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Phytochemical contents and antioxidant activities of Thai herbal tea from leaves of *Morus alba* and *Citrus hystrix*

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ABSTRACT

A herbal tea composed of *Morus alba* L. (leaves): *Citrus hystrix* DC. (leaves); 1: 1 (w:w) was extracted using aqueous media. Phytochemicals screening determined total phenolic content (TPC), total flavonoid content (TFC), and the identification of flavonoids and phenolic acids of the extract using High-Performance Liquid Chromatography (HPLC). Antioxidant activities, including 2,2-diphenyl-1-picrylhydrazy (DPPH) and ferric reducing antioxidant power (FRAP) were also assayed. The results showed that the TPC and TFC of the extract were 8.18 ± 0.26 mg GE/g Ext and 6.79 ± 0.75 mg RE/g Ext, respectively. The HPLC analyses of flavonoid and phenolic compounds indicated the presence of catechin, myricetin, quercetin, kaempferol, gallic acid, syringic acid, and coumaric acid as bioactive compounds. Antioxidant activities for DPPH and FRAP were 3.51 ± 0.03 mg TE/g Ext and 15.64 ± 0.36 mM Fe^{2+} E/g Ext, respectively. This finding indicates that Thai herbal tea derived from leaves of *M. alba* and *C. hystrix* has an inhibitory free radical effect and has the potential to promote health following herbal tea consumption.

Keywords: herbal tea, *Morus alba*, *Citrus hystrix*, phytochemistry, antioxidant

INTRODUCTION

Free radicals are one of the causes of many illnesses in humans, especially chronic diseases such as cancer, heart disease, cerebrovascular disease, hypertension, kidney disease, cataract, and gout through multiple mechanisms (Chan and Lim, 2006). Generally, reactive oxygen species (ROS) are a class of compounds that are formed from oxygen metabolism. These highly reactive molecules, such as the hydroxyl radical (•OH), peroxide (ROO•), and superoxide radicals ($O_2^{\bullet-}$), can cause severe damage to cells and tissues during various diseases which are linked to heart disease, carcinogenesis, and many other health issues. Synthetic antioxidants such as butylated hydroxyl anisole (BHA), propyl gallate (PG), butylated hydroxyl toluene (BHT), which have been used to prevent oxidation, have been found to cause internal and external bleeding in rats and guinea pigs at high dose (Iqbal et al., 2015). Nowadays, researchers are interested in using natural antioxidants such as bioactive flavonoids, which are of great importance due to their indigenous origin and strong efficacy to trap and scavenge free radicals (Borneo et al., 2009). In recent years, numerous studies have reported that phytochemicals from plants have antioxidant potential, which are risk factors for many diseases (Duthie and Crozier, 2000).

Herbal teas (more accurately known as tisanes) are mixtures of dried leaves, seeds, grasses, nuts, bark, fruits, flowers, or other plant parts that give them their flavor and provide therapeutic benefits to the body form of herbs (Ravikumar, 2014). The herbal tea in this study was a combination of dried leaves consisting of *Morus alba* and *Citrus hystrix*. This herbal tea developed by Kingyok Registered Ordinary Partnership, Borabue District, Maha Sarakham Province, Thailand (Kingyok ROP) as an alternative to conventional health care, with effects such as lowering blood sugar levels lowering blood lipids, lowering blood pressure, helping relaxation and detoxifying the body

M. alba (Family: Moraceae) known as mulberry, is a deciduous tree widely cultivated in subtropical, tropical, and temperate environments (Mahmoud et al., 2017). It has long been used in traditional medicines in many countries, including Thailand (Soonthornsit et al., 2017). The leaves have been used in Thai traditional medicine as an antipyretic, antitussive, sedative, antidiabetic, and to improve eyesight. Phytochemical composition in mulberry leaves consists of terpenoids, alkaloids, chalcones (including morachalcones B, C),

flavonoids (including astragalin, cyclomulberrin, isoquercitrin, kaempferol, morusin, quercetin, rutin, roseoside, and scopolin), anthocyanins, phenolic acids (including m-coumaric acid, syringic acid, and vanillic acid), stilbenoids (including mulberrosides A, B, F) and coumarins (Chan et al., 2016). Several recent studies have shown that mulberry leaves have antioxidant properties (Thabti et al., 2012), α-glucosidase inhibitory activity (Hwang et al., 2016), antidiabetic (Hansawasdi and Kawabata, 2006), antianxiety (Yadav et al., 2008), anti-inflammatory (Choi and Hwang, 2005), anti-atherosclerotic, anti-obesity, hypolipidemic effects, cognitive-enhancing effects and skin-whitening properties (Chan et al., 2016).

C. hystrix (Family: Rutaceae) is a native species in Asia, especially in tropical regions, and known as Kaffir lime. It is evergreen, aromatic, and distinctive with double-shaped compound leaves (Ratseewo et al., 2016). The leaf of Kaffir lime is a common spice used as a condiment in various Thai and Malaysian recipes (Butryee et al., 2009). In Thailand, it is used in traditional medicine by using leaves as a medicine antitussive, expectorant and carminative. The important biological constituents found in kaffir lime leaves are volatile compounds such as citronellal, linalool, β-cubebene, β-pinene, myrcene, limonene, γ-terpinene, ρ-cymene, terpinolene, copaene, caryophyllene, citronellyl acetate, citronellol, geranyl acetate and δ-cadinene (Lawrence et al., 1971). In addition, it contains coumarins, alkaloids, carbohydrates, flavonoids, glycosides, phenols, steroids, and tannins (Ali et al., 2015). Pharmacological effects of Kaffir lime leaves have been shown to include antioxidant (Ratseewo et al., 2016), hepatoprotective effect (Abirami et al., 2015), and anti-cancer activity (Tunjung et al., 2015).

This herbal tea was further developed by Kingyok ROP to select a herbal tea for health care. This study performed phytochemical screening (using TPC, TFC, and HPLC) and assayed for antioxidant activity (DPPH and FRAP assay) as a preliminary pharmaceutical investigation.

MATERIALS AND METHODS

Plant material and extraction

The herbal tea was composed of *M. alba* L. (leaves) and *C. hystrix* DC. (leaves); mixed in ratio 1:1 (w:w). Herbal tea was supplied by Kingyok ROP. All the fresh materials were cleaned and dried at 45 °C for 48 h in a hot air oven and then powdered. For extraction (Ext), 1 g of tea in powder form was extracted by immersing in 75 ml water (1:75 w/v) at 80 °C for 15 min. Extracts were filtered through a 0.45 μm membrane filter. The extracts were separated into two parts. Part 1 was stored at 4 °C for analysis using TPC, TFC, DPPH, and FRAP assay, which were compared with a single tea (*M. alba* leaf tea and *C. hystrix* leaf tea). Part 2 was stored at -18 °C for analysis by HPLC (Nammatra et al., 2021).

Total phenolic content (TPC) determination

Total phenolic content was determined according to a modified procedure (Singleton et al., 1999). The extract from herbal tea (100 μL) was oxidized with 500 μL of 0.2 N Folin-Ciocalteu's reagent and neutralized by adding 400 μL of 7.5% Na_2CO_3. The absorbance was measured at 765 nm by UV-Vis Spectrophotometer after mixing and incubated at room temperature for 30 min. The results were expressed as gallic acid equivalent (mg GAE/g Ext).

Total flavonoid content (TFC) determination

Flavonoid content was estimated using the aluminum chloride colorimetric method (Sharma and Agarwal, 2015). The extract from herbal tea (500 μL), 2,000 μL distilled water, and 150 μL 5% $NaNO_2$ solution were added. After 6 min, 150 μL 10% $AlCl_3$ solution was added and kept for another 6 min. To this reaction mixture, 2,000 μL 4% NaOH solution and 200 μL water were added to make up the final volume of 5,000 μL. The reaction mixture was mixed well and allowed to stand for 15 min after which absorbance was recorded at 510 nm. The total flavonoid content (TFC) was calculated from a standard rutin equivalent (mg RE/g Ext).

High-Performance Liquid Chromatography (HPLC) analysis

Analysis to compare the amount of some flavonoid and phenolic acid compounds from herbal tea that was beneficial to the health used the High-Performance Liquid Chromatography (HPLC) method according to Jorjong et al. (2015). HPLC–DAD system (Shimadzu, Japan), comprising of Shimadzu LC-20AC pumps, a SPD-M20A diode array detector, and an Apollo C-18 column (Alltech Associates, Deerfield, IL, USA) (4.6 mm x 250 mm, 5 μm) protected with guard column Inertsil ODS–3 (4.0 mm x 10 mm, 5 μm; GL Science Inc., Tokyo, Japan) were used for the analysis of flavonoids and phenolic acids. The mobile phase for flavonoids used acetonitrile/deionized water (2/97.8, v/v) containing 0.2% phosphoric acid (solvent A) and acetonitrile/deionized water (97.8/2, v/v) containing 0.2% phosphoric acid (solvent B) at a flow rate of 0.6 ml/min and 40°C column temperature. The UV–Vis spectra were detected at 254 nm. (Butkhup and Samappito, 2008). The mobile phase for phenolic acids consisted of acetonitrile (solvent A) and

phosphoric acid in deionized water pH 2.58 (solvent B) at a flow rate of 0.8 ml/min and 40 °C column temperature. The flavonoid compounds detected were catechin, myricetin, quercetin, and kaempferol, and the phenolic compounds were gallic acid, syringic acid, and coumaric acid, and all were analyzed by comparing the retention time and spectrum as well as standard addition.

Antioxidant activity by DPPH assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of extract from herbal tea was estimated by the reduction of the reaction color of DPPH solution and sample extracts by the method according to Thaipong et al. (2006). DPPH was dissolved in ethanol at 0.039 mg/mL. The extract was diluted with distilled water to yield sample solutions at various concentrations. 100 µL of the sample solution was added to 900 µL DPPH (0.1 mM) to give the working solution. After a 30 min reaction kept in the dark at ambient temperature, the absorbance of the solution was measured at 517 nm. Trolox (6-hydroxy-2,5,7,8-tetramethychlorman-2-carboxylic acid) was used as a positive control for comparison, and solvent mixed with 0. 1 mM DPPH solution was taken as a negative control. The percent scavenging was calculated by the following formula:

DPPH scavenging activity (%) = $[(A_0-A_S) / A_0] \times 100$

A_0 of control was the absorbance of the solvent mixed with DPPH solution, and As was the absorbance of the extract solution. DPPH radical scavenging was indicated as mg Trolox equivalent (mg TE)/g extraction.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted according to a previously described method by Benzie and Strain (1996). The working solution was prepared by mixing 25 mL of acetate buffer pH 3.6 (3.1 g of $CH_3COONa.3H_2O$ and 16 mL of CH_3COOH) to a concentration of 300 mM, 2. 5 mL TPTZ solution (10 mM TPTZ in 40 mM HCl), and 2. 5 mL of 20 mM $FeCl_3.6H_2O$ solution and equilibrated at 37 °C before use. Samples had a concentration of 1500 µg/mL (200 µL) and could react with 2.5 ml of the working solution for 30 min in the dark at 37 °C. Absorbance was measured at 593 nm (n=3) using a UV-Vis spectrophotometer. Ferrous sulfate ($FeSO_4$) was used as a standard to establish a standard curve. The FRAP antioxidant activity was expressed as mM of Fe^{2+} equivalents per g of sample (mM Fe^{2+}E/g Ext).

Statistical analysis

All assays were expressed as mean ± standard deviation (SD) from five separate experiments (n =5). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Total phenolic and total flavonoid contents

The total phenolic contents (TPC) were estimated using gallic acid, and total flavonoid contents (TFC) were estimated using rutin as standard. The results showed that the TPC and TFC of extract from HT were 5.89 ± 0.07 mg GAE/g Ext and 8.38 ± 0.33 mg RE/g Ext, respectively (Table 1), indicating a moderate total phenolic and total flavonoid content in herbal tea extract. These were significantly higher contents than *M. alba* leaf tea (TPC = 5.48 ± 0.18 mg GAE/g Ext and TFC = 7.26 ± 0.45 mg RE/g Ext), but were significantly lower than *C. hystrix* leaf tea (TPC = 20.21 ± 0.06 mg GAE/g Ext and TFC = 12.03 ± 0.24 mg RE/g Ext). Furthermore, other research has reported important total phenolic and flavonoid content in aqueous extracts derived from herbal tea from the leaves of *M. alba* and *C. hystrix*. Thabti et al. (2012) found that the aqueous extract of *M. alba* leaves showed TPC (759 ± 74 mg GAE/100 g DW) and TFC (717 ± 45 mg RE/100 g DW), and these levels were greater than in methanolic extract (TPC = 560.00 ± 97.23 mg GAE/100 g DW and TFC = 283.13 ± 4.10 mg RE/100 g DW). Radojković et al. (2012) reported that the ethanol extract of *M. alba* leaves had TPC at 66.766 ± 0.749 mg CAE /g and TFC at 33.303 ± 0.059 mg RE/g. There is also a report that methanol and ethanol extracts of *C. hystrix* leaves had TPC levels of 1.40 ± 0.32 mg GAE/g and 1.37 ± 0.32 mg GAE/g, and TFC were 2.58 ± 0.71 mg RE/g and 2.30 ± 0.56 mg RE/g, respectively (Ali et al., 2015). In 2016, Ratseewo et al. (2016) reported that the aqueous extracts obtained from steamed *C. hystrix* leaves had TPC at 22.18 ± 0.06 mg GAE/g and TFC was 11.84 ± 0.02 mg RE/g.

Phenolic compounds from plants, such as flavonoids, have antioxidant potential, which is a risk factor for disease (Duthie and Crozier, 2000). Furthermore, phenolic compounds are considered secondary metabolites, and these phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are diversified and are thought to have positive effects on human health (Saeed et al., 2012). The aqueous extraction is a method used to brew tea and not harmful to humans. Nevertheless, aqueous extraction showed a low level

of total phenolics due to most flavonoids being non-polar, making them less readily extractable with water, which is a strong polar solvent (Mojulat and Surugau, 2018). Furthermore, the aqueous extraction may result in lower total phenol levels than other methods or possess phenolic compounds that contain a smaller number of active groups than the other solvents (Do et al., 2014).

Table 1. Total phenolic contents (TPC) and total flavonoid contents (TFC) of extract from herbal tea and single tea.

Samples	TPC (mg GE/g Ext)	TFC (mg RE/g Ext)
HT	5.89 ± 0.07^{b}	8.38 ± 0.33^{b}
M. alba leaf tea	5.48 ± 0.18^{bc}	7.26 ± 0.45^{c}
C. hystrix leaf tea	20.21 ± 0.06^{a}	12.03 ± 0.24^{a}

HT= Herbal tea was composed of *M. alba* L. (leaves) and *C. hystrix* DC. (leaves); mixed in ratio 1:1 (w:w).
TPC was measured with gallic acid equivalents (mg GE/g Ext). TFC was measured with rutin equivalent (mg RE/g Ext).
Values in the columns with different superscript letters are significantly different ($P < 0.05$).

Flavonoids and Phenolic acids

A partial analysis of the flavonoids and phenolic acid compositions of the healthful herbal tea was performed using reversed-phase HPLC. Generally, the same flavonoid and phenolic acid compounds were present in all plant species, but there were differences in relative levels (Jorjong et al., 2015). In this study, partial flavonoid content analysis of the aqueous extract from herbal tea were catechin, myricetin, quercetin, and kaempferol. The results showed that the content of catechin, myricetin, quercetin, and kaempferol were 2324.77 ± 34.43 μg/g Ext, 170.36 ± 5.82 μg/g Ext, 6176.80 ± 11.72 μg/g Ext and 382.16 ± 3.54 μg/g Ext, respectively (Table 2). Previously, research has shown that the leaf extract of *M. alba* contained flavonoid constituents consisting of astragalin, atalantoflavone, cyclomulberrin, isoquercitrin, kaempferol, kaempferol 3-O-β-D-rutinoside, morusin, quercetin, quercetin 7-O-β-D-glucopyranoside, rutin, roseoside, skimmin and scopolin (Chan et al., 2016). The leaf extract of *Citrus hystrix* contains many flavonoids, such as myricetin, peonidin, cyanidine, quercetin, luteolin, hesperetin, apigenin and isorhamnetin (Butryee et al., 2009).

Gallic acid, syringic acid, and coumaric acid comprised the phenolic acid content of the aqueous extracts. The results showed that the content of gallic acid was higher than syringic acid and coumaric acid, which were 1,053.00 ± 76.30 μg/g Ext, 195.54 ± 9.72 μg/g Ext, and 182.81 ± 11.03 μg/g Ext, respectively (Table 3). Past studies have reported that the leaf extracts of *M. alba* have 5-O-caffeoylquinic acid, m-coumaric acid, p-coumaric acid, ferulic acid, gallic acid, hydroxybenzoic acid, protocatechuic acid, protocatechuic aldehyde, syringaldehyde, syringic acid, and vanillic acid (Chan et al., 2016), caffeoylquinic acid and caffeic acid (Thabti et al., 2012). The extract from the peel of *C. hystrix* has gallic acid, caffeic acid, p-coumaric acid, and ferulic acid (Wijaya et al., 2017). It is possible that the extraction of mixtures of more than one plant species together causes changes in the content and type of phytochemical. This study can indicate that bioactive compounds from leaves might be potential natural sources for the development of antioxidant function in dietary food.

Table 2. Flavonoid content (μg/g Ext) of extract from herbal tea.

Samples	Catechin (μg/g Ext)	Myricetin (μg/g Ext)	Quercetin (μg/g Ext)	Kaempferol (μg/g Ext)
HT	2324.77 ± 34.43	170.36 ± 5.82	6176.80 ± 11.72	382.16 ± 3.54

HT= Herbal tea was composed of *M. alba* L. (leaves) and *C. hystrix* DC. (leaves); mixed in ratio 1:1 (w:w).

Table 3. Phenolic acid content (μg/g Ext) of extract from herbal tea.

Samples	Gallic acid (μg/g Ext)	Syringic acid (μg/g Ext)	Coumaric acid (μg/g Ext)
HT	1053.00 ± 76.30	195.54 ± 9.72	182.81 ± 11.03

HT = Herbal tea was composed of *M. alba* L. (leaves) and *C. hystrix* DC. (leaves); mixed in ratio 1:1 (w:w).

Antioxidant activity

In this experiment, the radical scavenging activities of extract from herbal tea were measured using two different assays, namely DPPH and FRAP, as shown in Table 4. The antioxidant activity in aqueous extract of HT for DPPH was 3.51 ± 0.03 mg TE/g Ext, while FRAP radical scavenging activity of HT gave a moderate antioxidant capacity in this study which showed a reduction of ferrous ion (Fe^{2+}) radical of 15.64 ± 0.36 mM Fe^{2+} E/g Ext. These were significantly higher contents than *M. alba* leaf tea (DPPH = 3.13 ± 0.12 mg TE/g Ext and FRAP = 13.57 ± 1.10 mM Fe^{2+} E/g Ext), but were significantly lower than *C. hystrix* leaf tea (DPPH = 9.39 ± 0.18 mg TE/g Ext and FRAP = 21.21 ± 1.65 mM Fe^{2+} E/g Ext). This was consistent with the analysis of the total phenolic (TPC) and flavonoids (TFC), which found that kaffir lime tea had the highest values of both. Due to phenolic and flavonoid compounds from plants were antioxidant properties (Duthie and Crozier, 2000).

This result related well with the study of Radojković et al., (2012), reporting that an ethanol extract of *M. alba* showed high antioxidant activity with IC_{50} value by DPPH assay of 0.0124 mg/mL. In the same year, Chan et al. (2012) report that the aqueous extract with microwave drying from tea of shredded leaves of *M. alba* had antioxidant activity by both DPPH (1920 ± 23 mg AA/ 100 g) and FRAP (865 ± 25 mg GAE/ 100 g). Thabti et al. (2012) reported that the aqueous leaf extract of *M. alba* contained antioxidant activity with an IC_{50} value by DPPH assay of 5.59 ± 0.14 mg/mL. According to the study of Butryee et al. (2009) the aqueous extract of boiled *C. hystrix* leaves can be antioxidant both DPPH (39 ± 18% RSA) and FRAP (49.61 ± 21.14 μmole Fe^{2+} E/g). Laohavechvanich et al. (2010) reported that the aqueous extract of boiled *C. hystrix* leaves had an antioxidant activity with IC_{50} value by DPPH assay of 11.9 mg/mL. Furthermore, Ratseewo et al. (2016) found that the aqueous extract of boiled *C. hystrix* leaves showed high antioxidant activity with the FRAP method at 583 ± 18.17 μmol $FeSO_4$/g. Consistent with past studies, the antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelating of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation (Ali et al., 2015).

Table 4. DPPH and FRAP radical scavenging activities of extract from herbal tea and single tea.

Samples	DPPH (mg TE/g Ext)	FRAP (mM Fe^{2+} E/g Ext)
HT	3.51 ± 0.03^{b}	15.64 ± 0.36^{b}
M. alba leaf tea	3.13 ± 0.12^{bc}	13.57 ± 1.10^{c}
C. hystrix leaf tea	9.39 ± 0.18^{a}	21.21 ± 1.65^{a}

HT= Herbal tea was composed of *M. alba* L. (leaves) and *C. hystrix* DC. (leaves); mixed in ratio 1:1 (w:w).
DPPH radical scavenging activity was used Trolox as a positive control for comparison.
FRAP radical scavenging activity was reducing of ferrous ion (Fe^{2+}) radical from herbal tea.
Values in the columns with different superscript letters are significantly different ($P < 0.05$).

CONCLUSIONS

The aqueous extract of herbal tea from mixed in leaves of *M. alba* and *C. hystrix* have antioxidant activity based on TPC, TFC, DPPH, and FRAP assays. Flavonoid compounds identified by HPLC consisted of quercetin, catechin, kaempferol, and myricetin. Phenolics detected were gallic acid, coumaric acid, and syringic acid. It can be concluded that this herbal tea has antioxidant effects with the potential to be consumed as an herbal tea for health care. However, there is a need to clarify the major active substance, in vivo and in clinical research in the next study, and study-related other biological activities and other extraction methods.

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