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Evaluation of Ultrasonically Assisted Extraction of the Thai Polyherbal Formulation, Mahajak on Antioxidation and Anti-Inflammation Activities

Adisak Thomudtha[1], Pornpun Laovachirasuwan[2], Prasob-orn Rinthong[2]*

[1]*Ph.D. candidate in Doctor of Philosophy in Pharmacy Program, Faculty of Pharmacy, Mahasarakham University, Maha Sarakham, 44150, Thailand*
[2]*Pharmaceutical Chemistry and Natural Product Research Unit, Faculty of Pharmacy, Mahasarakham University, Maha Sarakham, 44150, Thailand*

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ABSTRACT

Mahajak polyherbal formula is a traditional oil preparation described in the official Thai medicinal textbook. The present study compared biological activities of Mahajak which was prepared by a traditional method and ultrasonically assisted extraction (UAE). The Mahajak samples obtained from four preparative methods including traditional preparation (TP), UAE with sonication time for 10, 20 and 30 minutes (UAE10, UAE20 and UAE30) were determined for *in vitro* antioxidant and anti-inflammatory activities. GC-MS and principal component analysis were performed to compare their chemical profiles. Results demonstrated the UAE samples did not show difference in the antioxidant activities from that of the TP samples. However, the UAE technique yielded the significance of higher effectiveness on the anti-inflammatory activities (p<0.05) as UAE10 and UAE20 showed the most potent anti-inflammatory activity (IC_{50} of 63.12 ± 4.12 and 62.37 ± 2.82 µg/mL, respectively) as compared to TP (IC_{50} = 74.27 ± 3.54 µg/mL). The GC-MS fingerprints of all UAE samples presented similar GC-MS profiles to that of TP. Ten major chemical components of Mahajak formula were identified and their contents were higher in the UAE samples. In conclusion, the results of this study demonstrated the impact of UAE on Mahajak formula production. The UAE technique improved the percentage yielding and anti-inflammatory activity of Mahajak preparations but not the effect on the chemical components.

***Keywords*:** Ultrasonic, Principal component analysis, Cluster analysis, Herbal medicine extraction, Aromatization.

Introduction

Mahajak polyherbal formula is a traditional oil preparation described in the official Thai medicinal textbook, "King Narai's Medicinal Formulas" since Ayutthaya period of the Thai Kingdom.[1,2] It was topically applied for skin pruritus, muscle pain and wound. This formulation consists of nine herbal ingredients including sesame oil, kaffir lime (*Citrus hystrix* DC., Rutaceae), cumin (*Cuminum cyminum* L., Apiaceae), fennel (*Foeniculum vulgare* Miller subsp. var. *vulgare*, Apiaceae), garden cress (*Lepidium sativum* L., Brassicaceae), black cumin (*Nigella sativa* L., Ranunculaceae), dill (*Anethum graveolens* L., Apiaceae), long pepper (*Piper retrofractum* Vahl., Piperaceae) and camphor. The preparative method was a combination between deep-fried and infused aromatization techniques.[3] At present, Mahajak is a popular topical formulation used by Thai traditional practitioners to relieve pain and to reduce skin inflammation.[4] Previous research publications have supported antioxidant and anti-inflammatory properties of the herbal ingredients of Mahajak.[5-14] However, a production process of Mahajak is time-consuming, hence considered a bottleneck in the supply chain.

At present, ultrasonically assisted extraction (UAE) is widely employed for the extraction of biologically active compounds in aromatized vegetable oil.[15,16] By applying this technique, processing time of herbal extraction was reduced and the extract yielded high phytochemical contents.[17,18] The UAE allowed for production of high-quality oil with fast and cost-effective technology. The present study aimed to compare biological activities of Mahajak between the traditional preparative method and UAE application. The *in vitro* antioxidant and anti-inflammatoryeffects of Mahajak samples were investigated. The GC-MS fingerprints and cluster analysis were performed to characterize and compare their phytochemical profiles.

*Corresponding author. E mail: prasoborn.r@msu.ac.th
Tel: +668 1872 2737

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Materials and Methods

Plant materials

Herbal constituents of Mahajak formula, sesame oil, dried kaffir lime peels, cumin, fennel, garden cress seeds, black cumin, dill, long pepper and camphor were purchased from herbal stores in Bangkok, Thailand (December, 2020) and were authenticated by an expert in Pharmacognosy (Bhanubong Bongcheewin) from Faculty of Pharmacy, Mahidol University, Thailand. All voucher specimens were deposited at the Herbarium of Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Thailand (Table 1).

Preparative methods of Mahajak samples

In this study, Mahajak samples were prepared following a traditional preparative method and UAE. Cumin, fennel, garden cress seed, black cumin, dill and long pepper were blended into powder and sieved through mesh No. 14. For the traditional preparative method, 926 mL sesame oil was heated, and added with 480 g kaffir lime peels to fry until scorched. The liquid part was collected and infused for aromatization with each of 7.5 g cumin, fennel, garden cress seed, black cumin and dill, 15 g long pepper and 30 g camphor, respectively for 15 minuites.[3] The preparation was filtered through sterile gauze to obtain Mahajak traditional preparation (TP).

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For the UAE preparation, 480 g kaffir lime peels, each of 7.5 g cumin, fennel, garden cress seed, black cumin and dill, 15 g long pepper and 30 g camphor were mixed thoroughly and added with 926 mL sesame oil. Ultrasonic technique was applied at a frequency of 25 kHz and a speed of 1,000 rpm. The extraction was filtered through sterile gauze and collected for 3 time periods; 10, 20 and 30 minutes as the UAE samples (UAE 10, UAE 20 and UAE 30, respectively).

Antioxidant activity determination

Antioxidant activities of Mahajak samples were evaluated using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging tests. Each sample was prepared in the form of solution with concentrations ranging from 3. 125to 100 µg/mL, using 10% DMSO in absolute ethanol. The DPPH assay was performed following Amid *et al*.[19] The ABTS radical scavenging activity was determined according to the protocol described by Payet *et al*.[20] Triplicate experiments were performed and ascorbic acid was used as a positive standard. The inhibitory percentages on both DPPH and ABTS radicals of Mahajak samples were calculated and results were expressed as IC_{50}.

Anti-inflammatory activity and cell viability determination

The *in vitro* anti-inflammatory activity and cell viability tests were performed on the murine macrophage-like RAW 264.7 cell line model. The anti-inflammatory activities of Mahajak samples were determined on the nitric oxide production model.[21] The RAW 264.7 cells were obtained from an expert (Rujiluk Rattarom) from Faculty of Pharmacy, Mahasarakham University, Thailand. Briefly, the murine RAW 264.7 cells were cultured in RPMI-1640 medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin (100 µg/mL), streptomycin (100 µg/mL), and 10% FBS. The cells were harvested with trypsin-EDTA, then were diluted to suspend in a fresh medium before plating for experiments. The cells were seeded in a 96-well plate with $1x10^5$ cells/well and allowed to adhere for 1 hour. The medium was replaced with a fresh medium containing 5 µg/mL of lipopolysaccharide (LPS), together with testing samples (at concentration of 100, 50, 20, 10, and 1 µg/mL). After an incubation for 24 hours, the nitric oxide production was determined by the measurement of accumulation of nitrite in the culture supernatant using the Griess reagent. Absorbance of the resultant solution was measured with a microtitre plate reader at 550 nm. Percentage inhibition was calculated and IC_{50} values were determined graphically (n = 4).

RAW 264.7 cells viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) colourimetric method. After 24-hour incubation with test samples, 10 µL MTT solution (5 mg/mL in PBS) was added to the wells. After 4-hour incubation, the medium was removed and isopropanol containing 0.04 M HCl was added to dissolve the formazan produced by the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds or extract were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. Indomethacin was used as a positive control.

GC-MS analysis

GC-MS was analyzed using a quartz capillary column. The heating program used an initial temperature of 40°C, increasing to 280°C at a rate of 5°C/min which was maintained for 60 minutes until the analysis was completed. The carrier gas was helium, the inlet temperature was 240°C, the split ratio was 40:1, and the injection volume was 10 L. Mass spectrometry conditions included a standard electron ionization (IE) source (70eV), an ion source temperature of 180°C, and an interface temperature of 240°C. The quadrupole mass analyzer had a scan range of 20 –700 amu and a scan speed of 4.0 scans/sec.

Principal component analysis and cluster analysis

The GC-MS data on Mahajak samples were subjected to a principal component analysis and cluster analysis, using STATA, version 14.0 software (College Station, TX) to evaluate similarity in the chemical components.

Statistical analysis

The results were shown in terms of mean ± standard deviation (SD). Statistical significance of the results was determined using analysis of variance (ANOVA), followed by the studentized range test. A confidence limit of $p<0.05$ was fixed for interpretation of the results using PRISM, version 2.01 software (San Diego, CA).

Results and Discussion

Yielding percentages

All obtained Mahajak samples had brownish yellow color. Table 2 shows yielding percentages of Mahajak samples. The UAE 20 and UAE 30 had a significantly higher yield than that of TP and UAE 10 ($p<0.05$).

Antioxidant, anti-inflammatory and cytotoxicity activities

Table 3 shows antioxidant, anti-inflammatory and cytotoxicity of Mahajak samples. The IC_{50} of Mahajak samples were in the range of 90.38-99.42 µg/mL when determined using DPPH and ABTS tests. The antioxidant activities of TP samples did not show the difference from those obtained from the UAE preparation methods ($p>0.05$). Ascobic acid was performed the potent antioxidant properties than that of all mahajak samples.

The anti-inflammatory activity was determined by LPS-induced inflammatory assays. The UAE 10 and UAE 20 samples showed the highest anti-inflammation activity when compared to TP and UAE 30 samples ($p<0.05$) with IC_{50} of 63.12 ± 4.12 and 62.37 ± 2.82 µg/mL, rspectively. Indomethacin, however, exhibited a higher potency on antioxidant properties than that of all Mahajak samples. For cell viability, determined by the MTT assay, the percentage of cell survival treated with Mahajak samples at concentration 100 µg/mL was higher than 90%.

Table 1: Herbal constituents of Mahajak formula

Herbal constituents of Mahajak formula			Part of use	Voucher specimen No.
Kaffir lime	*Citrus hystrix* DC	Rutaceae	Peels	PBM 005278
Cumin	*Cuminum cyminum* L.	Apiaceae	Seeds	PBM 005273
Fennel	*Foeniculum vulgare* Mill subsp.	Apiaceae	Seeds	PBM 005274
Garden cress	*Lepidium sativum* L.	Brassicaceae	Seeds	PBM 005275
Black cumin	*Nigella sativa* L.	Ranunculaceae	Seeds	PBM 005276
Dill	*Anethum graveolus* L.	Apiaceae	Seeds	PBM 005277
Long pepper	*Piper retrofractum* Vahl.	Piperaceae	Fruits	PBM 005278

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Table 2: Yielding percentages of Mahajak samples

Mahajak samples	% Yield
TP	27.6 ± 2.54[a]
UAE 10	29.6 ± 1.18[a]
UAE 20	36.9 ± 1.41[*b]
UAE 30	38.3 ± 1.90[*b]

[*] Statistically significant difference as compared to TP ($p < 0.05$).
[a,b] Statistically significant difference ($p < 0.05$).

GC-MS fingerprints

GC-MS analysis characterized 41, 41, 33, and 29 phytochemical components of TP, UAE 10, UAE 20 and UAE 30, respectively (Table 4). The components identified were mainly ketones, alkenes, benzenes and aldehydes, accounting for more than 97 % of the chromatographic area. The major components identified in the TP samples were beta-pinene (0.51-1.19 %), grandlure III (1.08-1.62 %), D-camphor (51.99-81.28%), palmitic acid (0.42-1.55%), cis-9-hexadecenal (5.70-11.65%), octadecanoic acid (0.56-1.56%), oxypeucedanin (1.10-1.85 %), gamma-tocopherol (0.3-1.54 %), sesamin (1.77-4.10 %) and gamma-sitosterol (0.40-1.54%). The GC chromatographic areas of these ten major compounds increased when the UAE technique was applied to Mahajak preparation.

Principal component analysis

Ten major components of Mahajak samples were characterized using principal component analysis. Tables 5 and 6 show the cumulative contribution of the first and second principal components (prin1 and prin2) attained to 94.35%. This contribution basically reflected all original variables were selected as the effective components from data analysis. Prin1 occupied 77.62% of total variation and it was negatively correlated with palmitic acid, cis-9-hexadecenal and octadecanoic acid and positively correlated with beta-pinene, grandlure III, D-camphor, oxypeucedanin, gamma-tocopherol, sesamin and gamma-sitosterol. Three of the relative contents of palmitic acid, cis-9-hexadecenal and octadecanoic acid reduced while the relative contents of beta-pinene, grandlure III, D-camphor, oxypeucedanin, gamma-tocopherol, sesamin and gamma-sitosterol increased when all UAE-applied Mahajak samples were compared to TP samples.

Prin2 was negatively correlated with the content of D-camphor and oxypeucedanin and positively correlated with the content of beta-pinene, grandlure III, palmitic acid, cis-9-hexadecenal, octadecanoic acid, gamma-tocopherol, sesamin and gamma-sitosterol. As compared to the UAE-applied Mahajak samples, it had a lower relative content of D-camphor and oxypeucedanin and had a higher relative content of beta-pinene, grandlure III, palmitic acid, octadecanoic acid, gamma-tocopherol, sesamin and gamma-sitosterol.

Table 3: Antioxidant, anti-inflammatory and cytotoxicity activities of Mahajak samples

Samples	IC_{50} (µg/mL)			% Cell viability
	DPPH	ABTS	LPS-induced NO	(at concentration 100 µg/mL)
TP	92.08 ± 2.57[*]	98.71 ± 0.49[*]	74.27 ± 3.54[*a]	95.04 ± 3.08
UAE 10	93.06 ± 0.85[*]	93.72 ± 5.04[*]	63.12 ± 4.12[*b]	97.37 ± 2.20
UAE 20	90.38 ± 3.86[*]	92.71 ± 2.21[*]	62.37 ± 2.82[*b]	95.36 ± 4.82
UAE 30	95.78 ± 2.89[*]	99.42 ± 0.71[*]	69.89 ± 1.40[*a]	95.62 ± 1.64
Ascorbic acid	22.99 ± 0.52	19.53 ± 2.76	-	-
Indomethacin	-	-	37.24 ± 1.53	98.34 ± 3.53

[*]Statistically significant difference when compared to ascorbic acid or indomethacin ($p < 0.05$). [a,b] Statistically significant difference within the column ($p < 0.05$).

Table 4: GC-MS fingerprints of Mahajak samples

Retention time (min)	Compounds	% Peak areas			
		TP	UAE 10	UAE 20	UAE 30
5.24	Alpha-pinene	0.06	0.06	0.05	0.05
5.85	Sabinene	0.51	0.55	0.39	0.59
5.92	Beta-pinene	0.51	0.81	0.62	0.83
6.71	D-limonene	0.47	0.4	0.31	0.41
7.17	Gamma-terpinene		0.05	0.07	
7.32	Trans-sabinene hydrate acetate		0.17	0.13	0.17
7.68	Fenchone	0.05	0.08	0.08	0.07
7.8	Linalool		0.15	0.18	0.15
7.85	Cis-2-norbornanol	0.1	0.12	0.12	0.13
7.93	Grandlure III	1.08	1.57	1.6	1.56
7.99	Isofenchol	0.13	0.19	0.17	0.19
8.15	Camphor	0.11	0.16	0.1	0.12
8.57	D-camphor	51.99	71.42	77.31	78.73
8.75	Borneol	0.14	0.18	0.2	0.2
8.88	Endo-borneol	0.37	0.48	0.53	0.6
9.03	Terpinene 4-acetate		0.07	0.13	0.07

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Retention time (min)	Compounds	% Peak areas			
		TP	UAE 10	UAE 20	UAE 30
9.1	[(Z)-(5,5,6-trimethyl-2-bicyclo[2.2.1]heptanylidene)amino]thiourea		0.03	0.04	
9.23	Terpineol	0.1	0.27	0.3	0.28
9.32	Endo-isocamphone	0.31	0.45	0.52	0.51
9.73	Beta-citronellol		0.12		
11.86	Copaene	0.08	0.04	0.04	0.07
12.037	Beta-cubebene		0.05		0.07
12.04	Germacrene D	0.11			
12.48	Caryophyllene	0.06	0.06	0.07	0.08
13.26	Tau-cadinol acetate	0.07		0.06	0.08
13.73	D-cadinene	0.09	0.07	0.06	0.07
14.07	Elemol	0.06			
15.61	Heptadecane	0.28			
16.64	N-octadecane	0.11			
17.61	N-nonadecane	0.09			
17.87	Methyl hexadecanoate	0.16			
18.17	Palmitic acid	1.55	0.79	0.62	0.49
18.25	3-methyl-5-ethyl-4-propylidene-cyclohex-2-ene		0.23		
18.4	Ethyl hexadecanoate		0.22		
19.2	Methyl (9Z,11E)-octadeca-9,11-dienoate	0.12			
19.23	Methyl oleate	0.14	0.15	0.09	
19.51	Cis-9-hexadecenal	11.65	9.9	6.88	5.7
19.66	Octadecanoic acid	1.56	1.1	0.78	0.61
20.49	Octadecane (CAS) n-octadecane	0.05	0.03		
20.68	2,2,4,4,7,7-hexamethyl-1,3,3a,7a-tetrahydroindene		0.06	0.05	
21.01	(Z)-octadec-9-enamide	0.26	0.29	0.42	
21.09	Icosane	0.1	0.12		
21.66	Nonacosane	0.15	0.05		
21.92	Oxypeucedanin	0.88	1.57	1.3	1.85
22.27	Tetratriacontane	0.08			
22.74	Butoxy-cyclohexyl-dimethylsilane	0.14			
22.84	2-monolinolein	0.2			
23.13	Citronellyl palmitate		0.12		
23.78	Pentadecane, 8-hexyl- (CAS) 8-n-hexylpentadecane	0.03			
25.53	Piperidine		0.26		
27.07	Gamma-tocopherol	0.3	1.54	0.54	0.38
29.27	Sesamin	1.77	4.1	3.89	3.95
32.63	Gamma-sitosterol	0.4	1.54	1.35	1.07

Cluster analysis

Figure 1 shows similarity of chemical profiles of Mahajak samples based on a hierarchical analysis. The dendogram indicated that the data set could be divided into three groups (Fig 1). The UAE 20 shows high similarity with UAE 30 samples with the lowest average distance while the UAE 10 and TP samples were separated.

Mahajak, a Thai traditional medicine described in an official textbook.[22] has been used in Thailand until the present. This study aimed to apply the UAE, a modern extraction technology on Mahajak production and evaluate its properties. The UAE improves diffusion process of the extraction solvent through facilitating swelling and

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hydration on the pores of plant cell wall, hence, permits the penetration of extraction solvent into cell greater than that by conventional methods and provides the effectively releasing intracellular product of the plant.[23] Previous paper showed the UAE exhibited a pronounced extraction yield of lycopene from red grapefruit (*Citrus paradise* Macf.) and reduced extraction time within 30 minutes.[24] Even though the present study did not show the UAE Mahajak samples to be different in the antioxidant activities as compared with the TP samples, it demonstrated the UAE technique contributed a significantly higher effectiveness on the anti-inflammatory activitites, where UAE 10 and UAE 20 exhibited the most potent anti-inflammatory activity. The UAE technique had an additional advantage in that it reduced production time as compared to the TP. The UAE 20 and UAE 30 samples demonstrated a high percentage of yielding as ultrasonicated time was a key factor on Mahajak UAE production. Intensification of the extraction process using ultrasound has been attributed to the cavitation phenomena leading to high shear forces in the media. Ultrasound also exerts the mechanical effect that has a strong impact on the solid surface, increases solvent penetration into cell and increases the contact surface area between the solid and liquid phases.[25] Based on the obtained data, ultrasonicated time for 20 minutes with ultrasonic power of 200 W was an optimal choice for Mahajak UAE preparation.

Concerning the phytochemical constituents of Mahajak formula, the GC-MS fingerprints presented a similar profile between all UAE and TP samples. Ten major compounds of Mahajak formula were identified and their contents were higher in the UAE samples. In addition, principal component analysis showed both positive and negative correlations of these major compounds

Table 5: Principal component analysis of ten major compounds of Mahajak samples

Compounds	Peak areas				Eigen vectors	
	TP	UAE 10	UAE 20	UAE 30	Prin1	Prin2
Beta-pinene	0.51	0.81	0.62	0.83	0.3022	0.1850
Grandlure III	1.08	1.57	1.6	1.56	0.3504	0.0677
D-camphor	51.99	71.42	77.31	78.73	0.3512	-0.1270
Palmitic acid	1.55	0.79	0.62	0.49	-0.3539	0.1208
Cis-9-hexadecenal	11.65	9.9	6.88	5.7	-0.2948	0.4401
Octadecanoic acid	1.56	1.1	0.78	0.61	-0.3298	0.3041
Oxypeucedanin	0.88	1.57	1.3	1.85	0.3260	-0.0115
Gamma-tocopherol	0.3	1.54	0.54	0.38	0.1317	0.7191
Sesamin	1.77	4.1	3.89	3.95	0.3523	0.1281
Gamma-sitosterol	0.4	1.54	1.35	1.07	0.3066	0.3329

Table 6: Eigen values of the principal components and their contribution and cumulative contribution

Components	Eigen value	Difference	Proportion	Cumulative contribution
Prin1	7.7619	6.0883	0.7762	0.7762
Prin2	1.6733	1.1082	0.1763	0.9435
Prin3	0.5650	0.5650	0.0565	1.0000
Prin4	0	0	0.0000	1.0000
Prin5	0	0	0.0000	1.0000
Prin6	0	0	0.0000	1.0000
Prin7	0	0	0.0000	1.0000
Prin8	0	0	0.0000	1.0000
Prin9	0	0	0.0000	1.0000

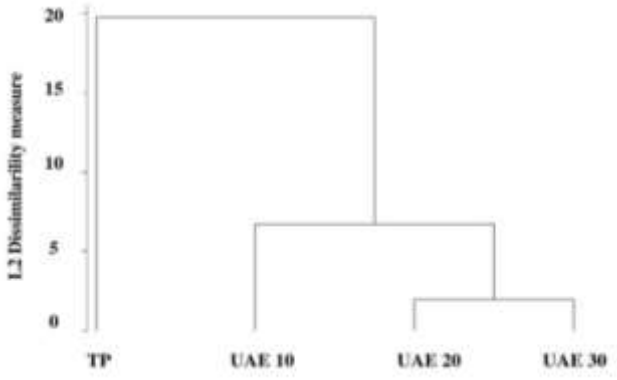

Figure 1: Dendogram of Mahajak samples analysis

Conclusion

This study revealed the impact of UAE on Mahajak formula production. The UAE technique improved yielding and demonstrated anti-inflammatory activity of Mahajak preparation despite no significant effect on the chemical components

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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