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Ocimum sanctum Linn. as a natural source of skin anti-ageing compounds

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ABSTRACT

Ocimum sanctum Linn., widely used in food preparation, has been reported for several biological beneficial activities and thus potential for treatment of various health conditions. However, the biological activities related to skin anti-ageing, including anti-collagenase, anti-elastase, and anti-hyaluronidase activities, have not been reported so far. Therefore, this study aimed to investigate the skin anti-ageing activity of *O. sanctum* extracts. The aerial part of *O. sanctum* was extracted by fractionated extraction using *n*-hexane, ethyl acetate, and ethanol, respectively. Rosmarinic acid and total phenolic content was investigated. Antioxidant activity was determined by *in vitro* methods. Anti-inflammatory activity was investigated according to the inhibition of nuclear factor kappa B (NF-κB) expression and interleukin-6 (IL-6) secretion. The anti-ageing activity was investigated by *in vitro* inhibition of collagenase, elastase, and hyaluronidase activities. The ethanolic extract of *O. sanctum*, which had the highest yield (6.5%), contained the highest rosmarinic acid (19.3% w/w) and the highest total phenolic content (50.2 ± 0.6 mg gallic acid/g sample). Additionally, it possessed the most potent antioxidant activity with the Trolox equivalent antioxidant capacity of 270.1 ± 15.1 μM/mg, equivalent concentration of 459.3 ± 91.4 μM/mg, and inhibition against 2, 2-diphenyl-1-picrylhydrazyl radical of 34.0 ± 0.7%. The ethanolic extract also showed the highest anti-inflammatory activity with the inhibition against IL-6 secretion and NF-κB expression of 54.7 ± 3.1% and 79.3 ± 9.6%, respectively. Moreover, it also exerted the highest inhibition against matrix metallopeptidase-1 and hyaluronidase with the inhibition of 77.7 ± 9.0% and 98.1 ± 0.1%, respectively. Rosmarinic acid was found as the major compound responsible for those anti-ageing activities. Therefore, the ethanolic extract of *O. sanctum* is an attractive natural source of anti-skin ageing ingredient for further applications in cosmetic and/or cosmeceutical industry.

1. Introduction

There are various factors, both intrinsic and extrinsic, affected skin ageing. Consequently, the results would be a deterioration of dermal extracellular matrix, including collagen, elastin, hyaluronic acid, etc. and finally lead to sagging skin and wrinkles (Farage et al., 2008). As the proportion of ageing population continues to increase (Duault et al., 2018), the dermatological concerns of the aged grow in medical importance. There are several ways to maintain the skin youthfulness, including iontophoresis, laser, derma roller, mesotherapy injection, botox injection, etc. However, these methods could cause skin injury since they are invasive methods. The topical formulations are more expansively used because of the lower price, ease of application, and no injury comparing to a medical device treatment. Additionally, natural compounds are also popular for using as an active ingredient in the topical formulations since they have various desirable biological activities and are safe for using on the skin.

Ocimum sanctum Linn., commonly known as “Queen of herb”

Abbreviations: NF-κB, nuclear factor kappa B; IL-6, interleukin 6

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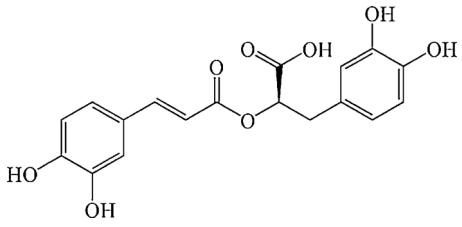

Fig. 1. Chemical structure of rosmarinic acid.

(Sharma et al., 2016), has been used in various food recipes in many countries, especially in Asia. Additionally, it has been reported for several biological beneficial activities and thus potential for treatment of bronchitis, bronchial asthma, malaria, diarrhea, dysentery, skin diseases, arthritis, painful eye diseases, chronic fever, insect bite, etc. (Prakash and Gupta, 2005). The major constituents of *O. sanctum*, including eugenol, ursolic acid, linalool, and rosmarinic acid (Fig. 1), have been reported to exert anti-inflammatory and antioxidant activities (Kelm et al., 2000). Therefore, *O. sanctum* would be an attractive plant for cosmetic product developments. However, the biological activities related to skin ageing, including anti-collagenase, anti-elastase, and anti-hyaluronidase activities, have not been reported before. Therefore, this study would be the first report of the anti-ageing activity of *O. sanctum* extracts. Moreover, this study also investigated the content of rosmarinic acid in *O. sanctum* extracts and determined the inhibitory activities against oxidation, inflammation, collagenase, elastase, and hyaluronidase of rosmarinic acid and *O. sanctum* extracts.

2. Materials and methods

2.1. Plant materials

The aerial parts of *O. sanctum* were purchased from local market in Chiang Mai, Thailand. The plants were authenticated and herbarium specimen number 023230 was deposited at the official Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand.

The plants were thoroughly washed with tap water and dried in the oven at 50 °C overnight (~12 h). The dried plant materials were then grounded into fine powder by using a Moulinex® blender, model number LM2070BD (Moulinex SA, Bagnolet, France) and kept at room temperature until further use.

2.2. Chemical materials

Rosmarinic acid, Trolox, indomethacin, dexamethasone, gallic acid, Folin–Ciocalteu reagent, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ammonium thiocyanate (NH_4SCN), collagenase from *Clostridium histolyticum* (ChC – EC.3.4.23.3), *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA), elastase from porcine pancreatic (PE–E.C.3.4.21.36), N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (AAAPVN), hyaluronidase from bovine testis (E.C. 3.2.1.3.5), sodium chloride (NaCl), calcium chloride ($CaCl_2$), ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$), 2,4,6 tripyridyl-s-triazine (TPTZ), Folin-Lowry's reagent, bovine serum albumin (BSA), polysorbate 20 (Tween 20), octylphenol ethoxylate (Triton X-100), glycerol, alcian blue 8GX, tricine, gelatin, hyaluronic acid, sodium phosphate, and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco modified eagle medium (DMEM), RPMI-1640, penicillin/streptomycin, L-glutamine, secondary antibody conjugated with HRP, and trypan blue were purchased from Invitrogen™ (Grand Island, NY, USA). Fetal bovine serum (FBS), newborn calf serum, and antibiotic-antimycotic 100X solution was purchased from GIBCO (Grand Island, NY, USA). NP-40 was purchased from Shell Chemical Co. (NY, USA). Sodium dodecyl sulfate (SDS), polyvinylidine fluoride (PVDF) transfer membrane, Coomassie blue and protein markers were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Rabbit polyclonal anti-NF-κB IgG, p105/p50 (Phospho-Ser337) was purchased from OriGene Inc. (Rockville, MD, USA). Rabbit polyclonal anti-GAPDH IgG and lipopolysaccharide (LPS) were purchased from Cell Signaling Technology® (Boston, MA). Protease inhibitors was purchased from Amresco® (Solon, OH, USA). SuperSignal® West Pico Chemiluminescent was purchased from Pierce (Rockford, IL, USA). Acetic acid, formic acid, hydrochloric acid, sodium deoxycholate, and Tris base were purchased from Fisher Chem Alert (Fair Lawn, NJ, USA). HPLC grade acetonitrile and bromophenol blue were purchased from Merck (Darmstadt, Germany). Ethanol, ethyl acetate, methanol, *n*-hexane, and dimethyl sulfoxide (DMSO) were purchased from Labscan (Dublin, Ireland).

2.3. Preparation of plant extracts

The dried powder of *O. sanctum* was macerated in *n*-hexane for 24 h (3 cycles) with the assists of heat (50 °C) and agitation by aluminum hot plate stirrer (Velp Scientific Inc., Milano, Italy). The weight proportion of dried plant material and solvent was 1:5. The pooled solvent from 3 extractions was filtered through no.1 Whatman filter paper (Merck KGaA, Darmstadt, Germany) and then removed under vacuum using rotary evaporator (Eyela, Tokyo, Japan) until dryness at 50 °C. The plant residue was macerated again in ethyl acetate (24 h, 3 cycles) and ethanol (24 h, 3 cycles), respectively. Three fractionated extracts, including *n*-hexane extract, ethyl acetate extract, and ethanolic extract were obtained. All extracts were kept in the refrigerator until further use.

2.4. Determination of rosmarinic acid content by high performance liquid chromatography (HPLC)

HPLC analyses were performed using an HP1100 system with a thermostatically controlled column oven and a UV detector set at 330 nm (Hewlett Packard, Palo Alto, CA, USA). A reversed phase column, Luna 5 u C18(2) (250 mm × 2.0 mm id, 5 µm) (Phenomenex, Torrance, CA, USA), was connected with a guard column (4.0 mm × 3.0 mm id, 5 µm) (Phenomenex, Torrance, CA, USA). A gradient mobile phase system consisting of 0.5% formic acid (phase A) and acetonitrile (phase B) at a flow rate of 1.0 mL/min and 20 µL of sample was injected. The gradient elution program was: 90% A (0–1 min), 75% A (2–12 min), 0% A (13–17 min), and 90% A (18–20 min). Samples and mobile phases were filtrated through a 0.45 mm Millipore filter, type GV (Millipore, Bedford, MA) prior to the HPLC injection.

Prior to the determination of rosmarinic acid content, the HPLC method has been validated by means of limit of detection (LOD), limit of quantification (LOQ), linearity, and percent recovery. The signal-to-noise ratio (S/N) of three and ten, which are generally accepted for determining LOD and LOQ, were 15.6 ng and 31.2 ng, respectively. The linear regression equations for rosmarinic acid indicated good correlation in the linearity range of 1.56–100 µg/mL (r^2 = 0.995). The percent recovery was found to be between 97.7–100.6% indicating that the HPLC method was reliable for the determination of rosmarinic acid content.

2.5. Determination of the total phenolic content by Folin–Ciocalteu Method

Total phenolic contents of *O. sanctum* extracts were determined by Folin–Ciocalteu method that has been described in the previous studies (Chaiyana et al., 2017) which was modified from the method of Li et al. (2007). Total phenolic content was expressed as mg/g gallic acid equivalents (GAE), calculated according to the standard curve constructed by using various concentration of gallic acid. All experiments were done in triplicate.

2.6. Antioxidant activity determination

2.6.1. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The scavenging activity against ABTS radical cations ($ABTS^{\cdot+}$) of rosmarinic acid and each *O. sanctum* extracts was determined by using ABTS assay according to a method that has been described in the previous studies (Chaiyana et al., 2017) which was modified from the method of Pellegrini et al. (2003). Trolox equivalent antioxidant capacity (TEAC) was calculated according to the standard curve constructed by using $ABTS^{\cdot+}$ scavenging activity of various concentrations of Trolox. All experiments were done in triplicate.

2.6.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The scavenging activity against DPPH radicals (DPPH ˙) of rosmarinic acid and each *O. sanctum* extracts was determined by using DPPH assay according to a method that has been described in the previous studies (Chaiyana et al., 2017b) which was modified from the method of Blois (1958). The scavenging effect of each samples was calculated by using the following equation; % scavenging effect = $\{[(a\text{-}b)\text{-}(c\text{-}d)]/(c\text{-}d)\} \times 100$, when a is an absorbance of $DPPH^{\cdot}$ solution without sample, b is an absorbance of solvent, c is an absorbance of $DPPH^{\cdot}$ solution with sample, and d is an absorbance of sample solution without $DPPH^{\cdot}$. All experiments were done in triplicate.

2.6.3. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power of rosmarinic acid and each *O. sanctum* extracts was determined by FRAP assay according to a method that has been described in the previous studies (Chaiyana et al., 2017b) which was modified from the method of Saeio et al. (2011). The results were expressed as EC_1, which were the concentration of the sample that was equivalent to that of 1 mM $FeSO_4$. All experiments were done in triplicate.

2.7. Anti-inflammatory activity determination

2.7.1. Determination of NF-κB level by Western blot analysis

2.7.1.1. Human leukemic monocyte lymphoma U937 cell culture and condition. U937 cells, grown in RPMI-1640 culture medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 IU/mL penicillin, were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.7.1.2. Western blot analysis. Western blot analysis was used to determine the effect of *O. sanctum* extracts on the level of transcription factor NF-κB in U937 cells. The experiments were done according to the previous study with slight modifications (Chaiyana et al., 2017a). Indomethacin, a well-known nonsteroidal anti-inflammatory drug (NSAID), was used as a positive control. All experiments were done in triplicate.

2.7.2. Determination of IL-6 secretion

2.7.2.1. Mouse monocyte macrophage RAW 264.7 cell culture and condition. RAW 264.7 cells (American Type Culture Collection, ATCC-TIB-71) stimulated with LPS were used to examine the effect of *O. sanctum* extracts on the secretion of IL-6. The cell culture was performed according to a method described previously by Mueller et al. (2010). Cells which were not treated with LPS served as a negative control, whereas, the cells treated with LPS served as a positive control.

2.7.2.2. Enzyme-linked immunosorbent assay (ELISA). The IL-6 secretion in aliquoted supernatants was determined by ELISA according to the manufacturer's protocol (R&D Systems).All incubation steps were performed at room temperature. The optical density at 450 nm, corrected by the reference wavelength 570 nm, was measured with a Genios Pro microplate reader (Tecan, Crailsheim, Germany).

2.7.2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. The viability of LPS-stimulated RAW 264.7 cells was investigated by an MTT assay. After transferring the supernatant for ELISA analysis, MTT dye solution was added and the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 2 h. The supernatant was then removed and the cells were lysed by DMSO. The optical density at 570 nm, corrected by the reference wavelength 690 nm, was measured using a Genios Pro microplate reader (Tecan, Crailsheim, Germany).

2.7.2.4. Calculation of IL-6 secretion. The calculated concentrations of cytokines were normalized to MTT values to reduce any variation from differences in cell density (Mueller et al., 2010). Secreted IL-6 from the Raw 264.7 cells were treated with only LPS served as positive control and were defined as 100%. The results of *O. sanctum* extracts were then calculated as a percent of this value. Additionally, %inhibition was calculated by subtracting IL-6 secretion from 100%. Dexamethasone was used as a positive control for IL-6 secretory inhibition. Three independent experiments were performed on individual days.

2.8. Anti-ageing activity determination

2.8.1. Collagenase activity determination by spectrophotometric method

The collagenase inhibitory activity of *O. sanctum* extracts was determined by spectrophotometric methods according to the previous study of Thring et al. (2009) with some modifications. Percentage of inhibitory activity against the enzyme was calculated using the following equation; % inhibition = $(1—a/b) \times 100$, when a is an absorbance of the mixture with *O. sanctum* extracts and b is an absorbance of the mixture without *O. sanctum* extracts.

2.8.2. Metalloproteinases activity determination by gel electrophoresis

2.8.2.1. Albino Swiss mouse embryo fibroblasts 3T3 cell culture and condition. The 3T3 cells were used to examine the effect of *O. sanctum* extracts on MMP-2 and MMP-9 activity. The cells were grown in DMEM culture medium supplemented with 10% (v/v) fetal calf serum, 1% antibiotic-antimycotic 100× solution, and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. All culture medium in the culture flask (2 mL) were replaced with new culture medium every 24 h. The culture medium collected during day 7 to day 10 were kept in the freezer (−20 °C) for the further determination of MMP-2 and MMP-9 expressions.

2.8.2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The expression levels of MMP-2 and MMP-9 were carried out by using SDS-PAGE. The culture medium from 3T3 cells that were treated with *O. sanctum* extracts at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h was collected. An equal volume of sample buffer, composing of 0.025% (w/v) bromophenol blue, 4% (v/v) SDS, 25% (v/v) of 0.5 M Tris−HCl pH 6.8, and 20% (v/v) glycerol in distilled water, was added and the sample mixture was then loaded into each well of SDS-polyacrylamide gel containing 0.1% (w/v) gelatin. Consequently, voltage was applied directly to the Tris/glycine/SDS running buffer. After the separation, SDS-polyacrylamide gel was removed and soaked in reaction buffer, composed of 0.1 M Tris−HCl pH 8.0, 5% (v/v) Triton X-100, and 200 mM NaCl in DI water, for 1 h at 37 °C. After that, 1.0 mL of 1.0 M $CaCl_2$ was added to the reaction buffer and incubated at the same condition for another 23 h. The gel was then washed with DI water and incubated in fixing buffer, composed of 50% (v/v) methanol and 12% (v/v) acetic acid in DI water, for 30 min at 37 °C. After that, the gel was washed with DI water and stained by using staining buffer, composed of 0.025 g of Coomassie blue, 40% (v/v) methanol, and 7% (v/v) acetic acid in DI water, for 1 h. Finally, the gel was destained by using destaining buffer, composing of 40% (v/v) methanol and 7.0% (v/v) acetic acid in DI water, until the protein markers were clearly appeared. The expression of MMP-2 and MMP-9

was then calculated by using ImageJ 1.51J8 program (Wayne rasband, NIH, USA).

2.8.3. Elastase inhibitory activity determination by spectrophotometric method

The elastase inhibitory activity of *O. sanctum* extracts was determined by spectrophotometric methods according to the previous study of Thring et al. (2009) with some modifications. Percentage of inhibitory activity against the enzyme was calculated using the following equation; %inhibition = (1—a/b)×100, when a is an absorbance of the mixture with *O. sanctum* extracts and b is an absorbance of the mixture without *O. sanctum* extracts.

2.8.4. Hyaluronidase activity determination by gel electrophoresis

The expression of hyaluronidase level was carried out by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 0.1 g of hyaluronidase from bovine testis (E.C. 3.2.1.3.5) dissolved in 20% (v/v) of 0.15 M NaCl, was incubated with or without *O. sanctum* extracts for 48 h at 37 °C. An equal volume of sample buffer, composed of 0.025%w/v bromophenol blue, 4% (v/v) SDS, 25% (v/v) of 0.5 M Tris−HCl, pH 6.8, and 20% (v/v) glycerol in distilled water, was added. The sample mixture was then loaded into each well of SDS-polyacrylamide gel containing 0.17% (w/v) hyaluronic acid. Consequently, the voltage was applied directly to the Tris/glycine/SDS running buffer to separate the hyaluronidase. After the separation, SDS-polyacrylamide gel was removed and soaked with the washing buffer, composing of 50 mM Tris−HCl, pH 8.0, 2.5% (v/v) Triton X-100, and 100 mM NaCl in DI water, for 1 h at 37 °C. After that, the gel was then incubated in reaction buffer, composing of 10% (v/v) of 0.2 M acetate buffer, pH 5.0 and 90% (v/v) of 0.15 M NaCl, at 37 °C with continuously shaking at 60 rpm for 16 h. The gel was then washed with DI water and stained by using staining buffer, composed of 0.5% (w/v) alcian blue 8GX and 3% (v/v) acetic acid in DI water, for 1 h. Finally, the gel was de-stained by using de-staining buffer, composed of 50% (v/v) methanol and 1.0% (v/v) acetic acid in DI water, until the protein markers clearly appeared. The expression of hyaluronidase was then calculated by using ImageJ 1.51J8 program (Wayne rasband, NIH, USA).

2.9. Statistical analysis

All data were presented as a mean ± standard deviation (SD). Statistical significance was assessed by the one-way analysis of variance (ANOVA) followed by post-hoc tests using the SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The probability values of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered significant.

3. Results and discussion

3.1. O. sanctum extracts

All *O. sanctum* extracts were greenish semisolid mass. Ethanolic extract showed the highest yield (6.5%), followed by ethyl acetate extract (2.1%) and *n*-hexane extract (2.0%), respectively.

3.2. Rosmarinic acid content of O. sanctum extracts

The HPLC chromatograms of *O. sanctum* extracts are shown in Fig. 2. There were several peaks detected in ethanolic extract and ethyl acetate extract, whereas, no peak could be detected in *n*-hexane extract. The likely explanation that rosmarinic acid could not be extracted by *n*-hexane was supported by the hydrophilicity of rosmarinic acid (Huang et al., 1997). The partition coefficient of rosmarinic acid was very low (log P = 0.71, 20°C, pH 5.71) when *n*-hexane was used as an organic phase (Boyadzhiev and Dimitrova, 2006).

The major peak of ethanolic extract and ethyl acetate extract,

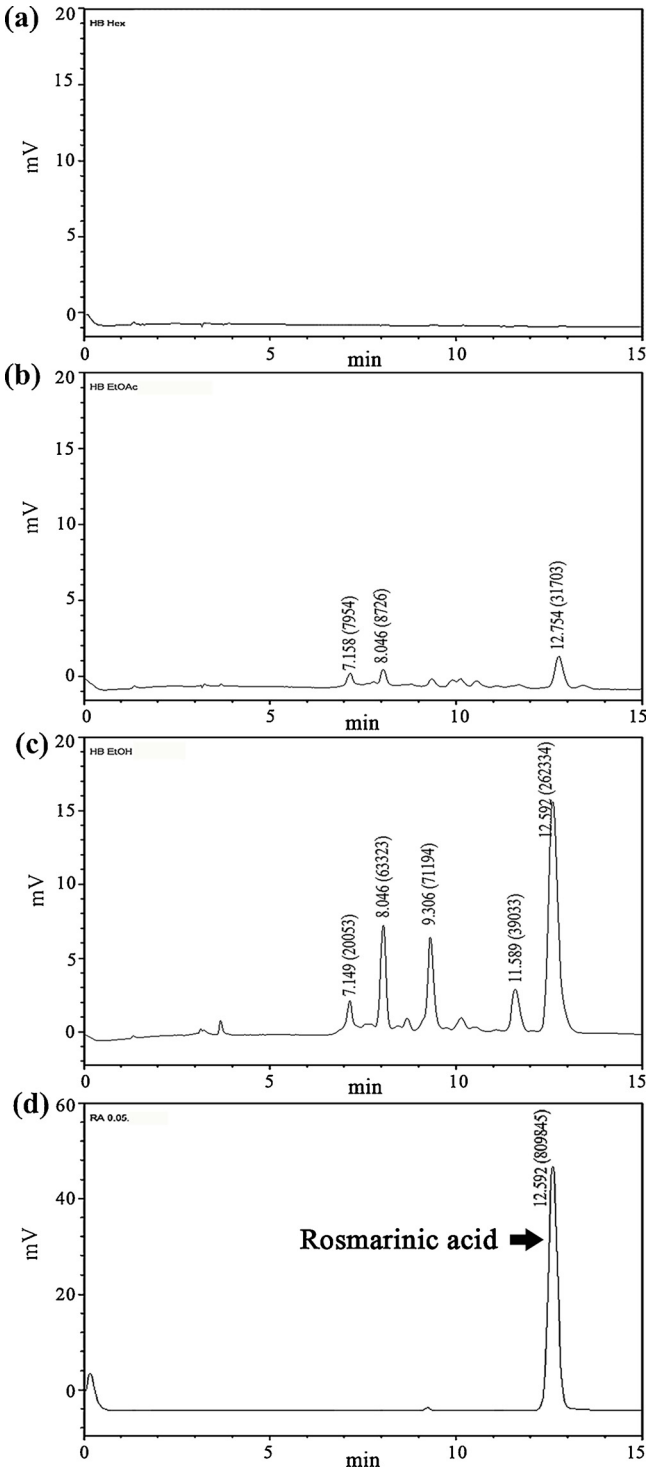

Fig. 2. HPLC chromatogram of (A) *n*-hexane extract, (B) ethyl acetate extract, (C) ethanolic extract, and (D) rosmarinic acid.

detected at the retention time of 12.592 and 12.754 min, corresponded well to that of rosmarinic acid (12.592 min). Therefore, rosmarinic acid was noted as the major constituent of ethanolic extract and ethyl acetate extract. The results also revealed that rosmarinic acid content were 19.3% (w/w) and 6.9% (w/w) in ethanolic extract and ethyl acetate extract, respectively. They were in a good agreement with the previous study which reported that rosmarinic acid was the predominant phenolic acid presented in *O. sanctum* (Sundaram et al., 2012). Generally, rosmarinic acid is a compound found mainly in the

Table 1
Total phenolic content and antioxidant activity of *O. sanctum* extracts compared with rosmarinic acid.

Sample	Total phenolic content (mg GAE/g)	Antioxidant activity		
		ABTS assay (TEAC:μM/mg)	DPPH assay[a] (%inhibition)	FRAP assay (EC_1:μM/mg)
n-Hexane extract	9.6 ± 3.3	111.9 ± 14.6	1.9 ± 1.8	99.4 ± 62.9
Ethyl acetate extract	21.5 ± 1.0	91.0 ± 12.1	4.7 ± 2.2	97.3 ± 41.3
Ethanolic extract	50.2 ± 0.6	270.1 ± 15.1	34.0 ± 0.7	459.3 ± 91.4
Rosmarinic acid	ND	1,638.9 ± 22.3	93.6 ± 4.8	1,774.3 ± 5.3

ABTS = 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH = 2,2-diphenyl-1-picrylhydrazyl, FRAP = ferric reducing antioxidant power, GAE = gallic acid equivalents, TEAC = Trolox equivalent antioxidant capacity, EC_1 = equivalent concentration, ND = not determined.
[a] The final concentration of *O. sanctum* extract and rosmarinic acid in DPPH assay was 0.1 mg/mL.

family of Lamiaceae, such as rosemary, sage, oregano, summer savory, basil, etc. (Zheng and Wang, 2001). The present study revealed that rosmarinic acid was also a main component in ethanolic and ethyl acetate extract of *O. sanctum*. Therefore, rosmarinic acid is suggested for use as a biological marker for the quantitative determination of *O. sanctum* extracts and could be employed in the standardization of herbal preparations containing *O. sanctum*.

3.3. Total phenolic content of O. sanctum extracts

Ethanolic extract showed the highest total phenolic content, followed by ethyl acetate extract and *n*-hexane extract, respectively (Table 1). The results were corresponding well to the rosmarinic acid content since ethanolic extract which contained the highest rosmarinic acid also showed the highest total phenolic content. Therefore, rosmarinic acid was found to be a major phenolic compound in *O. sanctum*, especially ethanolic extract and ethyl acetate extract. Rosmarinic acid is a phenolic compound with log *P* value of 0.71 (Boyadzhiev and Dimitrova, 2006; Fadel et al., 2011), therefore, it could be dissolved well in semi-polar solvents and efficiently extracted by using ethanol. This would be the reason why there was no rosmarinic acid in *n*-hexane extract which was extracted by non-polar solvent. But there should be more types of phenolic compounds other than rosmarinic acid in *n*-hexane extract, such as propanoic acid, apigenin, cirsimaritin, isothymusin, isothymonin, carnosic acid etc. that led to the detection of phenolic compounds in *n*-hexane extract (Kosar et al., 2003).

3.4. Antioxidant activity of O. sanctum extracts

Ethanolic extract possessed the highest antioxidant activity in all assays, including ABTS, DPPH, and FRAP (Table 1). Therefore, the mechanisms of ethanolic extract on oxidation inhibition were various. ABTS and DPPH were associated with electron-transfer reaction, whereas, FRAP was associated with the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Somwongin et al., 2018). The highest antioxidant activity of ethanolic extract correlated well with the total phenolic and rosmarinic acid content. Therefore, phenolic compounds, especially rosmarinic acid, played an important role in the antioxidant activity of *O. sanctum* extracts. The results were in a good accordance with a previous study that reported that rosmarinic acid was identified as the dominant radical scavengers in the extracts of Lamiaceae plants (Kosar et al., 2003). Moreover, rosmarinic acid were also the most potent antioxidant among various hydroxycinnamic acids (a class of phenolic compounds) due to the presence of two catechol structures that conjugated with a carboxylic acid group (Sánchez-Campillo et al., 2009).

3.5. Anti-inflammatory activity of O. sanctum extracts

The inhibitory activity against IL-6 secretion are shown in Fig. 3. Dexamethasone, a well-known anti-inflammatory agent, could suppress the IL-6 secretion with the inhibition of 34.4 ± 4.8%. Among *O. sanctum* extracts, ethanolic extract was the most potent against IL-6 secretion with the IC_{50} value of 81.3 ± 35.5 ng/mL, followed by *n*-hexane extract (143.5 ± 18.3 ng/mL) and ethyl acetate extract (311.8 ± 164.5 ng/mL), respectively. Although rosmarinic acid has been reported for the significant diminishing of IL-6 release both *in vitro* and *in vivo* (Vostálová et al., 2010; Jiang et al., 2009), the inhibitory activity of *O. sanctum* extracts was superior. The likely explanation might be due to other components which also possessed the inhibitory activity against IL-6 secretion, such as apigenin, cirsimaritin, carnosic acid, etc. (Wang et al., 2012; Shin et al., 2017). Moreover, the synergistic effect from various components, such as rosmarinic acid and phenolic compounds, might be another reason for the higher anti-inflammatory effect of *O. sanctum* extracts.

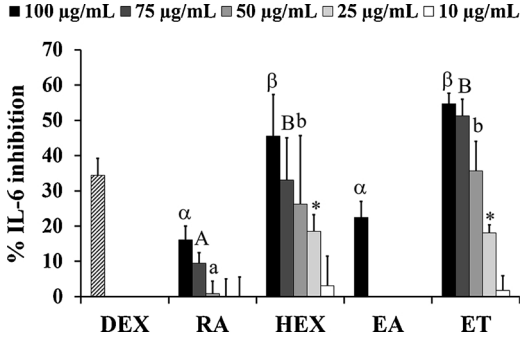

Fig. 3. Effect of dexamethasone (DEX), *n*-hexane extract (HEX), ethyl acetate extract (EA), ethanolic extract (ET), and rosmarinic acid (RA) on IL-6 inhibitory activity in Raw 264.7 cells. Raw 264.7 cells were treated with 10 μM DEX, HEX, EA, ET, and RA for 2 h. IL-6 secretion was determined by ELISA. LPS was served as positive control (100%). DEX was used as a positive control for IL-6 inhibitory secretion. α denotes significant difference from β ($P < 0.05$) among different extracts at the concentration of 100 μg/mL (■). A denotes significant difference from B ($P < 0.05$) among different extracts at the concentration of 75 μg/mL (■). a denotes significant difference from b ($P < 0.05$) among different extracts at the concentration of 50 μg/mL (■). Asterisk (*) denotes significant difference among different extracts at the concentration of 25 μg/mL (■) ($P < 0.05$). Data are the means ± SD of three independent experiments.

On the other hand, ethanolic extract and ethyl acetate extract possessed significant inhibition against NF-κB secretion with the inhibition of 20.7 ± 9.6% and 21.0 ± 6.1%, respectively ($P < 0.05$) (Fig. 4). However, *n*-hexane extract showed no effect on NF-κB levels. Indomethacin, a positive control, was identified as a potential compound that could suppress NF-κB expression with the inhibition of 49.0 ± 9.3% ($P < 0.001$). Rosmarinic acid, the major component of *O. sanctum* extracts, was a potent inhibitor against NF-κB with the inhibition of 36.8 ± 5.6% ($P < 0.001$). Therefore, rosmarinic acid from *O. sanctum* extracts was the main compound for NF-κB suppression. The results were in line with the previous study suggested that rosmarinic acid could inhibit the NF-κB secretion by blocking the NF-κB transcription factor (p50/p65) activation and inhibiting the phosphorylation of p-IκB-α (Jiang et al., 2009).

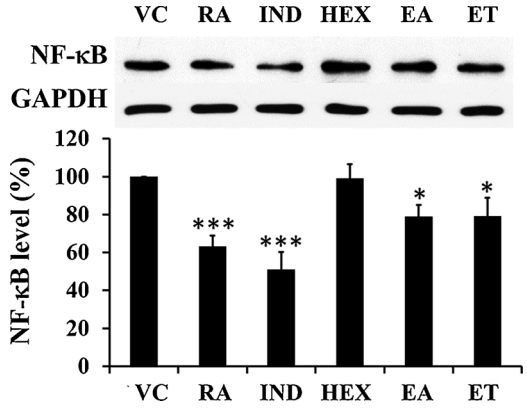

Fig. 4. Effect of rosmarinic acid (RA), indomethacin (IND), *n*-hexane extract (HEX), ethyl acetate extract (EA), and ethanolic extract (ET) on NF-κB protein expressions in U937 cells. The levels of NF-κB protein after treatments with 10 μg/mL of RA, IND, HEX, EA, and ET in U937 cells for 48 h were detected by Western blotting. DMSO (1%) treated cells were used as vehicle control (VC). GAPDH was used as a loading control. The protein levels were analyzed using a densitometer. The data present the means ± SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.6. Anti-ageing activity determination

The inhibitory activities of *O. sanctum* extracts against MMP-1 are shown in Fig. 5. Ethanolic extract possessed the significantly highest inhibition in all concentrations and also showed comparable results to that of rosmarinic acid at low concentration (80 μg/mL). Therefore, ethanolic extract would be the most potent against MMP-1. Collagenase from *Clostridium histolyticum*, also known as MMP-1, was able to hydrolyze triple-helical collagen in both physiological and *in vitro* conditions (Thring et al., 2009). The loss of collagen is considered as the characteristic histological finding in aged skin since collagen is the major protein of the extracellular matrix (ECM) (Baumann, 2007). Thus an inhibition of MMP-1 leads to the decrease of collagen break-down and retardation of skin ageing.

In contrary, ethanolic extract possessed the least inhibitory effect against MMP-2 and almost had no effect against MMP-9, whereas, ethyl acetate extract possessed the highest inhibitory activity against MMP-2

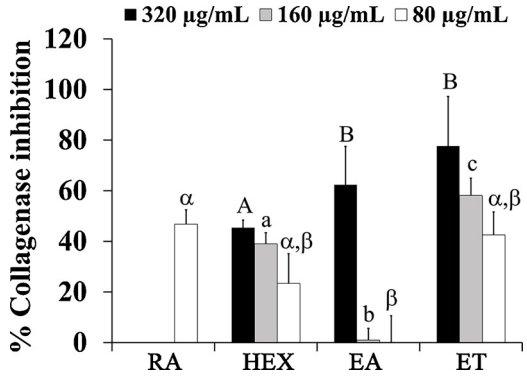

Fig. 5. Effect of rosmarinic acid (RA), *n*-hexane extract (HEX), ethyl acetate extract (EA), and ethanolic extract (ET) on collagenase inhibitory activity. The RA, HEX, EA, and ET at the concentrations of 80, 160, and 320 μg/mL were determined the collagenase activity by spectrophotometric analysis. α denotes significant difference from β ($P < 0.05$) among different extracts at the concentration of 80 μg/mL (■). a denotes significant difference from b and/or c ($P < 0.05$) among different extracts at the concentration of 160 μg/mL (■). A denotes significant difference from B ($P < 0.05$) among different extracts at the concentration of 320 μg/mL (□). The data present the means ± SD of three independent experiments.

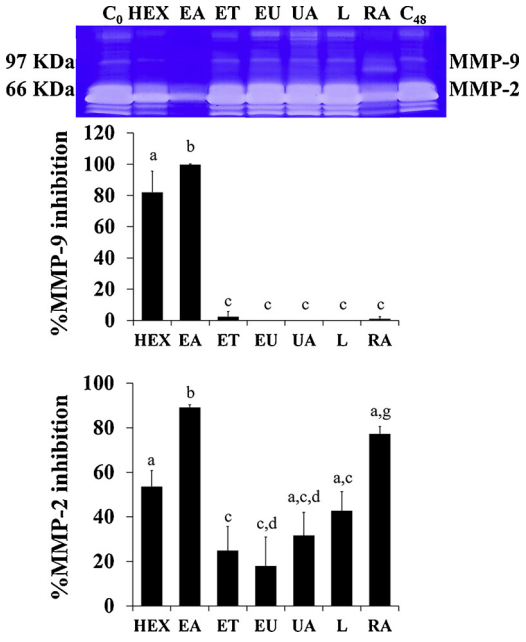

Fig. 6. Effect of *n*-hexane extract (HEX), ethyl acetate extract (EA), ethanolic extract (ET), eugenol (EU), ursolic acid (UA), linalool (L), and rosmarinic acid (RA) on MMP-2 and MMP-9 inhibitory activity in 3T3 cells. 3T3 cells were treated with HEX, EA, ET, EU, UA, L, and RA at the same concentration (1 mg/mL) for 48 h and detected MMP-2 and MMP-9 by SDS-PAGE. DMSO was used as a loading control at 0 h (C_0) and 48 h (C_{48}). The letter a–g denotes significant difference among different extracts and compounds ($P < 0.05$). The data present the means ± SD of three independent experiments.

and MMP-9 (Fig. 6). The likely explanation might be due to different hemopexin-like C-terminal domain, which is necessary for substrate recognition and also for inhibitor binding (Fridman et al., 2003). Tyr994 in collagen-binding domain of *C. histolyticum* collagenase is critical residue for the interaction with collagen and its hydroxyl group could form the hydrogen bonds which would produce the enzyme–collagen complex before the cleavage of peptide bond (Uesugi et al., 2009), whereas, MMP-2 and MMP-9 contained three contiguous fibronectin type II-like domains that are inserted within their catalytic domain and the groove in hemopexin domain contributed to the substrate binding (Morgunova et al., 1999).

There are several types of MMPs and only four types are related to the degradation of ECM in human skin, including MMP-1 (collagenase), MMP-2 (92 kDa gelatinase), MMP-3 (stromelysin 1), and MMP-9 (72 kDa gelatinase) (Jenkins, 2002). MMP-1 is the key enzymes that is capable of cleaving interstitial fibrillar collagen, which is the essential structural component of all connective tissues (Iyer et al., 2006). MMP-2 and MMP-9, which are commonly known as Gelatinase A and B, have a potential to degrade both collagen and elastin fiber network (Jenkins, 2002). MMP-9 exhibits prominent elastin and fibrillin degradation leading to the loss of skin resilience and suppleness, whereas, MMP-2 exhibits prominent collagen III degradation leading to negative impact on surrounding fibroblasts and the loss of dermis tensile properties (Jenkins, 2002; Waller and Maibach, 2006). Therefore, both ethanolic extract and ethyl acetate extract had a potential to be used for anti-ageing.

Elastin, one of the ECM produced by dermal fibroblast, plays an important role in skin elasticity because of its unique elastic recoil properties (Thring et al., 2009). Degradation and disorganization of elastin, which is primarily attributable to the elevation in dermal elastase activity, would lead to the sagging skin (Jenkins, 2002). Therefore, the compounds that could inhibit elastase activity would be a promising compound for anti-ageing. *n*-Hexane extract and ethyl acetate extract possessed comparable inhibitory activity against

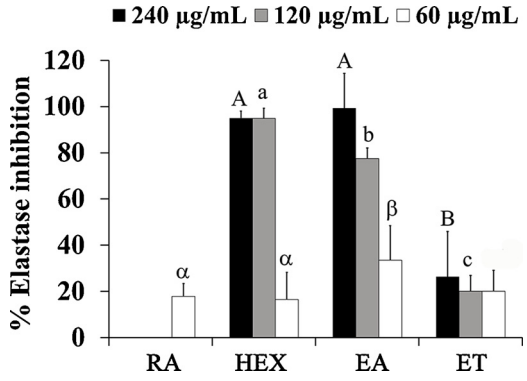

Fig. 7. Effect of rosmarinic acid (RA), *n*-hexane extract (HEX), ethyl acetate extract (EA), and ethanolic extract (ET) on elastase inhibitory activity. RA, HEX, EA, and ET at the concentration of 60, 120 and 320 µg/mL were determined by spectrophotometric method. α denotes significant difference from β ($P < 0.05$) among different extracts at the concentration of 60 µg/mL (■). a denotes significant difference form b and/or c ($P < 0.05$) among different extracts at the concentration of 120 µg/mL (■). A denotes significant difference from B ($P < 0.05$) among different extracts at the concentration of 240 µg/mL (□). The data present the means ± SD of three independent experiments.

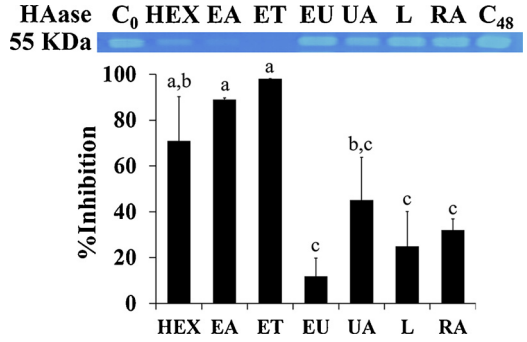

Fig. 8. Effect of *n*-hexane extract (HEX), ethyl acetate extract (EA), ethanolic extract (ET), eugenol (EU), ursolic acid (UA), linalool (L), and rosmarinic acid (RA) on hyaluronidase inhibitory activity. HEX, EA, ET, EU, UA, L, and RA at the same concentration (1 mg/mL) at 48 h and detected hyaluronidase (HAase) by SDS-PAGE. DMSO was used as a loading control at 0 h (C_0) and 48 h (C_{48}). The letter a, b, and c denote significant difference ($P < 0.05$) among different extracts and compounds. The data present the means ± SD of three independent experiments.

elastase with the IC_{50} value of 82.7 ± 30.9 and 77.9 ± 16.6 µg/mL, respectively, whereas, ethanolic extract possessed lower inhibitory activity (IC_{50} value = 270.3 ± 35.2 µg/mL) (Fig. 7). At low concentration (60 µg/mL), *n*-hexane extract and ethanolic extract possessed comparable results to that of rosmarinic acid, whereas, ethyl acetate extract exhibited the significantly highest inhibitory activity. Therefore, ethyl acetate extract would be the most suitable extract for anti-elastase since it could inhibit the enzyme even at lower concentration. Additionally, there should be any other compounds other than rosmarinic acid that were responsible for anti-elastase activity.

Hyaluronic acid is a high molecular weight glycosaminoglycan with markedly hydrophilic properties naturally found in both epidermis and dermis, so it could act as a natural moisturizing factor that maintain the skin moisture. The marked disappearance of hyaluronic acid in the skin lead to dramatic histochemical change observed in ageing skin (Farage et al., 2008). The enzyme hyaluronidase is the key factor that controls the turnover of hyaluronic acid in human skin (Papakonstantinou et al., 2012). Therefore, the compounds which could inhibit hyaluronidase might be useful for skin ageing.

The inhibitory activities of *O. sanctum* extracts against hyaluronidase investigated by SDS-PAGE are shown in Fig. 8. All *O. sanctum* extracts showed great inhibitory activities which were comparable to each other ($P > 0.05$). The likely explanation might be due to the high concentration of the extracts used in the assay (1 mg/mL) which led to the maximum plateau inhibition. Interestingly, the pure compounds, including eugenol, ursolic acid, linalool, and rosmarinic acid at the same concentration (1 mg/mL) possessed lower inhibitory activity. These findings might be used to confirm the synergistic effect of natural components in the *O. sanctum* extracts.

4. Conclusions

This study is the first report investigating skin anti-ageing activity of *O. sanctum* extracts. Among various fractionated extracts of *O. sanctum*, ethanolic extract possessed the most potent antioxidant activity with TEAC value of 270.1 ± 15.1 µM/mg, EC_1 value of 459.3 ± 91.4 µM/mg, and $DPPH^{\cdot}$ inhibition of 34.0 ± 0.7%. Additionally, ethanolic extract significantly suppressed the NF-κB expression (79.3 ± 9.6%). Furthermore, it inhibited IL-6 secretion, MMP-1, and hyaluronidase activity with the values of 54.7 ± 3.1%, 77.7 ± 9.0%, and 79.3 ± 9.6%, respectively. Rosmarinic acid, a major component of ethanolic extract (19.3%), was responsible for those biological activities which were related to skin anti-ageing, including antioxidation, anti-inflammation, and inhibition of the degradation of hyaluronic acid and collagen fiber. Therefore, ethanolic extract would be an attractive natural extract along with a very high potential for further development of anti-skin ageing products. Moreover, ethanolic extract showed the highest yield (6.5%) which would be good for the cosmetic industry due to the cost effectiveness. Although all solvent has already been removed from the extracts, ethanolic extract could be more preferable for using in the cosmetic products than any other organic solvents.

Conflict of interest

There are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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