CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journalhomepage:**http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

DETERMINATION OF ESSENTIAL OIL COMPOSITION, PHENOLIC CONTENT, AND ANTIOXIDANT, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF MARIGOLD (*CALENDULA OFFICINALIS* L.) CULTIVATED IN ALGERIA

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https://doi.org/10.34302/crpjfst/2019.11.2.8

Article history:
Received:
30 December 2018
Accepted:
10 April 2019

Keywords:
Calendula officinalis L.;
Essential oil;
Phenolic compounds;
Antioxidant activity;
Biological activity.

ABSTRACT

Microwave-assisted hydrodistillation (MAHD) was used as an ecofriendly method to extract the essential oils from flowers and leaves of *Calendula officinalis* L. cultivated in Algeria. The results obtained were compared with the conventional extraction method, hydrodistillation (HD), and analyzed by gas chromatography-flame ionization detector (GC-FID) and GC-MS. For flowers oils, 33 compounds were identified with HD method *vs* 20 compounds with MAHD method. For leaves, 26 compounds were identified with HD method *vs* 19 compounds identified with MAHD method. It is interesting to note, furthermore, that the use of MAHD method during 90 min allowed us to obtain relatively similar yields than HD method during 180 min. The main abundant volatile constituent was *α*-cadinol with 31.9±0.71% for HD *vs* 39.7±0.26% for MAHD in leaves oils and 32.3±0.26% for HD *vs* 37.1±0.30% for MAHD in flowers oils. The oxygenated sesquiterpens was the most represented group of natural compounds contributing to the chemical composition in all oils. In the other hand, extraction of total phenolic compounds (TPC) and total flavonoids (TFC) was affected by the solvent type and, thus, 100% methanol was the better extraction solvent for both leaves and flowers. Highest levels were obtained from leaves. The highest antioxidant activity was recorded for leaves extract with 100% methanol. These values indicated a weak antioxidant activity compared to antioxidant standards. A correlation was established between the phenolic and flavonoids contents and the antioxidant activity of the crude extracts. A moderate to great antibacterial activity was observed against $Gram^+$ bacteria. Any antibacterial activity was detected against fungi strains and $Gram^-$ bacteria.

1.Introduction

Pot marigold (*Calendula officinalis* L.) is an annual or biennial plant belonging to the Asteraceae family (Rotblatt 2000).

Native from Europe, Southern Africa, Western Asia and USA, *Calendula officinalis* L. is cultivated in temperate regions all over the world for their ornamental and medicinal

purposes. The leaves are also used in paint coating , cosmetic and nylon industries (Muuse *et al.,* 1992). In folk therapy, this species have been considered as values remedies against gastrointestinal ulcers, dysmenorrhea, fevers and conjunctivitis diseases (Lim 2014). It is mostly known for their high wound-healing properties (Nicolaus *et al.,* 2017). In fact, it has been reported that the preparation of leaves applied in the form of compresses (Dei Cas *et al.,* 2015) , the oil and ointments from flowers (Jarić *et al.,* 2018) and the tinctures or infusions made with aerial parts were employed to relieve ,wounds , bruises, minor burns and skin damaged (Arora *et al.,* 2013). Furthermore, many pharmacological investigations have demonstrated the biological effects of *Calendula officinalis* L. including anti-microbial (Gazim *et al.,* 2008b) (Efstratiou *et al.,* 2012) (Chebouti *et al.,* 2014) (Shankar *et al.,* 2017), anti-inflammatory (Ukiya *et al.,* 2006, Amoian *et al.,* 2010), hypoglycemic (Yoshikawa *et al.,* 2001) antioxydant (Ćetković *et al.,* 2004) ,anti-leishmanial (Nikmehr *et al.,* 2014) and genotoxic effects (Bakkali *et al.,* 2005).

Over the past decades, the essentials oils from medicinal plants have been isolated mainly by using hydrodistillation, steam distillation, maceration or expression (Ferhat *et al.,* 2007). However, these conventional methods present some disadvantages such as the loss or the degradation of some thermo-labile components due to the long extraction time and elevated temperatures (Luque de Castro *et al.,* 1999). For these reasons, the various novel extraction techniques including hydrodiffusion , supercritical fluid extraction, ultrasound-assisted extraction and pressurized solvent extraction have been developed and investigated in order to decrease considerably the processing time, increase the extraction yield and enhance the quality of the extracts (Chan *et al.,* 2011) (J. Mason *et al.,* 2011). Among them, microwave assisted extraction in combination with hydrodistillation (MAHD) has been recognized as efficient alternative for isolation a complex mixture of bioactive compounds from plant sources (Farhat *et al.,* 2017; Thach *et al.,* 2013).

Concerning *Calendula officinalis* L., numerous authors have been reported that the chemical composition of essential oil have been conducted in which volatile constituents were extracted by hydrodistillation(HD) (Chalchat *et al.,* 1991), headspace-solid phase microextraction (HS-SPME) (Gazim *et al.,* 2008a) , and supercritical CO_2 (Crabas *et al.,* 2003). However, according to our knowledge, no data are available about the essential oils from *C. officinalis* L. cultivated in Algeria.

Driven by this goal, we reported in this present study, for the first time, the chemical composition of the essential oil of leaves and flowers from *Calendula officinalis* L. extracted by microwave-assisted hydrodistillation. This extraction method was compared to hydrodistillation (as the reference method), in terms of extraction yields, extraction time and aromatic composition of the essential oils. On another hand, we focused our study to evaluate the influence of the extraction solvent type (methanol, ethanol absolute and ethyl acetate) on the phenolic and flavonoid contents and on the antioxidant and antimicrobial activities.

2.Materials and methods

2.1.Reagents

6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) ; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) , acide 2'2, azino-bis-(3-éthylebenzothiazoline)-6-sulfonique ($ABTS^{\cdot+}$), Folin-Ciocalteu reagent, aluminium trichloride ($AlCl_3$) , carbonate sodium (Na_2CO_3), Butylhydroxytoluene (BHT) , galic acid ,quercetine , persulfate ($K_2S_2O_8$) , extra pure sodium carbonate decahydrated (Na_2CO_3) were purchased from Sigma-Aldrich (Steinheim, Germany). Mueller-Hinton (MH) and Sabouraud dextrose agar (SDA) were obtained from Merck (Darmstadt, Germany).

All the solvents were of analytical grade. Alkane standards solutions (C_7-C_{27}) were obtained from Fluka-chemika (Buchs, Switzerland).

2.2.Vegetal material

The aerials parts of *Calendula officinalis* L. were cultivated and harvested from Birtouta area (36°38′59″ North, 2°59′56″ East) during mars 2017 at the flowering period. The authentication of the species was conducted in the department of Biology, University of Sciences and Technology Houari Boumediene, USTHB, Algiers.

2.3.Hydro-distillation apparatus

The vegetal material (400 g) was subjected to hydrodistillation with Clevenger- apparatus during180 min. The essential oils extracted from flowers and leaves were collected and stored in amber vials at 4° C prior to analysis. The extraction time was selected following the previous

2.4.Microwave-assisted hydrodistillation (MAHD) procedure

MAHD was performed at atmospheric pressure using a household microwave oven (MW8123ST, Samsung, United Kingdom) connected with Clevenger-apparatus with a maximum delivered power of 1550W in 100-W increments. The vegetal material (400 g) was immerged in a 2 L flask containing distillate water (1 L) and heating using a fixed power of 588W during 90 min. The flask was introduced in the oven cavity of microwave, and the Clevenger apparatus was used on the top, outside the oven, to collect the volatile extracts which were stored at 4° C until being analyzed. The experiments were conducted in triplicate under the same conditions and the mean value was reported.

2.5.General chromatographic conditions

The constituents of the oils were analyzed using Hewlett-Packard 6890 series Gas Chromatographic (GC) system (Agilent Technologies, Palo Alto, CA, USA) equipped with flame ionization detector (FID) set at 280°C. The separation was achieved using HP-5MS apolar capillary column (30 m × 250 µm× 0.25 µm film thickness). This column consists of 95 % dimethylsilicone with 5 % phenyl groups. The column temperature was initially programmed at 60° C for 8 min and increased to 250° C at 2° C/min, then finally held isothermally for 20 min. The carrier gas was Helium at a flow rate of 0.5 $mL.min^{-1}$ in split mode with an injection volume of 1 µL.

Gas chromatography-Mass spectrometry (GC-MS) analyses were performed using Hewlett-Packard 6890series GC system coupled with a mass spectrophotometer MSD 5973C and equipped with the same apolar capillary column .Temperature programming is the same as that used in the analysis by GC-FID. Helium was carrier gas with a flow rate 1.5 mL/min. The injected volume was 0.2 µL and the split ratio was 1:50. Injector temperature was 250° C. The injected MS conditions were: MS source temperature: 230° C and MS quadrapole temperature: 150° C, the ionization mode used was electronic impact at 70 eV over a scan range of 29–550 atomic mass units.

In order to calculate the retention indices , the homologous *n*-alkanes C_7-C_{27} was injected in the same conditions as the essential oils in GC-FID and GC-MS. The identification of volatile components was established by comparing their GC Kovats retention indices (KI) with those available in the literature and by matching their recorder mass spectral fragmentations patterns with those stored in the Wiley 9 and NIST 7N mass spectral library and others published index data (Adams 2007). Relative percentage amounts of each component are based on the peak areas obtained with GC-FID.

2.6.Analyzes of phenolic compounds

2.6.1 Extraction of phenolic compounds

Powdered air-dried (40 g) flowers or leaves were macerated in solvents (200 mL) with different polarity: 100% methanol, absolute ethanol and 100% ethyl acetate at room temperature for 24 h. The suspension, thus obtained, was passed through the filter paper N°1 (Whatman Ltd., UK) and concentrated under the vacuum on a rotary evaporator (Laborita 4001) at 40° C. The final extracts were stored at +4° C until utilization.

2.6.2.Phenolic and flavonoid contents

The total phenolic contents of the extracts were evaluated using the Folin-Ciocalteu method (Singleton *et al.,* 1999). An aliquot (250 µL) of the extracts, previously dissolved in the ethanol was added at Folin-Ciocalteu solution (1250 µL). After 3 min of reaction, a solution of sodium carbonate (1000 µl) at a concentration of 75 $g.L^{-1}$ was added and the mixture was shaken a few seconds on the Vortex. The absorbance measurements were determined spectrophotometrically at 765 nm. Gallic acid was used as a standard. Total phenolic content was expressed as milligrams gallic acid equivalent (GAE) per gram extract of dry mass (mg $GAE.g^{-1}$ DM).

The trichloride aluminum method was used to quantify the total flavonoids contents of the samples(Menaceur *et al.,* 2013) . To do this, an aliquot (1000 µL) of the extract dissolved in the ethanol was mixed with of solution of trichloride aluminum $AlCl_3$ (1000 µL, 2% w/v).The absorbance was measured at 420 nm after 1 h of incubation at room temperature. The quercetin was considered as standard for the calibration curve. The flavonoids contents were expressed as milligrams of quercetin equivalent (QE) per gram extract of dry mass (mg $QE.g^{-1}$ DM). The experiments were carried out in triplicate.

2.7.Antioxidant activity

2.7.1.Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging effect

The DDPH free radical scavenging assay was used to evaluate the antioxidant activity of the obtained extracts according to the method previously described with minor modifications (Brand-Williams *et al.,* 1995). Briefly, an ethanolic solution (25µL) of each sample at different concentrations (100-200-400-600-800 and1000 $\mu g.mL^{-1}$) were added to 975 µL of DPPH-ethanol solution (60 µM). After 30 min of incubation in the dark at room temperature, the absorbance was recorded at a wave length of 517 nm by using a spectrophotometer OPTIZEN 3220 UV. Butyl hydroxytoluene (BHT) was used as a standard antioxidant.

2.7.2.ABTS free radical scavenging activity

The ABTS test was assessed using the method reported by Ling *et al* (Ling *et al.,* 2009) . The ABTS radical cation was produced by reacting 7 mM ABTS stock solution and 2.45 mM $K_2S_2O_8$. The obtained mixture was kept in the dark at room temperature during 12-16 h prior use. The ABTS solution was diluted with ethanol in order to have a maximum absorbance of 0.703± 0.025 at 734 nm. An aliquot of 20 µL of the samples at various concentrations was added at 980 µL of diluted ABTS solution. The decrease of absorbance measurement was monitored at 734 nm in the 6th min after adding the sample to the ABTS solution. The synthetic vitamin E (Trolox) was used as an antioxidant standard.

For both tests, the radical scavenging activity was calculated according to the following equation: % of radical scavenging activity = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorption values of the blank sample and Abs_{sample} is the absorbance of the tested sample. The results were expressed as IC_{50} corresponding to the efficient concentration of the sample required to inhibit 50% of the free radical. The

experiments were carried out in triplicate and the IC_{50} values were reported as means ±SD.

2.8.Determination of antimicrobial activity

The antibacterial activity of the crude extracts was evaluated following the paper disk diffusion method described by Bauer *et al* (Bauer *et al.,* 1966) with slight modifications. For the experiments, the samples were tested against three $Gram^+$ bacteria: *Bacillus subtilis* ATCC6633, *Listeria monocytogenese, Staphylococcus aureus* MRSA 639c and two $Gram^-$ bacteria: *Escherichia coli* ATCC43300 and *Pseudomonas aeruginosa* ATCC 9027. Two pathogenic fungal strains: *Aspergillus carbonarius* M333 and *Umbulopsis ramanniana* NRRL1829 were also investigated. The bacterial and fungal strains were cultured in Mueller-Hinton (MH) at 37° C and Sabouraud dextrose agar (SDA) at 30° C respectively. Amoxicillin (25 µg/disc) was used as the positive control while a disc impregnated with 25 µL of DMSO was considered as the negative control. The appropriate agar mediums (MH or SDA) poured into Petri dishes were seeded with the cultures of microbial inoculum (10^6 CFU/mL) using a sterile cotton swab. Afterwards, the DMSO-extract solutions (25 µL) were added on the filter discs which were placed in the surface of Petri dishes. After staying at 4° C in the refrigerator during 1 h for diffusion, the Petri dishes were incubated during 24h for the bacteria strains and 48h for the fungi strains. Antibacterial activity was evaluated by measuring the diameter of the inhibition zones.

The extracts that showed antibacterial activity were screened for determination of minimum inhibitory concentration (MIC) by broth microdilution following National Committee for Clinical Laboratory Standards Guidelines with some modifications(Wikler 2009). A serial dilutions ranging between 7.8 to 500 $\mu g.mL^{-1}$ were carried out from an initial solution previously prepared in DMSO with concentration of 1000 $\mu g.mL^{-1}$. Then each solution (0.5 mL) was added to agar medium (5 mL) (MH or SDA). After that, the obtained mixture was poured into the Petri dishes which were inoculated with inocula strains of 10^6 $CFU.mL^{-1}$ and incubated immediately at 37° C during 24H for the bacteria strains and 48H for fungi strains. Amoxicillin and DMSO served as a positive and negative control respectively. The minimum inhibitory concentration (MIC) is defined as the lowest concentration which exhibited no growth. The assays were carried out in triplicate.

2.9.Statistical analysis

The experiments were done in triplicate. The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) with XLSTAT software on Microsoft Excel 2007. The results were expressed as mean ± standard deviation (SD). The Student's t-test at $p<0.05$ were considered statistically significant. Correlation was calculated according Pearson's method.

3.Results and discussions

3.1. Quantitative and qualitative analyzes of essential oils

Essential oils were studied in leaves and flowers.

3.1.1.Effect of the extraction methods on the essential oils yields

The overall yields were 0.045 ± 0.004 % (w/w) for HD *vs* 0.044±0.002% (w/w) for MAHD and 0.025±0.003% (w/w) for HD *vs* 0.023±0.003% (w/w) for MAHD for the essential oils extracting from flowers and leaves respectively (Figure 1). The oils yields obtained in 90 min) using MAHD was relatively similar to that obtained after 180 min for HD method. Moreover, the extraction time required to reach an extraction temperature of 100° C to get the first essential oil droplet in only 5 min compared to 30 min for HD. It should be noted, furthermore, that the obtained

yields are lower than those reported in the survey literature (0.1-0.97%) (Chalchat *et al.*, 1991) (Gazim *et al.*, 2008a) (Khalid and El-Ghorab 2006).

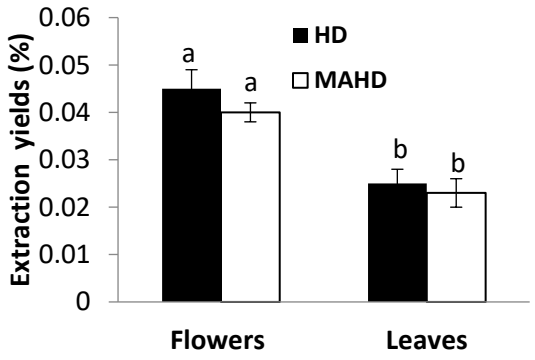

Figure 1. Extraction yields of essential oils from Calendula officinalis L. flowers and leaves with respect to extraction method.

Different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD. Error bars indicate standard deviation (SD).

3.1.2. Effect of the extraction methods on the chemical composition of the essential oils

The identified compound, their relative percentage, the experiments and literature retention indices are compiled in Table 1 and Table 2 for flowers and leaves essential oils respectively using HD and MAHD methods.

The analysis of the essential oils from flowers by GC and GC-MS allowed us to identify 33 compounds and 20 compounds representing 79.3% and 78.4% of the volatile oils constituents extracted by HD and MAHD respectively. The oils extracted were characterized in both methods by the predominance of two chemical families namely oxygenated sesquiterpens and sesquiterpens hydrocarbons. These compounds are responsible for the main biological activities of the vegetal matrice and their amounts depend closely to the used extraction method (Amorati *et al.*, 2013). The oil obtained by HD was little more concentrated in sesquiterpens hydrocarbons compounds compared with MAHD (22.6% HD *vs* 21.1% for MAHD). Oppositly , the oxygenated sesquiterpens compounds were more abundant in the oil extracted by MAHD (57.2%) than HD (53.7%). It was observed , in addition, that the monoterpens hydrocarbons compounds (such as *α*-pinene, *α*-phellandrene and *α*-terpinene) which represent 2.8% of the oils and the oxygenated monoterpens compounds (0.2%) such as (*α*-E)-ionone present with a relatively low percentage with HD method were absent or detected only at traces level using MAHD. The main abundant compounds were *α*-cadinol (37.1±0.26% for HD *vs* 32.3±0.3% for MAHD) followed by epi-*α*-cadinol (16±0.26% for HD *vs* 0% for MAHD) , epi-*α*- Muurolol (0% for HD *vs* 15.1±0.45% for MAHD) , *δ*-cadinene (13.1±0.52% for HD and 13.1±0.51% for MAHD), Torreyol (2.7±0.36% for HD *vs* 0.2±0.01% for MAHD) and Germacrene D (1.4±0.10% for HD *vs* 1.9±0.10% for MAHD).

Concerning the essential oils from leaves, 26 compounds were identified representing 80.1% of the oil extracted using HD while 19 compounds representing 88% were identified in oil extracted using MAHD. The major compounds identified and their respective percentage composition using both methods as follow as: *α*-cadinol (31.9±0.7% for HD *vs* 39.7±0.26% for MAHD), epi-*α*-cadinol (16.3±0.43% for HD *vs* 15.3±0.44% for MAHD), *δ*-cadinene (8.9±0.27% for HD *vs* 18±0.44% for MAHD), Torreyol (3.2±0.35% for HD *vs* 2.1±0.10% for MAHD) and *α*-Humulene (1.9±0.66%for HD *vs* 1.1±0.10% for MAHD). The established comparison between the chemical composition of the essential oils extracted by HD and MAHD methods showed a better abundance of oxygenated monoterpénes.

Table 1. Chemical composition of essential oils extracted by hydrodistillation (HD) and microwave-assisted hydrodistillation (MAHD) from flowers of *Calendula officinalis L.*

No	Compounds	KI[(a)]	KI [(b)]	HD (%)[(c)]	MAHD (%)[(c)]
1	*α*-Pinene	941	939	2±0.11	Tr
2	*α*-Phellandrene	1005	1002	0,1±0.01	-
3	*δ*-3-Carene	1013	1011	0,1±0.02	-
4	*α*-Terpinene	1018	1017	0,1±0.01	Tr
5	*p* –Cymene	1024	1024	0,1±0.01	-
6	*(Z-β)* Ocimene	1040	1037	0,1±0.01	Tr
7	*y* –terpinène	1062	1059	0,3±0.03	-
8	*Cis* Sabinene hydrate	1067	1070	Tr	-
9	*β*-bourbonene	1386	1388	0,1±0.02	0,1±0.01
10	*β* –Cubebene	1390	1388	0,1±0.01	-
11	*α*-gurgunene	1399	1409	0,3±0.02	0,4±0.03
12	*E*-Caryophyllene	1411	1419	1,1±0.10	-
13	*(E-α*) Ionone	1423	1430	0,2±0.01	-
14	*β* –copaene	1429	1432	-	0,1±0.03
15	*Cis* Cadina-1(6),4-diene	1464	1463	0,1±0.02	-
16	*Trans* Cadina-1(6),4-diene	1470	1476	0,2±0.01	0,2±0.05
17	*y* –Muurolene	1481	1469	0,8±0.02	-
18	Germacrene D	1484	1485	1,4±0.10	1,9±0.10
19	*β* –Selinene	1489	1490	0,2±0.02	-
20	*Trans*. Muurola-4(4),5- diene	1493	1493	0,2±0.03	0,3±0.03
21	*α*-Muurolene	1499	1500	2±0.50	-
22	cubebol epi	1502	1494	-	2,7±0.26
23	*y*-Cadinene	1506	1513	2±0.15	4±0.20
24	*δ*-Cadinene	1513	1518	13,1±0.52	13,1±0.51
25	*α*-Cadinene	1527	1530	0,5±0.02	0,8±0.02
26	*α*-calacorene	1545 [(d)]	1545	0,5±0.03	0,1±0.01
27	Murool-5-en-4-*β*-ol-cis	1552	1551	-	0,3±0.02
28	Murool- 5-en-4-*α*-ol-cis	1559	1561	-	0,5±0.07
29	Nerolidol	1560 [(e)]	1563	0,3±0.03	-
30	*β*-calacorne	1563	1565	-	0.1±0.01
31	Palustrol	1568	1568	0,3±0.01	0,3±0.03
32	caryophyllene oxide	1586	1583	-	Tr
33	Cubeban-11-ol	1596	1595	0,5±0.02	-
34	Guiol	1605	1600	0,2±0.02	-
35	*β*-Oplopenone	1609	1607	0,1±0.02	-
36	Cubenol (1,10-di-epi-)	1616	1619	0,4±0.05	0.8±0.02
37	Cubenol 1-epi-	1629	1628	0,9±0.05	-
38	epi *α* –Cadinol	1636	1640	16±0.26	-
39	*epi-α*-Muurolol	1644	1642	-	15,1±0.45
40	*α*-Muurolol (=Torreyol)	1646	1646	2,7±0.36	0,3±0.01
41	*α*-Cadinol	1655	1645	32,3±0.26	37.1 ±0.30
42	*Trans*- calamenen-10-ol	1672	1669	-	0,20±0.01
	Extraction time			180	90
	Yields			0.045±0.02	0.044±0.04
	Monoterpens hydrocarbons(%)			2.8	0
	Oxyenated monoterps (%)			0.2	0
	Oxygenated sesquiterpens (%)			53.7	57.3
	Sesquiterpens hydrocarbons (%)			22.6	21.1
	Total volatile compounds (%)			79.3	78.4

Note: [(a)]Experimental retention index relative to C_7–C_{27} *n*-alkanes on the HP5-MS (apolar capillary column), [(b)] literature retention index. [(c)] Percentage calculated by GC–FID on non-polar HP5—MS capillary column, [(d)] Gazim *et al. (*2008a), [(e)] Okoh *et al. (*2008), tr: traces (<0.1 %), - : absence of compound.

Compounds using HD (0.1%) than MAHD (0%) while a higher amounts of sesquiterpens hydrocarbons compounds were present in the essential oil extracted using MAHD compared to HD with a percentage of 27.4% and 24.6% respectively. Indeed, many volatile compounds including *α*-cadinene (0.9±0.01% for HD *vs* 1.3±0.25% for MAHD) and *α*-muurolene (3.4±0.40% for MAHD and 2.7±0.40% for HD) were present in low amounts with HD method or were absent such as *trans* cadina-1,4-diene (1±0.41% for MAHD *vs* 0% for HD) and *β*-copaene (0.2±0.02% for MAHD *vs* 0% for HD).

As the flowers oils, a greater proportion of the oxygenated sesquiterpens was observed in the leaves oil extracted by MAHD compared to HD (55.4% HD *vs* 60.6% for MAHD). This difference in the essential oil composition is probably due to the high absorption of microwave by these polar compounds in MAHD more than in HD extraction which favorise a more easily extraction of these compounds compared with others class of compounds such as monoterpens hydrocarbons that have lower dipolar moments (Ferhat *et al.,* 2006).

Furthermore, it is interesting to note that the quantities of the target compounds depend of the used extraction methods. According to the data values seen in the Table 2, the oxygenated sesquiterpens compounds of essential oil from leaves such as cubeban-11-ol and cubenol (1,10-di-epi) extracted by MAHD were present with a percentage of 1.2±0.66% and 0.6±0.020% respectively, or in the case of HD , these volatile compounds were present at 0.9±0.040% and 0.5±0.010% respectively. Considering another compounds such as Torreyol and β-oplopenone, the essential oil obtained by HD showed the presence of these compounds with a percentage of 3.2±0.35% and 0.1±0.01% respectively against 2.1±0.10% and 0% respectively in the MAHD extract. In the same way, the study of the chemical composition of essential oils from flowers (Table 1) reveals the presence of epi *α*-cadinol at 16±0.26% and 1-epi cubenol at 0.9±0.05% using MAHD whereas these volatile compounds were absent in the oil obtained by HD. Otherwise, *α*-muurolene and E-caryophyllene present at 2±0.50% and 1.1±0.10% respectively in the HD extracts were absent in the oils extracted by MAHD.

In addition, in both oils, some volatile constituents such as caryophyllene oxide and *α*-cubebene not detected using conventional extraction process were detected only at trace level by MAHD methods.

Regarding the aforementioned data, our finding was in agreement with those available in the literature. Indeed, the main component identified in this investigation in all oils (*α*-cadinol) was similar to that reported by Chalchat *et al* (Chalchat *et al.,* 1991) who studied *C.officinalis* L. from the French Central Massif. It has been reported also that the main components of essential oil from the Egyptian Pot Marigold cultivated under presowing low temperature were *α*-cadinol (up to 64.4%) following by *Δ*-cadinene, *δ*-cadenene and nerolidol (Khalid and El-Ghorab, 2006).

However, although the volatile compounds identified in this study were also mentioned by numerous authors, significant qualitative and quantitative differences were noted. This variation is probably due of the process factors, environmental conditions and/or genetic factors that affect the yield and quality of the essential oils (Duarte *et al.,* 2017). The essential oils from Brazilian Pot Marigold were dominated by sesquiterpens hydrocarbons (68.0 % of total area) and sesquiterpenols (27.0 % of total area) in which *δ*-cadinene (22.5%) and *α*-cadinol (20.4%) were the major constituents (Gazim *et al.,* 2008a). Flowers oils from Bucharest (Romania) obtained by steam distillation was

Table 2. Chemical composition of essential oils extracted by hydrodistillation (HD) and microwave-assisted hydrodistillation (MAHD) from leaves of *Calendula officinalis* L.

No	Compounds	KI[(a)]	KI[(b)]	HD (%)[(c)]	MAHD (%)[(c)]
1	Bornyl acetate	1285[(d)]	1288	0,1±0.01	-
2	*α*-cubebene	1388	1386	-	Tr
3	*β* –bourbounene	1390	1388	0,1±0.02	-
4	*α*-gurjunene	1408	1409	0,3±0.02	0,2±0.03
5	caryophyllene E	1418	1419	1±0.17	0,4±0.040
6	*β*-copaene	1430	1432	-	0,2±0.02
7	*β*-gurjunene	1434	1433	0,1±0.01	0,2±0.04
8	α-humulene	1452	1454	1,9±0.66	1,1±0.10
9	*Cis* cadina-1-(6),4-diene	1463	1469	-	0,4±0.02
10	*γ*-muurolene	1473	1479	1.8±0.20	Tr
11	*Trans.*cadina-1-(6),4-diene	1476	1471	-	1±0.49
12	*β*-selinene	1487	1490	0.4±0.01	-
13	*α*-muurolene	1500	1500	2,7±0.40	3,4±0.40
14	γ-cadinene	1513	1513	4,5±0.26	-
15	*δ*-cadinene	1523	1523	8,9±0.27	18±0.44
16	*Trans* cadina-1,4-diene	1534	1530	-	1±0.40
17	*α*-cadinene	1537	1538	0,9±0.01	1,3±0.25
18	*α*-calacorene	1543	1538	0,6±0.05	-
19	muurol-5-en-4-*β*-ol cis	1550	1551	0,3±0.02	-
20	muurol-5-en-4-*α*-ol cis	1561	1561	0,4±0.04	-
21	*β*-calacorene	1566	1565	0,6±0.02	-
22	Palustrol	1567	1568	0,3±0.02	-
23	cubeban-11-ol	1594	1595	0,9±0.40	1,2±0.66
24	*β*-oplopenone	1609	1607	-	0,5±0.01
25	cubenol(1,10-di-epi)	1616	1619	0,5±0.010	0,6±0.020
26	*α*-corocalene	1623	1623	0,6±0.10	-
27	cubenol 1-epi	1629	1628	1,2±0.10	1,2±0.20
28	epi *α*-cadinol	1641	1644	16,3±0.43	15,3±0.44
29	Torreyol	1646	1642	3,2±0.35	2,1±0.10
30	*α*-cadinol	1648	1654	31,9±0.26	39,7±0.71
31	calamenen-10-one-10-nor	1704	1704	0,3±0.01	-
32	Oplopenone	1740	1740	0,1±0.01	-
	Extraction time			180	90
	Yields			0.025±0.03	0.023±0.02
	Monoterpens hydrocarbons(%)			0	0
	Oxyenated monoterps (%)			0.1	0
	Oxygenated sesquiterpens (%)			55.4	60.6
	Sesquiterpens hydrocarbons (%)			24.6	27.4
	Total volatile compounds (%)			80.1	88

Note: [(a)]Experimental retention index relative to C_7–C_{27} n-alkanes on the HP5-MS (apolar capillary column), [(b)] literature retention index, [(c)]Percentage calculated by GC–FID on non-polar HP5-MS capillary column . [(d)] Gazim et al,. (2008a), tr: traces (<0.1 %), - : absence of compound.

characterized by a appreciable amounts of *α*-muurolene representing 41.5% of total area (Rădulescu *et al.*, 2000) of the plant, the essential oils of *Calendula officinalis* L. from South Africa were characterized by a greater proportion of *α*-cadinol, *α*-cadinene, *T*-muurolol, epi-bicyclosesquiphellandrene , limonene, 1.8 cineole and trans-*β*-ocimene belonging to class of monoterpens (Okoh *et al.*, 2007). The same author mentioned overwise, the effect of drying on the volatile composition of the oils: fresh flowers oil were dominated by *α*-thujene (26.9%), *T*-muurolol (24.9%) and δ-

cadinene (13.1%) as the main compounds. On the other hand, died leaves was dominated by 1,8-cinéole (29.4%), *α*-thujene (17.8%), *β*-pinene (6.9%) and *δ*-cadinene (9.0%) while the fresh leaves was found to be rich in T-muurolol (40.9%), *α*-thujene (19.2%) and *δ*-cadinene (11.4%) (Okoh *et al.*, 2008).

Many volatile constituents identified in this investigation were tested for their anticancer properties. Among of them , *α*-cadinol, showed selective toxicity against human colon adenocarcinoma cells (Sylvestre *et al.*, 2006) (He *et al.*, 1997). Otherwise, it was reported that the essential oils extracted from other medicinal plants containing *α*-pinene, *p*-cymene , *α*-copaene and *δ*-cadinene as volatile components have a potential antimicrobial and antifungal activities (Bel Hadj Salah-Fatnassi *et al.*, 2017).

3.2. Determination of phenolic content and evaluation of antioxidant and antibiological activities of samples extracts

3.2.1.Effect of solvent on total phenolic content (TPC) and total flavonoid content (TFC)

Three solvent systems were used (methanol, ethanol and ethyl acetate) for phenolic and flavonoid extractions from leaves or flowers of *Calendula officinalis* L. In the case of leaves, the extract obtained by 100 % methanol showed the highest total phenolic content, TPC (81.4 mg GAE.g^{-1} DM) and the highest total flavonoids content, TFC (24.11 mg QE.g^{-1}). Values obtained with 100 % ethanol were close but statistically different ($p<0.05$). TPC (46.16 mg GAE.g^{-1} DM) and TFC (19.75 mg QE.g^{-1}) obtained by extraction with 100 % ethyl acetate were lower.

In the case of flowers, the same extraction efficiency was recorded for the three solvents for both TPC and TFC but with low yields except for TPC extracted with ethyl acetate(49.6 *vs* 46.16 mg GAE.g^{-1} DM for leaves) (Figure2).

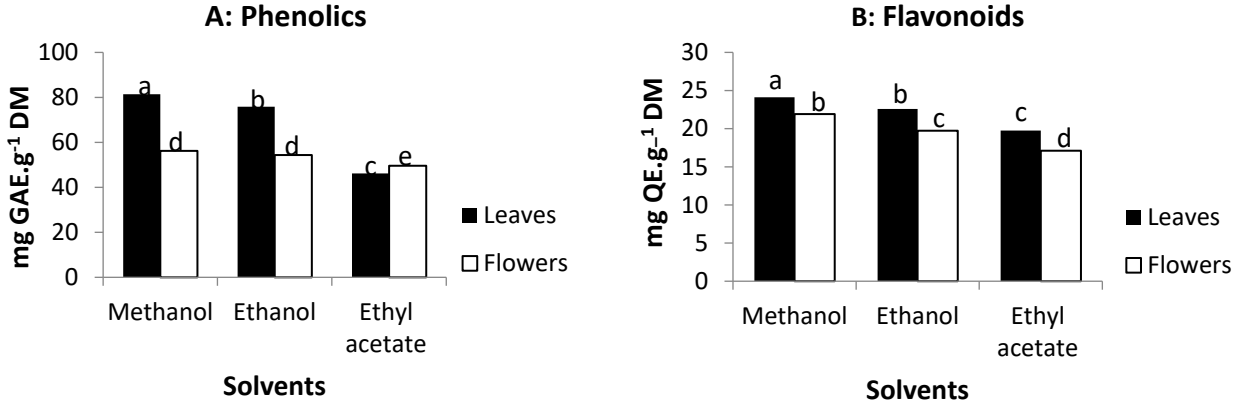

Figure 2. Total phenolics (A) and total flavonoids (B) contents of *Calendula officinalis* L. leaves and flowers according to extraction solvents.

For each graph, different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD. Error bars indicating standard errors (SD) are smaller than the symbol size.

The general results suggest that the values obtained were affected by the type and polarity of extracting solvents. Indeed, this parameter plays a key role in the efficiency of the process because its influence the solubility of the target compounds and the penetrability into the matrix (Rostagno and Prado, 2013). As corroborate by numerous

authors, in our case , methanol was the best extracting solvent due to its higher polarity and good solubility for phenolic component from plants followed by ethanol and ethyl acetate (Belwal *et al.*, 2016; Roby *et al.*, 2013).

3.2.2. Evaluation of antioxidant activity of samples extracts

Various techniques are available to screen the antioxidant activity of vegetal matrices. However, the use of only one method was not efficient to identify all possible mechanisms characterizing an antioxidant. Thus, two complementary in vitro assays namely the DPPH and ABTS free radicals scavenging were selected to evaluate the potential antioxidant activity of methanol , ethanol and ethyl acetate extracts from flowers and leaves of *Calendula officinalis* L.

The obtained results showed that all the investigated samples extracts exhibits the ability to scavenge the DPPH free radical. Indeed, the IC_{50} which corresponding of the required concentration of an extract to inhibit the free radical by 50% present the values ranging between 149.10±1.36 µg.mL^{-1} and 312.86±1.40 µg.mL^{-1} *(*Table 3*)*. The highest DPPH-activity was observed for methanol extract with IC_{50} values of 149.10±1.36 µg.mL^{-1} and 175.21±1.57 µg.mL^{-1} respectively for leaves and flowers and the lowest one for acetate ethyl extract with IC_{50} of 285.52±1.14 µg.mL^{-1} and 312.86±1.40 µg.mL^{-1} respectively for leaves and flowers. Globally, the leaves showed a better antioxidant activity compared with flowers. Regarding the ABTS test, the same results were obtained with the highest IC_{50} values of 146.29±1.17 µg.mL^{-1} for leaves methanol extract and 168.44±1.76 µg.mL^{-1} for flowers methanol extract. Oppositely, the lowest IC_{50} values were 280.91±1.1µg.mL^{-1} and 307.22±1.06 µg.mL^{-1} for ethyl acetate extracts from leaves and flowers respectively. It should be noted, furthermore, that all the tested extracts exhibits a lowest antioxidant activity compared to the positive control BHT(IC_{50}=28.12±0.14 µg.mL^{-1}) and Trolox (IC_{50}=10.14±0.11µg.mL^{-1}) respectively for DPPH and ABTS tests.

Table 3. Effect of phenolic compound extracts of *Calendula officinalis* L. on antioxydant activities.

Samples extracts	IC_{50} DPPH	IC_{50} ABTS
Leaves extracts		
Methanol	149.10±1.36^{a}	146.29±1.17^{b}
Ethanol	187.94±1.23^{c}	184.83±1.62^{d}
Ethyl acetate	285.52±1.14^{e}	280.91±1.1^{f}
Flowers extracts		
Methanol	175.21±1.57^{g}	168.44±1.76^{h}
Ethanol	196.48±1.22^{i}	192.12±1.26^{j}
Ethyl acetate	312,86±1,40^{k}	307,22±1,06^{l}
Standards		
BHT	28.12±0.13	--
Trolox	--	10.41±0.11

-- not applied

Different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD.

It should be noted , that the differences noted between the antioxidant activities potential of these crude extracts maybe attributed at the quality and quantity of the phenolics compounds present in the extracts (Mokrani and Madani, 2016; Decker 1997)

3.2.3. Comparison between DPPH and ABTS methods

The DPPH and ABTS scavenging assays resulted in close values obtained through the two methods for the same organ and the same solvent but those obtained by DPPH method were slightly highest indicating a lower inhibition capacity compared to ABTS. Our observations are in line with those indicated by some authors which report that ABTS inhibition for samples from vegetables, fruits and beverages is higher than DPPH inhibition (Ghouila *et al.*, 2016; Floegel *et al.*, 2011). This is explained by the fact that the DPPH method is characterized by a lower sensitivity. The reaction of DPPH radicals with most active molecules is slower than ABTS radicals (Binsan *et al.*, 2008; Martysiak-Żurowska and Wenta, 2012) By mixing all data obtained for both organs and the three solvents, it resulted a

very high correlation (r=0.9997) between the two methods for determining the antioxidant activity (DPPH and ABTS) (Figure 3). These results suggest that the two methods led to similar antioxidant activity of *Calendula officinalis* extracts. Vamanu and Nita (2013) have reported the same observation for mushroom extract but with a lower correlation.

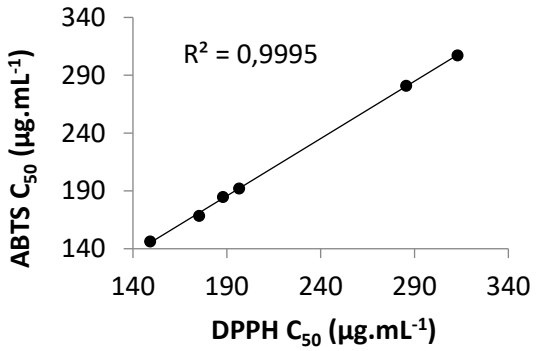

Figure 3. Correlation and regression of DPPH radical scavenging activity versus ABTS radical scavenging activity.
All values are issued from all phenolic compounds extracts (organ and solvent).

3.2.4. Relation between phenolic contents and antioxidant activity

A strong relationship between antioxidant capacity (DPPH and ABTS) and total phenolic or flavonoid contents was found (Table 4). This indicates that quantities of antioxidant molecules which were present in the extracts increased linearly with increasing concentrations. These results indicate also that flavonoids were the major contributors to the antioxidant properties of the studied plant. Correlation between phenolic compounds content and antioxidant activity has been reported by several authors and for many species. (Dudonné *et al.,* 2009; Li *et al.,* 2009; Piluzza and Bullitta 2011)

Table 4. Correlation of total phenolic and flavonoid contents with antioxydant activities (DPPH or ABTS).

	TPC Leaves	TPC Flowers	TFC Leaves	TFC Flowers
DPPH	0.9912	0.9925	0.9973	0.9471
ABTS	0.9909	0.9522	0.9943	0.9522

Values represent coefficient correlation (r) calculated according Pearson's method.

3.2.4. Antibiological activity

The prepared extracts from flowers and leaves of *Calendula officinalis* L. were screened against the selected Gram$^+$ bacteria, Gram$^-$ bacteria and fungi strains using the disc diffusion assay (Table 5). It has been observed that the effectiveness of the samples extracts depends of the tested microorganisms, the type of solvents used and the plant organ. Our finding showed that no antibacterial activity was detected against Gram$^-$ bacteria and fungi strains. The resistance of Gram$^-$ bacteria to inhibitory effect of the tested extracts could be attributed to lipopolysaccharides in their outer membrane, which make them inherently resistant to external agents, such as hydrophilic dyes, antibiotics and detergents (Hayouni *et al.*, 2007).

Otherwise, the tested extracts were globally most efficient against Gram$^+$ bacteria with a diameter of the growth inhibition zone ranging for 8.03±0.15 mm to 5.33±0.41 mm corresponding at moderate activities.

Methanol flowers extracts were efficient while tested against *Bacillus subtilus* ATCC6633 and *Listeria monocytogenes* with a diameter of the growth inhibition zone of 12.37±0.23 mm and 8.13±0.15 mm respectively . The same observation was done for the leaves extracts with an inhibition zone diameter of 10.9±0.78 mm and 12.16±0.12 mm against the same bacteria. However, any antibacterial activity was observed against *Staphylococcus aureus* 693c for both matrices. Ethanol extract from flowers present a moderate antibacterial activity with an inhibition zone diameter of 11.2±0.26 mm and 10.8±0.60 mm while no activity was observed against *Listeria monocytogenes*. In the same

way, tested against the same bacteria, ethanol leaves extracts didn't show any inhibition zone.

Oppositely, a moderate antibacterial activity was recorded against *Bacillus subtilus* ATCC6633 and *Staphylococcus aureus* 693c with an inhibition zone diameter of 10.9±0.78 mm and 12.16±0.12 mm respectively. Concerning ethyl acetate flowers extract, the antibacterial activity was observed only against *Bacillus subtilus* ATCC6633 with an inhibition zone diameter of 10.7±0.35 mm. As for the leaves extracts, an antibacterial activity was recorded against all the tested bacteria with a growth inhibition zone diameter of 8.1 ±0.36 mm, 10.1±0.20 mm and 8.03±0.15 mm for *Bacillus subtilus* ATCC6633, *Listeria monocytogenes* and *Staphylococcus aureus 693c* respectively. It should be noted furthermore, that among all the extracts, leaves methanol extract present the highest growth inhibition zone diameter with 25.33±0.41mm against *Bacillus subtilus* ATCC6633 corresponding at a great antibacterial activity. The amoxicillin exerted the strongest inhibitory effect against the tested microorganisms compared to all extracts. The Gram$^-$ bacteria and the fungi strains were not susceptible to amoxicillin at a concentration of 25 µg per disc.

Table 5. Effect of phenolic compound extracts of *Calendula officinalis* L. on growth inhibition zone diameter sizes (mm).

	Leaves extracts (25 µL)			Flowers extracts (25 µL)			Amoxicillin
	Methanol	Ethanol	Ethyl acetate	Methanol	Ethanol	Ethyl acetate	
Gram$^+$							
B. subtilis	25.33±0.41^a	10.9±0.78^b	8.1±0.36^c	12,37±0.23^d	11.2±0.26^e	10.7±0.35^e	45.13±0.21^f
L. monocytogenese	11.16±0.35^a	--	10.1±0.20^b	8.13±0.15^c	--	--	28.43±0.40^d
S. aureus	--	12.16±0.12^a	8.03±0.15^b	--	10.8±0.60^c	--	34.33±0.49^d
Gram$^-$							
E. coli	--	--	--	--	--	--	--
P. aeroginosa	--	--	--	--	--	--	--
Fungi							
A. *carbonarius*	--	--	--	--	--	--	--
U. *ramaniana*	--	--	--	--	--	--	--

-- no effect

For each line, different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD.

The minimum inhibitory concentration (MIC) was estimated for the extract which showed the interesting antibacterial activity against the Gram$^+$ bacteria. A strong antibacterial activity was presented by a low value of MIC (Coulidiati *et al.*, 2009). Globally, perusals of table 6 showed that the IMC values varied from 7.80 to125 µg.mL^{-1}. The lowest minimal inhibitory concentration corresponding to strongest antimicrobial activity was observed for methanol extract from leaves against the standard strain *Bacillus subtilus* ATCC6633 with IMC of 7.8µg.mL^{-1}. The ethanol extract from flowers and leaves observed a relatively appreciable effectiveness against *Staphylococcus aureus* 693c with IMC values of 31.25µg.mL^{-1} and 15.62 µg.mL^{-1} respectively. Also, the leaves methanol extract present a moderate antibacterial activity against *Listeria monocytogenes* with IMC of 15.62µg.mL^{-1}. For each bacteria strain used, in both vegetal matrice, the lowest antibacterial activity was noted or was not detected for ethyl acetate extract.

This observation could be explained by the quantity of the polyphenols and flavonoids present in the extracts which influence the effectiveness of the extracts against microorganisms (Rodríguez-Vaquero *et al.*, 2013). In fact, the finding literature noted that

the bioactive compounds such as phenolic compounds, flavonoids, tannins and alkaloids are one of the most important antimicrobial agent present in the plant (Levy, 1994). Some of them act by altering the biochemical systems of microorganisms , binging their protein molecules or causing inflammation of the cells in order to inhibits their life process. (Garrod 1995)

Table 6. Results of minimum inhibitory concentration (MIC) in µg.mL^{-1} of crude extracts against microorganisms (Gram^{+} bacteria)

Microorganisms	**Flowers extracts**			**Leaves extracts**			**Amox.**
	Methanol	Ethanol	Ethyl acetate	Methanol	Ethanol	Ethyl acetate	
B. subtilis	62.5	62.5	125	7.8	62.5	125	7.8
L. monocytogenese	125	--	--	15.62	--	125	15.62
S. aureus	--	31.25	--	--	15.62	62.5	15.62

-- Not detected

4. Conclusions

The present study was aimed to investigate, for the first time, the effects of the use of MAHD extraction method on the chemical composition of the essential oils from leaves and flowers of *Calendula officinalis* L. cultivated in Algeria. This modern and green method allow us to obtain relatively similar extraction yields compared with conventional method (HD) while reducing the extraction time and saving substantial energy. In all extracted oils, the oxygenated sesquiterpens was the dominant family and the α-cadinol the main compound, present with different relatives amounts depending on the isolation methods. It showed that the phenolic and flavonoids contents are affected by the solvent type. Thus, in general, methanol was the better extraction solvent for both leaves and flowers of *Calendula officinalis*. On another hand, the potential antioxidant of methanol, ethanol and ethyl acetate extracts from leaves and flowers was conducted by capturing free DPPH and ABTS radicals in comparison with adequate positive controls BHT and Trolox respectively. Our finding observed the influence of the polarity of solvents on the biological activities of the tested extracts. In fact, methanol extract presented the highest antioxidant activity, for both matrices, while the ethyl acetate extract observed the lowest one. These general observations could be correlated with the total amount of phenolics and flavonoids present in the extract.

The antibacterial activity of the extracts against pathogenic microorganisms was evaluated using the disc diffusion and broth microdilution methods. The comparison was done with a standard antibiotic (Amoxicillin). A moderate to great antibacterial activity was observed against Gram^{+} bacteria. The efficiency of this antibacterial effect depends on extraction solvent and plant organ. Any antifungal and antibacterial activity against Gram^{-} bacteria was detected.

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Acknowledgements

The authors gratefully acknowledge Pr. Nabila Amirouche from the Laboratory of Biology and Physiology of Organisms (LBPO), Faculty of Biological Sciences, (USTHB), Algiers, Algeria, for the botanical identification.