Effect of Heating Condition and pH on Stability of Total Phenolic Content and Antioxidant Activities of Samui (*Micromelum Minutum*) Extract

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Keywords: Temperature, pH, Total Phenolic Content, Antioxidant, Samui (*Micromelum Minutum*) Extract.

Abstract: Samui (*Micromelum minutum*) leaf, commonly consumed in southern Thailand has a high potential of antioxidant. In this study, this plant was extracted with three various solvents; water, ethanol (95%) and acetone (60%), freeze-dried and then, re-dissolved in water. The factorial design was applied to evaluate the effect of pH (ranged from 5-7) and heat condition (60-80°C) on the stability of bioactive activity. The total phenolic content (TPC), DPPH radical scavenging, ABTS radical scavenging, ferric ion reducing antioxidant power (FRAP) and antioxidation by TBARs method were determined. The result indicated the influence of the solvent. Acetone extract seemed to be more effective compared to ethanolic and aqueous extracts. In addition, pH and heat conditions significantly affected the stability of TPC and antioxidant capacities ($p \leq 0.05$). Generally, the TPC and antioxidant capacities were higher at higher pH. On the other hand, increasing heat conditions significantly deteriorated the TPC and antioxidant capacities. In conclusion, all studied factors influenced the stability of bioactive compounds and their activities which need to be considered when applying the extract into food.

1 INTRODUCTION

Plants are rich source of phytochemicals, for instance, phenolic compounds such as flavonoids, and tannins. All act as antioxidants which have been linked with several health benefits due to their medicinal properties and high nutritional value. Antioxidants also control, reduce or inhibit lipid oxidation caused by reactive oxygen species in foods, therefore, enhancing their shelf-life and quality (Cherkupally, et al, 2017; Altemimi, et al, 2017). Phytochemicals have been recognized their antioxidant potential which prevents oxidation of fat as well as consuming this type of vegetables may help prevent chronic non-communicable diseases (Thomas, et al, 2016).

Samui (*Micromelum minutum*) is a traditional vegetables commonly found in Southern Thailand. Apical bud and young leaves are popular in various main dishes as well as fresh consumption. It consists of several phytochemicals such as phenol, coumarin, alkaloids, and beta-sitosterol (Bunyapraphatsara, Chokchaijareunporn and Herbs, 2000; Areekul, 2552; van Valkenburg and Bunyapraphatsara, 2001). This coumarin had been reported it properties for inhibition of the cancer cells such as A549 (lung), ACHN (renal), H727 (lung), MCF-7 (breast) and HL-60 (leukemia) (Sakunpak, A., et al, 2013). This plant showed high potential sources of phenolic content and antioxidant compounds (Areekul and Promkraiwan, 2009; Friedman and Jurgens, 2000). In the previous study, Samui extract at a concentration of 500 ppm was effective in inhibiting the oxidation reaction in the water-based emulsion (Soto, et al, 2019).

The antioxidant efficiency of the extract can be changed through several factors including temperature and pH. Generally, heating causes an acceleration of the initiation reactions and hence decreases in the antioxidant activity. In addition, the pH also affects the decomposition of important substances, the stability of the phenolic compounds, and the antioxidant activity (Promkraiwan, 2009; Maisuthisakul, et al, 2007). In food production, it is a fact that food undergoes to the processing step including pH adjustment and heating process. Therefore, the understanding of the stability of plant extracts is necessary in order to apply them to food. The objective of this study was to evaluate the

Krungkri, W. and Areekul, V.
Effect of Heating Condition and pH on Stability of Total Phenolic Content and Antioxidant Activities of Samui (Micromelum minutum) Extract.
DOI: 10.5220/0009980800002964
In *Proceedings of the 16th ASEAN Food Conference (16th AFC 2019) - Outlook and Opportunities of Food Technology and Culinary for Tourism Industry*, pages 126-132
ISBN: 978-989-758-467-1

stability of phenolic compound and their antioxidant activity extracted from various solvents at different pH and heating conditions.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemical were purchased;Acetone (CH_3COCH_3 ;MacronFine Chemicals,USA), Methanol (Lab-scan, Ireland), Ethanol (Lab-scan, Ireland), 2,2-diphenyl-1-picrylhydrazyl (DPPH, $C_{18}H_{12}N_5O_6$; Aaldrich, USA), Folin–ciocalteu reagent (VWR, Prolabo, Ecuador), Sodium carbonate (Na_2CO_3; Ajax Finechem, Australia), Hydrochloric acid (HCl; J.T. Baker, USA), Gallic acid ($C_7H_6O_5$; Sigma-Aldrich, Germany), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, $C_{14}H_{18}O_4$; BBL, USA), Linoleic acid ($C_7H_6O_5$; Sigma-Aldrich, Germany), Thiobarbituric acid (Merck, Germany), Trichloro - acetic acid (Merck, Germany), 2,2'-Azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid)(Sigma,USA) and 2,4,6-Tripyridyl-s-triazine (TPTZ) (Sigma,USA)

2.1.2 Plant Materials

Samui (Surat Thani, Thailand) was purchased from local market in Surat-thani province Apical bud and young leave was selected, cleaned with water, and then dried in the hot air dryer at a temperature of 40°C until the final moisture content below 10% The sample was ground and sifted through a 40 mesh sieve. The powdered plant specimens was put into polyethylene bags (PE), vacuum sealed and stored at -20°C.

2.2 Preparation of Extracts

Three solvents were used to extract: water, ethanol (95%) and acetone (60%) The ground plant was weighed 5 gram and mixed with 100 ml of each solvent, stirred continuously with a temperature-controlled shaker (25 ± 2°C), at a speed of 200 rpm for 12 hours. The extract was then filtered with Whatman Filter No. 4. The filtrate was evaporated at a temperature of 35°C. After that, the freeze-drying was performed. The freeze dried sample was kept at -18°C.

2.3 Study of Heat Stability and pH

Thirty mg. of freeze-dried samples were re-dissolved in 30 ml DI water. Extract According to the method selected from Article 2.2 and then studied the effect of 3 levels of acidity, 5, 6 and 7 by dissolving 30 mg powder plant with DI 30 ml of water. And adjust the pH with a concentration of 0.3-0.4 molar acetate buffer at pH 6-7. Then divided into 3 parts, bringing 10 ml samples into the test tube Soak into the bath, After adjusting the pH, each sample was submerged into water-bath at three different conditions as following; 60, 70 and 80°C for 30, 15 and 3 min, respectively. After heat treatment, the sample was immediately cooled down in the ice-water and determined for all chemical analysis.

2.4 Chemical Analysis

2.4.1 Total Phenolic Content

Total phenolic contents of samples were determined by the Folin–Ciocalteu method (Shaghaghi, et al, 2008). Briefly, aliquots of 40 µl of samples and standards were mixed with 100 µl of deionized water, 20 µl of Folin–Ciocalteau reagent, and 40 µl of 10%sodium carbonate (Na_2CO_3). After incubation at room temperature for 30 min in the dark, the absorbance of the reaction mixture was measured at 765 nm against a deionized water blank by a microplate reader (Biochrom, EZ Read 2000). Using standard curve of Gallic acid solutions, the total phenolic contents of samples were determined in triplicates.

2.4.2 DPPH Radical-Scavenging Activity Assay

The radical-scavenging activity was determined by the DPPH method (Murakami, 2004). Briefly, aliquots of 50 µl of sample were mixed with 150 µl of 0.22M DPPH in ethanol (final concentration of 95%). The mixture was shaken vigorously and left to stand for 30 min at room temperature in the dark. Controls or blanks were prepared without the sample solution. The absorbance at 517 nm by DPPH was measured with a microplate reader (Biochrom, EZ Read 2000). Using standard curve of Trolox solutions, the DPPH in samples were calculated.

2.4.3 ABTS+ Radical Scavenging Activity

The radical-scavenging activity was determined by the $ABTS^+$ method (Zhou and Yu, 2004). Briefly, aliquots of 50 µl of sample solution were mixed with

100 µl of 5 M $ABTS^+$. The mixture was shaken vigorously and left to stand for 5 min at room temperature in the dark. Controls or blanks were prepared without the sample solution. The absorbance at 734 nm was measured with a microplate reader (Biochrom, EZ Read 2000). The $ABTS^+$ of samples were calculated using a standard curve of Trolox solutions, the $ABTS^+$ in samples were calculated.

2.4.4 Ferric Reducing/Antioxidant Power

The Ferric reducing/antioxidant power was determined (Benzie and Strain, 1996). Briefly, aliquots of 10 µl of sample solution were mixed with 300 µl of FRAP. The mixture was shaken vigorously and left to stand for 8 min at room temperature in the dark. The absorbance at 593 nm was measured with a microplate reader (Biochrom, EZ Read 2000). The FRAP of samples were calculated using a standard curve of Trolox solutions, the FRAP in samples were calculated.

2.4.5 Ant-Thiobarbituric Acid Reactive Substances

Anti-Thiobarbituric acid reactive substances was determined by the Anti-TBARs method (McDonald and Hultin, 1987). Briefly, aliquots of 0.2 mL of sample and standards were mixed with 0.8 ml of 1% linolenic acid, Leave in a water bath at a temperature of 50 ± 1°C for 18 hours. 2 mL of TCA-TBA-HCl solution to boil for 15 minutes, Rest to cool before spinning, at a speed of 5,500 rpm for 5 min, the absorbance at 520 nm by Anti-TBARs was measured with a microplate reader (Biochrom, EZ Read 2000). Using standard curve Butylated hydroxyanisole (BHA) solutions, the Anti-TBARs in samples were calculated.

2.5 Statistical Analysis

The results were expressed as the mean ± standard deviation (SD) calculated using Microsoft Excel. Data were analyzed for analysis of variance using SPSS program using a variance (ANOVA) followed by the two-tailed Duncan's multiple range test (DMRT). P-values less than 0.05 were considered significant ($p<0.05$).

3 RESULTS AND DISCUSSION

3.1 Total Phenolic Content

This experiment studied the stability of bioactive compounds extracted from three different solvents under the various pH and heating condition. The result is shown in Figure 1. TPCs in treated samples ranged between 36.22–78.82 mg GAE/g dry basis which significant lower compared with control (88.39 mg GAE/g dry basis). The was found that using different solvents affected the stability of extracted compounds. The higher TPC indicated the higher stability of phenolic compounds. For this experiment, the acetone extract provided the highest stability of phenolic compounds, followed by ethanol and water, respectively ($p\leq0.05$). Sweet potato leaf polyphenol had high retention under pH 5-7 and mind heat condition while increasing heating temperature and/or increasing acidity or alkalinity had a great impact on its stability (Sun, et al, 2017).

The effects of pH and heating condition also pronounced on the stability of phenolic content ($p\leq0.05$). However, the heating condition had higher effect. As increasing heating temperature, TPC retention is lower. Generally, heating causes an acceleration of the initiation reactions and hence decreases in the phenolic. High temperature and long-term heat treatment should be avoided (Evans, et al, 1996). The result showed that control had highest TPC indicating the presence of H+ and heat treatment may make the phenolic unstable. The hydrolyzation of phenolic acid under alkaline and strong acidic condition will induce the decrease of the number of phenolic (Medina, et al, 2007). From the result, the highest remaining TPC was found in the acetone extract at pH 7.0 with lowest heat condition.

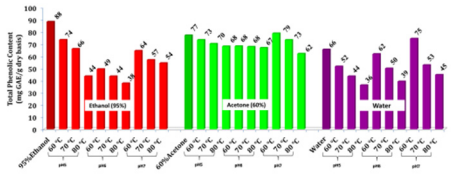

Figure 1: Effect of heating condition and pH on stability of total phenolic content of Samui extract.

3.2 DPPH Radical-Scavenging Activity Assay

The stability of Samui extracts from three various solvents on DPPH radical-scavenging activity is shown in Figure 2. DPPH values significantly decreased in all samples after treated with pH and

heating condition from 111.02 mg Trolox/g dry basis (control) to 42.12-91.56 mg Trolox/g dry basis ($p < 0.05$). This result was similar to TPC result where acetone extract had the highest stability followed by ethanol and water ($p<0.05$), respectively.

The effect of pH and heating condition also pronounced on the stability of plant extract ($p \leq 0.05$). However, the heating condition had higher impact compared with pH. As increasing heating temperature, DPPH retention was lower. This could be due to the decomposition of the antioxidant compound associated with the phenolic compounds. The lowest total phenolic content was attained under high heat (Sulaiman, 2017). The result showed that the control sample had highest DPPH indicating the presence of the antioxidant activity related to the number of phenolic hydroxyl and the electron-donating ability of molecules (Ruenroeng- klin, et al, 2008).

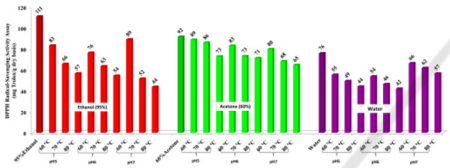

Figure 2: Effect of heating condition and pH on stability of DPPH radical-scavenging activity assay of Samui extract.

3.3 $ABTS^+$ Radical Scavenging Activity

Same as the result of TPC and DPPH, the control sample had the highest $ABTS^+$ and after treatment, all samples had significantly lower $ABTS^+$ (Table 1) except the ethanolic extract. At pH 7, the $ABTS^+$ of ethanolic extract (65.38-79.54 mg Trolox/g dry basis) was higher than that of control 61.77 mg Trolox/g dry basis. This may occur when some polyphenol from reaction of $ABTS^+$ radicals and the antioxidants added to the medium. Therefore, effective in the $ABTS^+$ radical scavenging activity (Huyut, et al, 2017).

In addition, the phytochemical stability of aqueous extract was the lowest. pH and heating condition also affected the stability of $ABTS^+$ ($p \leq 0.05$). However, as increasing heating temperature, $ABTS^+$ retention is lower resulting from high temperature influencing on the inhibition the process of hydrogen or electron donation (Sulaiman, 2017). The $ABTS^+$ radical is soluble in both aqueous and organic solvents. Thus, $ABTS^+$ method evaluates the antioxidant activity of both hydrophilic and lipophilic compounds from the process of hydrogen or electron donation (Aliakbarlu, et al, 2018). From the result, the highest remaining $ABTS^+$ was found in the ethanolic extract at pH 7.0 with lowest heat condition.

Table 1: Effect of heating condition and pH on stability of $ABTS^+$ radical scavenging activity of Samui (*Micromelum minutum*) extract.

Solvent	pH	Temp	ABTS mg Trolox/g dry basis
95%Ethanol			28.55 ± 0.17^{d}
	5	60	28.17 ± 1.28^{de}
		70	26.13 ± 0.11^{fg}
		80	23.63 ± 1.20^{ijk}
	6	60	25.78 ± 0.09^{fgh}
		70	24.14 ± 0.09^{hij}
		80	22.86 ± 0.90^{ikl}
	7	60	41.63 ± 0.50^{a}
		70	37.23 ± 1.43^{b}
		80	33.99 ± 0.31^{c}
60%Acetone			26.74 ± 0.44^{ef}
	5	60	24.19 ± 0.31^{hij}
		70	22.24 ± 0.12^{kl}
		80	20.46 ± 0.31^{m}
	6	60	25.47 ± 0.77^{fgh}
		70	23.65 ± 0.42^{ijk}
		80	21.68 ± 0.18^{lm}
	7	60	28.11 ± 1.64^{de}
		70	24.75 ± 2.02^{ghi}
		80	23.39 ± 0.41^{ijk}
Water			24.58 ± 0.29^{ghij}
	5	60	13.73 ± 1.09^{op}
		70	13.18 ± 0.73^{opq}
		80	10.93 ± 1.51^{q}
	6	60	14.82 ± 1.17^{no}
		70	13.81 ± 0.60^{op}
		80	11.90 ± 0.08^{qr}
	7	60	15.93 ± 0.64^{n}
		70	14.60 ± 0.51^{nop}
		80	12.95 ± 0.34^{pq}

Means with different superscript letters (a–z) in the same column differ significantly ($p< 0.05$).

3.4 Ferric Reducing/Antioxidant Power

The value of FRAP (Table 2) from ethanolic extract significantly decreased from 277.62 mg Trolox/g dry basis (control) to 103.32-233.78 mg Trolox/g dry basis ($p < 0.05$). The result from acetone and aqueous extract were similar. In addition, higher pH and higher heating condition affected their stabilities. The antioxidant potential of the extract was ascertained from FRAP assay based on their ability to reduce TPTZ-Fe^{3+} complex to TPTZ-Fe^{2+}. TPTZ-Fe^{2+} is an intensive blue color and can be monitored at 593 nm. Reducing power is associated with antioxidant

activity and may serve as a significant reflection of the antioxidant activity (Tinrat, 2016). From the result, the highest remaining FRAP was found in the water extract at pH 5.0 with the lowest heat condition.

Table 2: Effect of heating condition and pH on stability of Ferric reducing/antioxidant power of Samui (*Micromelum minutum*) extract.

Solvent	pH	Temp	FRAP mg Trolox/g dry basis
95%Ethanol			256.87±0.36^{c}
	5	60	241.88±0.83^{d}
		70	227.42±2.30^{e}
		80	205.66±1.51^{g}
	6	60	201.37±1.55^{gh}
		70	179.68±1.32^{jk}
		80	154.22±1.03^{m}
	7	60	179.58±1.17^{jk}
		70	129.62±1.48^{o}
		80	117.85±1.41^{p}
60%Acetone			274.43±2.28^{b}
	5	60	217.69±0.62^{f}
		70	178.25±1.82^{k}
		80	142.97±1.46^{n}
	6	60	198.35±1.55^{ghi}
		70	191.79±0.24^{hi}
		80	167.32±1.25^{l}
	7	60	222.70±0.87^{ef}
		70	199.54±1.19^{gh}
		80	153.42±0.87^{m}
Water			299.47±0.86^{a}
	5	60	272.58±1.02^{b}
		70	230.76±1.02^{e}
		80	199.13±1.30^{gh}
	6	60	205.80±1.02^{gh}
		70	188.94±1.24^{ij}
		80	152.78±0.81^{m}
	7	60	173.82±0.84^{kl}
		70	127.26±0.84^{o}
		80	104.16±0.55^{q}

Means with different superscript letters (a–z) in the same column differ significantly ($p< 0.05$).

3.5 Ant-Thiobarbituric Acid Reactive Substances

The values of ant-TBARs radical scavenging activity are shown in Table 3. Ant-TBARs decreased in all samples after treated with pH and heating condition. However, it was noted that acetone extract had less stability when exposed to pH and heat treatment which is not similar to the result of TPC and other antioxidant capacities. Ant-TBARs follow lipid oxidation by product analysis as produce from the propagation of lipid oxidation such as hydrogenperoxide compound and radical of hydrocarbon that reacts with thiobarbituric acid and form to complex compound (pink to red of color). If extraction leads to high of Ant-TBARs, it is well inhibit lipid oxidation (Maisuthisakul, et al, 2007). The highest remaining Ant-TBARs was found in the water extract at pH 7.0 with lowest heat condition.

Table 3: Effect of heating condition and pH on stability of Ant-Thiobarbituric acid reactive substances of Samui (*Micromelum minutum*) extract.

Sovent	pH	Temp	Ant-TBARs mg BHT/g dry basis
95%Ethanol			1127.61±0.77^{b}
	5	60	838.91±1.55^{def}
		70	759.62±0.44^{fgh}
		80	719.31±1.99^{h}
	6	60	873.02±1.55^{de}
		70	812.97±1.17^{efg}
		80	744.29±1.33^{gh}
	7	60	1252.00±2.63^{a}
		70	1216.50±0.92^{a}
		80	1054.08±2.63^{bc}
60%Acetone			1001.72±0.39^{c}
	5	60	469.10±1.11^{lmn}
		70	417.79±1.69^{mn}
		80	409.42±0.87^{n}
	6	60	500.85±1.37^{klm}
		70	477.32±1.61^{lmn}
		80	521.14±8.99^{jkl}
	7	60	627.16±0.65^{i}
		70	598.17±2.83^{ij}
		80	555.29±0.74^{ijkl}
Water			1008.22±0.39^{c}
	5	60	556.54±0.64^{ijkl}
		70	546.19±1.06^{ijkl}
		80	528.23±0.87^{jkl}
	6	60	592.01±1.06^{ij}
		70	577.95±0.73^{ijk}
		80	555.85±1.75^{ijkl}
	7	60	915.58±1.43^{d}
		70	832.36±1.64^{ef}
		80	803.48±1.14^{efg}

Means with different superscript letters (a–z) in the same column differ significantly ($p< 0.05$).

4 CONCLUSIONS

In conclusion, the extraction solvent affected on the stability of Samui (*Micromelum minutum*) extracts. Acetone extract seem to be more stability in TPC and antioxidant capacities except of TBARs when

compared to ethanolic and aqueous extracts. Additionally, pH and heat conditions significantly affected the stability of TPC and antioxidant capacities ($p \leq 0.05$). Generally, TPC and antioxidant capacities were generally higher at higher pH. On the other hand, the increasing heat condition significantly deteriorated the TPC and antioxidant capacities. All factors influenced the stability of bioactive compounds and their activities which need to be considered when applying the extract into food.

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