

Exploring the synergistic effect of Celery and Chinese kale on Antioxidant and Anti-Pancreatic Lipase and Anti-Acetylcholinesterase activities

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ABSTRACT

It has been well known that vegetables provided numerous health benefits and mixed recipe derived local vegetables have been used in traditional folklore. Therefore, this study aimed to observe the synergistic effect of the mixed water extract of Celery and Chinese kale on chemical compositions using GC-MS analysis and the biological activity including antioxidant activity and inhibited acetylcholinesterase (AChE) and suppressed pancreatic lipase by *in vitro* study. The GC-MS analysis of the chemical compounds showed the presence of Pyrrolidine, N,N- Dimethylaminoethanol, 2,3-Butanediol, Dimethyl trisulfide, Pentane, 3-methyl-, 3H-1,2,4-Triazol-3-one, 1,2-dihydro-, p-Cresol, 3-Butylisobenzofuran-1(3H)-one, Senkyunolide, n-Hexadecanoic acid, and Phytol. The Mixed extract significantly increased in total phenolic compound but no different in flavonoid content when compared with the extract alone. The biological activity showed that the potentials of antioxidant activity, inhibited pancreatic lipase activity and Anti-AChE activity of the mixed extract significantly increased than extract alone. The combination between celery and chinese kale extract showed high potential in antioxidant activity, inhibition of pancreatic lipase and AChE activity due to its syngenetic effect. Therefore, the combined extract is suitable for developing the functional food with positive health effects that extend beyond their nutritional value such as nutraceuticals products with mitigating hyperlipidemia and improving cognitive function.

KEYWORDS: *Apium graveolens* L.; *Brassica oleracea* L.; Antioxidant activity; Lipase; Acetylcholinesterase; GC-MS analysis

INTRODUCTION

At present, the accumulative line of scientific evidence revealed that health-promoting phytochemicals and essential minerals of fruit and vegetables significantly improved health benefits. The several reports have received considerable attention due to their potential human benefit effects including antioxidant, anti-inflammatory, antimicrobial, antiallergic and anticarcinogenic capacities [1]. These effects derived from plant bioactive compound such as phenolic compounds, flavonoids, anthocyanins, carotenoids and glucosinolates [2]. In addition, increasing suggestions of health benefits may be realized from plant-derived colors possessing exceptionally high antioxidant capacity such as green pigment of chlorophylls [3]. It is well known that several green vegetables in Thailand have been long-term used as functional color-food to serves as new functional food with potential protection and treatment in various diseases.

Celery or *Apium graveolens* L. belongs to the family *Apiaceae* (Umbelliferae). It has characteristic aromatic odor because of essential oil and volatile compounds, which are largely confined to the green leaves of plant [4]. Mostly celery is composed of flavonoids and phenols including apigenin, tannins, isoquercitrin, phytic acid and graveobioside [5,6]. Celery is a medicinal plant due to its effect which contributing role on antioxidant activity [7,8], antinociceptive potentials and antiinflammatory properties [9].

Chinese kale or *Brassica oleracea* var. *alboglabra* is widely distributed in southern China and Southeast Asia. Chinese kale is highly nutritious because of phytochemical constitutions, including glucosinolates, carotenoids, vitamin C, and phenolic compounds [10,11], and its abundance in health-promoting antioxidants and essential minerals [12]. The previous study reported the crucial role on health-promoting effect by the effect of cytotoxicity [13],

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antioxidant, anti-inflammatory, antimicrobial, antiallergic and anticarcinogenic capacities [11,14]. Herbal medicines, especially oriental medicine, are characterized by the use of mixtures of several herbs (multiherbs) in a single formula [15]. The effect of the multiherbs is more potential greater than used alone [16] because the therapeutic effects of these herbal products may arise from synergistic actions of herbal ingredients [17]. Therefore, this study aimed to develop a functional food product with the high potential of the combined extract between celery and chinese kale. Moreover, the chemical composition in the combined extract was evaluated by liquid chromatography–mass spectrometry (LC-MS) analysis and the biological activities to promote the health benefit effect including antioxidant, anti-pancreatic lipase and anti-AChE activities were also explored in *in vitro* study.

MATERIALS AND METHODS

Plant Materials and Development the Recipe of the Combined Extract

Local indigenous vegetables including Celery and Chinese kale were collected from Royal Project Foundation Farm, Chiang Mai Province during November 2019 to January 2020. The leaves and rhizomes were cleaned and cut into small pieces and dried under 40 °C. Then, the ground powder was macerated with distilled water at room temperature for 72 hour and the extracts were filtered with Whatman No. 1 filter paper. The filtrate was dried by freezing dryer and the powder was kept at -20 temp. before using. In this study, we developed the recipe product by combining the water extract of Celery and Chinese kale at a ratio of 4:1 respectively, based on our pilot data which showed that this ratio showed the highest potential for antioxidant capacity.

Determination of the Bioactive Compounds by Liquid Chromatography–mass Spectrometry (LC-MS) Analysis

The GC-MS analysis was evaluated the bioactive compounds of the mixed extracts by Agilent Technologies GC systems with GC-7890B (Agilent Technologies, Santa Clara, CA, USA) equipped with DB-5MS column (length 30m, diameter 0.250mm, film thickness 0.5µm). Analysis conditions are 5min, at 50 °C, 1min at 10 °C for column temperature, 240 °C for injector temperature, helium is the carrier gas and split ratio is 5:4. The sample (1µl) is evaporated in a split less injector at 300 °C. Run time is 53min. The compounds are identified by gas chromatography coupled with mass spectrometry. The structure of the compounds of the extract are ascertained by the interpretation of mass spectrum of GC-MS using the database of National Institute Standard and Technology (NIST). The mass spectrum of the unknown component is compared with the spectrum of the known components stored in the NIST library [18].

Determination of the Total Phenolic and Flavonoid Contents

The total phenolic content of the combined extract was determined by the Folin-Ciocalteu method [19]. In brief, an aliquot of 20µl of extract and gallic acid was added into test-tube containing 1.58ml of distilled water and 1.0ml of Folin-Ciocalteu phenol reagent which dissolved in 1 volume of Folin-Ciocalteu phenol (Sigma, MO, USA) with 2 volumes of distilled water. After incubating for 8min, 0.3ml of 20% Na₂CO₃ (w/v in distilled water) was added. Then, it was incubated for 2hr at room temperature in dark room. Absorbance was measured at 765nm with a Spectronic™ GENESYS™ 20 spectrophotometer (Thermo electron corporation, IL, USA). The total phenolic content was expressed in µg of GAE/mg extract. Total

flavonoid contents were estimated according to the aluminum chloride method [20]. Briefly, 0.5ml of the combined extract and quercetin were mixed with 1.5ml of 50% alcohol, 0.1ml of 10% AlCl₃, 0.1ml of 1M potassium acetate and 2.8ml of distilled water into a test-tube. The test-tube were vortexed for 10s and incubated at room temperature for 5min. The absorbance was measured at 415nm using a Spectronic™ GENESYS™ 20 spectrophotometer (Thermo electron corporation, IL, USA). The flavonoid content was expressed in µg of quercetin/mg extract.

Determination of Antioxidant Activity

Ferric Reducing Antioxidant Power (FRAP): Ferric reducing antioxidant power assay (FRAP) was evaluated as described earlier [21]. Briefly, the FRAP reagent was prepared by 25ml of 300mM acetate buffer pH 3.6, 2.5ml of 10mM tripyridyltriazine (TPTZ) solution in 40mM hydrochloric acid (HCl), and 2.5ml of 20mM FeCl₃•6H₂O solution. An aliquot of 50µl of the mixed extract at different concentration was mixed with 1450µL of the FRAP reagent and 100µl of distilled water. They were incubated at 37 °C for 10 minutes in water bath. The absorbance of reaction was measured at 593nm by a Spectronic™ GENESYS™ 20 spectrophotometer (Thermo electron corporation, IL, USA). The results were expressed IC₅₀ value of the sample.

2,20-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS): Free radical scavenging activity of the extract was determined by ABTS radical cation decolorization assay [22]. ABTS•+ cation radical was produced by the reaction between 7mM ABTS in water and 2.45mM potassium sulfate (1:1), stored in the dark at room temperature for 12-16h before use. ABTS•+ solution was then diluted with methanol to obtain an absorbance at 734nm using a Spectronic™ GENESYS™ 20 spectrophotometer (Thermo electron corporation, IL, USA). After an added 5µl of combined extract to 3.995ml of diluted ABTS•+ solution, the absorbance was measured at 30min after the initial mixing. The results were expressed IC₅₀ value of the sample.

Determination of Acetylcholinesterase (AChE) Inhibition

The evaluation of anti-AChE activity in the extract was performed with the colorimetric method utilizing acetylthiocholine iodide (ATCI) as a substrate [23]. The rate of production of thiocholine is determined by the continuous reaction of 5, 5-dithiobis-2-nitrobenzoate (DTNB) ion to produce the yellow anion of 5-thio-2-nitro-benzoic acid. In brief, 25µl of 15mM ATCI, 75µl of 3mM DTNB and 50µl of 50mM Tris-HCl, pH 8.0, containing 0.1% ovine serum albumin (BSA) and 25µl of the tested phytochemicals in the combined extract were added in to the 96 well plates, and the absorbance was measured at 405nm after 5 minutes of incubation at room temperature.

Then, 25µl of 0.22U/mL of AChE (Sigma-Aldrich Corporation, St. Louis, MO, USA) was added, the absorbance was measured again after 5 minutes of incubation at room temperature. Percentage of inhibition was calculated by comparing the rate of enzymatic hydrolysis of ATCI for the samples to that of the blank (50% aqueous methanol in buffer). Galanthamine (1-32mM) supplied by Sigma-Aldrich was used as a reference standard.

Determination of Pancreatic Lipase Inhibition

Pancreatic lipase inhibitory activity was determined by slightly modified titrimetric method [24]. In brief, porcine pancreatic lipase enzyme solution was immediately prepared at concentration 5mg/ml in 0.1M sodium phosphate buffer, pH 8.0. The substrate

was prepared by 4.5mg of P-Nitrophenylbutyrate (PNPB) with dissolved in 200 μ l of N,N-dimethyl formamide and final volume was made up by 10ml of 0.1M sodium phosphate buffer, pH 8.0. The reaction solution was mixed by 10 μ l of pancreatic lipase, 150 μ l of p-nitrophenyl palmitate solution and 40 μ l of 0.1M sodium phosphate buffer. Then, 25 μ l of test solution and standard was incubated with 50 μ l of enzyme solution for 30 minutes at 37 °C. The inhibition of Lipase activity was determined by 400nm of the absorbance using a microplate reader.

STATISTICAL ANALYSIS

Data are presented as the means \pm S.E.M. and were analyzed statistically using one-way ANOVA followed by a post hoc (LSD) test. The results were considered to be statistically significant at a *p*-value <0.05.

RESULTS

The Bioactive Chemical Compounds in the Combined Extract by GC-MS Study

Mass Spectrometer (GC-MS) is a important tool to evaluate potential to supply the definitive, qualitative and quantitative

information on molecules based on their structural compositions.

In this, interpretation of mass spectrum of GC-MS was done using database of National Institute Standard and Technology (NIST). The mass spectrum of unknown component was compared with the spectrum of the known component stored in the NIST library and major components were identified by with authentic standards and by with recorded from computerized libraries.

The GC-MS spectrum confirmed the presence of various components with different retention times as illustrated in Figure 1. These mass spectra are fingerprint of that compound which can be identified from the data library. The compound name, probability, and biological activity of the combined extract were shown in the Table 1. The major compounds were Pyrrolidine, N,N- Dimethylaminoethanol, 2,3-Butanediol, Dimethyl trisulfide, Pentane, 3-methyl-, 3H-1,2,4-Triazol-3-one, 1,2-dihydro-, p-Cresol, 3-Butylisobenzofuran-1(3H)-one, Senkyunolide, n-Hexadecanoic acid, and Phytol.

The results of GC-MS study specify that the water extract of the combined plants contains various bioactive compounds. Therefore, these contribute as medicinal bioactive with promoting health benefit.

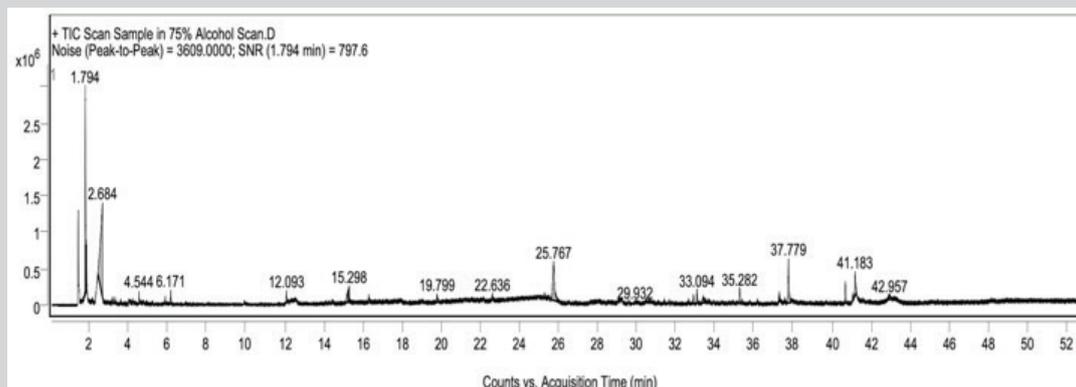


Figure 1: GC-MS analysis of the combined extract.

Table 1: The chemical composition of the combined extract exploring by GC-MS analysis.

Name of the Compound	Retention Time	Molecular Formula	Probability	Biological Activity
Pyrrolidine	4.028	C ₄ H ₉ N	82.75	Antioxidant
N,N-Dimethylaminoethanol	4.544	C ₄ H ₁₁ No	85.81	Antioxidant
2,3-Butanediol	6.171	C ₄ H ₁₀ O ₂	84.28	Phytohormone and Antioxidant responses
Dimethyl trisulfide	12.094	C ₂ H ₆ S ₃	93.33	Antioxidant and Hepatoprotective effects
Pentane, 3-methyl-	14.426	C ₆ H ₁₄	81.17	Antioxidant and Antimicrobial
3H-1,2,4-Triazol-3-one, 1,2-dihydro-	15.293	C ₂ H ₃ N ₃ O	81.29	Antidepressant agents
p-Cresol	25.766	C ₇ H ₈ O	81.73	Antioxidant
3-Butylisobenzofuran-1(3H)-one	31.429	C ₁₂ H ₁₄ O ₂	80.66	Neuroprotective
Senkyunolide	32.914	C ₁₂ H ₁₆ O ₂	80.11	Antioxidant
n-Hexadecanoic acid	37.769	C ₁₆ H ₃₂ O ₂	80.43	Anti-inflammatory
Phytol	40.682	C ₂₀ H ₄₀ O	87.41	Antioxidant

The Total Phenolic and Flavonoid Contents of the Combined Extract

The phenolic and flavonoid contents in the extract of Celery and Chinese kale and the combined extract were shown in the Table 2. The results showed that total phenolic and flavonoid content of Celery extract was $111.60 \pm 2.05 \mu\text{gGAE}/\text{mg}$ extract and $19.04 \pm 0.59 \mu\text{g}$ quercetin/mg extract, respectively and Chinese kale extract was $135.35 \pm 4.27 \mu\text{gGAE}/\text{mg}$ extract and $18.92 \pm 0.47 \mu\text{g}$

quercetin/mg extract, respectively. The combined extract expressed at $162.37 \pm 0.88 \mu\text{gGAE}/\text{mg}$ extract and $20.45 \pm 0.73 \mu\text{g}$ quercetin/mg extract, respectively.

Interestingly, The combined extract significantly increased the total phenolic compound than celery extract ($p < 0.05$) but no difference of Chinese kale. However, the flavonoid content of the combined extract showed no significant changes when compared to extract alone.

Table 2: The total phenolic and Flavonoid contents in Celery extract, Chinese kale and combined extract.

Extract	Total Phenolic (g GAE/mg extract)	Flavonoid (g quercetin/mg extract)
Celery	111.60 ± 2.05	19.04 ± 0.59
Chinese kale	135.35 ± 4.27	18.92 ± 0.47
Combined extract	$162.37 \pm 0.88^*$	20.45 ± 0.73

Note: Data expressed as Mean \pm S.D. (N=4)

GAE: Gallic acid equivalent

*, **, ***: $p < 0.05, 0.01, 0.001$; compared to Celery

#, ##, ###: $p < 0.05, 0.01, 0.001$; compared to Chinese kale

The Effect on Antioxidant Activities

This study determined antioxidant activity by colorimetric tests using spectrophotometric techniques. In general, spectrophotometric techniques are simple, rapid and not expensive, which probably explains their widespread use in antioxidant screening. In addition, since this techniques rely on the reaction of a radical, radical cation or complex with an antioxidant molecule, the most common methods for the *in vitro* determination of antioxidant capacity employed ABTS and FRAP assay. The results as showed in Table 3 showed that IC_{50} values for FRAP and ABTS assay

of celery extract were $2.335 \pm 0.02 \text{mg}/\text{ml}$ and $1.128 \pm 0.031 \text{mg}/\text{ml}$, respectively and Chinese kale extract were 4.007 ± 0.04 and $16.951 \pm 0.14 \text{mg}/\text{ml}$, respectively.

Moreover, the combined extract showed the IC_{50} values of FRAP and ABTS assay at 1.591 ± 0.08 and $0.212 \pm 0.06 \text{mg}/\text{ml}$, respectively. Interestingly, The IC_{50} values of ABTS assay of the combined extract showed significantly lower than the extract alone ($p < 0.05$ compared to celery and $p < 0.05$ chinese kale). However, the IC_{50} values of FRAP showed no significant changes when compared to extract alone.

Table 3: IC_{50} values of Antioxidant activity including FRAP and ABTS of Celery extract, Chinese kale extract and combined extract.

Extract	IC_{50} FRAP (mg/ml)	IC_{50} ABTS (mg/ml)
Celery	2.335 ± 0.002	0.357 ± 0.01
Chinese kale	4.007 ± 0.002	16.951 ± 0.14
Combined extract	1.591 ± 0.08	$0.212 \pm 0.06^{*}\#$

Note: Data expressed as Mean \pm S.D. (N=4)

IC_{50} : concentration is inhibited by 50%

FRAP: Ferric Reducing Antioxidant Power

ABTS: 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt

*, **, ***: $p < 0.05, 0.01, 0.001$; compared to celery

#, ##, ###: $p < 0.05, 0.01, 0.001$; compared to Chinese kale

The Effect on Inhibition of Acetylcholinesterase Activity and Lipase Activity

The results were shown in the Table 4. The inhibition of lipase and AChE activity was expressed in IC_{50} value. The IC_{50} values for inhibition of lipase activity of the Celery extract, Chinese kale and combined extract were $0.079 \pm 0.007 \text{mg}/\text{ml}$, $1.01 \pm 0.06 \text{mg}/\text{ml}$ and $0.048 \pm 0.002 \text{mg}/\text{ml}$, respectively. In addition, the IC_{50} values for inhibition of AChE activity of the Celery extract, Chinese kale and combined extract were $1.35 \pm 0.06 \text{mg}/\text{ml}$, $1.35 \pm 0.06 \text{mg}/\text{ml}$ and $0.020 \pm 0.004 \text{mg}/\text{ml}$, respectively. Interestingly, the effect of the combined extract showed significantly higher mitigating AChE activity than extract alone ($p < 0.01$ compared to celery and $p < 0.01$ chinese kale) but no changes in the pancreatic lipase inhibition.

DISCUSSION

To date, numerous lines of evidence have demonstrated that the imbalance of oxidative stress homeostasis gives rise to the enhanced oxidative stress which plays the crucial role on metabolic syndrome related cognitive disorders [25,26]. Therefore many reports have demonstrated that the local indigenous vegetables which possessing antioxidant activity are reported to possess anti-metabolic syndrome and enhancing cognitive function [27].

All data obtained from *in vitro* study of this study indicated that combination of Celery extract and Chinese kale extract showed higher potential than the extract alone. According to traditional medicine, the polyherbal formulation or multiherb have been long-term used to provide more benefits in the management of various

ailments [28,29]. This was also in agreement with the present results which clearly demonstrated that the combined extract of Celery and Chinese kale provided optimum benefit concerning about antioxidant activities and capacity of the inhibited lipase and AChE activity more than extract alone. These effects may be occurred by the synergistic effect when chemical substances or

biological structures interact resulting in an overall effect and multiple components in the combined extract offer great potential for synergistic actions that increasing bioactive activities. In recent years, it has become evident that synergistic effects of herbal combinations can be achieved by acting on multiple targets and improving bioavailability in crude extracts [30].

Table 4: IC₅₀ values of inhibition of lipase and AChE activity in Celery extract, Chinese kale extract and combined extract.

Extract	IC ₅₀ of Inhibition of lipase activity (mg/ml)	IC ₅₀ of Inhibition of AChE activity (mg/ml)
Celery	0.079±0.007	1.35±0.06
Chinese kale extract	1.01±0.06	1.51±0.02
Combined extract	0.048±0.002	0.020±0.004***##

Note: Data expressed as Mean±S.D. (N=4)

IC₅₀: concentration is inhibited by 50%

*, **, ***: $p < 0.05, 0.01, 0.001$; compared to celery

#, ##, ###: $p < 0.05, 0.01, 0.001$; compared to Chinese kale

In addition, the dietary polyphenols have been shown to significantly alleviate several manifestations of metabolic syndrome [31,32]. Especially, phenolic compounds have some potential efficacy for preventing obesity by inhibiting the activity of enzymes related to fat metabolism as pancreatic lipase, lipoprotein lipase and glycerophosphate dehydrogenase [33,34]. Moreover, various phenolic acids and flavonoid derived plant have potential on lowering AChE activity [35]. According to the results, the combined extract presented Phytol, a bioactive compound, which is a member of the group of branched-chain unsaturated alcohols [36]. Phytol is the product of chlorophyll metabolism in plants and able to reduce the production of free radicals leading to its antioxidant properties [37]. Moreover, 3-n-Butylphthalide (NBP) or 3-butyloisobenzofuran-1(3H)-one was found in the combined extract. It was reported that it showed important role on potent neuroprotective effect. Peng et al. found that L-3-n-butylphthalide (L-NBP) attenuated learning and memory deficits induced by chronic cerebral hypoperfusion in rats [38]. The present study demonstrated that the combined extract has potential to suppress AChE activity which could relate the improving cognitive and learning and memory function. In addition, 2,3-Butanediol which was found in the combined extract contributed on phytohormone and antioxidant responses [39]. The phytohormone significantly downregulated lipid accumulation [40]. The current results suggested the extract could inhibit the lipase activity leading decreased lipid metabolism which intern reduces the absorption of dietary fat.

However, in order to be used in pharmacological aspects and the way interacts with human system needs to be further explored to recommend its safe, effective possible mechanism and widespread use of in medicine. Therefore, the further study including toxicity and in vivo study will be evaluated to confirm its effect.

CONCLUSION

The study suggested that synergistic effect of the combined extract which contained Celery and Chinese kale has more capability than using extract alone. The GC-MS study showed chemical compounds that reputed on various bioactive activity. The present bioactive compounds served as antioxidant activity leading to effectively reduce lipase enzyme and AChE neurotransmitter or might be directly affected of phytochemical compound on health benefits. Therefore, the synergistic effect could affect multitargets

that contributing on both beneficial effects of metabolic syndrome and cognitive impairment. However, the possible underlying mechanisms will explore in animal model. In addition to assure the consumption safety of novel food, the toxicity will also be investigated.

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