioxidants. However, they have shown very well antioxidant capacities. GEE, which had lower total phenolic content than RWE, showed antioxidant activities in the case of ABTS + radical cation scavenging capacity and reducing power than RWE. For this reason, it was expected to get an interaction in various chemical antioxidant activities when RWE and GEE were mixed to obtain outstanding antioxidant properties. Therefore, RWE and GEE mixtures as combined extracts with various ratios were applied to investigate their antioxidant activities. It might be explained that the interaction was performed under various ratios when the study was carried out.

3.3 The interaction effects of combined extracts

The mixture ratios of RWE and GEE were mixed at 7 ratios of 5:1, 3:1, 2:1, 1:1, 1:2, 1:3 and 1:5 v/v. The antioxidant capacities of combined extracts using four assays such as DPPH, ABTS, FRAP, and FIC were carried out. Then, the interaction of chemical antioxidant activity with the various ratios of combined extracts was investigated, and their results are shown in Table 4–7. The observed (O) antioxidant capacities of the combined extracts were compared to the expected (E) value, and the combination index (CI) was calculated to classify a type of interaction.

Firstly, the interactions of DPPH+ free radical antioxidant activity of the combined extracts are shown in Table 4. Increasing the ratio of RWE in CRGE showed a low IC₅₀ value. Moreover, the observed IC₅₀ value (O) of ratio 5:1 was significantly different from the expected value (P < 0.05), which demonstrated the lowest IC₅₀ as compared with other ratios. Furthermore, the results showed a slight synergism effect at the ratio of 5:1 while the other ratios of combined extracts showed a nearly additive effect. In addition, no ratios showed antagonistic interaction. Yao *et al.*, (2009) stated that the interaction between the same structure of antioxidant molecules could produce a synergistic antioxidant effect. For example, a suitable solvent can decrease the enthalpy of dissociation of the hydroxyl O-H bond of ferulic acid glyceryl ester via stacking, thereby lowering the activation energy of the reaction between an antioxidant molecule and DPPH.

Table 4 The interaction of DPPH antioxidant capacity of the CRGE

Ratio	IC ₅₀ (μg/ml)		CI	Effect
	O	E		
5:1	1.064 ± 0.01*	1.212 ± 0.04	0.89	Slight synergism
3:1	1.119 ± 0.10	1.244 ± 0.03	0.91	Nearly additive
2:1	1.258 ± 0.15	1.276 ± 0.03	1.00	Nearly additive
1:1	1.299 ± 0.10	1.341 ± 0.03	1.00	Nearly additive
1:2	1.459 ± 0.04	1.406 ± 0.03	1.06	Nearly additive
1:3	1.469 ± 0.03	1.438 ± 0.03	1.04	Nearly additive
1:5	1.558 ± 0.01	1.402 ± 0.11	1.07	Nearly additive

Values are the mean of 3 replicates ± SD (n=3). The asterisk (*) indicates a significant difference between the observed value and expected value (P<0.05). O, observed value; E, expected value; CI, combination

Secondly, the interactions of ABTS + cation scavenging capacity of the CRGE are shown in Table 5. The observed value in the range of ratio 3:1 to 1:3 was significantly different from expected ABTS values (P<0.05). However, the synergetic effect of ABTS + cation scavenging capacity of the CRGE was not found. According to Heo *et al.* (2007) research, the observed combination of two and three phenolic compounds in the ABTS assay system does not show a synergistic antioxidant effect.

Table 5 The interaction of ABTS + radical cation scavenging capacity of the CRGE

Ratio	IC ₅₀ (mg/ml)		CI	Effect
	O	E		
5:1	0.184 ± 0.003	0.176 ± 0.008	1.06	Nearly additive
3:1	0.155 ± 0.005*	0.172 ± 0.007	0.93	Nearly additive
2:1	0.154 ± 0.003*	0.167 ± 0.006	0.95	Nearly additive
1:1	0.150 ± 0.003*	0.158 ± 0.005	0.98	Nearly additive
1:2	0.143 ± 0.004*	0.148 ± 0.003	0.99	Nearly additive
1:3	0.138 ± 0.002*	0.144 ± 0.003	0.98	Nearly additive
1:5	0.133 ± 0.002	0.154 ± 0.021	0.97	Nearly additive

Values are the mean of 3 replicates ± SD (n=3). The asterisk (*) indicates a significant difference between the observed value and expected value (P<0.05). O, observed value; E, expected value; CI, combination index.

The interactions of reducing the power of the CRGE are shown in Table 6. The results showed that at the ratios of 5:1, 3:1, and 1:3, the observed FRAP value showed a significant difference from expected values with significant differences ($P < 0.05$). The increased RWE ratio at 5:1 and 3:1 showed a slight synergistic effect, while other ratios showed only a nearly additive effect.

Table 7 shows the interactions of FIC with the CRGE. The observed values of all ratios were not significantly different ($P > 0.05$). Furthermore, only the combined extract ratio of 2:1 showed slight synergism, while other ratios showed nearly additive. Recently, the synergistic antioxidant effects of polyphenolic compounds by enhanced chelation of phenolic compounds with metal ions have been generally recognized (Lv *et al.*, 2021).

Table 6 The interaction of reducing power of the CRGE

Ratio	FRAP (mmol Fe (II) Equiv./g extract)		CI	Effect
	O	E		
5:1	0.714 ± 0.030*	0.644 ± 0.025	1.14	Slight synergism
3:1	0.737 ± 0.016*	0.670 ± 0.023	1.14	Slight synergism
2:1	0.726 ± 0.003	0.696 ± 0.021	1.09	Nearly additive
1:1	0.750 ± 0.011	0.747 ± 0.017	1.05	Nearly additive
1:2	0.780 ± 0.024	0.799 ± 0.013	1.04	Nearly additive
1:3	0.783 ± 0.007*	0.824 ± 0.011	0.99	Nearly additive
1:5	0.831 ± 0.016	0.783 ± 0.095	1.00	Nearly additive

Values are the mean of 3 replicates ± SD (n=3). The asterisk indicates a significant difference between the observed value and the expected value ($P < 0.05$). O, observed value; E, expected value; CI, combination index.

Table 7 The interaction of FIC of the CRGE

Ratio	FIC (mg EDTA Equiv./g extract)		CI	Effect
	O	E		
5:1	2.633 ± 0.10	2.617 ± 0.06	1.09	Nearly additive
3:1	2.471 ± 0.04	2.488 ± 0.02	1.10	Nearly additive
2:1	2.328 ± 0.08	2.365 ± 0.04	1.11	Slight synergism
1:1	1.872 ± 0.03	2.119 ± 0.06	1.01	Nearly additive
1:2	1.712 ± 0.03	1.872 ± 0.02	1.03	Nearly additive
1:3	1.446 ± 0.04	1.749 ± 0.06	0.91	Nearly additive
1:5	1.363 ± 0.03	1.626 ± 0.04	0.90	Nearly additive

Values are the mean of 3 replicates ± SD (n=3). O, observed value; E, expected value; CI, combination index.

In conclusion, there is an additive interaction in RWE and GEE regarding the chemical antioxidant activity. It should be noted that the majority of the combinations showed an additive interaction, and some ratios exhibited synergistic effects. In the DPPH and FRAP assays, the combination at ratio of 5:1 showed the synergistic result. To obtain synergistic effects, additional ratios with GEE or other herb extracts should be performed. Additional research is required to determine the possible underlying mechanisms behind the interaction. Furthermore, their antioxidant activity should be compared with the cell culture approach.

4. Conclusion

It could be concluded that both RWE and GEE showed an antioxidant potential and an appreciable quantity of antioxidant compounds with antioxidant activities. From studying phytochemical profiling by HPLC, the RWE consisted of gallic, protocatechuic, caffeic, coumaric, rosmarinic acids, and the flavonoid compound as apigenin. CRGE showed the antioxidant properties with various mechanisms via four different chemical antioxidant assays. Therefore, the combination of RWE and GEE could potentially play a role in determining the antioxidant capacities. Furthermore, the synergistic effects of the combined extract could be used to produce novel natural antioxidants in nutraceutical formulations that provide valuable protection against oxidative diseases and the agents that can be used in various food products.

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