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Investigation of *in vitro* wound healing properties of *Salvia sclarea* and *Citrus aurantium* essential oil combinations

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ABSTRACT: *Salvia sclarea* L. and *Citrus aurantium* L. are used ethnobotanically for wound treatment. The aim of this study was to evaluate phytochemical composition of *Salvia sclarea* L. and *Citrus aurantium* L. leaf essential oils and wound healing activities of their combinations. The phytochemical analysis was performed gas chromatography (GC) and mass spectroscopy (MS). Major components of *S. sclarea* leaf essential oil was confirmed both by GC-FID and GC/MS as 67% linalyl acetate, 20.2% linalool, 2.6%α-terpineole, respectively. The major components of *C. aurantium* leaf essential oil was also confirmed as 55% linalyl acetate, 23.4% linalool, 5.4%α-terpineole, respectively. Combinations of essential oils were loaded into Carbopol gel formulations. Wound healing activity of essential oil and combinations was investigated using *in vitro* scratch assay. As a result of the study, it was determined that the most effective wound healing concentration for *Salvia sclarea* essential oil was 50 µg/mL. The most effective concentration of *Citrus aurantifolia* essential oil was determined as 12.5 µg/mL. The aim of the study is to investigate the wound healing activities of the tested essential oils individually and in combination. The fact that no activity is observed in empty formulations is evidence that the activity of the loaded formulations does not come from empty formulations. It was observed that the loaded formulations showed better effects than the control Madecassol. Studies need to be confirmed by working under *in vivo* conditions.

KEYWORDS: *Salvia sclarea*; *Citrus aurantium*; wound healing; essential oil.

1. INTRODUCTION

Salvia sclarea L. is a plant belonging to the Lamiaceae family, known by names such as paskulak, clary sage and salba. It is popularly used to treat colds, mouth sores, digestive problems and wounds [1-2]. *In vitro* studies have concluded that *Salvia* sp. is a natural antimicrobial and can be used to treat wounds. According to the results of the previous *in vitro* wound study conducted with *Salvia haenkei* extract, it was observed that the extract had a 25.1% effect compared to the control group. The wound healing effect of *Salvia multicaulis* was evaluated in the *in vivo* model and all concentrations were found with wound healing potential compared to the control group. It was also observed that it stimulates angiogenesis in fibroblast cells. Additionally, it was demonstrated that *S. sclarea* essential oil is effective in wound healing [3-6].

Citrus aurantium L. is generally known as bitter orange. Its leaves, fruit peels, fruits and essential oil are used for many purposes. It has many traditional uses, including anxiety, obesity and wound treatment [7]. Previous studies showed that fruit peel essential oils heal ulcer wounds in rats [8]. In addition, the wound healing effect of formulations prepared from extracts obtained from *C. aurantium* fruit peel was detected in rats [9].

Medicinal extracts/essential oils accelerate the wound healing process by showing antimicrobial, anti-inflammatory and antioxidant effects; It is also known that they have a positive effect on this process by providing a humid environment [10-12]. In addition, it was shown in previous studies that some essential oils contribute to collagen fibril formation [13].

In this study, essential oils of *Salvia sclarea* L. and *Citrus aurantium* L. leaves, which are known to have potential effects on wound healing, were used. It is known that different preparations of these oils have

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antimicrobial and antioxidant effects in ethnobotanical and experimental studies, and some types promote healthy cell proliferation and angiogenesis. To the best of our knowledge this is the first extensive comparative *in vitro* wound healing activity evaluation of *S. sclarea* and *C. aurantium* essential oils. The *in vitro* cytotoxic effects on healthy cell lines such as HEK293 cells were screened for loaded formulations. Therefore, the hypothesis of this study is to develop a formulation of these two plants and their combinations, which are known as traditionally used in wound treatment, and to evaluate the results in wound treatment.

2. RESULTS

2.1. Phytochemical analysis

As a result of GC/MS analysis of *Salvia sclarea* essential oil, the major compounds were identified as linalyl acetate (67%), linalool (20.2%), α-terpineol (2.6%), geranyl acetate (1.7%), germacrene D (1.3%), β-caryophyllene (1.2%), geraniol (1.1%), respectively. 97.3% of the essential oil components were identified, 23 components in total. The main components of *Citrus aurantium* essential oil were identified as linalyl acetate (55%), linalool (23.4%), α-terpineol (5.4%), geranyl acetate (4%), geraniol (2.8%), neryl acetate (2.3%), (E)-β-oxymene (1.3%), myrcene (1.2%), nerol (1.1%), respectively. 22 different components were identified in the essential oil (Table 1).

Table 1. GCMS analysis results of tested essential oils

RRI	Component	*S. sclarea* %	*C. aurantium* %
1032	*α*-Pinene	0.1	0.1
1035	*α*-Thujene	-	tr
1118	*β*-Pinene	0.1	0.8
1132	Sabinene	tr	0.1
1159	*δ*-3-Carene	-	0.3
1174	Myrcene	0.3	1.2
1203	Limonene	0.2	0.6
1218	*β*-Phellandrene	-	tr
1246	(*Z*)-*β*-Ocimene	tr	0.4
1255	γ-Terpinene	tr	tr
1266	(*E*)-*β*-Ocimene	tr	1.3
1280	*p*-Cymene	0.1	-
1290	Terpinolene	-	0.3
1497	*α*-Copaene	0.5	-
1535	*β*-Bourbonene	tr	-
1553	Linalool	20.2	23.4
1565	Linalyl acetate	67.0	55.0
1611	Terpinen-4-ol	-	tr
1612	*β*-Caryophyllene	1.2	0.8
1706	*α*-Terpineol	2.6	5.4
1726	Germacrene D	1.3	-
1733	Neryl acetate	0.2	2.3
1755	Bicyclogermacrene	-	0.1
1765	Geranyl acetate	1.7	4.0
1773	*δ*-Cadinene	0.1	-
1808	Nerol	tr	1.1
1857	Geraniol	1.1	2.8
2008	Caryophyllene oxide	0.4	-
2144	Spathulenol	0.2	-
	Total	**97.3**	**100**

2.2. Scratch assay and cytotoxicity

In this study, the wound healing effects of *Salvia sclarea* and *Citrus aurantium* essential oils at different concentrations (12.5, 25, 50 µg/mL) were observed. The results were evaluated by comparing the data

obtained with the positive control (Madecassol) and blank formulations. According to the results of the study, *Salvia sclarea* essential oil showed a very effective example of proliferation and cell migration and almost completely closed the wound within 24 hours. The most effective dose for *Salvia sclarea* was found as 50 µg/mL. However, as the concentration of *Citrus aurantium* essential oil increased, the number of cells decreased. Therefore, the most effective dose of *Citrus aurantium* essential oil was determined as 12.5 µg/mL.

After experiments were carried out with each essential oil, the loaded rates into the formulation were determined according to the test results. In the filled formulation, the healing effects of 50 µg/mL *Salvia sclarea* + 12.5 µg/mL *Citrus aurantium* essential oils on the wound were checked. It was observed that it had a greater wound healing effect compared to the control groups. It was determined that the individual effects of essential oils are higher than their combined effects. The results are given in Figure 1 and Figure 2.

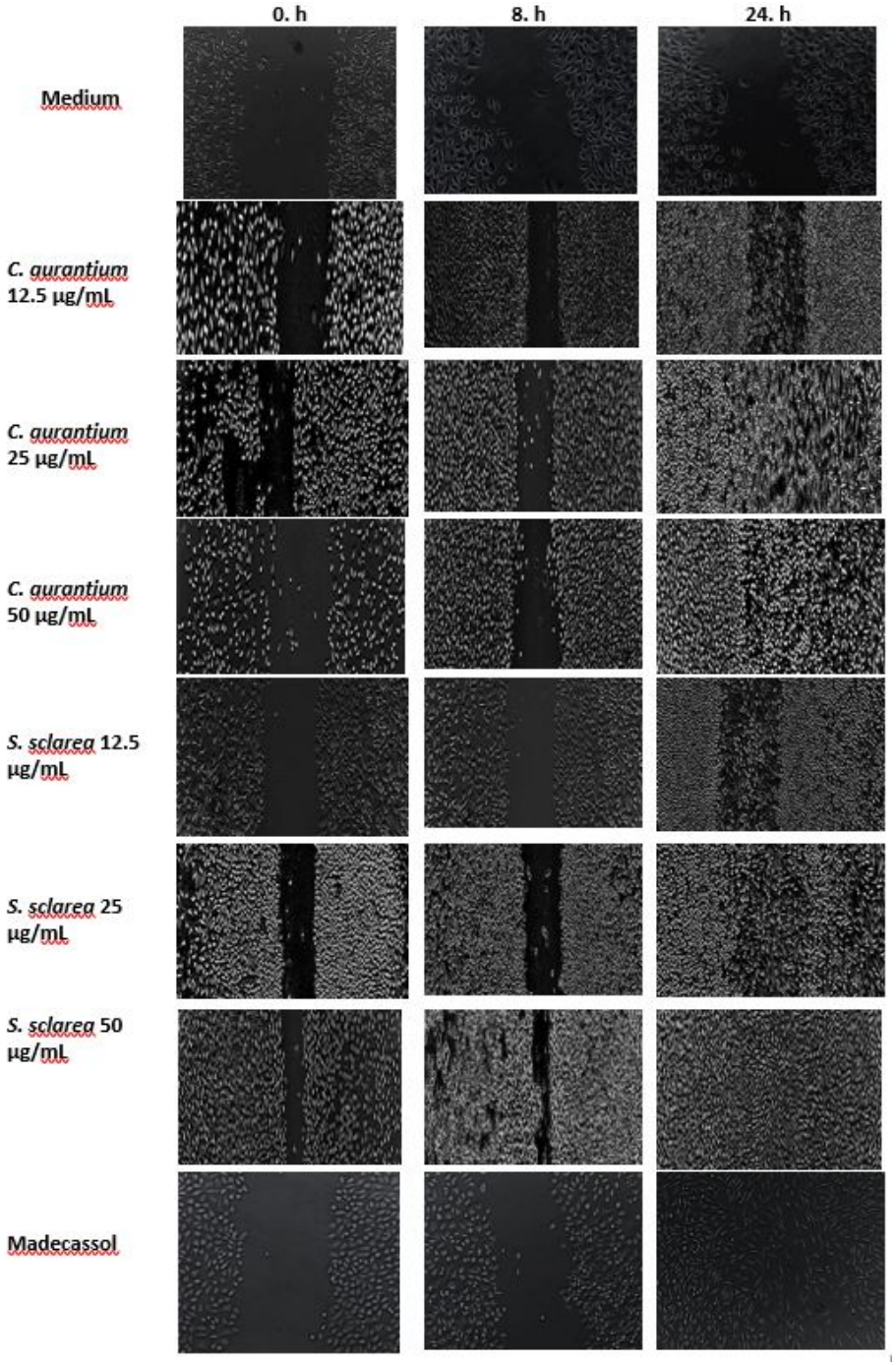

Figure 1. Wound Healing Images of Essential Oils Applied at 0 - 8 - 24 Hours

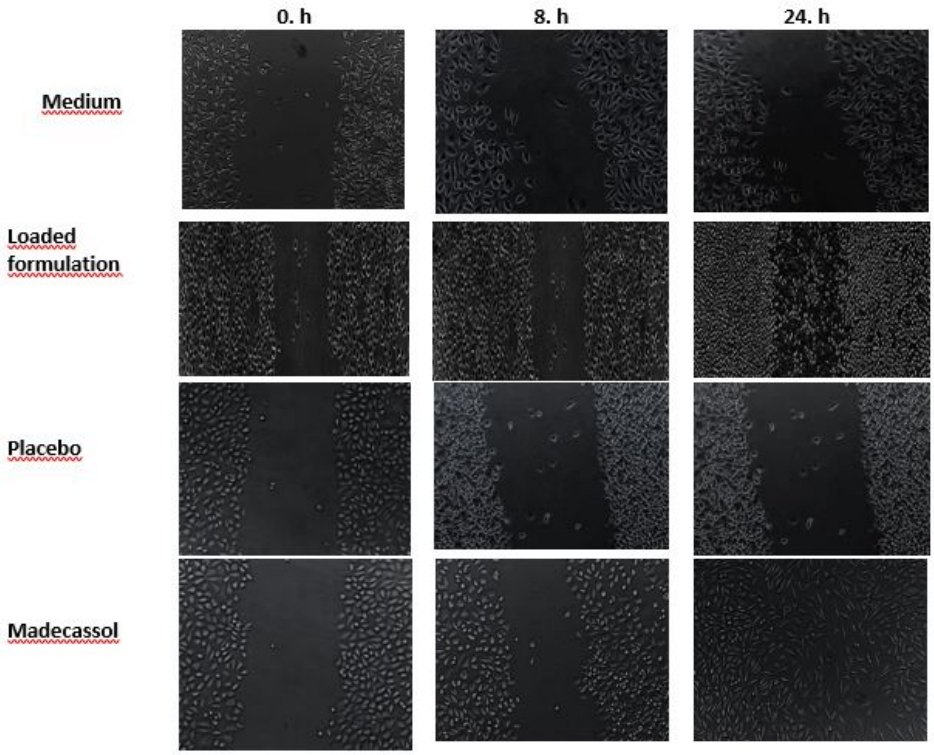

Figure 2. Wound Healing Images of Formulations Applied at 0 - 8 - 24 Hours

In this study, the loaded and empty gel was applied at a concentration of 2 mg/mL on healthy mouse fibroblast cells (L929) and did not show toxicity even at the maximum concentration. It is important for safe use that it does not have a toxic effect.

3. DISCUSSION

Since wound treatment is a complex process involving various aspects with significant influence on healing, this is an unfulfilled clinical challenge in healthcare. According to traditional definitions, hemostasis, inflammation, proliferation, and tissue remodeling are four separate stages of wound healing. The arrangement of these stages in wound healing relies on factors such as cytokines, growth factors, proteases, eicosanoids, kinins, and cellular metabolites [10].

In vitro studies conducted on wounds with *Salvia sclarea* concluded that its essential oil is an antimicrobial agent and that it can be used in the process of treating wounds. In addition, according to the results of an *in vitro* wound study conducted with another species such as *Salvia haenkei* extract, it was observed that it had a 25.1% greater effect on keratinocyte cells compared to the negative control group. The wound healing effect of *Salvia multicaulis* was evaluated in the *in vivo* model and all concentrations were found to have wound healing potential compared to the negative control group. It was also observed that it stimulates angiogenesis in fibroblast cells. Considering previous studies, it was proven that the essential oil of the *S. sclarea*, which grows in Turkish cities such as Erzurum and Tunceli, is effective in wound healing, like other plants in this genus [3-5, 14]. When literature studies are examined, it was revealed that the *Salvia* genus is effective in the wound healing process. To the best of our knowledge, one of the unique features of this study is that no previous study was conducted on the cell migration and proliferation ability of *Salvia sclarea* essential oil. As a result of the antimicrobial and antioxidant properties of the plant, the data obtained from this study in the *in vivo* environment must be confirmed.

Citrus aurantium essential oil has antibacterial, antifungal, antioxidant and antidiabetic properties. A previous study showed that *C. aurantifolia* extract is effective in blood vessel formation by increasing angiogenesis and increases proliferation in fibroblast cells [15-16]. It was revealed that *Citrus* polyphenols can accelerate the healing of mouth wounds [17]. Another study demonstrated that *Citrus aurantium* essential oil induces healing activity in mucous glands by increasing the formation of new blood vessels in an *in vivo* model [8]. In addition, *Citrus aurantium* essential oil and its major component limonene were previously studied in

detail on stomach wounds and were shown to be effective [18]. Literature research showed that the *Citrus* genus is currently used in the wound healing process because it increases proliferation in wounds. Proliferation values of the essential oil of *Citrus aurantium* fruits and leaves were studied and the proliferation rate was found to be over 100% at 0.1 mg/mL [19].

Lack of toxic effects is important for safe use. Many studies conducted in this field showed that *Citrus aurantium* and *Salvia sclarea* essential oils contain components that are cytotoxic to cancer cells and can be used in the development of anticancer agents. However, it does not have a toxic effect on healthy cells [20-21].

To the best of our knowledge, this study is the first study in the literature. The investigation of the wound healing activities of *Salvia sclarea* L. and *Citrus aurantium* L. oils individually and in combination is the original aspect of the study. The fact that no activity is observed in empty formulations is evidence that the activity of the loaded formulations does not come from empty formulations. It was observed that the loaded formulation was more effective and closed the wound opening better at the 24th hour than the positive control Madecassol.

4. CONCLUSION

As a conclusion, the *in vitro* characterization properties of oils obtained from *Salvia sclarea* and *Citrus aurantium* leaves and their combinations were evaluated for the first time based on evidence. Their cytotoxicity was investigated with the MTT assay in healthy cell lines, and their effects on wound treatment were evaluated with the scratch assay in an *in vitro* model. Considering the synergistic potential of the oils, the combined concentration was determined and their synergies were examined. As a result of the *in vitro* results, the standardized oils determined were loaded into the carrier systems with effective doses to make a carbopol gel formulation, and the *in vitro* effects of the loaded formulations were investigated with the same methods. At the same time, patentable findings were obtained and prototypes were developed. Further biological and phytochemical investigations are recommended to evaluate their potential as wound healer therapeutics.

5. MATERIALS AND METHODS

5.1. Chemicals

The standard chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and the solvents were purchased from Merck. Methyl thiazole tetrazolium (MTT) reagent were purchased from Sigma-Aldrich (Germany).

5.2. Plant material

Commercial *Salvia sclarea* and *Citrus aurantium* essential oils were supplied by Art de Huile Company, İstanbul, Türkiye. Voucher samples are deposited at IMEF Herbarium (Herbarium No: IMEF 1191-1192).

5.3. GC-FID and GC/MS Analyses

The Agilent 6890N GC system was used. Simultaneous automatic injection was carried out using the same conditions in two identical columns [HP-Innowax FSC column (60 m × 0.25 mm, 0.25 µm film thickness, Agilent, Walt & Jennings Scientific, Wilmington, Delaware, USA)] in the Agilent 5975 GC/MSD system. Relative percentages (%) of the compounds were calculated using the FID chromatograms. For the idenfication and characterization in-house "Baser Library of Essential Oil Constituents" and various GC/MS Libraries such as MassFinder 3 Library, where authentic samples or the relative retention index (RRI) of n-alkanes were also considered [22].

5.4. Scratch assay

Cell culture wound model experiment was conducted to examine cell migration and proliferation ability according to treatments. Cells were seeded in 24-well plates at 1.5×10^5 cells and 0.4 mL medium per well and grown to 100% confluency after 24 h of culture. Three horizontal lines were drawn for each one, which were used to take photographs of the scratch wound at the same spot at different times. Cells were washed twice with phosphate-buffered saline (PBS) and replaced with 0.5 mL of high-glucose DMEM containing 10% FBS. Photographs of the scratch-shaped wound were taken at 10x magnification using a microscope (AxioCam, Germany) at 0-8-24 hours. Images were monitored for the width of the scratch area at different time intervals

(0.8 and 24 hours) and analyzed with an image analysis software (Image J.2.0 software, USA) to calculate wound closure [23].

5.5. MTT Assay

Cell viability was essentially determined using the MTT assay as described by (The studied cells were situated at 5 ×10^4 cells into each well of 96-well tissue culture plates (Nunc, Denmark) and incubated for 24 h. After this procedure, all the tested materials were dissolved in DMSO (0.5%) individually and added to culture wells at varying concentrations (1–1000 µg/mL). After 24 h of incubating period, 30 µL MTT solution (0.5 mg/mL in Phosphate Buffered Saline) was added to each well and the cells were incubated for 4 h at 37°C. Purple formazan crystals were generated via the reduction product of the MTT agent by the mitochondrial dehydrogenase enzyme of intact cells. These crystals were dissolved in 150 µL DMSO and the absorbance was read by Spectramaxi3 (OD570 nm). The percentage of living cells was calculated based on the medium control. [24].

$$\text{Viability \%} = (\text{Absorbance}_{\text{material}} / \text{Absorbance}_{\text{control}}) \times 100$$

5.6. Carbopol gel preparation

To prepare the base gel, a previously reported methodology was used with slight modifications [25]. 0.5 g Carbopol (dispersed in water), 7 g glycerin, and 20 g isopropyl alcohol were blended with slowly stirring using a mechanical stirrer, at 25 °C and 3.5 g of triethanolamine in water was transferred. The mixture was further filled with water up to 100 g weight. Following, the blend was stirred constantly until the formation of a clear gel. In addition, a hydroxypropyl cellulose gel (2%) was prepared. The Carbopol gel and hydroxypropyl cellulose gel were mixed (50:50) ratio and 5% of the essential oils were added following by gently stirring. Similarly, a blank gel was also prepared.

5.7. Statistical analysis

All repeated experiments were conducted in triplicate. Statistical analysis were determined by using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA; Version 8.4.3). The data were expressed as mean ± standard deviation (SD).

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