RESEARCH AND DEVELOPMENT OF AN ANTI-CELLULITE PRODUCT FROM THAI HERBAL COMPRESS

NGAMRAYU NGAMDOKMAI

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in (Pharmaceutical Sciences)
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has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Pharmaceutical Sciences of Naresuan University

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Academic Paper	Ph.D. Dissertation in Pharmaceutical Sciences, Naresuan University, 2021
Keywords	anti-cellulite product, volatile oil, natural products, lipolysis, herbal compress

ABSTRACT

Cellulite is associated with a complex array of adipocytes under the skin and the vascular system. Herbal compresses previously developed from Thai traditional medicines have been proven to have an anti-cellulite effect in healthy volunteers within 2 weeks of treatment. One particular herbal compress had previously been identified as having specific anti-cellulite properties, and the objectives in this research study were 1) to investigate the mechanisms of the cellulite reducing chemical components of that herbal compress, 2) develop a topical preparation containing the essential oils found in extracts obtained from the ingredients of that herbal compress, 3) investigate the stability and quality control methods of the formulation developed and 4) study the effectiveness on cellulite reduction and safety of the developed formulation in female participants. Eight essential oils in, and two water extracts from, the ingredients of the herbal compress, together with nine monoterpenoid constituents, were tested on the 3T3-L1 adipocytes. The vasodilatory effect on rat aortae was also studied. The adipocytes were induced by dexamethasone, 3-isobutyl-1- methylxanthine and insulin. At all concentrations tested, all essential oils, water extracts and their monoterpenoid constituents significantly inhibited lipid accumulation activity ($p < 0.05$) and decreased the number of triglycerides when compared to untreated cells ($p < 0.01$). In addition, our results showed that the mixed oil distilled from the mixed ingredients of the herbal

compress could relax the isolated rat aorta (EC50 = 14.74 ± 2.65 µg/mL). Overall, all essential oils, extracts, and chemical constituents tested showed adipogenesis inhibition and lipolysis induction effects on the cultured adipocytes with the mixed oil demonstrating vasorelaxation activity, successfully demonstrating the anti-cellulite effects of the extracts from the herbal compress.

To formulate a more convenient dosage form for administering the extracts, a gelled emulsion or an 'emgel' was prepared. The caffeine bio-active marker was characterized by HPLCs and the monoterpenes (camphene, camphor, 3-carene, α-citral, β-citral, limonene, β-myrcene, α-pinene, β-pinene and terpinene-4-ol) were characterized by headspace GCMS. The stability study was carried over a 12-week period. Changes in the compositions were monitored every 2-weeks during storage at 4, 25 and 50°C. The anti-cellulite herbal emgel that was developed was physically stable under accelerated testing. The shelf-life of the emgel, when kept at room temperature, was estimated to be 31 months, using the Q10 method.

The emgel was tested in a double-blind, placebo-controlled trial with 18 women aged 20–50yr with severe cellulite. The emgel was applied to the thigh skin twice daily for 12 weeks, and the appearance of cellulite (the primary outcome), thigh circumferences, skin firmness, and cutaneous blood flow (secondary outcomes) were assessed at baseline, 2, 4, 8 and 12 weeks with a 2-week follow-up. Reduced cellulite severity scores were seen at each testing time point, from 13.4±0.3 (baseline) to 12.1±0.3 (week 2) and 9.9±0.6 (week12). All secondary outcomes improved with both the placebo and the herbal emgels suggesting that the ingredients in the base-formulation might be responsible. During the testing period participant were queried, their diaries were analysed, and they underwent physical monthly inspections. No adverse events were reported or identified during this period. The herbal emgel safely improved the appearance of cellulite, while the base emgel may play a role for other endpoints. In conclusion, the research and development of anti-cellulite product containing extracts and volatile oils from Thai herbal compress completed successfully and the efficacy and safety of the ingredients of the formulation were demonstrated in both *in vitro* and *in vivo* tests.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my advisor Associate Professor Dr. Kornkanok Ingkaninan for the continuous support of my Ph.D. studies and related research, for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis.

As well, I would like to thank my co–advisors: Associate Professor Dr. Neti Waranuch, Associate Professor Dr. Krongkarn Chootip, and Dr Norman Scholfield for their suggestions, unlimited encouragement, and unstinting help during this period. I am most grateful to my thesis committee: Associate Professor Dr. Assistant Professor Katechan Jampachaisri for her insightful consulting about statistical analysis and her overall encouragement, Additionally, I would like to thank Dr. Nitra Nuengchamnong for her teaching of GCMS and her suggestions regarding this. Without they precious support it would not be possible to conduct this research.

I am grateful for the financial support, I have received from the Royal Golden Jubilee Program (Grant No. PHD/0008/2560, 2017); the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, and National Research Council of Thailand (NRCT).

I thank my lab mates and friends for the stimulating discussions and for all the fun we have had in the last five years.

Finally, I would like to express my gratitude to my family for their spiritual support throughout the composition of my thesis and throughout my life.

I would also like to thank Mr Roy I. Morien of the Naresuan University Graduate School for his time and effort in editing my theses, and published papers, for correct English grammar and expression.

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CHAPTER I

INTRODUCTION

Background and rationale

Cellulite describes the appearance of overlying subcutaneous fat, usually around the thighs and buttocks, of post-pubescent females. Excessive fat accumulation in these deposits causes it to invade the dermis which disrupts the tissue architecture resulting in the orange peel-like appearance of the skin (1-2). Cellulite develops in most women even though they are not necessarily overweight or obese, which carries no health risk, but has an aesthetically unpleasing appearance. Anti-cellulite products have become popular especially those based on natural ingredients. Cellulite treatments include a wide range of herbal extracts or methylxanthines delivered by subdermal injection, topical creams and wraps, dietary supplements, or mechanical interventions such as massaging, laser or light therapy, and liposuction. While they may be efficacious, the invasive approaches carry some risks. Adipogenesis or lipogenesis, the differentiation process of adipocytes from precursor cells, provides constant renewal of adipocytes, and contributes to the increase of adipose tissue mass, and insulin plays a predominant role in the adipogenic process. Lipolysis, on the other hand, is the decomposition of the chemical that causes fat to be released from the adipose tissue by hydrolysis of the ester bonds in triglycerides (3-4).

Herbal compresses are also known as Luk-Pra-Kob in Thai, or just herbal balls. A herbal compress is composed of several herbs bundled together in a muslin ball with a short handle. Such compresses have traditionally been used in massage treatment in Thailand to relieve pain, inflammation or used by postpartum women.

In a previous study, we modified a Thai traditional herbal compress to an anti-cellulite product (5). This particular herbal compress can be used to give an anti-cellulite effect along with a relaxing sensation. The formulation contained *Zingiber officinale* Roscoeoe rhizomes (ginger), *Piper nigrum* L. fruit (black pepper), *Piper retrofractum* Vahl. fruit (java long pepper), *Camellia sinensis (*L.*)* Kuntze leaf *(*tea), and *Coffea arabica* L. seed (coffee) as principal ingredients together with some

auxiliary herbs i.e. *Zingiber montanum* (J. Koenig) Link ex A.Dietr. rhizomes (plai), *Curcuma longa* L. rhizomes (turmeric*)*, *Cymbopogon citrates* DC. Stapf., leaves (lemon grass), *Citrus hystrix* DC., fruit peels (kaffir lime), while the flavoring agents were camphor and salts. The herbal compress containing these herbal ingredients were selected based on the anti-cellulite, vasodilation, and anti-inflammation effects, and its effects of increasing microvascular and lymphatic flow, and/or stimulating lipolysis and reducing lipogenesis (6). The anti-cellulite effects of the herbal compress were determined using a double-blind, randomized placebo-controlled trial conducted on 21 female participants aged 20 to 55. The participants received treatment with the herbal compress on one thigh and with the placebo compress on the other thigh, and the treatment was carried out twice a week (30 min/treatment) for 8 weeks. The results showed that the herbal compress could significantly reduce thigh circumference, skin fold thickness and the severity of cellulite within 2 weeks (7). However, its mechanism and ingredients responsible for reducing cellulite were not known.

In the current study, we investigated the effects of the extracts and essential oils from the herbal ball, and some of their chemical constituents on adipogenesis in 3T3-L1 cells, and also investigated its vasorelaxant effects. The extracts and essential oils from the ingredients of the herbal compress were also used as active ingredients in the formulation in order to develop a topical formulation from the ingredients of the anti-cellulite herbal compress which might fulfill the needs of a broader group of users. The stability and physical characteristics of the developed formulation were studied. The chemical constituents in the extracts and essential oils, as well as in the formulation, were determined by headspace gas chromatography-mass spectrometry (HS-GCMS) and high-performance liquid chromatography (HPLC) for quality control purposes. The safety and efficacy of the newly developed anti-cellulite formulation were determined using a double-blinded, randomized placebo-controlled trial with female participants.

Objectives of the study

1. To investigate the effects of the extracts and essential oils and their monoterpenoid constituents obtained from the ingredients of the herbal compress on adipocytes differentiation in 3T3-L1 adipocytes and to investigate the vasorelaxant effect of the mixed oils on rat isolated aortae.

2. To develop a topical preparation containing the extracts and essential oils obtained from the ingredients of the herbal compress.

3. To investigate the stability and quality control methods of the formulation developed.

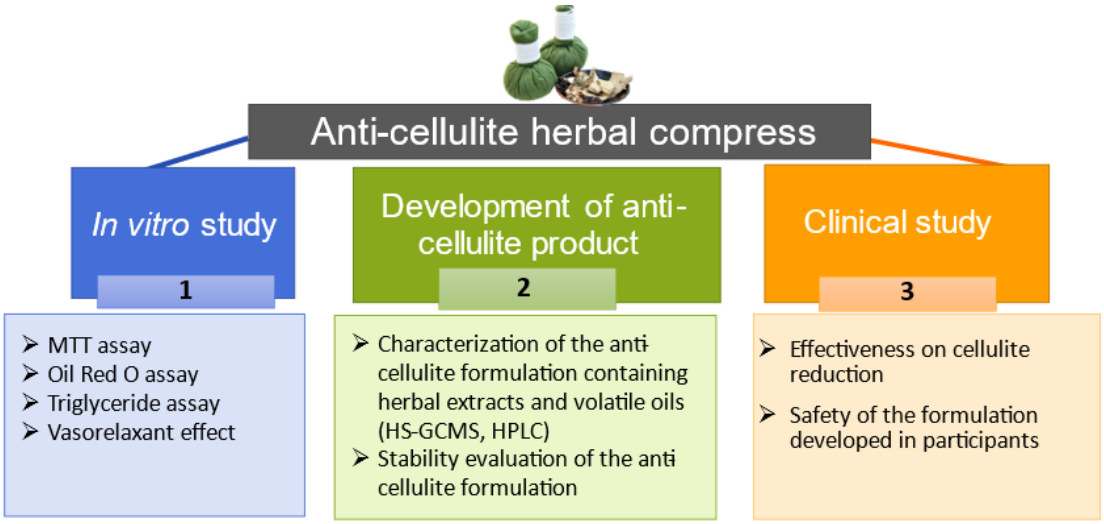
4. To study effectiveness on cellulite reduction and safety of the developed formulation on female participants.

Scope of the study

The essential oils of the aromatic herbal components in the herbal compress were prepared using the water distillation method. The tea and coffee extracts were extracted by hot water and the extracts were then lyophilized. The chemical constituents in the essential oils and the extracts were qualitatively and quantitatively analyzed using a Headspace GC-MS and HPLC. Bioactive markers for quality control study were identified and the method of quantitative analysis of the markers was validated. The essential oils and extracts, and some of the bioactive markers, were investigated for their effects on adipogenesis in 3T3-L1 cells using the Oil Red O assay and a triglyceride colorimetric assay kit. The vasorelaxant effect of the mixture of the essential oil that were distilled from the powdered form of all ingredients, was tested on the aortae isolated from rats.

An anti-cellulite topical preparation was formulated using the extracts and essential oils as active ingredients. The stability of the formulation was then studied. A blind randomized placebo-controlled trial was conducted. Eighteen female participants aged 20-55 who had cellulite were treated with the developed formulation applied on one thigh and the placebo applied on the other thigh over a period of three months. Anti-cellulite efficacy was evaluated using measurements of weight and body mass index, thigh circumference, severity of cellulite, skin firmness, and blood flow. The participants were given a diary to record their activities during the study, and

their satisfaction on the formulations was recorded using a questionnaire. The scope of the study is illustrated in the schematic below.



Expected outputs of the study

1. A prototype of an anti-cellulite topical formulation containing ingredients from the Thai herbal compress
2. A petty patent of the anti-cellulite product
3. Publications in scientific journals

Expected outcomes of the study

1. The mechanism of the ingredients of the herbal compress on adipocytes differentiation in 3T3-L1 adipocytes.
2. The new anti-cellulite topical formulation using ingredients originated from the herbal compress.
3. The information of quality and stability of the anti-cellulite formulation developed.
4. The efficacy and safety data of anti-cellulite formulation developed.

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CHAPTER II

LITERATURE REVIEW

Cellulite

More than 150 years ago, cellulite was first documented in French medical literature. There are several terms that refer to cellulite e.g. adiposis edematosa, incipient cellulite or status protusus cutis, full-blown cellulite or dermopanniculosis deformans, nodular liposclerosis, gynoid lipodystrophy and edematofibrosclerotic panniculopathy (1-2).

Cellulite is characterized by an orange peel like appearance, or dimpling, or a nodular, cottage cheese like appearance, or lumpy bumpy impressions on the skin surface around the thighs, buttocks, and occasionally the lower abdomen and upper arms in both men and women. Many women are self-conscious as a result of it. Cellulite, however, is neither an illness nor a painful condition. It is distinct from obesity, even though it is thought to be linked to obesity (2-5).

Incidence

Cellulite affects up to 85-90 percent of women, according to various studies, and it is most common in post-pubescent women over the age of 20. It affects people of all ethnicities, though it appears to be more frequent in Caucasian than Asian women. Cellulite may affect even the healthiest of women. Males can have cellulite as well, especially if they have testosterone deficiency or are on estrogen medication. (6-7).

The origins of the cellulite

Many reports define the major etiologies of cellulite in various ways, which may be summarized as follows:

1. Gender: Nürnberger and Müller first observed major differences in the architecture of the male and female subcutaneous connective tissue. When the subcutaneous fat herniates, it visibly protrudes the epidermis (8). (Figure 2)

2. Ethnicity: Caucasian women are more prone cellulite than Asian women (8).

3. Diet; Eating high carbohydrate diet stimulates hyperinsulinemia and encourages lipogenesis that leads to an increase in body fat content, thereby enhancing cellulite (2, 8).

4. Lifestyle: prolonged sitting or standing may interrupt normal blood flow that leads to more stasis and causes modifications in the microcirculation of cellulite-risk areas (2, 8).

5. Hormone: endocrine factors which intervene in the level of adipose tissue (i.e. insulin, glucagon, corticoids and thyroid), sex hormones and pregnancy (during periods of changes which is related to an increase in some hormones, such as the prolactin and insulin) and increasing overall fluid volume can affect lipogenesis (1, 9-10).

6. Pregnancy, which is linked to an increase in specific hormones including prolactin and insulin, as well as an increase in total fluid volume, promotes cellulite through lipogenesis and fluid retention (8).

Even though the causes of cellulite are debatable, there is evidence from many dermatologists that supports these causal factors.

Anatomy

Cellulite is a degenerative condition in which the hypodermis undergoes changes, resulting in uneven undulations on the skin overlaying the afflicted regions. Cellulite is caused by a series of processes involving the epidermis, dermis, and subcutaneous tissue (Figure 1) (11).

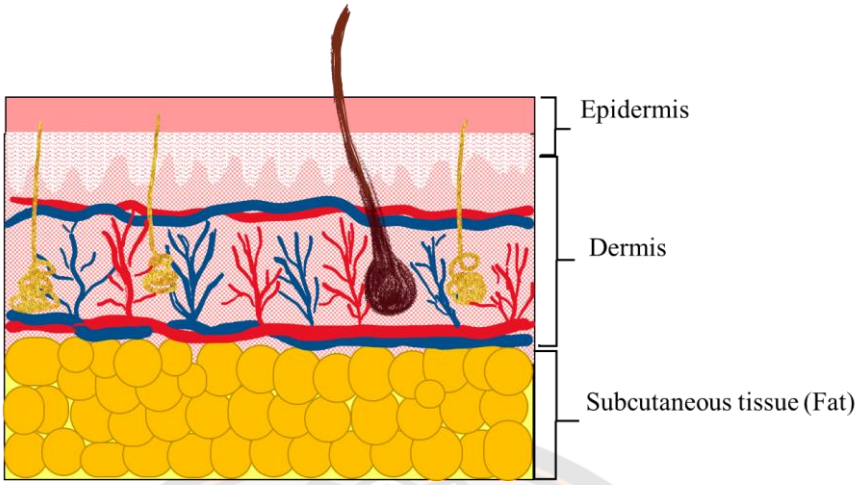


Figure 1 Anatomy of skin

Epidermis is the outer layer of the skin that lacks blood vessels. It receives oxygen and nutrients from the lower layers. It continually creates new skin cells to replace dead cells on the surface. The epidermis also produces melanin, which colors the skin.

Dermis is the middle layer of the integument. It consists of loose connective tissue and houses a number of accessory structures of the skin and connects to the epidermis.

Hypodermis or subcutaneous tissue is found beneath the dermis. It is a subcutaneous layer (under the skin). The hypodermis is composed of loose, fibrous connective tissue, which is richly supplied with lymphatic and blood vessels and nerves. It is much thicker than the dermis. It contains coils of ducts of sudoriferous (sweat) glands, and the bases of hair follicles (12).

Adipose tissue or body fat is loose connective tissue containing mostly adipocytes, which are derived from preadipocytes. Its primary function is to store energy as lipids. The lipid reserves are permanently being renewed, which is a sign of a particularly active cell metabolism with a consequent rapid turnover of lipids. This metabolism consists of 3 phases: lipogenesis, storage of lipids in the form of triglycerides, and lipolysis. Several functions have been attributed to adipose tissue: thermoregulation, thermal and mechanical isolation, and the production and storage of energy (11-14). Adipocytes are large, vacuolated cells, almost filled with

triglycerides. They are grouped together in lobules and surrounded by conjunctivo-vascular walls.

In women, these walls are vertical and perpendicular to the fascia, whereas in men they are slanted. This explains why the “orange peel” effect is mainly a female problem. The connective part includes reticulin or reticular fibers, collagen fibers and reticulo-endothelial cells. Vascularization is ensured by the system of 1 artery and 2 veins for each lobule and by numerous capillaries which run through the lobules and which surround each adipocyte (1, 12-15). As well as this extensive vascularization there is a rich lymphatic network and an innervation made up of sympathetic fibers and nerves which run up through the hypodermis before reaching the overlying dermis.

Biosynthesis of triglycerides

Triglycerides principally arise from glycerol, (as alpha-glycerophosphate (α-Glycero-P)) and fatty acids as Acyl-CoA.

α-Glycero-P comes from anaerobic glycolysis. About 25 g of glucose are metabolized in this way every 24 hours. α-Glycero-P can also come from pyruvate produced during the metabolism of amino-acids. The existence of pyruvate enables the maintenance of lipogenesis during prolonged fasting and chronic hypoglycemia.

Fatty acids from the diet are absorbed by the gut, changed into lipoprotein and then delivered to the cells of the body by being transported in the blood. The adipocyte possesses an enzymatic membrane complex, the lipoprotein lipase, which hydrolyzes the lipoproteins to release the fatty acids. The effects of this enzyme are elevated in post-prandial periods by insulin stimulation and lessened during prolonged fasting or muscular exercise (14, 16-18).

Stored fat is in the form of triglycerides, which means that to reduce the amount of fat stored, lipolysis must be promoted. Lipolysis is the medical term for the breakdown of fat, which is the degradation process of triglycerides from adipocyte by lipoprotein lipase, leading to the formation of fatty acids and glycerol. Lipase located on the fat cell membrane can be activated or inhibited by catecholamines (2, 19-20).

Lipolysis process

The lipolysis process is controlled by the nervous system and plasma insulin levels. Signal molecules that control this nervous system, such as adrenaline, are called catecholamines. Adrenaline is also known as epinephrine, in which adrenergic receptors interact with these compounds. Catecholamines can stimulate lipolysis via G_s proteins on β_3-adrenergic receptor. They can also inhibit lipolysis via G_i proteins on α-adrenergic receptors.

Cellulite is more often found in specific anatomical sites (thighs and buttocks) because of the presence of adrenergic receptors in these sites (20-21).

Activation of lipolysis is mediated by an increment of cyclic AMP (cAMP) concentration. An increased cAMP level can be stabilized by inhibiting phosphodiesterase, leading to the activation of the protein kinase-A (PKA) and hormone-sensitive lipase (HSL). HSL hydrolyses triglycerides to free fatty acid (FFA) and monoacylglycerols. In the last step of hydrolysis, monoacylglycerols are decomposed into glycerol and FFA.

FFA is a source of fuel for the generation of ATP. Oxidation of FFA involves the formation of fatty acyl-coenzymeA (acyl-CoA), and translocates across mitochondrial membrane, which subsequently undergoes β-oxidation in mitochondrion. In each turn of this cycle, acetyl CoA and a fatty acyl-CoA with two fewer carbon atoms, are produced (1, 11).

In short, lipolysis can be stimulated by β-adrenergic receptor agonists, α_2-adrenergic receptor antagonists, phosphodiesterase inhibitors and β-oxidation stimulators.

Several factors influence lipolysis or lipogenesis and contribute to a decrease or increase in the thickness of the fatty tissue. Insulin stimulates lipogenesis, which is enhanced by estrogen and prolactin, and decreased by progesterone, luteinizing hormone (LH), testosterone and glucocorticoids. A hypercalorific diet rich in carbohydrates stimulates lipogenesis, because it increases lipoprotein lipase (LPL) activity. Physical exercise decreases the plasma concentration of insulin. Catecholamines stimulate lipolysis through the activation of adenylcyclase. Methylxanthines, papaverine and tiratricol also increase lipolysis through the inhibition of phosphodiesterase.

Regulation of lipolysis

Lipolysis generates free fatty acids which supply the muscular cells with energy and permits the storing of glucose in the gluco-dependent tissues. Lipolysis also supplies glycerol, which is used in the hepatic neoglucogenesis pathway.

Lipolysis co-exists with lipogenesis, but one of the two must predominate: the regulation of adipose tissue metabolism (lipolysis in particular) will depend on the nutritional state and on hormonal stimulations.

The maintaining of body weight (i.e. the adipose mass) depends on the well-being of each of the regulatory systems.

For the daily food intake, lipogenesis is dependent on the amount of fatty acids and glycerophosphate in the body: fats and carbohydrates are the source of these cellular substrates. Secreted insulin stimulates lipoprotein lipase and the absorption of glucose into the cell. The glucose is then transformed into α-glycerophosphate. Under normal alimentary conditions, the metabolism tends to favour lipogenesis.

If, however, the daily food intake happens to decrease, lipolysis would take over and fatty acids and released glycerol would supply the energy and glucose needed by the cells (13-14, 16-18).

Development of cellulite

Cellulite occurs with alterations to the adipose tissue and microcirculations that result from blood and lymphatic disturbances. This brings about fibrosclerosis of the connective tissue in the epidermis, dermis and hypodermis or subcutaneous tissue. When these fat cells become larger, their hypertrophy compresses the blood vessels, which decreases blood flow and results in water infiltrating the hypodermis.

The pathophysiology of cellulite that shows as an increasing concentration of glycosaminoglycans (GAG) in the skin overlying cellulite areas, presumably leading to a rise in the amount of water retained in the skin in this disease, was first published in 1987 (11, 22-23). The increased risk of cellulite in women bearing the D allele of the angiotensin-converting enzyme (ACE) gene I/D genotype is likely to be related increases of the vasoconstrictor angiotensin II formation in the subcutaneous adipose tissue, ultimately resulting in a reduced local blood flow (24). These effects induce changes that may lead to vascular compression, local edema, microvascular

dysfunction, tissue hypoxia, inflammation, and fibrosis. Cellulite results from the protrusion of the adipose tissue into the dermis. This may also result in moderate local inflammation that is chronic and self-sustaining (25). Mechanisms for the development of cellulite are illustrated in Figure 2.

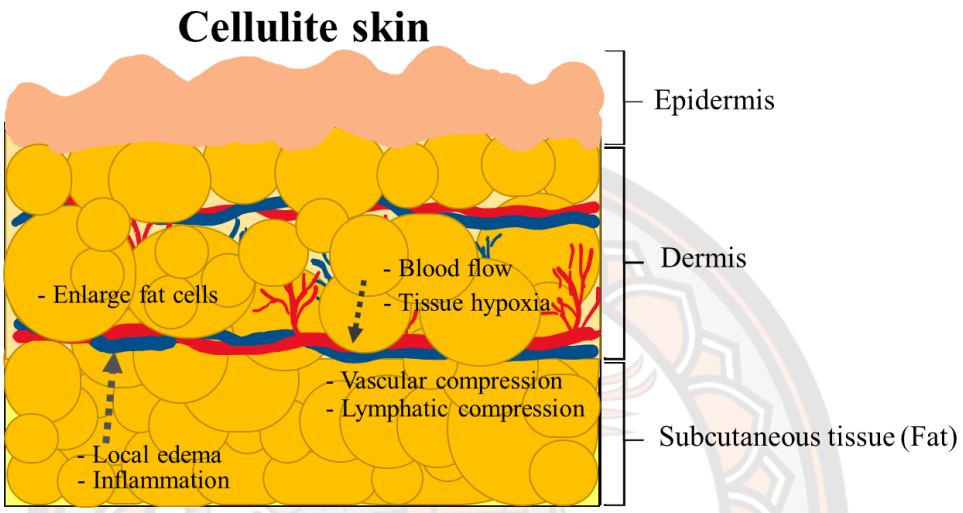


Figure 2 The formal arrangement of the dermis and hypodermis of hypertrophied adipocyte tissue

Cellulite Pathophysiology

Various cellulite pathophysiology ideas have been proposed in recent years. However, a review of the literature found very few studies to validate these currently popular theories and treatments of cellulite. One paper that was found emphasised the point that a thorough understanding of cellulite pathophysiology is necessary for successful treatment modalities to be developed (11). The main theories attempt to explain the anatomical or physiological changes of cellulite, e.g. edematous fibrosclerotic panniculopathy, gynoid lipodystrophy, etc. The four leading hypotheses that explain the physiology of cellulites are; the architectural differences of subcutaneous tissue between genders, altered connective tissue septae, vascular changes and inflammatory factors (4, 15).

Symptoms of cellulite

There are several skin features, which are very often reported by patients. Besides the characteristic orange peel appearance and alterations in arms, abdomen, knees, thigh and trochanters seen in cellulite, subjective symptoms characterized by alterations in subcutaneous tissues may also appear. The following alterations may be found: (11, 20, 26).

1. Heaviness and tension at lower limbs
2. Cold feet
3. Cramps
4. Paraesthesias
5. Pain
6. Dry skin
7. Altered sensitivity
8. Tiredness

Cellulite diagnosis

The diagnosis of potentially supplementary illnesses is also aided by a thorough patient history. The following is crucial information: (11, 26):

1. medical and family history
2. obesity/diet
3. diabetes
4. hepatitis
5. lipid and endocrine alterations
6. bowel habits and conditions
7. menstrual periods and estro-progestagen therapies
8. bone fractures
9. systemic diseases
10. surgical history
11. exercise
12. nutrition
13. food or drug allergies
14. smoking and alcohol use
15. previous therapies

Cellulite stages

Cellulite is classified into four separate categories, each with a severity level ranging from 0 to 3. The patient should be evaluated in the standing position with relaxed muscles. On the other hand, if the patient shows visible dimpling, they should be asked to contract their muscles or the pinch test should be used to distinguish between scores 0 and 1 (pinch test by squeezing the skin between the thumb and index finger).(27). The patient should be in the supine posture (laying) with relaxed muscles while evaluating for scores 2 and 3. (Table 1).

Table 1 The Nürnberger and Müller cellulite classification scale (28-29)

Grade/Stage	Standing	Supine	Pinch test
0	---	---	---
1	---	---	Dimpling present
2	Dimpling present	---	Dimpling present
3	Dimpling present	Dimpling present	Dimpling present

Hexsel et al. (27) evaluated the five essential morphological characteristics of cellulite using standardized pictures of 55 individuals in 2009. In that study, a dermatologist with competence in cellulite evaluation used the cellulite severity scale to analyze all patients in a standing position with relaxed gluteus muscles (CSS). The CSS is a validated, easy to apply tool used to assess cellulite grading (30) and comprises the assessment of the most important clinical and morphologic aspects of cellulite: (A) number of evident depressions; (B) depth of depressions; (C) morphologic appearance of skin surface alterations; (D) grade of laxity, flaccidity, or sagging skin; and (E) the first cellulite classification by Nürnberger and Müller (31). Each morphologic feature of cellulite was scored on a scale of 0 to 3, resulting in a total score ranging from 1 to 15. In the Hexsel study, a new photonumeric severity scale was developed and validated and the new classification of cellulite was established which is determined by the severity scores, as illustrated in Table 2.

Table 2 Scoring system of the cellulite severity scale and new classification of cellulite proposed by Hexsel, et al. (2009)

Cellulite severity scale	New classification
1–5	Mild
6–10	Moderate
11–15	Severe

Cellulite treatment

Cellulite can be treated in three ways, according to the literature review: (21):

1. Decreasing adipocyte contents
2. Strengthening and protecting fundamental substances of connective tissue
3. Improvement of microcirculation

Various cellulite treatments can be categorized into three groups; topical, instrumental and oral method; and can also be divided into four main categories (15, 21).

1. Attenuation of aggravating factors,
2. Physical and mechanical methods,
3. Pharmacological agents and,
4. Laser

Pharmacological agents used for cellulite treatments

Both oral and topical administrations can transport active substances to target fat cells and improve fundamental substances.

Topical management

To apply topical treatments to reduce the appearance of cellulite, the concentration and pharmacokinetics of the active drugs as well as the nature of the delivery vehicle must be considered. Vehicles can be in the form of gels, ointments, foams, creams, and lotions, all of which aim to efficiently deliver active product into the skin.

The main barrier to drug penetration is the stratum corneum, the cornified outermost layer of the epidermis. Formulations for topical use may include “permeation enhancers” which significantly increase cutaneous penetration when included in the formulation. Skin enhancers can be common solvents (water, alcohol, and methyl alkylsulphoxide) or surfactants. They may also be phospholipid molecules called phytosomes, which increase their lipid solubility when attached to the active drug.

Liposomes, which are specifically constructed lipid vesicles loaded with active medicines, are used in an unique percutaneous delivery technique. According to their mechanism of action, topical anti-cellulite preparations can be divided into four major groups. (Table 3) (11).

Table 3 Examples of agents for cellulite, based on mechanisms of action (11)

1.	**Agents that accelerate the flow of blood via the microvasculature**
	Centella (*Centella asiatica*), Butcher’s broom (*Ruscus aculeatus*), Common ivy (*Hedera helix*), Ground ivy (*Glechoma hederaceae*), Ginkgo (*Ginkgo biloba*), Horse chestnut (*Aesculus hippocastanum*), Papaya (*Carica papaya*), Pineapple (*Ananas sativus*, *Ananas comosus*), Rutin, Pentoxyfylline, Red grapes (*Vitis vinifera*), Silicium, artichoke (*Cynara scolymus*), Sweet clover (*Melilotus officinalis*),
2.	**Agents that lipogenesis inhibitors and lipolysis promoters**
	Methylxanthines (aminophylline, caffeine, theobromine, theophylline), β-adrenergic agonists (adrenaline, isoproterenol), α-adrenergic antagonists (dihydroergotamine, piperoxan, phentolamine, yohimbine)
3.	**Agents that restore the normal structure of the dermal and subcutaneous tissue**
	Ascorbic acid (vitamin C), Bladderwrack (*Fucus vesiculosus*), Retinol (vitamin A)
4.	**Agents that inhibit or eliminate the production of free radicals**
	Ascorbic acid (vitamin C), Ginkgo (*Ginkgo biloba*), α-tocopherol (vitamin E), Red grapes (*Vitis vinifera*)

Consumers can acquire topical methods easily, and they do not require the services of experienced advisers. Several active constituents work in different ways to enhance lipolysis, inhibit lipogenesis, and improvement of cellulite appearance.

Consumers may choose from a broad choice of items due to their convenience of use and acceptable efficacy. The active ingredients are discussed as shown in Table 4.

Table 4 Representative of medicinal plants and their active constituents used for anti-cellulite treatment (11)

Plant	**Active constituent**
Algae or Bladderwrack (*Fucus vesiculosus*)	Iodine
Algae (*Fucus vesiculosus*)	Polysaccharides, bromine and iodine
Barley (*Hordeum vulgare*)	Catechin, tocopherol, and lutein
Bearberry (*Uva ursi*)	Glycoside arbutin (mild diuretic)
Blackcurrent (*Ribes higram* L.)	Flavonoids
Bitter orange (*Citrus aurantium* L. *var. amara*)	Naringin, hesperidin, neohesperidin
Butcher's broom (*Ruscus aculeatus*)	Ruscogenin, neoruscogenin (bioflavanoids)
Cayenne (*Capsicum annuum* L.)	Capsaicin
Centella (*Centella asiatica*)	Pentacyclictriterpenes (Asiatic acid, Madecassic acid, Asiacoside and Madecasspsode)
Chilli (*Capsicum frutescens* L.)	Capsaicin, cryptoxanthin, capsorubin, carotenoid
Coffee (*Coffea spp*.)	Caffeine
Cola (*Cola nitida*)	Polyphenolic compounds (Tannic acid, Catechin, Epicatechin)
Cypress (*Cupressus sempervirens* L.)	Cetechin derivatives
Cypress (*Cupressus sempervirens* L.)	Cetechin derivatives
Garcinia (*Garcinia atroviridis* Griff)	(-)- Hydroxycitric acid, tartaric acid, malic acid, ascorbic acid
Gingko (*Gingko biloba*)	Ginkgo-flavonoid glucosides, terpenoids

Plant	**Active constituent**
Ginseng (*Zingiber officinali*)	Ginsenosides
Ginger(*Zingiber officinalis* Rosc.)	Gingerols, shogaols
Grape vine(*Vitis vinifera*)	Leucocyanidines(bioflavanoids)
Green tea (*Thea sinensis*)	Flavanoids, methylxanthines
Hawthorne berry (*Crataegus* spp.)	Bioflavanoids
Horsetail (*Equisetum arvensis*)	Silicon
Horse chestnut (*Aesculus hippocastanum*)	Aescin, Escin(seeds)
Ivy (*Hedera helix*)	Falcarinol
Lady's thistle (*Silybum marianum* GAERTN)	Flavanolignans
Lady's Mantle (*Alchemilla vulgaris* L.)	Tannins

From Table 4, and 5 above, these plants contain chemicals compounds beneficial for cellulite treatment. They minimize cellulite appearance through many mechanisms as suggested below (32). These mechanisms include:

1. Decrease of adipocyte contents: The stimulated lipolysis process, one of which uses a 3β-adrenergic agonist and α2-adrenergic inhibitor. There are a number of families of pharmacological actives which include xanthines and xanthine derivatives which are beta-adrenergic agonists, which work by inhibiting phosphodiesterase, which finally leads to the conversion of triglycerides into free fatty acids and glycerol. Caffeine is the most popularly used ingredient in anti-cellulite products and is one of the xanthines classification. Other examples include theobromine, theophylline, and aminophylline. α2-adrenergic inhibitors that work in a similar way include yohimbine, α- yohimbine, piperoxine, phetolamine and dihydroergotamine.

Table 5 Representative of chemical substances used for anti-cellulite treatment (32)

Chemical substances	Mechanisms
Coenzyme A	Induce active transport through mitochondria
L-carnitine	Induce active transport through mitochondria
Vitamin A	Stimulate turnover of skin
Retinoic acid	Stimulate turnover of skin
Xanthines and xanthine derivatives	Phosphodiesterase inhibitor
Co-enzyme Q10	Antioxidant
Vitamin C	Antioxidant
Vitamin E	Antioxidant

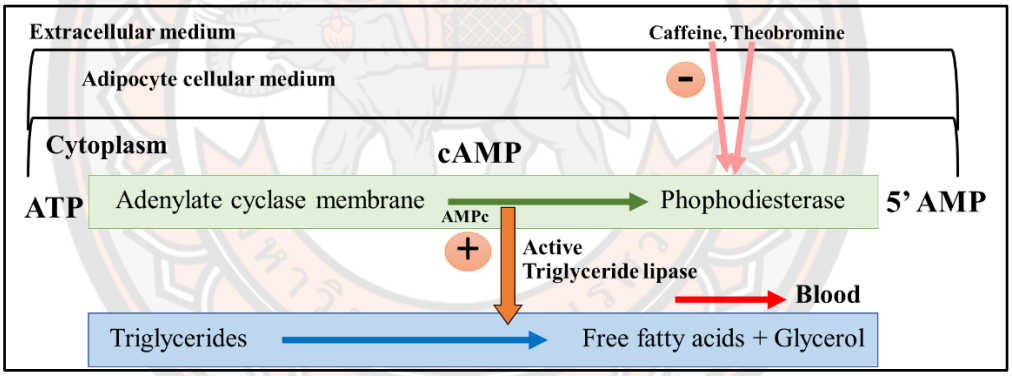


Figure 3 Action of caffeine and theobromine on lipolysis regulation

Other herbal extracts that are claimed to promote lipolysis include cayenne extract, and ginger extract claiming to have thermogenic effects. A study of body shaping cream containing capsicum, ginger, and garcinia extracts at 1% w/w in 20 volunteers for a period of 28 days illustrated a significant decrease of the average perimeter of the waistline (-6.55 + 0.52 cm) in all participants. However, there was a non-significant decrease of body weight (-0.43 $\pm$ 0.28 kg) in 77% of the volunteers (33).

2. Protection and enhancement of fundamental substances: both the vasculature and the septa in the hypodermis play a vital role in the development of cellulite.

All connective tissue, including collagen, elastin, fibronectin and various glycosaminoglycans (GAGs), can be adversely affected by matrix metalloproteinases (MMPs) which are often released by cells in response to inflammation, such as collagenases and elastases. Therefore, a goal of a slimming formulation is to target inflammation and MMP deactivation, as well as directly strengthen the connective tissue (34). To summarize, the factors contributed to the loss of skin elasticity are: 2.1) Free radicals, 2.2) Skin enzymes (mainly elastase), 2.3) Slow turnover of the skin.

The reduction of free radicals can be achieved by using antioxidants, which helps improve inflammation, and the resultant release of MMPs that target the destruction of connective tissues. Antioxidant scavengers can also be found in various foods and plant extracts. For example, grape seed extract, green and white tea extracts, vitamin C, vitamin E, coenzyme Q10 (co-Q10), genistein, lycopene, carotenoids such as beta-carotene and lutein, pomegranate extract, alpha lipoic acid, resveratrol and pine bark extract. Other sources include spices such as garlic, onion, turmeric, sage, thyme, and oregano. Another mechanism helps to stimulate turnover of extacellular matrix to produce healthy collagen or elastin can be achieved by using retinoic acid, retinols, vitamin A, vitamin C. In a study using 0.3% retinol over 6 months period showed an improvement in cellulite in 12 out of 19 subjects (35).

However, another study could not find any change in the cellulite appearance but observed an increase in skin elasticity and a decrease in its viscosity, which used a plant complex combining lady’s thistle, alchemilla or yarrow, horsetail, and germinated seeds, which contain corresponding actives, silybin, tannins, silicon, and peptides which variously exhibit inhibition of elastase enzymes. Elsewhere, positive results in increased skin elasticity as well as decreased wrinkle by 36.7% were observed. Other herbal sources include Asiatic centella extract, which consists of chemically asiaticosideo (40%), madecassic acid (30%) and Asiatic acid (30%). An *in vitro* study demonstrated that these act on fibroblasts, stimulating collagen and mucopolysaccharide synthesis.

3. Improvement of microcirculation: another hypothesis regarding the appearance of cellulite is that it is a result of decreased microcirculation, which occur as a direct result of the deterioration of the dermal vasculature. The mechanisms to achieve this are to strengthen the walls of veins, to improve blood flow, and to reduce water retention.

Rutin is a citrus flavanoid glycoside found in buckwheat, the leaves and petioles of the Rheum species, and the fruit of the Brazilian Fava D'Anta tree. It acts as a capillary-strengthener as well as acts to slow down lipogenesis. Citrus bioflavonoids from lemons, grapefruits and oranges, as well as fruit bioflavanoids such as hesperidin, eriocitrin and flavonones from grapes, plums, apricots and cherries, also strengthen capillaries and regulate their permeability. Butcher's broom extract and horsetail extract are also known to strengthen walls of arteries and veins.

Other plant extracts that assist blood circulation include bilberry extract, ginkgo biloba, hawthorne berry, ivy, and ginseng. The current knowledge of individual and combination topical therapies used to reduce cellulite (32) and chemicals substances found in natural extracts can be generally grouped according to their activities, as shown in Table 6.

Table 6 Summary of case studies using topical approach for cellulite treatment (32, 36)

Active compound	Evaluation	No. (subjects)	Period	Results
1.5% theophylline of gel	Thigh circumference above knee 25 cm	40	4 wks	-1.69 + 1.43 cm
10% aminophylline	Thigh circumference	28	49 days	-1.25 inches
1% w/w ginger, capsicum, garcinia extracts	Waistline circumference	20	4 wks	-6.55 + 0.52 cm
caffeine, L-carnitine, coenzyme A	Thigh circumference above knee 20 cm	3	4 wks	-1.26 cm
Caffeine, ivy, butcher's broom	Tactile examination and Ultrasonic echography on thigh	27	1 month	All 27 showed decreased thickness of subcutaneous fat
0.3% retinol	Laser Droppler velocimetry on thigh	19	6 months	12 positive results, 7 no improvement
5% herbal extracts	Image analysis	6	45 days	-36.7% decrease in wrinkle
2% aminophylline with 10% glycolic acid	circumferences, ultrasound measurements, subcutaneous fat depth Clinical evaluation of cellulite Self-evaluation survey	23	12 wks	cellulite appearance improved (37)
0.25% Visnadine + 0.5% Ginko biloba, 1% Escin)	thigh circumference, skin plastoelasticity, contact thermography, ultrasound measurements, spectrophotometric analysis, clinical evaluation of cellulite	25	4 wks	the use of synergistic botanical standardized extracts, through the exploitation of different mechanisms of action and acting on different biological targets, provides visible and measurable results in the improvement of cellulite signs and symptoms

Active compound	Evaluation	No. (subjects)	Period	Results
7% caffeine solution	% of patients with reduction/rise of thigh and hip circumferences % of changes in perivascular dermic oedema (functional capillary density, dermal capillaries diameter), Self-evaluation survey	134	1 month	reduction of thigh circumferences in more than 80% of the cases and reduction of hip circumference in 67.7%. FCD, DPD, and CD did not change significantly after treatment.(38)
Cryotherapy with camphor and menthol gel	circumference: arm, waist, hip, abdomen and thigh, cutaneous folds (tricipital, subscapular, medial axillary, pectoral, suprailiac, abdominal, femoral), body fat percentage, body self-image scale	36	average of 8,45 applications, three or four times a week, with a one- or two-day interval between applications.	isolated, decreased medial axillary, pectoral and abdominal skinfold and arm body perimeter measure, but did not change significantly the others body perimeter measures, fat percentage and body self-image in young women.

Hydro-distillation

To isolate essential oils by hydro-distillation, the aromatic plant material is packed in a still and a sufficient quantity of water is added and brought to the boil. Alternatively, live steam is injected into the plant container. The boiling water or steam free the essential oils from the oil glands in the plant tissue. The vapor mixture of water and oil is condensed by indirect cooling with water and the condensed distillate flows into a separator, where the oil separates automatically from the distillate water (39).

Three Types of Hydrodistillation

There are three types of hydrodistillation for isolating essential oils from plant materials:

1. Water distillation
2. Water and steam distillation
3. Direct steam distillation

Herbal compress

Luk-Pra-Kob, or herbal ball, is another name for the herbal compress. It is form into a ball with a small handle from a muslin bag that is filled with various herbs. Herbal compresses have long been utilized in Thai massage therapy to reduce pain and inflammation, as well as in postpartum mothers suffering from the consequences of delivery. Our previous study (40) discussed a modification to the herbal compress that included plants with anti-cellulite, vasodilation, and anti-inflammation properties, as well as certain conventional auxiliary herbs and flavoring additives. The main components of this composition are ginger, black pepper, java long pepper, tea, and coffee (Table 7). In that study, 21 women (20-55 years old) with cellulite (grade>2) were treated with steamed placebo or herbal compresses twice weekly on one or both thighs for 8 weeks with a 2-week washout period. The herbal compress therapy reduced their Nürnberger-Müller cellulite scores from 12.6±2.0 to 9.9±2.4 (means ± SEM) in contralateral placebo-treated thighs after 8 weeks ($p<0.0001$; effect size=1.16, CI=0.48-1.83). The herbal compresses were shown to be effective in reducing cellulite and thigh size.

Table 7 The ingredients of the anti-cellulite herbal compress (40)

Classification	Ingredient		
	Botanical name (Common name)	Amount (% w/w)	Part used
Constituents of traditional compresses (50 % w/w of whole recipe) Herbs reducing inflammation or showing other benefits to skin			
	Curcuma longa Linn. (Turmeric) [41]	5.0	Rhizome
	Zingiber montanum (J.Koenig) Link ex A.Dietr.(Plai) [42]	5.0	Rhizome
	Cymbopogon citratus Stapf. (Lemon grass)[43]	10.0	Stalk
	Citrus hystrix DC. (Makrut lime) [44]	10.0	Peel of fruit
	Cinnamomum camphora (L.) Presl. (Camphor) [45]	13.0	Bark
	NaCl (salt)	7.0	NA
Herbal drugs selected for potential anti-cellulite action (50% of whole recipe) Herbs reducing inflammation, increasing microvascular and lymphatic flow, and/or stimulating lipolysis and reducing lipogenesis			
	Zingiber officinale Rosc. (ginger) [46]	20.0	Rhizome
	Piper nigrum Linn. (black pepper) [47]	7.5	Fruit
	Piper retrofractum Vahl. (java long pepper) [48]	7.5	Fruit
	Camellia sinensis (L.) Kuntze (tea) [49]	7.5	Leaves
	Coffea arabica Linn. (coffee) [50]	7.5	Seed

Volatile oils and extracts of the principal ingredients of the anti-cellulite herbal compress

To formulate the herbal compress for anti-cellulite application, nine essential oils and two extracts which have been reported to influence cellulite were selected as the active ingredients (principal herbs). The seven essentials oils and two plants are listed in Table 8. and the chemical profiles of the anti-cellulite herbal compress oil which was composed of camphor, camphene, citral, 3-carene, citronellal, caffeine, limonene, myrcene, α-pinene, β-pinene, sabinene, and terpinen-4-ol, are presented in Table 9.

Table 8 Essential oil monographs are present in anti-cellulite herbal emgel of ginger essential oil (51-54)

Scientific name:	*Zingiber officinale* Roscoe	
Family name:	Zingiberaceae	
Part of use:	rhizome	
Description:	odour, aromatic; taste, pungent (54)	
Method of Extraction:	steam distillation	
Volatile oil content:	not less than 2% v/w (54)	
Essential Oil Characteristics:		
○ Color	clear to pale yellow	
○ Aroma	base, middle	
○ Viscosity	thin	
Chemical composition:	**Monoterpenes** (%trace); limonene 1-2%, alpha-pinene 1-2% camphene 1-2% sabinene 4-10%	**Sesquiterpenes** (%trace); zingiberene 14-50% β-sesquiphelllandrene 10%
	Monoterpenols (%trace); linalool 1-3%	
Therapeutic properties:	Analgesic, anti-inflammatory, antirheumatic, carminative, rubefacient, stomachic, stimulant, vasodilator, anti-emetic, anti-spasmodic, expectorant, immunostimulant, laxative	
Bioactive markers	camphor, camphene, 3-carene, citronellal, limonene, myrcene, α-pinene, β-pinene, and terpinen-4-ol	
Precautions	Has very low dermal toxicity, when tested at 4% in petrolatum it produced no irritation after 48 hours closed patch test on human subjects. However, in hypersensitive individuals, ginger may produce dermatitis. Do not use on babies / children (too warming)	

Table 9 Essential oil monographs are present in anti-cellulite herbal compress emgel of black pepper essential oil (55-57)

Scientific name:	*Piper nigrum* L.	
Family name:	Piperaceae	
Part of use:	fruit	
Description:	odour, aromatic; taste, pungent (58)	
Method of Extraction:	steam distillation	
Volatile oil content:	not less than 1% v/w (58)	
Essential Oil characteristics:		
○ Color	clear to pale yellow	
○ Aroma	top, middle	
○ Viscosity	thin	
Chemical composition:	**Monoterpenes** (%trace); limonene 5-10%, 3-carene 5-7%, camphene 1-2%, sabinene 1-14%, myrcene 2-3%	**Sesquiterpenes** (%trace); β-caryophyllene 30-35%, zingiberene 5-10%
Therapeutic properties:	analgesic, anti-spasmodic, carminative, rubefacient, stomachic, diaphoretic, laxative, stimulant, anti-microbial, tonic	
Bioactive markers	camphor, camphene, citral, 3-carene, citronellal, limonene, myrcene, α-pinene, β-pinene, sabinene, and terpinen-4-ol	
Precautions	Non-toxic, non-sensitising. Can be irritant to skin and mucus membranes in high concentrations. Oxidises readily on exposure to air. Believed to antidote homeopathic remedies	

Scientific name:	*Piper retrofractum* Vahl.	
Family name:	Piperaceae	
Part of use:	fruit	
Description:	odour, aromatic; taste, pungent (58)	
Method of Extraction:	steam distillation	
Volatile oil content:	not less than 1% v/w (58)	
Essential Oil characteristics:		
o Color	clear to pale yellow	
o Aroma	top, middle	
o Viscosity	thin	
Chemical composition:	**Monoterpenes** (%trace); limonene 33%, alpha-pinene 30%, sabinene 1-5%, myrcene 3-5%	**Sesquiterpenes** (%trace); caryophyllene 15-20%
Therapeutic properties:	analgesic, anti-spasmodic, carminative, rubefacient, stomachic, diaphoretic, laxative, stimulant, anti-microbial, tonic	
Bioactive markers	camphor, camphene, citral, 3-carene, citronellal, limonene, myrcene, α-pinene, β-pinene, sabinene, and terpinen-4-ol	
Precautions	Non-toxic, non-sensitising. Can be irritant to skin and mucus membranes in high concentrations. Oxidises readily on exposure to air.	

Table 10 Essential oil monographs are present in anti-cellulite herbal compress emgel of turmeric essential oil (61-64)

Scientific name:	*Curcuma longa* L.
Family name:	Zingiberaceae
Part of use:	rhizome
Description:	odour, aromatic; taste, pungent (58)
Method of Extraction:	steam distillation
Volatile oil content:	not less than 1% v/w (58)
Essential Oil characteristics:	
○ Color	clear to yellow
○ Aroma	top, middle
○ Viscosity	thin
Chemical composition:	**Monoterpenes** (%trace); limonene 33%, alpha-pinene 30%, sabinene 1-5%, myrcene 3-5% **Sesquiterpenes** (%trace); ar-turmerone 40-55%
Therapeutic properties:	anti-inflammatory, anti-arthritic, anti-microbial, antifungal, anti-oxidative, rubefacient, stimulant (nerves, uterus and heart muscles), tonic, vermifuge
Bioactive markers	camphor, 3-carene, citronellal, myrcene, α-pinene, β-pinene, and terpinen-4-ol
Precautions	less side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature. Turmeric is known to cause skin irritation if taken in large doses or undiluted.

Table 11 Essential oil monographs are present in anti-cellulite herbal compress emgel of plai essential oil 65-70)

Scientific name:	*Zingiber montanum* (J.Koenig) Link ex A.Dietr.	
Family name:	Zingiberaceae	
Part of use:	rhizome	
Description:	odour, aromatic;(58)	
Method of Extraction:	steam distillation	
Volatile oil content:	not less than 1% v/w (58)	
Essential Oil characteristics:		
○ Color	clear to pale yellow	
○ Aroma	top, middle	
○ Viscosity	thin	
Chemical composition:	**Monoterpenes** (%trace); sabinene 30-60%, γ-terpinene 5-8%, terpinen-4-ol 22-30%, α-pinene 1-2%, β-pinene 2-3%	**Sesquiterpenes** (%trace); β-phellandrene 1-2%
Therapeutic properties:	anti-inflammatory, anti-arthritic, anti-microbial, antifungal, anti-oxidative, analgesic, anti-spasmodic, stimulant the immune system, rejuvenating, treating bruise, sprain, musculoskeletal pain, regulate the blood, stimulate urination, release gas and relaxing properties.	
Bioactive markers	camphor, 3-carene, myrcene, α-pinene, β-pinene, and terpinen-4-ol	
Precautions	Plai essential oil is considered non-toxic, non-sensitizing and non-irritating. Always best to spot test first though on sensitive skin.	

Table 12 Essential oil monographs are present in anti-cellulite herbal compress emgel of lemon grass essential oil (71-74)

Scientific name:	*Cymbopogon citratus* DC. Stapf	
Family name:	Poaceae	
Part of use:	stalk	
Description:	odour, aromatic, fresh (58)	
Method of Extraction:	steam distillation	
Volatile oil content:	not less than 1% v/w (58)	
Essential Oil characteristics:		
o Color	clear to pale yellow	
o Aroma	top, middle	
o Viscosity	thin	
Chemical composition:	**Monoterpenes** (%trace); limonene 3-6%, β-myrcene 0.8-20% **Aldehydes** (%trace); citral 75-80%, geranial 40-50%, neral 25-30%, β-pinene 1-2%, citronella 0.2-1.32%,	**Monoterpenols** (%trace); linalool 1-3%, geraniol 5-10%
Therapeutic properties:	anti-inflammatory, anti-arthritic, anti-microbial, antifungal, anti-oxidative, analgesic, anti-hypolipidemic, anti-spasmodic, stimulant the immune system, rejuvenating and relaxing properties.	
Bioactive markers	camphor, citral, 3-carene, citrolnella, limonene, myrcene, β- and β-pinene	
Precautions	A common side effect of lemongrass oil is rash. Lemongrass may also cause irritation and burning if not properly diluted when used on the skin. Caution is advised in patients with diabetes or hypoglycemia and in those taking drugs, herbs, or supplements that affect blood sugar. Serum glucose levels may need to be monitored by a healthcare provider. Medication adjustments may be necessary.	

Table 13 Essential oil monographs are present in anti-cellulite herbal compress emgel of kaffir lime essential oil (75-77)

Scientific name:	*Citrus hystrix* DC.	
Family name:	Rutaceae	
Part of use:	fruit peel	
Description:	odour, aromatic, fresh (58)	
Method of Extraction:	steam distillation	
Volatile oil content:	not less than 1% v/w (58)	
Essential Oil characteristics:		
o Color	clear to pale yellow	
o Aroma	top, middle	
o Viscosity	thin	
Chemical composition:	**Monoterpenes** (%trace); sabinene 30-50%, limonene 17-24%, β-myrcene 1-2%, camphene 0.1-2%, terpinen-4-o 1-3%	**Monoterpenols** (%trace); linalool 0.6-1%
Therapeutic properties:	anti-inflammatory, anti-arthritic, anti-microbial, antifungal, anti-oxidative, stimulating, activation oil; useful for alleviating depression and stress.	
Bioactive markers	camphor, camphene, citral, 3-carene, citronellal, limonene, myrcene, α-pinene, β-pinene, sabinene, and terpinen-4-ol	
Precautions	kaffir lime peel essential oil needs to be used cautiously. For instance, this essential oil is highly potent and, hence, it should never be consumed directly. Direct consumption of this essential oil may lead to vomiting, nausea, light-headedness, dizziness as well as other undesirable side effects. It is advisable that you always dilute this essential oil by blending it with water or any other suitable diluting agent. Aside from this, there are no reports of unwanted side effects due to the use of kaffir lime essential oil. As in the case of any other herbal remedy, you should essentially check with a professional medical practitioner prior to using this essential oil or making any major modification in your supplement regime.	

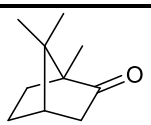
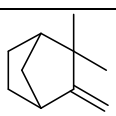
Table 14 Tea and coffee are present in anti-cellulite herbal compress emgel

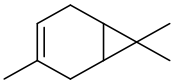
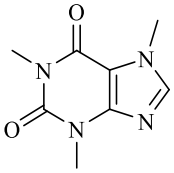
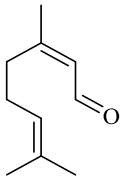
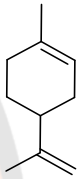
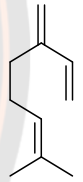
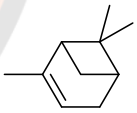
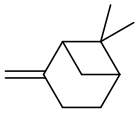
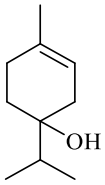
Scientific name:	*Camellia sinensis* (L.) Kuntze
Family name:	Theaceae
Part of use:	leaf
Method of Extraction:	Water extraction
Chemical composition:	caffeine not less than 1.5 % (w/w) (77)
Bioactive markers	caffeine increase lipolysis (78-79), decrease lipogenesis (80-81)

Coffee

Scientific name:	*Coffea arabica* L.
Family name:	Rubiaceae
Part of use:	seed
Method of Extraction:	Water extraction
Chemical composition:	caffeine not less than 1% (w/w) (82)
Bioactive markers	caffeine stimulates lipolysis (81, 83-84), decrease lipogenesis (80-81)

Table 15 Chemical structure and molecular information of some monoterpenes found in essential oils

Compound Name	**CAS#**	**MW**	**Formula**	**Chemical structure**
Camphor	76-22-2	152.2334	$C_{10}H_{16}O$	
Camphene	79-92-5	136.2340	$C_{10}H_{16}$	

Compound Name	CAS#	MW	Formula	Chemical structure
3-carene	13466-78-9	136.2340	$C_{10}H_{16}$	
Caffeine	58-08-2	194.1906	$C_8H_{10}N_4O_2$	
Citral	5392-40-5	152.2334	$C_{10}H_{16}O$	
Limonene	138-86-3	136.2340	$C_{10}H_{16}$	
Myrcene	123-35-3	136.2340	$C_{10}H_{16}$	
α-pinene	80-56-8	136.2340	$C_{10}H_{16}$	
β-pinene	127-91-3	136.2340	$C_{10}H_{16}$	
Terpinen-4-ol	562-74-3	154.2493	$C_{10}H_{18}O$	

Gas chromatography/mass spectrometry (GC/MS)

Gas chromatography/mass spectrometry (GC/MS) is a synergetic combination of two powerful analytical techniques. There is a GC which separates components of a mixture as a function of time, depending on the boiling point and the

polarity of these components. After separation, the MS provides information that helps to identify (structural identification) the components in the mixture.

Evaluation of GC-MS Analyses

Chromatograms obtained by gas chromatography (GC)/mass spectrometry (MS) are plots of the signal intensity against the retention time, as with classical GC detectors. Nevertheless, there are considerable differences between the two types of chromatogram arising from the fact that data from GC-MS analyses are in three dimensions. The elution of each individual substance can be detected by evaluating the mass spectra using a ‘maximizing masses peak finder’ program and can be shown by a marker. Each substance’s specific ion shows a local maximum at these positions, which can be determined by the peak finder. The mass spectra of all the analytes detected are shown in a three-dimensional representation for the purposes of screening. For further evaluation, the spectra can be examined individually.

Headspace techniques

The gas phase (in equilibrium or not with the matrix) above a solid or liquid sample is referred to as "headspace" (HS). Headspace is used to analyze volatile and semi-volatile organics in solid, liquid, and gas samples. This is placed in a closed container (usually a vial sealed with a septum). One of the most elegant possibilities for instrumental sample preparation and sample transfer for GC-MS systems is the use of the headspace technique (Figure 4). All the typically costly processes, such as sample extraction, clean-up, and concentration, are skipped here (85).

Using the headspace technique, the volatile substances in the sample are separated from the matrix. Under the conditions of the analysis, the latter is not volatile. Sample jars that are firmly closed, such as those used for the static headspace technique, can commonly be filled at the sampling location. The partition of the extremely and moderately volatile chemicals between the matrix and the gas phase above the sample is used to extract the analytes. After the partition equilibrium has been set, the gas phase contains a qualitatively and quantitatively representative cross-section of the sample and is therefore used for analysing the components to be

determined. All involatile components remain in the headspace vial and are not analysed.

Volatile components from complex sample mixtures can be extracted from non-volatile sample components and isolated in the headspace or vapor portion of a sample vial. An aliquot of the vapor in the headspace is delivered to a GC system for separation of all of the volatile components (85).

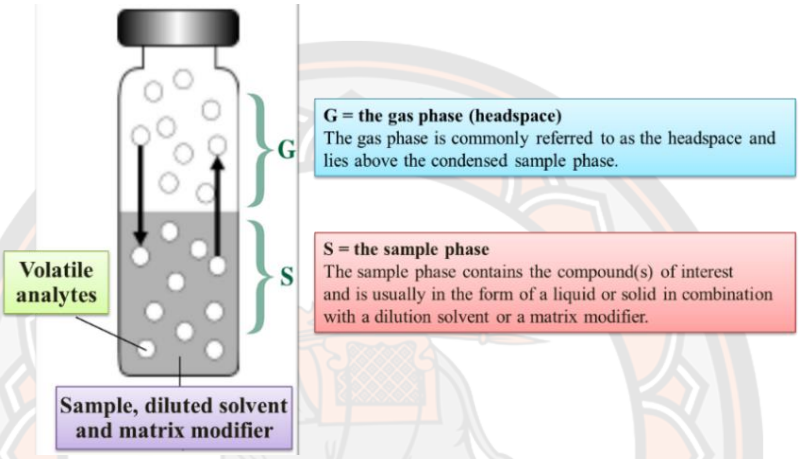


Figure 4 Phases of the headspace vial

The investigating of adipogenesis inhibition and lipolysis stimulation

The 3T3-L1 cell is obtained from murine Swiss 3T3 cells produced from disaggregated Swiss 3T3 mouse embryos aged 17 to 19 days, which display a fibroblast-like morphology (Figure 5). The 3T3-L1 murine fibroblast cell line has frequently been employed as an *in vitro* model for the evaluation of drugs that inhibit adipogenesis or stimulate lipolysis. When grown under the right conditions, this cell can grow into adult adipocytes or mature adipocytes (86-87).

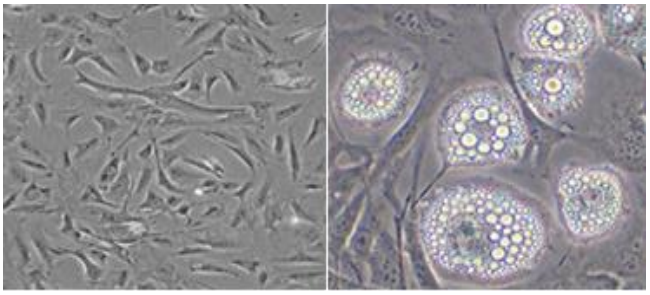


Figure 5 3T3-L1 preadipocyte (left) and adipocyte morphology (right)

In general, 3T3-L1 cells with a fibroblast phenotype can convert to adipocytes, but this requires a combination of differentiation inducers, often known as medium differentiation induction (MDI). These inducers include insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX). In the presence of bovine fetal serum, DEX and IBMX increase intracellular cAMP levels.

To study the effect of the samples on lipid accumulation of 3T3-L1, the mature adipocytes were incubated with varying concentrations of tested compounds or vehicle control. The lipid accumulation and lipolytic activity were determined using several techniques such as Oil Red O staining, and triglyceride assays.

Oil Red O staining assay

Principle of the assay: Oil Red O is a lysochrome (fat-soluble dye) diazo dye (Figure 6A) used for the staining of neutral triglycerides. Oil Red O is a red powder and commonly used to identify cell morphology during the differentiation stage of preadipocytes, as they physiologically convert from proliferative-competent cells into mature adipocytes. Under light microscopy, adipocyte-derived lipid droplets that are stained with Oil Red O appear bright red/orange, leaving the remainder of the non-stained cellular constituents (nuclei appear blue color) (Figure 6B) (24). Total lipid accumulations in adipocytes are measured at 500 nm, after the dye is conveniently extracted from the lipid droplet.

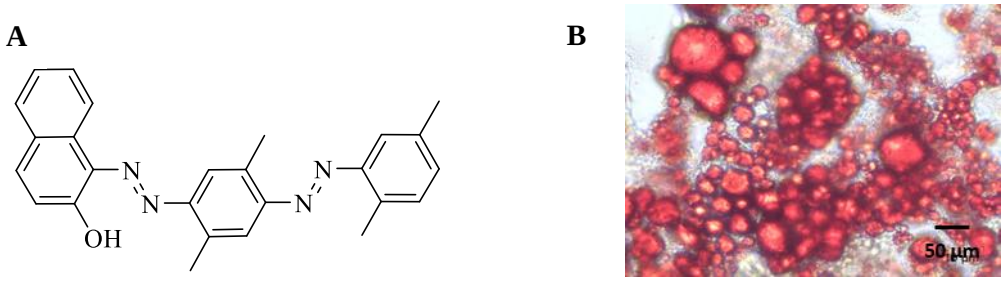


Figure 6 (A) Chemical structure of Oil Red O and (B) 3T3-L1 adipocytes after staining with Oil Red O solution

Lipolysis assay

Principles of the assay: the lipolysis pathway consists of three steps which are catalyzed at each step by lipase enzymes. The enzymes hydrolyze triglycerides into diglyceride, monoglyceride, and glycerol. Fatty acid is produced at all three steps (Figure 7). The triglyceride colorimetric assay uses the enzymatic hydrolysis of triglycerides by lipase to produce glycerol and free fatty acid. Glycerol released is subsequently measured by a coupled enzymatic reaction system. The glycerol phosphorylated by adenosine triphosphate (ATP) forms glycerol-3-phosphate (G-3-P) and adenosine-5′-diphosphate (ADP) in a reaction catalyzed by glycerol kinase (reaction 2). G-3-P is then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2) (reaction 3). A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine (4-AAP) and sodium *N*-ethytl-*N*-(3-sulfopropyl)m-anisidine (ESPA) with H_2O_2, producing a brilliant purple color (reaction 4). All these reactions are shown in Figure 9. The absorbance is measured at 530-550 nm.

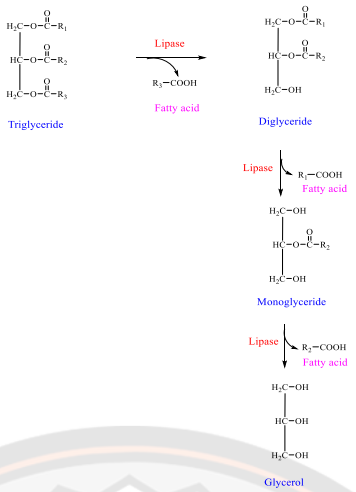


Figure 7 Hydrolysis reaction of triglycerides

$$\text{Triglycerides} \xrightarrow{\text{lipoprotein lipase}} \text{Glycerol + Fatty acid} \quad (1)$$

$$\text{Glycerol + ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate (G-3-P) + ADP} \quad (2)$$

$$\text{G-3-P + } O_2 \xrightarrow{\text{glycerol phosphate oxidase}} \text{dihydroxyacetone phosphate (DAP) + } H_2O_2 \quad (3)$$

$$2H_2O_2 + \text{4-AAP + ESPA} \xrightarrow{\text{peroxidase}} \textbf{Quinoneimine dye} + 4H_2O \quad (4)$$

Figure 8 Triglyceride assay reactions

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CHAPTER III

EFFECTS OF ESSENTIAL OIL AND SOME CONSTITUENTS FROM INGREDIENTS OF ANTI-CELLULITE HERBAL COMPRESS ON 3T3-L1 ADIPOCYTES AND RAT AORTAE

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Abstract

Cellulite is associated with a complex of adipocytes under the skin and vascular system. A herbal compress previously developed was proven to have an anti-cellulite effect in healthy volunteers within 2 weeks of treatment. However, its mechanism and ingredients responsible for reducing cellulite were not known. The purpose of this study was to investigate the activity of eight essential oils in, and two water extracts from, the ingredients of the herbal compress together with nine monoterpenoid constituents on the 3T3-L1 adipocytes**.** The vasodilatory effect on rat aortae was also studied. The adipocytes were induced by dexamethasone, 3-isobutyl-1-methylxanthine and insulin. At all concentrations tested, all essential oils, water extracts and their monoterpenoid constituents significantly inhibited lipid accumulation activity ($p<0.05$) and decreased the amount of triglycerides when compared to untreated cells ($p<0.01$). In addition, our results showed that the mixed oil distilled from the herbal compress mixed ingredients could relax the isolated rat aorta (EC_{50} = 14.74 ± 2.65 µg/ mL). In conclusion, all essential oils, extracts and chemical constituents tested showed effects on adipogenesis inhibition and lipolysis induction on the cultured adipocytes with the mixed oil demonstrating vasorelaxation activity, all of which might be the mechanisms of the anti-cellulite effects of the herbal compress.

Introduction

Cellulite is associated with excessive fat accumulation and increases in size and number of adipocytes under the skin, which is caused by genetic, dietary, behavior and hormones. Cellulite is usually found around the thighs and buttocks of post-pubescent females. The increase in these cellulite deposits causes them to invade the dermis which disrupts the tissue architecture, microcirculation, skin elasticity and dermal thickness resulting in the orange peel-like appearance of the skin (1, 2).

There are basically two pathways which can be targeted to achieve cellulite reduction. First, the inhibition of lipogenesis to prevent fat storage in the adipocytes, and, second, the induction of lipolysis which is the metabolic pathway through which lipid triglycerides are hydrolyzed into a glycerol and three fatty acids. Essentially, this is the process of the decomposition of the chemical that causes fat to be released from

the adipose tissue by the hydrolysis of the ester bonds in the triglycerides of the fatty tissue under the skin. As well, the enlarged fat cells, evident as cellulite, lead to the alteration in the microvascular network of the fat tissue, resulting in water retention, which results in the compression of the vascular vessels and in cellular changes (3, 4). There are known compounds, such as retinol, that improve the appearance of cellulite by increasing the microcirculation (5). The mixture of retinol, caffeine, and ruscogenin could increase microcirculation on the thigh of 46 women who showed moderate degrees of cellulite (6). The use of herbal compresses is popular in traditional Thai therapies, such as in traditional message and spa. These compresses contain herbs bundled within a cloth to form a ball which is warmed and applied to relieve muscle pains, stress and strains. In previous studies (7, 8), we modified a Thai traditional herbal compress to use as an anti-cellulite product. The formulation contained *Zingiber officinale* Roscoe rhizomes (ginger), *Piper nigrum* L. fruit (black pepper), *Piper retrofractum* Vahl. Fruit (java long pepper), *Camellia sinensis* (L.) Kuntze leaf (tea) and *Coffea arabica* L. seed (coffee) as the principal ingredients together with some auxiliary herbs i.e. *Zingiber montanum* (J. Koenig) Link ex A.Dietr. rhizomes (Cassumunar ginger or plai), *Curcuma longa* L. rhizomes (turmeric), *Cymbopogon citratus* DC. Stapf. leaves (lemon grass), *Citrus hystrix* DC. fruit peels (kaffir lime), with camphor and salts added for both scent and skin penetration. In those prior studies, the anti-cellulite effects of the herbal compress were determined via a double-blinded, randomized placebo-controlled trial conducted on 21 female volunteers aged 20 to 55 over an 8-week test period. The results showed that the herbal compress could significantly reduce thigh circumference, skin fold thickness and the severity of cellulite within 2 weeks. However, the mechanisms of this action and the bioactive constituents responsible for such an action were not identified.

In our current study, we distilled the essential oils of the herbal compress and each ingredient, with the tea leaves and coffee beans being extracted by water. All samples were tested, together with their major monoterpenoid constituents (camphor, camphene, citral, 3-carene, limonene, myrcene, alpha-pinene, beta-pinene, and terpinene-4-ol), for their anti-cellulite effects. The effects of these samples on lipid accumulation were demonstrated, in vitro, on the mouse adipocyte cell 3T3L1 model

and the inhibiting of adipogenesis and stimulation of lipolysis was observed and measured. Further, the vasorelaxant effect of the mixture of the essential oil, distilled from the powdered form of all ingredients, was tested on the aortae isolated from rats.

Materials and Methods

Chemicals and plant materials

Keratinocyte serum-free medium (KSFM) and supplements (2.5 μg of recombinant human epidermal growth factor and 25 mg of bovine pituitary extract), high glucose Dulbecco's Modified Eagle's Medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), phosphate buffered saline (PBS) and antibiotics were purchased from GIBCO (Grand Island, NY, USA), while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), DMSO, Oil Red O reagent and human recombinant insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA. Dexamethasone and IBMX were purchased from Merck (Kenilworth, NJ, USA). Caffeine, camphene, camphor, 3-carene, α-citral, β-citral, limonene, β-myrcene, α-pinene, β-pinene and terpinene-4-ol were purchased from Sigma-Aldrich (Buchs, Switzerland). Adrenaline was purchased from MARCH (Bangkok, Thailand). The ingredients of the herbal compress i.e., ginger (rhizome), black pepper (fruit), java long pepper (fruit), turmeric (rhizome), plai (rhizome), lemongrass (stalk) and kaffir lime (fruit peel) were purchased in Phitsanulok, Thailand. Specimens of all nine herbs were collected and authenticated by comparing with voucher lots available in the Biological Sciences Herbarium, Naresuan University, Phitsanulok or comparing with botanical illustrations. Roasted coffee beans (Arabica100% Coffman®) were produced by Coffman International. Co., Ltd., Thailand and the tea (Three Horses®) was purchased from Three Horses Tea Co., Ltd., Thailand.

Extraction

Essential oil extraction: The essential oil of each seven plant ingredients as well as the mixed oil were extracted using hydro-distillation. The plant materials were cut into small pieces, dried at 45–50 °C and ground into powder. The powder of each plant (50 g) was placed in a round-bottomed flask with 500 mL of distilled water. For the mixed oil, the 150 g of mixture of seven plant herbal compress ingredients in the

ratio that reported in previous study (1) was placed in a round-bottomed flask with 1500 mL of distilled water. The distillation apparatus was set to 100 °C for 3 to 5 h of distillation (2-3). Tea and coffee water extraction: Tea and coffee (100 g) were boiled in 400 mL of distilled water for 3 times, and filtered through a filter cloth, followed by 5 min of centrifuging, then the supernatant was lyophilized and stored at −20 °C in screw cap bottles.

Cell culture

The protocol was approved by Naresuan University Institutional Review Board for human keratinocyte and fibroblast cells (Approval number 608/59), and for 3T3-L1 preadipocytes and adipocytes (Approval number 0044/61). 3T3-L1 preadipocyte cell line was obtained from ATCC (Manassas, VA, USA). Keratinocyte cells were cultured in KFSM supplemented with 5 µg/mL epidermal growth factor human recombinant, 50 µg/mL bovine pituitary extract and 1% P/S solution (4). Fibroblast and 3T3-L1 preadipocyte cells were cultured in DMEM, 10% FBS (fibroblast), or 10% BCS (3T3-L1), 3.7 g/L sodium bicarbonate and 1% P/S solution. The cells culture condition was maintained at 37 °C and humidified in an atmosphere of 5% CO_2.

Adipocyte differentiation (adipogenesis assay)

For adipocyte differentiation, the 3T3-L1 pre-adipocytes were plated in 96 well plates at a density of 2×10^3/well and cultured in DMEM supplemented with 10% BCS and 1% P/S solution. Two days after confluence; day 0, the media was removed and the fresh differentiation media i.e., DMEM with 10% FBS, 1 µM dexamethasone, 0.5 mM IBMX and 5 µg/mL insulin was added and maintained for 2 days at 37 °C in an atmosphere of 5% CO_2. The media was replaced with fresh DMEM containing insulin every second day. By day 9, more than 90% of the cells had differentiated into lipid droplets (5-6).

Cell viability

Human keratinocyte and fibroblast cells were separated from human foreskins. The keratinocytes were seeded at 2×10^4 cells/well and the fibroblast cells were seeded at 2×10^4 cells/well. The 3T3-L1 pre-adipocytes were seeded at 1×10^4 cells/well and 3T3-L1 pre-adipocytes, for differentiation to the adipocytes, were seeded at 2×10^3 cells/well in a 96-well plate. The essential oils and extracts and their

major compounds were dissolved in 100% DMSO and the culture medium was then replaced with 100 µL serial dilutions (0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250 and 500 µg/mL) of the extracts for keratinocytes, fibroblast, and pre-adipocytes, and 12.5, 25, 50, 100, 200 and 500 µg/mL for the adipocytes. The cells were then incubated with the essential oils/extracts at 37 °C, and 5% CO_2, for 24 h. The viability of the differentiated cells adipocyte and post-confluent adipocytes was ascertained by treating them with a sample solution in differentiation medium every 2 days for 9 days, after which the viability was assayed. Where the final concentration of DMSO was less than 1% *v*/*v* in the cell culture medium, the cells were added to each well with 50 µL of MTT working solution (1 mg/mL) in PBS (pH = 7.4) and incubated for a further three hours. The solution was measured at a test wavelength of 595 nm by microplate reader (7-9).

Quantification of lipid content by lipid accumulation

The accumulation of lipids in the cells was quantified by Oil-Red-O assay. The inhibition of essential oils and extracts, and their major monoterpenoids, were evaluated for their preventive and treatment effects against adipogenesis. Various concentrations of the essential oils and extracts (12.5 to 200 µg/mL) were added to the differentiation media (with insulin) on days 3, 5 and 7. After day 7, the lipid droplets in the mature adipocytes were stained then visualized through Oil Red O staining and photographed. To evaluate the treatment effect, the samples were incubated with the mature adipocyte on day 9 for 24 h and their effects were stained with Oil Red O on day 10 (10-11).

Oil-Red-O staining

After the lipid droplets were stained and showed through the Oil Red O staining of 3T3-L1, the adipocytes were treated with different concentrations of the extracts, as described above. The cells were washed twice with PBS, fixed with 10% formalin for 8 min and left for 1 h at room temperature when they were washed again with 60% Isopropanol and stained with freshly prepared Oil Red O solution diluted with 3 parts of 0.5% Oil Red O in 2 parts of distilled water, for 45 min at room temperature. The cells were again washed twice with distilled water to remove the excess stain and then were dried. The cells were then examined under a microscope. After 10 min, the Oil Red O staining was extracted by isopropanol. The absorbance

was measured using a microplate reader at 500 nm (6, 12) and examined under a microscope (Nikon) and the images were captured.

Determination of riglyceride (TG) content

Next, 3T3-L1 adipocytes were induced for differentiation in the same fashion as stated in session 3.5. At day 9, they were treated with samples of essential oils, extracts and monoterpenoids, or positive controls of caffeine and adrenaline, and incubated at 37 °C, and 5% CO_2 for 24 h. The cells were then collected and lysed using sonication. The total triglyceride contents in the cells were determined using the Triglyceride Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) (20,36,37).

Vasorelaxant effects of mixed oil

This study was approved and conducted in accordance with the guidelines of the Naresuan University Animal Care and Use Committee (NUACUC; Animal Ethics Approval Number: NU-AE601021). After anesthetizing the male Wistar rats, using intraperitoneal injection of thiopental sodium (100 mg/kg), the rats' aortae were excised and kept in cold physiological Krebs' solution (mM): NaCl 122 mM; KCl 5 mM; (N-(2-hydroxyethyl) piperazine N'-(2-ethanesulfonic acid)) HEPES 10 mM; KH_2PO_4 0.5 mM; NaH_2PO_4 0.5 mM; $MgCl_2$ 1 mM; glucose 11 mM; and $CaCl_2$ 1.8 mM (pH = 7.4). After removal of the superficial connective tissues, each aorta was cut into ring segments, 3–4 mm in length, which were then mounted in standard 10 mL organ baths continuously aerated (95% O_2:5% CO_2) and filled with Krebs-Hensleit (KH) buffer (pH = 7.4) at 37 °C. A Mac Lab A/D converter (Chart V5, A.D. Instruments, Castle Hill, NSW, Australia) was used to measure the isometric tension of the force transducers which were connected with intra-luminal wires. The resting tension of the aortic rings was maintained at 1 g and the rings were equilibrated for 60 min to ensure a stable contraction with 10 µM phenylephrine (PE). Presence of the endothelial lining was evaluated by observing >70% relaxation with 10 µM acetylcholine (Ach) after stable contraction with PE (13).

The experiment was conducted on endothelium intact rat aortae equilibrated at 1 g initially and pre contracted with 10 µM PE. A stable contraction plateau was observed which was then followed by the cumulative addition of mixed oil from 1 µg/mL, 3 µg/mL, 10 µg/mL, 30 µg/mL, 100 µg/mL and 300 µg/mL. Each

concentration was incubated until the relaxation was stable. Identical concentrations of DMSO alone were added to serve as the negative control group at the same time interval as the addition of the mixed oil, in order to ensure that the relaxation was rendered by the mixed oil rather than the DMSO. The vessel was washed with physiological Kreb's solution after complete relaxation had been observed at the highest concentration of the mixed oil. To evaluate the vessels' integrity, 80 mM K^+ solution was added. The immediate contraction plateau of each vessel was observed, signifying the vessel's viability throughout the experimental protocol. The % relaxation was calculated as % contraction in response to PE.

Gas chromatography-mass spectrometry analysis of monoterpenoid constituents in mixed oil

GC-MS analysis used an Agilent 7890B, Gas Chromatography System-5977B coupled to an Agilent 5977B MSD model mass spectrometer (Agilent Technologies, Singapore). Mixed oil was prepared by dissolving 5 mg into 1 mL of methanol and injected into a capillary column HP-5 5% Phenyl Methyl Silox (30 m × 250 μm × 0.25 μm; Agilent 19091S-433) with a constant flow rate of Helium 1.0 mL/min. The injector was set at 250 °C and performed by split mode with a split ratio of 100:1 (in 1.0 μL). The GC oven temperature was initially set at 70 °C for 5 min, then increased to 100 °C at a rate of 3 °C/min and held for 3 min, then increased to 250 °C at a rate of 20 °C/min and held for another 1 min, with a total run time of 26.5 min (Figure 14).

Monoterpenoid constituents of the mixed oil were identified by mass spectrometry in full scan mode using mass analyzer and confirmed by comparing their spectra to those of the NIST MS search 2.2 library. The mass spectrometer was operated in the electron impact ionization mode (70 eV), with a scan range of 50 to 550 amu. The interested constituents of mixed oil from the anti-cellulite herbal compress and their relative peak areas are listed in Table 17.

Table 16 The GC-MS retention times and the relative peak areas of the interested monoterpenoid constituents of mixed oil from the anti-cellulite herbal compress

Monoterpenes Constituents [1]	Retention Time (min)	Relative Area (%) [2]
α-pinene (1)	5.044	6.13
Camphene (2)	5.478	3.73
β-Pinene (3)	6.383	20.99
β-myrcene (4)	6.774	2.63
3-carene (5)	7.495	8.34
D-limonene (6)	8.221	20.70
Camphor (7)	13.036	8.38
Terpinene-4-ol (8)	14.630	20.93
β-citral (9)	17.791	3.84
α-citral (10)	19.108	4.33

Note: [1] Relative area (%) obtained by area of the interested peak/total area of 10 interested peaks × 100.

[2] The number in the brackets of represent the peak in Figure 14.

Statistical analysis

All adipogenesis experiments, each with a set of 3 wells, were carried out in triplicate. Data were statistically evaluated by a one-way analysis of variance (ANOVA). Determination of significant differences ($p < 0.05$) between means was supported by Tukey's multiple comparison test (GraphPad Prism software version 8.0, San Diego, CA, USA). Values are given as mean ± standard error of the sample animals. The EC_{50} values and Emax values to achieve maximum relaxation were obtained by concentration-response curve fitting using GraphPad Prism software version 8.0, San Diego, CA, USA.

Results and Discussion

Cell viability

Notably, 3T3-L1 adipocytes are widely used in assays of adipogenesis because they can tolerate an increased number of passages and homogeneously respond to treat-ments (9,10). Prior to our study of the effects of essential oils/extracts and their major monoterpenoid constituents on adipogenesis and lipolysis of 3T3-L1 adipocytes, we tested the viability of the various concentrations (1–500 μg/mL) of the samples on the preadipocytes and adipocytes.

In addition, the viability of the samples on keratinocyte, fibroblast was studied to provide safety information. The highest dilution that resulted in more than 80% cells being viable was considered to be the non-toxic concentration to be used in our further studies on adipogenesis and lipolysis of 3T3-L1 adipocytes. Table 18 illustrates the non-toxic levels of the tested concentrations.

Table 17 Non-toxic concentrations of 7 essential oils, mixed oil, tea and coffee extracts, and their major constituents in anti-cellulite herbal compress selected from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

Samples	Concentrations (μg/mL)			
	KR [1]	FB [2]	PA [3]	A [4]
1. Lemon grass oil	31.25	31.25	31.25	62.5
2. Ginger oil	62.5	125	62.5	125
3. Black pepper oil	125	125	125	125
4. Long pepper oil	125	250	125	250
5. Tea water extract	125	250	125	250
6. Turmeric oil	125	250	125	250
7. Cassumunar ginger oil	250	250	250	250
8. Coffee water extract	250	250	250	250
9. Kaffir lime oil	250	250	250	250
10. Mixed oil	250	250	250	250
11. Camphor	ND	ND	200	200

Samples	Concentrations (µg/mL)			
	KR [1]	FB [2]	PA [3]	A [4]
12. Camphene	ND	ND	200	200
13. Citral	ND	ND	200	200
14. 3-carene	ND	ND	200	200
15. D-limonene	ND	ND	200	200
16. β-myrcene	ND	ND	200	200
17. α-pinene	ND	ND	200	200
18. β-pinene	ND	ND	200	200
19. Terpinene-4-ol	ND	ND	200	200
20. Caffeine	1 mM (194.2 µg/mL)			
21. Adrenaline	0.1 mM (18.3 µg/mL)			

Note: [1] Keratinocyte; [2] Fibroblast; [3] 3T3-L1 preadipocyte; and [4] adipocyte cells. (ND = not determined).

Preventive and treatment effects of essential oils/extracts and their major monoterpenoid constituents on adipogenesis of 3T3-L1 cells

Adipogenesis is a complex process by which pre-adipocytes transform into adipocytes. Oil red O staining is the most commonly used method for distinguishing adipocytes from other cells and has recently been used as a quantitative method to assess different degrees of adipocyte differentiation (14). In our study, the pre-adipocytes were treated with dexamethasone, 3-isobutyl-1-methyl xanthine (IBMX) and insulin to induce the differentiation. After nine days, the formation of adipocytes was evaluated. The preventive and treatment effects of the essential oils distilled from the ingredients of the herbal compress and the aqueous extracts of tea and coffee on adipogenesis were studied at the concentrations that were non-toxic to the cells. To investigate the preventive effect, the samples were added to the media on day 3, 5 and 7 after the initiation. Their effects on 3T3-L1 adipocyte differentiation were observed via lipid accumulation oil red O staining on day 9. To evaluate the treatment effect, the samples were incubated with the mature adipocyte on day 9 after the initiation and their effects were measured on day 10.

The results showed that all samples prevented adipogenesis in a dose-dependent manner. Of the samples tested, lemon grass oil demonstrated inhibition of lipid accumulations at a concentration of 12.5 µg/mL that was 23 ± 6%, which was the lowest effective concentration of all samples tested (Figure 9A). As well as lemon grass, another promising sample was ginger oil, which gave 33 ± 5% inhibition at the concentration of 50 µg/mL. The remaining essential oils and extracts tested showed around 30% inhibition at the concentration of 100–200 µg/mL, whereas the positive controls i.e., 18 µg/mL adrenaline and 194 µg/mL caffeine expressed 24 ± 5% and 25 ± 2% inhibition, respectively. Manaharan et al. 2016 reported that ginger oil at various concentrations (50 to 800 µg/mL) significantly decreased lipid content in mature adipocytes in a dose-dependent fashion (11). Coffee and tea have shown anti-obesity and anti-adiposity activities in adipocytes in some previous studies (15-16). Both coffee extracts and their major constituents, namely caffeine, caffeic acid, chlorogenic acid and trigonelline, increased glycerol release while also reducing the accumulation in the 3T3-L1 cells during adipocytic differentiation. Also, the expression of the peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor that controls the differentiation of adipocytes, is inhibited by the consumption of coffee. Also, as reported by (17-18), the main theaflavin of tea, polyphenols and theaflavin-3,3′-digallate (TF3), demonstrated an anti-adiposity effect in mature adipocytes through the activation of the AMPK pathway. Further, Goto et al. (19) showed that several bioactive terpenoids, which are derived from herbal and dietary plants, function as PPAR modulators as regulators of carbohydrate and lipid metabolism. However, the anti-adipogenic effects of the essential oils distilled from lemongrass, black pepper, long pepper, turmeric and cassumunar ginger, as well as the mixed oil from herbal compresses, are reported here for the first time.

The lipid accumulation, after the mature adipocytes had been treated with samples of the essential oils and tea and coffee extract, was evaluated to ascertain the effect of the treatment. Figure 9B shows that lemon grass (25 µg/mL), ginger (50 µg/mL), black pepper (100 µg/mL), long pepper (50, 100 µg/mL) and mixed oil (100, 200 µg/mL) significantly inhibited lipid accumulation in the range of 12–24%. Interestingly, the positive controls i.e., 18 µg/mL adrenaline and 194 µg/mL caffeine showed the same range of % lipid accumulation inhibition (21% and 17%,

respectively). All samples tended to decrease intracellular lipids in a concentration-dependent manner. The maximum inhibition of lipid accumulation (24%) was observed from 100 µg/mL long pepper oil. It is noted that the positive controls as well as all test samples could reduce lipid accumulations on 3T3-L1 adipocytes in both preventive and treatment experiments where the degree of reduction was greater in the preventive experiments. The samples that clearly showed significantly higher % lipid accumulation in the preventive experiments when compared to the treatment experiments were turmeric, cassumunar ginger, tea and mixed oil ($p < 0.05$).

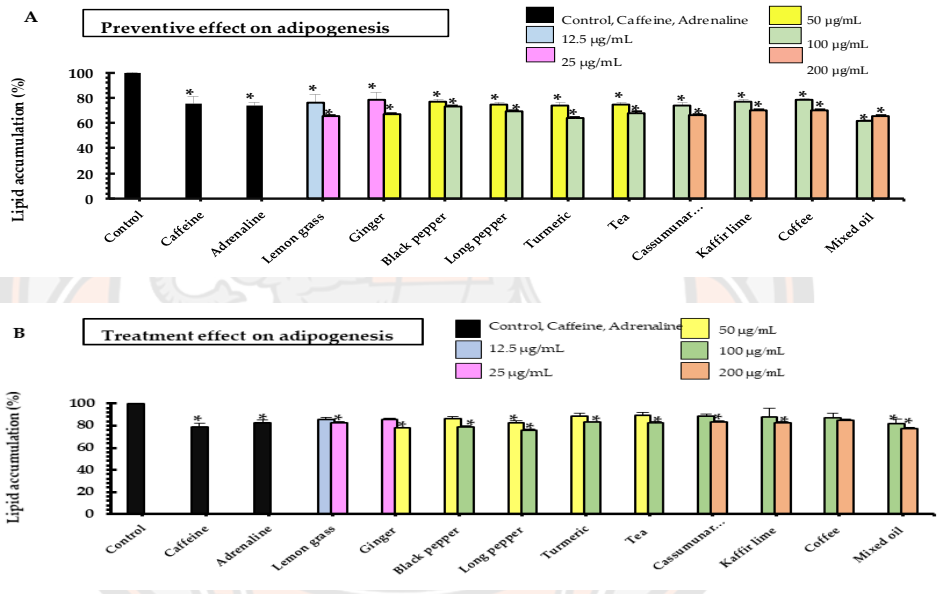


Figure 9 Lipid accumulation in 3T3-L1 adipocytes after treated with essential oil, extracts and positive controls (adrenaline 0.1 mM or 18.3 µg/mL and caffeine 1 mM or 194.2 µg/mL); (A) in the preventive experiments where the pre-adipocytes were treated with the samples during the differentiation on days 3, 5 and 7, and (B) in the treatment experiments where the samples were added after the pre-adipocytes were differentiated to adipocytes (on day 9) and incubated for one day. The lipid accumulation was measured by Oil Red O assay, and the results are expressed as the mean ± SEM of triplicate tests. Data expressed in percentage in comparison with control. One-way ANOVA showed significant value, * $p < 0.05$ as compared to control

Nine monoterpenoid constituents of the herbal compress ingredients were tested for their preventive and treatment effects on adipogenesis of 3T3-L1 adipocytes (Figure 10). The results showed that all samples significantly inhibited lipid accumulation as compared to the control cells in both preventive and treatment method, although most samples tended to have a higher preventive effect than the treatment effect. The significant difference between preventive effects and treatment effects are shown in citral (50, and 100 µg/mL, $p < 0.001$), and 3-carene (100 µg/mL, $p < 0.05$). For the effective effect, the highest inhibition of lipid accumulation was observed in limonene at the concentration of 100 µg/mL, with 47 ± 2% inhibition. The limonene compound significantly decreased lipid accumulation more than the caffeine (194.2 µg/mL, 38 ± 1% inhibition) and adrenaline (18.3 µg/mL, 34 ± 3% inhibition). For the treatment effect of the intracellular lipid accumulation, these results indicated that the nine major monoterpenoid constituents inhibited lipid accumulation (Figure 10B). These data also show that camphor, camphene, citral, 3-carene, alpha-pinene in the concentration of 100 µg/mL as well as limonene, myrcene, beta-pinene and terpinene-4-ol in the concentrations of 50 and 100 µg/mL significantly inhibited lipid accumulation by 20–33% where caffeine (18 µg/mL) showed 18 ± 1% inhibition and adrenaline (194 µg/mL) showed 21 ± 3% inhibition. The highest % inhibition of lipid accumulation was observed in limonene at 100 µg/mL (38 ± 4%), which was significantly higher than caffeine and adrenaline ($p < 0.05$). A previous animal study (18) showed the preventive effects of limonene on hyperglycemia and dyslipidemia in high-fat diet-induced obesity mice. In addition, limonene was reported to be effective in regulating the peroxisome proliferator-activated receptor (PPAR)-α signaling and liver X receptor (LXR)-β signaling. The microscopic pictures of 3T3-L1 adipocytes after stained with Oil Red O in preventive and treatment experiments are shown in Figure 11.

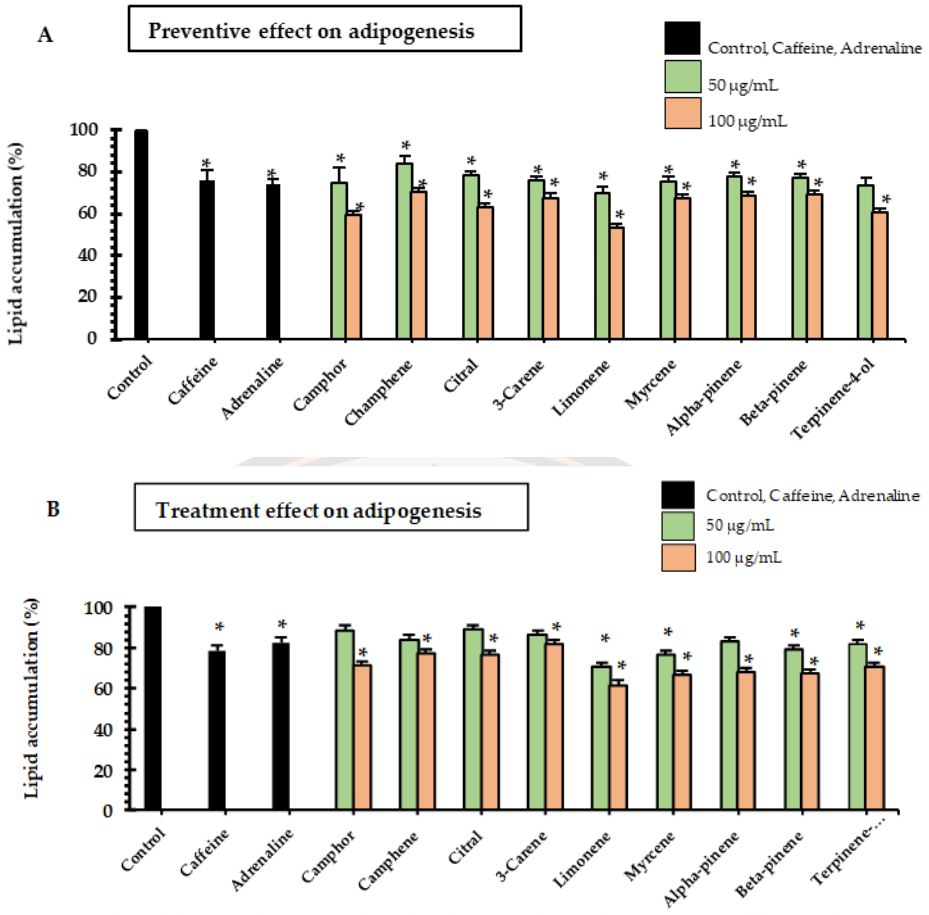
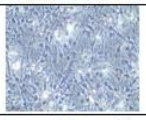
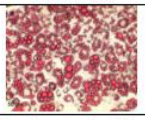
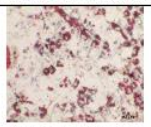
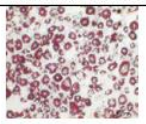
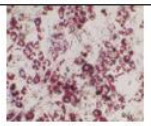
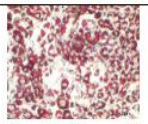
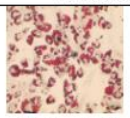
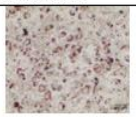
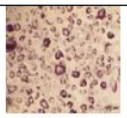
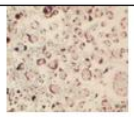
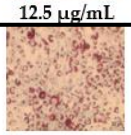
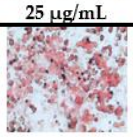
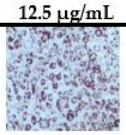
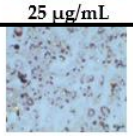
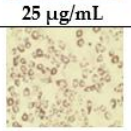
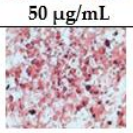
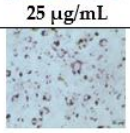
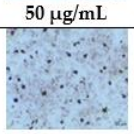
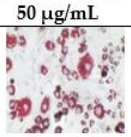
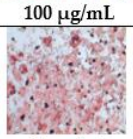
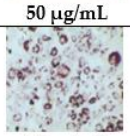
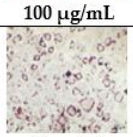
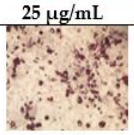
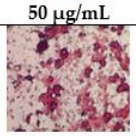
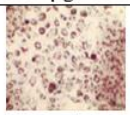
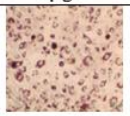
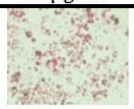
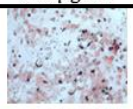
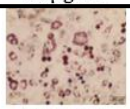
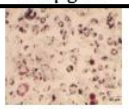
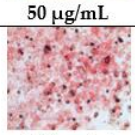
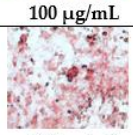
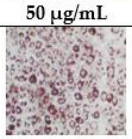
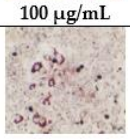
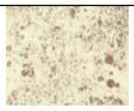
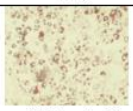
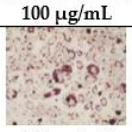
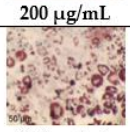


Figure 10 Lipid accumulation in 3T3-L1 adipocytes after treated with monoterpenoid constituents of the herbal compress ingredients and positive controls (adrenaline 0.1 mM or 18.3 µg/mL and caffeine 1 mM or 194.2 µg/mL); (A) in the preventive experiments where the pre-adipocytes were treated with the samples during the differentiation on days 3, 5 and 7, and (B) in the treatment experiments where the samples were added after the pre-adipocytes were differentiated to adipocytes (on day 9) and incubated for one day. The lipid accumulation was measured by Oil Red O assay, and the results expressed as the mean ± SEM of triplicate tests. Data expressed in percentage in comparison with control. One-way ANOVA showed significant value, * $p < 0.05$ as compared to control

	Undifferentiated	Control
		
	Preventive effect	**Treatment effect**
(1) Caffeine	 1mM Caffeine	 1mM Caffeine
(2) Adrenaline	 0.1mM Adrenaline	 0.1mM Adrenaline
(3) Lemon grass oil	 12.5 µg/mL  25 µg/mL	 12.5 µg/mL  25 µg/mL
(4) Ginger oil	 25 µg/mL  50 µg/mL	 25 µg/mL  50 µg/mL
(5) Black pepper oil	 50 µg/mL  100 µg/mL	 50 µg/mL  100 µg/mL
(6) Long pepper oil	 25 µg/mL  50 µg/mL	 25 µg/mL  50 µg/mL
(7) Tea water extract	 50 µg/mL  100 µg/mL	 50 µg/mL  100 µg/mL
(8) Turmeric oil	 50 µg/mL  100 µg/mL	 50 µg/mL  100 µg/mL
(9) Cassumunar ginger oil	 100 µg/mL  200 µg/mL	 100 µg/mL  200 µg/mL
(10) Coffee water extract	 100 µg/mL  200 µg/mL	 100 µg/mL  200 µg/mL

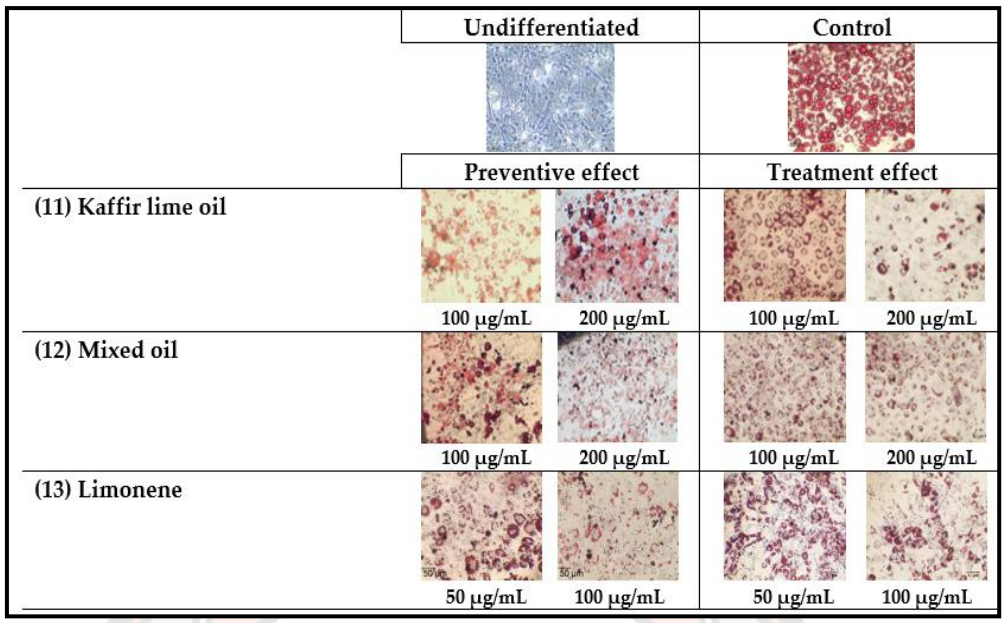


Figure 11 (1-13) Representative photographs (20×) of 3T3-L1 adipocytes stained with Oil Red O after treatment with the essential oils, extracts and limonene in comparison with undifferentiated and control cells. To study the preventive effect, the mature adipocytes were treated with samples on days 3, 5 and 7 after the initiation and the oil red O staining was conducted on day 9. For treatment effect, the samples were incubated with the mature adipocyte on day 9 and their effects were measured on day 10. Notably, 1 mM (18.3 µg/mL) caffeine and 0.1 mM (194.2 µg/mL) adrenaline were used as positive controls.

Effects of essential oils/extracts and heir major monoterpenoid constituents on triglyceride accumulation of adipogenesis of 3T3-L1 cells

In addition to inhibition of adipocyte differentiation and mature adipocyte, we also determined the effect of the test samples on triglyceride accumulation of 3T3-L1 adipocytes causing the in vitro lipolysis effect. Excessive amounts of triglyceride accumulation in the adipocyte are related to an increased risk of a variety metabolic disease. In our study, treatment of cells with seven essential oils, mixed oil and tea and coffee extracts decreased triglyceride accumulation in differentiated 3T3-L1 cells. The amount of intracellular triglyceride accumulated in adipocytes was significantly

decreased at all concentration levels tested (Figure 12A, B) when compared to the effect in the untreated control cells.

The lowest concentration that significantly decreased triglyceride content was observed in lemon grass oil (25 µg/mL, 53 ± 3% triglyceride content). Five essential oils that demonstrated the most prominent decrease for the triglyceride content were long pepper oil (100 µg/mL, 42 ± 6% triglyceride content), black pepper (100 µg/mL, 47 ± 5% triglyceride content), coffee (200 µg/mL, 47 ± 7% triglyceride content), kaffir lime (200 µg/mL, 50 ± 5% triglyceride content) and mixed oil (200 µg/mL, 50 ± 2% triglyceride content). A total of 1 mM of Caffeine decreased the triglyceride contents by 41 ± 2% and 0.1 mM adrenaline decreased triglyceride accumulation by 67 ± 4% (relative to the control).

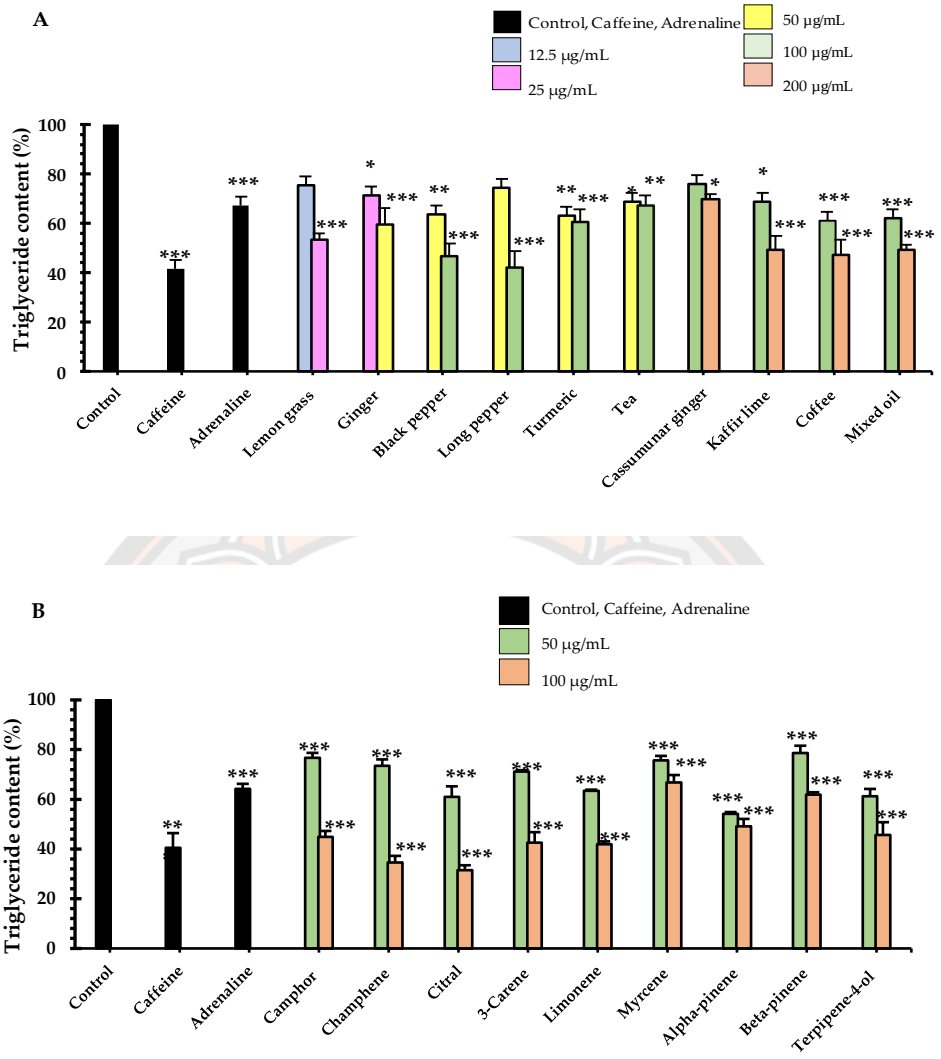


Figure 12 The effects of (A) the essential oils/extracts, and (B) monoterpenoid constituents on triglyceride content of 3T3-L1 adipocytes after treatment for 24 h.Intracellular triacylglycerol content was determined using enzymatic colorimetric methods. We used 1 mM (18.2 µg/mL) caffeine and 0.1 mM (194.3 µg/mL) as positive controls. Values are expressed as mean ± SE of three independent experiments. One-way ANOVA showed significant value, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control cells (untreated).

For nine monoterpenoid constituents on lipolysis of lipid accumulation in adipocytes, at day 10, were measured using intracellular triglyceride content, as shown in Figure 12B. The maximum significant decrease of triglyceride content was

identified in the presence of citral (100 µg/mL concentration, 32 ± 1% triglyceride content) and camphene (100 µg/mL, 35 ± 2% triglyceride content), relative to the control. In addition, two constituents: camphene and citral at 100 µg/mL concentrations, decreased triglyceride content more than caffeine ($p < 0.01$). We also found that nine constituents had decreased triglyceride content more than adrenaline. These were camphor, camphene, citral, 3-carene, limonene, myrcene, alpha-pinene, beta-pinene and terpinene-4-ol at 100 µg/mL concentration. Interestingly, triglyceride determination of camphene, citral and limonene showed similar decreases in total lipid accumulation in mature adipocytes as compared to the 1% dimethyl sulfoxide (DMSO) control. Our results concur with the animal study reported in (20), which showed that there was a significant inhibition of differentiation of preadipocytes to mature adipocytes was observed, and it was evident from reduced lipid accumulation in the cells. Cellular lipid content was decreased by 18% by camphene at 10 µM, by 29% at 50 µM and by 37% at 100 µM, when compared with the control cells treated with DMSO. Previous work has also found that 30 µM citral exhibits significant inhibition of total triglyceride accumulation using the triglyceride determination kit by 30%, while 40 µM showed a 50% inhibition, and 50 µM showed 80% inhibition (10). In another study (21), a quick screening on the lipolytic effect of monoterpenes in 3T3-L1 adipocyte was conducted with the result that 1µM of limonene stimulated lipolysis by 17%. Caffeine and adrenaline were used as a positive control in this study. Figure 12A, B show that caffeine at the concentration of 1 mM or 194 µg/mL increased lipolysis with a triglyceride accumulation reduction of 41 ± 2% triglyceride content. Adrenaline at the concentration of 0.1 mM or 18 µg/mL also decreased triglyceride accumulation (67 ± 4% triglyceride content). These results are supported by a previous report that showed that caffeine inhibited triglyceride content by 11%, 22% and 34% at the concentration of 1, 5 and 10 µg/mL (22). In addition, adrenaline (1 µM) stimulated lipolysis for about a 30% glycerol release. Total glycerol content in the medium indicates the lipolytic effect of adrenaline in 3T3-L1 adipocytes (23). Previous studies demonstrated that lemon grass oils are rich in citral (24-25), and also that citral inhibited the formation of intracellular lipid accumulation in a concentration-dependent manner (10–50 µM) for 30, 40 and 50 µM concentrations of citral (10). It should be noted that the present research has shown the potent impact on

the lipolytic effect of nine essential oils, mixed oil and their major constituents, which are monoterpenes, The potential anti-cellulite activity of the seven essential oils, mixed oil and tea and coffee water extracts, and their nine major monoterpenoid constituents, extracted from the anti-cellulite herbal compress that we had developed, were assayed on the 3T3-L1 cell lines of preadipocyte, a commonly used cell model for adipose cell biology research. Furthermore, anti-cellulite effects can be exerted by reducing the size and number of intracellular lipid accumulations in adipocytes and assayed triglyceride accumulations. It is suggested that further work be undertaken to study the molecular mechanisms and to substantiate the effectiveness of bioactive compounds as anti-lipogenesis or lipolysis substances, and the beneficial anti-cellulite activities that we have shown to be demonstrated by the herbal compress.

Vasorelaxant effects of mixed oil on rat aortae

Our previous clinical study showed that the anti-cellulite herbal compress improved cellulite appearance, as assessed by measurement of thigh circumferences, skin thickness and severity of cellulite (1). Blood flow enhancement via vasodilation could be one of the mechanisms activated by the anti-cellulite herbal compress. Therefore, the mixed oil derived from hydrodistillation of the herbal compress was tested on isolated rat aortae. This test revealed a concentration-dependent (1–300 μg/mL) vasorelaxant effect of mixed oil on the phenylephrine pre-contracted endothelium-intact vessel (EC_{50} = 14.74 ± 2.65 μg/mL and E_{max} = 99.51 ± 0.49%, Figure 13). The vascular action of the herbal compress could be attributed to some of the major constituents of mixed oil, in particular monoterpenes i.e., α-pinene, camphene, β-pinene, β-myrcene, 3-carene, D-limonene, camphor, terpinene-4-ol β-citral and α-citral, which were displayed on chromatographic profiles shown in a GC-MS chromatogram (Figure 14). The mechanism of vasorelaxant actions of the mixed oil could involve the endothelium dependent pathway i.e., nitric oxide release, as reported by several studies evaluating vascular actions of the volatile oils and plants containing similar monoterpene profiles (7,26,27).

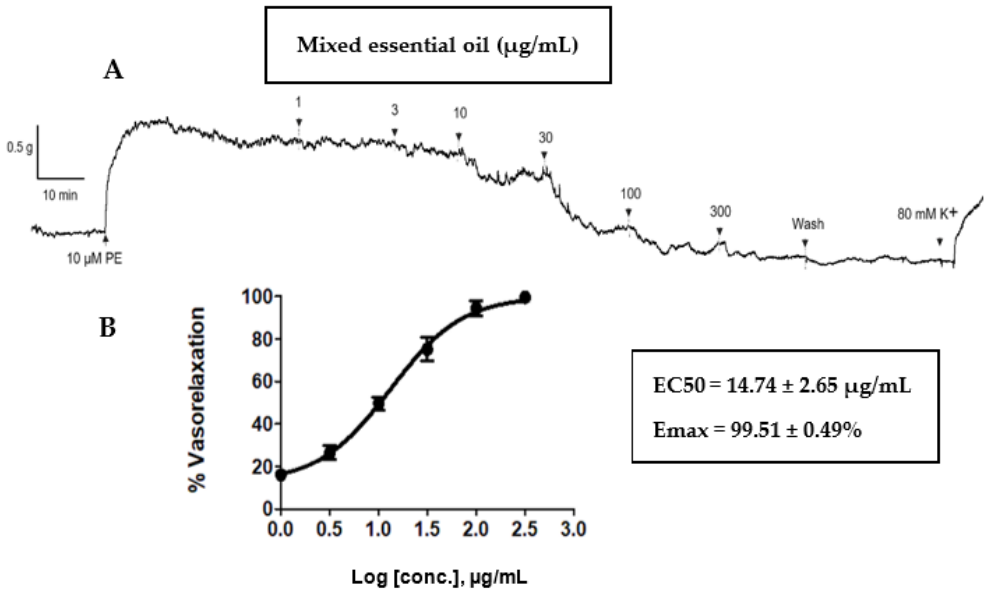


Figure 13 (A) A typical trace of the vasorelaxant effect of the mixed oil distilled from the herbal compress mixed ingredients (0–300 µg/mL) on endothelium-intact aortae. (B) Concentration-relaxation curves for mixed oil from anti-cellulite herbal compress (1–300 µg/mL) on endothelium-intact aortic rings. All data points are means ± SEMs (n = 5).

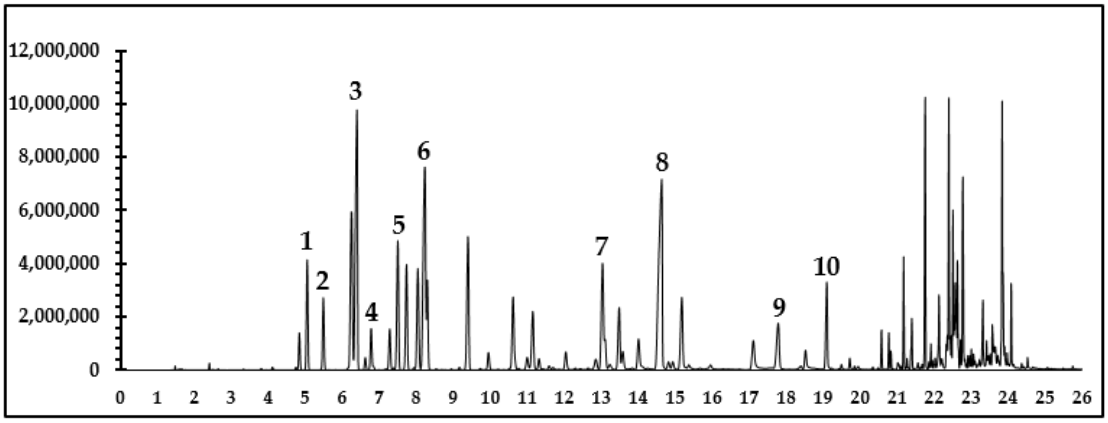


Figure 14 GC-MS total ion chromatogram of the mixed oil distilled from the herbal compress mixed ingredients. The peaks of the main constituents were identified by comparing the molecular mass from mass spectra data of each compound with the NIST library (Version 2.2) as (1) α-pinene, (2) camphene, (3) β-pinene, (4) β-myrcene, (5) 3-carene (6) D-limonene, (7) camphor, (8) terpinene-4-ol, (9) β-citral and (10) α-citral.

Conclusions

This study presents the preclinical effects, on cellular lipid accumulation, triglyceride content and the vasodilatation effect of on rat aortae, of the essential oils and extracts obtained from Thai traditional herbal compresses and their constituents. These findings demonstrate the abilities of the test samples to decrease lipid accumulation resulting in the inhibition of adipocyte differentiation and increasing lipolysis on 3T3-L1 adipocyte cells. The mixed oils showed vasodilatory effects on rat aortae via endothelium-dependent release of vasodilators. Our study is the first to report on the anti-cellulite mechanisms of Thai traditional herbal compresses, including prevention of lipid accumulation and increasing blood flow. Our findings allow us to confidently suggest that the anti-cellulite activity of volatile oils and their monoterpenes constituents, or combinations of them, are useful in the treatment for cellulite.

Acknowledgements

We would like to acknowledge Royal Golden Jubilee Program (RGJ-PhD) (0008/2560) for the scholarship of NN, the Center of Excellence for Innovation in Chemistry (PERCH-CIC) and Cosmetics and Natural Products Research Center (COSNAT), Naresuan University on research facility support. We also wish to thank Roy I. Morien of the Naresuan University Graduate School for his efforts in editing the English grammar and expressions in this paper.

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CHAPTER IV

DEVELOPMENT, CHARACTERIZATION AND STABILITY EVALUATION OF THE ANTI-CELLULITE EMGEL CONTAINING HERBAL EXTRACTS AND ESSENTIAL OILS

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Abstract

Recently, the herbal compress has been successfully developed and applied for cellulite treatment. The aim of this study was to formulate a more convenient dosage form of herbal ap-plication containing the extracts and essential oils of the herbal ingredients from the original formula. In addition, we aimed to characterize and evaluate stability of the developed dosage form. A gelled emulsion or an 'emgel' incorporated with the herbal extracts and essential oils was successfully prepared. The bio-active marker monoterpenes (camphene, camphor, 3-carene, α-citral, β-citral, limonene, β-myrcene, α-pinene, β-pinene and terpinene-4-ol) were characterized by headspace GCMS and caffeine was characterized by HPLC. The stability study was carried out over a period of 12 weeks at 4, 25 and 50°C. Changes in the compositions were monitored every 2 weeks. The anti-cellulite herbal emgel that was found to be physically stable under accelerated testing condition. Using Q10 method, the shelf-life of the emgel, when kept at 25 °C was esti-mated to be 31 months.

Keywords: topical formulation; anti-cellulite, cosmetic; monoterpenenoids; accelerated stability

Introduction

Natural ingredients are preferrable in cosmetics due to their safety image (1). Previously, the anti-cellulite treatment by applying warmed traditional herbal compress on the cellulite area for 15 min has been proven to be effective (2). The herbal compress comprising aromatic herbs i.e. ginger (*Zingiber officinale* Roscoe), black pepper (*Piper nigrum* L.), java long pepper (*Piper retrofractum* Vahl.), plai (*Zingiber montanum* (J. Koenig) Link ex A.Dietr.), turmeric (*Curcuma longa* L.), lemon grass (*Cymbopogon citratus* DC. Stapf.) and kaffir lime (*Citrus hystrix* DC.) and herbs containing xanthine alkaloids i.e. tea (*Camellia sinensis* (L.) Kuntze) and coffee (*Coffea arabica* L.). Recently, the more convenient dosage form, anti-cellulite emgel, was freshly prepared and clinically tested. The effectiveness was already proved and reported (3). The anti-cellulite mechanisms of the essential oils including monoterpenoids constituents of the aromatic herbs as well as extracts of tea and coffee have been elucidated *in vitro* as reduction of lipid accumulation and vasorelaxant (4).

The aim of this study was to validate headspace-gas chromatography/mass spectrometry (HS-GCMS) and high-performance liquid chromatography (HPLC) methods for monoterpenoids and caffeine determination of the main constituents in the anti-cellulite emgel, and evaluate product stability after storage over a period of 12 weeks at 4, 25 and 50°C.

Materials and Methods

Chemicals and standards

Methanol and water were of LC-MS grade and purchased from RCI Labscan Ltd., (Bangkok, Thailand). Reference standards, camphor (purity ≥95%), camphene (purity ≥95%), caffeine anhydrous (purity > 99%), citral (purity ≥98%), 3-carene (purity ≥90%), limonene (purity ≥97%), myrcene (purity ≥90%), α-pinene (purity ≥98%), β-pinene (purity ≥99%), and terpinen-4-ol (purity ≥95%) were products from Sigma-Aldrich (St. Louis, MO, USA.). Internal standard, Menthol (purity ≥98%) was from TCI (Shanghai, China). The standard homologous series of n-alkanes (C8-C40) was obtained from Sigma-Aldrich (St. Louis, MO, USA.) analytical.

Essential Oils and Extracts

Essential oils of ginger, black pepper, java long pepper, plai, turmeric, lemon grass and kaffir lime were purchased from Thai-China Flavours and Fragrances Industry Co., Ltd (Ayutthaya, Thailand). They were mixed in the ratio equivalent to the composition of the herbs used in the herbal compress formula (4) and called a “mixed oil”.

Tea of Three Horses brand, and roasted coffee of Arabica 100% Coffman brand, were purchased in Phitsanulok, Thailand. Ground tea leaves and coffee beans were separately extracted by boiling water for 20 min. After filtration and 5 min of centrifuging, the aqueous solutions were lyophilized to provide tea and coffee extracts which then stored at -20 °C until used.

Preparation of Anti-Cellulite Emgel

The anti-cellulite emgel composed of 5% mixed oil, 5%camphor, 0.05% tea extract and 0.05% coffee extract. The other ingredients and their function were listed in Table 19. Camphor was dissolved in the mixed oil, while tea and coffee extracts were added to the carbopol gel and mixed with other ingredients resulted in the formation of an emgel. The base sample (without the mixed oil) was prepared with similar materials and under identical conditions for formulation.

Table 18 Anti-cellulite emgel ingredients

Anti-cellulite emgel	Function
deionized water	diluent
carbopol 940	gelling agent
disodium EDTA	chelating agent
propylene glycol	moisturizing agent
glycerin	moisturizing agent
phenoxyethanol and chlorphenesin	preservatives
triethanolamine (TEA)	pH adjuster
PEG-40 hydrogenated castor oil	solubilizer
rice bran oil	emollient
tea extract	active ingredient
coffee extract	active ingredient
mixed oil	active ingredient
camphor	active ingredient

Headspace-gas chromatography/mass spectrometry (HS-GCMS) and high performance liquid chromatography (HPLC) analyses

The HS-GCMS and HPLC methods for determination of chemical ingredients in the anti-cellulite emgel were developed and validated in term of limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, and precision (AOAC, 2012) (8). Placebo emgel was used as a blank sample.

HS-GCMS instruments and chromatographic conditions

HS analysis was performed with the Agilent G4556-64000 network. HS auto sampler Agilent PN 7697A was used to directly introduce samples automatically into the Agilent 7890B Gas Chromatography System-5977B MSD model mass spectrometer. After the vials had been pressurized with carrier gas, then the emgels HS sample were injected into a capillary column HP-5 (5% Phenyl Methyl Silox), (30 m x 250 μm x 0.25 μm; Agilent 19091S-433). The carrier gas was helium (He) with a constant flow rate of 1.3 mL/min. The GC oven temperature was initially set at 70°C for 3 min, then increased to 100°C at a rate of 3°C/min and held for 3 min, then increased to 250°C at a rate of 20°C/min and held for 1 min, with a total run time of 22 min. Mass spectrometry analysis was carried out using an Agilent mass selective detector model 5977B MSD coupled to the gas chromatograph, selected ions mode (SIM) of specific m/z ratios for each ten interested compounds and one internal standard. The mass spectrometer was operated in the electron impact ionization mode (70 eV), with a scan range of 50 to 550 amu.

HS-GCMS method validation

Injections of standard solutions over a range of LOQ were used to test the method's linearity. Three replicates were performed at each level of validation samples. The calibration curves were obtained with the average of peak area ratios of three replicates. To find the correlation coefficient (r^2) value, six concentration levels of standard solutions were analysed. Precision was determined using the repeatability between 6 replicate samples of placebo emgel and spiked with the concentrations of QC1, QC2, and QC3 of the calibration curve and reported as a coefficient of variation (percentage). The accuracy was calculated as the percent recovery from three replicates of samples spiked with the same concentration that used for the determination of precision. Intra-day and inter-day precision and accuracy were performed at three concentrations.

Sample preparation for HS-GCMS

Mixture standard solutions in methanol at concentrations of 500, 250, 125, 62.5, 31.2, and 15.6 μg/mL (camphene, myrcene) and 2500, 1250, 625, 312.5, 156.2, and 78.1 mg/mL (camphor, alpha-citral, beta-citral, 3-carene, limonene, α-pinene, β-

pinene, and terpinen-4-ol) were prepared for generating the calibration curve. Menthol in methanol (2 mg/mL) was used as the internal standard.

For preparing the validation samples, 1 mg of a blank matrix (placebo emgel) and a blank sample (blank vial) were spiked with the internal standard and prepared in a headspace vial of 20 mL and then injected into each batch of samples to demonstrate that no cross-contamination and interference during the analysis.

Qualitative analyses of constituents in the anti-cellulite emgel using HS-GCMS method

Volatile compounds of anti-cellulite emgel were analysed by using HS-GCMS method, stated above, and identified by comparing their spectra to those of the NIST MS search 2.2 library, also by comparison with their GC Kovats retention indices (RI) to those of standard compounds and data from literature. The linear RIs for all components were determined by co-injecting the samples with a solution containing the homologous series of n-alkanes (C8-C20). Caffeine was identified by retention time of standard compound.

HPLC instruments and chromatographic conditions

Chromatographic analysis was performed using a Shimadzu SCL-10A HPLC system equipped with Shimadzu SPD-10A UV/Vis detector, LC-10AT pump, SIL-20AC HT auto-samplers, CTO-10ASVP column oven. Chromatographic separation was performed on a Phenomenex Synergi 4u Hydro-RP 80A column (150 x 4.60 mm, 4 µm particle size) connected to a Phenomenex C18 (10 x 4.6 mm, 5 µm) guard column maintained the temperature at 35°C. The isocratic mobile phase was methanol and water (40:60 v/v), at a flow rate of 1.0 mL/min. The injection volume was 10 µL and the eluates were monitored at 275 nm. The total run time was 8 min.

HPLC method validation

The method was validated according to the ICH guidelines by determining linearity, LOD, LOQ, precision and accuracy. The linearity range of the standards was determined on seven concentration levels that ranged from 0.3125 – 20 µg/mL. Calibration curves were measured in every analysis day and each sample was determined in triplicate. The standard curves were plotted by areas under curve of caffeine standard. The linearity of calibration curve was assessed by calculating the coefficient of determination (r^2). LOD and LOQ under the present chromatographic

conditions were determined by injecting the standard solutions until a signal-to-noise ratio of each compound was 3 for LOD and 10 for LOQ. The intra-day precision of the method was analyzed from the measurement of two concentration levels of caffeine for six times within one day. Precision is represented by the relative standard deviation (%RSD) calculated from standard deviation/mean × 100. Inter-day precision was validated by measuring %RSD for three consecutive days at two concentration levels, 1.875 and 7.5 μg/mL (n = 3, each level). The accuracy and recovery were determined by spiking the known concentration of the standard solution to coffee extract to obtain two different concentrations (1.875 and 7.5 μg/mL). These experiments were done in triplicate. The accuracy is presented as percent recovery of the spiked concentration which was calculated from ((measured standard concentration-standard concentration in the non-spiked sample / standard concentration spiked) × 100.

Caffeine standard solutions for HPLC method

The stock solution of caffeine standard was freshly prepared by dissolving in methanol to obtain the concentration of 10 mg/mL. This solution was further diluted with water to make standard calibration curves, LOD and LOQ. The solutions were then filtered through nylon syringe filters with a 0.45 μm pore size.

Determination of marker compounds in anti-cellulite emgel by HS-GCMS and HPLC

Ten monoterpenoids in anti-cellulite emgel were quantitatively determined by HS-GCMS, and caffeine was analyzed by HPLC analysis.

Sample preparation for HS-GCMS

Each sample, 1 mg of the anti-cellulite emgel was weighted in a HS vial of 20 mL and covered with aluminium crimp cap with silicon septum. 10 μl of internal standard was added in each sample vial.

Sample preparation for HPLC

A 20 mg sample of the anti-cellulite emgel was weighted in a vial of 1.5 mL and dissolved in methanol then vortexed for 1 min. The sample solutions were filtered through nylon syringe filters with a 0.45 μm pore size.

Accelerated stability study of anti-cellulite emgel

The anti-cellulite emgel was stored at an ambient temperature, 4°C (±2°C), and at 50°C (±2°C). The physical properties included color, odor, pH, viscosity, and phase separation as well as chemical markers were determined every 2 weeks for 12 weeks. Heating-cooling cycle test was performed by alternating conditions between 4 °C (±2 °C) for 48 hours and 45 °C (±2 °C) 48 hours of each cycle for 6 cycles. The studies were conducted in triplicate.

Shelf-Life Prediction by Q10 Method

The Q10 approach is a tool for forecasting product shelf-life. It is assumed that the ratio of times to equal harm at two temperatures, which are usually 10°C apart, is constant (6,7,9). The shelf life of the anti-cellulite emgel at 25 °C was calculated using the following from the equation:

$$t_{80}(\mathrm{T_2}) = \frac{t_{80}(\mathrm{T_1})}{\mathrm{Q}^{(\triangle \mathrm{T}/10)}}$$

Where t_{80} (T2) denotes the shelf-life at 25 °C and t_{80} (T1) denotes the shelf-life at 50 °C.

Microbial stability studies

Microbial testing of anti-cellulite emgel was carried out using a preservation test with plate method. Five types of microorganisms i.e *Staphylococcus aureus* TISTR 1466, *Pseudomonas aeruginosa* ATCC 25783, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, and *Aspergillus niger* were added in the anti-cellulite emgel formulation. The criteria of acceptance, and the consideration of preservative stability were measured according to USP 29 Chapter 51 Antimicrobial Effectiveness (10).

Statistical analysis for quality control studies

Data was expressed as the average ± standard deviation (SD). Statistical analysis was conducted using analysis of variance (ANOVA) and Student's t-test by GraphPad.

Results and Discussion

Anti-Cellulite Emgel Formulation

Anti-cellulite emgels were formulated by using a carbomer (Carbopol 940) combined with an emulsion to create the delivery base for hydrophobic substances. A mixture of seven essential oils of herbal ingredients in the compress (mixed oil) in combination with tea and coffee extracts were chosen as active ingredients of the anti-cellulite formulation (5). The problem of the short chain hydrocarbon molecules of the essential oils (e.g., alpha-pinene, camphene, myrcene, and terpinen-4-ol) being incompatible with the emulsion droplets in the formulation, was to be resolved, by introducing a carrier oil with a long lipid tail into the emulsion system as a diluent for the essential oils. The carrier oil also enhanced the viscosity of the oil combination and promoted the development of emulsions. The carrier oil for this emulsion system was a virgin cold pressed rice bran oil. These mixed oil and two extracts were well incorporated into the preliminary developed stable emgel base. The emulsion-base gel containing the ingredients was formulated. The formulation are shown in Table 20.

Texture, color, odor, pH (at 25°C) and viscosity (spindle no. 5, 20 rpm, 25°C) of all formulations were observed during the stability tests. Measurements were taken before and after 24 days of storage at 4°C followed by six cycles of heating and cooling at 4 and 45°C for 48 hours per cycle. A formulation kept at 25 °C was used as a control.

Table 19 Anti-cellulite emgel formula

Ingredient	Formula
deionized water	66.00
carbopol 940	0.80
disodium EDTA	0.10
propylene glycol	2.00
glycerin	6.00
phenoxyethanol and chlorphenesin	1.00
triethanolamine (TEA)	1.00
PEG-40 hydrogenated castor oil	5.00
rice bran oil	8.00
tea extract	0.05
coffee extract	0.05
mixed oil	5.00
camphor	5.00

Chemical Characterization of The Anti-Cellulite Emgel

Twenty-nine constituents of anti-cellulite emgel were characterized by HS-GCMS (Table 21). The monoterpenes from the essential oil constituents were detected as major constituents. The rank by %peak area were camphor (100.0%), δ-Curcumene (21.6%), limonene (8.8%), β-sesquiphellandrene (8.6%), α-curcumene (6.6%), β-bisabolene (6.4%), sabinene (6.3), tumerone (5.8%), alpha-citral (5.2%), and beta-citral (4.6%).

Table 20 The volatile composition of anti-cellulite emgel analysed by using HS-GCMS

Peak No.	RT (min)	RI [1]	Identified compounds	Relative area (%)
1	1.66	562	trimethoxyborane	2.57
2	3.67	939	alpha-pinene	2.06
3	3.86	955	camphene	2.81
4	4.14	978	sabinene	6.31
5	4.21	984	beta-pinene	4.38
6	4.31	992	beta-myrcene	2.03
7	4.57	1009	alpha-phellandrene	1.14
8	4.67	1014	3-carene	3.93
9	4.75	1019	alpha-terpinene	1.22
10	4.95	1029	limonene	8.84
11	5.03	1033	eucalyptol	1.39
12	5.49	1058	gamma-terpinene	1.64
13	6.13	1092	alpha-terpinolene	1.55
14	7.71	1152	camphor	100.00
15	8.49	1180	(Internal standard) menthol	31.01
16	8.63	1185	gamma-terpinene	4.29
17	8.92	1195	alpha-terpineol	1.83
18	9.45	1227	(preservative) phenoxyethanol	7.95
19	9.73	1250	beta-citral	4.60
20	10.19	1277	alpha-citral	5.22
21	11.75	1373	caryophyllene	2.26
22	12.15	1395	alpha-curcumene	6.66
23	12.20	1400	germacrene D	1.87
24	12.23	1404	delta-curcumene	20.43
25	12.28	1409	beta-selinene	3.15
26	12.32	1413	beta-bisabolene	6.41
27	12.43	1424	beta-sesquiphellandrene	8.46
28	13.25	1511	tumerone	5.81
29	13.42	1530	curlone	1.26

Note: [1] Comparison with kovats retention index (RI), the NIST library (Version 2.2) comparison using GCMS (SCAN) analysis

HS-GCMS Method Validation

Selectivity

These values demonstrate that no interfering peaks occurred in the corresponding retention time of each analyte (Figure 15). The selected ions mode (SIM) and retention time (RT) for compounds of interested on the HS-GCMS analysis were (1) α-pinene (Specific m/z ratios; 91, 92, 93), RT 3.611, (2) Camphene (Specific m/z ratios; 79, 93, 121), RT 3.810, (3) β-pinene (Specific m/z ratios; 69, 91, 93), RT 4.166, (4) β-myrcene (Specific m/z ratios; 69, 91, 93), RT 4.257 (5) 3-carene (Specific m/z ratios; 79, 91, 93), RT 4.605, (6) D-limonene (Specific m/z ratios; 91, 136), RT 4.895, (7) Camphor (Specific m/z ratios; 69, 81, 95), RT 7.588, (7) Menthol (Specific m/z ratios; 71, 81, 95), RT 8.420, (8) Terpinen-4-ol (Specific m/z ratios; 43, 71, 111), RT 8.560, (9) β-citral (Specific m/z ratios; 41, 69, 94), RT 9.747, (10) α-citral (Specific m/z ratios; 69, 84, 94), RT 10.16.

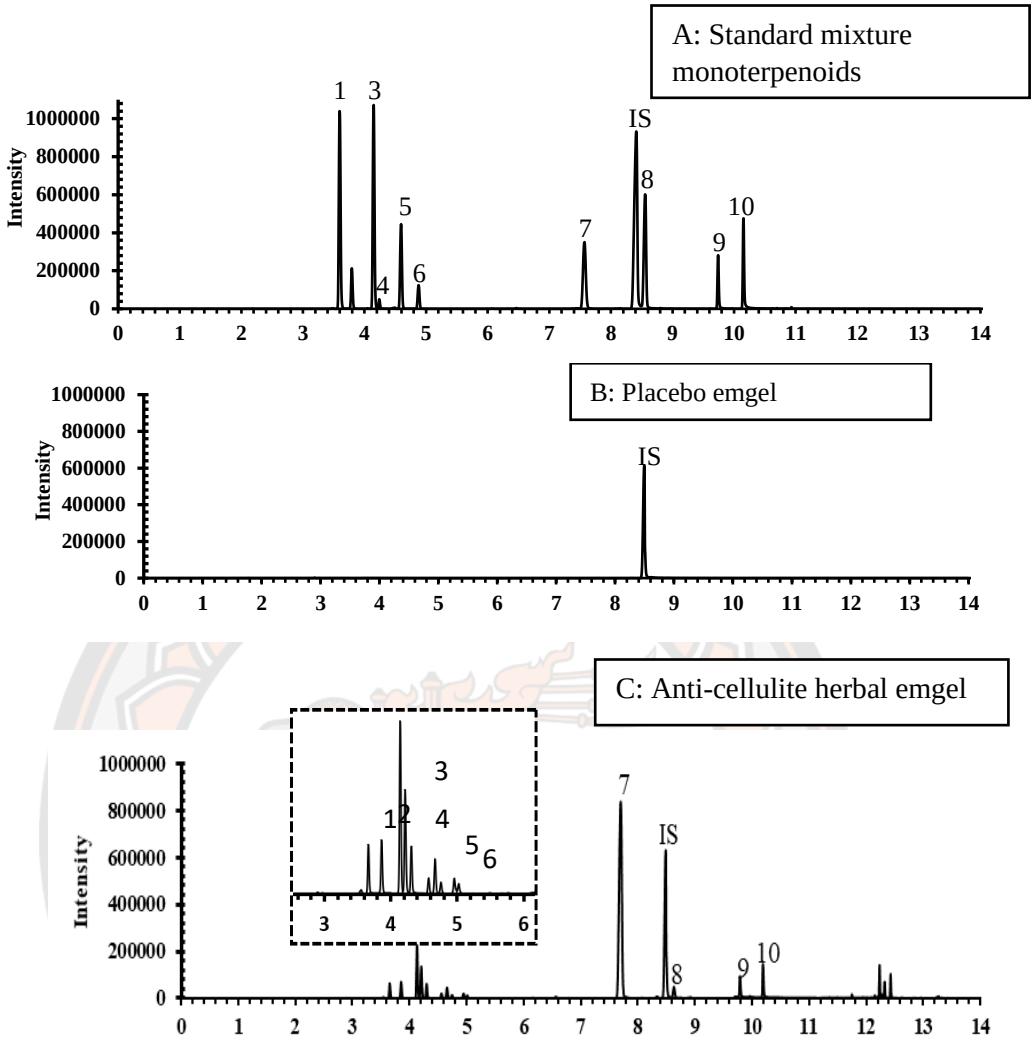

Figure 15 HS-GCMS (SIM) total ion chromatograms of (A) Standard monoterpenoid mixture (2.5µg/mL), (B) placebo emgel (20 mg), and (C) anti-cellulite herbal emgel. The peaks of ten constituents were identified by comparison with standard references as (1) α-pinene, (RT 3.61), (2) camphene, (RT 3.81), (3) β-pinene, (RT 4.16), (4) β-myrcene, (RT 4.25), (5) 3-carene (RT 4.61), (6) D-limonene, (RT 4.89), (7) camphor, (RT 7.59), (8) terpinene-4-ol, (RT 8.55), (9) β-citral, (RT 9.75), and (10) α-citral (RT 10.16)

Linearity

The range of linearity of the ten constituents with their LOD and LOQ are shown in Table 22. The r^2 for the calibration curves for all compounds were > 0.990. For all substances, the signals were linear over concentration ranges suggesting that the method was appropriate for analyzing these compounds in the same sample.

Table 21 Correlation coefficient (r^2), linear range, LOD, and LOQ of ten monoterpenoids in anti-cellulite emgel analysed by HS-GCMS

Analytes	RT	(r^2)	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
alpha-pinene	3.611	0.9997	39.1 – 1250.	13.0	39.1
camphene	3.810	0.9989	62.5 - 2000	20.8	62.5
beta-pinene	4.166	0.9982	39.1 - 1250	13.0	39.1
myrcene	4.257	0.9978	62.5 - 2000	20.8	62.5
3-carene	4.605	0.9987	39.1 - 1250	13.0	39.1
limonene	4.895	0.9974	39.1 - 1250	13.0	39.1
camphor	7.588	0.9989	62.5 - 2000	20.8	62.5
terpinene	8.420	0.9964	39.1 - 1250	13.0	39.1
beta-citral	8.560	0.9988	62.5 - 2000	20.8	62.5
alpha-citral	9.747	0.9976	62.5 - 2000	20.8	62.5

Precision and accuracy

Intra-day and inter-day precision and accuracy at three concentrations were studied. Both precision and accuracy were within reasonable limits (% RSD was less than 15 percent and % accuracy was between 85 and 110 %) (AOAC, 2012) (Table 23).

The accuracy was determined by spiking the three different concentrations of ten monoterpenoid standards. The recovery in the range of 94 -107 (%RSD ≤ 4.21) was obtained in Table 23.

Table 22 Intra-day and inter-day precision, and accuracy of HS-GCMS method for determination of ten monoterpenoid standards assessed at three different concentration levels (n = 3) in three consecutive days. Accuracy was expressed as the percent recovery of ten monoterpenoid standards in three different concentrations in placebo emgel

Analytes	Concentration levels /Spiked mount (µg/ml)	Intra-day (n=3)		Inter-day (n=9)		Accuracy (n=9)
		Measured concentration (µg/mL) ± SD	Precision (%RSD)	Measured concentration (µg/mL)	Precision (%RSD)	Recovery (%)
Alpha-pinene	120	126.62 ± 4.07	3.21	124.45 ± 3.54	2.85	105.5
	190	190.75 ± 1.38	0.73	190.90 ± 1.03	0.54	98.6
	1000	1013.42 ± 5.56	0.54	1004.97 ± 10.63	1.06	98.8
Camphene	125	131.09 ± 2.41	1.84	128.40 ± 3.88	3.02	97.4
	300	284.46 ± 6.33	2.23	277.07 ± 11.66	4.21	101.8
	1600	1561.43 ± 7.74	0.49	1568.28 ± 9.42	0.60	99.4
Beta-pinene	120	109.23 ± 3.17	2.91	107.70 ± 2.83	2.63	100.5
	190	192.64 ± 4.88	2.53	193.65 ± 4.11	2.12	100.4
	1000	1013.34 ± 9.99	0.98	1012.33 ± 9.53	0.94	100.0
Myrcene	125	112.72 ± 4.11	3.64	113.48 ± 2.846	2.50	100.6
	300	273.69 ± 3.84	1.40	272.10 ± 8.16	2.99	101.1
	1600	1436.74 ± 4.31	0.30	1442.68 ± 11.36	0.78	106.7
3-carene	120	124.26 ± 4.42	3.56	122.61 ± 4.22	3.44	94.4
	190	189.28 ± 3.96	2.09	186.85 ± 3.96	2.45	102.0
	1000	924.90 ± 6.15	0.66	927.38 ± 5.33	0.57	102.6
Limonene	120	130.95 ± 3.20	2.44	129.40 ± 2.91	2.91	105.5
	190	194.62 ± 5.02	5.02	191.83 ± 5.34	2.78	102.1
	1000	1025.84 ± 9.62	0.94	1025.34 ± 13.53	1.32	98.3
Camphor	125	134.15 ± 5.09	3.79	134.26 ± 5.09	3.79	98.2
	300	286.33 ± 8.94	3.12	291.26 ±9.32	3.20	96.7
	1600	1718.85 ± 2.75	0.16	1718.85 ± 11.69	0.68	97.8

Analytes	Concentration levels /Spiked mount (µg/ml)	Intra-day (n=3)		Inter-day (n=9)		Accuracy (n=9)
		Measured concentration (µg/mL) ± SD	Precision (%RSD)	Measured concentration (µg/mL)	Precision (%RSD)	Recovery (%)
Terpinene-4-ol	120	114.58 ± 2.17	1.89	112.78 ± 4.19	3.71	100.4
	190	177.21 ± 4.89	2.76	176.40 ± 3.47	1.97	107.5
	1000	942.44 ± 7.19	0.76	934.46 ± 10.47	1.12	101.6
Beta-citral	125	107.75 ± 0.95	0.89	108.02 ± 2.31	2.14	98.3
	300	314.81 ± 9.63	3.06	311.76 ± 8.18	2.62	96.2
	1600	1649.47 ± 13.03	0.79	1663.07 ± 14.08	0.85	99.0
Alpha-citral	125	128.02 ± 4.96	3.88	127.39 ± 4.07	3.19	99.5
	300	329.87 ± 7.54	2.28	329.24 ± 5.74	1.74	99.0
	1600	1642.30 ± 12.91	0.79	1645.17 ± 11.29	0.69	99.0

HPLC Method Validation

The HPLC method for determination of the caffeine was developed and validated according to the ICH guidelines.

Selectivity

The separation was done on C18 column with isocratic elution of 40% methanol in water. The tailing factor and resolution of caffeine standard met the ICH guidelines. The identification of the caffeine standard was confirmed from the retention time of with the sample (Figure 24).

Linearity

The calibration equations, linearity, limit of detection (LOD) and limit of quantitation (LOQ) values are presented in Table 24. The HPLC chromatograms of caffeine standard, placebo, and anti-cellulite emgel are shown in Figure 16 (A-C).

Table 23 Correlation coefficient (r^2), linear range, LOD, and LOQ of caffeine standard in anti-cellulite emgel analysed by HPLC

Analyte	RT (min)	(r^2)	Linear range (µg/mL)	LOD (ng/mL)	LOQ (ng/mL)
Caffeine	4.015	1.00	0.3125-20	156.250	15.625

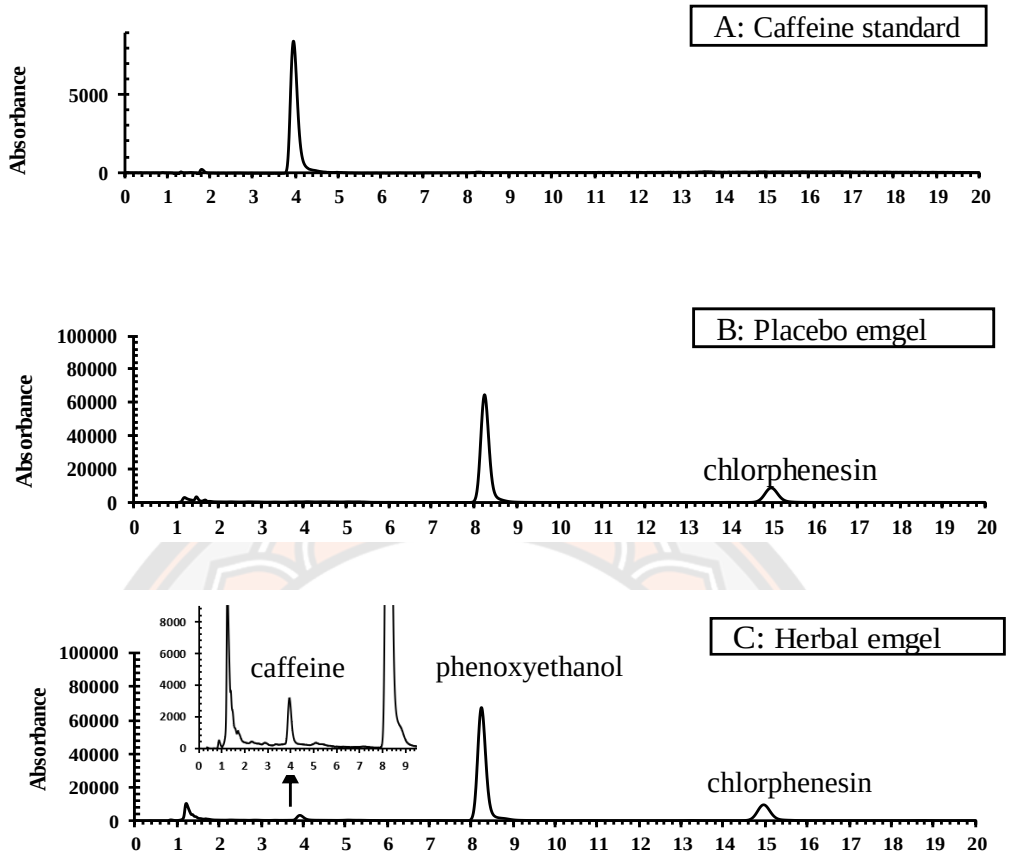

Figure 16 HPLC chromatograms of (A) caffeine standard (2.5µg/mL), (RT 4.015), (B) placebo emgel (20mg/mL), (C) anti-cellulite emgel (20mg/mL)

Precision and accuracy

Intra-day and inter-day precision and accuracy at three concentrations were performed. Both precision and accuracy were within reasonable limits (% RSD was less than 15 percent and % accuracy was between 85 and 110 %) (AOAC, 2012). (Table 25). The %RSD which reflected the intra-day and inter-day precisions of the caffeine standard was not more than 2.5.

The accuracy was determined by spiking the two different concentrations of caffeine standard (1.8 and 7.5 ppm) at 0.1 mg/mL of coffee extract. The recovery in the range of 98 -103 (%RSD $\leq$ 6.32) was obtained. All data are shown in Table 25.

Table 24 Intra-day and inter-day precision, and accuracy of HPLC method for determination of caffeine standard assessed at two different concentration levels (n = 3) in three consecutive days. Accuracy was expressed as the percent recovery of caffeine standard in two different concentrations in coffee extract

Standard	Concentration levels /Spiked mount (µg/ml)	Intra-day precision (n=6)		Inter-day precision (n=9)		Accuracy (n=9)
		Measured concentration (µg/mL) ± SD	Precision (%RSD)	Measured concentration (µg/mL) ± SD	Precision (%RSD)	Recovery (%)
Caffeine	1.88	1.86 ± 0.03	1.70	1.81 ± 0.05	2.51	101.0
	7.50	7.49 ± 0.19	2.52	7.32 ± 0.18	2.51	100.6

Quantitation of Interested Constituents in Anti-Cellulite Emgel Using HS-GCMS

The ten interested constituents were analyzed in the anti-cellulite emgel by HS-GCMS (SIM) (Table 26). The major monoterpene in the formula was camphor (251.0 µg/mg).

Table 25 The contents of ten monoterpenoid constituents in anti-cellulite emgel analysed by HS- GCMS (n=3)

Anti-cellulite emgel	Monoterpenoids presented in the formulation (ug/mg) Average ± S.D.
Alpha-pinene	85.2 ± 0.6
Camphene	50.8 ± 1.8
Beta-pinene	88.4 ± 1.1
Myrcene	53.3 ± 4.5
3-carene	46.7 ± 1.8
Limonene	36.8 ± 6.7
Camphor	251.0 ± 3.2
Terpinene-4-ol	104.3 ± 2.6

Anti-cellulite emgel	Monoterpenoids presented in the formulation (ug/mg) Average ± S.D.
Beta-citral	65.6 ± 1.3
Alpha-citral	75.0 ± 2.1

Determination of Caffeine Content in Tea, Coffee Material, and Anti-Cellulite Emgel by HPLC

The caffeine concentrations of the coffee, tea, and anti-cellulite emgel were analysed by HPLC. For the anti-cellulite emgel, a content of 0.05% of each tea and coffee extracts in the formulation contained approximately 1% of caffeine (Table 27).

Table 26 The content of caffeine in anti-cellulite emgel were analysed by HPLC (n=3)

Sample	Caffeine content Ave ± S.D. (µg/g)
Coffee extract (freeze dried)	45.0 ± 0.4
Tea extract (freeze dried)	64.2 ± 1.1
Anti-cellulite emgel with tea and coffee extract in this formulation	48.1 ± 2.3

Physical Stability of Anti-Cellulite Emgel

Physical stability is important to consider while maintaining the lipophilic chemicals in the emulsion formulae and subsequently in cosmetic products and prevent or mitigate deterioration during storage. The anti-cellulite emgel was evaluated for qualities such as color, odor, pH, viscosity, and phase separation. The anti-cellulite emgel had a homogeneous texture with butter milk color and characteristic odor of herbal essential oil. After storage for 12 weeks (Table 28), the physical properties of anti-cellulite emgel remained similar to baseline while after heating-cooling stability study and after storage at 50 °C for 3 months, there was only minor change in the appearance of anti-cellulite emgel.

Table 27 The physical stability of anti-cellulite emgel at initiation day and after stored at 4°C, 25 °C, 50 °C for 3 months and after 6-heating cooling cycles

Conditions	Physical examination	pH	Viscosity (Cp)	Separation
Initiation	Smooth texture, pale brown	6.89 ± 0.02	1715 ± 5.29	No phase separation
4°C	Smooth texture, pale brown	6.61 ± 0.04	1681 ± 6.81	No phase separation
25 °C	Smooth texture, pale brown	6.68 ± 0.05	1632 ± 5.50	No phase separation
50 °C	Smooth texture, pale brown (darker and smell, slightly stronger)	6.63 ± 0.02	1616 ± 5.03	No phase separation
Heating-Cooling 6 cycles 45°C/ 4°C	Smooth texture, pale brown (slightly darker and smell, slightly stronger)	6.65 ± 0.03	1654 ± 3.21	No phase separation

Chemical stability evaluation using GCMS and HPLC

The active monoterpenoids and caffeine in the anti-cellulite emgel were determined using our validated HS-GCMS and HPLC methods. The anti-cellulite emgel were stored for 12 weeks at 4 °C, RT, 50 °C. After 8 weeks storage at 50 °C, the active monoterpenoids in emgel formulation retained more than 80% of the initiation (Figure 17) whereas caffeine retained more than 80% after 12 weeks (Figure 17).

Microbiological stability evaluation

The durability of preservatives is a critical element in ensuring microbial efficiency. To demonstrate the microbiological stability, we conducted a preservation challenge test. Our results shown in Table 29, indicate the acceptance criteria required by the method for acceptability in each time (Day 0, Day 7, Day 14, and Day 28). The

anti-cellulite emgel was resistant to microbial proliferation, which could pose a risk if used improperly.

Table 28 Preservation efficacy of anti-cellulite emgel

Microbes	Log_{10} CFU/g			
	Day 0	**Day 7**	**Day 14**	**Day 28**
Staphylococcus aureus	6.0	3.3	<2.0	<2.0
Pseudomonas aeruginosa	5.9	3.0	<2.0	<2.0
Escherichia coli	6.0	<2.0	<2.0	<2.0
Candida albicans	4.4	<2.0	<2.0	<2.0
Aspergillus niger	4.6	<2.0	<2.0	<2.0

Note: * (<) Analytical units with a log_{10} concentration less than 2.0 log_{10} CFU/g are acceptable.

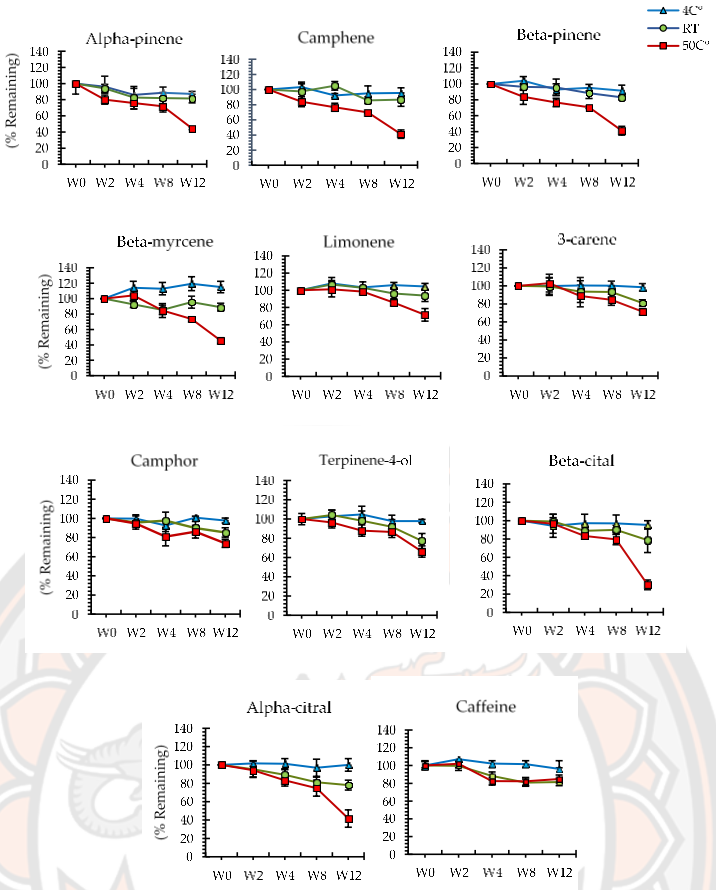

Figure 17 The percentages for the relationship between the time of storage and temperature at room temperature (25°C), refrigerator (4°C), and oven (50°C) conditions. Change in percentage of interested constituents as camphor, camphene, citral, 3-carene, limonene, myrcene, alpha-pinene, beta-pinenen, terpinene-4-ol and caffeine for 12 weeks

Calculation of monoterpenoids and caffeine accelerated shelf life by Q10 method

The accelerated stability test, which predicts the potential behavior of the anti-cellulite emgel, is quite informative. The shelf life of anti-cellulite emgel was calculated using the Q10 equation (6,7), which was considered a cornerstone in estimating the storage life of monoterpenoids with heat-sensitive degradation. The storage life of the monoterpenoids in the anti-cellulite emgel was estimated to be 31

months, while caffeine was 46 months in the same concentration of anti-cellulite herbal emgel.

Calculation of Monoterpenoids and Caffeine Accelerated Shelf Life by Q10 Method

The accelerated stability test, which predicts the potential behavior of the anti-cellulite emgel, is quite informative. The shelf life of anti-cellulite emgel was calculated using the Q10 equation (6,7), which was considered a cornerstone in estimating the storage life of monoterpenoids with heat-sensitive degradation. The storage life of the monoterpenoids in the anti-cellulite emgel was estimated to be 31 months, while caffeine was 46 months in the same concentration of anti-cellulite herbal emgel.

Conclusions

An anti-cellulite herbal emgel was successfully formulated. HS-GCMS and HPLC methods were developed and validated to quantitatively determine of the monoterpenoid and caffeine constituents in the formulation. The emgel was physically stable after 3 month-storage at 4° C, RT, and 50° C. The caffeine content showed no significant changes and passed the acceptance criteria ≥80% at all temperature test while monoterpenes showed some degree of degradation at 50° C after 2 months. The shelf-life of the emgel was, consequently calculated as 31 months by using Q10 method.

Patents

The petit patent for the anti-cellulite herbal emgel product was obtained from Department of Intellectual Property (DIP), (Thailand), no. 17425, date 11 March 2021.

Author Contributions

Conceptualization, N.N., K.I., K.C. and N.W.; methodology and experimental design, N.N., N.C., K.C., N.N.(Nitra Neungchamnong), K.I. and N.W.; software, N.N.; validation, N.N., N.C., K.I., and N.W.; formal analysis, N.N., N.C., N.N.(Nitra Neungchamnong) and N.W.; investigation, N.N. N.C., and K.I.; resources, N.W., K.C. and K.I.; data curation and interpretation, N.N., N.N.(Nitra Neungchamnong), K.I. and

N.W.; writing original draft preparation, N.N., K.I., and N.W. ; writing review and editing, N.N., K.I., and N.W.; visualization, N.N., K.C., N.C., N.N. (Nitra Neungchamnong), and N.W.; supervision K.I., K.C. and N.W.; project administration, K.I.; funding acquisition, K.I. and N.W. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by: (i) Royal Golden Jubilee Program (RGJ-PhD) (0008/2560) for the scholarship of NN, (ii) Thailand Center of Excellence for Life Sciences (TCEL) (TC-A2/62), (iii) National Research Council of Thailand (NRCT) (DBG6080005, IRN61W0005) and (iv) Center of Excellence for Innovation in Chemistry (PERCH-CIC), the Ministry of Higher Education, Science, Research and Innovation. No funding agency influenced or restricted any part of the trial, its data, nor will they see the manuscript prior to publication.

Acknowledgments

We would like to acknowledge the Royal Golden Jubilee Program, PERCH-CIC, NRCT, TCELs and Cosmetics and Natural Products Research Center (COSNAT). We would like to extend our appreciation to Roy I. Morien of the Naresuan University Graduate School for his assistance in editing the English grammar, syntax, and expression in the paper.

Conflicts of Interest

All authors completed the ICMJE disclosure form and reported the following: KI is PI for the funding listed above, has received an honorarium from the University, and KI, NN, KC, and NW are named in the petit patent (Thailand) lodged by TCEL, no. 12872

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CHAPTER V

EFFICACY OF AN ANTI-CELLULITE HERBAL EMGEL: A RANDOMIZED CLINICAL TRIAL

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Abstract

Cellulite describes unsightly skin overlying subcutaneous fat around thighs and buttocks of post-pubescent females. A monoterpene herbal ‘emgel’ was tested in a double-blind, placebo-controlled trial with 18 women aged 20–50yr with severe

cellulite. Appearance of cellulite (primary outcome), thigh circumferences, skin firmness, and cutaneous blood flow (secondary outcomes) were assessed at baseline, 2, 4, 8 and 12 weeks with a 2-week follow-up. Herbal emgel massaged into the thigh skin twice daily reduced cellulite severity scores from 13.4±0.3 to 9.9±0.6, and by 25% by image analysis at week12. All secondary outcomes improved with both placebo and herbal emgels suggesting that ingredients in the base-formulation were active on these targets or that massaging was responsible. Participant queries, their diaries, and monthly inspections found no adverse events. The herbal emgel safely improved the appearance of cellulite, while the base emgel may be pharmacologically active for other endpoints. Further studies on active constituents and their mechanism of action are needed to explore mechanisms of actions.

Introduction

Cellulite describes the lumpy, folded, dimpling, or ‘orange-peel’ appearances of the skin overlying subcutaneous fat, usually around the thighs and buttocks, of post-pubescent females. Gynoidal fat is regarded as an attractive feature in women but when excessive, it is perceived as ugly. It is associated with excessive fat accumulation in these deposits but its etiology is subjected to several theories (1). In most superficial fat, especially gynoidal, lipid accumulation is promoted by estradiol via its estrogen receptor alpha (ERa) while fat cells produce estrogen, particularly in obesity. Gynoidal fat is mobilised in pregnancy and lactation but ERa resistance in cellulite may prevent lipid release.

In the obese pathology, the nutritive superficial vasculature and lymphatic vessels become dysfunctional. Excessive fat becomes inflammatory causing fibrosis thereby preventing connective tissue remodelling that accommodates increased dermal and subcutaneous fat deposition that causes the characteristic lumpiness or dimpling (2).

Cellulite develops in most women who are not necessarily overweight or obese, causes no apparent health risk, but is aesthetically unpleasing. Accordingly, anti-cellulite treatments have proliferated including products based on herbal ingredients. The latter include a wide range of herbal extracts including methylxanthines delivered by subdermal injection, topical creams and wraps, dietary supplements, or alternatively,

mechanical interventions such as massaging, laser or light therapy, and liposuction. While they may be efficacious, the invasive approaches carry some risks (2-3). Adipogenesis or lipogenesis, the differentiation of adipocytes from pre-adipocytes and mesenchymal cells, provides constant renewal of adipocytes and contributes to the increase of adipose tissue mass, and insulin plays a predominant role in the adipogenic process. On the other hand, lipolysis and its oxidation safely reduces the adipocyte fat load (4-5).

The Thai herbal compress is a muslin cloth that encapsulates 100-200g of mixed herbs and when steamed are used to relieve pain, inflammation, in postpubertal women. In a previous study, the compress contents were selected to target the main tissue pathologies of cellulite: inflammation, dermal fibrosis, poor microvascular and lymphatic function, and refractory lipolysis (6). These compresses reduced cellulite, skin-fold thickness and circumferences of treated thighs in a placebo controlled trial conducted on 21 premenopausal women for 8 weeks (7). In addition, we demonstrated *in vitro* that essential oils and some herbal compress ingredients reduced lipogenesis and increased lipolysis in 3T3-L1 adipocytes, and relaxant in rat aortae (8).

However, herbal compresses are expensive to produce, treatment is labour-intensive, and needs dedicated premises. The original compress formulation relied on solid constituents whose active ingredients were leached out by steaming and pressing over the affected skin areas, contents that are incompatible with any self-administered topical lotion. Instead, the essential oils comprising mainly monoterpenes from the original formula were emulsified into an aqueous base along with preservatives and a gelling agent to produce a gelled emulsion or an ‘emgel’ which adheres to the skin without running off during ~10min of self-massaging. This project aimed to test the anti-cellulite efficacy, and safety of this emgel in a fully blinded, single arm, randomised, placebo controlled trial in women having cellulite.

Materials and Methods

Formulations

Products in this study were an emulsion and gel mixed to produce an ‘emgel’. The herbal emgel was formulated from selected essential oils in term of anti-cellulite agent in treatment. All essential oils of the herbal emgel i.e., ginger

(rhizome), black pepper (fruit), java long pepper (fruit), turmeric (rhizome), plai (rhizome), lemongrass (stalk) and kaffir lime (fruit peel) were purchased from Thai-China Flavours and Fragrances Industry Co., Ltd., Thailand. Tea (leaves) (Three Horses®) was purchased from Three Horses Tea Co., Ltd., Bangkok, Thailand. Coffee (seed) were purchased by Coffman International. Co., Ltd., Bangkok, Thailand. Selected other excipients assisted pharmacological action (Table 30).

Tea and Coffee Extracts: 100 g of tea or coffee were extracted by boiling in 3 changes of 400mL of distilled water, the combined 1200ml of solution filtered through a cloth, centrifuged (100g, 5min), the supernatant was removed and lyophilized and stored at −20 °C in screw cap bottles. The ratio of 0.05% tea and 0.05% coffee (aqueous extracts) were present in emgel treatment. The dimethylxanthines in tea and coffee extracts were used to inhibit phosphodiesterase, reduce lipogenesis, and activate lipolysis, helping to restore the normal structure of the subcutaneous tissue and acting as anti-free radicals.

Mixed Oil: The herbal emgel was developed with mixed oil from an anti-cellulite herbal compress by our previous study (8). This contained ginger, black pepper, long pepper, turmeric, cassumunar ginger, lemon grass and kaffir lime, and tea and coffee extracts. The main ingredients of the herbal emgel formulation comprised 5.0% mixed oil containing the putative active monoterpenes (camphene, camphor, 3-carene, α-citral, β-citral, limonene, β-mrycene, α-pinene, β-pinene, and terpinene-4-ol, 5.0% camphor) and an emulsifier (89.9), rice bran oil, and deionized water. The placebo emgel contained the same base formulation without the essential oil/extracts (Table30). It was formulated by the Cosmetics and Natural Products Research Center (Faculty of Pharmaceutical Sciences, Naresuan University).

Table 29 Composition of the emgels used to treat cellulite

Ingredient	Herbal emgel %W/W	Placebo emgel %W/W
Carbopol	0.8	0.8
Disodium EDTA	0.1	0.1
Propylene glycol	2.0	2.0
Glycerol	6.0	6.0
Microcare (Phenoxyethanol + Chlophenesin)	1.0	1.0
Triethanolamine (TEA)	1.0	1.0
Rice bran oil	8.0	8.0
PEG-40 hydrogenated castor oil	5.0	5.0
Tea extract	0.5	0
Coffee extract	0.5	0
Mixed herbal essential oils	5.0	0
Camphor	5.0	0
Demineralized water (aqua)	65.1	76.1

Quality Control for Treatment Products Analysis of Ten Monoterenoids by Headspace GC-MS, Caffeine by HPLC Headspace GC-MS Conditions

For placebo and herbal emgels, 2 mg of the treatment products were weighed in a headspace vial of 20 ml and sealed using a silicone/PTFE septum and then incubated at 140 °C for 5 min. The transfer line temperature was maintained at 250 °C.

Monoterpenoids in the herbal emgel were analysed using a gas chromatograph (Agilent 7890B) and a mass spectrometer (Agilent 5977B MSD). The resultant vapour phase injected into the GC/MS in split injection mode (ratio 50:1). The treatment products in vial had been pressurized with carrier gas, then injected into a capillary column HP-5 5% phenyl methyl silox (30 m x 250 μm x 0.25 μm; Agilent 19091S-433: 93.92873). Helium as the carrier gas flowed at 1.3 mL/min. The initial temperature was held at 75 °C for 1 min and raised at 8 °C/min to reach 100 °C and held for 4 min. The temperature gradient continued at 25 °C/min until 250 °C. The

temperatures of the injector port and detector were 230 and 250 °C, respectively. The total run time was 14.1 min. To identify monoterpenoids, the mass spectrometer was operated in selective ion monitoring (SIM) mode (100 ms dwell times). For GC–MS analysis, electron ionization (EI) system was used with ionization energy at 70 eV.

Study Protocol

This was of a single arm, double-blind design where the treatment was randomly allocated to one or other thigh and the placebo applied to the contralateral side and reported according to the CONSORT 2010 guidelines. The participants applied the allocated placebo emgel and then the herbal emgel twice daily (morning and evening) to each thigh for 12 weeks. Morphometric and instrument measurements were carried out before the first application (baseline) and thereafter at 2, 4, 8, and 12 weeks during treatment, and after the 2-week follow-up without treatment.

Ethical Approval

The protocol was approved by the Institutional Review Board and Ethics Committee, Naresuan University, in accordance with the Helsinki Declaration (2013) and Good Clinical Practices (IRB No. 128/ 2019, dated April 03, 2019) (COA No.128/2019) and registered with the Thai Clinical Trials Registry, number TCTR20160302001.

Study Site and Setting

The recruitment and clinical study were conducted from May to August inclusively, 2019. The study was performed in dedicated testing rooms at the Cosmetics and Natural Products Research Center (Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand, 65000).

Cohort Size

Twenty participants were needed based on a previous study that generated significance at <0.05. Two sample comparison of proportions power calculated as 80% for total sample size equal to 46 legs (n=23). Also suggest a minimum of 18 participants (36 legs).

Outcomes

The primary endpoint was the degree of cellulite scored visually from photographs, and independently by image analysis of photographs.

The secondary endpoints were thigh circumferences, cutaneous blood flow, skin firmness, and participant comments.

Participant Specifications

Twenty women who displayed cellulite on the thighs.

Inclusion Criteria

Women aged 20-55 who had cellulite on their thighs. Grade ≥2.0 on the Nürnberger and Müller scale, due to subcutaneous fat accumulation, and had a BMI (body mass index) of 20-29, were included.

Exclusion Criteria

Pregnancy, lactation, coagulation disorders, scars, local infections or marks that obscured cellulite on the thighs, systemic diseases, a history of dermatitis and/or allergic reactions to herbs, neuropathy, disorders of the skin or its vasculature, use of hormone contraceptives, anti-histamines, steroids, or had non-steroidal anti-inflammatory drugs within 3 days of beginning the study, anti-cellulite treatment within the past 3 months, or major surgery within the past year (9-10).

Recruitment

Participants were found by advertisements posted around Naresuan University. Potential participants read the information sheet, had their cellulite assessed, and given a ~15 min briefing. Twenty applicants meeting the selection criteria, signed the informed consent form, were enrolled, and given a diary to log events during the trial.

Criteria for Withdrawal

During the study, participants were withdrawn from the study if they had an adverse reaction or irritation that could result from the intervention, any illness that prevented any period of participation of the study, used additional treatments for cellulite over the thighs, participated in any other study, became pregnant, or wanted to discontinue the treatment.

Randomization

Each participant was allocated an ID code and treatment allocated to either the left or right thigh by block of four randomization, with the contralateral thigh receiving the placebo treatment.

Blinding and Allocation Concealment

The ID allocation table was securely stored by Miss Nunguthai Suphrom (NS), Faculty of Science, who had no role in the treatment formulations, their storage, their distribution to participants, the measurements, nor data analysis. NS secured the allocation table and attached appropriate sachet labels that identified the participant ID and to which leg the sachet contents were to be applied. Every measurement was conducted by the same technician throughout the entire trial. All evaluators and technicians were paid, were blinded to allocations, were not authors, and had no other conflicts of interests. All data analyses were deferred until every participant had completed the trial.

Baseline Characteristics

All participants were ethnic Thais and classed by the Asian classification as over-weight (23.0-24.9 kg/m²) or obese (25.0-29.9 kg/m^2), had similar lifestyles, and lived within a 5 km radius of the Cosmetics and Natural Products Research Center at Naresuan University.

Monitoring

Treatment constituents used are common topically applied compounds used in cosmetics. Every day for 1 week and thereafter every 2 weeks for the remainder of the trial each participant was queried about allergies, soreness, or any other health issues arising from the treatment or general health, adherence, variations of daily routines, emgel usability and its odour. Their diaries were examined, and any problems with the trial discussed at every visit. The personal mobile telephone numbers of responsible trial staff were provided for '24/7' contact. Participant body weights and blood pressures were recorded at each visit and the thighs and other skin areas examined for rashes.

Participant Experience

The flow diagram for participant passage through the trial is shown in Figure 19.

Application of The Emgels

Participants applied the emgels twice daily, at 0600-1000hr after a morning shower and at 1800-2200hr after an evening shower, for the 84 consecutive days (12 weeks). Self-treatment began with a shower and thorough drying. They then applied one complete sachet of emgel (5 g) uniformly around the entire circumference of their

upper legs from 3 cm above the patella to the inguinal region using gentle circular motions using their palm, until a residual thin oily remained that aided skin penetration (~3min per thigh). Participants refrained from showering or washing their thighs for at least 30 min thereafter.

Measurement of Clinical Outcomes

Measurements were made at baseline, 2, 4, 8 and 12 weeks, and at another 2 weeks without treatment (week 14). Participants were asked to wear shorts ('hot pants') at visits. During the 14-week study, they were asked to maintain their normal routines and diets, and to avoid anti-cellulite products. Participants were paid expenses of 400 Thai Baht at each attendance (~US$12). Evaluations were conducted in clinical testing laboratories, each dedicated to particular tests under controlled temperature (24°C±1°C) and humidity (55% ±2%, limits 65%). Each visit was scheduled at the same time-of-day. Participants were asked to acclimatise for 30 min in similar conditions and their weights and blood pressures recorded. Then, every participant went through the following series of tests, conducted always by the same technician for east test.

Imaging

The participant stood upright on a rotatable platform with the heel of the leg under test positioned over the axis of rotation and bearing most of the body weight. The other foot was placed ~12cm in front and 20cm lateral of the study leg. The thigh aspect under study was illuminated by a white light beam with a 15^{o} dispersion and adjusted to an elevation of ~70^{o} with respect to the participant vertical axis. The light source was positioned at platform level to reduce illumination gradients introduced by a divergent beam. The dedicated camera (auto-exposure, 100mm focal length, 24Mpixels and 12-bit depth, Canon EOS 800D) had a fixed position. Each thigh was photographed from posterior, lateral, and anterior aspects and then the contralateral thigh similarly photographed.

Each image was analysed using 2 methods:

Visual Appearance: Grey scale images were presented separately to three trained judges who graded the severity of cellulite from 0 to 3 according to the Nurnberger and Muller cellulite classification scale (9).

Image Analysis: A region of interest (ROI) was selected from raw images (4000x3000 pixels) that only included one thigh. From this, another ROI measuring 4x60mm (width x height), referred to the thigh skin, was extracted and vertical line scans converted to 8-bit 2D arrays over the 4mm width which were then averaged into a single 8-bit vertical array. This array was smoothed using a 'simple' algorithm (averaging ~10pixels shifting 1 pixel at a time along the array) that yielded a pixel array that filtered out features <1mm in size (Pxarray1). To create an illumination baseline, Pxarray1 was further smoothed by 20pixels by removing <20mm feature (Pxarray2). Pxarray2 was subtracted from Pxarray1 and summed deviations from the resultant array gave a measure of undulations representing cellulite. Thus, smooth featureless skin yielded a value of 0 and thighs showing visible cellulite had values 200-10000. The software automatically selected 11 more adjacent 4x60 pixel ROIs each time shifted horizontally, and the same algorithm applied each time. Finally, the 12 results were summated into an overall score.

In this simple analysis, cellulite is only apparent as horizontal or diagonal ridges and troughs because illumination beam is vertical from the bottom. A more serious limitation were the black lines drawn on the skin used to measure thigh circumferences. During the 14 weeks, this line becomes less distinct and more difficult to digitally ablate. That restricted image sampling to a 80x60mm area.

Thigh Circumferences

At the first visit, reference points were marked on the anterior thigh surfaces 10 and 20 cm above the centre of the patella. 'Velcro' tape was positioned so that each point around the thigh was 10 or 20 cm vertically above the patella centre. This tape was used as a guide rule to draw a circumferal line on the skin around the thigh with an indelible pen. This line was then used to position a glass fibre flexible tape to measure thigh circumference at '10' and '20cm' at each visit.

Skin Firmness

This was measured using a Cutometer® MPA 580 (Courage and Khazaka, Germany) at 3 repetitions on the posterior thighs (11-12). The instrument yields a value depending on how much skin is pulled into the probe and varies between 0 (a fluid surface) and 1(a non-stretchable solid surface). Typical values for skin are close

to 1.0 (100%) that provide an indication of cutaneous collagen, elasticity, and shape recovery.

Cutaneous Blood Flow

Blood flow through the cutaneous microcirculation of 6x6cm areas of posterior thighs was imaged by laser Doppler flowmetry using a PeriCam PSI system (Perimed AB, Järfälla, Sweden) using laser speckle contrast analysis. The speckle pattern in the illuminated area is monitored using a 1388 x 1038-pixel CCD camera. Blood perfusion is calculated by analyzing the variations in the speckle pattern in each image pixel. These were averaged for the whole 6x6cm area and provided an estimated of the heterogeneity of cutaneous blood flow (13).

Participant Self-Assessments

A self-assessment questionnaire was collected on day 0 (baseline), which queried the participants view about their skin and cellulite status.

Another questionnaire presented at the week 12 visit sought participant opinions about their subjective perception of formulation efficacies applied to each leg.

At week 14 they completed a further questionnaire seeking their views on the subjective features of the applied formulations, their efficacy, smell, and overall observations about the treatments.

Diary Record

Participants were given a diary containing trial information, scheduled appointment dates, and into which they entered any contemporaneous comments about treatments to each leg, adverse events, and treatment variances.

Data Analyses

The clinical data obtained at each time-point were compared with baseline for each group and, also between groups in the search for statistically relevant differences. For continuous outcomes, means ± SD were calculated. Analysis of variance with repeated measures were performed to test effects of placebo and herbal products over time. Also, thigh circumferences, skin thicknesses, skin firmness, and blood flow microcirculation in each intervention group was compared with baseline using Bonferroni's post-hoc test. The satisfaction scores at the trial end were analyzed

by Wilcoxon's test ($P < 0.05$) and the number of participant comments from diary recorded was calculated.

Results

Major constituents of mixed essential oil, tea and coffee extracts

Ten constituents of the herbal gel (containing 5% mixed essential oils) were identified by headspace GC/MS) and characterised by comparing their MS fragmentation patterns and comparator retention times of standard compounds (Figure 18A). Identities were confirmed by mass spectra retrieved from the National Institute of Standards and Technology (NIST MS search 2.2 library) spectral database. These were absent from the placebo emgel that contained no essential oils (red trace, Figure 18A). Caffeine was also apparent in the herbal emgel (from 0.5% of each tea and coffee aqueous extracts) and identified by retention times using HPLC (Figure 18B).

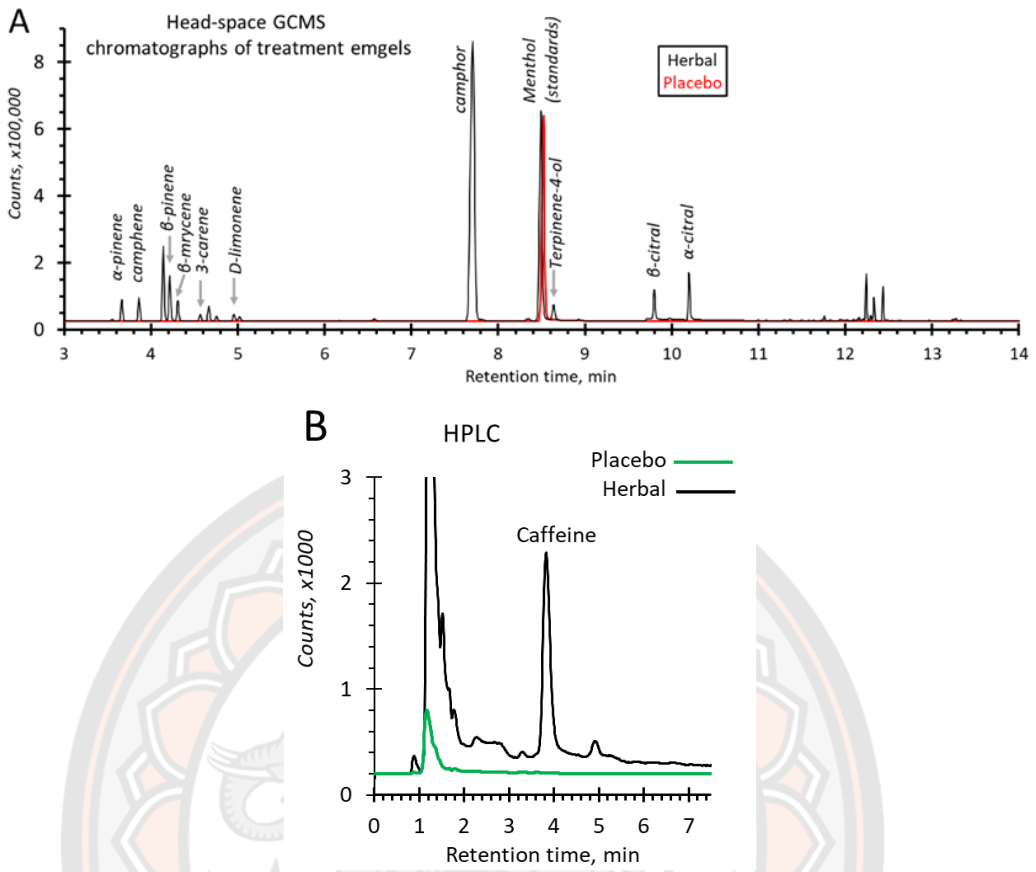

Figure 18 Chromatographic analysis of mixed essential oil, and tea and coffee extracts. (A) Headspace GC-MS (SIM) total ion chromatogram of the main mixed essential oil constituents (labelled) of in the herbal emgel (2 mg injected) (black trace), and the pla-cebo emgel (red trace, displaced by 2sec), (B) HPLC chromatogram of caffeine (Reten-tion time, 3.93 min)

Participants

Of 55 women answering the advertisement, the 20 who fitted the selection criteria were enrolled, randomised and followed the protocol (Figure 19). One participant was withdrawn because of an influenza infection (week 7) and another who developed seasonal allergy (week 8). Both women self-medicated to treat their conditions. Neither were considered to arise from the cellulite treatment. Eighteen participants completed the trial. Nobody reported protocol violations and all sachets were returned empty suggesting 100% adherence to the treatment. Their baseline thigh characteristics showed matching of all outcome parameters (Table 31).

Table 30 Baseline data

Parameters		Placebo emgel	Herbal emgel
		Means ± SD	
Respondents to advertisements		55	
Participants enrolled		20	
Participants completing the trial		18	
Female		100%	
Age		29.9 ± 8.8 y (22-53)	
Body weight		60.7 ± 12.1 kg	
Body mass index		23.5 ± 3.4 kg/m2	
Cellulite grading*		grade 3.0 ± 0	grade 3.0 ± 0
Cellulite Severity Scale (CSS)**		12.9 ± 0.7 (Severe)	13.4 ± 0.8 (Severe)
Cellulite by image analysis ‡		8356 ± 3736	8675 ± 3592
Thigh circumference, at 10 cm (Lower)		47.1 ± 4.0 cm	47.6 ± 4.4 cm
	at 20 cm (Upper)	55.4 ± 4.6 cm	55.5 ± 4.6 cm
Skin firmness,	anterior thigh	0.93 ± 0.02	0.93 ± 0.03
	posterior thigh	0.95 ± 0.02	0.95 ± 0.01
Blood flow,	posterior thigh	0.40±0.12 PU/mmHg	0.42±0.13 PU/mmHg

Note: * Nürnberger and Müller scale (9)

** Classification of cellulite based on the results of visual scores (Mild 1-5; Moderate 6-10; Severe 11-15) ‡ Image analysis yields a unitless values reflecting the depth of undulations over the skin.

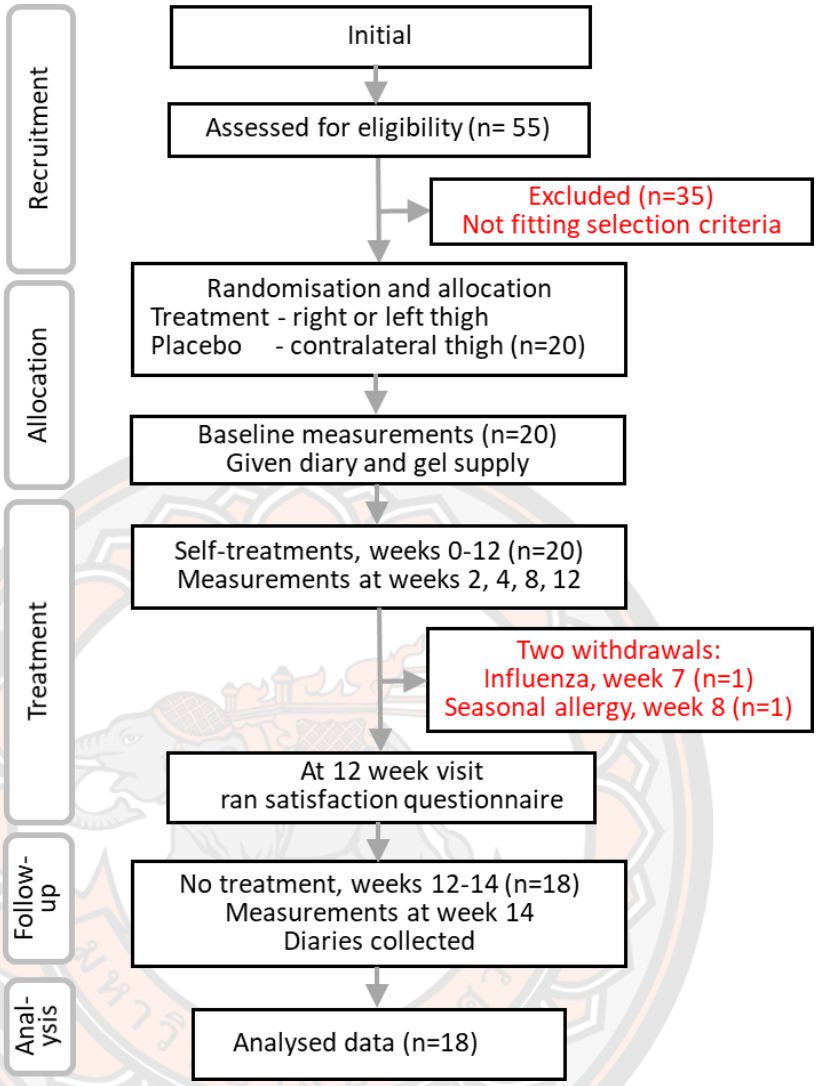

Figure 19 Participant experience through the trial

Visual estimation of cellulite

Three trained evaluators graded monochrome images, similar to examples shown in Figure 20. Treatment with the herbal emgel clearly diminished the observed cellulite over the time-course of the trial (Figure 21) compared to a small reduction produced by the placebo treatment. Cessation of treatment allowed the cellulite to become partly re-established. The herbal emgel reduced the cellulite grade at week 12 compared to baseline: 1.8 compared to 2.6 found previously (7).

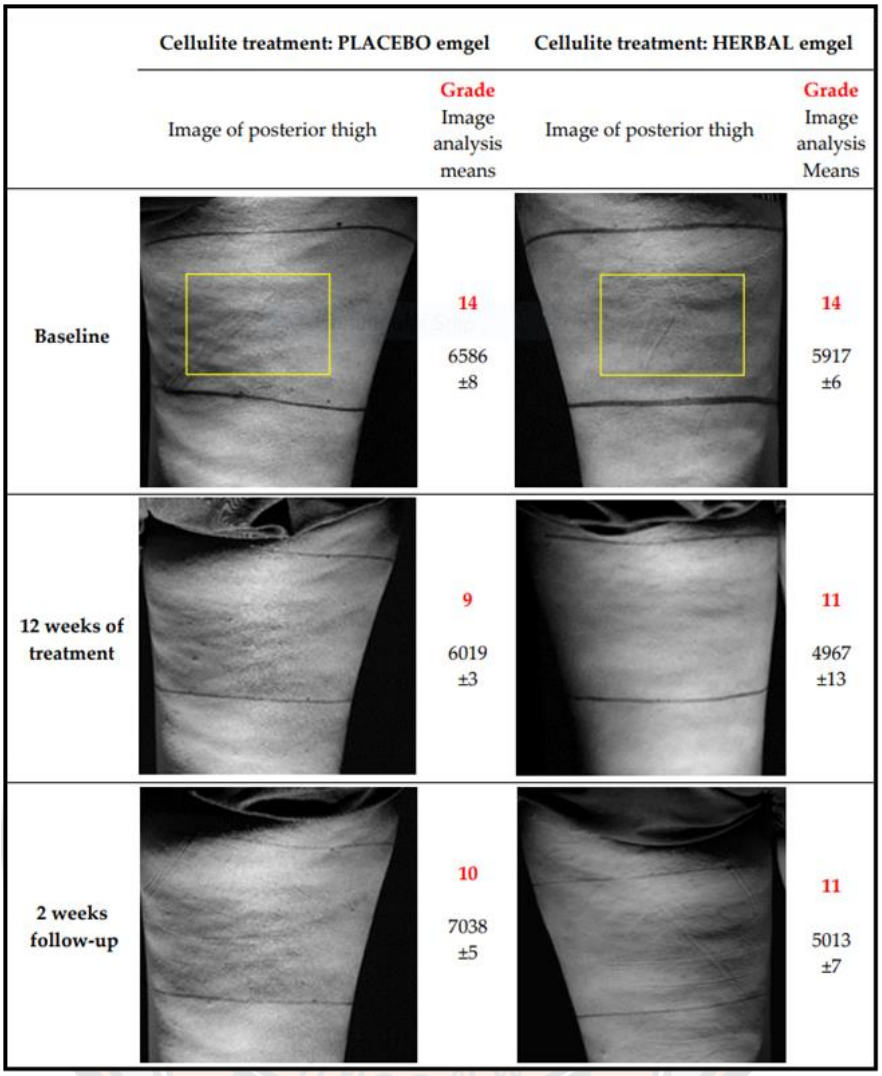

Figure 20 Representative photographs from a single participant showing thighs in posterior aspect as seen by three evaluators whose estimate is given as cellulite grades. The image analysis used the areas bounded by the yellow boxes and the unitless values were generated by 3 scans of each image to give means±SEMs

Cellulite by image analysis

Reading of the same photographs also showed a decline in cellulite reaching about the same level compared to baseline (compare Figures 21B and C). However, the placebo treatment did not show any apparent fall in the cellulite value using this measurement tool compared with visual grading. These differing details created by

the two measuring systems probably arise from the restricted region of interest imposed by the image analysis algorithm (see methods) creating larger variations.

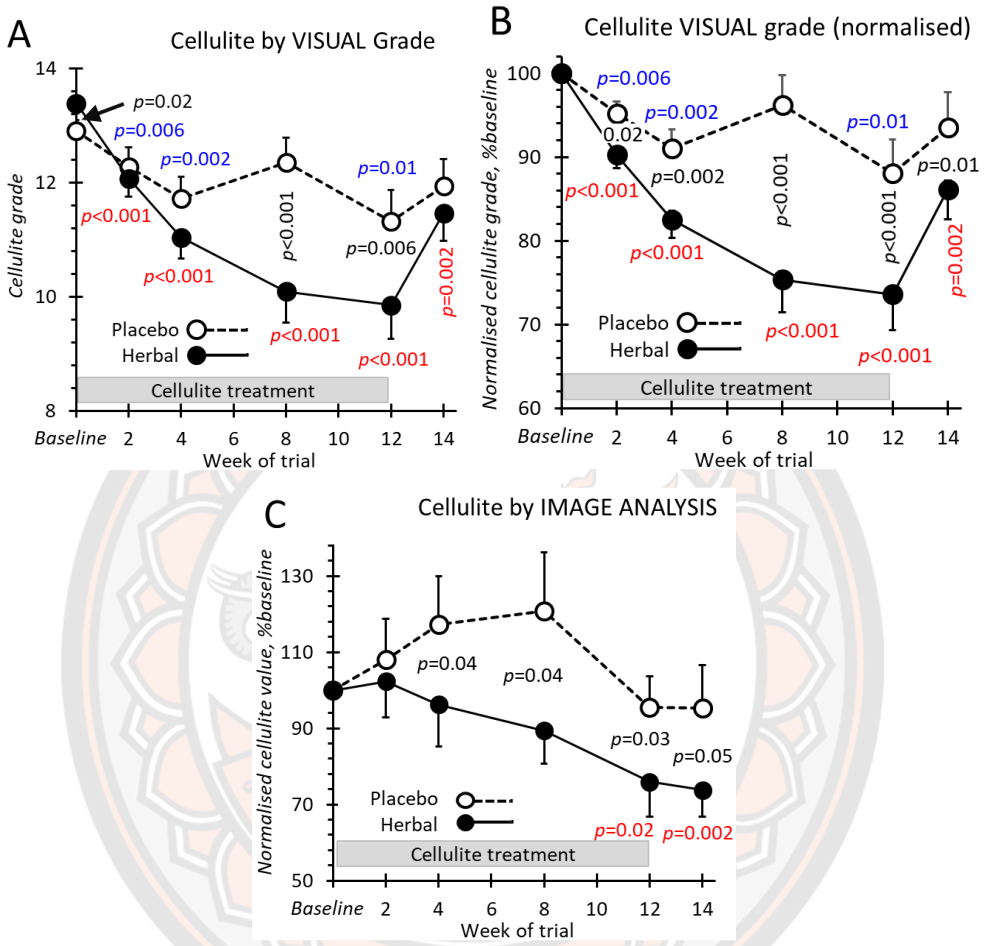

Figure 21 Cellulite changes during the 14 week trial. (A) Cellulite grades estimated by three blinded independent evaluators. (B) Cellulite grades expressed as percentages of the grade be-fore treatment (baseline). (C) Normalised cellulite values measured by image analysis. Values are means ± SEMs. P-values in red compare values for herbal gel with corresponding baseline values, those in blue compare values for placebo gel. P-values in black compare herbal and placebo emgel values at the same time point. All statistical testing was performed on data nor-malised to baseline values for each participant. An absent value indicates p>0.1. All points are means ± SEM.

Thigh circumferences

Throughout the 12 weeks of treatment, both lower and upper thighs appeared to become slightly thinner with both placebo and herbal emgel treatments by 0.8-1.0cm beyond week 2 (Figure 22A-B). There was an apparent additional ~0.5cm reduction in circumference beyond week2/4 with the herbal emgel but failed to reach a significance threshold. Nevertheless, this was probably a genuine action of herbal emgel since: (i) this circumference shrinkage was similar to the previous study (7) and (ii) that all values for herbal emgel were lower at the time points when an effect would be expected (4-14 weeks) and at both 10 and 20 cm above the knees.

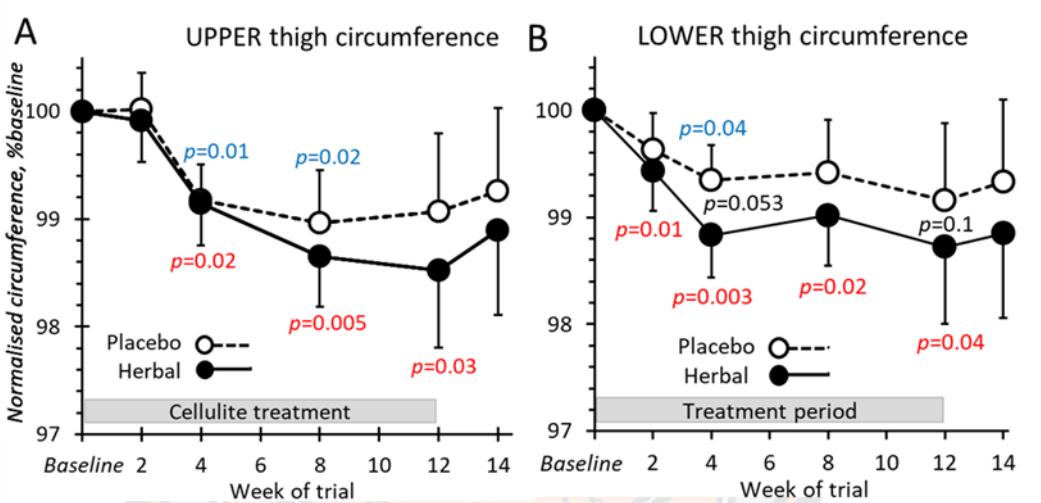

Figure 22 Thigh circumferences 20 (upper) (A) or 10 cm (lower) (B) above the knee treated with either placebo or herbal emgels. The measurements expressed as the ratio corresponding ration at time 0 (baseline). For upper thighs, actual baseline values for placebo and herbal emgels were 55.4±1.1 and 55.6±1.1 cm respectively and 47.1±0.9 and 47.6±1.0 cm for the lower thigh. P-value color coded as Figure 21

Skin firmness

Skin firmness for the posterior thigh was unaffected by either placebo or herbal emgel (Figure 23A). For the anterior site, both treatments promoted skin firmness but without any superior action by the herbal emgel (Figure 23B).

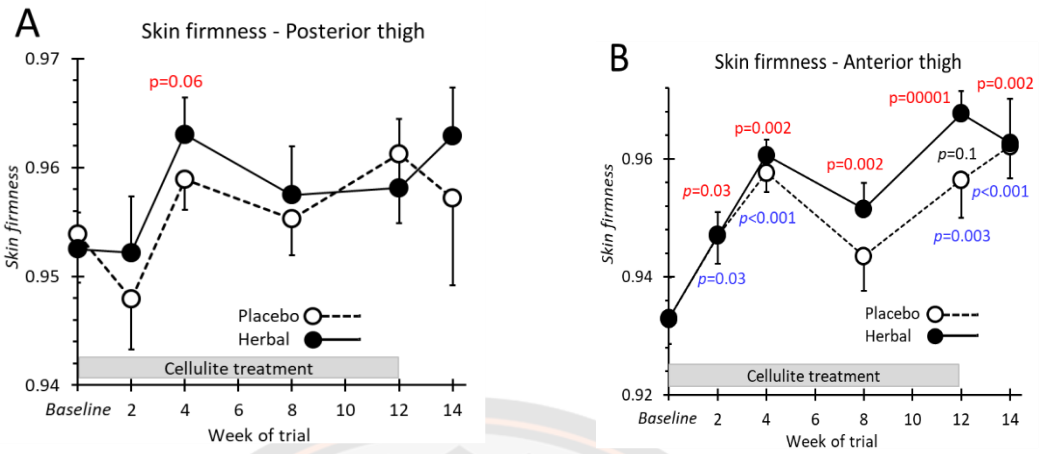

Figure 23 Skin firmness recorded from the posterior (A) and anterior (B) aspects of the thighs. All p-values colour coded as figure 21

Cutaneous blood flow

Cutaneous blood flow was measured by laser Doppler from 8 regions of each thigh (one from each of 4 sides at 10 cm and another 4 at 20 cm above the knees). For all the sites tested, cutaneous blood flow increased with both placebo and herbal emgel treatments after week 2 compared to baseline (Figure 24). There was insufficient strength to demonstrate any effect on cutaneous blood flow with herbal emgel.

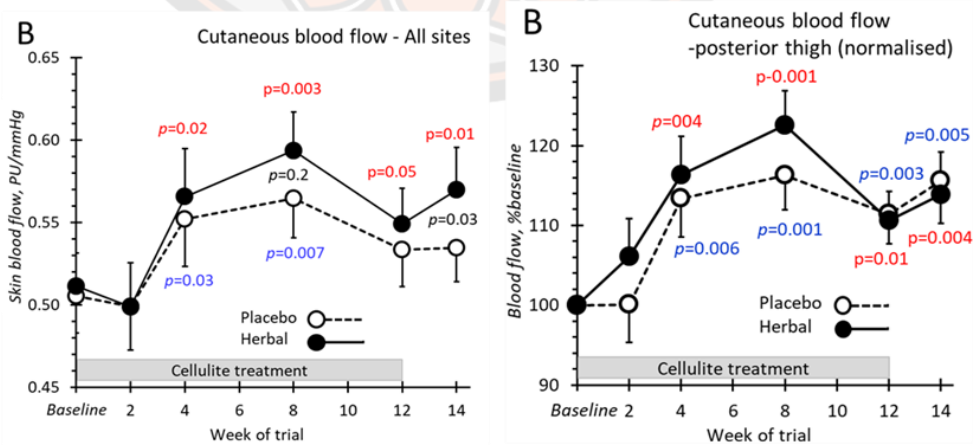

Figure 24 Cutaneous blood flow by laser Doppler flowmetry. All p-values colour coded as figure 21

Participant comments

Before treatment, participants were questionnaired about their thighs and gave fairly low evalulations that did not discriminate between legs (Table 32).

Table 31 Pre-trial participant self-evaluation questionnaire about their thighs

Questions **before treatment** about each thigh	**Satisfaction evaluations** [a]		
	Left thigh	**Right thigh**	
	Mean±SD	**Mean±SD**	**P-value** [b]
The skin feels smooth	2.06±0.80	2.06±0.80	0.500
The skin feels tight	1.56±0.70	1.56±0.70	0.500
My thigh appears to be large	1.72±0.75	1.72±0.75	0.500
The thigh cellulite is severe	1.78±0.81	1.56±0.70	0.193

Note: [a] Responses were scored 1 to 5, where 1=Strongly disagree, 5= Strongly agree, and 3=Neutral.

[b]*P*-value by Wilcoxon signed rank test (2-tailed) comparing left and right thigh.

On completing the trial, 45 spontaneous diary entries were extracted and 16 positive comments were made about the placebo treated thighs compared to 29 about herbal emgel treated thighs, most commonly about firmer skin and reduced cellulite (Table 33).

Table 32 Summary of 45 spontaneous free-text comments extracted from participant diaries entered during the 12-week treatment. Translated by sense rather than verbatum

Generalised comments about thighs	Placebo thigh	Herbal thigh
	N comments (%)	N comments (%)
My skin seems firmer	4 (22%)	10 (56%)
My cellulite appears to be reduced	5 (28%)	8 (44%)
My thighs appear to be thinner	2 (11%)	4 (22%)
My thigh skin appears to be smoother	5 (28%)	7 (39%)
Total comments	16	29

After the trial, participants were presented with another questionnaire that sought self-assessments about specific properties and actions of the emgels. For the properties, they discerned little difference between the two emgels, but some preferences for the herbal emgel actions (Table 34).

Table 33 Self-evaluation questionnaire about perceptions on cellulite-related properties after 12 weeks of treatment

The Question Appearing on the Questionnaire—Translated From Thai	Satisfaction score [a]		*P*-value [b]
	Placebo emgel	Herbal emgel	
	Mean+SD	Mean±SD	
About the emgels			
Gel texture good	3.83+0.79	3.50±1.04	-0.14*
Emgel fragrance pleasant	3.39±1.04	3.11±1.02	-0.21*
Gel well absorbed by the skin	3.11±1.13	3.06±1.26	-0.44*
Overall satisfaction with emgel	3.61±0.50	3.56±0.92	-0.41*
Effects of the emgels			
Your thighs are smoother	3.44±0.98	3.78±0.80	0.14
Your thighs feel firmer	3.17±1.04	3.44±1.25	0.24
Your thighs feel smaller	2.83±1.10	3.33±1.08	0.09
Your cellulite appears to be reduced	3.00±1.14	3.61±0.85	0.04
Overall satisfaction with the emgel	3.61±0.85	4.11±0.92	0.04

Note: [a] The responses were given a numerical value of 1 to 5, where 1=Strongly disagree, 5= Strongly agree, and 3=Neutral. [b]*P*-value determined using Wilcoxon signed rank test (2-tailed). *These values in **red** indicate that herbal emgel is worse than placebo emgel, **back** better than.

Monitoring and adverse events

No adverse events were reported via questionnaires, diaries, or verbal communication. No reddening, swelling, nor irritation were observed around treatment areas by trial personnel at the time of testing.

For clinical monitoring throughout the trial, body weights and BMIs showed no population changes throughout the study but there was a noticeable blood pressure decline (Table 35). Some recruits (n=10) were postgraduate students who reported stress and sleep deprivation, but the twice daily self-treatment helped relaxation. For this group, BP begin at 114/81 falling to 104/74 at week 12 and for other participants corresponding BPs were 109/74 to 105/72. None suffered from dizziness, faintness or postural hypotension and these values were normal for females of this age group, so was not cause for concern. Blood pressures at follow-up were the same as they were at the end of the treatment period.

Table 34 Clinical monitoring parameters of participants

Parameter	Baseline	12 weeks	p-value
	Means ± SD		
Body weight (kg)	60.4±12.3	60.7±13.3	NS
Body mass index (kg/m^2)	23.4±3.3	23.5±3.9	NS
Systolic blood pressure, mm Hg	112.0±12.3	104.6±9.5	0.006
Diastolic blood pressure, mm Hg	78.3±9.7	73.4±7.8	0.01

Discussion

The primary endpoint, the appearance of the thigh skin, was improved when treated with the anti-cellulite herbal formulation, as assessed visually and by image analysis while the placebo was without effect. The secondary outcomes were thigh

circumference, skin firmness, and cutaneous blood flow. For treatment with both placebo and herbal emgels, all three parameters improved while the herbal emgel alone had little further effect over and above the placebo action. A similar placebo effect was observed previously for both cellulite appearance and thigh circumference using herbal compresses (7).This suggests that herbal emgel is acting on the cellulite appearance alone independent of these other three actions. In explaining these three placebo actions, the following mechanism may operate: (i) a constituent(s) of the placebo emgel is active, or (ii) a compound in the herbal emgel is transdermally absorbed when applied to one thigh (8% of the body surface) thereby acting systemically on both legs. All these are credible possibilities acting alone or in consort but our data is unable to distinguish their contributions. Nevertheless, these arguments support the notion that the improved appearance of cellulite arises from the localised treatment, an assertion supported by our own (7) and several other studies (14-17).

Thigh skin is 2.4mm thick, while thigh subcutaneous fat is another 12.4mm (18). Dermal adipocytes arise from a myofibroblast lineage, are immuno-active in skin wounding, proliferate with cold exposure and dedifferentiate back to myofibroblasts and influence extracellular matrix remodelling (19). Thus, topically applied anticellulite agents are unlikely to directly influence subcutaneous fat unless systemically absorbed, but could realistically interact with dermal fat.

We detected an improved cutaneous blood flow but could not demonstrate a selective effect of the herbal through the topical route. Given this and that the herbal is vasodilatory in vitro (8), a dedicated trial on blood flow is needed. For this, nutritive and thermally-activated blood flow need discriminating, blood flow measured in deeper layers (limited to ~0.7mm with our equipment), and the possible role of systemic absorption and action are explored.

Our and other studies achieve only a ~20% cellulite reduction suggesting scope for further improvement (20). While targeting adipocyte lipid turnover is one strategy, only long-term solutions like moderating excessive energy intake or increasing lipid utilisation by oxidative phosphorylation in muscle or mitochondrial uncoupling in beige/brown adipocytes need to be part of or accompany phytopharmacological interventions.

Conclusions

The primary outcome showed a clear amelioration of cellulite of the thigh posterior aspects confirmed by the participants feeling that the herbal monoterpene emgel improved the feel and appearance of their treated leg. Both the herbal and placebo emgels improved the secondary outcomes (thigh circumference, and skin firmness and blood flow) suggesting emgel constituents mediated primary and secondary outcomes. However, the treatment effect sizes are limited without accompanying fat load reductions and increased fat oxidation see with lifestyle changes.

Funding

This research was funded by: (i) Royal Golden Jubilee Program (RGJ-PhD) (0008/2560) for the scholarship of NN, (ii) Thailand Center of Excellence for Life Sciences (TCEL) (TC-A2/62), (iii) National Research Council of Thailand (NRCT) (DBG6080005, IRN61W0005) and (iv) Center of Excellence for Innovation in Chemistry (PERCH-CIC), the Ministry of Higher Education, Science, Research and Innovation. No funding agency influenced or restricted any part of the trial, its data, nor will they see the manuscript prior to publication.

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, 2013 guidelines, and approved by the Institutional Review Board of Naresuan University for human research (Approval number 1039/61).

Informed consent statement

All participants were fully briefed about the study and their involvement, and all signed the informed consent form as approved by the IRB. Informed consent was obtained from all subjects involved in the study.

Data availability statement

The data presented in this study will be available on request from the corresponding author.

Acknowledgments

We acknowledge with gratitude the Royal Golden Jubilee Program, PERCH-CIC, NRCT, TCELs and Cosmetics and Natural Products Research Center (COSNAT).

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CHAPTER VI

CONCLUSION

Cellulite involves an array of pathologies involving dermal and subcutaneous adipocytes, associated connective tissue, the microvasculature, and inflammation. Herbal compresses previously developed from Thai traditional medicines have been proven to have an anti-cellulite effect in healthy volunteers within 2 weeks of treatment. One particular herbal compress had previously been identified as having specific anti-cellulite properties, and the objectives in this research study were 1) to investigate the mechanisms of the anti-cellulite chemical components of these herbal compresses, 2) develop a topical preparation containing the essential oils found in extracts obtained from the ingredients of the herbal compresses, 3) investigate the stability and quality control methods of the formulation developed and 4) study the effectiveness on cellulite reduction and safety of the developed formulation in female participants.

Firstly, this study presents the preclinical effects, on cellular lipid accumulation, triglyceride content and the vasodilatation effect of on rat aortae, of the essential oils and extracts obtained from Thai traditional herbal compresses and their constituents. These findings demonstrate the abilities of the test samples to decrease lipid accumulation resulting in the inhibition of adipocyte differentiation and increasing lipolysis on 3T3-L1 adipocyte cells. The mixed oils showed vasodilatory effects on rat aortae via endothelium-dependent release of vasodilators.

Secondly and thirdly, an anti-cellulite herbal emgel was successfully formulated. HS-GCMS and HPLC methods were developed and validated to quantitatively determine of the monoterpenoid and caffeine constituents in the formulation and investigate the stability and quality control of the bioactive maker. The emgel was physically stable after 3 month-storage at 4°C, RT, and 50°C. The caffeine content showed no significant changes and passed the acceptance criteria ≥80% at all temperature test while monoterpenes showed some degree of degradation

at 50°C temperature after 2 months. The shelf-life of the emgel was, consequently calculated as 18 months by using Q10 method.

Fourthly, the clinical study demonstrated to measure treatment efficacy of herbal emgel on cellulite, containing volatile oils and extracts of the traditional Thai herbal compress was tested in a double-blind, placebo-controlled trial with 18 women aged 20-50 year with severe cellulite. The primary outcome showed a clear amelioration of cellulite of the thigh posterior aspects confirmed by the participants feeling that the herbal monoterpene emgel improved the feel and appearance of their treated leg. Both the herbal and placebo emgels improved the secondary outcomes (thigh circumference, and skin firmness and blood flow) suggesting emgel base constituents mediated primary and secondary outcomes. However, the treatment effect sizes were limited. Without accompanying reductions of dietary calorie intake and increased fat oxidation promoted by lifestyle changes, the overall anti-cellulite action will continue to be limited.

In conclusion, the research and development of anti-cellulite product containing extracts and volatile oils from Thai herbal compress completed successfully and the efficacy and safety of the ingredients of the formulation were demonstrated in both *in vitro* and *in vivo* tests.

Limitations of the studies

In vitro studies:

In vitro protocols provide proof of principle. Since the study cells are isolated from the in vivo environment where body fluids, other cells, growth factors, nerve and hormonal actions, the presence of extracellular matrix proteins, different drug concentrations, etc. may exert influences that are difficult to replicate in vitro. For most human pathologies, these can be studied in more detail in experimental animals. However, there is little prospect of developing animal model for cellulite. Therefore, potential treatments have to be tested directly in humans.

Clinical studies:

1. While we recorded all adverse events, hidden changes in skin properties such as its ability of resist infection, increased risk of skin cancers, ability to repair wounds, effect on hair growth, etc. were not studied.

2. The study cohort was drawn from a population having similar lie-styles so the results cannot be generalised.

3. Participating in a rigorous and structured trial may also have an impact on the outcome which may not be seen in the ‘real world’.

4. The sensitivity of the questionnaire; many studies' limited scope leaves missed vital information about the intervention's effects on quality of life, and costs.

5. Difficult to find out the same smell of the herbal emgel product for placebo without effective effect.

6. Non-adherence to treatment was difficult verify

7. Since the formation was applied to about 8% of the body surface and that the active ingredients have to be absorbed through the skin to produce the therapeutic effect, there is a strong possibility that these compounds reach concentrations that have pharmacological effects throughout the body that may be adverse.

Future studies

1. Ways of increasing the effect size need exploring by treating the cellulite accompanied by reduced lipid load by reduced dietary energy load and increased oxidation through exercise.

2. The likelihood of cutaneous absorption of some formulation constituents will appear in the circulation and the following investigations are needed:

2.1 Are circulating constituents responsible for the anticellulite action

2.2 Can these compounds cause unexplored adverse events.?

3. The fate of FFA release needs determining, particularly measuring plasma FFA concentrations.

4. Safety studies should specifically explore any effects on cell biology that could potentially interfere with skin function.

5. A reporting system should be set in place to monitor adverse events associated with formulation use

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