

Composition of the Essential Oil of *Coristospermum cuneifolium* and Antimicrobial Activity Evaluation



Authors

Alessandro Venditti¹, Claudio Frezza², Giulia Salutari¹, Mirella di Cecco³, Giampiero Ciaschetti³, Alessandra Oliva⁴, Massimiliano De Angelis⁴, Vincenzo Vullo⁴, Manuela Sabatino⁵, Stefania Garzoli⁵, Federico Peppi⁵, Rino Ragno⁵, Mauro Serafini², Armandodoriano Bianco¹

Affiliations

- 1 Dipartimento di Chimica, Università di Roma “La Sapienza”, Rome, Italy
- 2 Dipartimento di Biologia Ambientale, Università di Roma “La Sapienza”, Rome, Italy
- 3 Ente Parco Nazionale della Majella, Sulmona, Italy
- 4 Dipartimento di Sanità Pubblica e Malattie Infettive, Università di Roma “La Sapienza”, Rome, Italy
- 5 Dipartimento di Chimica e Tecnologie del Farmaco, Università di Roma “La Sapienza”, Rome, Italy

Key words

Coristospermum cuneifolium, Apiaceae, essential oil composition, ethnopharmacology, comparison within the genus, antimicrobial activity

received 03.05.2017

revised 01.07.2017

accepted 24.07.2017

Bibliography

DOI <https://doi.org/10.1055/s-0043-118131>

Planta Med Int Open 2017; 4: e74–e81

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ISSN 2509-9264

Correspondence

Dr. Alessandro Venditti

Dipartimento di Chimica

Università di Roma “La Sapienza”

Piazzale Aldo Moro 5

00185 Rome

Italy

Tel.: +39/064/99 13 622, Fax: +39/064/99 13 841

alessandro.venditti@gmail.com

ABSTRACT

For the first time, the chemical composition and antimicrobial evaluation of *Coristospermum cuneifolium* (previously named *Ligusticum lucidum* subsp. *cuneifolium*) essential oil obtained from the aerial parts are reported in this work. Approximately 85% of the total constituents were identified by GC-MS analysis, evidencing the presence of 12 chemical components which belong to several classes of natural compounds. Most of them are reported for the first time in the *Ligusticum* genus (s.l.) and in the Apiaceae family. Their presence was able to provide a rationale for essential oil use in the field similar to those obtained from other species of the *Ligusticum* genus (s.l.). Moreover, the huge presence of aromatizing and flavoring components, accounting for 44.4% of the essential oil composition, might make *C. cuneifolium* a useful natural source of aromatic components for the food and cosmetic fields. In addition to this, a deep comparison of the essential oil of this species with that of other entities within the *Ligusticum* genus (s.l.) was performed and discussed on a chemotaxonomic basis.

The essential oil was tested for its antimicrobial activity at both high and low inoculum ($\sim 5 \times 10^5$ and $\sim 5 \times 10^3$ cfu/mL, respectively) against several bacterial and fungal strains, including methicillin-susceptible *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 14053), methicillin-resistant *S. aureus* (clinical strain), carbapenem-susceptible *Klebsiella pneumoniae* (clinical strain), carbapenem-resistant *K. pneumoniae* (clinical strain), and carbapenem-resistant *Acinetobacter baumannii* (clinical strain).

A high potency against *C. albicans* was shown, with an absence of growth at the concentration of 3.01 mg/mL; similarly, for methicillin-susceptible *S. aureus* and methicillin-resistant *S. aureus*, a reduction of 1.73 and 2 log₁₀ cfu/mL at the concentration of 3.01 mg/mL was observed. With regard to gram-negative microorganisms, only slight potency against *A. baumannii* was shown, whereas no activity was found against *E. coli* and *K. pneumoniae*.

Introduction

Coristospermum cuneifolium (Guss.) Bertol. [synonym of *Ligusticum lucidum* Mill. subsp. *cuneifolium* (Guss.) Tammaro] is a perennial herbaceous plant belonging to the Apiaceae family. From the systematic point of view, this plant was originally classified as *Ligusticum*, a genus which is no longer recognized as valid since it resulted in not being a monophyletic genus from both the morphological and genetic profile [1]. For this reason, it was decided to use the name *Coristospermum*, and the subspecies *L. lucidum* subsp. *cuneifolium* has been upgraded to the species rank and classified as *C. cuneifolium* [2].

The name of the genus derives from the greek terms κορυστός (*korystos*) and σπέρμα (*spérma*), which together mean “full of seeds”, while the name of the species derives from Latin and refers to the typical wedge-shaped leaves.

From the morphological point of view, this species is characterized by an erect stem that is fully branched, striated, and fluted. The leaves are long in the lower part of the plant, and linear and lanceolate in the upper portion. The inflorescence is formed by a composed umbel and is constituted by small white flowers that bloom between June and July. Lastly, the fruits are oblong (► **Fig. 1**) [3].

This species is endemic in the Italian territory, especially in central regions of Italy such as Latium, Abruzzo, and Molise [4]. There are only a couple of articles in the literature on entities belonging to the *Coristospermum* genus because of its relative recent recognition as an autonomous genus, and these mainly report on the genetic diversity of isolated populations [5] and karyological aspects [6]. There is, instead, only a single work reporting on the activities and chemical composition of *L. lucidum* subsp. *cuneifolium* (botanical denomination no longer valid, but referred to as *C. cuneifolium*) solvent extract showing several pharmacological properties, among which the anti-inflammatory and antioxidant ones are the most well known and important [7]. By consequence, many of the *Ligusticum* species are used in the folk medicine of several countries, especially China and America. In particular, *Ligusticum striatum* DC. is used in China to treat pain and to cure several hematological disorders such as thrombosis and ischemia due to its high cardiovascular, neuroprotective, and anti-fibrotic properties



► **Fig. 1** *C. cuneifolium* (Guss.) Bertol.

[8–10]. Indeed, *Ligusticum porteri* J. M. Coult. & Rose is still used by native Americans to cure the flu, colds, and toothaches [11] but, to date, there is no record of the use of *C. cuneifolium* in local ethno-medical traditions.

From a literature survey on essential oil (EO) from plants of the *Ligusticum s.l.* genus, many have seen that numerous reports indicated several pharmacological activities, i. e., analgesic, cicatrizing, antipyretic, antioxidant, insecticidal, and anticonvulsant [12–14]. Several publications report on the *Ligusticum s.l.* genus EO composition and its associated pharmacological properties [15–17], while no information was found regarding *C. cuneifolium* EO (CCEO) and associated pharmacological activity.

With the aim to verify the presence of certain chemical components that might justify the use of *C. cuneifolium* in the ethnopharmacological field [similarly as for other *Ligusticum* (s.l.) species] and to complete our previous study on the *C. cuneifolium* non-volatile phytochemical pattern [18], herein the CCEO isolation is reported along with its chemical composition analysis and antimicrobial activity evaluation against several bacterial and fungal strains.

Finally, a detailed comparison of the CCEO chemical composition and other EOs obtained from species of the *Ligusticum* (s.l.) genus is reported.

Results and Discussion

The CCEO GC-MS analysis led to the identification of 12 compounds. This corresponds to 84.2% of the total area of the gas chromatogram (► **Table 1**).

Compounds **1**, **2**, **4**, **7**, and **9** are sesquiterpenes, compounds **3** and **5** are organic acids, compound **6** is a diketone, compound **8** is a trisubstituted benzaldehyde, compound **10** is a bicyclic monoterpene lactone, compound **11** is a substituted benzyl alcohol and, lastly, compound **12** is an acyclic diterpene alcohol. The structures of the main components are reported in ► **Fig. 2**.

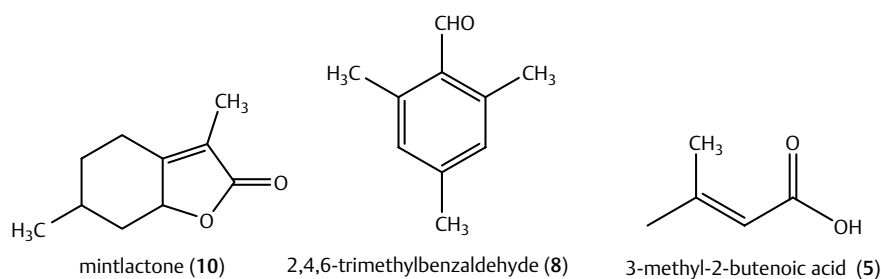
β -farnesene (**1**), β -caryophyllene (**2**), β -copaene (**4**), caryophyllene oxide (**7**), spathulenol (**9**) mintlactone (menthactone) (**10**), and phytol (**12**) represent new constituents of the EO for the *C. cuneifolium* species, 2,4,6-trimethyl-benzaldehyde (**8**) is a new constituent of the EO for the *Ligusticum s.l.* genus, 3-methyl-butanoic acid (**3**), 3-methyl-2-butenoic acid (**5**), 2,5-bornanedione (**6**), and 2,4,6-trimethylbenzyl alcohol (**11**) are, instead, new constituents of the EO also for the Apiaceae family.

β -Farnesene (**1**) was already evidenced in *Ligusticum s.l.* [15, 19, 20] and, in general, represents one of the major constituents of the EO of plants belonging to the Apiaceae family [21–23]. This compound was reported to have insecticidal properties [24]. β -Caryophyllene (**2**) was already reported in the *Ligusticum s.l.* genus [19] and has anti-inflammatory, gastroprotective, and anesthetic properties [25–27].

3-Methyl-butanoic acid (**3**; common name isovaleric acid) and 3-methyl-2-butenoic acid (**5**) (also known as 3-methyl-crotonic acid) were found only in traces in the EOs of plants belonging to the Berberidaceae, Lamiaceae, and Asteraceae families [28, 29] and were recognized for the first time during the present study as constituents of the EO obtained from an Apiaceae species. It is interesting to note that these compounds resulted instead as being present in quite a high amount in CCEO, accounting for 3.5 and 10.6%, re-

► **Table 1** Essential oil composition of *C. cuneifolium* and comparison among *C. cuneifolium* and other species of the *Ligusticum* s.l. genus.

Compounds	<i>C. cuneifolium</i>	Retention index	Area %	<i>L. striatum</i> [11]	<i>L. mutellina</i> [15]	<i>L. porteri</i> [16]	<i>L. sinense</i> [37]	<i>L. jeholense</i> [37]	<i>L. grayi</i>
β-Farnesene (1)	0.8%	1682	0.8	0.18%	1.6%	0.3%	–	–	–
β-Caryophyllene (2)	5.2%	1629	5.2	–	3.1%	–	–	–	–
3-Methylbutanoic acid (3)	3.5%	1686	3.5	–	–	–	–	–	–
β-Copaene (4)	0.5%	1512	0.5	–	0.1%	–	–	–	–
3-Methyl-2-butenoic acid (5)	10.6%	1821	10.6	–	–	–	–	–	–
2,5-Bornanedione (6)	2.5%	2006	2.5	–	–	–	–	–	–
Caryophyllene oxide (7)	9.8%	2035	9.8	–	1.9%	–	–	–	–
2,4,6-Trimethylbenzaldehyde (8)	14.8%	2065	14.8	–	–	–	–	–	–
Spathulenol (9)	7.1%	2152	7.1	0.26%	5.1%	–	3.3%	0.9%	–
Mintlactone (10)	24.3%	2316	24.3	–	–	–	–	–	–
2,4,6-Trimethylbenzyl alcohol (11)	3%	2340	3.0	–	–	–	–	–	–
Phytol (12)	2%	2620	2.0	1.14%	–	–	–	–	–
Total identified			84.1	–	–	–	–	–	–



► **Fig. 2** Structures of the three major constituents of the essential oil of *C. cuneifolium*.

spectively. The former has a very pungent smell and seems to have anticonvulsant properties [30], while for the latter, no pharmacological properties are reported in the literature but, in medicine, its presence is linked with a disease known as organic aciduria [31].

On the other side, β -copaene (**4**) was already evidenced, even if in small traces, in *Mutellina purpurea* (Poir.) Reduron, Charpin & Pimenov [19] [considered as syn. of *Ligusticum mutellina* (L.) Crantz] and in other entities of the family, i. e., *Ferula glauca* L. [32]. It was reported to exhibit antioxidant effects on human lymphocyte cultures [33].

The oxygenated monoterpene 2,5-bornanedione (**6**) resulted in being a compound more typical of the Lamiaceae family [34]. This compound, which is an oxidation product of camphor, showed many aromatizing properties [34].

Caryophyllene oxide (**7**) was previously identified only in *L. mutellina* [19]. From the pharmacological point of view, this compound presents strong anti-inflammatory and cytotoxic properties [35, 36].

2,4,6-Trimethyl-benzaldehyde (**8**), also known as mesitaldehyde, was already found only in entities belonging to the Apiaceae family, even in small concentrations. In particular, its presence was reported in *Eryngium corniculatum* Lam. [37], *Eryngium foetidum* L.

[38], *Trachydium roylei* Lindl. [39], and *Prangos ferulacea* (L.) Lindl. [40]. It also has aromatizing properties and was present as one of the main components in the CCEO with 14.8% of the total composition.

Indeed, 2,4,6-trimethylbenzyl alcohol (**11**), also known as mesityl-methanol, was never recognized as an EO component from species of the Apiaceae family, and it accounted for 3.0% of the total composition in CCEO. Also, this compound is a good flavoring agent. Its presence is chemically related to compound **8**, which represents its oxidized form.

Spathulenol (**9**) represents one of the major constituents of the EOs of a plant belonging to the *Ligusticum* s.l. genus [15] and to the Apiaceae family, which was also identified in *T. roylei* Lindl., *Kundmannia anatolica* Hub. Mor., and *Seseli rigidum* Waldst. Kit. [39, 41, 42]. This compound was reported to exert strong antibacterial properties [43].

Mintlactone (menthalactone; **10**) was originally identified as a minor component of *Mentha piperita* [44–46] and *Mentha aquatica* [47] EOs and, more recently, its presence was also evidenced in aged red wines [48]. It is a very interesting odorant compound related to the *p*-menthane bicyclic γ -lactone (3,6-dimethyl-4,5,6,7-tetrahydro-benzo[b]-furan-2(*H*)-one) derivatives that are widely

used as flavoring agents in foods and cosmetics [49–52]. For this reason, in the literature there are a great number of works reporting on organic synthetic approaches [53–56] for the preparation of mintlactone and its isomers. The presence of mintlactone (**10**) in CCEO (as well as for *Ligusticum* s.l.) is herein reported for the first time, and it is worth to note that it accounted for 24.1 % of the total composition, therefore, *C. cuneifolium* may be regarded as an abundant natural source of this compound.

Lastly, phytol (**12**) was already evidenced in the *Ligusticum* s.l. genus [57] as well as in the Apiaceae family [58] and showed anti-inflammatory, antimicrobial, and diuretic properties [59].

► **Table 1** shows a comparison between the CCEO chemical composition and those cited in the literature for other species of the *Ligusticum* s.l. genus, focusing only on overlapping compounds.

As can be seen, seven compounds were exclusively found in *C. cuneifolium*: 3-methyl-butanoic acid (**3**), 3-methyl-2-butenoic acid (**5**), 2,5-bornanedione (**6**), 2,4,6-trimethyl-benzaldehyde (**8**), mintlactone (menthalactone) (**10**), and 2,4,6-trimethylbenzyl alcohol (**11**).

Spathulenol (**9**) was, instead, the most common compound and, in fact, it was found in all of the compared species besides *L. porteri* and *L. grayi* Coult. & N. E. Rose, both coming from the USA. Anyway, in the studied species (*C. cuneifolium*), the total percentage of this compound was much more abundant compared to any other *Ligusticum* s.l. species. This might be one peculiarity of the plant growing in Majella National Park and might be used as distinctive marker of this area.

The other distinctive trait is the presence of mintlactone (**10**) as a major component of the EO, which, to the best of our knowledge, has not been previously recognized in any *Ligusticum* s.l. EOs. This additional peculiarity, as well as the total absence of ligustilide and related butyl-phthalides which, on the contrary, are often recognized as the principal components of the EOs from *Ligusticum* s.l. species, contributed to the very unique chemotype observed in *C. cuneifolium*.

From a chemosystematic point of view, the total absence of butyl-phthalidic derivatives and the presence of peculiar components not yet demonstrated in *Ligusticum* s.l. genus is further evidence to enhance the recent upgrade to the species rank of *C. cuneifolium*, also on the phytochemical basis.

The EO composition from the species *L. mutellina*, harvested in Poland, is more similar to that of *C. cuneifolium*, likely indicating a similar environmental and climatic influence of growth area. β -Farnesene (**1**) is the only component present in almost every species listed in ► **Table 1** with similar relative abundance in all of them. The EO composition of *L. grayi* [60] is the only one not presenting any constituent in common with that of *C. cuneifolium*.

The activities of reference compounds and CCEO are reported in ► **Table 2, 3**, respectively. As expected, both methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) were susceptible to vancomycin and rifampin, whereas a high level of meropenem resistance was observed in clinical strains of *Klebsiella pneumoniae* and *Acinetobacter baumannii*.

CCEO showed high potency against *Candida albicans* both at high and low inoculum, with an absence of growth at a concentration of 3.01 mg/mL. Likely, compared with the starting inoculum (5.30 log₁₀ cfu/mL), we observed a reduction of 1.73 and 2 log₁₀ cfu/mL for MSSA and MRSA at the concentration of 3.01 mg/mL, re-

spectively; at a lower inoculum (3.69 log₁₀ cfu/mL), the potency of CCEO was bactericidal only against MRSA, with an absence of growth after 24 h incubation. At high inoculum, no activity was observed against *Escherichia coli* and *K. pneumoniae* (both carbapenem susceptible and resistant strains); interestingly, CCEO demonstrated a slight potency against a clinical strain of carbapenem-resistant *A. baumannii*, with a reduction of 1.75 log₁₀ cfu/mL compared with the initial inoculum.

The antimicrobial activity of *C. cuneifolium* might be very interesting when considering its possible use as a part of the treatment of infections caused by MSSA, MRSA, and, in particular, against *C. albicans*, both at high and low inoculum.

The activity against carbapenem-resistant *A. baumannii* deserves further investigation in order to evaluate the role of CCEO in the setting of multidrug-resistant *A. baumannii* infections, which represent a global threat due to the limited available therapeutic options and the high mortality [61].

From an analytical point of view, CCEO resulted in being constituted by 12 compounds, namely, β -farnesene (**1**), β -caryophyllene (**2**), 3-methyl-butanoic acid (**3**), β -copaene (**4**), 3-methyl-2-butenoic acid (**5**), 2,5-bornanedione (**6**), caryophyllene oxide (**7**), 2,4,6-trimethyl-benzaldehyde (**8**), spathulenol (**9**), mintlactone (**10**), 2,4,6-trimethylbenzyl alcohol (**11**), and phytol (**12**). Seven of them are new constituents of CCEO, one is a new constituent of the EO for the *Ligusticum* s.l. genus and five are new constituents of the EO oil for the Apiaceae family. The presence of all these peculiarities in the CCEO composition, together with the absence of ligustilide and related compounds that are widely distributed among the *Ligusticum* genus, may give additional evidence, from a chemosystematic standpoint, to support the upgrade of *C. cuneifolium* to an autonomous species.

The presence of these components also provide a rationale for the use of CCEO in the ethnopharmacological field, similar to that reported for the EOs from other species of the *Ligusticum* (s.l.) genus, due to their outstanding medical properties. In addition, the huge presence of components widely known for their aromatizing and flavoring properties (i. e., **6, 8, 10**, and **11**, which accounted for 44.4 % of the total composition) might make the studied species a useful natural source of aromatic components for the food and cosmetic fields.

When making a comparison, it emerged that seven compounds were exclusively present in *C. cuneifolium* and that the EO composition showed several similarities with that reported for *L. mutellina*. This fact would lead us to believe that these species have very similar environmental conditions. Indeed, spathulenol (**9**) was also present in the majority of the *Ligusticum* species. It is interesting to note that **9** might be regarded as a sort of a marker compound because it has been found in CCEO at a much higher concentration, but we cannot exclude that it represents a peculiarity of the plants growing in Majella National Park without further studies on different populations.

Last but not least, the observed antimicrobial properties of CCEO provide useful information for its possible role in the therapy of infections caused by MSSA, MRSA, and, in particular, *C. albicans*, whereas the activity against a carbapenem-resistant strain of *A. baumannii* deserves further investigation.

► **Table 2** Activity of antimicrobials commonly used in clinical practice against different bacterial strains at high (A) and low (B) inoculum.

Strain	MIC/MBC (mg/L)							
	A				B			
	VAN	MEM	RIF	FLU	VAN	RIF	MEM	FLU
MSSA (ATCC 29213)	0.50/1.00	–	0.03/0.07	–	0.50/0.50	0.03/0.03	–	–
MRSA*	1.00/1.00	–	0.07/0.07	–	0.50/0.50	0.07/0.07	–	–
<i>Candida albicans</i> (ATCC 14053)	–	–	–	0.25/1.00	–	–	–	0.25/0.50
CS <i>Klebsiella pneumoniae</i> *	–	0.25/0.25	–	–	–	–	0.03/0.03	–
CR <i>Klebsiella pneumoniae</i> *	–	16/32	–	–	–	–	16/32	–
<i>Escherichia coli</i> (ATCC 25922)	–	0.1/0.25	–	–	–	–	0.03/0.03	–
CR <i>Acinetobacter baumannii</i> *	–	256/256	–	–	–	–	32/32	–

* Clinical strain. MSSA: methicillin-susceptible *S. aureus*; MRSA: methicillin-resistant *S. aureus*; CS: carbapenem susceptible; CR: carbapenem resistant. VAN: vancomycin; RIF: rifampin; MEM: meropenem; FLU: fluconazole

► **Table 3** Activity of *C. cuneifolium* against different bacterial strains at high (A) and low (B) inoculum.

Strains	A			B		
	$\Delta\log_{10}$ cfu/mL 3.01 mg/mL*	$\Delta\log_{10}$ cfu/mL 0.30 mg/mL*	MBC (mg/mL) ^o	$\Delta\log_{10}$ cfu/mL 3.01 mg/mL [§]	$\Delta\log_{10}$ cfu/mL 0.30 mg/mL [§]	MBC (mg/mL) ^o
MSSA (ATCC 29213)	1.73	1.52	>3.01	1.69	0.11	>3.01
MRSA**	2	NA	>3.01	3.69	1.15	3.01
<i>Candida albicans</i> (ATCC 14053)	5.30	NA	3.01	3.69	0.52	3.01
CS <i>Klebsiella pneumoniae</i> **	NA	NA	–	NT	NT	NT
CR <i>Klebsiella pneumoniae</i> **	NA	NA	–	NT	NT	NT
<i>Escherichia coli</i> (ATCC 25922)	NA	NA	–	NT	NT	NT
CR <i>Acinetobacter baumannii</i> **	1.75	NA	>3.01	NT	NT	NT

* Difference between the starting inoculum (5.30 log₁₀ cfu/mL) and the number of residual viable colonies after 24 h incubation; ** clinical strain; §difference between the starting inoculum (3.69 log₁₀ cfu/mL) and the number of residual viable colonies after 24 h incubation; ^ominimal bactericidal concentration. MSSA: methicillin-susceptible *S. aureus*; MRSA: methicillin-resistant *S. aureus*; CS: carbapenem susceptible; CR: carbapenem resistant. NA: not active; NT: not tested

Materials and Methods

Plant material

A sample of the *C. cuneifolium* plant aerial parts (500 g) was harvested during July 2015 in the territory of the Majella National Park in Abruzzo and, more precisely, in the town of Santa Eufemia a Majella (Pescara province; geographical coordinates: 42°10'04" N, 14°05'97" E) at 1500 m a.s.l. of altitude.

The botanical identification was performed by the botanists of the park (Dr. Mirella Di Cecco and Dr. Giampiero Ciaschetti) by means of available literature [3, 4]. The plant materials were dried at room temperature with the use of a desiccator immediately after the harvest. A specimen is stored in the Sapienza University of

Rome Chemistry Department for further reference under voucher number CC08072015B.

Chemicals

The following reagents and material were utilized: distilled water for the steam distillation process; diethyl ether for the liquid/liquid extraction of the EO from the oil/water biphasic distillate; methanol as the solvent used for the GC-MS analysis; anhydrous sodium sulfate to dry the organic extracts. All the solvents having RPE analytical purity grade, if not differently specified, were purchased from Sigma-Aldrich. Antimicrobial agents were provided as purified powders by the manufacturer (Sigma-Aldrich). Stock solutions at different concentrations were prepared in sterile and pyrogen-free 0.9% saline or water according to manufacturer's instructions.

The following antimicrobials were used as references: vancomycin (VAN, Sigma-Aldrich, purity >99%) and rifampin (RIF, Sigma-Aldrich, purity ≥97%) for gram-positive microorganisms, meropenem (MEM, Sigma-Aldrich, purity ≥98%) for gram-negative bacteria, and fluconazole (FLU, Sigma Aldrich, purity ≥98%) for fungi.

Biologicals

For the determination of *C. cuneifolium* antimicrobial activity, the following microorganisms were used: MSSA (ATCC 29213), *E. coli* (ATCC 25922), *C. albicans* (ATCC 14053), MRSA (clinical strain), carbapenem-susceptible *K. pneumoniae* (clinical strain), carbapenem-resistant *K. pneumoniae* (clinical strain), and carbapenem-resistant *A. baumannii* (clinical strain). Until further analyses, bacteria were stored on a cryovial bead preservation system (Microbank, Pro-Lab Diagnostics) at -80 °C.

Inoculum was prepared by spreading one cryovial bead on a blood agar plate and then incubation overnight at 37 °C. One colony was resuspended in 5 ml tryptic soy broth (TSB) and incubated at 37 °C without shaking. Overnight cultures were then adjusted to a turbidity of 0.5 McFarland, corresponding to $\approx 1 \times 10^8$ cfu/mL.

Instruments

A 20 L steel apparatus was used for steam distillation according to the method described in the next paragraph. For separation and identification of the constituents of the EO, a GC-MS/GC-FID TurboMass Clarus 500 from Perkin Elmer Instruments was used, characterized by a Stabilwax fused silica gel capillary column (Restek; 60 m long, 0.25 mm I.D., 0.25 µm film thickness). Helium was used as the carrier gas (1.0 mL/min) and the oven temperature was kept at 60 °C for 5 min and raised up to 220 °C with a speed of 5 °C/min and kept constant at 220 °C for 30 min. MS spectrometry was performed at 70 eV with a mass range from 30 to 350 m/z.

The main components of the EO were identified by comparison of their MS spectra with those present in the NIST and Wiley libraries. A second confirmation was achieved by calculating the GC retention indices (RI). The relative abundances of the separated compounds were achieved utilizing the same instrumentation with a FID detector.

Isolation of *coristospermum cuneifolium* essential oil

Similarly as previously reported [62, 63], dried plant material (200.0 g) was subjected to steam distillation. After 8 h, the accumulated oil/water double phase (800 mL) was extracted 3 times with 100 mL of diethyl ether. The unified organic layers were dried over anhydrous sodium sulfate (Na₂SO₄), filtered, and deprived of the solvent in vacuum to furnish oils (28.6 mg of CCEO, yield 0.0143%). The prepared oils were stored in tightly closed dark vials until further analysis.

Coristospermum cuneifolium essential oil antimicrobial activity evaluation

The CCEO sample was dissolved in a 25% DMSO water solution, with the highest tested concentration of 3.01 mg/mL. The used concentration of DMSO did not interfere with bacterial and fungal viability (data not shown).

Antimicrobial activity determination

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined for each reference compound (► **Table 2**) at high and low bacterial inoculum ($\sim 5 \times 10^5$ and $\sim 5 \times 10^3$ cfu/mL, respectively). For CCEO, only the MBCs (at high and low inoculum) were evaluated, as the starting solution was opalescent. The decision to test both high and low inoculum was based on the likely bacterial amount in case of systemic and local infections, respectively (► **Table 3**). Cation-adjusted Mueller Hinton (CAMHB) and Sabouraud broths were used for bacteria and fungi, respectively.

Briefly, two fold serial dilutions of CCEO and each antimicrobial agent were prepared in 2 mL Mueller Hinton broth (MHB) in borosilicate glass tubes and incubated for 18 h at 37 °C. For antimicrobials agents, the MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth whereas MBC, for both references and CCEO, was defined as ≥99.9% (i. e., ≥ 3 -log₁₀ CFU/mL) reduction of the initial bacterial count at each time point [64]. Furthermore, CCEO antibacterial activity was assessed as the difference between the starting inoculum and the number of residual viable colonies after 24 h of incubation (expressed as Δ log₁₀ cfu/mL) [65].

Conflicts of Interest

The authors declare no conflict of interest.

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