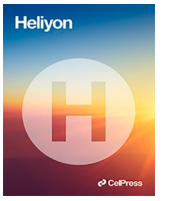
Heliyon 9 (2023) e13063

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Synergistic effect of Trikatuk, a traditional Thai formulation, on antioxidant and alpha-glucosidase inhibitory activities

Thanutchaporn Nutmakul [a,*], Savita Chewchinda [b]

[a] Graduate Program in Nutrition, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, 10400, Thailand
[b] Department of Food Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok, 10400, Thailand

ARTICLE INFO

Keywords:
Synergistic effect
Polyherbal
Traditional medicine
Antidiabetic
Piperine
Ginger

ABSTRACT

Oxidative stress and hyperglycemia are known to be responsible for several diseases, including diabetes. To prevent these diseases, efforts are ongoing to identify novel antioxidants with hypoglycemic effects. Trikatuk is a traditional Thai formulation composed of three herbs in equal quantities: the fruits of *Piper nigrum* and *Piper retrofractum* and the rhizomes of *Zingiber officinale*. This formulation has been reported to have antioxidant activity and the three individual herbs have been traditionally used for managing diabetes. Therefore, the present study aimed to investigate the phytochemical contents, antioxidant activity (DPPH and ABTS radical scavenging assays), and alpha-glucosidase inhibitory activity of Trikatuk. As Trikatuk is a combination of herbs, the synergistic effect and two different preparation methods from powder or extract mixtures were also explored. The results revealed that Trikatuk, particularly prepared from a mixture of extracts, exhibited high contents of piperine, total phenolics and total flavonoids, as well as significant antioxidant and alpha-glucosidase inhibitory activities. Trikatuk also exhibited additive or synergistic effects in all experiments. Overall, Trikatuk is potentially an alternative traditional medicine and dietary supplement offering antioxidant activity and diabetes management.

1. Introduction

Free radicals have been reported to be involved in the pathogenesis of many diseases such as insulin resistance, diabetes, and cardiovascular disease. Physiologically, they are essential for cell functions, such as intracellular signaling, inflammation and immune function. However, when the production of free radicals exceeds the elimination capacity of the antioxidant defense systems, oxidative stress will occur and lead to cell dysfunction, apoptosis, and even cell death. Antioxidant compounds that inhibit or reduce free radicals, thus protect against cell damage, and reduce the incidence of several diseases [1].

As well as arising from the action of free radicals, oxidative stress can also be caused by chronic hyperglycemia, which induces cell injury and dysfunction, pancreatic β-cell damage, and insulin resistance, and leads to the onset of diabetes. Chronic hyperglycemia is also often associated with several complications in diabetic patients, including retinopathy, nephropathy, neuropathy, and risk factors for cardiovascular diseases, infections, and progression of cancer cells [1,2]. In particular, postprandial hyperglycemia has been reported to be associated with mortality and cardiovascular disease. The treatment of postprandial hyperglycemia using acarbose, an alpha-glucosidase inhibitor, has also been associated with a reduction in cardiovascular events [3]. Alpha-glucosidases are enzymes

* Corresponding author.
E-mail address: thanutchaporn.nut@mahidol.ac.th (T. Nutmakul).

https://doi.org/10.1016/j.heliyon.2023.e13063
Received 3 October 2022; Received in revised form 1 December 2022; Accepted 16 January 2023
Available online 18 January 2023

located in the brush border of the enterocytes and are responsible for hydrolyzing carbohydrates into glucose. The inhibition of alpha-glucosidases can thus retard liberation of glucose from carbohydrates, delay glucose absorption and reduce postprandial hyperglycemia. However, alpha-glucosidase inhibitors have been reported to cause gastrointestinal side effects, such as diarrhea, abdominal pain, and flatulence [4]. Therefore, efforts to discover novel alpha-glucosidase inhibitors with minimal side effects are of continuing interest.

Traditional medicine, also known as complementary and alternative medicine, is used for the prevention and treatment of many diseases because of its effectiveness, availability, and accessibility [5]. Based on the holistic approach of traditional medicine, prescriptions often contain various herbs as a formulation, with each herb providing its particular function to provide combination and synergistic effects for treating diseases [6]. Indeed, several herbs used in traditional medicine have been reported to be potential sources of antioxidants and alpha-glucosidase inhibitors [4]. Several studies have also reported on the synergistic antioxidant and alpha-glucosidase inhibitory activities of herbal combinations [7–9]. Therefore, polyherbal formulations used in traditional medicines might be candidates for investigation.

Trikatuk is a well-known Thai traditional formulation and has long been widely used. It is composed of three herbs in equal quantities, the fruits of *Piper nigrum* L. and *Piper retrofractum* Vahl and the rhizomes of *Zingiber officinale* Roscoe [10,11]. This formulation is similar to a well-known Ayurvedic formulation, Trikatu. However, the difference between these two formulations is the use of *Piper longum* L. instead of *Piper retrofractum* in Trikatu [12]. Traditionally, Trikatuk has been used for adjusting the balance of the body elements, improving food digestion and absorption, for providing anti-tussive and diaphoretic effects, and for treating influenza and flatulence [10]. Although it has long been used in Thai traditional medicine, only a few studies have reported on its functional effects, such as antioxidant, cholinesterase inhibitory [11], and anti-inflammatory activities [13]. The use of individual herbs in Trikatuk have been reported for diabetes management in Thai traditional medicine with scientific evidence supporting the observed effects [14]. From these studies, Trikatuk might exhibit antidiabetic effects and any synergistic antioxidant and alpha-glucosidase inhibitory activities should therefore be investigated.

A combination of herbs including Trikatuk is commonly prepared by two methods: combining herb powders according to a formula ratio to produce the formulation before extraction; or by combining extracts of each component herb according to a formula ratio. These two methods have been reported to produce formulations which exhibit different potencies [7,13]. Thus, the effect of preparation methods needs to be investigated.

The present study, therefore, aims to evaluate the potential of Trikatuk for development as a traditional medicine or dietary supplement for providing antioxidants and managing diabetes. The contents of phytochemicals, and antioxidants, and their alpha-glucosidase inhibitory activities of ethanolic extracts of Trikatuk and its component herbs will be analyzed. The synergistic effect and the influence of preparation methods will also be explored.

2. Materials and methods

2.1. Reagents and chemicals

Dimethyl sulfoxide (DMSO), piperine analytical standard, Folin-Ciocalteu phenol reagent, ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), acarbose, alpha-glucosidase from *S. cerevisiae*, and 4-nitrophenyl α-D-glucopyranoside (pNPG) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA); aluminum chloride, ascorbic acid, and potassium persulfate from Ajax Finechem (Taren Point, NSW, Australia); gallic acid from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); anhydrous sodium carbonate from CARLO ERBA Reagents (Emmendingen, Germany); and Dulbecco's Phosphate Buffered Saline (PBS) from Gibco Thermo Fisher Scientific (Waltham, MA, USA). All other chemical reagents were of analytical grade.

2.2. Extraction and preparation

The crude raw materials, the fruits of *P. nigrum* (PN) and *P. retrofractum* (PR), and rhizomes of *Z. officinale* (ZO), were purchased from a local traditional drug store in January 2022 (Fig. 1). The voucher specimens (TNRA2201-03) were deposited at the office of the Graduate Program in Nutrition, Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand. The crude materials were ground to a fine powder then equal portions of each (1:1:1, w/w) were mixed to make the Trikatuk (TK). The powders of PN, PR, ZO and TK were macerated with 95% ethanol for 72 h at room temperature with occasional shaking. Each ethanolic extract was then filtered through a

Fig. 1. The crude raw materials of Trikatuk, a Thai traditional formulation.

filter paper and dried using a rotary evaporator. All dried extracts were aliquoted and stored in a sealed container at −20 °C until use.

To determine the synergistic effect and the influence of preparation methods, each dried extract was dissolved in DMSO or ethanol to make a stock solution at a concentration of 10 mg/mL. The stock solutions were combined in an equal ratio to obtain the combinations as follows: PN + PR (1:1, v/v), PN + ZO (1:1, v/v), PR + ZO (1:1, v/v), PN + PR + ZO (1:1:1, v/v).

2.3. Determination of phytochemical contents

2.3.1. Determination of piperine content

The piperine content was determined in TK, PN, and PR by HPLC analysis as previously reported with a slight modification [15]. The HPLC analysis was performed with a Prominence HPLC system (Shimadzu Co., Kyoto, Japan) equipped with binary pumps (LC-20AD), an auto sampler (SIL-20A HT), a column oven (CTO-20A) and a photodiode array detector (SPD-M20A). The chromatographic separation was performed on a Shimadzu Shim-pack GIST C18 column (4.6 × 250 mm, 5 μm particle size). The isocratic elution consisted of 48% of acetonitrile and 52% of 1% acetic acid in deionized water. The flow rate was 1.0 mL/min throughout the run. The column temperature was maintained at a constant temperature of 40 °C.

The standard piperine was dissolved with 50% acetonitrile in deionized water and diluted to the appropriate concentrations to establish a standard curve. The ethanolic stock solutions of TK, PN, and PR were also diluted with 50% acetonitrile in deionized water to the appropriate concentrations. Both the standard and samples were filtered through a 0.45 μm membrane filter then 10 μL of each was injected into the system. The piperine peak was detected at 341 nm.

2.3.2. Determination of total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent method [16]. The ethanolic stock solutions of each sample were diluted with deionized water to 1 mg/mL then 20 μL of each sample were mixed with 50 μL of the Folin-Ciocalteu reagent (diluted 1:10 with deionized water) in 96-well plates then left to stand for 3 min. Then 80 μL of 7.5% sodium carbonate solution were added followed by incubation for 2 h in the dark at room temperature. The absorbance was measured using a microplate reader (Tecan, Männedorf, Switzerland) at 765 nm. Gallic acid was used to establish the standard curve. The TPC was expressed as milligrams of gallic acid equivalent per gram of the extract (mg GAE/g Extract).

2.3.3. Determination of total flavonoid content

The total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method [16]. The ethanolic stock solutions of each sample were diluted with ethanol to 1 mg/mL then 100 μL of each sample were mixed with 100 μL of 2% aluminum chloride solution in 96-well plates. After incubation for 10 min at room temperature, the absorbance was measured at 415 nm. Quercetin was used to establish the standard curve. The TFC was expressed as milligrams of quercetin equivalent per gram of the extract (mg QE/g Extract).

2.4. Determination of antioxidant activity

The antioxidant capacity was determined by ABTS and DPPH radical scavenging assays as previously reported with a slight modification [16].

2.4.1. ABTS radical scavenging assay

The $ABTS^+$ radical was prepared by mixing 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution in equal volumes, and allowing the mixture to react for 12–16 h in the dark at room temperature. Before the assay, the $ABTS^+$ radical was diluted in methanol until the absorbance reached a value of 1.100 ± 0.020 at 734 nm. The ethanolic stock solutions of each sample were diluted with ethanol to 1 mg/mL then 10 μL of each sample were mixed with 200 μL of the $ABTS^+$ radical solution in 96-well plates. After incubation for 6 min in the dark at room temperature, the absorbance was measured at 734 nm. Trolox was used to establish the standard curve. The results were expressed as milligrams of Trolox equivalent antioxidant capacity per gram of the extract (mg TEAC/g Extract).

2.4.2. DPPH radical scavenging assay

The DPPH radical was freshly prepared by diluting with methanol to 152 μmol/L. The ethanolic stock solutions of each sample were serially diluted to various concentrations with ethanol then 100 μL of each sample were mixed with 100 μL of the DPPH radical solution in 96-well plates. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 517 nm. Ascorbic acid was used as a standard reference. The percentage of DPPH radical scavenging capacity was calculated as follows:

$$\%\ \text{DPPH radical scavenging capacity} = [(A_c - A_s)/A_c] \times 100,$$

where A_c is the absorbance of the DPPH solution without the sample and A_s is the absorbance of DPPH react with the sample. The results from each sample were expressed as an IC_{50} value (the concentration of the sample required to scavenge 50% of DPPH radicals).

2.5. Determination of alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibitory activity was determined as previously reported with a slight modification [17]. The DMSO stock

solutions of each sample were serially diluted to various concentrations (0.125–8 mg/mL) with deionized water. Acarbose was used as a positive control and was diluted to various concentrations (0.25–4 mg/mL) with deionized water. In the 96-well plates, 20 μL of each sample were mixed with 20 μL of 0.5 U/mL alpha-glucosidase and 60 μL of PBS (pH 6.9). The mixture was pre-incubated at 37 °C for 10 min before adding 20 μL of 5 mM pNPG as a substrate and further incubation at 37 °C for 20 min for the reaction to take place. The absorbance of p-nitrophenol was measured at 405 nm. The percentage of alpha-glucosidase inhibitory activity was calculated as follows:

$$\%\ \text{Alpha} - \text{glucosidase inhibitory activity} = [(A_c - A_s) / A_c] \times 100$$

where A_c is the absorbance of alpha-glucosidase solution without the sample and A_s is the absorbance of alpha-glucosidase reacting with the sample. The results from each sample were expressed as an IC_{50} value (the concentration of the sample required to inhibit 50% of alpha-glucosidase activity).

2.6. Determination of synergistic effect

The synergistic effect of the different combinations was determined by comparing the theoretical value and the value obtained from the experiment. The theoretical value was calculated by using the additive contributions of each individual in the combination as follows:

Theoretical value of A + B =

$$\text{Theoretical value of A} + \text{B} = \left(\frac{\text{Exp. value of A}}{2}\right) + \left(\frac{\text{Exp. value of B}}{2}\right) \quad (1)$$

$$\text{and Theoretical value of A} + \text{B} + \text{C} = \left(\frac{\text{Exp. value of A}}{3}\right) + \left(\frac{\text{Exp. value of B}}{3}\right) + \left(\frac{\text{Exp. value of C}}{3}\right) \quad (2)$$

This calculation method is easy and has commonly been used for determining the types of interactions in herbal combinations [8, 18].

The type of interaction of ABTS radical scavenging capacity was considered as synergistic when the theoretical value was >5% below the experimental value, as antagonistic when the theoretical value was >5% above the experimental value, and as additive when the theoretical and experimental values differed by < 5%.

For the DPPH radical scavenging capacity and alpha-glucosidase inhibitory activity, the type of interaction was considered as synergistic when the theoretical IC_{50} value was >5% above the experimental IC_{50} value, as antagonistic when the theoretical IC_{50} value was >5% below the experimental IC_{50} value, and as additive when the theoretical and experimental IC_{50} values differed by < 5%.

2.7. Statistical analysis

All experiments in the present study were performed in triplicate and repeated three times. The data are reported as average ± standard deviation from the three experiments. Regression was used to calculate the IC_{50} values. The data were analyzed by analysis of variance (ANOVA) with 95% significance, followed by Tukey's test ($P < 0.05$). The relationship between the phytochemical contents, including piperine, TPC, and TFC and the bioactivities, including DPPH and ABTS radical scavenging capacities, and α–glucosidase inhibitory activity was analyzed using Pearson's correlation. All the statistical analyses were performed using SPSS (version 18.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Extraction yield and piperine content

The yield of ethanolic extracts and piperine contents of TK and the component herbs are shown in Table 1. The yield of TK was 5.133% and of the component herbs, PR exhibited the highest yield (9.034%), followed by PN (5.448%), and ZO (3.793%).

The piperine content was determined in TK, PN, and PR, with PN exhibiting the highest content (35.14%) followed by PR (26.79%) and TK (22.58%). The average contents of piperine in PN and PR were used to calculate the piperine contents in the combinations by

Table 1
Extraction yields and piperine content of Trikatuk and its component herbs.

Ethanolic extracts	Yields (%)	Piperine contents[a] (mg /g Extract)
Trikatuk (TK)	5.133	225.75 ± 2.41
Piper nigrum (PN)	5.448	351.40 ± 2.86
Piper retrofractum (PR)	9.034	267.93 ± 3.09
Zingiber officinale (ZO)	3.793	ND

[a] Values represent average ± standard deviation. ND, Not determined.

using Equations (1) and (2). The calculated piperine contents in PN + PR, PN + ZO, PR + ZO, and PN + PR + ZO were 30.97%, 17.57%, 13.40%, and 20.64%, respectively.

3.2. Phytochemical contents

The total phenolic content (TPC) and total flavonoid content (TFC) in TK, its component herbs, and their combinations were determined (Table 2). Comparing the preparation methods, TK which was derived from the powder mixture had a TPC significantly lower than that derived from the extract mixture, PN + PR + ZO. Of the component herbs, the highest TPC was found in ZO, followed by PN and PR. The combinations of two component herbs revealed that PN + ZO and PR + ZO exhibited a similar TPC which was significantly higher than that of PN + PR. The TPC of these combinations exhibited various differences with respect to their theoretical values (see Equations (1) and (2)), as indicated by the rate of increase in the phenolic content (RIPC). The RIPC for PN + PR + ZO, PN + ZO, and PR + ZO increased by 22.14%, 7.10%, and 23.09%, respectively, while that for PN + PR decreased by 12.03%.

Regarding the total flavonoid content, TK also exhibited a lower TFC, which was not significantly different from that of PN + PR + ZO. Of the component herbs, the highest TFC was also found in ZO, followed by PN and PR. The combinations revealed that PN + ZO exhibited the highest TFC, higher than ZO, followed by PR + ZO, and PN + PR. The TFC of these combinations also increased with respect to their theoretical values, as indicated by the rate of increase in the flavonoid content (RIFC). The RIFC of PN + PR + ZO, PN + PR, PN + ZO, and PR + ZO increased by 8.93%, 18.13%, 16.22%, and 12.68%, respectively.

3.3. ABTS and DPPH radical scavenging capacities

The antioxidant capacity was determined by the ABTS and DPPH radical scavenging assays (Table 3). The results of the ABTS assay were correlated with the TPC values. The antioxidant capacity of TK was significantly weaker than that of PN + PR + ZO. Of the component herbs, ZO exhibited the strongest antioxidant capacity, followed by PN and PR. The combinations revealed that PN + ZO exhibited the strongest antioxidant capacity, followed by PR + ZO, and PN + PR. The types of interaction for PN + PR + ZO, PN + ZO, and PR + ZO were additive while that for PN + PR was antagonistic. This suggested that ZO might contribute an additive effect on the ABTS radical scavenging capacity.

For the DPPH assay, the results are presented as IC_{50} values and were correlated with the TFC. The antioxidant capacity of TK was also significantly weaker than that of PN + PR + ZO. Of the component herbs, ZO exhibited the strongest antioxidant capacity, which was comparable to that of ascorbic acid, a positive control, followed by PN and PR. The combinations revealed that PN + ZO and PR + ZO exhibited a comparable radical scavenging capacity, significantly stronger than that of PN + PR. However, the types of interaction of all the combinations were synergistic. This suggested that all component herbs might exert a synergistic effect on the DPPH radical scavenging capacity.

3.4. Alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibitory activity is presented as IC_{50} values (Table 4). The inhibitory activity of TK was significantly weaker than that of PN + PR + ZO. Of the component herbs, PN exhibited the strongest inhibitory activity, significantly stronger than that of acarbose, a positive control. The inhibitory activity of PR was stronger, but not significantly different than that of ZO. The combinations revealed that PN + PR exhibited the strongest inhibitory activity, followed by PN + ZO and PR + ZO. The inhibitory activities of PN + PR and PN + ZO were also stronger than that of acarbose. The types of interaction of PN + PR and PN + ZO were synergistic while those of PR + ZO and PN + PR + ZO were additive. This suggested that PN might contribute synergistic effects on the alpha-glucosidase inhibitory activity.

Table 2
Experimental and theoretical values of total phenolic (TPC) and total flavonoid (TFC) contents of ethanolic extracts of Trikatuk, its component herbs and their combinations.

Extracts	Total phenolic content (mg GAE/g Extract)			Total flavonoid content (mg QE/g Extract)		
	Experimental	Theoretical	RIPC[a] (%)	Experimental	Theoretical	RIFC[b] (%)
PN	30.70 ± 0.72^{d}	–	–	$2.62 \pm 0.43^{a,b}$	–	–
PR	11.52 ± 1.06^{f}	–	–	0.81 ± 0.07^{c}	–	–
ZO	85.66 ± 1.85^{a}	–	–	$3.30 \pm 0.33^{a,b}$	–	–
TK	20.85 ± 1.21^{e}	–	–	$2.36 \pm 0.43^{a,b}$	–	–
PN + PR + ZO	52.07 ± 1.44^{c}	42.63	22.14	$2.44 \pm 0.58^{a,b}$	2.24	8.93
PN + PR	18.57 ± 1.86^{e}	21.11	- 12.03	$2.02 \pm 0.55^{b,c}$	1.71	18.13
PN + ZO	62.31 ± 1.81^{b}	58.18	7.10	3.44 ± 0.75^{a}	2.96	16.22
PR + ZO	59.81 ± 0.89^{b}	48.59	23.09	$2.31 \pm 0.39^{a,b}$	2.05	12.68

The different superscript letters denote significantly different mean values ($P < 0.05$, Tukey's test).
Values represent average ± standard deviation of triplicate experiments (n = 3).
[a] The rate of increase in the Total phenolic content.
[b] The rate of increase in the Total flavonoid content.

Table 3
Experimental and theoretical values of ABTS and DPPH radical scavenging capacities of ethanolic extracts of Trikatuk, its component herbs and their combinations.

Extracts	ABTS radical scavenging capacity (mg TEAC/g Extract)			DPPH radical scavenging capacity IC_{50} value (μg/ml)		
	Experimental	Theoretical	Effect	Experimental	Theoretical	Effect
PN	73.98 ± 3.31^{f}	–	–	228.09 ± 7.81^{d}	–	–
PR	11.04 ± 0.92^{h}	–	–	1290.64 ± 33.61^{f}	–	–
ZO	370.68 ± 5.78^{a}	–	–	61.20 ± 1.21^{a}	–	–
TK	130.61 ± 5.03^{e}	–	–	231.50 ± 12.06^{d}	–	–
PN + PR + ZO	159.34 ± 2.86^{d}	151.90	Add	165.05 ± 15.20^{c}	526.64	Syn
PN + PR	39.46 ± 6.06^{g}	42.51	Ant	414.50 ± 2.56^{e}	759.36	Syn
PN + ZO	215.52 ± 4.87^{b}	222.33	Add	107.23 ± 2.70^{b}	144.64	Syn
PR + ZO	195.16 ± 2.38^{c}	190.86	Add	122.98 ± 6.74^{b}	675.92	Syn
Ascorbic acid	–	–	–	23.79 ± 3.79^{a}	–	–

Values represent average ± standard deviation of triplicate experiments (n = 3).
The different superscript letters denote significantly different mean values ($P < 0.05$, Tukey's test).

Table 4
Experimental and theoretical values of alpha-glucosidase inhibitory activity of ethanolic extracts of Trikatuk, its component herbs and their combinations.

Extracts	Alpha-glucosidase inhibition IC_{50} value (mg/mL)		
	Experimental	Theoretical	Effect
PN	0.29 ± 0.13^{a}	–	–
PR	5.67 ± 0.35^{d}	–	–
ZO	6.73 ± 0.27^{d}	–	–
TK	5.81 ± 0.32^{d}	–	–
PN + PR + ZO	4.07 ± 0.65^{c}	4.23	Add
PN + PR	0.78 ± 0.29^{a}	2.98	Syn
PN + ZO	$1.59 \pm 0.69^{a,b}$	3.51	Syn
PR + ZO	6.43 ± 0.48^{d}	6.20	Add
Acarbose	$2.95 \pm 1.05^{b,c}$	–	–

Values represent average ± standard deviation of triplicate experiments (n = 3).
The different superscript letters denote significantly different mean values ($P < 0.05$, Tukey's test).

3.5. Correlations between phytochemical contents and biological activities

The relationship between phytochemical contents, including piperine, total phenolic and total flavonoid contents and biological activities including ABTS and DPPH radical scavenging capacities and alpha-glucosidase inhibitory activity were analyzed using Pearson's correlation coefficient (Table 5). The piperine content was significantly negatively correlated with the TPC and TFC values, ABTS radical scavenging capacity, and alpha-glucosidase inhibitory activity and significantly positively correlated with the DPPH radical scavenging capacity. This suggested that extracts with a high piperine content would have low TPC and TFC values and exhibit low ABTS and DPPH radical scavenging capacities but strong alpha-glucosidase inhibitory activity. TPC was significantly positively correlated with the TFC value, and the ABTS radical scavenging capacity but significantly negatively correlated with the DPPH radical scavenging capacity. This suggested that extracts with a high TPC might also have a high TFC and exhibit strong ABTS and DPPH radical scavenging capacities. The ABTS radical scavenging capacity was also significantly negatively correlated with the DPPH radical scavenging capacity and significantly positively correlated with the alpha-glucosidase inhibitory activity. This indicated that extracts with a strong ABTS radical scavenging capacity might also exhibit a strong DPPH radical scavenging capacity, but a weak inhibitory activity for alpha-glucosidase.

Table 5
Pearson's correlation coefficients of relationships between piperine content, total phenolic content, total flavonoid content, ABTS radical scavenging capacity, DPPH radical scavenging capacity, and alpha-glucosidase inhibitory activity.

	Piperine	TPC	TFC	ABTS	DPPH
TPC	**−0.858**[b] (P = 0.000)				
TFC	**−0.453**[a] (P = 0.026)	**0.697**[b] (P = 0.000)			
ABTS	**−0.927**[b] (P = 0.000)	**0.945**[b] (P = 0.000)	**0.693**[b] (P = 0.000)		
DPPH	**0.413**[a] (P = 0.045)	**−0.670**[b] (P = 0.000)	**−0.791**[b] (P = 0.000)	**−0.669**[b] (P = 0.000)	
Alpha-glucosidase inhibitory activity	**−0.677**[b] (P = 0.000)	0.290 (P = 0.169)	−0.134 (P = 0.532)	**0.427**[a] (P = 0.037)	0.109 (P = 0.612)

[a] Correlation is significant at the 0.05 level (2-tailed).
[b] Correlation is significant at the 0.01 level (2-tailed).

4. Discussion

Trikatuk, a Thai traditional formulation, has long been used for adjusting the balance of the body elements, improving food digestion and absorption, providing anti-tussive and diaphoretic effects, and treating influenza and flatulence [10]. A well-known Ayurvedic formulation, Trikatu, is also used for management of digestive disorders, coughs, asthma and obesity and is combined with other herbs to enhance its bioavailability [12]. These two formulations have similar names and component herbs. This might have arisen because traditional Thai knowledge was created through the selection, adoption, adaptation and utilization of traditional medicines after past contact with countries such as India and China [19]. Several herbal formulations have been adopted and adapted from Ayurvedic medicine, including Trikatu. Thai Trikatuk is composed of the fruits of the Java long pepper or *P. retrofractum* as substitutes for the Indian long pepper or *P. longum,* which are used as components of Ayurvedic Trikatu, possibly because of their similar appearance and because *P. retrofractum* is a plant cultivated in Thailand [20]. Ayurvedic Trikatu has been studied intensively and reported to possess many pharmacological properties such as antioxidant, antihyperlipidemic, antimicrobial, and anti-inflammatory activities [12]. Unlike Thai Trikatuk, only a few studies have reported antioxidant, cholinesterase inhibitory [11], and anti-inflammatory activities [13]. In the present study, we have reported on the total phenolic and total flavonoid contents, antioxidant activities, and alpha-glucosidase inhibitory activity as well as the synergistic effect of Trikatuk for the first time.

The term antioxidant is applied to substances or molecules that can delay or prevent the irreversible damage to other substances or macromolecules and promote health benefits, because oxidative stress causes several pathophysiological processes [21]. Phenolic compounds, particularly flavonoids, are known as antioxidants because of their ability to scavenge free radicals and chelate metal ions [22]. Therefore, the measurement of total phenolic and total flavonoid contents is important for predicting the antioxidant capacity of each extract. In the present study, the TPC, TFC and antioxidant activity against DPPH and ABTS radicals of TK, its component herbs, and their combinations were determined. The TPC of the extracts could be classified into three categories: low (<10 mg GAE/g Extract), medium (10–50 mg GAE/g Extract) and high (>50 mg GAE/g Extract) [23]. Therefore, the TPC in PN + PR + ZO was classified as high, while that in TK was medium. Of the component herbs, only ZO was classified as high with the highest TPC, whereas PN and PR were classified as medium. This corresponded with the experimental results on the combinations, where combinations with ZO produced a high TPC while PN + PR exhibited a medium TPC. For the TFC, as a part of the phenolic compounds, the results were associated with TPC at the lower level. Of the component herbs, ZO exhibited the highest TPC and TFC. Previous studies have reported many phenolic compounds in ZO, mainly gingerols, shogaols, and paradols. Other phenolic compounds include zingerone, gingerenone, and 6-dehydrogingerdione [24], and flavonoids such as quercetin, rutin, catechin, epicatechin, kaempferol, and naringenin [25].

The antioxidant capacity was evaluated by the ABTS and DPPH assays which are simple, rapid, inexpensive, acceptable, and widely used [21,26]. DPPH is the free radical most commonly used in antioxidant screening assays where the capacity of each extract could be classified as very strong (IC_{50} < 50 μg/mL), strong (IC_{50} 50–100 μg/mL), moderate (IC_{50} 101–150 μg/mL), and weak (IC_{50} > 150 μg/mL) [27]. Overall, the Trikatuk formulations prepared by both methods exhibited a weak antioxidant capacity. Of the component herbs, only ZO was classified as strong, while PN and PR exhibited a weak antioxidant capacity. Combinations with ZO thus produced a moderate antioxidant capacity, while that of PN + PR was weak. The results from the DPPH assay were similar to those from the ABTS assay. ZO, with the highest TPC and TFC values, exhibited the strongest ABTS and DPPH radical scavenging capacities. The Pearson's correlation coefficients also revealed that TPC and TFC were significantly correlated with the ABTS and DPPH radical scavenging capacities. Similar to our results, many studies have consistently reported a correlation between antioxidant activity estimated by ABTS and DPPH assays and the TPC and TFC values [23,26,28,29]. This suggests that ZO, with the highest TPC and TFC values, could be the main contributor to the antioxidant function of Trikatuk.

Alpha-glucosidases, enzymes active in the final step of carbohydrate metabolism, catalyze the hydrolysis of the alpha-glycosidic bonds of disaccharides and oligosaccharides into glucose. Inhibiting alpha-glucosidases thus retards glucose liberation from carbohydrates, delays glucose absorption and reduces postprandial hyperglycemia [4]. Postprandial hyperglycemia has been reported to be associated with mortality and cardiovascular disease and its treatment using acarbose, an alpha-glucosidase inhibitor, has been associated with a reduction in cardiovascular events [3]. In the present study, the combination of PN + PR + ZO or Trikatuk prepared from the extract mixture exhibited alpha-glucosidase inhibitory activity comparable to that of acarbose. PN and combinations including PN also exhibited a stronger alpha-glucosidase inhibitory activity than acarbose. Notably, piperine, the main compound of PN and PR, was significantly correlated with alpha-glucosidase inhibitory activity. This has been confirmed by a molecular docking study of piperine against alpha-glucosidase [30]. This suggests that the alpha-glucosidase inhibitory activity of Trikatuk might originate from the piperine present in PN and PR.

Synergism is the principal and characteristic basis of traditional medicine that plays an important role in improving efficacy or reducing toxicity because a traditional medicine prescription usually contains various herbs in its formulation. The different components of a prescription exert a synergistic effect by acting on multiple targets at the same time or improving the pharmacokinetic processes, resulting in a better therapeutic effect than a single herb [6]. In the present study, we have provided evidence that Trikatuk exhibited not only antioxidant activity, but also an inhibitory effect on alpha-glucosidase activity. Most of the combinations investigated in the present study also exhibited additive or synergistic effects. Only the combination of PN and PR exhibited an antagonistic effect on ABTS radical scavenging capacity, possibly because the piperine in PN and PR enhanced the production of hydroxyl radicals at high concentrations, even though it exhibited antioxidant activity at a low concentration [31].

Trikatuk is composed of three herbs in equal quantities. The two methods commonly used for preparing these polyherbal combinations are combining the herb powder according to the formula ratio before extraction or combining the extracts of each component herb according to the formula ratio. These two different methods have been reported to exhibit different efficacies [7,13]. A study on

the antioxidant activities of eight traditional Chinese herb used in pairs investigated the influence of the combination method and found that some herb pairs prepared from a powder mixture exhibited greater antioxidant activities than those prepared from an extract mixture and *vice versa* [7]. A study on the anti-inflammatory effect of Trikatuk also revealed that Trikatuk prepared from a powder mixture exhibited a better anti-inflammatory activity than Trikatuk prepared from an extract mixture [13]. However, the results of the present study revealed that Trikatuk prepared from an extract mixture (PN + PR + ZO) exhibited significantly higher TPC and stronger antioxidant and alpha-glucosidase inhibitory activities than Trikatuk prepared from a powder mixture (TK). Therefore, for the further development of Trikatuk as a traditional medicine or a dietary supplement for antioxidant and antidiabetic purposes, we suggest using Trikatuk prepared from an extract mixture.

5. Conclusions

The present study has reported for the first time the synergistic effect on DPPH radical scavenging capacity and the additive effect on ABTS radical scavenging and alpha-glucosidase inhibitory activities of Trikatuk, a traditional Thai medicine. The different methods for preparing herbal combinations have been compared, showing that Trikatuk prepared from an extract mixture exhibited better results than that prepared from a powder mixture. This result is useful for the further development of Trikatuk as an antioxidant and as an alpha-glucosidase inhibitor. Of the component herbs, *Z. officinale* appeared to contribute the antioxidant function, because of its high TPC, TFC and antioxidant activities. *P. nigrum* and *P. retrofractum* appeared to contribute the alpha-glucosidase inhibitory activity because of their high piperine contents and potent alpha-glucosidase inhibitory activity. These results were supported by the analysis of the correlation between the phytochemical contents and biological activities. Based on our results, we strongly suggest that Trikatuk has a good potential for development as a traditional medicine or a dietary supplement for managing diabetes because of its potent antioxidant and alpha-glucosidase inhibitory activities. Although the antidiabetic effect is a novel property of Trikatuk, the antidiabetic assay performed in the present study used alpha-glucosidase from yeast not from mammals. Therefore, further studies on animal models and humans are needed to investigate and confirm the efficacy and safety of Trikatuk.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

Authors' contributions

TN: Conceptualization, Investigation, Data collection and analysis, Visualization, Writing - Original Draft, Writing - Review & Editing.

SC: Methodology, Resources, Data analysis, Writing - Original Draft

Acknowledgments

The authors would like to thank the Faculty of Pharmacy, Mahidol University for research facility support and Miss Bussakorn Chootsungnern for assisting with the experiments. We thank Philip Creed, PhD, from Edanz (https://www.edanz.com/ac) for editing a draft of this manuscript.

References

[1] S. Mahjoub, J. Masrour-Roudsari, Role of oxidative stress in pathogenesis of metabolic syndrome, Casp. J. Intern. Med. 3 (1) (2012) 386–396.
[2] B. Giri, S. Dey, T. Das, M. Sarkar, J. Banerjee, S.K. Dash, Chronic hyperglycemia mediated physiological alteration and metabolic distortion leads to organ dysfunction, infection, cancer progression and other pathophysiological consequences: an update on glucose toxicity, Biomed. Pharmacother. 107 (2018) 306–328, https://doi.org/10.1016/j.biopha.2018.07.157.
[3] P.J. Pinés Corrales, V. Bellido Castañeda, F.J. Ampudia-Blasco, Update on postprandial hyperglycemia: the pathophysiology, prevalence, consequences and implications of treating diabetes, Rev. Clin. Esp. 220 (1) (2020) 57–68, https://doi.org/10.1016/j.rce.2018.06.015.
[4] A.M. Dirir, M. Daou, A.F. Yousef, L.F. Yousef, A review of alpha-glucosidase inhibitors from plants as potential candidates for the treatment of type-2 diabetes, Phytochemistry Rev. 21 (2021) 1049–1079, https://doi.org/10.1007/s11101-021-09773-1.
[5] H. Yuan, Q. Ma, L. Ye, G. Piao, The traditional medicine and modern medicine from natural products, Molecules 21 (5) (2016) 559, https://doi.org/10.3390/molecules21050559.
[6] H. Yuan, Q. Ma, H. Cui, G. Liu, X. Zhao, W. Li, G. Piao, How can synergism of traditional medicines benefit from network pharmacology? Molecules 22 (7) (2017) 1135, https://doi.org/10.3390/molecules22071135.
[7] W.J. Yang, D.P. Li, J.K. Li, M.H. Li, Y.L. Chen, P.Z. Zhang, Synergistic antioxidant activities of eight traditional Chinese herb pairs, Biol. Pharm. Bull. 32 (6) (2009) 1021–1026, https://doi.org/10.1248/bpb.32.1021.
[8] J. Vinholes, M. Vizzotto, Synergisms in alpha-glucosidase inhibition and antioxidant activity of *Camellia sinensis* L. Kuntze and *Eugenia uniflora* L. ethanolic extracts, Pharmacogn. Res. 9 (1) (2017) 101–107, https://doi.org/10.4103/0974-8490.197797.
[9] I. Rahayu, P.H. Heng, K.H. Timotius, *In vitro* antioxidant properties and α-glucosidase inhibition of combined leaf infusions from *Psidium guajava* L., *Syzygium polyanthum* L., and *Annona muricata* L, Pharm. J. 11 (6) (2019) 1269–1277, https://doi.org/10.5530/pj.2019.11.197.

[10] T.K. Lim, Piper retrofractum, 4, Fruits, in: Edible Medicinal and Non-medicinal Plants, Springer Netherlands, 2012, pp. 351–357, https://doi.org/10.1007/978-94-007-4053-2_42.
[11] P. Tappayuthpijarn, C. Sattaponpan, I. Sakpakdeecharoen, A. Ittharat, Cholinesterase inhibitory and antioxidant activities of Thai traditional remedies potentially used for Alzheimer's disease, Thai J. East Asian Stud. 17 (1) (2012) 18–25. https://so02.tci-thaijo.org/index.php/easttu/article/view/51093.
[12] R. Kaushik, J. Jain, A.D. Khan, P. Rai, Trikatu - a combination of three bioavailability enhancers, Int. J. Green Pharm. 12 (3) (2018) S437–S441.
[13] S. Nuaeissara, A. Itharat, W. Pipatrattanaseree, S. Panthong, Anti-inflammatory activity and major compounds of the traditional Thai medicines, Triphala, Trikatuk, and their combined formulae, Sci. Technol. Aliment. 27 (1) (2022) 180–189. https://ph02.tci-thaijo.org/index.php/SciTechAsia/article/view/240315.
[14] C. Andrade, N.G.M. Gomes, S. Duangsrisai, P.B. Andrade, D.M. Pereira, P. Valentão, Medicinal plants utilized in Thai Traditional Medicine for diabetes treatment: ethnobotanical surveys, scientific evidence and phytochemicals, J. Ethnopharmacol. 263 (2020), 113177, https://doi.org/10.1016/j.jep.2020.113177.
[15] S. Shrestha, N. Chaudhary, R. Sah, N. Malakar, Analysis of piperine in black pepper by high performance liquid chromatography, J. Nepal Chem. Soc. 41 (1) (2020) 80–86, https://doi.org/10.3126/jncs.v41i1.30492.
[16] V.H. Sato, S. Chewchinda, W. Parichatikanond, B. Vongsak, *In vitro* and *in vivo* evidence of hypouricemic and anti-inflammatory activities of *Maclura cochinchinensis* (Lour.) Corner heartwood extract, J. Tradit. Complement. Med. 10 (1) (2020) 85–94, https://doi.org/10.1016/j.jtcme.2019.03.003.
[17] S. Sekhon-Loodu, H.P.V. Rupasinghe, Evaluation of antioxidant, antidiabetic and antiobesity potential of selected traditional medicinal plants, Front. Nutr. 6 (2019) 53, https://doi.org/10.3389/fnut.2019.00053.
[18] S.A. Makanjuola, V.N. Enujiugha, O.S. Omoba, D.M. Sanni, Combination of antioxidants from different sources could offer synergistic benefits: a case study of tea and ginger blend, Nat. Prod. Commun. 10 (11) (2015) 1829–1832, https://doi.org/10.1177/1934578X1501001110.
[19] K. He, Traditional Chinese and Thai medicine in a comparative perspective, Compl. Ther. Med. 23 (6) (2015) 821–826, https://doi.org/10.1016/j.ctim.2015.10.003.
[20] C. Suwanphakdee, D.A. Simpson, T.R. Hodkinson, P. Chantaranothai, A synopsis of Thai piper (Piperaceae), Thai Forest Bull. 48 (2) (2020) 145–183, https://doi.org/10.20531/tfb.2020.48.2.08.
[21] J.d.S. Mendonça, R.d.C.A. Guimarães, V.A. Zorgetto-Pinheiro, C.D.P. Fernandes, G. Marcelino, D. Bogo, K.d.C. Freitas, P.A. Hiane, E.S. de Pádua Melo, M.L. B. Vilela, V.A.d. Nascimento, Natural antioxidant evaluation: a review of detection methods, Molecules 27 (11) (2022) 3563, https://doi.org/10.3390/molecules27113563.
[22] S.B. Nimse, D. Pal, Free radicals, natural antioxidants, and their reaction mechanisms, RSC Adv. 5 (35) (2015) 27986–28006, https://doi.org/10.1039/C4RA13315C.
[23] M. .d.S.M. Rufino, R.E. Alves, E.S. de Brito, J. Pérez-Jiménez, F. Saura-Calixto, J. Mancini-Filho, Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil, Food Chem. 121 (4) (2010) 996–1002, https://doi.org/10.1016/j.foodchem.2010.01.037.
[24] Q.Q. Mao, X.Y. Xu, S.Y. Cao, R.Y. Gan, H. Corke, T. Beta, H.B. Li, Bioactive compounds and bioactivities of ginger (*Zingiber officinale* Roscoe), Foods 8 (6) (2019) 185, https://doi.org/10.3390/foods8060185.
[25] A. Ghasemzadeh, H.Z.E. Jaafar, A. Rahmat, Identification and concentration of some flavonoid components in Malaysian young ginger (*Zingiber officinale* Roscoe) varieties by a high performance liquid chromatography method, Molecules 15 (9) (2010) 6231–6243, https://doi.org/10.3390/molecules15096231.
[26] N. Bibi, M.H. Shah, N. Khan, A. Al-Hashimi, M.S. Elshikh, A. Iqbal, S. Ahmad, A.M. Abbasi, Variations in total phenolic, total flavonoid contents, and free radicals' scavenging potential of onion varieties planted under diverse environmental conditions, Plants 11 (7) (2022) 950, https://doi.org/10.3390/plants11070950.
[27] J. Sukweenadhi, O. Yunita, F. Setiawan, M.T. Kartini Siagian, A.P. Danduru, C. Avanti, Antioxidant activity screening of seven Indonesian herbal extract, Biodiversitas 21 (5) (2020) 2062–2067, https://doi.org/10.13057/biodiv/d210532.
[28] A. Floegel, D.-O. Kim, S.-J. Chung, S.I. Koo, O.K. Chun, Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods, J. Food Compos. Anal. 24 (7) (2011) 1043–1048, https://doi.org/10.1016/j.jfca.2011.01.008.
[29] M.F.A. Bakar, N.E. Ahmad, F.A. Karim, S. Saib, Phytochemicals and antioxidative properties of Borneo indigenous Liposu (*Baccaurea lanceolata*) and Tampoi (*Baccaurea macrocarpa*) fruits, Antioxidants 3 (3) (2014) 516–525, https://doi.org/10.3390/antiox3030516.
[30] E. Magaña-Barajas, G.V. Buitimea-Cantúa, A. Hernández-Morales, V.d.R. Torres-Pelayo, J. Vázquez-Martínez, N.E. Buitimea-Cantúa, *In vitro* α-amylase and α-glucosidase enzyme inhibition and antioxidant activity by capsaicin and piperine from *Capsicum chinense* and *Piper nigrum* fruits, J. Environ. Sci. Heal. B. 56 (3) (2021) 282–291, https://doi.org/10.1080/03601234.2020.1869477.
[31] R. Mittal, R.L. Gupta, *In vitro* antioxidant activity of piperine, Methods Find. Exp. Clin. Pharmacol. 22 (5) (2000) 271–274, https://doi.org/10.1358/mf.2000.22.5.796644.