Wild Chamomile [*Cladanthus mixtus* (L.) Chevall.] Collected from Central-Northern Morocco: Phytochemical Profiling, Antioxidant, and Antimicrobial Activities

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Abstract: We investigated phytochemicals and biological activities in *Cladanthus mixtus* essential oil (CMEO) and various extracts. To this end, flowers CMEO microwave-extracted was subjected to chemical analysis using GC-MS. Antimicrobial activities of CMEO and various extracts obtained by maceration and Soxhlet were tested against four microbial strains using agar well diffusion assay and microtitration method. Antioxidant activities were determined, for extracts, using DPPH. CMEO chemical composition revealed 44 compounds. Santolina alcohol (40.7), germacrene D (8.9), and α-pinene (5.7%) were the main constituents. The best records of yield, total phenolics, and flavonoids were obtained with Soxhlet and methanol for extracts. Important antimicrobial activities were recorded in CMEO and extracts. For CMEO, MICs ranged from 10.17 (*Bacillus subtilis* ATCC 3366) to 13.83 μg/mL (*Escherichia coli* ATCC 25922). Among extracts, Met-OH was the most efficient with MICs ranged from 11.17 (*Candida albicans* ATCC 10231) to 15.83 μg/mL (*E. coli*). Met-OH extract presented the highest antiradical activity ($IC_{50} = 55.50$ μg/mL), while the n-hexane displayed the lowest one ($IC_{50} = 259$ μg/mL). Based on these outcomes, CMEO and various extracts from *Cladanthus mixtus* flowers could be suggested for their use as potential natural preservatives to enhance foods shelf-life, herbal formulations, and also as antiseptics and disinfectants.

Keywords: *Cladanthus mixtus*; phytochemicals; green extraction; santolina alcohol; biological activity.

1. Introduction

It is widely evidenced that there is a global emergence of resistant microorganisms, which is increasingly restricting the potency of the currently available drugs [1], resulting in a failure in the management and treatment of infections [2]. Therefore, the prevalent antibiotic's effectiveness has been reported to decrease steadily, leading to a 'post-antibiotic' era, as

reviewed in Reardon (2014) [3]. Moreover, according to several estimations, a persistent rise in antibiotic resistance would lead to about 10 million deaths yearly by 2050, as evidenced in O'neill (2014) [4] and Mendelson (2015) [5]. Besides, this situation is getting worse, causing the misuse and overuse of antibiotics. In this context, there is a pressing need to explore novel strategies to fight drug-resistant microorganisms. Because microbes' multidrug resistance became a major concern since it represents a global threat to public health, the natural products screening to search for new antimicrobial agents has become imperative [6,7]. In fact, recently, plants and associated secondary metabolites have received much attention from the scientific community, emphasizing their therapeutic potentials [8,9]. In this regard, many plants used in traditional medicine to treat different illnesses have been demonstrated to be more effecient, not expensive compared to conventional drugs. They have less or even no side effects [10].

An essential part of the chemical species (especially secondary metabolites) produced by plants is to protect them against pathogens [1]. In this context, various plant extracts and related compounds have been reported to have important antimicrobial powers both in vitro and in vivo [11].

Besides, Morocco is deemed to be one of the most important floristic areas in Northern Africa. This enormous plant diversity is due to its geographical position, climate, diverse geology, topography, and ecoregion [12]. The Moroccan flora is estimated to encompass 978 endemic taxa, over half of the North African endemic plant species [13]. This endemic richness is ascribed to the presence of mixed and clear differentiated environments, as highlighted in Ranko *et al*. (2013) [12].

The *Cladanthus* genus (formerly known *Chamaemelum* then *Ormenis*), which belongs to the Asteraceae cosmopolitan family, comprises 130 species widespread in Mediterranean areas (including Morocco), South Africa, Southwest Asia, and Western Europe [14]. Among them, Moroccan chamomile is botanically known as [*Cladanthus mixtus* (L.) Chevall.] with several previous synonyms [= *Ormenis mixta* (L.) Dumort.; *Chamaemelum mixtum* (L.) All. *Anthemis mixta* L.]. It is a spontaneous annual plant (10–40 cm tall with fragrant white/ yellow flowers) that grows widely in northern Morocco and along the Atlantic coast on sandy soils [15,16]. It is mainly used for essential oil (EO) extraction. Morocco is the main supplier of EOs of this species in the international market. *Cladanthus mixtus* (*C. mixtus*) EOs are used both in perfume and pharmaceutical industries, according to Benjilali and Zrira (2005) [17]. Likewise, the infusion of leaves and flowers are also used in Moroccan folk medicine thanks to its numerous health-healing properties, including antiseptic, anti-inflammatory, antibacterial, antispasmodic, and sedative effects [18–21].

Despite its valuable EOs and pharmacological properties, *C. mixtus* has received little attention regarding its chemical composition. Under the different previous scientific names, some studies have addressed the phytochemistry and biological properties of *C. mixtus* extracts [22–24]. Elouaddari *et al*. (2019a) [21] reviewed *C. mixtus* EOs chemical composition and related biological activities. Following the authors' review, EOs yield ranges from 0.06 to 1.56% and varies greatly among geographical areas. Higher yields were reported to be in dry environments, while the wetter ones displayed relatively lesser yields. According to the same authors, phytochemicals found in *C. mixtus* EOs encompass 264 compounds with wide variations depending on factors such as environments, plant parts (leaves, flowers, or stems), extraction technology, etc conditions, among others. These compounds are distributed, in decreasing order, as follows: Oxygenated monoterpenes (30–45.3%), monoterpene hydrocarbons (15–24.5%), sesquiterpene hydrocarbons (14–33.9%), oxygenated

sesquiterpenes (4–11.7%), and others (traces–4.5%). This biochemical richness is behind numerous properties like antimicrobial activities, anticorrosive, and cytotoxic activity against human cervical cancer cell lines.

To the best of our knowledge, no detailed information about phytochemicals and associated biological activities in *C. mixtus* growing in central-northern Morocco hence the originality of this research work, which had as objectives, (i) to assess antioxidant and antimicrobial activities of aerial parts from *C. mixtus*, (ii) to investigate the EO phytochemical composition of this species, and (iii) to compare extracts obtained using various solvents in terms of yield, total phenolics, total flavonoids, and their antioxidant and antimicrobial activities.

2. Materials and Methods

2.1. Chemicals, solvents, and reagents.

All reagents and solvents used were of the highest purity. 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, methanol, n-hexane, ethanol, ethyl acetate, aluminum chloride ($AlCl_3$), sodium carbonate, sodium sulfate, Folin–Ciocalteu, dimethyl sulfoxide (DMSO), gallic acid (GA), quercetin (Q), and potassium acetate were used in this work. These chemical reagents were acquired from Stigma-Aldrich (St. Louis, MO). All other chemicals and solvents used were of analytical grade.

2.2. Plant material and extracts preparation.

Firstly, the plant species has been botanically authenticated by Prof. A. Ennabili from the Higher School of Technologies (SMBA University, Fez, Morocco). At full blooming, flowers (the aerial parts) of *C. mixtus* were collected in May 2018 from the Mezraoua region (15 km from Taounate province, 34°31'48" N, 4°42'36" W). This region belongs to Central-northern Morocco, characterized by a Mediterranean climate humid in winter and semi-arid in summer. The sampled plants were dried in a dark room to avoid the photo-oxidation, then crushed using an electric grinder to a fine powder subjected to extraction via maceration. Briefly, 25 g of powder were used for extraction using various solvents separately (ethanol, ethyl acetate, methanol, and n-hexane). The extraction was achieved throughout 72 h. The obtained extracts were then filtered through Whatman No. 1 filter paper. The residue was re-extracted twice. The combined extracts of every sample were evaporated, at a temperature of 45 °C, using a rotary evaporator (Heidolph Collegiate, LV28798826, New Jersey, USA). The obtained samples were used to study their antioxidant and antimicrobial activities [25].

2.3. EOs isolation.

C. mixtus EOs were isolated using two different methods: Microwave and hydrodistillation, as described below. The yields of oils were calculated and expressed (in %) per weight of the dried plant material. After that, only EOs obtained via microwave were subjected to phytochemical profiling using gas GC-MS. According to published literature [26,27], the microwave method has several advantages over traditional alternatives such as shorter isolation time (15 min versus 3 h required for hydrodistillation), environmental impact (lower energy cost), a cleaner method (since no residue generation and no solvents used),

enhances antimicrobial and antioxidant activity, and provides more valuable EOs (higher amount of oxygenated phytocompounds).

2.3.1. Microwave-assisted extraction.

Solvent-free microwave extraction has been performed following Lucchesi *et al*. (2004) [26] in a Milestone "DryDist" microwave laboratory oven. This is a multimode microwave reactor 2.45 GHz with 10^3 W as maximum power. An external infrared sensor controlled the temperature. 100 g of plant material were heated, at atmospheric pressure, using a fixed power density of 1 W g^{-1} for 15 min without solvents or water. The direct interaction of microwaves with biological water (present in the fresh plant material) fosters the release of EOs contained inside the plant tissues. The mixture of hot ''crude juice'' and in situ water move, thanks to earth gravity downwards, on a spiral condenser where it condensed. In a receiving flask, oily condensate was collected continuously. At the end, EO was collected, dried via anhydrous sodium sulfate, and stored at 4 °C until used.

2.3.2. Hydrodistillation.

To isolate EO from *C. mixtus*, dried aerial parts (flowers) were subjected to hydrodistillation using a Clevenger-type apparatus. Three independent distillations involving 100 g of plant material were carried out by boiling, for three hours, in a 1-liter flask topped by a column of 60 cm length connected to a refrigerant as described in Jennan *et al*. (2018) [28]. Separation of the obtained EO from the water was done using decantation. EO was then dried over anhydrous sodium sulfate and kept in amber vials at 4 °C until use.

2.4. Phytochemical profiling of EO using gas GC-MS.

The EO analysis, achieved through microwave extraction, was carried out according to Talbaoui *et al*. (2016) [29]. It was carried out using a TRACE GC ULTRA equipped with non-polar VB5 (95% methyl polysiloxane, and 5% phenyl), capillary column (30 m × 0.25 mm i.d. and 0.25 µm as a film thickness), directly coupled to a mass spectrometer (Polaris Q) (EI 70 eV). The temperatures of the injector and detector were set at 250 and 300 °C, respectively. The oven temperature was programmed to increase at 4 °C/min from 40 to 180 °C and at 20 °C/min for 180–300 °C. The gas carrier was helium with a flow rate of 1 mL/min; the sample (1 µL) was injected according to a splitless mode.

2.5. Total phenolics and flavonoids determination.

2.5.1. Determination of total phenolics content (TPC).

TPC was determined using the Folin-Ciocalteu method [30] with slight modifications. Briefly, 100 µL of extracts (1 mg/mL) were added to 500 µL of 1:10 Folin–Ciocalteau reagent (prepared before use). After 4 min, 400 µL of sodium carbonate 7.5% (m/v : 75mg/mL) were added. The mixture was incubated for 90 min at room temperature; the optical density was then read at 765 nm using a Cary 50 UV-Visible Spectrometer (Varian, Mulgrave, Australia). A standard curve was prepared using gallic acid (GA, 5 mg/mL) with various concentrations ranging from 0 to 150 µg/mL. The results were expressed as GA equivalents (GAE) per g of sample (dry matter, DM).

2.5.2. Determination of total flavonoid content (TFC).

TFC was determined using the aluminum trichloride colorimetric (AlCl₃) method [31] with slight modifications. Briefly, 250 µL of extracts (2 mg/mL) were mixed with 50 µL of AlCl3 (10%, m/v), 750 µL of absolute ethanol, 1.4 mL of deionized water, and 50 µL of potassium acetate (1 M). The mixture was incubated at room temperature for 30 min; the absorbance was then measured at 415 nm. Quercetin was used to carry out the standard curve. The results were expressed as a gram of quercetin equivalents (QE) per g of sample (dry matter, DM).

2.6. Antioxidant activities assessment.

The ability to trap the radical DPPH (2,2-diphenyl-1- picrylhydrazyl) was determined by the standard method described in Brand-Williams *et al*. (2015) [32] with some modifications. Briefly, at least 0.2 mL from various concentrations (30–480 μg/mL) of *C. mixtus* extracts were added to 1.8 mL of the DPPH methanolic solution at 0.11 mM. After incubation in darkness at room temperature (23 ± 2 °C) for 30 min, the absorbance was measured at 517 nm against a DPPH methanolic blank solution. The DPPH radical scavenging activity (also known as antioxidant activity, AA) have been calculated based on the following equation:

$$\% \ (\text{AA}) = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \right] \times 100$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of the control reaction (containing all reagents, except the test sample) and $\text{Abs}_{\text{sample}}$ sample is the absorbance of extracts at 517. Ascorbic acid was used as a positive control, and the concentration of extracts that inhibit 50% (IC_{50}) of DPPH was computed based on the percentage graph inhibition following the extract concentration using the exponential equation.

2.7. Determination of antimicrobial activities.

Microbial strains used here are of significant concern in problems health and food spoilage as previously described in our previous works [33,34]. EO and extracts obtained using various solvents were tested for antimicrobial activities against the following strains: *Escherichia coli* ATCC 25922 (*E. coli*), *Staphylococcus aureus* ATCC29213 (*S. aureus*), *Bacillus subtilis* ATCC 3366 (*B. subtilis*), and *Candida albicans* ATCC 10231 (*C. albicans*).

2.7.1. Disc diffusion assay.

The antimicrobial activities of the extracts were examined following the disk diffusion method [35]. Briefly, microbial strains were cultured overnight at 37 °C on Luria-Bertani broth. After that, an inoculum consisting of 0.5 McFarland was prepared. A 100 µL of bacterial inoculum was inoculated onto Petri dishes containing a sterile medium of Luria-Bertani agar. Sterile filter paper discs (diameter consisting of 5 mm) were deposited on the medium and impregnated with 10 µL of extract solution (500 mg/mL of dimethyl sulfoxide [DMSO] at 2%). The control was carried out with discs containing 10 µL of DMSO at 2%.

2.7.2. Minimum inhibitory concentration (MIC) of plant extracts and Eos.

To determine MICs, the agar dilution method was used as recommended by the Natural Committee for Clinical Laboratory Standards [36]. All tests were performed in nutrient broth

for bacterial strains. Various concentrations (7.5, 10, 12.5, 15, and 20 μg) from dry extracts and EO were added separately to 1 mL nutrient broth tubes containing 105 CFU/mL of live bacterium's cells. To spread the extracts and EOs throughout the broth, the tubes (contained 10 mL broth) were incubated in an incubator shaker. The highest dilution (the lowest concentration), displaying no visible bacterial growth, was considered MIC. After that, cells from the tubes showing no growth were cultured on nutrient agar plates to determine whether the inhibition was permanent or reversible.

2.8. Statistical analyses.

All determinations and measurements were achieved, at least in triplicates. The combined analyses of variance (ANOVA) were computed to assess magnitude effects of extraction techniques and solvents used on yield, TFC, and TFC. Quantitative differences were assessed by the general linear procedure followed by Duncan's test. Data statistical analyses were performed using the SPSS package version 23 (IBM, Armonk, NY, USA). Values were expressed as means ± standard deviations (SD). Differences were considered significant at 5% as a probability level. Principal component analysis (PCA) was performed on mean values to discriminate among solvents and extraction techniques using STATGRAPHICS package version XVIII (Statpoint Technologies, Inc., Virginia, USA).

3. Results and Discussion

3.1. Results.

3.1.1. Chemical composition of EO.

The yield of EOs (of a yellowish color) obtained from *C. mixtus* flowers by both and microwave and hydrodistillation was expressed as the percentage of plant dry weight. EO yield obtained by microwave (0.79 ± 0.06%) was higher than that achieved using hydrodistillation (0.53 ± 0.09%). The obtained chromatogram (Fig. 1) for EO chemical composition was characterized by 44 chemical compounds (Table 1) accounting for 96.9% of the total chemical composition.
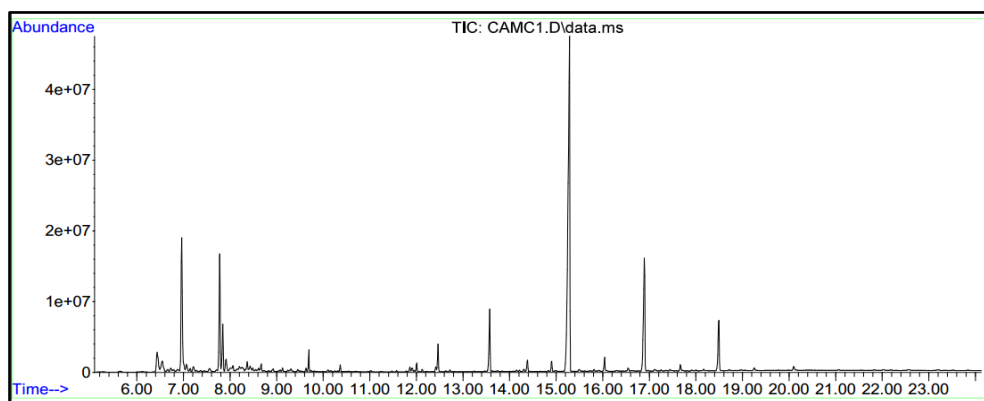
Figure 1. A representative typical GC-MS total ion current (TIC) chromatogram of essential oil isolated using microwave extraction from aerial parts (dried flowers) of *C. mixtus* harvested from central-northern Morocco.

Nineteen of them were in concentrations over 1%, and the remaining 25 constituents were in concentrations lower than 1%. The major twenty compounds (≥ 1%), in a decreasing order, were santolina alcohol (40.7%), germacrene D (8.9%), α-pinene B (5.7%), Artemesia

alcohol (4.3%), (E)-β-Farnesene (4.0%), Limonene (3.1%), yomogi alcohol (3%), caryophylladienol (2.8%), camphor (2.5%), santolina acetate (2.2%), pinocarvone (1.9%), β-caryophyllene (1.7%), germacrene D-4-ol (1.6%), epi-α-muurolol (1.6%), α-cadinol (1.4%), (E)-pinocarveol (1.3%), epi-α-Cadinol (1.2%), δ-cadinene (1.1%), and bornyl acetate (1%). From these outcomes, it seems that *C. mixtus's* EO was dominated by oxygenated monoterpenes (mainly santolina alcohol), monoterpene hydrocarbons (α-pinene and limonene), and sesquiterpene hydrocarbons (mostly germacrene D and (E)-β-Farnesene).

Table 1. Chemical compounds (determined using GC-MS) of essential oil (%) isolated using microwave extraction from aerial parts (dried flowers) of *C. mixtus* collected from central-northern Morocco. Compounds are listed in the elution order. tr: trace (< 0.1%).

No.	Compounds	Retention index	Relative percentage
1	α-Pinene	939	5.7
2	Camphene	954	0.2
3	Sabinene	971	0.1
4	β-Pinene	979	0.3
5	Myrcene	982	0.5
6	Yomogi alcohol	986	3.0
7	p-Cymene	1025	0.1
8	Limonene	1028	3.1
9	1,8-Cineole	1030	0.3
10	Santolina alcohol	1037	40.7
11	Artemesia alcohol	1080	4.3
12	Campholenal	1121	0.1
13	(E)-pinocarveol	1139	1.3
14	Camphor	1146	2.5
15	Pinocarvone	1155	1.9
16	Borneol	1166	0.3
17	Santolina acetate	-	2.2
18	Terpinen-4-ol	1181	0.3
19	α-Terpineol	1199	0.2
20	Hexyl isovalerate	-	0.1
21	Bornyl acetate	1289	1
22	δ-Elemene	1340	Tr
23	Silphiperfolene	-	Tr
24	(Z)-Jasmone	-	Tr
25	Phenethyl acetate	-	0.1
26	α-Copaene	1377	0.1
27	β-Maaliene	-	0.4
28	β-Elemene	1392	0.6
29	β-Caryophyllene	1419	1.7
30	(E)-β-Farnesene	1456	4
31	α-Humulene	1480	0.4
32	Germacrene D	1485	8.9
33	Bicyclogermacrene	1502	0.2
34	α-Muurolene	1503	0.8
35	δ-Cadinene	1520	1.1
36	(Z)-Nerolidol	1536	0.3
37	Germacrene D-4-ol	1570	1.6
38	Caryophyllene oxide	1579	0.9
39	Caryophylladienol	-	2.8
40	epi-α-Cadinol	-	1.2
41	epi-α-Muurolol	-	1.6
42	α-Muurolol	1639	0.6
43	α-Cadinol	1650	1.4
44	β-Bisabolol	1670	Tr
Total			**96.9**

3.1.2. Analyses of variance (ANOVA).

Table 2 summarizes the mean squares from the combined analyses of variance for yield, TPC, and TFC. From these outcomes, it has been shown that extraction technique, solvent, and

their interaction impacted significantly, at least at 5% as a probability level, the yield, TPC, and TFC. However, the principle variability source was solvent for the three parameters. Regarding yield, solvent effects accounted for about 73% of the total variance. However, the extraction technique's effects were of lesser magnitude and explained 25% of the total variance. Similar trends were observed in TPC and TFC variability; solvent effects were the most important and explained around 95% of the total variance; meanwhile, the effects of extraction technique were less important and explained only 3%. Finally, the extraction technique × solvent interaction was also of a lesser extent since it explained around 1% of the three parameters' variance (yield, TPC, and TFC).

Table 2. Mean squares from the combined analyses of variances for yield (%), total phenolics content (TPC, mg GAE/g DM), and total flavonoids content (TFC, mg QE/g DM). Df = degree of freedom, GAE = Gallic acid equivalents, QE = Quercetin equivalents, and DM = Dry matter. * Significant at 0.05 probability level; ** Significant at 0.01 probability level; *** Significant at 0.001 probability level.

Source of variation	Df	Yield	P-Value	TPC	P-Value	TFC	P-Value
Extraction technique (ET)	1	81.33***	0.0001	7.72**	0.008	15.44***	0.0005
Solvent (S)	3	231.58***	0.0002	482.24***	0.0007	272.17***	0.0006
Replicate (S)	8	0.97	0.4813	0.75	1	0.68	1
ET × S	3	4.51*	0.0333	4.28*	0.0091	5.69*	0.0091
Residual	9	0.93		0.91		0.90	
Total (corrected)	24						

3.1.3. Mean comparison of yield, TPC, and TFC for solvents and extraction techniques.

Mean values of yield, TPC, and TFC for both extractions techniques (maceration and Soxhlet) and various solvents (ethanol, ethyl acetate, methanol, and n-hexane) are given in Table 3. As evidenced in these outcomes, significant differences (p < 0.05) were observed between both extractions techniques and among the four solvents used. Among solvents, extracts from methanol showed the highest yield, TPC, and TFC for both extractions techniques. The lowest values of yield, TPC, and TFC were achieved using n-hexane. Between extractions techniques, the Soxhlet technique displayed the highest yield (23.3%), TPC (36.21 mg GAE/g DM), and TFC (26.80 mg QE/g DM) in the case of methanol. In contrast, the lowest records of these parameters were obtained using maceration and n-hexane.

Table 3. Mean values of yield (%), total phenolics content (TPC, mg GAE/g DM), and total flavonoids content (TFC, mg QE/g DM) for various *C. mixtus* extracts obtained by two extraction techniques (maceration and Soxhlet) using four types of solvents namely ethanol (E), ethyl acetate (EA), methanol (M), and n-hexane (H). Within the same column, values followed by the same letter are not significantly different at 5% as a probability level. GAE = Gallic acid equivalents, QE = Quercetin equivalents, and DM = Dry matter.

		Maceration				Soxhlet		
		Yield	TPC	TFC		Yield	TPC	TFC
M		21.10 ± 0.82[a]	34.70 ± 0.60[a]	24.85 ±0.65[a]		23.30 ± 0.80[a]	36.21 ± 1.24[a]	26.80 ± 0.80[a]
E		15.72 ± 0.24[b]	30.41 ± 1.67[b]	16.79 ±0.93[b]		19.76 ± 0.81[b]	33.15 ± 1.80[b]	18.43 ± 1.41[b]
EA		9.43 ± 0.54[c]	25.17 ± 0.62[c]	13.38 ±0.85[c]		15.42 ± 1.19[c]	27.13 ± 0.84[c]	14.03 ± 0.84[c]
H		6.69 ± 0.39[d]	15.39 ± 0.74[d]	9.04 ±1.30[d]		9.19 ± 0.54[d]	16.39 ± 1.75[d]	11.23 ± 1.88[d]

Extracts from ethanol and ethyl acetate presented better scores, after methanol, of yield, TPC, and TFC. Therefore, different solvents' ability to achieve a good yield and extract bioactive compounds (TPC and TFC) was in the following order: methanol > ethanol > ethyl acetate and finally n-hexane.

3.1.4. Mean comparison of antioxidant activities and IC_{50}.

Antioxidant activities mean values for different extracts and ascorbic acid (control) are shown in Fig. 2. As evidenced in these outcomes, large variations were observed among antioxidant activities in the investigated extracts. Extracts from all solvents proved to reduce radical DPPH; however, ascorbic acid had the best performance in scavenging this radical. As extracts' concentration increases, there was a significant decrease ($p < 0.05$) in DPPH absorbance at 517 nm, translated into a discoloration from purple to yellow. Indeed, at 40 µg/mL, ascorbic acid showed the capacity to inhibit more than half (56.53%) of DPPH. At the same concentration (40 µg/mL), inhibition percentages of Me-OH, Et-OH, Ethyl Acetate, and n-hexane were 38.16, 31.22, 17.55, and 11.47%, respectively. Similarly, at 480 µg/mL, ascorbic acid inhibition percentage reached 100%, while Me-OH, Et-OH, Ethyl Acetate, and n-hexane were 93.36, 79.15, 75.62, and 70.48%, respectively. Antioxidant activities seemed to follow a pattern of dose-dependent type. As compared to ascorbic acid (control), methanol was the most efficient regarding DPPH reduction, while n-hexane presented the smallest scavenging activity.
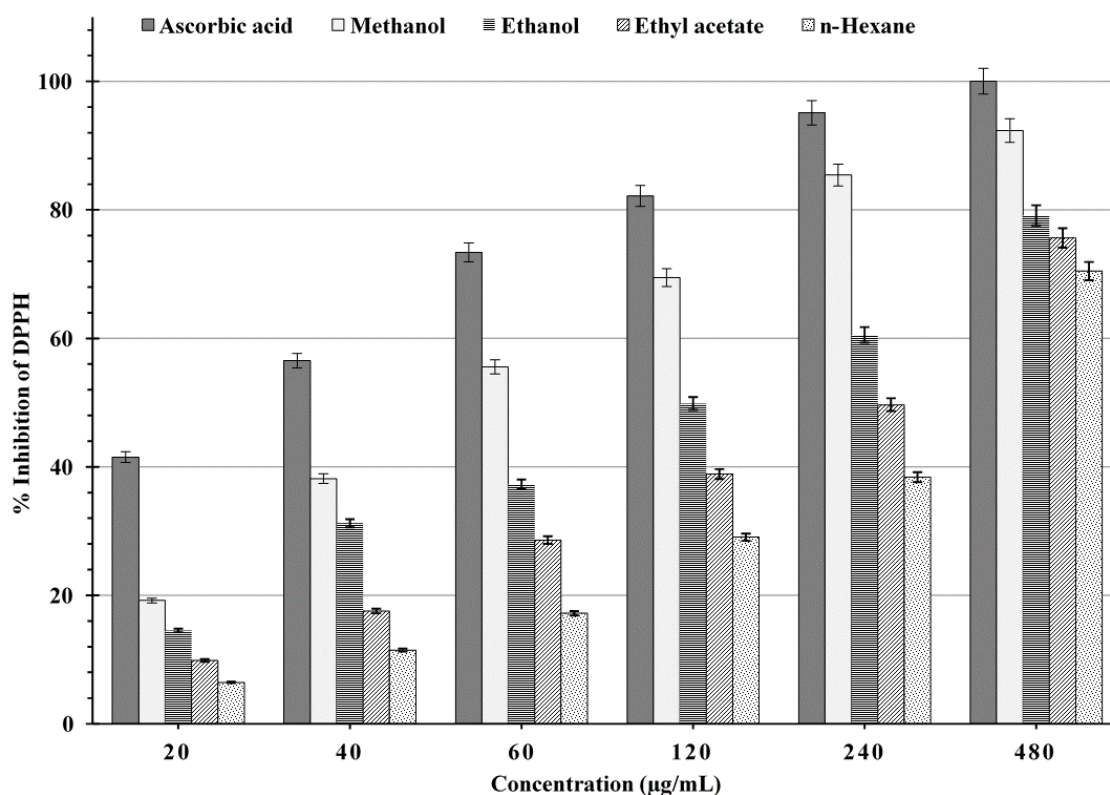
Figure 2. Antioxidant activities expressed as inhibition percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) of various extracts (methanolic, ethanolic, ethyl acetate, and n-hexane) obtained from aerial parts (dried flowers) of *C. mixtus.* Ascorbic acid was used as a control. Results are presented as mean values of three replicates.

The IC_{50} value is defined as the concentration of a given extract that inhibits 50% of the DPPH radical. The IC_{50} was determined, for various extracts, based on percentage inhibition of DPPH versus the concentrations of extracts and ascorbic acid (positive control). The Met-OH extract presented the highest antiradical activity (IC50 = 55.50 µg/mL) followed by the Et-OH extract (IC_{50} = 121.5 µg/mL), the ethyl acetate extract (IC_{50} = 240.9 µg/mL), and the n-hexane extract as the lowest antiradical power (IC_{50}= 259 µg/mL).

3.1.5. Mean comparison of antimicrobial activities and MIC.

Mean values of antimicrobial activities (inhibitory zone diameter, mm) and MICs (µg/mL) for various solvents and EOs are summarized in Table 4. As demonstrated by the Duncan's test, there were significant variations ($p < 0.05$) among the investigated parameters for various solvents and tested microbial strains. The best scores of inhibition zone diameter were achieved with methanol for all tested strains regarding antimicrobial activities. At the same time, the lowest values of this parameter were recorded in the case of n-hexane. Extracts from ethanol and ethyl acetate presented medium values between methanol and n-hexane. Concerning microbial strains, *C. albicans* presented the greatest values of inhibition zone diameter but only in methanol and ethanol (33.67 and 30 mm, respectively), while *S. aureus* (a Gram-positive bacterium) presented the best records of this parameter with ethyl acetate (27.33 mm) and n-hexane (21.50 mm). *E. coli* (a Gram-negative bacterium) was found to display the smallest inhibition zone diameter records under various solvents.

Table 4. Mean values of antimicrobial activities measured as inhibition zone diameter (IZD, mm) and minimal inhibitory concentration (MIC, in µg/mL) of various extracts (methanolic, ethanolic, ethyl acetate, and hexane) obtained by Soxhlet extraction and essential oil from *C. mixtus* against four microbial strains (*E. coli, S. aureus, B. subtilis,* and *C. albicans)*. Values are averaged for three replicates with standard deviation. Within the same column and for the same parameter, values followed by the same letter are not significantly different at 5% as a probability level.

	E. coli	*S. aureus*	*B. subtilis*	*C. albicans*
IZD (in mm)				
Methanol	27.33 ± 0.76^a	32.33 ± 1.04^a	30.17 ± 0.76^a	33.67 ± 1.52^a
Ethanol	23.50 ± 2.29^b	29.83 ± 2.28^b	26.50 ± 1.32^b	$30.00 \pm 1.80_b$
Ethyl Acetate	20.00 ± 1.32^c	27.33 ± 1.04^c	20.83 ± 1.29^c	26.00 ± 2.18^c
n-Hexane	13.50 ± 1.32^d	21.50 ± 1.80^d	17.67 ± 2.08^d	17.17 ± 1.04^d
MIC (in µg/mL)				
Methanol	15.83 ± 0.29^d	11.50 ± 0.50^d	12.33 ± 0.76^c	11.17 ± 1.04^{de}
Ethanol	17.00 ± 0.50^c	12.33 ± 0.29^c	13.00 ± 0.50^c	12.67 ± 0.29^c
Ethyl acetate	18.00 ± 0.50^b	15.17 ± 0.29^b	15.50 ± 0.50^b	14.83 ± 0.77^b
n-Hexane	20.83 ± 0.76^a	17.17 ± 0.29^a	17.50 ± 0.50^a	16.00 ± 0.50^a
Essential oil	13.83 ± 1.26^e	10.50 ± 0.50^e	10.17 ± 0.29^d	10.67 ± 1.04^e

Concerning MICs (Table 4), extracts from various sovents as well as EO showed a wide range of variability. Also, MIC's greatest records were found in n-hexane extracts followed by ethyl acetate, ethanol, and methanol for all studied microorganisms. As compared to the four solvents used, EO showed the smallest records of MIC against all bacterial strains. It is worthy of highlighting that among microbial strains, *E. coli* presented the best scores of MIC (lower antimicrobial activity) for EO (13.83 mm) as well as methanol (15.83 mm), ethanol (17 mm), ethyl acetate (18 mm), and n-hexane (20.83 mm). In contrast, *B. subtilis and S. aureus* had the lowest MIC (higher antimicrobial activity) from EO (10.17 mm) and ethanol (12.33 mm), respectively. Moreover, *C. albicans* was found to present the smallest values of MIC for extracts from methanol (11.17 mm), ethyl acetate (14.83 mm), and n-hexane (16 mm).

3.1.6. Principal component analysis (PCA).

PCA was used as a multivariate statistical method to discriminate among solvents used for extraction and extractions techniques based on various dependant variables (yield, TPC, TFC, MIC, and antimicrobial activities). The two first principal components (PCs) were retained because they explained that over 99% of the total variability.
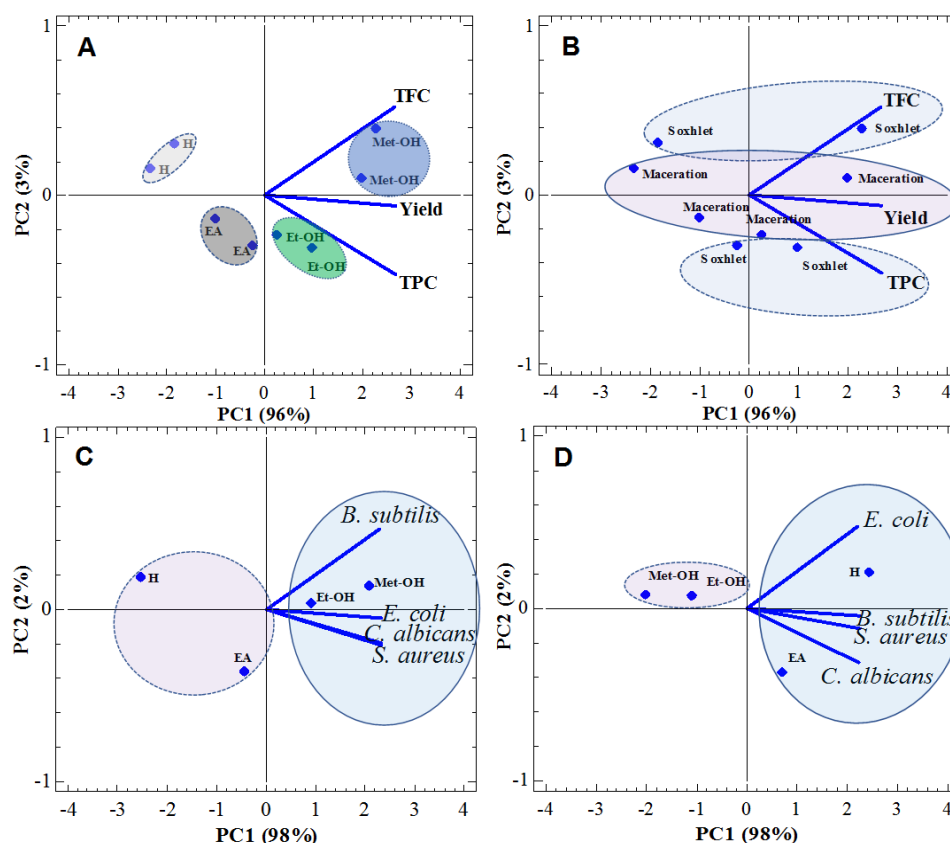
Figure 3. Principal component analysis (PCA) projections on PC1 and PC2. Eigenvalues are symbolized as blue segments representing parameters that most affect each principal component. Plotted points are mean values of each studied parameter of various extracts (methanolic, ethanolic, ethyl acetate, and n-hexane) obtained from aerial parts (dried flowers) of *C. mixtus*. H = n-hexane, Met-OH = Methanol, Et-OH = Ethanol, EA = Ethyl Acetate, TPC = total phenolics content, TFC = total flavonoids content.

Points plotted on the surface determined by axis 1, and 2 (Fig. 3A) are related to solvents, which seem to be distributed along with the first component (PC1). Toward the positive side of PC1, Me-OH and Et-OH interacted with higher yield scores, TPC, and TFC. In contrast, both n-hexane (H) and ethyl acetate (EA) were projected on the negative side of PC1 with low records of these parameters. Similarly, points plotted on the plan determined by axis 1 and 2 were linked to extraction techniques (Fig. 3B). It seems that both techniques were distributed along with the second component (PC2), Soxhlet interacted with both TPC and TFC on the negative and positive sides of PC2, respectively.

Fig. 3C presents the distribution of solvents (independent variables) versus antimicrobial activities as dependant variables on the surface determined by PC1 and PC2. From these outcomes, solvents were distributed according to PC1 (98%). Both Me-OH and Et-OH were associated with higher records of antimicrobial activities for all tested strains. In contrast, H and EA together were associated with negatives values of PC2 and antimicrobial activities. Finally, Fig. 3D summarizes interactions between solvents as independent variables on one hand and MIC from all tested strains as dependant variables on the other hand. Solvents seemed to be plotted following the first component PC1, with over 98% of the total variability. Both extracts from H and EA were linked with higher MIC records on the positive side of PC1. On the contrary, extracts from Me-OH and Et-OH interacted, on the negative values of PC2, with smaller MIC values.

3.2. Discussion.

In this work, we reported EO chemical profiling, antioxidants, and antimicrobial activities of flower extracts of Moroccan chamomile (*C. mixtus)*. It is well documented that chemical composition depends upon several factors like environmental conditions under which plants are grown, genetic differences, the plant part used for EOs isolation, among others [21]. In addition, other parameters, such as harvest season and extraction methods, widely affect the extraction yield of the EOs/extracts, their composition, and, therefore, their bioactivity [37].

A literature review shows that EO yield varies widely within the same plant species depending upon several factors such as plant parts used for extraction, phenological stage, the geographical area under which plants are grown, harvest season, extraction techniques, and conditions like temperature, duration, among others [38,39]. These factors impact not only EO yield but also the chemical composition. In our results, EO yield was found to be 0.79 ± 0.06 (Microwave) and $0.53 \pm 0.09\%$ (Clevenger). Solvent-free microwave extraction is based on the combination of low microwave heating and distillation that is performed at atmospheric pressure. The advantages of this method over hydrodistillation include rapidity in attaining the extraction temperature of 100°C for the first EO droplet, the high yield of EO, the lower energy requirements, and the high purity of the oil extracted [26]. EO yield values, found in our results, were in agreement with yield reports for *C. mixtus* collected from several areas in Morocco and abroad [21,40–43]. Elouaddari *et al*. (2015) [16] investigated *C. mixtus* EO from leaves and flowers. They found that EO is mainly concentrated in flowers. As recently reviewed by Elouaddari *et al*. (2019a) [21], *C. mixtus* EO yields vary in the range 0.06–1.56%.

As for yield, EO chemical composition varies widely qualitatively and quantitively. It is well established that EOs are complex mixtures; the chemical composition within the same species vary widely. A total of 264 components were identified, in the literature, in *C. mixtus* EO as outlined in Elouaddari et al. (2019b) [24], with the predominance of the oxygenated monoterpenes. As highlighted in the results section, we identified and quantified 44 compounds. The five major components were the following: Santolina alcohol was the major component (40.7% of the chemical composition), followed by Germacrene D (8.9%), α-Pinene (5.7%), Artemisia alcohol (4.3%), (E)-β-Farnesene (4%). Several authors reported similar results regarding the major component of EO isolated from *C. mixtus* flowers. Indeed, Zrira *et al*. (2007) [40] found that santolina alcohol as the major component EO isolated from various plant parts (whole plant, leaves, flowers, and stems) collected from western Morocco, with flowers having the score (40.2%). Likewise, Toulemonde and Beauverd (1984) [44], Lis-Balchin *et al*. (1998) [45], Satrani *et al*. (2007) [46], and Darriet *et al*. (2012) [41] found similar chemical composition to our findings with the dominance of santolina alcohol. On the contrary, other authors found that this alcohol is present in smaller quantities or even absent [20,21,42,43]. *C. mixtus'* EO from eastern Morocco (Oujda) was found to have trans-β-farnesene as the main component (35.5–50.5%) as outlined in Elouaddari *et al*. (2013) [42] and Elouaddari *et al*. (2014) [43]. 2-Methyl-2- trans-butenyl methacrylate (32.0–35.2%) was the main constituent for samples collected in western Morocco (Bouznika) as demonstrated by several reports [21,42,43]. Elouaddari *et al*. (2013) [42], while investigating EO' composition from various areas in western Morocco, they found that camphor (14.4–29%) was the main constituent together with trans-β-farnesene (8.3%), and 2-tridecanone (21.5%). It is worthy to note that the occurrence of some oxygenated monoterpenes (santolina alcohol, Artemisia alcohol, and yomogi alcohol) were also described in similar amounts in other species belonging

to Asteraceae family such as *Achillea ageratum* [47]. These differences, within the same species, highlighted in the EO composition of *C. mixtus* flowers, could be attributed to several factors such as pedo-climatic differences, plant phenology, extraction technique, and conditions.

As presented in the results section, significant differences were found among solvents and between extraction methods regarding extract yields, TPC, and TFC. These outcomes were consistent with published literature for chamomiles and other species [48–51]. Extracts from Me-OH and Et-OH performed better than n-hexane and ethyl acetate. However, ethanol is preferred to extract bioactive compounds, mainly due to its lower toxicity [52]. Similar trends were outlined in several reports on Chamomiles and other species [33,34,49,50]. As demonstrated by previous similar works, Me-OH and Et-OH are the better solvents in extracting phenolic compounds from chamomile extracts thanks to their higher polarity and good solubility of phenolic components contained in plant materials as demonstrated by Roby *et al*. (2013) [49]. Likewise, in a study involving thirteen chemotypes belonging to German chamomile (*Matricaria recutita* L.) collected in South-central Italy, Formisano *et al*. (2015) [50] found similar values of yield, TPC, and TFC for methanolic extracts achieved maceration.

Moreover, as compared to Soxhlet extraction, maceration allowed lower records of yield extracts, TPC, and TFC in agreement with other works [53,54]. In this context, as explained in Markom *et al*. (2007) [55], Soxhlet extraction carried out at solvents' boiling temperature, the viscosity and surface tension are lower than those at lower temperatures (maceration). Therefore, the solvent can reach, far more efficiently, the active sites within the solid matrix, thus promoting better solubilization resulting in a higher yield and phenolics content when compared to maceration. However, extraction via maceration has several advantages over Soxhlet. One of the most important advantages of maceration is conserve thermolabile compounds' integrity thanks to the low extraction temperature and saving time, material, and the constituents' diversity [56].

Concerning antimicrobial activities, wide variations in terms of inhibition zone diameter and MIC were highlighted among microbial strains on one hand and various extracts on the other hand. Mean values reported in our work were comparable to those found in the literature for *C. mixtus* collected in Morocco and abroad [24,41,42]. As highlighted in the results' section, the most important records of inhibition zone diameter were records in yeast strain (*C. albicans*), followed by Gram-positive bacteria (*B. subtilis* and *S. aureus*), and the Gram-negative one (*E. coli*) especially for extracts from Me-OH and Et-OH. Such findings agreed with other published works [24,57], who found that yeast strains show their superiority over both kinds of bacteria (Gram-positive and Gram-negative) for both EOs and methanolic extracts.

Most works carried out regarding the antimicrobial activities of EOs have demonstrated Gram-positive bacteria to be more sensitive than Gram-negative bacteria. This sensitivity difference is due to the presence of an outer membrane containing hydrophilic lipopolysaccharides encompassing the bacterial peptidoglycan layer in Gram-negative bacteria, which acts as a barrier towards macromolecules as well as hydrophobic compounds and limits the diffusion of hydrophobic compounds into the bacterium cytoplasm [58,59]. As for microbial strains, significant differences in antimicrobial activities were outlined among solvents with the superiority of Me-OH and Et-OH. Following our results, Elshobary *et al*. (2020) [60] found that low polarity solvents such as hexane show lower activities mainly due to the difference in the solubility of the bioactive compound [61].

Results of MICs confirmed the effectiveness of various extracts and EO against studied microbial strains. For solvents, lower MIC values were found in Met-OH and Et-OH extracts, suggesting that their biochemical content has strong antimicrobial activities since they can inhibit microbial growth even at low concentrations. Effects of various solvents (with different polarities) and different extraction techniques on the chemical composition and biological activities have been investigated in German chamomile extracts, showing that these parameters can deeply modify EO and extract composition and MIC against several pathogen strains [62,63]. These works' outcomes demonstrated that higher polarity solvents are more effective in extracting bioactive compounds and, therefore, showing the ability to inhibit microbial growth at lower concentrations (lower MIC). A similar trend was observed for IC_{50}; compared to n-hexane and ethyl acetate, extracts from Met-OH and Et-OH reduced 50% of DPPH at lesser concentrations. According to Roby *et al*. (2013) [49], IC_{50} is the moles of phenolic compounds divided by moles of DPPH necessary to reduce by 50% the absorbance of DPPH. The lower the IC_{50}, the higher is the antioxidant activity. The difference in antioxidant activities among solvents observed in our results were consistent with Yazdani *et al*. (2019) [64].

Results regarding the difference between solvents and extractions techniques were graphically elucidated using PCA. This multivariate statistical approach is widely used as a discriminative tool in many fields such as chemometric and agronomy [65–67]. In our previous works, PCA was successfully used to separate environments and genotypes based on fruit quality traits, including phenolics content [68–71]. As stated in the results' section, the first component (PC1 = 96%, Fig. 3A), separated solvents confirming, thus, the ANOVA outcomes that solvent was the main variability source. A similar trend was observed in the case of antimicrobial activity, and MIC (Fig. 3C and D), that variability in these data was attributed, to a large extent, to solvents PC1 (98%) followed by microbial strains (PC2 = 2%).

The observed biological activities could be ascribed to the main constituents such as santolina alcohol, germacrene D, and α-pinene, as suggested in several reports [20,47,72,73]. Furthermore, synergistic effects of other minor constituents cannot be disregarded, as discussed in El Abdouni Khayari *et al*. (2016) [47].

The outcomes of the present work demonstrated that EO and various extracts from *C. mixtus* display good antioxidant and antimicrobial activities. The production of such EO and bioactive components from *C. mixtus* and their use as potential natural preservatives could be of important economic value. However, supplementary investigations involving more detailed in vitro and *in vivo* assays seem to be needed to determine which components of the EO or extracts give the best biological activities. Overall, this paper presents valuable information regarding the composition and antimicrobial activity of *C. mixtus* EO and their various extracts from northern Morocco. Besides its traditional uses, *C. mixtus* might be explored for eventual applications in functional foods and nutraceuticals.

4. Conclusions

Better records of yield, TPC, TFC, and associated biological activities were achieved with Me-OH (higher polarity), especially when combined with Soxhlet as an extraction technique. These outcomes were better elucidated by PCA, which allowed clear discrimination of solvents and extraction techniques mainly through the first two components accounting for over 98% of the total data variability. EO and various extracts from *C. mixtus* flowers showed potential antimicrobial and antioxidant activities linked to an essential biochemical richness. The three main compounds (> 5%) found in *C. mixtus* EO were santolina alcohol (40.7%),

followed by germacrene D (8.9%), and α-pinene (5.7%). Wide variations in *C. mixtus* EO were found in peer-reviewed literature depending on several factors (geographical area, phenology, techniques used, among others). Therefore, EO producers must be aware of these variations. Results of our study suggested the exploitation of *C. mixtus* flower EO and extracts as natural preservatives to enhance the shelf life of herbal formulations as well as nutraceuticals.

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Conflicts of Interest

The authors declare no conflict of interest.

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