*SHORT REPORT*

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# *In vitro* ACE2 and 5-LOX Inhibition of *Rosmarinus officinalis* L. Essential Oil and its Major Component 1,8-Cineole

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**Abstract:** In this present study, *Rosmarinus officinalis* L. essential oil, and its major component 1,8-cineole was evaluated *in vitro* for angiotensin converting enzyme 2 (ACE2), as well as for 5-lipoxygenase (5-LOX) enzyme inhibitory activity. The essential oil composition was confirmed both by GC-FID and GC/MS, where 1,8-cineole (62.7%), α-pinene (12.6 %), and camphor (8.3 %) were identified as the main constituents. Activity studies were performed at concentrations of 20 $\mu$g/mL for essential oil, and 5 $\mu$g/mL for the major compound 1,8-cineole, which were compared experimentally with standards. The essential oil was evaluated using a fluorometric multiplate based enzyme inhibition kit, where the ACE2 inhibition of Rosmarinus aetheroleum was 20%, while the 5-LOX inhibition was observed as 81.1%, respectively. In addition, the major constituent 1,8-cineole also showed remarkable ACE2 inhibition with 89.2%, and 5-LOX inhibition with 37.2%, respectively. As a result, the cineole chemotype rosemary essential oil, and its major constituent 1,8-cineol may have antiviral potential applications against coronaviruses due to ACE2 enzyme inhibition with anti-inflammatory effects. Further *in vivo* studies are needed to confirm the efficacy of essential oils and their constituents.

**Keywords:** *Rosmarinus officinalis* L.; essential oil; ACE2; 5-LOX; 1,8-cineole. 

## 1. Plant Source

The test materials of the present study namely; 1,8-cineole, 5-lipoxygenase enzyme from soybean, and nordihydroguaiaretic acid (NDGA) DMSO were acquired from Sigma Aldrich, if not otherwise

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stated. Commercial Rosmarini aetheroleum was kindly provided by Doallin Ltd., İstanbul, a voucher sample is deposited at IMEF Herbarium (Herbarium No: IMEF 1145).

## 2. Previous Studies

*Rosmarinus officinalis* L., of Lamiaceae is a popular and well known cultivated medicinal aromatic plant. Thus, traditionally rosemary is often used since ancient times as spice, ornamentally, for fragrances *etc*. *R. officinalis* preparations are used as antimicrobial, memory support, diuretic, expectorant, among others [1-2].

In previous studies, *R. officinalis* essential oil was investigated for its phytochemistry and different biological properties such as anti-hyperglycemic, anti-hyperlipidemic, cytotoxic, anticholinergic, antimicrobial, and antioxidant activities [3-6]. The constituents of *R. officinalis* essential oils are diverse based on their chemotypes, and cultivars such as 1,8-cineole, camphor, verbenone, borneol, bornyl acetate, and α-pinene among other secondary metabolites. Consequently, 13 different *R. officinalis* chemotypes were suggested based on the relative percentages of 1,8-cineole, camphor, borneol, verbenone, bornyl acetate, and α-pinene, respectively [7,8]. The remarkable antioxidant activity of the essential oil was evaluated previously by different methods [8]. It is also known for its wide spectrum antimicrobial potential, and therefore utilized as a food preservative beside other applications [9]. *R. officinalis* essential oil was also investigated for its anti-inflammatory potential in previous *in vivo* studies [10].

The purpose of this present study was to evaluate the *in vitro* angiotensin converting enzyme 2 (ACE2), and 5-lipoxygenase (5-LOX) enzyme inhibitory activities of the *R. officinalis* essential oil, and its major component 1,8-cineole, comparatively, with a potential utilization as an anti-covid19 agent, to the best of our knowledge for the first time.

## 3. Present Study

*GC-FID and GC/MS Analysis:* Agilent 6890N GC system was used for the GC-FID analyses. Whereas the Agilent 5975 GC-MSD system was used for GC/MS. The temperature of the FID detector was set to 300°C. Concurrent auto-injection was performed in two identical columns using the same conditions in the GC/MS system. Relative percentages (%) were calculated using FID chromatograms (see Table 1). Relative retention indices were used to characterize the essential oil components. This process was held either by authentic samples or analyzing relative retention index (RRI) of n-alkanes, along with GC/MS Library, MassFinder 3 Library, in-house “Başer Library of Essential Oil Constituents” [7].

Overall, 99.9% of the essential oil composition was characterized, and confirmed. The compounds are listed in Table 1, with their relative percentages. Main components were found as 1,8-cineole (62.7%), α-pinene (12.6 %), and camphor (8.3 %), respectively. Out of 13 different *Rosmarinus officinalis* chemotypes our results comply with the 1,8-cineole chemotype [7]. In addition, the essential oil composition was compatible with the European Pharmacopoeia.

*ACE2 Enzyme Inhibition Assay:* The test substances were initially dissolved in DMSO < 1% (v/v). The enzyme inhibition experiment was carried out in accordance with the manufacturer's instructions for the kit "Angiotensin II Converting Enzyme (ACE2) Inhibitor Screening Kit (BioVision, K310)" and the enzyme inhibition of the substances was measured with $E_x$/ $E_m$ = 320/420 nm wavelength using a multimode microplate reader (SpectraMax i3) at fluorescence mode. The enzyme inhibition of the test substances was calculated by comparing with standards included in the kit. In the presence of an ACE2-specific inhibitor, the enzyme loses its peptidase activity, resulting in a decrease in fluorescence intensity. Due to this decrease, % inhibition values were calculated as mean values for all samples resulting from duplicate data.

Initially, the *R. officinalis* essential oil, and its major component 1,8-cineol was evaluated for their *in vitro* ACE2 enzyme inhibition potential. While *R. officinalis* essential oil (20 µg/mL) inhibited ACE2 by 20%, the main compound of the oil 1,8-cineole (5 µg/mL) showed 89.2% inhibition, respectively.

The COVID-19 pandemic is currently an important and definitive health problem in the world. Since the interaction of the COVID-19 virus spike, and ACE2 enzyme is necessary for the infection. Thus, any agent that interrupts its interaction, and the human monoclonal antibody based on the receptor

binding domain, also the recombinant human ACE2 protein (rhuACE2) have withdrawn attention as potential targets [11,12]. Essential oils are well known for their wide antimicrobial spectrum including their antiviral effects. It is reported that, *R. officinalis* essential oil showed antiviral potential against herpes viruses [13–15]. In previous studies, 1,8-cineole was investigated against different virus types, and found to be highly effective, especially against rhinoviruses and herpes viruses as reported [16,17]. Recent *in-silico* studies also stated that 1,8-cineole might be effective on coronaviruses [18]. In addition to the *in silico* studies, the *in vitro* findings obtained in this present study also show that 1,8-cineole may be an effective compound against COVID-19.

**Table 1.** The composition of *R. officinalis* essential oil

| **RRI** | **Compound** | **%** |
|---|---|---|
| 1014 | Tricyclene | 0.1 |
| 1032 | α-Pinene | 12.6 |
| 1072 | α-Fenchene | 0.1 |
| 1076 | Camphene | 3.4 |
| 1118 | β-Pinene | 1.7 |
| 1174 | Myrcene | 0.5 |
| 1203 | Limonene | 3.0 |
| 1213 | 1,8-Cineole | 62.7 |
| 1280 | *p*-Cymene | 3.9 |
| 1384 | α-Pinene oxide | t |
| 1452 | 1-Octen-3-ol | 0.1 |
| 1497 | α-Copaene | 0.1 |
| 1532 | Camphor | 8.3 |
| 1553 | Linalool | 0.6 |
| 1611 | Terpinen-4-ol | 0.2 |
| 1612 | β-Caryophyllene | 0.2 |
| 1682 | δ-Terpineol | 0.3 |
| 1683 | *trans*-Verbenol | 0.2 |
| 1706 | α-Terpineol | 1.0 |
| 1719 | Borneol | 0.7 |
| 1729 | *cis*-1,2-epoxy-Terpin-4-ol | 0.2 |
| 1804 | Myrtenol | 0.1 |
| 1864 | *p*-Cymen-8-ol | 0.1 |
| | Total | 99.9 |

RRI: Relative retention indices calculated against *n*-alkanes; %: calculated from FID data; t: Trace (< 0.1 %)

*LOX Enzyme Inhibition Assay:* The 5-lipoxygenase (5-LOX) assay was measured by modifying the spectrophotometric method of Baylac and Racine [19]. The reaction was initiated by the addition of linoleic acid solution, the change of absorbance at 234 nm was observed for 10 min. All the kinetic experiments were performed in triplicate. The concentration of the tested essential oil was 20 $\mu$g/mL, where the pure compounds were tested at 5 $\mu$g/mL. All tests and control assays were corrected by blank experimental data for non-enzymatic hydrolysis. The absorbance change per minute was determined. The percentage of inhibition (%I) was calculated as the absorbance change per minute of enzyme activity (without inhibitor) compared to absorbance change per minute of the test sample. Nordihydroguaiaretic acid (NDGA) was used as positive control. Experiments were performed in triplicates, and results are given as mean.

The 5-LOX enzyme inhibition of rosemary essential oil tested in this study was measured as 81.13% at a concentration of 20 $\mu$g/mL. In addition, the major constituent 1,8-cineole showed 37.17% inhibition, whereas compared the positive control NDGA showed 91.15 % inhibition at 5$\mu$g/mL concentration. In previous studies, the anti-inflammatory effect of the *R. officinalis* was reported [20,21]. In particular, the anti-inflammatory effect of rosemary essential oil, and various essential preparations

were studied, by different *in vitro* and *in vivo* protocols [22]. As it is well known, lipoxygenases are a mediator of inflammation; and the inhibition is a pathway in relieving the inflammation [23].

In this present study, the anti-inflammatory activity of the essential oil, and its major constituent 1,8-cineole was studied for their *in vitro* lipoxygenase enzyme inhibition potential, as it may affect many pathologies. In previous studies, the lipoxygenase enzyme inhibition of rosemary essential oil was studied, where different results were observed [24]. Although this difference is acceptable for the rosemary species with different chemotypes, it can be suggested, that the relatively high inhibition value of the essential oil of rosemary may be due to the synergy of the components, since its major component 1,8-cineol showed lower inhibition compared to the rosemary essential oil itself.

There is also a perspective for the utilization of essential oils against microbial infections, and in the management of coronaviruses [14, 15, 18]. As it is known, monocytes allow viruses to spread to all organs, and tissues by migrating to the tissues where they become infected resident macrophages. Monocytes and macrophages infected with SARS-CoV-2 can produce multiple types of pro-inflammatory cytokines, and chemokines that contribute to local tissue inflammation, and a dangerous systemic inflammatory response called cytokine storm. Both local tissue inflammation, and cytokine storm play a fundamental role in the development of COVID-19-related complications such as acute respiratory distress syndrome (ARDS), which is the main cause of death in COVID-19 patients. Potential therapeutic interventions can be highlighted to alleviate inflammatory reactions in possible approaches to the treatment of COVID-19 while explaining monocytes and macrophage responses during severe coronavirus infections [12, 25].

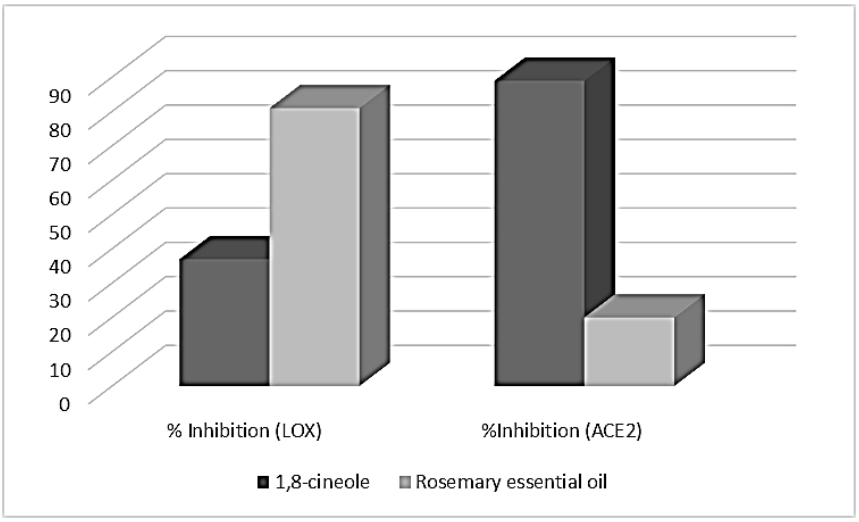


**Figure 1.** 5-LOX and ACE2 enzyme inhibitions of rosemary essential oil, and 1,8-cineole

As a conclusion, in this present study, the rosemary essential oil, and its major component 1,8- cineole were evaluated for their potential *in vitro* ACE2 and 5-LOX enzyme inhibitory activities. Especially in terms of ACE2 enzyme inhibition, to the best of our knowledge, rosemary essential oil, and 1,8-cineole were previously not reported experimentally. In addition, remarkable results were observed for both enzyme inhibitions, and compared with 1,8-cineol, which is the major component of the tested oil. Based on the first *in vitro* experimental results, it can be suggested that *R. officinalis* essential oils may be further evaluated as a potential antiviral, anti-inflammatory agent against coronaviruses. To the best of our knowledge, this is the first comparative experimental *in vitro* report both on the ACE2 and LOX-5 enzyme inhibition of the *R. officinalis* essential oil and its major component 1,8-cineole.

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