



# Chemical composition, cytotoxic effect and antimicrobial activity of *Stachys koelzii* Rech.f. essential oil against periodontal pathogen *Prevotella intermedia*

Parvin Ramak<sup>a</sup>, Gholam Reza Talei<sup>b,\*</sup>

<sup>a</sup> Research Division of Natural Resources, Lorestan Agricultural and Natural Resources Research and Education Center, AREEO, Khorramabad, Iran

<sup>b</sup> Head of Virology Department, Razi Herbal Medicines Research Center, Medical School, Lorestan University of Medical Sciences Khorramabad, Iran

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## ABSTRACT

*Prevotella intermedia* is associated with periodontal diseases and endodontic infections. Periodontitis can be suppressed by utilizing the antiseptics, which target the infectious bacteria. The member of *Stachys* sp. has been used traditionally in the form of decoction or infusion for management of infectious diseases. The subject of this article was to evaluate the chemical composition, antimicrobial and cytotoxic effect of *Stachys koelzii* essential oil and its main components against *Prevotella intermedia*. GC-FID and GC-MS analysis were used to determine the chemical composition. The antimicrobial effects of *S. koelzii* essential oil was evaluated by micro-broth dilution assay. Time kill curve assays, leakage of cytoplasmic materials and anti-biofilm effects were determined. Its cytotoxic effect was evaluated by MTT assay. Essential oil with main components of  $\alpha$ -pinene, trans-caryophyllene and 1,8-cineole inhibited *P. intermedia* with MIC and MBC values of 0.1 and 0.2 mg/mL. Its biofilm formation was higher than  $\alpha$ -pinene, followed by trans-caryophyllene and 1,8-cineole. Essential oil and its main components increased the leakage of cytoplasmic components. Essential oil showed cytotoxic effect on HeLa cell lines with IC<sub>50</sub> 0.06 mg/mL. The cytotoxic effect of  $\alpha$ -pinene on healthy cell lines was higher than essential oil. *S. koelzii* essential oil can be used in mouthwash formulations and its efficacy should be evaluated in large clinical studies.

## 1. Introduction

*Prevotella intermedia* as anaerobic Gram negative bacteria is associated with periodontal diseases, and endodontic infections (root canal, periapical lesions and apical periodontitis). Invasion of *P. intermedia* to human buccal epithelial cells allows access to nutrients. Increase in gingival cervical fluid stimulates the growth of *P. intermedia*, which results in plaque and biofilm formation [6]. Therefore, periodontitis and other related oral diseases can be suppressed by utilizing antiseptics, which target *P. intermedia*. The use of chemical disinfectants in oral health is associated with some adverse effects [35] and the risk of microbial resistance [16]. Thus, the use of natural antiseptics in oral cavity have been interested for scientists [29]. Natural products especially medicinal plants are multifunctional in their biological activities and donate different biological activities to product, including anti-inflammatory, antimicrobial and biofilm killing effects [16,37]. The members of *Stachys* L. with common name of “woundwort”, “hedge nettle” are used traditionally as antiseptics for treatment of infections. *Stachys* L. is one of the largest genera of Labiatae family, which comprises about 300 species of herbaceous, annuals and perennial plants in

the world. It has 34 herbaceous plants in Iran, which 15 species are endemic [25]. The origin of *Stachys* name is from Greek, means an “ear of grain”, which refers to inflorescence spikes in this genus. *Stachys* L. is traditionally used as decoction or infusion for treatment of vaginal tumors, rheumatic disorders, asthma, ulcers, abdominal pains, fever and inflammatory condition. In Anatolian ethnobotany, *Stachys* sp. are used as tea due to the antibacterial activities of its essential oil [3,28]. In Serbia, *S. officinalis* aerial parts is used to treat respiratory (whooping cough, bronchitis, asthma), and urogenital tract diseases (cystitis, glomerulonephritis and menometrorrhagia) [19]. *S. annua* is traditionally used as febrifuge, anti-catarhal, vulnerary and tonic in Italy [40]. Flavonoids, fatty acids, iridoids, phenolic acids, and essential oils are the main secondary metabolites of *Stachys* L [7]. Anti-inflammatory, antinociceptive [12], antioxidant [10], antimicrobial [19], anxiolytic [32], neuroprotective [21], anti-allergic [33] effects of some species of *Stachys* have been confirmed. The use of *Stachys* sp. as tea according to traditional believes due to antimicrobial activities of its essential oil content can be a rational reason for evaluating the biological activities of *Stachys* sp. especially the antimicrobial activities.

*Stachys koelzii* is used in traditional medicine as infusion for

\* Corresponding author. Lorestan University of Medical Sciences, Anooshirvan Rezaei Square, 6813833946, Khorramabad, Lorestan, Iran.  
E-mail address: [talei.gh@lums.ac.ir](mailto:talei.gh@lums.ac.ir) (G.R. Talei).

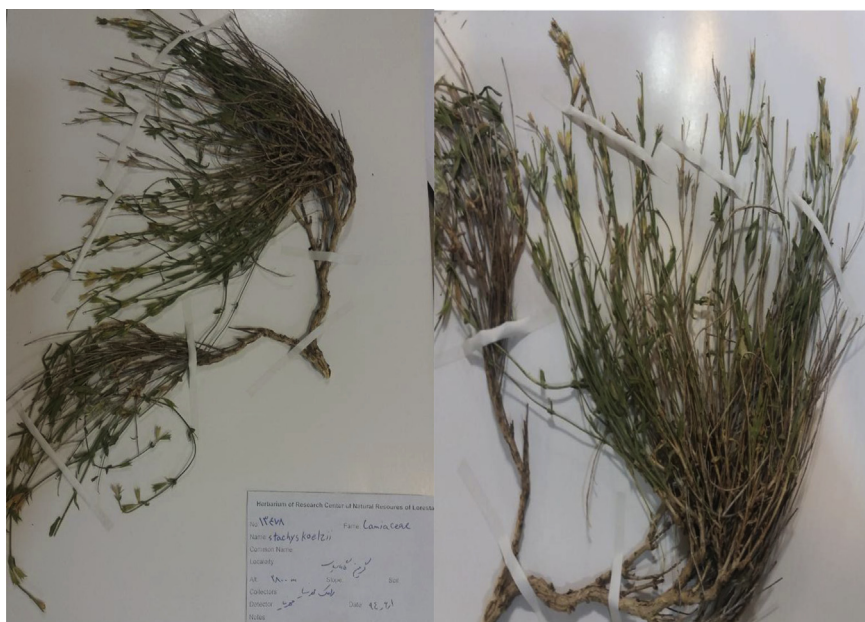


Fig. 1. The herbarium sample of *S. koelzii* (Herbarium number: LUR 13478).

treatment of oral diseases [26]. Also, there is no study on chemical composition, essential oil, antibacterial and cytotoxic effects of this plant, therefore, the antibacterial and anti-biofilm effects of *Stachys koelzii* essential oil was evaluated against periodontal pathogen *P. intermedia* according to folklore. Furthermore, the chemical composition of *S. koelzii* essential oil and the role of its three main components in antibacterial effects along with its cytotoxic effect were evaluated.

## 2. Materials and methods

### 2.1. Plant material

*Stachys koelzii* aerial parts at full flowering stage were collected from Garin Mountain, the largest flat land (altitude 2900 m above sea level), in Lorestan province, western of Iran in August 2015. The plant materials were identified by Dr. M. Mehrnia and the voucher specimen was authenticated under number LUR 13478 in the herbarium of Lorestan Agricultural Research and Natural Resources Center, Khorramabad, Iran (Fig. 1). *Stachys koelzii* Rech. f. is perennial, cushion-like and woody at base. Stems are thorny, 10–20 cm long, thick at base and become thinner going upwards. They are covered with long white wool-like hairs (up to 2 mm). Leaves are without petioles, lanceolate, 8–16 (length) × 2–5 (width) mm, narrow at both ends. The calyx is 8–10 × 3–5 mm; tubular, covered in white dense wool-like hair and sub sessile to sessile glandular hairs, teeth triangular to lanceolate and 3–6 mm long. Corolla with pale violet upper edge and light yellow lower edge, emerges a little bit out of calyx tube. Achenes oblong in outline, 1–1.5 × 0.8–1.0 mm long and smooth surface. Flowering and fruiting happens between late March and early June [13,30].

### 2.2. Essential oil extraction

For essential oil extraction, 500 g of finally ground dried aerial parts of *S. koelzii* was subjected to hydro-distillation method in Clevenger-type apparatus for 3 h. The extracted oil with yellow color was separated and dried over dry sodium sulfate. The yield of essential oil was calculated on the base of the dry weight (w/w). It was stored at +4 °C until the analysis.

### 2.3. Analysis the chemical composition of essential oil by GC and GC-MS

Chemical composition of essential oil was analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Approximately 0.2 µL of diluted essential oil in ether (1/10) was injected into the GC-FID on a HP 6890 gas chromatograph, which equipped with a FID detector and an HP-5 fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The split ratio was adjusted to 50:1. Helium as the carrier gas was used with a flow rate of 0.9 mL/min. The temperature of column was initiated from 50 °C. Then, it was raised to 200 °C with rate of 5 °C/min. The injector and FID detector temperatures were 230 and 260 °C, respectively.

GC-MS analysis was carried out in an Agilent 6890 gas chromatograph interfaced with an Agilent 5973 MSD using helium as the carrier gas in the same condition of GC-FID. Components of oil were identified by comparison with Retention indices (RI) relative to homologous series of n-alkanes and by computer search using libraries of Wiley 275.L and Wiley 7n.1, as well as comparison of the fragmentation pattern of the mass spectra with data published in the literature [1].

### 2.4. Chemical compounds

The analytical grade of 1,8-cineole, (+)- $\alpha$ -pinene and (–)-trans-caryophyllene with cat no: C80601, P45680, C9653 were purchased from Sigma-Aldrich Co. (St. Louis, MO).

## 3. Antibacterial evaluation of essential oil and main components

### 3.1. Micro-broth dilution assay

The antibacterial activity of essential oil and main components (1,8-cineole, (+)- $\alpha$ -pinene and (–)-trans-caryophyllene) was evaluated against *Prevotella intermedia* ATCC 49046 by micro-broth dilution assay. The bacterial strain was cultured in Tryptic Soy Broth (TSB) supplemented with 5 µg/mL hemin, 1 µg/mL vitamin K<sub>1</sub> and incubated at 37 °C under anaerobic conditions. The bacterial culture was suspended twice in PBS buffer (pH = 7.2) and its turbidity was adjusted to 1 × 10<sup>6</sup> CFU/ml. The essential oil and main components were dissolved in dimethylsulfoxide (DMSO) and then two fold serially dilutions were prepared in 96 micro-titer plates. Tinidazole was used as positive

control. The bacterial suspension was added to each well and the plates were incubated at 37 °C under anaerobic condition for 48 h. Minimal inhibitory condition (MIC) and Minimal bactericidal concentration (MBC) was defined as the lowest concentration of compounds which inhibited or killed the bacteria, respectively [4].

### 3.2. Time-kill curve assay

For evaluating the time kill curve for each compound against *P. intermedia*, the turbidity of suspended bacteria in PBS was adjusted to  $1 \times 10^7$  CFU/mL  $1 \times$ ,  $2 \times$ , and  $4 \times$  MIC values of each compound were added to bacterial suspension, then was incubated at 37 °C under anaerobic condition. The CFU of bacteria was calculated at different time intervals, by culturing the bacterial suspension on TSA. The results were reported in triplicate [36].

### 3.3. The anti-biofilm effect evaluation

The anti-biofilm effect of essential oil and main components were evaluated by crystal violet staining method. *P. intermedia* ( $1 \times 10^6$  CFU/mL) suspension was added to serial dilution of compounds as micro-broth dilution. The plates were incubated at suitable condition for 48 h. The untreated cells were then prepared as control. After incubation, the culture media in each well were removed, washed. The biofilm formation was evaluated by staining the dried wells by crystal violet (0.1%) for 15 min. The wells were washed with distilled water and dried. 200  $\mu$ L of ethanol was added to wells and read at the absorbance of 570 nm using a micro-plate reader. The experiments were performed in triplicate [23].

### 3.4. Measurement the leakage of cytoplasmic materials

For this purpose, *P. intermedia* ( $1 \times 10^9$  CFU/mL) suspension was incubated with MIC concentration of compound under suitable condition. Untreated bacterial suspension was used as the control. About 2 mL of bacterial supernatant was centrifuged at different intervals (0, 1, 2 and 4 h), and the cells were removed. The release of cellular nucleic acids, and the concentration of proteins were measured at the absorbance of 260 nm by a UV-VIS spectrophotometer (UV 1800, Shimadzu, Japan) and ELISA micro-plate reader according to the Bradford's method, respectively. The experiments were performed in triplicate [5].

### 3.5. Cytotoxicity effect of essential oil and main compounds

The cytotoxic effect of compounds were evaluated against human cervical carcinoma HeLa cell line (ATCC CCL-2) and the human normal healthy lymphocyte cell line (ECACC 91112124) by MTT assay. The cell lines were cultured and incubated at 37 °C in atmosphere containing 5% CO<sub>2</sub>.

The concentration of  $5 \times 10^4$  cell lines were seeded in wells of micro-titer plates, the cells were suspended in 100  $\mu$ L of RPMI medium supplemented with 10% FBS in a 96-well plate and were incubated for 24 h. Different concentrations of diluted essential oil and main components were added to each wells and re-incubated for further 24 h. About 10  $\mu$ L of MTT (0.05 mg) was added to each well and then plates were incubated for 4 h. The broth media was discarded, and dried, 100  $\mu$ L DMSO was added using an ELISA micro-plate reader at 490 nm. The concentration of each compound which inhibited the cell growth by 50%, was defined as IC<sub>50</sub> (mg/mL). The cell viability curves were calculated with regard to the control cells. The experiment was performed in triplicate [31].

### 3.6. Statistical analysis

The data from three replicates of all experiments was recorded as means  $\pm$  SD (Standard Deviation).

## 4. Results and discussion

### 4.1. Essential oil's yield

The yield of essential oil for *S. koelzii* aerial parts at full flowering stage was 0.5% (w/w). Up to now, oil yield for *S. koelzii* was the highest extraction yield for *Stachys* sp. The oil yield for *S. obtusifolia*, *S. Lavandulifolia*, *S. setifera* ssp. *Iranica*, *S. chrysantha*, *S. candida*, *S. byzantine*, *S. annua*, *S. tymphaea* were 0.145% [2], 0.25% [15], 0.18% [14], 0.18%, 0.12% [34], 0.25% [24], 0.014%. [40], 0.04% (w/w) [39], respectively. The geographical region, extraction method and the organ have essential role in oil's yield. It was reported that *S. palustris* aerial parts from Hungary and France had the yield of 0.02 and 0.05% w/w, respectively [41]. The yield of oil in hydro-distillation method was higher than steam distillation for *S. persica* (0.3% vs. 0.2%) [18]. The yield of essential oil for *S. byzantina* leaves and stem were 0.1 and 0.08%, respectively [22]. Therefore, among different species of *Stachys*, *S. koelzii* could be a good source of essential oil.

### 4.2. Chemical composition of *S. koelzii* aerial parts essential oil

Chemical composition of *S. koelzii* essential oil showed the presence of 35 compounds, which represented 99.97% of total oil composition.  $\alpha$ -pinene (36.71%), 1,8-cineol (20.53%) and trans-caryophyllene (12.34%) were the main components of essential oil, followed by camphene (6.47%), borneol acetate (5.02%), and  $\beta$ -pinene (3.51%) (Table 1).

There is no report on chemical composition of *S. koelzii* essential oil.  $\alpha$ -pinene as the first main components of *S. koelzii* essential oil has been reported as the first main component (53%) of *S. gaziantepeensis* aerial

**Table 1**

The chemical composition of *Stachys koelzii* flowering aerial parts essential oil.

Compound	Retention Index	Area	Retention time	Percent
<b><math>\alpha</math>-pinene</b>	932	3232719775	6.276	<b>36.71</b>
camphene	948	569451345	6.596	6.47
$\beta$ -pinene	977	308776840	7.27	3.51
$\beta$ -myrcene	980	173379691	7.591	1.97
phellandrene	1005	106899106	7.968	1.21
$\alpha$ -terpinene	1018	68229694	8.288	0.77
<b>1,8-Cineole</b>	1031	1807994271	8.762	<b>20.53</b>
trans- $\beta$ -Ocimene	1043	26944378	9.111	0.31
$\gamma$ -Terpinene	1059	68977439	9.425	0.78
$\alpha$ -terpinolene	1084	23227264	10.225	0.26
cis-Ocimene	1125	1696259	10.517	0.02
trans-sabinene hydrate	1132	20447560	10.665	0.23
Camphor	1139	126210330	11.928	1.43
Pinocarpone	1162	16210855	12.46	0.18
borneol	1180	93876110	12.689	1.07
4-Terpineol	1189	21095883	12.969	0.24
Myrtenol	1194	18125153	13.586	0.21
Borneol acetate	1285	442171427	16.203	5.02
cis-Carane	1297	20687332	16.392	0.23
Myrtenyl acetate	1306	26585968	17.375	0.30
Bicycloelemene	1330	18624992	17.695	0.21
$\alpha$ -Copaene	1353	7940634	18.078	0.09
$\alpha$ -Ylangene	1379	40330969	18.781	0.46
$\alpha$ -Cubebene	1396	30795885	18.924	0.35
$\beta$ -bourbonene	1400	16862447	19.221	0.19
Isocaryophyllene	1407	18733747	19.907	0.21
<b>trans-Caryophyllene</b>	1428	1086781459	20.439	<b>12.34</b>
$\beta$ -Gurjunene	1432	101838054	20.69	1.16
Aromadendrene	1440	101278262	20.907	1.15
$\alpha$ -Humulene	1516	79562665	21.347	0.90
Germacrene B	1557	43402859	22.593	0.49
delta-Cadinene	1569	31777884	23.376	0.36
Caryophyllene oxide	1581	37266969	25.211	0.42
Hexadecane	1600	11791421	25.439	0.13

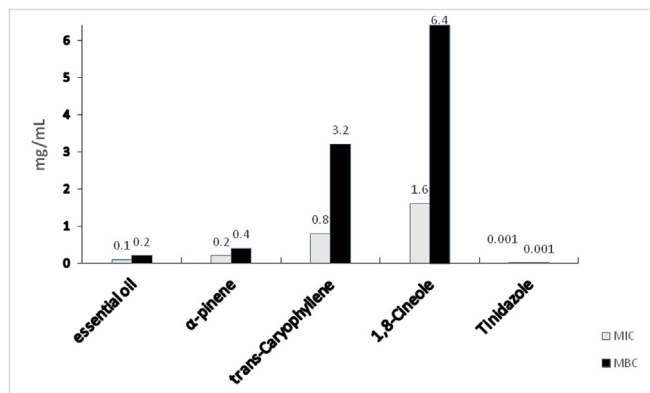


Fig. 2. Antibacterial effects of *S. koelzii* essential oil and its main components against *P. intermedia*.

parts essential oil [17].

Germacrene was the main component of *S. tymphaea* [39], *S. annua* [40], *S. byzantina* [22,24] oils, but the results of our chemical profiles showed no similarity to any other *Stachys* sp. essential oil. Germacrene is present in low amount in *S. koelzii* essential oil.

### 4.3. Antibacterial effect of *S. koelzii* essential oil against *P. intermedia*

In micro-broth dilution assay, essential oil showed the best antibacterial activity with MIC and MBC values of 0.1 and 0.2 mg/ml, followed by  $\alpha$ -pinene. The antibacterial effects of  $\alpha$ -pinene was lower than that of trans-caryophyllene and 1,8-cineole. It is believed that the synergistic effects between components has been responsible for antibacterial effects of *S. koelzii* essential oil. The antibacterial effects of essential oil was weaker than antibiotic (Fig. 2).

The results of time kill curve assay and anti-biofilm effects of *S. koelzii* essential oil were in accordance with micro-broth dilution result. According to Fig. 3, the log viable count of bacterium in control group increased during the time intervals, while reduction in log viable counts of bacterium were observed in treated groups. The most reduction in log viable counts of bacterium was in essential treated group, followed by  $\alpha$ -pinene, trans-caryophyllene. 1,8-cineole had the lowest effect on reduction of viable counts.

In screening the anti-biofilm effects of compounds, essential oil inhibited the biofilm formation higher than its main components. The anti-biofilm effect of  $\alpha$ -pinene was higher than trans caryophyllene, followed by 1,8-cineole (Fig. 3).

The antibacterial activity of *Stachys* sp. have been the subject of some different studies, while *S. koelzii* essential oil's antibacterial activity was not the subject of any research article.

*S. candida* with manoyl oxide (12.07%), caryophyllene oxide

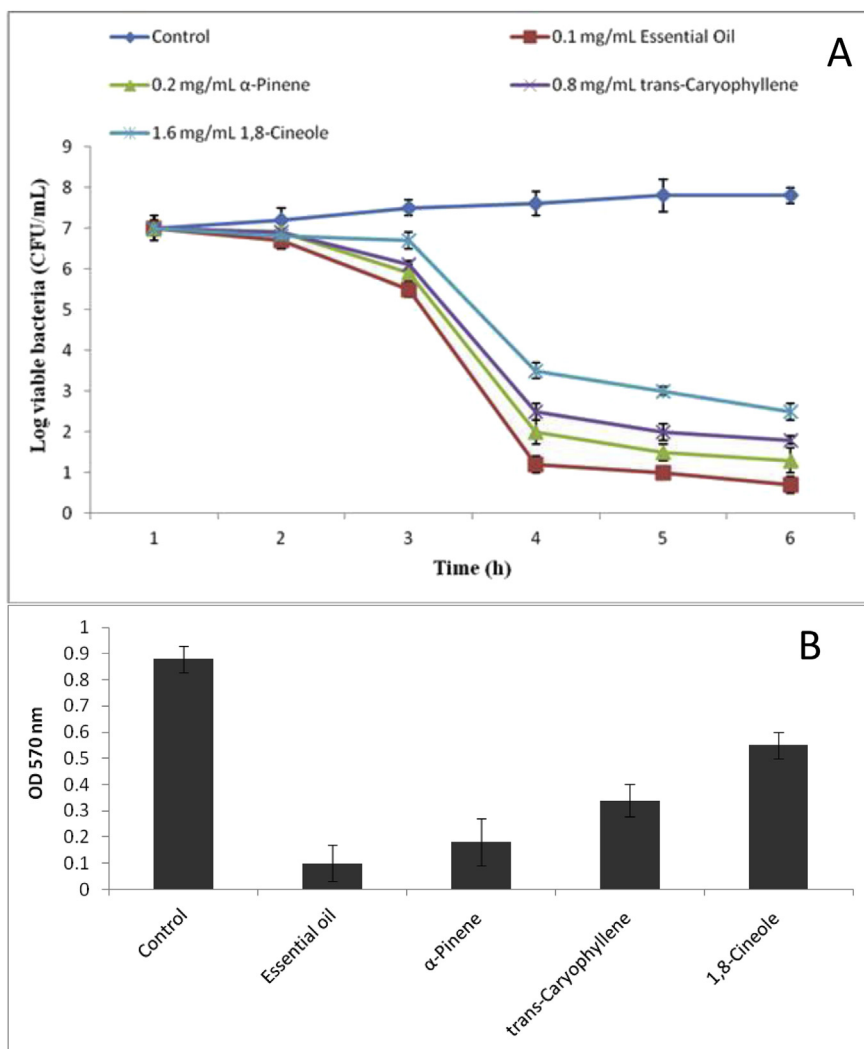


Fig. 3. A) Time kill curve assay of *S. koelzii* essential oil and its main components against *P. intermedia*, B) The anti-biofilm effects of *S. koelzii* essential oil.

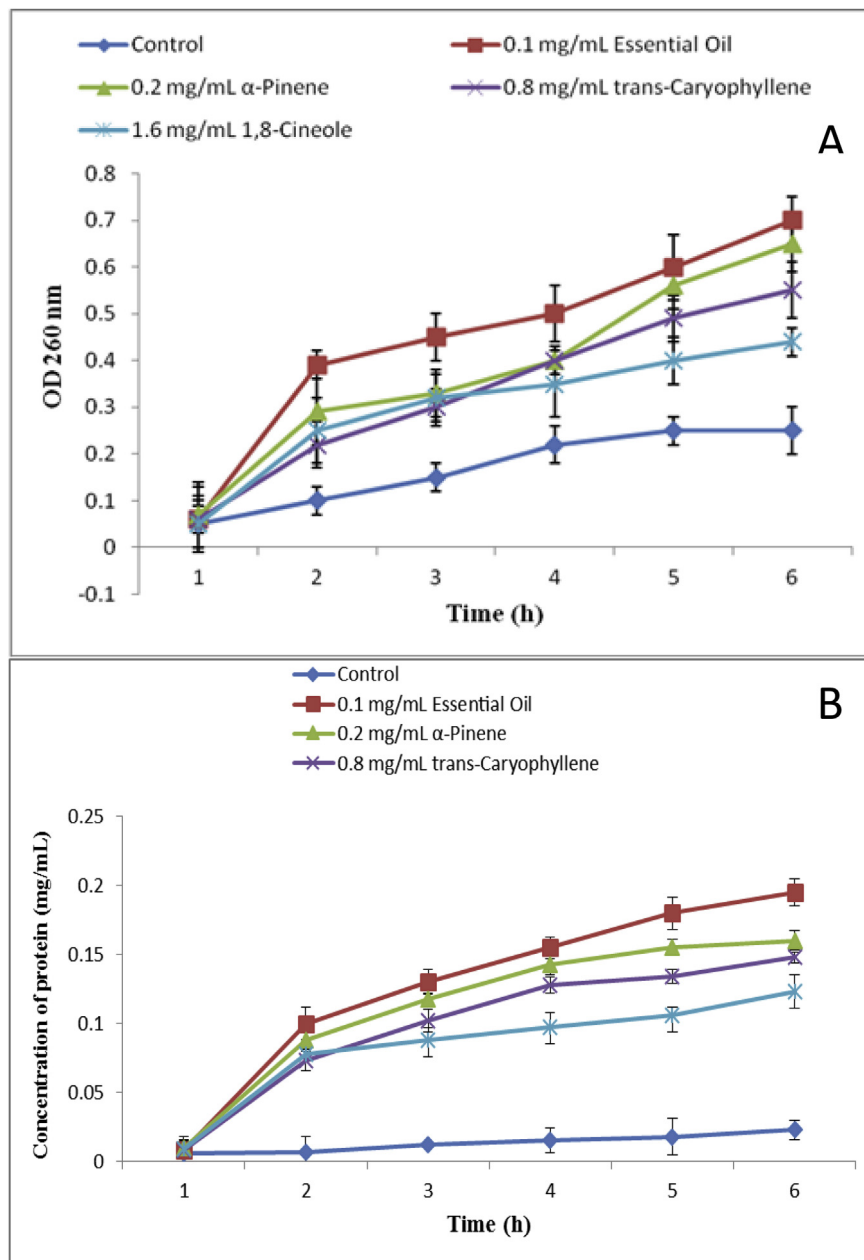


Fig. 4. Effect of the essential oil and three main compounds on leakage of nucleic acid (A) and protein (B) from *P. intermedia*.

Table 2

Cytotoxic effects (mg/mL) of *Stachys koelzii* aerial parts essential oil by MTT assay.

	essential oil	α-pinene	trans-Caryophyllene	1,8-Cineole
HeLa cell line	0.06	1.23	2.54	2.76
lymphocyte cell line	4.31	2.41	3.72	3.22

(11.50%) and (E)- caryophyllene (9.58%) as main components has been proved to be active against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 227853). *S. chrysantha* with main components of α-cadinol (16.24%), caryophyllene oxide (11.63%), epi-α-muurolol (11.25%) and (E)-caryophyllene (6.33%) was inactive against bacteria [34]. *S. officinalis* aerial parts essential oil

exhibited the best antifungal activity against *Aspergillus niger* (MIC, MFC = 2.5 and 5 mg/m), *Candida albicans* (MIC = MFC = 5 mg/ml) [19]. *S. byzantina* leaves and stem essential oils showed antibacterial activity against *Enterococcus faecalis*, *P. aeruginosa*, *K. pneumoniae*, and *Bacillus subtilis*. These essential oils showed moderate activity against *C. albicans* and *S. aureus*. *Escherichia coli* was resistant to leaf and stem *S. byzantina* essential oils [22]. *S. tymphaea* essential oil with main components of germacrene D (30.0%) and (E)-β-farnesene (12.4%) showed no antimicrobial effects against *S. aureus* and *E. coli* in disc diffusion method [39]. It seems that the antimicrobial effects of *Stachys* sp. is related to their chemical composition and it should be evaluated for each species. *S. koelzii* essential oil had acceptable antibacterial effects against oral pathogenic *P. intermedia*.

#### 4.4. Measurement the leakage of cytoplasmic materials

Treatment of bacterial cells with essential oil increased the leakage

of cytoplasmic materials in time dependent manner, followed by  $\alpha$ -pinene, trans-caryophyllene, and 1,8-cineole. Untreated cells had the lower leakage of cytoplasmic materials (Fig. 4).

#### 4.5. Cytotoxic effects of *Stachys koelzii* essential oil

Because MTT assay is easy enough, this method is used for cytotoxic effects of essential oil. Although, the MTT assay may not always be the best choice, but it can be useful to quantify the activation level of cells, independent of proliferation of bacteria and eukaryotes [9]. MTT assay as quantitative method is outlined in regulatory standards (ISO 10993-5, 2009), and have appeared in literature related herbal extracts [8,20,27,38]. Therefore, due to common use of this method and our equipment limit, this method is used as an acceptable method for cytotoxic effects.

The IC<sub>50</sub> evaluation of essential oil and its main components showed the higher cytotoxic effect of essential oil towards the human tumor cell line in comparison with healthy human cells line (Table 2). In fact, the essential oil inhibited the carcinoma cell lines with IC<sub>50</sub> of 0.06 mg/mL, followed by  $\alpha$ -pinene and trans-caryophyllenes. 1,8-cineole inhibited the HeLa cell lines in higher IC<sub>50</sub> than the others. The cytotoxic effect of  $\alpha$ -pinene on healthy lymphocyte cell line was higher than the others components.

The cytotoxic effect of *S. annua* subsp. *annua* aerial parts essential oil with phytol (9.8%), germacrene D (9.2%), spathulenol (8.5%) as main components were confirmed against HCT116, A375, MDA-MB 231 human tumor cell lines [40]. The cytotoxic effects of *S. byzantina* (stem), *S. palustris* (stems, folium, flowers), *S. recta* (stems), *S. germanica* (flowers) methanolic extract against MCF-7 (breast adenocarcinoma), HeLa (cervix adenocarcinoma), and A431 (skin epidermoid carcinoma) showed that MCF7 was inhibited by all extracts, while HeLa and A431 showed sensitivity to *S. recta* and *S. palustris* stems methanol extract [11]. These results suggest lower adverse side effects of the *S. koelzii* essential oil on healthy normal cell lines and acceptable cytotoxic effects against adenocarcinoma cell line.

#### 5. Conclusion

*S. koelzii* essential oil with oil's yield higher than the other studied *Stachys* sp. and the different chemical profile with main components of  $\alpha$ -pinene, trans-caryophyllene, 1,8-cineole was the subject of this study. *S. koelzii* oil with acceptable antibacterial and anti-biofilm effects against oral pathogenic *P. intermedia* had higher antibacterial and anti-biofilm higher activity than  $\alpha$ -pinene, followed by trans-caryophyllene and 1,8-cineole, which they increased the leakage of cytoplasmic membranes. The acceptable cytotoxic effects of essential oil against HeLa cell line, and lower cytotoxic effect on normal healthy cell line confirmed the traditional uses of *S. koelzii* as valuable antiseptic, which can be used in mouthwash or oral healthcare products after confirming their safety and effectiveness in toxicological animal models and clinical studies.

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