Iranian Journal of Pharmaceutical Sciences
2021: 17 (3): 63-70
www.ijps.ir

Original Article

The Analgesic Effect of *Salvia reuterana*

Hamidreza Monsef Esfahani[a], Keyvan Amirshahrokhi[b], Atiyeh Mashmool[a], Mahdi Moridi Farimani[c], Ahmadreza Dehpour[d], Mansour Miran[e,*]

[a]Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, [b]Department of Pharmacology and Toxicology, School of Pharmacy, Ardabil University of Medical Sciences, Ardabil, Iran, [c]Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran, [d]Department of Pharmacology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran, [e]Department of Pharmacognosy and Biotechnology, School of Pharmacy, Ardabil University of Medical Sciences, Ardabil, Iran.

Abstract

Salvia reuterana, commonly known as Maryam Goli Esfahani, is a member of the Labiateae family. In Iranian folk medicine, aerial parts of *S. reuterana* have been used as sedative and anxiety. Evaluation of various extracts of the plant for their analgesic activity revealed that treatment of mice with *n*-hexane extract (500 mg/kg, i.p.) significantly increased the latency time as compared to the control group. Fractionation of the hexane extract of *S. reuterana* led to the isolation of sclareol as the main compound (0.19% w/w). Column chromatography was used to isolation of compounds from *S. reuterana* and a spectroscopic method including NMR was used to identification of the isolated compound. Evaluation of the analgesic effect of sclareol using a hot plate, tail-flick, and formalin tests in mice confirmed the potent analgesic effect of sclareol as an effective compound of *S. reuterana*. These results showed that the *n*-hexane extract of aerial parts of *S. reuterana* and its main constituent sclareol showed significant analgesic activity in different rodent nociceptive behavioral tests.

Keywords: Analgesic Activity, Formalin Test, Hot Plate Test, *Salvia reuterana*, Sclareol, Tail-Flick Test.

Corresponding Authors: Mansour Miran, Department of Pharmacognosy and Biotechnology, School of Pharmacy, Ardabil University of Medical Sciences, Ardabil, Iran,
Email: m.miran@arums.ac.ir
Tel: +45 33522437
Cite this article as: Monsef Esfahani H, Sarmadian H, Amirshahrokhi K, Mashmool A, Moridi Farimani M, Dehpour A, Miran M, *The Analgesic Effect of Salvia reuterana*, 2021, 17(3): 63-70.

1. Introduction

Salvia is the largest genus in the Lamiaceae family, consisting of around 1000 species. This genus of plants is widely distributed in America and Asia. *Salvia* species are one of the most important medicinal plants widely used to treat different disorders, including pains, epilepsy, cancers, tuberculosis, hepatitis, and menstrual disorders [1]. It has been shown that *Salvia* species exert significant effects on the central nervous system (CNS) [2]. *Salvia lavandulaefolia* was reported to show memory

enhancing effect. *Salvia leriifolia* has a neuroprotective effect in an animal model of ischemia. It has been reported that some species of *Salvia* such as *S. aethiopis, S. miltiorrhiza,* and *S. leriifolia* exhibited potent analgesic properties in animal models of hot-plate and tail-flick tests. Study the mechanism of action of *Salvia* species revealed that their pharmacologic effects on CNS may mediate by GABA, glutamate, and opioid receptors [2].

Numerous diterpenoids, typically abietane s and rearranged abietanes with bioactivities, including anticancer, cardiotonic , and topoisomerase inhibitor properties, have been isolated from Salvia species [3].

S. reuterana is endemic to Iran and is known as Maryam Goli Esfahani in Persian. This species grows in the most areas of Iran such as Golestan, West Azerbaijan, Kermanshah, Tehran, Esfahan and Fars provinces [3, 4]. *S. reuterana* is a rich source of sclareol and its derivatives including 14α-hydroxy-15-chlorosclareol, 14α-hydroxy-15-acetoxysclareol, 6β-hydroxy-14α-epoxysclareol, 6β-hydroxysclareol, 14α-epoxysclareol, 6β, 14α-dihydroxy-15-acetoxysclareol, and 14α, 15-dihydroxy sclareol [3, 5]. Moreover, germacrene D, bicyclogermacrene and β-caryophyllene are the main compounds in the essential oil of *S. reuterana* [6].

It has been demonstrated that extract of *S. reuterana* shows a remarkable anxiolytic effect. Indeed *S. reuterana* has been suggested as a sedative and anxiolytic medicinal plant [7]. The aim of the present study was to investigate the effect of *n*-hexane, ethyl acetate, and methanol extracts of aerial parts of *S. reuterana* on pain response using a hot plate and tail-flick tests in mice.

2. Material and Methods

2.1. Animal

Experiments were performed on adult and healthy NMRI mice weighing 20 to 25 g. Animals were kept in our animal house under controlled conditions (22 ± 2°C, 12 h light/dark cycle) and allowed free access to water and a standard diet. All animal processes were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and with the approval of our university Ethics Committee.

2.2. Experimental Design

Twenty-five mice were divided into five groups (n= 5); (1) Normal control group: mice received saline and DMSO (20 µl, i.p.); (2) Morphine group: mice received morphine (6.25 mg/kg, i.p.) dissolved in saline; (3) Hexane group: mice received hexane extract of *S. reuterana* (500 mg/kg, i.p.); (4) Ethyl acetate group: mice received ethyl acetate extract of *S. reuterana* (500 mg/kg, i.p.); (5) Methanol group: mice received methanol extract of *S. reuterana* (500 mg/kg, i.p.). The dose of *S. reuterana* extracts used in this study was selected based on our preliminary experiments. We used several dosages of the extracts and 500 mg/kg was the lowest dose

that produced a significant analgesic effect in mice.

2.3. Nociceptive Behavioral Tests

2.3.1. Hot Plate Test

The hot plate test was done according to the method described by Eddy and Leimbach (1953). Mice were placed on a hot plate with the temperature set to 55 ± 1 °C. The first signs of nociception, including paw licking or jumping, were recorded, and the mouse was immediately removed from the hot plate. A cut-off time of 60 seconds was used to prevent tissue damage in mice. If the mouse showed no response within 60 seconds, we removed the mouse from the hot plat apparatus.

2.3.2. Tail-Flick Test

The tail-flick test was done according to the method described by D'Amour and Smith in (1941). We used a tail-flick test apparatus to measure the analgesic effect of *S. reuterana*. An intense light beam was focused on a small spot on the tail of the mouse, and the time required for the mouse to flick its tail to the side was recorded. A cut-off time of 10 seconds was set to prevent tissue damage in mice. When the animal did not flick his tail in the cut-off time, the light beam turned off automatically to prevent tissue damage of mice.

2.3.3. Formalin Test

The formalin test was done according to the method described by Dubuisson and Dennis (1977). In this method, a dilute solution of formaldehyde in 0.9 % saline (50 µl of 3% formalin) was injected subcutaneously into the dorsal surface of the mice's hind paw. The mice were then placed on a glass plate under a glass funnel. In a formalin test, the behaviors indicative of pain include shaking the paw and licking or biting the injected paw. The pain response is the amount of time that mice spend licking the injected paw. There are two distinct periods or phases of licking behavior; the early or acute phase lasting the first 5 min and the late or chronic phase lasting from 15 to 45 min after formalin injection. Two groups of mice were used for the formalin test; mice in normal control group received saline 0.9%, and mice in sclareol group received sclareol (20 mg/kg, i.p.). Formalin was injected 30 min after receiving sclareol, and the test was started.

2.4. Statistical Analysis

Results were analyzed statistically by one-way analysis of variance (ANOVA) followed by Tukey's posthoc test. Data were expressed as means ± standard error (SEM). Differences between groups were considered to be significant when $P < 0.05$.

2.5. Plant Material

S. reuterana aerial parts were collected from the northern hilly areas of Tehran, Iran, in 2015. Before analysis, the plant material was dried in the shade and stored in the dark at 20° C before analysis. A voucher specimen ((MPH-1321) has been deposited in the herbarium of the Medicinal Plant and Drug Research Institute (MPH) of Shahid Beheshti University, Tehran, Iran.

2.6. Extraction and Isolation

Stock extracts were prepared with 50 g powdered aerial parts of the plant and extracted by n-hexane, ethyl acetate and methanol solvents, respectively. 500 mg of each extract was dissolved in 10 mL of normal saline, and 20 µL of DMSO was used to increase the solubility of the extracts. In order to isolate the constituents from the active extract, the air-dried aerial parts of *S. reuterana* (500 g) were extracted with n-Hexane (3 × 1.5 L) by maceration at room temperature. The extract was concentrated in a rotary evaporator to afford 15 g of a dark gummy residue. A portion of the extract (10 g) was subjected to a silica gel column chromatography (230-400 mesh, 250g) with a gradient of hexane–EtOAc (100/0 to 0/100) to give 5 fractions. Fraction 3 (1.5 g, washed with hexane–EtOAc 50:50) was separated on a silica gel column chromatography (70-230 mesh, 40 g) and eluted with a gradient of hexane – CH3Cl (65:35) as isocratic, to afford 950 mg compound 1.

2.7. General Experimental Procedure

NMR spectra were recorded at a target temperature of 18 °C on a Bruker Avance III 500 MHz spectrometer operating at 500.13 MHz for ^{1}H, and 125.77 MHz for ^{13}C.

3. Result and Discussion

3.1. Hot Plat and Tail-Flick Tests

To evaluate the analgesic activity of *S. reuterana*, the mice were treated with *n*-hexane, ethyl acetate, and methanol extracts at a dose of 500 mg/kg (single dose, i.p.). Morphine (6.25 mg/kg) was injected into a group of mice as the positive control group. After 30 min, mice were tested for the hot plate and tail-flick tests for the pain response. As shown in Figures 1A and 1B, the hot plate and tail-flick tests results revealed that the treatment of mice with *n*-hexane extract of *S. reuterana* (500 mg/kg, i.p.) significantly increased the latency time as compared to the control group. The analgesic effect of ethyl acetate and methanol extracts was not significant as compared to the control group. Therefore, the *n*-hexane extract of *S. reuterana*

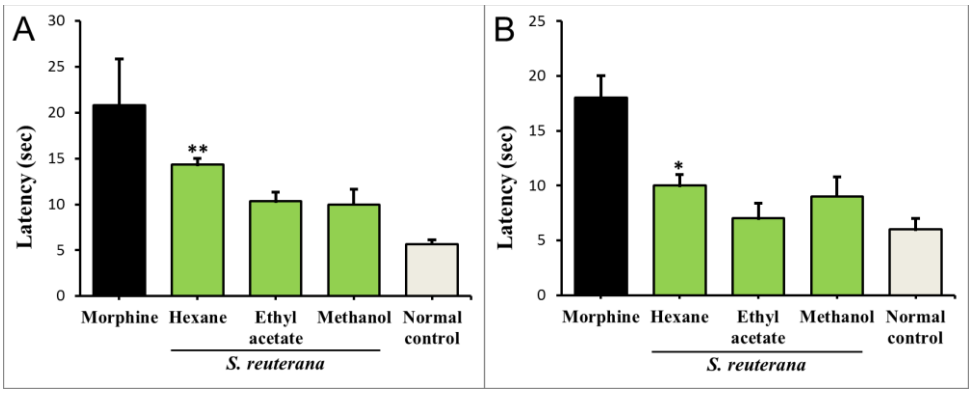

Figure 1. The analgesic effect of the hexane, ethyl acetate and methanol extract from *S. reuterana* (500 mg/kg, i.p.) on the hot plate (A) and tail flick (B) tests. Data were expressed as means ± SEM. **P* < 0.05, ***P* < 0.01 as compared to the control group.

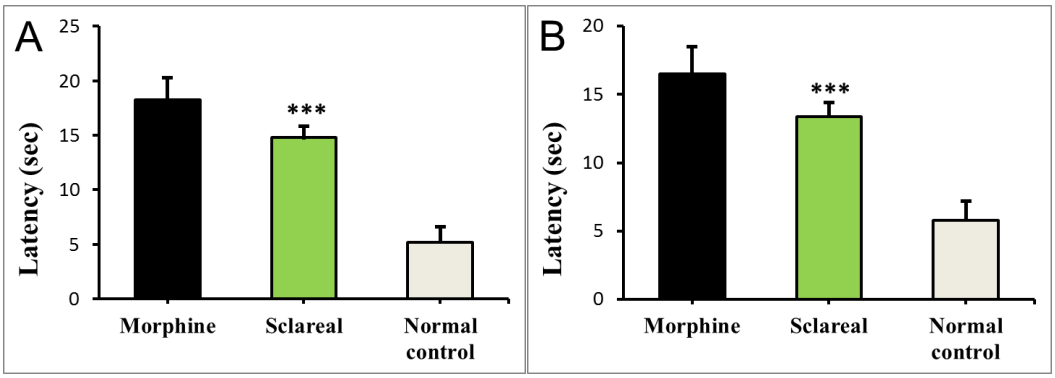

Figure 2. The analgesic effect of sclareol (20 mg/kg, i.p.) on the hot plate (A) and tail flick (B) tests. Data were expressed as means ± SEM. ****P* < 0.001 as compared to the control group.

was used to isolate its effective compound. Sclareol was isolated as the effective compound in the *n*-hexane extract of *S. reuterana*.

As shown in Figures 2A and 2B, the results from the hot plate and tail-flick tests revealed that treatment of mice with sclareol (20 mg/kg, i.p.) significantly increased (P < 0.001) the latency time as compared to the control group.

3.2. Formalin Test

As shown in figure 3, the results from the formalin test showed that treatment of mice with sclareol (20 mg/kg, i.p.) significantly reduced the formalin-induced pain in both early (p < 0.01) and late (p < 0.05) phases.

3.3. Identification of Sclareol

The ^{13}C-NMR spectrum of compound **1** indicated 20 carbon resonances at δ_c 72.5 and 73.0 showed the presence of two hydroxyl groups. The ^{1}H-NMR spectrum showed five methyl groups at δ_H 0.80, 0.87, 0.92, 1.11 and 1.21. Resonances at 4.04 (dd. J= 2, 10 Hz), 5.20 (dd, J=2, 17 Hz) and 5.95 (dd, J= 10.7, 17.3Hz) represent monosubstituted olephin group. Therefore, the structure of compound **1** was identified as sclareol (figure 4) using ^{1}H-NMR

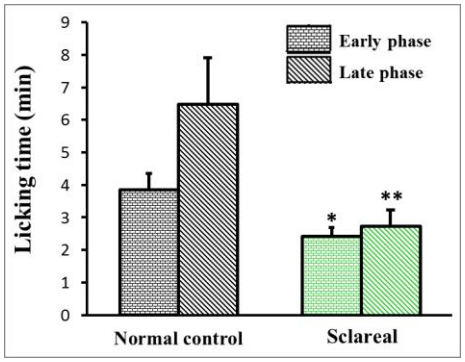

Figure 3. The analgesic effect of sclareol (20 mg/kg, i.p.) on the formalin test. Data were expressed as means ± SEM. **P* < 0.05 as compared to the early phase of the control group. ***P* < 0.01 as compared to the late phase of the normal control group.

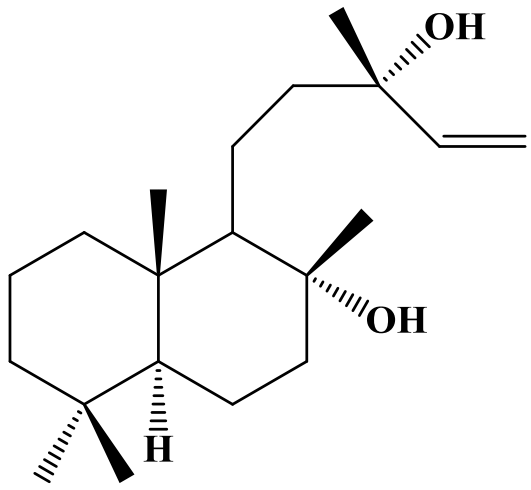

Figure 4. Structure of sclareol.

and ^{13}C-NMR, as well as by comparison with the literature [8].

The hot plate and tail-flick tests are commonly used models for the evaluation of acute thermal pain response in animals. Both methods are generally used for testing the effectiveness of centrally acting analgesic agents. Paw-licking and jumping are the two parameters measured in the hot plate test. Licking is considered as a rapid response to a painful thermal stimulus, and jumping is a late and elaborated response. Our findings showed that the *n*-hexane extract of *S. reuterana* significantly increased pain tolerance in mice. So, the *n*-hexane extract was selected to isolate the active compound. Finally, sclareol was isolated as the active and significant compound.

To evaluate the analgesic effect of sclareol, we treated mice with sclareol (20 mg/kg, i.p.). This group of mice was tested for the pain response by the hot plate, tail-flick, and formalin tests. Our results showed that sclareol significantly increased the tolerance of pain in mice. This finding demonstrated the analgesic effect of sclareol as an effective compound of *S. reuterana*.

The formalin test is a chemical model to continuously assess pain induced by injured tissue. In this method, the response to formalin-induced pain shows an early and a late phase. The early phase is due to the peripheral stimulus and a direct effect on nociceptors. The late phase appears to be an inflammatory response induced by the release of inflammatory mediators [9]. We used formalin test to assess the analgesic effect of sclareol further. Our findings showed that Sclareol significantly reduced formalin-induced pain in mice's early and late phases. Indeed, this result confirmed the potent analgesic effect of sclareol as an effective compound of *S. reuterana*.

Salvia species were found to possess significant effects on CNS. It has been reported that the ethanolic extract of *S. haematodes* Wall exhibited remarkable analgesic activity [10]. It has been found that chloroform extract of *S. wiedemannii* Boiss. (500 mg/kg) showed significant analgesic activity in comparison to morphine [11]. The analgesic and antipyretic effects of *S. africana-lutea* were demonstrated using acetic acid-induced writhing and hot plate tests in mice and rat [12]. Another species of *Salvia* including *S. aegyptiaca*, *S. aethiopis*, *S. leriifolia*, *S. mexicana* and *S. divinorum* were evaluated because of their analgesic activity [2].

S. reuterana is a source for labdane diterpenes such as sclareol and related compounds. Our previous researches led to the isolation and identification of several new labdane diterpenoids from aerial parts of *S. reuterana* [3, 5]. Diterpene compounds have been reported to possess analgesic activity. It has been shown that, a furan labdane diterpene, known as Marrubiin, is the main analgesic compound of *Marrubium vulgare*. [13]. Grayanotoxins were diterpenoids isolated from the leaves of *Rhododendron micranthum* and showed significant analgesic effects in an acetic acid-induced writhing test [14]. Phytochemical investigations on the leaves of *Rhododendron auriculatum* led to the isolation of sixteen diterpenoids with significant analgesic activity

[15]. Neorogioltriol is a brominated diterpene with analgesic activity that isolated from *Laurencia glandulifera* [16]. Aethiopinone is an o-naphthoquinone diterpenoid from *Salvia aethiopis* roots. Aethiopinone showed analgesic, anti-inflammatory, and antipyretic effects in the animal model of tail immersion test [17]. There are various studies about the pharmacological and therapeutic properties of sclareol. [18-25]. It has been shown that sclareol has a therapeutic effect against rheumatoid arthritis in experimental models. Sclareol at 5 and 10 mg/kg doses reduced paw swelling and relieved arthritic severities in mice. Sclareol reduced inflammation by decreasing inflammatory cytokines (TNF-α, IL-1β, IL-6) and downregulation of nuclear factor-κB (NF-κB) mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways [26]. For the first time, our study demonstrated that sclareol (20 mg/kg) as the active compound of *S. reuterana* showed remarkable antinociceptive effects in mice.

4. Conclusion

This study demonstrated that treating mice with n-hexane extract of aerial parts of S. reuterana (500 mg/kg, i.p.) showed significant analgesic activity in hot plate, tail-flick, and formalin tests. Fractionation of the hexane extract of *S. reuterana* led to the isolation of sclareol as the main compound. Evaluation the analgesic effect of sclareol in mice confirmed the potent analgesic effect of sclareol as an effective compound of S. reuterana. We recommend that further studies are needed to clarify the molecular mechanism underlying the analgesic effect of sclareol.

References

[1] Wu YB, Ni ZY, Shi QW, Dong M, Kiyota H, Gu YC, Cong B. Constituents from *Salvia* species and their biological activities. *Chem. Rev*. (2012) 112(11): 5967-6026.

[2] Imanshahidi M, Hosseinzadeh, H. The pharmacological effects of *Salvia* species on the central nervous system. *Phytother Res*. (2006) 20(6): 427-37.

[3] Moridi Farimani M, Miran M. Labdane diterpenoids from *Salvia reuterana. Phytochemistry*. (2014) 108: 264-269.

[4] Rechinger KH. Flora Iranica; Akademische druck und verlagsanstalt. Graz, Austria. (1987) 150, 403-476.

[5] Moridi Farimani M, Miran M, Nejad Ebrahimi S. New diterpenoids from the aerial parts of *Salvia reuterana. Iran. J. Pharm. Res*. (2019) 18(1): 406-411.

[6] Esmaeili A, Rustaiyan AH, Nadimi M, Larijani K, Nadjafi F, Tabrizi L, Chalabian F, Amiri H. Chemical composition and antibacterial activity of essential oils from leaves, stems and flowers of *Salvia reuterana* Boiss. grown in Iran. *Nat. Prod. Res*. (2008) 22(6): 516-520.

[7] Rabbani M, Sajjadi S.E, Jafarian A, Vaseghi G. Anxiolytic effects of *Salvia reuterana* Boiss. on the elevated plus-maze model of anxiety in mice. *J. Ethnopharmacol*. (2005) 101: 100-103.

[8] Koval'skaya S, Kozlov N, Kulcitki V, Aricu A, Ungur N. Transformation of sclareol under ritter reaction conditions. *Russ. J. Org*. (2013) 49. [9] Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: An evaluation of the method. *Pain*. (1992) 51(1): 5-17.

[10] Akbar A, Tariq M, Nisa M. Pharmacological studies on *Salvia haematodes* Wall. *Acta Trop*. (1985) 42 (4): 371–374.

[11] Ustun O, Sezik E. Analgesic Activity of *Salvia wiedemannii* Boiss. Used in Turkish folk medicine. *Rec. Nat. Prod*. (2011) 5: 328-331.

[12] Amabeoku GJ, Eagles P, Scott G, Mayeng I, Springfield E. Analgesic and antipyretic effects of *Dodonaea angustifolia* and *Salvia africana-lutea*. *J. Ethnopharmacol*. (2001) 75(2): 117-124.

[13] Meyre-Silva C, Yunes RA, Schlemper V, Campos-Buzzi F, Cechinel-Filho V. Analgesic potential of marrubiin derivatives, a bioactive diterpene present in *Marrubium vulgare* (Lamiaceae). *Farmaco*. (2005) 60(4): 321-326.

[14] Sun N, Qiu Y, Zhu Y, Liu J, Zhang H, Zhang Q, Yao G. Rhodomicranosides A−I, analgesic diterpene glucosides with diverse carbon skeletons from *Rhododendron micranthum*. *Phytochemistry*. (2019) 158: 1-12.

[15] Sun N, Feng Y, Zhang Q, Liu J, Zhou H, Zhang H, Yao G. Analgesic diterpenoids with diverse carbon skeletons from the leaves of *Rhododendron auriculatum*. *Phytochemistry*. (2019) 168: 112113.

[16] Chatter R, Kladi M, Tarhouni S, Maatoug R, Kharrat R, Vagias C, Roussis V. Neorogioltriol: A brominated diterpene with analgesic activity from *Laurencia glandulifera. Phytochem. Lett*. (2009) 2(1): 25-28.

[17] Hernandez-Perez M, Rabanal RM, De La Torre M C, Rodriguez B. Analgesic, anti-inflammatory, antipyretic and haematological effects of aethiopinone, an o-naphthoquinone diterpenoid from *Salvia aethiopis* roots and two hemisynthetic derivatives. *Planta. Med*. (1995) 61(6): 505-509.

[18] Wu PC, Chuo WH, Lin SC, Lehman CW, Lien CZ, Wu CS, Lin CC. Sclareol attenuates the development of atopic dermatitis induced by 2,4-dinitrochlorobenzene in mice. *Immunopharmacol. Immunotoxicol*. (2019) 41(1): 109-116.

[19] Noori S, Hassan ZM, Mohammadi M, Habibi Z, Sohrabi N, Bayanolhagh S. Sclareol modulates the Treg intra-tumoral infiltrated cell and inhibits tumor growth *in vivo*. *Cell Immunol*. (2010) 263(2): 148-153.

[20] Mahaira LG, Tsimplouli C, Sakellaridis N, Alevizopoulos K, Demetzos C, Han Z, Dimas K. The labdane diterpene sclareol (labd-14-ene-8, 13-diol) induces apoptosis in human tumor cell lines and suppression of tumor growth *in vivo* via a p53-independent mechanism of action. *Eur. J. Pharmacol*. (2011) 666(1): 173-182.

[21] Borges GSM, Silva JdO, Fernandes RS, de Souza ÂM, Cassali GD, Yoshida MI, Ferreira LAM. sclareol is a potent enhancer of doxorubicin: Evaluation of the free combination and co-loaded nanostructured lipid carriers against breast cancer. *Life. Sci*. (2019) 232: 116678.

[22] Dimas K, Kokkinopoulos D, Demetzos C, Vaos B, Marselos M, Malamas M, Tzavaras T. The effect of sclareol on growth and cell cycle progression of human leukemic cell lines. *Leuk. Res*. (1999) 23(3): 217-234.

[23] Campos DR, Celotto AC, Albuquerque AAS, Ferreira LG, Monteiro A, Coelho EB, Evora PRB. The diterpene sclareol vascular effect in normotensive and hypertensive rats. *Arq. Bras Cardiol*. (2017) 109(2): 117-123.

[24] Park JE, Lee KE, Jung E, Kang S, Kim YJ. Sclareol isolated from *Salvia officinalis* improves facial wrinkles via an antiphotoaging mechanism. *J. Cosmet. Dermatol*. (2016) 15(4): 475-483.

[25] Crusco A, Whiteland H, Baptista R, Forde-Thomas JE, Beckmann M, Mur LAJ, Hoffmann KF. Antischistosomal properties of sclareol and its heck-coupled derivatives: Design, synthesis, biological evaluation, and untargeted metabolomics. *ACS Infect. Dis*. (2019) 5(7): 1188-1199.

[26] Tsai SW, Hsieh MC, Li S, Lin SC, Wang SP, Lehman CW, Lin CC. Therapeutic potential of sclareol in experimental models of rheumatoid arthritis. *Int. J. Mol. Sci*. (2018) 19 (5): 1351.