Preparative Method of Mahajak Oil Formula and Formulation Development using Ethosome Technology

Adisak Thomudtha

A Thesis Submitted in Partial Fulfillment of Requirements for

degree of Doctor of Philosophy in Pharmacy

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for Doctor of Philosophy (Pharmacy)

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The examining committee has unanimously approved this Thesis, submitted by Mr. Adisak Thomudtha , as a partial fulfillment of the requirements for the Doctor of Philosophy Pharmacy at Mahasarakham University

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TITLE Preparative Method of Mahajak Oil Formula and Formulation Development using Ethosome Technology

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ABSTRACT

Mahajak is a polyherbal formula described in the official Thai medicinal textbook, "King Narai's Medicinal Formulas," since the Ayutthaya period of the Thai Kingdom. It consists of nine herbal ingredients, including sesame oil, kaffir lime (*Citrus hystrix* DC.), cumin (*Cuminum cyminum* L.), fennel (*Foeniculum vulgare* Miller subsp. var. *vulgare*), garden cress (*Lepidium sativum* L.), black cumin (*Nigella sativa* L.), dill (*Anethum graveolens* L.), long pepper (*Piper retrofractum* Vahl.), and camphor. Mahajak is topically applied for skin pruritus, muscle pain, and wounds. The conventional preparative method of Mahajak was a combination of deep-fried and infused aromatization techniques. However, these production process is time-consuming and considered a bottleneck in the supply chain.

In recent years, ultrasonically assisted extraction (UAE) is widely employed for the extraction of biologically active compounds in aromatized vegetable oil. Applying this technique reduces the processing time of herbal extraction and produces the extract with high yielding phytochemical contents. UAE also allows for the production of high-quality oil with fast and cost-effective technology.

The present study aimed to compare the biological activities of Mahajak samples which prepared by 3 methods, including conventional preparative method (CMOF), modified conventional preparative method (MMOF), and UAE method using sonication times of 10, 20, and 30 minutes (UMOF10, UMOF20, and UMOF30). All samples were evaluated for *in vitro* antioxidant activities using DPPH and ABTS methods, anti-inflammatory activities using nitric oxide assays, anti-bacterial activities for *Staphylococcus aureus* DMST 8440 and *Streptococcus pyogens* DMST 17020 using disc diffusion and micro broth dilution methods. The percentage yield and the chemical profiles of all samples were determined using GC-MS and principal component analysis.

The results showed that the antioxidant activities of the MMOF and UAE samples (UMOF10, UMOF20, and UMOF30) were not significantly different from those of the CMOF sample. The IC_{50} of Mahajak samples, determined using DPPH and ABTS tests, ranged from 90.38-99.42 µg/mL. Ascorbic acid exhibited stronger

antioxidant properties than all Mahajak samples. However, the UAE technique was found to be more effective in terms of anti-inflammatory activities (p<0.05), as UMOF10 and UMOF20 showed the most potent anti-inflammatory activity (IC_{50} of 63.12 ± 4.12 and 62.37 ± 2.82 µg/mL, respectively) compared to CMOF (IC_{50} = 74.27 ± 3.54 µg/mL). For the antibacterial activity determination, all Mahajak samples exhibit the inhibition zone against *S. pyogens* DMST 17020 and *S. aureus* DMST 8440 ranging from 8.53–10.30 mm. The minimum inhibitory concentration (MIC) were observed at the concentration of 15.38 and 18.18 µL/mL, respectively. The minimum bactericidal concentration (MBC) were more than 15.38 and 18.18 µL/mL, respectively.

In addition, the UMOF20 and UMOF30 samples had higher percentages of oil recovery than that of CMOF, MMOF, and UMOF10 (p<0.05). The GC-MS analysis indicated all UAE samples presented similar fingerprints to that of CMOF. Ten major chemical components of Mahajak samples, including betapinene, grandlure III, D-camphor, palmitic acid, cis-9-hexadecenal, octadecanoic acid, oxypeucedanin, gamma-tocopherol, sesamin, and gamma-sitosterol were identified and their contents were higher in the UAE samples.

The study also developed an ethosome of Mahajak using the classical cold method. The ethosomal system used in the formulations contained 2-4% (w/w) phospholipids, 20-40% (w/w) ethanol, and aqueous phase to 100% (w/w). The optimized MOF sample (UMOF20) was formulated into nine formulas (F1-F9) as the ethosomal dosage form. The entrapment efficiency and physicochemical properties of each formulation were evaluated by varying the concentrations of phospholipids and ethanol. Among the nine formulations, UMOF20 ethosome F1 was found to be the optimal formulation with a robust system having a pH of 4.77±0.02, vesicle size of 225.00 ± 3.60 nm, polydispersity index (PDI) of 0.35±0.01, zeta potential of -11.87±0.55, and percent entrapment efficiency (EE) of sesamin and sesamolin at 94.13±0.21 and 99.14±0.07, respectively. .

Stability studies were conducted on the most satisfactory formulation of UMOF20 F1 ethosome at temperatures of 40 ± 2°C for 90 days. The stability of ethosomes can be determined by monitoring the vesicle size, size distribution, zeta potential, pH, and physical characteristics of ethosomes. The vesicle size, PDI, zeta potential, and pH were 239.12 ± 3.65 nm, 0.35 ± 0.01, -11.8 ± 0.32 mV, and 4.56 ± 0.10, respectively. Based on these results, it was concluded that UMOF20 ethosome F1 was stable. However, after the stability test at 60 and 90 days, the vesicle size, PDI, and zeta potential increased, indicating that the ethosome system tended to decrease in stability.The appropriate UMOF20 F1 ethosome provided significantly higher skin permeation parameters, such as Q_{24} (23.46±0.06 µg/cm^2), *Jss* (1.92±0.08 µg/cm^2/h), and *Kp* (0.05±0.002 X10^{-3} cm/h), compared to UMOF20 Q_{24} (0.55±0.02µg/cm^2), *Jss* (undetected), and *Kp* (undetected). Overall, the study demonstrated the potential of using UMOF20 ethosome F1 as a stable and effective delivery system for the active ingredients in Mahajak formulas.

In conclusion, the results of this study revealed that UAE technique has an

impact on Mahajak production. It was found to improve the percentages of oil recovery and demonstrate the anti-oxidant, anti-inflammatory, and anti-bacterial properties of the Mahajak preparation, although it had no significant effect on the chemical components. Additionally, the study developed an ethosomal formulation containing the MOF formula.

Keyword : Mahajak Oil Formula, Ultrasonically Assisted Extraction, Ethosome

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Adisak Thomudtha

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CHAPTER I
INTRODUCTION

1.1 Introduction and significance

Mahajak oil formula (MOF) is a polyherbal formulation No. 78 in oil dosage forms of the King Narai's Medicine textbook. It is indicated for (1) puts in the ears for treatment ears hemorrhoids, vertigo, ulcer and pruritus of the skin (2) relieves muscle pain due to spasm and fatigue (3) treats wound injuries from the knife. This formulation consists of sesame oil (*Sesamum indicum* L., Lamiaceae), kaffir lime peel (*Citrus hystrix* DC., Rutaceae), cumin (*Cuminum cyminum* L., Apiaceae), fennel (*Foeniculum vulgare* Miller subsp. var. *vulgare*, Umbelliferae), garden cress seed (*Lepidium sativum* L., Brassicaceae), black cumin (*Nigella sativa* L., Ranunculaceae), dill (*Anethum graveolens* L., Apiaceae), long pepper (*Piper retrofractum* Vahl., Piperaceae) and camphor (*Cinnamomum camphora* (L.) J. Presl., Lauraceae).[1] The chemical compounds which exerted biological activities of MOF are sesamin, sesamol and sesamolin [2, 3] (sesame oil), limonene, citronellal and β-pinene [4, 5] (bergamot peel), cumin aldehyde[6] (cumin), trans-anethole[7] (fennel), β-amyrin and benzyl nitrile [8, 9] (garden cress seed), thymoquinone[10] (black cumin), D-carvone, Dillapiole and D-limonene[11] (dill), piperine[12] (long pepper) and D-camphor[13] (camphor). In addition, the herbal constituents of MOF show all antioxidant, antiinflammatory and antibacterial activities. [2, 3, 5-7, 10, 11, 13-20]

MOF has been used by Thai people since the Ayutthaya period until nowadays. The preparative method is followed by King Narai's Medicine textbook as the first step, sesame oil was fried until hot and then mixed kaffir lime peel to fry continuing until scorched. Filtered the liquid part and collected the filtrate. The last step mixed each of 7.5 g cumin, fennel, garden cress seed, black cumin and dill, 15 g of long pepper and then 30 g of camphor. [1] However, the preparation process of MOF has been unclear in some steps such as frying time of bergamot peel in sesame oil, the temperature for mixing some herbals in fired sesame oil and stirring time to soluble camphor completely. Moreover, the chemical compositions and pharmacological evidence to support the ethnopharmacology of MOF are not established.

Infused aromatization is a traditional method of preparation. [21] Herbal materials and flavoring agents are finely ground and mixed with oil. The mixture is left at room temperature for a defined time and with periodic shaking. [22] Then, the liquid part is filtered to remove the solid parts and ready to use. [21] In order to speed up the process, possible changes of this aromatization method include infusion in vacuum conditions or nitrogen atmosphere and heating at moderate temperature or ultrasound assisted extraction (UAE). [21, 23] Recently, UAE methods were being widely employed for the extraction of various biologically active compounds in aromatized vegetable oils. [23, 24] The application of ultrasound as a laboratory based technique for assisting the extraction of plant material was widely reported in the literature. UAE of capsaicinoids from chili peppers (*Capsicum annuum* L., Solanaceae) in flavored olive oil showed higher capsaicinoids content than the conventional extraction and could allow the production of high quality oils, with fast and cost-effectively methods. [25] UAE of aromatic plants directly into the olive oil reduced the time of the whole extraction process from 24 hours or several weeks to only a few minutes. In addition, even several hours of exposure of oil to ultrasound did not alter the oxidative stability of studied oil. Within several minutes of ultrasound maceration of studied garden sage, laurel and rosemary, high quality products with high polyphenolic content were obtained, together with carotenoid and chlorophyll. [23] Basil leaves were directly put in the olive oil and ultrasounds were applied to the mixture in order to accelerate the diffusion of the basil aromas in the olive oil. The processing time was reduced from days to a few minutes when comparing traditional maceration and ultrasound assisted aromatization. [26] UAE is considered to be a technique to increase phytochemical content of MOF and reduces the time of preparative process.

Ethosomes were introduced for the first time by Touitou et al. in 1997 which enhanced skin delivery of drugs and identified with safety profiles for in vitro and in vivo performance. [27, 28] Ethosomal systems are small vesicular systems composed mainly of a phospholipid, water and high ethanol. The structure exhibits lipid bilayers which enclose part of the surrounding water that contains a relatively high concentration of ethanol (20- 45%). [28] The presence of ethanol in the vesicles confers greater flexibility than conventional liposomes. This combined with the permeation

enhancing the effect of ethanol itself, leads to a higher penetrated amount and deeper penetration of the drug through the skin. [28-33] There are some studies to support herbal medicine loaded ethosomes. *Curcuma longa* extract loaded ethosomal creams were applied to human volunteers and showed promising results as either photoprotective or antiwrinkle agents. [34, 35] *Sesamum indicum* L., Lamiaceae seed extract loaded ethosomal formulation was successfully prepared by loading phospholipids and ethanol. Ethosomes were a very promising carrier for the transdermal delivery of *S. indicum* L. seed extract revealed from higher entrapment efficiency and better stability profile. [36] Lycopene rich extract loaded ethosomal formulation that epicutaneous application was able to decrease the level of anthralin induced ear swelling in a way that was highly comparable to the positive control and show anti-oxidant and antiinflammatory activities. [37]

The present study aims to prepare MOF using three preparative methods: conventional, modified conventional, and UAE preparative methods. The phytochemical composition, antioxidant, anti-inflammatory, and antibacterial activities of all obtained MOF samples are investigated. The optimized preparative method is selected and developed further into an ethosomal formula of MOF.

1.2 Research objectives

1.2.1 To investigate the antioxidant, antiinflammatory and antibacterial activities of MOF samples

1.2.2 To determine the chemical profile of MOF samples by using GC-MS technique.

1.2.3 To develop and evaluate the characterization of the optimized MOF ethosomes.

1.3 Research hypothesis

1.3.1 MOF samples which prepared by the difference preparative methods will show different biological activities and contained the different chemical contents.

1.3.2 Ethosomal technology will enhance skin permeation and increase the stability of MOF formulation.

1.4 Scope and limitation of the study

1.4.1 The MOF samples are prepared by 3 preparative methods as (1) conventional method (2) modified conventional method (3) UAE method.

1.4.2 The MOF samples are investigated their biological activities as followed: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2 ,2 '-azino-bis (3 -ethylbenzthiazoline-6 -sulphonic acid) (ABTS) method for antioxidant activity, LPS induced nitric oxide production for anti-inflammatory activity. Micro broth dilution and disc diffusion methods are performed for antibacterial activity.

1.4.3 The MOF samples are investigated the chemical fingerprints and chemical content by GC-MS analysis.

1.4.4 The optimized MOF sample is selected from their anti-inflammatory and antibacterial activities, respectively.

1.4.5 The optimized MOF sample is formulated to ethosomal dosage by using the classical cold method. The ethosomal system comprised of 2 - 4 % phospholipids, 20-40 % ethanol and aqueous phase to 100 %.

1.4.6 The MOF ethosomes were evaluated for various parameters using different techniques. The parameters included vesicle size, polydispersity index (PDI), zeta potential, entrapment efficiency (EE), chemical marker content, stability, and skin permeability. The evaluation techniques included electron microscopy for vesicle morphology, zetasizer for PDI, zeta potential measurement, and vesicle size determination, ultracentrifugation technique for determining EE, GC-MS for chemical content, stability testing for stability, and Franz diffusion by cell diffusion technique for skin permeability study.

1.5 Research of variables

1.5.1 Determination of antioxidant, anti-inflammatory and antibacterial activities of MOF samples.

1.5.1.1 Independent variables

The independent variables are the MOF samples which prepared by 3 preparative methods as

(1) Conventional preparative method

(2) Modified conventional preparative method

(3) UAE preparative method using extraction time 10 minutes

(4) UAE preparative method using extraction time 20 minutes

(5) UAE preparative method using extraction time 30 minutes

1.5.1.2 Dependent variables

The dependent variables are biological activities of the MOF sample as

The IC_{50} values for antioxidant and anti-inflammatory activities

The inhibition zones for antibacterial activity

The minimum inhibition concentration (MIC) for antibacterial activity

1.5.2 Determination chemical compositions of MOF samples.

1.5.2.1 Independent variables

The independent variables compose of MOF samples are

(1) Conventional preparative method

(2) Modified conventional preparative method

(3) UAE preparative method using extraction time 10 minutes

(4) UAE preparative method using extraction time 20 minutes

(5) UAE preparative method using extraction time 30 minutes

1.5.2.2 Dependent variables

The contents of chemical marker of MOF samples

1.5.3 Determination of the optimized ethosomal dosage form of MOF.

1.5.3.1 Independent variables

The independent variables are MOF ethosome formulations.

1.5.2.2 Dependent variables

The dependent variables are the characterization of MOF ethosomes as

Vesicle size,

Polydispersity index,

Zeta potential,

Percent entrapment efficiency

The cumulative amount at times (Qt) of chemical marker content.

1.6 Expected result

The anticipated outcomes of this study are:

1.6.1 Research data of the antioxidant, anti-inflammatory and antibacterial activities of MOF samples.

1.6.2 Chemical profile and the chemical contents of MOF samples.

1.6.3 The good characterization and stable MOF ethosomal formulation.

1.7 Definition

1.7.1 MOF consists of sesame oil (*Sesamum indicum* L., Lamiaceae) Tha-nan 600, kaffir lime peel (*Citrus hystrix* DC., Rutaceae) 480 g each 7.5 g of cumin (*Cuminum cyminum* L., Apiaceae), fennel (*Foeniculum vulgare* Miller subsp.var.*vulgare*, Umbelliferae), garden cress seed (*Lepidium sativum* L., Brassicaceae), black cumin (*Nigella sativa* L., Ranunculaceae), dill (*Anethum graveolens* L., Apiaceae), long pepper (*Piper retrofractum* Vahl., Piperaceae) 15 g and camphor (*Cinnamomum camphora* (L.) J. Presl., Lauraceae) 30 g. The herbal materials were purchased from an herbal store in Bangkok and authenticated by Assist. Prof. Bhanubong Bongcheewin, Ph.D., Faculty of Pharmacy, Mahidol University, Thailand.

1.7.2 In Thai traditional scriptures, Tha-Nan is different from Tha-nan Luang, which is a unit of volume equivalent to 1 liter. Tha-nan Luang is used to measure out crude drugs for preparing medicines instead of the traditional Tha-nan. This difference might affect the efficacy of the prepared drug.

To calculate the calibrated volume of Tha-nan, the traditional Tha-nan size number is multiplied by 1.548, and the resulting factor is then used to obtain the volume in milliliters. Therefore, in this study, Tha-nan 600 equals 928.8 ml in theory. However, from the experiment, it was found that Tha-nan 600 equals 925.78 ±18.03 mL. In this study, sesame oil was used, and the amount used was 926 mL for Tha-nan 600, which was slightly lower than the calculated theoretical value.

1.7.3 Thian-Tung-Ha is the combination of 5 herbal medicines as cumin, fennel, garden cress seed, black cumin and dill.

1.7.4 The conventional preparative method is prepared by following King Narai's Medicine textbook. First fried sesame oil until hot and then mixed kaffir lime peel fried continuing until scorched, the second filtration in order to separate bergamot peel then collect the filtrate and final mixed each 7.5 g powder of cumin, fennel, garden cress seed, black cumin and dill, 15 g powder long pepper and follow by camphor 30 g.

1.7.5 The modified conventional preparative method is prepared by sonicated the mixture after mixing Thian-Tung-Ha with camphor at 20 kHz with a speed of 1000 rpm for 10 minutes.

1.7.6 UAE preparative method is prepared by sonicated all herbal ingredients in sesame oil at 20 kHz with a speed of 1000 rpm for 10, 20 and 30 minutes.

1.7.7 The optimized MOF sample is considered from their anti-inflammatory and antibacterial activities, respectively.

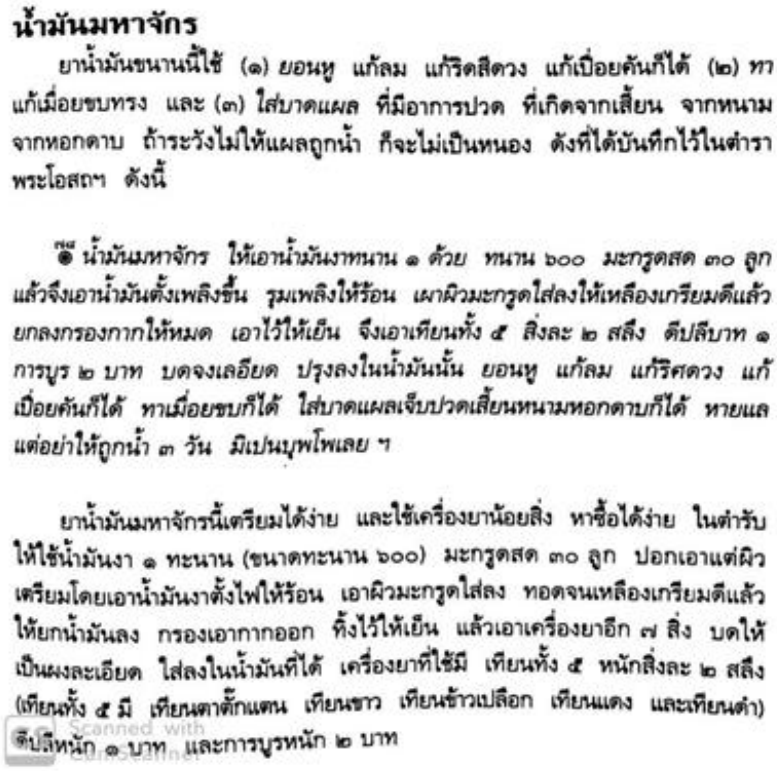
1.7.8 Ethosome is a small vesicular system composed mainly of a phospholipid, water and high ethanol. The ethosome structure exhibits lipid bilayers, which enclose part of the surrounding water. The inner layer is a water soluble compartment containing ethanol.

1.7.9 The optimized ethosome of MOF is considered from the particle size less than 300 nm, a narrow size distribution, zeta potential more than +30 mV or less than -30 mV and entrapment efficiency more than 50%.

CHAPTER II
LITERATURE REVIEW

2.1 Mahajak oil formula (MOF)

The Mahajak oil formula (MOF) is a recipe for a Thai drug that appears in "King Narai's Medicine" and has several indications: (1) it is used by putting it in the ears to treat ear hemorrhoids, vertigo caused by Wayo dhatu disturbance, ulcers, and pruritus, (2) it relieves muscle pain and fatigue, and (3) it is used to treat knife injuries and prevent wound contact with water during the use of this formula. The recipe consists of several herbal medicines (Table 1), including 600 Tanan of sesame oil (*Sesamum indicum* L., Lamiaceae), and 480 g of Kaffir lime peel (*Citrus hystrix* DC., Rutaceae). Additionally, 7.5 g each of cumin *(Cuminum cyminum* L., Apiaceae), fennel (*Foeniculum vulgare* Miller subsp. var. vulgare, Umbelliferae), garden cress seed (*Lepidium sativum* L., Brassicaceae), black cumin (*Nigella sativa* L., Ranunculaceae), dill (*Anethum graveolens* L., Apiaceae), and 15 g of long pepper (*Piper retrofractum* Vahl., Piperaceae), as well as 30 g of camphor (*Cinnamomum camphora* (L.) J. Presl., Lauraceae), are added. To prepare the formula, the sesame oil is first fried until hot and then mixed with the Kaffir lime peel to continue frying until scorched. The liquid part is then filtered and collected. The final step is to mix 7.5 g each of cumin, fennel, garden cress seed, black cumin, and dill, along with 15 g of long pepper and 30 g of camphor. [(1)]



น้ำมันมหาจักร

ยาน้ำมันขนานนี้ใช้ (๑) *ยอนหู* แก้ลม แก้ริดสีดวง แก้เปื่อยคันก็ได้ (๒) *ทา* แก้เมื่อยขบทรง และ (๓) *ใส่บาดแผล* ที่มีอาการปวด ที่เกิดจากเสี้ยน จากหนาม จากหอกดาบ ถ้าระวังไม่ให้แผลถูกน้ำ ก็จะไม่เป็นหนอง ดังที่ได้บันทึกไว้ในตำรา พระโอสถฯ ดังนี้

๏ น้ำมันมหาจักร ให้เอาน้ำมันงาหนาน ๑ ด้วย หนาน ๖๐๐ มะกรูดสด ๓๐ ลูก แล้วจึงเอาน้ำมันตั้งเพลิงขึ้น รุมเพลิงให้ร้อน เผาผิวมะกรูดใส่ลงให้เหลืองเกรียมดีแล้ว ยกลงกรองกากให้หมด เอาไว้ให้เย็น จึงเอาเทียนทั้ง ๕ สิ่งละ ๒ สลึง ดีปลีบาท ๑ การบูร ๒ บาท บดจงเลอียด ปรุงลงในน้ำมันนั้น ยอนหู แก้ลม แก้ริศดวง แก้เปื่อยคันก็ได้ ทาเมื่อยขบก็ได้ ใส่บาดแผลเจ็บปวดเสี้ยนหนามหอกดาบก็ได้ หายแล แต่อย่าให้ถูกน้ำ ๓ วัน มิเปนบุพโพเลย ฯ

ยาน้ำมันมหาจักรนี้เตรียมได้ง่าย และใช้เครื่องยาน้อยสิ่ง หาซื้อได้ง่าย ในตำรับให้ใช้น้ำมันงา ๑ ทะนาน (ขนาดทะนาน ๖๐๐) มะกรูดสด ๓๐ ลูก ปอกเอาแต่ผิว เตรียมโดยเอาน้ำมันงาตั้งไฟให้ร้อน เอาผิวมะกรูดใส่ลง ทอดจนเหลืองเกรียมดีแล้ว ให้ยกน้ำมันลง กรองเอากากออก ทิ้งไว้ให้เย็น แล้วเอาเครื่องยาอีก ๗ สิ่ง บดให้เป็นผงละเอียด ใส่ลงในน้ำมันที่ได้ เครื่องยาที่ใช้มี เทียนทั้ง ๕ หนักสิ่งละ ๒ สลึง (เทียนทั้ง ๕ มี เทียนตาตั๊กแตน เทียนขาว เทียนข้าวเปลือก เทียนแดง และเทียนดำ) ดีปลีหนัก ๑ บาท และการบูรหนัก ๒ บาท

Figure 1 The recipe and preparative method of MOF in the King Narai's Medicine textbook[(1)]

Tha-Nan in Thai traditional scriptures was different from Tha-nan Luang, the latter being a unit of volume equivalent to 1 l. Tha-nan Luang was used to measure out crude drugs for preparing medicines instead of traditional Tha-nan, which might affect prepared drug efficacy. The calibrated volume of Tha-nan was calculated by multiplying the traditional Tha-nan size number with 1.548, then converting factor, to obtain the volume in ml, so this study Tha-nan 600 equals 928.8 ml in theory. However, from the experiment, it was found that Tha-nan 600 equals 925.78 ±18.03 mL.[(38)] Bath is the Thai traditional weighing for preparing Thai traditional medicine, 1 Bath consists of 4 Salunge, 1 Bath to obtain 15 g in the metric system. [(1)] Thian-Tung-Ha is the combination of 5 herbal medicines are cumin, fennel, garden cress seed, black cumin and dill. [(1)]

Table 1 The herbal constituents of MOF [1, 38]

Herbal medicines	Quantity	Parts of used
Sesamum indicum L.	926 ml	oil
Citrus hystrix DC	480 g	peel
Cuminum cyminum L.	7.5 g	seed
Foeniculum vulgare Mill.	7.5 g	seed
Lepidium sativum L.	7.5 g	seed
Nigella sativa L.	7.5 g	seed
Anethum graveolus L.	7.5 g	seed
Piper retrofractum Vahl	15 g	fruit
Cinnamomum camphora (L.) J. Presl.	30 g	oil

2.2 Herbal ingredients of MOF

2.2.1 Black sesame seeds, *Sesamum indicum* L, Lamiaceae [39]

1) Botanical description

Sesamum is a genus that belongs to the Pedaliaceae family. [39, 40] black sesame seeds are mainly grown and cultivated in Africa, China, India, and South America. [41] Thus, *S.indicum* is found mostly in subtropical and tropical regions. black sesame seeds are one of the oldest cultivated plants. Its agricultural use is dated back to more than 5000 years ago, in an Indian subcontinent known as Harappa Valley. [40] It is an annual herbaceous plant that usually grows to a height of 10–120 cm and occasionally to as much as 180 cm. The seeds ripen in a fruit capsule that reaches a length of approximately 2–8 cm, its width varying between 0.5 and 2 cm. Sesame seeds are typically around 3–4 mm long, ovate, and flattened, measuring around 2 mm wide and 1 mm thick. The size, shape and color of seeds can vary considerably according to botanical species. The color of the seeds of the world's most widely traded sesame species is creamish-white. Other common colors include yellowish-brown, brown, golden-yellow, reddish gray and black.[42]

2) Chemical constituents

Sesame seeds provide highly stable oil and nutritious protein and meal, and are used in confectionery foods. They are also an ingredient in Ayurvedic oils under the Indian system of medicine. Sesame has been reported to possess anti-aging properties, a hypocholesterolemic effect, and alleviation of symptoms of alcohol withdrawal, among others.[43-45] Phytochemical investigation of sesame has revealed the presence of biologically active compounds, namely lignans and lignan glucosides. [46] Medicinal properties of raw sesame oil are due to the presence of 0.5-1.1 % sesamin, 0.2-0.6 % sesamolin and trace amounts of sesamol [47] which the chemical structures are shown as Fig. 2. Several studies have shown that sesamin, the major compound present in sesame, inhibits cholesterol absorption and synthesis in rats. [48] Other compounds, such as sesamol and sesaminol, are reported to be responsible for the increased stability of sesame oil. [49]

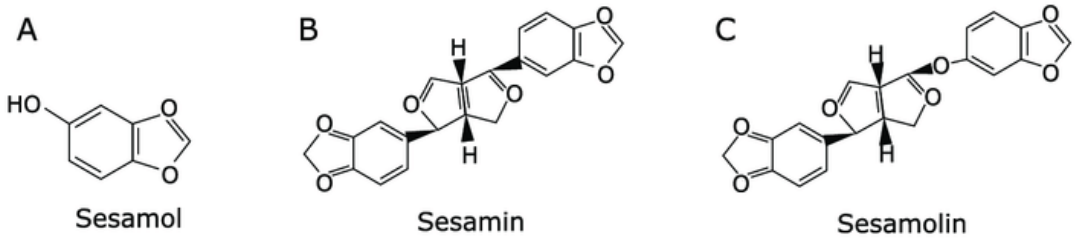


Figure 2 The chemical structures of the chemical compounds of sesame seed:
(A) sesamol; (B) sesamin; (C) sesamolin [50]

3) Biological activities

(1) Antioxidant

The reports demonstrated that the sesame oil extract and α-tocopherol had DPPH radical scavenging activity expressed as IC_{50} value of 0.026 mg/mL and 0.031 mg/mL, respectively. The IC_{50} value is inversely proportional to antioxidant activity. Based on the IC_{50} value, sesame seed oil can be categorized as an oil with considerable antioxidant potential. Additionally, the ABTS radical scavenging activity expressed as percentage discoloration at a 2.0% (w/v) concentration of the oil extract and α-tocopherol was 58.0% and 46.0%, respectively. [51]

(2) Antiinflammatory activity

A. Ruckmani et al. reported that the sesame seed extract had anti-rheumatoid activity comparable to diclofenac and methotrexate in the Freund's complete adjuvant-induced arthritis in rats model. The ethanolic extract of sesame seed at 800 mg/kg had a better effect than at 400 mg/kg. The mechanism of action was attributed to the inhibition of the release of pro-inflammatory cytokines, namely IL-6 and TNF-α, and the inflammatory mediators. [52]

(3) Antibacterial activity

In a study on the effect of oil pulling on dental caries-causing bacteria, the results revealed that the minimum inhibitory concentration (MIC) of sesame oil against *Salmonella typhi* was 10 µl/mL. However, for other organisms, the MIC values were in the range of 350-500 µl/ml. Sesame oil showed the best antimicrobial activity and was also equal to standard Kanamycin, and it showed the highest zone of inhibition against *S. typhi*. It was reported that sesame oil had antibacterial activity against *Streptococcus mutans*, *Lactobacilli acidophilus*, and total bacteria. [53]

Mahendra Kumar C and Singh SA reported that the order of antimicrobial activity of the three compounds was sesamol > sesamin > sesamolin. The MIC for sesamol was 2 mg/mL against *Bacillus cereus* and *Staphylococcus aureus*, but it only inhibited 80% of the growth of *Pseudomonas aeruginosa* at a concentration of 2 mg/mL. Sesamin and sesamolin showed relatively less antimicrobial activity. Sesamin inhibited 69%, 69%, and 59% of growth at a concentration as high as 2 mg/mL, while sesamolin showed 61%, 62%, and 53% of growth inhibition at the same concentration against *Bacillus cereus*, *Staphylococcus aureus*, and *P. aeruginosa*, respectively. [54]

In a study on the antimicrobial activity of sesame oil, it was reported that the oil exhibited a potent MIC value of 10 µl/mL against *Salmonella typhi*. Sesame oil was found to have the best antimicrobial activity, with a zone of inhibition ranging from 15-25 mm, which was equivalent to the standard Kanamycin (19-40 mm), and it also showed the highest zone of inhibition against *S. typhi*. [55]

2.2.2 Kaffir lime, *Citrus hystrix* DC, Rutaceae [39]

1) Botanical description

Kaffir lime is a thorny bush that can reach a height of 2 to 11 m (6 to 35 ft) and possesses aromatic, distinctly shaped double leaves. These hourglass-shaped leaves are composed of the leaf blade and a flattened stalk, known as a petiole. The fruit of the kaffir lime is rough and green, and it turns yellow as it ripens. It is distinguished by its bumpy exterior and small size, measuring approximately 4 cm (2 in) in width. [56]

2) Chemical constituents

The compound responsible for the characteristic aroma of kaffir/makrut lime was identified as (–)-(S)-citronellal, which is contained in the leaf oil up to 80%. Minor components include citronellol (10%), nerol, and limonene (as shown in Fig. 3). It is noteworthy from a stereochemical perspective that kaffir/makrut lime leaves only contain the (S) stereoisomer of citronellal, while its enantiomer, (+)-(R)-citronellal, was found in both lemon balm and, to a lesser extent, lemongrass. However, citronellal is only a trace component in the essential oil of the latter. Kaffir lime fruit peel contains an essential oil comparable to that of lime fruit peel oil, with main components including limonene and β-pinene. [4, 57]

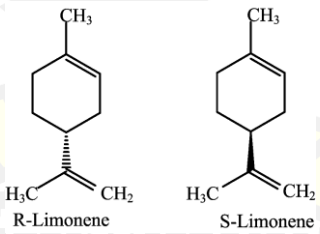


Figure 3. Chemical structure of limonene [58]

3) Biological activities

(1) Antioxidant activity

In a study on the fractionation of phenolic and flavonoid compounds from Kaffir lime peel extracts and evaluation of antioxidant activity, the aim was to investigate the effect of solvent polarity (hexane, ethyl acetate, and n-butanol) employed during fractionation of ethanolic crude extract and assess its antioxidative

activity to neutralize DPPH radical. The crude extract exhibited the lowest IC_{50} value, which was around 0.09 mg/mL. Following the crude extract, the n-butanol fraction showed the second-lowest position to neutralize the same DPPH solution, with an IC_{50} value reported around 0.44 mg/ml. The ethyl acetate fraction, residue, and hexane fraction had IC_{50} values of 1.54, 5.3, and 53.83 mg/mL, respectively. Ascorbic acid was selected as the positive control. [59]

Wungsintaweekul J et al. reported that the methanol extracts of Kaffir lime, from both the leaves and peel, exhibited potent antioxidant activity when tested by the radical-scavenging DPPH method, with IC_{50} values of 24.6 and 66.3 mg/mL, respectively. [60]

(2) Antiinflammatory activity

Coumarins, including bergamottin, oxypeucedanin, and 5-[(6',7;-dihydroxy-3',7'-dimethyl-2-octenyl)oxy-]-psoralen, were found in the methanolic extract of fruits and exhibited as inhibitors of lipopolysaccharide and interferon-induced nitric oxide generation in RAW 264.7 cells. Bergamottin showed higher inhibitory activity (IC_{50} value of 14 µM) than the other two coumarins and was comparable to that of a synthetic L-arginine analogue inhibitor of iNOS (N-(iminoethyl)-L-ornithine) (IC50 value of 7.9 µM). Bergamottin has the potential to inhibit the LPS/IFN-g-triggered iNOS expression pathways and/or iNOS enzyme activity. [61]

(3) Antibacterial activity

In the study, the inhibitory activity of volatile oils from Kaffir lime peel against eight bacteria and three fungi was investigated. The results showed that the volatile oils exhibited inhibitory activity against *Mycobacterium phlei* with MICs of 3.5 mg/mL. [60]

The ethyl acetate extract of kaffir lime peel showed a broad spectrum of inhibition against all gram-positive bacteria, yeast and molds including *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Saccharomyces cerevisiae* var. sake and *Aspergillus fumigatus* TISTR 3180. It exhibited MIC values of 0.28 and 0.56 mg/mL against *Sac. cerevisiae* var. sake and *B. cereus*, respectively while the minimum bactericidal concentration (MBC) values against both microbes were 0.56 mg/ml. The MIC values of the extract against *L. monocytogenes*, *A.*

fumigatus TISTR 3180 and *S. aureus* were 1.13 mg/mL while the MBC values against *L. monocytogenes* as well as *A.fumigatus* TISTR 3180 and *S. aureus* were 2.25 and 1.13 mg/mL, respectively. [5]

The antibacterial activities of Kaffir lime leaf and fruits oil were tested by disc-diffusion and broth microdilution methods against 411 isolates of groups A, B, C, F, G streptococci, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* (methicillin-resistant and -sensitive *S. aureus*) and *Acinetobacter baumannii*, obtained from patients with respiratory tract infections. Kaffir lime leaf and fruits oil were both effective against all the pathogens with MIC ranges of 0.06 – 68 mg/mL and 0.03 – 17.40 mg/mL, respectively. [62]

2.2.3 Cumin, *Cuminum cyminum* L., Apiaceae [39]

1) Botanical Description

Cuminum cyminum L., from the Apiaceae family, is an aromatic herb whose dried seeds are commonly used as a spice. It is a slender and branched herb that can reach up to 13-15 cm in height. The leaves are divided into long and narrow segments, similar to fennel but much smaller, and have a deep green color. The flowers are small and can be rose or white in color, arranged in stalked umbels with only four to six rays. The oblong-shaped seeds are thicker in the middle and compressed laterally, measuring about 5 mm in length. They resemble caraway seeds but are lighter in color and bristly instead of smooth. Additionally, the seeds are almost straight, instead of being curved. [63]

2) Chemical constituents

The major compounds found in cumin essential oil of Egyptian cultivars were cumin aldehyde (35.25%), tetradecene (12.25%), γ-terpenene (12%), β-ocimene (9.72%), p-mentha-2-en-ol (9%), α-terpinyl acetate (5.32%), α-terpinolene (3%), lmonine (0.5%), myrcene (0.2%), β-pinene (0.9%) and α-pinene (0.19%). [64] Cumin seeds contain a high percentage of oil content (45%) and protein (23%). They have a bitter flavor and a warm aroma due to their high content of essential oil. The chief constituent and the most abundant compound found was thymoquinone. The amino acid profile includes lysine, leucine, isoleucine, valine, alanine, and glycine, and additional components were starches, lignans, alkaloids, organic acids, and poisonous glucosides. The seeds also contained minor quantities of minerals such as sodium, iron, zinc, copper, phosphorus, and calcium, as well as vitamins like vitamin C, vitamin B complex, and folic acid. [65]

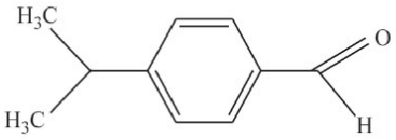


Figure 4. Cumin aldehyde major chemical compound in *C. cyminum* L.[66]

3) Biological activities

(1) Antioxidant activity

The phenolic content in cumin was found to be higher at 539.13 ± 0.65 µmol GAE/g. The cumin essential oils exhibited the highest radical scavenging activity with an IC_{50} value of 26.05 ± 0.16 mg/mL, which was lower than Trolox (29.12 ± 0.05 mg/mL) by DPPH method. To determine the EOs reductive abilities, the transformation of Fe3+ – Fe2+ in the presence of oils was investigated. Cumin EO showed the highest reducing power at 341.65 ± 0.32 µmol Fe2+/g EO, which was greater than Trolox (321.05 ± 1.9 µmol Fe2+/g EO). [67]

Allahghadri T et al. studied the antioxidant activity of cumin seed oil and found that the oil exhibited higher antioxidant activity compared to that of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). The cumin essential oil also exhibited dose-dependent scavenging of DPPH radicals with an IC_{50} value of 5.4 µg/mL. [68]

(2) Antiinflammatory activity

The anti-inflammatory effects of cumin essential oil (CuEO) were investigated in lipopolysaccharide- (LPS-) stimulated RAW 264.7 cells and the underlying mechanisms were studied. A mitochondrial-respiration-dependent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) reduction assay showed that CuEO did not exhibit any cytotoxic effect at the tested concentrations (0.0005–0.01%). Real-time PCR tests showed that CuEO significantly inhibited the mRNA expressions of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), interleukin-1, and IL-6. Moreover, western blotting analysis revealed that CuEO blocked LPS-induced transcriptional activation of nuclear factor-kappa B (NF-κB) and inhibited the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). The results indicated that CuEO exerted anti-

inflammatory effects in LPS-stimulated RAW264.7 cells via the inhibition of NF-κB and mitogen-activated protein kinases ERK and JNK signaling. [69]

(3) Antibacterial activity

The antibacterial effect of cumin seed extract was tested against several pathogens using agar diffusion and dilution methods. The results showed that *E. coli*, *S. aureus*, and *S. faecalis* were sensitive to various oil dilutions, while *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were resistant. The complete death time on exposure to cumin oils was 30, 90, and 120 minutes for *E. coli*, *S. aureus*, and *S. faecalis*, respectively. Based on the results, it can be concluded that *E. coli* is the most vulnerable and S. faecalis is the least vulnerable microorganism to the oils under study. The disc diffusion method and determination of minimal inhibitory and bactericidal concentrations (MIC and MBC) also indicated that *E. coli* is the most sensitive microorganism, with the lowest MBC value of 1 µl/mL. [68]

The antibacterial activity of cumin seed extracts was investigated against 10 Gram-positive and Gram-negative bacteria using the disc diffusion method. Standard procedures were used to determine MIC and MBC. The highest inhibition zone was observed against *E. coli*, with a diameter of 16.67 ± 0.47 mm at 250 mg/mL. Ethanol, methanol, and acetone extracts showed inhibition zones of 15.00 ± 0.82 mm, 15.33 ± 0.47 mm, and 15.67 ± 0.82 mm, respectively, against *Bacillus subtilis*, *Sarcina lutea*, and *Klebsiella pneumonia*. MIC and MBC values ranging from 20 to 50 mg/mL and 40 to 60 mg/mL, respectively, were recorded against the tested bacteria. [70]

2.2.4 Fennel, *Foeniculum vulgare* Mill., Apiaceae [39]

1) Botanical description

Foeniculum vulgare Mill., belonging to the Apiaceae family, is a perennial herb that grows upright and branches out, reaching up to 6.6 ft. (2 m) in height. Its foliage is soft and feathery, almost hair-like, and its stem is erect, cylindrical, bright green, and smooth, giving the impression of being polished. The plant has multiple branched leaves that are cut into the finest of segments, growing up to 40 cm long. The leaves are finely dissected, and the ultimate segments are filiform or threadlike, about 0.5 mm wide. The plant produces bright golden flowers in large, flat terminal umbels, with thirteen to twenty rays, blooming in July and August. The

flowers are small, yellow, and found in large flat-topped umbels. The fruits of *Foeniculum vulgare* Mill. are oblong to ovoid, measuring 0.12–0.2 inches (3–5 mm) in length and 1.5–2.0 mm in width. [71]

2) Chemical constituents

Fennel has been reported to contain 6.3% moisture, 9.5% protein, 10% fat, 13.4% minerals, 18.5% fiber, and 42.3% carbohydrates. The minerals and vitamins present in fennel include calcium, potassium, sodium, iron, phosphorus, thiamine, riboflavin, niacin, and ascorbic acid. The major components of fennel seed essential oil have been reported to be trans-anethole, fenchone, estragol (methyl chavicol), and alpha-phellandrene (Fig. 5). The other classes of phytochemicals present in fennel are phenols and phenolic glycosides. [7]

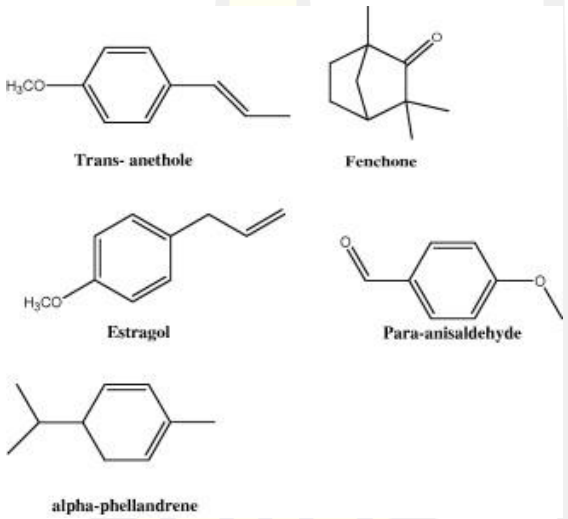


Figure 5 Major chemical compounds of *F. vulgare* Mill. seed essential oil. [7]

3) Biological activities

(1) Antioxidant activity

The methanolic extract of fennel fruit has also been reported to exhibit antioxidant activity by reducing the malondialdehyde level in the fennel fruit methanol extract group compared to the control group. Moreover, the essential oil and acetone extracts of *F. vulgare* have been reported to exhibit strong antioxidant activity compared to butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).[72]

Anwar F et al. reported that fennel seed extracts contain appreciable levels of total phenolic content (627.21–967.50 GAE, mg/100 g) and total flavonoid content (374.88–681.96 CE, mg/100 g). Fennel essential oil and extracts also exhibited good DPPH radical scavenging activity, showing IC_{50} values of 32.32 and 23.61–26.75 μg/mL, respectively, and inhibition of peroxidation at 45.05 and 48.80–70.35%, respectively. [73]

(2) Antiinflammatory activity

The inhibitory effects of fennel fruit on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells were investigated under non-cytotoxic conditions (100 μg/mL). The 80% methanol extract was then partitioned successively with hexane, methylene chloride, ethyl acetate, and n-butanol, and the fractions were also examined for their anti-inflammatory effects. Among them, the hexane, methylene chloride, and ethyl acetate fractions inhibited nitric oxide (NO) and prostaglandin E2 (PGE2) production in LPS-stimulated macrophages. The methylene chloride and ethyl acetate fractions also suppressed the productions of interleukin (IL)-1β and IL-6 by down-regulating their mRNA levels in LPS-stimulated RAW 264.7 cells. Furthermore, the ethyl acetate fraction strongly suppressed tumor necrosis factor (TNF)-α at the protein and mRNA levels in LPS-stimulated RAW 264.7 cells. These observations suggest that the anti-inflammatory actions of fennel are due to the inhibition of the productions of NO, PGE2, and pro-inflammatory cytokines. [74]

(3) Antibacterial activity

The antimicrobial activity of the essential oil extracted from fennel seeds, as measured by the minimum inhibitory concentration (MIC), can be divided into six categories. The first category has the lowest MIC value of 0.5 ppm and includes *Staphylococcus aureus* and *Streptococcus-B- haemolyticus*. The second category had an MIC value of 1.0 ppm and includes *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae,* and *Shigella shiga*. The third category had an MIC value of 1.5 ppm and includes *Klebsiella sp*. and *Sarcina lutea.* The fourth category had an MIC value of 2.0 ppm and includes *Salmonella typhi* and *Shigella boydii*. The fifth category had an MIC value of 5.0 ppm and includes only one microorganism, *Shigella sonnie*. The sixth category includes many microorganisms with high MIC values. [75]

The results of the disc diffusion method indicated that fennel essential oils exhibited considerable antimicrobial activity against all strains tested, especially against Gram-positive bacteria. *B. subtilis* and *A. niger* were found to be the most sensitive microorganisms tested, with the largest inhibition zones (29 and 28 mm) and the lowest MIC values (62.6 and 80.6 μg/mL), respectively. On the other hand, the least activity was observed against *E. coli*, with the smallest inhibition zones (14 mm) and the highest MIC value (259.3 μg/mL). [73]

2.2.5 Garden cress, *Lepidium sativum* L., Brassicaceae [39]

1) Botanical description

Lepidium sativum L., Brassicaceae, popularly named as garden cress, a member of the Brassicaceae family. It is an erect and herbaceous annual plant that grows from 15 to 45 cm in height. Long racemes of *L. sativum* has small white flowers, with broad or obovate pods which emarginated at the apex and winged. [14]

2) Chemical constituents

Garden cress seeds contain 24% oil which composed mainly of a-linolenic acid (ALA) 32 % and linolenic acid (LA) 12 %. This oil was reactively stable owing to its high content of antioxidants and phytosterols. [76] GC- MS analysis of the fixed oil revealed the presence of 16 components. Major constituents are β-amyrin 31.33 % , 9,12,15-octadecatrienoic acid methyl ester 15.97 % , 9-octadecenoic acid methyl ester 11.93 %,α-amyrin 9.32 % , 11-eicosenoic acid methyl ester 6.64%, 9,12– octadecadienoic acid 6.03 % , hexadecanoic acid methyl ester 5.24 % (Fig. 6). [9] Also, it was found to contain glucotropoeoline, 4-methoxyglucobrassicin, sinapine, sinapic acid, calmodulin, sinapoyglucose, ester of caffeic, p-coumaric, ferulic, quinic acids, protein, minerals, vitamins, 5-4-dihydroxy-7,8,3,5-tetramethoxyflavones, 5-3-dihydroxy-7,8,4trimethoxyflavones and 5-3-dihydroxy-6,7,4trimethoxyflavones. The plant seeds contain mainly alkaloids. For example, glucotropeaolin, N, N-dibenzylthiourea, lepidine, N, N-dibenzzyl urea, sinapic acid and choline ester (sinapin); also contain calcium iron, carotene, riboflavin, uric acid, cellulose, phosphorus, thiamine and niacin. Seed oil found to contain stearic, palmitic, linoleic behenic, oleic, arachidic, lignoceric acids, benzyl isothiocyanate, benzyl cyanide, sterol and sitosterol, which mainly can be used in treating dysentery and diarrhea. The aerial parts contained 27-diol-27-benzoate, stigmast-5-en-3β as one of the key chemical constituents. [20]

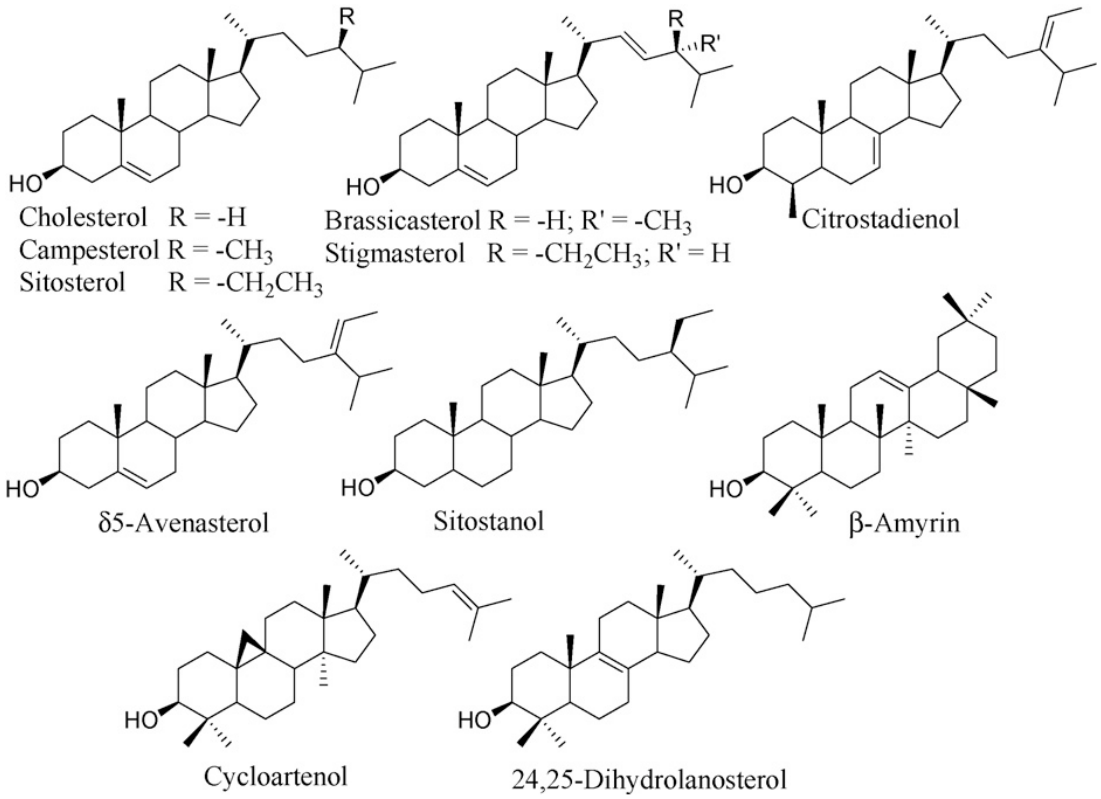


Figure 6 Chemical constituents of *L. sativum* Linn. seeds [77]

3) Biological activities

(1) Antioxidant activity

Antioxidant activity of garden cress seed oil was determined using free radical scavenging activity (DPPH) by adding different concentrations of oil to DPPH. DPPH remaining amount was assessed at 30 min with k level at 520 nm, then the % of inhibition was calculated. Garden cress seed oil showed dose-dependent scavenging of DPPH, with an IC_{50} value of 40 mg/mL. [14]

Chatoui K et al. reported that the IC_{50} values of the methanol extracts of *L. sativum* (LS) were 925.22 ± 0.02 ppm whereas the α-tocopherol (standard) was found to be 50.04 ppm. The methanol extracts of LS contained flavonoids, saponins, tannins, alkaloid and terpenoids. The results revealed that methanol extracts had significant antioxidant. [78]

(2) Antiinflammatory activity

Two new acylated flavonol glycosides named kaempferol-3-O-(2-O-sinapoyl)-β-d-galactopyranosyl-(1→2)-β-d-glucopyranoside-7-O-α-l-rhamnopyranoside (1) and quercetin-3-O-(6-O-benzoyl)-β-d-glucopyranosyl-(1→3)-β-d-galactopyranoside-7-O-α-l-rhamnopyranoside (2), were isolated together with six known compounds from the seeds of garden cress . Their structures were elucidated on the basis of spectroscopic analysis and chemical methods. In vitro 1 and 2 inhibited nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, with IC_{50} values of 25.36 and 25.08 μM, respectively. The result revealed that two new acylated flavonol glycosides showed in vitro antiinflammatory activity. (79)

(3) Antibacterial activity

Microdilution method was used to test the antimicrobial effect of *L. sativum* seed oil (LSO) against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Klebsiella pneumoniae* and *Candida albicans*. The results clearly showed that bacteria and fungi tested were susceptible to LSO, for all of which the MIC was 47.5 mg/mL, except *S. enterica*, which showed a higher MIC of 90 mg/mL. The MBC of LSO was found to be equivalent to 100 mg/mL for inhibiting the growth of all bacteria and fungi. This comparable antimicrobial activity against the tested gram-negative and gram-positive bacteria and the fungus reveals that LOS exhibits broad-spectrum antimicrobial action. (14)

This study aimed to distinguish that the effect of seeds extracts on some microorganisms and the possibility of absolute concentration could achieve that. Using standard organisms, such as *E. coli, K. pneumoniae, salmonella paratyphi, Staphylococcus aureus, Enterococcus faecalis, P. aeruginosa* and *C. albicans*, inoculated them in different concentrations of the extract, using different solutions base, such as water, crude, hexane, chloroform and ethanol, high sensitivity determined by the size of the zone in the certain dilution, which presented in plate of each plate cultured, was in 50 mg/mL 5%. More effective appeared with *E.coli* in crude solution and *K. pneumoniae* in hexane solution as each zone diameter was 16 mm, while the low diameter of sensitivity was obtained with *C. albicans* with 7 mm. MIC and MBC for each organism were ranged from 50 mg/mL to nill. (80)

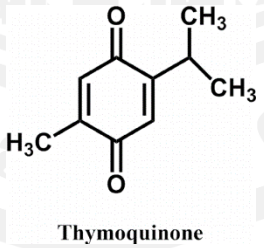
2.2.6 Black cumin, *Nigella sativa* L. Ranunculaceae [39]

1) Botanical description

Nigella sativa L., Ranunculaceae, Black cumin is a small prostrate annual herb about 45 cm high 2-3 slender leaves pinnatisect, 2-4 cm long cut into a linear segment, segments oblong. Flowers pale, blue on solitary long peduncles, seeds trigonous and black in color. The plant has a rather stiff, erect, branching stem, bears deeply-cut grayish-green leaves and terminal grayish-blue flowers, followed by odd, toothed seed vessels, filled with small somewhat compressed seeds, usually three-cornered, with two sides flat and one convex, black or brown externally white and oleaginous, strong agreeable aromatic odor, like that of nutmegs, and a spicy, pungent taste. The flowers are delicate and usually colored pale blue and white, with 5–10 petals. The fruit is a large and inflated capsule composed of 3–7 united follicles, each containing numerous seeds. It has a pungent bitter taste and a faint smell of strawberries. [81]

2) Chemical constituents

The seeds reported to contain a fatty oil rich in unsaturated fatty acids, mainly linoleic acid 50-60 %, oleic acid 20 %, eicosadienoic acid 3 % and dihomolinoleic acid 10 %. Saturated fatty acids (palmitic, stearic acid) amount to about 30% or less. α-sitosterol is a major sterol, which accounts for 44 % and 54 % of the total sterols in Tunisian and Iranian varieties of black seed oils respectively, followed by stigmasterol. [10] Major phenolic compounds of black cumin seed extracts obtained by supercritical carbon dioxide are thymoquinone, inactivated, thymohydroquinone, and thymol (Fig. 7). [82]



Thymoquinone

Figure 7 Chemical structure of thymoquinone. [83]

3) Biological activities

(1) Antioxidant activity

Black cumin seeds were evaluated antioxidative potential by using two separate methods, inhibition of free radical DPPH and superoxide radicals scavenging activities assay. In the first case, the IC_{50} value of the oil was 12.76 ± 1.1 µg/ml. The ethyl acetate extract (IC_{50} = 16.34 ± 1.2 µg/mL) exhibited the strongest activity among the extracts. In the superoxide radicals scavenging activities assay, the ethyl acetate extract was superior to other extracts (IC_{50} = 21.76 ± 1.2 µg/mL). Furthermore, the total phenolic compounds were determined and expressed as gallic acid equivalent. The results of this study suggest that these plant seeds could serve as a source of natural antioxidants and preservative agents with potential applications in food and pharmaceutical industries. [84]

(2) Antiinflammatory activity

Black cumin seeds were evaluated the potential effects of thymoquinone (TQ) on inflammatory responses, we initially quantified its effect on the secretion of NO and PGE2 in LPS-stimulated macrophage-like RAW264.7 cells. At a concentration of 25 µM, TQ strikingly ($p < 0.01$) suppressed LPS-, pam3CSK-, and Poly(I:C)-mediated NO production in a dose-dependent manner by up to 97% inactivated RAW264.7 cells and by 95% in peritoneal primary macrophages. Under the same conditions, TQ also effectively ($p < 0.01$) decreased LPS-triggered PGE2 release by up to 99% at 25 µM. The standard compounds L-NAME and indomethacin dose dependently reduced the secretion of NO and PGE2 under the same conditions. TQ treatment maintained intact viability of RAW264.7 cells, primary macrophages, and HEK293 cells at the concentrations that suppressed NO and PGE2 release, in contrast to NSC95395 (2,3-bis-[(2-hydroxyethyl)thio]-1,4-naphthoquinone). This suggests that the NO and PGE2 inhibitory effects of TQ are not due to non-specific toxicity. Meanwhile, there was no remarkable inhibition of NO production under TQ treatment conditions without LPS stimulation. [85]

(3) Antibacterial activity

Ugur AR et al. reported that the MIC values of black cumin oil against *Staphylococcus aureus* American Type Culture Collection (ATCC) 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and

Pseudomonas aeruginosa ATCC 27853 standard strains were 0.5 μg/mL, 2 μg/mL, 34 μg/mL, and 64 μg/mL, respectively. The black cumin oil showed excellent antibacterial activity against clinical isolates of methicillin-resistant *S. aureus* and methicillin-resistant coagulase-negative Staphylococci with very low MIC range of < 0.25-1.0 μg/mL. (86)

The crude extracts of black cumin seeds were tested in varying dilutions against strains of *Bacillus cereus* 2156, *B. subtilis* 2920, *E. coli* 2065, *Staphylococcus epidermidis* 2493, *Klebsiella pneumoniae* 2957, *Pseudomonas aeruginosa* 5029, *Salmonella typhmurium* 2501, *Enterobacter aerogens* 5139 using agar well diffusion technique in swabbed Mueller Hinton agar plates under standard laboratory conditions. Extract in ethanol and n-hexane showed remarkable dose dependant antibacterial activity against the tested strains as evident from the zones of inhibition. No activity of the extract was observed against *P. aeruginosa* 5029 and *E. aerogens* 5139. The most sensitive strain was *S. epidermidis*. No cross resistance was noticed with any of the tested antibiotics. The MIC and MBC value of the black cumin ethanol extract against *S.epidermidis* was 1.0 and 4.0 mg respectively. (87)

2.2.7 Dill, *Anethum graveolus* L., Apiaceae (39)

1) Botanical description

Anethum graveolens L., Apiaceae, is commonly known as dill and its Thai vernacular name is Phak chi Lao or Thian ta takkataen. It is a biennial or annual aromatic herb belonging to Apiaceae (Umbelliferae) family, grows up to 30-120 cm height. Compound leaf with a divide margin shows a thread-like shape. The stems are hollow. Inflorescences are arranged in umbels. Flowers have a pale yellow color. The fruit is a schizocarp, comprises a pair of carpels that split apart as two mericarps at the mature stage. (11)

2) Chemical constituents

Dill contained essential oils, fatty oil, moisture 8.39 %, proteins 15.68 %, carbohydrates 36 %, fiber 14.80 %, ash 9.8% and mineral elements such as calcium, potassium, magnesium, phosphorus, sodium, vitamin A and niacin. Fruits of dill contain 1 - 4% essential oil comprising of major compounds: carvone 30 - 60 % (Fig. 8.), limonene 33 %, α-phellandrene 20.61 %, including pinene,

diterpene, dihydrocarvone, cineole, myrcene, paramyrcene, dillapiole, isomyristicin, myristicin, myristin, apiol and dillapiol. *A. graveolens* essential oil also contained furanocoumarin, 5-(4”-hydroxy-3”methyl-2”- butenyloxy)-6, 7-furocoumarin, oxypeucedanin, oxypeucedanin hydrate and falcarindiol. [88, 89] The total phenol and total flavonoid contents of dill extract was 105.2 mg of gallic acid equivalents/g of the dried extract and 58.2 mg of catechin equivalents/g of the dried extract, respectively. [90]

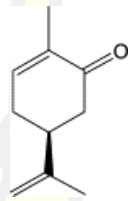


Figure 8 Chemical structure of carvone.[91]

3) Biological activities

(1) Antioxidant activity

Dill seeds extract were evaluated antioxidant effect by the in vitro assay based on ABTS+ radical scavenging capacity. The result showed stronger scavenging capacity with an IC_{50} of 2.68 mg/mL wish was equivalent to 0.89 mM of Trolox. The concentration of 8 mg/mL of the extract was equivalent to 0.85 mM of Trolox. The standard used (Trolox) shows an IC_{50} of 0.59 mM by the DPPH method. [92]

The results of DPPH radical scavenging activity revealed that methanol extract was found to be most effective in all the tested extracts and antioxidant activity increased with an increase in the concentration of tested components. The higher activity of methanol extract of dill seed was related to its high phenolic and flavonoid content. Methanol extract exhibited the highest antioxidant activity with IC_{50} value of 0.30 mg/mL followed by dichloromethane and hexane extracts having IC_{50} value of 0.46 and 0.95 mg/mL respectively. However, the antioxidant activity of the various extracts was found to be lower than that of ascorbic acid IC_{50} of 0.08 mg/mL. [93]

(2) Antiinflammatory activity

The ant-inflammatory of flowers dill was evaluated by the amount of NO production in LPS induced macrophages. After stimulation with LPS 100 ng/mL for 18 h, NO production in the culture media increased markedly, to as high as 16.9 mM in the RAW 264.7 cells. The NO levels were 15.6, 12.3, 8, and 3 mM respectively in the LPS-stimulated RAW 264.7 cells that were pretreated with 5, 25, 50, and 100 mg/mL AGF. In other words, the concentration of NO decreased by 7.7, 27.2, 52.6, and 82.2% respectively as compared to that produced in response to treatment with LPS alone. Accordingly, AGF markedly inhibited the production of NO in a concentration-dependent manner in LPS-stimulated RAW 264.7 cells. [94]

(3) Antibacterial activity

Essential oil fraction of dried fruit dill extracts, deodorized hot water fraction and methanol fraction of dill plant were evaluated for their antibacterial activity against *E. coli*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae*, *S. aureus*, *S. epidermidis* and *S.typhi* by disc-diffusion and micro-well dilution methods. The essential oil fraction exhibited activity against five pathogenic bacteria *E. faecalis*, *K. pneumoniae*, *S. aureus*, *S. epidermidis* and *S. typhi* with inhibition zone of 7.8-11.3 mm in diameter while the deodorized hot water fraction and methanol fraction were inactive. The most sensitive strain was *S. epidermidis* with MIC of 0.25 mg/mL.[95]

Dill seed essential oil exhibited antimicrobial activity against *S. aureus* (ATCC 25923), *C. albicans* (ATCC 90028), *E. coli* (ATCC 25922) and *A. flavus* except *P. aeruginosa* (ATCC 27853) with an inhibition zone of 16-30 mm in diameter. The MIC of essential oil ranged from 5.99-59.47 μg/mL, the lowest concentration of essential oil 5.99 μg/mL restrained *S. aureus* and *E. coli*. [96]

2.2.8 Long Pepper, *Piper retrofractum* Vahl. [39]

1) Botanical description

This plant is a climbing vine with stems of about 3–4 mm in diameter. Its leaves have blades that are glabrous, lanceolate, with acuminate apex and asymmetric base, and are about 10–12 cm long and 3–3.5 cm wide. The vine is dioecious, with male spikes of about 5 cm long and female spikes about 4 cm long and 0.5–1 cm wide and part of the ovaries are attached on the axis. Its berries are spherical and arranged densely on the axis. [97] The fruits of long pepper have been used for their

anti-flatulent, expectorant, antitussive, antifungal, uterus-contractile, sedative-hypnotic, appetizing and counter-irritant properties in traditional medicine. (98)

2) Chemical constituents

Phytochemical investigated indicated that dried fruit of long pepper contained piper-octadecalidine, piperine (Fig. 9), pipernonaline, guineensine, methyl piperate, N-isobutyl-2E,4E,8Z-eicosatrienamide and β-sitosterol. (99)

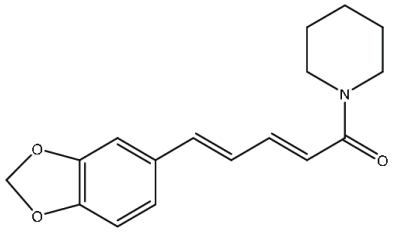


Figure 9 Chemical structure of piperine. (100)

3) Biological activities

(1) Antioxidant activity

The study, antioxidant activities of different solvent extracts of long pepper using DPPH Assay. The results showed that the DPPH free radicals were scavenged by all plant extracts in a concentration dependent manner. Moreover, the IC_{50} values for DPPH radicals with methanol, ethyl acetate and n-hexane extract of the long pepper were found to be 101.74; 66.12 and 57.66 ppm, respectively. Interestingly, the IC_{50} value of n-hexane extract 57.66 ppm was lower than ascorbic acid 66.12 ppm, indicating that n-hexane extract was a more potent scavenger of free radicals than methanol and ethyl acetate extracts. , our results suggested that n-hexane extract of long pepper might contain potent antioxidant compounds. (101)

The antioxidant activity of the methanolic extract of long pepper was investigated by using the DPPH assay. The result showed that the DPPH activity of the extracts was increased in a dose dependent manner, which was found in the range of 0 -31.53 % as compared to ascorbic acid 0 - 43.19 %. The IC_{50} values of methanol extract in DPPH radical was obtained to be 101.74 ppm. Meanwhile, the IC_{50} value of the ascorbic acid was found to be 66.12 ppm. This result indicates that methanol extract of long pepper was mild antioxidant activity. (102)

(2) Antiinflammatory activity

Piperine displays antipyretic, analgesic, insecticidal and antiinflammatory activities. It is the first amide to be isolated from Piper species. In the process of identifying non-steroidal antiinflammatory small molecules from piperine showed inhibits adhesion of neutrophils to the endothelial monolayer. The inhibition of neutrophils to endothelial monolayer by piperine was due to its ability to block the tumor necrosis factor-alpha (TNF-alpha) induced expression of cell adhesion moleculesthe i.e. ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and E-selectin as analyzed by cell-ELISA and confirmed by flow cytometry. Further, inhibition of ICAM-1 by piperine was reversible. As nuclear factor-kappaB (NF-kappaB) was known to control the transcriptional regulation of cell adhesion molecules hence, the effect of piperine on NF-kappaB in the cytoplasm and in the nucleus of endothelial cells was measured. The inhibition pattern for VCAM-1 by piperine was first evident at 2.5 μg/mL concentration with maximal inhibition at 40 μg/mL concentration. [103]

(3) Antibacterial activity

The ethanolic and methanolic extracts of long pepper showed promising activities as they inhibited the growth of the test organisms at a very low concentration. The highest activity of ethanolic and methanolic extracts was found against *E. coli* MIC=0.59 mg/mL. These were also identified as the maximum activities against these test organisms. High antibacterial activity of 9.38 mg/ml was observed for ethanolic extract against *P. aeruginosa* and methanolic extract against *S. aureus*; 18.75 mg/ml of methanolic and ethanolic extracts against *E. faecalis* and *S. aureus*, respectively; and 75.00 mg/mL for methanolic extract against *P. aeruginosa*. [104]

2.2.9 Camphor, *Cinnamomum camphora* (L.) J. Presl. [39]

1) Botanical description

The camphor tree (*C. camphora*) is a broad-leaved, evergreen tree. The alternate leaves are shiny dark green above and lighter green below and have wavy margins with three distinct yellow veins. A distinctive odor of camphor is emitted when the leaves are crushed. [105] The flowers are inconspicuous and the fruit is a black pea-sized berry. The camphor tree grows in full sun or partial shade and it is

drought tolerant but not particularly cold tolerant. It invades hardwood forests, upland pine and scrub woods, fence rows and urban green spaces. Camphor tree is a widely distributed and cultivated in southern China as a common Chinese medicinal plant. It is used in Chinese folk medicine for the treatment of rheumatic arthritis, muscular strains, abdominal pain, rheumatism, twist, cough, bronchitis. [106]

2) Chemical constituents

The oil's high eugenol content also makes it valuable as a source of this chemical for subsequent conversion into isoeugenol, another flavoring agent. Major oil constituents of *C. camphor* are camphor (Fig. 10), linalool, borneol, camphene, dipentene, terpeneol, safrole and cineole. [105]

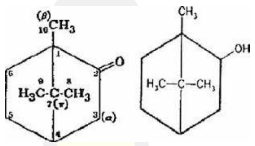


Figure 10 Chemical structures of camphor (left) and borneol (right). [13]

3) Biological activities

(1) Antioxidant activity

Lee HJ et al. reported that ethyl acetate and butanol extracts of camphor displayed strong anti-oxidative activity with IC_{50} values of 14 and 15 μM, respectively, when tested by the DPPH and xanthine oxide (XO) assays. [107]

From the study, antioxidant activities of crude extracts from peel and seed of camphor the result showed that the fractions obtained by n-hexane, ethyl acetate, n-butanol or water of peel and seed had significant antioxidant activity. The IC_{50} value of n-butanol, water and ethyl acetate fraction of the peel exhibited 48.82, 221.32 and 647.85 μg/mL scavenging activity in DPPH free radical. The IC_{50} value of n-butanol, water and ethyl acetate fraction of the peel exhibited 1135.47, 438.69 and 3127.09 μg/mL in metal chelating ability on ferrous ions assays. the n-butanol fraction of peel displayed the best antioxidant activity in ABTS assay. [108]

(2) Antiinflammatory activity

Lee HJ et al. reported that the total crude extract 100 μg/mL prepared with 80% methanol extract and its fractions 100 μg/mL obtained by solvent partition with hexane and ethyl acetate extract significantly blocked the production of interleukin (IL)-1β, IL-6 and the tumor necrosis factor (TNF)-α from RAW264.7 cells stimulated by lipopolysaccharide (LPS) up to 20–70 %. The hexane and ethyl acetate extracts 100 μg/mL also inhibited nitric oxide (NO) production in LPS/interferon (IFN)-γ-activated macrophages by 65 %. The methanol extract 100 μg/mL as well as two fractions 100 μg/mL prepared by a solvent partition with n-butanol and ethyl acetate strongly suppressed the prostaglandin E2 (PGE2) production in LPS/IFN-γ-activated macrophages up to 70%.[107]

(3) Antibacterial activity

The essential oil from camphor leaves showed antimicrobial activity for all tested microorganisms, except for *P. aeruginosa*. Higher antimicrobial activities over the tested bacterium were observed for *Xanthomonas campestris* (33.0 mm), and lower activities were obtained for *Yersinia enterocolitica* (10.5 mm). For the MIC, it was observed that all microorganisms presented susceptibility to the essential oil. For Gram-positive bacteria, the MIC varied from 1.00 mg/mL (*Streptococcus mutans*) to 1.75 mg/mL (*Staphylococcus epidermidis*). In the case of Gram-negative bacteria, the MIC was 0.625 mg/mL for *Citrobacter freundii* and 2.50 mg/ml for *Shigella flexneri*. [109]

Pinoresinol from the leaves of camphor was used to test antibacterial activities against five food-related bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *Salmonella enterica*). Results indicated that 62.5 μg/mL pinoresinol completely inhibit the growth of five bacteria. Much effort was focused on elucidating the mechanism of antibacterial action of pinoresinol against *P. aeruginosa* and *B. subtilis* by determination of cell permeability and observing the changes of cell microstructure using scanning electron microscope (SEM). The leakage of the soluble saccharides and proteins was detected and up to 13.89 and 21.76 μg/mL, respectively for *P. aeruginosa* and 11.08 and 19.05 μg/mL, respectively for *B. subtilis*. Measurements of the release of the soluble saccharides and proteins confirmed the disruptive action of pinoresinol on the cytoplasmic membrane. From the result can conclude that pinoresinol from camphor leaves shows antibacterial activity [110]

2.3 Ultrasound-assisted extraction (UAE)

Ultrasound is a key technology in achieving the objective of sustainable ‘‘green” chemistry and extraction. Ultrasound is well known to have a significant effect on the rate of various processes in the chemical and food industry. [111] UAE is a process that combines the use of acoustic energy with that of an extraction solvent to isolate target compounds from various plant matrices. [112] The enhancement of the mass transfer is brought about by the acoustic cavitation induced in a liquid medium, which is one of the beneficial effects of this technology. [113] Some of the major advantages of UAE, when compared to the conventional extraction methods, are low energy consumption, reduced treatment time, less solvent usage, increased safety of the operators, and improved extraction yield. The increase of extraction efficiency, alongside the reduction of the extraction time, is determined by two mechanisms, ultrasonic cavitation and mechanical mixing effect. Ultrasonic waves generate a mechanical effect in the solvent, resulting in faster movement of molecules and a disruption of the plant cell walls when the cavitation bubbles collapse on the surface of the extraction material, which leads to higher penetration of the solvent into the solid matrix. The extraction process is also enhanced by localized heating (thermal effect) that stimulates the diffusion of the targeted compound into the solvent. [111, 114]

2.3.1 Influencing parameters of UAE

2.3.1.1 Physical parameters

1) Impact of ultrasound physical characteristics: power and frequency

Several studies show that high ultrasonic power causes major alterations in materials by inducing greater shear forces (depending on the nature and properties of the medium); however, in the food industry, this parameter is usually optimized in order to use the minimum power to achieve the best results. [115] Generally, the highest efficiency of UAE, in terms of yield and composition of the extracts, can be achieved by increasing the ultrasound power, reducing the moisture of food matrices to enhance solvent-solid contact and optimizing the temperature to allow a shorter extraction time. However, some studies showed the power variation can result in a certain selectivity of target molecules, where the ratio of some molecules is a function of the applied power. [116, 117]

2) Intensity

Ultrasonic intensity (UI) is expressed as the energy transmitted per second and per square meter of the emitting surface. This parameter is directly correlated to the amplitude of the transducer and consequently to the pressure amplitude of the sound wave. With the increase in the pressure amplitude, the bubble collapse will be more violent. To achieve the cavitation threshold, a minimum value of UI is required. Regarding extraction, the determination of UI is a relevant input value impacting strongly on extraction efficiency. [118]

3) Shape and size of ultrasonic reactors

Since ultrasound waves are reflected upon reaching a solid surface, the shape of the reaction vessel used for extraction using an ultrasonic bath is crucial. For this reason, a flat-bottomed vessel such as a conical flask would be the optimal choice to minimize the reflection of waves. [119] The thickness of the vessel should also be kept minimal to reduce attenuation. It is necessary to calculate the optimum reactor dimensions and the position of the emitter in relation to the transducer to achieve maximum energy transfer to the medium.[120]

2.3.1.2 Medium parameters

1) Solvent

The choice of solvent in UAE is determined by the solubility of the target metabolites, as well as physical parameters such as viscosity, surface tension, and vapor pressure. These physical parameters can affect the acoustic cavitation phenomenon, particularly the cavitation threshold. [121] The initiation of cavitation in a liquid requires that the negative pressure during the rarefaction cycle overcomes the cohesive forces between the molecules composing the liquid. An increase in viscosity or surface tension leads to an increase in these molecular interactions, significantly raising the cavitation threshold. Therefore, when working with samples of high viscosity, the amplitude needs to be increased to obtain the necessary mechanical vibrations that will result in cavitation. This is because as the viscosity of the sample increases, so does the resistance of the sample to the movement of the ultrasonic device, such as the tip of an ultrasonic probe. [122]

2) Temperature

The temperature strongly impacts the properties of the solvent used in UAE. Increasing the temperature causes a decrease in both viscosity and surface tension, and also increases the vapor pressure. This rise in vapor pressure results in more solvent vapors entering the bubble cavity, leading to the formation of numerous cavitation bubbles that collapse less violently, ultimately reducing the sonication effects. [122] As a consequence, higher temperatures may reduce the sonochemical effects due to the collapse of cavitation bubbles. Therefore, to favor sonochemical effects, lower temperatures are preferred, and temperature control is usually applied to limit temperature rise.[123] In the case of UAE, some authors report a beneficial effect of temperature rises from 20 °C to 70 °C compared to non-sonicated extractions. [124] This effect has been justified by an increase in the number of cavitation bubbles and a larger solid-solvent contact area, enhancements of solvent diffusivity with consequent enhancement of desorption and solubility of the interest compounds. However, this effect is decreased when the temperature is near the solvent's boiling point and most authors report a beneficial effect of low temperature (below 30 °C) in the case of UAE. [125]

3) Presence of dissolved gases and external pressure

Generally, in the field of extraction, the composition of gases dissolved in the solvent is not controlled. If the external pressure is increased, then a greater acoustic pressure is required to induce cavitation. But once the cavitation threshold is reached under external pressure (>1 atm), the intensity of the cavitation bubble collapse is higher than without pressure and consequently, an enhancement in sonochemical effects is obtained. [126, 127]

4) Matrix parameters

The solubility and stability of the target compounds in the chosen solvent and temperature of the liquid medium can influence the final yield of the extraction. Likewise, since the extractive system is a heterogeneous and complex porous media, the size of the cavitation bubble has an effect on the efficiency of the extraction. Other parameters related to the solid-liquid extraction such as the solid/liquid ratio and particle size of the material are relevant to the efficacy of extraction. The extraction yields may vary also due to plant material's structure, plasticity or compositional differences which will result in different degrees of impacts from cavitation effects. [128]

2.3.2 UAE techniques application for infusion aromatization

Aromatization is a chemical reaction in which an aromatic system is formed from a single nonaromatic precursor. Typically aromatization is achieved by dehydrogenation of existing cyclic compounds, illustrated by the conversion of cyclohexane into benzene. Aromatization includes the formation of heterocyclic systems. [129] The usual method of aromatization of oils is solid-liquid maceration, which was used by Pitenis, and other works studying the effect of the addition of essential oils or extracts of herbs or spices in quality indices and oxidative stability and sensory profiles. [130] Aromatization with natural products is a very common practice in the olive oil industry. Virgin olive oil is widely appreciated and consumed for its taste and aroma as well as for its nutritional benefits. [131]

UAE methods were being widely employed for the extraction of various biologically active compounds in aromatized vegetable oils (Table 2). The application of ultrasound as a laboratory based technique for assisting the extraction of plant material was widely reported in the literature. [23]

Table 2 UAE techniques application in bioactive compound extractions

Aromatic plants	**Experimental remarks**
Olive leaves *Olea europaea* L.	Solid-liquid oil enrichment (oil to solid material ratio 10:1 (mL/g), 20 min, 25 °C) assisted by ultrasound (225 W, 50% amplitude, duty cycle 0.5 s) produced edible oils with better quality than non-ultrasonicated oils.[132]
Basil leaves *Ocimum basilicum* L.	Ultrasound-assisted aromatization of 1L of olive oil with fresh basil leaves of different amounts. The essential oil contained in the basil leaves was directly extracted into the olive oil without any intermediate stage, which led to an aromatized olive oil in few minutes compared to several hours required in the conventional maceration.[26]

Table 2 (Continue)

Aromatic plants	Experimental remarks
Olive leaves *Olea europaea* L.	Olive oil enrichment with phenolic compounds (e.g., oleuropein) from olive leaves by ultrasonic maceration (60 W, 16 °C and 45 min). The highest total phenolic content (414.3 ± 3.2 mg of oleuropein equivalent/kg of oil), oleuropein (111.0 ± 2.2 mg/kg of oil) and α-tocopherol (55.0 ± 2.1 g/kg of oil) concentrations obtained by optimized ultrasound-assisted extraction proved its efficiency compared to the conventional solid-liquid extraction.[133]
Sea buckthorn pomace *Hippophae rhamnoides*	Ultrasound-assisted extraction (power 0.67 W/g oil and 35 °C) has been used to greatly improve the direct enrichment of edible oils (sunflower, rapeseed, olive, and soya) with carotenoids from sea buckthorn pomaces in terms of quantity and process time (from 33.83 mg/L extract in 90 min obtained by conventional extraction to 51.64 mg/L extract in only 20 min by ultrasound). [134]
Carrot *Daucus carota* L.	Ultrasound-assisted extraction (carrot/oil ratio 2:10, 22.5 W, 40 °C and 20 min) using sunflower as alternative solvent to hexane obtained the highest β-carotene yield (334.75 mg/L) in 20 min, while conventional solvent extraction obtained a similar yield (321.35 mg/L) in 60 min. [135]
Carrot residue (obtained after juice extraction) *Daucus carota* L.	Extraction using ultrasonic horn (oil to solid material ratio 20:0.3, 100 W, 50 min, 50 °C), the maximum extraction yield of β-carotene was 83.32 % while that was 64.66 % when using the ultrasonic bath. [136]
Pomegranate peels *Punica granatum* L.	Sunflower and soy oil were used as alternative solvents to study the effect of various parameters on the yield between ultrasound and conventional extraction, in which the optimal conditions for achieving maximum yield of carotenoids from pomegranate peels were oil to solid material ratio 10:1, 30 min, 51.5 °C, 58.8 % of amplitude level and sunflower oil solvent. [137]

2.4 Gas chromatography-mass spectrometry fingerprint of herbal ingredients of MOF

2.4.1 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography (GC), is a type of chromatography in which the mobile phase is a carrier gas, usually, an inert gas such as helium or a un-reactive gas such as nitrogen and the stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside glass or metal tubing, called a column. The capillary column contains a stationary phase; fine solid support coated with a nonvolatile liquid. The solid can itself be the stationary phase. The sample is swept through the column by a stream of helium gas. Components in a sample are separated from each other because some take longer to pass through the column than others. Mass spectrometry (MS), the detector for the GC is MS. As the sample exits the end of the GC column it is fragmented by ionization and the fragments are sorted by mass to form a fragmentation pattern. Like the retention time (RT), the fragmentation pattern for a given component of the sample is unique and therefore is an identifying characteristic of that component. It is so specific that it is often referred to as the molecular fingerprint. GC-MS is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. GC can separate volatile and semi-volatile compounds with great resolution, but it cannot identify them. MS can provide detailed structural information on most compounds such that they can be exactly identified, but it cannot readily separate them. [138] Gas chromatography has a very wide field of applications. But, its first and main area of use is in the separation and analysis of multi-component mixtures such as essential oils, hydrocarbons, and solvents. [139, 140] Intrinsically, with the use of the flame ionization detector and the electron capture detector (which have very high sensitivities) gas chromatography, can quantitatively determine materials present at very low concentrations. It follows that the second most important application area is in pollution studies, forensic work and general trace analysis. Because of its simplicity, sensitivity, and effectiveness in separating components of mixtures, gas chromatography is one of the most important tools in chemistry. It is widely used for quantitative and qualitative analysis of mixtures, for the purification

of compounds, and for the determination of such thermochemical constants as heats of solution and vaporization, vapor pressure, and activity coefficients. Knowledge of the chemical constituents of plants is desirable not only for the discovery of therapeutic agents but also because such information may be of great value in disclosing new sources of economic phytocompounds for the synthesis of complex chemical substances and for discovering the actual significance of folkloric remedies. [139, 141, 142] GC-MS was used to determine chemical composition in MOF. Hence, herbal medicines in MOF were reviewed chemical compounds by GC-MS for prediction the chemical composition in MOF as shown in Table 3.

Table 3 GC-MS analysis of herbal ingredients in MOF

Herbal ingredients	**Chemical profiles by GC-MS analysis**
S. indicum L.	The seed kernels of *S. indicum* L. were extracted with ethanol and yield of components determined by Gas Chromatography/Mass Spectrometry (GC/MS). The prevailing compounds found in ethanolic extract were carvacrol 0.04 %, sesamol 0.11 %, 4-Allyl-2-methoxy-phenol 0.0 4%, palmitic acid 1.08 %, cis-9-hexadecenal 85.40 %, lineoleoyl chloride 0.52 %, palmitic acid β-monoglyceride 0.40 %, dihydro-aplotaxene 0.61 %, oleoyl chloride 1.11 %, (+)-sesamin 4.73 %, 1,3-benzodioxole, 5-[4-(1,3-benzodioxol-5-yloxy)tetrahydro-1 H,3 H-furo [3,4-c]furan-1-yl], [1 S-(1,3,4,6α.), 2.01 %], 6-nitrocholest-5-en-3-yl acetate 0.22%, ergost-5-en-3β-ol 2.35 % and 24-propylidenecholesterol 0.16 %. [143]
C. hystrix DC.	The major constituents of ethyl acetate extract from kaffir lime peel were limonene 31.64 %, citronellal 25.99 % and β-pinene 6.83 %, whereas β-pinene 30.48 %, sabinene 22.75 % and citronellal 15.66 % appeared to be major components of the hydro distillate essential oil. [5]

Table 3 (Continue)

Herbal ingredients	Chemical profiles by GC-MS analysis
C. cyminum L.	The major compounds in cumin essential oil of Egyptian cultivars were cumin aldehyde 35.25 %, tetradecene 12.25 %, γ-terpenene 12 %, β-ocimene 9.72 %, p-mentha-2-en-ol 9 %, α-terpinyl acetate 5.32 %, αterpinolene 3 %, lmonine 0.5 %, myrcene 0.2 %, β-pinene 0.9 % and α-pinene 0.19 %.(64)
F. vulgare Mill.	The major constituents of the essential oil tested were trans-anethole 69.87 %, fenchone 10.23 %, estragole 5.45 % and limonene 5.10 %. Regarding the groups of chemical constituents represented, the fennel essential oil mainly consisted of oxygenated monoterpenes 87.30 %, followed by monoterpene hydrocarbons 7.88 % and sesquiterpene hydrocarbons 0.35 %. Trans-anethole, fenchone and estragole were the main oxygenated monoterpenes, while limonene was the major monoterpene. (73)
L. sativum L.	GC- MS analysis of the fixed oil revealed the presence of 16 components. Major constituents are β-amyrin 31.33 % , 9,12,15-octadecatrienoic acid methyl ester 15.97% , 9-octadecenoic acid methyl ester 11.93 %,α-amyrin 9.32 % , 11-eicosenoic acid methyl ester 6.64%, 9,12–octadecadienoic acid 6.03% , hexadecanoic acid methyl ester 5.24 % .(9)
N. sativa L.	The essential oil resulted in the identification of 38 components representing 84.65 % of the total amount. The major component was p-cymene 36.2 % followed by thymoquinone 11.27 %, αthujene 10.03 %, longifolene 6.32 % and carvacrol 2.12%. (144)

Table 3 (Continue)

Herbal ingredients	Chemical profiles by GC-MS analysis
A. graveolens L.	A total of 20 compounds was identified and showed that α-Phellandrene 38.85%, p-cymene 14.72 %, γ-terpinene 13.96 %, α-pinene 9.61%, limonene 6.45%, germacrene D 6.01 %, dill ether 3.38 %, β -myrcene 2.51 %, and α – thujene 2.06 % were the major constituents of essential oil. (145)
P. retrofractum Vahl.	Essential oil of *P. retrofractum* fruits was analyzed by GC and GC-MS and 20 components were identified. The main constituents were β-caryophyllene 5.3 %, β-bisabolene 6.4 %, α-curcumene 7.0 %, pentadecane 10.9 %, caryophyllene oxide 7.4 %, heptadec-8-ene 24.6 % and heptadecane 15.1 %.(146)
C. camphora (L.) J. Presl.	The essential oil obtained by hydrodistillation was investigated by GC and GC-MS.The main components of the essential oil were identified to be D-camphor 40.54 %, linalool 22.92 %, cineole 11.26% and 3,7,11-trimethyl-3-hydroxy-6,10-dodecadien-1-yl acetate 4.50 %.(147)

2.5 Application of ethosomes in herbal medicines

2.5.1 Introduction of the ethosomes

Ethosomes were introduced for the first time by Touitou et al. in 1997, that exhibits enhanced skin delivery of drugs and identified with safety profiles for in-vitro and in-vivo performance. (27, 28) Ethosomes are soft malleable vesicles contain phospholipids, alcohol (ethanol and isopropyl alcohol) in relatively high concentration and water. These soft vesicles represent novel vesicles carriers for enhanced delivery through the skin. The size of the ethosomes vesicles can be modulated from tens of nanometers to microns. Unlike classical liposomes, ethosomes were shown to permeate through the stratum corneum barrier and were reported to possess significantly higher transdermal flux in comparison to liposomes. (148) It has been

shown that the physicochemical characteristics of ethosomes permit this vesicular carrier to transport active substances more effectively through the stratum corneum (SC) into the deeper layers of the skin than conventional liposomes. [149] Ethosomes have become an area of research interest in herbal formulation due to its enhanced skin permeation and improved entrapment efficiency (EE). As plant drugs are considered safe due to their natural origin, they exhibit promising therapeutic effects. However, most of the phytoconstituents fail to achieve bioavailability due to poor absorption. The reasons may be the large molecular sizes and low lipid solubility which causes poor absorption of phytoconstituents resulting in reduced bioavailability. Incorporation of these plant actives or extracts into vesicular carriers vastly improves their absorption and consequently bioavailability. [150, 151] There are some studies to support herbal medicines loaded ethosomes. Curcuma longa extract loaded ethosomal creams were applied to human volunteers and showed promising results as either photoprotective or antiwrinkle agents. [34, 35] The sesame seed extract loaded ethosomal formulation was successfully prepared by loading phospholipids and ethanol. The study confirmed that ethosomes are a very promising carrier for the transdermal delivery of sesame seed extract revealed from higher entrapment efficiency (EE) and better stability profile. [36] Lycopene rich extract loaded ethosomal formulation that epicutaneous application was able to decrease the level of anthralin induced ear swelling in a way that was highly comparable to the positive control and show anti-oxidant and antiinflammatory activities. These results support the idea that the lycopene-rich extract may be a good alternative to the expensive commercial lycopene for incorporation into advanced topical delivery systems. [37]

2.5.2 Type of ethosomal systems

2.5.2.1 Classical ethosomes

Classical ethosomes are a modification of classical liposomes and are composed of phospholipids, a high concentration of ethanol up to 45% w/w and water. Classical ethosomes were reported to be superior over classical liposomes for transdermal drug delivery because they were smaller and had negative ζ-potential and higher entrapment efficiency. Moreover, classical ethosomes showed better skin permeation and stability profiles compared to classical liposomes. [28, 152]

2.5.2.2 Binary ethosomes

Binary ethosomes were developed by adding another type of alcohol to the classical ethosomes. The most commonly used alcohols in binary ethosomes are propylene glycol (PG) and isopropyl alcohol (IPA). [153]

2.5.2.3 Transethosomes

This ethosomal system contains the basic components of classical ethosomes and an additional compound, such as a penetration enhancer or an edge activator (surfactant) in their formula. These novel vesicles were developed in an attempt to combine the advantages of classical ethosomes and deformable liposomes (transfersomes) in one formula to produce transethosomes. [154]

2.5.3 Structure of ethosomes

The main difference between ethosomes and liposomes is in their composition. Ethosome comprises various types of phospholipid structures, water, and low molecular weight alcohol (ethanol or isopropyl alcohol) in high concentrations that provide malleability to the vesicle membrane (Fig. 11). [155] The ethosomal lipids are in a more-fluid state than liposomes containing the same ingredients without ethanol. Thus the ethanol can act as a “mixing” agent for lipid vesicles and provide vesicles with softness characteristics, which allow them to increase their distribution in different skin layers. [28] However, because of their high ethanol concentration, the lipid membrane is packed less firmly than conventional vesicles but has equivalent solidity, allowing a more malleable structure and enhance drug distribution ability in stratum corneum lipids. In the cases of drugs with high solubility, the presence of ethanol in ethosomes can exhibit high encapsulation efficiency and improved drug loading. [156]

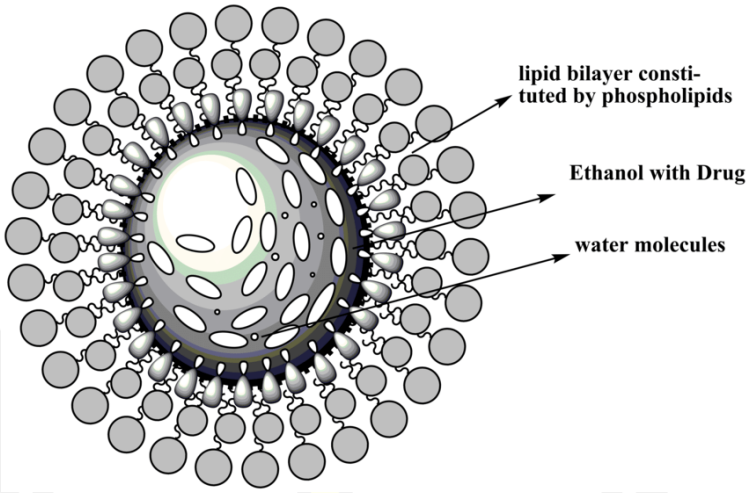


Figure 11 Structure of ethosomes (embodying ethanol and drug molecules). [157]

The non-aqueous phase (alcohol and glycol combination) may range between 20 to 70%.[28, 158] and polyglycols like propylene glycol, are used as a skin penetration enhancer. Various phospholipids which are used as vesicle forming component are phosphatidylcholine, phosphatidic acid, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol (PI). In addition, non-ionic surfactants such as polysorbate 80 and sorbitan monolaurate can be combined with the phospholipids in these preparations. Cationic lipids like cocamide, POE alkyl amines, dodecylamine, cetrimide, etc can be added too. [36, 158] Cholesterol used at a range of 0.1% - 1% provides stability to the vesicle membrane. Such a composition enables the delivery of a high concentration of active ingredients through the skin. Drug delivery can be modulated by altering alcohol: water or the alcohol-polyol: water ratio. In addition, soybean phosphatidylcholine (Phospholipon 90), ethanol, drug, and distilled water can be served for the production of Ethosomes. [159] The size of these vesicles should be reduced to 200 nm or 300 nm to be suitable for this route of administration. [160] The different additive employed in the formulation of ethosome as shown in Table 4

Table 4 Different additive employed in the formulation of ethosomes [36, 159]

Class	Example	Uses
Phospholipid	Soya phosphatidyl choline Egg phosphatidyl choline Dipalmityl phosphatidyl choline Distearyl phosphatidyl choline	Vesicles forming component
Polyglycol	Propylene glycol	As a skin penetration enhancer
Alcohol	Ethanol Isopropyl alcohol	For providing the softness for vesicle membrane As a penetration enhancer
Cholesterol	Cholesterol	For providing the stability to the vesicle membrane
Dye	Rhodamine-123 Rhodamine red Fluorescence Isothiocyanate(FITC) 6- Carboxy fluorescence	For characterization study
Vehicle	Carbopol Đ934, HPMC	As a gel former
Surfectants	polysorbate 80 sorbitan monolaurate	Emulsifier

2.5.4 Influence of high alcohol content

Ethanol is an established efficient permeation enhancer and is present in quite high concentration (20-50%) in ethosomes. The synergistic effect of the combination of relatively high concentration of ethanol (20-50%) in vesicular form in ethosomes was suggested to be the main reason for their better skin permeation ability. The high concentration of ethanol (20-50%) in ethosomal formulation could disturb the skin lipid bilayer organization. Therefore, when integrated into a vesicle membrane, it could give an ability to the vesicles to penetrate the SC. Furthermore, due to high ethanol concentration, the ethosomal lipid membrane was packed less tightly than conventional vesicles but possessed equivalent stability. This allowed a

softer and malleable structure giving more freedom and stability to its membrane, which could squeeze through small openings created in the disturbed SC lipids. In addition, the vesicular nature of ethosomal formulations could be modified by varying the ratio of components and chemical structure of the phospholipids. [159]The effect of ethanol concentration as shown in Table 5.

Table 5 Effect of ethanol concentration in different ethosomal reports

Ethanol Percentage	**Study**	**Results**
30%	Comparative study of minoxidil ethosomes with other ethanolic hydroethanolic solution or phospholipid ethanolic micellar solution of minoxidil and comparative study of transdermal delivery between testosterone ethosomal patch and other commercially available patches. [28]	Dramatic enhancement in the delivery of minoxidil and increased entrapment the efficiency of testosterone.
30%	Characterization of novel ethosomal carrier containing trihexyphenidyl HCl (THP) and to the investigation of the delivery of THP from ethosomes versus classic liposomes. [30]	Significant enhancement in the delivery of THP from ethosomal system.

Table 5 (Continue)

Ethanol Percentage	Study	Results
40%	Comparative study of dermal delivery of minoxidil (Mx), a lipophilic drug from ethosomes versus classic, liposomes characterization on grounds of shape, lamellarity, particle size and entrapment efficiency percentage (EE), by transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM). [33]	Efficient delivery of a fluorescent substance into the skin in terms of quantity and depth, than either liposomes or hydroalcoholic solutions.
30%	Skin delivery of ketotifen (KT) by deformable liposomes, under non-occlusive conditions. [161]	Improved skin delivery of nonentrapped KT. Ethosomes, having KT both inside and outside the vesicles showed superior skin deposition.
-	Investigation of phase and packing properties of dipalmitoylphosphatidylcholine vesicles or multibilayers in the presence of ethanol. [162]	Ethanol induces hydrocarbon interdigitation and increases intermembrane separation in the gel phase; it also broadens the interface and finally increases the range and the extent of the lipid head group salvation

2.5.5 Mechanism of penetration

The skin structure consists of the epidermis or topmost layer of the skin, the dermal-epidermal junction, the dermis, and the subcutaneous fat as shown in Figure 12.[163] The epidermis is the outermost layer and is seen on the surface of the skin. The main cells of the epidermis are the keratinocytes, which synthesize the protein keratin. The keratinocytes develop at the bottom and rise to the top, where they are shed from the surface as dead cells. [164] The outer most portion of the epidermis known as the stratum corneum. It consists of 10 to 25 layers of dead, extended, fully keratinized corneocytes, which are inserted in a matrix of lipid bilayers. It has been shown that the stratum corneum is the main barrier to penetration through the skin. It is relatively waterproof and, when undamaged, obstructs most bacteria, viruses, and other foreign substances from entering the body. The keratinocytes in the stratum corneum are dead squamous cells that are no longer multiplying. [164] The dermis consists is mostly made of dense irregular connective tissue and is much thicker than the epidermis. The dermis is responsible for the tensile strength of the skin. Its main roles are to regulate temperature and to supply the epidermis with nutrient-saturated blood. Much of the body's water supply is stored within the dermis. [164, 165]The subcutaneous layer (It is also called hypodermis) lies below the dermis. The subcutaneous layer is primarily composed of fat and connective tissue. It performs as a protective cushion and helps to insulate the body by monitoring heat gain and heat loss. Not all authors regard this layer as a part of the skin, but it definitely has a strong impact on the way the skin looks. [164, 165]

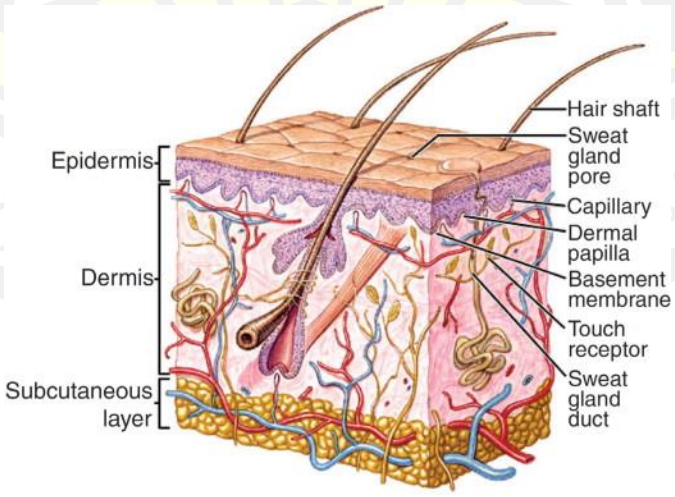


Figure 12 Structure of human skin [166]

The basic advantage of ethosomes over liposomes is the increase permeation of drugs. The mechanism of penetration of the ethosomes in and through the skin is not yet completely clear. But it is suggested that the drug absorption probably occurs in the following two phases

2.5.5.1 Ethanol effect; according to the first mechanism, ethosomal formulations contain ethanol in their composition that interacts with intercellular lipid molecules in the polar head group region, thereby increasing their fluidity and decreasing the density of the lipid multilayer, which results in an increase in membrane permeability.

2.5.5.2 Ethosomes effect; the high alcohol content is expected to results in increased skin permeability. So the ethosomes permeates very easily inside the deep skin layers, where it got combined with skin lipids and releases the drugs into the deep layer of skin. [167]

2.5.6 Method of preparation

Preparation of ethosomes grounds on simple and easy scale-up techniques without entailment of any sophisticated instruments at the pilot and industrial level both. The preparation of ethosomes comprises two basic methods ‘‘cold’’ and ‘‘hot’’.[148] This study focuses review literature classical cold method.

2.5.6.1 Cold method

This is one of the most widely used techniques for the preparation of ethosomes, which consists of two basic and simple setups. In the first setup, phospholipid and other lipid material are dissolved in ethanol at room temperature by vigorous stirring with the use of mixer such as Heidolph mixer with continuous addition of polyols such as propylene glycol, etc. with constant stirring followed by heating at 30 °C in a water bath. In the second setup, water is to be heated at 30 °C in a separate vessel, both mixtures (obtained from first and second setup) are to be blended together following 5 min stirring in a covered vessel. [168] The vesicle size of ethosomal formulation can be decreased to desire extend using sonication or extrusion method. Finally, the formulation is stored under refrigeration.[155, 168, 169]

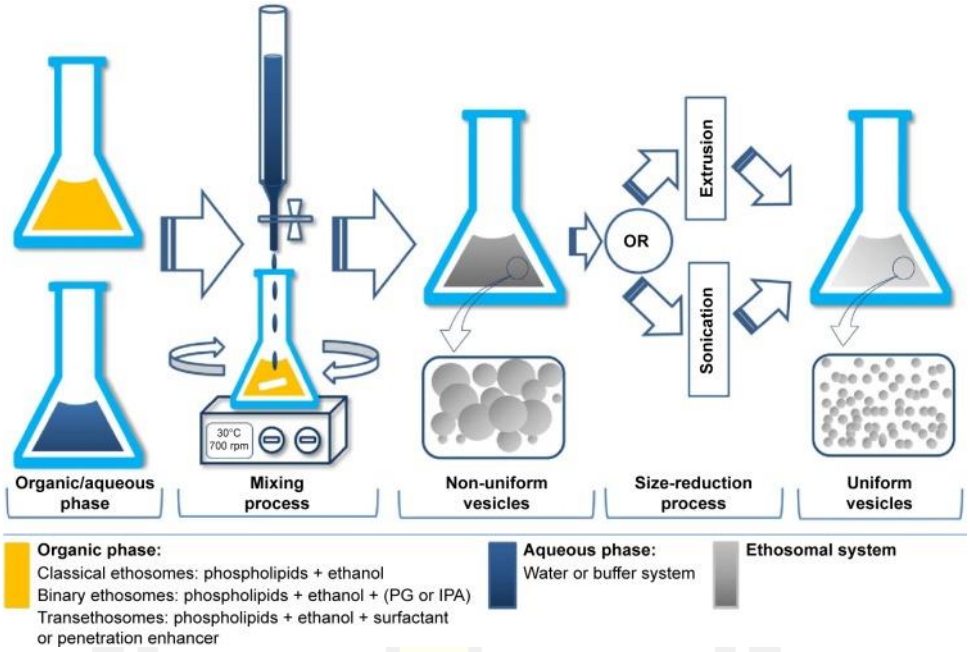


Figure 13 The classical cold method for ethosomal preparation [160]

2.5.7 Method of Characterizations of Ethosomal Formulation.

2.5.7.1 Vesicle shape

Visualization of ethosomes can be done using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Visualization by electron microscopy reveals an ethosomal formulation exhibited vesicular structure 300-400 nm in diameter. The vesicles seem to be malleable as evident by their imperfect round shape. [170]

2.5.7.2 Vesicle size and Zeta potential

Particle size and zeta potential can be determined by dynamic light scattering (DLS) using a computerized inspection system and photon correlation spectroscopy (PCS). Size ranges between nanometers and microns which is influenced by the composition of the formulation, e.g. ethosomes prepared with 30% ethanol and 2% phospholipid (PL), data showed that a narrow particle size distribution with an average size of 153±4 nm. In the ethanol concentration range of 20–45%, the size of the vesicles increased with decreasing ethanol concentration, with the largest particles in preparations containing 20% ethanol (193±8 nm) and the smallest in preparations containing 45% ethanol (103±9 nm). [171]

2.5.7.3 Drug entrapment

The entrapment efficiency of ethosomes can be measured by the ultracentrifugation technique. [170] The ability of ethosomes to efficiently entrap lipophilic and hydrophilic drugs can be explained by the high degree of lamellarity and the presence of ethanol in the vesicles. In addition, ethosomal formulations possess greater entrapment capability than liposomes. [172]

2.5.7.4 Transition Temperature

The transition temperature of the vesicular lipid systems can be determined by using differential scanning calorimetry which also detects ethanol-skin phospholipid interaction, a characteristic attributed to the fluidizing effect of ethanol on the phospholipid bilayers. [173]

2.5.7.5 Drug content

The drug content of the ethosomes can be determined using a UV spectrophotometer. This can also be quantified by a modified high performance liquid chromatographic or gas chromatography-mass spectrometry (GC-MS) method. [148]

2.5.7.6 Surface tension measurement

The surface tension activity of the drug in aqueous solution can be measured by the ring method in a Du Nouy ring tensiometer. [148]

2.5.7.7 Stability studies

The stability of vesicles can be determined by assessing the size and structure of the vesicles over time. Mean size is measured by DLS and structure changes are observed by TEM.[148]

2.5.7.8 Skin permeation studies

The release of MOF ethosomes was determined using Franz diffusion cell technique. The formulated ethosomes were placed in the donor compartment, and an osmosis cellophane membrane was placed between the donor and acceptor compartments. The acceptor compartment was filled with phosphate buffer of pH 7.4 and maintained at a temperature of 37 ± 1°C with stirring at 500 rpm using a magnetic bead fitted to a magnetic stirrer. Sample aliquots of 1 ml were withdrawn at defined time intervals, and the same volume of medium was replaced to maintain sink conditions. The collected samples were analyzed using GC-MS. [174]

Table 6 Characterizatic summarization of ethosomes. [170]

Parameter	Importance	Method
Size and shape	Determine skin penetration	SEM, TEM, DLS
Zeta potential	Stability of vesicles	Zeta Meter
Entrapment efficiency	Suitability of method	Ultracentrifugation
Drug content	Important in deciding the amount of vesicle preparation to be used	UV, HPLC, GC-MS
Stability studies	To determine the shelf life of vesicle formulation	SEM, TEM, HPLC
In vitro dissolution	Determine the drug release rate from vesicle	Franz diffusion cell
Skin permeation	Determines the rate of drug transport through skin	CLSM

2.5.8 Advantages of ethosomal drug delivery. [28, 170, 175, 176]

2.5.8.1 Delivery of large molecules (peptides, protein molecules) is possible.

2.5.8.2 It contains non-toxic raw material in the formulation.

2.5.8.3 Enhanced permeation of drugs through the skin for transdermal drug delivery.

2.5.8.4 Ethosomal drug delivery system can be applied widely in Pharmaceutical, Veterinary, Cosmetic fields.

2.5.8.5 High patient compliance: The ethosomal drug is administrated in the semisolid form (gel or cream) hence producing high patient compliance.

2.5.8.6 A simple method for drug delivery for comparison of Iontophoresis and Phonophoresis and other complicated methods.

2.5.8.7 The Ethosomal system is passive, non-invasive and is available for immediate commercialization.

2.5.8.8 High market attractiveness for products with proprietary technology. Relatively simple to manufacture with no complicated technical investments required for the production of ethosomes.

2.5.9 Disadvantages of ethosomal drug delivery. [176, 177]

2.5.9.1 They require high blood levels. It is limited only to potent molecules, those requiring a daily dose of 10 mg or less.

2.5.9.2 It is not a means to achieve rapid bolus type drug input, rather it usually designed to offer slow, sustained drug delivery.

2.5.9.3 Adequate solubility of the drug in both lipophilic and aqueous environments to reach dermal microcirculation and gain access to the systemic circulation.

2.5.9.4 The molecular size of the drug should be reasonable that it should be absorbed percutaneously.

2.5.9.5 The adhesive may not adhere well to all types of skin.

2.5.9.6 It may not be economical.

2.5.9.7 Poor yield.

2.5.9.8 Skin irritation or dermatitis due to excipients and enhancers of drug delivery systems.

CHAPTER III
METHODOLOGY

3.1 Contents

3.1.1 Chemical and equipment

3.1.2 Preparation method of MOF

3.1.2.1 Traditional method

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3.1.3 In vitro assays

3.1.3.1 Antioxidant activities

1) DPPH assay

2) ABTS assays

3.1.3.2 Antiinflammatory activity

Nitric oxide assays

3.1.3.3 Antibacterial activities

1) Disc diffusion method

2) Broth dilution method

3.1.4 Chemical content analysis by GC-MS

3.1.5 Ethosoms preparation

3.1.5.1 Quality assurance of ethosomes dosage form

1) vesicle size

2) size distribution

3) zeta potential

4) percentage entrapment

5) stability of ethosomes

3.1.5.2 Evaluating the permeability of ethosomes dosage form

3.1.6 Statistical analysis

3.2 Chemicals and equipments

3.2.1 Herbal materials

Sesame oil	*Sesamum indicum* L.
Kaffir lime peel	*Citrus hystrix* DC.
Cumin	*Cuminum cyminum* L.
Fennel	*Foeniculum vulgare* Miller. subsp.var.*vulgare*
Garden cress seed	*Lepidium sativum* L.
Black cumin	*Nigella sativa* L.
Dill	*Anethum graveolus* L.
Long pepper	*Piper retrofractum* Vahl.
Camphor	*Cinnamomum camphora* (L.) J. Presl.

3.2.2 Microbial strains and Cell lines

3.2.2.1 *Staphylococcus aureus* DMST 8440 (MSU, Thailand)

3.2.2.2 *Streptococcus pyogenes* DMST 17020 (MSU, Thailand)

3.2.2.3 The murine monocytic macrophage RAW 264.7 (MSU,Thailand)

3.2.3 Chemical reagents

3.2.3.1 Ethanol (AR grade, Merck, Germany)

3.2.3.2 Methanol (HPLC grade, Merck, Germany)

3.2.3.3 Dimethyl sulfoxide (DMSO) (AR grade, Merck, Germany)

3.2.3.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) (AR grade,Flukka, Switzerland)

3.2.3.5 Ascorbic acid (AR grade, Merck, Germany)

3.2.3.6 Ferrous sulfate (AR grade, Merck, Germany)

3.2.3.7 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

3.2.3.8 Potassium persulfate (AR grade, Merck, Germany)

3.2.3.9 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (AR grade, Merck, Germany)

3.2.3.10 Agar media (Himedia, India)

3.2.3.11 Hydrochloric acid (AR grade, Merck, Germany)

3.2.3.12 15% glycerol (AR grade, Merck, Germany)

3.2.3.13 Broth medium (Merck, Germany)

3.2.3.14 Lipopolysaccharide (LPS) (Sigma-Aldrich, Germany)

3.2.3.15 RPMI-1640 medium (Merck, Germany)

3.2.3.16 Dimethylformamide (DMF) (AR grade, Merck, Germany)

3.2.3.17 Glacial acetic acid. (AR grade, Sigma-Aldrich, Germany)

3.2.3.18 Sodium dodecyl sulfate (SDS) (AR grade, Sigma-Aldrich, Germany)

3.2.3.19 McFarland standard (Himedia, India)

3.2.3.20 Antibiotic disks and Paper disc

3.2.3.21 Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)

3.2.3.22 Sodium carbonate (AR grade, Sigma-Aldrich, Germany)

3.2.3.23 Soya phosphatidylcholine (AR grade, Merck, Germany)

3.2.3.24 Sorbitan laurate (Span®20) (Merck, Germany)

3.2.3.25 Sorbitan monooleate (Tween®20) (Merck, Germany)

3.2.4 Equipments

3.2.4.1 Balance 4 position (Sartorius LE 2445, Germany)

3.2.4.2 Balance 5 position (Sartorius LE 2445, Germany)

3.2.4.3 pH meter (Sartorius LE 2445, Germany)

3.2.4.4 Centrifuge (Multifuge X1 Pro Centrifuge Series, Japan)

3.2.4.5 Ultrasonicator probe (Sonics, USA)

3.2.4.6 Gas Chromatograph-Mass Spectrometer (GC-MS) (Shimadzu , Japan)

3.2.4.7 UV-visible spectrophotometer (Jasco V530, Japan)

3.2.4.8 Franz diffusion cells (Logan Instrument, China)

3.2.4.9 Diffusion Cell Apparatus (Logan Instrument, China)

3.2.4.10 Mixed cellulose esters (MCE) membrane (Merck, Germany)

3.2.4.11 Ultrasonic bath (NineLife, Thailand)

3.2.4.12 Ultracentrifuge (Beckman Coulter, USA)

3.2.4.13 Inverted microscope (Nicolet™ iN™ 5 FTIR Microscope, Japan)

3.2.4.14 Hotplate magnetic stirrer (IKA, (Merck, Germany)

3.2.4.15 Zetasizer (Malvern Panalytical, USA)

3.2.4.16 Constant climate chamber (Memmert, Germany)

3.2.4.17 Incubator (Thermo Fisher Scientific, Japan)

3.2.4.18 Autoclave (Remel™ Autoclave Bag, Thermo Scientific™, Japan)

3.3 Preparative methods of MOF

Plant materials

The herbal ingredients used in the MOF formula, including fresh kaffir lime peels, cumin, fennel, garden cress seeds, black cumin, dill, and long pepper, were purchased from herbal stores in Bangkok in January 2020. To ensure their authenticity, the ingredients underwent authentication by an expert in Pharmacognosy, Bhanubong Bongcheewin, from the Faculty of Pharmacy at Mahidol University in Thailand.

In addition, camphor and sesame oil were procured from Aektrong Chemicals Co., Ltd., also located in Thailand. All voucher specimens were deposited at the Herbarium of the Department of Pharmaceutical Botany at the Faculty of Pharmacy, Mahidol University. Details of the voucher specimens are available in Table 7.

Table 7 Herbal constituents of MOF formula

	Plant materials		**Part of use**	**Voucher specimen No.**
Kaffir lime	*Citrus hystrix* DC	Rutaceae	Fresh peels	PBM 005278
Cumin	*Cuminum cyminum* L.	Apiaceae	Dried seeds	PBM 005273
Fennel	*Foeniculum vulgare* Mill subsp.	Apiaceae	Dried seeds	PBM 005274
Garden cress	*Lepidium sativum* L.	Brassicaceae	Dried seeds	PBM 005275
Black cumin	*Nigella sativa* L.	Ranunculaceae	Dried seeds	PBM 005276
Dill	*Anethum graveolus* L.	Apiaceae	Dried seeds	PBM 005277
Long pepper	*Piper retrofractum* Vahl.	Piperaceae	Dried fruits	PBM 005278

MOF samples were prepared using 3 methods (triplicated) as following

3.3.1 Conventional method

The conventional method was prepared by following the King Narai's Medicine textbook step by step as [1]

(1) Ground cumin, fennel, garden cress, black cumin, dill, long pepper and camphor to fine powder, then sieving through mesh No.40.

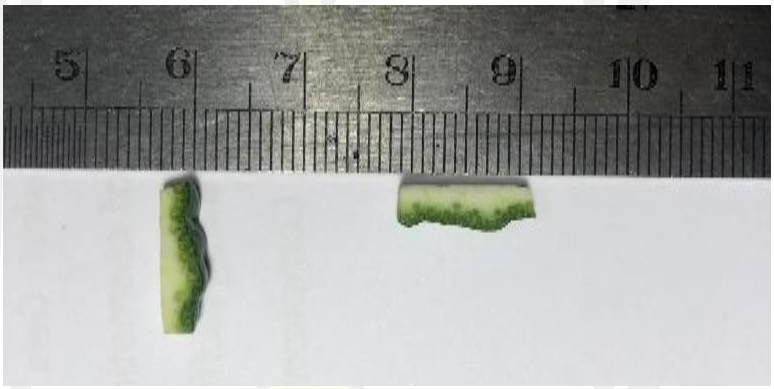


Figure 14 Kaffir lime peel cut in piece that used in this study.

(2) Cut kaffir lime peel in piece sized 1.00 – 1.50 x 0.20 - 0.50 cm. as shown in Figure 14

(3) Put 92.60 mL sesame oil in a fired pot (8-inch diameter) and heat at the temperature 100-120 °C.

(4) Fried 48.0 g kaffir lime peel in sesame oil at the temperature 100 -140 °C for 30 minutes until scorched as shown in Figure 15

Figure 15 Kaffir lime peel, frying until scorched

(5) Filtered the liquid part through sterile gauze and collected the filtrate.

(6) Leave the filtrate cool down to the temperature 30 °C.

(7) Put 0.75 g cumin, fennel, garden cress, black cumin and dill powder, 1.50 g long pepper powder and 3.0 g camphor powder with the filtrate.

(8) Stirred the mixture at 60 rpm for 10 minutes.

(9) Filtered the liquid part through sterile gauze and recorded the volume of MOF samples. (Figure 16)

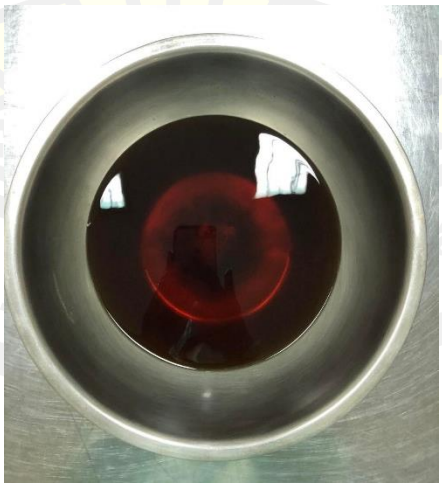


Figure 16 The CMOF sample obtained from a traditional preparative method.

3.3.2 The modified conventional method

The modified conventional method was followed by the same process as conventional method in the step (1) – (7) of the traditional method. Then ultrasonicated of the mixer 10 minutes by a sonicator as a condition 20 kHz with a speed of 1000 rpm for 10 minutes. Filtered the liquid part through sterile gauze and recorded the volume of MOF samples.

3.3.3 Ultrasound-assisted extraction (UAE) method

In the present study, MOF preparative method using UAE was investigated the effect of the mixing time on chemical constituents of MOF. The mixing time was varied with 3 periods as 10, 20 and 30 minutes. UAE preparative method was performed step by step as

(1) Ground cumin, fennel, garden cress, black cumin, dill, long pepper and camphor to fine powder, then sieving through mesh No.40.

(2) Cut kaffir lime peel in piece sized 1.00 – 1.50 x 0.20 - 0.50 cm.

(3) Put 48.0 g kaffir lime peel in 92.60 mL sesame oil.

Figure 17 Ultrasonicated MOF at 20 kHz.

(4) Put 0.75 g cumin, fennel, garden cress, black cumin and dill power, 1.50 g long pepper powder and 3.0 g camphor in (3).

(5) Ultrasonicated the mixture as a condition 20 kHz with a speed of 1000 rpm vary mixing times as 10, 20 and 30 minutes, respectively.

(6) Filtrated the liquid part through sterile gauze and recorded the volume of MOF samples.

3.4 *In vitro* assays

3.4.1 Antioxidant activities

Sample preparation was prepared by dissolved MOF samples in DMSO 1 mg/mL as a stock solution.

3.4.1.1 DPPH scavenging assay

The assay method was determined by the method described by Amic et al. [178] All samples, blank and standard ascorbic acid for calibration curve were performed in triplicate.

1) Method

(1) In each well of a 96-well plate, added the concentration of each sample from the stock solution as 20 µL.

(2) Used the DMSO 20 µL as a blank sample and or the standard ascorbic acid for calibration curve were used 20 µL (10-100 µg/mL) sample solutions.

(3) Added 180 µL 0.1 mM DPPH radical solutions for 15 minutes incubated at room temperature, then measured the absorbance at 517 nm.

(4) The radical scavenging activity can be measured in the sample and calculated % inhibition as the following equation:

$$\% \text{ inhibition} = 100 \times (A_{blank} - A_{sample})/A_{blank}$$

Where A_{blank} = absorbance of blank, A_{sample} = absorbance of the sample

(5) From the ascorbic acid calibrate curve, measured the ascorbic acid equivalent antioxidant capacity of the sample.

3.4.1.2 ABTS radical scavenging activity

ABTS assay was determined according to the method described by Payet et al. [179] All samples, blank and calibrated were performed in triplicate.

1) Method

(1) All samples, blank and calibrated were performed in triplicate.

(2) In each well of a 96-well plate, add each sample which concentration from stock solution as 20 µL.

(3) Use the solvent (20 µL) as a blank sample.

(4) For the calibration curve, use 20 µL of ascorbic acid (1 – 100 µg/mL) stock solutions.

(5) Start in the reaction by adding 280 µL ABTS working solution, start time for 5 min. from the first addition, incubate at room temperature.

(6) Measure the absorbance of each well plate at 734 nm.

(7) The radical scavenging activity of the sample is measured and calibrated as the following equation:

$$\% \text{ inhibition} = 100 \times (A_{blank} - A_{sample})/A_{blank}$$

Where A_{blank} = absorbance of blank, A_{sample} = absorbance of the sample

3.4.2 Antiinflammatory activity

3.4.2.1 Nitric oxide assays

The effect of extracts on NO production by murine macrophage-like RAW 264.7 cell lines was determined using a method modified from that Makchuchit et al. [180]

Method

(1) All samples, blank and calibrated were performed in triplicate.

(2) The murine monocytic macrophage RAW 264.7 cell line was 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 and diluted to a suspension in fresh medium before plating for experiments.

(3) Cells were seeded in 96-well plate with $1x10^5$ cells/well and allowed to adhere for 1 h.

(4) After that, the medium was replaced with fresh medium containing 5 µg/mL of lipopolysaccharide (LPS), together with test samples (preparative of MOF in DMSO) at 100, 50, 20, 10 and 1 µg/mL.

(5) Incubated for 24 h.

(6) Nitric oxide production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent.

(7) The absorbance of the resultant solution was measured with a microtitre plate reader at 550 nm.

3.4.2.2 Macrophage preparation

The RAW 264.7 cells were kindly provided by Rujiluk Rattarom, Ph.D., Faculty of Pharmacy, Mahasarakham University, Maha Sarakham, Thailand. They 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 and diluted to a suspension in fresh medium before plating for experiments.

3.4.3 Antibacterial activity

3.4.3.1 Microbial strain and growth media

The test organisms used were collected from the stock cultures of the Faculty of Pharmacy, Mahasarakham University. The standard strains of *Staphylococcus aureus* DMST 8440 and *Streptococcus pyogens* DMST 17020 were employed in the study.

S. aureus DMST 8440 were grown on nutrient broth (Tryptic soy broth, TSB) at 37 °C incubate for 12–16 h.

S. pyogenes DMST 17020 (group A streptococci, GAS) were grown on nutrient broth (Todd-Hewitt broth supplemented with 0.2% yeast extract, THY) at 37 °C incubate for 24 h. [181, 182]

Turbidity was adjusted to match that of a 0.5 McFarland standard ($1x10^8$ CFU/mL).

3.4.3.2 Screening of MOF samples using disk diffusion technique

The disk diffusion test was performed using the standard procedure as described by Jorgensen et al. [183]

(1) The inoculum suspension of each bacterial strain was swabbed on the entire surface of the agar.

(2) Sterile 6-mm filter paper discs were aseptically placed on agar surfaces, and MOF samples were immediately added to discs in volumes of 15 µL.

(3) A 20 µL aliquot of 20% DMSO was also added to a sterile paper disc as a negative control, whereas a disc containing 10 µg cloxacillin was placed in the plate as a positive control.

(4) The plates were left at ambient temperature for 15 minutes to allow excess prediffusion of extracts prior to incubation at 37 °C for 24 h. Diameters of inhibition zones were measured.

(5) Each experiment was done in triplicate.

3.4.3.3 Determination of the minimum inhibitory concentration using micro broth dilution test

The dilution test was performed to determine minimum inhibitory concentrations (MICs) using the standard procedure as described by Jorgensen et al. (183)

(1) One hundred microliters of broth media were added in each well of a microtiter plate.

(2) The inoculum suspension (20 µL) of each bacterial strain was then added in each well containing broth media.

(3) Different amounts of the MOF samples a concentration of 200 µL/mL in 20% DMSO (e.g. 2, 4, 6, 8, 10 and 12 µL) were added to the broth cultures (120 µl) to obtain final concentrations of 3.27 , 6.45, 9.52, 12.5, 15.38 and 18.18 µL/mL respectively.

(4) The negative and positive controls were also performed using 20% DMSO and cloxacillin.

(5) Triplicate wells were run for each concentration of MOF samples.

(6) The plates were incubated at 37°C for 24 h, and the turbidity was measured at 620 nm using the microplate reader.

(7) The lowest concentration that inhibited the visible growth of the tested organisms was recorded as the MIC, which was determined by the absorbance value in the range of 0.2 - 0.4.

3.4.4 GC-MS analysis for MOF samples

The present study employed GC-MS analysis to determine the chemical profile of MOF samples. The study included five groups of MOF samples, and each group was analyzed in triplicate. The GC-MS conditions used were based on the method described by Qin D-M et al. and included the use of a quartz capillary column with an initial temperature of 40°C. The temperature was then increased to 280°C at a rate of 5°C/min, and this temperature was maintained for 60 minutes until the analysis was completed. Helium was used as the carrier gas, and the inlet temperature was 240°C, with a split ratio of 40:1 and an injection volume of 1 µL. The mass spectrometry conditions included a standard electron ionization (IE) source with a voltage of 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. By using these conditions, the study was able to determine the chemical profile of the MOF samples. [184]

3.4.5 Principal component and cluster analysis

The present study employed principal component analysis (PCA) and cluster analysis using STATA (Statistical/Data analysis) v14.0 software to evaluate the influence of different MOF preparative methods. PCA is a statistical technique that reduces the dimensionality of a dataset while retaining as much variation as possible. It identifies the underlying factors or components that explain the maximum variation in the dataset. Cluster analysis, on the other hand, is a statistical method used to group similar objects or observations into clusters.

In this study, PCA and cluster analysis were used to identify any differences or similarities in the chemical profile of MOF samples prepared using different preparative methods. By using STATA v14.0 software, the study was able to analyze the data and generate visual representations of the results. The analysis helped to evaluate the influence of different MOF preparative methods on the chemical profile of the samples and identify any potential patterns or clusters within the data.

3.5 Ethosomes preparation of MOF

The optimized MOF sample was formulated into nine different ethosomal dosage forms in triplicate using the classical cold method. The ethosomal system used in the formulations contained 2-4% (w/w) phospholipids (soya phodphatidylcholine, SPC) 20-40% (w/w) ethanol, and aqueous phase to 100% (w/w). The different formulations were prepared by varying the concentrations of phospholipids and ethanol, and the entrapment efficiency and physicochemical properties of each formulation were evaluated.

as shown in Table 8.

Table 8 Composition of ethosomal formulations

Formulation code	**Phospholipid (%w/w)**	**Ethanol (%w/w)**	**MOF (mL)**	**Span 20 (mL)**	**Tween 20 (mL)**	**Water (mL) q.s. to**
F1	2	20	7.5	1	0.5	100
F2	2	30	7.5	1	0.5	100
F3	2	40	7.5	1	0.5	100
F4	3	20	7.5	1	0.5	100
F5	3	30	7.5	1	0.5	100
F6	3	40	7.5	1	0.5	100
F7	4	20	7.5	1	0.5	100
F8	4	30	7.5	1	0.5	100
F9	4	40	7.5	1	0.5	100

3.5.1 Cold method [28]

The formulation of the ethosomes was followed by the classical cold method technique. The ethosomal system is comprised of 2 – 4% phospholipids (soya phosphatidylcholine, SPC), 20 – 40% ethanol, 1 mL of Span 20, 0.5 mL of Tween 20 and the formulation was adjusted to 100 % w/w with distilled water. Phospholipids, MOF and Span 20 were dissolved in ethanol this mixture was heated to 30°C ± 1°C in

a water bath while the aqueous phase was prepared by dissolving Tween 20 in distilled water then heated to 30°C ± 1°C. The resulting aqueous solution was added slowly in a fine stream to the lipidic solution in the center of the vessel with constant stirring using magnetic stirrer at 700 rpm in a closed vessel. The temperature was kept 30°C throughout the experiment. The mixing was continued for additionally 5 min. The prepared ethosomes were then sonicated at 4°C using a probe sonicator at 20 kHz for three cycles of 5 minutes each, with an interval of 5 minutes between each cycle. Finally, the ethosomes were refrigerated.

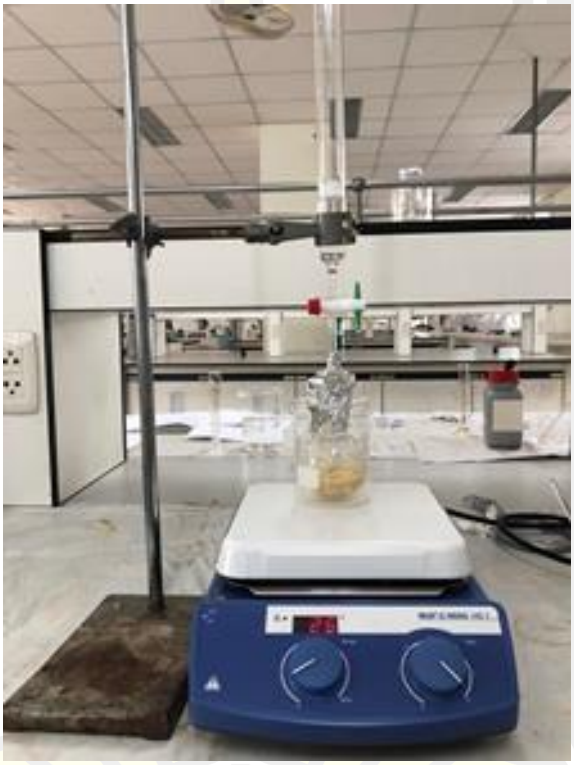


Figure 18 UMOF20 ethosome preparation by classical cold method.

3.5.2 Quality assurance of ethosomes dosage form

3.5.2.1 Vesicle shape

The inverted microscope was used to characterize the shape of the ethosomal vesicles.

3.5.2.2 Vesicle size and Zeta potential

The size, size distribution, and zeta potential of ethosomes were determined by Zetasizer. Before measurements, the vesicular suspension was diluted with the appropriate medium. The water was used to dilute MOF ethosomes.

3.5.2.3 Entrapment efficiency (EE)

Ultracentrifugation is a technique used to separate and purify particles based on their density, size, and shape. In the case of MOF ethosomes, ultracentrifugation was used to separate the vesicles from the suspension and determine the entrapment efficiency of the major chemical components, sesamin and sesamolin.

The entrapment efficiency is a measure of how much of the active ingredient is retained within the MOF ethosomes. It is calculated using the following equation:

$$\% \text{ Entrapment efficiency} = (Dt-De)/Dtx100$$

Where:

De = Amount of major chemical content in supernatant liquid

Dt = Theoretical amount of major chemical content used to prepare the formulation

To determine the entrapment efficiency of the MOF ethosomes, the vesicles were separated from the suspension using ultracentrifugation at 20,000 rpm for 90 minutes at 4°C. After centrifugation, the sediment and supernatant liquids were separated, and the amount of major chemical composition in the supernatant liquid was determined using an indirect method.

Using the values obtained from the analysis, the entrapment efficiency can be calculated by plugging in the values into the equation above. The resulting percentage will indicate how much of the major chemical content was retained within the MOF ethosomes, and how much was released into the supernatant liquid.

3.5.2.4 Stability study

The ability of ethosomal formulations to retain the major chemical content was checked by keeping the preparations at temperatures 40 ± 2°C for

different periods of time 30, 60 and 90 days. The stability of ethosomes can also be determined properties by monitoring the size, size distribution, zeta potential and physical characteristic of ethosomes.

3.5.2.5 Evaluating the permeability of MOF ethosomes

The in vitro skin permeation of the optimized ethosome formulation and control formulation were measured through the synthetic membrane using Franz diffusion cells. The effective diffusion area of the diffusion cell was 1.77 cm^2. The receptor compartment was filled with 11 mL of phosphate buffer saline solution pH 7.4 (PBS).In the donor compartment, 1 mL of each sample was applied on the mixed cellulose esters (MCE) membrane. The synthetic membrane was maintained between the donor and the receptor compartment. The Franz diffusion cells were maintained at 37ºC with stirring at 500 rpm throughout the experiment.At defined time intervals 0.5, 1, 2, 3, 4, 6, 8, 12, 15, 20 and 24 h, 1 mL sample of the receiver medium was withdrawn through the sampling port of the diffusion cell. An equal volume of fresh PBS was replaced into the receptor compartment after each sampling.All withdrawn samples were analyzed by the GC-MS method in triplicate as the same condition followed by Qin D-M et al. The control system was used as an optimized MOF sample.

3.6 Statistical analysis

The statistical significance of the data generated in the study was tested using ANOVA (analysis of variance) followed by the studentized range test. ANOVA is a statistical test that is used to analyze the differences between the means of two or more groups, while the studentized range test is a post-hoc test used to determine which groups are significantly different from each other.

A confidence limit of $p < .05$ was fixed for the interpretation of the results, which means that there is a 95% probability that the differences observed between the groups are not due to chance.

The software PRISM (GraphPad, Version 2.01, San Diego, CA) was used to conduct the statistical analysis. PRISM is a commonly used software for statistical analysis and graphing in the life sciences.

CHAPTER IV
RESULT

4.1 Preparative methods of MOF

In this study, the researchers prepared MOF samples using three different methods: conventional, modified conventional, and UAE (ultrasonic-assisted extraction). The ingredients used for all three methods were cumin, fennel, garden cress seed, black cumin, dill, long pepper, kaffir lime peels, camphor, and sesame oil.

For the conventional method, the sesame oil was heated and then mixed with the kaffir lime peels to fry until scorched. The liquid part was collected and infused with the other ingredients for 15 minutes before being filtered through sterile gauze to obtain CMOF sample.

For the modified conventional method, the preparative process was followed as the conventional method, but ultrasonication was applied for 10 minutes before filtering the liquid part through sterile gauze to obtain the MOF sample.

For the UAE method, the ingredients were mixed together and then subjected to ultrasonicate for 3 time periods; 10, 20 and 30 minutes to obtain UMOF 10, UMOF 20 and UMOF 30, respectively. After filtration through sterile gauze, all samples had a liquid form that resembles brownish-yellow oil with as shown in Figure 19.

The percentages of oil recovery of all MOF samples were recorded in the range of 44.65 – 61.82 (Table 9). The results showed that UMOF20 and UMOF30 samples had significantly higher percentages of oil recovery than CMOF, MMOF, and UMOF10 (p<0.05). However, it should be noted that only the percentages of oil recovery of UMOF20 and UMOF30 were significantly higher than those of the CMOF and MMOF samples.

Overall, the results suggest that the UAE method, particularly UMOF30, is the most effective in terms of percentages of oil recovery when compared to the conventional and modified conventional methods.

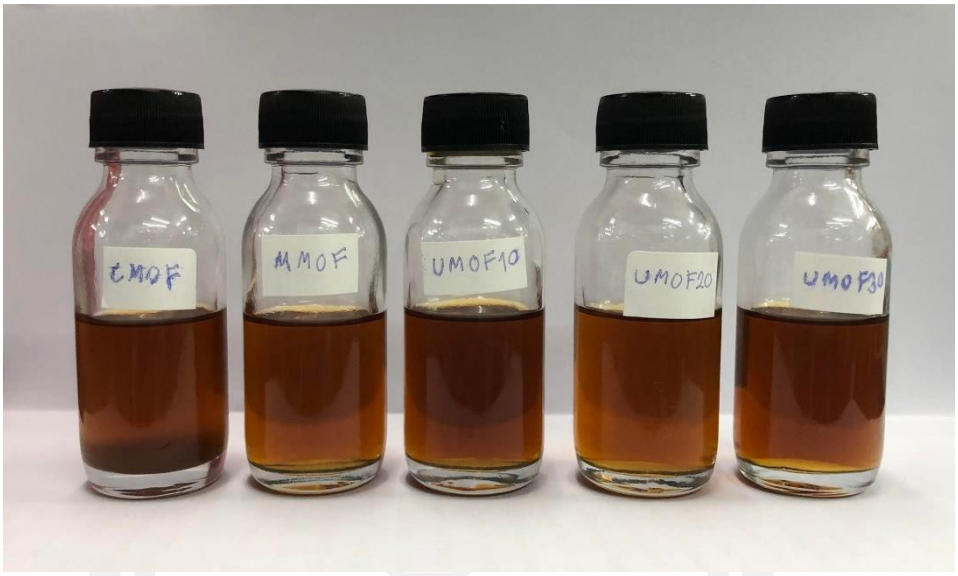


Figure 19 Characteristic of MOF samples

Table 9 Percentages of oil recovery of MOF samples

Samples (n=3)	Percentages of oil recovery
CMOF	44.65 ± 4.14[a]
MMOF	45.14 ± 1.74[a]
UMOF10	47.75 ± 1.87[a]
UMOF20	59.75 ± 2.22[*b]
UMOF30	61.82 ± 3.24[*b]

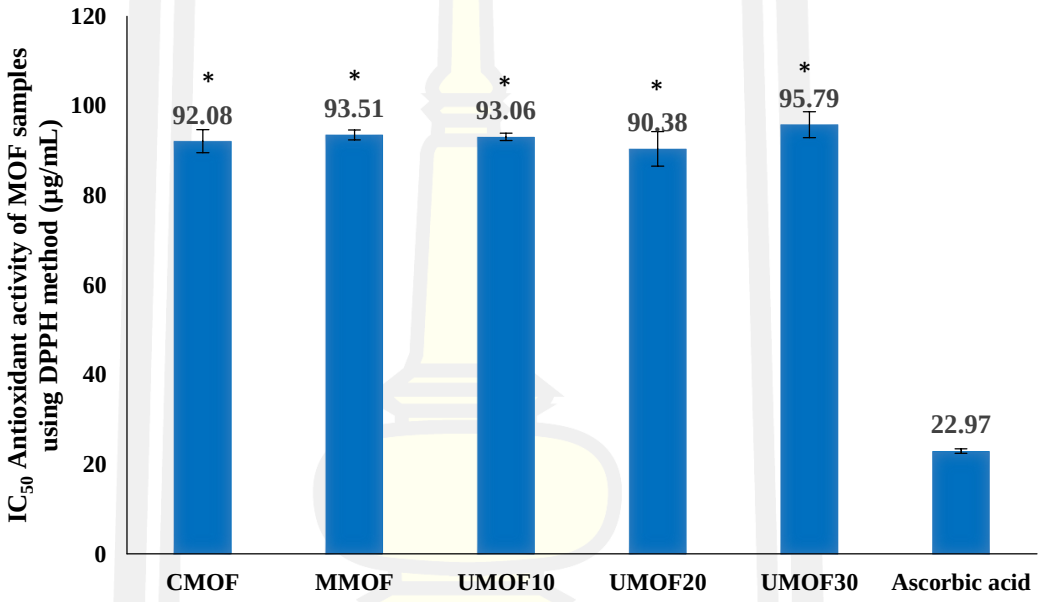
[*] Statistically significant difference as compared to CMOF (p<0.05).

[a,b] Statistically significant difference within the samples (p<0.05).

4.2 *In vitro* assays

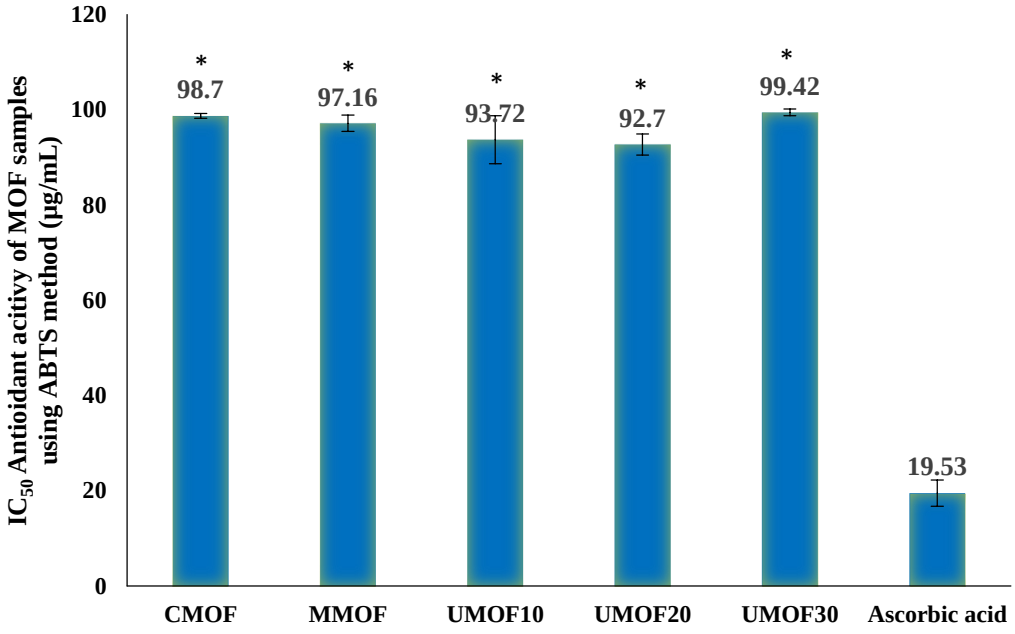
4.2.1 Antioxidant activities of MOF samples using DPPH and ABTS assays.

The IC_{50} values of all MOF by the DPPH or ABTS radicals assays were in the range of 90.38 - 99.42 µg/mL as shown in Figure 20-21. The antioxidant activity of all sample were no significant differences (p>0.05) and ascorbic acid exhibited the potent antioxidant activity more than of MOF samples.



*represent statistically significance compared with ascorbic acid. ($p < 0.05$)

Figure 20 IC_{50} values of MOF samples on DPPH reaction

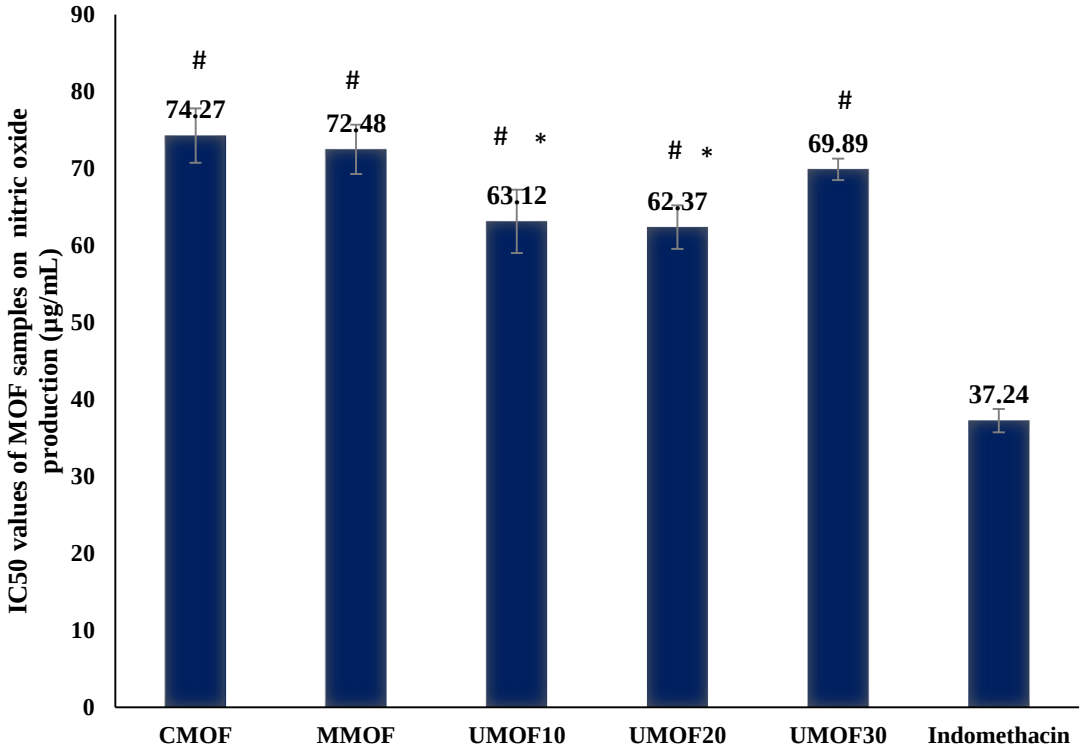


*represent statistically significance compared with ascorbic acid. ($p < 0.05$)

Figure 21 IC_{50} values of MOF samples on ABTS reaction

4.2.2 Anti-inflammatory and cytotoxicity activities using nitric oxide method

LPS was used to induce inflammatory condition and NO were measured to investigate the anti-inflammation activity of MOF samples. It was found that the IC_{50} values of MOF samples on NO production were in the range of 62.37 ± 2.82 - 74.27 ± 3.54 µg/Ml as shown in Figure 22. The UMOF10 and UMOF20 samples showed the highest anti-inflammatory activity with a significant difference when compared to CMOF and UMOF30 samples (p<0.05). The IC_{50} values of UMOF10 and UMOF20 were 63.12 ± 4.12 and 62.37 ± 2.82 µg/mL, respectively. Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), exhibited higher potency than all MOF samples in terms of anti-inflammatory activity (p<0.05).



* represents statistically significance compared with CMOF ($p < 0.05$)

#represents statistically significance compared with Indomethacin ($p < 0.05$)

Figure 22 IC_{50} values of MOF samples on NO production

The MTT assay is a commonly used assay to assess cell viability and proliferation based on the ability of viable cells to convert a yellow tetrazolium salt into a purple formazan product. This statement means that when the MTT assay was used to evaluate the cell viability of cells treated with MOF samples at a concentration of 1- 100 µg/mL, the percentage of surviving cells was greater than 90%.was shown in Table 10.

Table 10 Cytotoxic determination of MOF samples

	Percent cell viability			
Samples	**1 µg/mL**	**10 µg/mL**	**50 µg/mL**	**100 µg/mL**
CMOF	96.55 ± 5.34	93.53 ± 8.89	97.24 ± 8.43	99.63 ± 1.67
MMOF	94.62 ± 4.38	97.39 ± 1.73	95.70 ± 2.63	96.87 ± 4.44
UMOF10	98.87 ± 1.63	93.37 ± 2.76	95.92 ± 6.09	95.94 ± 3.67
UMOF20	97.55 ± 1.76	96.20 ± 2.80	94.90 ± 3.74	94.50 ± 4.98
UMOF30	98.74 ± 1.35	96.34 ± 3.55	98.30 ± 4.34	97.97 ± 2.64
Indomethacin	99.43 ± 1.49	100.70 ± 1.92	99.83 ± 0.52	98.02 ± 1.08

The MOF samples were tested for their antioxidant, anti-inflammatory, and cytotoxic activities in vitro, and the IC_{50} values of MOF samples for DPPH and ABTS assays were in the range of 90.38 - 99.42 µg/mL. UMOF10 and UMOF20 samples showed higher anti-inflammatory activity than CMOF and UMOF30 samples, with IC_{50} values of 63.12 ± 4.12 and 62.37 ± 2.82 µg/mL, respectively. Additionally, the cell viability of MOF samples at a concentration of 1 - 100 µg/mL was higher than 90% in the MTT assay. Overall, the MOF samples exhibited promising antioxidant and anti-inflammatory activities without significant cytotoxicity at a concentration of 100 µg/mL was shown in Table 11.

Table 11 Antioxidant, anti-inflammatory and cytotoxicity activities of Mahajak samples

Samples	IC_{50} (µg/mL)			% Cell viability (at concentration 100 µg/mL)
	DPPH	ABTS	LPS-induced NO	
CMOF	92.08 ± 2.57 *	98.71 ± 0.49 *	74.27 ± 3.54 *a	95.04 ± 3.08
MMOF	93.51 ± 1.13	97.16 ± 1.73	72.48 ± 3.21	96.87 ± 4.44
UMOF 10	93.06 ± 0.85 *	93.72 ± 5.04 *	63.12 ± 4.12 *b	97.37 ± 2.20
UMOF 20	90.38 ± 3.86 *	92.71 ± 2.21 *	62.37 ± 2.82 *b	95.36 ± 4.82
UMOF 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).

4.2.3 Antibacterial activity using disc diffusion and microdilution method.

The study aimed to evaluate the antibacterial properties of MOF samples against *Staphylococcus pyogenes* DMST 17020 and *Staphylococcus aureus* DMST 8440 using several methods. The disc diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) were utilized in the investigation.

The disc diffusion method involves placing paper discs that have been impregnated with the MOF sample on a culture plate. The culture plate contains bacterial cells that have been previously spread on the surface of the agar medium. The presence of the MOF samples on the discs results in the diffusion of the MOF molecules into the agar medium. This diffusion leads to the formation of a zone of inhibition around the disc where bacterial growth is prevented. The diameter of the zone of inhibition is a measure of the antibacterial activity of the MOF sample.

The minimum inhibitory concentration (MIC) is the lowest concentration of the MOF sample that inhibits bacterial growth. The MOF sample is tested by preparing a series of dilutions and adding them to wells in a microtiter plate. A bacterial suspension is then added to each well, and the plate is incubated. The lowest

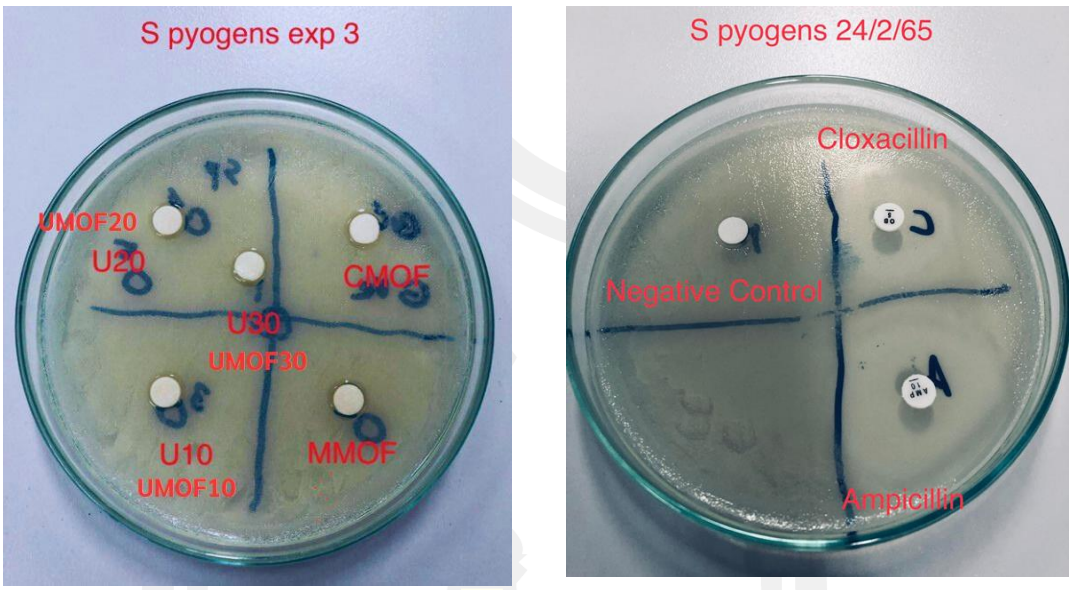
concentration of the MOF sample that inhibits bacterial growth is determined by examining the wells for bacterial growth.

The minimum bactericidal concentration (MBC) is the lowest concentration of the MOF sample that kills the bacterial cells. The MOF sample is tested using a similar method as the MIC determination. However, after the incubation period, an aliquot from each well that does not show any visible bacterial growth is transferred onto a fresh agar medium to determine if the bacterial cells were killed or not.

In conclusion, the study investigated the antibacterial activity of MOF samples against *Staphylococcus pyogenes* DMST 17020 and *Staphylococcus aureus* DMST 8440 using the disc diffusion method, MIC, and MBC. These methods provide different measures of the antibacterial activity of MOF samples, which can be useful for developing new antimicrobial agents., as shown in Table 12 and Figure 23-25.

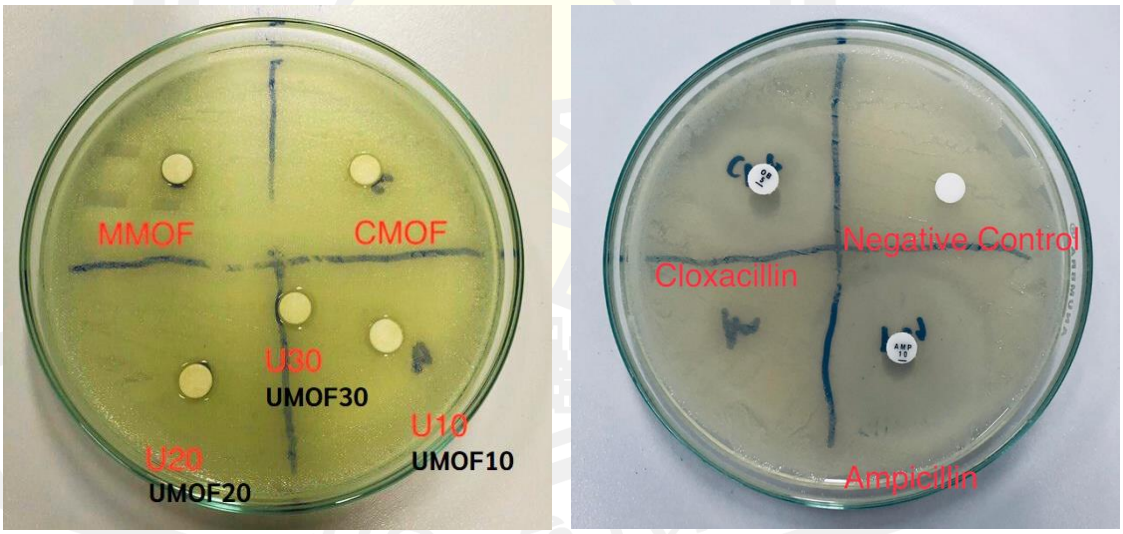
Table 12 Antibacterial activities of MOF samples against bacterial test organisms by disc diffusion and microdilution method

MOF samples	**Diameter of zone of inhibition (mm)**		**The MIC (µL/mL)**		**The MBC (µL/mL)**	
	S. pyogens DMST 17020	*S. aureus* DMST 8440	*S.pyogens* DMST 17020	*S.aureus* DMST 8440	*S. pyogens* DMST 17020	*S. aureus* DMST 8440
CMOF	8.67±0.416	8.13±0.115	15.38	18.18	>15.38	>18.18
MMOF	8.53±0.416	8.33±0.115	15.38	18.18	>15.38	>18.18
UMOF10	9.06±0.231	9.06±0.230	15.38	18.18	>15.38	>18.18
UMOF20	10.3±0.264	9.06±0.115	15.38	18.18	>15.38	>18.18
UMOF30	9.47±0.987	9.20±0.200	15.38	18.18	>15.38	>18.18
AMPICILLIN	32.00±0.200	30.67±0.305				
CLOXACILLIN	23.37±2.514	25.00±0.529				



(A) (B)

Figure 23 Disc diffusion test of MOF samples against *S. pyogens* DMST 17020 (A) zone of inhibition diameter (B) negative control and positive control (Cloxacillin and Ampicillin)



(A) (B)

Figure 24 Disc diffusion test of MOF samples against *S. aureus* DMST 8440 (A) zone of inhibition diameter (B) negative control and positive control (Cloxacillin and Ampicillin)

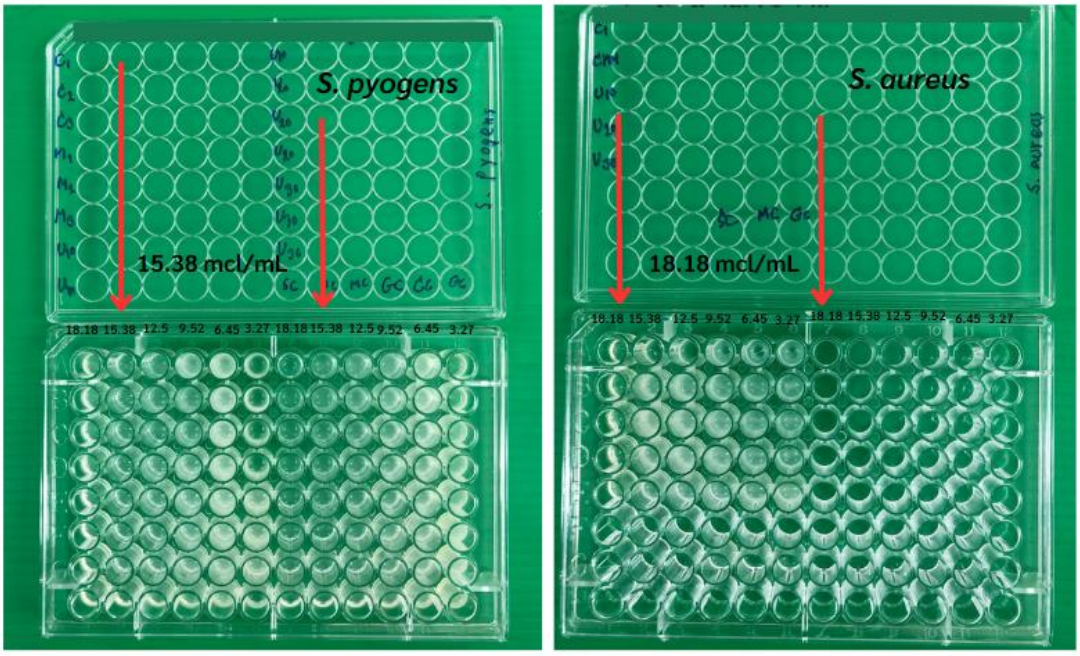


Figure 25 Minimum inhibitory concentration (MIC) values of MOF samples against *S. pyogens* DMST 17020 and *S. aureus* DMST 8440

The study found that the MOF samples exhibited antibacterial activity, with inhibition zones against *S. pyogens* DMST 17020 in the range of 8.53-10.30 mm and against *S. aureus* DMST 8440 in the range of 8.13-9.12 mm, respectively, when a disc containing 15 µL of MOF samples was used. Minimum inhibitory concentration (MIC) values of 15.38 and 18.18 µL/mL and minimum bactericidal concentration (MBC) values of more than 15.38 µL/mL and 18.18 µL/mL were obtained for *S. pyogenes* DMST 17020 and *S. aureus* DMST 8440, respectively. Therefore, the study suggests that MOF samples have potential as a treatment for skin infections

4.3 GC-MS analysis for MOF samples

4.3.1 Chemical profile of MOF samples

GC-MS analysis was conducted on the MOF samples to identify their phytochemical components. The analysis revealed a total of 41, 33, 40, 33, and 29 components for CMOF, MMOF, UMOF10, UMOF20, and UMOF30, respectively (Table 13). The majority of the identified components were ketones, alkenes, benzenes, and aldehydes, which accounted for more than 97% of the chromatographic area.

Among the identified components, the major ones included 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 were found to increase when the UAE technique was applied to the MOF preparation. The percentage composition of these compounds varied in different MOF samples.

Table 13 GC-MS fingerprints of MOF samples

Retention time (min)	**Compounds**	**% Peak areas**				
		CMOF	**MMOF**	**UMOF10**	**UMOF20**	**UMOF30**
5.24	Alpha-pinene	0.06	0.11	0.06	0.05	0.05
5.85	Sabinene	0.51	0.75	0.55	0.39	0.59
5.92	Beta-pinene	0.51	1.19	0.81	0.62	0.83
6.71	D-limonene	0.47	0.66	0.4	0.31	0.41
7.17	Gamma-terpinene		0.04	0.05	0.07	
7.32	Trans-sabinene hydrate acetate		0.03	0.17	0.13	0.17
7.68	Fenchone	0.05	0.08	0.08	0.08	0.07
7.8	Linalool			0.15	0.18	0.15
7.85	Cis-2-norbornanol	0.1	0.15	0.12	0.12	0.13
7.93	Grandlure III	1.08	1.62	1.57	1.6	1.56
7.99	Isofenchol	0.13	0.22	0.19	0.17	0.19
8.15	Camphor	0.11	0.14	0.16	0.1	0.12

Table 13 (Continue)

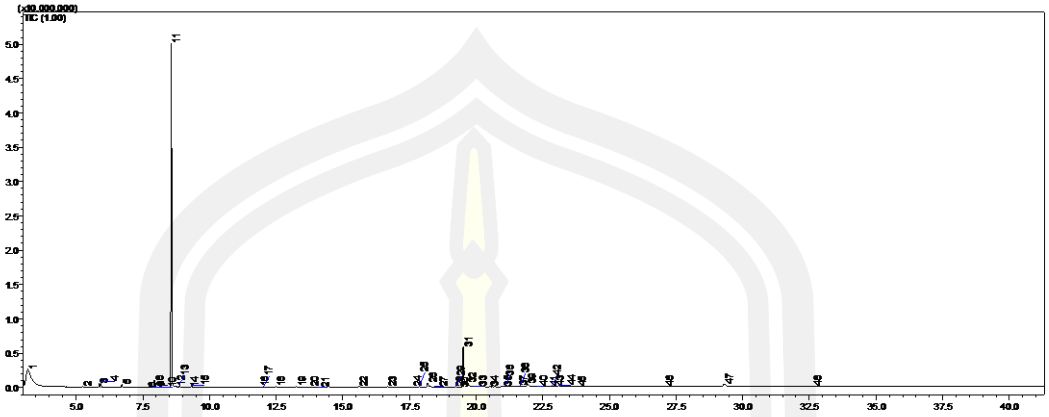
Retention time (min)	Compounds	% Peak areas				
		CMOF	MMOF	UMOF10	UMOF20	UMOF30
8.57	D-camphor	51.99	81.28	71.42	77.31	78.73
8.75	Borneol	0.14	0.22	0.18	0.2	0.2
8.81	4-methyl-1-propan-2-ylbicyclo[3.1.0]hexan-3-one		0.02			
8.88	Endo-borneol	0.37	0.6	0.48	0.53	0.6
9.03	Terpinene 4-acetate			0.07	0.13	0.07
9.1	[(Z)-(5,5,6-trimethyl-2-bicyclo[2.2.1]heptanylidene)amino]thiourea			0.03	0.04	
9.23	Terpineol	0.1	0.13	0.27	0.3	0.28
9.32	Endo-isocamphone	0.31	0.5	0.45	0.52	0.51
9.73	Beta-citronellol			0.12		
11.86	Copaene	0.08	0.09	0.04	0.04	0.07
12.035	Beta.-Copaene		0.11			
12.037	Beta-cubebene			0.05		0.07
12.04	Germacrene D	0.11				
12.48	Caryophyllene	0.06	0.09	0.06	0.07	0.08
12.92	Alpha-Humulene		0.03			
13.26	Tau-cadinol acetate	0.07			0.06	0.08
13.73	D-cadinene	0.09	0.11	0.07	0.06	0.07
14.07	Elemol	0.06	0.08			
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	0.42	0.79	0.62	0.49
18.25	3-methyl-5-ethyl-4-propylidene-cyclohex-2-ene		0.08	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	0.07	0.15	0.09	
19.51	Cis-9-hexadecenal	11.65	4.68	9.9	6.88	5.7
19.66	Octadecanoic acid	1.56	0.56	1.1	0.78	0.61

Table 13 (Continue)

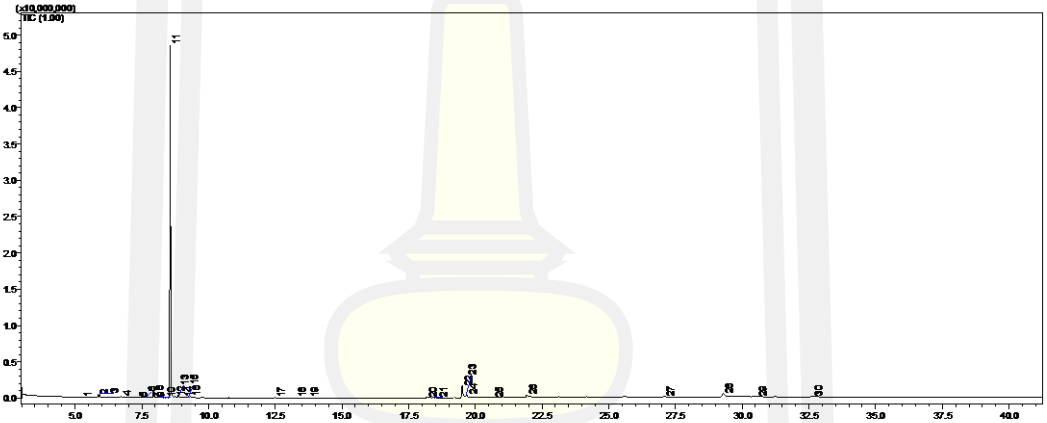
Retention time (min)	Compounds	% Peak areas				
20.49	Octadecane (CAS) n-octadecane	0.05		0.03		
20.68	2,2,4,4,7,7-hexamethyl-1,3,3a,7a-tetrahydroindene			0.06	0.05	
21.01	(Z)-octadec-9-enamide	0.26		0.29	0.42	
21.09	Icosane	0.1		0.12		
21.66	Nonacosane	0.15		0.05		
21.92	Oxypeucedanin	0.88	1.1	1.57	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		0.56	0.26		
27.07	Gamma-tocopherol	0.3	0.34	1.54	0.54	0.38
29.27	Sesamin	1.77	2.6	4.1	3.89	3.95
32.63	Gamma-sitosterol	0.4	0.74	1.54	1.35	1.07

The GC-MS analysis of the MOF samples obtained from the conventional, modified conventional, and UAE preparation methods showed very similar chromatograms. This suggests that the preparation methods did not significantly affect the chemical composition of the MOF samples, as presented in Figure 26.

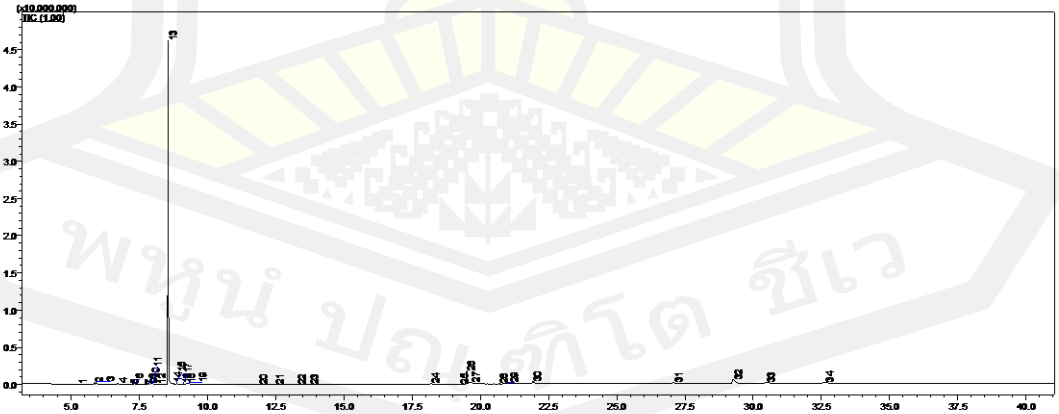
(A) CMOF



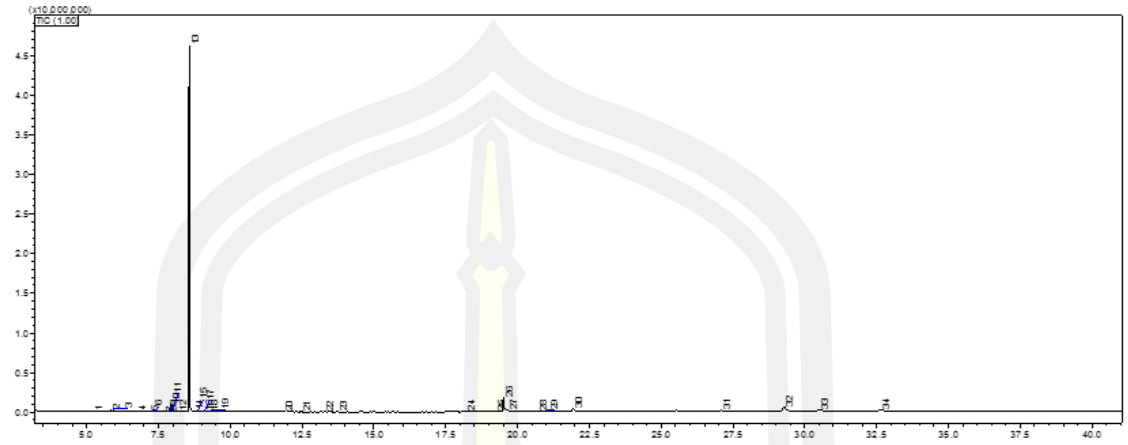
(B) MMOF



(C) UMOF10



(D) UMOF 20



(E) UMOF 30

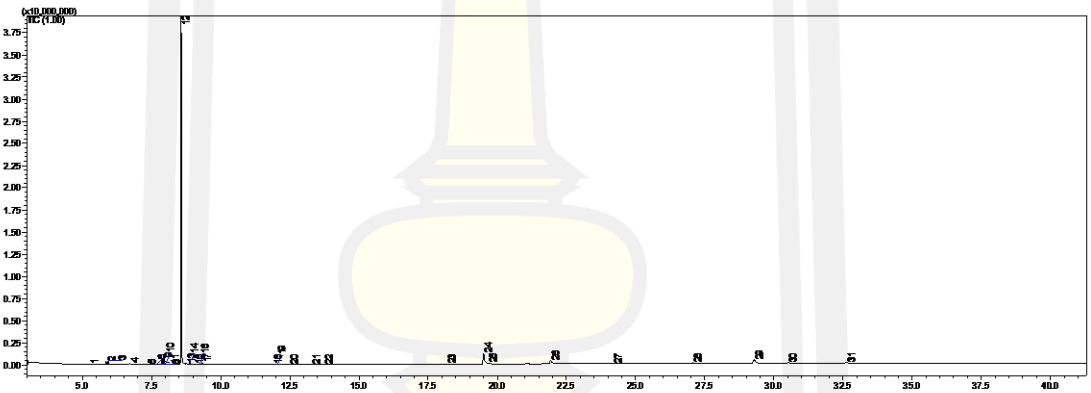


Figure 26 GC-MS Chromatogram of MOF samples. (A) CMOF, (B) MMOF, (C) UMOF1, (D) UMOF20 and (E) UMOF30

4.3.2 Principal components analysis

The ten characteristic components of the MOF samples are beta-pinene, grandlure III, D-camphor, palmitic acid, cis-9-hexadecenal, octadecanoic acid, oxypeucedanin, .gamma.-tocopherol, (+)-sesamin and gamma.-sitosterol that occurred in high quantities was used for the principal component analysis (Table 14), eigenvalues of the principal components, their contribution, and cumulative contribution (Table 15). The cumulative contribution of the first and second principal components (prin1 and prin2) was 89.79%, reflecting the originality of all variables selected as effective components during data analysis.

The first principal component (prin1) accounted for 64.00% of the total variation (Table 15) and was negatively correlated with palmitic acid, cis-9-hexadecenal, and octadecanoic acid, whereas it was positively correlated with beta-pinene, grandlure III, D-Camphor, oxypeucedanin. gamma.-tocopherol, (+)-sesamin and gamma.-sitosterol. This indicated that three of the relative contents of palmitic acid, cis-9-hexadecenal, and octadecanoic acid were reduced, while the relative contents of cis-9-hexadecenal, octadecanoic acid, n-hexadecanoic acid, and octadecanoic acid increased when the MOF samples were prepared by the modified and UAE methods compared to the conventional preparative method.

However, the second principal component (prin2) was negatively correlated with the content of beta-pinene and D-camphor and positively correlated with the content of grandlure III, palmitic acid, cis-9-hexadecenal, octadecanoic acid, oxypeucedanin, .gamma.-tocopherol, (+)-sesamin, and gamma.-sitosterol. This could be explained that as compared to other preparative methods modified and the UAE method resulted in a lower relative content of beta-pinene and D-camphor and higher relative contents of grandlure III, palmitic acid, cis-9-hexadecenal, octadecanoic acid, oxypeucedanin,. gamma.-tocopherol, (+)-sesamin and gamma.-sitosterol.

Table 14 Principal component analysis of ten major compounds of MOF samples

Compounds	**Fraction[a] (%)**					**Eigenvectors**	
	CMOF	MMOF	UMOF10	UMOF20	UMOF30	Prin1[b]	Prin2
beta-Pinene	0.51	1.19	0.81	0.62	0.83	0.2566	-0.3502
Grandlure III	1.08	1.62	1.57	1.6	1.56	0.3872	0.0273
D-Camphor	51.99	81.28	71.42	77.31	78.73	0.3867	-0.1166
Palmitic acid	1.55	0.42	0.79	0.62	0.49	-0.3822	0.1165
cis-9-Hexadecenal	11.65	4.68	9.9	6.88	5.7	-0.3258	0.3460
Octadecanoic acid	1.56	0.56	1.1	0.78	0.61	-0.3646	0.2325
Oxypeucedanin	0.88	1.1	1.57	1.3	1.85	0.2759	0.2826
.gamma.-Tocopherol	0.3	0.34	1.54	0.54	0.38	0.0842	0.5294
(+)-Sesamin	1.77	2.62	4.1	3.89	3.95	0.3054	0.3756
gamma.-Sitosterol	0.4	0.74	1.54	1.35	1.07	0.2618	0.4518

[a] Fraction of component in MOF samples (%) = peak area of a component in MOF samples/total peak area of identified components in MOF samples.

[b] Prin1 and Prin2 represent the first and the second principal components.

Eigenvalues of Prin1 and Prin2 are 6.40 and 2.54, respectively. Eigenvalues greater than 1 are suitable for selecting the principal component. The proportion and cumulative contribution of Prin1 and Prin2 from ten major compounds in MOF samples, as shown in Table 15

Table 15 Eigenvalues of the principal components and their contribution and cumulative contribution

components	Eigenvalue	difference	proportion	cumulative
Prin1[a]	6.40224	3.85955	0.6400	0.6400
Prin2	2.54069	1.81744	0.2541	0.8941
Prin3	0.72325	0.38743	0.0723	0.9664
Prin4	0.33581	0.33581	0.0336	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
Prin10	0	0	0.0000	1.0000

[a] Prin represents the principal component, the order is from the first to the ten.

4.3.3 Cluster analysis

The dendrogram in Figure 27 was used to diagnose and characterize the correlation among the CMOF, MMOF, and UAE preparative methods (UMOF10, UMOF20, and UMOF30) by showing the dissimilarity of chemical profiles of MOF samples. The cluster analysis was performed using the average distance method, which grouped the dataset into four clusters.Cluster 4 (CL4) was formed by UMOF20 and UMOF30, with the lowest average distance, indicating that both UMOF20 and UMOF30 have the highest similarity in their chemical profiles. CL3 was formed by MMOF and CL4, CL2 was formed by UMOF10 and CL3, and CL1 was formed by CMOF and CL2. CL3, CL2, and CL1 showed inferior average distances, indicating a chemical profile similar to that before CL1.

The cluster analysis revealed that the chemical profile of MMOF, UMOF10, UMOF20, and UMOF30 are similar, which suggests that the preparation of MOF by ultrasonic extraction at times 10, 20, and 30 minutes showed similar chemical profiles. Figure 18 shows the similarity in the chemical profiles of the MOF samples based on hierarchical analysis.

Overall, the dendrogram indicated that the dataset could be divided into four groups, and UMOF20 showed high similarity with the UAE 30 samples with the lowest average distance, while the UMOF10 and CMOF samples were separated.

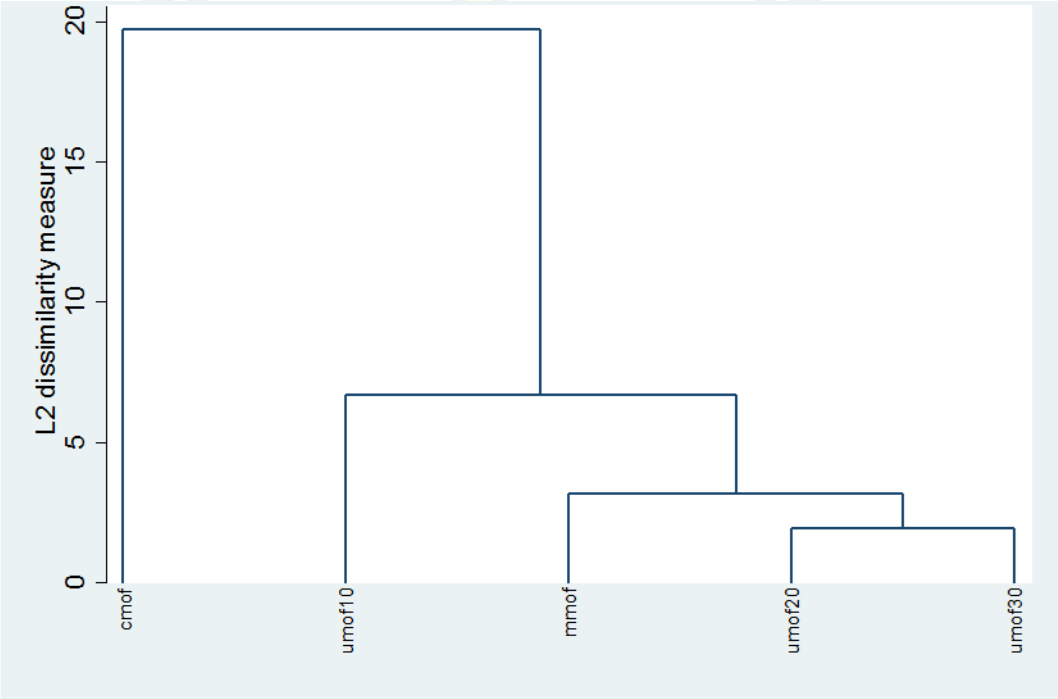


Figure 27 Dendogram for cluster analysis of the main compounds of MOF samples. There are four clusters: CL4 formed by UMOF20 and UMOF30, CL3 formed by MMOF and CL4, CL2 formed by UMOF10 and CL3, and CL1 formed by CMOF and CL2.

The study results showed that UMOF20 had a significantly different percentages of oil reovery compared to all other groups of MOF samples except for UMOF30. Additionally, UMOF20 exhibited better anti-inflammatory activity with a lower IC_{50} value. However, there was no statistically significant difference in antioxidant and antibacterial activity tests between the preparation methods of MOF samples. The chromatograms of UMOF20 were also similar to CMOF samples. Based on these findings, UMOF20 was chosen for further development using ethosomal technology.

4.4 Ethosomes preparation of MOF

4.4.1 Quality assurance of ethosomes dosage form

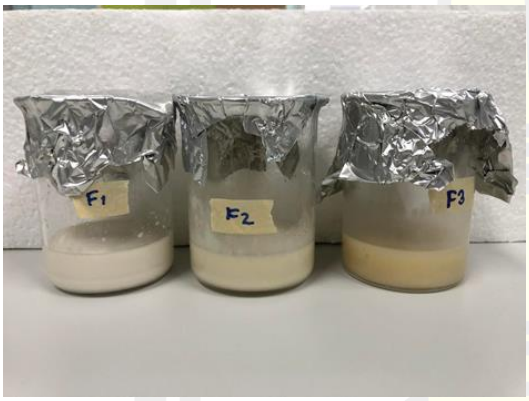
Using the classical cold method, the optimized MOF sample (UMOF20) was formulated into 9 formulas to ethosomal dosage form.[168] Sonication was used to reduce the size of ethosomal systems. The ethosomal system is comprised of 2 – 4% soya phosphatidylcholine (SPC), 20–40% ethanol, 1 mL of Span 20, 0.5 mL of Tween 20 and the formulation was adjusted to 100 % w/w with distilled water. The characterization of the prepared ethosome was evaluated in terms of physical appearance, particle size, size distribution, zeta potential, and entrapment efficiency. The physical appearance and vesicle morphology of the ethosome were visualized as shown in Table 16, Figure 28, and Figure 29 , respectively. The prepared ethosome has a milky, white liquid appearance. The microscopic evaluation showed the morphology of ethosomes. The ethosome vesicles were spherical shape (Figure 29).

The physical appearance and vesicle morphology of ethosomes are shown in Table 1 and Figures 19 and 20, respectively. The pH for all the formulations was in the range of 4.31 ± 0.05 – 4.77 ± 0.02 at 25°C as shown in Table 16.

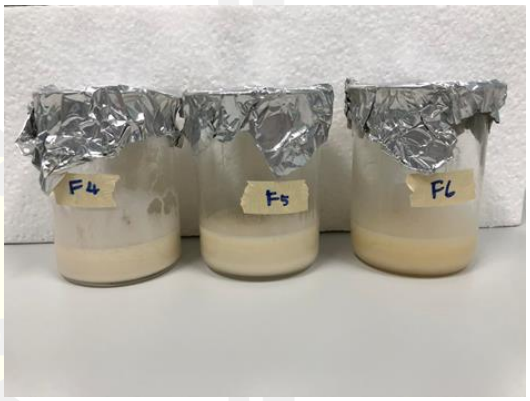
Table 16 Physical characteristics of MOF ethosomal batches

Formulation code	Physical appearances	pH
F1	Milky white liquid	4.77±0.02
F2	Milky white liquid	4.37±0.01
F3	Milky white liquid	4.46±0.01
F4	Milky white liquid	4.62±0.01
F5	Milky white liquid	4.65±0.04
F6	Milky white liquid	4.31±0.05
F7	Milky white liquid	4.53±0.01
F8	Milky white liquid	4.62±0.04
F9	Milky white liquid	4.44±0.04

Data represented as mean±SD, n=3.



(A) Formulations F1-F3



(B) Formulations F4-F6

(C) Formulations F7-F9

Figure 28 Physical characteristics of MOF ethosomal batches

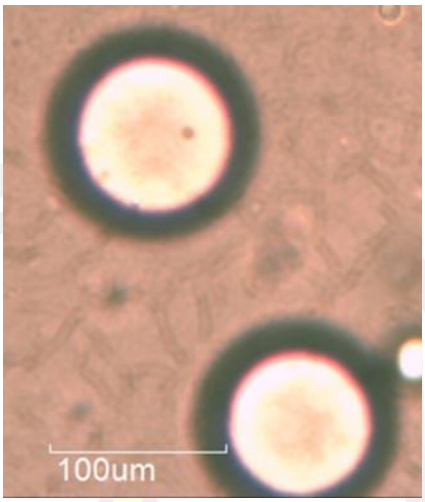
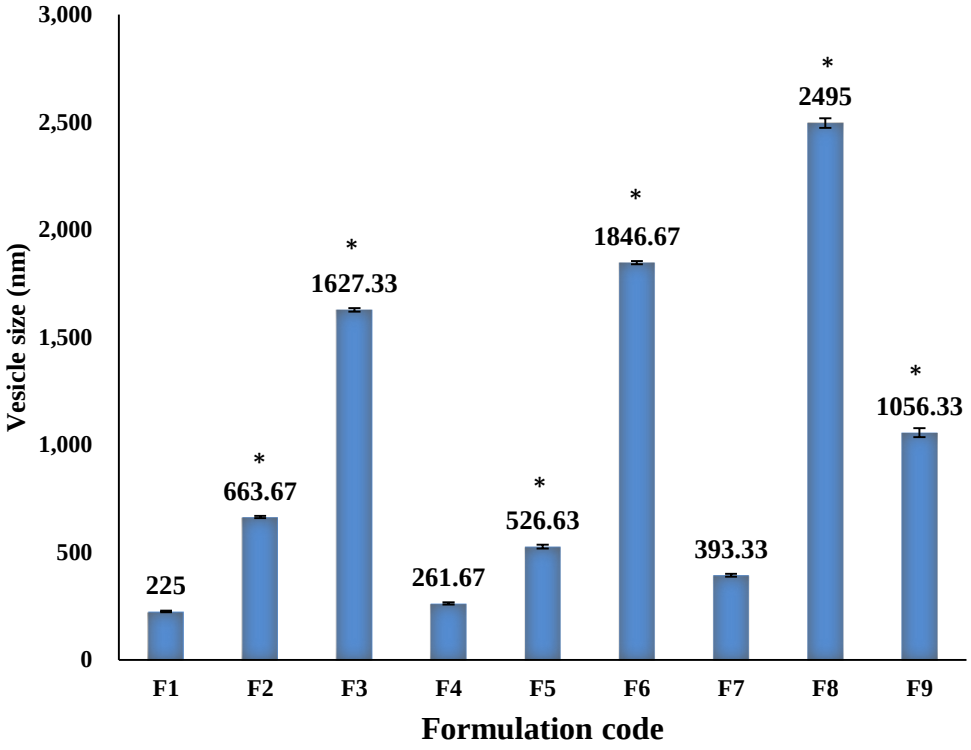


Figure 29 The microscopic of UMOF 20 F1 ethosomes

4.4.1.1 Vesicle size

Among the 9 formulations of ethosomes containing UMOF20, only F1 and F4 had vesicle sizes within the desired range of less than 300 nm. The vesicle size of F1 was 225.00 ± 3.60 nm, while the vesicle size of F4 was 261.67 ± 5.13 nm. The other formulations did not meet the size criteria and showed statistically significant differences compared to the optimized ethosome formulation (p<0.05), as shown in Figure 30.

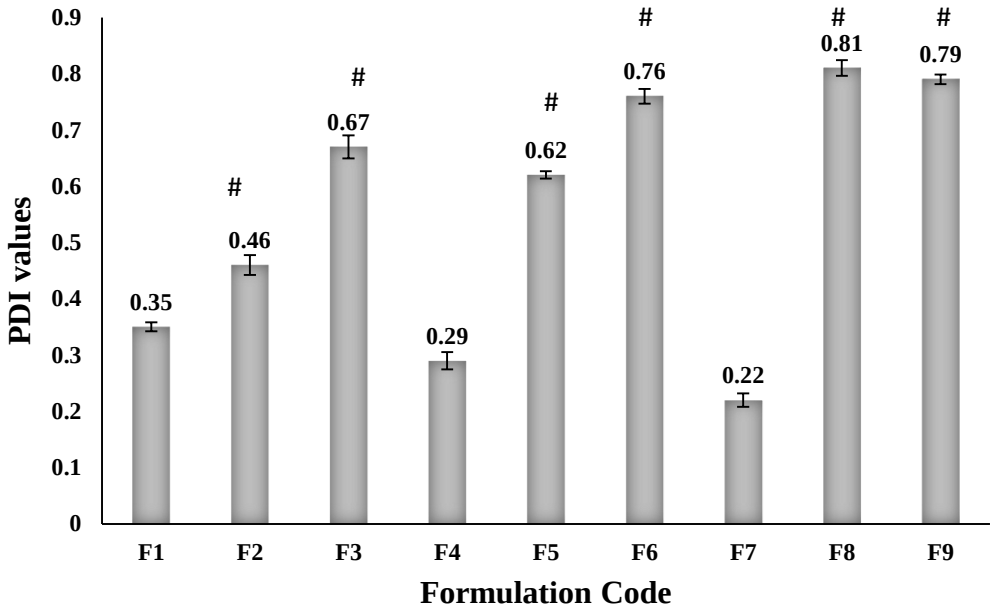


* represents statistically significance compared with F1 and F4 ($p < 0.05$)

Figure 30 Vesicle size of UMOF20 ethosomes.

4.4.1.2 Size distribution

The homogeneity of prepared ethosomes based on their vesicle size distribution was evaluated using polydispersity index (PDI). The formulated batches showed a range of PDI values from 0.22±0.01 to 0.81±0.01, as shown in Figure 31, indicating a narrow to broad size distribution. Optimized ethosomes such as F1, F4, and F7 exhibited PDI values less than 0.5, which is acceptable. Therefore, the optimized ethosomes containing UMOF20 were considered to have a narrow size distribution.



[#] represents statistically significance compared with F1, F4 and F7 ($p < 0.05$)

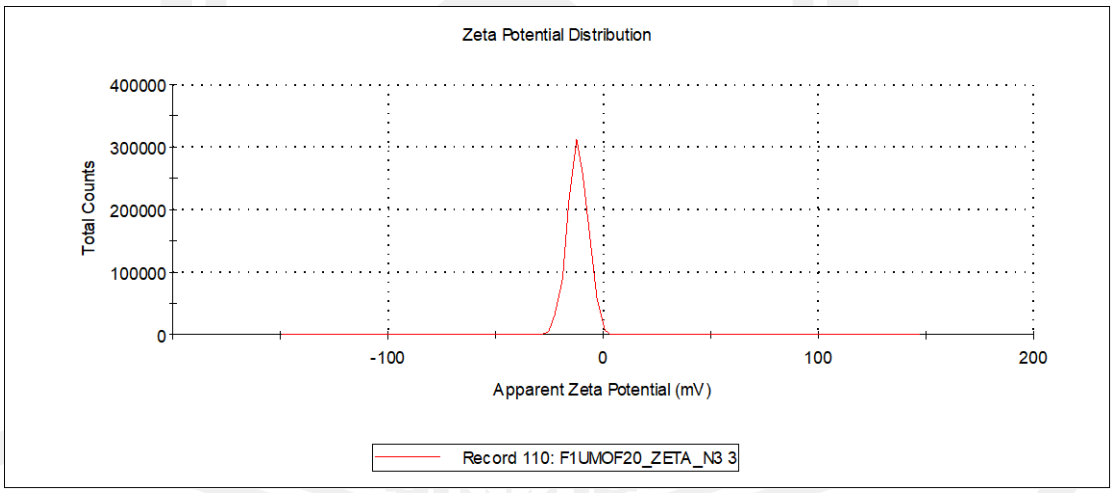
Figure 31 PDI values of the UMOF20 ethosomes.

4.4.1.3 Zeta potential

The zeta potential (ZP) was determined using a Zetasizer as it is an important parameter affecting stability. Generally, particles with zeta potential values greater than +30 mV or less than −30 mV are considered stable as they prevent aggregation between vesicles due to electrostatic repulsion and indicate the physical stability of vesicular systems. The value of the optimized ethosomal formulation (F1) was found to be -11.87±0.55 mv. Among the formulations, it was found that F1 had the lowest zeta potential and the best stability (statistically significant, $p < 0.05$) when compared to other formulations. The result of zeta potential from UMOF20 ethosomes is shown in Figure 32.

* represents statistically significance compared with F1 and F7 ($p < 0.05$)

Figure 32 Zeta potential of the UMOF20 ethosomes.



Apparent Zeta potential 12 mV of UMOF20 F1 ethosome

Figure 33 Zeta potential of UMOF20 F1 ethosome

4.4.1.4 Entrapment efficiency

The ultracentrifugation technique was used to measure the entrapment efficiency (EE) of UMOF20 ethosomes using an indirect method. In the ethosome system, all formulations (F1-F9) had a percent entrapment efficiency (% EE) of more than 80% for sesamin and sesamolin. The ranges of % EE of ethosomes from F1-F9 were between 88.21 ± 3.05% - 94.13 ± 0.21% of sesamin and 88.80 ± 3.05% - 99.14 ± 0.07% of sesamolin, respectively. It was found that F1 had the highest % EE of 94.13 ± 0.21 sesamin and 99.14 ± 0.07 sesamolin, respectively, but this was not statistically significant (p>0.05) compared to other formulations. The results of this study are shown in Figure 33.

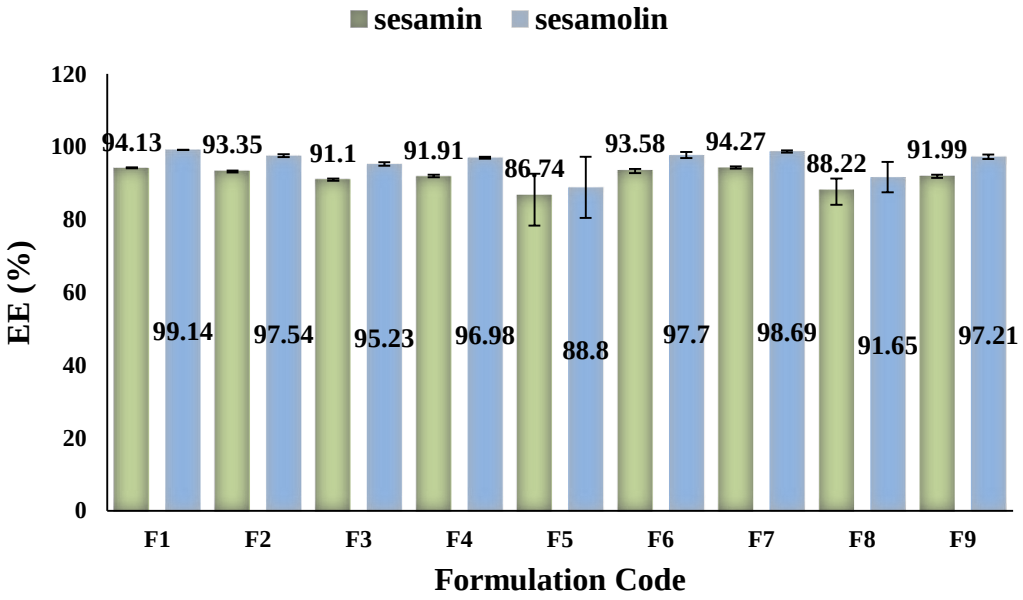


Figure 34 Percent entrapment efficiency (% EE) of the UMOF20 ethosomes.

The optimized ethosomes containing UMOF20 were found to have a particle size of less than 300 nm with a narrow size distribution, a zeta potential of more than +30 mV or −30 mV, and an entrapment efficiency of more than 50%. Based on these criteria, F1 was identified as the optimized ethosome formulation, which included 2% phospholipid, SPAN20: Tween20 (1:0.5 mL ratio) as the lipid component, and 20% ethanol as the dispersion medium. F1 resulted in an ethosome with a vesicular size of 225.00±3.60 nm (polydispersity index PI = 0.35±0.01), a zeta potential of -11.87±0.55 mV, and an efficient entrapment of 94.13±0.21 % of sesamin and 99.14±0.07 % of sesamolin, as shown in Table 17

Table 17 Vesicle size, polydispersity index and zeta potential of MOF ethosomal batches

Formulation code	Vesicle Size (nm)	PDI	Zeta potential (mV)	EE (%) Sesamin	EE (%) Sesamolin
F1 (n=3)	225.00±3.60	0.35±0.01	-11.87±0.55	94.13±0.21	99.14±0.07
F2 (n=3)	663.67±5.50	0.46±0.01	-1.18±0.03	93.35±0.08	97.54±0.40
F3 (n=3)	1627.33±8.08	0.67±0.02	-1.10±0.04	91.09±0.12	95.23±0.50
F4 (n=3)	261.67±5.13	0.29±0.01	-7.87±0.37	91.91±0.38	96.98±0.26
F5 (n=3)	526.63±8.67	0.62±0.02	-0.61±0.39	86.73±5.80	88.80±8.40
F6 (n=3)	1846.67±7.64	0.76±0.01	-0.92±0.19	93.58±0.35	97.69±0.82
F7 (n=3)	393.33±6.02	0.22±0.01	-1.04±0.35	94.26±0.35	98.69±0.27
F8 (n=3)	2495.00±22.71	0.81±0.01	-0.49±0.12	88.21±3.05	91.64±4.15
F9 (n=3)	1056.33±20.50	0.79±0.01	1.03±0.01	94.13±0.28	97.21±0.60

Data represented as mean±SD, n=3. PDI: Polydispersity index, EE: Entrapment efficiency

4.4.1.5 Stability of ethosomes

A quality control study of the UMOF20 ethosomes' nine formulas showed that F1 is an optimized ethosome that meets the criteria of the ethosome system. As a result, F1 was chosen for the stability test. The ability of ethosomal formulations to retain the stability was tested by keep the preparations at 40 ± 2 °C (accelerated storage condition) for various time periods (30, 60, and 90 days). The stability of ethosomes can also be determined by monitoring the vesicle size, size distribution, zeta potential, pH and physical characteristics of ethosomes. The result is shown in Table 18.

Table 18 The stability of UMOF20 ethosome F1 was tested under accelerated storage conditions at 40 ± 2 °C for 30 days, 60 days, and 90 days.

Periods of time (days)	Vesicle Size (nm)	PDI	Zeta potential (mV)	pH
30	239.12±3.65	0.35±0.01	-11.8±0.32	4.56±0.10
60	474.38±3.79[a]	0.52±0.02[a]	-0.10±0.04[a]	4.94±0.04
90	894.89±4.50[a, b]	0.89±0.04[a, b]	1.75±0.04[a]	4.67±0.04

[a]represents statistically significance compared with periods of time 30 days ($p < 0.05$)

[b]represents statistically significance compared with periods of time 30 and 60 days ($p < 0.05$)

The stability of the most satisfactory formulation of UMOF20 ethosome F1 was evaluated at 40 ± 2°C for 90 days. After 30 days, the ethosome appeared as a milky liquid with no separation. The vesicle size, PDI, zeta potential and pH were 239.12 ± 3.65 nm, 0.35 ± 0.01, -11.8 ± 0.32 mV, and 4.56 ± 0.10 respectively. Based on these results, it was concluded that UMOF20 ethosome F1 was stable. However, after the stability test at 60 and 90 days, the vesicle size, PDI and zeta potential were increased, indicating that the ethosome system tended to decrease in stability.

The stability test results indicate that the UMOF20 ethosome F1 is stable for the first 30 days, as there were no significant changes in the physical appearance and important parameters. However, after 60 and 90 days, there were significant increases ($p<0.05$) in vesicle size, PDI, and zeta potential, which indicate that the ethosome system was losing stability.

4.4.2 Evaluating the permeability of MOF ethosomes

4.4.2.1 *In vitro* skin permeation studies

In this study, in vitro skin permeation of the optimized ethosome formulation UMOF20 F1 was experimented using a modified Franz-type diffusion cell with a mixed cellulose esters (MCE) membrane. The ability of the ethosomal carrier to deliver sesamin through the MCE membrane was evaluated. The membrane

permeation of UMOF20 F1 was tested and compared with UMOF20, and the results are shown in Figure 22 and Table 21.

It is evident from the permeability profile that throughout the 24-hour assay period, the cumulative amount of sesamin that permeated through the MCE membrane in the UMOF20 F1 ethosome formulation was 23.46±0.06 μg/cm^2 (58.13±0.16 %), which was significantly higher (p<0.05) than that from UMOF20, which was only 0.55±0.02 μg/cm^2. In fact, very little sesamin was detected from UMOF20 in the receptor fluid throughout the experiment.

Furthermore, the steady state flux Jss (2.3907μg/cm^2/h) and the skin permeation coefficient Kp (0.06 ±0.09 x10^{-3}cm/h) from ethosome were significantly higher (p<0.05) than these from UMOF20 formulation. These results prove that the UMOF20 F1 ethosome was more effective in delivering sesamin than the UMOF20 formulation, as shown in Table 19.

Table 19 *In vitro* skin permeation parameters of sesamin from the UMOF20 ethosome F1 and UMOF20

Formulations	**Q_{24} (μg/cm^2)**	**%Q_{24}**	**Jss (μg/cm^2/h)**	**Kp(X10^{-3} cm/h)**
UMOF20 ethosome F1	23.46±0.06[a]	58.13±0.16[a]	1.92±0.08[a]	0.05±0.002[a]
UMOF20	0.55±0.02	1.40±0.05	UD*	UD*

Each data represents the mean±SD (n = 3). UD*, undetected; J_{ss}, steady state flux; K_p, permeation coefficient.

[a]represents statistically significance compared with UMOF20 (p < 0.05)

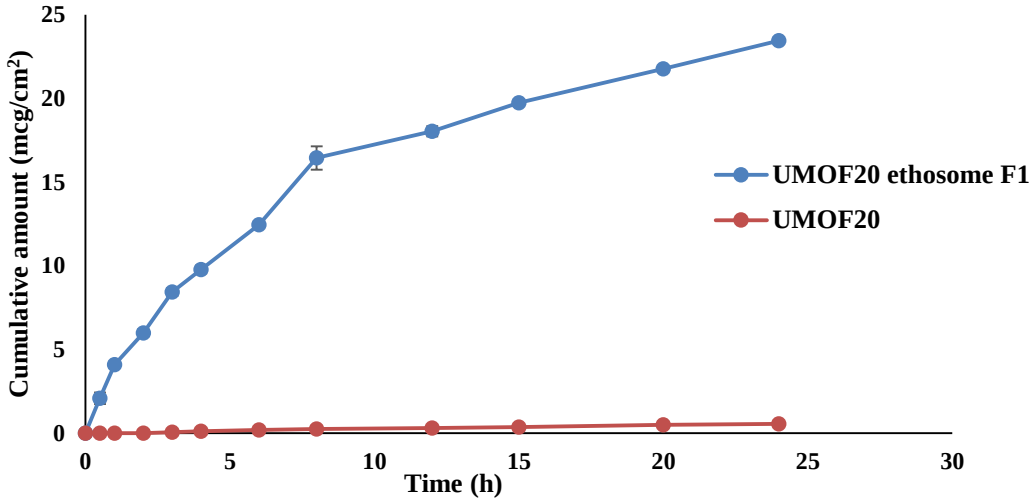


Figure 35 In vitro cumulative amount-time profiles of sesamin permeated across mixed cellulose esters (MCE) membrane, UMOF20 ethosome F1 and UMOF20. Each point represents the mean±SD (n=3).

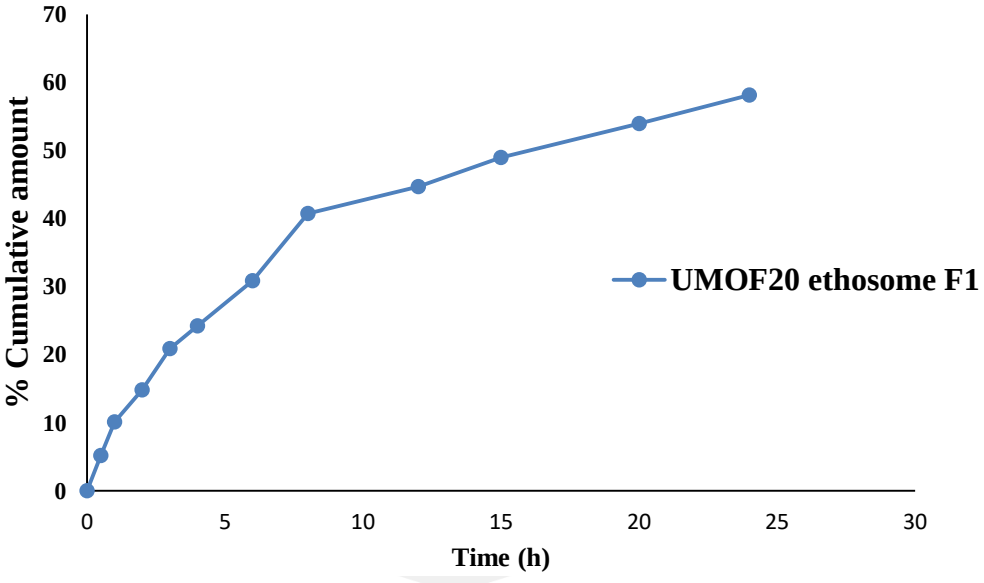


Figure 36 Percent cumulative amount-time profiles of sesamin permeated across mixed cellulose esters (MCE) membrane, UMOF20 ethosome F1 and UMOF20. Each point represents the mean±SD (n=3).

CHAPTER V
DISCUSSION

5.1 Preparative methods of MOF

In this study, the preparation of MOF formulations using three methods, namely conventional, modified conventional, and UAE method, was investigated. The results showed that UMOF20 and UMOF30 had significantly higher percentages of oil recovery than CMOF ($p<0.05$). Moreover, UMOF20 and UMOF30 showed the highest percentages of oil recovery among all MOF samples ($p<0.05$, % oil recovery 59.75 ± 2.22, and 61.82 ± 3.24 for UMOF20 and UMOF30, respectively). Furthermore, UMOF20 demonstrated significantly higher percentages of oil recovery than CMOF, MMOF, and UMOF10 ($p<0.05$). There are several possible possibility for this result. Firstly, the intensification of the extraction process using ultrasound has been attributed to the cavitation phenomena leading to high shear forces in the media.[186] Secondly, ultrasound also exerts a mechanical effect that has a strong impact on the solid surface, therefore increasing solvent penetration into the cell and increasing the contact surface area between the solid and liquid phase.[186] To improve the diffusion process, the ultrasound wave will facilitate swelling and hydration, so that the pores of the cell wall become large. [187] During sonication, ultrasound produces cavitation bubbles from ultrasonic waves that permit penetration of the extraction solvent into the plant cell wall greater than conventional methods, effectively releasing the intracellular product of the plant. [188] Many studies showed UAE increase the yield after the extraction process, the quantity of caffeic acid (85.71%), rutin (3550%), and rosemarinic acid (468.62%) in extract increased in comparison with the conventional method (solvent extraction), ultrasound enhance extraction yield of gallic acid from *Labisia pumila* up to 1.23-fold compared to the conventional method.[189-190] UAE showed a pronounced greater extraction yield of all-trans-lycopene from red grapefruit (*Citrus paradise* Macf.) and reduced extraction time effectively with a peak value at 30 min.[191]

Overall, the study found that the use of UAE in the preparation of MOF formulations resulted in higher percentages of oil recovery compared to conventional and modified conventional methods. Specifically, UMOF30 showed the highest percentages of oil recovery across all methods, while UMOF20 also demonstrated a significantly higher percentages of oil recovery than other MOF samples. The reason for this result is likely due to the intensification of the extraction process using ultrasound, which leads to high shear forces in the media and a greater penetration of the extraction solvent into the plant cell wall. This, in turn, effectively releases intracellular products and increases the yield of the desired compound. This finding is consistent with many other studies that have also reported increased yield and extraction efficiency with the use of UAE compared to conventional methods.

5.2 *In vitro* assays

5.2.1 Antioxidant activity of MOF samples using DPPH and ABTS method

The MOF samples are investigated their biological activities as followed: DPPH and ABTS) method for antioxidant activity. The results showed that all MOF samples exhibited anti-oxidation properties. The IC_{50} of MOF samples was in the range of 90.38 - 99.42 µg/mL when determined using DPPH and ABTS tests.

The antioxidative activity of the MOF samples was likely due to the antioxidative activity of all the herbs in the formulation. For example, sesame oil the most ingredient in the MOF formula has anti-oxidation activity due to various compounds such as sesamol, sesamin, and sesamolin. These compounds have been shown to scavenge free radicals and prevent lipid peroxidation, which can lead to cell damage and contribute to the development of various diseases. In addition to sesame oil, other herbs, and ingredients in the MOF formulation may also contribute to its antioxidant activity. [50-51]

For instance, the herbs in MOF formula such as D-camphor and beta-pinene have been found to possess antioxidant properties. D-camphor has been shown to scavenge free radicals and prevent lipid peroxidation in vitro, while beta-pinene has been shown to have a strong antioxidant capacity in various cell-based assays. Moreover, the combination of multiple herbs in the MOF formulation may also have a

synergistic effect, enhancing the overall antioxidant activity. Overall, the MOF formulation appears to have promising antioxidant activity, which may have potential health benefits.[108-109]

5.2.2 Anti-inflammatory and cytotoxicity activities using nitric oxide method

Sesame oil, one of the herbal constituents in MOF formula, has been reported to possess anti-inflammatory, antioxidant and antibacterial activities.[51-55] It contains sesamin and sesamol, which have been shown to inhibit the production of pro-inflammatory cytokines and reduce oxidative stress in various studies.[50,52] D-camphor and beta-pinene, which are also present in Mahajak, have been reported to possess anti-inflammatory and analgesic properties.[52]

For MOF preparation, the UAE technique enhanced the phytochemical extraction and increased the anti-inflammatory activity. The current study found that UAE application significantly increased the content of sesamin, D-camphor and beta-pinene which attributed to the anti-inflammatory effect. These data support previous studies that have reported the use of UAE for preparing herbal formulations. The conditions used in these studies involved sonication for 20-30 min at a frequency of 20-40 kHz. These conditions enhanced the extraction of sesamin and D-camphor, resulting in their increased pharmacological activities. sesamin exhibited antioxidation activity as determined by ROS inhibition assay and anti-inflammatory activity as determined by nitric oxide assay. Both D-camphor and sesamin exhibited antibacterial activity against *S. pyogens* and *S. aureus*, as determined by disc diffusion and microdilution methods.[192-193]

Overall, the use of UAE in the preparation of herbal formulations can enhance the bioactive compounds and increase their pharmacological activities, including anti-inflammatory, antioxidant, and antibacterial activities.

5.2.3 Antibacterial activity using disc diffusion and microdilution method

MOF samples were investigated the antibacterial activity by disc diffusion and microdilution methods. For disc diffusion test, each disc contained 15 µL of each MOF sample. Results showed that the MOF samples exhibited inhibition zones against *S. pyogens* DMST 17020 and *S. aureus* DMST 8440 in the range of 8.53-10.30 and 8.13-9.12 mm, respectively. All of MOF samples showed similar MIC values of *S. pyogenes* DMST 17020 and *S. aureus* DMST 8440 as 15.38 and 18.18 µL/mL, respectively. MBC values were more than 15.38 and 18.18 µL/mL, respectively.

The narrow inhibition clear zones observed in this study may be due to the high lipophilic polarity of the MOF samples. Poor water solubility of sesame oil can lead to uneven distribution across the agar surface, as reported in previous studies. [194,195] The previous study documented that MOF formula did exhibit the narrow inhibition clear zones against *S. aureus* and had a MIC value greater than 5 mg/mL against *S. aureus.*[196] It also reported that the herbal constituents present in MOF samples demonstrated antibacterial activities against *S. pyogens* and *S. aureus* when evaluated using disc diffusion and broth dilution methods. [197-201] However, herbal constituents in MOF samples were mixed with sesame oil, which has a lipophilic formulation with low polarity, resulting in narrow inhibition clear zones against *S. pyogens* and *S. aureus* with inhibition clear zones in the range of 8.13-9.12 mm due to uneven distribution across the agar surface when tested for antibacterial activity.[195] The study results also showed inhibition clear zones that were close to sesame oil (10-20 mm.).[197] Additionally, MIC values were different compared to the herbal constituents in the MOF sample due to differences in preparation methods and extraction solvents. GC-MS analysis revealed sesamin had the highest percentage peak area in the MOF samples and the evidence supported their antibacterial activities against *S. pyogens* and *S. aureus.*[197]

5.3 GC-MS analysis for MOF samples

5.3.1 Chemical profile of MOF samples

GC-MS analysis characterized 41, 33, 40, 33, and 29 phytochemical components of CMOF, MMOF, UMOF10, UMOF20 and UMOF30, respectively. 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 MOF 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

The results of the GC-MS analysis indicate that the MOF samples obtained using the traditional, modified, and UAE preparation methods have similar chemical profiles. This suggests that the preparation methods used did not significantly alter the chemical composition of the MOF samples.

GC-MS is a powerful analytical technique used to identify and quantify the chemical components of a sample. The fact that the chromatograms of the different MOF samples were very similar suggests that the MOF samples contain similar chemical components regardless of the preparation method used.

5.3.2 Principal components analysis (PCA)

PCA is a statistical technique used to analyze and identify patterns in large datasets. In this study, it used to analyze the ten major chemical compounds identified in the MOF samples by GC-MS.

The results of the PCA analysis suggest that there are two principal components (prin1 and prin2) that can explain the variability in the chemical composition of the MOF samples. Prin1 is the principal component with the highest probability of explaining the variation in the data.

The analysis suggests that there is a likelihood that preparing MOF samples using the different methods (CMOF, MMOF, and UAE) will result in the presence of the ten major chemical compounds in varying amounts. The PCA analysis will show a positive value for any substances found in abundance, indicating that they contribute to the variability in the chemical composition of the samples. Conversely, the analysis will show a negative value for any substances found in low abundance or not detected in the sample, indicating that they do not significantly contribute to the variability in the data.

Overall, the results of the PCA analysis provide insights into the chemical composition of the MOF samples and how it varies depending on the preparation method used. For example, prin1 occupied 64.00% of total variation and was negatively correlated with palmitic acid, cis-9-hexadecenal, and octadecanoic acid whereas it was positively correlated with beta-pinene, grandlure III, D-camphor, oxypeucedanin, .gamma.-tocopherol, (+)-sesamin and gamma.-sitosterol. It indicated that three of the relative contents of palmitic acid, cis-9-hexadecenal, and octadecanoic acid reduce while the relative content of cis-9-hexadecenal, octadecanoic acid, n-hexadecanoic acid, and octadecanoic acid increased when the MOF samples were prepared by the modified and UAE methods as compared to the conventional preparative method. However, the second principal component (prin2) was negatively correlated with the content of beta-pinene and D-camphor and positively correlated with the content of grandlure III, palmitic acid, cis-9-hexadecenal, octadecanoic acid, oxypeucedanin, .gamma.-tocopherol, (+)-sesamin, and gamma.-sitosterol. This could be explained that as compare to other preparative methods modified and UAE method resulted in the lower relative content of beta-pinene and D-camphor and higher relative contents of grandlure III, palmitic acid, cis-9-hexadecenal, octadecanoic acid, oxypeucedanin, gamma.-tocopherol, (+)-sesamin and gamma.-sitosterol.

5.3.3 Cluster analysis

Cluster analysis is a statistical technique used to group similar data points together based on their similarity. In this study, cluster analysis was used to analyze the % peak area of the ten major chemical compounds identified in the MOF samples from GC-MS analysis.

The results of the cluster analysis suggest that the preparation patterns of MOF samples in each method (CMOF, MMOF, and UAE) can affect the % peak area of the ten major chemical compounds to be different or similar. For example, UMOF20 and UMOF30 obtained from the UAE preparation method showed similar amounts of the ten major chemical compounds. This is in contrast to CMOF, which is prepared using a conventional method and has a different set of ten major chemical compounds.

This finding suggests that the preparation method used can have an impact on the chemical composition of the MOF samples, which may in turn affect their biological activity. However, further studies are needed to investigate the biological activity of the identified chemical compounds and how it varies depending on the preparation method used.

Overall, the cluster analysis provides insights into the chemical composition of the MOF samples and how it varies depending on the preparation method used.

5.4 Ethosomes preparation of MOF

5.4.1 Quality assurance of ethosomes dosage form

5.4.1.1 Vesicle size

Ethosomes of UMOF20 have been prepared by cold method and evaluated.[168] The microscopic evaluation showed the morphology of ethosomes. Shapes most of the ethosome vesicles were observed as spherical. (Figure 20). It is evident from the results that the size of the vesicles increased with increasing concentrations of soya phosphatidylcholine (SPC) from 2 % to 4%. This observation supports the findings that the concentration range of SPC in an ethosomal formulation is 0.5%-5% is suitable for ethosomal system. Increasing SPC concentration will increase vesiculae size slightly or moderately.[202] Particle size in Table 19 ranged in

size from 225.00±3.60 nm to 1627.33±8.08 nm for formulations with concentrations of (2%, F1-F3) SPC, formulations with concentrations of SPC (3%, F4-F6) ranged in size from 261.67±5.13 nm to 1846.67±7.64 nm while formulations with concentrations of SPC (4%, F7-F9) ranged in size from 393.33±6.02 nm to 2495.00±22.71 nm. As a result, increasing concentrations of SPC (1–4%) led to significant increases ($p< 0.05$) in the vesicles' size. A previous study found that increasing the concentration of SPC increased mean particle size. With a formulation containing 2 % SPC, small vesicles were formed. The size of ethosomes doubled when the concentration of SPC was increased twofold. [203] When SPC concentration was increased, the vesicles grew larger, which was likely due to the phospholipids stiffening them and causing the formation of a thicker matrix structure. Many reported similar results.[202-204] Vesicles relatively smaller than 300 nm were capable of delivering their drugs into deeper layers of skin to a certain extent, whereas the highest drug delivery needed particles smaller than 70 nm.[205]

Ethosomal dispersions were produced with increasing amounts of ethanol (20%, 30%, and 40%) and SPC concentration (2%, 3%, and 4%). In the study, although keeping the concentration of SPC at 2%, it was found that by increasing the concentration of ethanol from 20% to 40%, the size of the vesicles increased which was observed vesicle size in between 225 – 1,627 nm from F1-F3 and the optimum ethanol concentration for this study is 20%. However, increasing ethanol concentration beyond the optimum level would cause the bilayer to be leaky and lead to a slight increase in vesicular size and a slight decrease in entrapment efficiency, and further increases in ethanol concentration would solubilize the vesicles. [206] Ethanol is an efficient penetration enhancer. It plays an important role in ethosomal systems by giving the vesicles unique characteristics in terms of size, zeta potential, stability, entrapment efficacy, and enhanced skin permeability. Ethanol concentrations in ethosomal systems have been reported to range from 10% to 50%. [207] The solvent properties and edge activation mechanism of ethanol result in some steric stability, which in turn modifies the net charge of the systems. These findings were in agreement with those of Garg et al. and Yang et al. [208] Dayan and Touitou found that the negative charge of the empty ethosomes increases with increasing ethanol concentration.[207] Ethanol acts as a negative charge provider for the surface of

ethosomes, thereby avoiding aggregation of the vesicular system due to electrostatic repulsion. Additionally, ethanol was also reported to have stabilizing effects. Electrostatic repulsion prevents the vesicular system from becoming aggregated because of the negative charge provided by ethanol. The vesicular charge is shifted from positive to negative due to the high ethanol concentration in ethosomes.[207, 209] The lowest ethanol concentration (20%) was advantageous because zeta potential values were improved as a result of its concentration. The surface charge and ethosome stability were both improved when zeta potential values were in the range of -11.87±0.55 to -1.04±0.35mv (F1, F4, and F7). However, as the ethanol concentration rose to 40%, the zeta potential decreased -from 1.10±0.04 to 1.03±0.01 (F3, F6, and F9) to low values (unfavorable). Ethanol concentration significantly enhanced vesicles "zeta potential" ($p < 0.05$) as ethanol concentrations were found to be between 10% and 25% (mid-concentration range). According to Ogiso et al., The skin permeation properties of negatively charged vesicles were superior to positively charged ones. The ethanol (10–25%) content in these nanocarriers is primarily responsible for the interpretation of the negative charge of the zeta potential in ethosomal systems.[210] The polar head groups of phospholipids are negatively charged by ethanol, resulting in electrostatic repulsion. As a result, ethosomal vesicles would be less likely to aggregate, increasing the stability of these nanocarriers.[211]

5.4.1.2 Size distribution

The PDI (polydispersity index) is a measure of the size distribution of particles in a sample. A PDI value of 0 indicates a completely uniform size distribution, while higher values indicate a broader distribution. In this study, PDI was used to evaluate the homogeneity of prepared ethosomes based on their vesicle size distribution. [212-213]

The values obtained for PDI in the formulated batches were between 0.35 and 0.79. These values indicate a fairly uniform and homogeneous particle size distribution, with PDI values less than 0.5 being considered acceptable. A PDI value of 0.35 ± 0.00, as reported for one of the formulations (F1), indicates a highly homogeneous population of vesicles, which may result in a smaller mean particle size of 225.00 ± 3.60.

Overall, the PDI values obtained in this study suggest that the formulated ethosomes have a relatively narrow to broad size distribution, with most formulations showing a fairly uniform and homogeneous particle size distribution. This is an important characteristic for drug delivery systems, as a uniform size distribution can lead to improved stability and efficacy of the formulation.[210]

5.4.1.3 Zeta potential

The zeta potential (ZP) is an important parameter that affects the stability of vesicular systems and is determined using a zeta sizer. Generally, particles with zeta potential values above +30 mV or below −30 mV are considered stable because it prevents aggregation between vesicles due to electrostatic repulsion and indicates the physical stability of the formulation.[214] A high zeta potential prevents the aggregation between vesicles and thus enhances the physical stability of the formulation. However, the formulated ethosomal formulation showed a negative zeta potential (-1.08–11.87 mV) caused by the net charge of the lipid composition in the formulation. The negative zeta potential is responsible for enhancing the percutaneous permeation of the drug. The optimized ethosomal formulation (F1) had a zeta potential value of -11.87±0.55 mV, which was achieved by using soya phosphatidylcholine (SPC) at 2% and ethanol at 20% . A study investigated the effect of SPC concentration on the zeta potential of transethosomes prepared using sesame oil. They found that increasing the concentration of SPC from 0.5% to 2% led to a significant increase in the zeta potential of the transethosomes.[215] Another study also found that increasing the SPC concentration in transethosomes led to an increase in the zeta potential of the particles. However, the result suggested that above a certain concentration (around 2%), further increases in SPC concentration did not significantly affect the zeta potential.[216] A study investigated the effect of ethanol concentration on the zeta potential of transethosomes prepared using sesame oil. The results showed that increasing the concentration of ethanol from 10% to 20% led to a significant increase in the zeta potential of the transethosomes. This suggests that the higher ethanol concentration resulted in a more uniform and stable lipid bilayer structure, which in turn led to a higher zeta potential.[215]Another study found that increasing the ethanol concentration in transethosomes prepared using SPC led to an increase in the zeta potential of the particles. However, the authors noted that beyond

a certain concentration (around 20%), further increases in ethanol concentration did not significantly affect the zeta potential.[216]

It has been observed that the skin has a negative surface charge, which is favorable for stabilizing and improving the transdermal penetration of drugs. This is due to the electrostatic repulsion between the negative charge on the skin surface and the SPC nano-transfersomes, as found by Kateh Shamshiri et al. [217] Therefore, the negative zeta potential of the ethosomal formulation may be beneficial for enhanced drug delivery through the skin.

5.4.1.4 Percentage entrapment

The delivery potential of ethosomal system is directly affected by its sesamin and sesamolin carrying capacity which is determined in terms of entrapment efficiency, EE. The EE of ethosomes was determined for all the formulations (F1-F9). The ranges of EE of ethosomes were between 88.21 ± 3.05 % - 94.13 ± 0.21 % of sesmin and 88.80 ± 3.05 % - 99.14 ± 0.07 % of sesamolin respectively. The ethanol concentration in the ethosome system should not be too high, and generally, should be kept below 40%. As increasing concentration of ethanol results in leaking of the drug from the lipid bilayer due to which EE decreases therefore, ethanol concentration only up to 40% was considered for this experiment. [168, 218]

The formulation F1 with 20% ethanol concentration was having EE of 87.61 ± 1.53%, beyond 30% and 40% ethanol concentration the EE was found to be declining (F2, F3, F5. F6,F8 and F9). Higher EE with an appropriate amount of ethanol is possibly due to increased solubility of sesamin and sesamolin of UMOF 20 in ethanol present in the ethosomal core. This is in accordance with the previous study.[219-220] The result suggested that 2 % SPC is optimal concentration along with 20% ethanol concentration for better EE and any increase or decrease in concentration of SPC or ethanol reduces the EE of ethosomes. Ali M. Nasrle et al. found that SPC would be the lipid of choice for the optimization study's subsequent formulations. As a result, as lipid concentration dropped, EE% increased. Furthermore, the addition of soya lecithin to ethosomal formulations improved their ability to encapsulate the drug. Despite this, the ethanol concentration, sonication time, or penetration enhancer type had no significant effect.[211]

In summary, the entrapment efficiency (EE) of sesamin and sesamolin in the ethosomes was evaluated for all formulations (F1-F9) and ranged between 88.21 ± 3.05% - 94.13 ± 0.21% for sesamin and 88.80 ± 3.05% - 99.14 ± 0.07% for sesamolin. The optimal concentration of ethanol for the formulation was found to be 20%, as higher concentrations resulted in decreased EE. Additionally, the optimal spc concentration was found to be 2%, and any deviation from this concentration resulted in a reduction in EE. SPC was found to be the optimal lipid for the formulation, and its addition improved the ability of the ethosomes to encapsulate the drug. The ethanol concentration, sonication time, or penetration enhancer type did not have a significant effect on the EE of the ethosomes.

5.4.1.5 Stability study

Stability studies were conducted on the most satisfactory formulation, UMOF20 ethosome F1, at a temperature of 40 ± 2°C for 90 days. After 30 days, the results showed that the ethosome remained a milky liquid with no separation, and its parameters were as follows: vesicle size of 239.12±3.65 nm, PDI of 0.35±0.01, zeta potential of -11.8±0.32, and pH of 4.56±0.10. Based on these physical characteristics and important parameters, it was concluded that UMOF20 ethosome F1 was stable. However, after the stability test at 60 and 90 days, it was found that the vesicle size tended to increase (239.58-895.33 nm), resulting in an increase in the PDI (0.35-0.89), and the zeta potential tended to increase (-11.8-1.75). All these observations suggested that the ethosome system tended to become less stable. Transethosomes are sensitive to temperature and can undergo phase transitions that can affect their stability. High temperatures can cause the vesicles to become less stable and may lead to drug leakage, while low temperatures can cause the vesicles to become too rigid and less permeable. It is recommended to store transethosomes at a cool temperature (e.g. 4-8°C) and avoid exposure to high temperatures or temperature fluctuations. [221] Another study found that pH values between 4.5-5.5 were optimal for stability ethosome containing sesamin, with significant sesamin leakage occurring at both lower and higher pH values.[222] To improve the stability of the system in future experiments, a stabilizer may be added, including: (1) Cholesterol, a rigid steroid molecule that enhances the stability and efficiency of drugs in ethosomal systems, preventing leakage and reducing vesicular permeability and vesicular fusion that impact efficiency entrapment (EE); (2) Propylene glycol (PG), a widely used

penetration enhancer, which, when incorporated in ethosomal systems at a concentration range of 5%–20%, influences the ethosomal properties of size, entrapment efficiency, permeation, and stability. The incorporation of PG in ethosomal systems leads to a further reduction in particle size in comparison to systems without PG. A significant decrease in particle size from 103.7±0.9 nm to 76.3±0.5 nm was achieved when PG concentration was increased from 0% to 20% w/w. Other researchers have suggested that the presence of ethanol and PG in ethosomes provides better solubility of drugs, leading to higher entrapment efficiency and improved drug distribution throughout the vesicle. [223-224] However, more studies are required to evaluate the stability of ethosomal systems for long-term storage, particularly when they are incorporated in dosage forms.

It is important to note that stability studies are critical for determining the shelf-life and efficacy of drug delivery systems. In the case of UMOF20 ethosome F1, the stability tests revealed a decrease in stability over time, which indicates the need for a stabilizer to be added to the system. The addition of stabilizers like cholesterol and propylene glycol has been shown to enhance the stability and efficiency of drugs in ethosomes. However, more studies are needed to evaluate the long-term stability of ethosomal systems when incorporated into dosage forms. Overall, stability studies are crucial for ensuring the effectiveness of drug delivery systems and must be carefully considered in the development and optimization of such systems.[225]

5.4.2 Evaluating the permeability of MOF ethosomes

From the membrane permeation study, these results were proved that the UMOF20 ethosome F1 was more effective in delivering sesamin than UMOF20 formulation. sesamin, a major lignan constituent of sesame oil, has limited permeation through Mixed Cellulose Ester (MCE) membrane due to its relatively large molecular weight (354.35 g/mol) and low solubility in water.[224] Previous studies have found that drugs with lipophilic properties generally have low permeation properties through the cellulose membrane. However, using Strat-M™, which is similar to human skin, gives good results and is suitable for the drug with lipophilic properties.[224] It has been found that transethosome sesame oil may have limited permeation through Mixed Cellulose Ester (MCE) membrane due to the low solubility of sesame oil in water and the hydrophobic nature of the transethosomes.[225]

The better permeability of ethosome UMOF20 F1 compared to UMOF20 carriers sesamin may be attributed to the addition of ethanol into the formulation. Ethanol has long been used as a skin permeation enhancer for topical and transdermal delivery. It could increase sesamin solubility leading to an enhancement of the entrapment efficiency of sesamin in the formulation. Ethanol could alter the stratum corneum's barrier property and increase the thermodynamic activity due to the evaporation of ethanol. However, the skin permeation is not only the effect of ethanol but also depends on phospholipids and skin lipids. [161]Touitou et al. suggested a hypothetical model of ethosome to enhance the penetration of drugs through the stratum corneum lipid. It was explained that the better skin permeation of ethosome may be due to the synergistic mechanism between high concentrations of ethanol, phospholipid vesicles, and skin lipids.[168] The stratum corneum lipids at physiological temperature are densely packed and highly conformationally ordered. Ethanol interacts with lipid molecules in the polar head group region, which results in increasing fluidity and may finally lead to increased membrane permeability. In addition, it may provide the vesicles with soft flexible characteristics that are easy to penetrate into deeper layers of the skin. The malleable ethosome vesicle can forge paths in the disordered stratum corneum.[206] Moreover, the negative charge of the ethosome surface, which is attributed to the ethanol content, may also play a role in enhancing skin permeation. The negative charge on the surface of the ethosome repels the negatively charged skin surface, reducing electrostatic interactions between the ethosome and skin. This leads to increased penetration of the ethosome through the stratum corneum. Additionally, the small size of the ethosome vesicles (less than 200 nm) may also contribute to their enhanced skin permeation properties. The small size of the vesicles allows them to penetrate more easily through the intercellular spaces of the stratum corneum, leading to increased skin penetration. Finally, the ability of ethosomes to enhance the permeation of lipophilic drugs through the skin suggests that they may be useful for delivering a wide range of lipophilic drugs for topical and transdermal applications.[224-226]

CHAPTER VI
CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Mahajak oil formula (MOF), a Thai traditional medicine described in an official textbook.[1] has been used in Thailand until the present. This study aimed to apply the UAE, a modern extraction technology on MOF production and evaluate its properties. The UAE improves diffusion process of the extraction solvent through facilitating swelling and 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. Previous study showed the UAE exhibited a pronounced extraction yield and reduced extraction time within 30 minutes. Even though the present study did not show the UAE Mahajak samples to be different in the antioxidant activities as compared with the CMOF samples, it demonstrated the UAE technique contributed a significantly higher effectiveness on the anti-inflammatory activitites, where the UAE technique are UMOF10 and UMOF20 exhibited the most potent anti-inflammatory and anti-bacterial activities . The UAE technique had an additional advantage in that it reduced production time as compared to the CMOF. The UMOF20 and UMOF30 samples demonstrated a high percentages of oil recovery 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. 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 MOF samples, the GC-MS fingerprints presented a similar profile between all UAE and CMOF samples. Ten major compounds of MOF samples 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. All MOF samples show anti-oxidant activity and anti-bacterial activity.This study revealed the impact of UAE on MOF production. The UAE technique improved percentages of oil recovery and demonstrated anti-inflammatory activity of MOF preparation despite no significant effect on the chemical components.

This is an important finding that demonstrates the potential of UAE as a modern extraction technology for traditional medicines. The higher content of major compounds in UAE samples indicates that the UAE technique can effectively extract the active constituents of MOF formula. The anti-oxidant and anti-bacterial activity of all MOF samples also suggests that the MOF has potential therapeutic applications.

However, it is important to note that the lack of significant effect on chemical components should be further investigated in future studies, as it may have implications for the standardization and quality control of the MOF formula. Overall, this study highlights the importance of modernizing traditional medicine production methods to improve their effectiveness and efficiency, while also preserving their cultural and historical value.

This study was able to develop an ethosome containing the MOF formula. which will be developed as a skin delivery system for the treatment of pain and anti-bacterial in wound healing according to the knowledge of Thai traditional medicine. From the experiment, it was found that UMOF20 ethosome F1, which consisted of MOF 7.5 ml, phospholipid 2% w/w, SPAN20: Tween20 (1:0.5 ml ratio) as a lipid component, and 20% w/w ethanol as a dispersion medium is the optimized ethosome. The physical appearance is a milky white liquid. Upon microscopic examination, it exhibits a robust ethosome system with a pH 4.77. The optimized ethosome containing UMOF20 was considered from the particle size less than 300 nm, a narrow. size distribution, the zeta potential of more than +30 mV or -30 mV, and entrapment efficiency of more than 50%. It gave ethosome with 225.00±3.60 nm vesicular size (polydispersity index PI = 0.35±0.01), a zeta potential of -11.87±0.55 mV, and efficient entrapment of 94.13±0.21 % of sesamin and 99.14±0.07 % of sesamolin.

The development of an optimized ethosome containing the MOF formula is a significant achievement in this study. The use of an ethosome as a skin delivery system is a promising approach for enhancing the efficacy of traditional medicines. The optimized ethosome with a particle size less than 300 nm, a narrow size distribution, and high entrapment efficiency of sesamin and sesamolin indicates its potential for effective skin delivery. The physical appearance and pH of the optimized ethosome also suggest its suitability for topical use. Overall, this study provides valuable insights into the development of a skin delivery system for Mahajak oil formula, which can potentially be used for the treatment of pain and wound healing in line with the principles of Thai traditional medicine.

Stability studies were carried out for the most satisfactory formulation of UMOF20 ethosome F1 at temperatures of 40 ± 2°C for 90 days. After 30 days, the experimental results showed that the physical appearance of the ethosome is milky liquid with no separation. The parameter value showed vesicle size 239.58±2.64, PDI 0.35±0.01, zeta potential -11.8±0.02 and pH 4.57±0.02. From the above physical characteristics and important parameters, it can be concluded that UMOF20 ethosome F1 is stable. Moreover, it was found that after the stability test at 60 and 90 days, the ethosome system tended to decrease in stability. To increase the stability of the system in the next experiment a stabilizer of that system may be added, such as cholesterol and poly glycol. However, more studies are required to evaluate the stability of ethosomal systems for long-term storage, particularly when they are incorporated in dosage forms.

The appropriate ethosome provided significantly higher skin permeation parameters such as Q_{24} (23.46±0.06 μg/cm2), Jss (2.3907μg/cm2/h), and Kp (0.06 $x10^{-3}$cm/h), compared to UMOF 20. This incident could be confirmed that the UMOF20 as a lipophilic formula, which has insoluble in water, might remain on stratum corneum lipid and not penetrate the skin. These results indicated that the ethosome could enhance skin permeation that leading to efficiency in the topical and transdermal delivery system.

The stability studies carried out on the most satisfactory formulation of UMOF20 ethosome F1 showed that the formulation was stable at 40 ± 2°C for 90 days with no separation and maintained its physical appearance as a milky liquid. The

vesicle size, PDI, zeta potential, and pH remained within an acceptable range after 30 days of stability testing. However, the stability of the system decreased after 60 and 90 days of testing, suggesting the need for a stabilizer to improve the stability of the system during long-term storage. The ethosome formulation also demonstrated significantly higher skin permeation parameters than UMOF20, indicating its potential use in topical and transdermal delivery systems.

6.2 RECOMMENDATION

Based on the findings of the study, the following recommendations can be made to improve the yields (percentages of oil recovery), anti-oxidation, anti-inflammation, and antibacterial properties of the MOF samples:

1. Use ultrasonic extraction technique: The study found that ultrasonic extraction (UAE) improved the yields of the Mahajak formula, and increased the anti-inflammatory and antibacterial activities of the MOF samples. Therefore, using UAE technique can be a good option to improve the yields and properties of MOF samples.

2. Optimize ultrasonication time and power: The study identified that ultrasonication time and power were the key factors that affect the yields and properties of MOF samples. Therefore, it is recommended to optimize these parameters to obtain the best results.

3. Quality control of Hebal medicine in MOF samples: The quality of the raw materials used in the preparation of MOF samples can have a significant impact on their properties. Therefore, it is recommended to use high-quality raw materials to obtain MOF samples with higher yields, anti-oxidation, anti-inflammation, and antibacterial properties. To ensure the quality of MOF samples in herbal medicine, the following aspects should be considered based on the quality control requirement protocol of herbal medicine in the Thai Herbal Pharmacopoeia:

Identity: The identity of the plant material used in the herbal medicine should be verified through macroscopic and microscopic examination, chemical tests, and DNA analysis.

Purity: The herbal medicine should be free from contaminants such as heavy metals, pesticides, and microbial organisms.

Quality: The quality of the herbal medicine should be evaluated based on its chemical composition, physical properties, and therapeutic efficacy.

Stability: The stability of the herbal medicine should be evaluated under various storage conditions, including temperature, humidity, and light exposure.

Safety: The safety of the herbal medicine should be evaluated through toxicity testing and clinical trials.

Packaging and labeling: The packaging and labeling of the herbal medicine should comply with the regulations set by the Thai Food and Drug Administration.

4. Conduct further research: While the present study has shown promising results, further research is needed to determine the optimal conditions for the preparation of MOF samples, as well as to investigate their safety and efficacy in vivo

Based on the study's findings, it is recommended that quality control measures be implemented for the UMOF20 ethosome preparation to ensure its consistency and effectiveness. Quality control can be achieved through the following steps:

1. Characterization of the ethosome product: The UMOF20 ethosome should be characterized using techniques such as gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) to confirm the presence and quantity of the active compounds.

2. Stability testing: The stability of the UMOF20 ethosome should be evaluated to determine its shelf life and to ensure that the active compounds do not degrade over time.

3. Microbial testing: The UMOF20 ethosome should be tested for microbial contamination to ensure its safety for use.

4. Formulation optimization: The formulation of the UMOF20 ethosome should be optimized to enhance its properties such as permeability, stability, and skin compatibility.

Implementing these quality control measures can help to ensure the consistency, safety, and effectiveness of the UMOF20 ethosome preparation.

For skin permeation test, it would be beneficial to conduct skin permeation tests to evaluate the ability of UMOF20 ethosomes to penetrate the skin barrier and

deliver the active components to the desired target site. The study can use in vitro skin permeation models such as Franz diffusion cells to measure the permeation kinetics of the UMOF20 ethosome. In addition, it would be useful to compare the skin permeation profile of the UMOF20 ethosome with that of other formulations, such as the CMOF, to assess the potential advantages of using the UMOF20 ethosome. Furthermore, the study can also consider conducting *ex vivo* skin imaging studies using confocal microscopy or other imaging techniques to visualize the penetration and distribution of the UMOF20 ethosome within the skin layers. Such studies can provide valuable information on the skin permeation behavior of the UMOF20 ethosome.

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APPENDIX

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Original Research Article

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

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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.

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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

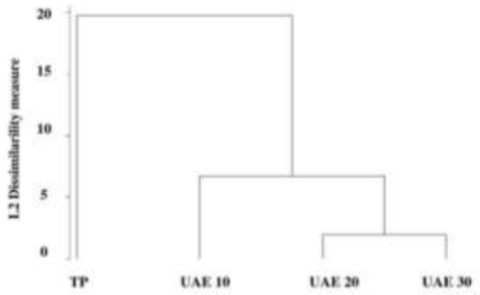

Figure 1: Dendogram of Mahajak samples analysis

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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