A Standardized Composition Comprised of Extracts from Rosmarinus officinalis, Annona squamosa and Zanthoxylum clava-herculis for Cellulite

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ABSTRACT

Background: Cellulite, characterized by changes in the skin morphology presented as dimpled or puckered skin appearance, is highly prevalent among postadolescent women. Cellulite management ranges from topical cream applications to invasive procedures. While some interventions showed improvements in physical appearances of affected areas, so far, none have reversed the condition to a full recovery. These unsuccessful measures signify the intricate nature of cellulite etiology highlighting its complexity leading to the possibility for a combination treatment approach to target multiple mechanisms. Materials and Methods: We screened our plant library for extracts that reduces cellular lipid accumulation, improve microcirculation, possess high total antioxidant capacity, significant anti-platelet aggregation, and anti-inflammatory activities using lipid accumulation assay in 3T3-L1 cells, Croton oil-induced hemorrhoid test in rats as a model for microcirculation, anti-platelet aggregation assay, nitric oxide (NO) inhibition assay, and 1,1-diphenyl-2-picrylhydrazyl assay. Results: Three known botanicals such as Rosemary officinalis, Annona squamosa and Zanthoxylum clava-herculis were identified as lead extracts in these tests. Treatment of 3T3 cell with A. squamosa at 1 μg/ml resulted in 68.8% reduction in lipid accumulation. In croton oil-induced hemorrhoid study, Z. clava-herculis reduced the recto-anus coefficient by 79.6% at 6 mg/kg indicating improvement in microcirculations. Similarly, R. officinalis caused inhibition of 82%, 71.8%, and 91.8% in platelet aggregation, NO production and free radical generation at 31.25 μg/ml, 6.2 μg/ml, and 40 μg/ml concentrations suggesting its anti-oxidant, and anti-inflammatory activities. Conclusions: Data depicted here suggest that formulation of these well-known botanicals at a specific ratio perhaps may yield a composition with a much wider spectrum of mechanisms of actions to impact the multiple pathways involved in cellulite onset, continuation, or exacerbations.

Key words: Annona squamosa, cellulite, Rosemary officinalis, Zanthoxylum clava-herculis

SUMMARY

- Cellulite represents one of the main esthetic concerns of women with a likely cause of psychological insecurities. Its pathophysiology involves multiple pathways that include vascular, adipose tissues, inflammation, structural and physiological.
- Treatment strategies for cellulite comprises increasing microcirculation flow, reducing lipogenesis, promoting lipolysis, free radicals scavenging or formation reduction, anti-inflammation and other invasive procedures.
- We screened our plant library for extracts that reduces cellular lipid accumulation, improves microcirculation, possesses high total antioxidant capacity, inhibits platelet aggregation, and moderates inflammation.
- Botanical extracts from Rosmarinus officinalis, Annona squamosa and Zanthoxylum clava-herculis were identified as leads and formulated to yield a standardized composition designated as UP1307 and suggested its usage for cellulite.

INTRODUCTION

Cellulite, characterized by changes in the skin morphology presented as dimpled or puckered skin appearance around the posterolateral region, is highly prevalent among women. Records suggest that about 85% of post-adolescent women have some degree of cellulite. It represents one of the main esthetic concerns of women with a likely cause of psychological insecurities that may lead to a desperate need for cosmetic or treatment interventions.

Well-defined specific etiology of cellulite has remained elusive for decades. Through time, substantial speculations have been reported describing the complex nature and the tangled co-presence of multiple factors involving in the initiation, perpetuation, and exacerbation of the condition rendering multiple definitions. It is a multifactorial condition where its occurrence believed to be associated with mainly...
lipoxygenase, and promoting lipolysis, (c) preventing the free radical formation or scavenging free radicals, and (d) modulating with anti-inflammatory and anti-edematous agents have been proposed.

Varieties of formulations have been currently dispensed for cellulite. Some of these preparations contain methylxanthines (caffeine, aminophylline, theophylline, and theobromine), retinol (Vitamin A), alpha-tocopherol (Vitamin E), and ascorbic acid (Vitamin C) as the major active constituents. Extracts from Ginkgo biloba, Centella asiatica, Ruscus aculeatus, Ananas sativus, Chenopodium quinoa seed, and Yuzu seed are also available for a similar indication. However while some interventions showed improvements in physical appearances of affected areas, so far, none have reversed the condition to a full recovery. These unsuccessful measures further signify the intricate nature of the condition highlighting its complexity leading to the discussion of a combination therapy to target multiple mechanisms. In this regard, we screened our plant library for extracts that reduce cellular lipid accumulation, possess the high total antioxidant capacity, significant anti-platelet aggregation and anti-inflammatory activities. Three known botanicals such as Rosemary officinalis (RM504-C), Annona squamosa (RM606) and Zanthoxylum clava-herculis (UP342-C) were identified in these tests as leads and formulated at a specific ratio to yield a composition designated as UP1307.

Significant reports have been documented describing the various traditional and contemporary ethnopharmacological applications of these three medicinal plants. For example, A. squamosa L. (family: Annonaceae), also known as sugar apple, or custard apple, is the most widely grown Annona fruit tree in tropical and subtropical areas comprising of about 130 genera and more than 2300 species. The plant is a native of the West Indies and is currently cultivated throughout India mainly for its edible fruit. This plant is widely known to possess several medicinal properties with various phytochemical and biological activities. Partially purified flavonoids of aqueous Annona leaves extract to possess antimicrobial and insecticidal activity.

Annonaceous acetogenins constitute a series of natural products isolated exclusively from Annonaceae plants with a broad range of biological activities such as anticancer, cytotoxic, antiparasitic, pesticidal, and immunosuppressive activities. Rosmarinus officinalis L. is an evergreen perennial shrub native to Europe and cultivated in many parts of the world. Rosemary leaves are often used as spices and flavoring agents because of the desirable flavor and antioxidant activity of the dried leaves of the plant. There is an increasing interest in the pharmaceutical properties of rosemary, being used in conventional medicine to improve memory and relieve pain, for its antimicrobial, hepatoprotective, anti-inflammatory, anti-tumorigenic, or chemo-preventive activities. The major bioactive component of rosemary extract, carnosol and carnosic acid (CA) have showed a wide range of activities, which include adipogenesis inhibition, glucose and lipid metabolism regulation, weight gain reduction, cholesterol levels and glycemia improvement, liver steatosis improvement and gastric lipase inhibition yielding body weight and plasma lipids management. CA also inhibits cytokine-induced adhesion molecules expression and monocyte adhesion to endothelial cells through a mechanism that involves nuclear factor-kB (NF-kB), which could be involved in the anti-inflammatory properties of this compound. The cayenne may undergo an oxidative degradation and rearrangement cascade, giving rise to other rosemary antioxidant compounds such as carnosol, rosmanol, galdosol, and rosmarinone.

Similarly, Zanthoxylum clava-herculis L and Zanthoxylum americanum Mill, commonly known as Southern and Northern prickly ash, respectively, belong to the Yellow Wood (Rutaceae) family and has a long historical use as a botanical remedy. Zanthoxylum extract also referred to the toothache tree, conventionally been used by the Native American as a remedy for toothaches where the root-bark and berries having been listed in the old version of the United States Pharmacopoeia. The bark is greatly recommended for chronic rheumatism, toothache, ulcers, colic, skin sores, diarrhea, indigestion, and circulatory problems. The bark of Prickly Ash contains aporphine alkaloids (magnoflorine, laurifoline, and liliodenine), benzo-phananthridine alkaloids (cherlythrine), ligan (asarinin), coumarins (xanathoxylentin and xanthyletin), long chain amides (herculin), and essential oil. In the present study, we evaluated each component of the composition on suggested key mechanisms of action of cellulite (such as adipogenesis, vasorelaxation, platelet aggregation, inflammation, and free radical formation) and recommended further investigation on their composition for the potential use in cellulite.

MATERIALS AND METHODS

Materials

Annona squamosa L

The sugar apple fruit extract is manufactured through a series of proprietary processing steps which have been validated and performed in accordance with Good Manufacturing Practice (GMP). Annona dried whole fruit was ground to a particle size of no larger than 2 ml. Dried ground sugar apple fruit (100 kg) was then transferred to a supercritical fluid extractor (pressure 25 Mpa, temperature 50°C, CO₂ flow 70 ml/min, 2 h) and ethyl alcohol (95% food grade ethanol, quantity is 30% of the raw material) was added. The supercritical fluid CO₂ extract was evaporated under vacuum. The extraction yield is 3.7%. The contents of two biomarkers were quantified by high-performance liquid chromatography (HPLC) method with Squamocin not <1%, and Kaurenoic acid content not <4% in the extract. Squamocin and Kaurenoic Acid in the A. squamosa fruit extract were quantified with a Luna C-18 reversed-phase column (Phenomenex, 10 μm, 250 mm × 4.6 mm) in an Agilent HPLC system at 210 nm.

Zanthoxylum clava-herculis

A total of 800 kg of fresh Z. clava-herculis L. (Prickly Ash) tree bark was dried, cut, crushed, and then extracted with approximately 3-fold volume (2400 L) of 90% ethyl alcohol in water (v/v); the extraction was carried on at 90°C for 8 h. The ethanol solution was filtered to obtain the supernatant which was then concentrated with an evaporator under vacuum at 40°C. This extraction and concentration procedure was repeated three times. The extraction solutions were then combined and concentrated together. The concentrated solution stayed for 24 h in the refrigerator to obtain a supernatant. The supernatant was vacuum-dried to obtain 87.5 kg of Prickly Ash EtOH extract powder. The extraction yield was about 10.9% (w/w). Two biomarkers—Magnoflorine and Laurifoline in Prickly Ash extract were quantified with a Luna C18 reversed-phase column (Phenomenex, 5 μm, 250 mm × 4.6 mm) in an Agilent 1200 HPLC system at 275 nm.
**Rosemary officinalis**

Dried 29 kg of Rosemary leaf was extracted with approximately 10-fold volume (300 L) of 95% ethyl alcohol at 40°C. Above-described procedure was repeated two times. The extraction solutions were concentrated until the volume became 1/30 the original volumes. The concentrated solutions were then combined and evaporated again to reduce the volume of concentrated solution until 1/90 the volume of the original extraction solution was achieved. The concentrated solution was left at room temperature for 24 h to allow separation into a supernatant and precipitate-laden. Concentrated supernatant was dried by vacuum drying to obtain rosemary extract that contained between 30% and 60% CA. CA was quantified with a Luna C18 reversed-phase column (Phenomenex, 5 μm, 250 mm × 4.6 mm) in an Agilent HPLC system at 206 nm.

**Lipid accumulation assay in 3T3-L1 cells**

3T3 L1 mouse embryo fibroblasts were purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 10% bovine calf serum until confluent. Two days after postconfluence (D0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 5 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μM dexamethasone for 2 days (D2). Cells were then maintained in 10% FBS/DMEM medium with 5 μg/mL insulin for another 2 days (D4), followed by culturing with 10% FBS/DMEM medium for 4 days (D8). Test samples were treated from day 0–8 of adipogenesis with RM606 at 0.0625 μg/mL, 0.125 μg/mL, 0.25 μg/mL, 0.35 μg/mL, 0.5 μg/mL, and 1 μg/mL concentrations, and medium was changed every 2 days. On day 8, lipid droplets on cells were stained with Oil Red-O and measured at 510 nm. Tumor necrosis factor-α (TNF-α) at a concentration of 10 ng/mL was used as a positive control.

**Croton oil induced haemorrhoid**

Male SD rats (6 weeks old, approximately 140 g) were purchased from Harlan Sprague–Dawley and allowed to acclimate for 1 week. The rats were maintained in a pathogen-free facility in accordance with the National Research Council of Laboratory Care and use guidelines. Each experiment was performed with age-matched rats 7–8 weeks old. The croton oil-induced hemorrhoid model in rats was performed according to the method published by Nishiki et al.[26] In brief, a cotton swab with a diameter of 4 mm soaked with 0.16 mL of inducer (deionized water: 18% 30% and 60% CA. CA was quantified with a Luna C18 reversed-phase column (Phenomenex, 5 μm, 250 mm × 4.6 mm) in an Agilent HPLC system at 206 nm.

**Anti-platelet aggregation assay**

Whole blood was collected from male Sprague–Dawley rats and then transferred into 15 mL test tube containing 1 mL of anticoagulant citrate/dextrose solution (ACD, 85 mM trisodium citrate, 83 mM dextrose, and 21 mM citric acid). Blood was centrifuged at 170 ×g for 7 min. to obtain platelet-rich plasma, which was further centrifuged at 1200 ×g for 7 min to remove residual erythrocytes. This platelet-rich plasma was centrifuged twice more at 350 ×g with a washing buffer for 10 min to remove the ACD solution, and then platelet precipitates were adjusted to (3 × 10^5/mL) for aggregation assay in Tyrode buffer (137 mM of NaCl, 12 mM of NaHCO3, 5.5 mM of glucose, 2 mM of KCl, 1 mM of MgCl2, 0.3 mM of NaHPO4, and pH 7.4). The washed platelets were preincubated at 37°C for 2 min with RM504-C or vehicle (<0.1%) and then stimulated with collagen. Collagen is a well-known agonist for platelet aggregation and thrombus formation. The reaction mixture was further incubated for 5 min with stirring at 170 ×g. Aggregation was monitored by measuring light transmission in an aggregometer (Chronologic, Haverton, PA, USA).

**Nitric oxide inhibition assay**

Overproduction of NO in macrophages is a hallmark of inflammation. Therefore, we determined the effect of RM504-C (6.25–100 μg/mL) in LPS (0.1 μg/mL) stimulated RAW264.7 cells. RAW264.7 (Korean Cell Line Bank, South Korea) were cultured and maintained in DMEM (Dalseogu, South Korea) enriched with 10% heat-inactivated FBS (WelGene Co., South Korea), 100 μg/mL streptomycin, and 100 U/mL penicillin (Lonza, MD, USA) in a humidified atmosphere of 5% CO2 at 37°C. Cultured RAW264.7 cells (4×10^5) were pretreated with or without SCME (1,3,10μg/mL) for 30 min and then stimulated with lipopolysaccharide (LPS, 0.1 μg/mL) for 18 h in a 96 well plate. The cell culture supernatant (100 μL) was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid (H3PO4) and 0.1% N-1-naphthylenediamine dihydrochloride in deionized distilled water) and then subjected to plate reading at 540 nm in an ELISA reader. The accumulated nitrite in the culture medium was quantified using a standard of NaNO2 (1 mM).

**1,1-Diphenyl-2-picrylhydrazyl assay**

The stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to examine the free radical scavenging activity of Rosemary (RM504-C) extract samples. In brief, a 0.2 mM solution of DPPH was prepared in DMSO, which was mixed with each extract sample at concentrations of 1, 5, 10, 20, and 40 μg/mL. After a 30 min incubation in the dark, absorbance was measured at 517 nm with a spectrophotometer. A decrease in solution absorbance indicates a decrease of DPPH and an anti-oxidant effect. The anti-oxidant activity is expressed as a percent inhibition.

**RESULTS**

**Effect of Annona squamosa extract (RM606) on lipid accumulation**

Dose correlated reduction in cellular lipid accumulation was observed when 3T3 cells were treated with RM606 at 0.0625 μg/mL to 1 μg/mL concentration. Compared to control, percent inhibition of 32.1%, 45.6%, and 68.8% were observed for RM606 at 0.35, 0.5, and 1 μg/mL, respectively [Figure 1] with an IC50 value of 0.489 μg/mL. The positive control, TNF-α showed 77.1% inhibition compared to control.

**Effect of Zanthoxylum clava-herculis (UP342-C) on microcirculation improvement**

The edema developed linearly until 7-8 h after application of croton oil and the severity of the edema was sustained for more than 24 h. Vehicle alone did cause significant swelling of the recto-anus as compared with the rats without the extract [Figure 2]. The average RAC of vehicle-treated croton oil-induced rats was 1.66 which was 40.7% higher than that of the normal control (RAC = 1.18) [Figure 2]. However, treatment with UP342-C extract reduced the swelling of the recto-anus by 79.5%, 67.5%, and 42.1%, for dosages 6 mg/kg, 2 mg/kg, and 0.67 mg/kg, respectively [Figure 2]. The same preparation was tested at 6 mg/kg for topical application and found a 93.1% RAC inhibition with statistical significance when compared to vehicle-treated croton oil-induced rats with P = 0.02 (USP NO. 6210680B1) indicating its effect in microcirculation.
Anti-platelet aggregation effects of *Rosmarinus officinalis* (RM504-C)

CA enriched rosemary extract (RM404-C) showed a dose-dependent inhibition of collagen-induced platelet aggregation in rat platelets [Figure 3]. The highest inhibition, 82%, in platelet aggregation was observed when RM404-C was used at 31.25 μg/ml concentration.

Nitric oxide inhibition effects of rosemary extract (RM504-C)

Nitric oxide (NO) overproduction can serve as proinflammatory mediator at pathologic conditions to induce and/or exacerbate inflammation. Data in Figure 4 showed that RM504-C was a potent inhibitor of NO production suggesting its anti-inflammatory activity. Dose-dependent inhibition of NO was observed in this assay. At concentrations as low as 6.2 μg/ml, a 71.8% inhibition in production of NO was observed. Complete inhibition in NO production was noted for concentrations above 12.5 μg/ml. In the unstimulated control group, no production of NO was observed. Controls are unstimulated cells expected to have minimal to no production of nitric oxide. Cells were treated with RM504-C at 6.25–100 μg/ml concentrations. Data expressed as mean ± standard deviation of triplicates.
38.4%, 85.2%, and 91.8% were observed for RM504-C at 1, 5, 10, 20, and 40 μg/ml, respectively [Figure 5].

**DISCUSSION**

The present study depicts preclinical data from cellular lipid accumulation, microcirculation improvement, anti-platelet aggregation, NO inhibition, and DPPH assays for three well-known medicinal plant extracts such as *R. officinalis*, *A. squamosa* and *Z. clava-herculis*. The significance of these findings in association with cellulite have been discussed.

Cellulite is a nonpathologic skin condition mainly known for its negative impacts on quality of life with no mortality or morbidity. However, it takes a serious psychological toll on affected women. Blood and lymphatic microcirculation damage are believed to give rise to structural changes in the fatty layer and surrounding collagen matrix at cellulite pathophysiology. Subsequently, subcutaneous edema could also appear as a sequel as a result of superficial microcirculation insufficiency due to altered permeability of blood vessels. Cellulite management ranges from topical cream applications to invasive procedures, including laser-assisted lipolysis and liposuction. Despite the significant progress in the field of combating cellulite, currently, there are no treatment options successfully curing the underline causes. Among the various predisposing factors that influence cellulite, both microvascular and lymphatic circulation impairment are considered the primary etiological factors. As a result, significant numbers of products are commercially available in various formulations targeting microcirculation. For example, *C. asiatica* extracts for microcirculation and an anti-inflammation,[32] *R. aculeatus* for improving lymphatic drainage,[33] *Carica papaya* as anti-edema,[34] and Common ivy (*Hedera helix*) for lymphatic drainage[35] are some of the topically applicable botanical extracts with limited success in treating cellulite. These materials believed to impose their desired outcome by reducing blood viscosity, inhibiting platelet aggregation, moderating vascular permeability, and improving vascular tone, which may lead to improved microcirculation. Aligning with these proposed underlying mechanisms, in the present study, significant inhibition in platelet aggregation (as a result of *R. officinalis*) and improved microcirculation by *Zanthoxylum clava-herculis* were observed for constituents of the composition suggesting their beneficial usage for improved microcirculation. Recently, it has also been shown that the major bioactive component of *Zanthoxylum* extract, Magnoflorine, to have a dual target effect in NF-κB inhibition and β2-adrenoceptor agonist activities both of which have a primary contribution in the pathophysiology of cellulite.[32] While the former activity modulates the inflammatory pathway, the later may lead to smooth muscle relaxation and hence vasodilation which could have a measurable impact in improving microcirculation rendering their application in cellulite management.

Asserting the multifactorial causative agents of cellulite, adipogenesis, and lipolysis play a major role at the cellulite-prone areas in the pathophysiology of cellulite. Physiologic and biochemical properties of adipose tissue in these areas have been shown to be different from subcutaneous adipose tissue elsewhere in both hormonal responsiveness and metabolic activities. Among the few neurotransmitters that affect the acute lipolysis of adipose tissues (e.g., catecholamine) work through the activation of the beta-adrenergic receptor.[32,33] Stimulation of these receptors as an agonist has a positive effect on lipolysis. For instance, β-agonists such as methylxanthines (e.g., caffeine and aminophylline) preparation are widely available on the market for topical use for cellulite believed to impose their activity through acting directly on adipose cells, promoting lipolysis, inhibiting phosphodiesterase, increasing levels of cyclic adenosine monophosphate and/or activating the triglyceride lipase enzyme and breaks down triglycerides into free acids and glycerol.[24,34] Here again, the β1-adrenoceptor agonist alkaloid extract from *Zanthoxylum* (e.g., magnoflorine) may share similar mechanisms in promoting lipolysis situating the extract with additional benefit in cellulite intervention. Most importantly, in our study, extract from *A. squamosa* showed a direct impact on adipogenesis as demonstrated by the significant reduction in lipid accumulation in 3T3 cells. In fact, during our discovery process, we screened more than 5300 plants extracts using the fat cells isolated from the human adipose tissues and identified acetogenins as the primary actives from *A. squamosa* with an IC50 of 1 ng/ml in inhibition of adipogenesis. Substantiating our findings, Gokaraju et al. have reported the effect of the major active component of *Squamosa* extract, acetogenins, in down regulating adipogenesis differentiating markers and perilipin protein revealing its inhibiting adipogenesis and/or accelerating lipolysis activities reinforcing its usage in cellulite modulation.[36]

There are reasonable assumptions for the presence of inflammatory processes and hence free radical generation or vice versa at the time of deposition of dystrophic adipose tissues during the pathogenesis of cellulite.[27] For example, abnormal changes in the endothelium and edema formation are the two possible pathological signals suggestive of the existence of these phenomena. Moreover, the isolation of macrophages and monocytes from the biopsies of fibrous septae of patients with cellulite were further considered as an indication of involvement of chronic low-grade inflammation in the pathophysiology of cellulite.[28] In these regards, extracts from all the three components of the composition, *Rosmarinus officinalis*, *A. squamosa*, and *Zanthoxylum americanum*[29] have been previously reported to possess anti-inflammatory activities that strengthen their usage in cellulite intervention. In particular, the free radical scavenging and inhibition of NO activities of *Rosmarinus officinalis* have an extract that have been demonstrated in our assays attest its strong anti-oxidant and anti-inflammatory activities. These findings further fortify the argument that each component of the composition has a significant share in addressing one or multiple pathways among the suggested possible mechanisms of action in cellulite pathology. Some topical formulations composed of *G. biloba* as anti-inflammatory and anti-oxidant,[1] Red grapes (*Vitis vinifera*) as an antioxidant,[30] and *Carica papaya* as anti-inflammatory[31] are examples of plant extracts with a similar mechanism of actions indicated for cellulite.
The current study is not without a limitation. While statistically significant results were reported in all the disclosed assays for each extract tested, for some, no positive controls were used. As a result, the relative potency of the extracts cannot be determined against known standard references.

CONCLUSIONS

Collectively, botanical formulations applied topically as anti-cellulite intend to reduce lipogenesis, activate lipolysis, restore subcutaneous tissue normal structure, and reduce free radical generation or scavenge free radicals, prevent inflammation, increase microcirculation, and modulate actomyosin and actin cytoskeleton, thus increasing microcirculation. Hence, we suggest further mechanism based elucidation of their composition for cellulite management and validation of hypothesis in human clinical study.

Acknowledgments

The authors would like to express their best gratitude to Dr. Eujin Hyun, Dr. Edward, Dr. Wenwen Ma, and Dr. Min Chu, and Unigen team for their incalculable support for the completion of this research.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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