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Essential oil characterization, phytochemical analysis, antioxidant and synergistic antibacterial effects of ethanolic extract from *Calendula arvensis* located in the Algerian Aurès area

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Abstract

In this study, we investigated the volatile composition of the Algerian medicinal plant *Calendula arvensis*, as well as the antioxidant evaluation, total phenolics and flavonoids contents, and synergistic antibacterial effect of its ethanolic extract (CAEE) with chemical antibiotics against some bacterial strains. The results of the GC-MS and GC-FID analyses revealed the existence of 67 compounds, with the major constituents being δ-cadinene (11.6%), α-muurolol (9.3%), cubeban-11-ol (9.1%), α-cadinol (8.4%), *epi*-α-bisabolol (7.5%), phytol (7.3%), *epi*-cubebol (4.0%), α-bisabolol (3.9%), and neophytadiene (3.8%). Certain volatile components, including neophytadiene, geijerene, α-terpineol, cogeijerene, γ-himachalene, 5-hydroxycalamenene, methyl docosanoate, heptacosane, methyle tetracosanoate, nonacosane, uncontane, and muurol-5-en-4-α-ol skeletons, were detected for the first time in *C. arvensis* oil. In addition, a remarkable synergistic effect was reported for CAEE against *E. coli*, resulting in the re-sensitization of the *E. coli*-resistant strain to penicillin. Indeed, phenols and flavonoids were also presented in CAEE with moderate concentrations (17.7 µg GAE/mg and 12.9 µg QE/mg, respectively) and correlated positively with its moderate antioxidant capacity. These findings imply that this species might be a valuable source of antibacterial and antioxidant chemicals in the future.

Keywords: *Calendula arvensis*, essential oil, GC-MS, antibacterial synergistic effect, antioxidant evaluation.

Introduction

Calendula arvensis (Vaill.) L., an annual herbaceous species known as "field marigold," is widespread all throughout northern Africa, Europe, and SW Asia (Gravina *et al*., 2022). It is a member of the Asteraceae family. Additionally, *C. arvensis* is frequently used as food, with the leaves serving as a crucial ingredient in making a traditional soup (Motti *et al*., 2020). In addition, it is frequently used in folk medicine to treat wounds and contusions and is renowned for its antibacterial and astringent qualities (Khouchlaa *et al.,* 2023). For maintaining skin firmness, reducing skin irritation, and repairing damaged tissues, preparations of their blossoms are advised (Addis *et al*., 2020). Science spent a lot of time studying this species. The antioxidant and antimicrobial properties of flower extracts have been studied (Abudunia *et al*., 2017). These two biological properties were based on the fact that synthetic antioxidant drugs, which are currently used to scavenge reactive free radicals and protect organisms from numerous pathologies, may carry potential toxicological risks (Shakeri *et al*., 2012). To overcome this, a combination of two or more antibacterial agents frequently uses their synergistic effect, particularly the synergistic effect of plant extracts with antibiotics, because this kind of synergism can be utilized to reduce the side effects of antibiotics by reducing the dose and to overcome the emergence of antibiotic-resistant bacteria (Chang *et al*., 2022). In the current study, we concentrated on isolating and characterizing the volatile components and evaluating the antioxidant potential of the aromatic and therapeutic species *C. arvensis* from the Algerian Aurès region, as well as the phytochemical analysis of its crude ethanolic extract (CAEE), with antibacterial synergistic effects in combination with antibiotic drugs.

Materials and methods

Collection of plant material

C. arvensis was collected in November 2015 from the Algerian Aurès zone (Ouled Fadel, Touffana, 50 km east from Batna, 35° 29′ 03″ nord, 6° 37′ 29″ east, altitude: 1026 m). Voucher specimen (CA/103/VAR/03-15) of the plant material was identified and deposited in the herbarium of the VARENBIOMOL Research Unit, University of Mentouri brothers, Constantine1.

Essential oil extraction

A Kaiser Lang apparatus was used to steam distill the fresh plant material (840 g), which was crushed into small pieces and subjected to the process for three hours (yield (*w/w*), 0.004%). The resulting essential oil was weighted (35.9 mg), dried over anhydrous sodium sulfate, and stored at 4°C until analysis.

Preparation of ethanolic extract

The air-dried material of *C. arvensis* (30 g) was extracted three times with a mixture of ethanol and water (80:20) at room temperature. The crude ethanolic extract was concentrated to dryness under decreased pressure after being filtered through cotton. The CAEO and CAEE yields were estimated in relation to the weight of the plant using the following formula: Yield (%) = (W_1 × 100) / W_2; where; W_1: weight of the obtained sample; W_2: Weight of the air-dried materials.

GC-FID Analysis of essential oil

The obtained essential oil was analyzed on an Agilent Gas Chromatograph (GC-FID) Model 6890, equipped with a HP-5MS fused silica capillary column (5%-diphenyl-95%-dimethylpolysiloxane, 25 m x 0.25 mm, film thickness 0.25 µm), programmed from 50°C (5 min) to 250 °C at 3°/min, and held for 10 min. Injector and flame ionization detector temperatures were 280 and 300 °C, respectively. The oil was diluted in acetone (3.5%, *v/v*) and injected in split mode (1/60). Helium was used as a carrier gas (1.0 mL/min). Solutions of standard alkanes were analyzed under the same conditions to calculate retention indices (RI) with the Van del Dool and Kratz equations (Ourzeddine *et al*., 2017; Kebbi et *al.,* 2020).

GC-MS analysis of essential oil

Mass spectrometry was performed on an Agilent gas chromatograph-mass spectrometer (GC-MS) Model 7890/5975, equipped with an HP-5MS capillary column (25 m x 0.25 mm, film thickness 0.25 µm) programmed with the same conditions as for GC-FID. The mass spectrometer (MS) ionization was set in positive electron impact mode at 70 eV, and the electron multiplier was set at 2200 V. Ion source and MS quadrupole temperatures were 230 °C and 180 °C, respectively. Mass spectral data were acquired in the scan mode in the *m/z* range of 33–450. The essential oil constituents were identified by matching their mass spectra and retention indices (RI) with those of reference compounds from libraries (Adams, 1995; McLafferty and Stauffer, 1991). The proportions of the identified compounds were calculated by internal normalization.

Phytochemical screening of CAEE

Test for saponins: A few milligrams of CAEE were dissolved in distillate water, and then stirred for a few minutes. The formation of foam indicates the presence of saponins (Dubale *et al*., 2023).
Test for tannins: The addition of $FeCl_3$ (1%) allows the indication of the presence/or absence of tannins. The appearance of a dark blue-green color indicates the presence of gallic tannins, and the greenish-blue color indicates the presence of tannins catechetical (Alqethami *et al*., 2021).
Test for flavonoids: The appearance of a dark yellow color after the addition of NaOH (10%), which will be colorless after the addition of the HCl acid drops (37%), indicates the presence of flavonoids (Tojola et *al.,* 2019).

Test for glycosides: After adding 2 mL of concentrated H_2SO_4, the appearance of a reddish brown color indicates the presence of a steroidal part of the glycoside (Tojola *et al*., 2019).

Test for reducing sugars: 5 mL of the Fehling reagent were added to 5 mL of sample. This mixture was then heated for 3 min, and the appearance of a brick-red precipitate confirms the presence of reducing sugars (Edeoga *et al*., 2005).

Test for steroids: 1.5 mL of CAEE was mixed with 2 mL of chloroform and concentrated H_2SO_4. Two layers were formed (aqueous and organic). After adding concentrated H_2SO_4, the appearance of a red color in the lower chloroform layer indicates the presence of steroids (Gul *et al.*, 2017).

Test for phenolics: The addition of a few drops of $FeCl_3$ (2%) to 2 mL of sample led to the apparition of a dark-blue or dark-green color in the presence of phenolics (Edeoga *et al*., 2005).

Test for mucilages: The formation of a precipitate following mixing 1 mL of sample and 5 mL of absolute alcohol indicates the presence of mucilages (Karumi *et al*., 2004).

Test for carotenoids: 3 mL of HCl and 3 mL of H_2SO_4 were added to 10 mL of sample. The appearance of a green-blue color indicates the presence of carotenoids (Edeoga *et al*., 2005).

Test for iridoids: Few drops of HCl were added to 2 mL of sample. The mixture was then heated for a few minutes, and the appearance of a blue color indicates the presence of iridoids (Tojola *et al*., 2019).

Test for anthocyans: The presence of anthocyans was proved after the appearance of a red color when mixing a few drops of HCl with 5 mL of sample (Karumi *et al*., 2004).

Test for reducing compounds: 5 mL of $C_2H_4O_2$ containing traces of $FeCl_3$ and 5 mL of H_2SO_4 containing traces of $FeCl_3$ were added to 1 mL of sample. The formation of red-brown and blue-green phases indicates the presence of reducing compounds (Balamurugan *et al*., 2019).

Test for alkaloids: Few milligrams of CAEE were mixed with 5 mL of HCl (2N). The mixture was then heated and filtered. After, a few drops of Wagner's reagent dissolved in 100 mL distilled water were added, and a brown colored precipitate indicates the presence of alkaloids (Raaman, 2006).

Thin layer chromatography (TLC) analysis of CAEE

TLC is an easy method for testing herbal extracts. However, it should only be used as a first step in developing structural hypotheses. Based on the current findings, more research is underway to isolate the metabolites (Nortjie *et al*., 2022). Briefly, 2–5 µL of the extract were applied twice using a capillary spotter to the TLC plate (20 x 20 cm) (Table 1), 1.5 cm from the bottom. They were then developed in a solvent system designed to separate the desired substances in a trough chamber to a height of 80 mm and saturated with the solvent vapor for 30 minutes. Spots were seen independently after the solvent migration under UV light at 365 nm (for the flavonoids) or using visualization reagents (for tannins and saponins). The developed plates were dried in an air heater to evaporate the solvents from the plates. After that, the colors of the spots were noted, and Rf values were calculated according to the following formula:

Retention factor (*Rf*) = Distance traveled by a compound /distance traveled by the solvent.

Table 1: Mobile phases and visualization reagents used for the separation of flavonoids, saponins, and tannins in *C. arvensis* ethanolic extract

Compounds	Solvent system	Visualisation methods
Flavonoids	Test 1: Toluene / Ethyl acetate / Dichloromethane (6 / 4 / 1, *v/v/v*)	UV lamp at 365 nm
	Test 2: Butanol / Acetic acid / Distilled water (4 / 1/ 5, *v/v/v*)	
Tannins	Toluene / acetone / acetic acid (5 / 5 / 1, *v/v/v*)	Ferric chloride (2%)
Saponins	Toluene / Methanol / Dichloromethane (4 / 5 / 1, *v/v/v*)	Sulfuric acid then drying at 110 °C for 10 min

Assessment of total phenolic content TPC

The TPC was estimated according to the Folin-Ciocalteu protocol (Singleton *et al*., 1999). Different dilutions (500–7.81 µg/mL) were prepared from gallic acid. Thus, the CAEE was prepared at 1 mg/mL. Then, 250 µL of aliquots were mixed with 500 µL of the Folin-Ciocalteu reagent (1N). The mixture was incubated for 4 minutes at room temperature, and then 250 µL of Na_2CO_3 (20%) was added. The mixture was incubated in the dark and at room temperature for two hours. Afterwards, the absorbance was measured at 765 nm by a UV spectrophotometer. The procedure was carried out three times, and the results are expressed in µg GAE/mg of extract.

Assessment of total flavonoids content TFC

The aluminum trichloride ($AlCl_3$) technique was utilized to calculate the TFC, and quercetin served as the standard. After preparing the CAEE in methanol (1 mg/mL), 1 mL of an aluminum trichloride solution (2%) was added. For one hour, the mixture was incubated at room temperature and in the dark. After that, a UV spectrophotometer was used to detect the absorbance at 420 nm. The experiment was carried out three times, and the results were expressed as µg QE/mg extract from the quercetin calibration curve (500-7.81 µg/mL), which was examined under the identical conditions (Nurcholis *et al*., 2021; Fadel *et al*., 2020).

Antioxidant assay (DPPH radical scavenging test)

The antioxidant assay of CAEE was evaluated using the DPPH radical method (Baliyan *et al*., 2022). Indeed, 100 µL of the extract aliquots were added to 3 mL of already prepared DPPH (0.04 mg/mL).

At the same time, a negative control was prepared by mixing 100 µL of methanol with 3 mL of the methanolic solution of DPPH. After 30 minutes of incubation at room temperature and in the dark, the absorbance was measured at 517 nm. Ascorbic acid has been tested under identical circumstances as an antioxidant standard. The experiment was conducted twice, and the anti-free radical activity was calculated using the equation shown below: % inhibition = [(Ac – As)/Ac] x 100, where Ac is the blank control absorbance and As is the sample absorbance at 517 nm. All experiments were carried out in duplicate, and the results were expressed as IC_{50} values from a calibration curve using Microsoft Excel.

Antibacterial synergistic effect assay

The antibacterial synergistic effect of penicillin (30 µg) was tested by the disk diffusion method on Muller-Hinton agar (Mgbeahuruike *et al*., 2019). Five bacterial strains were employed, four of which were negative gram-positive (*Escherichia coli*, ATCC 25922, *Klebsiella pneumonia*, ATCC 700603, *Enterobacter spp.*, and *Pseudomonas aeruginosa*, ATCC 27853) and one of which was positive gram-negative (*Staphylococcus aureus*, ATCC 25923). All tested bacteria were obtained from the Ibn-Ennafis laboratory situated in Khenchela (the city of Algeria). Standard discs of penicillin (30 µg) were impregnated with 10 µL of samples from CAEE dissolved in dimethylsulfoxide (DMSO) at 100 mg/mL and transferred aseptically on Muller-Hinton agar medium, which had been previously inoculated with 100 µL of bacterial inoculum prepared in sterile saline water (equivalent to 0.5 McFarland standard). After 24 h of incubation at 37°C, zone inhibition diameters were assessed. The synergistic effect was evaluated by comparing the obtained result inhibition to three controls: discs exclusively impregnated with tested antibiotics, discs prepared by sterile Wattman paper N°3 of 6 mm in diameter, impregnated only with the crude extract dissolved in DMSO at 100 mg/mL (10 µL/disc), and discs impregnated with DMSO used as negative controls. The test was performed in triplicate, and the results were expressed as the mean (±) standard deviation. Bacterial strains presenting inhibition zone diameters (D) less than 8 mm, $9 \leq D \leq 14$ mm, $15 \leq D \leq 19$ mm, and $D > 20$ mm were considered, respectively, resistant (-), sensitive (+), highly sensitive (++), and extremely sensitive (+++).

Results and Discussion

The essential oil of our plant *C. arvensis* was subjected to GC-MS and GC-FID analysis (Table 2 and Fig. 2), which found 67 compounds with a total percentage of 96.5%, of which the percentage of identified compounds was 92.2%, while unidentified compounds had a percentage of 3.3%. The major compounds were, respectively, δ-cadinene (11.6%), α-muurolol (9.3%), cubeban-11-ol (9.1%), α-cadinol (8.4%), *epi*-α-bisabolol (7.5%), phytol (7.3%), *epi*-cubebol (4.0%), α-bisabolol (3.9%), and neophytadiene (3.8%).

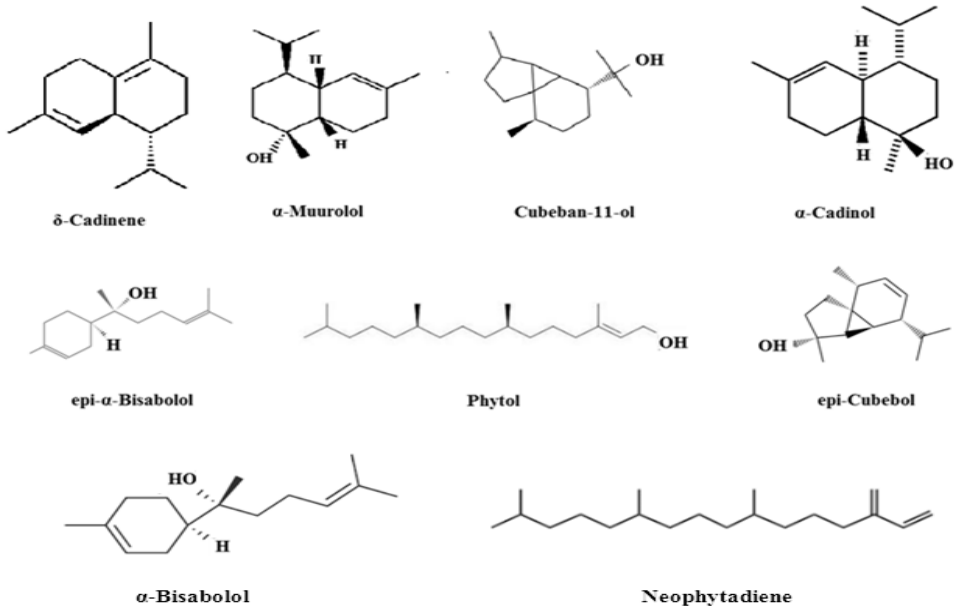

Fig. 1 Chemical structures of the major volatile components from *C. arvensis*

In Algeria, a published study carried out on *C. arvensis* from many locations has shown variability in the volatile chemical composition and revealed high amounts of znigiberenol 1 and β-curcumene (Belabbes *et al*., 2017). In fact, a French sample was found to be rich in δ-cadinene and α-cadinol (Paolini *et al*., 2010). However, the major volatile constituents from a Turkish *C. arvensis* were α-selinene and α-pinene (Tosun *et al*., 2012). Additionally, the first two main components of the Turkish sample were δ-cadinene and *epi*-cubebol (Huseyin *et al*., 2020). Indeed, the abundance of τ- and δ-cadinol in *C. arvensis* oil was recently reported in another Turkish sample (Okan *et al*., 2024). To our knowledge, firstly, no *C. arvensis* oil was known to be highly rich in neophytadiene, and second, it is the first time that our *C. arvensis* contained geijerene, α-terpineol, cogeijerene, γ-himachalene, 5-hydroxycalamenene, methyl docosanoate, heptacosane, methyle tetracosanoate, nonacosane, uncontane, muurol-5-en-4-α-ol, and menthol skeletons. In fact, since our sample specimen was actually collected in the Aurès region, which is distinguished by a semi-arid climate with significant seasonal temperature differences, fertile soil, and various altitudes, these conditions may have a greater impact on the sample's chemical variability, without ignoring other potential influences like extraction techniques, genetics, and the time of collection (Feng *et al*., 2021).

Table 2: Chemical composition of *C. arvensis* essential oil

Peak N°	RT	[b]RI	[a]Components	%
1	7.0190	1029	Unknown	0.02
2	7.0500	1030	Limonene	0.07
3	7.8060	1073	*cis*-Hydrate sabinene	0.06
4	8.3980	1107	Linalool	0.04
5	8.7190	1127	*cis-p*-Menth-2-en-1-ol	0.09

6	8.9770	1143	*trans-p*-Menth-2-en-1-ol	0.04
7	9.0120	1146	Geijerene	0.07
8	9.0620	1149	Unknown	0.07
9	9.4680	1174	Menthe-1,5-dien-8-ol	0.03
10	9.6120	1184	Terpinene-4-ol	0.21
11	9.7180	1190	*p*-Cymene-8-ol	0.06
12	9.8440	1198	α-Terpineol	0.05
13	9.9960	1209	Decanal	0.09
14	10.049	1212	*cis*-Piperitol	0.12
15	10.957	1275	Unknown	0.01
16	11.148	1288	Cogeijerene	0.06
17	11.781	1335	Unknown	0.02
18	11.984	1350	α-Cubebene	0.11
19	12.052	1355	Unknown	0.02
20	12.222	1368	Unknown	0.01
21	12.377	1379	α-Copaene	0.45
22	12.533	1391	β-Cubebene	0.18
23	12.790	1411	α-Gurjunene	0.27
24	12.956	1424	β-Caryophyllene	0.27
25	13.210	1444	Selinene	0.26
26	13.332	1454	*cis*-Muurola-3,5-diene	0.21
27	13.413	1461	Sesquisabinene	0.24
28	13.604	1476	*trans*-Cadina-1(6),4-diene	0.34
29	13.641	1479	γ-Muurolene	0.31
30	13.752	1488	γ-Himachalene	1.07
31	13.813	1492	Viridiflorene	1.14
32	13.849	1495	*epi*-Cubebol	4.00
33	13.923	1501	α-Muurolol	9.30
34	14.038	1511	β-Bisabolene	0.40
35	14.076	1514	Unknown	0.36
36	14.115	1518	γ-Cadinene	0.88
37	14.177	1523	δ-Cadinene	11.63
38	14.227	1527	Bisabolene	0.43
39	14.259	1530	Cadina-1,4-diene	0.27
40	14.343	1537	α-Cadinene	0.48
41	14.415	1543	*cis*-α-Bisabolene	0.98
42	14.553	1555	α-Agarofurane	0.33
43	14.806	1576	Unknown	0.29
44	14.877	1582	Spathulenol	0.58
45	14.992	1592	Gleenol	0.17
46	15.043	1596	Muurol-5-en-4-α-ol	1.57
47	15.127	1604	Cubeban-11-ol	9.07
48	15.216	1612	Ledol	2.26
49	15.290	1618	Unknown	0.90

50	15.462	1634	1-*epi*-Cubenol	2.79
51	15.627	1648	*epi*-α-Cadinol	2.76
52	15.644	1650	*epi*-α-Muurolol	3.39
53	15.788	1663	α-Cadinol	8.44
54	15.918	1674	β-Bisabolol	0.37
55	16.104	1691	*epi*-α-Bisabolol	7.48
56	16.126	1693	α-Bisabolol	3.88
57	17.301	1804	5-Hydroxycalamenene	0.18
58	17.645	1838	Neophytadiene	3.76
59	20.265	2099	Phytol	7.30
60	21.919	2299	Tricosane	0.32
61	23.530	2499	Pentacosane	0.46
62	23.752	2528	Methyl docosanoate	0.52
63	25.003	2696	Unknown	0.64
64	25.032	2700	Heptacosane	1.32
65	25.247	2731	Methyletetracosanoate	0.40
66	26.431	2901	Nonacosane	1.29
67	27.740	3101	Uncontane	0.37
Oil yield				0.004
Total				95.56
Total identified				92.23
Total not identified				03.33

[a]Compounds are listed in order of their RI.
[b]RI (retention index) measured relative to *n*-alkanes using HP-5MS column.
RT (retention time).

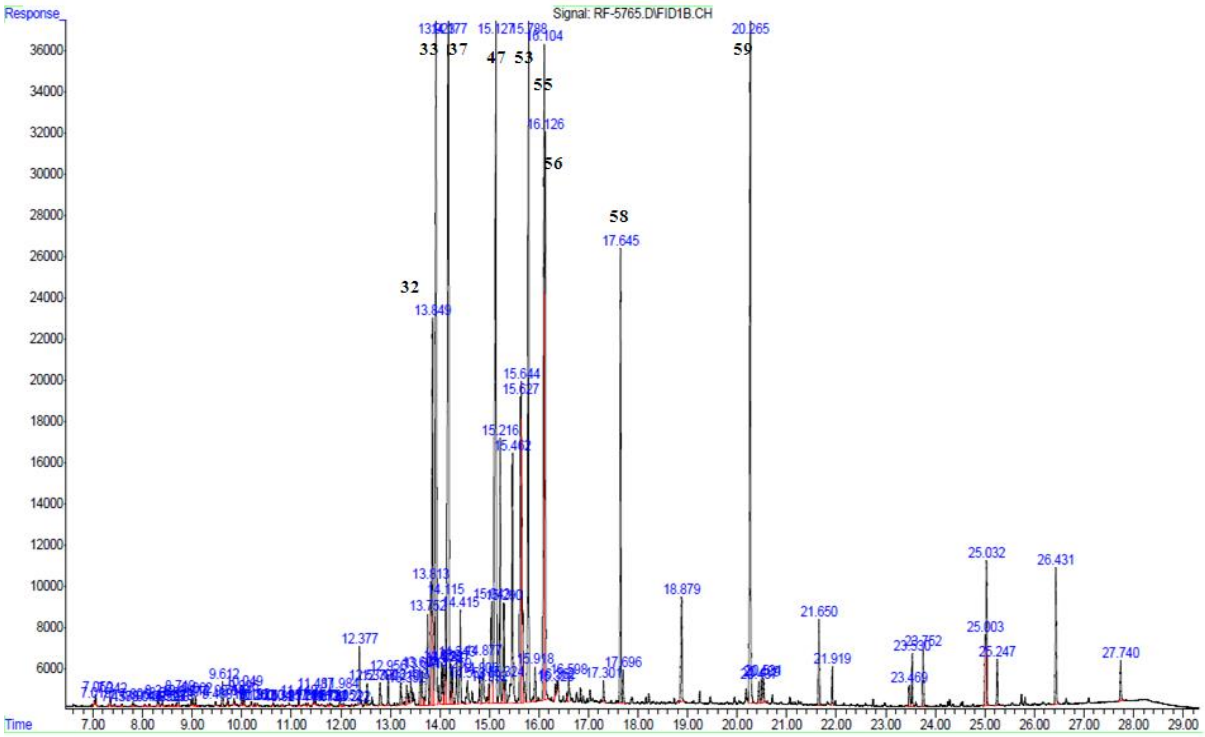

Fig. 2 GC-FID chromatogram of *C. arvensis* oil indicating the main volatile components.

The results from the phytochemical screening of CAEE revealed the presence of different chemical compounds such as flavonoids, saponins, steroides, polyphenols, reducing sugars, carotenoids, and alkaloids (Table 3).

Table 3: Secondary metabolites classes in CAEE

***C. arvensis* ethanolic extract, yield = 27.8%**					
Constituents	**Intensity**	**Constituents**	**Intensity**	**Constituents**	**Intensity**
Saponins	+	Steroides	+++	Anthocyans	-
Flavonoids	++	Polyphenols	++	Irridoids	-
Tannins	-	Mucilages	-	Alkaloids	+
Glycosides (Reducing sugars)	+++	Carotenoids	++	Reducing compounds	-

Keys: (-) not detected (absent); (+) slightly present; (++) moderately present; (+++) highly present.

The pre-mentioned factors may also have an impact on the percentage yields (Dai *et al*., 2010; Chew *et al*., 2011; Costa *et al*., 2012). Results from phytochemical tests were in accordance with the previous published works, where they confirmed the richness of *C. arvensis* in various classes of phytoconstituents such as triterpene alcohols, triterpene saponins, flavonoids, carotenoids, and

polysaccharides (Arora *et al*., 2013). Indeed, all tested phytochemicals are known to be biologically active compounds, and they are responsible for different pharmacological activities, including antibacterial (Dumenil *et al*., 1980), hemolytic, anti-inflammatory (Chemli *et al*., 1990), and antimutagenic (Elias *et al*., 1990). Thus, the presence of these phytochemical compounds in the CAEE may be the reason for its use in folk medicine as disinfectant, antispasmodic, diuretic, anti-inflammatory, anticancer, and antipyretic (Arora *et al*., 2013). The diverse phytoconstituents tested in CAEE require their separation and testing through thin-layer chromatography. In the present study, the results of thin layer chromatography using different solvent systems revealed the presence of promising spots, as shown in Figs. 2 and 3 and Table 4.

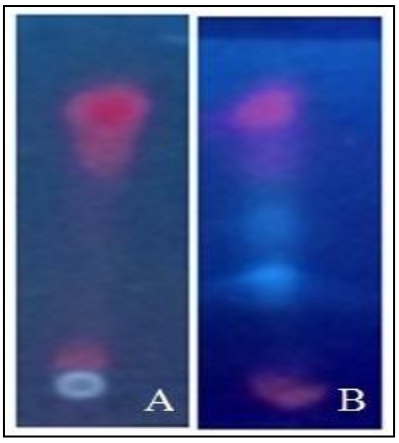

Fig. 3 TLC observations under UV lamp of flavonoids from CAEE; A: toluene / ethyl acetate / dichloromethane (6/4/1, *v/v/v*), B: butanol / acetic acid / distilled water (4/1/5, *v/v/v*)

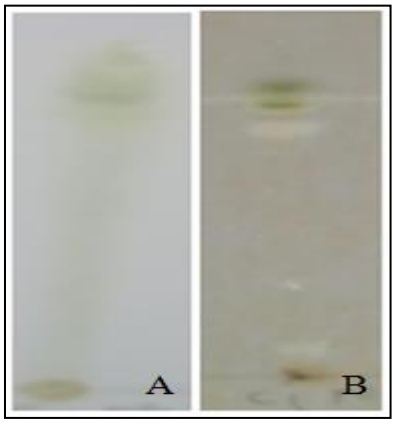

Fig. 4 TLC observations from CAEE of saponins (A) and tannins (B)

Table 4: Rf values and colors of CAEE spots detected for flavonoids, tannins, and saponins by TLC

Compounds	Solvent system	Visualisation methods	Colors of spots	Rf values
Flavonoids	Toluene/ Ethyl acetate /Dichloromethane (6 / 4 / 1, *v/v/v*)	Under UV $_{365\ nm}$	Red	0.76
	Butanol / Acetic acid / Distilled water (4 / 1 / 5, *v/v/v*)	Under UV$_{365\ nm}$	Blue-white	0.36
			Blue	0.54
			Purple	0.86
			Red	0.92
Tannins	Toluene / acetone / acetic acid (5 / 5/ 1, *v/v/v*)	Visualisation by $FeCl_3$ (2%)	Brownish-grey	0.67
Saponins	Toluene / Methanol / Dichloromethane (4 / 5 / 1, *v/v/v*)	Visualization by H_2SO_4 then drying at 110 °C for 10 min.	Green	0.88

TLC flavonoids analysis giving a single spot only (red, Rf: 0.76) using the first solvent system (toluene, ethyl acetate, and dichloromethane 6 / 4 / 1, *v/v/v*) (Fig. 2A) While five spots were recorded using the second solvent system butanol, acetic acid, and distilled water (4 / 1 / 5, *v/v/v*) with different colors visualized under UV light at 365 nm wavelength (blue, white, blue, purple, and red) and diverse Rf values (0.36, 0.54, 0.86, 0.92), respectively (Fig. 2B). Indeed, the blue spots could be due to the presence of 5-deoxyisoflavones, 7,8-dihydroxy-flavanones, anthocyanidins-3-glycosides, methylated isoflavones, flavanones, chalcones, and methylated flavones (Gwatidzo *et al*., 2018). Markham (1982) and Marcel (2012) have shown that anthocyanidins-3-glycosides give red spots, whereas the appearance of purple spots indicates the existence of flavones and chalcones. Concerning saponins, only one spot was recorded using toluene, methanol, and dichloromethane (4, 5, 1, *v/v/v*) with an Rf value of 0.88, and a green color appeared in the H_2SO_4 spray reagent (Fig. 3A). Finally, for tannins, one tested compound was separated by preparative TLC using toluene, acetone, and acetic acid (5, 5, 1, *v/v/v*) with Rf values of 0.67 and a brownish-gray color, which is visualized under ferric chloride solution (2%) reagent spray, specific reagents for tannins detection (Fig. 3B) (Rehana *et al*., 2014). The TLC method is the best choice for the detection of compounds present in plants. The combination of solvents with variable polarity in diverse proportions can be used for the partition of pure secondary metabolites from plant extract. The disparity in Rf values in different solvent systems indicates the presence of various types of phytoconstituents in the extract, gives a very important idea of their polarity, and also helps in the selection of a suitable solvent system for the segregation of pure compounds by column chromatography from this plant extract (Solanki *et al*., 2019; Gujjeti *et al*.,

2013). Furthermore, phenolics such as flavonoids are known to have an interest in human nutrition and health because they are responsible for several biological activities, including antioxidant, anti-allergic, anti-inflammatory, and antimicrobial properties (Chung *et al*., 1998). All these biological activities of phenolic components forced their quantification. The total phenol and flavonoid contents of the ethanolic extract were estimated according to the equations demonstrated in Fig. 5. The TPC and TFC of CAEE were estimated by Folin-Ciocalteu and $AlCl_3$ assays, and the results were 17.7 µg GAE/mg and 12.9 µg QE/mg, respectively (Table 5). In fact, these values were lower than those reported by Ercetin *et al.* (2012) for TPC and TFC (118.2 µg GAE/mg and 74.14 µg QE/mg, respectively) in the flower methanolic extract of *C. arvensis*. This variation can be due to plant growth circumstances, such as soil, geographic site, organ developmental conditions, level of maturity, and genetics, or to the extraction methods and the nature of solvents used (Agata *et al*., 2009).

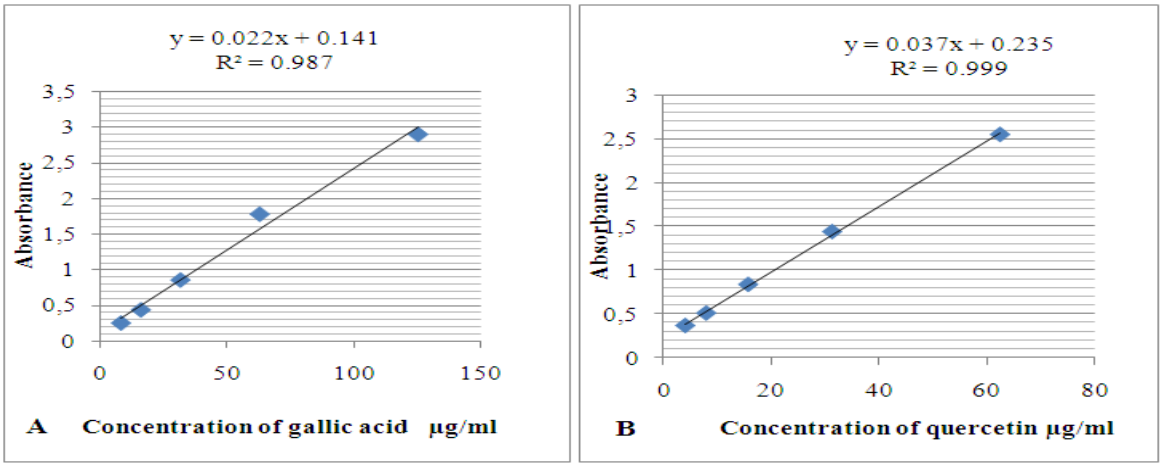

Fig. 5 Calibration graphs for total phenol content (A) and total flavonoids content (B) (µg/mL)

Using the DPPH radical-scavenging technique, the antioxidant potency of CAEE and the standard ascorbic acid was assessed. The fundamental idea behind this test is to gauge an extract's ability to scavenge the radical DPPH that is created in solution when an atom or an electron is donated (Tepe *et al*., 2005). According to the findings (Table 5 and Fig. 6), CAEE demonstrated a high value when compared to the reference ascorbic acid (IC_{50} values of 15.76 and 0.20 mg/mL, respectively). CAEE demonstrated a moderate antioxidant capability since a lower IC_{50} value indicated stronger antioxidant activity. Additionally, it appears that the overall phenol and flavonoid concentrations and antioxidant capability are related. Our findings concurred with those of Ercetin *et al*. (2012), who found that *C. arvensis* extracts had a moderate capacity of DPPH scavenging action.

Table 5: Antioxidant assay, total phenol, and flavonoid contents of CAEE (data were expressed as mean values ± SD, n = 2)

Tested samples	Total phenolic content µg GAE/mg	Total favonoid content µg QE/mg	DPPH radical scavenging IC_{50} (mg/mL)
C. arvensis (ethanolic extract)	17.67 ± 2.39	12.94 ± 2.19	15.76 ± 7.19
Ascorbic acid			0.20 ± 0.0042

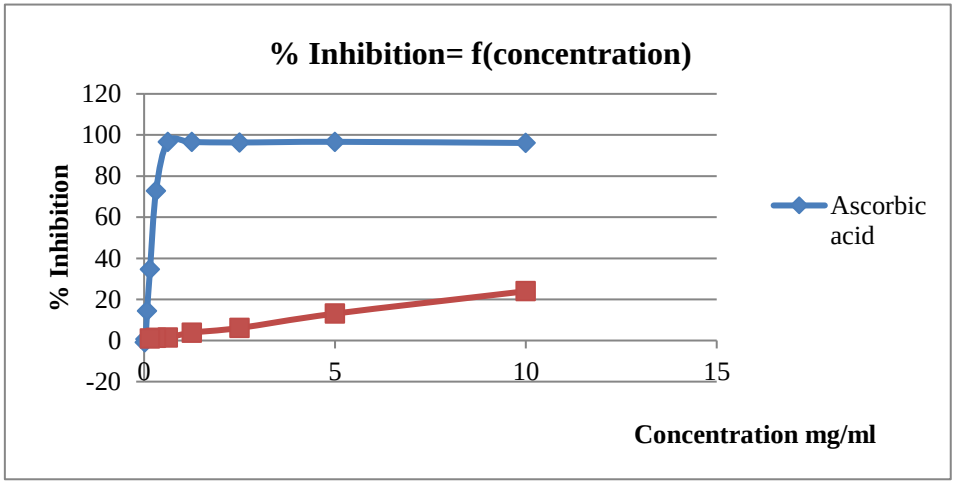

Fig. 6 DPPH radical-scavenging assay of CAEE

In order to investigate the synergistic impact, four resistant bacterial strains were evaluated for the inhibitory effect caused by combinations of CAEE and penicillin. According to this section's results (Table 6), this combination was antagonistic against *S. aureus* (00 mm for the combination vs. 09 and 00 mm for CAEE and penicillin, respectively). The *E. coli* strain that was resistant to penicillin was, however, re-sensitized by the combination (10 mm vs. 0 mm for CAEE and 00 mm for penicillin) due to a noteworthy possible synergistic inhibitory effect that was seen. The findings indicated that in the situations of *P. aeruginosa, K. pneumonia,* and *Enterobacter* sp. combinations, no synergistic impact was seen.

Table 6: Bacterial strains sensitivity against CAEE, penicillin and their combinations

	Sensitivity of bacterial strain			
Bacterial strains	**CAEE (100 mg/mL)**	**Penicillin (30 µg)**	**Combination between CAEE and penicillin**	**Interaction**
S. aureus	R (9 mm)	R	R	Antagonistic*
Enterobacter sp	R	R	R	No synergestic effect
P. aeruginosa	R	R	R	No synergestic effect
K. pneumoniae	R	R	R	No synergestic effect
E. coli	R	R	S (10 mm)	Potential synergistic**

Antagonistic*: the simultaneous effect of two agents leads to the partial reduction or complete (cancellation) inhibition of the action of one of them; Potential Synergistic**: the effect of the two agents simultaneously is greater than the sum of the effects of agents separately; R: resistant (-); S: sensitive (+)

The synergistic effects of multiple plant extracts in conjunction with various antibiotics against both gram-positive and gram-negative bacteria have been demonstrated by scientists in several in vitro investigations. According to Silva *et al.* (2019), ampicillin (β-lactam), kanamycin, and gentamicin (aminoglycosides) demonstrated synergism with the ethanol extract of *Plectranthus ornatus* leaves against *S. aureus*. The ethanolic extract of *Salvia officinalis* showed the same synergistic effects. In a different research, Dawoud *et al*. (2013) combined Rehum methanol extract with several antibiotics (gentamycin, ceftasidine, tobramycin, cefoperazone, and spictinomycin) and showed a possible antibacterial synergistic activity against *S. aureus*. The ethanol extract of *Punica granatum* rind showed very good synergistic activity with ciprofloxacin, resulting in the re-sensitization of the *Klebsiella pneumoniae*-resistant strain (Stefanović *et al*., 2018). It is well known that plant extracts possess the ability to enhance the activity of an antibiotic in combination with it. That ability of plant secondary metabolites reflects in the modification or blocking of resistance mechanisms so that re-sensitization of resistant bacterial strains to antibiotics as well as reducing the antibiotic doses consequently decreases the side effects of antibiotics (Stefanović *et al*., 2018). It is considered that bioactive metabolites from medicinal herbs change and inhibit the mechanisms of resistance in pathological bacterial strains and thus exhibit a synergistic effect with antibiotics (Abreu *et al*., 2012; Gibbons *et al*., 2005). According to several published studies in the literature, a variety of mechanisms of synergistic action, including alteration of active sites on bacterial cells, inhibition of enzymes located on the cell membrane that catalyze the degradation or modification of antibiotics, improvement of membrane permeability, and inhibition of efflux pumps, result from the combination of active compounds from plants and antibiotics in resistant bacteria. Intriguingly, it has been reported that the synergistic interaction of plant extract with antibiotics led to a drop in the minimal dose necessary for effective antibacterial activities, which is significant since it may lower the risk of side effects and treatment costs (Olgica *et al*., 2017).

Conclusion

The current work included preliminary phytochemical assays and documented the volatile content and synergistic effects of *C. arvensis* from the Algerian Aurès region. The findings indicated that CAEE contains a variety of substances, including phenols, flavonoids, steroids, glycosides, saponins, and carotenoids. In addition, a noteworthy synergistic impact of CAEE against *E. coli* was determined, leading to the re-sensitization of the *E. coli* resistant strain to penicillin and antagonistic combination in the case of *S. aureus*. GC-MS analysis revealed a variability in the volatile composition and the appearance of different compounds in our *C. arvensis* oil, including neophytadiene, gigerin, α-terpineol, kujigerin, γ-hemachalene, 5-hydroxycalamine, methyl docosanoate, heptacosane, methyl tetracosanoate, nonacosane, uncontan, and morol-5-n-4-α-ol structures. Furthermore, a number of factors, including extraction techniques, collection timing, geographic and environmental

circumstances, might be implicated in this variation. In fact, we believe that these findings can improve our understanding of the synergistic characteristics and volatile content of *C. arvensis* and inspire researchers to look into more phytochemical and biological studies on this species.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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