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CHEMICAL COMPOSITION AND ANTI-INFLAMMATORY ACTIVITY OF ESSENTIAL OILS FROM RESIN OF *COMMIPHORA* SPECIES

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ABSTRACT. Essential oils (EOs) were prepared by the hydro-distillation technique from the resins of four *Commiphora* species and analyzed by GC-MS. Major constituents of EOs were α-copaene (22.71%), β-caryophyllene (28.03%) and β-caryophyllene oxide (13.89%) for *C. sphaerocarpa*; α-pinene (29.1%) for *C. africana*; hexadecane (14.1%) for *C. habessinica* and δ-cadinene (31.5%) for *C. schimperi*. We investigated the anti-inflammatory effects of EOs in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages by measuring nitric oxide (NO). The effect in mRNA or protein level after EO treatment were evaluated by RT-PCR and Western blot analysis, respectively. Among four *Commiphora* species, *C. sphaerocarpa* EO demonstrated a significant inhibition of LPS by 27.2±3.6% at 10 μg/mL and 62.3±5.2% at 20 μg/mL. *C. sphaerocarpa* EO inhibited LPS mediated *i*NOS over expression in both protein and mRNA level with dose dependent manner. It inhibited phosphorylation of ERK1/2, p38, ATF2. The enhanced anti-inflammatory activity of the EO of the plant was due to HO-1 expression by ROS dependent Nrf2 activation in RAW264.7 cells. These findings indicate *C. sphaerocarpa* EO inhibits the pro-inflammatory responses by inhibiting MAPK/ATF2, and triggering ROS/Nrf2/HO-1 signaling. Therefore, *C. sphaerocarpa* EO could have potential for useful therapeutic candidate preventing and treating inflammatory diseases.

KEY WORDS: GC-MS, Anti-inflammatory, *C. africana*, *C. habessinica*, *C. sphaerocarpa*, *C. schimperi*

INTRODUCTION

Inflammation is a complex biological response process by various immune cells including macrophages for the protection against agents such as irritants, viruses and bacteria [1]. Macrophages play an important function in inflammatory responses through the production of various cytokines [2]. In the genus *Commiphora* (Burseraceae) about half of the species are endemic to the eastern African countries such as Ethiopia, Kenya and Somalia [3]. *Commiphora* species exudates fragrant oleo gum resin naturally from the bark. The exudate contains gum, resin and volatile oils.

Different *Commiphora* species including from *C. myrrha* [4, 5], *C. guidottii* [6], *C. quadricincta* [7], *C. holtziana* [4, 8], *C. sphaerocarpa* and *C. kataf* [4] were reported to contain volatile oils composed of components such as α-pinene, camphene, β-pinene, myrcene and limonene. The chemical constituents of essential oils from different *Commiphora* species were reported to show differences mainly in their sesquiterpenoids composition such as β-elemene, α-copaene, α-humulene, β-selinene and germacrene B [9].

Among *Commiphora* species, *Commiphora mukul* is the most frequently investigated for its pharmacological activity where its crude ethyl acetate extract solvent fractions and isolated

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compounds showed anti-inflammatory property. Pregnane type steroids: *Z*-guggulsterone, *E*-guggulsterone, triterpenoids: myrrhanol A and myrrhanone A reported from the resin displays anti-inflammatory activities. The mechanism of action ranges from NO inhibition to multiple inflammation-related proteins and signal pathways such as reactive oxygen species (ROS), tumour necrosis factor alpha (TNF-a), prostaglandin E2 (PGE2), nuclear factor kappa b (NF-kB) and mitogen-activated protein kinase (MAPK)/[10, 11].

Sesquiterpene fractions obtained from *Commiphora molmol*, yielded furanodien-6-one and methoxyfuranoguaia-9-en-8-one as major compounds which exhibited antimicrobial activities against various microbial strains [12]. We reported cholesterol and lathosterol from the resin of *C. habessinica* [13]. The chloroform fraction significantly inhibited cell proliferation of four cancer cell lines, with dose-dependent relation *in vitro* and a strongest effect was reported on A549 cell lines. A mixture of cholesterol : lathosterol (47.9% : 52.1%) exhibited a moderate cytotoxicity which is greater than the individual compound towards A549 and A2780 with IC_{50} of 13.77 and 20.36 μg/mL, respectively that might be due to synergetic effect [13]. Recently we reported a new tricyclic triterpene acid commonly called commafric A from the resin of *C. africana* which has a significant anti-proliferative activity against A549 cell lines [14]. Methanol extract of *C. schimperi* gum-resin was reported to have antibacterial effects on some Gram-positive bacteria [15].

Various parts of species belonging to the genus *Commiphora* are largely utilized in for treatment of various diseases of humankind and cattle such as healing burn (in human beings), wound (in cattle) and to eradicate cattle ticks and are employed in cleansing women's body [16]. Exudate of *Commiphora africana* was reported to treat wounds traditionally and those of *Commiphora habessinica* was locally used to treat eye pain [17]. In addition to the above-mentioned uses, the smokes of gum-resins from *Commiphora* species are also used by followers of both the Orthodox Christian (in church and at home) and Muslim religions during their prayers, essentially to 'calm and collect' their nerves. It is also used as incense during coffee ceremonies at home [18]. The resin is *C. sphaerocarpa* is used traditionally to treat stomach complaints in Bale region, Ethiopia. The commercially known resin called myrrh is obtained from the resin of *Commiphora myrrha.* Resins obtained from several different *Commiphora* species are marketed as myrrh. Therefore, some previous chemical studies [19-21] on myrrh, were based on materials obtained from the market. Consequently, such reports lack information on botanical source of the investigated samples. To the best of our knowledge, there was no studies on the potential of anti-infammatory activity on essential oils from the resins of *C. africana*, *C. habessinica*, *C. sphaerocarpa* and *C. schimperi*. In this study we analyzed the chemical composition and evaluated anti-inflammatory activities of four resin oils extracted from botanically identified *Commiphora* species.

EXPERIMENTAL

Cell culture media and chemicals

Dulbecco's Modified Eagle medium (DMEM) and LPS (*Escherichia coli* 055:B5) were purchased from Gibco Inc. (NY, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. We purchased SB203580, PD98059 from Calbiochem (San Diego, CA). SB216763 and N-acetyl cysteine (NAC) Sigma–Aldrich. Antibodies against iNOS, activating transcription factor 2 (ATF2) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA) and those against IκB-a, p65, ERK1/2, phospho-ERK1/2(Thr202/Tyr204) and Actin were purchased from Cell Signaling (Bervely, MA, USA). Other chemicals were purchased from Sigma-Aldrich, Korea unless specified. C_8-C_{20} and C_7-C_{40} (n-alkane standard solutions were purchased form Sigma Aldrich, Korea. Chloroform and anhydrous sodium sulfate were purchased from Daejung Chemicals and Metals Co., Ltd, and Duksan Pure Chemical Co. Ltd, Korea, respectively.

Plant materials

Resins of *Commiphora africana* (A. Rich.) Engl., *Commiphora habessinica* (Berg) Engl.; and *Commiphora schimperi* (Berg) Engl. were collected from the natural vegetation of Borena zone (4° 52' 59.99" N and 38° 04' 60.00" E), Oromia regional state, Ethiopia, in October, 2016. The plant materials of *C. sphaerocarpa* Chiov. were collected from Sof Omar (6° 54' 59.99" N, and 40° 44' 59.99" E), Bale, Ethiopia, in October 2016. The plants authentication was done by Mr. Shambel Alemu and voucher specimens (*Commiphora habessinica:* 072771, *C. africana*: 072801, *C. Sphaerocarpa:* 072820, *C. Schimper:* 072823) were deposited at the National Herbarium of Addis Ababa University, Addis Ababa. These samples were collected from natural vegetation and there is no permission required to conduct research works in Ethiopia.

Extraction of the volatile oils

C. africana (22 g), *C. habessinica* (36 g), *C. sphaerocarpa* (16 g) and *C. schimperi* (46 g) resins were powdered using an electric grinder and extracted using hydro-distillation for 3 h in a modified Clevenger apparatus. The essential oils were collected, dried over anhydrous Na_2SO_4 and kept in a refrigerator at -4 ºC until further analysis. Yields of the oils were 2.93, 0.05, 0.61 and 0.03% for *C. africana*, *C. habessinica*, *C. sphaerocarpa* and *C. schimperi* resins, respectively.

GC-MS analysis

GC-MS analysis of essential oils was performed by a GC (7890B, Agilent Technologies, USA) coupled with an MS (5977A Network, Agilent Technologies) in the central analytical lab in Andong National University. The GC had an HP- 5MS column ((30 m × 250 µm internal diameter (i.d.) and 0.25 µm). Helium was used as a carrier gas (flow rate 1 mL/min). The injector temperature was 230 °C and the injection mode was split mode (4:1). The initial oven temperature was 40 ºC for 3 min and raised up from 40 to70 ºC at the speed of 4 ºC/min and held at isothermal for 3 min and raising with no holding time to 80 ºC at 1 ºC/min, 100 ºC at 4 ºC/min then to 180 ºC at 10 ºC/min and then 20 ºC/min until it reached 280 ºC and held at this temperature for 2 min with the total run-time of 60 min. Mass spectra were recorded in electron-impact mode, with ionization energy of mode at 70 eV, scanning the 33-500 m/z range. The volatile compounds in the oils were identified by comparing the mass spectra of the compounds in the oils with those in the database of NIST11 GC-MS libraries, the relative retention indices (RRI) relative to C_8-C_{20} and C_7-C_{40} *n*-alkanes and literature data [22-24].

Cell culture and treatment

Mouse macrophage cell line, RAW264.7 was obtained from Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 ºC and 5% CO_2 humidified atmosphere. The solvent used to dissolve the EOs of resins of *Commiphora* species was dimethyl sulfoxide (DMSO). After dissolving the oils in the solvent, cells treatment was followed. DMSO was used as a vehicle and the final DMSO concentration was not exceeded 0.1% (v/v).

Measurement of nitric oxide (NO) production

The inhibitory effect of *Commiphora* resin EOs on the formation of NO in LPS-stimulated RAW264.7 cells was determined using literature reported [25]. RAW264.7 cells were inoculated in 12-well plate for 24 h. Cells were first treated with *Commiphora* resin EOs at the

aforementioned concentrations for 2 h followed by co-treatment with LPS (1 μg/mL) for more 18 h. Then the media (200 μL) was mixed with equal amount of Griess reagent (1% sulfanilamide and 0.1% N-1-(naphthyl)ethylenediamine, dil. HCl in 2.5% phosphoric acid). The mixture was then incubated for more 5 min at 23 °C before the absorbance measurement at 540 nm.

Measurement of intracellular ROS

After the treatment, RAW264.7 cells were washed with 1 × phosphate-buffered saline (PBS) three times and harvested. Then, the cells were incubated with 2.5 μM of DCFH-DA for 30 min. After 30 min, the cells were washed three times with 1 × PBS. Intracellular ROS generation was measured by using UV/Visible spectrophotometer (Human Cop., Xma-3000PC, Seoul, Korea) (excitation, 485 nm; emission, 530 nm).

Western blot analysis

Western blot analysis was performed according to the previously reported method [26]. RAW264.7 cells were washed three times with cold 1 × phosphate-buffered saline and lysed at 4 °C for 30 min using cold radioimmunoprecipitation assay buffer (Boston BioProducts, Ashland, MA, USA) containing protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Sigma-Aldrich). The solution was then centrifuged at 15,000 rpm for 10 min, and the supernatant was separated from the precipitate for protein quantitation using BCA method (Thermo Fisher Scientific, Waltham, MA USA). The protein was separated on SDS-PAGE for about 1 h at 150 V and then transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 2 h at 100 V. PVDF membranes were blocked using 5% non-fat dry milk in tris-buffered saline containing 0.05% Tween 20 (TBS-T) by stirring at room temperature for 1 h. The specific primary antibodies in 5% non-fat dry milk dissolved with TBS-T buffer were treated with PVDF membranes and reacted while stirring at 4 °C overnight. Then, PVDF membranes were washed three times with TBS-T buffer, and then treated with the secondary antibodies in 5% non-fat dry milk dissolved with TBS-T buffer for 1 h at room temperature. Chemi luminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized using LI-COR C-DiGit Blot Scanner (Li-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Results are expressed as mean ± SD (standard deviation). The one-way ANOVA followed by Dunnett's test was used for statistical analysis. Differences with $*p$ or $\#p$-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Essential oil chemical analysis

Ethnobotanical information we gathered during field trips, showed resins of different *Commiphora* species were used traditionally for treatment of various disorders. For example, the resin of *C. sphaerocarpa* is a popular traditional medicine for treatment of stomach ache in the area of collection. However, there were no detailed studies on their chemical composition and biological activities on the resins of these plants. Even though the plants belong to the same genus, there are variations in the percentage oil yield of the resins. Yields of the essential oils of the resin were 2.93, 0.05, 0.61 and 0.03% for *C. africana*, *C. habessinica*, *C. sphaerocarpa* and *C. schimperi* resins, respectively. The highest percentage yield was obtained for *C. africana* which is followed by *C. sphaerocarpa*. The percentage yield of the oil of *C. africana* is about five times

compared to that for *C. sphaerocarpa* resin. The lowest oil yield was obtained for *C. schimperi* resin. The percentage yield of the resins might be due to the difference in the type of compounds they constitute, soil type and environmental factors.

Table 1 shows the chemical composition of the constituents of *Commiphora* resin essential oils. Totally, 27, 30, 30 and 44 volatile components were identified, with 96.3, 88.9, 92.7 and 92.2% of the composition for *C. africana*, *C. habessinica*, *C. sphaerocarpa* and *C. schimperi* essential oils, respectively. The main constituents of *C. africana* essential oils were α-pinene (29.1%), *cis*-verbenol (12.7%), verbenone (12.1%), and β-pinene (6.2%) (Table 1, Figure 1). The composition of *C. habessinica* essential oils were verbenone (5.2%), tetradecane (6.4%), hexadecane (14.1%) (Table 1, Figure 2). These analysis results were compared with resin essential oils of commonly known *Commiphora* species. The resin of *C. myrrha* (myrrh) oil was reported to constitute oxygenated sesquiterpenes such as furanoeudesma1,3-diene (34.0%), furanodiene (19.7%) and lindestrene (12.0%) as major compounds [27]. In this study the hydro-distilled oil of the resin of *C. sphaerocarpa* showed the presence of sesquiterpenes such as β-caryophyllene (28.0%), α-copaene (22.71%) and β-caryophyllene oxide (13.9%) as major constituents (Table 1. Figure 3). *C. sphaerocarpa* resin essential oil was also reported to have various sesquiterpenes such as α-copaene (5.3%), β-elemene (6.7%), α-gurjunene (7.0%), β-selinene (8.0%), α-selinene (11.0%), α-guaiene (6.0%), T-cadinol (7.0%), curzerenone and furanodienone (13.0%) [4]. The percentage composition α-copaene in this study is higher than the previous report [4]. Additionally, there is some variation in percentage composition of other constituents of the plant which might be due to various factors such as seasonal variation of sample collected [28] and environmental conditions [29]. The essential oil of resin samples of commonly known *C. mukul* was reported to have α-cadinol (7.25-14.5%). Other predominant constituents in the *C. mukul* oil include α-pinene, α-cubebene, α-copaene, β-caryophyllene, *trans*-caryophyllene, α-humulene, γ-cadinene, δ-cadinene and cembrene A [30] and most of these compounds were similar to those of the resin of *C. shpaerocarpa* EO in this study. In the current study we found *C. schimperi* essential oils contains δ-cadinene (31.5%), hexadecane (11.6%), pentadecane (8.1%) as major components (Table 1, Figure 4).

Table 1.Chemical composition of the essential oils from *C. africana* (A), *C. habessinica* (B), *C. sphaerocarpa* (C) and *C. schimperi* (D) resins.

RI (Cal)	RI (lit)	Compounds	A (%)	B (%)	C (%)	D (%)	Identification
		Monoterpenes	**91.6**	**13.6**	-	**5.4**	RI lit, MS
		Monoterpenes hydrocarbons	**45.4**	**0.4**	-	-	RI lit, MS
925	924	α-Thujene	0.2	-	-	-	RI lit, MS
932	932	**α-Pinene**	**29.1**	0.4	-	-	RI lit, MS
945	946	Camphene	0.9	-	-	-	RI lit, MS
951	953	Thuja-2,4 (10)-diene	0.2	-	-	-	RI lit, MS
971	968	β-Thujene	4.8	-	-	-	RI lit, MS
973	974	**β-Pinene**	**6.2**	-	-	-	RI lit, MS
1019	1020	*p*-Cymene	2.2	-	-	-	RI lit, MS
1024	1024	Limonene	1.6	-	-	-	RI lit, MS
1056	1054	γ-Terpinene	0.2	-	-	-	RI lit, MS
		Oxygenated monoterpenes	**46.2**	**13.1**	-	**5.4**	
1060	1159	*p*-Menth-8-en-1-ol	1.6	-	-	-	RI lit, MS
1091	-	*p*-Menth-2-en-1-ol	0.9	-	-	-	RI lit, MS
1117	1122	α-Campholenal	0.9	-	-	-	RI lit, MS
1132	1135	*trans*-Pinocarveol	2.7	0.7	-	-	RI lit, MS
1134	1141	Camphor	2.3	0.6	-	-	RI lit, MS
1138	1137	***cis*-Verbenol**	**12.7**	-	-	0.8	RI lit, MS
1141	1140	*trans*-Verbenol	0.8	-	-	-	RI lit, MS

RI (Cal)	RI (lit)	Compounds	A (%)	B (%)	C (%)	D (%)	Identification
1153	1158	*trans*-Pinocamphone	0.3	-	-	-	RI lit, MS
1155	1160	Pinocarvone	0.5	-	-	-	RI lit, MS
1165	1158	*trans*-3-Pinanone	1	-	-	-	RI lit, MS
1170	1174	Terpinen-4-ol	3.8	3.3	-	0.8	RI lit, MS
1178	1184	*p*-Cymen-8-ol	0.5	-	-	-	RI lit, MS
1185	1186	α-terpineol	1.8	3.2	-	0.8	RI lit, MS
1186	1195	Myrtenal	1.1	-	-	-	RI lit, MS
1190	1194	Myrtenol	2.2	-	-	-	RI lit, MS
1200	1204	**Verbenone**	**12.1**	5.2	-	3.1	RI lit, MS
1212	1215	*trans*-Carveol	0.4	-	-		RI lit, MS
1237	1239	Carvone	0.3	-	-		RI lit, MS
1295	1289	Thymol	0.3	-	-		RI lit, MS
		Sesquiterpenes	**3.0**	-	**90.6**	**38.1**	
		Sesquiterpene hydrocarbons	**2.9**	-	**75.3**	**35.8**	
1345	1345	α-Cubebene	-	-	0.2	-	RI lit, MS
1345	1350	α-Longpinene	0.2	-	-	-	RI lit, MS
1361	1379	β-Patchoulene	-	-	0.2	-	RI lit, MS
1370	1374	**α-Copaene**	**1.6**	-	**22.7**	-	RI lit, MS
1381	1387	β-Bourbonene	-	-	0.3	-	RI lit, MS
1387	1389	β-Elemene	-	-	0.2	-	RI lit, MS
1424	1417	**β-Caryophyllene**	-	-	**28.0**	-	RI lit, MS
1426	1432	*trans*-α-Bergamotene	1.0	-	0.3	-	RI lit, MS
1431	1430	β-Copaene	-	-	0.1	-	RI lit, MS
1457	1464	α-Humulene	-	-	3.3	-	RI lit, MS
1472	1458	Alloaromadendrene	-	-	3.0	-	RI lit, MS
1479	1478	γ-Muurolene	-	-	2.7	-	RI lit, MS
1482	1481	4,11-selinadiene	-	-	3.0	-	RI lit, MS
1489	1489	β-Selinene	-	-	1.0	-	RI lit, MS
1490	1493	γ -Maaliene	-	-	0.3	-	RI lit, MS
1495	1498	α-Selinene	-	-	0.4	-	RI lit, MS
1509	1500	α-Muurolene	-	-	0.8	0.8	RI lit, MS
1513	1513	γ -Cadinene	-	-	0.7	1.5	RI lit, MS
1518	1537	α-Cadinene	0.1	-	-	-	RI lit, MS
1518	1520	7-epi-a-selinene	-	-	3.1	-	RI lit, MS
1527	1522	**δ-Cadinene**	**-**	**-**	4.7	**31.5**	RI lit, MS
1537	1533	*trans*-Cadinadiene-1,4	-	-	0.1	2.0	RI lit, MS
1547	1544	α-Calacorene	-	-	0.2	-	RI lit, MS
		Oxygenated sesquiterpene	**0.1**	-	**15.3**	**2.3**	
1572	1577	Spathulenol	0.1	-	-	1.1	RI lit, MS
1586	1590	Globulol	-	-	-	1.2	RI lit, MS
1589	1582	**β-Caryophyllene oxide**	-	-	**13.9**	-	RI lit, MS
1607	1608	α-Humulene epoxide II	-	-	1.0	-	RI lit, MS
1653	1652	α-Cadinol	-	-	0.4	1.1	RI lit, MS
		Aliphatic hydrocarbons	-	**72.6**	**1.9**	**47.6**	
1055	-	Undecane, 5,7-dimethyl-	-	0.4	-	-	MS
1096	1100	Undecane	-	0.4	-	-	RI lit, MS
1196	1200	Dodecane	-	2.2	-	0.7	RI lit, MS
1210	-	Undecane, 2,6-dimethyl-	-	1.0	-	-	MS
1215	-	Cyclohexane, 2-butyl-1,1,3-trimethyl-	-	0.9	-	-	MS
1255	-	Decane, 2,3,4-trimethyl-	-	1.0	-	-	MS
1260	-	Dodecane, 2-methyl-	-	1.5	-	-	MS
1283	-	6-Methyltetralin	-	0.6	-	-	MS

RI (Cal)	RI (lit)	Compounds	A (%)	B (%)	C (%)	D (%)	Identification
1295	1300	Tridecane	-	3.8	-	1.4	RI lit, MS
1309	-	1,4-Dimethyltetralin	-	0.8	-	0.5	MS
1313	-	Undecane, 2-methyl-	-	0.7	-	-	MS
1328	-	Tridecane, 2-methyl-	-	-	-	0.4	MS
1334	-	3,5-Dimethyldodecane	-	-	-	0.7	MS
1336	-	1,4-Dimethyltetralin	-	0.8	-	-	MS
1337	-	Dodecane, 2,6,10-trimethyl-	-	-	-	1.1	MS
1346	-	Tetracontane, 3,5,24-trimethyl-	-	-	-	0.4	MS
1346	-	Dodecane, 2,5-dimethyl-	-	0.9	-	-	MS
1351	-	Tridecane, 4,8-dimethyl-	-	-	-	0.7	MS
1371	-	Cyclododecane, ethyl-	-	-	-	0.7	MS
1372	-	Dodecane, 2,6,10-trimethyl-	-	1.7	-	-	MS
1375	-	Tetradecane, 5-methyl-	-	-	-	0.8	MS
1379	-	Dodecane, 2,6,11-trimethyl-	-	-	-	2.4	MS
1379	-	2-Methyl-*Z*-4-tetradecene	-	0.5	-	-	MS
1383	-	5,6-Dimethyltetralin	-	1.0	-	0.8	MS
1395	1400	Tetradecane	-	**6.4**	0.2	**3.6**	RI lit, MS
1399	-	Decane, 3,8-dimethyl-	-	0.7	-	0.4	MS
1441	-	Cyclododecane, ethyl-	-	0.6	-	0.7	MS
1443	-	Cyclotetradecane	-	0.4	-	-	MS
1453	-	Tetradecane, 4-methyl-	-	0.5	-	0.7	MS
1458	-	10-Methylnonadecane	-	2.2	-	-	MS
1465	-	Tetradecane, 3-methyl-	-	0.7	-	0.9	MS
1487	-	Cyclopentadecane	-	0.2	-	0.6	MS
1495	1500	**Pentadecane**	-	**11.1**	-	**8.1**	RI lit, MS
1541	-	Pentadecane, 7-methyl-	-	0.7	-	-	MS
1545	-	Decane, 4-cyclohexyl-	-	1.1	-	-	MS
1547	-	Tridecane, 5-methyl-	-	-	-	0.5	MS
1547	-	2-methyltetracosane	-	1.1	-	-	MS
1553	-	Pentadecane, 4-methyl-	-	0.8	-	0.8	MS
1558	-	Pentadecane, 2-methyl-	-	1.5	-	-	MS
1565	-	Pentadecane, 3-methyl-	-	1.0	-	1.1	MS
1579	-	1*Z*-5*E*-7-Dodecatriene	-	-	0.6	-	MS
1594	1600	**Hexadecane**	-	**14.1**	0.9	**11.6**	RI lit, MS
1639	-	Hexadecane, 7,9-dimethyl-	-	0.6	-	0.5	MS
1643	-	Pentadecane, 2,6,10-trimethyl-	-	1.4	-	-	MS
1648	-	Cyclopentane, undecyl-	-	0.5	-	-	MS
1652	-	Hexadecane, 4-methyl-	-	0.5	-	-	MS
1658	-	Hexadecane, 2-methyl-	-	0.5	-	0.6	MS
1665	-	Hexadecane, 3-methyl-	-	0.5	-	0.6	MS
1694	1700	**Heptadecane**	**-**	**5.0**	0.2	**4.1**	RI lit, MS
1700	-	Pentadecane, 2,6,10,14-tetramethyl-	-	0.7	-	-	MS
1752	-	Nonadecane, 4-methyl-	-	-	-	0.2	MS
1793	1800	Octadecane	-	1.3	-	1.1	RI lit, MS
1893	1900	Nonadecane	-	-	-	0.5	RI lit, MS
1992	2000	Eicosane	-	0.4	-	0.4	RI lit, MS
		Others	**1.7**	**2.8**	**0.3**	**1.1**	
1140	-	1,3-Cycloheptadiene	-	1.0	-	-	MS
1156	-	6-Methyl-3,5-heptadiene-2-one	0.2	-	-	-	MS
1159	-	1,3-Cyclopentadiene, 5,5-dimethyl-	0.7	-	-	-	MS

RI (Cal)	RI (lit)	Compounds	A (%)	B (%)	C (%)	D (%)	Identification
1196	-	Cyclohexanol, 2-methylene-6-methyl-	0.8	-	-	-	MS
1266	-	1-Iodo-2-methylundecane	-	0.9	-	-	MS
1269	-	Oxalic acid, decyl 2-ethylhexyl ester	-	0.9	-	-	MS
1373	-	1-Octadecanesulphonyl chloride	-	-	-	1.1	MS
1623	-	Benzenepropanoic acid	-	-	0.3	-	MS
		Total identified	**96.3**	**88.9**	**92.7**	**92.2**	

A, B, C and D stands for *C. africana, C. habessinica, C. sphaerocarpa* and *C. schimperi* resin essential oils respectively. RI (Cal) = based on HP 5MS capillary column (non-polar column) and alkane standards (C_8-C_{20} and C_7-C_{40}) according to [22, 23].

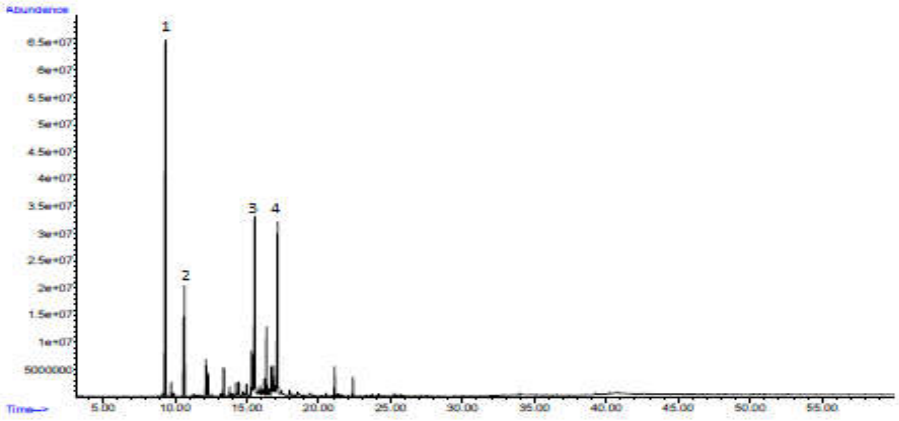

Figure 1. GC of the hydrodistillate of *C. africana*, **1**: α-pinene (29.1%), **2**: β-pinene (6.2%), **3**: *trans*-verbenol (12.7%), and **4**: D-verbenone (12.1%).

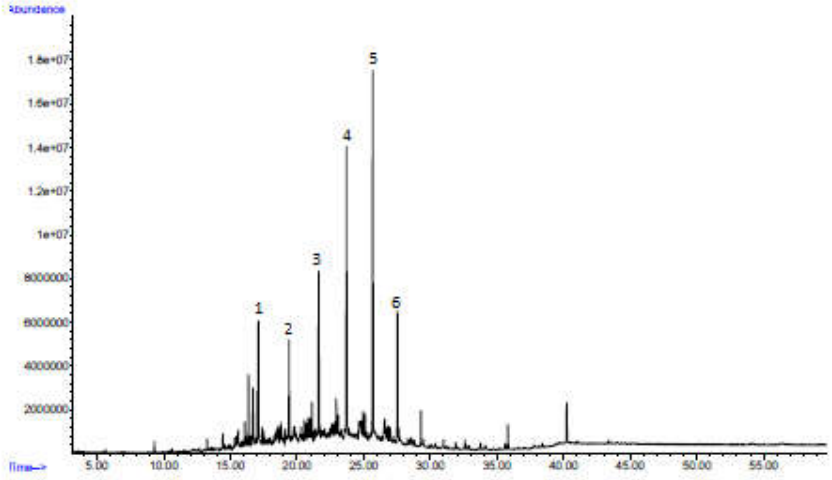

Figure 2. GC of the hydrodistillate of *C. habessinica*, **1**: D-verbenone (5.24 %), **2**: decane, 2,3,6-trimethyl-(3.79%) **3**: tetradecane (6.35%) **4**: pentadecane (11.1%) **5**: heptadecane, 2,6,10,15-tetramethyl-(14.08%) and **6**: heptadecane (5.01%).

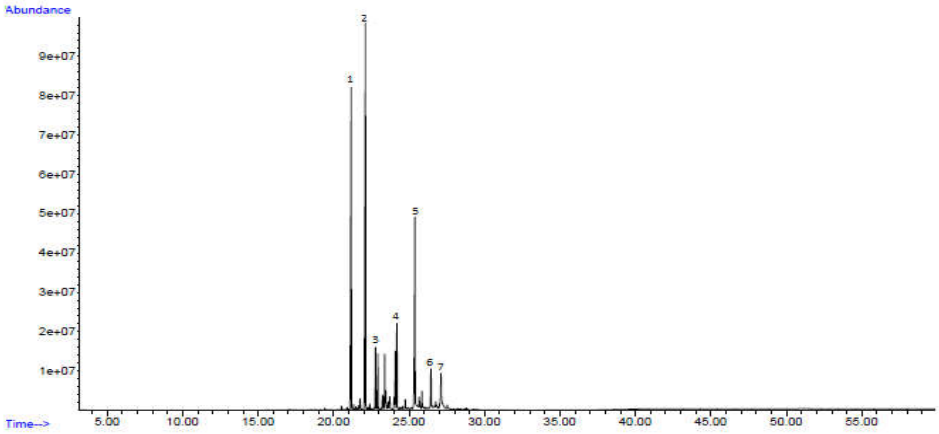

Figure 3. GC of the hydrodistillate of *C. sphaerocarpa*, **1**: α-copaene (22.71%), **2**: caryophyllene (28.03%), **3**: humulene (3.27%), **4**: β-cadinene (4.7%), **5**: caryophyllene oxide (13.9%), **6**: (+)-epi-bicyclosesquiphellandrene (2.73%), and **7**: syn-tricyclo[5.1.0.0(2,4)]oct-5-ene, 3,3,5,6,8,8-(4.12%).

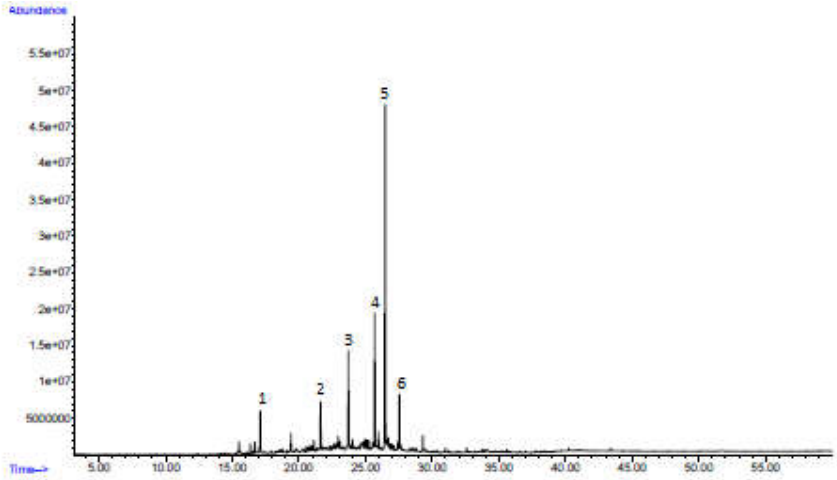

Figure 4. GC of the hydrodistillate of *C. schimperi*, **1**: verbenone (3.13%), **2**: tetradecane (3.61%), **3**: pentadecane (8.06%), **4**: hexadecane (11.6%), **5**: δ-cadinene (31.52%, and **6**: heptadecane (4.06%).

Activities of essential oil samples on the production of NO in LPS-induced RAW264.7 macrophages

Inflammation response is very important process of the body response during injury, infection or irritation. It can be classed as both acute and chronic inflammations. Chronic inflammation is a persistent one and involving localized in number of mediator molecules, which cause progressive damage to the body [31]. The release of those mediators on their part play roles in causing various

diseases which include arthritis, cancer, heart disease, Alzheimer's disease, diabetes and asthma. Various parts of traditional medicinal plants have been used for treatment of different diseases caused by inflammation such as healing of wounds and others. However, there have not been detailed investigations about their immunological effects. The present study investigated the influence of each volatile oil on NO production by treating the oils on macrophages in which inflammation was caused by bacterial LPS (Figure 5).

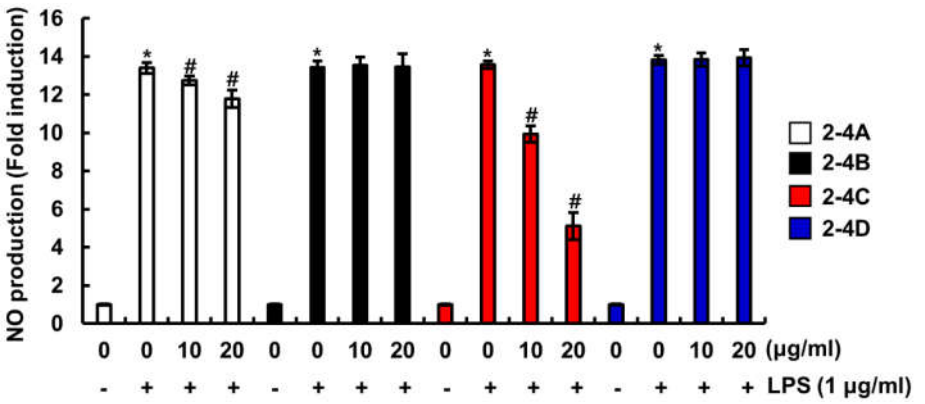

Figure 5. Effect of *C. africana* (2-4A), *C. habessinica* (2-4B), *C. sphaerocarpa* (2-4C) and *C. schimperi* (2-4D) resins essential oils on NO production in LPS-stimulated RAW264.7 cells. Cells (1.8×10^5 cells/mL) were stimulated by LPS (1 μg/μL) for 24 h in the presence of *Commiphora* essential oils (10 and 20 μg/mL), (-) cells without LPS and (+) cells with LPS (1 μg/mL).

To compare the anti-inflammatory effects of *C. africana*, *C. habessinica*, *C. sphaerocarpa* and *C. schimperi*, the NO production inhibitory activity was evaluated by pretreatment with EOs at 10 and 20 μg/ml. *C. africana* resin EO slightly inhibited LPS-mediated NO production, but *C. sphaerocarpa* dramatically inhibited LPS-induced NO production in RAW264.7 cells. The presence of sesquiterpenes (90.6%) such as α-copaene (22.7%), β-caryophyllene (28.0%) and its oxygenated sesquiterpene, β-caryophyllene oxide (13.9%) in high content in *C. sphaerocarpa* EO might be the major contributor for inhibition of LPS-mediated NO production in RAW264.7 cells. This statement is supported by a study on anti-inflammatory effect of inhibition of 5-LOX catalyzed leukotrienes production inhibition of β-hexosaminidase by caryophyllene oxide [32] and a study on caryophyllene showed inhibition of NF-Kb [33], reduction of edema and TNF-α concentrations [34]. Chavan *et al.* [35] reported the compound from *Annona squamosa* which had analgesic effects [35].

A study by Tipton *et al.* [36] on myrrh oil largely containing sesquiterpene compounds exhibit considerable anti-inflammatory activity by inhibiting the transcription factors responsible for the transcription of genes encoding numerous cytokines, one of which being IL-6. Remarkably sesquiterpene lactones such as partenolide [37], pseudoguaianolides, guaianolides, germacrolides, heliangolides, melampolides and 4,5-dihydrogermacranolides, reported from natural products possessed anti-inflammatory effect caused by inhibiting NF-kB by targeting the lkB kinase complex [38]. It is known that inflammation can induce adverse effects such as cancer; a study reported that β-caryophyllene and β-caryophyllene oxide showed anticancer effects on growth and proliferation of several cancer cells. β-caryophyllene oxide changes several important pathways for cancer development, such as MAPK, PI3K/AKT/mTOR/S6K1 and STAT3 [39].

However, NO production by LPS was not changed in the treatment of *C. habessinica* and *C. schimperi* Eos (Figure 5). Both EOs constitute aliphatic hydrocarbons as major in their oils with *C. habessinica* (72.6%) and *C. schimperi* (48.3%). The slight inhibition of LPS-induced NO production in RAW264.7 cells by *C. africana* resin EO might be due to the presence of monoterpenes (91.6%) in major amount in the mixture. On the other hand, *C. sphaerocarpa* resin EO strongly inhibited the NO production with increment of concentration might be due to the presence of sesquiterpenes as major constituents. Thus, we selected *C. sphaerocarpa* resin EO for the further study. There were reports which showed production of NO in higher concentration is connected with immunological and inflammatory diseases [40].

Effects of C. sphaerocarpa EO on iNOS expression in LPS-induced RAW264.7 macrophages

To evaluate whether inhibitory effect of *C. sphaerocarpa* against LPS-mediated NO production results from the regulation of iNOS expression; we investigated whether *C. sphaerocarpa* EO affects iNOS expression in LPS-induced RAW264.7 cells. As a result, *C. sphaerocarpa* EO dose-dependently inhibited LPS-mediated iNOS overexpression in both protein and mRNA level (Figure 6). The protein level expression suppressed by more than 20% and 60% at a concentration of 10 and 20 µg/mL, respectively. On the other hand, mRNA production slightly reduced to 31.8% at 10 µg/mL and moderately at 20 µg/mL to 37.5% (Figure 6).

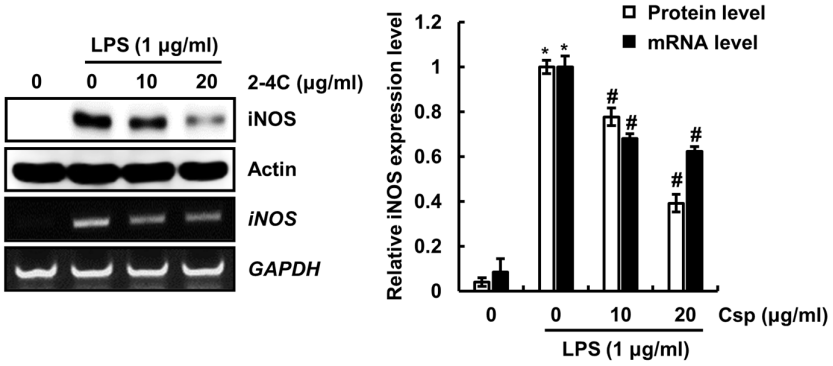

Figure 6. Effect of *C. sphaerocarpa* resin EO on the expression of iNOS in LPS-stimulated RAW 264.7 macrophage cells, Actin and GAPDH was used as the control protein. The protein levels of iNOS were detected by Western blot analysis. Values given are the mean ± SD (n = 3). *p < 0.05 compared to LPS treatment without resin EOs.

Effect of C. sphaerocarpa EO on LPS-stimulated IκB-α degradation and the nuclear accumulation of NF-κB p65 in RAW264.7 cells

It was reported that under normal conditions, NF-κB is sequestered in cytoplasm by inhibition of κB-kinase complex inhibitor (IκB) [41]. However, IκB degradation by the inflammatory stimuli such as LPS results in the release of NF-κB from its inhibitory complex, which leads to the nuclear translocation of NF-κB. Thus, we investigated the inhibitory effects of *C. sphaerocarpa* EO to IκB-α degradation (Figure 7A) which leads to nuclear accumulation of NF-κB p65 (Figure 7B). Our result showed the treatment of LPS alone degraded IκB-α and induced the nuclear accumulation of NF-κB p65 compared with the control cells. On the other hand, the *C. sphaerocarpa* resin EO did not inhibit LPS-mediated IκB-α degradation and the nuclear accumulation of NF-κB p65 in RAW264.7 cells. These findings indicate that inhibitory effect of

C. sphaerocarpa EO against LPS-mediated NO and iNOS production may be independent on NF-kB signaling pathway (Figure 7).

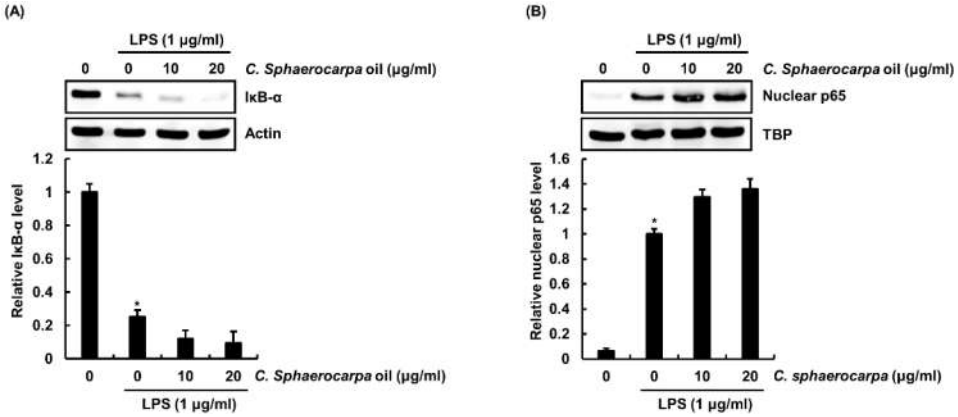

Figure 7. Effect of *C. sphaerocarpa* EO (2-4C) on LPS-mediated (A) IκB-α degradation and (B) The nuclear accumulation of NF-κB p65 in RAW264.7 cells. Actin and TBP used as a positive control.

Effects of C. sphaerocarpa essential oil on LPS-mediated phosphorylation of ERK1/2 and p38 - stimulated RAW264.7 cells

There are reports showing that inflammatory stimuli such as LPS phosphorylate extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 as MAPKs, which induces the expression of inflammatory mediators. Therefore, we evaluated whether *C. sphaerocarpa* EO affects LPS-mediated phosphorylation of ERK1/2 and p38. As a result, we observed that *C. sphaerocarpa* EO inhibited the phosphorylation of ERK1/2 and p38 compared to LPS treatment alone, which shows that *C. sphaerocarpa* EO may suppress activation of MAPK (Figure 8A). MAPK activation leads to the nuclear accumulation of activating transcription factor 2 (ATF2), which causes the production of the inflammatory mediators. As a result, we observed that LPS treatment alone induces ATF2 phosphorylation and accumulated ATF2 to the nucleus. On the contrary, *C. sphaerocarpa* EO inhibited ATF2 phosphorylation and subsequent ATF2 nuclear accumulation (Figure 8B and C). These results show inhibition of MAPK activation by *C. sphaerocarpa* EO might be due to the mitigation of ATF2 nuclear accumulation, which results *C. sphaerocarpa* resin EO exhibiting anti-inflammatory activity.

Effect of C. sphaerocarpa EO on HO-1 by ROS dependent Nrf2 activation and ZnPP in LPS-activated RAW264.7 cells

The overexpression of inducible heme oxygenase-1 (HO-1) blocks the production of pro-inflammatory mediators such as NO and iNOS and knockdown induced severe inflammation in the animal model and it may have anti-inflammatory effect. Thus, we evaluated whether *C. sphaerocarpa* affects HO-1 expression in RAW264.7 cells. *C. sphaerocarpa* resin EO dose-dependently induced HO-1 overexpression in RAW264.7 cells. NF-E2-related factor 2 (Nrf2) was reported to be correlated with HO-1 expression (Figure 9A). Although Nrf2 is sequestered in cytoplasm under normal condition, external stimuli induce Nrf2 activation through the nuclear accumulation of Nrf2. Indeed, overexpression of HO-1 by natural products has been reported to be involved in Nrf2 activation. Reactive oxygen species induce Nrf2 activation, which contributes

to HO-1 expression. To evaluate that HO-1 expression by *C. sphaerocarpa* results from ROS-dependent Nrf2 activation, RAW264.7 cells were pretreated with NAC as ROS scavenger and then co-treated with *C. sphaerocarpa* EO. As shown in (Figure 9B), the presence of NAC inhibited Nrf2 nuclear accumulation and HO-1 expression induced by *C. sphaerocarpa* EO in RAW264.7 cells which demonstrate that *C. sphaerocarpa* EO mediated HO-1 expression and may result from ROS-dependent Nrf2 activation. Next, we evaluated whether *C. sphaerocarpa* EO increases ROS level in RAW264.7 cells and found that it increased ROS level in RAW264.7 cells which might be explained due to the presence of β-caryophyllene, β-cayophyellene oxide [34, 35] and α-humulene in the plant oil (Figure 9C). α-Humulene was reported to decrease in cellular GSH level and enhance in ROS formation [42]. In our study even though the relative percentage of α-humulene is low (3.3%) in the plant oil, it might also be the possible reason for the anti-inflammatory effect of *C. sphaerocarpa* EO. In a related study, Bao *et al.* [43] also reported cadinane sesquiterpenes such as myrrhterpenes A and B from myrrh (the resin of *Commiphora myrrha*) to have anti-inflammatory activity which support our findings.

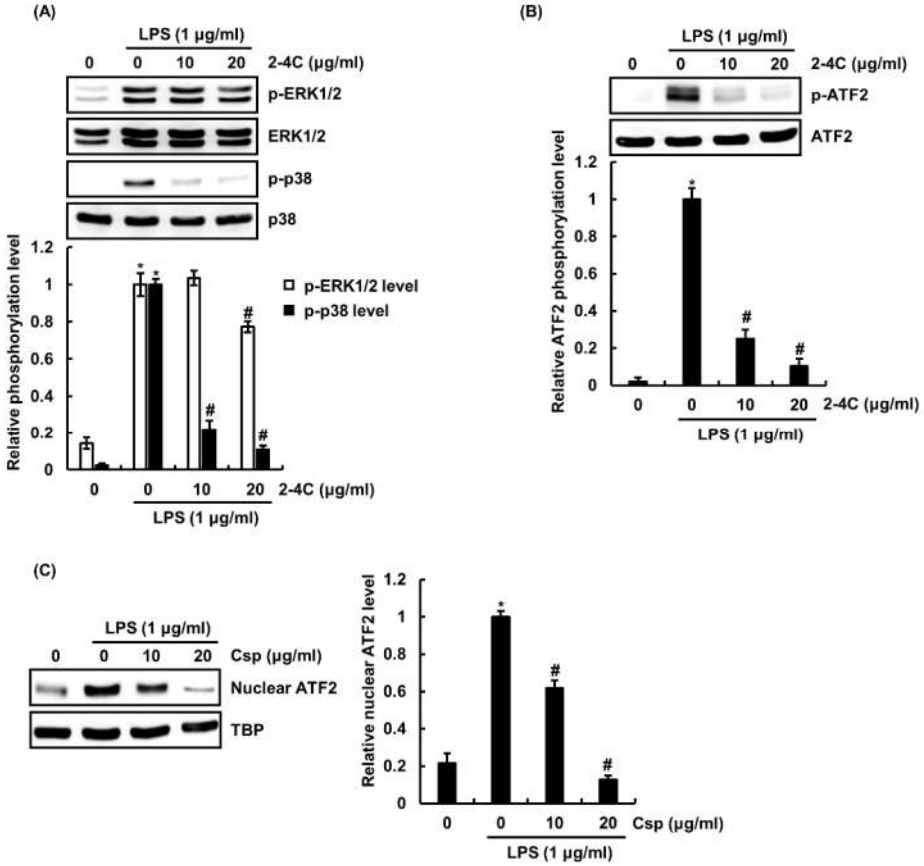

Figure 8. Effect of *C. sphaerocarpa* EO (2-4C) on phosphorylation of ERK1/2, p38 (A) and ATF2 nuclear accumulation (B and C) in LPS-induced RAW264.7 cells. The levels of p-ERK1/2, p-p38 and p-ATF2 were determined using the Western blot method. The cells were stimulated with LPS (1 μg/μL) in the presence of *C. sphaerocarpa* EO (at 10 and 20 μg/mL).

Furthermore, we tested the inhibitory effect of *C. sphaerocarpa* EO against NO production by ZnPP as a HO-1inhibitor to investigate whether HO-1 expression by *C. sphaerocarpa* EO is responsible to their anti-inflammatory effects. The inhibition of HO-1 by ZnPP decreased the inhibitory effect of *C. sphaerocarpa* EO against NO production in LPS-induced RAW264.7 cells (Figure 9D) which shows that HO-1 might be one of the molecular targets responsible for the observed anti-inflammatory effects of *C. sphaerocarpa* resin EO compared with EO of resins of other *Commiphora* species.

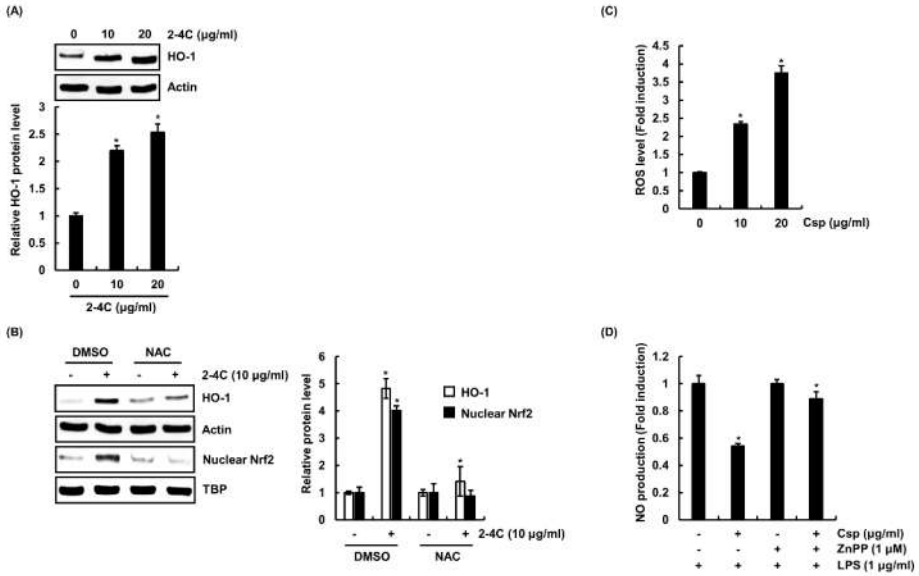

Figure 9. Effect of *C. sphaerocarpa* EO (2-4C) on (A) HO-1 expression in RAW264.7 cells; (B) The presence of NAC inhibited Nrf2 nuclear accumulation and HO-1 expression; (C) Increased ROS level in RAW264.7 cells; (D) The inhibition of HO-1 by ZnPP decreased the inhibitory effect of *C. sphaerocarpa* EO against NO production in LPS-stimulated RAW264.7 cells.

CONCLUSION

We found the anti-inflammatory activity of *Commiphora* resin EOs depended on the type and number of chemical compounds present in the plant. The anti-inflammatory effect increases in the order with EOs rich in aliphatic hydrocarbons, monoterpenes, and sesquiterpenes, respectively. Thus, EO of *C. sphaerocarpa* resin was especially rich in sesquiterpenes namely α-copaene, β-caryophyllene, and β-caryophyllene oxide and showed significantly higher for anti-inflammatory activities than *C. africana* EO rich in monoterpenes. The EO of *C. sphaerocarpa* resin exerted anti-inflammatory activity by inhibiting NF-κB and MAPK signaling pathways and the activation of Nrf2/HO-1 signaling pathways. The EOs from the other two *Commiphora* species showed no anti-inflammatory effect. Sesquiterpenes such as α-copaene, β-caryophyllene. β-caryophyllene oxide and α-humulene might be employed for inflammatory diseases treatment either in a pure form or synergistically though further *in-vivo* dose dependent anti-inflammatory effects investigation of these sesquiterpenes is required. The observed anti-inflammatory effects of the EO of the resin of *C. sphaerocarpa* proves claims of local people use of the resin during

the stomach complaints. There are more than 52 *Commiphora* species reported to be available only in Ethiopia, a detailed chemical composition and mechanisms associated with their anti-inflammatory effects of EOs from these *Commiphora* resins are largely unknown and require due attention. We reviewed the ethnobotanical, phytochemical, and pharmacological activities of resins of different *Commiphora* species and indicated they have a lot of prospects to be used as traditional medicines and in pharmaceutical industries [44]. A preliminary clinical study conducted on myrrh showed its anti-inflammatory and anti-plaque activities [45]. However, further investigations should still be carried out to confirm their safety and efficacy resins and essential oils of different *Commiphora* species.

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