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Pharmacological characterisation of *Seseli gracile* Waldst. & Kit. (Apiaceae) essential oil

Farmakološka karakterizacija etarskog ulja *Seseli gracile* Waldst. & Kit. (Apiaceae)

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Abstract

Background/Aim. Phytochemical and pharmacological investigations of essential oils isolated from plant species of the genus *Seseli* have been intensified recently. These plant species have long-term use in nutrition and traditional medicine in the treatment of various disorders. Volatile secondary metabolites of *Seseli gracile* Waldst. & Kit (*Apiaceae*) have not been pharmacologically examined so far. The aim of the conducted research was to assess the antiradical, antimicrobial and spasmolytic activities of *S. gracile* essential oil isolated from the aerial parts of the plant. **Methods.** The antiradical activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test, antimicrobial activity using broth microdilution method with standard strains of Gram (+), Gram (-) bacteria and yeast, while spasmolytic activity was evaluated on isolated rat ileum, pre-contracted with a high concentration of potassium. **Results.** The results showed moderate antiradical (SC_{50} = 78.60 μg/mL) and good spasmolytic activity (IC_{50} = 271.4 nL/mL) of *S. gracile* essential oil. In the range of tested concentrations, minimal inhibitory concentration (MIC) was determined only for the strain of *Escherichia coli* (500 μg/mL). **Conclusion.** Results obtained in this study justify the need for further studies to elucidate exact molecular mechanism underlay this spasmolytic effect of *S. gracile* herb essential oil.

Key words:
apiaceae; oils, volatile; pharmacology; plant extracts; muscle relaxation; antioxidants; anti-infective agents.

Apstrakt

Uvod/Cilj. Etarska ulja izolovana iz vrsta roda *Seseli* su u poslednje vreme intenzivno farmakološki i fitohemijski proučavana. Biljke iz ovog roda se koriste već duži niz godina u ishrani i tradicionalnoj terapiji različitih oboljenja. Farmakološka aktivnost etarskog ulja izolovanog iz *Seseli gracile* do sada nije ispitivana. Stoga je cilj ovog istraživanja bio da se ispita antiradikalska, antimikrobna i spazmolitična aktivnost etarskog ulja izolovanog iz nadzemnih delova biljne vrste *S. gracile*. **Metode.** Antiradikalska aktivnost je utvrđena testom neutralizacije 2.2-difenil-1-pikrilhidrazil (DPPH) radikala, antimikrobna, bujon-mikrodilucionom metodom uz upotrebu standardnih sojeva Gram (+), Gram (-) bakterija i gljivica, a spazmolitična na izolovanom ileumu pacova, prethodno kontrahovanom visokom koncentarcijom kalijuma. **Rezultati.** Rezultati pokazuju umerenu antiradikalsku (SC_{50} = 78.60 μg/mL) i dobru spazmolitičnu aktivnost (IC_{50} = 271.4 nL/mL) etarskog ulja *S. gracile*. U opsegu testiranih koncentracija utvrđena je minimalna inhibitorna koncentracija (MIC) samo za soj *Escherichia coli* (500 μg/mL). **Zaključak.** Dobijeni rezultati opravdavaju potrebu za budućim istraživanjima koja bi razjasnila tačne mehanizme kojima se ostvaruje spazmolitički efekat etarskog ulja nadzemnog dela *S. gracile*.

Ključne reči:
apiaceae; etarsko ulje; farmakologija; ekstrakti, biljni; mišić, relaksacija; antioksidanti; antiinfektivni agensi.

Introduction

Although many species from genus *Seseli* (Apiaceae) are distributed worldwide, there are some endemic species that are narrowly distributed, sometimes only at the locations where the taxon was first described [1, 2]. Different *Seseli* species contain various pharmacologically active compounds: coumarins [3], flavonoids [4], polyacetylenes [5], sesquiterpene

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compounds [6]. Many plants of this genus species are traditionally used worldwide as food [7], spices [8] and remedies for treating various disorders [9] including gastrointestinal [10] and even neurological [11] ones. They also contain secretory structures in different plant organs for essential oil (EO) deposition. The chemical composition of EOs isolated from different plant organs greatly varies [12]. Until recently, EO contribution to total demonstrated pharmacological effects of the isolates from various *Seseli* species was unknown [13]. A possible explanation for this was pronounced pharmacological effects and promising therapeutic potential of coumarins as the main group of secondary metabolites in *Seseli* species [14]. *Seseli (S.) gracile* Waldst. & Kit. (Apiaceae*)* is endemic species present in Serbian and Romanian flora [15]. The first study of *S. gracile* volatile fraction showed that it was not a rich source of EO like some other species from the same genus [12, 16]. This could be the reason why its chemical composition was unknown until recently [17]. According to the literature, none of the previous studies investigated the pharmacological effects of *S. gracile* EO.

Methods

Chemicals

Chemicals used were: sodium chloride, potassium chloride, calcium chloride, magnesium chloride, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate, glucose and boric acid from Lach-Ner, s.r.o (Brno, Czech Republic); acetylcholine chloride, phenylephrine hydrochloride, *n*-hexane (CHROMASOLV®, for HPLC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,3,5-triphenyltetrazolium chloride (TTC) and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, U.S.A.); ethanol absolute (for HPLC) from Fisher Scientific (Loughborough, UK) and carboxymethyl cellulose, sodium salt from Across Organics (Geel, Belgium). Ampicillin, amikacin and nystatin were purchased from Galenika, Belgrade, Serbia; Müeller-Hinton broth and Sabouraud dextrose broth were purchased from Institute of Immunology and Virology Torlak, Belgrade, Serbia.

Plant material

Aerial parts of *S. gracile* were collected in 2015 from the natural habitat on Mali Štrbac in the Danube/Đerdap gorge (Serbia) in a flowering phase (July) and identified by Professor Branislava Lakušić. A voucher specimen was deposited in the Herbarium of the Department of Botany, Faculty of Pharmacy, Belgrade under the accession number HFF3702.

EO isolation

The EO from air-dried and powdered plant material was isolated by hydro distillation in a Clevenger-type apparatus, using 1 mL of *n*-hexane as a collecting solvent according to the European Pharmacopoeia 7.0 procedure.

Chemical analysis

Equipment and operating conditions of essential oil chemical analysis are presented in Table 1.

The linear retention indices (RI) were determined in relation to a homologous series of n-alkanes (C_9–C_{24}) under the same operating conditions.

Identification of the compounds was based on comparison of their RI, retention times (RT), mass spectra (MS) and flame ionization detector (FID) values with those obtained from authentic samples and/or the NIST/NBS, Wiley libraries and the literature [18].

Relative percentages of the identified compounds were computed from the gass chromatography (GC)-FID peak area.

Animals

Male normotensive Wistar rats (200–300 g) were housed in standard laboratory conditions (4–8 animals in a cage), at room temperature and 12-hour light-dark cycle with unrestricted access to food and water. All procedures conducted in this pharmacological test were approved by the local Ethics Committee and are in compliance with the Euro-

Table 1

Equipment and operating conditions of essential oil chemical analysis

Equipment	Operating conditions
GC system	Agilent 6890N
Detectors	5975 MSD and FID
Column	HP-5 MS column (30 m x 0.25 mm x 0.25 μm)
Operating conditions	
Injection volume	2 μL
Injection temperature	200 °C
Split ratio	10 : 1
Carrier gas	Helium
Gas flow rate	1.0 mL/min (constant flow mode)
Column temperature	60–280 °C (linearly programmed) with 3 °C/min rate; held at 280 °C for 5 min
Transfer line temperature	250 °C
FID detector temperature	300 °C
m/z range of EI mass spectra	35–550

GC – gas chromatography; FID – flame ionization detector; EI – electron ionization.

pean Council Directive of November 24, 1986 (86/609/EEC).

Antioxidant activity (DPPH radical assay)

Three aliquots of the EOs were mixed with 0.4 mL of 0.5 mM DPPH in absolute ethanol, and the final volume was adjusted to 2 mL. All mixtures were vigorously stirred for 30 s and left for 30 min in dark at room temperature. Zero point four millilitres of 0.5 mM DPPH diluted up to 2 mL of absolute ethanol was used as the control. The absorbance of samples and controls were measured at 517 nm immediately after incubation. Scavenging (SC) of DPPH radical was calculated using the equation: SC (%) = 100 (A_0 - As)/A_0, where A_0 is the absorbance of the control, and As is the absorbance of the tested sample. The SC_{50} value represented the concentration of the EO that caused 50% of DPPH radical scavenging. Results were compared with the activity of L-ascorbic acid [19].

Antimicrobial activity

Antimicrobial activity was tested by the broth microdilution method and expressed as the minimal inhibitory concentrations (MIC). Standard strains of three Gram (+) bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633), four strains of Gram (−) bacteria (*Escherichia (E.) coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Salmonella enterica* subsp. *enterica* serovar *Abony* NCTC 6017 and *Pseudomonas aeruginosa* ATCC 27853) and two strains of yeasts *Candida (C.) albicans* ATCC 10231 and *C. albicans* ATCC 10259 were used in this study.

The assay was performed in 96-well microtiter plates with test strains suspended in Müller-Hinton and Sabouraud broth for the bacteria and yeast, respectively, to make the final density of 5×10^5 cfu/mL. 2,3,5-triphenyltetrazolium chloride (TTC) was added as the indicator of bacterial growth, while the growth of *C. albicans* was estimated by monitoring the formation of a precipitate or opalescence. Microorganisms and TTC were used as a positive control. Serial doubling dilutions from 31.25 to 500.00 μg/mL of the investigated EO were prepared and tested in duplicate against each organism. First EO samples were dissolved in DMSO)and then diluted with Müller-Hinton and Sabouraud broth. The final concentration of DMSO in the tested samples was lower than 1%. The plates were incubated at 37 °C for 24 h for the bacteria and 48 h for *C. albicans*. As standards, antibiotics ampicillin, amikacin and nystatin were used [19].

Spasmolytic activity

To avoid any anaesthetic substances impact on the further pharmacological assay, animals were sacrificed by stunning and exsanguination. Ileum was isolated, cleaned from perivascular tissues, and quickly cut into rings (3–5 mm) [20]. These rings were suspended by triangle stainless steel hooks connected to a force transducer (Ugo Bazile model) in organ baths containing 10 mL Tyrode's solution (mmoL/L: NaCl, 136.89; KCl, 2.68; $CaCl_2$, 1.80; $MgSO_4$, 1.05; NaH_2PO4, 0.42; $NaHCO_3$, 11.90; glucose, 5.5). This solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2 at 36 °C. The rings were incubated for 60 minutes (stabilization period) and after that, they were gradually stretched to the tension of 1 g. Tonic contractions of isolated ileum segments, elicited by depolarizing KCl solution (80 mM) were registered using an isotonic transducer (Ugo Basile S.R.R. model 7003) and displayed on data acquisition program LabScribe2.

Sample preparation

EO dissolved in 0.5% carboxymethyl cellulose sodium salt (Na-CMC) water solution was added directly to the organ bath in a cumulative manner (0.1–400 nL/mL). Higher EO concentrations were added only after achieving maximum response effect of the following lower concentration or 10 minutes after an absence of the response. The spasmolytic effect was expressed as the concentration (IC_{50}) which causes 50% relaxation of contraction induced by KCl (100% contraction). The EO-induced relaxation was compared with the vehicle effect.

Statistics

The one-way analysis of variance (ANOVA) with post-hoc Bonferroni test was used for determination of statistically significant differences between the spasmolytic effects of tested sample and vehiculum.

Results

Chemical analysis

S. gracile essential oil chemical analysis results are presented in Table 2. Twenty-two compounds were identified in the analysed sample representing 99.31% of total essential oil. Monoterpenes, with a content of 94.1%, were the mayor constituents. *S. gracile* essential oil is characterized by high amount of terpinolene (40.55%) followed by γ-terpinene (23.34%) and *p*-cymene (9.62%).

Antioxidant activity (DPPH radical assay)

Antioxidant activity of *S. gracile* EO was determined using DPPH radical assay. The results of DPPH antiradical activity of analysed EO and L-ascorbic acid were presented in Figure 1. The concentration of the sample that caused 50% of DPPH radical scavenging (SC_{50}) was 78.6 μg/mL. Although *S. gracile* EO exhibited lower antioxidant activity than L-ascorbic acid (referent antioxidant substance), it could be considered as significant.

Antimicrobial activity

S. gracile EO has exhibited none or weak antimicrobial potential (Table 3). In the range of tested concentrations,

Table 2

***Seseli gracile* essential oil tested sample chemical composition**

Compound	KI	%
α -Thujene	929.0	0.30
α - Pinene	936.2	2.17
Sabinene	975.8	1.67
ß- Pinene	980.8	6.26
Myrcene	992.4	1.24
α -Phellandrene	1007.3	0.28
p-Cymene	1028.0	9.62
Limonene	1031.1	3.65
ß-*(Z)*-Ocimene	1038.1	2.86
ß-*(E)*-Ocimene	1048.2	0.59
γ-Terpinene	1063.4	23.34
Terpinolene	1095.9	40.55
1,3,8-*p*-Menthatriene	1136.4	0.37
trans-*p*-Mentha-2,8-dien-1-ol	1145.8	0.74
cis-*p*-Mentha-2,8-dien-1-ol	1167.8	0.53
cis-Carveol	1177.4	0.29
p-Cymen-9-ol	1186.3	1.19
α-Copaene	1375.9	0.20
(E)-Caryophyllene	1419.9	0.63
ß-*(E)*-Farnesene	1456.1	1.28
trans-Muurola-4(14),5-diene	1481.5	0.32
δ-Cadinene	1523.8	1.22
Total identified		99.31
Monoterpenes		94.10
Sesquiterpenes		3.66
Other		1.55

KI – Kovat's retention indices determined relative to two series of n-alcanes on HP-5 MS column.

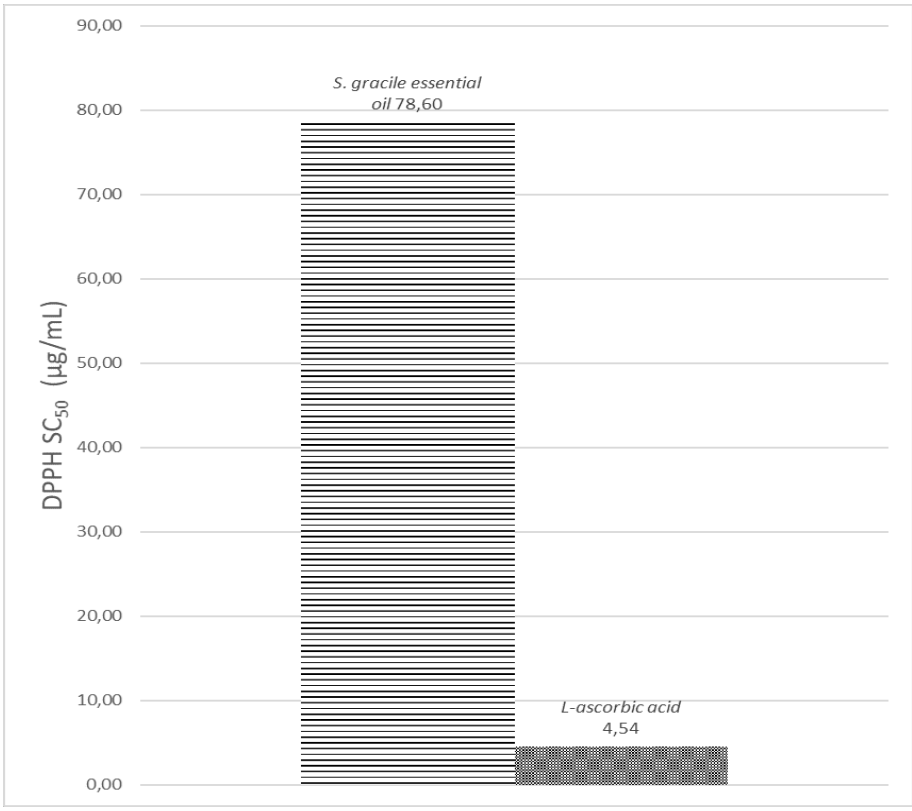

Fig. 1 – Antioxidant activity of *S. gracile* essential oil compared to L-ascorbic acid. DPPH – 2,2-diphenyl-1-picrylhydrazyl; SC_{50} – scavenging concentration 50%.

Table 3

Antimicrobial effects of *Seseli gracile* essential oil (SGEO) and standard antibiotics on tested strains of bacteria and yeasts

Microorganisms		(SGEO)	Ampicillin	Amikacin	Nystatin
		MIC (μg/mL)			
Gram (+)	*Staphylococcus aureus* ATCC 25923	> 500	0.5	2	n.t.
	Enterococcus faecalis ATCC 29212	> 500	0.5	n.t.	n.t.
	Bacillus subtilis ATCC 6633	> 500	n.t.	n.t.	n.t.
Gram (-)	*Escherichia coli* ATCC 25922	500	2	5	n.t.
	Klebsiella pneumoniae ATCC 13883	> 500	4	n.t.	n.t.
	Salmonella abony NCTC 6017	> 500	n.t.	n.t.	n.t.
	Pseudomonas aeruginosa ATCC 27853	> 500	3	0.5	n.t.
Yeast	*Candida albicans* ATCC 10231	> 500	n.t.	n.t.	3
	Candida albicans ATCC 10259	> 500	n.t.	n.t.	5

MIC – minimal inhibitory concentrations; n. t. – not tested.

MIC was determined only for the strain of *E. coli* (500 μg/mL). The EO did not affect the growth of other microorganisms (MIC > 500 μg/mL).

Spasmolytic activity

Essential oil of *S. gracile* showed dose-dependent relaxation effect on isolated ileum, previously contracted with a high concentration of K^+ (C_{KCl}= 80 mM). EO concentration which caused 50% of inhibition was 271.4 nL/mL. Concentration-response curves of the relaxation effect of EO and vehiculum are presented in Figure 2 and show that the relaxation effect originates from the EO.

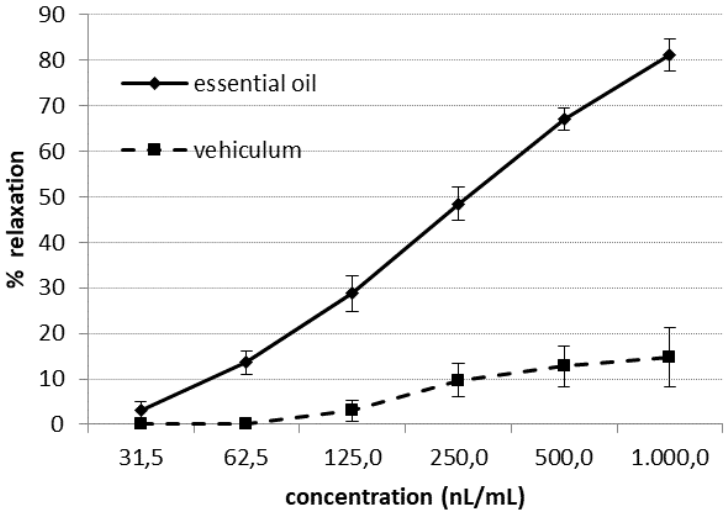

Fig. 2 – Concentration-response curve for relaxation of induced effect by increasing concentrations *(*0.1–400 nL/mL*)* of essential oil (solid line curve) and by vehiculum (0.5% carboxymethyl cellulose sodium salt) added in the same manner as an essential oil (dashed line curve). Data are given as the mean ± standard error of the mean. The concentration curves are significantly different (one-way ANOVA, $p < 0.001$; *post-hoc* Bonferonni test, $p < 0.001$).

Discussion

As previously shown, EO isolated from aerial parts of *S. gracile* is characterized by a high amount of monoterpenes (with content over 90%). Its major constituents were terpinolene (> 40.0%) followed by γ-terpinene (> 20.0%) and *p*-cymene (9.62%) [17].

Some similarities regarding monoterpenes content were observed when comparing the chemical composition of EOs isolated from aerial parts between *S. gracile* and other members of *Seseli* genus. Monoterpenes are major constituents of EOs isolated from aerial parts of *S. rigidum* [21], *S. campestre* [22] and *S. tortuosum* [23] as well as from *S. rigidum* fruit [24]. Moreover, in abovementioned EOs monoterpenes were mostly represented with α-pinene (35.9–57.4%). Previous studies also showed that in EOs isolated from aerial parts of other *Seseli* species, terpinolene was not usually the most abundant compound. On the other hand, γ-terpinene was detected in EOs isolated from fruits of *S. petraeum* [25], *S. rigidum* [24], *S. globiferum* [26] and leaves of *S. bocconi* [27] as one of the major components.

Content of terpinolene and γ-terpinene in analyzed EO of *S. gracile* and their potency for various pharmacological

activities, such as antioxidant, antimicrobial and antispasmodic were positively correlated [28–30].

Antioxidant activity (DPPH radical assay)

EOs isolated from the aerial parts of different *Seseli* species did not exhibit significant antioxidant potential [24, 26]. Chemical analysis of *S. gracile* EO showed *γ*-terpinene and terpinolene were the most abundant components, representing 63.89% of total oil [17]. Tepe et al. [31] showed that *γ*-terpinene, as a second most abundant compound of *Clinopodium vulgare* L. (Lamiaceae) EO significantly contributed to its total antioxidant activity with IC_{50} = 122 μg/mL. The study which tested DPPH antiradical activity of twenty-one citrus EOs components demonstrated that *γ*-terpinene and terpinolene had a very similar radical-scavenging effect, but much stronger than that of a Trolox (referent antioxidant substance). Further analysis of structure-activity relation showed that the presence of 1,4-cyclohexadiene moiety and conjugated system enhances antiradical activity [32]. Considering that DPPH radical scavenging activity was not detected for *p*-cymene [31], the antioxidant potential of *S. gracile* EO mainly depends on the content of *γ*-terpinene and terpinolene [31].

Essential oil isolated from aerial parts of *S. gracile* did not exhibit significant antimicrobial activity. Only Gram (-) *E. coli* strain showed a slightly higher sensitivity to the tested sample with MIC value of 500 μg/mL. *Melaleuca alternifolia* (Maiden & Betche) Cheel (Myrtaceae) EO is good source of monoterpenes *γ*-terpinene and terpinolene with a content of 23.0% and 3.1%, respectively [33]. Carson and Riley[30] showed that *γ*-terpinene and terpinolene exhibited significant antimicrobial activity against *Staphylococcus aureus* with MICs 4.0% and 2.0% (expressed as v/v), respectively. Moreover, terpinolene also exhibited significant antimicrobial activity against *E. coli* and *C. albicans* with MICs, 4.0% and 8.0% (v/v), respectively, in the same study. Considering that in the abovementioned study, *γ*-terpinene and terpinolene demonstrated significant individual antimicrobial effects, it was expected that *S. gracile* EO should exhibit strong antimicrobial activity. The lack of expected activity cannot be fully explained unless additional research is conducted. A partial explanation for the absence of activity may be related to the potential antagonistic effect of the major components, the effect already confirmed for the mixture of terpinen-4-ol and *γ*-terpinene. Results of Cox et al.[34] have indicated a decrease in antimicrobial activity of a mixture of these compounds on *S. aureus* versus their individual effects on the same bacterial strain.

Spasmolytic activity

Several EOs rich in monoterpenes showed spasmolytic activity. The most abundant component of *S. gracile* EO, terpinolene showed the ability to inhibit serotonin-induced contraction of the isolated ileum of the rat [35]. The second most abundant component of *S. gracil*e EO *γ*-terpinene exhibited antispasmodic activity on the isolated rabbit jejunum with an IC_{50} = 8.6 μg/mL [36]. EO isolated from *Poliomintha longiflora* A. Gray *(*Lamiaceae*)* exhibits antispasmodic activity based on the presence of *p*-cymene and carvacrol. *p*-Cymene showed good spasmolytic activity on carbachol-induced contractions with an IC_{50} value of 9.85 μg/mL [37]. According to the literature data, more than 70% of *S. gracile* EO possesses the potential for smooth muscle relaxation. However, without further research, it remains unknown whether these components in the mixture have a synergistic effect or, as in the case of *α*- and *β*-pinene, a weaker spasmolytic effect than the collection of individuals [38].

Conclusion

The results from this study suggest moderate DPPH antiradical and good spasmolytic activity of *S. gracile* herb essential oil. Despite expectations based on the data of EO chemical composition analysis, it was not possible to confirm significant antimicrobial activity. These results justify the need for further pharmacological investigations of *S. gracile* EO and its most abundant compounds.

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