THE INVOLVEMENT OF ANTIOXIDANT AND SUN PROTECTION FACTORS

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1. The involvement of antioxidant and sun protection factors

The aim of this work was to study the effectiveness of different levels of antioxidant on sun protection factors (SPF). The optimal extraction condition based on antioxidant activity and sun protection factor was estimated using the factorial design. The relationship and their interaction of the extraction factors affacting antioxidant activity, was represented as a model equation. The model equation was derived from the experimental data provided the most fit which high coefficient of determination (higher than 0.96). The most significant factor of the extraction based on antioxidant activity and sun protection factor was the percentage of ethanol (extraction solvent). In addition, this work demonstrated the weakness of linear relationship between antioxidant activity and SPF values by Pearson Correlation Coefficient of 0.078. The lowest effective concentration at 50% (EC_{50}) was at 7.20 µg/mL. The highest sun protection factor of the *A.catechu* sunscreen gel was at 14.02 $\pm$ 0.02, without chemical sunscreen ingredients. Eventually, the results represented that *A.catechu* was applicable for being a great candidate for applying in sunscreen products.

2. Keywords: Factorial design, antioxidant, sun protection factor, *Acacia catechu* (L.f.) Willd

3. Introduction

A.catechu is a medicinal plant belonging to the Fabaceae family which is also called pea family or legume family (Hashmat et a., 2013, lPatel et.al., 2009). *A.catechu* is widely distributed in Asia. This plant has several pharmacological properties. The heartwood of *A.catechu* is used in melancholia, conjuctivits, haemaptysis, caterrah, cough, prusitue, leprosy, leukoderma, skin diseases, helminthiasis, norexia, diarrhea, dysentery, foul ulcers and wounds, haemoptysis, haematemsis, haemorrages, fever, anaemia, diabetes and pharyngodynia (Devi et.al., 2010). The chief constituents of the heartwood *A.catechu* are catechin and catechu tannic acid along with small proportions of brown coloring matter. It also contains tannin, flavotannin, gallotannin, phloratannin, etc. As the *A.catechu* contain many potent flavonoids having great anti-oxidant property (Hashmat et.al., 2013 & Hazra et.al., 2010). (+)-Catechin, is a substance, which diminish or arrest the action of some hormones (Azed et.al., 2001). The property of catechin in human skin is supposed to be the active ingredient for the treatment of leukoderma. Catechins are a biologically important flavonoids. Catechins from heartwood of *A.catechu* also have been used in both supplements and prescription medical food. According to the major components from natural sources, especially flavonoids such as catechins, this plant may have a potential for the treatment and prevention of UV-mediated diseases such as sunburn and skin cancer. However, there are no scientific reports available to clearly reveal the effectiveness of the different levels of antioxidant activity on SPF. While, statistical experiment design (ED), full factorial design can reveal the involvement of each factor of the extraction on its sun protection factor.

The ultimate goals of this research are the optimization the procedure to extract of flavonoids from *A.catechu* based on SPF values using a two-level factorial design and establishing a relationship between antioxidant and the SPF values.

4. Review of literature

A.catechu is a small to moderate sized plant widely distributed throughout Asia deciduous. It is a thorny tree which grows up to 15 m (50 ft) in height. It is utilized with betel leaves for chewing. Catechu (or cutch), a hot water extract of red heartwood of *A.catechu* is brown material with bitter taste. There are several medicinal values. The aqueous extract of *A.catechu* has a significant usefulness on both cell mediated and humoral immunity (Ismail et.al., 2009). Moreover, the aqueous by virtue of its antioxidant might be useful in the treatment of cancer (Patil et.al., 2003). Catechins from heartwood of *Acacia catechu* (L.f.) also has been used in both supplements and prescription medical food. A supplemental 90-day oral toxicity study was conducted in Hsd: SD rats to determine the potential of UP446 to produce toxicity. The results reveal that a dose of 1,000 mg/kg/day was identified as no-observed-adverse-effect-level (Yiman et.al., 2010).

The main chemical constituent of *A. catechu* are catechin, epecatechin, epigallocatechin, epicatechingallate, phloroglucin, protocatechuic acid, quarcetin, poriferasterol glycosides, lupenone, procyanidin, kaemferol, L-arabinose, D-galactose, D-rhamnose andaldobiuronic acid, afzelchin gum ,mineral and taxifolin (Hashmat et.al., 2013). Catechin: biologically highly active. It is used as a haemostatic agent. (Azed et.al., 2001), epicatechin, epicatechin-3-*O*-gallate, flavonol dimmers and flavonol glycosides (Shen et.al., 2006). Flavonoids are secondary plant metabolites in various vegetables, fruits and herbal plants. Flavonoids show important pharmacological activities such as anti-allergy, antimicrobial activities (Al-Oqail et.al., 2012; Dua, Garg & Mahajan, 2013), anti-inflammatory, anti-viral, anti-microbial activities (Chirumbolo, 2010), anti-cancer (Nam et.al., 2016, Chidambara, Kim, Vikram, & Patil, 2012; Xie et.al., 2012), and antioxidant (Masuoka et.al., 2012 Brunetti et.al., 2013). Additionally, antioxidants from natural sources are found to be potential for the treatment and prevention of UV-mediated diseases. 4-Nerolidylcathecol, the major antioxidant of *Pothomorphe umbellata* root extract, is also stable when exposed to UV-B irradiation. The alpha-tocopherol preservation and photoprotective effects of the *Pothomorphe umbellata* are due to its antioxidant activity (Siliva et.al., 2005). Catechins are also a group of important sunscreen from natural sources (Lee et.al., 2008). Thus, an increasing interest in the use of natural sources in sunscreens to provide supplemental photoprotective action and the activity is ongoing to discover products that can increase the sun protection factor (SPF) and stability. Sun exposure to human skin or solar ultraviolet (UV) radiation may cause several skin damages. These damages include sunburn, skin cancer, oxidative stress as well as photoaging depending on the amount and type of the UV rediation and the individual exposure. The global warming and ozone depletion are also the factor for irritating mankind. While, natural sources may provide the possibilities for the treatment and prevention of UV-mediated diseases. Additionally, the systematic study using statistical experimental design (ED), a two-factorial design would be used to estimate the effects of all interactions for the extraction, and the analysis of variance would reveal the significant of antioxidant activity on sun protection properties. Therefore, a two-level factorial design and the analysis of variance was employed to evaluate the best procedure of the extraction based on sun protection properties and antioxidant activity.

5. Materials and methods

5.1 Experimental design for extraction

A full factorial design was employed for the screening of significant factors and their interaction affecting total flavonoids and *in vitro* sun protection factor. Five variables (*i.e*. the concentration of the extracting agent (percentage of ethanol, EtOH) (X_1), the extracting temperature (X_2), the material ratios (the weight of *Acacia catechu*: volume of the extracting agent) (X_3), the extracting time (X_4) and number of the extraction (X_5) that have an impact on the extraction of flavonoids were evaluated. A two-level factorial design was employed using upper and lower limits: percentage of 95% EtOH (X_1, 70 and 100 %), the extraction temperature (X_2, 60 and 70 °C), the material ratios (X_3, 1:10 and 1:15), the extracting time (X_4, 8 and 12 hours) and number of the extraction (X_5, 2 and 4 times). The key responses to determine the optimal conditions are the percentage of total flavonoids in the plant extracts, and *in vitro* sun protection factor. The condition that led to the maximization of the responses will be desirable. Additionally, A 2^5 (32 experiments) factorial design was performed randomly (n = 3). The interaction between variables was studied and the model equations were derived from the experimental data using the Design Expert (6.0.5) program. The dried chips of red heartwood of *A.catechu* were immersed under the extracting agent and extracted as the conditions from the experimental design. Each extracting process was material extracted, filtered, evaporated, and the residue was investigated its antioxidant and the sun protection factors. The involvement and the condition that led to the best property was considerable.

5.2 Determination of antioxidant activity

For 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) assay, an aliquot of samples will be mixed with 4.5 mL DPPH reagent (0.006 mM of DPPH redical in methanol) and the final volume was adjusted to 5.0 ml with water. After 10 minutes incubation, the disappearance of DPPH radical colour upon radical reduction was monitered by measuring the absorbance at 517 nm using UV/VIS spectrophotometer with deionized water in reagent as a control blank. The percentage of the remaining DPPH radical was calculated and plotted against sample and represented the results as the effective concentration at 50 % (EC_{50}).

5.3 Formulations

Gel formulations containing sample extracts with final concentration of 0.1% (w/w) and 1.5% (w/w) of carbomer 940 were prepared by neutralizing and dispersion of carbomer with triethanolamine under constant stirring. The hydrogels were kept at room temperature (Siliva V.V.da *et.al*. 2005). Then, the extracts were weighted and small amounts of the gel formulation were added under constant stirring. The gel was once more neutralized with trietanolamine under constant stirring. The final pH of the formulation was 5.0.

5.4 Determination of the *in vitro* sun protection factor

The gel formulations were dissolved in methanol UVsolv: water (6:4). Scans of the samples in solution were run from 320 to 290 nm. Ten commercial sunscreens, were used for calculation of the correction factor (CF). Sunscreen ingredients, (v/v) diluted to 0.2 μl/ml were used as standards. Equations were proposed by Mansur et.al (Siliva V.V.da et.al.,2005) and used to calculate the SPF.

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times abs(\lambda) \quad \ldots\ldots\ldots\ldots(1)$$

Where CF is correction factor, determined by ten commercial sunscreens with known SPF; EE (λ) the erythemal efficiency spectrum; *I* (λ) the solar simulator spectrum as measured with a calibrated spectrometer; $\sum_{290}^{320} EE(\lambda) \times I(\lambda)$ = 290-320 nm in 5 nm increments; abs (λ) is the spectrometer measure of the sunscreen absorbance. Table 1 shows the normalized values of the product function used in these studies.

Table 1. The normalized product function used in the calculation of SPF data

λ (nm)	EE×*I* (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
	=1.000

EE: erythemal efficiency spectrum; *I*: solar simulator intensity spectrum
(Sayre et al., 1979).

6. Results

6.1 Determination of antioxidant activity

Table 2 summarizes the antioxidant activity of each extracts. All values are means of three replicated determinations. The lowest and highest amounts of effective concentration at 50 % (EC_{50}) from the extracts were 7.20 and 13.41, respectively. The results revealed that higher antioxidant activity (about 2-fold) were obtained from the different extraction conditions.

6.2 Determination of the *in vitro* sun protection factor

Ten commercial sunscreens were calculated the correction factor using equation 1 and presented in Table 2. The final average correction factor was 39.60. The average correction factor was used to calculate SPF values. SPF values of each extract at the different extraction are represented in Table 3. The highest and lowest SPF values of the *Acacia catechu* (L.f.) Willd extract gels were 3.97 ± 0.02 and 14.02 ± 0.02, respectively. As results, 3.5-fold improvement in SPF values were obtained under the different extraction conditions. Moreover, the SPF values from *A.catechu* were higher than the SPF values from *P.umbellate* reported by Silva et.al., 2005 (Silva et.al., 2005) reported that SPF value of *P.umbellate* extract gel was 3.35 ± 0.02 and the SPF value of the isolated 4-NC was 4.00 ± 0.59, suggesting the presence of additional compounds with sunscreen activity in the extract.

6.3 Statistical analysis

The responses from the experimental design were analyzed in randomized order to transform systematic error into random error. Multiway factorial ANOVA showed that the measured and predicted values were in good agreement and no outlier was identified. In a model with significant *p*-value ($p < 0.05$), a high regression coefficient demonstrates significant effect of independent variables on the corresponding responses.

The model equations derived from the experimental data that gave the most fit for each response are shown as the following.

(EC_{50}) = $+ 10.04 - 0.026 X_1 - 0.13 X_2 - 0.25 X_3 - 0.32 X_4 - 0.34 X_5$ ……………………………..……(2)
$- 0.23 X_1 X_2 - 0.41 X_1 X_3 + 1.01 X_1 X_4 - 0.18 X_1 X_5 + 0.35 X_2 X_3 - 0.34 X_2 X_4 - 0.19 X_2 X_5$
$- 0.073 X_3 X_4 + 0.33 X_3 X_5 + 0.097 X_4 X_5 + 0.059 X_1 X_2 X_3 - 0.62 X_1 X_2 X_3 + 0.29 X_1 X_2 X_5$
$- 0.049 X_1 X_3 X_5 - 0.23 X_1 X_4 X_5 + 0.020 BCD + 0.57 BCE + 0.57 BDE - 0.42 CDE + 0.69 ABCE$
(*p*-value = 0.0204, coefficients of determination = 0.9584)

SPF = $+7.49 - 1.28 X_1 + 0.43 X_2 - 0.59 X_3 + 0.061 X_4 + 0.43 X_1 X_2 + 0.086 X_1 X_3$ ……(3)
$- 0.50 X_2 X_3 - 0.36 X_2 X_4 + 0.32 X_3 X_4 + 0.63 X_2 X_3 X_4 - 0.013 X_1 X_2 X_3 X_5 + 0.79 X_1 X_2 X_4 X_5$
$- 0.76 X_1 X_2 X_3 X_4 X_5$
(*p*-value < 0.0001, coefficients of determination = 0.8405)

Total flavonoid contents and *in vitro* SPF values from the extractions under full factorial design are summarized in Table 3. In addition, Pearson correlation coefficient of 0.078 revealed the weakness of a linear relationship between antioxidant activity and SPF values. According to the effect estimated values (the coefficients of the equation), the most significant factor for antioxidant activity and SPF was the percentage of ethanol.

Table 2 EC_{50} and SPF of each extract from full factorial design experiments

No.	X_1 (%)	X_2 (°C)	X_3 (mL/g)	X_4 (h.)	X_5	EC_{50} (µg/mL)	SPF
1	100	70	10	12	2	10.31	5.07 ± 0.00
2	100	60	15	8	4	7.20	3.97 ± 0.02
3	100	70	10	8	4	7.71	6.41 ± 0.02
4	100	60	15	12	2	13.17	5.31 ± 0.01
5	100	60	10	8	2	8.94	4.16 ± 0.01
6	100	60	10	12	4	13.41	5.58 ± 0.02
7	100	60	15	12	4	8.21	5.71 ± 0.00
8	100	60	10	12	2	13.23	6.56 ± 0.01
9	100	70	15	8	4	10.71	5.10 ± 0.02
10	100	60	15	8	2	8.64	4.97 ± 0.01
11	100	70	15	12	4	10.33	6.83 ± 0.01
12	100	60	10	8	4	10.14	6.51 ± 0.00
13	100	70	15	12	2	8.68	7.23 ± 0.02
14	70	60	10	8	4	11.82	6.93 ± 0.00
15	70	60	15	8	4	12.71	8.42 ± 0.02
16	100	70	10	12	4	8.20	8.01 ± 0.01

No.	X_1 (%)	X_2 (°C)	X_3 (mL/g)	X_4 (h.)	X_5	EC_{50} (μg/mL)	SPF
17	70	70	15	8	4	11.37	6.23 ± 0.01
18	70	60	10	8	2	11.71	8.05 ± 0.01
19	70	70	10	12	4	9.91	6.78 ± 0.05
20	70	70	15	12	4	9.09	8.57 ± 0.02
21	70	70	15	8	2	12.92	6.93 ± 0.01
22	70	70	15	12	2	9.07	7.28 ± 0.01
23	100	70	15	8	2	7.89	6.49 ± 0.01
24	70	60	10	12	2	8.81	8.07 ± 0.01
25	70	60	15	12	2	7.97	7.98 ± 0.02
26	70	60	15	12	4	8.60	9.33 ± 0.00
27	70	70	10	8	4	7.73	14.02 ± 0.02
28	100	70	10	8	2	13.41	11.40 ± 0.02
29	70	60	15	8	2	10.04	10.05 ± 0.01
30	70	60	10	12	4	8.01	11.33 ± 0.01
31	70	70	10	8	2	12.84	9.23 ± 0.02
32	70	70	10	12	2	8.41	11.20 ± 0.01

X_1 = percentage of ethanol, EtOH), X_2 = the extraction temperature, X_3 = volume of the extracting solvent per 1 g of Acacia catechu, X_4 = the extracting time and X_5 = number of the extraction

6. Disscussion

For the sake of simplicity, the effects of individual main factors and observed trends are summarized in Table 4. For instance, antioxidant activity can be improved by increasing the percentage of ethanol, volumes of the extracting solvent, and decreasing the extracting time, the extraction temperature and number of the extraction. Sun protection factors can be improved by increasing the percentage of ethanol, volumes of the extracting solvent, number of the extraction and decreasing the extraction temperature and the extracting time. In addition, significant factors of both responses at $P \leq 0.05$ were observed as the percentage of ethanol and the extraction temperature under the model equation. The significant factors provided the same trends of both responses. Our results are similar to that reported by Ebrahimzadeh et.al., 2014. Ebrahimzadeh et.al.,(2014) no correlation was found between antioxidant activity and SPF. However, the optimal conditions for the extraction based on the responses could be considered under the model equations from this work.

Table Summary of factor effects on the key responses

	X_1 (%)	X_2 (°C)	X_3 (mL/g)	X_4 (h.)	X_5
Increasing of SPF	↑	↓	↑	↓	↑
Decreasing of EC_{50}	↑	↓	↑	↓	↓

X_1 = percentage of ethanol, EtOH), X_2 = the extraction temperature, X_3 = volume of the extracting solvent per gram of Acacia catechu, X_4 = the extracting time and X_5 = number of the extraction

7. Conclusion

This current study is one of Thai National Research with emphasis on the standardization and quality control of natural products. Additionally, this is a first report on the analysis of the extraction effects as the predicted model equations for the extraction on antioxidant activity and SPF values. The use of factorial design for the development of the extraction is powerful as it enables full control of the effects of the design variables. Results from the factorial design experiment indicated that the percentage of ethanol (the solvent for the extraction) and the extraction temperature were the main affecting factors and their interactions were significant of antioxidant activity and sun protection property. Additionally, the *in vitro* SPF test of this work confirmed the efficacy of *A.catechu* sunscreen. Thus, *A.catechu* can further improve for being a promising sunscreen product.

8. Biography

Oraphan Anurukvorakun was born in Phang-Nga Province, Thailand**.** She received the B.Sc. degree in Chemistry from Rajabhat Institute Suratthani, Thailand, in 1999, the M.Sc. degree in Pharmaceutical chemistry and Phytochemistry, Mahidol University, Thailand in 2004 and the Ph.D.degree in Pharmaceutical chemistry and Phytochemistry, Mahidol University, Thailand in 2008. She is currently a lecturer at the Department of Cosmetic Science, Faculty of Science and Technology, Phranakorn Rajabhat University. Her main areas of research interest are natural products, analytical techniques such as capillary electrophoresis, high performance liquid chromatography, mass spectrometry and hyphenated techniques.

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