Original Article

ฤทธิ์ต้านออกซิเดชันของสารสกัดถั่วเหลืองจากการสกัดแบบดั้งเดิม

Antioxidant Activities of The Soybean Extracts Obtained by Classical Extraction

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บทคัดย่อ

คนไทยส่วนใหญ่บริโภคสารสกัดถั่วเหลือง ซึ่งมักเตรียมโดยการสกัดด้วยน้ำต้มเดือดเป็นประจำ แต่ยังไม่มีรายงานผลการศึกษาทางด้านสารอาหารที่เป็นองค์ประกอบ การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบความสามารถในการเป็นสารต้านอนุมูลอิสระและหาปริมาณของไอโซฟลาโวนในสารสกัดถั่วเหลืองที่ได้จากการสกัดแบบดั้งเดิมนี้ จากการวิเคราะห์สารสกัดถั่วเหลืองด้วยวิธี HPLC พบว่ามีไดเซอีนและเจนิสเทอีนเป็นองค์ประกอบหลัก ศึกษาการทดสอบฤทธิ์ต้านอนุมูลอิสระของสารสกัดถั่วเหลืองด้วยวิธี DPPH และ ABTS รวมทั้งวิเคราะห์หาปริมาณสารประกอบฟีนอลและหาปริมาณไอโซฟลาโวนทั้งสองด้วย Folin-Ciocalteau และ HPLC จากวิธี DPPH assay พบว่าไดเซอีนและเจนิสเทอีนมีค่า IC_{50} เท่ากับ 0.41 และ 0.39 mg/ml ตามลำดับ เปรียบเทียบกับสารมาตรฐาน trolox ซึ่งมีค่า IC_{50} เท่ากับ 0.28 mg/ml เมื่อทดสอบด้วยวิธี ABTS assay ทั้งไดเซอีนและเจนิสเทอีนมีความสามารถต้านอนุมูลอิสระ $ABTS^{\bullet+}$ ด้วยค่า IC_{50} เท่ากับ 0.55 และ 0.53 mg/ml ตามลำดับและแรงกว่า trolox (IC_{50} = 0.88 mg/ml) จากการหาปริมาณสารประกอบฟีนอลในสารสกัดถั่วเหลืองพบว่าปริมาณไดเซอีนและเจนิสเทอีนเป็นสัดส่วนประมาณครึ่งหนึ่งของปริมาณสารประกอบฟีนอลทั้งหมด ซึ่งจากผลการทดลองนี้สามารถใช้เป็นข้อมูลในการนำสารสกัดถั่วเหลืองที่ได้จากการสกัดแบบดั้งเดิมเป็นสารต้านอนุมูลอิสระจากธรรมชาติได้

คำสำคัญ: สารสกัดถั่วเหลือง ไดเซอีน เจนิสเทอีน การสกัดแบบดั้งเดิม ฤทธิ์ต้านอนุมูลอิสระ

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Abstract

Many Thai people consume soybean extracts obtained by classical boiled water extraction daily. However, there is no scientific data on their composition of phytonutrients. The present study aims to assess the antioxidant power and composition of isoflavones in soybean extracts from classical extraction. HPLC analyses of the extracts showed daidzein and genistein as the major components. The antioxidant activities of the soybean extracts were evaluated by free radical scavenging (DPPH and ABTS) assays. Total phenolic and isoflavone contents were also determined by using Folin-Ciocalteau reagent and developed HPLC methods, respectively. The IC_{50} values of daidzein, genistein and reference standard, trolox, in DPPH radical scavenging assay were 0.41, 0.39 and 0.28 mg/ml, respectively. Daidzein and genistein were also found to scavenge the $ABTS^{\bullet+}$ with IC_{50} values of 0.55 and 0.53 mg/ml, respectively, and the activities were higher than trolox (IC_{50} = 0.88 mg/ml). Measurement of total phenolic contents of the soybean extracts showed that daidzein and genistein account for about half part of total phenolics in soybean extracts. These results obtained in this study clearly indicate that the soybean extracts obtained by classical extraction has a significant potential to use as a natural antioxidant agent.

Keywords: soybean extracts, daidzein, genistein, classical method, antioxidant activities
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Introduction

Antioxidants play important roles in the scavenging of free radicals and chain breaking of the oxidation reactions. The prevention of oxidative stress related diseases in the human body and the inhibition of oxidative reactions in foods, pharmaceutical and cosmetic products are some of the useful potential functions of antioxidants. The two commonly used synthetic antioxidant, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been restricted because of their toxicity and DNA damage induction (Thompson and Moldeus, 1988). Due to these limitations, natural antioxidants from plant extracts have been widely interested due to their safety.

Soybeans are one of the most produced commodities worldwide. They have attracted increasingly attention owing to their nutritional and health-related beneficial aspects. Soy isoflavones, daidzein and genistein, are phytochemicals of prominent interest for some of these beneficial health effects (Rostagno et al., 2002; Rostagno et al., 2005; Georgetti et al., 2008). Isoflavones are a subclass of flavonoids and also called phytoestrogens due to their weak estrogenic activity with potential protective effect against some hormone related diseases (Rostagno et al., 2007; Penalvo et al., 2004). Several studies have shown that soy isoflavones play an important role in the reduction of cardiovascular disease risk as well as prevention of several hormonally influenced cancers, menopausal symptoms and osteoporosis. Earlier studies indicated that soy isoflavones, especially genistein, have the antiphotocarcinogenic properties and can also significantly decrease UV-induced cutaneous erythrema and skin ulceration in human skin (Kim et al., 2004; Draelos, 2007; Chiu and Kimball, 2003). Furthermore, isoflavones in soymilk can reduce hair growth and hair follicle dimensions (Seiberg et al., 2001). These findings have encouraged soy isoflavones as possible topical alternative agent and surge of interest from the cosmetic industry. Their ability

to act as antioxidants may also serve to prevent oxidative damage in living tissue, since they have hydroxyl groups in rings A and/or B, and are thus capable of donating hydrogen to free radicals (Lazarus and Baumann, 2001; Rostagno et al., 2004; Liggins et al., 1998; Dixon and Ferreira, 2002). However, the degradation of the phenolic substances, including daidzein and genistein, are degraded when exposed to high temperature.

Generally, consumption of soybean extract as soymilk in Thailand was prepared by using classical extraction techniques at 100°C for 1 hour. Thus, the aim of this work was to evaluate the effect of high temperature during preparation of soymilk on free radical-scavenging activity, polyphenolic and isoflavone contents of the aqueous extracts. According to literature data, this is probably the first study where the total phenolic content and radical- scavenging activities of daidzein and genistein from soybean extracts obtained by classical extraction were compared.

Materials and methods

1. Plant materials and chemicals

Seeds of Glycine max (L.) Merr. were acquired from a local producer in Nonthaburi, Thailand (Rhai-Thip Co. Ltd). Two isoflavone standards, genistein and daidzein, were purchased from Sigma-Aldrich (St.Louis, MO). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). 1,1-diphenyl 2-picryl hydrazyl (DPPH), 6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (trolox), 2,2,-Azino-bis(3-ethylbenzothizoline-6-sulfonic acid, diammonium salt (ABTS) and Folin-Ciocalteu's phenol reagent were purchased from Sigma, Germany.

2. Preparation of soybean extract by using classical extraction

Soybeans were macerated in water for 8 hours and then blended with water (soybeans 1 g / water 12 ml). The crude extract was filtered and boiled for 1 hour. Water was removed by using freeze drying prior to storage at -20°C. The dried extract was used for the assessment of antioxidant activity. The isoflavones content of the extracts were determined by using high performance liquid chromatrography in comparison to standards genistein and daidzein.

3. Determination of soy isoflavone contents by using developed high performance liquid Chromatography (HPLC)

The developed HPLC method for quantification of genistein and daidzein has been validated and shown to be reliable, accurate, precise, and linear. The validity of the method has met the requirement of AOAC guidelines (Sobharaksha et al., 2011-in press). Therefore it can be used as an accurate routine procedure for the determination of soy isoflavone contents in soybean extracts with short retention times (13.8 and 14.5 minutes respectively) and simple.

A Finnigan modular LC system with a Model P4000 dual pump equipped with a Rheodyne 7725i injector linked to a 20 µl loop and a Model UV 6000 photodiode array detector was used for analysis by liquid chromatography. A Phenominex C_{18} column (250 x 4.6 mm I.D., particle size 10 µm) was used for chromatographic separations. The chromatographic data were obtained by a PC system, and software ChromQuest from Thermo Fisher Scientific was used to acquire and process the data. Gradient elution was needed for complete separation of the analysis. The mobile phase consisted of two eluents: (A) 0.1% acetic acid in deionized water and (B) 0.1% acetic acid in methanol. The analyses were preformed under gradient elution conditions. The system was maintained at 50% B for 5 minutes with the flow rate of 1 ml/min, then, increased to 80% in 5 minutes with the flow rate of 0.5 ml/min and held at 80% for another 7 minutes with the flow rate of 1 ml/min. At

the end, the system was set to increase solvent B from 80% to 100% within 2 minutes, holding these conditions for 11 minutes and then returned to the original condition, 50% B, for 10 minutes. Total run was time 40 minutes including 10 minutes stabilization time. The chromatographic analysis was performed at an ambient temperature and detection wavelength set to 254 nm. Injection of 20 µl was effected with a SGE Analytical Science 100 µl syringe.

4. Determination of total phenolic contents

Total phenols in the soybean extracts were determined by Folin-Ciocalteau reagent using gallic acid as a standard (Kerget et al., 2005). 100 µl solution of soybean extract was added to 500 µl of Folin-Ciocalteau reagent. After 10 minutes, 400 µl of 7.5% Na_2CO_3 was added and the mixture was incubated at 50°C for 5 minutes. The absorbance of the blue color that developed was read at 760 nm. The content of total phenols was expressed as gallic acid equivalents (GAE) in mg per g extract. All analyses were run in triplicate and mean values were calculated. The calibration equation of gallic acid was $y = 10.043x - 0.1724$ ($R^2 = 0.999$).

5. Determination of antioxidant activities

5.1 Free radical-scavenging activity of soybean extract using DPPH (1,1-diphenyl 2-picryl hydrazyl)

The free radical-scavenging activity of the soybean extract was measured in terms of hydrogen donating on radical-scavenging ability using the stable radical DPPH (Brand-Williams et al., 1995). 0.20 mM solution of DPPH in ethanol was prepared and 950 µl of this solution was added to 50 µl of extract solution in water at different concentrations. The mixture was shaken vigorously and was allowed to stand for 60 minutes at room temperature. The absorbance of the resulting solution was measured at 517 nm. The inhibition percentage of free radical by sample was calculated using the following formula: % inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$, where $A_{control}$ was the absorbance of the control (blank, without extract) and Asample was the absorbance in the presence of the extract.

All the tests were performed in triplicate and the graph was plotted with the mean values. Radical-scavenging ability was calculated as IC_{50} (concentration causing 50% inhibition) and expressed as trolox equivalent antioxidant capacity (TEAC) in mg trolox per g extract as follows:

$TEAC\ (mg\ trolox/g) = (IC_{50(trolox)} / IC_{50(sample)}) \times 10^3$

The IC_{50} of trolox used for calculation of TEAC was 0.28 mg/ml.

5.2 ABTS cation radical scavenging assay

The antioxidant activity was measured by 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid free radical cation ($ABTS^+$) decolorization assay. The stock solution of 7 mM ABTS and 140 mM potassium persulfate ($K_2S_2O_8$) were mixed for 16-18 hours in dark and low temperature. The solution was diluted with ethanol to give 0.7±0.05 absorbance at 750 nm. The soybean extract was dissolved in ethanol to give an appropriate concentration. An aliquot of 20 µl of each ethanolic extract solution was added to 980 µl of $ABTS^+$ radical cation solution. The mixture was shaken vigorously and the absorbance was measured at 750 nm. The radical scavenging assay was calculated using the following formula: % Inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$, where $A_{control}$ was the absorbance of the control (blank, without extract) and A_{sample} was the absorbance in the presence of the extract.

All the tests were performed in triplicate and the graph was plotted with the mean values. Radical-scavenging ability was calculated as IC_{50}

and expressed as trolox equivalent antioxidant capacity (TEAC) in mg trolox per g extract as follows: TEAC (mg trolox/g) = ($IC_{50(trolox)}$ / $IC_{50(sample)}$) x 103. The IC_{50} of trolox used for calculation of TEAC was 0.88 mg/ml.

Results and discussion

1. The content of total phenols

It is considered that the phenolic compounds contributed to overall antioxidant activities of soybean extract. Total phenolics content of soybean extract was assessed using the Folin-Ciocalteu reagent, which is a mixture of phosphomolybdate and phosphotungstate. In one gram of soybean extract, the amounts of isoflavones, daidzein and genistein, in soybean extract, were 1.28±1.24 and 1.33±1.67 mg per g extract, respectively and 4.79±0.43 mg gallic acid equivalent per g extract of phenols was detected. The results indicated that both isoflavones account for about half part of total phenolics in soybean extracts. It is accepted as an indication of antioxidant potential because they act in soybean extracts as antioxidants.

2. Inhibition of DPPH radical

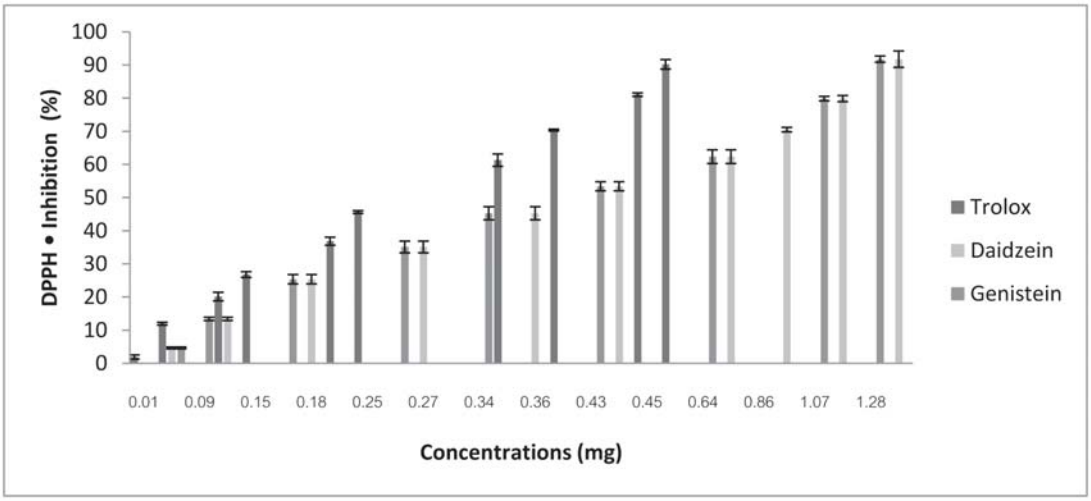
The antioxidant activity of the soybean extracts were evaluated by the hydrogen-donor ability to DPPH· method. The DPPH radical is considered to be a model for a lipophillic radical. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products. DPPH· is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Liu and Yao, 2007). The reduction capability of DPPH· is determined by the decrease in its absorbance at 517 nm induced by antioxidants. Now DPPH· has been widely used in assessment of radical scavenging activity because of its ease and convenience.

The scavenging effect of soybean extracts and trolox on DPPH radical was compared on the bases of their concentrations providing 50% inhibition (IC_{50}). A higher DPPH· radical-scavenging activity is associated with a lower IC50 value. The results showed that the DPPH activity of soybean extracts was found to increase in dose dependent manner (Figure 1). The main phenolic substances, daidzein and genistein, in the soybean extracts at the used concentrations displayed a noticeable effect on DPPH· radical-scavenging activity. The IC_{50} values were found to be 0.30 g and 0.28 mg for soybean extract and trolox, respectively (Table 1). The amount of daidzein and genistein in the soybean extract, which providing potential effect of DPPH· activity as 50% of free radicals inhibition, were quantified by the HPLC assay with the values of 0.41 and 0.39 mg, respectively (Figure 2). Isoflavones in the soybean extracts showed the similar scavenging activity to trolox in the range of 0.05-0.44 mg, respectively. Antioxidant activities of the extract and isoflavones were shown in the terms of TEAC values as in Table 2. Since TEAC is a measurement of the effective antioxidant activity of the extract, a higher TEAC would imply greater antioxidant activity of the sample. Thus, the results indicated that both isoflavones had relatively high TEAC and genistein had the antioxidant potential greater than

Table 1 Effect of soybean extracts concentration on different radical-scavenging activities.

Soybean (g)	Soybean extract (g)	Daidzein (mg)	Genistein (mg)	DPPH$^{\bullet}$ Inhibition (%)	ABTS$^{\bullet+}$ Inhibition (%)
0.18	0.03	0.05	0.05	4.65 ± 0.27	-
0.36	0.06	0.10	0.09	13.39 ± 0.5	-
0.78	0.13	0.18	0.17	25.35 ± 1.41	6.46 ± 0.79
1.20	0.20	0.27	0.26	35.08 ± 1.78	15.81 ± 0.92
1.56	0.26	0.36	0.34	45.24 ± 1.97	29.45 ± 0.93
1.98	0.33	0.44	0.43	53.41 ± 1.38	44.87 ± 1.64
3.00	0.50	0.67	0.64	62.32 ± 2.06	55.15 ± 1.34
3.96	0.66	0.90	0.86	70.50 ± 0.67	68.36 ± 1.75
4.98	0.83	1.11	1.07	79.84 ± 0.93	82.96 ± 1.83
6.00	1.00	1.33	1.28	91.76 ± 2.47	95.41 ± 1.24

Values are given as mean ± SD of three replicates.



daidzein.

Figure 1 Free radical scavenging activity of daidzein and genistein, compare to trolox, by DPPH method. (Results are means ±SD of three replicates).

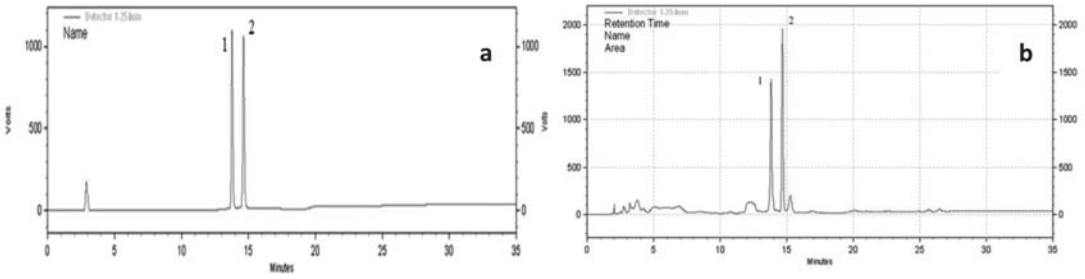


Figure 2 HPLC chromatograms of isoflavones, genistein (1) and daidzein (2), standard mixture (a) and isoflavones in the soybean extracts (b).

3. ABTS radical scavenging

The ABTS assay was employed to measure the antioxidant activity of the soybean extract because it does not require sophisticated analytical equipment and provides a good estimate of the antioxidant activity of pure compounds and complex matrices (Antolovich, 2002). The soybean extract displayed antioxidant activities as it was able to scavenge the $ABTS^{\bullet+}$ radical cation. The soybean extract had a scavenging activity on $ABTS^{\bullet+}$ radicals in a dose dependent manner (0.03-1 g). Nonetheless, when compared to trolox, the ABTS radical scavenging effect of the extract was found to be low. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract.

As shown in Figure 3, the scavenging power of all the soybean extracts increased with increasing amount of sample. The IC_{50} values were found to be 0.41 g and 0.88 mg for soybean extracts and trolox, respectively (Table 1). The concentration of daidzein and genistein in the soybean extract, needed for 50% inhibition, was quantified by the HPLC assay and found to be 0.55 and 0.53 mg, respectively. The scavenging effect of the extract and isoflavones were determined in the terms of TEAC values as in Table 2. It is interesting to find that daidzein and genistein had relatively high TEAC and exhibited antioxidant potential greater than trolox.

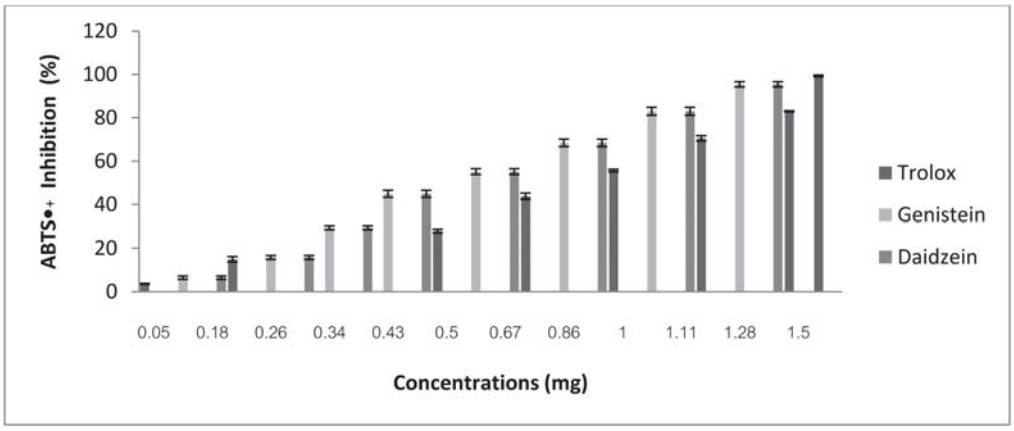


Figure 3 Free radical scavenging activity of genistein and daidzein, compare to trolox, by ABTS method. (Results are means ± SD of three replicates).

Table 2 Antioxidant activities of the soybean extracts and its major components (daidzein and genistein) in DPPH and ABTS radical scavenging activities.

Sample	DPPH assay		ABTS assay	
	IC_{50} (mg)	TEAC	IC_{50} (mg)	TEAC
Soybean extract	301.00	0.93	414.80	2.12
Daidzein	0.41	682.90	0.55	1,600.00
Genistein	0.39	717.95	0.53	1,660.30

TEAC = Trolox equivalent antioxidant capacity

IC_{50} = concentration causing 50% inhibition

Conclusion

The total phenolic contents and radical-scavenging activities of daidzein and genistein from soybean extracts, which were prepared by using classical extraction techniques at high temperature, were compared. The results showed that the soybean extracts still contained high level of total phenolic compounds, including daidzein and genistein, and were capable of inhibiting free radicals to terminate the radical chain reaction. Furthermore, a significant and linear relationship was found between the antioxidant activities and phenolic contents, indicating that phenolic compounds could be major contributors to antioxidant activity. The main phenolic compounds, daidzein and genistein, in soybean extracts showed strong antioxidant activity by inhibiting $ABTS^{\bullet+}$ stronger than $DPPH^{\bullet}$ when compared with standard trolox. Therefore, the soybean extracts obtained by classical extraction can be used as an easily accessible source of natural antioxidants with consequent health benefits.

References

Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. *Analyst* 2002; 127: 183-198.

Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft und-Technologie* 1995; 28: 25-30.

Chiu A, Kimball AB. Topical vitamins, minerals and botanical ingredients as modulators of environmental and chronological skin damage. *Br J Dermatol* 2003; 149: 681-691.

Dixon RA, Ferreira D. Molecules of interest: Genistein. *Phytochemistry* 2002; 60: 205-211.

Draelos ZD. Skin lightening preparations and the hydroquinone controversy. *Dermatol Ther* 2007; 20: 308-313.

Georgetti SR, Casagrande R, Souza CRF, Oliveira WP, Fonseca MJV. Spray dry of the soybean extract: effects on chemical properties and antioxidant activity. *LWT-Food* Science and Technology 2008; 41: 1521-1527.

Kerget MS, Kotnik P, Hadolin M, Hras AR, Simonic M, Knez Z. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem* 2005; 89: 191-198.

Kim S, Lee J, Kim W, Park W, Sim Y, Lee S. Protective effects of dietary soy isoflavones against UV-induced skin-aging in hairless mouse model. *J Am Coll Nutr* 2004; 23(2): 157-162.

Lazarus MC, Baumann IS. The use of cosmeceutical moisturizers. *Dermatol Ther* 2001; 14: 2003-2007.

Liggins J, Bluck LJC, Coward WA, Bingham SA. Extraction and quantification of daidzein and genistein in food. *Anal Biochem* 1998; 264: - 17.

Liu Q, Yao H. Antioxidant activities of barley seeds extracts. *Food Chem* 2007; 102: 732-737.

Peñalvo JL, Nurmi T, Adlercreutz H. A simplified HPLC method for total isoflavones in soy products. *Food Chem* 2004; 87: 297-305.

Rostagno MA, Araujo JMA, Sandi D, Supercritical fluid extraction of isoflavones from soybean flour, *Food Chem* 2002; 78: 111-117.

Rostagno MA, Palma M, Barroso CG. Pressurized liquid extraction of isoflavones from soybeans. *Anal Chim Acta* 2004; 522: 169-177.

Rostagno MA, Palma M, Barroso CG. Solid-phase extraction of isoflavones. *J Chromatogr A* 2005; 1076: 110-117.

Rostagno MA, Palma M, Barroso CG. Microwave assisted extraction of soy isoflavones. *Anal Chim Acta* 2007; 588: 274-282.

Seiberg M, Liu JC, Babiarz L, Sharlow E, Shapiro S. Soymilk reduces hair growth and hair follicle. *Exp Dermatol* 2001; 10: 405-413.

Sobharaksha P, Indranupakorn P, Luangtana-anan M. Determination of isoflavones in soybeans under various extraction methods by developed high performance liquid chromatography. *Thai J Hosp Pharm* 2011; 21(1). Article in press.

Thompson D, Moldeus P. Cytotoxicity of butylated hydroxyanisole and butylated hydroxytoluene in isolated rat hepatocytes. *Biochem Pharmacol* 1988; 37(11): 2201-2207.