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Synergistic effect on the antioxidant and antibacterial activity of essential oils of *Artemisia campestris* L. and *Citrus aurantium*

[Efecto sinérgico sobre la actividad antioxidante y antibacteriana de los aceites esenciales de *Artemisia campestris* L. y *Citrus aurantium*]

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Abstract: The study evaluated the antibacterial and antioxidant activity of the combination of *Artemisia campestris* L. ssp *campestris* and *Citrus aurantium* essential oils. The antioxidant activity was evaluated based on the DPPH free radical scavenging assay and the FRAP assay. The binary combinations were set up with improved antioxidant activity. The antibacterial effect was determined against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli*), and the yeast *Candida albicans* using the disk diffusion method. The results of the inhibition zone diameter showed an antagonistic effect for the binary combinations against *Escherichia coli* and *Bacillus subtilis*, while no inhibition zone diameter was reported for *Staphylococcus aureus* and *Candida albicans*. These results suggest the importance of studying antibacterial activities and finding proportions with synergistic or antagonistic effects for food preservation systems.

Keywords: Essential oils; Antioxidant activities, Antibacterial activities; Synergistic, GC-MS

Resumen: El estudio evaluó la actividad antibacteriana y antioxidante de la combinación de los aceites esenciales de *Artemisia campestris* L. ssp *campestris* y *Citrus aurantium*. La actividad antioxidante se evaluó mediante el ensayo de captura de radicales libres DPPH y el ensayo FRAP. Se establecieron combinaciones binarias con actividad antioxidante mejorada. El efecto antibacteriano se determinó frente a bacterias Gram-positivas (*Staphylococcus aureus* y *Bacillus subtilis*), bacterias Gram-negativas (*Escherichia coli*) y la levadura *Candida albicans* utilizando el método de difusión en disco. Los resultados del diámetro de la zona de inhibición mostraron un efecto antagónico para las combinaciones binarias contra *Escherichia coli* y *Bacillus subtilis*, mientras que no se reportó zona de inhibición para *Staphylococcus aureus* y *Candida albicans*. Estos resultados sugieren la importancia de estudiar las actividades antibacterianas y encontrar proporciones con efectos sinérgicos o antagónicos para sistemas de preservación de alimentos.

Palabras clave: Aceites esenciales; Actividades antioxidantes; Actividades antibacterianas; Sinérgico; GC-MS

INTRODUCTION

The essential oils (EOs) are complex mixtures of VCs extracted from flowers, leaves, stems, and roots of the plants. Particular attention was given to EOs due to their broad ranges of biological activities including the antimicrobial, antioxidant, anti-inflammatory properties among many others. Essential oils are generally known to be phenolic compounds, which can scavenge free radicals and chelate metal ions, preventing oxidative damage. The main antioxidant activity comes from these compounds (Garg *et al*., 2010; Tazeen *et al*., 2017). Antimicrobial activity for the essential oils could be reasoned with their complex chemical compositions, often terpenes, terpenoids, or other volatile compounds.(Chouhan *et al*., 2017). Interestingly, a combination of different essential oils can cause synergistic effects, enhancing their overall efficacy against a wider range of microorganisms (Aldoghaim *et al*., 2018).

Looking for synergistic effects of combined essential oils, the latter represents one of the most promising strategies to enhance the biological activity of essential oils and to overcome the problem of multi-resistant microorganisms (Chouhan *et al*., 2017). Recent investigations have focused on the antioxidant and antibacterial synergism of medicinal plant essential oils. These studies have shown that the unique chemical composition and interaction between various compounds present in essential oils can result in enhanced antimicrobial and antioxidant activities.

The food processing industry is faced with the urgent challenge of preserving food supplies due to the risk of micro-organism contamination and oxidation spoilage, potentially reducing drastically the shelf life and quality of a wide variety of foods. Artificial preservatives and antioxidants are commonly used to arrest these problems. The problem of their toxicity and health hazards, however, has been raised (Benamar-Aissa *et al*., 2023). As such, greater efforts have been devoted to the design of natural preservatives that would inhibit the proliferation of micro-organisms and slow oxidation reactions without affecting the safety and quality of foods (Tiwari *et al*., 2009). The preference for the application of natural preservatives over their synthetic counterparts has been brought into focus due to their compatibility with living organisms, capacity for biodegradation, low toxicity, and cost-saving (Mogoşanu *et al*., 2016a; Mogoşanu *et al*., 2016b). The design of new and more effective antimicrobial blends, along with understanding the interaction between components of crude essential oils, is an important area of research that can enable the application of essential oils to combat the problem of multidrug-resistant microorganisms (Chouhan *et al*., 2017). The current study investigates the synergistic effect of the antioxidant and antibacterial activities of the essential oils extracted from *Artemisia campestris* L (wormwood) and *Citrus aurantium* (bitter orange). *Artemisia campestris*, from the family *Asteraceae*, is known to contain a high amount of cineole, a monoterpene with established antimicrobial and antioxidant activities. According to Aldoghaim *et al*. (2018), *Citrus aurantium*, known as bitter orange, is also rich in limonene, another terpene compound that shows potential antimicrobial and antioxidant activities. The interactions between these two plants essential oils have not been widely studied in terms of synergy, and the understanding of such combined effects will be of great value for the development of natural, multifunctional preservatives and antimicrobial agents.

MATERIALS AND METHODS

Plant material

The aerial parts (including stems and leaves) of *Artemisia campestris ssp. campestris* (AC) were collected in February 2019, in a region of Laghouat: AFLOU (34°06'27 N. 02°06'03 ‘E,1403 m). The plucked fruits of *Citrus aurantium* (CA) were collected in March 2019, from trees growing in the centre of Laghouat. The plants were identified by Mr Brahim Guit, a doctor in the agronomy department at the University of Djelfa, and Professors Yousfi Mohamed and Ouinten Mohamed. After collection, the plant samples were air-dried in the dark at room temperature for 15 days. *Citrus aurantium* barks were used fresh to extract the EOs. The control specimens (AC-AP02/19 and CA-P01/19) were deposited in the herbarium of the Basic Sciences Research Laboratory at the University of Laghouat, Algeria.

Essential oil extraction

Fresh *Citrus aurantium* fruit peels and dried aerial parts of *Artemisia campestris* L. were subjected to hydrodistillation for 3 hours, using a Clevenger-type apparatus. Water was used as the solvent for the extraction process. Once extraction was complete, each essential oil sample was dried over anhydrous

sodium sulphate and stored in sealed bottles in the dark at -20°C.

Figure No. 1
Photo of *Artemisia campestris* L. *ssp. Campestris*

Essential oil analysis

The oils were analysed by two methods: the first was gas chromatography equipped with a flame ionisation detector (GC-FID), while the second was gas chromatography coupled to mass spectrometry (GC/MS).

GC-FID chromatographic conditions

The essential oil samples were analysed using a Chrompack CP 9002 gas chromatograph equipped with a flame ionisation detector (FID) and a DB-5 capillary column (30 m × 0.32 mm, film thickness 0.25 µm); injector and detector temperatures were maintained at 250°C and 280°C, respectively. A solution volume of 0.5 µL, prepared by diluting 10% (vol) of essential oil in dichloromethane (CH_2Cl_2), was injected in split mode (50:1), Helium was used as carrier gas with a flow rate of 1 mL/min. The temperature of the column was programmed as follows: initial isothermal phase of 50°C for 3 minutes, then a temperature rise at a rate of 2°C/min until 250°C was reached, finally an isotherm of 10 minutes was maintained at this last temperature. The percentages of constituents were calculated by electronic integration of the FID peak areas (surface area under the peaks), without recourse to correction factors. Linear retention indices (LRIs) were calculated for separate compounds relative to a homologous series of (C9-C25) n-alkanes using the following formula:

$$\boldsymbol{LRI} = 100 \times \left[\frac{tr(x) - tr(Cn)}{tr(Cn+1) - tr(Cn)}\right] + 100 \times n$$

tr(x) retention time of the product framed by the retention times of the alkanes tr(Cn) and tr(Cn+1)

GC/MS chromatographic conditions

The volatile compounds (essential oil) are isolated and were analysed by GC/MS, using a Shimadzu GC/MS-QP2010 ultra instrument fitted with a fused DB-5 capillary column (the same as that used and described in the GC-FID analysis). The oven temperature was programmed as follows: a temperature rises from 50°C to 250°C at a rate of 3°C/min, followed by an isothermal stationary phase lasting 10 minutes. The injector temperature was 250°C and the detector temperature 280°C. The split ratio was 100:1. The carrier gas was helium (purity 99.995%) at a flow rate of 1.2 mL/min. Mass spectrometer conditions were as follows: ionisation voltage 70 eV; ion source temperature 150°C; electron ionisation mass spectra were acquired in the 50-550 m/z range.

Antioxidant activity

The present study aims to evaluate the antioxidant activity of various essential oils using the DPPH and FRAP assays. The 1,1-diphenyl-2 picrylhydrazyl and Ferric Reducing Antioxidant Power assays are widely used methods to assess the antioxidant activity of various plant-derived compounds, including essential oils. These assays measure the ability of a compound to scavenge free radicals and reduce ferric ions,

respectively, providing insights into the overall antioxidant capacity

Scavenging free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH•)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH-) radical was one of the first radicals used to study the structure/antioxidant activity relationship of bioactive compounds. Since then, certain modifications have been made and an important parameter has been introduced: the determination of the IC_{50} inhibitory concentration, defined as the effective substrate concentration resulting in a 50% reduction in the free radicals initially introduced (Brand-Williams *et al*., 1995). In this test, the antioxidants reduce the violet-coloured diphenyl-picrylhydrazyl radical to a yellow compound, diphenyl-picrylhydrazine, whose colour intensity is inversely proportional to the capacity of the antioxidants present in the reaction medium (Sánchez-Moreno, 2002). The method described by Tepe *et al*. (2005), was used to measure this activity. A volume of 120 µL of dilutions of the extracts was added in the presence of 1 mL of ethanolic solution of DPPH at a concentration of 120 µM (Gourine *et al*., 2010). In parallel, a negative control (without extract) was prepared. After 30 min incubation in the dark at room temperature, absorbance was measured at 517 nm using a Shimadzu UV/Vis 1601 spectrophotometer. The percentage inhibition (I%) was calculated using the following formula:

$$\boldsymbol{I}(\%) = \left(1 - \frac{A\ sample}{A\ blank}\right) \times 100$$

With:

I(%): percentage inhibition

A_{blank}: absorbance of the negative control (without extracts)

A_{Sample}: absorbance of the sample tested

Ferric ion reducing power (FRAP)

The FRAP method developed by Benzie & Strain (1996), involves the reduction of a ferric tripyridyltriazine complex [(Fe(III)-TPTZ)$_2$] to a ferrous tripyridyltriazine complex [(Fe(II)-TPTZ)$_2$] by an antioxidant (AH), at a pH of 3.6 to maintain the solubility of the iron. During the reduction of the ferric complex to the ferrous complex, an intense blue coloration appears very quickly with an absorption maximum at 593 nm. This method is based on the ability of extracts to reduce ferric iron (Fe_3^+) to ferrous iron (Fe_2^+). This mechanism is known to be an indicator of electron-donating activity, characteristic of the antioxidant action of polyphenols (Yıldırım *et al*., 2001). First, an aqueous acetic acid/sodium acetate buffer solution at 300 µM pH=3.6 was prepared. The 10 mM TPTZ reagent, diluted in 40 mM HCl, was prepared extemporaneously. The second 20 mM $FeCl_3$ reagent was also prepared extemporaneously. Finally, the FRAP working solution is obtained by mixing 2.5 mL of TPTZ solution, 2.5 mL of $FeCl_3$ solution and 25 mL of buffer solution. This solution must be kept in a 37°C bath. This protocol is based on the method developed by Benzie & Strain (1996), with a few modifications (Pulido *et al*., 2000). The test consists of mixing 100 µL of diluted extract with 3000 µL of FRAP working solution maintained at 37°C in glass haemolysis tubes. Absorbance was measured at 593 nm after incubating the reaction in a thermostatic bath at 37°C in the dark for exactly 30 minutes for the essential oils. A calibration curve was prepared using different concentrations of ascorbic acid; the results were expressed relative to ascorbic acid from the calibration curve, then expressed as AEAC ascorbic acid equivalents. Antioxidant activity expressed as an AEAC value and defined as the concentration of vitamin C in mg/ml that confers similar antioxidant activity to a given extract sample of 1 mg/mL concentration.

Synergistic effects between essential oils

Synergistic effects are determined by the type of interaction between the essential oils. Different doses of *Artemisia campestris* extracts were mixed with varying doses of *Citrus aurantium* extract (ratio 1/9, 3/7, 5/5, 7/3, 9/1) and the antioxidant activity of the mixture was determined using IC_{50} for DPPH and by AEAC for the FRAP test, these values are compared by the antioxidant activity of the individual essential oils.

Antibacterial activity

The agar diffusion method (Balouiri *et al*., 2016; Sateriale *et al*., 2020) was used to assess the antimicrobial effect of the essential oil alone and in mixture against the four microbial strains (Table No. 1). Briefly, the target strains were incubated overnight at 37°C in Mueller Hinton Agar (MHA). The overnight suspension, prepared in sterile saline with a final density of 0.5 McFarland (106 colony-

forming units per millilitre, CFU/mL), was spread evenly with sterile buffer onto MHA plates. Next, filter paper discs (6 mm in diameter) previously soaked in the EO samples (5 µL) were placed on the inoculated agar surface. The plates were kept at 4°C for 2 h to allow dispersion and then incubated for 24 h at 37°C for growth of the target strains. The diameters of the zones of inhibition (DI) around the discs were measured in millimetres using a calliper. A standard disc containing amoxicillin (20 µg/disc) was used as positive control, while sterile water or dimethyl sulphoxide (DMSO) was used as a negative control. *Artemisia campestris* extracts were mixed with doses of *Citrus aurantium* extracts (ratio 5/5) to determined synergistic effects.

Table No. 1
Strains used in the various antimicrobial activity tests

Microorganisms	Gram	Code	Conservation laboratory
Escherichia coli	Negatif	ATCC 8739	MNHN
Staphylococcus aureus	Positif	ATCC 6538	MNHN
Bacillus subtilis		ATCC 6633	LAPSAB
Candida albicans	Yeast	ATCC 10231	LAPRONA

RESULTS AND DISCUSSION

Essential oil yields

Table No. 2 shows the EO yield for the two EOs of *Artemisia campestris* and *Citrus aurantuim*, respectively. While the yields of AC ranged between 0.11 to 0.98%, that of CA was 1.67%. It is evident that the yields of essential oil are higher in the AC samples collected from the Aflou region, while, for the AC species, there is hardly any difference between the yields according to region or locality of origin. For example, in the AF2 sample, EO yielded an average of 0.46%; this value is slightly higher than that obtained for the AF1 sample, whose average value was 0.11%. The highest yield of 0.98% was recorded for sample AF3.

These differences in values for EO between the two are based on climatic conditions during harvesting, soil type, geographical location, and plant species. Comparison with yields reported in the literature reveals some differences. Indeed, for the species AC, essential oil yields obtained between 0.11-0.98%, are much higher than those reported for Lithuania, ranging from 0.03 to 0.08% (Judzentiene *et al*., 2010). Our plant has a lower yield than that of Akrout *et al.* (2009), who worked on *Artemisia campestris* L. originating from Beni-Khedache (a mountainous area in southern Tunisia). According to Akrout *et al.* (2009), the EO yield of *Artemisia campestris* from Tunisia is 1.2%. According to an investigation by Touil *et al.* (2017), the lowest EO yield of *Artemisia campestris* L. was recorded for samples collected in spring (0.37%), while the highest level was recorded for those collected in summer, with an average of (0.41%). As a result, the EO performance of a specific species is influenced by both internal factors (such as growth phases) and external factors (such as soil and climatic conditions and extraction techniques) (Touil *et al*., 2017).

Chemical composition of essential oils

Two chromatographic profiles (representative of each species) obtained by GC/FID are shown as examples in Figure No. 2. The chemical structures of these main compounds are shown in Figure No. 3. The results also show that the *Citrus aurantium* EO sample is mainly composed of limonene, with a content of 95.9%. The total percentages for the components identified ranged from 90.45 to 91.73% for the species *Artemisia campestris* L. (Table No. 2). The results also showed that the EO samples were rich in monoterpene hydrocarbons with ranges varying from 35.70 to 41.73%. The results of the EO analysis identified nine majority compounds (Table No. 1). The EOs from the Aflou region consist mainly of α-Pinene (8.51-15.41%) and limonene (3.46-12.81%) accompanied by other compounds at relatively low levels: Sabinene (1.79-4.68%), β-Pinene (3.45 7.58%), β-Myrcene (1.77-7.02%), cis-β-Ocimene (0.8-5.02%)γ-Terpinene (0.72-1.5%) and Spathulenol (1.19-1.72%).

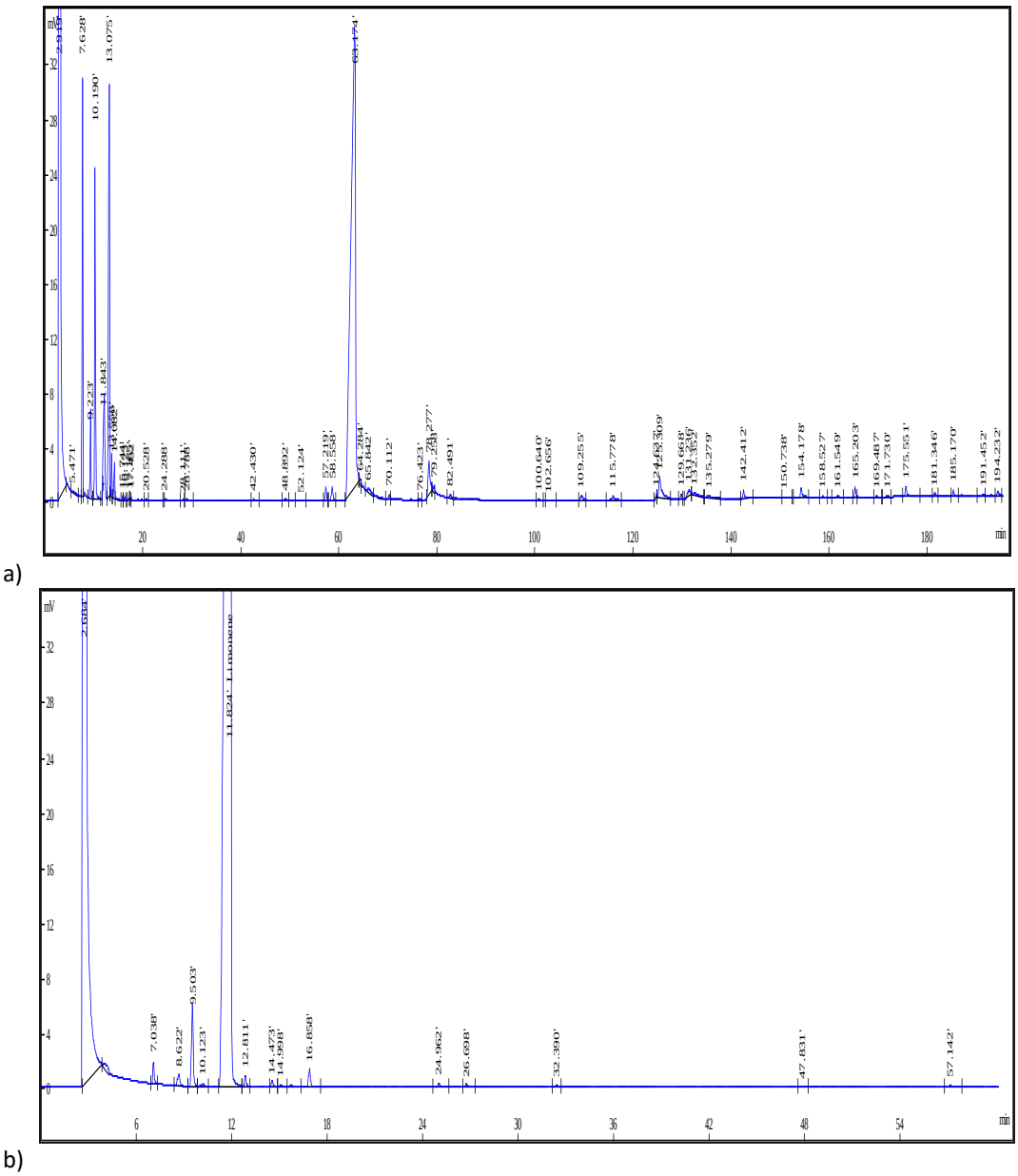
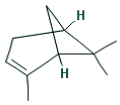
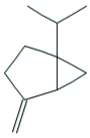


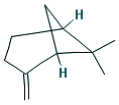
Figure No. 2
Chromatographic profiles obtained by GC/FID of the EOs of *Artemisia campestris* L. (a) and *Citrus aurantium* (b)



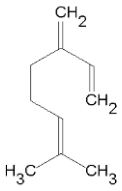
α-Pinene



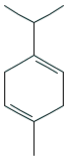
Sabinene



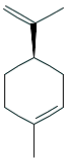
β-Pinene



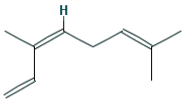
Myrcene



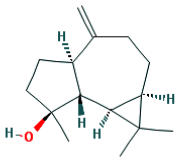
γ-Terpinene



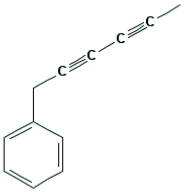
Limonene



cis-β-Ocimene



Spathulenol



Capillene

Figure No. 3
Semi-developed chemical structure of the majority compounds identified by GC and GC/MS in EO for the species *Artemisia campestris* L. and *Citrus aurantium*

Table No. 2
Percentage of the major components of the essential oil of *Artemisia campestris* L *ssp. eu-campestris* and *Citrus aurantium* species collected in different regions of Algeria

Compounds		AF1	AF2	AF3	CA
α-Pinene	932	**8,51**	**15.41**	**10.40**	1.75
Sabinene	969	1.79	4.68	3.32	-
β-Pinene	974	7.58	3.45	5.56	-
β-Myrcene	988	1.77	7.024	4.25	-
Limonene	1024	**12.81**	**3.46**	**10.56**	**95.9**
cis-β-Ocimene	1040	0.8	5.02	4.64	-
γ-Terpinene	1057	0.72	1.50	1.2	-
Capillene	1493	**55.17**	**50,00**	**49.2**	-
Spathulenol	1577	1.72	1.19	1.32	-
Total identified		90.87	91.73	90.45	97.65
Yield		0.11	0.46	0.98	1.67
Monoterpenic hydrocarbons		35.7	41.73	41.25	97.65
Homomonocyclic aromatics		55.17	50.00	49.2	-

In addition, Aflou region EOs also showed high values of homomonocyclic aromatic compounds represented by the same component, capillene. This paper reports for the first time high percentages ranging between 49.2 and 55.17%. The work of Boukhalkhal *et al*. (2018), is the first that identified capillene as the main component in *Artemisia campestris ssp. eu-campestris* in the Aflou region (Algeria). Although capillene has been reported in other *Artemisia* species, this study marks its first identification as a major component in *Artemisia campestris ssp. eu-campestris* from this specific region. The study also mentions capillin as a minor compound found for the first time in this subspecies. The two subspecies of *Artemisia campestris* L. studied in Boukhalkhal *et al*. (2018), *ssp. eu-campestris* and *ssp.glutinosa* Both presented different essential oil chemical profiles. *Artemisia campestris ssp. eu-campestris* is mainly characterized by monoterpene hydrocarbons, with the predominance of capillene, identified for the first time in this Algerian subspecies. *ssp. glutinosa* is also dominated by monoterpene hydrocarbons, but with a different composition than those of *ssp. eu-campestris*. A lower variability was recorded in this subspecies between the studied populations. This is the first report of capillene and capillin in the essential oils of *Artemisia campestris ssp. eu-campestris* from Aflou region by Boukhalkhal *et al*. (2018). More precisely, this is the first report concerning the EO of this Algerian subspecies. This is also stated in the paper as a first report in this plant, since capillene reached values up to 46.93% in the completely flowering plant.

A great deal of variability has been observed in the percentages of the main constituents of *Artemisia campestris* EOs. This is due to a number of factors, the most important of which are the climate, the soil, the harvesting period and the method of storage and extraction. Genetic factors and the vegetative cycle also play a part in the chemical diversity of *Artemisia campestris* L. volatile oils.

The chemical composition of AC essential oil can vary according to several factors such as geographical location, environmental conditions and the specific subspecies or variety, Benamar-Aissa *et al*. (2024), identified fifty-nine compounds, representing 96.85% of the total composition of *Artemisia campestris* EO analysed in their study. The main components were monoterpene hydrocarbons (75.96%), with α-pinene the most abundant (13.81%), followed by β-pinene (9.18%) and limonene (7.21%). Oxygenated monoterpenes represented 10.13% of the EO, with notable amounts

of camphor (2.23%) and terpinen-4-ol (1.12%). Sesquiterpene hydrocarbons and oxygenated sesquiterpenes were present in smaller quantities (1.69% and 6.01% respectively).

Consequently, different essential oil chemotypes have been reported in the literature. Abidi *et al*. (2018), studied a population of *Artemisia campestris* growing in Tunisia and found β-pinene (36.4%) and 2-undecanone (14.7%) as the main volatile components. Other Tunisian populations of A. campestris were analysed by Younsi *et al*. (2017), who detected germacrene D (16.38%), β pinene (16.33%) and limonene (9.17%) as the main essential oil compounds. Dib *et al*. (2017), studied the essential oil composition of a population growing in Morocco, finding spathulenol (10.2%) as the main component. Judzentiene and Budiene (Judzentiene & Budiene, 2014) studied natural populations growing in Lithuania and found germacrene D (9.8-31.2%) as the main volatile compound in all samples analysed. On the other hand, Houicher *et al*. (2016), reported a similar chemical profile for a natural population growing in Algeria, with α-pinene (18.7%), β-pinene (16.8%), β-myrcene (17.3%) and germacrene D (10.3%) as the main constituent of the essential oil.

Other studies have reported different major components, highlighting the variability of this species. For example, Akrout *et al*. (2003), found β-pinene (24.2-27.9%), p-cymene (17.4-22.3%) and α-pinene (4.1-11.0%) as dominant compounds in *Artemisia campestris* EO from south-eastern Tunisia. Lis *et al*. (2015), identified germacrene D (20.3-30.1%), β pinene (3.7-15.4%) and γ-humulene (6.6-9.8%) as main constituents. This variation in chemical composition highlights the importance of taking into account the origin and specific characteristics of the plant material when analysing *Artemisia campestris* EO. After a comparison of previous reports, it was found that the composition of EO from Morocco (ssp. unspecified) (Dib *et al*., 2017) and the Boussaâda region in Algeria (ssp. unspecified) (Belhattab *et al*., 2011) are completely different from the current study. As a result, the essential oil of *A. campestris* in Mediterranean countries showed distinct chemical patterns and variations in the proportion of chemical components in the EO. In France, γ terpinene, capillene, 1 phenyl-2,4 pentadiyne, and spathulenol were the most common components identified in the aerial parts. Ar-curcumene, caryophyllene oxide, p-cymene, β-pinene, germacrene D bicyclogermacrene, and myrcene were the ones found in Italy.

According to the literature, in *Artemisia*, acetylenic compounds (capillene and capillin) have already been identified in the essential oils of *Artemisia capillaris* (Harada & Iwasaki, 1982; Yano, 1983) and *Artemisia dracunculus* (Pappas & Sturtz, 2001). Moreover, capillene was detected in *Artemisia campestris* L. *ssp. glutinosa* with a content that depended on the part studied in a different phenological state (vegetative, before anthesis, full flowering and seed-bearing) ranging from 8.9 to 33.1% (Juteau *et al*., 2002). The maximum value was reported for the seed-bearing stage (33.1%). At full flowering, this compound represented a value of 22.3%. In the present study, high proportions of capillene (49.2-55.17%) were recorded in the EO of *ssp. campestris*. It is also the first time that this plant has shown a capillene content of up to 55.17% at the beginning of the vegetative cycle.

Evaluation of antioxidant activity

The *in vitro* antioxidant activity of the EOs from all the individual *Artemisia campestris* L. samples and in combination with *Citrus aurantium* EOs was assessed by the two tests adopted previously. The results of these tests are summarised in Table No. 3.

Measurement of anti-free radical power using the DPPH test

The results were expressed as the percentage of inhibition (I %) in Table No. 3 and represented by curves in Figure No. 4. The results show that the individual EOs from the different *Artemisia campestris* L. samples studied have fairly high antioxidant powers. These powers are confirmed by the high values of the inhibition percentages I% in relation to the low dilution ratios. These values allow us to evaluate and compare the efficacy of our EOs. The I% inhibition percentages of antioxidant activity are proportional to the concentration (dilution ratios) of the individual and combined EOs.

In the light of the results obtained, we can see that the individual EOs in samples AF1, AF2 and AF3 showed fairly high antioxidant powers. The minimum IC_{50} value recorded for the Aflou 3 sample represented the highest antioxidant activity (IC_{50}=5.80 mg/mL). In general, EO from *ssp. eu-campestris* showed higher antioxidant activity compared to *ssp. glutinosa*, (IC_{50}=13.07 - 31.94

mg/mL), particularly for the Aflou samples (rich in capillene) (Boukhalkhal *et al*., 2018). Previous studies examining the antioxidant activity of *Artemisia campestris* essential oils using the DPPH assay were predominantly from countries in north-west Africa (Mediterranean region): Tunisia (Akrout *et al*., 2009; Akrout *et al*., 2011), Morocco (Dib *et al*., 2017) and Algeria (Dob *et al*., 2005). Comparison of our results obtained with individual essential oils with published data (in most cases, the ssp. is not provided) was particularly difficult, as various reaction parameters were used, compared with several modified protocols that employed different initial DPPH concentrations, different solvents such as 'methanol, ethanol, etc, and different reaction times '30 min, 60 min, etc.'. In addition, as noted in previous reports, some inconsistencies were also noted; For example, the IC_{50} values for *Artemisia campestris* EO reported twice by the same author (following precisely the same protocol) (Akrout *et al*., 2009; Akrout *et al*., 2011) showed a wide disparity: 1.875 and 94.5 mg/mL. On first analysis, it seems that the IC_{50} values from the literature are difficult to compare (IC_{50}=1.875 mg/mL (Akrout *et al*., 2009), 94.5 mg/mL (Akrout *et al*., 2011), 0.69 mg/mL (Dib *et al*., 2017).

Table No. 3
Percentage inhibition (I %) of antioxidant activity of essential oils

Samples	Organ	Report	Colour reactions			
			DPPH•		FRAP(AEAC) mg/L	
			Individual	combined	Individual	combined
AF 1	Dry leaf	10/90	9%	28%	6,38	5,66
		30/70	26%	44%	8,2	7,12
		50/50	42%	14%	11,12	10,54
		70/30	58%	24%	20,54	20,83
		90/10	69%	50%	33,33	28,89
			DPPH•		FRAP(AEAC) mg/L	
			Individual	combined	Individual	combined
AF 2	Dry leaf	10/90	19%	21%	5,66	26,06
		30/70	30%	41%	7,12	36,55
		50/50	44%	52%	10,54	50,07
		70/30	52%	64%	20,83	69
		90/10	58%	60%	28,89	80,9
			DPPH•		FRAP(AEAC) mg/L	
			Individual	combined	Individual	combined
AF 3	Dry leaf	10/90	18%	17%	6,67	22,28
		30/70	18%	40%	7,15	25,3
		50/50	47%	48%	10,41	34,06
		70/30	60%	60%	21,83	41,92
		90/10	68%	68%	29,89	42,80
			DPPH•		FRAP (AEAC) mg/L	
			Individual		Individual	
CA	Bark	10/90	1.5%		1.9	
		30/70	2.5%		3.8	
		50/50	5.6%		3.8	
		70/30	7.8%		1.07	
		90/10	10.1%		7.56	

However, the combination of EO AF1/CA showed a decrease in antioxidant capacity at all dilution ratios, compared with the individual effect of EO AF1 already obtained, which is reflected in the appearance of an antagonistic effect between the two associated EOs. This is perhaps directly linked to the nature of the chemical structures of the individual terpene compounds making up EO AF1 are those responsible for the high antioxidant capacity. In comparison, the antioxidant capacity of combined EO AF2/CA (IC_{50}=4.6 mg/L) is much higher than the antioxidant capacity of EO AF2 (IC_{50}=6.8 mg/L) taken individually with all dilution ratios studied, allowing us to conclude that there is a synergistic effect when EO AF2 and EO CA are combined. The AF3/CA combination shows significant activity (IC_{50}=5.5mg/L) but is significantly different from that of the most active combination (AF2/CA) and can therefore be considered a synergistic intermediate or moderate antioxidant effect. This can be explained by the different terpene composition of the EOs on the one hand (distribution of individual compounds with antioxidant activities), and by the positive synergy or antagonism resulting from a combination of different essential oils with *Citrus aurantium* EO on the other. In fact, chemical structure is an important determinant of antioxidant potential, and high antioxidant activity is explained by the presence of molecules containing radical-scavenging groups in their structures. The effective concentration representing 50% inhibition (IC_{50}) calculated from the graph for the various samples is shown in Table No. 4.

The study by Benamar-Aissa *et al*. (2024), examined the antioxidant activity of binary blends of *Artemisia campestris* and *Citrus aurantium* extracts using the DPPH and β-carotene bleach tests. For the binary blend of CA and AC essential oils, the DPPH test revealed a synergistic effect, meaning that the combined antioxidant activity was greater than the sum of the individual activities. As the proportion of CA essential oil increased from 0% to 100%, antioxidant activity, measured by IC_{50}, initially rose rapidly and then more slowly. The most effective blend was around 33.33% CA and 66.67% AC, showing a significantly lower IC_{50} than pure CA essential oil and only slightly higher than pure AC essential oil. It is important to note that this study did not include FRAP analysis. Antioxidant activity was assessed using DPPH and β-carotene bleach assays only.

Table No. 4
IC_{50} values of *Artemisia campestris* L. extracts determined by the DPPH test

Extracts	IC50 (mg/mL)
AF1	6,20±0,183
AF2	6,80±0,166
AF3	5,80±0,156
CA	5,84±0,022
AF1/AC	9,10±0,157
AF2/AC	4,60±0,201
AF3/CA	5,50±0,157

Ferric ion reducing activity (FRAP)

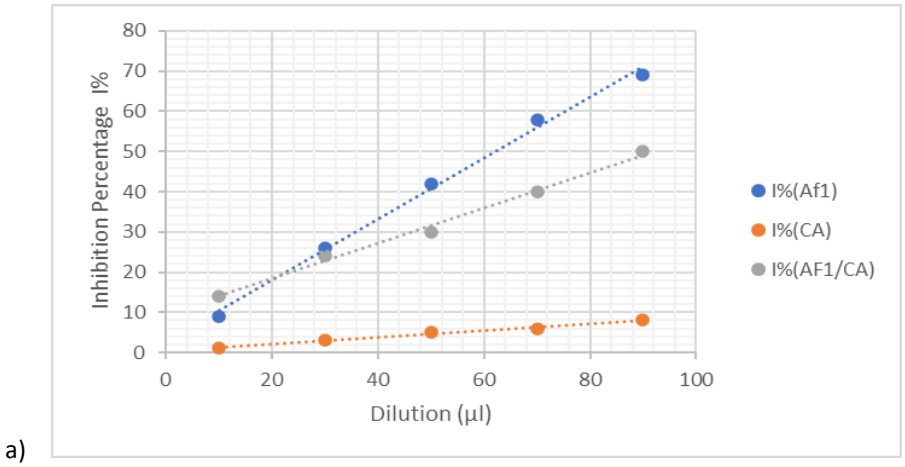
The antioxidant activity of individual and associated EOs was assessed using the FRAP method, a simple, rapid and reproducible assay that is universal and can be applied to plants, plasmas and organic and aqueous extracts alike. The presence of reducing agents in extracts causes the reduction of Fe_3^+/ferricyanide complex to the ferrous form, so Fe_2^+ can be assessed by measuring and monitoring the increase in blue color density in the reaction medium at 593 nm. Many current publications have indicated that there is a direct relationship between antioxidant activity and the reducing power of components in some plants. The results of the FRAP test, expressed as AEAC values (g/L), are summarized in Table No. 3 and shown in Figure No. 5. The FRAP activity profiles obtained (Figure No. 5) reveal that all the combined and individual EOs tested have significant

antioxidant activities. However, the combination of EO AF1/CA shows a similarity in $Fe3^{+}$ ion reducing power to the individual effect of EO AF1 at all dilution ratios studied. It can therefore be said that the combination does not promote a synergistic effect. In comparison, the reducing power of Fe_2^{+} ions in EO AF2/CA combined is much higher than the antioxidant power of EO AF2 taken individually with all dilution ratios studied, allowing us to conclude that there is a synergistic effect when EO AF2 and EO CA are combined. The AF3/CA combination has a reducing activity which is significantly different from that of the most active combination (AF2/CA) and can therefore be considered a moderate antioxidant synergistic effect.

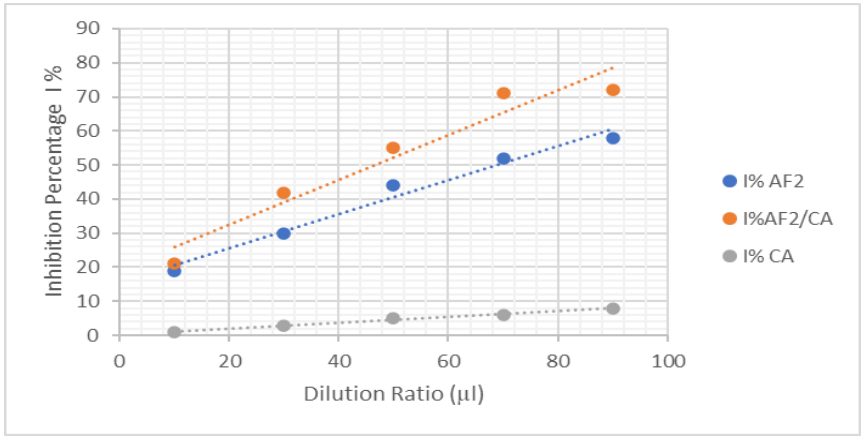
Furthermore, the chemical composition of essential oils of *Artemisia campestris ssp. campestris* from the Aflou province and their antioxidant activity power have been investigated by Boukhalkhal *et al*. (2018), α-pinene, capillene, and β-myrcene are the most important components that are associated with the highest antioxidant activity as measured by DPPH and FRAP tests, according to the results of principal component analysis.

The results of the aromatograms generated by the essential oils studied show that for *Escherichia coli* bacteria, the essential oil AF2 has the best activity with a diameter of 13.02 mm, for AF1 12.15 mm, while AF3 has the lowest activity with a diameter of 7.55 mm. And for the *Staphylococcus aureus* bacterium, we find that the essential oil AF2 once again presents the best activity with a diameter of 24.79 mm, while AF1 and AF3 have diameters of 13.95 mm and 13.62 mm respectively. On the other hand, for *Bacillus subtilis* bacteria, AF1 showed the best activity overall, with a diameter of 65.59 mm, while AF2 had a diameter of 22.17 mm and AF3 a diameter of 10.79 mm. These oils have an inhibition effect on all strains with a variation in diameters (Figure No. 6). On the other hand, the essential oils combined with *Citrus aurantium* showed significant results, while the AF2 /AC combination for both Bacillus subtilis and *Escherichia coli* bacteria also recorded low inhibition diameters compared with individual EO AF2 (*Bacillus subtilis* = 14.40 mm and *Escherichia coli* = 7.15 mm). For *Staphylococcus aureus* bacteria, however, no diameter was recorded, so it remained inactive and for the *Escherichia coli* bacteria, the combination of EO AF3/AC has the following small diameters: 6.81 mm and 12.34 mm. This means that the combination of AF2/AC oils has the best results of the association.

We conclude that the individual essential oils present greater inhibition diameters for the three bacteria than the combined essential oils, where diameters are low, allowing us to predict that the association does not favor synergy of antibacterial activity. For fungi, the inhibition diameters for EO AF3 are the highest at 40.1 mm, compared with EO AF 2 (39 mm) and AF 1 (23 mm), and there is a complete absence of inhibition diameters for essential oils combined with *Citrus aurantium*. The study by Benamar-Aissa *et al*. (2024), examined the antimicrobial activity of binary blends of *Artemisia campestris* and *Citrus aurantium* essential oils against five microbial strains: *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa* and *C. albicans*. The blends were prepared in three different ratios (1:3, 1:1 and 3:1) of AC essential oil to CA essential oil. The results showed a strong synergistic effect against S. aureus in all three blend ratios. This means that the combined antimicrobial activity of AC and AC essential oils was significantly greater than the sum of their individual activities. For *E. faecalis*, synergy was observed at ratios 1:3 and 1:1, while an additive effect (combined activity equal to the sum of individual activities) was observed at ratio 3:1. However, the mixtures showed no consistent improvement in antimicrobial activity against the other micro-organisms tested. Against *E. coli*, *P. aeruginosa* and *C. albicans*, the blends generally showed either an additive effect, or no significant change from the activity of the weakest essential oil. In some cases, the mixture even produced a smaller zone of inhibition than the CA essential oil alone. These results suggest that the synergistic antimicrobial effect of the CA and CA essential oil blend is more pronounced against Gram-positive bacteria (*S. aureus* and *E. faecalis*) than against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) or the yeast C. albicans. The specific mechanisms underlying these interactions warrant further study.



a)



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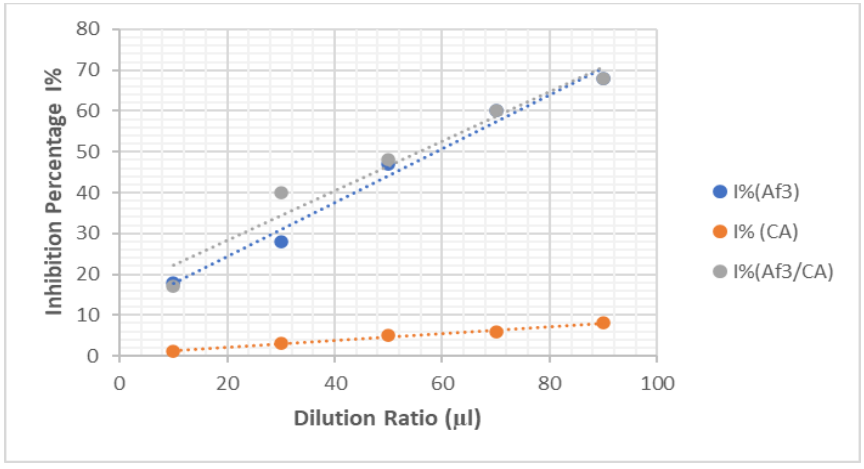


c)

Figure No. 4
Curves showing the antioxidant power of individual essential oils and associated with CA
a) Aflou 1, b) Aflou 2, c) Aflou 3

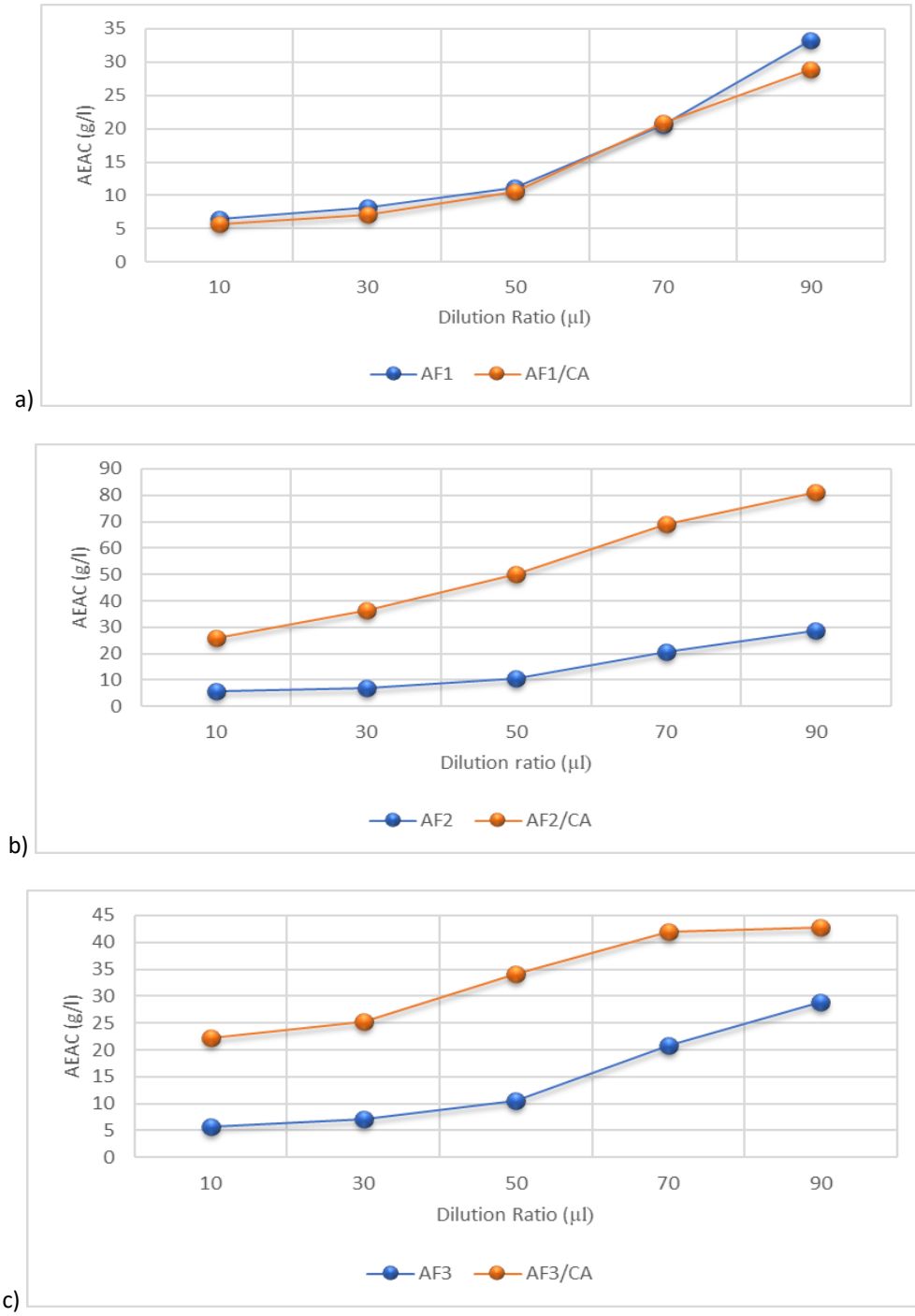


Figure No. 5
Curves showing the antioxidant power of individual essential oils and associated with CA
a) Aflou 1, b) Aflou 2, c) Aflou 3

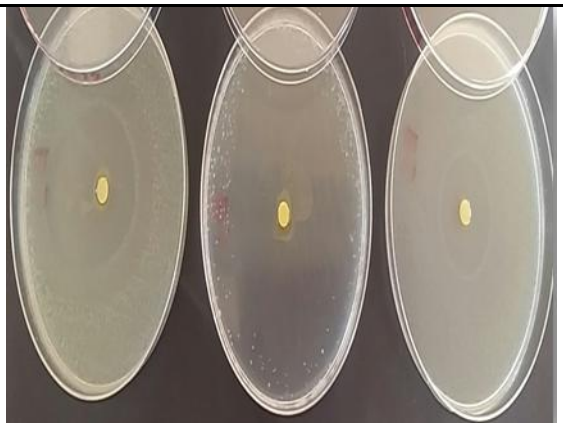
Study of antibacterial activity

Tests are carried out on three bacterial strains to determine which preparation contains the most inhibitory Gram-positive and two Gram-negative bacteria. The results of the AC essential oil inhibition tests are shown in Table No. 5.

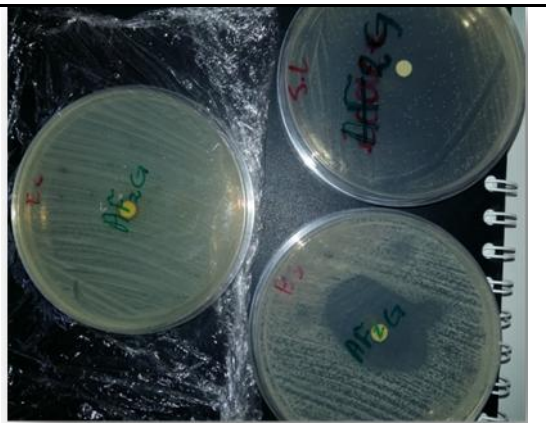
Table No. 5
Diameter values of inhibition zones (mm) of essential oils for bacterial strains

Essential oils	**Bacteria**			**Fungus**
Samples	Escherichia coli	Staphylococcus aureus	Bacillus subtilis	*Candida albicans*
AF1	12,15 ± 0.05	13,95 ± 0.14	65,59 ± 0.09	23 ± 0.11
AF2	13,02 ± 0.25	24,79 ± 0.35	22,17 ± 0.15	39 ± 0.26
AF3	7,55 ± 0.10	13,62 ± 0.23	10,79 ± 0.42	40,1 ± 0.38
AF1/CA	-	-	-	-
AF2/CA	7,15 ± 0.62	-	14,40 ± 0.20	-
AF3/CA	6,81 ± 0.33	-	12,34 ± 0.32	-
AMP: 10 µg	10.35 ± 0.070	41.75 ± 0.777	6 ± 0.01	

- no action



AF1



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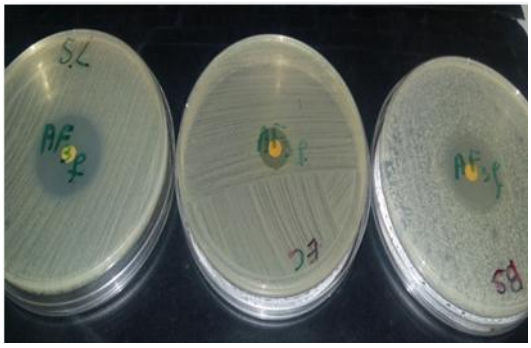
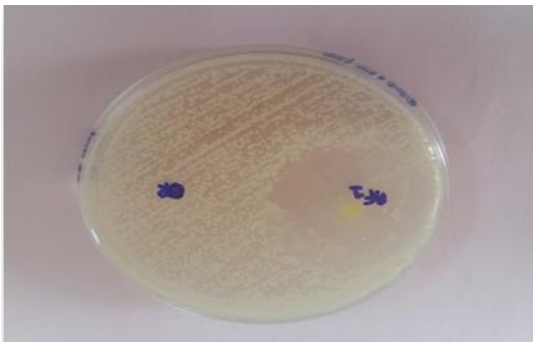


AF3



AF1 Combined CA

Figure No. 6
Results on the three strains of essential oil AF1, AF2, AF3, and AF1 Combined CA

CONCLUSION

The purpose of this study is to try to make *Citrus aurantium* extracts more valuable as food additives, preservatives, or antioxidants because it is a common plant with affordable extracts. According to the primary findings of this study, there is a potential prospect that *Citrus aurantium* extract could potentially be used as an additive or as a diluting agent for essential oils. It may also have synergistic effects on antibacterial activity and antioxidant properties. As a result, the findings demonstrated the significance of researching how the ratios of extracts in the mixture alter antibacterial activity and identifying the range of ratios that can be used in a food preservation system, neither antagonistically or synergistically, with negligible or no negative impacts.

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