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Ethnobotanical study and valorization of bioactive compounds of food interest of some native *Lamiaceae* of western Algeria: case of *Calamintha candidissima* (Munby) Benth. and *Micromeria inodora* (Desf.) Benth.

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ABSTRACT

The purpose of this study was to characterize *Clinopodium candidissimum* (Munby) Kuntze and *Micromeria inodora* (Desf.) Benth. (*Lamiaceae*) natives of western Algeria by the investigation of ethno-medicinal uses by ethnobotanical investigations, phytochemistry by the quantification of minerals, the rate of polyphenols and flavonoids, the composition of essential oils obtained by hydrodistillation by gas chromatography - mass spectrometry (GC-MS), the aromatic profile by solid phase micro-extraction in the headspace (HS-SPME) coupled with GC-MS, also NMR and LC-MS^n and HPLC-DAD-MS^n were performed for polar fractions. The *in vitro* bioactivity of different extracts was evaluated by several tests, namely: antioxidant potential by three methods (DPPH, ABTS and CUPRAC); enzymatic inhibition potential by cholinesterases, α-glucosidase and α-amylase, In addition, our extracts were examined for their possible antimicrobial activity using the method of disk diffusion against several strains. Informants reported no traditional knowledge of *M. inodora*, while for *C. candidissimum*, therapeutic and culinary uses were recorded for traditional uses of Nepeta albida, which is morphologically similar to our plant.

A high level of minerals, particularly iron, manganese, zinc, boron, copper, calcium and potassium has been confirmed. Levels of phenolic compounds and flavonoids in the methanolic extracts were 63.33 (µg GAeq.mg^{-1}Ext) and 11.19 (µg Qeq.mg^{-1}Ext) for *M. inodora*; 58.79 (µg eq AG/mg Ext) and 40.93 (µg eq Q/mg Ext) for *C. candidissimum*. For *M. inodora*, NMR and LC-MS^n analyses revealed the presence of aglyconic and glycosylated flavonoids, phenylpropanoid derivatives and acids triterpenoids. Analysis of the oil identified 66 components, primarily oxygenated mono- and sesquiterpenes (38.2% and 32.0%, respectively). The aromatic profile by HS-SPME-GC-MS revealed 45 compounds, among which oxygenated monoterpenes appeared to be the most abundant (65.8%).

For *C. candidissima*, qualitative analyses by ^{1}H-NMR and qualitative analyses by HPLC-MS^n revealed the presence of aglyconic and glycosylated flavonoids (3.1%), phenyl propanoids (3.6%), and other compounds. On the other hand, HS-SPME-GC-MS identified 38 volatile constituents, among which the oxygenated, pulegone monoterpenes (44.8%) was the most abundant. Analyses of antioxidant activity showed moderate capacity. The potential for enzymatic inhibition indicates a lack of activity. Antimicrobial activity has shown that all strains tested are sensitive to our extracts.

Overall, these data indicate that *C. candidissimum* and *M. inodora* may be a potential source of antimicrobial and bioactive compounds and a starting point for further research on these plant species.

Keywords: *Clinopodium candidissimum*; *Micromeria inodora*; Ethnobotanical survey; HS-SPME, GC-MS; NMR; HPLC-MS^n; Biological activities.

RÉSUMÉ

Cette étude visait à caractériser *Clinopodium candidissimum* (Munby) Kuntze et *Micromeria inodora* (Desf.) Benth. (*Lamiaceae*) natives de l'ouest Algérien par l'investigation des usages ethno-médicinal par des enquêtes ethnobotaniques, la phytochimie par la quantification des minéraux, le taux des polyphénols et des flavonoïdes, la composition des huiles essentielles obtenue par hydrodistillation par chromatographie en phase gazeuse - spectrométrie de masse (GC-MS), le profile aromatique par micro extraction en phase solide dans l'espace de tête (HS-SPME) couplée à la GC-MS, aussi des analyses RMN et LC-MS^n et HPLC-DAD-MS^n ont été realisés pour des fractions polaires. La bio-activité *in vitro* de différentes extraits a été évaluée par plusieurs tests à savoir : le potentiel antioxydant par trois méthodes (DPPH, ABTS et CUPRAC) ; le potentiel d'inhibition enzymatique par la cholinestérases, α-glucosidase et α-amylase, En outre, nos extraits ont été examinés pour leur éventuelle activité antimicrobienne en utilisant la méthode de diffusion de disque contre plusieurs souches. Les informateurs n'ont fait état d'aucune connaissance traditionnelle sur *M. inodora*, tandis que pour *C. candidissimum*, des utilisations thérapeutiques et culinaires ont été enregistrées concernant les utilisations traditionnelles de Nepeta albida, qui est morphologiquement similaire à notre plante.

Un taux élevé de minéraux, en particulier du fer, manganèse, zinc, bore, cuivre, calcium et potassium a été confirmé. Les teneurs en composés phénoliques et en flavonoïdes des extraits méthanoliques sont respectivement de 63,33 (µg GAeq.mg^{-1}Ext) et 11,19 (µg Qeq.mg^{-1}Ext) pour *M. inodora* ; 58,79 (µg eq AG/mg Ext) et 40,93 (µg eq Q/mg Ext) pour *C. candidissimum*. Concernant *M. inodora*, les analyses RMN et LC-MS^n ont révélé la présence de flavonoïdes aglyconiques et glycosylés, de dérivés phénylpropanoïdes et d'acides triterpénoïdes. L'analyse de l'huile a permis d'identifier 66 composants, principalement des mono et sesquiterpènes oxygénés (38,2 % et 32,0 %, respectivement). Le profile aromatique par HS-SPME-GC-MS a révéler 45 composés, parmi lesquels les monoterpènes oxygénés semblaient être les plus abondants (65,8 %).

Pour *C. candidissima*, les analyses qualitatives par ^{1}H-RMN et quali-quantitatives par HPLC-MS^n ont révélé la présence de flavonoïdes aglyconiques et glycosylés (3,1%), de phényl-propanoïdes (3,6%), et d'autres composés. D'autre part, la HS-SPME-GC-MS a permis d'identifier 38 constituants volatils, parmi lesquels les monoterpènes oxygénés, pulegone (44,8%) était le plus abondant. Les analyses de l'activité antioxydante ont révélé des capacités modérées. Le potentiel d'inhibition enzymatique indique une absence d'activités. L'activité antimicrobienne a montré que toutes les souches testées sont sensibles aux nos extraits.

Globalement, ces données indiquent que *C. candidissimum* et *M. inodora* pourraient être une source potentielle de composés antimicrobiens et bioactifs, et elles représentent un point de départ pour des recherches plus approfondies sur ces espèces végétales.

Mots-clés : *Clinopodium candidissimum* ; *Micromeria inodora* ; Enquête ethnobotanique ; HS-SPME, GC-MS ; NMR ; HPLC-MS^n ; Activités biologiques.

ملخص

هدفت هذه الدراسة إلى توصيف *Clinopodium candidissimum* (Munby) Kuntze و *Micromeria inodora* (Desf.) Benth. (*Lamiaceae*) موطنها غرب الجزائر من خلال التحقيق في الاستخدامات الطبية العرقية من خلال دراسة استقصائية عن النباتات العرقية ، والكيمياء النباتية من خلال القياس الكمي للمعادن ، ومعدل البوليفينول والفلافونويد ، وتكوين الزيوت الأساسية التي تم الحصول عليها عن طريق التقطير المائي عن طريق الكروماتوغرافيا في الطور الغازي - قياس الطيف الكتلي (GC-MS) ، تم إجراء التنميط العطري عن طريق الاستخراج الجزئي للطور الصلب في فراغ الرأس (HS-SPME) إلى جانب GC-MS ، وكذلك تم إجراء NMR و $LC\text{-}MS^n$ و HPLC- $DAD\text{-}MS^n$ للكسور القطبية.

تم تقييم النشاط الحيوي في المختبر لمستخلصات مختلفة من خلال عدة اختبارات: إمكانات مضادات الأكسدة بثلاث طرق (DPPH و ABTS و CUPRAC) ؛ إمكانية التثبيط الأنزيمي بواسطة الكولينستراز ، α-glucosidase و α-amylase ، بالإضافة إلى ذلك ، تم فحص مستخلصاتنا من أجل نشاطها المحتمل كمضاد للميكروبات باستخدام طريقة نشر القرص ضد العديد من السلالات. أبلغ المشاركون في الاستقصاء عن عدم وجود معارف إستخدامات تقليدية عن *M. inodora*، بينما تم تسجيل إستخدامات علاجية و في الطهي ل *C. candidissimum* التي تتماثل مع الإستخدامات التقليدية ل Nepeta albida التي تتشابه شكليًا مع نباتنا.

تم تأكيد وجود نسبة عالية من المعادن، وخاصة الحديد والمنغنيز والزنك والبورون والنحاس والكالسيوم والبوتاسيوم. محتويات المركبات الفينولية والفلافونويد في المستخلصات الميثانولية هي على التوالي 63.33 ميكروجرام مكافئ من حمض الجاليك لكل مليغرام من المستخلص و11.19ميكروجرام مكافئ من كيرسيتين لكل مليغرام من المستخلص بالنسبة ل *M. inodora* و 58.79ميكروجرام مكافئ من حمض الجاليك لكل مليغرام من المستخلص و 40.93ميكروجرام مكافئ من كيرسيتين لكل مليغرام من المستخلص ل*C. candidissimum* .

وفيما يتعلق بـ *M. inodora*، كشفت تحليلات NMR و $LC\text{-}MS^n$ عن وجود الفلافونويد aglyconiques و glycosylés ومشتقات phénylpropanoïdes وأحماضtriterpénoïdes. سمح تحليل الزيت الأساسي بتحديد 66 مكونًا، معظمها من الأكسجين الأحادي وsesquiterpènes (38.2٪ و 32.0٪ على التوالي). كشف تحليل HS-SPME-GC-MS عن وجود 45 مركبًا متطايرًا، من بينها monoterpènes oxygénés الأكثر وفرة (65.8٪).

بالنسبة لـ *C. Candissima* ، كشفت التحليلات النوعية ^{1}H-NMR و $HPLC\text{-}MS^n$ للمستخلصات عن وجود مركبات الفلافونويد aglyconic و glycosylated (3.1٪) ، و phenyl-propanoids (3.6٪) ، و مركبات أخرى.

من ناحية أخرى، سمح تحليل HS-SPME- GC-MS بتحديد 38 مكونًا متطايرًا، من بينها monoterpènes oxygénés، كان pulegone الأكثر وفرة (44.8٪).

كشفت تحليلات النشاط المضاد للأكسدة عن قدرات معتدلة على مستويات مختلفة في جميع مستخلصات الميثانوليك. أظهرت إمكانات تثبيط الإنزيم للمستخلصات النباتية: الكولينستريز و α- glucosidase وα- amylase عن عدم وجود أنشطة معتبرة مضادة للكولينستراز ومضادة للسكري لكلا النباتين. أظهر النشاط المضاد للميكروبات أن جميع السلالات التي تم اختبارها حساسة لمستخلصاتنا.

بشكل عام، تشير هذه البيانات إلى أن *C. candidissimum* و *M. inodora* يمكن أن يكونا مصدرًا محتملاً لمضادات الأكسدة ومضادات الميكروبات والمركبات النشطة بيولوجيًا، وهما يمثلان نقطة انطلاق لمزيد من الأبحاث حول هذا النوع من النباتات.

الكلمات المفتاحية: *Clinopodium candidissimum* ؛ *Micromeria inodora* ؛ دراسة استقصائية عن النباتات العرقية ؛ HS-SPME، GC-MS ؛ NMR; $HPLC\text{-}MS^n$ ؛ الأنشطة البيولوجية.

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%	Percent
± SEM	Standard error of mean
°C	Degree Celsius
^{13}C	Carbon-13
^{1}H	Proton
a.s.l.	Above sea level
$A_{0.5}$	Concentration indicating 0.500 absorbance intensity
ABTS	2,2′-Azino bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AChE	Acetylcholinesterase
Al	Aluminum
$AlCl_3$	Aluminum trichloride
AMB	Amphotericin B
AMC	Amoxicillin
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
B. cereus	*Bacillus cereus*
BChE	Butyrylcholinesterase
BHA	Butylated hydroxyl anisole (2-tert-Butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole)
C	Carbon
C. albicans	*Candida albicans*
C. candidissimum	*Clinopodium candidissimum* (Munby) Kuntze
C.	*Clinopodium*
C. nepeta	*Clinopodium nepeta*
CC	Column chromatography
CF	Chloroform fraction
$CHCl_3$	Chloroform
CLSI	Clinical and Laboratory Standards Institute
COSY	Correlation spectroscopy
CRO	Ceftriaxon
Cu^{+}	Cuprous ion
Cu^{2+}	Copper (II) ion
$CuCl_2$	Copper chloride
CUPRAC	Cupric Reducing Antioxidant Capacity
CZ	Cefazolin
DCM	Dichloromethane
DM	Dry matter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl free radicals
DTNB	5,5'-dithiobisnitrobenzoate
DW	Dry weight
E. coli	*Escherichia coli*
EA	Ethyl acetate
ESI	Electrospray Ionisation
$FeCl_3$	Iron chloride
FFAA	Fatty acid

FOX	Cefoxitin
g	Gramme
GAE	Gallic acid equivalent
GC-EI-MS	Gas chromatography coupled with electron ionization mass spectrometry detection
GC-MS	Gas chromatography- mass spectrometry
GPS	Global Positioning System
h	Hour
H_2SO_4	Sulfuric acid
$H_3PMo_{12}O_{40}$	Phosphomolybdic acid
$H_3PW_{12}O_{40}$	Phosphotungstic acid
HCA	Hierarchical cluster analysis
HCl	Hydrogen chloride
HPLC-DAD-MS^n	High Performance Liquid Chromatography-Diiode Array Detector-Mass Spectrometry
HPLC-MS/MS	High performance liquid chromatography tandem mass
HPLC-MS^n	High Performance Liquid Chromatography-Mass Spectrometry
HS-SPME	head-space solid phase micro extraction
Hz	Hertz
IC_{50}	Concentration providing 50 percent inhibition activity
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
IZD	Inhibition zone diameter
KOH	Potassium hydroxide
l.r.i.	Linear retention indices
LC MS^n	Liquid chromatography–mass spectrometry
LOD	Limit of detection
LOQ	Limits of quantification
LRI	Linear retention indices
m	Meter
M	Molar concentration
M.	*Micromeria*
M. inodora	*Micromeria inodora* (Desf.) Benth.
m/z	Mass to charge ratio
MAE	Microwave-assisted extraction
MBC	Minimal Bactericidal Concentration
MeOH	Methanol
MFC	Minimal Fungicidal Concentration
Mg	Magnesium
mg	Milligramme
MH	Mueller-Hinton Agar
mHz	MegaHertz
MIC	Minimum Inhibitory Concentration
Min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
Mo_8O_{23}	Molybdenum
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

N	North
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NC	Neocuproine
ND	Not determined
NH_4Ac	Ammonium acetate
NIST	National Institute of Standards and Technology
nm	Nanometer
NMR	Nuclear magnetic resonance
N°.	Number
OD	Optic density
P. aeruginosa	*Pseudomonas aeruginosa*
PCA	Principal components analysis
PDMS	Poly-dimethylsiloxane
pH	Potential of Hydrogen
PNPG	4-N-nitrophenyl-a-D-glucopyranoside
ppb	Parts per billion
ppm	Part per million
psi	Pounds of force per square inch of area
PTFE	Polytetrafluoroethylene
r.t.	Room temperature
R^2	Coefficient of determination
RF	Resistance Factor
rpm	Rotation per minute
Rt	Retention time
s	Second
S.	*Satureja*
S. aureus	*Staphylococcus aureus*
S. calamintha	*Satureja calamintha*
SD	Standard deviation
SDA	Sabouraud Dextrose Agar
SPME	Supelco Solid Phase Micro-extraction
SSO	Sahara and Sahel Observatory
T.	*Thymus*
TDDS	Turbo detection datascanning
TTC	Triphenyl tetrazolium chlorid
UV	Ultraviolet
V	Volt
v/v	volume per volume
vs.	Versus
W	West
w/w	weight by weight relationship
W_8O_{23}	Tungsten blue oxides
WHO	World health organization
α	Alfa
δ	Chemical shift in ppm
µg GAeq.mg^{-1}Ext	Microgram of Gallic Acid Equivalent per milligram of extract
µg	Microgramme
µg Q_{eq}.mg^{-1}Ext	Microgram of Quercetin Equivalent per milligram of extract

µl	Microliter
µm	Micrometer

INTRODUCTION

INTRODUCTION

Ethnobotanical study is of undeniable interest for the discovery of new plants, which remain an important source of medicines, either because the compounds they contain are valuable active ingredients, either because chemists have been able to change the structure of some of these principles to make them less toxic, more effective, or to give them better bioavailability. The use of substances of plant origin for the prevention or treatment of various diseases in humans has existed for thousands of years. The use spectrum of substances of plant origin concerns a large number of diseases in humans.

In a logic strategy of medicinal plants studies, the valorization is not limited to the level of ethnopharmacological knowledge and plant biology, but also the phytochemical study which is mandatory to identify all necessary information. Nature has been a source of therapeutic agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, many based on their use in traditional medicine **(Murugan and Mohan, 2011)**. Since antiquity medicinal plants are used to relieve and cure the human diseases, their therapeutic properties are due to the presence of hundreds even thousands of natural bioactive compounds called secondary metabolites. Plants have occupied a prominent place and have been used for man a privileged point of contact with nature and health. Popularity of herbal remedies is increasing, they have been tested by our ancestors, of whose virtues confer a significant place in traditional therapy **(Tabuti et *al.*, 2003)**. According to the WHO statistics, about 80% of African populations use traditional medicine for their primary health care **(WHO, 2003)**. In recent years, there has been a remarkable rise of medicinal plants use, probably due to their local abundance, cultural significance and inexpensive procurement **(Thomford et *al.*, 2015)**. Among the scientific disciplines interested in herbal medicine, ethnobotanics are considered as a field focused on the study of the indigenous and native knowledge on how plants are recognized, used and managed.

Algeria, with its large area and diversified climate has a rich heritage of plants used in traditional pharmacopoeia to treat several diseases, which is a source of rich and abundant medicinal matter. On the other hand, Algeria with its history and its strategic location has benefited from different cultures. Important knowledge of plant medicine, currently used in traditional Algerian medicine, originated in the medical heritage of Muslim civilization, transmitted from generation to generation **(Chériti et *al.*, 1995)**. In Oran, the second most important city of Algeria the appeal to the herbalist to cure diseases, which resist to the conventional medicine became recurring in this region. Not only because it happens that healing

plants are sometimes more efficient than the medicine but also and especially because the cost of medicine is very expensive many inhabitants of Oran do not any more manage to keep up with that.

Several work has been done in Algeria on plants of medicinal and food interest which have been evaluated for their richness in secondary metabolites and for their antioxidant, antimicrobial and antidiabetic activities **(Djeridane et *al*., 2006)**. In spite of these numerous studies on plants collected in Algeria, there are still many plants not evaluated in terms of their biological activities the case of two plants *Lamiaceae* native of Western Algeria (Oran region): *Micromeria inodora* (Desf.) Benth and *Clinopodium candidissimum* (Munby) Kuntze.

Numerous phytochemical studies have been carried out on both plants but informations on traditional therapeutic uses remains poorly documented. This study aims to contribute to the valorization of this two plant in order to fill the non-mentioned gaps in terms of ethnobotany. More specifically, the aim is to evaluate the variation in knowledge of the uses of the species according to the sex, age and academic levels of the interviewed natives and to determine the convergence of use of the different parts of the plant between individuals. This study aimed to evaluate the ethnobotanical informations related to the use of *Clinopodium candidissimum (Munby) Kuntze* and *Micromeria inodora* (Desf.) Benth. in Oran region for medicinal purposes. Furthermore, mineral quantifications, total phenolic and flavonoids contents have been assessed for the two studied plants.

In order to promote Algerian medicinal and edible plants also to further contribute to the phytochemistry of this scarcely known species, in this study we aimed at characterising the volatile composition and the non-volatile polar constituents of the studied plants aerial parts, using different extractive and analytical approaches. A headspace solid phase micro-extraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) method was used for the characterisation of *C. candidissimum* volatiles, while integrated NMR and HPLC-DAD-MS^n approaches allowed the identification and quantification of polar constituents.

In addition, we investigate an exhaustive characterization of the aroma profile and the non-volatile constituents of the polar and apolar extracts of *M. inodora* aerial parts by means of different techniques, namely gas chromatography-mass spectrometry (GC–MS) analysis of the hydrodistilled essential oil, head-space solid phase micro-extraction (HS-SPME) coupled with GC–MS for the volatile fraction and an integrated NMR and high-performance liquid chromatography with mass spectrometry detection (HPLC-MS) approach for the characterization of the crude extracts.

Antioxidant potential was assessed for both plants by three methods (DPPH, ABTS and CUPRAC); *in vitro* enzyme inhibition potential of plant extracts cholinesterases (AChE) and (AChB), α-glucosidase and α-amylase assays were investigated. Moreover, the methanolic extracts were screened for their possible antimicrobial activity using disc diffusion method against several strains (*Staphylococcus aureus* (MRSA) ATCC 34300, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231 and *Candida albicans* ATCC 10237).

Our thesis will be articulated in the following way:

- A first part which is devoted to the bibliographic synthesis is divided into three chapters:
- The first chapter describes the medicinal plants, knowledge on phytotherapy and traditional medicine by plants, in particular the active principles, phytochemical properties and the studied plants (*C. candidissimum* and *M. inodora*);
- The second chapter is devoted to the different extraction and analysis techniques;
- The third chapter describe the biological activities of plants.
- The second part describes the experimental work and is divided into two chapters:
- The first chapter describes the materials and methods used in this study;
- The second chapter is devoted to the obtained results, their interpretations and discussions.
- Finally, a general conclusion summarize the whole study in highlighting interesting results and recommending future prospects.

LITERATURE REVIEW

Chapter I

Medicinal plants

I. Medicinal plants

I.1. Natural products

Nature has been a source of therapeutic and nutritional agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, many based on their use in traditional medicine **(Murugan and Mohan, 2011)**. Products came from natural origins can be called "natural products".

Natural products come from four major sources:

- An entire organism (e.g., a plant, an animal, or a microorganism) that has not been subjected to any kind of processing or treatment other than a simple process of preservation;
- Part of an organism (e.g., leaves or flowers of a plant, or an isolated animal organ);
- An extract of an organism or part of an organism;
- Pure compounds isolated from plants, animals, or microorganisms, (e.g., alkaloids, flavonoids, glycosides, steroids, sugars, terpenoids, etc).

Nevertheless, in most cases the term natural products refer to secondary metabolites (small molecules produced by an organism that are not strictly necessary for the living of the organism). Natural products can be from any terrestrial or marine source: plants, animals, or microorganisms. The databases of natural products have recorded more than two hundred thousand compounds from almost all part of the world **(Satyajitet *al.*, 2006)**.

Research into the chemical and biological properties of natural products over the past two centuries has not only yielded drugs for the treatment of human illnesses, but have provided the motivation for the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major field for the discovery of novel and more effective therapeutic agents. Natural products not only served as the main source of medicine for mankind, but played a vital role in the history of medicine, with the plants represented a rich source among them in the ancient time. The structure determination and biological activity screening of natural products, particularly those with a history of medicinal use, taking clues from folklore medicines. Plant preparations have a very special characteristic that distinguish them from chemical drugs and any other natural sources, a single plant may contain a considerable number of bioactive compounds and a combination of plants even more. This complexity is one of the most important challenges to phytoscientists attempting to identify a single bioactive phytocompound or chemical group in the enormous molecules that comprises a single crude extract **(Mendonca-Filho, 2006)**.

I.2. Herbal Medicine, Aromatherapy and Food Additives

Throughout the ages, humans have relied on nature for their basic needs, for the production of food, shelter, clothing, transportation, fertilizers, flavours and fragrances, and medicines **(Cragg and Newman, 2005)**. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and probably thousands of years of use **(Heinrich et *al.*, 2004)**. Among the substances that were used are oils of *Cedrus* species (cedar) and *Cupressus sempervirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh) and *Papaver somniferum* (poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation. In ancient Egypt, bishop's weed (*Ammi majus*) was reported to be used to treat vitiligo, a skin condition characterized by a loss of pigmentation **(Staniszewska et *al.*, 2003; Beissert and Schwarz, 2002)**. More recently, a drug (β-methoxypsoralen) has been produced from this plant to treat psoriasis and other skin disorders, as well as T-cell lymphoma **(Beissert and Schwarz, 2002)**.

The interest in nature as a source of potential chemotherapeutic agents continues, natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world today **(Farnsworth et *al.*, 1985; Cragg and Newman, 2005)**. In the last 40 years, many potent drugs have been derived from flowering plants; including for example *Dioscorea* species (diosgenin), from which all anovulatory contraceptive agents have been derived; reserpine and other anti- hypertensive and tranquilizing alkaloids from *Rauwolfia* species; pilocarpine to treat glaucoma and 'dry mouth', derived from a group of South American trees (*Pilocarpus spp.*) in the *Citrus* family; two powerful anti-cancer agents from the Rosy Periwinkle (*Catharanthus roseus*); laxative agents from *Cassia sp.* and a cardiotonic agent to treat heart failure from *Digitalis* species **(Newman et *al.*, 2000)**.

The probable undiscovered pharmaceuticals for modern medicine has often been cited as one of the most important reasons for protecting forests. Therefore, the high annual extinction rate is a matter for concern. Modern allopathic medicine has its roots in ancient medicine, and it is likely that many important new remedies will be discovered and commercialized in the future, as it has been till now, by following the leads provided by traditional knowledge and experiences. While European traditions are particularly well known and have had a strong influence on modern western pharmacognosy, almost all societies have well-established herbal

traditions, some of which have hardly been studied at all. The study of these traditions will not only provide an insight into how the field has developed but it is also a fascinating example of our ability to develop a diversity of cultural practices **(Cragg and Newman, 2005)**.

In some countries, the use of medicinal plants is often associated with witchcraft and superstition, because people do not have the scientific insight to explain and predict the curative action of plants **(Gurib-Fakim, 2006)**.

I.2.1. Phytotherapy

Herbalism (also herbal medicine or phytotherapy) is the study of botany and use of plants intended for medicinal purposes or for supplementing a diet. Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Modern medicine recognizes herbalism as a form of alternative medicine, as the practice of herbalism is not strictly based on evidence gathered using the scientific method. Modern medicine makes use of many plant-derived compounds as the basis for evidence-based pharmaceutical drugs. Closely related to herbalism, phytotherapy is the intended medical use of plants and plant extracts for therapeutic purposes **(Capasso et *al.*, 2003; Amirkia and Heinrich, 2015)**. A possible differentiation with herbalism is that phytotherapy may require constituents in the plant extract be standardized by adhering to a minimum content of one or several active compounds in the therapeutic product **(Amirkia and Heinrich, 2015; Bouyahya et *al.*, 2022)**.

Phytotherapy is distinct from homeopathy and anthroposophic medicine, and avoids mixing plant and synthetic bioactive substances. Modern phytotherapy may use conventional methods to assess herbal drug quality, although more typically relies on modern processes like high-performance liquid chromatography (HPLC), gas chromatography (GC), ultraviolet/visible spectrophotometry or atomic absorption spectroscopy to identify species, measure bacteriological contamination, assess potency, and create certificates of analysis for the material **(Gad et *al.*, 2013**; **Güzel et *al.*, 2015)**.

I.2.2. Aromatherapy

The use of plants for therapeutic purposes has always been in human life, and is still valid despite the vast power and responsibility of the pharmaceutical chemistry, mainly based on the active principles of synthesis. Nowadays, use of alternative and complementary therapies with mainstream medicine has gained the momentum. Aromatherapy is one of the complementary therapies, which use essential oils as the major therapeutic agents to treat several diseases. The essential or volatile oils are extracted from the flowers, barks, stem, leaves, roots, fruits and other parts of the plant by various methods. This therapy is a natural

way of healing a person's mind, body and soul **(Worwood, 2000; Pichersky and Gershenzonky, 2002)**.

Literature survey reveals that this therapy has gained a lot of attention in the late 20th century and has become very popular in the 21st century due to its importance, popularity and widespread use; it is recognized as aroma science therapy **(Esposito et *al.*, 2014)**. These aroma molecules are very potent organic plant chemicals that make the surroundings free from disease, bacteria, virus and fungus **(Liu et *al.*, 2013)**. The penetration potential of these oils to reach the subcutaneous tissues is one of the important characters of this therapy.

I.2.3. Food Additives

Food safety is a global issue with significant implications for human health. The WHO annually reports that unsafe food results in the illnesses of at least 2 billion people worldwide, which can be deadly. Food additives are substances added to foods to keep them fresh or to enhance their colour, flavour or texture. Some additives have been used for centuries; for example, preserving food by pickling (with vinegar), salting, as with bacon, preserving sweets or using sulfur dioxide as with wines. With the advent of processed foods in the second half of the twentieth century, many more additives of plant-based origin have been introduced to food industry. The significance of medicinal aromatic and spice plants in food additives is raised by their antimicrobial (bactericidal and fungicidal) properties, owing to which they make highly valued preservatives for fresh vegetable or meat preparations as well as canned products. Given the consumer demand for natural preservatives and/or consumer negative response to chemical compounds, it is imperative that more research is focused on the application of plant antimicrobials to food safety **(Davidson, 2005)**.

I.3. Traditional herbal medicine and ethnobotanical studies

Traditional medicine comprises medical aspects of traditional knowledge that developed over generations with in the folk beliefs of various societies before the era of modern medicine. Traditional medicine refers to health practices, approaches, skills, knowledge and beliefs incorporating plants, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination of treat, diagnose and prevent illnesses or maintain well-being. It has been defined by the world health organization **(WHO, 2008)** as "the sum total of all knowledge and practice whether explicable or not used in the diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to

generation. Whether verbally or in writing. This system of healthcare also knows as folk's medicine ethnomedicine or indigenous medicine.

Plants have been utilized as medicines for thousands of years **(Samuelsson, 2004)**, these medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations **(Balick and Cox, 1996; Samuelsson, 2004)**. The specific plants to be used and the methods of application for particular ailments were passed down through oral tradition. Eventually information regarding medicinal plants was recorded in herbal phamacopoeias **(Balunas, 2005)**.

The fast current globalization has led to lifestyle changes, which can lead to big losses of traditional knowledge **(Cámara-Leret et *al.*, 2014)**. The multidisciplinary nature of ethnobotany makes it the perfect tool to collect rapidly disappearing traditional knowledge. Next to ensuring traditional knowledge does not get lost, ethnobotanical research helps showing the needs and priorities of local people when new systems or policies for biodiversity conservation or management strategies are developed **(Parada et *al.*, 2009; Beltrán-Rodríguez et *al.*, 2014; Bouasla and Bouasla, 2017)**.

Ethnobotany is part of the discipline ethnobiology, which studies the dynamic relationships among peoples, biota and environments **(Ethnobiology Working Group, 2003)**. The term 'ethnobotany' was first used in 1895 by the American botanist John William Harshberger, but the discipline of ethnobotany dates back long before the term was introduced **(Pardo de Santayana et *al.*, 2010)**. Harshberger defined ethnobotany as 'the use of plants by aboriginal people'. Since Harshberger's time, the definition of ethnobotany has evolved to include both anthropology and botany: ''studying the dynamic relation between people and plants'' **(Martin, 1995; Cotton, 1996)**.

Along with the definition, the discipline of ethnobotany also changed a lot during the past years and has grown from its initial focus on the use and management of plants, to the addition of socio-cultural and economic aspects and the perceptions, concepts, views and values of the local people **(Balick and Cox, 1996; Cruz-Garcia, 2014)**. This has led to the more multidisciplinary nature of ethnobotany that not only includes anthropology and botany, but also ecology, economics, linguistics, geography, agriculture, pharmacology, etc. **(Cruz-Garcia, 2014; Martin, 1995)**.

The application of plants in traditional medicine has a long history, and the use of plants as medicine is as old as human civilization. Historically, medicinal plants have gained considerable recognition for the prevention and treatment of different sicknesses. Numerous biologically active components have been isolated from plants known in traditional medicine.

It is estimated that out of the 122 plant-derived drugs, 80% have been developed based on ethnomedical information **(Fabricant and Farnsworth, 2001)**. The main idea of ethnobotany is to investigate human-plant relationships by recording and preserving local community knowledge **(McClatchey et *al*., 2008)**. Based on ethnobotanical studies, new bioactive compounds or new plant uses are continually being identified or rediscovered **(Atanasov et *al*., 2015)**.

I.3.1. Origin and Development of Ethnobotanical Study

Plants provide food and medicine besides protecting the environment and are very important for survival of people. Human beings have adapted to the present life style of crop cultivation for food from hunting gathering that occurred nearly 10,000 years ago **(Cohen, 1976).** According to **Cotton (1996)**, ethnobotany encompasses all studies that concern the mutual relationships between plants and traditional people. Among the relationships of humans with plants, indigenous knowledge on traditional medicine is one. Thus, people depend on plants not only for food but also for preparation of remedies. The focus of ethnobotany is on how plants have been or are used, managed and perceived in human societies and includes plants used for food, medicines, rituals, social life and others. The relationship between plants and human cultures is not limited to the use of plants for food, clothing and shelter but also includes their use for religious ceremonies, ornamentation and health care **(Tabuti et *al*, 2003; Khan et *al*, 2007; Tahira *al*, 2014)**.

Ethnobotanical research documents the knowledge on cultural interaction of people with plants, and figures out how local people have traditionally used plants for various purposes and how they incorporate plants into their cultural tradition and religion **(Balick and Cox, 1996)**. Ethnobotanical studies are now growing and in fast progress throughout the world. One of the main motivating forces behind this expansion is the increasing awareness of the considerable practical and social value of traditional knowledge. Ethnobotany is to define local community plant resources needs utilization and management. Therefore, the conservation of ethnobotanical knowledge as part of living cultural knowledge and practices between communities and the environment is essential for biodiversity conservation **(Martin, 1995)**.

Ethnobotany is also help to save foreign exchange. Moreover, the development of medicinal plants in primary health care not only will save the foreign exchange but also will aid in conserving the national heritage **(Abiyot Birhanu et *al*., 2006; Berhan et *al*., 2006)**. Medicinal plants play a key role in the development and advancement of modern studies by serving as a starting point for the development of novelties in drug . According to **Martin (1995)**, ethnobotanists often have to work without the support of colleagues in order to establish

close relationships with communities. However, in order to achieve more detailed and reliable results, ethnobotanical studies need participation of various disciplines such as plant taxonomy, anthropology, linguistics, economic botany and others and 'ethnobotanists' haves the role of ''explorer'' and they have a great responsibility to share the information they collect from the local people with the great collection of human knowledge. Note that identification of new drugs based on traditional medicinal plants in the areas of pharmaceuticals is among the potential application of ethnobotanical inquiry in recent decade **(Arihan and Mahin, 2007)**.

I.3.2. African traditional medicine

African traditional medicine is ancient and perhaps the most diverse of all medicinal systems. Africa is considered to be the cradle of humankind, with a rich biological and cultural diversity and marked regional differences in healing practices. Unfortunately, even today the systems of medicines are poorly recorded. The documentation of medicinal uses of African plants is becoming increasingly urgent because of the rapid loss of the natural habitats of these plants due to human activities. The African continent is reported to have one of the highest rates of deforestation in the world **(Green and Sussman, 1990)**.

African traditional medicine in its varied forms is holistic, involving both the body and the mind. The healer typically diagnoses and treats the psychological basis of an illness before prescribing medicines to treat the symptoms. Well known African medicinal plants include *Acacia senegal* (gum arabic), *Agathosma betulina* (buchu), *Aloe ferox* (Cape aloes), *Aloe vera* (north African origin), *Artemisia afra* (African wormwood), *Aspalathus linearis* (rooibos tea), *Boswellia sacra* (frankincense), *Catha edulis* (khat), *Commiphora myrrha* (myrrh), *Harpagophytum procumbens* (devil's claw), *Hibiscus sabdariffa* (hibiscus, roselle), *Hypoxis hemerocallidea* (African potato), *Prunus africana* (African cherry) **(Neuwinger, 2000; Newman et *al.*, 2000)**.

I.3.3. Classical Arabic and North African traditional medicine

The oldest written information in the Arabic traditions comes from the Sumerians and Akkadians of Mesopotamia, thus originating from the same areas as the archeological records of Shanidar IV **(Heinrich et *al.*, 2004)**. The earliest documented record, which presumably relates to medicinal plants, found in the grave of the Neanderthal man from Shanidar IV, an archeological site in Iraq. Pollen of several species of plants, presumably used as medicines, was discovered among which are: *Centaurea solstitialis* (*Asteraceae*), *Ephedra altissima* (*Ephedraceae*), *Althea sp*. (*Malvaceae*) amongst others. Although this may not be a finding with direct bearing on the culture of Shanidar, these species or closely related ones from the same genus, are still important today in the phytotherapy of Iraq and also known from other

cultural traditions. These species may well have been typical for the Neanderthal people and may also be part of a tradition for which Shaidar IV represents the first available record **(Cragg and Newman, 2005)**.

The Middle East is known as the cradle of civilisation and many plants cultivated nowadays were domesticated in this region. The Babylonians, Assyrians and Sumerians recorded herbal remedies in cuneiform writing on numerous clay tablets. Of special interest is the Code of Hammurabi, a comprehensive set of civil laws carved in stone and commissioned by the King of Babylon and which lists several medicinal herbs **(Spiegel and Springer, 1997)**.

Similar documents have survived several millennia in Egypt. The Egyptians documented their knowledge (including medical and pharmaceutical) in wall paintings of tombs dating from the Old Kingdom and on papyrus which is made from *Cyperus aquaticus*. During the Dark and Middle Ages, the monasteries in countries such as England, Ireland, and Germany were responsible for preserving the remains of Western knowledge. However, it was the Arabs who were responsible for the preservation of much of the Greco- Roman expertise, and for expanding it to include the use of their own resources, together with the Chinese and Indian herbs, till then unknown to the Greco-Roman world. The Arabs were the first to establish privately owned drug stores in the 8th century. The Persian pharmacist, physician, philosopher and poet, Avicenna, contributed much to the sciences of pharmacy and medicine throughout works such as Canon medicinae, regarded as the "final codification of all Greco-Roman medicine". Canon medicinae included elements of other cultures healing system and forms the basis for a distinct Islamic healing system known today as Unani-Tibb **(Sheehan and Hussain, 2002).**

Among the famous medicinal plants of the Middle East and Egypt are: *Allium cepa* (onion), *Astracantha gummifera* (tragacanth), *Carthamus tinctorius* (safflower), *Carum carvi* (caraway), *Ferula assafoetida* (asofoetida), *Lawsonia inermis* (henna), *Papaver somniferum* (opium poppy), *Peganum harmala* (syrian rue), *Prunus dulcis* (almond), *Punica granatum* (pomegranate), *Rosa* x *damascena* (damask rose), *Ricinus communis* (castor oil plant), *Salvadora persica* (toothbrush tree), *Senna alexandrina* (senna), *Sesamum indicum* (sesame), *Trachyspermum ammi* (ajowan), *Trigonella foenum-graecum* (fenugreek) and *Vitis vinifera* (grape) **(Neuwinger, 2000; Gurib-Fakim, 2006)**.

Drug discovery from medicinal plants Drug discovery from medicinal plants has evolved to include numerous fields of inquiry and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist, or plant ecologist who collects and identifies the plants of interest. Collection may involve species with known

biological activity for which active compounds have not been isolated (i.e traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program. It is necessary to respect the intellectual property rights of a given country where plants of interest are collected **(Baker et *al.*, 1995).** Phytochemists (natural product chemists) prepare extracts from the plant material, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compounds through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant drug discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets.

Numerous methods used to acquire compounds for drug discovery include isolation from plants and other natural sources; synthetic chemistry; combinatorial chemistry, and molecular modeling **(Ley and Baxendale, 2002; Geysen et *al*., 2003; Lombardino and Lowe, 2004)**. Despite the recent interest in molecular modelling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, the natural products, and particularly that of medicinal plants, remain an important source of new drugs, drug leads, and chemical entities **(Newman et *al.*, 2003; Butler, 2004)**.

I.4. Folk and evidence-based medicine from a modern perspective

Since the end of the 20th century, the popularity of traditional medicine has grown rapidly. In addition, during recent years there has been a growing interest in alternative treatments. There are several reasons for this, for example, conventional medicine does not always lead to the desired result; misuse and/or abuse of synthetic medicines often cause side effects, but folk medicine practitioners recommend “natural” products as safe; many people worldwide do not have adequate and regular access to treatment with medicine methods and tools. In many cases, the use of herbal medicines in scientific evidence-based medicine is based on folk and traditional medicine, and the use of herbal medicines is not always scientifically proven with laboratory testing or clinical trials **(European Medicines Agency, 2017)**.

Folk medicine is the foundation of modern evidence-based medicine; it is an empirical method of treatment with a long history, which eliminates the symptoms of the disease without finding out the true cause, this knowledge has been transmitted from generation to generation. Folk medicine varies from nation to nation and is especially popular in rural areas. Until the 19th century, it was the most important method of treatment. As modern medicine developed and became more accessible, the importance of folk medicine decreased. At the same time, scientific interest in folk medicine is growing, as ethnopharmacology is considered an essential

tool for obtaining new biologically active substances. The list of herbal drugs used in human medicine is shorter than the known number of plant species. Interestingly, only a small part of the therapeutic effects of plants have been scientifically studied, so in most cases the use of plants is based on tradition and long-standing use **(Petrovska, 2012)**.

I.5. Medicinal Plants as a Future Source of New Drugs

The process of drug discovery is so long involves the identification of candidates, synthesis, characterization, screening, and assays for therapeutic efficacy. Despite competition from different drug discovery methods, natural products are still providing their fair share of new clinical candidates, new drugs and new drug leads. These compounds were still a significant source of new drugs, especially in the anticancer, antihypertensive, anti-infectives, immuno-suppression, and neurological disease therapeutic areas, and some of them have since progressed further into clinical trials or onto the market **(Butler, 2004)**.

It is considered that because of the structural and biological diversity of their constituents, medicinal plants offer a unique and renewable resource for the discovering of potential new drugs and biological entities **(Lahlou, 2007)**. Therefore, plant-based metabolites can be predicted to remain an essential component in the search and development for new, safe and economical medicaments. Unfortunately, as a result of ongoing climate changes and anthropogenic factors, a significant decrease in global vegetative species in future is predicted, endangering the sources of potential new drugs from nature and prompting urgent actions **(Maclean and Wilson 2011; Thomas et *al.* 2004)**.

I.6. Importance of Medicinal Plants

There has been an increasing concern, in the health sector, due to the growing resistance of bacteria to the available antibiotics. As result, it is very important to develop new strategies that could discover new therapeutic agents by exploring new agents derived from plants **(Monteiro et *al.*, 2012)**. Bioactive compounds in plants are compounds produced by them that have pharmacological or toxicological effects in any other organism. Although nutrients can show pharmacological or toxicological effects when consumed at high dosages (e.g. vitamins and minerals), nutrients in plants are generally not included in the term bioactive plant compound. The typical bioactive compounds in plants are produced as secondary metabolites. Thus, a definition of bioactive compounds in plants could be the secondary plant metabolites showing pharmacological or toxicological effects in man and animals **(Aksel, 2010)**.

I.7. Nutritional and Proximate Value of Medicinal Plants

Natural surroundings is providing plentiful vegetal-capital for all organisms, which have therapeutic benefits. The significant standards of certain herbs have been printed however, a bulky sum of these still persist unknown. Therefore, it is a need to discover their usages and to check for medicinal and biological studies to establish their healing charecters **(Mushtaq et *al.*, 2009)**. The basic nutritional importance of plants is assessed by their component of protein, carbohydrate, fats and oils, minerals, vitamins and water which are accountable for the development and growth in living organism **(Waziri and Akinniyi, 2011; Waziri and Saleh, 2015).** Carbohydrates, fats and protein are the indispensable nutrition for existence. Quality and quantity of proteins in the seeds are the basic aspects, which is imperative in the assortment of plants for nutritious worth, taxanomic classification and plant development courses **(Shinwari et *al.*, 2002; Nisar et *al.*, 2009)**. Alongside with pharmacological and phytochemical significance, every plant has its peculiar nutrient composition. These nutrients are vital for the physical and biological functioning of the body. Such nutrition like carbohydrates, fats and proteins play a significant part in nourishing plants as well as human needs of energy and life sustaining processes **(Adnan et *al.*, 2010)**.

I.8. Secondary metabolites in plants

Secondary metabolites are organic compounds naturally occurring in plants, which derive through methylation, hydroxylation, glycosylation or other biochemical reactions from primary metabolites (carbohydrates, proteins, amino acids, lipids) **(Korkina 2007)**. Secondary metabolites could be classified into several categories according to various features, for example based on their chemical structure, biosynthetic pathway or their solubility in different solvents, etc. Major popular classification is related to the presence or absence of nitrogen in their chemical structure **(Gershenzon, 2002)**. Thus secondary metabolites form two major groups: (1) nitrogen containing—alkaloids, non-protein amino acids, amines, cyanogenic glycosides, and glucosinolates; and (2) nitrogen not containing—terpenes (mono-, sesqui-, di-, tri-, tetraterpenes, steroids, saponins), phenolics (phenolic acids and phenylpropanoids), polyketides and polyacetylenes **(Figure 01)**.

Secondary metabolites are involved in variety of plant physiological and developmental processes such as the formation of root nodules; germination of pollens, pigmentation of leaves and petals; contribution in stress responses as signaling molecules or scavengers of free radicals, which are formed after UV irradiation and invasion of pathogens **(Gershenzon, 2002; Edreva, 2005; Korkina, 2007; Edreva et *al.*, 2008; Buer et *al.*, 2010; Samanta et *al.*, 2011).** Plant

secondary metabolites possess biological activities, which are important for human life and health and therefore are used in traditional and modern medicine, food industry, perfumery and cosmetics **(Havsteen, 2002; Korkina, 2007; Dinkova-Kostova, 2008; Jansen et *al*., 2008; Zhang and Björn, 2009; Caputi and Aprea, 2011; Wijesinghe and Jeon, 2011)**.

Many alkaloids are neurotransmitters, which are used in medicine as antiarrhythmic, antihypertensive, anesthetic, analgesic, antipyretic compounds. Terpenes, terpenoids, plant-derived phenylpropanoids (especially flavonoids) and their derivatives are among the most common biologically active components in food, wines, beer, spices, aromas, fragrances, and essential oils. Taking in account their protective properties, secondary metabolites are of great medicinal interest, especially as free radical scavengers and antioxidants, anticancer, anti-virus, antibacterial, antiprotozoal, anti-inflammatory agents and UV screeners (**Muniz, 2006)**.

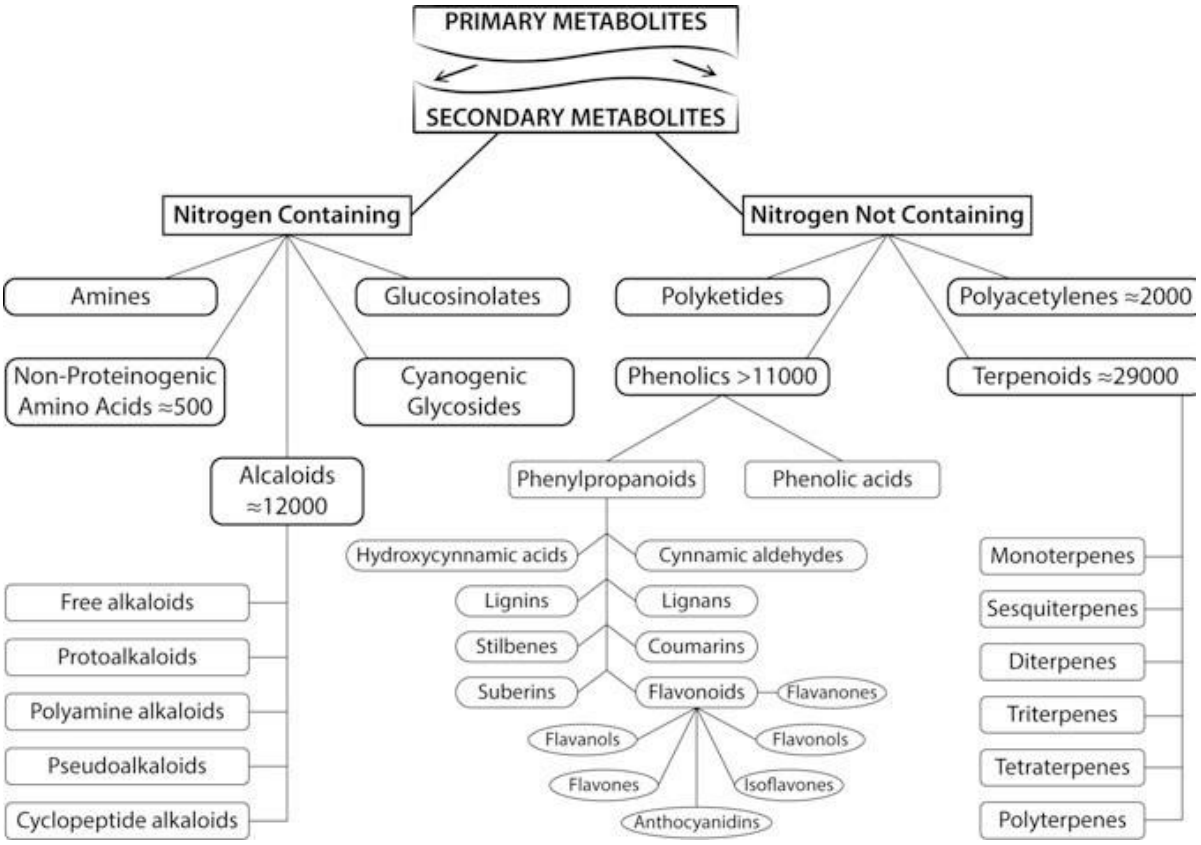


Figure 01. Major secondary metabolites subgroups **(Gershenzon, 2002)**

Plants have so-called "secondary" metabolites as opposed to primary metabolites such as proteins, carbohydrates and lipids. These compounds differ according to the species and, although their roles are still poorly known, it is clear that they are involved in the relationships between the plant and the living organisms that surround it. They are probably essential elements in the coevolution of plants with living organisms, such as parasites, pathogens and

predators, but also pollinators and disseminators. These different relationships have led to an extreme diversification of secondary compounds. It is considered that less than 10% of the species of higher plants that currently populate the planet have been explored for their chemical and biological properties. Secondary metabolites can be classified into several major groups: among them, phenolic compounds, terpenes and steroids as well as nitrogenous compounds including alkaloids. Each of these classes contains a very large diversity of compounds that have a very wide range of activities in human biology **(Krief, 2003)**.

I.8.1. Phenolic compounds (Polyphenols)

The name "polyphenols" or "phenolic compounds" covers a vast group of more than 8,000 molecules, divided into about ten chemical classes that all have one thing in common: the presence in their structure of at least one aromatic ring with 6 carbons, itself carrying a variable number of hydroxyl functions (OH). There are many classes of polyphenols: phloroglucinols, quinones, stilbenoids, coumarins, anthocyanins, tannins, flavonoids, acid-phenols... **(Figure 02)**.

These structures can also be acylated, glycosylated, resulting in a wide variety of structures and polarities. Phenolic compounds are a therapeutically and economically interesting family. They are exploited in herbal medicine and in specialties for vasculoprotective (flavonoids, anthocyanins, tannins), antispasmodic (phloroglucinols) properties and are of great interest for their antioxidant potential **(Abedini, 2013)**.

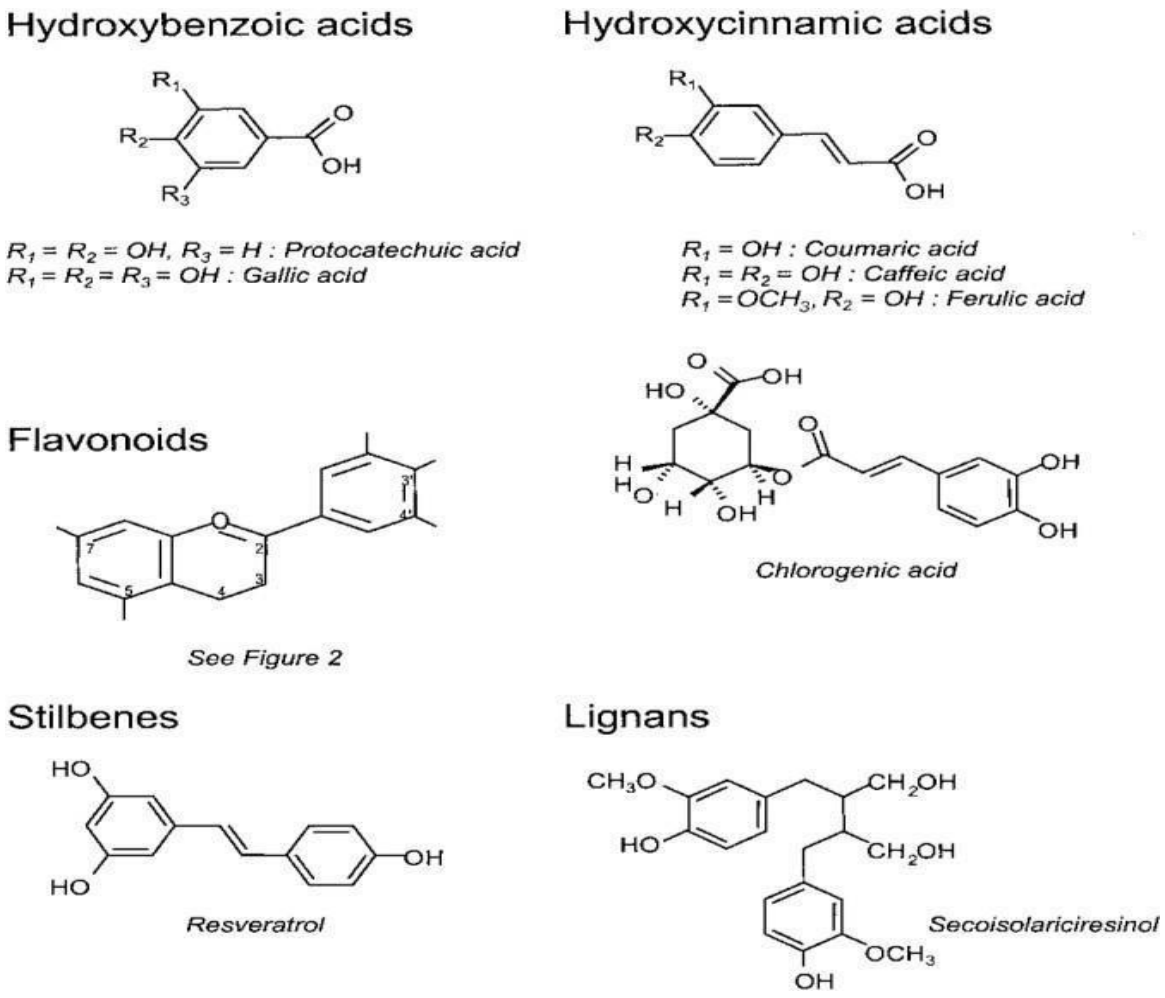


Figure 02. Chemical structures of polyphenols **(Manach et *al.*, 2004)**

I.8.2. Flavonoids

These are pigments that allow the coloration of flowers, fruits and sometimes leaves. When they are not directly visible, they contribute to the coloration by their role of co-pigments. They are polyphenols with a basic structure in C6-C3-C6, consisting of two aromatic rings, connected by an oxygenated heterocycle.

They are classified according to the degree of oxidation of the central pyranic ring. They are also distinguished by the number and position of hydroxyl groups, by the existence or not of substituent on the genin. All flavonoids have a common biosynthetic origin and therefore have the same basic structural element. They can be grouped into different classes according to the degree of oxidation of the central pyranic nucleus, the nucleus B linked to the heterocycle C can be in the positions 2 or 3.

- In position 2: the flavonoid is called flavan.

- In position 3: the flavonoid is designated by the term isoflavan.
- If the position 4 of the flavan carries a carbonyl group, the molecule is called flavanone.
- If the C2-C3 bond in the flavanone skeleton is additionally unsaturated, the compound is called flavone.
- If the preceding skeleton is substituted in position 3 by a hydroxyl group, it is designated by the name of flavonol.

Occupying a prominent place in the phenol group, flavonoids are ubiquitous secondary metabolites of plants **(Abedini, 2013)**.

I.8.3. Tannins

Tannins are secondary metabolites of certain vascular land plants. They are found in all parts of the plant (root, bark, leaves, seed coat, cork, unripe fruit, galls, etc.). They are molecules of phenolic nature of water-soluble polyphenols of molecular mass between 500 and 3000 kDa, which one of the roles is to protect the plants from the attack of certain parasites and herbivores. In some species, tannins reach very high levels allowing industrial exploitation (such as tea whose leaves contain 15 to 25% of tannins). However, the tannin content normally found in most plant tissues, such as fruits and leaves, is between 2 and 5% of fresh weight. Tannins are soluble in water, alcohols and acetone but insoluble in organic solvants. Their solubility varies with the degree of polymerization **(Brillouet et *al.* 2013)**.

Chemically there are two main types of tannins that differ in structure as well as in biogenetic origin, condensed tannins and hydrolyzable tannins.

- Condensed tannins (or proanthocyanidins) are found in the bark, roots and leaves of most plant species and in fruits and seeds. Condensed tannins are dimers, oligomers or polymers of flavonoids, specifically flavan-3-ols.
- Hydrolyzable tannins have a more restricted taxonomic distribution mainly in trees and herbaceous dicotyledonous plants and in many foods such as pomegranates, strawberries, raspberries, blackberries and nuts. They are mainly derived from phenolic acids, such as gallic acid or ellagic acid, hence their subdivision into gallotanins or gall tannins and ellagitanins or ellagic tannins. Associated with D-glucose, gallic acid forms an ester, pentagal- loylglucose, a precursor of hydrolyzable tannins **(Brillouet et *al.* 2013)**.

The anti-nutritional and adverse effects of tannins on dietary protein digestibility are explained by the ability of these molecules to combine with dietary proteins, rendering them unassailable by proteolytic enzymes. In addition, tannins can directly inactivate digestive enzymes.

I.8.4. Terpenoid compounds

Terpenoids are the oldest family of natural products. The terms terpenoids, terpenes and isoprenoids are often used interchangeably and come from turpentine, an essential oil obtained by distillation of conifer resin **(Phillips and Croteau, 1999)**. The word terpene refers to unsaturated hydrocarbons derived from isoprene while, Terpenoids are the most structurally, stereochemically and functionally diverse family of natural products with over 55,000 molecules identified to date in all life forms **(Christianson, 2008)**. Their structures **(Figure 03 and 04)** range from simple linear hydrocarbon chains to complex arrangements of carbon rings **(Connolly and Hill, 1991)** .While some terpenoids perform primary metabolic functions essential for growth and reproduction in many organisms, the majority function as secondary metabolites and contribute to species' adaptation to their ecological niche **(Harborne, 1991, Benabdelkader.2012.)**.

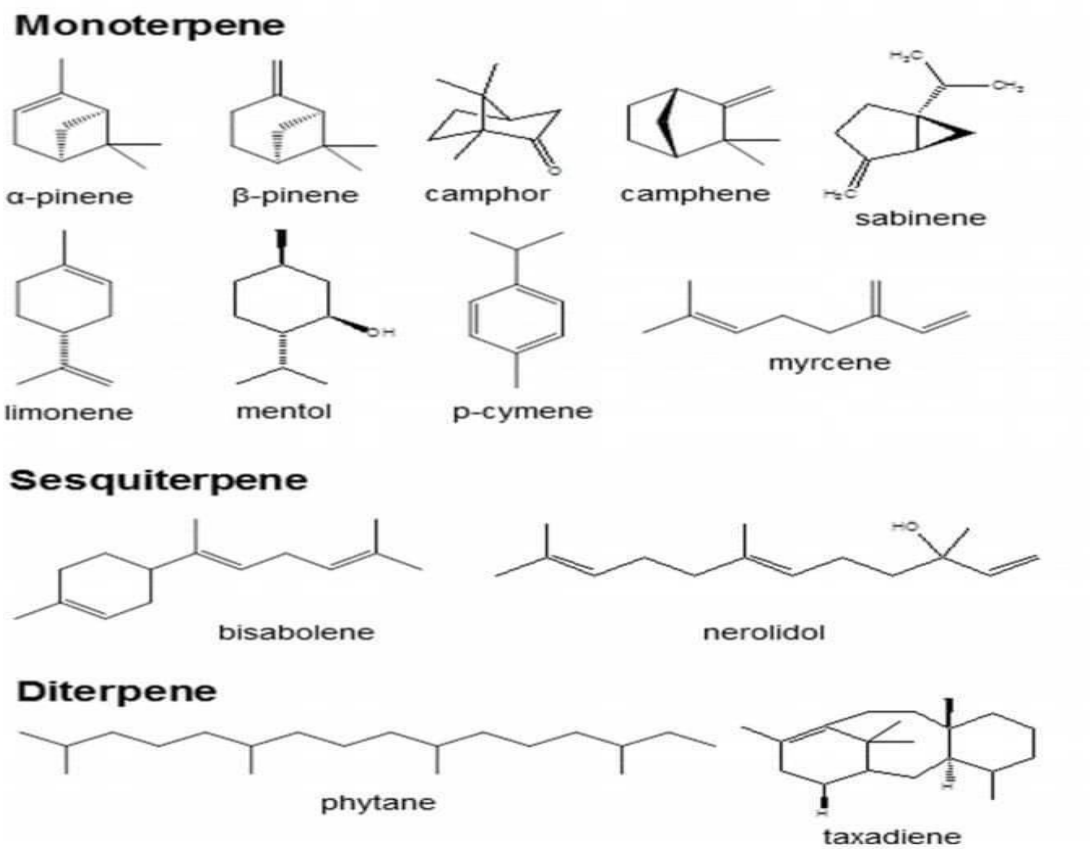


Figure 03. Structure of multiple forms of terpenes **(Cho, Kyoung Sang,2017)**

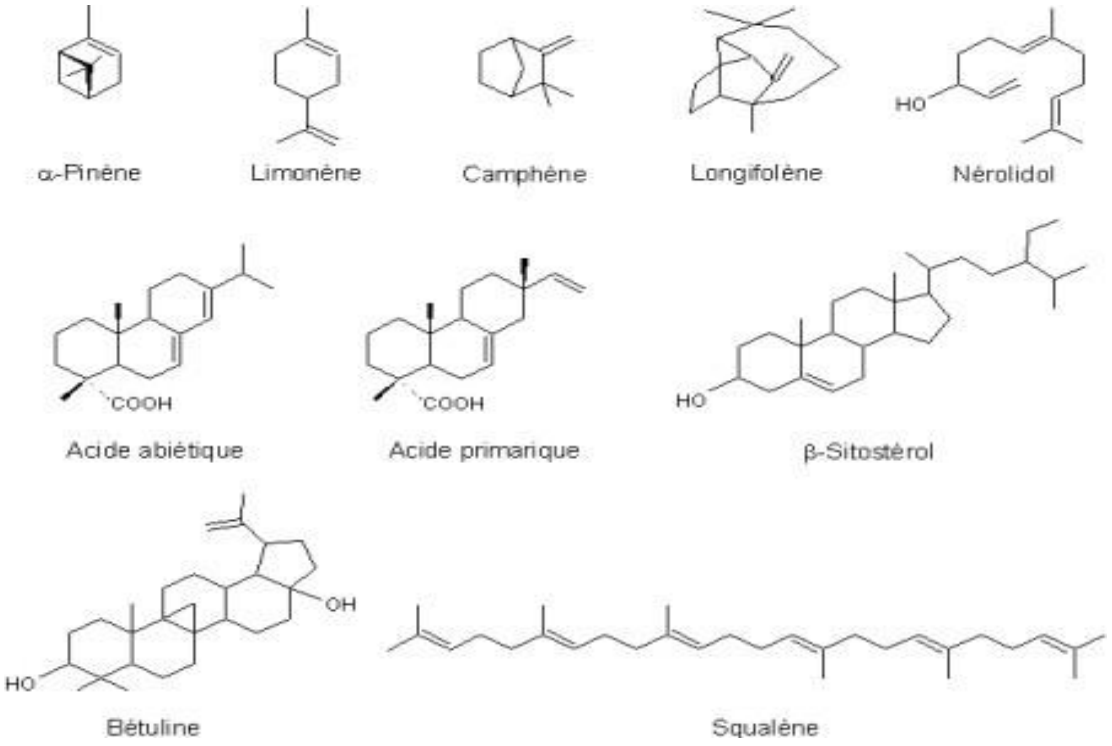
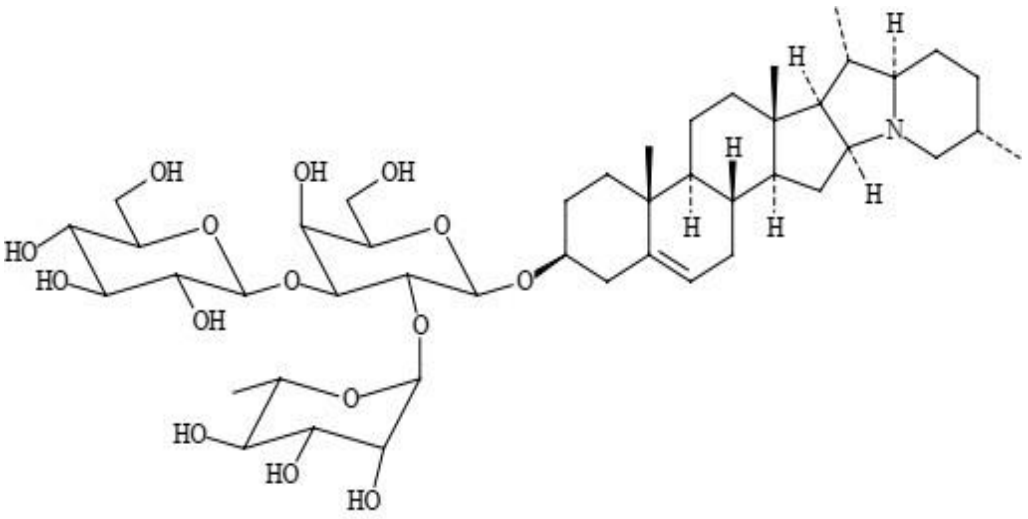


Figure 04. Structure of terpenes and terpenoids **(Candelier, 2013)**

I.8.5. Alkaloids

Alkaloids are natural and organic substances originating mainly from plants and containing at least one nitrogen atom in their chemical structure, with a variable degree of basic character. Since the identification of the first alkaloid - namely morphine - from opium in 1806, more than ten thousand alkaloids have been isolated from plants. Alkaloids are mainly extracted from flowering plants, but are also found in some animals such as ants, frogs and ladybugs. They are relatively stable compounds that are stored in plants as products of different biosynthetic pathways, mostly from amino acids such as lysine, ornithine, tyrosine and tryptophan. Some structures are relatively simple, while others are quite complex **(Figure 05 and 06)**.

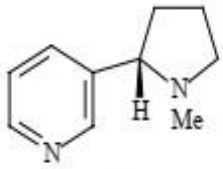
Alkaloids can be found in all parts of the plant, but depending on the species of the plant, they accumulate only in the bark, roots, leaves or in fruits. The part in which alkaloids accumulate is not necessarily the part where they are synthesized (**Muniz, 2006; Aniszewski, 2007)**.



1

Solanine

(*Solanum Tuberosum*)



2

Nicotine

(*Nicotiana Tabacum*)

Figure 05. Structure of alkaloids **(Muniz, 2006)**

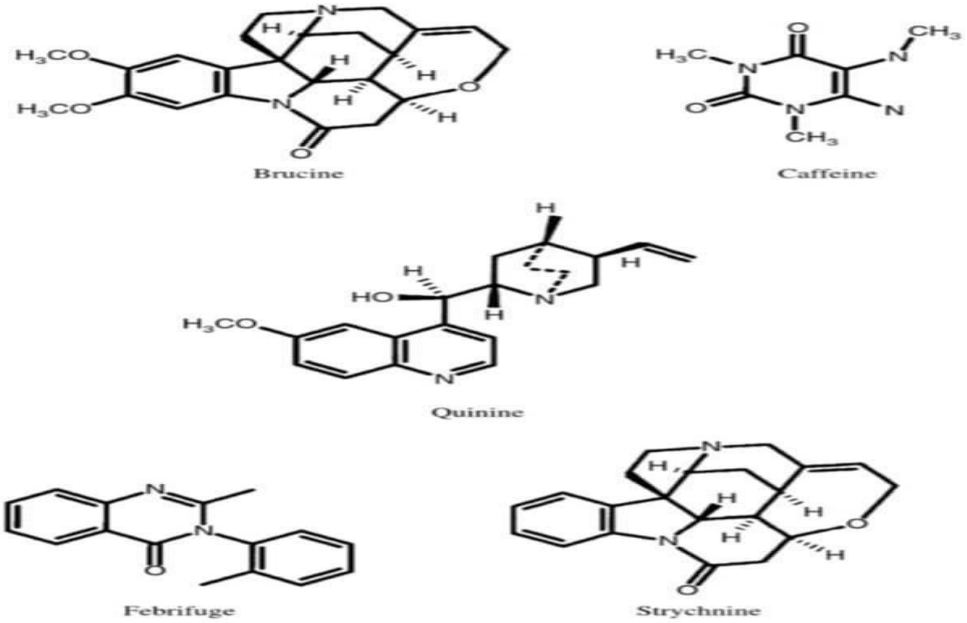


Figure 06. Multiple structures of alkaloids **(Tadeusz, 2007)**

I.9. Synthesis and role of plant secondary metabolites

In plants, as a result of metabolic processes, many different kinds and types of organic compounds or metabolites are produced. These metabolites are grouped into primary and secondary metabolites. The primary metabolites like chlorophyll, amino acids, nucleotides, simple carbohydrates or membrane lipids, play recognised roles in photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation. The secondary metabolites also differ from primary metabolites in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or a taxonomically related group of species, whereas the basic primary metabolites are found throughout the plant kingdom **(Taiz and Zeiger, 2006)**. During the past few decades, experimental and circumstantial evidence has made it clear that many secondary metabolites do indeed have functions that are vital for the fitness of a plant producing them. The main roles are:

- Defence against herbivores (insects, vertebrates);
- Defence against fungi and bacteria;
- Defence against viruses;
- Defence against other plants competing for light, water and nutrients;
- Signal compounds to attract pollinating and seed dispersing animals;
- Signals for communication between plants and symbiotic micro-organisms (e.g. N-fixing Rhizobia or mycorrhizal fungi);
- Protection against UV-light or other physical stress **(Wink et *al.*, 1999)**.

They have also provided an invaluable resource that has been used to find new drug molecules **(Gurib-Fakim, 2006)**.

I.10. Application of metabolomics in natural products

Metabolomics is defined as an omics technology that aimed to comprehensive analysis of many small-molecular metabolites as possible in a target system **(Vignoli et *al.*, 2019)**. It is the large-scale study that relates biological end points to multiple altered metabolite concentrations provides a wealth of biological information on complex systems. Natural products are identified as chemical compounds or substances that synthesized by a living organism and have high structural diversity with extraordinary complexity and unique therapeutical and biological properties, which have shaped their utility over thousands of years. Natural products have been used in both traditional and modern medicine to treat or prevent

from numerous diseases. The search for new drugs reestablished natural products as golden mark for drug discovery and the starting point to provide basic bioactive compounds that help reduce side effects and increase bioavailability **(Sarker and Nahar, 2012)**. In addition, research in natural products stimulated the revolution of new powerful technologies in discovering new bioactive molecules. These modern technologies including metabolomics open the opportunity to understand the activity mechanism of these natural products and thus can also be practically used for the development of potential novel drugs **(Croteau et *al.*, 2000; Harvey, 2008)**.

The aim of natural product research is the identification of bioactive metabolites while metabolomics seeks to give interpretation of complex metabolic mixture datasets. Metabolomics is an area of investigation that is regarded as a systematic tool for studying complex metabolite mixtures, it intends for high throughput analysis of identified and novel compounds in endogenous metabolite mixtures of biological samples **(Zhao et *al.*, 2018)**. Therefore, this approach is the best choice for analyzing active compounds without prior isolation or purification within the active mixtures of natural products. In other words, the application of metabolomics in natural product analysis tries to restyle natural product discovery from the current 'grind and find' approach to targeted, hypothesis-driven discovery model. In the screening of natural products, metabolomics data analysis could be used in two key methods, using qualitative or quantitative approaches. NMR spectra or MS spectra are both capable of being used as qualitative approaches that enable the comprehensive investigation of metabolic profiles of analytes to generate an all-inclusive spectrum which can be exploited for further component information by a sophisticated statistical algorithm such as principal component analysis (PCA) **(Cuperlovic-Culf and Culf, 2016)**. However, in quantitative analysis, profile data are first used to assign and quantify metabolite concentrations (relative or absolute) and these data can help the mechanistic identification and elucidation of these metabolites **(Clarke and Haselden, 2008)**.

I.11. Plants investigated in this study

I.11. 1. *Lamiaceae* family

The family *Lamiaceae* or *Labiatae*, also called *Labiaceae,* is considered one of the main Mediterranean families with species **(El-Gazzar and Watson, 1970; Guignard, 1996)**. This family of dicotyledonous angiosperm plants includes about 258 genera and 6970 species **(Botineau, 2010)**. It is a cosmopolitan family, ranking 3rd and 13th in the world in terms of species variation **(Nurtazina et *al.*, 2016)**. It is known for its great economic value due to its

richness in compounds pharmacologically active, in essential oils and by horticultural species **(Nurtazina et *al*.,2016 ; Trivellini et *al*., 2016 ; Božović and Ragno, 2017)**. Several species of this family are known as: rosemary, oregano, sage, mint and lavender, which are widely exploited by the cosmetic, food and pesticide industries **(Venkateshappa and Sreenath, 2013; Khaled-Khodja et *al*., 2014 ; Trivellini et *al*., 2016)**.

The plants are often herbaceous, or under shrubs with glandular hairs, usually aromatic. Their stems are square, some species are erect, others recumbent bearing opposite or whorled leaves. The bisexual flowers, irregular grouped in the axil of the leaves in more or less elongated inflorescences or in more or less dense terminal inflorescences. The calyx is synsepal, bilabiate and carries 5 to 15 protuberant veins. The corolla has a very developed tube, with two lobes forming an upper lip and three lobes forming a lower lip. The dry fruit separates into four articles each containing one seed **(Guignard, 1998; Guignard, 2001; Carr, 2004)**.

The family *Lamiaceae* is well represented in the flora of Algeria with 183 taxa including 19 endemic. It comes in fourth position after the *Asteraceae* (557 taxa), *Poaceae* (456 taxa) and *Fabaceae* (455) **(Dobignard and Chatelain, 2012)**. **Quezel and Santa (1963)**, report 27 genera and 140 species. Most of the species of this family are frequently used as condiments, and for culinary and medicinal purposes worldwide **(Bianchi, 2014)**. The majority of the *Lamiaceae* plants possess pleasant odor and have pharmaceutical and medical applications. The fragrance is mainly related to the presence of external glandular structures in most of these plants and this morphologic feature is particularly developed in plants belonging to the *Lamiaceae* family **(Giuliani and Maleci Bini, 2008; Venditti et *al.*, 2013; Venditti et *al*., 2014; Venditti et *al*., 2015; Venditti et *al*., 2016)**. It is one of the most widely used families in the world as a source of spices and powerful extracts antimicrobial, antifungal, anti-inflammatory and antioxidant **(Gherman et *al.*, 2000)**. A very large number of genera of the *Lamiaceae* family are sources rich in terpenenoids, flavonoids, glycosylated iridoides and phenolic compounds **(Benayache, 2013)**.

This family is an important source of essential oils and actives compounds, used in aromatherapy and perfumery even if synthetic perfumes tend to replace these essences, perfumery luxury continues to use these plants by distilling them, in order to extract the precious fragrance they contain and maintain the quality of its products. The cosmetics industry also uses *Lamiaceae* for their moisturizing and often antiseptic properties **(Lahsissene et *al.*, 2009)**.

I.11.2. The genus *Micromeria* Benth.

The genus *Micromeria* (*Lamiaceae*) includes worldwide-distributed polymorphic flowering herbs, subshrubs, and shrubs predominantly growing in rocky ground **(Slavkovska**

et *al.*, 2001). Plants belonging to the *Micromeria* genus are rich in essential oils such as pulegone, isomenthone, and menthone **(Başer et *al.*, 1995; Slavkovska et *al.*, 2001; Duru et *al.*, 2004; Güllüce et *al.*, 2004; Karousou et *al.*, 2012; Radulović and Blagojević,2012; Šavikin et *al.*, 2010; Telci and Ceylan, 2007; Vladimir-Knežević *al.*, 2000).**

Micromeria Benth. genus as currently understood belongs to the *Lamiaceae* family and contains about 70 species with a range of distribution extending from the Himalayan region to the Macaronesian archipelago (with Madeira, Cape Verde and the Canary Islands) and from the Mediterranean to South Africa and Madagascar **(Kubitzki and Bayer, 2003; Meimberg et *al.* ,2006)**.

Micromeria was first detected by **Bentham** in **1829.** This name is retained at the expense of the domestic names *xenopoma* wild (1811) and *zygis* desv. (1825) for reasons of nomenclatural stability. The same is true for the names *Sabbatia* moench (1794) and *piperella* presl. (1826), which were rejected because the first is a homonym of another genus of the family *Gentianaceae* given by **Adanson (1763)** and the second is a *nomen nudum* **(Babu, 1969).** The name is derived from the Greek *mikros* (small) and *meris* (part), alluding to the size of the leaves **(Ferrari, 1984)**. Based on their morphological characteristics and their phylo- genetic relationships, species of *Micromeria* are grouped into three sections: *Cymularia*, *Eumicromeria* and *Pseudomelissa* **(Boissier et *al.*, 1867)**.

In Algeria, eight taxa of the genus *Micromeria* are retained, including one endemic taxa and a hybrid **(Dobignard and Chatelain, 2012)**. These are *M. X bourlieri, M. debilis, M. fontanessi, M. gracea subsp. Gracea, M. hochreutineri, M. inodora, M. juliana and M. nervosa*.

Also, four species of *Micromeria* are found: *M. fontanessi*, *M. pulmosa*, *M. nervosa*, and *M. inodora*. On the other hand, a recent study published by **Jean-Paul Peltier** that concerns the plant biodiversity of southwest Morocco revealed the existence of five species of *Micromeria* endemic to Morocco and Algeria: *M. arganietorum*, *M. debilis*, *M. hochereutineri*, *M. macrosiphon*, and *M. monantha* **(Šavikin et *al.*, 2010)**.

Two other species were identified by **Battandier and Trabut (1897)**, they are *M. microphyla* and *M. graeca*. A total of eleven species of *Micromeria* have been recorded in the Algerian territory **(Radulović and Blagojević., 2012)**.

I.11.2.1. *Micromeria inodora* (Desf.) Benth.

Micromeria inodora (Desf.) Benth. **(Figure 07)** is an odorless plant that spreads on rocks, limestone in the western Mediterranean region particularly localized in particular, on the western Mediterranean coastline, especially southern Spain, western Algeria, Morocco and the

Balearic Islands. In Tlemcen *M. inodora* is present all along the coastline from Marsa Ben M'Hidi to Rechgoun **(Benomari et *al.*, 2016)**.

The taxa is known throught several synonyms: *Satureja fontanesii* Briq. = *Micromeria barceloi* Willk. = *Satureja Barceloi* (Willk.Pau.) = *Thymus inodorus* (Desf.). In Algeria, this plant is known among the ancient population under the name of «*Tazira hmeur*» in Berber or « *Zaeter el Hamir* » in Arabic **(Quézel and Santa, 1963)**.

- **Botanical description**

It is a subshrub with erect, much-branched stems 10-50 cm, with calyx 4 mm glabrescent and pink corolla, large lower lip (10-12 mm), (6-7 mm) wide. Small leaves (2-3 mm), sessile, acicular, with rolled margins, tomentose underside; flowers axillary, solitary, briefly pedicellate **(Quézel and Santa, 1963)**.

Figure 07. Morphological aspects of *Micromeria inodora* (Desf.) Benth.

- **Botanical classification**

According to **Quézel and Santa (1962)**, the classification of *Micromeria inodora* (Desf.) Benth. in the plant kingdom is as follows:

Kingdom: *Plantae* (plant)

Phylum: Spermaphytes (phanerogams)

Subphylum: Angiosperms

Class: Magnoliopsida

Subclass: Metachlamydeae (gamopetales)

Order: *Lamiales*

Sub-order: Verbeninae

Family: *Lamiaceae* (labiae)

Genus: *Micromeria*

Species: *Micromeria inodora* (Desf.) Benth.

- **Therapeutic property**

The *Micromeria* species are generally consumed as tea herbs and they are broadly used as aromatic culinary supplements and possess medicinal value due to their bioactive secondary metabolites such as terpenoids, phenols, and essential oils. In traditional medicine *Micromeria* species have been used to treat colds and fever, heart disorders, headaches, wounds and skin infections **(Formisano et *al.*, 2014; Ali-Shtayeh et *al.*, 1998; Telci and Ceylan, 2007**). They were also reported to have anti-inflammatory, antispasmodic, stimulating, antibacterial **(Ali-Shtayeh et *al.*, 1998; Duru et *al.*, 2004; Stojanović et *al.*, 2006; Formisano et *al.*, 2007; Abdelwahab et *al.*, 2015; Brahmi et *al.*, 2017)**, and antioxidative and biological effects **(Couladis et *al.*, 2003; Güllüce et al., 2004; Benomari et *al.*, 2016).** The leaf decoct of *Micromeria* plants is used to treat stomachache, cold, fever, and wounds **(Benomari et *al.*, 2016).**

They contain a wide range of phytocompounds such as terpenoids, iridoids, phenoles and flavonoids such as rosmarinic acid, chlorogenic acid, apigenin, thymonin and acetylated flavone glycoside **(Başer et al., 1996; Tzakou and Couladis, (2001); Samec et *al.*, 2015; Azab, 2016; Kalaki kordkolaei et *al.*, 2019)**.

Chemically the essential oils of most species of the genus *Micromeria* have been characterized by the abundance of oxygenated monoterpenes such as piperitone, piperitone, piperitone oxide, linalool, thymol, limonene, verbenone, α-pinene camphene pulegone and borneol **(JoséPérez-Alonso et *al.*, 1996; Mallavarapu et *al.*, 1997; Marinković et *al.*, 2002; Mastelić et *al.*, 2005).**

I.11.3. The genus *Calamintha* (Munby.)

Lamiaceae is an important family of dicotyledonous plants, it includes about 4000 species and nearly 210 genera **(Naghibi et *al.*, 2015)**. The genus *Satureja* consists of about 200 species of herbs and shrubs, often aromatic. It is widely represented in the Mediterranean region, Asia and boreal America **(Cantino et *al.*, 1992)**.This genus is characterized by aerial parts with a distinctive taste and can be added to several culinary preparations or used in traditional medicine, to treat various disease **(Güllüce et *al.*, 2003)**.

A number of species of perennial plants of the *Lamiaceae* family native to Europe and Western Asia (more precisely Iran and Central Europe) are called calaments. Calaments belong to the subgenera: *Acinos, Micromeria, Clinopodium* and *calamintha*, which are grouped together in the same genus *Saturja* **(kerbouche , 2009)**.

I.11.3.1. Clinopodium candidissimum (Munby) Kuntze (=*Calamintha candidissima* (Munby.) Briq.)

Clinopodium candidissimum (Munby) Kuntze **(Figure 08)** is a strictly endemic species of Algeria **(Quezel and Santa, 1962; Melnikov, 2017)**. This plant has been originally described in Algeria from Oran province by Munby as *Melissa candidissima* Munby, but later it has been reported with several synonyms, in particular *Calamintha candidissima* (Munby) Benth. and *Satureja candidissima* (Munby) Briq. **(Engler et *al.*, 1897; GBIF Secretariat, 2021).** Despite the fact that **Dobignard and Chatelain** retain *Calamintha candidissima* (Munby) Benth in their synonymic indexes of the flora of North Africa **(Dobignard and Chatelain, 2012)**, several works **(Govaerts, 1999; Govaerts, 2003; Melnikov, 2016; Melnikov, 2017)** retain rather *Clinopodium candidissimum* (Munby) Kuntze as an accepted name. This plant grows spontaneously between oleanders and rocky lawns in and around Oran, and it is known to the population as« *Zaater cheleuh*» and «*Nabta elbida*» **(Quezel and Santa, 1962)**.

Among their Synonyms: *Satureja candidissima* (Munby.).Briq., *Melissa candidissima* (Munby.) [1847], *Calamintha candidissima* (Munby.) Straighth. , *Calamintha candidissima* (Munby.) var *laxiflora* Faure et Maire. [1848] **(Quezel and Santa, 1963)**.

- **Botanical description**

The plant is covered except in the inflorescence with a thick whitish velvety tomentum. Ovoid leaves, shortly pedicelled pinkish flowers of 8-12 mm. Calyx and inflorescence glabrous **(Quezel and Santa, 1963)**.

Figure 08. Morphological aspects of *Clinopodium candidissimum* (Munby) Kuntze

- **Botanical classification**

The classification of *Clinopodium candidissimum* (Munby) Kuntze in the plant kingdom is as follows:

***Domain**: Biota*

***Kingdom**: Plantae* Haeckel.*, 1866*

***Sub-kingdom**: Viridaeplantae*

***Division:** Magnoliophyta* Cronquist, Takhtajan & W. Zimmermann.*, 1966*

***Class**: Equisetopsida* C.Agardh.*, 1825*

***Subclass** : Magnoliidae* Novák ex Takht.*, 1967*

***Super-Order:** Asteranae* Takht.*, 1967*

***Order:** Lamiales* Bromhead.*, 1838*

***Family:** Lamiaceae* Martinov.*, 1820*

***Genus:** Clinopodium*

***Species:** Clinopodium candidissimum* (Munby) Kuntze

- **Therapeutic property**

C. candidissimum was studied for the first time by **Attou (2017)**, where a survey on its traditional uses was conducted, this plant called by the local population in western Algeria by "Nabta el bida" was effective in cases of flu, intestinal worms, infections, and as a dressing for the healing of burns and wounds.

The genus *Satureja* is characterized by the presence of essential oils, flavonoids, tannins, phenolic acids (rosmarinic acid, caffeic acid) and saponins **(Kirimer et *al.*, 1991; Vârban et *al.*, 2009)**. The belonging of *Calamintha candidissima* (Munby.) Briq. to the family *Lamiaceae*, and the richness of its essential oil in pulegone and monoterpenes gives this plant several biological properties such as antimicrobial, insecticide, larvicide and herbicide, spasmolytic, against gastrointestinal disorders such as indigestion and diarrhea, anti-inflammatory and analgesic **(Attou, 2017)**. It fights flatulence, stimulates digestion, tonic, antiseptic, antispasmodic, and treats colic. It has a warming effect and is recommended in case of respiratory tract infections (bronchitis) **(Iserin, 2001; Chevallier and Larousse, 2001)**.

It is used in cooking to flavor sauces or to prepare a traditional Algerian dish from the Sid safi region, called "Rfiss" which is a preparation with semolina, butter, salt, water and the fresh cut plant, after cooking it is cut into pieces and mixed with milk or melted butter, the same recipe can be made with *Satureja calamintha* Scheele. *ssp. nepeta* **(Attou, 2017)**.

Chapter II
Different extraction andanalysis techniques

II. Different extraction and analysis techniques of plant

II.1. Extraction techniques of actives compounds from plants

Medicinal plants are the richest bio-resource of drugs from traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction of the bioactive plant constituents has always been a challenging task for the researchers **(Tiwari et *al.*, 2011)**.

Extraction, as the term is used pharmaceutically, is the separation of medicinal active compounds from plants using selective solvents through standard procedures. The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to achieve the therapeutically desired compounds and to eliminate unwanted material by treatment with a selective solvent. The solvent attracts the soluble molecules and leave the insoluble material behind **(Das, 2010)**. There are many protocols available to extract bioactive molecules from medicinal plants such as maceration, infusion, percolation, digestion, hot continuous extraction (Soxhlet), microwave-assisted extraction, boiling, and ultrasonic extraction (sonication) **(Chuo *al*., 2022)**.

Selection of procedures and solvents for extraction of the particular components of the plants depends on the nature of the desired molecules. Variation in extraction methods usually depends on some factors such as length of the extraction period, solvent used, Temperature, particle size of the plant tissues, the solvent-to-sample ratio. Successful Identification of biologically active compounds from plant material is significantly dependent on the type of solvent used in the extraction procedure. Solvent characteristics need to be carefully considered when deciding on the extraction protocol to follow. Solvents have to be easily removed from the extract; therefore, the highly volatile solvents are preferred. Other factors such as physical and chemical properties of the compounds of interest have to be taken into consideration as they may affect the extraction procedure. Standardization of extraction procedures contributes significantly to the final quality of the herbal drug **(Handa, 2008)**.

Standard steps in the extraction of plant material start with size reduction of dried plant material, the purpose of this step is to break the cell wall in order to increase the surface are exposed to the solvent. The next step is the treatment with the desired solvent followed by the filtration of the extract. The final step is the concentration and drying process to remove the solvent and obtain the dried crude extract. This step can be achieved by evaporating the solvent under reduced pressure followed by a freeze-dried or the use of a low temperature oven **(Tiwari et *al.*, 2011)**.

With the increasing demand for herbal medicinal products, nutraceuticals, and natural products for primary healthcare worldwide, medicinal plant extract manufacturers and essential oil producers have started using the most appropriate extraction techniques. Different methods are used to produce extracts and essential oil of defined quality with the least variations. Herbs and medicinal plants have been used for centuries as source of a wide variety of biologically active compounds. The plant crude material or its pure compounds are extensively used to treat diverse ailments by generations of indigenous practitioners **(Swamy and Akhtar, 2019)**. They are currently the subject of much research interest, but their extraction as part of phytochemical and biological investigations presents specific challenges that must be addressed throughout the solvent extraction **(Jones and Kinghorn, 2006)**. Natural products provide unlimited opportunities for new drug discovery because of the unmatched availability of chemical diversity **(Cos et *al*., 2006)**.

Extraction is separating the medicinally active mixture of many naturally active compounds usually contained inside plant materials (tissues) using selective solvents through the standard procedure **(Handa, 2006)**. It can also be defined as the treatment of the plant material with solvent, whereby the medicinally active constituents are dissolved and most of the inert matter remains undissolved. Thus, the purpose of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular marc known as residue **(Azwanida, 2015)**. The obtained product is a relatively complex mixture of metabolites, in liquid or semisolid state or (after removing water) in dried powder form, and are intended for oral and/or external uses. Extraction is based on the difference in solubility between the solute, other compounds in the matrix, and the solvent used to stabilize **(Omeroglu et *al*., 2019)**.

In general, there are three common type of extractions: liquid/solid, liquid/liquid and acid/base. The extraction of these active compounds needs appropriate extraction methods that consider the plant parts used as starting material, the solvent used, extraction time, particle size and the stirring during extraction **(Visht and Chaturvedi, 2012; Jurinjak et *al*.,2018)**. Extraction methods include solvent extraction, distillation method, pressing and sublimation according to the extraction principle; solvent extraction is the most widely used method **(Harborne, 1998)**.

The plant part used and solvent, as starting material and the extraction procedure are three basic parameters reported that influence the quality of an extract **(Pandey and Tripathi, 2014)**. Proper extraction procure is the first step towards isolating and identifying the specific compounds in crude herbal material. It plays a significant and crucial role in the outcome; successful extraction begins with careful selection and preparation of plant sample and thorough

review of the appropriate literature for indications of which protocols are suitable for a particular class of compounds or plant species **(Jones and Kinghorn, 2006)**.

For instance, if the components are volatile or prone to degradation, they can first be frozen and homogenized with liquid nitrogen. The extraction, in most cases, involves soaking the plant material in solvent for some specific time. Reported properties on an excellent extraction solvent include low toxicity, preservative action, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, and inability to cause the extract to be complex or dissociate **(Omeroglu et *al*., 2019)**.

The principle of solid–liquid extraction is that when a solid material comes in contact with the solvent, the soluble components in the solid material are dissolved in, and move to the solvent. In solvent extraction, the mass transfer of soluble ingredients to the solvent takes place in a concentration gradient. The mass transfer rate depends on the concentration of ingredients, until equilibrium is reached. After that, there will no longer be a mass transfer from plant material to the solvent. In addition, heating the solvent can also enhance the mass transfer because of better solubility **(Omeroglu et *al*., 2019)**.

Moreover, the concentration gradient changes if fresh solvent replace the solvent equilibrium with the plant material **(Handa, 2006)**. Properties required for an excellent extracting solvent (or a mixture of solvents) include removal, inert, non-toxic, free from plasticizers, not easily inflammable, and no or less chemical interaction **(Visht and Chaturvedi, 2012)**. The selection of solvent is therefore crucial for solvent extraction, solubility, selectivity, cost, and safety should be taken into account in selecting solvent **(Harborne, 1998)**.

The factors affecting the choice of solvent are quality of phytochemicals to be extracted, rate of extraction, diversity of metabolites extracted, the toxicity of the solvent in the bioassay process, and the potential health hazard of the extractants and ease of subsequent handling of the extract. Obtaining maximum yield and the highest quality of the targeted compounds is the central goal of the extraction process **(Omeroglu et *al*., 2019)**. Extraction methods are usually chosen per the properties of targeted active compounds, the water content of the plant material, and the objectives of extraction. Initially, natural bioactive compounds are extracted using various extraction techniques, and their bioactivities are identified using *in vitro* and *in vivo* testing **(Patel et *al*., 2019; Chuo *al*., 2022**). A successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction. Since the extract will contain traces of residual solvent, the solvent should not interfere with the bioassay **(Pandey and Tripathi, 2014)**.

Various conventional (classical) and non-conventional (innovative) methods can extract plant materials. Variation in extraction procedures usually depends on key factors as extraction time, temperature, particle size of tissues, solvent-to-sample ratio and the pH of solvent.

II.1.1. Classical and/or conventional techniques

The commonly employed extraction methods (long been used) are primarily based on liquid–solid extraction. They are ordinarily easy to operate and are based on heat and/or solvents with different polarities.

II.1.1.1. Maceration

This process is conducted by soaking the plant materials (coarse or powered) in a closed stoppered container in a solvent allowed to stand at room temperature for 2-3 days with frequent stirring to obtain plant extracts. A sealed extractor is used to avoid solvent evaporation at atmospheric pressure. The process is intended to soften and break the plant's cell walls to release the soluble phytoconstituents. The mixture is then pressed or strained by filtration or decantation after a specific time **(Azwanida, 2015; Handa et *al.*, 2008)**.

Maceration is the simplest and still widely used procedure. The extraction procedure in this stationary process works on principle of molecular diffusion, which is a time consuming process. Maceration ensures dispersal of the concentrated solution accumulation around the particles' surface and brings fresh solvent to the surface of particles for further extraction **(Zhang and Li, 2010)**.

II.1.1.2. Digestion

This is a kind of maceration in which gentle heat is applied during the maceration extraction process. The temperature does not alter the active ingredients of plant material, so there is greater efficiency in the use of menstruum (solvent or mixture of solvent used for extraction). It is used when the moderately elevated temperature is not objectionable and the solvent efficiency of the menstruum is increased thereby **(Pandey and Tripathi, 2014)**. The most used temperatures are between 35 and 40°C, although it can rise to no higher than 50°C. The plant part to be extracted is placed in a container with the pre-heated liquid to the indicated temperatures, is maintained for a period that may vary between half an hour to 24 hours, shaking the container regularly. This process is used for the herbal material or plant parts that contain poorly soluble substances or polyphenolic compounds **(Kamil Hussain et *al.*, 2019)**.

II.1.1.3. Infusion

Infusion is a simple chemical process used to extract plant material that is volatile and dissolves readily or release its active ingredients easily in organic solvents. Infusion and decoction use the same principle as maceration; both involve soaking the plant material in

boiled or cold water, which is then allowed to steep in the liquid. The maceration time for infusion is, however shorter. The liquid may then be separated and concentrated under a vacuum using a rotary evaporator. Infusion finds its application in tea preparation and consumption prescribed in psychophysical asthenia, diarrhea, bronchitis, asthma, etc **(Kamil Hussain et *al*., 2019)**.

II.1.1.4. Lixiviation (elution)

The word "lixiviation" (comes from the Latin lixivium, "lessive"); this extraction is carried out with cold or boiled, fresh and new solvent, always. Extraction of components is done using water as solvent **(Azwanida, 2015)**.

II.1.1.5. Decoction

The current process involves boiling the plant material in water to obtain plant extracts. Heat is transferred through convection and conduction, and the choice of solvents will determine the type of compound extracted from the plant material. The sample is boiled in a specified volume of water for a defined time (15 to 60 minutes.) It is then cooled, strained, filtered, and added enough water through the drug to obtain the desired volume. This method is suitable for extracting thermostable (that does not modify with temperature) and water-soluble compounds, hard plant materials and commonly resulted in more oil-soluble compounds than maceration **(Azwanida, 2015)**.

II.1.1.6. Tincture

It is the extraction of plant material in alcohol. Usually, the plant material (fresh) and ethyl alcohol are taken at the ratio of (1:5). Because of the alcohol content, the tinctures can be stored at room temperatures without decomposing **(Rasul, 2018)**.

II.1.1.7. Percolation

It is conducted by passing the boiled solvent through the plant material at a controlled and moderate rate (e.g. 5–7 drops per min) until the extraction is complete before evaporation. The concentrated plant extracts are commonly collected at the bottom of the vessel. To obtain a significant amount of extract, successive percolations can be performed by refilling the percolator with fresh solvent and pooling all extracts together. This procedure is mostly used to extract active compounds in the preparation of tinctures and fluid extracts. Its major disadvantage is that large volumes of solvents are required, and the procedure can be time-consuming and may require skilled persons **(Kamil Hussain et *al*., 2019)**.

II.1.1.8. Steam distillation and hydrodistillation

Steam and hydrodistillation methods are usually used to extract volatile compounds, including essential oil, insoluble in water, from various aromatic and medicinal plants. This is

conducted by boiling the plant materials in water to obtain essential oils after vapor condensation. Steam distillation occurs at a temperature lower than the boiling point of the ingredients. The method is useful for thermos-sensitive bioactive compounds e.g., natural aromatic compounds. The heat leads to breakage in the sample's pores and then enables the release of the target compound from a matrix. As Raoult's law states that while mixing two immiscible liquids, the boiling point will be reduced. Therefore, in the mixture of volatile compounds having a boiling point between 150 and 300°C and water having a boiling point at about 100°C (at atmospheric pressure), the mixture evaporation will be getting closer to that of the water **(Rassem et *al.*, 2016; Omeroglu et *al.*, 2019)**.

There are similarities between the hydrodistillation and the steam distillation principles. In brief, plant material is immersed in water or a proper solvent followed by heating to boiling under atmospheric pressure in the alembic. In a condenser, essentials oils vapors and water undergo a liquefaction process, and they are then separates from water/solvent after collection of the condensate in the decanter. The principle of extraction is based on isotropic distillation, hydrodistillation with water immersion, direct vapor injection, and water immersion and vapor injection are the three main types of hydrodistillation, the distillation time depends on the plant material being processed **(Rassem et *al.*, 2016)**.

II.1.1.9. Hot continuous extraction or Soxhlet extraction

In this method, finely ground sample is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, set in the thimble chamber of the Soxhlet apparatus. **Franz von Soxhlet (1879)** developed the first Soxhlet apparatus. Extraction solvents are heated in a round bottom flask, vaporized into the sample thimble, condensed in the condenser, and dripped back. When the liquid content reaches the siphon arm, the liquid content is emptied into the bottom flask again, and the process is continued **(Azwanida, 2015)**. The disadvantages include no possibility of stirring, and a large amount of solvent is required, this method is unsuitable for thermo labile compounds as prolonged exposure (long extraction time) to heat may lead to their degradation. It constitutes an official classical method used to determine different foods' fat content **(Wang and Weller, 2006; Pandey and Tripathi, 2014; Omeroglu et *al.*, 2019)**.

II.1.2. Innovative (non-conventional) techniques

There is steady progress in the development of extraction technology in recent years, they are also known as advanced techniques with the most recently developed.

II.1.2.1. Microwave-assisted extraction (MAE)

Microwaves are part of the electromagnetic spectrum of light with a range of 300 MHz to 300 GHz, and wavelengths of these waves range from 1 cm^{-1} to 1 m^{-1}. These waves are made

up of two perpendicular oscillating fields, which are used as energy and information carriers **(Mandal et *al.*, 2007)**.

In this extraction process, the use of microwave energy results in faster heating. Due to the exposure of each molecule to the microwave field, its direct effects include, thermal gradients reduction, volume generation due to heat, equipment size reduction, because of the higher process rates, and thus increase in productivity, through better usage of the same equipment process volume **(Alupului et *al.*, 2012)**. MAE is a feasible green solvent extraction procedure as it uses water or alcohol at elevated temperature and controlled pressure conditions **(Figure 09)**.

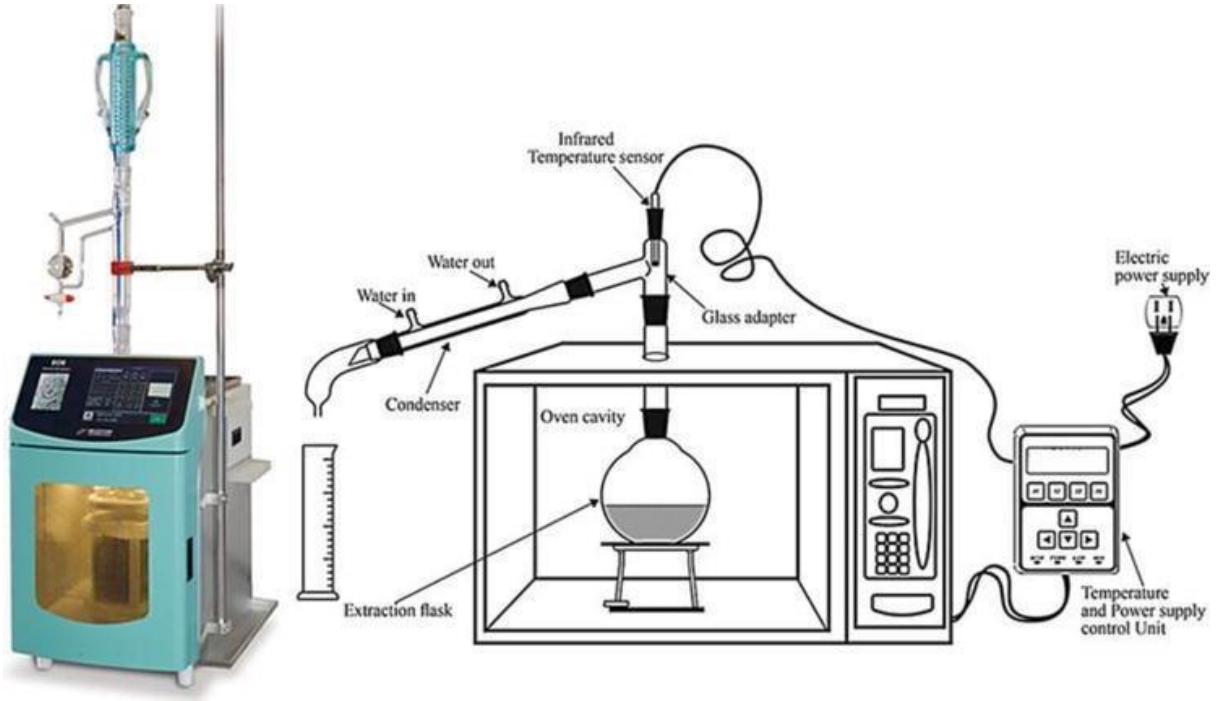


Figure 09. Schematic representation of microwave-assisted extraction equipment **(Castro-López et *al.*, 2016)**

This procedure has demonstrated various benefits like ease to handle and understand steadiness. Many studies reported that MAE has higher yields and is significantly faster than conventional methods for extracting active substances from plant materials. MAE can be presented as a potential alternative to the traditional soli-liquid extraction techniques **(Handa, et *al.*, 2008; Castro-López et *al.*, 2016; Chuo et *al.*, 2020)**.

II.1.2.2. Ultrasound-assisted extraction (UAE) or sonication extraction

This extraction method involves using ultrasound with frequencies ranging from 20 to 2000 KHz; this increases the permeability of cell walls and produce cavitation. Although the

process is helpful in some cases, its large-scale application is limited due to its high cost. The most noticeable disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy on the active components of the medicinal plants through the formation of free radicals and consequently undesirable changes on the drug molecules **(Handa, 2006)**. The schematic representation of the equipment is given below **(Figure 4)**.

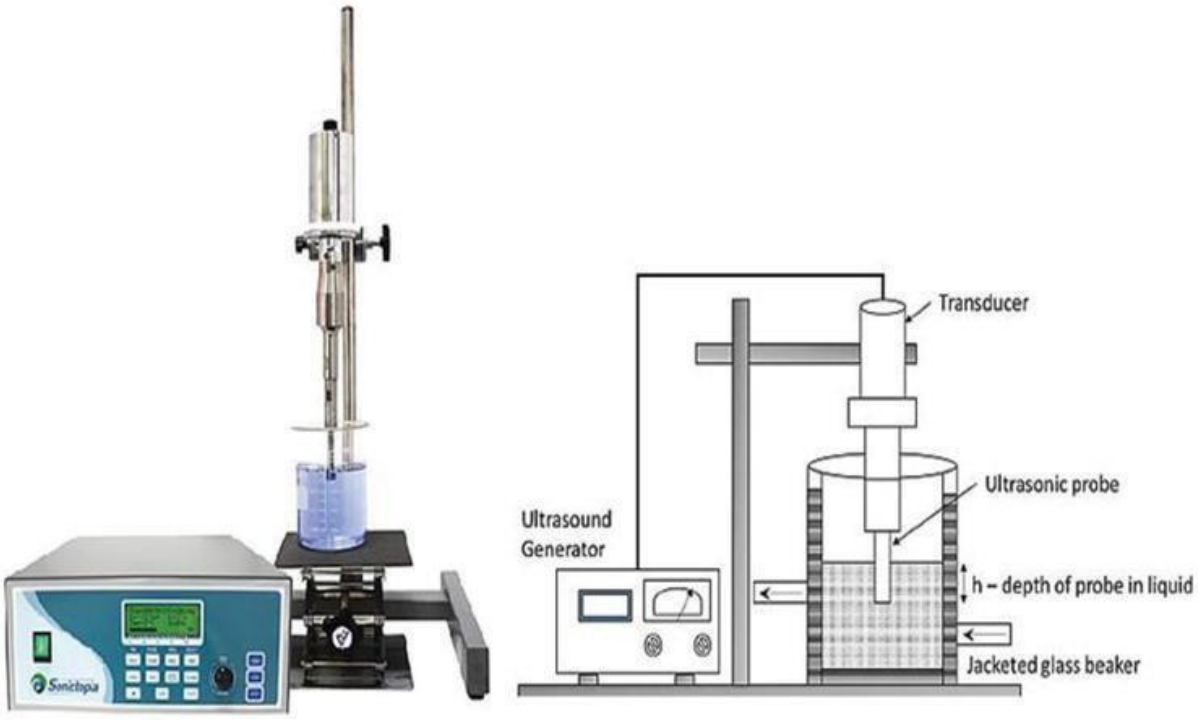


Figure 10. Schematic representation of an ultrasound-assisted extraction equipment **(Castro-López et *al.*, 2016)**

II.2. Isolation and purification of natural products

Bioactive compounds in plants can be classified according to different criteria; it could be based on clinical function, their pharmacological or toxicological effects and that relevant to the clinician, pharmacist or toxicologist. It might be a botanical categorisation based on families and genera of the plants producing the bioactive compounds. It could be useful to categories them according to biochemical pathways and chemical classes. The main chemical groups of bioactive compounds in plants are flavonoids, glycosides, tannins, resins, lignans, and alkaloids **(Ramawat et *al.*, 2009)**.

Plant extracts are mixtures of complex molecules that have different physical, chemical and biological properties. It is not easy to obtain pure bioactive compounds with only one isolation procedure; more than one protocol is usually applied to isolate them to homogeneity. The initial step is the separation of the extracted components into various fractions of similar

properties; these fractions can be obtained by elution with a particular solvent in various chromatography techniques. These techniques based on separation of substances between a stationary and a mobile phase. The mobile phase moves close to the stationary phase and the mixture to be separated move together with the mobile phase, the isolation occurs due to the different interactions of the mixture with both phases **(Czaplicki, 2013)**.

Chromatography is one of the most important techniques in the isolation and purification of natural products. The older techniques comprised of thin layer chromatography (TLC), column chromatography (CC) and flash chromatography. The latest techniques include high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC). Selection of isolation protocols of the known molecules can be carried out based on the general features of the molecules itself such as, size, stability, charge and solubility **(Sarker et *al.*, 2006)**.

However, it is more difficult to design an isolation protocol for unknown components. In this case, the nature of the crude extracts is an important factor to be considered. It is also worthwhile carrying out qualitative tests for the presence of various types of compounds, such as, phenolics, steroids, alkaloids, flavonoids, as well as analytical thin-layer chromatography (TLC), or HPLC profiling in order to determine the general profile of the extracts. In the most cases, it is not easy to apply a single separation technique to isolate individual compounds from a crude mixture due to its complexity. Hence, the crude extract is initially separated into various detached fractions containing compounds of similar polarities or molecular sizes. For initial fractionation of any crude extract, it is advisable not to generate too many fractions, because it may spread the target compound over several fractions and might avoid detection. Modern preparative, or semi preparative high-performance liquid chromatography (HPLC) can be used for finer fractionation and often guided by an on-line detection technique **(Sarker et *al.*, 2006)**.

II.3. Instrumentation and tools in metabolomics studies

The final step of natural product extraction is to identify, unequivocally, the molecule that is responsible for a given pharmacological effect. It is possible to determine the chemical composition through advanced techniques. Metabolomics platforms fundamentally fall into few multiple techniques; (HPLC), (HS-SPME), (NMR), (GC-MS), (LC-MS), (ICP-OES), which are the most often effective tools that are used to scrutinize the molecular constitution of a sample. These analytical instruments have been designed to provide fast, detailed, a notable, quantitative and reproducible data. It is important to note that, each instrument has advantages

and limitations as well and no sole instrument or method is able to spot all metabolites that exist in the sample. Thus, efforts have also been devoted to combine these different instruments (LC-NMR-MS) for ideal metabolite profiling and advance structure elucidation **(Liu and Locasale, 2017)**.

II.3.1. High-performance liquid chromatography (HPLC)

As mentioned earlier, prior to the 1970's, few reliable chromatographic technologies were commercially available for routine laboratory work. Before that, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. Later, high pressure liquid chromatography. (HPLC) began to emerge in the industries, it was developed in the mid-1970's and quickly improved with the development of column packing materials and the improvement of the online detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds **(Charles et *al.*, 2001)**.

HPLC is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification of HPLC, computers and automation was added, improvements in type of columns such as micro-column, affinity columns, all that lead to improve the reproducibility and reliability, and finally fast HPLC began to emerge **(Sailaja et *al.*, 2014)**.

HPLC system consists of a computer, solvent reservoirs, pumps, injector, column, detector, and fraction collector. The main part of the system is the column where separation occurs. Separation of components occurs as the analytes and mobile phase are pumped through the column (stationary phase). Types of HPLC generally depend on the phase system used in the process, these types including; normal phase HPLC (NP-HPLC), this method uses a polar stationary phase and a non-polar mobile phase; Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase; Size exclusion chromatography (SEC), also called as gel permeation chromatography mainly separates particles on the basis of size, this technique is widely used for the molecular weight determination of polysaccharides; In ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same

charge are excluded; Bio-affinity chromatography based on specific reversible interaction of proteins with ligands, because of specificity of the interaction, this type can result in very high purification in a single step **(Malviya et *al.*, 2010; Verma, 2014; Llorent-Martínez, 2015)**.

Among all available techniques, reversed-phase chromatography is the most commonly used separation modes due to its broad application range, especially with the separation of natural products from plant material. The reasons for this include the simplicity, versatility, and range of the reversed-phase method as it is able to handle compounds in a wide range of polarity and molecular mass. The separation mechanism in reverse phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic material in the stationary phase **(Prathap et *al.*, 2013)**.

II.3.2. Headspace solid phase microextraction (HS-SPME)

Extraction is a very important step when studying the composition of floral volatiles of a plant. For this purpose, the methods frequently used are steam distillation, solvent extraction, and headspace trapping **(Báez, 2011)**. In addition to being time-consuming, the first two techniques require the use of large quantities of solvents and can cause thermal artifacts **(Kim et *al.*, 2000)**. Therefore, headspace trapping methods such as headspace solid phase microextraction (HS-SPME) are increasingly used. Indeed, it is a technique that has several advantages, including the reduction of extraction time, the absence of the use of organic solvents, the possibility of automation, the facility of coupling with gas chromatography (GC), simplicity, sensitivity, and selectivity **(Mohammadhosseini, 2015)**. It is a technique that was introduced by **Arthur and Pawliszyn (1990)**, to analyze pollutants in water. Since then, its field of application has developed enormously, particularly in the analysis of the odorous components of many flowers and other plant materials **(Almeida et *al.*, 2006)**.

The SPME is based on the ab/adsorption of the compounds on a fiber, coated with a polymer, also known as the extraction phase. Several polymers of different polarities are currently available, and desorption of compounds is generally achieved by a thermal method in GC. Indeed, polar compounds are sorbed on polar fibers and nonpolar compounds on nonpolar fibers. However, it is impossible to perform repeated injections when the whole sample is desorbed in the injector **(Tholl et *al.*, 2006)**. For extraction, the fiber can be directly immersed in the liquid, in the presence of a liquid sample; in this case, it is called direct immersion (DI-SPME) or it can be exposed to the vapor phase in the presence of a solid, liquid, or gaseous sample, in which case it is called headspace SPME (HS-SPME). Apart from the nature of the

fiber, several other parameters can affect the SPME method such as extraction temperature and duration, equilibration time, desorption time, and agitation **(Mejía, 2002)**.

II.3.3. NMR-based approach

Nuclear magnetic resonance spectroscopy (NMR) is one of the most powerful analytical chemistry techniques for investigating natural products and characterizing their structure. NMR has developed into an essential tool for the determination of the structure of new compounds and natural product chemical analysis. This tool is applicable in a wide range of applications, from the characterization of synthetic products and the study of molecular structures biological systems to analyzing molecular interactions and motions **(Kim et *al.*, 2010)**.

Previous researchs with NMR have shown spectacular developments in biochemical analysis. The application of NMR for profiling or metabolomics research has two basic approaches. The first method is represented by spectral patterns (chemical changes and intensities) which are applicable for comparison and group studies with a note that the compounds are not initially found in this approach (an untargeted approach). Due to the statistical tools that are used including principal components analysis (PCA), this method sometimes is named as the chemometric analysis. On the other hand, the case where metabolites that are known to exist in the sample are identified and quantified using a reference spectral library is the case that represents the second approach (a targeted approach) **(Cox et *al.*, 2014; Dayrit and de Dios; 2017)**. One-dimensional (1D) and two-dimensional (2D) routine NMR methods can be utilized to estimate the complex structure of the compound by both improving the hardware and designing multi-pulse sequences.

II.3.3.1. One-dimensional NMR

NMR spectroscopy interacts with nuclei that serve as miniature magnets and an external magnetic field, providing an effective instrument for scrutinising the chemical bonding and nucleus environment. Hence, these phenomena are key that extend to the applicability of ^{1}H and ^{13}C NMR to natural products **(Cox et *al.*, 2014)**. In fact, ^{13}C NMR is known to be used less frequently than ^{1}H NMR due to its lower sensitivity and longer acquisition time. Nevertheless, ^{13}C NMR spectra characterized by its simplicity, less acute issues with overlapping peaks, unimpressionable to solvent effects, and finally more comparable across different magnetic field strengths. Moreover, the singlet nature of ^{13}C NMR signals is another factor that highly assist in the discrimination of the identity of the separated compounds. Substantially, the composite structures and the exiguous concentration of the detected natural products makes the

application of 1DNMR approach more challenging. Nonetheless, 1D HNMR in the untargeted mode is still preferred to be used alone or as a first-pass screening besides hierarchical cluster analysis (HCA) and PCA in order to provide the cluster and profile information **(Yan et *al.*, 2016)**.

II.3.3.2. Two-dimensional NMR

As mentioned before, due to the signal overlaps issues in 1D H-NMR spectra and in order to obtain more information about a molecule and its environment two-dimensional NMR techniques are usually used to overcome these limitations. Although, conventional one dimensional NMR is adequate for the different functional classes of small molecules to observe separate peaks, multiple overlapping resonances may make it difficult to interpret the NMR spectrum for larger, more complex molecules. The 2D methods include 1H J-resolved NMR (2D JNMR), ^{1}H-^{1}H correlation spectroscopy (2D COSY) and total correlation spectroscopy (2D TOCSY), ^{1}H-^{13}C heteronuclear single quantum coherence (2D HSQC), and ^{1}H-^{13}C heteronuclear multiple bond coherence (2D HMBC). The 2D information is represented with two frequency axes each representing a chemical shift and it is especially useful in determining the structure of a complicated molecule which is difficult to be determined using 1D NMR spectroscopy. The majority of ^{1}H NMR studies combine 1D 1HNMR for PCA analysis with two dimensional homonuclear (^{1}H-^{1}H) or heteronuclear (^{1}H-^{13}C) NMR methods for identification of natural product metabolites. However, metabolite analysis by using NMR technology alone does not accord with the sensitivity of MS based platforms, but the tremendously reproducible quantitative findings of NMR, will remain worthwhile in biochemical research, especially when coupled with MS based techniques such as LC-MS and GC-MS **(Simmler et *al.*, 2014; Dayrit and de Dios; 2017)**.

II.3.4. LC-MS approach

Broadly, liquid chromatography mass spectroscopy (LC-MS) is the term suggested to the techniques that integrate the separating power of high-performance liquid chromatography (HPLC) with the detection capability of mass spectrometry (MS). Such technique is heavily used in a wide range of sectors such as drugs discovery for its high degree sensitivity and selectivity in the analysis of labile analytes, analysis of more polar compounds without derivatization, and analysis of significantly higher masses **(Cox et *al.*, 2014)**. The application of LC-MS in the metabolite's separation target is based on the differences in their affinity (retention strength) for the stationary phase or mobile phase, followed by the detection of these

metabolites using UV, electrical conductivity, or fluorescence based on their characteristics. This detection method accurately qualifies substances based on retention time and quantitate substances based on peak intensity and peak region **(Grebe and Singh, 2011; Zhou et *al.*, 2012)**.

Mass spectrometry is an incredible diagnostic method used to quantify known materials, to recognize obscure metabolites inside the studied sample, and to elucidate the structure and substance properties of various particles. This tool essentially considers the impact of ionizing energy on particles. The concept of MS is to rely on chemical reactions in the gas phase in which the metabolites are expended during the formation of ionic and nonpartisan species. From the examined sample, a mass spectrometer produces several ions; it then divides them according to their particular mass-to-charge ratio (m/z), and then records the relative abundance of each form of ion. A mass spectrum of the molecule is thus produced. The tools of MS are mass spectrometers and data that called mass spectra, which can be presented in several different ways, allowing fast retrieval of the desired information about the analyte **(Watson and Sparkman, 2007)**.

Moreover, the development of high-throughput and quantitative Tandem mass spectrometry (MS/MS) offers extra data about specific ions. The definite ions in this approach are in a quadrupole filter according to their m/z during the first round of MS and are fragmented under a number of different dissociation methods. These fragments are then separated based on their individual m/z ratios in a second round of MS. MS/MS is widely used due to the possibility to match the resulted fragments with the predicted sequences that reported in databases **(Kang and Hur, 2012)**. In conclusion, LC with MS are enhanced synergistically, and they provide together more advantages such as the structural information, speed of analysis, convenience, analysis of multicomponent mixtures, accurate quantitation, evaluation of chromatographic peak purity **(Pitt et *al.*, 2009)**.

II.3.5. GC-MS approach

Gas chromatography mass spectrometry (GC-MS) is an instrumental tool that separates metabolites mixture (GC component) and identifies the separated metabolites at a molecular level (the MS component). GC-MS analysis starts with the volatilization of the sample in the gas chromatograph. Consequently, this volatilization leads to the vaporization of the analyzed sample (gas phase), followed by the separation of its different components through capillary column stuffed with a stationary (solid) phase **(Rifai et *al.*, 2018)**. The sample is pushed by an

idle bearer gas as argon, helium or nitrogen, resulted segments will be ionized by electrons or other chemical ionization sources in the mass spectrometer and accelerated through the instruments mass analyzer. At this point, ions are successfully separated dependent on their distinctive mass-to-charge (m/z) ratios. The ions detections, analysis, and the metabolites spectra that are represented by the peaks as a function of the distribution of their m/z ratios, are the final stride in this analysis. In the final mass spectra, the quantity of the analyzed metabolite is represented by the peak heights, and the appearance of several different peaks signifies that the sample used is a complex sample **(Garcia and Barbas, 2011; Gilbert et *al*., 2013)**.

GC-MS is widely applied for chemical analysis, and especially for drug discovery. The association of MS and GC-MS can be applied in both full scan MS or select ion monitoring (SIM) mode either to cover a wide range of m/z ratios or to gather data for specific masses of interest, respectively. This method enables high-efficiency chromatographic isolation and excellent quantification limits to be accomplished and requires the use of computer-aided mass spectral libraries for metabolite detection **(Garcia and Barbas, 2011; Rodrigues et *al.*, 2017)**.

II.3.6. Inductively coupled plasma optical emission spectroscopy (ICP-OES)

ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) is a multi-element technique (can analyze more than 70 elements), used mainly to perform quantitative analyses. ICP-OES can provide quantitative bulk elemental composition of a wide variety of sample types, including powders, solids, liquids and suspensions. Solid samples are usually dissolved or digested using a combination of acids. The fundamental principle of the ICP-OES analysis method consists in injecting the sample in the form of a liquid aerosol directly into the core of the plasma via the injector. The sample in liquid form is transformed into an aerosol by a nebulizer. This introduces the aerosol into a nebulization chamber whose role is to filter droplets larger than 10 μm. The analyte can then be detected and quantified with an optical emission spectrometer (OES), which measures the intensity of radiation emitted at the specific wavelength of the element from atoms or ions of thermally excited analyte. Intensity measurements are converted to elemental concentration by comparison with calibration standards, this technique is particularly powerful for quantitative chemical analysis **(Frayret et *al*., 2012)**.

Chapter III
Biological activities

III. Biological activities

Bioactivity is the ability of a compound or a series of compounds to harmonize biochemical and physiological functions of living matter. In the natural products field, the biological response of these multi-compound mixtures can be attributed to a set of compounds at the same time, with different mechanisms of action or with synergistic and/or antagonistic effects **(Segneanu et *al.*, 2017)**. Thus, the complexity of the multi-compound mixture in the plant extracts is a daunting challenge that faces the assessment of the bioactive compounds. Bioactivity profiling of a mixture of compounds desires to evaluate the selectivity of bioactive constituents in the context of target-based approaches. It is generally directed at finding bioactivities for a particular target, most often a drug target, and it is commonly used in different screening programs that aim at the identification of the compounds chemical functionalities as well as assessing the possibility of their isolation from different sample sources **(Torras-Claveria et *al.*, 2010)**.

Over the years, different strategies have been developed for bioactivity analysis of complex mixtures and identification of the bioactive components. A robust repertoire of techniques termed the bioassay-guided identification is the oldest and the most iterative methodology used in natural products research to detect new active principles **(Kellogg et *al.*, 2016; Perera et *al.*, 2019)**. Bioassay are scientific experiments utilized to measure the pharmacological activity or the potency of substances such as drugs and plant extracts by juxtaposing its effects on a living material with that of a standard preparation **(Ukwueze et *al.*, 2013)**. Usually, an integration of test methods can be carried out in the various stages of the discovery process for the purpose of uncovering new bioactive compounds from plants **(Harkati, 2011; Strömstedt et *al.*, 2014)**.

Due to the various types of phytoconstituent that are present in the plant material and belong to various chemical classes, medicinal plants play a crucial role in the treatment of several serious diseases **(Afrisham et *al.*, 2015)**. By design, bioassay screening is the dawning to highlight the most active compound in the complex extract obtained from those medicinal herbs, with the objective of searching for novel drug candidates to overcome health problems **(Salehi et *al.*, 2019)**.

III.1. Antioxidant Activity

III.1.1. Origin of oxidative stress

Free radicals, reactive oxygen species, oxidative stress and antioxidants are becoming increasingly familiar terms for professionals of the public. However, these concepts are not new

since they must be recall that **Gerschman and Hartman (1950)** were already talking about the oxygen toxicity and free radical theory to explain the aging process. In **1969**, the Americans **McCord and Fridovich** isolate from human red blood cells an antioxidant enzyme system Superoxide dismutases (SOD), thus demonstrating for the first time that our organization does produce reactive oxygen species that it needs to protect itself from, this discovery will be scientific research around the world on oxidative stress and antioxidants **(Favier, 2003)**.

Oxygen is an essential molecule in aerobic life processes, as final receptor of electrons in the body; O_2 is transformed into water molecules at level of the mitochondrial respiratory chain. This reduction reaction involves four electrons and is associated with the production of energy in the form of adenosine triphosphate (ATP) molecules, this mitochondrial process is not perfect. However, as 2-5% of the oxygen is transformed into reactive oxygenated species, in a first stage the free anion radical superoxide ($O_2^{\bullet -}$) is trained, which then leads to the production of other reactive oxygen species (ERO) such as hydrogen peroxide (H_2O_2), singulet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), acid hypochlorous (HOCl) and nitrated derivatives ($NO^{\bullet}$, ONO^{2-}) **(Pincemail et *al.*, 2002)**.

A free radical is an unstable chemical species (atom or molecule) that is highly reactive has an unpaired electron, and seeks to rip an electron from the neighboring molecule. To side of some enzymes (xanthine oxidase, NADPH oxidase), mitochondria is the source, in small quantities, these free radicals are not harmful. However, in significant quantities and/or if exposure is prolonged, these reactive oxygen species cause alterations including lipid peroxidation, protein carboxylation and/or fragmentation of DNA, or even cell death by apoptosis. Oxidizing stress is a state of profound imbalance between reactive oxygen species production and endogenous antioxidant system. Oxidizing stress can therefore be due to malfunction mitochondrial chain (ischemia–reperfusion, aging), activation of enzyme systems (xanthine oxidase, NADPH oxidase, glucose oxidase, and monoamine oxidase) and a release of free iron from chelating proteins (ferritin) or an oxidation of certain molecules (glucose, hemoglobin, catecholamines...) on the one hand and poor diet low in antioxidants contributing to decreased activity antioxidant on the other hand **(Pincemail et *al.*, 2002)**.

III.1.2. Consequences of oxidative stress

The consequences of oxidative stress will vary greatly depending on the dose and cell type, mild stress will increase cell proliferation and expression of adhesion proteins, medium stress will facilitate apoptosis, while high stress will cause necrosis and severe stress will disrupt the membrane causing. Other biological disturbances are observed following oxidative stress: decreased membrane fluidity, receptor abnormalities, decreased sensitivity to insulin,

disruption of cell immunity, fibrosis, lipid deposition, weakening muscle, or even neuronal death or the appearance of mutations. Abnormal biological molecules and over-expressing certain genes, stress oxidizing would be the main initial cause of developing many processes pathological such as atherosclerosis, cancerogenesis, aging, diabetes and pathologies nervous **(Pincemail et *al*., 2002; Favier, 2006)**.

III.1. 3. Antioxidants and Defence Systems

An antioxidant is any molecule at a relatively low concentration compared to oxidizable substrate, delays, inhibits or prevents the oxidation of this substrate. These compounds have the ability to react with reactive oxygen species and thus render them harmless. From many natural and synthetic antioxidants. Endogenous antioxidants may be enzymatic or non-enzymatic **(Neha et *al*., 2019)**.

III.1.3.1. Enzymatic antioxidants

The human organism has an enzymatic system, consisting mainly of three enzymes: superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). These enzymes have a complementary action on the radical cascade at the superoxide and hydrogen peroxide, ultimately leading to the formation of water and oxygen molecular **(Favier, 2003)**.

III.1.3.2. Non-enzymatic antioxidants

This group of antioxidants contains metal sequestration proteins, which act by decreasing the availability of pro-oxidants, such as Fe^{2+}/Fe^{3+} or Cu^{2+}/Cu^{+} (e.g. transferrin, ferritin, albumin, caeruloplasmin, etc.). On the other hand, there are small molecules that act either as cofactors of enzymes cited either as a clean antioxidant. Direct-acting antioxidants are capable of delivering electrons to the free oxygen so that it can be trapped, to attack biological structures. They can act as agents and can act as reducing agents able to pass their electrons to reactive oxygen species and eliminate them **(Pincemail et *al.*, 2002)**. These molecules come from either endogenous sources (glutathione, melatonin, acid uric, melanin, etc.), or from exogenous sources provided by food, e.g. carotenoids, vitamin E, vitamin C, phenolic compounds and especially acids phenolics, flavonoids, tannins and coumarin and essential oils **(Bruneton, 1999; Pincemail et *al.*, 2002; Yoo et *al.*, 2008; Neha et *al*., 2019)**. Synthetic include butyl hydroxytoluene (BHT), octyl gallate (OG), butylhydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ).

III.1.4. Antioxidant activity of phenolic compounds

Today, research is focused on extracting natural antioxidants that would be less toxic and more effective than synthetic antioxidants generally used to combat "oxidizing stress", which has a priori a strong effect. Synthetic antioxidants, such as butylhydroxytoluene (BHT)

and butylhydroxyanisole (BHA), are well known for their ability to stopper oxidation chain reactions of a lipid **(Avlessi et *al*, 2004)**. They have been shown to be able to increase the risk of cancer-causing diseases, causing swelling of the liver and influence liver system activities and cerebrovascular diseases **(Choi et *al.*, 2007)**. In this regard, flavonoids and other polyphenols have captured attention due to their non-toxicity and potential for implementation in the diets in humans **(Belščak-Cvitanović et *al.*, 2018)**. Polyphenols naturally present in food or formed during processes, are considered free radical eliminators **(Shahidi, 2003)**. The antioxidant properties of polyphenols vary with their chemical structure. The positions and degrees of hydroxylation play an important part in the antioxidant activity of polyphenols. The latter carry a catechol group (aromatic nucleus carrying two adjacent hydroxyl functions) have a potential high antioxidant. Thus, all flavonoids with 3' and 4' hydroxylation have significant antioxidant activity. In addition, additional hydroxylation in 5' as on myricetine will increase this activity compared to compounds like quercetin without them **(Shahidi and Naczk, 2003)**.

Nucleus B hydroxylation also plays an important role in antioxidant activity flavonoids while hydroxylation in 5 and 7 on cycle A has little influence. The glycosylation in 3 of flavonoids by mono- or disaccharides reduces their activity antioxidant compared to their aglycones (so rutoside is less active than the quercetin). It has been shown that chalcones, and in particular the 3,4- dihydroxychalcones were particularly effective, especially in comparison with their flavanones for isoflavones, hydroxylation in 4' and 5 is essential to a significant antioxidant activity as in the case of genistein. The same Thus, the antioxidant activity of phenolic acids depends on their degree of hydroxylation. In addition, hydroxycinnamic acids have been shown to be more antioxidant than corresponding benzoic acids **(Shahidi and Naczk, 2003; Belščak-Cvitanović et *al*., 2018)**.

III.1.5. DPPH Radical Scavenging Activity

Free radicals are byproducts that generated by several chemical processes, such as metabolism. Because of their unpaired electrons, free radicals are characterized by a high unstable nature that shove them to seek out other electrons. The oxidized molecules by the free radicals transformed into free radicals themselves and will seek to interact with another healthy molecule **(Lobo et *al.*, 2010)**. These deleterious reactions chain produce an oxidative stress which appears when the formation of free radicals overwhelms the detoxification potentiality of antioxidant defenses **(Ayepola et *al.*, 2014)**. These complications are the first reason behind the vicious damage of cells structure and thus damage DNA, proteins, and lipids **(Valko et *al*., 2007)**. Numerous scientific evidences have spotlighted the direct link between oxidative stress and the aging process and some acute pathologies as well as variety of chronic diseases

including cancer, atherosclerosis, Alzheimer's disease, Parkinson's disease, and diabetes mellituse **(Pham-Huy et *al*., 2008; Lobo et *al*., 2010)**.

High oxidative stress has been implicated in the diabetic state. Free radical production can launch lipid peroxidation of lipoproteins, which in turn stimulates inactivation of enzymes, glycation of protein, basement and other membranes, and it is associated with the development of long-term complications in diabetes. In addition to this, experimental evidence claimed that oxidative stress could also cause a damage of beta cells of the pancreas, which found to be one of the tissues that have the lowest levels of integral antioxidant defenses. In addition, free radical acts as a mediator of insulin resistance and its progression to glucose intolerance and installation of diabetes mellitus. Free radicals are suggested as an underlying mechanism that contributes in the metabolic abnormalities of diabetes, subsequently causing the complications of diabetes such as atherosclerosis, myocardial infarction, nephropathy, atherosclerotic, retinopathy, and neuropathy **(Sabu and Kuttan, 2002; Moussa, 2008; Tiwari and *al.*, 2013; Asmat et *al*., 2016)**. While free radicals have been linked to diabetes, antioxidants exhibit a therapy property to delay, prevent, and decrease risks of diabetes.

Antioxidants play a vital role as health protecting factors. Scientific evidence claims that antioxidants can reduce the risk of developing chronic diseases such as diabetes **(Kirtikar and Basu, 2006)**. There are several forms of antioxidants, including plant-derived polyphenols; and antioxidant that neutralize free radicals either by providing the additional electron required to make the pair, or by breaking down the free radical molecule to render it harmless **(Sheela et *al.*, 2013; Ayepola et *al*., 2014)**. In order to evaluate the antioxidant activity of a sample, DPPH assay method is the most popular among *in vitro* assays that have been developed for this purpose due to its simplicity, speed, and low cost **(Shekhar and Anju, 2014; De Torre et *al.*, 2019)**.

III.2. Enzyme inhibitory activities

III.2.1. Alzheimer’s and anticholinesterase activity

III.2.1.1. Alzheimer’s disease

Alzheimer’s disease, the most common form of dementia neurodegenerative, is characterized by progressive impairment of cognitive functions, behavioral disorders and decreased ability to perform basic activities of daily life **(Fang et *al.* 2016)**. Generally, patients with these disease present difficulties in remembering names, recent events, finding words and implement ideas. Thus, encounter problems of motor skills and orientation in space and time. Most seriously, they also have psychobehavioral disorders such as aggression, anxiety,

depression and hallucinations **(Lanari et *al*., 2006)**. Neuropathologically, Alzheimer's disease is associated with progressive loss of network of cholinergic neurons and synaptic dysfunction in different brain regions, but deeper still, in areas associated with cognition **(Habtemariam, 2019)**.

Pathophysiological, molecular, cellular and gene expression studies have revealed that Alzheimer's disease was a multifactorial syndrome derived from a complex set of factors involving synaptic acetylcholine deficiency and other related neurotransmitters, progressive accumulation of protein fragment β-neurotoxic amyloid (Aβ) (senile plaques) outside brain neurons and twisted strands of the tau protein (entanglement) inside the neurons, the oxidative stress, neural inflammation, etc. **(Houghton et *al*., 2006; Singh et *al*., 2010)**. Oxidative damage to neuronal substances and increased accumulation of iron in areas brain specific are considered major pathological aspects of Alzheimer's disease **(Weinreb et *al.* 2004)**.

III.2.1.2. Relationship between Oxidative Stress and Alzheimer's disease

For a number of reasons, the human brain appears particularly vulnerable to oxidative stress, which required the development of antioxidant defenses complex to maintain the oxidative balance. With advanced age, the oxidative balance, oxidative stress, which sometimes leads to diseases, especially sporadic or environmental-induced diseases, such as Alzheimer's disease, the cardiovascular disease and cancer **(Praticò and Mecocci, 2013)**. Alzheimer's disease has various factors etiological factors, including genetic and neurochemical factors described above. In addition, a common pathological feature of Alzheimer's disease is the oxidation of nucleic acids, proteins and lipids in neurons interact with polyunsaturated fatty acids in neurons, resulting in levels high levels of lipid peroxidation **(Praticò, 2008; Chen and Zhong, 2014)**. Increased levels of biomarkers of oxidative stress (carbonyls, MDA and 3-nitrotyrosine) in blood and changes in antioxidant enzyme activity (SOD and CAT) reflect stress oxidative in the brain. The underlying mechanisms proposed for initiating stress oxidative in Alzheimer's disease include the accumulation of the protein Aβ, hyperphosphorylation of tau protein, inflammation, mitochondrial dysfunction and accumulation of heavy metals **(Enogieru et *al.*, 2018)**.

III.2.1.3. Anticholinesterase activity

Brain nerve cells communicate by releasing substances chemicals called neurotransmitters. Acetylcholine is a synaptic neurotransmitter, which, by binding to the membrane cholinergic receptor, allows depolarization of the membrane and the transmission of impulses at neuromuscular junctions. Acetylcholinesterase (AChE) present in the central nervous system catalyzes the hydrolysis of Acetylcholine (ACh) to choline and acetic acid,

located primarily in the synaptic slit, and thus plays a crucial role in the regulation of nerve impulse transmission by ensuring rapid hydrolysis of acetylcholine at the central and peripheral cholinergic synapses, in order to allow cholinergic neurons to return to a resting state after activation by nerve impulses **(Dall'Acqua 2013)**.

AChE also called true cholinesterase, because it is intracellular, is identified in the plasma and synapses. This enzyme is contained in erythrocytes, tissues nerve and neuromuscular junction. Although AChE remains the enzyme primarily responsible for the transmission of cholinergic nerve impulses development of the MA, an increasingly significant involvement of the butyrylcholinesterase (BChE), non-specific cholinesterase or pseudo-cholinesterase, in co-regulation of cerebral ACh levels. In fact, it has recently been proposed to treat by molecules inhibiting both AChE and BChE **(Ballard, 2002)**. People with Alzheimer's disease have low levels of acetylcholine, which explains the cognitive impairment observed. The solution to increase acetylcholine levels at the synaptic level is then to decrease its degradation, this by inhibiting the action of AChE and/or BChE **(Houghton et *al*., 2006; Dall'Acqua, 2013)**.

The use of reversible and partial inhibitors of AChE and/or BChE is not one of the possible pharmaceutical approaches to treating symptoms of the disease Alzheimer's. Other neurotransmitters are involved and may also be important.

III.2.2. α- Amylase inhibitory activity assay

The α-amylase (α-1, 4-glucan-4-glucanohydrolase) enzyme is found in microorganisms, plants and particularly high activity in germinating cereals. α-amylase is produced in mammals both in saliva especially in the secretory granules of the cells of the salivary glands and pancreas. Porcine pancreatic αamylase is an endo-type amylase and is extracted from the pig pancreas. α-amylase has 496 amino acid residues and 170 water molecules. Porcine pancreatic αamylase consists of two isoenzymes. They have the same molecular weight but differ slightly in amino acid composition and isoelectric point. The enzyme requires one calcium ion and chloride ions for the integration and activation of the enzyme. The maximum enzyme activity occurs at pH-7. α-amylase catalyzes the hydrolysis of internal α-(1-4) glucosidic bonds in amylose and α-(1-6), α-(1-4) glycosidic bond in amylopectin through multiple attacks toward the non-reducing end **(Rick and Stegbauer, 1974)**.

III.2.3. α-Glucosidase inhibitory activity assay

One of the strategies to monitor blood glucose for type 2 diabetes mellitus is to either inhibit or reduce the production of glucose in the small intestine. Naturally, α-glucosidase enzymes aid the cleavage of carbohydrates to generate simpler sugars or monosaccharides for

intestinal absorption. However, an α-glycosidase inhibitor is one of the enzymes that is used for the treatment of diabetes by blocking the digestion of carbohydrates from the intestine, resulting in a decreased rate of hydrolytic cleavage of carbohydrate and thus, lowering blood glucose level throughout the day **(Pujiyanto et *al.*, 2012)**. It has been proved that α-glucosidase inhibitory activity is one of the best strategies to lower the postprandial rise in blood glucose and in turn help avoiding the onset of late diabetic complications. Varied types of potential α-glucosidase inhibitors have been extensively studied and developed to meet the needs of better blood glucose management. α-glucosidase inhibitors such as acarbose, miglitol, voglibose, and 1-deoxynojirimycin (DNJ) are currently commercialized as anti-glucosidase drugs **(Assefa et *al.*, 2020)**. However, due to the side effects of these synthetic α-glucosidase inhibitors, there is now a bulk of evidence that natural products are transpired to be a rich source for such inhibitors, thereby prompting researchers to explore an economical alternative to control the diabetes with higher safety profiles **(Kumar et *al.*, 2011)**.

As a classic strategy, the colorimetric assay is commonly used because of its practicability and brevity for α-glucosidase inhibitory activity screening. through this colorimetric assay strategy, the effect of α-glucosidase inhibitory activity can be deterred by using the α-glucosidase hydrolysis of p-nitrophenyl-α-D-glucopyranoside (PNPG) to produce p-nitrophenol (PNP) and then measuring ultraviolet absorption of PNP at 405 nm **(Zhang et *al.*, 2020)**.

III.3. Antimicrobial activity and antimicrobial resistance

III.3.1. General information on antimicrobial activity

Microbial infections are caused by different microorganisms and are responsible for the most deadly diseases and the most widespread epidemics in the world. The anti-infective therapy is based mainly on the use of antibiotics that selectively inhibit certain metabolic pathways of microbes (bacteria, fungi, protozoa) at very low doses, usually without toxic effects for higher organisms. Antibiotics, in the strict sense, are elaborate products by microorganisms (molds: *Penicillium* and *Aspergillus* or bacteria: genera *Streptomyce*s and *Bacillus*), but usually includes among them semisynthetic drifts and fully synthetic **(Nauciel, 2000).**

The activity of antimicrobials *in vitro* is determined by their concentration; they may cause their destruction (microbiocide effect) or prevent their multiplication (microbiostatic effect) by action at one or more metabolic stages essential to the life of the bacterium. Each of the antibiotics has its own spectrum of action: some with restricted spectrum are only effective against a limited group of microorganisms, some attack only gram-negative bacteria, others

attack only grams positive, finally others are broad-spectrum **(Levison, 2004)**. Thus, antibiotics act on: the synthesis of peptidoglycan (Beta-ctamines, Glycopeptides and Fosfomycins), membranes (Polypeptides), protein synthesis (Macrolides, Aminosides, Phenicolae, Tetracyclines), synthesis of nucleic acids (Quinolones, Sulfamides) and intermediate metabolism **(Nauciel, 2000)**. The action of antibiotics is influenced by many factors: microbial concentration, medium, interaction with another antibiotic, etc.

Phenolic compounds have important antimicrobial activities, probably due to their structural diversities. The antimicrobial properties of some classes of polyphenols were proposed, either to develop new preservatives or develop innovative therapies for the treatment of various microbial infections that consider increasing microbial resistance against conventional antibiotic therapy **(Daglia, 2012)**. In addition, antimicrobial activity in polyphenols that occur in medicinal plants has been studied against a wide range of microorganisms. Sites and number of hydroxyl groups on groups phenolics are assumed to be related to their relative toxicity to microorganisms **(Scalbert, 1991)**. Among polyphenols, flavan-3-ols, flavonols, and tannins received more attention due to their wide spectrum and higher antimicrobial activity in comparison with other polyphenols, and the fact that most of them are capable of remove several microbial virulence factors **(Daglia, 2012)**.

Phenolic compounds, originally, act on the cell membrane in function their hydrophobicity, alter permeability and cause leakage of contents or interfere with membrane proteins, resulting in disruption of structure **(Radulović et *al*., 2013)**. Flavonoids also inhibit synthesis of nucleic acids by inhibition of topoisomerase, energy metabolism and cell wall and membrane synthesis **(Cushnie and Lamb, 2011)**. Quinones have the potential to form an irreversible complex with nucleophilic amino acids in the proteins. The probable targets in the microbial cell are adhesives exposed to surface, cell wall polypeptides and membrane-bound enzymes **(Cowan, 1999)**. Proanthocyanidins have several modes of action such as destabilization cell membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism or deprivation of substrates necessary for growth microbial **(Daglia, 2012)**. Welsh activity is due to their strong affinity for iron and inactivation of membrane-bound proteins. Coumarines cause reduced cellular respiration **(Cowan, 1999)**.

III.3.2. Antibiotic resistance

Antibiotic resistance is a growing problem in practice medical. This is a phenomenon as old as the appearance of antibiotics. Over time, the number of antibiotic-resistant bacteria has increased and resistance to antibiotics have become a global threat to public health **(Stefanović, 2018)**. Antibiotic resistance may be natural or acquired; natural or intrinsic is a

species trait that affects all strains of the same species or bacterial genus. It is linked to its genetic heritage. Thus, the anaerobic bacteria are naturally resistant to aminoglycosides because the passage of aminoglycosides through the cytoplasmic membrane requires an active transport system absent in anaerobes. In addition, Gram-negative *bacilli* are naturally resistant to hydrophobic antibiotics because these molecules have difficulties to pass the outer membrane of their wall. *Klebsiella spp*. naturally produces beta lactamases; this enzyme is then present in the periplasmatic space of the bacterium and leads to destruction antibiotics such as penicillins A, before they can reach their target bacterial **(Nauciel, 2000)**.

In addition to natural resistance, there is also acquired resistance. An antibiotic, in as a selective agent, induces genetic changes in bacteria, contributing to development, selection and spread of resistant strains. This process of acquired resistance is supported by rapid mutation and resistance gene transfer. These (via plasmids, transposons) can be transferred between individuals of the same or related bacterial species, between members of the commensal or pathogenic microbiota and between different environmental habitats, spreading resistance **(Barbosa and Levy, 2000; El Astal et *al.*, 2005)**. Indiscriminate use broad-spectrum antibiotics, immunosuppressants, intravenous catheters, organ transplants and a persistent epidemic of human immunodeficiency (HIV) may also be the source of microbial strains reduced susceptibility to antibiotics and an increased number of resistant bacterial strains antibiotics **(Selvamohan et *al.*, 2012)**.

Experimental part

Chapter I

Materials and methods

I. Materials and methods

I.1. Objectives, site and duration of study

I.1.1. Objectives of study

To carry out the present study, a number of objectives were set:

- Conduct an ethnobotanical survey on two plants from *Lamiaceae* family, native of western Algeria: *Micromeria inodora* (Desf.) Benth. and *Clinopodium candidissimum* (Munby) Kuntze among traditional practitioners, herbalists and people who use or sell medicinal plants;
- Carry out a complete phytochemical screening;
- To proceed to the extraction of volatile (essential oils) and non-volatile (methanolic) extracts from the aerial part of plants studied;
- Analyze and identify the possible products detected in different fractions by the appropriate techniques (HPLC-MS^n , HS-SPME, GC-MS, NMR);
- Evaluation of biological activities (*In-vitro*): antioxidant, antimicrobial, anti-cholinesterase (anti-Alzheimer (AChE and BChE)), antidiabetic activity;
- Detect fractions or compounds with food interests from studied plants.

I.1.2. Site and duration of study

This experimental study was carried out during the period from 2016 to 2023 at:

- Biochemistry, Plant Protection, Biotechnology, Food Technology and Microbiology laboratories, Faculty of Nature and Life Sciences, University of Tiaret, Algeria.
- Organic Chemistry and Chromatography laboratory, Department of Chemistry, Faculty of Sciences, University of Muğla Sitki Koçman, Muğla, Turkey.
- Department of Pharmacy, University of Pisa, Via Bonanno 6, 56126, Pisa, Italy
- School of Pharmacy, University of Camerino, via S. Agostino 1, 62032, Camerino, Italy
- Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy

I.2. Plant material

I.2.1. Plant selection criteria

Plants have been used for nutritional purposes by people since the beginning of mankind. However, after discovering its medicinal properties, flora has become a useful source of compounds with important roles in the prevention and/or treatment of diseases, promoting better health. In fact, the ancestral use of herbal plants can be considered as the basis for the use

of naturally bioactive molecules depending on traditional medicine as primary health care, mainly through the use of plant extracts and their bioactive compounds.

In order to increase the chances of isolating new molecules and/or new therapeutic (or cosmetic) application routes, it is more judicious to choose a species that has been little or not studied until now. For the present work, *M. inodora* and *C. candidissimum* were chosen among the least studied species while taking into consideration their endemic character. Therefore, this work is a contribution to a better knowledge of these plant species.

The choice of our investigated plants is based on two criteria:

*First, Algeria is a good source of medicinal food plants; therefore, it is fortunate to have a variety of flora resources and ethnobotanical history, which reflect it as an ideal place for biological activity screening and a source of new pharmacological compounds.

*The second criterion is that there has been a significant gap between the fields of ethnobotany and phytochemistry.

Knowing that our country has an immense biodiversity of which each plant is characterized by a rather important reservoir of secondary metabolites with particular therapeutic and pharmacological characteristics which require to be exploited by the researches to determine a new natural bioactive substance which will be able to answer the various problems of the health and to be an alternative of the synthetic drugs.

I.2.2. Harvesting and botanical identification of plants

- ***Micromeria inodora* (Desf.) Benth.**

M. inodora aereal parts **(Figure 11)** were collected from individuals spontaneously growing in the region of Lions Mountain, Oran (North-West Algeria, 200 m a.s.l., N 35.798917, W 0.489923) in March 2016. Botanical determination was performed by Dr. MIARA Mohamed Djamel. (Lecturer at Department of Biology, Faculty of Nature and Life Sciences, University of Tiaret) using available literature **(Chermat et *al.*, 2015)** and an herbarium specimen was deposited in the Herbarium Universitatis Camerinensis, University of Camerino, Italy, under the codex CAME 27739, and available online on the an Archive system (http://www.anarchive.it/anArchive/index.jsp).

Figure 11. Morphological aspects of *M. inodora*

- ***Clinopodium candidissimum* (Munby) Kuntze**

A mixture of inflorescences, stems and leaves of *C. candidissimum* **(Figure 12)** was collected from individuals spontaneously growing in region of Djebel Murdjadjo, Oran (Northweastern Algeria, 400 m above sea level, GPS coordinates: Latitude: N 35.701930, Longitude 0.721408W) in March 2016. Botanical determination was performed by Dr. MIARA M.D. using available literature **(Quézel and Santa, 1962)** and a voucher specimen was deposited in the Herbarium Universitatis Camerinensis, University of Camerino (Italy), under the code "CAME 27740".

Figure 12. Morphological aspects of *C. candidissimum*

I.2.3. Drying, grinding and sieving of plants

After harvesting the plants material, were cleaned of impurities and then dried in the shade in a dry and ventilated place at room temperature protected from light, until the stabilization of their mass in order to avoid possible risks of oxidation of the polyphenols and to preserve the integrity of the molecules as much as possible. Then, the samples were collected in clean bags and stored in a dark and humid place. The dried aerial parts of the two studied plants were

grinded and sieved with an electric grinder until a very fine powder was obtained. This powder was kept at room temperature, stored in bottles protected from light and moisture until the use.

I.3. Methods

I.3.1. Experimental protocol

The experimental protocol summarizing the steps followed in our study is shown in **(Figure 13)**.

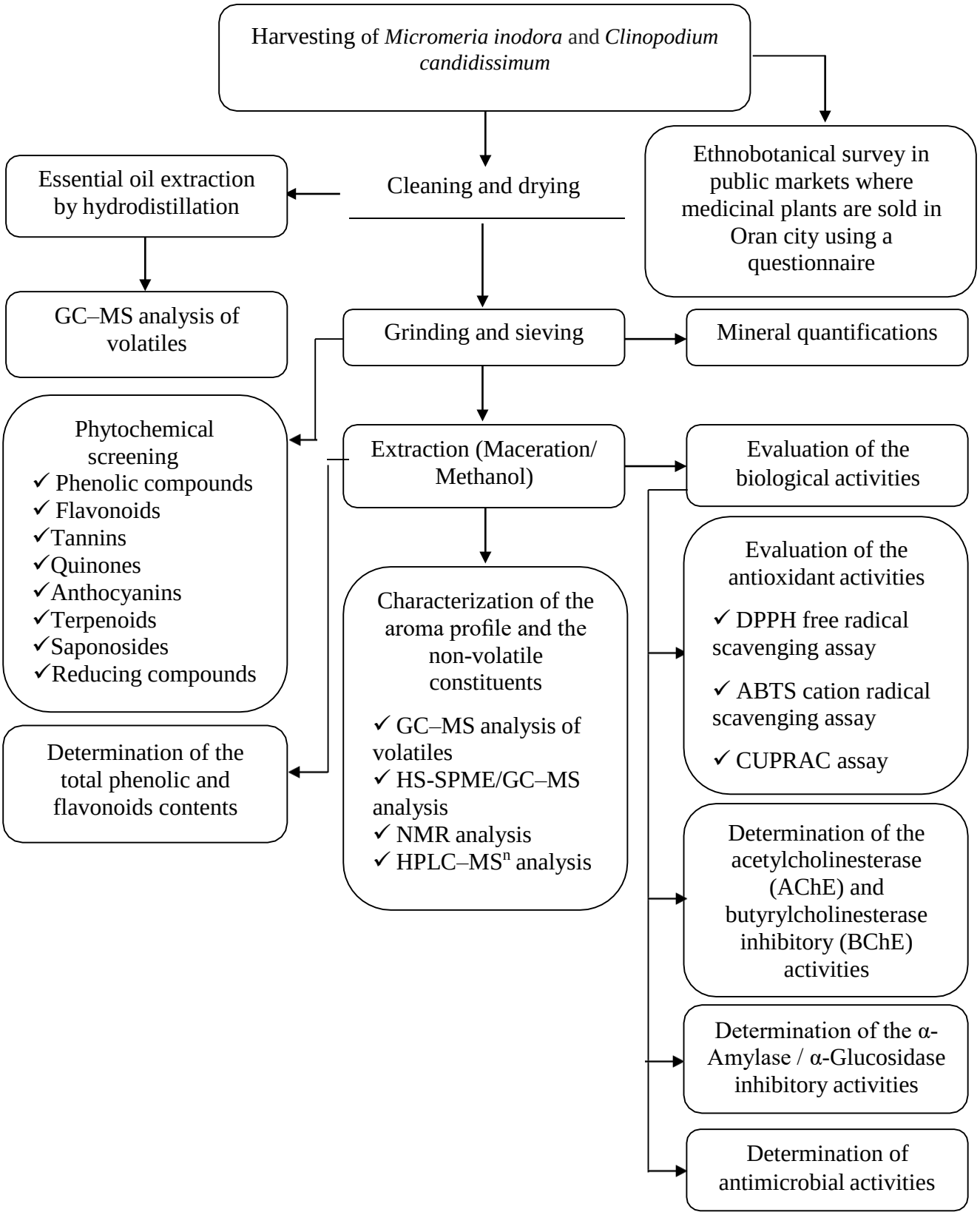


Figure 13. Experimental protocol

I.3.2. Ethnobotanical survey

Numerous phytochemical studies have been carried out on *C. candidissimum* and *M. inodora* but informations on traditional therapeutic uses remains poorly documented. To improve knowledge on the use of *M. inodora* and *C. candidissimum* an ethnobotanical survey in public markets where medicinal plants are sold in Oran city was conducted during the period of 4 months (February - May 2021) in collaboration with the inhabitants of the city and herbalists of medicinal plants (herbalists, healers, actors of traditional medicine,…) by using a questionnaire **(Appendix I)** to get an overview on various local traditional uses. A total of 100 questionnaires were collected, response calculations were performed for various variables.

The different sections of the questionnaire were:

- Profile of the respondent (age, gender, academic level);
- Vernacular names of *M. inodora* and *C. candidissimum*;
- Habitat and distribution;
- Harvested or purchased;
- Therapeutic use;
- Part used;
- Mode of preparation;
- Mode of administration and dosage.

This study reports the first ethnobotanical survey of these plant species in Oran region, the aim of this investigation is to contribute to the valorization and to evaluate the variation in knowledge related to the use of *M. inodora* and *C. candidissimum* which is widely used in traditional therapy for medicinal purposes according to interviewed natives and to determine the convergence of use of the different parts of the plant between individuals in order to fill the non-mentioned gaps in terms of ethnobotany.

- **Description of the study area**

Oran city is one of the most important in the Maghreb, it is a coastal city on the mediterranean sea located in northwestern Algeria, 432 km from the capital Algiers **(Figure 14)**, Oran area is at an altitude of 109 m from the sea level, situated between 35.6971° N, 0.6308° W. This city benefits from a classic Mediterranean semi-arid climate marked by a summer drought with soft winters a bright and clear sky. During the summer months, rainfall becomes scarce or even non-existent **(OSS, 2007)**. The study area has a flora of ecological and medicinal interest **(Trabut, 1887; Bensaid and Gasmi, 2008)**.

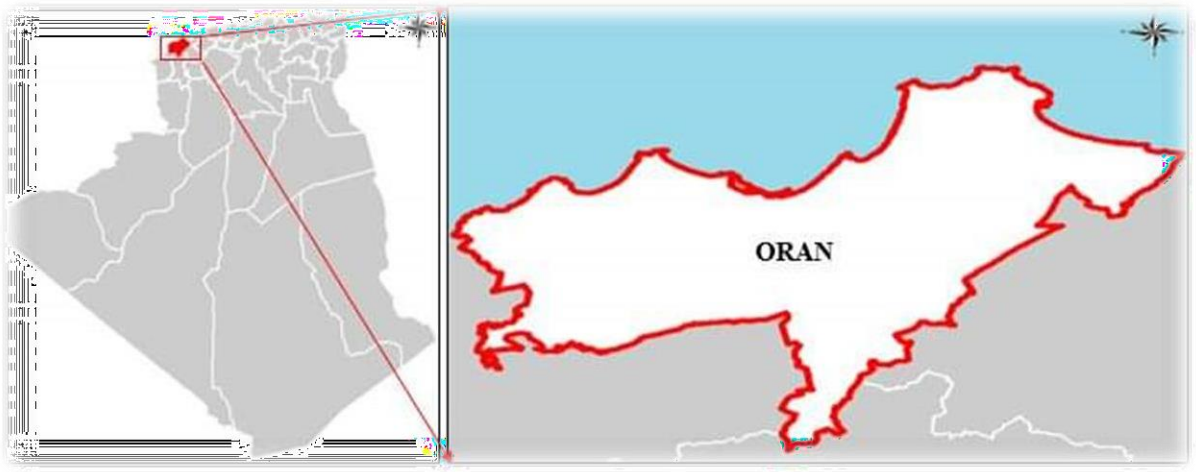


Figure 14. Location of the study area

I.3.3. Phytochemical screening of *C. candidissimum* and *M. inodora*

The phytochemical screening consists in carrying out qualitative phytochemical tests, based on coloring or precipitation reactions more or less specific to each class of chemical compounds belonging to secondary metabolisms of the studied plants.

A quantity of 5g of plant material was macerated in 50 mL of methanol and stirred for one hour in ambient air, the mixture was filtered, and the methanolic extract was submitted to various tests. The presence or absence of different classes of secondary metabolites contained in *C. candidissimum* and *M. inodora* methanolic extracts were qualitatively tested as follow:

- **Phenolic compounds**

A volume of 10 mL of hydrogen chloride (HCl) is added to 10 mL of methanolic infusion. A positive test is revealed by the red coloration in the presence of polyphenols **(Harborne, 1984)**.

- **Flavonoids**

The flavonoid detection reaction consists in treating 2.5 mL of the methanolic extract with 0.5 mL of concentrated HCl and a few small magnesium chunks (Mg). The presence of flavonoids is revealed if the pink or red color develops after 3 min **(Bruneton, 1993)**.

- **Tannins**

The presence of tannins is demonstrated by adding, to 1mL of the methanolic extract, 2 mL of distillated water and 2 to 3 drops of diluted iron chloride ($FeCl_3$) solution. A positive test

is revealed by the appearance of a blue-black color (gallic tannins), green or blue-green color (catechic tannins) **(Trease and Evans, 1987)**.

- **Quinones**

A solution of 1 mL of extract to be analyzed to which a few drops of sodium hydroxide (10% NaOH) turn yellow, indicating the presence of quinones **(Edeoga et *al.*, 2005)**.

- **Anthraquinones**

To 1mL of extract to be analyzed, a few drops of potassium hydroxide (10% KOH) are introduced; after shaking the solution turns red, indicating the presence of anthraquinones **(Edeoga et *al.*, 2005)**.

- **Terpenoids**

To 5mL of extract are added 2mL of Chloroform and 3mL of sulfuric acid (H_2SO_4). The formation of two phases and a brown color at interphase indicates the presence of terpenoids **(Edeoga et *al.*, 2005)**.

- **Saponosides**

The detection of saponosides is carried out by adding 1mL of extract to 2mL of hot water, after shaking (20 min) the appearance of a persistent foam more than 5 min, indicates the presence of saponosides **(Trease and Evans, 1987)**.

- **Reducing compounds**

The detection of reducing compounds consists in treating 1mL of the methanolic extract with 2 mL of distilled water and 20 drops of Fehling's liquor, then heating. A positive test is revealed by the formation of a brick-red precipitate **(Trease and Evans, 1987)**.

I.3.4. Preparation of extracts and essential oils

- **Methanolic extract preparation**

The methanolic extracts **(Figure 15)** of the studied plants (*C. candidissimum* and *M. inodora*) was prepared from 10 grams of ariel parts dried powder of the which were macerated three times with 100 mL of methanol (99.99%) for 24 h at room temperature in the dark, with agitation. Then the mixture was filtered on whatman N°.1 filter paper, the solvents were evaporated under low pressure (Rotary evaporator Buchi R-210), obtained crude extracts were kept at 4°C until use **(Bendif et *al.*, 2017)**.

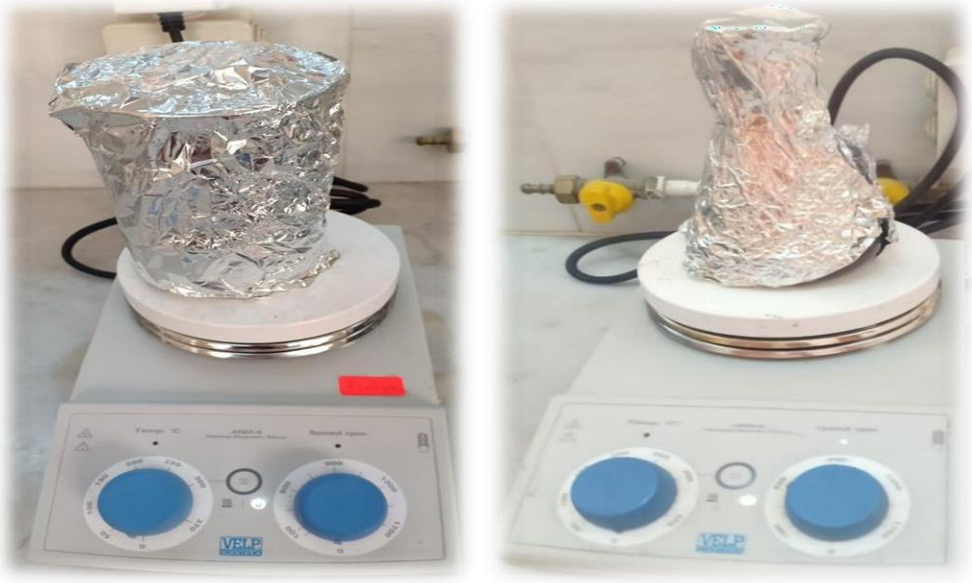


Figure 15: Preparation of the methanolic extracts of *C. candidissimum* and *M. inodora*

- **Determination of the extracts yield**

The yield is the mass of the extract determined after evaporation of the solvent; it is expressed as a percentage of the initial mass of the plant subjected to extraction. The yield was calculated by the following formula:

$$\mathbf{Y\ (\%) = M / M_0 \times 100}$$

M: the mass in grams of the resulting dry extract.

M0: the mass in grams of the plants studied.

- **Essential oil extraction by hydrodistillation**

Aerial parts of plants materials (*C. candidissimum* and *M. inodora*) were cut into small pieces and submitted to hydro-distillation for 3 h in a Clevenger type apparatus **(Clevenger, 1928)**. The essential oil was collected and dried over anhydrous Na_2SO_4. It was stored into dark sealed vials at 4°C before further investigations.

- **Determination of essential oil yield**

The extraction yield (%) is calculated by the formula below:

Y (%) = (Mass of essential oil / Mass of plant material used) x 100

I.3.5. Mineral contents

Many studies focused on the phytochemical screening of studied plants but to the best of our knowledge, there are no published reports on the mineral quantifications. The

digestion of *C. candidissimum* and *M. inodora* was performed on CEM MARS5 (USA) microwave digestion system. Approximately 0.5 g of dry sample (plant powder) was transferred to a Polytetrafluoroethylene (PTFE) digestion tube containing 6 mL of nitric acid (65%) and 2 mL of hydrogen peroxide. The operating conditions of the microwave oven were as follow: temperature (150-200°C), ramp (20 min), time (2 min), and the power (100%) for each step. The digested sample was cooled at room temperature, filtered, and the filtrate was diluted by adding 100 mL of ultra-pure water. The mineral concentration was determined by the inductively coupled plasma-optical emission spectrometry (ICP-OES) **(Tel Çayan et *al.*, 2017)**.

I.3.6. Determination of total phenolic content

The determination of total phenolic in methanolic extracts of the studied plants was performed by the Folin-Ciocalteu method **(Singleton et *al.*, 1999)**. 20 µl of each methanolic extract were mixed with add 1.58 mL of distilled water and 100 µl of Folin-Ciocalteu reagent diluted in distilled H_2O (v/v); this mixture was shaked vigorously and then leaved for 6 min before adding 300 µl of sodium carbonate at 7.5%. After 2 hours of incubation in the dark at room temperature, the absorbance of the solution was measured at 765 nm using UV-Visible spectrophotometer (Spectronic 20 Genysis TM). A calibration curve was established using gallic acid as standard at different concentrations. The results were expressed as microgram of Gallic Acid Equivalent (GAE)/mg of Dry Matter.

The Folin-Ciocalteu reagent is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), it is reduced by phenols to a mixture of tungsten blue oxides (W_8O_{23}) and molybdenum (Mo_8O_{23}) **(Ribéreau-Gayon et *al.*, 1972)**. This blue coloration whose intensity is proportional to the levels of phenolic compounds present in the medium gives an absorption maximum at 760 nm.

I.3.7. Determination of flavonoids content

The estimation of the total flavonoid content in the methanolic extracts of the studied plants was carried out by the method of **Bahorun et *al.* (1996)**, 1 mL of the methanolic extracts was added to 1 mL of 2% methanolic aluminium chloride solution, this mixture was incubated in the dark at room temperature for 15 min. The absorbance of the solution was measured at 430 nm using the UV-visible spectrophotometer (Spectronic 20 Genysis TM). A calibration curve was established using Quercitin as standard at different concentrations. The results were expressed as microgram of Quercitin Equivalent /mg of Dry Matter.

Flavonoids have a free hydroxyl group (OH), in position 5 which is likely to give with the CO group, a colored complex with aluminum chloride. Flavonoids form yellowish complexes by chelation of metals (iron and aluminum). This translates the fact that the metal (Al) loses two electrons to unite with two oxygen atoms of the phenolic molecule acting as electron donor **(Ribéreau-Gayon et *al.*, 1972)**.

I.3.8. Qualitative and quantitative analyses of the studied plants

In order to promote Algerian medicinal and edible plants also to contribute to the phytochemistry of this scarcely known species, in this work we aimed at characterizing the aroma profile and the non-volatile constituents of *M. inodora* and *C. candidissimum* aerial parts, using different extractive and analytical approaches. Gas chromatography-mass spectrometry (GC–MS) analysis of the hydrodistilled essential oil *M. inodora.* headspace solid phase micro-extraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) method was used for the characterisation of volatiles, while integrated NMR and high-performance liquid chromatography with mass spectrometry detection (HPLC- MS^n) approaches allowed the identification and quantification of the crude extracts.

- **GC–MS analysis of volatiles of *M. inodora***

For GC-EI-MS (Gas chromatography coupled with electron ionization mass spectrometry detection) analysis a Varian CP-3800gas-chromatograph equipped with a capillary DB-5 column (30 m × 0.25 mm and 0.25 µm coating thickness) coupled with a Varian Saturn 2000 mass detector were used. GC-MS analyses were achieved as described in **Bendif et *al.*, (2017)**, the analytical conditions were as follows: injector 220 °C, and transfer line 240 °C; oven temperature programmed from 60 to 240°C at 3°C /min (gradient length: 60 min); carrier gas helium at 1 mL/min; 0.2 µl of a 10 % *n*-hexane solution of the essential oil were injected in the split mode (split ratio 1:30) and using the following acquisition parameters: full scan; scan range: 35–300 *m/z*; scan time: 1.0 s; threshold: 1count. The constituents were identified by comparison of the retention times with those of authentic samples, comparing their linear retention indices (l.r.i.) relative to the series of *n*-hydrocarbons, and on computer matching against commercial **(Adams, 2007; NIST, 2014)** and home-made libraries built up from pure substances.

- **HS-SPME/GC–MS analysis**

For HS-SPME, the technique described by **Ascrizzi et *al.* (2017)** was applied. A Supelco Solid Phase Micro-extraction (SPME) device coated with poly-dimethylsiloxane (PDMS, 100

µm) was employed tosample the headspace of a mixture of ground aerial parts of the studied plants. Plant materials was inserted into a 4 mL glass vial for 1 h at r.t., to equilibrate. Then, the SPME fibre was used to sample the headspace for 30 min. GC-MS analyses were achieved as described in **Bendif et *al.*, (2017)** using aVarian CP-3800 gas-chromatograph coupled to a Varian Saturn 2000 mass spectrometer equippeda DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm). Chromatographic conditions: injector and transfer line temperatures 220 and 240 °C, respectively; oven temperature programmed from 60 to 240 °C at 3 °C /min; carrier gas (helium, 1 mL/min); splitless injection. Identification was performed by comparing retention times with those of reference pure compounds, comparing their linear retention indices (LRI) relative to the C_6– C_{28} series of *n*-hydrocarbons, and by matching their mass spectra against commercial **(Adams, 2007; NIST, 2014)** and home-made libraries built up from pure substances.

- **Preparation of extracts for NMR and HPLC-MS^n analyses**

Samples preparations was performed according to previously published protocols **(Dall'Acqua et *al.*, 2010; Bendif et *al.*, 2017).** Briefly, after grinding 3 g of *M. inodora* aerial parts with a blender, sonication of 500 mg of powder was done with 50 mL of methanol for 10 min. After centrifugation, the supernatant was removed, and the residue was extracted in the same way with another 50 mL of the same solvent for two more times. The supernatants were then collected in a round-bottom flask, and the extract concentrated under reduced pressure at 30°C using a rotary evaporator, obtaining 93.10 mg of crude extract (yield 18.62% w/w). The extracts were stored in dark glass vials at -20°C before chemical characterization. 500 mg of the residue was dissolved in methanol-water (1:9) (50 mL) and sonicated. Then, the solution was partitioned with *n*-hexane (20 mL for three times), dichloromethane (DCM; 20 mL for three times) and ethyl acetate (EA; 20 mL for 3 times).

For *C. candidissimum*, 50 g of dried aerial parts were ground with a blender, suspended in 150 mL of methanol and extracted at r.t. for 15 min, by using anultrasound bath. Suspended plant material was removed by centrifugation at 4,000 rpm for 10 min, and supernatant was collected in a round-bottom flask. The residue was extracted with other 50 mLof methanol in ultrasound bath for 15 min, and the same process was repeated twice. Supernatants were dried using a rotary evaporator at 35 °C to constant weight, in order to obtain a dried concentrated extract. This latter was stored in amber glass vials at -20 °C until analysis. The yield of the extraction was 8.8% w/w. 300 mg of the dried powdered extract were used for

NMR and HPLC-MS^n analyses. The remaining extract was partitioned with solvents at different polarity, in order to obtain purified fractions for NMR analysis. The powder was suspended in 50 mL of a methanol/water (1:9) mixture and dissolved by sonication for 10 min. The obtained solution was then partitioned using 20 mL of hexane in a separation funnel, and, after removal of the organic phase, it was partitioned with 20 mL of dichloromethane (DCM), 20 mL of ethyl acetate (EA), and 20 mL of butanol. For each solvent, extraction was repeated in triplicate. Finally, the obtained fractions were dried using a rotary evaporator and dried residues were stored at -20 °C until analysis.

- **NMR analysis**

Dried powdered extracts were dissolved in deuterated methanol and submitted to NMR analysis. NMR analyses were performed on a Bruker Avance III 400 MHz spectrometer, using standard pulse sequences. .^{1}H-NMR was obtained for MeOH, DCM, EA and *n*-hexane fractions for *M. inodora*. ^{1}H-NMR spectra were acquired for methanol, hexane, DCM, EA and butanol extracts for *C. candidissimum.*

- **HPLC–MS^n analysis of methanol extracts**

The phenolic constituents in the methanolic extract of the aerial parts of *M. inodora* were tentatively identified by comparison of their HPLC-MS^n fragmentation patterns with literature data and with freely available web MS databases (Metlin: https://metlin.scripps.edu/landingpage.php?pgcontent=mainPage;MoNA:https://mona.fiehnlab.ucdavis.edu/).

Secondary metabolites in methanol extract of *C. candidissimum* vegetative parts were tentatively identified by HPLC-DAD-MS^n, comparing the fragmentation patterns with literature data and with standard compounds, when available. Before analysis, the dried extract was dissolved in methanol at a concentration of 5 mg/mL, and the solution was filtered through a 0.45 µm Millipore filter. The HPLC-DAD-MS^n method used was the same as reported in **(Bendif et *al.*, 2017; Peron et *al.*, 2019).**

The HPLC-MS system consisted of a 212 Varian binary pump equipped with a Varian Prostar 430 autosampler, coupled to a Varian MS500 mass detector (Ion Trap), operating in both negative and positive Electrospray Ionisation (ESI) modes for *M. inodora*. However, a chromatographic system composed by an Agilent 1260 binary pump equipped with autosampler and DAD and coupled with a Varian 500 Ion Trap mass detector (MS) was used. MS was

equipped with an Electrospray Ionisation (ESI) source, operating in negative ion mode for *C. candidissimum*.

To achieve the separation of secondary metabolites, an Agilent Eclipse plus C_{18} column (2.1 × 150 mm, 3.5 µm) was used as stationary phase, while a mixture of acetonitrile (A) and 0.1% formic acid in water (B) was used as mobile phase. The elution gradient was as follows: 0 min, 10% A; 20 min, 54% A; 23 min, 100% A, and isocratic up to 32 min. Re-equilibration time was 8 min. Flow rate was 0.2 mL/min. ESI parameters were: needle voltage, ± 4500 V; capillary voltage, 70 V; RF loading, 100%; nebulizing gas pressure, 20 psi (nitrogen); drying gas pressure, 15 psi; drying gas temperature, 350 °C. Data were acquired in the *m/z* range 50–2000. The turbo detection datascanning (TDDS®) function was used to acquire data about the fragmentation patterns of eluted compounds, setting n = 3 levels of fragmentation.

For quantitative purposes, DAD was used for *C. candidissimum*. Flavonoids, phenolic acids and gallic acid derivatives were quantified using linear calibration curves obtained by analysing 0.5-100 µg/mL solutions of rutin, chlorogenic acid and gallic acid, respectively. Calibration curves were, respectively: $y = 51.5x - 183.22$ ($R^2 = 0.998$), $y = 90.54x - 32.84$ ($R^2 = 0.999$), and $y = 109.72x - 68.33$ ($R^2 = 0.999$). Limit of detection (LOD) for rutin, chlorogenic acid and gallic acid were 0.05, 0.02 and 0.02 µg/mL, respectively, while limits of quantification (LOQ) were 0.15, 0.06 and 0.06 µg/mL, respectively.

On the other hand, MS detector was used to achieve triterpenes quantification. The linear calibration curve was obtained by analysing 0.5-100 µg/mL solutions of ursolic acid, and its equation was $y = 651.2x - 26.9$ ($R^2 = 0.998$). LOD and LOQ for ursolic acid were 0.15 and 0.5 µg/mL, respectively.

I.3.9. Evaluation of the biological activities

I.3.9.1. Antioxidant activity

Antioxidant activities of *C. candidissimum* and *M. inodora* methanolic extracts were determined by DPPH free radical scavenging **(Blois, 1958; Tel-Çayan et *al.*, 2015)**, ABTS cation radical scavenging **(Tel-Çayan et *al.*, 2015**; **Re et *al.*, 1999)** and CUPRAC (cupric-reducing antioxidant capacity) assays **(Apak et *al.*, 2004)** spectrophotometrically by using a 96-well microplate reader, SpectraMax 340PC384 (Molecular Devices, Silicon Valley, California, USA). Softmax PRO v5.2 software (Molecular Devices, Silicon Valley) was used

for calculations and measurements of the bioactivities data, stock solutions of the samples (4000 µg/mL) were prepared using methanol (99.9%) as a solvent.

- **DPPH free radical scavenging activity**

The DPPH free radical scavenging activity of *C. candidissimum* and *M. inodora* methanolic extracts was performed using the developed method of **Blois (1958)**. Briefly, 40µl of methanol extracts at different concentrations were added to 160 µl DPPH (0.1 mM in methanol) and incubated for one hour in the dark and then the optical density was measured at 517 nm. BHA was employed as a positive antioxidant. For each sample, the measurements were performed in triplicate.

The DPPH free radical scavenging activity of the extracts was calculated using the formula given as follows:

$$\text{DPPH free radical scavenging activity (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

- **ABTS radical scavenging activity**

The ABTS free radical scavenging activity of *C. candidissimum* and *M. inodora* methanolic extracts was determined according to **Re et *al.* (1999)**. The ABTS was solved in distilled water to make 7 mM concentration, on which 2.45 mM potassium persulfate was added, and the mixture was kept overnight in the dark to prepare the ABTS cation radical. The resulting $ABTS^{\bullet+}$ solution was then diluted with distilled water to obtain 0.70 (±0.01) absorbance at 734 nm using a spectrophotometer (Shimadzu 1601, Japan). In each well of the microplate, 160 µL of this solution is added to 40 µL of sample at different concentrations. Methanol was used as a negative control; BHA was used as standard antioxidant. The absorbance was read at 734 nm after 10 min incubation at room temperature. For each sample, the measurements were carried out in triplicate. The $ABTS^{+}$ free radical scavenging activity of the extracts was calculated using the formula given in the DPPH assay.

- **CUPRAC (cupric-reducing antioxidant capacity) activity**

The cupric Reducing Antioxidant Capacity (CUPRAC) activity of *C. candidissimum* and *M. inodora* methanolic extracts was determined according to the method of **Apak et *al.* (2008)** modified by **Öztürk (2012)**. This method is based on the reduction of Cu^{2+} followed by the treatment of Cu^{+} with a chromogenic reagent Neocuproine (NC) (2,9 -dimethyl- 1,10-phenanthroline) in the presence of phenolic hydroxyls leading to the formation of a stable complex between neocuproin and copper ions (Cu^{+}) that absorbs at 450 nm. A mixture

consisting of 60 µL of NH_4Ac ammonium acetate (1 M, pH=7.0), 50 µL of 7.5 mM neocuproin, and 50 µL of copper chloride ($CuCl_2$, $2H_2O$) 10 mM was prepared, followed by 40 µL of sample solution at different concentrations was added to the initial mixture. After one hour of incubation at room temperature, the absorbance is read at 450 nm. BHA was the standard antioxidant used in this assay. For each sample, the measurements were carried out in triplicate. The results are expressed as absorbances and $A_{0.5}$ values (µg/mL) corresponding to the concentration indicating an absorbance intensity of 0.50 are calculated and compared to those of BHA used as positive control.

I.3.9.2. *In vitro* Anti-Alzheimer activity

In vitro spectrophotometric methods were used to determine acetylcholinesterase (AChE) and butyrylcholinesterase inhibitory (BChE) **(Ellman et *al.*, 1961; Topal, 2020)** activities of *C. candidissimum* and *M. inodora* methanolic extracts by using a 96-well microplate reader, SpectraMax 340PC384 (Molecular Devices, Silicon Valley, California, USA). Softmax PRO v5.2 software (Molecular Devices, Silicon Valley) was used for calculations and measurements of the bioactivities data, Stock solutions of the samples at 2000 µg/mL concentration were prepared using methanol (99.9%) as a solvent.

The Ellman test is a standard protocol for the determination of free thiols, it is based on the cleavage of acetylthiocholine by AChE and butyrylthiocholine by BChE to generate thiocholine which reacts with 5,5'-dithiobisnitrobenzoate (DTNB) to form the 5-thio-2-nitrobenzoate anion of yellow color. In the presence of an enzymatic inhibitor, this reaction allows to study the kinetic parameters and thus to determine the IC_{50} values (concentration decreasing 50% of the enzymatic activity).

In this method, the key substrates for the reaction were iodide acetylthiocholine iodide and butyrylthiocholine chloride, and electric eel AChE and horse serum BChE were used. Briefly, 20 µL of AChE enzyme (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) were incubated for 15 min at 25 °C with 150 µL of buffer with 0.1 M sodium phosphate (pH 8.0) and 10 µL of sample at different concentrations. The reaction was then started by adding 10 µL of iodide acetylthiocholine (7.1×10^{-4} M) or butyrylthiocholine chloride (2×10-4 M) and 10 µL of DTNB (5×10^{-4} M). The absorbance of the yellow 5-thio-2-nitrobenzoate anion produced is measured at 412 nm every five minutes for 15 min. The positive control used was galanthamine at the same concentrations as the samples. The percentage inhibition of AChE and BChE is determined relative to the blank (methanol with phosphate buffer pH=8) by the formula:

[(Enzyme activity without extract - Enzyme activity with the extract)/ Enzyme activity without extract] x100

I.3.9.3. α-Amylase / α-Glucosidase inhibitory activities

The α-amylase / α-glucosidase inhibitory activities of *C. candidissimum* and *M. inodora* methanolic extracts were determined spectrophotometrically **(Kim et *al.*, 2010; Quan et *al.*, 2019)** by using a 96-well microplate reader, SpectraMax 340PC384 (Molecular Devices, Silicon Valley, California, USA). Softmax PRO v5.2 software (Molecular Devices, Silicon Valley) was used for calculations and measurements of the bioactivity data.

- **Determination of α-amylase inhibitory activity**

α-Amylase inhibitory activity of the extracts was tested by using the method previously reported by **Quan et *al.* (2019)** with slight modifications in the use of incubation time, reagents and amounts of the used reagents and samples. 25 µL sample solution and 50 µL α-amylase solution (0.1 units/mL) in phosphate buffer (20 mM pH=6.9 phosphate buffer prepared with 6 mM NaCl) were mixed in a 96-well microplate. The mixture was pre-incubated for 10 min at 37 °C. After pre-incubation, 50 µL starch solution (0.05 %) was added and incubated for 10 min at 37 °C. The reaction was completed by addition of 25 µL HCl (0.1 M) and 100 µL Lugol solutions. 96-well microplate reader was used to measure absorbance at 565 nm. Acarbose was used as standard. The sample concentration providing 50 % inhibition activity (IC_{50}) was calculated from the graph of α-amylase inhibitory activity against sample concentrations.

- **Determination of α-glucosidase inhibitory activity**

α-Glucosidase inhibitory activity of the extracts was determined using the method previously reported by **Kim et *al.* (2010)** with slight modifications in the use of incubation time, reagents and amounts of the used reagents and samples. 50 µL phosphate buffer (0.01 M pH=6.9), 25 µL PNPG (4-N-nitrophenyl-a-D-glucopyranoside) in phosphate buffer (0.01 M pH=6.9), 10 µL sample solution and 25 µL α-glucosidase (0.1 units/mL) in phosphate buffer (0.01 M pH=6.0) were mixed in a 96-well microplate. The mixture was incubated for 20 min. at 37 °C. 90 µL sodium carbonate (0.1 M) was added into the microplate to end the reaction. A 96-well microplate reader was used to measure absorbance at 400 nm. Acarbose was used as standard. The sample concentration providing 50 % inhibition activity (IC_{50}) was calculated from the graph of α-glucosidase inhibitory activity against sample concentrations.

I.3.9.4. Determination of antimicrobial activities

Antimicrobial activity of *C. candidissimum* and *M. inodora* methanolic extracts were determined by disc diffusion test according to a modification method described by **Nicoletti et *al.* (2012),** following the Clinical and Laboratory Standards Institute (CLSI) guidelines **(CLSI, 2009)**, and all the equipment used was sterilized in autoclave at 121°C for 15 min.

The antibacterial activity was screened against several bacteria which are reference strains of the American Type Culture Collection (ATCC): $Gram^+$ (*Staphylococcus aureus* (MRSA) ATCC 34300, *Staphylococcus aureus* ATCC 6538 and *Bacillus cereus* ATCC 14579), $Gram^-$ (*Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9027) and a yeast (*Candida albicans* ATCC 10231 (CA_2) and *Candida albicans* ATCC 10237 (M_3)), the morphological particularity of these bacterial strains is summarized in **Table 01**.

Bacterial strains were subjected to a continuous overnight subculture at 37°C in nutrient agar and incubated at 37°C for 24 h to optimize their growth, streaked to ensure purity (in order to obtain a young culture and isolated colonies). *C. albicans* were grown on Sabouraud Dextrose Agar (SDA) for 24 h in petri dishes at a temperature of 37°C.

Briefly, cells were resuspended in saline ($1–2x10^8$ cells/mL for bacteria (0.5 Mc Farland) and 10^6 cells/mL for *Candida*) and spread on the petri dishes of Mueller-Hinton Agar (MH) for bacteria and Sabouraud Dextrose Agar (SDA) for *C. albicans* using a sterile cotton swab. Sterile whatman paper discs (6 mm in diameter) were placed on the surface of inoculated petri dishes and spotted with 10µL of the extract (we prepared a concentration range of 50 mg/mL, 100 mg/mL and 200 mg/mL solubilized in dimethylsulfoxide (DMSO)). The petri dishes of Mueller-Hinton Agar (MH) were incubated 24 h at 35 ± 1°C for bacteria and 24 h at 37°C for *C. albicans* were grown on Sabouraud Dextrose Agar (SDA).

The activity was done in triplicate and it was determined by measuring the diameter of the growth inhibition zone (inhibition zone diameter, IZD) visible around the paper disc and compared with reference diameters related to the antibiotics used. Reported IZDs are inclusive of the paper disc diameter (6 mm). IZDs higher than 6 mm indicated growth inhibition, while IZD =6 mm meant no activity. Indeed, the sensitivity of a germ is non-

existent for a diameter inferior or equal to 6 mm. It is limited for a diameter between 6 and 14 mm, and average for a diameter between 14 and 20 mm. For a diameter greater than or equal to 20 mm the germ is very sensitive **(Duraffourd et *al.*, 1990)**. Negative controls were set using wattman disks impregnated with DMSO (10 µL disks), and the positive controls were set up with: Amoxicillin (AMC) 30 µg, Cefazolin (CZ) 30 µg, Ceftriaxon (CRO) 30µg, Cefoxitin (FOX) 30µg and Amphotericin B (AMB) 20µg.

- **Determination of Minimum Inhibitory Concentration (MIC), Minimal bactericidal concentration (MBC) and Minimal fungicidal concentration (MFC)**

The minimum inhibitory concentration (MIC) was evaluated by a broth microdilution method according to **Okusa et *al.* (2007);** the plant extract was dissolved in DMSO (20 mg/250 µL) and diluted to 5 mL with Mueller-Hinton broth, the final DMSO concentration being 5%. This solution was transferred in 96-well plates (200 µL/well) and serially diluted with Mueller-Hinton broth. After incubation period (22h), 40 µL of a 2 mg/mL Triphenyl tetrazolium chlorid (TTC) indicator solution (indicator of microorganism growth) was added to every well and the plate was incubated at 37 °C for about 2 h **(Hammer et *al.*, 2003)**. The TTC indicator solution changes from clear to purple in the presence of bacterial activity. Whereas it remains clear when microbial growth was inhibited. The MIC was defined as the lowest concentration of methanolic extacts that showed no visible bacterial growth after incubation time (no color change (clear) of TTC).

The minimal bactericidal concentration (MBC) and Minimal fungicidal concentration (MFC) was determined by plating directly the content of wells with concentrations higher than the MIC value. The MBC and MFC values was determined when there was no colony growth from the directly plated contents of the wells. The MBC and MFC was considered as the lowest concentration of methanolic extacts that killed 99.9% of microorganisms in culture on the agar plate after incubation period.

The MBC/MIC and MFC/MIC ratios was also calculated to show the nature of antibacterial effect of methanolic extracts. When the ratio was less than or equal to 4, the methanolic extract was considered as bactericidal or fungicidal and when the ratio was higher than 4, it was considered as bacteriostatic or fungistatic methanolic extract **(Levison, 2004)**.

Table 01. Strains tested during the determination of antimicrobial activity

Strains		References	Preferential habitat	Pathogenicity
Gram$^+$	*Staphylococcus aureus* (MRSA)	ATCC 34300	Nose, back of the throat, armpits, skin folds of the groin, wounds, blood, urine	skin infections, bloodstream infections, surgical-site infections, and pneumonia, arthritis, pneumonia, sepsis
	Staphylococcus aureus	ATCC 6538	Skin, hair, dust, air mucous membranes, nasal cavity, contaminated food Nasopharynx Perineum	Skin infections, wounds burns, abscesses Osteitis, osteomyelitis Endocarditis Septicemia Pulmonary infections foodborne infections, nosocomial infections
	Bacillus cereus	ATCC 14579	Soil food, and marine sponges	Responsible of foodborne illnesses, causing severe nausea, vomiting, and diarrhea, skin infections
Gram$^-$	*Escherichia coli*	ATCC 8739	Fecal matter Contaminated food Wastewater	Urinary tract infections Wounds Septicemia Respiratory infections food infections, nosocomial Gastroenteritis
		ATCC 25922		
	Pseudomonas aeruginosa	ATCC 9027	Soil, water, plants Respiratory tracts Fecal matter	Pulmonary infections, Burns Wounds Septicemia food infections, nosocomial infections Eye, urine, gastrointestinal and pulmonary infections
Yeasts	*Candida albicans*	ATCC 10231 (CA_2)	Digestive, respiratory and genital mucosa, Digestive tract	Septicemia, Candidiasis, Visceral infections superficial candidiasis, skin infections
		ATCC 10237 (M_3)		

MRSA: Methicillin-resistant *Staphylococcus aureus*

ATCC: American Type Culture Collection (ATCC)

I.3.10. Statistical analysis

- The data from ethnobotanical survey, phytochemical screening, extractions yields, antibacterial activity, total polyphenols and flavonoids content were subjected to statistical analysis using the Microsoft Excel 2013 software and presented as mean ± SD of three replicate.
- The results of the activity assays (antioxidant, *in vitro* anti-Alzheimer (AChE) and (BChE), α-Amylase / α-Glucosidase inhibitory activities) and mineral contents were given as means ± standard error meaning. The results were evaluated using an unpaired t-test and one-way analysis of variance ANOVA. The differences were regarded as statistically significant at $p<0.05$. Minitab 16.2.1. Statistical software (MINITAB Inc, 2010), and Softmax PRO v5.2 software (Molecular Devices, Silicon Valley) were used for all calculations and measurements of the bioactivity data.

Chapter II
Results and discussion

II. Results and discussion

II.1. Ethnobotanical survey

Medicinal plants have been used by humans for the treatment of various diseases for thousands of years from past to present. Species of the *Lamiaceae* family are among the most used medicinal plants due to the wide variety of secondary components they contain, this study investigates ethnobotanical survey of two plants *Lamiaceae* native of Western Algeria among traditional practitioners, herbalists and people who use or sell medicinal plants in this area. It is the case of *Micromeria inodora* (Desf.) Benth and *Clinopodium candidissimum* (Munby) Kuntze.

II.1.1. Sociodemographic profile of the informants

- **Gender distribution**

In this study, one hundred people with ethnobotanical knowledge have been investigated. Among them, we find both men and women but it is men rating (74%), who are the most represented **(Figure 16).** Women are less represented with (26%); this finding is in good agreement with those reported in **Bouredja et *al.* (2020)**. The predominance of men can be justified by local customs and traditions, as this is probably a profession reserved for men. It seems that this situation can be generalized to the whole of North Africa in the majority of the studies consulted, with the exception of a few, notably that of **Ait ouakrouch (2015)** in Morocco with a percentage of 82% men. Other authors including **Boudjelal et *al.* (2013)**, report also a dominance of men among the people surveyed and they attribute this bias to the traditions and culture of the region where by women should not converse with strangers. This does not necessarily mean that men know more about medicinal plants than women, but reflects how access to information is influenced by the traditions of the community.

In the survey field, while women and men are equally responsible for the collection of medicinal plants, women do the drying, storage and preparation of recipes for the care of family members and the men reserve the task of collecting the plants in areas that are considered dangerous.

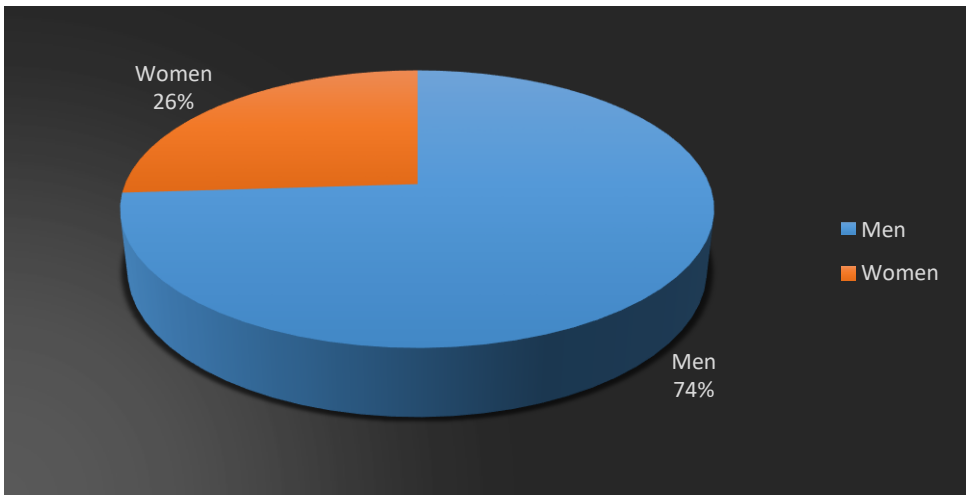


Figure 16. Distribution of the frequency of participants in the ethnobotanical survey by gender

- **Age distribution**

The distribution of informants with respect to age class shows the predominance of the age class of [30-40[years (39%). The age groups [40-50[, [50-60[, [20-30[, [60-70[and [70-80[are represented with respectively percentages: (23 %), (11%), (10%), (7%) and (6%). People >80 years had a percentage of (4%), while the very young (<20 years) were not represented **(Figure 17)**.

The majority of ethnobotanical studies in North Africa consulted report a predominance of interviewees with ages between 30 and 60 years old, illiterate people seem to be most knowledgeable about medicinal plant uses **(Hammiche and Maiza, 2006; Boudjelal et *al.*, 2013; Eddouks et *al.*, 2016; Lakhdari et *al.*, 2016; Ouelbani et *al.*, 2016; Miara et *al.*, 2018)**.

Generally, the use of plants in Algeria is spread at all the ages, old people are supposed to provide more reliable information, because they hold a good part of the ancestral knowledge that is transmitted orally. The transmission of this knowledge is currently in danger because it is not yet assured **(Anyinam, 1995)**.

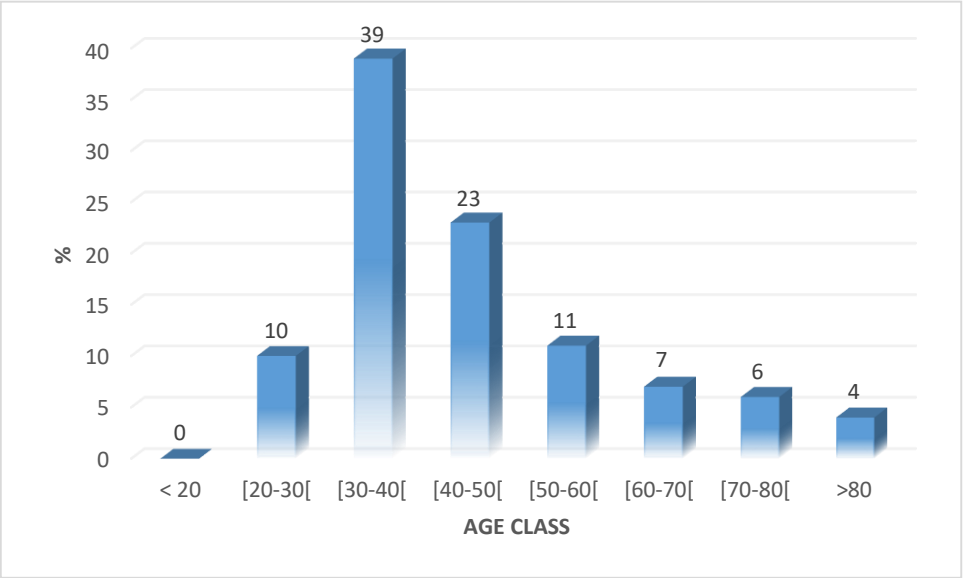


Figure 17. Distribution of the frequency of participants in the ethnobotanical survey by age

- **Level of education distribution**

Result shows that people with secondary level represent the highest rate (44%) followed by university level (28%), primary level (15%) and illiterate (13%) **(Figure 18)**, Similar findings were found in those reported in **Bouredja et *al.* (2020)**. This shows that there is a relationship between the educational level of informants and their ethnobotanical knowledge, as educational level increases ethnobotanical knowledge decreases, illiterate people seem to be most knowledgeable about medicinal plant uses this is probably due to the desire of young people to try natural therapies **(Boudjelal et *al.*, 2013; Miara et *al.*, 2018)**.

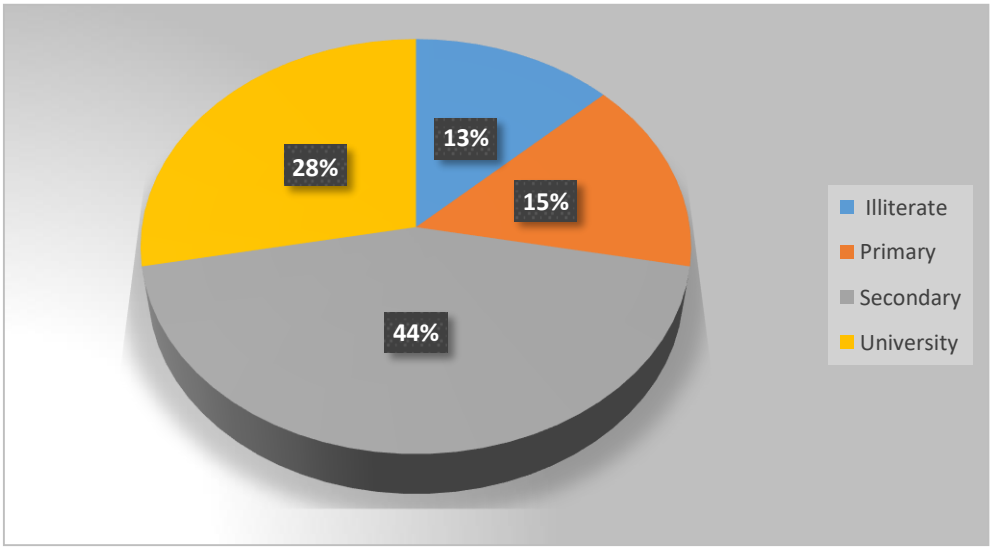


Figure 18. Distribution of the frequency of participants in the ethnobotanical survey by academic level

II.1. 2. Ethnobotanical survey result of *Micromeria inodora* (DESF.) Benth.

- **Vernacular name**

Study participants did not know *Micromeria inodora*, they confused this plant with others species and thought it was "*Zaitra*" =*Thymus ciliatus* (64%), Some of them thought it was a "*Chih*" = *Artemisia herba alba* (22%), others thought it was a "*Djertila*" (*Thymus Hirtus*, *Thymus Algeriensis*, *Thymus Ciliatus*) (2%), and others (30%) did not know the plant at all **(Figure 19)**.

In Algeria, this plant is known among the ancient population under the vernacular name of «Tazira hmeur» in Berber or « Zaeter el Hamir » in Arabic **(Quézel and Santa, 1963)**.

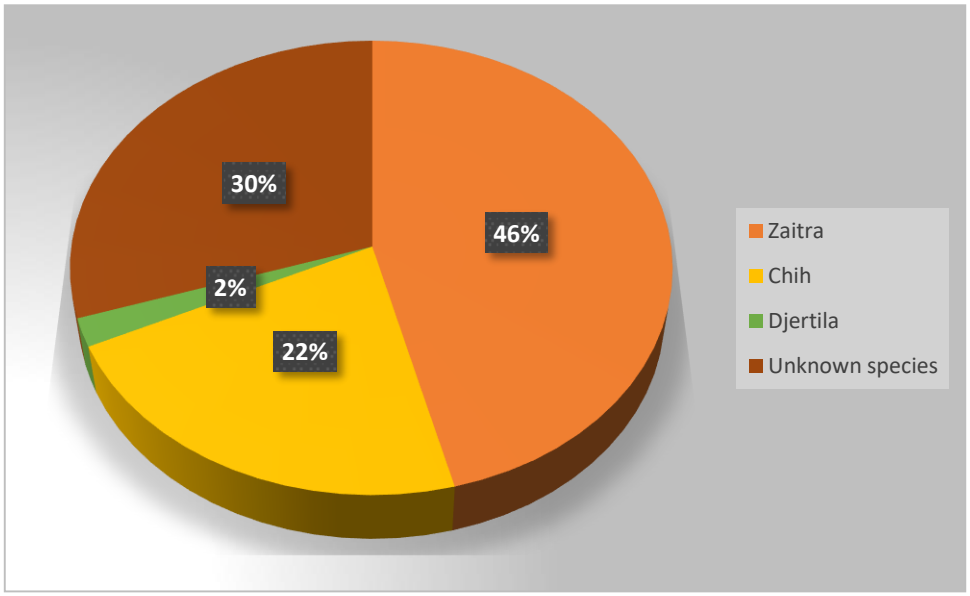


Figure 19. Distribution of frequency of vernacular name of *M. inodora*

Micromeria inodora was unknown to all the participants of this ethnobotanical survey, they confused it with other species. For this reason, the other section of this survey could not be discussed because informants didn't know about it.

In the literature, some promising medicinal uses have been reported for *Micromeria inodora* leaves decoction. They have been generally used in folk medicine as a substitute for mint, notably in the treatment of wounds, stomachache, colds, and fevers and as condiment **(Benomari et *al.*, 2016)**.

It's well known that *Micromeria* is part of a complex group of genera that comprises herbs, subshrubs, and shrubs with medicinal value, used in popular medicine against colds and

fever, heart disorders, headache, wound, skin infections, and as expectorant **(Formisano et *al*., 2014)**.

Micromeria genus are traditionally used as herbal teas and flavoring agents in the preparation of local foods in many parts of Turkey and also as an alternative for mint in the Turkish folk medicine **(Kirimer et *al*., 1993a; Kirimer et *al*., 1993b; Tabanca et *al*., 2001; Duru et *al*., 2004).** *Micromeria* species are generally consumed as tea herbs and they are broadly used as aromatic culinary supplements and also possess medicinal value due to their bioactive secondary metabolites and essential oils **(Lawrence, 1989, JoséPérez-Alonso et *al*., 1996)**. Other authors report also different species uses of this genus such as its ability to remove kidney stones and as powerful remedies against indigestion, skin burn and infections, cold, stomachache, headache, liver, heart and pulmonary diseases, toothache, eye inflammation along with chest pains in Turkey **(Baytop, 1984; Ali- Shtayeh et *al*., 1997; Başer et *al*., 1997; Tabanca et *al*., 2001; Bukvicki, et *al*., 2015)**. In traditional medicine, they have been used to treat colds and fever, heart disorders, headaches, wounds and skin infections **(Ali-Shtayeh et *al*., 1998; Telci and Ceylan, 2007; Formisano et *al*., 2014)**. They were also reported to have anti-inflammatory, antispasmodic, stimulating, antibacterial **(Ali-Shtayeh et *al*., 1997; Duru et *al*., 2004, Stojanović et *al*., 2005, Formisano et *al*., 2007; Abdelwahab et *al*., 2015; Brahmi et *al*,. 2017)**, antioxidative and biological effects **(Couladis et *al*., 2003; Güllüce et *al*., 2004; Benomari et *al*., 2016)**.

In some African countries like Algeria, a decoction prepared from the aerial parts of different *Micromeria* species can relieve painful stomachache and act as a strong herbal drug against cough, cold and fever. Moreover, it can improve and effectively heal infectious wounds, and its dried parts have been recommended as condiments for culinary purposes **(Brahmi et *al*., 2017)**.

II.1. 3. Ethnobotanical survey result of *Clinopodium candidissimum* (Munby) Kuntze

- **Vernacular name**

The majority of the participants in the study **(Figure 20)** know *C. candidissimum* by Nabta elbida (87%), while (5%) thought that it was Fliou (*Mentha pulegium*) and others (8%) did not know it at all.

In Algeria, this plant is known among the ancient population under the vernacular name of «Zaater cheleuh» and «Nabta elbida» **(Quezel and Santa, 1962)**.

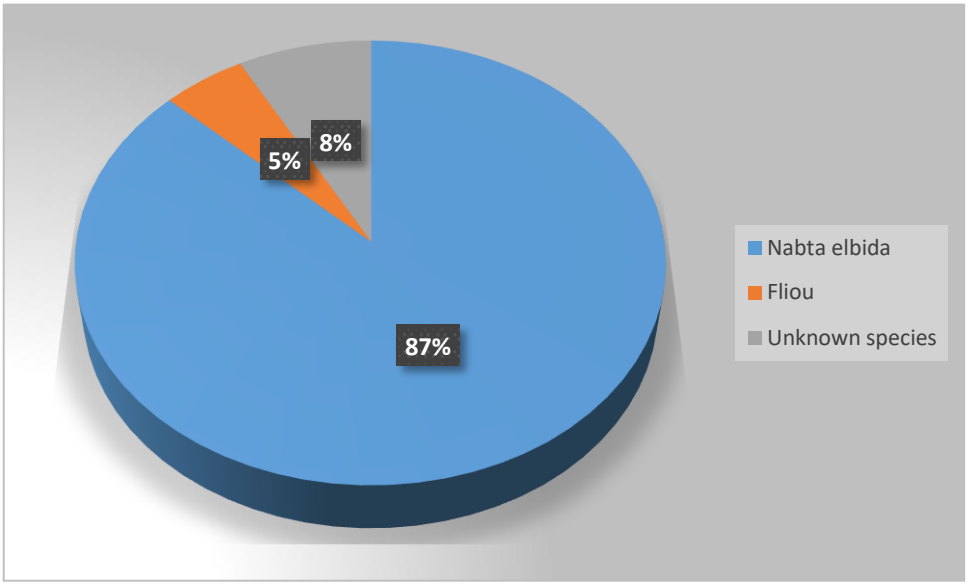


Figure 20. Distribution of frequency of vernacular name of *C. candidissimum*

- **Habitat and distribution, used Parts in preparation of remedies, harvested or purchased, method of preparation, administration mode and dosage use of *C. candidissimum***

All informants in this survey said that the plant is found in the region of Oran (87).*C. candidissimum* is a strictly endemic species of Algeria; it grows between oleanders and rocky lawns in Oran and its surroundings **(Quezel and Santa, 1962)**. The most widely used plant part of *C. candidissimum* for the preparations of remedy were aerial part, which accounted for (100%). Bioactive compounds are contained in the plant organs with different concentrations; people use the aerial part based on their traditional heritage that can explain the most uses of this part by the local population. All participants in this survey use only one technique which is infusion, the use of infusions is also a common mode of preparation **(Ouelbani et *al.*, 2016)**. The oral way is the only mode of administration used because it represents the simplest, most effective and rapid mode of administration, similar results were observed in ethnobotanical study in Morocco with (77%) **(El hafian et *al.*, 2014).** All participants buy the plant and use a small amount until healed **(Table 02)**.

Table 02. Distribution of frequency of habitat and distribution ,plant parts used in preparation of remedies, harvested or purchased, method of preparation, administration mode and dosage use of *C. candidissimum*

Parameters	Response	Number of citations
Habitat and distribution	Oran	87
Part used	Aerial	87
Harvested or Purchased	Purchased	87
Method of preparation	Infusion	87
Administration mode	Oral	87
Dosage	a small amount until healed	87

- **Therapeutic use**

Participants said that they use the plant in the same way as *Clinopodium nepeta*. In our research area, the therapeutic uses of *C. candidissimum* **(Figure 21)** was recorded especially to treat influenza, cough and rheum (92), respiratory diseases (2)**,** rheumatism (2). It is also used as a depurative plant that is especially suggested for purifying the blood (2) and warming the body (2).In addition to these therapeutic uses, it has other culinary uses such as the preparation of hrira herbs and is consumed as herbal tea.

C. candidissimum was studied for the first time by **Attou (2017)**, where a survey on its traditional uses was conducted. This plant called by the local population in western Algeria by "Nabta el bida" was effective in cases of flu and fever, gastrointestinal disorders, intestinal worms, antimicrobial and antifungal infections, and as a dressing for the healing of burns and wounds. It is used in cooking to flavor sauces, as it has a pleasant smell; or to prepare a traditional Algerian dish from the Sid safi region (Aïn Témouchent city in north-western Algeria), called "Rfiss" which is a preparation with semolina, butter, salt, water and the fresh cut plant, after cooking it is cut into pieces and mixed with milk or melted butter, the same recipe can be made with *Satureja calamintha* Scheele. *ssp*. *nepeta*. In Italy, *C. nepeta* is used for insect bites.

Several studies were found that species belonging to the family of *Lamiaceae* are used commonly to treat different diseases in many regions of the world. In the Mediterranean regions, the plants of genus *Calamintha* (syn. *Clinopodium*) are previously used as alimentary and medicinal plants **(Tuttolomondo et *al.*, 2014)**.

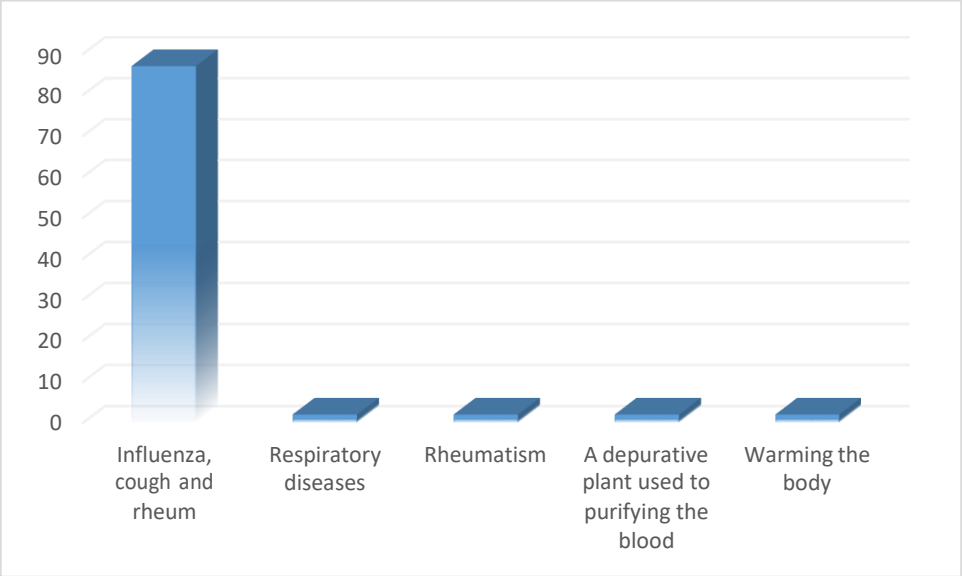


Figure 21. Distribution of different therapeutic use of *C. candidissimum*

Previous ethnobotanical studies from north Algeria indicated *Lamiaceae* as the family with the most medicinal plants cited **(Benarba et *al.* 2015; Boudjelal et *al.* 2013; Chermat and Gharzouli 2015; Meddour and Meddour-Sahar 2015; Miara et *al.* 2013; Miara et *al.* 2019).** Based on these reports and the current one, *Lamiaceae* could be considered the most botanical family explored in phyto-folk medicine in north Algeria.This work constitutes a source of information, which can serve as a basis for pharmacological studies in order to evaluate the therapeutic efficacy and safety of these two plants with traditional biological effect. There was a lack of information among herbalists regarding the adverse effects and potential toxicity of plants.

Currently, ethnobotanical studies on the traditional use of wild and cultivated plants have become increasingly popular and have contributed to bioeconomics and green knowledge. In all countries, different cultures have established their own ethnobotany systems based on beliefs that plants are a healthy and natural source of life and a cure for all people **(Edeoga et *al.*, 2005).** Algerian pharmacopoeia is an integral part of the local culture, which has continued to the present by passing from generation to generation among healers and herbalists through traditional scriptures; and clinical experience collected over hundreds of years. However, this knowledge still counted as traditional because it could not be formalized in forms or codex **(Boudjelal et *al.*, 2013)**. A successful way to keep the culture, knowledge and the plants associated with them relevant is to use the resulting knowledge and give it a new life by integrating traditional medicine into the modern health system using ethnobotany and ethnopharmacology.

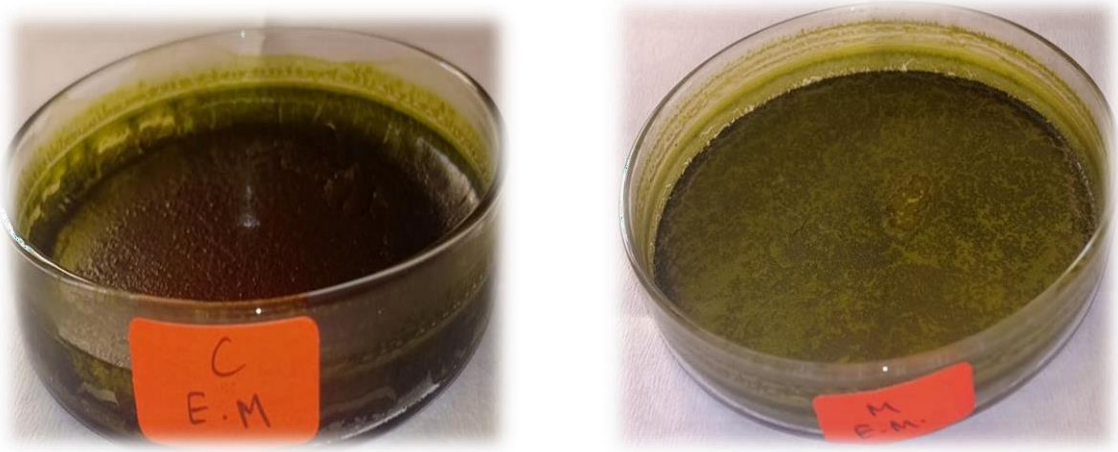
II.2. Phytochemical study

II.2.1. Characteristics of plant extracts

Extraction yield of the studied plants is represented in **Table 03**, it is expressed as a percentage relative to the initial mass of the plant subjected to the extraction. The methanolic extracts characteristics **(Figure 22)** was found to be brown solid powder extract with a yield of 12.38% for *M. inodora* and green solid crystal extract with a yield of 18.64% for *C. candidissimum*. Essential oils **(Figure 23)** were obtained from fresh aerial parts by hydrodistillation yielded (w/w) 0.1% for *M. inodora* and 1.9% for *C. candidissimum*.

Table 03. Plant extracts characteristics of *M. inodora* and *C. candidissimum*

Plant extracts		Characteristics	Color	Yield (%)
methanolic extract	*M. inodora*	Solid powder	Brown	**12.38**
	C. candidissimum	Solid crystal	Green	**18.64**
essential oil	*M. inodora*	Pungent smell	Transparent	**0.1**
	C. candidissimum	Fragrant and strong smell	Light yellow	**1.9**



a- *C. candidissimum* **b-** *M. inodora*

Figure 22. Physical appearance of methanolic extracts

b- *C. candidissimum* **b-** *M. inodora*

Figure 23. Essential oils of plants studied

Comparing our results with other studies, **Medair and Djelloul benchrif (2019)** revealed that the methanolic extract yield of *C. candidissimum* from Ain temouchent region was higher than our result (28.75%). Our extraction yield was higher than that found in the methanolic extract yield of *M. inodora* from Tlemcen region in Algeria (3,84±0,52 %) **(Adjdir, 2022)**. Other research works have shown weaker extraction yields than ours; **Bougandoura (2011)** found a yield of (8.58%) in *Satureja calamintha* extract and extraction values of (14.6 and 12%) were obtained for *S. calamintha subsp. nepeta* from the Annaba and Jijel regions, respectively (**Labiod, 2016)**.

These variations in the yield of extraction is dependent on several factors that can influence the performance of extraction, such as particle size, nature of the solvent, solvent quantity/volume ratio, techniques used and extraction conditions (pH, temperature, extraction time and the degree of agitation) which affect total phenol and flavonoid levels **(Lee et *al.*, 2003).**

Our yield value of *M. inodora* essential oil is consistent with that reported by **Benomari et *al.* (2016),** The yield range of yellow essential oils obtained by hydrodistillation from fresh aerial parts of *M. inodora* from western Algeria collected at the flowering stage from 24 locations widespread in the littoral of Tlemcen region was (w/w) 0.15 – 0.80%.Thus, *C. candidissimum* essential oil yield was higher than several studies, which report that *C. candidissimum* samples collected from Ain-Temouchent region give a yields range of essential oils from 0.93 to 1.6% **(Attou, 2017; Benyoucef et *al.*, 2020).**

This difference between the extraction yields obtained for the different species is due to climatic factors (heat, cold), geographical (altitude, nature of the ground, rate of exposure to the sun), extraction method and the nature of plants (harvest period and part used) which can influence the performance of extraction.

In our research study, essential oils biological activities was not evaluated because of the low yield (0.1%) of *M. inodora*, we did only the essential oils extraction of the studied plants by hydrodistillation and the GC/MS analysis.

II.2.2 Phytochemical Screening

Phytochemical screening results performed on the methanolic extracts of the two species studied were shown in **Table 04** and **Appendix II**. These phytochemical tests carried out allowed us to have a general idea of the chemical composition of the studied plants.

Table 04. Phytochemical screening results of *M. inodora* and *C. candidissimum* methanolic extracts

Plant / Compounds	*M. inodora*	*C. candidissimum*
Phenolic compounds	++	+++
Flavonoids	+	+++
Tannins	+ (Catechic)	+++ (Catechic)
Quinones	+++	++
Anthraquinones	-	+
Terpenoids	+++	+
Saponins	+	++
Reducing compounds	-	++

(+++): strongly positive reaction, (++): positive reaction, (+): weakly positive reaction, (-): negative reaction

Phytochemical screening performed on the methanolic extract of *M. inodora* revealed the presence of flavonoids, quinones, terpenoids, phenolic compounds, saponins and catechic tannins. We also noted the absence of anthraquinones and reducing compounds. Similar insights were found in those reported in **Adjdir (2022),** the phytochemical screening carried out on the extracts of *M. inodora* found the presence of flavonoids, tannins, alkaloids, saponosides and quinones, the absence of terpenoids, coumarins and anthraquinones was detected. **Bechiri-**

abbes (2016), report the presence of flavonoids, catechic tannins, alkaloids, saponosides and quinones in *M. inodora* extracts. Terpenoids, anthraquinones and reducing compounds were absent.

On the other hand, the qualitative analysis executed on the *C. candidissimum* methanolic extract indicate the presence of phenolic compounds, flavonoids, catechic tannins, quinones, saponins, terpenoids, anthraquinones and reducing compounds. Our findings are in good agreement with **Medair and Djelloul benchrif (2019)**, which revealed the presence of flavonoids, tannins, triterpenes, reducing compounds, saponosides and coumarins in the aerial part of *C. candidissimum* growing in western of Algeria Sidi Safi region Aïn-Témouchent city.

Relatively comparable results was found in several previous investigations carried out on the genus *Satureja* demonstrate the presence of flavonoids, tannins, phenolic acids and saponins **(De Pooter and Schamp, 1986; Perez-Alonso et *al.*, 1993; Ristorecelli et *al.*, 1996; Ildiko et *al.*, 2009; Varban et *al.*, 2009; Bougandoura and Bendimerad, 2013; Benkhedimallah and Kismun, 2014)**.

Our data show the richness of *C. candidissimum* and *M. inodora* with chemical substances and may represent a new potential source of bioactive compounds in therapy.

II.2.3. Mineral contents

Many studies focused on the phytochemical screening of *C. candidissimum* and *M. inodora* but to the best of our knowledge, there are no published reports on the mineral quantifications. It is worth mentioning that here, the elemental mineral contents of the studied plants aerial parts is reported in our work for the first time. The mineral composition was determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) results were shown in **Table 05**.

The analyses result was established to give nutrient values per (%) of dried weight for the macro-elements minerals (phosphorus, potassium, calcium and magnesium), and per ppm (parts per million) of dried weight for the micro-elements minerals (iron, copper, manganese, zinc and boron). Our finding revealed that the studied plants contains high amounts of minerals particularly iron, manganese, zinc, boron, copper, calcium and potassium. Iron was the most abundant micro-element in both aerial part of the studied plants, with concentration of (8243,02 ±164,86 ppm) for *M. inodora* and (2142,47 ±42,85 ppm) for *C. candidissimum*, followed by manganese, zinc, boron, copper (159,51 ±6,38; 87,48 ±2,62; 28,17 ±0,56 and 21,71 ±1,09 ppm)

for *M. inodora* and(88,15 ±3,53; 76,94 ±2,31; 23,93 ±0,48 and 12,4 ±0,62 ppm) for *C. candidissimum*, respectively.

Calcium was the most abundant macroelement in both aerial part of the studied plants, with a concentration of 2,52 ±0,25 % for *C. candidissimum* and 2,08 ±0,21% for *M. inodora*, followed by potassium, with a concentration of 1,34 ±0,04% for *C. candidissimum* and 0,58 ±0,02% for *M. inodora*. While phosphorus and magnesium concentrations were less than 0,4 % in both aerial part of the studied plants.

Statistically, the results of *C. candidissimum* and *M. inodora* mineral contents were significant for phosphorus iron, manganese, zinc, boron and potassium. However, Calcium, magnesium and copper contents are statistically not significant.

Table 05. Mineral contents of the aerial parts of *M. inodora* and *C. candidissimum*

Plants / **Mineral contents**	***M. inodora***	***C. candidissimum***
Phosphorus (%)	0,08 ±0,00*	0,18 ±0,00*
Potassium (%)	0,58 ±0,02*	1,34 ±0,04*
Calcium (%)	2,08 ±0,21	2,52 ±0,25
Magnesium (%)	0,25 ±0,02	0,38 ±0,03
Iron (ppm)	8243,02 ±164,86*	2142,47 ±42,85*
Copper (ppm)	21,71 ±1,09	12,4 ±0,62
Manganese (ppm)	159,51 ±6,38*	88,15 ±3,53*
Zinc (ppm)	87,48 ±2,62*	76,94 ±2,31*
Boron (ppm)	28,17 ±0,56*	23,93 ±0,48*

*****Values expressed herein are mean ± SEM of three parallel measurements $p<0.05$

The minerals mentioned above have a significant role in the functioning of the human body and this makes of *C. candidissimum* and *M. inodora* a good candidate for nutritional supplement products. More precisely, iron plays a crucial role in preventing anemia by synthesizing hemoglobin and myoglobin. It participates in many metabolic processes, including respiration and DNA synthesis; it is also responsible for enzymes formation and other iron-containing enzymes **(Abbaspour et *al.*, 2014)**. Calcium is an essential element for building bones and teeth and ensuring the proper functioning of muscles and nerves **(Beto, 2015)**. Potassium has a role in protecting against hypertension and improving bone health, it is an essential dietary nutrient and constitutes about 70% of the positive ions in the cells and is fundamental for regulating the acid-base and water balance of the cells. It is erucial to heart and

smooth muscle contraction, making it important for normal digestive and muscular function, while phosphorus is essential for skeletal mineralization **(Tel-Çayan et *al.*, 2017).**

Besides, Magnesium is involved in glucose homeostasis and has a significant impact on diabetes control **(Pham et *al.*, 2007**). It is a cofactor for many metabolic reactions and plays a vital role in bone mineralization and muscle relaxation **(Al Alawi et *al.*, 2018)**.

It is worth mentioning that manganese, zinc, copper are involved in tissue, cellular and subcellular functions, including immune regulation through humoral and cellular mechanisms, nerve conduction, muscle contractions, regulation of membrane potential, mitochondrial activity, and enzymatic reactions **(Siddiqui et *al.*, 2014)**, copper and zinc play a key role in maintaining antioxidant defenses **(Spears and Weiss, 2008; Negi et *al.*, 2012)**.

All of the minerals and trace elements measured are micronutrients that are essential for normal body function as they are beneficial for physiological functions **(Calabrese et *al.*, 1985)**. These elements are involved in many biochemical reactions; they are present as stabilizing components of enzymes and proteins and function as cofactors for many enzymes **(Siddiqui et *al.*, 2014)**.

Indeed our results obtained are agree with many studies previously mentioned that other plants from the same botanical family (*Lamiaceae*) such as oregano, thyme, rosemary and *M. fruticosa*; are riche in mineral compounds **(Kirimer, 1992; García-Galdeano et *al.*, 2020; Al-Nuri et *al.*, 2022)**. Our findings corroborated previous studies on the mineral content of *Thymus vulgaris*, as the Turkish variety of this plant also included highest concentrations of calcium, iron, potassium and magnesium, when compared to thirty-two Turkish medicinal herbs **(Özcan, 2004)**. Other studies revealed also the richness of *Thymus* species in mineral compounds, it was revealed that *T. zygis* subsp. *gracilis, T. pallidus, T. willdenowii* containing numerous mineral compounds with certain variability depending on plant species and plant parts **(Ouknin et *al.*, 2018)**. Moreover, other species belonging to *Thymus* genus such as *T. capitatus* and *T. broussonetii* containing different mineral compounds **(Kandil et *al.*, 1994)**. The variability of these components depends to different factors such as the genetic of specie, plant parts used, and climatic changes **(Bouyahya et *al.*, 2020; Mrabti et *al.*, 2021; Naceiri Mrabti et *al.*, 2021)**.

The mineral content of the samples belonging to different species of the same genus be different; it can be noted that it changes depending on genetic factors, geographical location, climatic factors, vegetation period, air pollution, and environmental factors.

II.2.4. Total phenolic and flavonoids contents

Total phenolic content of the crude methanolic extracts of the studied plants was determined by the Folin-Ciocalteu method, referring to the calibration curve of gallic acid used as standard **(Appendix III)**. The content are expressed in µg equivalent of gallic acid/mg of extract (µg $GA_{eq}.mg^{-1}Ext$), and determined by the equation of type: y = 0.010x + 0.0061 knowing that R^2 = 0.999.

The determination of flavonoids content in the methanolic extracts was carried out using the aluminum trichloride ($AlCl_3$) method, referring to the calibration curve of quercetin used as standard **(Appendix III)**. The content are expressed in µg quercetin equivalent/mg of extract (µg $Q_{eq}.mg^{-1}Ext$). The flavonoid levels of crude methanolic extracts of plants studied were obtained from the calibration curve, which follows an equation of the type: y = 0.0375x + 0.0069 knowing that R^2 = 0.999.

Total phenolic and flavonoids contents in the methanolic extracts of *M. inodora* and *C. candidissimum* were presented in **Table 06**. Results shows that the total phenolic and flavonoids contents was found to be 63.33 (µg $GA_{eq}.mg^{-1}Ext$) and 11.19(µg $Q_{eq}.mg^{-1}Ext$) for *M. inodora*; 58.79(µg $GA_{eq}.mg^{-1}Ext$) and 40.93(µg $Q_{eq}.mg^{-1}Ext$) for *C. candidissimum*, respectively.

Table 06. Total phenolic and flavonoids contents in the methanolic extracts of *M. inodora* and *C. candidissimum*

Extract / Contents	*M. inodora*	*C. candidissimum*
Total phenolics (µg $GA_{eq}.mg^{-1}Ext$)[a]	63.33	58.79
Total flavonoids (µg $Q_{eq}.mg^{-1}Ext$)[b]	11.19	40.93

(a): Microgram of Gallic Acid Equivalent per milligram of extract
(b): Microgram of Quercetin Equivalent per milligram of extract

Our findings are in disagreement with the previous studies which report higher values than our obtained results. **Adjdir (2022),** show that total phenolic and flavonoids contents in *M. inodora*. methanolic extract was found to be 288,67 ±2,57 (µg eq GA/mg of dry extract) and 79,90 ±7,34 (µg eq catechin /mg of dry extract), respectively. On the other hand, *M. inodora*. aqueous extract contains 229.19 ±10.05 (µg.Eq GA / mg) of total phenolic and 157.76 ±14,03 (µg.Eq catechin / mg extract) of flavonoids **(Bechiri-abbes, 2016)**. **Medair and Djelloul**

benchrif (2019), also determined that *C. candidissimum* methanolic extract contains 14.782 mg EGA / g DM of polyphenols and 4,546 mg EC /g DM of flavonoids.

Previous research works indicate that the ethanolic extract from the aerial part of *M. graeca* harvested in Algeria contains a content of polyphenols and total flavonoids of 430 (mg Eq AG /100 g of dry weight) and 190 (mg Eq Quercetin/100 g dry weight), respectively **(Brahmi et *al.*, 2017)**. While these values are clearly superior to those determined by **Vladimir-Knežević et *al.* (2011)**, on three species of the Croatia (*M. croatica, M. juliana* and *M. thymifolia*, which showed the richness of these plants in polyphenols (9.7% to 13.1%), in flavonoids (0.01 to 0.09%); in phenolic acids (5.3 to 6.8%) and tannins (3.1 to 6.1%).

Phenolic compounds have a significant capacity for adsorbing and neutralizing free radicals, quenching singlet oxygen, or decomposing peroxides because of the presence of hydroxyl substituents and their aromatic structure, which allows them to scavenge free radicals **(Villaño et *al.*, 2007)**. Phenolic compounds are also well known due to a variety of biological properties such as antiallergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antithrombotic, cardioprotective, and vasodilatory effects **(Ksouri et *al.*, 2007)**.

Several factors can influence the polyphenol and flavonoids contents in the plants such as the methods of extraction, geographical and climatic factors, genetic heritage, period of harvest, degree of maturation of the plant as well as the duration of storage **(Pedneault et *al.*, 2001)**.

II.2.5. Qualitative and quantitative analyses of the studied plants

II.2.5.1. *Micromeria inodora* (Desf.) Benth.

A. Essential oil analysis

Hydrodistillation of the aerial parts of *M. inodora* gave 0.1 % oil yield on a dry weight basis; its composition is reported in **Table 07**. This value is in concurrence with that mentioned by **Benomari et *al.* (2016)** for the same species growing in Algeria.

Table 07. Chemical composition of the essential oil from *M. inodora* aerial part

N.	Component[a]	RI exp.[b]	RI Lit.[c]	%[d]	Id[e]
1	α-pinene	941	939	0.2	Co-I, RI,MS
2	camphene	948	946	0.1	Co-I, RI,MS
3	β-pinene	981	979	tr[f]	Co-I, RI,MS
4	2,3-dehydro-1,8-cineole	992	991	0.3	RI,MS

5	*p*-cymene	1028	1026	0.1	Co-I, RI,MS
6	limonene	1032	1031	0.3	Co-I, RI,MS
7	1,8-cineole	1034	1033	0.3	Co-I, RI,MS
8	*p*-mentha-3,8-diene	1074	1072	Tr	RI,MS
9	*p*-cymenene	1091	1090	Tr	RI,MS
10	Nonanal	1104	1102	1.1	RI,MS
11	Camphor	1145	1144	7.0	Co-I, RI,MS
12	Borneol	1168	1166	0.2	Co-I, RI,MS
13	terpinen-4-ol	1179	1177	0.1	Co-I, RI,MS
14	α-terpineol	1191	1189	0.3	Co-I, RI,MS
15	*n*-decanal	1206	1203	0.4	Co-I, RI,MS
16	*trans*-carveol	1218	1217	0.2	Co-I, RI,MS
17	Pulegone	1239	1237	0.4	RI,MS
18	Carvone	1244	1243	0.2	Co-I, RI,MS
19	1-decanol	1272	1270	0.1	RI,MS
20	bornyl acetate	1287	1286	0.1	Co-I, RI,MS
21	(2*E*,4*E*)-decadienal	1316	1315	Tr	RI,MS
22	α-terpinyl acetate	1352	1350	29.1	RI,MS
23	(2*E*)-undecenal	1368	1366	Tr	RI,MS
24	α-copaene	1377	1376	0.1	Co-I, RI,MS
25	β-bourbonene	1385	1386	Tr	RI,MS
26	(*E*)-caryophyllene	1419	1419	1.3	Co-I, RI,MS
27	*cis*-muurola-3,5-diene	1448	1447	0.2	RI,MS
28	α-humulene	1455	1455	0.1	Co-I, RI,MS
29	*allo*-aromadendrene	1462	1460	2.4	RI,MS
30	*cis*-cadina-1(6),4-diene	1464	1462	1.3	RI,MS
31	9-*epi*-(*E*)-caryophyllene	1468	1467	1.3	RI,MS
32	germacrene D	1482	1483	0.3	RI,MS
33	β-selinene	1487	1487	0.5	RI,MS
34	*trans*-γ-cadinene	1514	1513	0.2	RI,MS
35	*trans*-calamenene	1523	1524	1.8	RI,MS
36	6-cadinene	1524	1524	0.3	RI,MS
37	α-cadinene	1538	1537	0.1	RI,MS
38	α-calacorene	1543	1544	0.2	RI,MS
39	*cis*-muurol-5-en-4-β-ol	1549	1550	0.1	RI,MS
40	1-*nor*-bourbonanone	1563	1561	0.2	RI,MS
41	Spathulenol	1577	1576	1.2	RI,MS
42	caryophyllene oxide	1582	1583	3.1	Co-I, RI,MS
43	Globulol	1584	1584	2.6	RI,MS
44	1,10-di-*epi*-cubenol	1615	1614	4.4	RI,MS
45	α-cadinol	1652	1653	2.6	RI,MS
46	*cis*-calamenen-10-ol	1661	1660	0.5	RI,MS

47	*trans*-calamenen-10-ol	1670	1668	0.3	RI,MS
48	Cadalene	1675	1675	0.7	RI,MS
49	*cis*-14-*nor*-muurol-5-en-4-one	1684	1682	13.8	RI,MS
50	*epi*-α-bisabolol	1685	1686	2.3	RI,MS
51	α-bisabolol	1686	1687	0.5	Co-I, RI,MS
52	*n*-heptadecane	1700	1700	0.1	Co-I, RI,MS
53	Pentadecanal	1716	1715	0.1	RI,MS
54	Aristolone	1763	1762	0.4	RI,MS
55	*n*-octadecane	1800	1800	0.2	Co-I, RI,MS
56	hexahydrofarnesyl acetone	1845	1845	1.4	RI,MS
57	*n*-nonadecane	1900	1900	0.7	Co-I, RI,MS
58	*n*-hexadecanoic acid	1969	1972	1.2	Co-I, RI,MS
59	Manooloxide	1990	1989	0.7	RI,MS
60	*n*-eicosane	2000	2000	0.2	Co-I, RI,MS
61	*n*-heneicosane	2100	2100	0.9	Co-I, RI,MS
62	*n*-docosane	2200	2200	0.1	Co-I, RI,MS
63	*n*-pentacosane	2500	2500	Tr	Co-I, RI,MS
64	*n*-heptacosane	2700	2700	Tr	Co-I, RI,MS
65	*n*-nonacosane	2900	2900	0.1	Co-I, RI,MS
66	*n*-hentriacontane	3100	3100	0.1	Co-I, RI,MS
	Total identified (%)			**89.1**	
	Essential oil content (%, v/w)			**0.1**	
	Monoterpene hydrocarbons			**0.7**	
	Oxygenated monoterpenes			**38.2**	
	Sesquiterpene hydrocarbons			**10.8**	
	Oxygenated sesquiterpenes			**32.0**	
	Non-terpene alkanes			**2.4**	
	Non-terpene aldehydes			**1.6**	
	Others			**3.4**	

[a] Compounds are listed in order of their elution from a HP-5MS column.

[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{32} alkanes.

[c] Linear retention index taken from Adams and NIST 14.

[d] Relative percentage values are means of three determinations with a RSD% in all cases below 19 %.

[e] Identification methods: Co-I, based on comparison of RT, RI and MS with those of analytical standard; MS, based on comparison with WILEY, ADAMS, FFNSC2 and NIST 08 MS libraries; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC 2 and NIST 08.

[f] Tr, % below 0.1 %.

The essential oil analysis allowed to identify 66 components which accounted for 89.1% of the total oil composition, mainly oxygenated mono and sesquiterpenes (38.2% and 32.0%, respectively) and sesquiterpene hydrocarbons (10.8%). On the contrary, monoterpene

hydrocarbons were scarcely represented in the oil (0.7%); non-terpene aldehydes and alkanes reached 1.6 and 2.4%, respectively. Overall, this finding is in good agreement with those reported in **Benomari et *al*. (2016)**, on a cumulative sample composed of 24 pooled essential oils from *M. inodora* collected from different areas of Algeria ,83 constituents were described which were dominated by oxygenated compounds (78.1%), oxygenated sesquiterpenes (46.9%) were higher than oxygenated monoterpenes (30.4%). It's should be noted that this study is the only one carried out on *M. inodora* essential oil.

Our findings revealed that the main components were the monoterpenes α-terpinyl acetate (29.1%) and camphor (7.0%), together with the sesquiterpene *cis*-14-*nor*-muurol-5-en-4-one (13.8%). Other minor monoterpene components were α-pinene (0.2%), limonene (0.3%) and borneol (0.2%). Finally, other representative sesquiterpenes were caryophyllene oxide and α-cadinol (3.1 and 2.6%, respectively).

A different profile reported rich of *trans*-sesquisabinene hydrate (20.9%), α-terpinyl acetate (19.8%), globulol (4.9%), caryophylleneoxide (4.3%), β-bisabolol (2.9%) and *trans*-7-*epi*-sesquisabinenehydrate (2.6%). Variations between our results and the essential oil composition could be due mainly to the different collections sites of the plant specimens and to seasonal variations related to the different harvesting times **(Benomari et *al.*, 2016)**.

B. Headspace analysis of aroma profile

The aroma profile of *M. inodora* aerial parts was analyzed by head-space solid phase micro extraction (HS-SPME) coupled with GC–MS. The SPME has the advantage to be a fast, simple and non-destructive method of pre-concentration that can be performed without solvents, with detection limits that can reach parts per billion (ppb) levels for some compounds.

Using this technique, the volatiles spontaneously emitted by the powdered aerial parts of *M. inodora* were studied. The 45 volatile compounds identified are reported in **Table 08**.

Table 08. Aroma profile (percentages of identified compounds) of *M. inodora* aerial parts obtained using SPME GC–MS

Nº.	Constituents[a]	RI exp.[b]	RI Lit.[c]	%[d]
1	α-pinene	941	939	2.7
2	Camphene	948	946	2.9
3	β-pinene	981	979	2.1
4	2,3-dehydro-1,8-cineole	992	991	2.7
5	α-phellandrene	1006	1005	1.0
6	p-cymene	1028	1026	0.9

7	1,8-cineole	1034	1033	10.2
8	γ-terpinene	1063	1061	0.1
9	cis-linalool oxide (furanoid)	1076	1074	0.3
10	terpinolene	1090	1088	0.9
11	nonanal	1104	1102	1.5
12	α-campholenal	1128	1126	0.4
13	nopinone	1139	1138	0.5
14	camphor	1145	1144	23.8
15	pinocarvone	1164	1162	0.6
16	4-terpineol	1179	1177	0.9
17	myrtenol	1195	1193	1.3
18	verbenone	1207	1204	0.7
19	pulegone	1239	1237	1.1
20	thymoquinone	1252	1252	0.3
21	linalyl acetate	1259	1257	0.3
22	(E)-anethole	1284	1283	1.4
23	bornyl acetate	1287	1286	1.4
24	2-hydroxy-5-methylacetophenone	1315	1316	1.3
25	α-terpinyl acetate	1352	1350	21.3
26	α-copaene	1377	1376	0.8
27	β-bourbonene	1385	1386	0.3
28	β-elemene	1392	1391	0.1
29	cyperene	1399	1398	0.3
30	α-gurjunene	1410	1409	1.4
31	β-caryophyllene	1419	1419	3.1
32	β-copaene	1431	1432	0.1
33	α-humulene	1455	1455	1.0
34	allo-aromadendrene	1462	1460	3.0
35	cis-muurola-4(14),5-diene	1463	1460	0.7
36	β-chamigrene	1476	1475	0.6
37	γ-muurolene	1479	1480	0.4
38	germacrene D	1482	1483	1.6
39	β-selinene	1487	1487	0.4
40	cis-β-guaiene	1491	1490	0.2
41	α-selinene	1495	1494	0.8
42	n-pentadecane	1500	1500	0.4
43	γ-cadinene	1514	1513	0.5
45	6-cadinene	1524	1524	1.1
	caryophyllene oxide	1582	1583	0.7
	Total identified (%)			**98.1**
	Monoterpene hydrocarbons			**10.6**
	Oxygenated monoterpenes			**65.8**
	Sesquiterpene hydrocarbons			**16.4**
	Oxygenated sesquiterpenes			**0.7**
	Phenylpropanoids			**1.4**
	Non-terpene derivatives			**3.2**

Although not directly comparable, these numbers were significantly lower than those reported in the essential oil studied **(Table 07)**. Overall, the powder of *M. inodora* was dominated by the monoterpenes fractions. Oxygenated monoterpenes appeared to be the most abundant with 65.8% of the total composition, followed by sesquiterpene hydrocarbons (16.4 %) and monoterpene hydrocarbons (10.6%). Among volatiles, the main emitted monoterpenes were camphor (23.8%), α-terpinyl acetate (21.3%) and 1,8-cineole (10.2%). Other components were present in smaller amounts, such as β-caryophyllene (3.1%). From a qualitative standpoint, the aroma profile detected by SPME was overlapping that of essential oil forthe monoterpene fraction but with a higher selectivity (76.4 *vs.* 49.0% for monoterpenes in headspace and essential oil, respectively). On the other hand, the polydimethylsiloxane (PDMS) fibre revealed to be less sensitive for the less volatile sesquiterpenes (17.1 *vs.* 42.8 %, respectively). It is worth mentioning that here; the aroma profile analysis of *M. inodora* aerial parts by head-space solid phase micro extraction (HS-SPME) coupled with GC–MS is reported in our work for the first time.

C. NMR analysis of *M. inodora* crude extracts

The ^{1}H-NMR analysis of the chemical composition of *M. inodora* aerial parts was performed on extracts obtained from plant material using solvents at different polarity. The extracts, after solvent removal, were dissolved in deuterated water. The spectrum of the methanol extract **(Figure 24)** shows signals that can be assigned to different classes of secondary metabolites. In the aromatic region, signals ascribable to hydroxycinnamic derivatives could be observed. The signals at δ 7.03 (s), 6.95 (d; J =7.0Hz) and δ 6.85 (d; J = 7.0) correspond to the position H-2, H-5 and H-6 of 1,3,4-trisubstitued aromatic ring, respectively, while those at δ 7.39 (d; 15.97 Hz) and 6.16 (d; 15.97 Hz) can be ascribed to the *trans* double bond. The same region of the spectrum shows signals assignable to flavonoid portions. Signals at δ 6.76−6.47 area scribable to H-6 and H-8 of flavonol glycosides. Other signals in the region δ 4.50–5.50 can be ascribed to anomeric proton signals, while crowded signals in the region δ 3.00–4.00 are supporting the presence of sugar residues. The aliphatic region shows clear signals ascribable to methyl groups (both secondary and quaternary), as well as superimposed multiplets suggesting the presence of CH and CH_2 that can indicate the presence of triterpenoids.

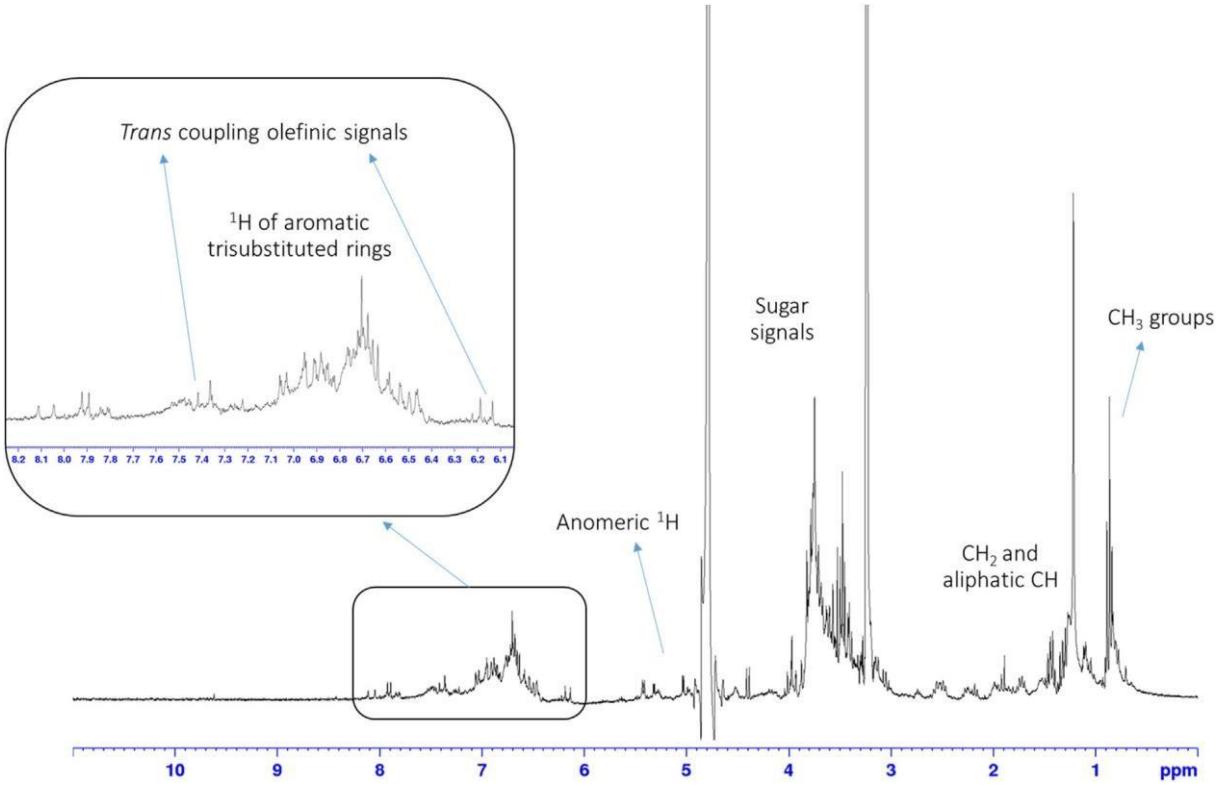


Figure 24. ^{1} H-NMR spectrum of the MeOH fraction

NMR of the *n*-hexane extract shows mainly signals attributable to fatty acids and lipids **(Figure 25)**. Singlets at δ 0.851 can be assigned to terminal methyl groups, while the signals at δ 1.25–1.35 can be related to methylene groups from the aliphatic chain of fatty acids. Broad signal at δ 5.10–5.30 support the presence of unsaturated fatty acids. Methylene vicinal to olefins are revealed at δ 1.99 and inter-olefins signals can be assigned at δ 2.75, while protons at δ 2.28 and 1.52 could be assigned to α and β carbonyl hydrogens, respectively. Minimal signals ascribable to phenolic compounds could be observed at δ 6.00–7.00.

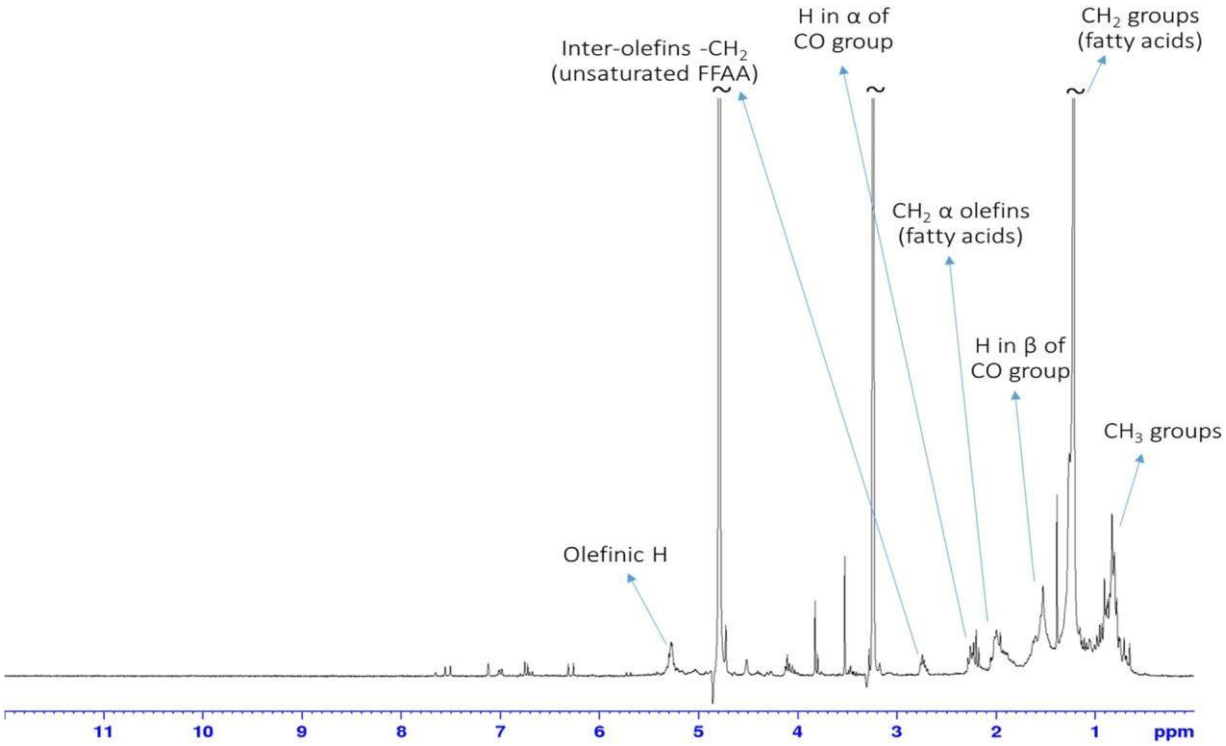


Figure 25. [1] H-NMR spectrum of the *n*-hexane fraction

NMR spectra of DCM and EA extracts appear similar to each other, and they support the presence of triterpenes. Characteristic signals are observable, due to the removal of the lipid signals that were extracted in the n-hexane fraction **(Figure 26)**. Signals at δ 5.15 (m) and 5.27 (m) are ascribed to olefinic protons, and singlets in the range δ 0.70–1.20 are assignable to triterpenes' methyl signals **(Choi et *al.*, 2005)**. Multiplets in the region between δ 2.25−1.12 are ascribable to methylene groups and aliphatic CH, while the doublet at δ 0.88 ppm (J = 6.90 Hz) is attributable to a secondary methyl group.

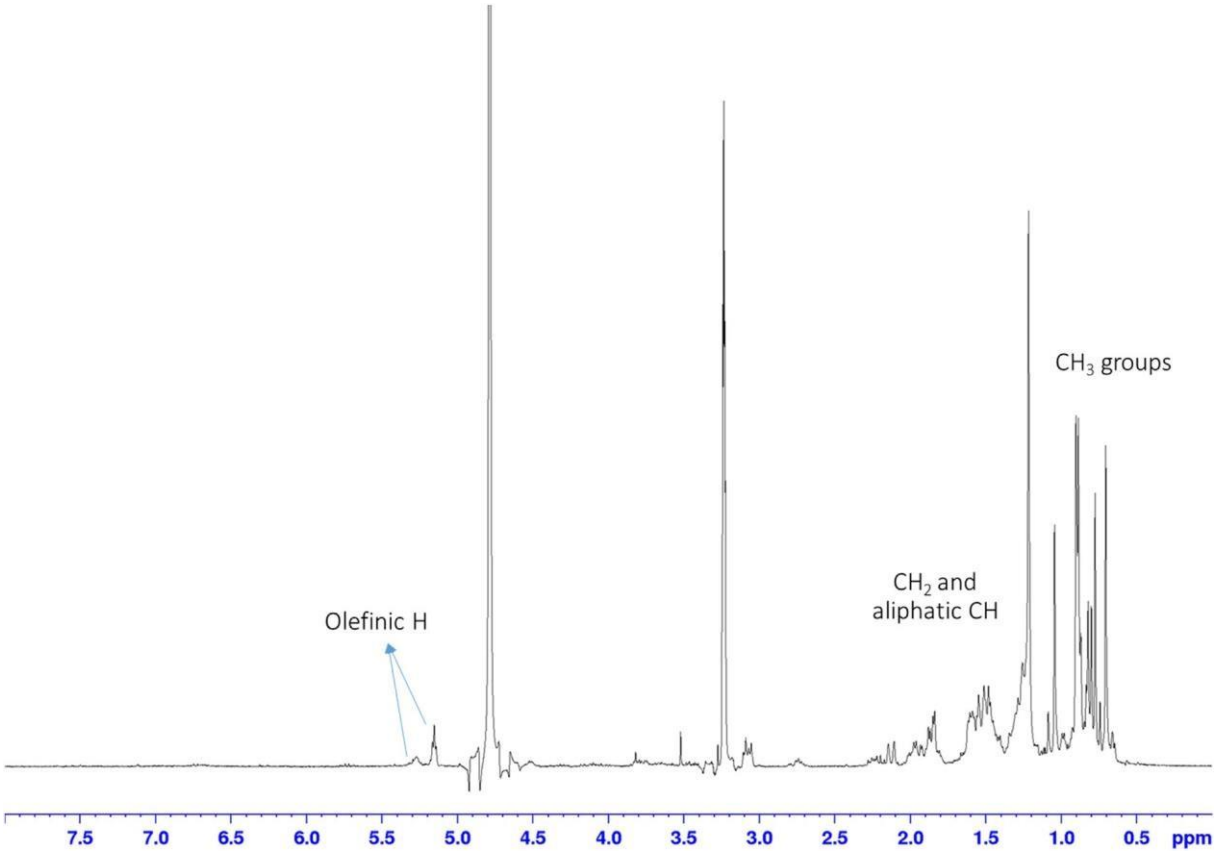


Figure 26. [1] H-NMR spectrum of the DCM fraction

D. HPLC–MS^n analysis of secondary metabolites from *M. inodora* methanol extract

The HPLC-MS^n analysis of the *M. inodora* methanol extract allowed to tentatively identify 38 constituents, by comparison of MS/MS spectra with literature. An exemplificative chromatogram is reported in **Figure 27**. Among the constituents, 19 were identified as flavonoids. In detail, luteolin derivatives, quercetin, kaempferol and apigenin derivatives was the most representative compounds of this class **(Table 09)**.

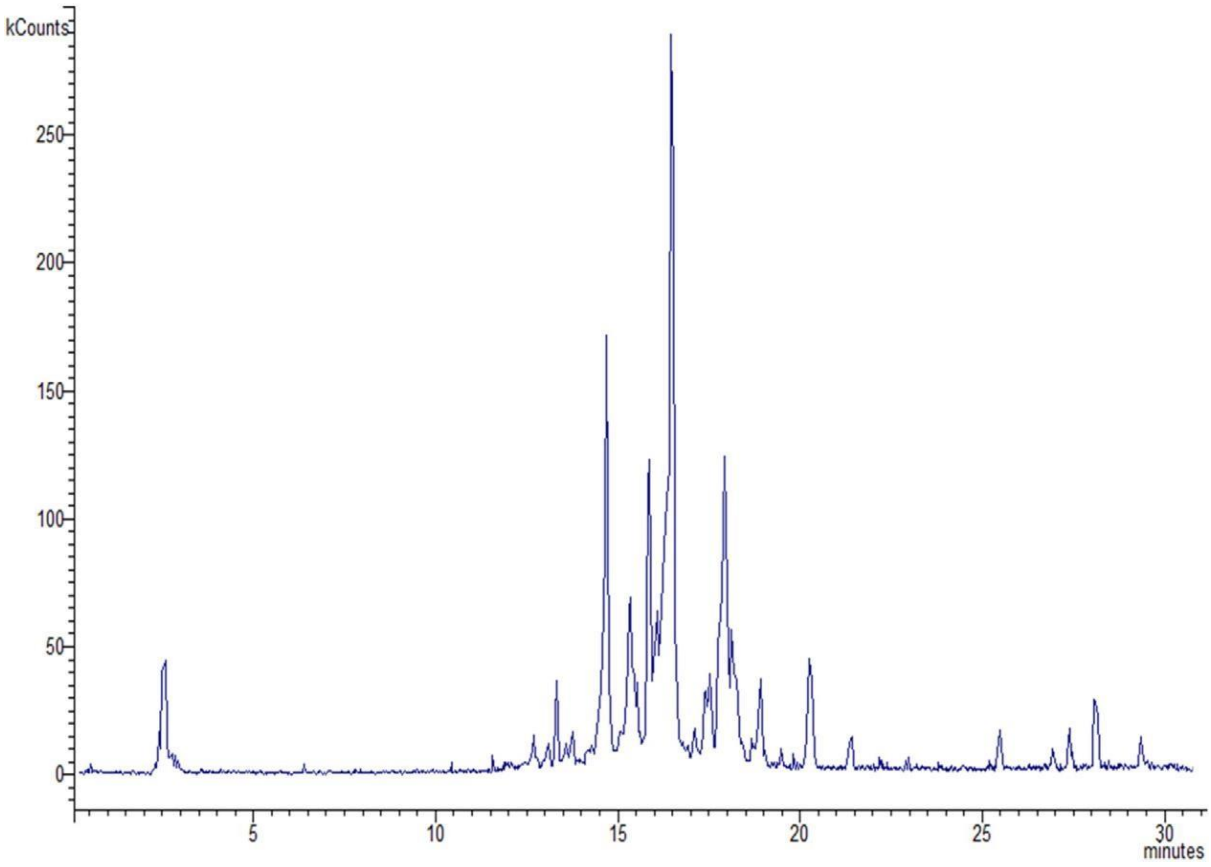


Figure 27. Intensity of base peak chromatogram obtained from the HPLC-MS^n analysis of *M. inodora* methanolic extract in ESI (-)

Table 09. Identification of phenolic constituents from the methanolic extract of *M. inodora* by HPLC-MS^n

Molecular ion (*m/z*)	Ionization mode	MS2	MS3	R.T. (min)	Tentative identification	Chemical class	Reference
269	–	225 224 183 151 117		21.1	Apigenin	Flavonoids	(Ye et *al*., 2012)
283	–	268	241 240 211	26.3	Calycosin	Flavonoids	(Ye et *al*., 2012)
287	+	269 241 161 153 135	127 111 97 85	13.6	Luteolin	Flavonoids	(Abu-Reidah et *al*., 2019)
299	–	284	257 256 227	21.6	Gliricidin	Flavonoids	(Ye et *al*., 2012)
301	+	286 257 229	258	14.8	Chrysoeriol	Flavonoids	(Abu-Reidah et *al*., 2019)
301	+	286 258 229 167	258	14.6	Diosmetin	Flavonoids	(Abu-Reidah et *al*., 2019)
343	–	328 313	298 285	25.4	Dihydroxy-trimethoxyflavone	Flavonoids	(Abu-Reidah et *al*., 2019)
359	+	326 298	298	27.2	Hydroxy-tetramethoxyflavone	Flavonoids	(Abu-Reidah et *al*., 2019)
389	+	374 373 359 341 328	343 331 316	29.1	Erianthin	Flavonoids	Metlin*
447	–	285	267 241 217 199 175 151	13.7	Luteolin 7-O-glucoside	Flavonoids	(Friščić et *al*., 2016)
449	–	287 269	151 135	12.4	Dihydroluteolin hexoside	Flavonoids	(Da Silva et *al*., 2014)
449	–	287 269	151 135	13.1	Dihydrokaempferol hexoside	Flavonoids	(Da Silva et *al*., 2014)
449	–	287	151 135	13.4	Eriodictyol-7-O-glucoside	Flavonoids	(Friščić et *al*., 2016)
461	–	285	267 241	14.4	Kaempferol-3-O-	Flavonoids	(Barreira et *al*.,

			217 199 175 151		glucuronide		2014)
477	–	301	255	13.6	Quercetin-7-O-glucuronide	Flavonoids	(Li et *al.*, 2016)
551	+	303	285 257 229	14.9	Quercetin malonyl-hexoside	Flavonoids	(Abu-Reidah et *al.*, 2019)
577	–	269	225 151	15.1	Apigenin-7-O-rutinoside	Flavonoids	(Gouveia et *al.*, 2012)
593	–	285	241 199 175 151	12.5	Luteolin-7-O-rutinoside	Flavonoids	(Gouveia et *al.*, 2012)
639	–	315 301		15.8	Isorhamnetin-3-O-di-glucoside	Flavonoids	(Brito et *al.*, 2014)
359	–	197 179 161 133	133	16.5	Syringic acid hexoside	Phenolic acids	(Abu-Reidah et *al.*, 2019)
369	–	223 197 179 161 133	133	16.4	Rosmarinic acid	Phenolic acids	(Barros et *al.*, 2013)
537	–	493 383 313 295		14.2	Lithospermic acid	Phenolic acids	(Abu-Reidah et *al.*, 2019)
597	–	295 197 179		14.3	Yunnaneic acid F	Phenolic acids	(Abu-Reidah et *al.*, 2019)
637	–	461 443		18.9	Leucoseptoside A	Phenolic acids	(Barros et *al.*, 2013)
718	–	359 341 295		14.0	Sagerinic acid	Phenolic acids	(Abu-Reidah et *al.*, 2019)
735	–	537		16.7	Danshensu derivative	Phenolic acids	(Gouveia et *al.*, 2009)
759	–	561		14.5	Danshensu derivative	Phenolic acids	(Gouveia et *al.*, 2009)
777	–	579		15.8	Danshensu derivative	Phenolic acids	(Gouveia et *al.*, 2009)
149	+	121 93 65		12.8	Anethole	Terpenoids	Metlin**

471	–	451 427 425 383		29.5	Masilinic acid or Corosolic acid I	Terpenoids	(Abu-Reidah et *al*., 2019)
493	–	359 313 295		18.5	Salvianolic acid A	Terpenoids	(Petreska et *al*., 2011)
717	–	519 359 357	339 321	17.9	Salvianolic acid B/E/L	Terpenoids	(Abu-Reidah et *al*., 2019)
104	+	60		2.5	Choline	Others	Metlin***
191	–	173 155 137		2.6	Quinic acid	Others	(Zeng et *al*., 2006)
305	–	225		12.6	Methyl-hydroxyjasmonate sulphate	Others	(Abu-Reidah et *al*., 2019)
311	–	293 275 235 223 183		26.9	15,16-dihydroxy-9,12-octadecadienoic acid	Others	(Yang et *al*., 2013)
327	–	229 211		20.2	Oxiranedioctanoic acid	Others	(Abu-Reidah et *al*., 2019)
329	–	311 293 229 211	211 167 231 175	21.3	Trihydroxyoctadeca −10(E)-dienoic acid	Others	(Abu-Reidah et *al*., 2019)
371	–	249 231	157 113	13.9	Deacetylasperulosid e	Others	
377	–	341 215	179 161	2.6	Sucrose	Others	(Friščić et *al*., 2016)
387	–	225 207 163	143	12.7	Tuberonic acid hexoside I	Others	(Abu-Reidah et *al*., 2019)

*Metlin ID: 51429. **Metlin ID: 43903. ***Metlin ID: 56.

Several studies have reported that *Micromeria* species are a source of flavonoid and glucosidic conjugates, being apigenin and luteolin derivatives the most abundant ones **(Tomas-Barberan et *al.*, 1991; Vladimir-Knežević et *al.*, 2011)**. Flavonoids have been reported as the responsible for several pharmacological activities attributed to many medicinal plants, notably antioxidant, antimicrobial, and enzyme inhibitory **(Nile et *al.*, 2018)**, hence *M. inodora* from Algeria could represent a novel evaluable source of these bioactives.

Other nine constituents were tentatively identified as phenolic acids **(Table 09)**. Rosmarinic acid is a secondary metabolite widely encountered in nature, and has been already detected as a main component of several *Micromeria* species **(Vladimir- Knežević et *al.*, 2011; Brahmi et *al.*, 2017; Sarikurkcu et *al.*, 2020)**. Furthermore, syringic acid hexoside, lithospermic acid, sagerinic acid and yunnaneic acid F have been identified in the leaves of *M. fruticosa* **(Abu-Reidah et *al.*, 2019)**. Among the terpenoids identified in the methanolic extract of *M.inodora*, two $[M-H]^-$ ions with *m/z* 471 and 717 have been already reported in *M. fruticosa*, but the authors could not precisely identify them by means of LC-MS data.

We tentatively identify the two ions as maslinic acid or corosolic acid I (*m/z* 471) and salvianolic acid B, E or L (*m/z* 717), respectively, comparing the MS^n spectra with available literature **(Abu-Reidah et *al.*, 2019)**. Among the remaining compounds detected in *M. inodora*, two were identified as derivatives of jasmonic acid, namely methyl-hydroxyjasmonate sulphate and tuberonic acid hexoside I, while other three were fatty acids, namely 15,16-dihydroxy-9,12-octadecadienoic acid, oxiranedioctanoic acid, and trihydroxyoctadeca-10(E)-dienoic acid.

II.2.5.2. *Clinopodium candidissimum* (Munby) Kuntze

A. Explorative NMR analysis

The NMR approach was used for a preliminary evaluation of the composition of *C. candidissimum* aerial parts fractions. To assess the presence of different classes of phytoconstituents, extraction of *C. candidissimum* was performed using methanol, and this crude extract was then fractionated with solvents at different polarity, i.e., hexane, dichloromethane (DCM), ethyl acetate (EA) and butanol. The superimposition of the 1H-NMR spectra obtained from the analyses of these fractions is reported in **Figure 28**.

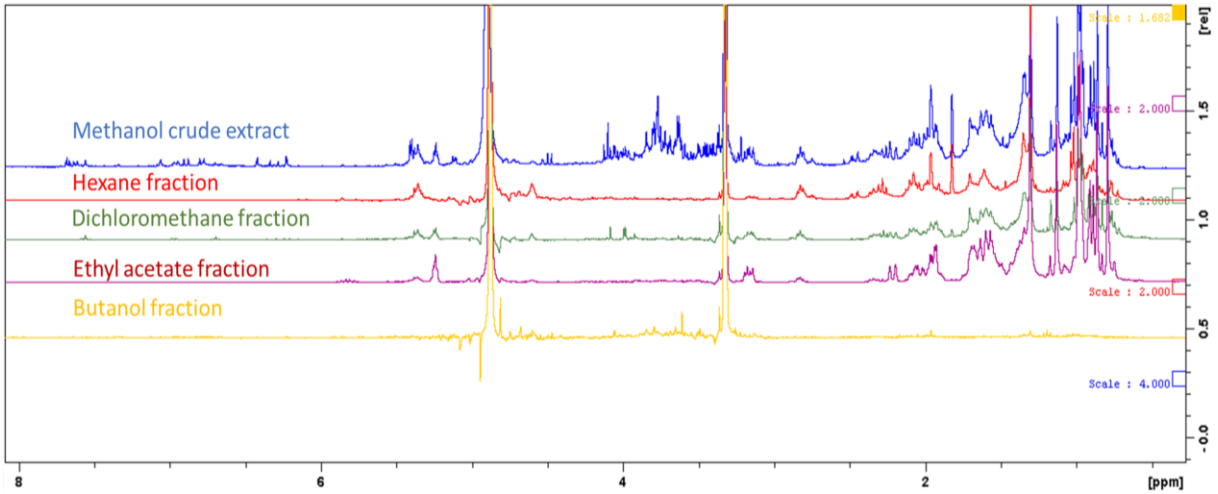


Figure 28. Superimposition of ^{1}H-NMR spectra obtained from the analysis of *C. candidissimum* methanol crude extract (blue trace), and fractions obtained from the liquid/liquid partition of methanol crude extract with solvents at different polarity, namely hexane (red trace), dichloromethane (green trace), ethyl acetate (violet trace), and butanol (yellow trace)

Several signals in the spectrum of methanol crude extract are ascribable to fatty acids, sugars as well as phenolic constituents. A large number of signals in the more shielded part of the spectrum support the presence of aliphatic compounds, thus suggesting the presence of a hydrocarburic portion of constituents ascribable to terpenoids. Furthermore, several signals are clearly visible in the aromatic part of the spectrum, suggesting the presence of aromatic derivatives and phenolics. Among these, signals ascribable to rutin are observed: namely, the aromatic signals ascribable to the catechol ring and the H-6/H-8, as well as those of the anomeric proton of glucopyranosyl and rhamnopyranosyl units **(Table 10)**. These are in good agreement with the literature, and with spectra of rutin acquired for comparison purposes **(Napolitano et *al.*, 2012)**.

Table 10. Main signals observed in the ^{1}H-NMR spectra of *C. candidissimum* extracts obtained using solvents at different polarities. Tentative assignments of identified compounds to specific chemical classes are also reported.

Extract	Compound(s)	^{1}H-NMR Resonance (ppm, multiplicity)	Assignment
MeOH	Rutin	7.65 d	H-6’
		7.67 d	H-2’
		6.96 dd	H-5’
		6.38 d	H-8
		6.22 d	H-6
		5.11 d	Anomeric H of glucose
		4.53	Anomeric H of rhamnose
DCM, EA	Ursolic acid	5.15 brs	H -12
		3.17 dd	H-3
		0.82 d	H-29
		0.89 d	H-30
		0.84 s	H -24
		0.76	H-26
		0.89 s	H-23
		1.05	H-25
		1.26	H-27
		2.35 m	H-18
Hexane, DCM	FFAAs	5.37 m	Olefine H of unsaturated FFAA
		2.27 t	CH_2 of FFAA vicinal to CO, or vicinal to sp2 C
		1.28 m	CH_2 of FFAA aliphatic chains
		0.85 t	Terminal CH_3 of FFAA

DCM: dichloromethane; EA: ethyl acetate; FFAA: fatty acid

Intense aliphatic signals supporting the presence of fatty acids as well as a series of sharp signals indicating the presence of quaternary methyl moieties of terpenoids are observed in the spectrum of the hexane fraction. These observations suggest a large presence of terpenoids. The spectrum of the DCM fraction presents a similar shape as hexane's one, but with a clearer aliphatic region (δ 0.90-2.00). This latter shows several sharp signals suggesting the presence of quaternary methyl groups ascribable to terpenes, such as triterpenes **(Sut et *al.*, 2018)**.

However, compared to hexane fraction, a lower amount of signals ascribable to fatty acid moieties is observed **(Figure 28 and Table 10)**. The spectrum of the EA fraction showed the presence of triterpenoids that mostly can resume the structure of ursolic acid, due to the presence of six quaternary and one secondary methyl groups, the deshielded methylene linked to a hydroxyl group, and the olefinic signal. These assignments are in good agreement with 2α-hydroxyursolic acid **(Choi et *al.*, 1991)**. The spectrum of the butanol fraction did not show significant signals.

Based on these data, we decided to proceed with HPLC-DAD-MS^n for the analysis of phenolics and triterpenoids, and with HS-SPME-GC-MS for the analysis of lower molecular weight (i.e., volatile) terpenoids.

B. HPLC-DAD-MS^n analysis of secondary metabolites in the crude methanol extract

HPLC-DAD-MS^n analysis of *C. candidissimum* aerial parts (a mixture of inflorescences, stems and leaves) was performed on the methanol crude extract, since it was the one containing the broadest range of phytochemicals, from phenolics to terpenes. HPLC-DAD-MS^n allowed to identify 30 secondary metabolites, whose details are reported in **Table 11**.

By using the HPLC-DAD technique, total contents of flavonoids, phenolic acids and gallic acid derivatives were assessed at 3.1%, 3.6% and 0.76% of whole extract, respectively. The total phenolic content was comparable to that already reported for *C. vulgaris* **(Khan et *al.*, 2018)**. Rutin resulted as the most abundant phenolic compound among those identified, followed by several caffeic acid oligomers, caffeoylquinic acid isomers and the gallic acid derivative hexa-O-galloyl-glucoside. Several phenolic constituents identified in *C. candidissimum* have been already reported by other authors in other *Calamintha spp.* For instance, the glycosylated flavonoids hyperoside and 6,8-C-dihexosylapigenin, and the phenolic acids chlorogenic acid, rosmarinic acid and salvianolic acid B have been detected in the leaves of *C. nepeta* from Italy **(Pacifico et *al.*, 2015)**.

Table 11. Identification of phenolic constituents from the crude methanol extract of *C. candidissimum* by HPLC-MS^n in negative ion mode [ESI-]

Chemical class	[M-H]$^-$	R.T. (min)	Main MS2 fragments	Main MS3 fragments	Tentative identification	mg/g*	Ref.
Flavonoids	283	29.12	268	240 239 211	Acacetin	0.07	(Pacifico et *al.*, 2015)
	305	16.48	225	224 207 182 181 165 163 135 133 207	Gallocatechin**	1.26	(Ozarowski et *al.*, 2013)
	343	28.31	328 313	298 285 270 215	5,6-dihydroxy-3',4',7-trimethoxy flavone	0.27	(Salih et *al.*,2017)
	373	28.87	358 343	328 300	Neobaicalein	0.08	(Zhang et *al.*, 2008)
	447	29.34	279	261 259 233	2″-Hydroxy-2″-(3″,4″,5″-trihydroxylphenyl) ethyl-3-(3-(5′,7′-dihydroxychromone)-3-hydroxybutanoate	0.29	(Pacifico et *al.*, 2018)
	463	19.81	301	271 255 179 151	Hyperoside**	1.72	(Pacifico et *al.*, 2015)
	489	20.95	327 285 255	255 227 211	Kaempferol-3-*O*-acetyl-hexoside	0.69	(Mekam et *al.*, 2019)
	505	20.09	463 337 301	271 255 179 151	Quercetin acetyl-hexoside	1.23	(Barros et *al.*, 2012)
	577	27.33	431 299 269 225	-	Apigenin 7-*O*-neohesperidoside	0.07	(Brito et *al.*, 2014)
	593	17.06	503 473 383 353	353 325 297	6,8-*C*-dihexosylapigenin	1.28	(Pacifico et *al.*, 2015)
	607	20.18	563 299	284	Diosmetin 7-*O*-neohesperidoside	2.21	(Brito et *al.*, 2014)

	609	18.98	301	286 257 242 199	Rutin**	19.36	-
	623	20.43	315 300	271 255	Isorhamnetin-3-*O*-rutinoside	1.51	(Li et *al.*, 2016)
	755	17.75	315 300	271 255	Isorhamnetin-3-*O*-[2-*O*-*β*-xylopyranosyl-6-*O*-*α*-rhamnopyranosyl]-*β*-glucopyranoside	0.97	(Lou et *al.*, 2001)
					Total flavonoids	**31.00**	
Phenolic acids	163	21.74	163 161 131 119	-	*p*-Coumaric acid	0.57	(Pereira et *al.*, 2015)
	353	15.78	191 179 191 173 135	111 93	Caffeoylquinic acid isomer	6.84	(Pacifico et *al.*, 2015)
	353	16.36	191 179 191 173 135	111 93	Caffeoylquinic acid isomer	3.22	(Pacifico et *al.*, 2015)
	359	20.82	223 197 179 161	133	Rosmarinic acid**	5.03	(Pacifico et *al.*, 2015)
	555	18.54	359 357 179 161	-	Salvianolic acid K	0.86	(Ziani et *al.*, 2019)
	717	20.64	555 519	357 339 295 247 163	Salvianolic acid B	4.51	(Pacifico et *al.*, 2015)
	895.5	20.02	851 794 697 555 357 339	-	Caffeic acid pentamer	8.39	(Pacifico et *al.*, 2015)
	1075	20.52	913 877 715 555 519	-	Caffeic acid hexamer	6.63	(Pacifico et *al.*, 2015)
					Total phenolic acids	**36.07**	

Gallic acid derivative	1091	18.49	939 787	-	Hexa-*O*-galloyl-glucoside	7.56	(Xiang et *al.*, 2019)
					Total gallic derivatives	**7.56**	
Triterpenes	471	22.02	453 407	-	Hydroxyursolic acid #	2.11	(Li et *al.*,2017)
	455	23.61	407	-	Ursolic acid**#	4.05	(Sun et *al.*, 2019)
					Total triterpenes	**6.16**	
Other	327	24.63	291 229 211 171	211 209	Oxo-dihydroxy-octadecenoic acid	NQ	(Llorent-Martínez et *al.*, 2015)
	329	24.04	311 293 229 211 171	211 209	Trihydroxyoctadecenoic acid	NQ	(Llorent- Martínez et *al.*, 2015)
	387	17.28	225 207 179 163	-	12-*O*-hexosyljasmonate	NQ	(Pacifico et *al.*, 2015)
	377	2.63	341	179 161 119 89	Disaccharide (HCl adduct)	NQ	(Jiménez- López et *al.*, 2017)
	539	2.57	503	383 341 323 221	Maltotriose	NQ	(Jin et *al.*,2018)

*Flavonoids amounts are expressed as rutin equivalents; phenolic acids amounts are expressed as chlorogenic acid equivalents; amounts of gallicderivatives are expressed as gallic acid equivalents; triterpenes amounts are expressed as ursolic acid equivalents.
**Compounds identified on the basis of comparison with standard available in the lab.

Confirmed by NMR data.

NQ: not quantified.

Regarding triterpenes, ursolic acids and its hydroxylated derivative were identified, and their amounts reached the 0.62% of the whole crude extract. Previously published phytochemical data on *Calamintha* genus have shown that ursolic acid and its derivatives are common constituents within this genus **(Khodja et *al.*, 2018)**. Conversely, our LC-MS results show that triterpenes are only minor constituents of *C. candidissimum* crude extract, although abundant signals attributable to the same class of phytochemicals were observed in the NMR data. These contrasting results suggest that further in depth studies are required for a more comprehensive elucidation of the triterpenes content of this plant. Chemical structures of the most abundant polar constituents identified in *C. candidissimum* are reported in **Figure 29**.

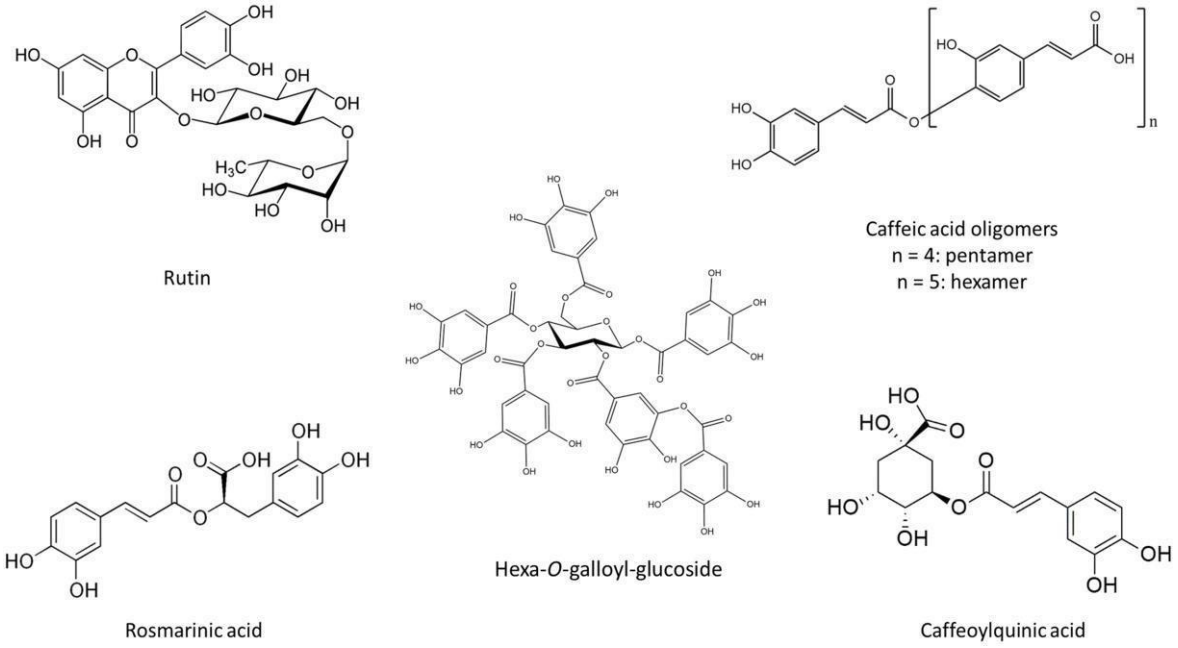


Figure 29. Chemical structures of the most abundant polar constituents identified in the methanol crude extract of *C. candidissimum* aerial parts by HPLC-MS^n analysis.

C. HS-SPME-GC-MS analysis

The analysis on the mixture of ground stems, leaves and flowers allowed to characterize 98.7% of the total volatiles emission and to identify 38 constituents **(Table 12)**. The composition of the volatile bouquet was dominated by oxygenated monoterpenes (66.2%), which included pulegone (44.8%), piperitenone (6.6%), isopulegone (5.8%) and neo-menthol (3.8%). Sesquiterpene hydrocarbons were found as the second major group of chemicals (27.4%), including germacrene D (16.2%) and bicyclogermacrene (3.0%). Chemical structures of the main volatiles identified in *C. candidissimum* are reported in **Figure 30**.

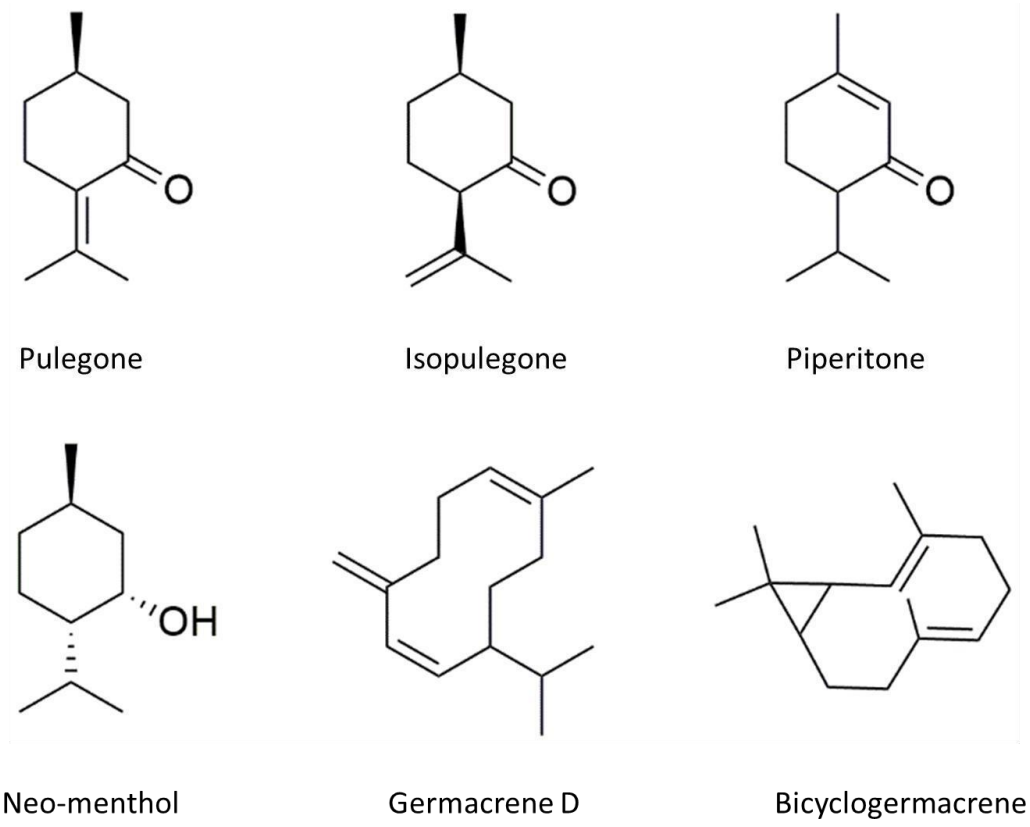


Figure 30. Chemical structures of the main volatile constituents of *C. candidissimum* arerial parts extracted and characterized by HS-SPME-GC-MS analysis

Table 12. Aroma profile of *C. candidissimum* aerial parts obtained by HS-SPME-GC-MS analysis

N.	Constituents	l.r.i.	Lit. Rib	%
1.	β-pinene	982	980	0.3
2.	p-cymene	1028	1027	0.4
3.	Limonene	1032	1031	1.1
4.	Linalool	1101	1099	0.1
5.	Camphor	1145	1144	1.0
6.	Menthone	1155	1153	0.6
7.	Menthofuran	1165	1164	0.3
8.	neo-menthol	1167	1166	3.8
9.	Isopulegone	1174	1175	5.8
10.	α-terpineol	1191	1190	1.4
11.	Decanal	1206	1204	0.2
12.	Pulegone	1239	1237	44.8
13.	Piperitone	1254	1253	0.5
14.	Isopiperitenone	1271	1272	0.1
15.	(E)-anethole	1284	1285	0.4
16.	p-cymen-7-ol	1290	1291	0.5
17.	Carvacrol	1301	1301	0.1
18.	iso-dihydrocarveol acetate	1327	1329	0.2
19.	δ-elemene	1340	1339	0.7
20.	Piperitenone	1342	1343	6.6
21.	piperitenone oxide	1365	1363	0.4

22.	α-copaene	1377	1377	0.6
23.	β-bourbonene	1385	1384	0.8
24.	β-cubebene	1391	1390	0.4
25.	β-elemene	1393	1391	1.0
26.	(Z)-jasmone	1395	1394	1.5
27.	α-gurjunene	1410	1409	0.2
28.	β-ylangene	1420	1421	1.6
29.	β-copaene	1430	1432	0.8
30.	Aromadendrene	1440	1441	0.3
31.	Alloaromadendrene	1462	1460	0.5
32.	cis-muurola-4(14),5-diene	1463	1461	0.3
33.	γ-muurolene	1479	1477	0.3
34.	germacrene D	1481	1480	16.2
35.	Bicyclogermacrene	1496	1494	3.0
36.	trans-γ-cadinene	1514	1513	0.2
37.	δ-cadinene	1524	1524	0.5
38.	Spathulenol	1577	1578	1.2
	Monoterpene hydrocarbons			1.8
	Oxygenated monoterpenes			66.2
	Sesquiterpene hydrocarbons			27.4
	Oxygenated sesquiterpenes			1.2
	Phenylpropanoids			0.4
	Non-terpene derivatives			1.7
	Total identified			98.7

[a] LRI relative to C_6-C_{28} n-alkanes on the DB-5 column
[b] LRI taken from (Adams &Sparkman 2007; NIST 2014)

No previous studies are present in the literature about the spontaneous volatile emission of this species. Even if the SPME results cannot be directly compared with essential oil composition, these results partially reflect already published data regarding the chemical composition of essential oils from the aerial parts of *C. candidissimum*, since several studies have indicated pulegone and menthone as the two major components. For instance, the constituents identified in *C. candidissimum*

essential oil by **Benyoucef et *al.* (2020)** were principally oxygenated monoterpenes, represented by pulegone (70.4%), menthol (5.2%) and isomenthone (4.5%). *Satureja calamintha subsp. nepeta* from Portugal has isomenthone, 1,8-cineole and trans-isopulegone as dominant components **(Marongiu et *al.*, 2010)**.

Finally, in a study involving wild *C. nepeta* from Corsica identified three chemotypes whose essential oils were characterised by menthone/pulegone, pulegone, piperitone and piperitenone oxides as main components, respectively **(Ristorcelli et *al.*, 1996;Baldovini et *al.*, 2000)**.

Within the *Satureja* genus, a great variability of the volatile profile has been reported, although carvacrol, thymol, p-cymene, borneol, b-caryophyllene, germacrene D, and caryophyllene oxide have been indicated as major compounds **(Azaz et *al.*, 2002)**. Differences between our results and those previously published may be due to the different analytical techniques used, the part of the plant studied, the age of the plant, the period of the vegetative cycle, or even to ecological and genetic factors.

II.3. Biological study

II.3.1. Antioxidant activity

There are many papers attempting to rank the antioxidant properties of plant materials using different methods and for better assessment of antioxidant properties, several methods should be used. Therefore, to evaluate antioxidant activities of *C. candidissimum* and *M. inodora* methanolic extracts DPPH free radical scavenging, ABTS cation radical scavenging and CUPRAC (cupric-reducing antioxidant capacity) assays were investigated **(Appendix IV)** spectrophotometrically by a 96-well microplate reader, using 400 µg portions of each extract and BHA was employed as a standard antioxidant. The results were established as 50% inhibition concentration IC_{50} (µg/mL) that means sample concentration providing 50 % activity (DPPH and ABTS) and $A_{0.5}$ which corresponding the concentration indicating 0.500 absorbance (CUPRAC).

Results for antioxidant potential of *C. candidissimum* and *M. inodora* methanolic extracts tested with three methods show a moderate antioxidant activities compared with those of standard antioxidant in all tests **(Figure 31, 32 and 33)**. The antioxidant activity of *M. inodora* methanolic extract was found to be (DPPH: IC_{50} = 63.17 ± 2.28 µg/mL; $ABTS^+$: IC_{50} = 31.08 ± 1.06 µg/mL; CUPRAC: $A_{0.5}$ = 67.10 ± 2.06 µg/mL). *C. candidissimum* extract revealed a reducing potential of (DPPH: IC_{50} = 47.93 ± 1.11 µg/mL; $ABTS^+$: IC_{50} = 36.47 ± 0.91 µg/mL; CUPRAC: $A_{0.5}$ = 58.31 ± 1.31 µg/mL). The good and highest scavenging activity was observed in the $ABTS^+$ cation radical scavenging assay. However, the CUPRAC assay supports the DPPH free radical scavenging inhibitory activity results. On the other hand, the methanolic extracts was not so active as compared

with the BHA standard (DPPH: IC_{50} = 3.44 ± 0.09 µg/mL; $ABTS^{+}$: IC_{50} = 1.88 ± 0.06 µg/mL; CUPRAC: $A_{0.5}$ = 5.62 ± 0.08 µg/mL).

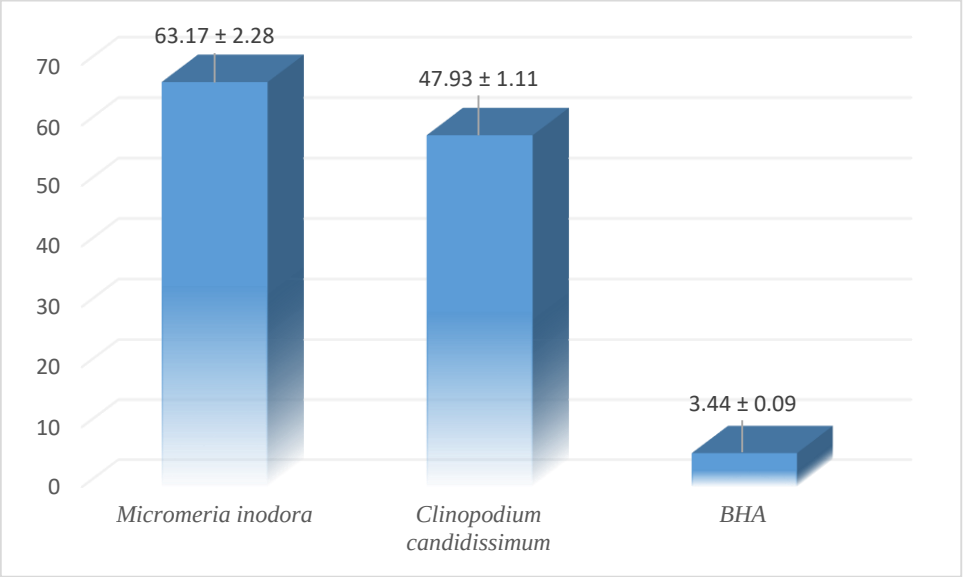


Figure 31. Antioxidant activity (IC_{50}, µg/mL) of *M. inodora* and *C. candidissimum* methanolic extracts and the standard BHA against the DPPH free radical scavenging

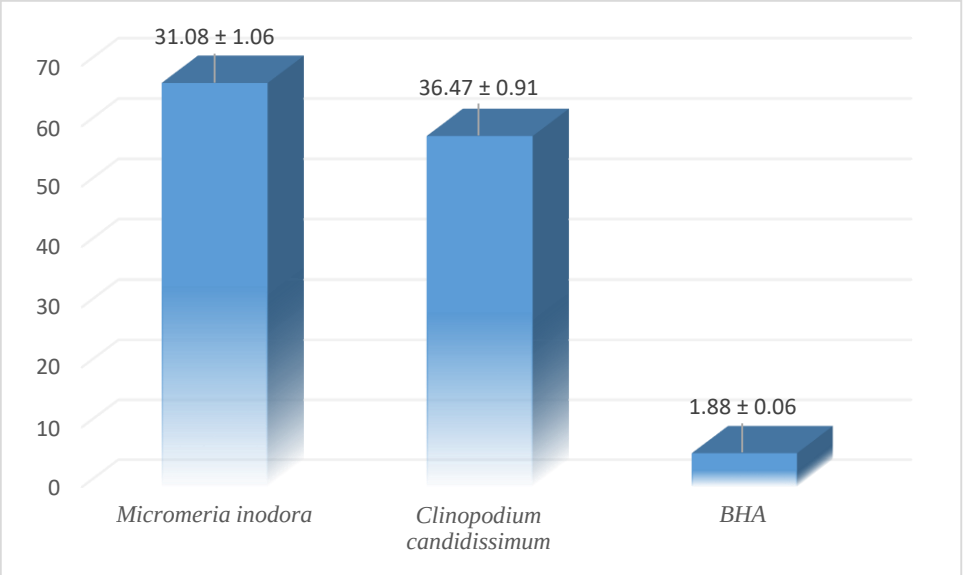


Figure 32. Antioxidant activity (IC_{50}, µg/mL) of *M. inodora* and *C. candidissimum* methanolic extracts and the standard BHA against the ABTS cation radical scavenging

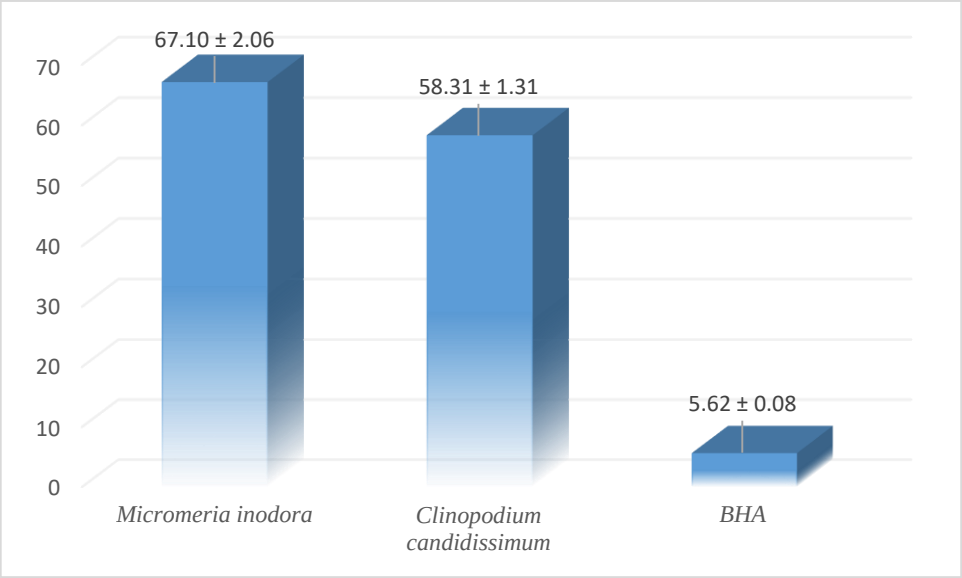


Figure 33. Antioxidant activity ($A_{0.5}$, µg/mL) of *M. inodora* and *C. candidissimum* methanolic extracts and the standard BHA against CUPRAC (cupric-reducing antioxidant capacity)

Previous report revealed that *M. inodora* exhibits important antioxidant activity, methanolic extract show an interesting antiradical effect at low concentration (IC_{50}= 9,9 ±0,001 µg/mL); aqueous extract exhibited a high antioxidant activity (IC_{50}= 9.66 ±0.65 µg/mL) **(Bechiri-abbes, 2016**; **Adjdir, 2022)**. Among the IC_{50} values cited in *Micromeria* species, *M. inodora* was the most active which revealed the lowest IC_{50} values **(Güllüce et *al.* 2004; Vladimir- Knežević et *al.* 2011; Abdelwahab et *al.* 2015)**.

A DPPH$^{\bullet}$ radical-scavenging activity was previously recorded for polar extracts of other *Micromeria* species. The ethanolic extracts of *M. croatica*, *M. juliana*, and *M. thymifolia* from Croatia and the methanolic extract of *M. fruticosa* ssp. *serpyllifolia* from Turkey exhibited considerable scavenging of DPPH$^{\bullet}$; *M. croatica* was the most active species (IC_{50} = 4.7 µg/mL) **(Kalodera et *al.*, 1994; Güllüce et *al.*, 2004; Vladimir- Knežević et *al.*, 2011; Šamec et *al.*, 2015)**. The ethanolic extract of *M. myrtifolia* from Lebanon was quite active towards DPPH$^{\bullet}$, whereas the essential oils and apolar (hexane and chloroform) extracts showed only marginal antiradical activities **(Formisano et *al.*, 2014; Sarikurkcu et *al.*, 2019)**. However, ethanol extract of *M. graeca* is potent in neutralizing $ABTS^{\bullet+}$ (IC_{50} = 30.5 ± 0.9 µg/mL) and DPPH$^{\bullet}$ (IC_{50} = 65.8 ± 2.4 µg/mL) radicals **(Brahmi et *al.*, 2017)**.

Vladimir- Knežević et *al.*, (2011) showed that the radical-scavenging activity of *Micromeria* species largely depends on their major compound, rosmarinic acid, a powerful free antioxidant.

The assessment of antioxidant potency using the DPPH free radical trapping method indicated that *C. candidissimum* methanolic extract have considerable antioxidant effectiveness with an IC_{50} of 6.81 mg/ml **(Medair and Djelloul benchrif, 2019)**. In the other hand, *C. candidissimum* essential oil seems to have antioxidant activity with an IC_{50} of 8.8 mg/ml and 272.523± 8.745 µg/ml **(Attou, 2017; Benyoucef et *al.*, 2020)**. Other study carried out by **Bougandoura and Bendimerad (2013)**, on *Satureja calamintha* shows that the aqueous and methanolic extracts have a moderate antioxidant activity; their IC_{50} was 1.876 and 2.075 mg/ml, respectively.

According to the analysis of results, we found that the extracts of two studied species exhibit a moderate antioxidant activity but *M. inodora* show a promising effect compared to *C. candidissimum*.

It should be noted that there is no previous studies on the antioxidant activities concerning the ABTS cation radical scavenging and the CUPRAC (cupric-reducing antioxidant capacity) assays of *M. inodora* and *C. candidissimum*. Therefore, our findings show that *M. inodora* and *C. candidissimum* can be interesting in food industry and in the prevention and therapies of diseases related to the oxidative stress.

II.3.2. Enzyme inhibition activity

To determine the enzyme inhibition potential of plant extracts, cholinesterases, α-glucosidase and α-amylase assays were used and the results are given in **Table 13**.

Table 13. Anticholinesterase and anti-diabetic activities (IC_{50} values) of *M. inodora* and *C. candidissimum* methanolic extracts

Extracts & compounds		Anticholinesterase activity		Anti-diabetic activity	
		AChE assay [a]	**BChE assay** [a]	**α-Amylase Inhibitory assay**[a]	**α-Glucosidase Inhibitory assay**[a]
		IC_{50} (µg/mL)	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**
M. inodora		*>200*	*>200*	*>400*	*>400*
C. candidissimum		*>200*	*>200*	*>400*	*>400*
Galantamine [b]	Standard	Nd	Nd	-	-
Acarbose[b]	Standard	-	-	Nd	Nd

[a] Values expressed herein are mean ± SEM of three parallel measurements. $p<0.05$
[b] Reference compound
Nd: not determined

The α-glucosidase and α-amylase enzyme inhibition activities of *M. inodora* and *C. candidissimum* methanolic extracts compared with those of standard drug acarbose were determined spectrophotometrically *in vitro* in a 96 well plate microplate reader, using 400 µg portions of each extract. According to the results, no significant α-glucosidase and α-amylase enzyme inhibition activities (IC_{50}>400 µg/mL) was observed in the plant extracts. The findings are in disagreement with the study of **Bechiri-Abbes (2016)** on the inhibitory effect of digestive enzymes by *M. inodora* aqueous extract which revealed an inhibitory effect *in vitro* of α-glucosidase (IC_{50} not determined) and α-amylase (IC_{50}= 666 µg/mL). To the best of ours knowledge, there is no previous study on the α-glucosidase and α-amylase enzyme inhibition activities of *C. candidissimum*.

The *in vitro* acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of *M. inodora* and *C. candidissimum* methanolic extracts were carried on spectrophotometrically in a 96 well plate microplate reader, using 200 µg portions of each extract and compared with those of standard drug galantamine used to treat mild Alzheimer's disease. The studied plants were unable to indicate anticholinesterase activity (AChE and BChE), no significant activity was observed in the methanolic extracts (IC_{50}>200 µg/mL).

AChE and BChE are related to Alzheimer's disease, there are some hypotheses about the formation of Alzheimer's disease but the exact reason is unclear. The lack of acetylcholine in the brain is the only known hypothesis. Therefore, acetylcholinesterase inhibitory drugs have been developed and used for the patients. *In vitro*, Ellman's method is frequently used to test any compounds inhibitory activity against the acetylcholinesterase enzyme. Scientists know that nature is full of various compounds that should be screened for acetylcholinesterase inhibitory activity using a simple and easy method **(Ullah et *al.*, 2022)**.

The search of new active agonists and antagonists of the acetylcholinesterase is nowadays the main target of several researchers; to the best of ours knowledge there is no previous study on the Anticholinesterase activity of *M. inodora* and *C. candidissimum*. In this context, it should be noted that a large number of species of the *Lamiaceae* family have been scarcely studied. The results obtained from *Micromeria* spp. have evaluated the anticholinesterase activity of the organic extracts of *M. juliana* (L.) Bentham ex Reichb. including light petroleum, acetone and methanol ones. Using 200 µg portions of each extract, anticholinesterase activities were found to be, respectively, -5.9 ± 4.1 µg/mL, 35.3 ± 3.1 µg/mL and -7.6 ± 6.8 µg/mL, using galantamine as a positive control (74.0 ± 0.8, IC50: 5.0 ± 0.1 µg/mL). In addition, the reported IC_{50} values were higher than 200 µg/mL for all the employed organic solvents. Furthermore, using the butyryl-cholinesterase (BChE) assay on 200

µg of the organic extracts, the activities were found to be respectively 40.9 ± 3.1 µg/mL, 52.4 ± 1.8 µg/mL and -6.2 ± 2.3 µg/mL, compared to galantamine used as positive control (75.0 ± 0.6 µg/mL, IC50: 50.8 ± 0.9 µg/ mL). In this relation, IC_{50} values of the light petroleum and methanol extracts were higher than 200 µg/mL, while the IC_{50} of the acetone extract was 185.6 ± 1.9 µg/ mL **(Öztürk et *al.*, 2009)**.

The characterized compounds in the report of **Öztürk et *al.* (2011)** were also tested for their anticholinesterase and antioxidant potentialities, together with the crude organic extracts. From the results obtained, three of the isolated compounds exhibited a medium-high acetylcholinesterase inhibitory activity, while the other compounds were found to be inactive.

A modest anti-cholinesterase activity was also observed in the chloroform and methanol extracts (500 µg/mL) from *M. fruticosa* subsp. *brachycalyx* P.H.Davis (syn. of *Clinopodium serpyllifolium* subsp. *brachycalyx* (P.H.Davis) Bräuchler) 39.50 ± 0.63% and 35.85 ± 2.89%, respectively, when compared with standard galantamine (93.14 ± 0.14%) **(Taskin et *al.*, 2020)**.

Many *Satureja* species have been reported for its AChE and BChE inhibitory activities, **Silva et *al.* (2009)** report that the inhibitory activity over AChE by *S. montana* essential oil was assessed as a potential indicator for the control of Alzheimer's disease. The supercritical non-volatile fractions, which showed the highest content of (+)-catechin, chlorogenic, vanillic, and protocatechuic acids, also inhibited selectively and significantly BChE. Another plant species evaluated for its AChE and BChE inhibitory activities is *S. parvifolia*. According to a study reported by **Cabana et *al.* (2013)**, three different polar extracts (decoction, ethanol, and hydrolate) of this species were studied. All extracts showed excellent AChE and BChE inhibitory capacities. **Orhan et *al.* (2008)** tested AChE and BChE inhibitory activities of the essential oil obtained from *S. cuneifolia*. They showed a very high inhibitory activity (over 80%) against AChE. *S. thymbra* was also evaluated for its cholinesterase inhibitory effects against AChE by **Öztürk (2012)** witch reveled a promising enzyme inhibition activity of the essential oil.

II.3.3. Antimicrobial activity

Antimicrobial activity of methanolic extracts of *C. candidissimum* and *M. inodora* were determined by disc diffusion test against several bacteria which are reference strains of the American Type Culture Collection (ATCC): $Gram^+$ (*Staphylococcus aureus* (MRSA) ATCC 34300, *Staphylococcus aureus* ATCC 6538 and *Bacillus cereus* ATCC 14579), $Gram^-$ (*Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9027) and a yeast (*Candida albicans* ATCC 10231 (CA2) and *Candida albicans* ATCC

10237 (M3)). The positive controls were set up with Amoxicillin (AMC) 30 µg, Cefazolin (CZ) 30 µg, Ceftriaxon (CRO) 30µg, Cefoxitin (FOX) 30µg and Amphotericin B (AMB) 20µg.

Activity was determined by measuring the diameter of the growth inhibition zone (inhibition zone diameter, IZD) visible around the paper disc and compared with reference diameters related to the antibiotics used. Reported IZDs are inclusive of the paper disc diameter (6 mm). IZDs higher than 6 mm indicated growth inhibition, while IZD equal to 6 mm meant no activity. Indeed, the sensitivity of a germ is non-existent for a diameter inferior or equal to 6 mm. It is limited for a diameter between 6 and 14 mm, and average for a diameter between 14 and 20 mm. For a diameter greater than or equal to 20 mm, the germ is very sensitive.

Antibiotic susceptibility testing involves investigating the sensitivity of strains to antibiotics. Report the values in (mm) of the zones of inhibition reached with the different strains studied. It should be noted that different strains of the bacteria studied react differently to the antibiotics tested, even if they are two strains of the same bacterial species, due to antibiotic resistance.

The minimum inhibitory concentration (MIC) was evaluated by a broth microdilution method, it is was defined as the lowest concentration of methanolic extacts that showed no visible bacterial growth after incubation time (no color change (clear) of Triphenyl tetrazolium chlorid (TTC) indicator of microorganism growth).

The minimal bactericidal concentration (MBC) and Minimal fungicidal concentration (MFC) was determined by plating directly the content of wells with concentrations higher than the MIC value. The MBC and MFC values was determined when there was no colony growth from the directly plated contents of the wells. The MBC and MFC was considered as the lowest concentration of methanolic extacts that killed 99.9% of microorganisms in culture on the agar plate after incubation period.

The MBC/MIC and MFC/MIC ratios was also calculated to show the nature of antibacterial effect of methanolic extracts. When the ratio was less than or equal to four, the methanolic extract was considered as bactericidal or fungicidal and when the ratio was higher than four, it was considered as bacteriostatic or fungistatic methanolic extract.

The findings of the present work revealed that the methanolic extract of the studied plants exhibited noticeable antimicrobial and antifungal activity against all tested strains with various inhibition diameters; this difference could be due to the difference in the cell wall structure of the tested bacteria.

Obtained outcomes are very promising for the methanolic extracts of *C. candidissimum* and *M. inodora*. The diameters of inhibition zones **(Appendix V)**, MIC **(Appendix VII)**, MBC and MFC values **(Appendix VIII)** and MBC/MIC and MFC/MIC ratio of the plants extracts against different pathogens are illustrated in **Table 14** for *M. inodora* and in **Table 15** for *C. candidissimum*. The result of the antibiotic susceptibility testing are shown in **Table 16**.

Results of the *M. inodora*. methanolic extract from **Table 14** showed that all tested strains are sensitive in all the concentration range of 50 mg/mL, 100 mg/mL and 200 mg/mL, IZD was (9 - 12.33mm) for the concentration of 50 mg/mL, (10 - 17 mm) for the concentration of 100 mg/mL and (11 - 17 mm) for the concentration of 200 mg/mL. *S. aureus* (MRSA) ATCC 34300 was the most sensitive for all the concentration range. The MIC values was 1000 µg/mL for *E. coli* ATCC 25922 and 2000 µg/mL for all the tested strains, MBC and MFC was found to be <2000 µg/mL for all the tested strains. MBC/MIC ratio was one for *P. aeruginosa* ATCC 9027, for the other strains was not determined, *M. inodora* methanolic extract was considered as bactericidal.

Table 14. Antibacterial activity of *M. inodora* methanolic extract

Extract \ Strains			*S. aureus* (MRSA) ATCC 34300	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 9027	*E. coli* ATCC 8739	*E. coli* ATCC 25922	*B. cereus* ATCC 14579	*C. albicans* (CA2) ATCC 10231	*C. albicans* (M3) ATCC 10237
Micromeria inodora	Diameter of inhibition zone (mm)	50 (mg/mL)	12,33 ±1,15	10,33 ±0,57	9 ±0	11 ±1	9,33 ±0,57	10,33 ±0,57	9,66 ±0,57	9 ±0
		100 (mg/mL)	17±0	12±0	14±0	13±0	10±0	11±0	13±0	13±0
		200 (mg/mL)	17±0	12±0	14±0	15±0	11±0	12±0	15±0	13±0
	MIC (µg/mL)		2000	2000	2000	2000	1000	2000	2000	2000
	MBC (µg/mL)		<2000	<2000	2000	<2000	<2000	<2000	-	-
	MFC (µg/mL)		-	-	-	-	-	-	<2000	<2000
	MBC/MIC ratio		Nd	Nd	1	Nd	Nd	Nd	-	-
	MFC/MIC ratio		-	-	-	-	-	-	Nd	Nd

*Values expressed herein are mean ± SEM of three parallel measurements
Nd: not determined

The study of **Benomari et *al.* (2016),** demonstrate a significant antibacterial effect of the essential oils of *M. inodora* on strains *S. aureus* ATCC 29213 (22mm, MIC=60 µg/mL), *B. cereus* ATCC 11778 (19 mm, MIC=500 µg/mL), and a moderate antifungal effect on *C. albicans* IP 444 (13mm, MIC=1000µg/mL). Other studies carried out on the essential oils of species of *Micrmeria* showed an interesting antibacterial and antifungal power on the strains Gram negative (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella enteritidis*), Gram positive (*B. subtilis*, *B. cereus*, *S. aureus*), and on yeasts (*C. albicans*) **(Stojanovic and Palic, 2008; Azab, 2016)**.

Another study found that *M. inodora* extracts exhibited low antibacterial activity in liquid and solid media. Only *S. aureus* and *E. coli* strains appeared to be the most sensitive (diameters of

inhibition zones = 6.5 mm, CMI= 110 mg/mL). These extracts showed no inhibitory effect on the growth of *Candida* strains **(Adjdir, 2022)**.

Brahmi et *al.* (2017), investigate the antibacterial activity of the *M. graeca* extract against four pathogenic bacteria, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538, and *S. aureus* 100459, the extracts were inactive against all test-microorganisms (MIC > 2000 µg/mL). **Ali-Shtayeh et *al.* (1997)**, reported that the freeze-dried water extract of *M. nervosa* (origin, Palestine) indicated lowest activity.

C. candidissimum methanolic extract showed that all tested strains are sensitive in all the concentration range **(Table 15)**; IZD was (9 -12 mm) for the concentration of 50 mg/mL, (10 - 14 mm) for the concentration of 100 mg/mL and (11 - 18 mm) for the concentration of 200 mg/mL. *E. coli* ATCC 8739 and *S. aureus* (MRSA) ATCC 34300 were the most sensitive strains. The MIC values was 1000 µg/mL for *E. coli* ATCC 8739 and *B. cereus* ATCC 14579 and the two *C. albicans* tested. For the other tested strains, the MIC was found to be 2000 µg/mL. MFC was found to be 1000 µg/mL for the *C. albicans* tested strains. MBC values 1000 µg/mL for *B. cereus* ATCC 14579 and 2000 µg/mL for the other strains. MBC/MIC ratio was two for *E. coli* ATCC 8739 and one for the other strains, MFC/MIC ratio was found to be one, *C. candidissimum* methanolic extract was considered as bactericidal and fungicidal.

Table 15. Antibacterial activity of *C. candidissimum* methanolic extract

Extract \ Strains			*S. aureus* (MRSA) ATCC 34300	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 9027	*E. coli* ATCC 8739	*E. coli* ATCC 25922	*B. cereus* ATCC 14579	*C. albicans* (CA2) ATCC 10231	*C. albicans* (M3) ATCC 10237
Clinopodium candidissimum	Diameter of inhibition zone (mm)	50 (mg/mL)	11,33 ±1,15	11,66 ±0,57	10,66 ±0,57	11 ±0	12 ±1	9 ±0	9,33 ±0,57	9,33 ±0,57
		100 (mg/mL)	14±0	13±0	12±0	14±0	12±0	10±0	13±0	12±0
		200 (mg/mL)	18±0	13±0	12±0	14±0	13±0	11±0	13±0	13±0
	MIC (µg/mL)		2000	2000	2000	1000	2000	1000	1000	1000
	MBC (µg/mL)		2000	2000	2000	2000	2000	1000	-	-
	MFC (µg/mL)		-	-	-	-	-	-	1000	1000
	MBC/MIC ratio		1	1	1	2	1	1	-	-
	MFC/MIC ratio		-	-	-	-	-	-	1	1

*Values expressed herein are mean ± SEM of three parallel measurements

In agreement with our results **Benyoucef *al.* (2020)**, report that *C. candidissimum* essential oil had moderate antimicrobial activities against all strains (*E. coli, P. aeruginosa, Salmonella typhi, S. aureus, Clostridium sporogenes, B. subtilis, Enterococcus faecalis, Lactobacillus rhamnosus, B.*

cereus) with MIC and MBC values between 1.5-6.2 µl/ml and 6.2-12.5 µl/ml, respectively. The essential oil had a bacteriostatic effect against *E. coli* only. A majority of the antimicrobial properties of essential oils are due to the presence of oxygenated terpenoids, especially phenolic terpenes, phenyl propanoids and alcohols. Several reports have shown that carvacrol exerts an antibacterial effect by disintegrating the outer membrane and disrupting the cytoplasmic membrane of bacteria **(Lambert et *al.*, 2001; Tabanca et *al.*, 2004)**.

Attou (2017), showed that *C. candidissimum* essential oil have a bacteriostatic effect against all susceptible strains. It is exerts a growth inhibition at concentrations not exceeding 6.25 µl/ml against $Gram^+$, the most sensitive being *S. aureus* (0.78 µl/ml) and the yeast *C. albicans*, the only bacterium $Gram^-$ sensitive to this essential oil, *E. coli* is inhibited at 12.5 µl/ml.

According to **Brahmi et *al.* (2017)**, nonpolar extracts from plants have been shown more effective antibacterial properties than polar extracts. This is often true for plants belonging to the genus *Micromeria*; in fact, the antibacterial activity of these plants is mostly due to their essential oils rather than polar compounds. **Ali-Shtayeh et *al.* (1997)**, reported that the antimicrobial activity of different *Micromeria* species extracts could be attributed to plants phenolic compounds.

The Antibiotic susceptibility testing results of this study**(Table 16)** show that there are resistant strains such as *P. aeruginosa* ATCC 9027, *S. aureus* (MRSA) ATCC 34300 and *Bacillus cereus* ATCC 14579, and very sensitive strains and extremely sensitive strains**(Appendix VI)**.

Table 16. Antibacterial activity of antibiotics against the tested strains

Strains / Antibiotics		*S. aureus* (MRSA) ATCC 34300	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 9027	*E. coli* ATCC 8739	*E. coli* ATCC 25922	*B. cereus* ATCC 14579	*C. albicans* (CA2) ATCC 10231	*C. albicans* (M3) ATCC 10237
Diameter of inhibition zone (mm)	Amoxicillin (AMC) 30 µg	40	22	00	34	20	15	-	-
	Cefazolin (CZ) 30 µg	00	23	00	34	25	00	-	-
	Ceftriaxon (CRO) 30µg	00	15	18	44	36	00	-	-
	Cefoxitin (FOX) 30µg	22	12	00	40	25	00	-	-
	Amph (AMB) 20µg	-	-	-	-	-	-	22	20

The use of antimicrobial agents is critical to the successful treatment of infectious diseases, although there are numerous classes of drugs that are routinely used to treat infections in humans; pathogenic microorganisms are constantly developing resistance to these drugs because of indiscriminate use of antibiotics **(Gibbons, 1992; Al-Bari et *al*., 2006)**. The use of higher plants and preparations made from them to treat infections is a longstanding practice in a large part of the population, especially in the developing countries, where there is dependence on traditional medicine for a variety of ailments **(Ahmad and Mohammad, 1998).**

Many naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against pathogens **(Deans and Ritchie, 1987; Kumar et *al*., 2006)**. Bacterial infectious diseases represent an important cause of morbidity and mortality worldwide. Therefore, the development of new antimicrobial agents for the treatment of bacterial infections is of increasing interest.

Our findings were very encouraging and motivating to pursue supplementary investigations in order to isolate and purify individual components that are conducting this act and furthermore to study *in vivo* biological activities.

CONCLUSION

CONCLUSION

Using medicinal plants for various treatments has been practiced for centuries due to its pharmaceutical, cosmetic and nutritional values. This traditional medicinal knowledge has been passed orally from generation to generation. Today's generation does not take much interest, and there is a threat of extinction of therapeutic expertise, thus it needs to be documented. In order to promote Algerian medicinal and edible plants, in this study we report the first ethnobotanical survey of *Clinopodium candidissimum* (Munby) Kuntze and *Micromeria inodora* (Desf.) Benth. (*Lamiaceae* native of Western Algeria) in Oran region which contribute to a better knowledge and to evaluate the ethnobotanical informations related to the use of these plants which is widely used in traditional therapy for medicinal purposes.

Ethnobotanical surveys among one hundred people have been carried out in Oran region, it was characterized by the predominance of men with (74%), the age class of [30-40[years with (39%) and people with secondary level represent the highest rate (44%). Informants reported no traditional knowledges on *M. inodora*, while, for *C. candidissima* therapeutic and culinary uses were recorded.

In addition, we performed a phytochemical screening of these two plant species, which highlighted the presence of flavonoids, quinones, terpenoids, phenolic compounds, saponins and catechic tannins. Mineral quantifications demonstrate that the studied plants aerial parts contains high amounts of minerals particularly iron, manganese, zinc, boron, copper, calcium and potassium. Total phenolic and flavonoids contents in the methanolic extracts was found to be 63.33 (µg $GA_{eq}.mg^{-1}Ext$) and 11.19(µg $Q_{eq}.mg^{-1}Ext$) for *M. inodora*; 58.79(µg $GA_{eq}.mg^{-1}Ext$) and 40.93(µg $Q_{eq}.mg^{-1}Ext$) for *C. candidissimum*, respectively.

For *M. inodora*, GC–MS analyses of volatiles extracted using either hydrodistillation or HS-SPME yielded different results, being the two extraction methods not comparable. The analysis of the essential oil obtained by hydrodistillation allowed to identify 66 constituents, among which oxygenated mono- and sesquiterpenes were the most abundant (38.2 % and 32.0 %, respectively) and sesquiterpene hydrocarbons (10.8 %). Main components were the monoterpenes α-terpinyl acetate (29.1 %) and camphor (7.0 %), and the sesquiterpene cis-14-nor-muurol- 5-en-4-one (13.8 %). On the contrary, the characterization of the volatile extract obtained by HS-SPME allowed to identify 45 constituents, among which camphor (23.8 %), α-terpinyl acetate (21.3 %) and 1,8-cineole (10.2 %) were the most representative. Explorative 1H-NMR analyses of methanolic extract of *M. inodora* showed the presence of glycosidic derivatives of phenylpropanoids, flavonoids and triterpenes. Multi-stage HPLC-MS analysis of

the same extract allowed to tentatively identify 41 of these compounds, mainly glycosylated and non-glycosylated flavonoids (19 compounds) and phenolic acids (9 compounds).

For *C. candidissimum*, the qualitative ^{1}H-NMR and quali-quantitative HPLC-MS^{n} analyses of fractions obtained with solvents at different polarity revealed the presence of aglyconic and glycosylated flavonoids (3.1%), phenylpropanoids (3.6%), gallic acid derivatives (0.76%), and triterpenoids (0.62%), among the others. On the other hand, HS-SPME-GC-MS allowed to identify 38 volatile constituents, among which the oxygenated monoterpenes pulegone (44.8%), piperitenone (6.6%), isopulegone (5.8%) and neo-menthol (3.8%), and the sesquiterpene hydrocarbons germacrene D (16.2%) and bicyclogermacrene (3.0%) were the most abundant.

Therefore, to evaluate antioxidant activities of *C. candidissimum* and *M. inodora* methanolic extracts DPPH free radical scavenging, ABTS cation radical scavenging and CUPRAC (cupric-reducing antioxidant capacity) assays were investigated. Antioxidant potential of *M. inodora* methanolic extract was found to be (DPPH: IC_{50} = 63.17 ± 2.28 µg/mL; $ABTS^{+}$: IC_{50} = 31.08 ± 1.06 µg/mL; CUPRAC: $A_{0.5}$ = 67.10 ± 2.06 µg/mL). *C. candidissimum* extract revealed a reducing potential of (DPPH: IC_{50} = 47.93 ± 1.11 µg/mL; $ABTS^{+}$: IC_{50} = 36.47 ± 0.91 µg/mL; CUPRAC: $A_{0.5}$ = 58.31 ± 1.31 µg/mL). The studied species exhibit a moderate antioxidant activity but *M. inodora* show a promising effect compared to *C. candidissimum*.

Enzyme inhibition potential of plant extracts *in vitro* anticholinesterase (AChE and AChB), α-glucosidase and α-amylase inhibitory activities were investigated. The studied plants were unable to indicate anticholinesterase activity (IC_{50}>200 µg/mL) and no significant α-glucosidase and α-amylase enzyme inhibition activities (IC50>400 µg/mL) was observed in the methanolic extracts.

Antimicrobial activity of methanolic extracts were determined by disc diffusion test against several strains (*Staphylococcus aureus* (MRSA) ATCC 34300, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231 and *Candida albicans* ATCC 10237).

All tested strains are sensitive in all the concentration range of 50 mg/mL, 100 mg/mL and 200 mg/mL. Concerning *M. inodora,* IZD was (9 -12.33mm) for the concentration of 50 mg/mL, (10 - 17 mm) for the concentration of 100 mg/mL and (11 - 17 mm) for the concentration of 200 mg/mL. S. aureus (MRSA) ATCC 34300 was the most sensitive for all the concentration range. The MIC values was 1000 µg/mL for *E. coli* ATCC 25922 and 2000

μg/mL for all the tested strains, MBC and MFC was found to be <2000 μg/mL for all the tested strains. MBC/MIC ratio was one for *P. aeruginosa* ATCC 9027, for the other strains was not determined. While for *C. candidissimum* methanolic extract, IZD was (9 -12 mm) for the concentration of 50 mg/mL, (10 - 14 mm) for the concentration of 100 mg/mL and (11 - 18 mm) for the concentration of 200 mg/mL. *E. coli* ATCC 8739 and S. aureus (MRSA) ATCC 34300 were the most sensitive strains. The MIC values was 1000 μg/mL for *E. coli* ATCC 8739 and *Bacillus cereus* ATCC 14579 and the two *C. albicans* tested. For the other tested strains, the CMI was found to be 2000 μg/mL. MFC was found to be 1000 μg/mL for the *C. albicans* tested strains. MBC values 1000 μg/mL for *Bacillus cereus* ATCC 14579 and 2000 μg/mL for the other strains. MBC/MIC ratio was two for *E. coli* ATCC 8739 and one for the other strains, MFC/MIC ratio was found to be one.

Obtained results showed the richness of *C. candidissimum* and *M. inodora* with chemical substances and may represent a new potential source of bioactive compounds in therapy. This can serve as a basis for pharmacological studies in order to evaluate the therapeutic efficacy and safety of these two plants with traditional biological effect.

Our findings show that endemic species contains valuable components endowed with possible biological activities, including antioxidant and volatile compounds, which could find wide practical application in various areas, such as formulation and production of food additives and healthy supplements. These findings could be useful for other research in particular; the combination of traditional knowledge with currently available research tools may open up new perspectives for drug discovery from natural sources.

Recommendation

Based on the findings of this study the following recommendations are suggested:

- The extracts of these plants should be further analyzed to isolate the specific bioctive compounds.
- Toxicity studies of the effective plants should also be done to determine the safety indices of the extracts. Clinical trials should be carried out to explore the potential of these plant extracts in the treatment of the infectious diseases.
- Determine the other biological activities of these plant extracts: anti-inflammatory, anticancer…….
- The assessment of the bioactivities of these plants extracts for *in vivo* research will be an interesting area to be investigated, in order to determine the optimum dosage that ensures the safety and efficacy of these plants for the management of health diseases.

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APPENDICES

Appendix I: Ethnobotanical survey sheet

IBN KHALDOUN UNIVERSITY- TIARET -

FACULTY OF NATURAL AND LIFE SCIENCES

DEPARTMENT OF NATURAL AND LIFE SCIENCES

ETHNOBOTANICAL SURVEY SHEET

Sheet number:

First and last name:

Age : ☐ <20 ☐ [20-30[☐ [30-40[☐ [40-50[☐ [50-60[

☐ [60-70[☐ [70-80[☐ <80

Gender: ☐ Male ☐ Female

Academic level: ☐ Illiterate ☐ Primary ☐ Secondary ☐ University

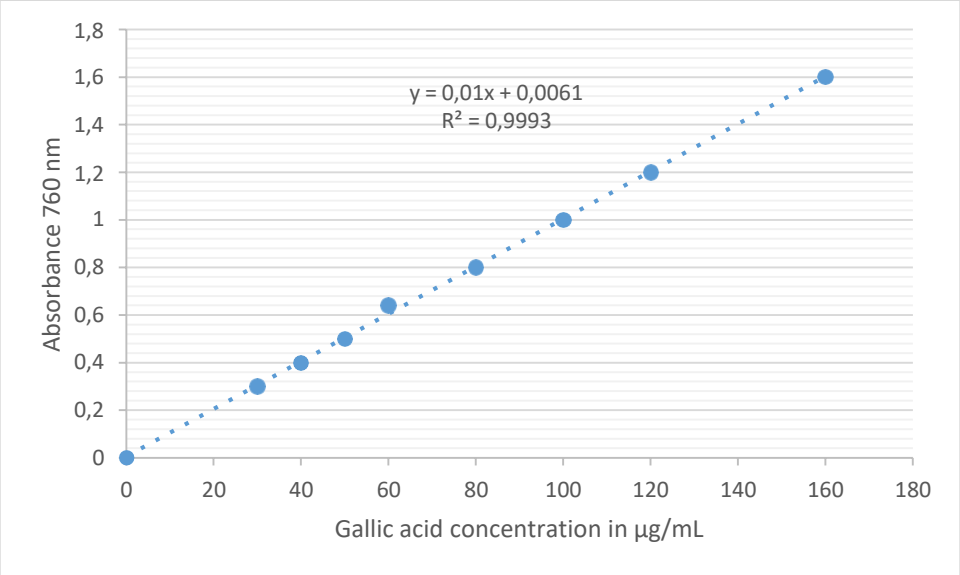
Medicinal species	***Micromeria inodora* (DESF.) Benth.**	***Clinopodium candidissimum (Munby) Kuntze***
Vernacular name		
Habitat and distribution		
Harvested or Purchased		
Therapeutic use		
Part used		
Method of preparation		
Method of administration		
Dosage		

Appendix II: Phytochemical screening results of *M. inodora* and *C. candidissimum* methanolic extract

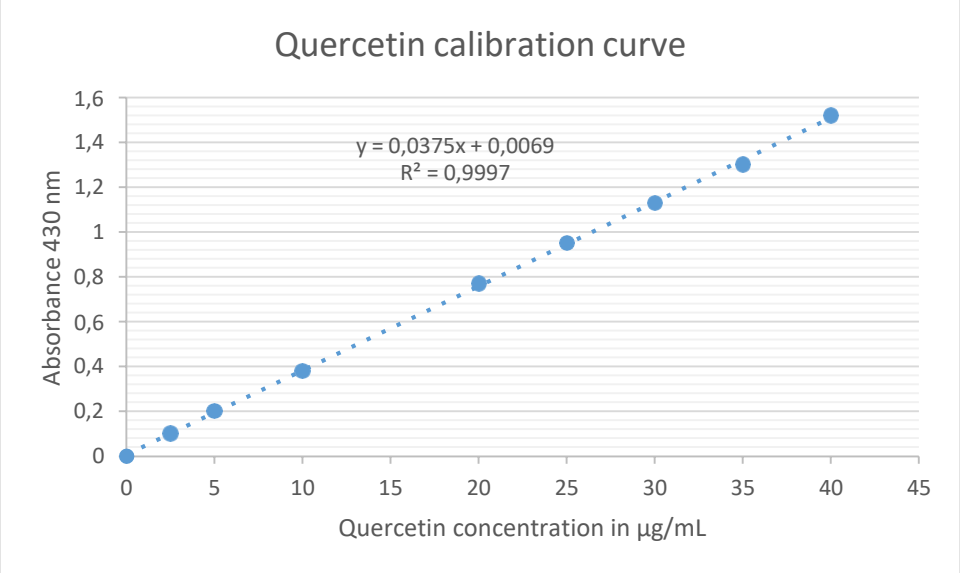
Phytochemical screening results of *M. inodora* methanolic extract

Phytochemical screening results of *C. candidissimum* methanolic extract

Appendix III: Calibration curves



Gallic acid calibration curve



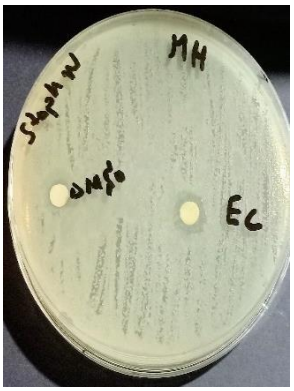
Quercetin calibration curve

Appendix IV: Antioxidant activities (IC_{50} and $A_{0.5}$ values) of the methanolic extracts of *M. inodora* and *C. candidissimum*

Extracts & compounds		Antioxidant activity		
		DPPH assay [a]	**$ABTS^+$ assay** [a]	**CUPRAC assay** [a]
		IC_{50} (µg/mL)	**IC_{50} (µg/mL)**	**$A_{0.5}$ (µg/mL)**
M. inodora		63.17 ± 2.28	31.08 ± 1.06	67.10 ± 2.06
C. candidissimum		47.93 ± 1.11	36.47 ± 0.91	58.31 ± 1.31
BHA[b]	Standard	3.44 ± 0.09	1.88 ± 0.06	5.62 ± 0.08

Abbreviation: BHA, 2-tert-Butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole
[a] Values expressed herein are mean ± SEM of three parallel measurements. $p<0.05$
[b] Reference compound

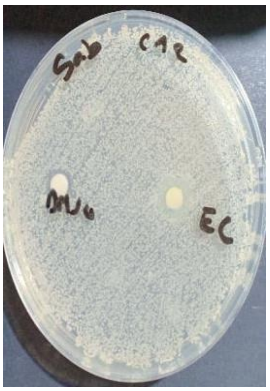
Appendix V: Antimicrobial activity of *C. candidissimum* and *M. inodora* methanolic extracts



S. aureus ATCC 6538

B. cereus ATCC 14579

S. aureus (MRSA) ATCC 34300

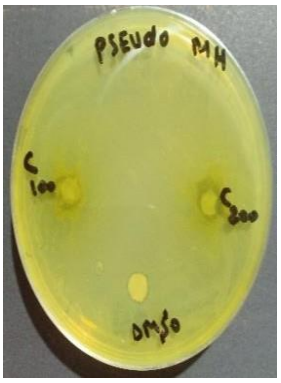


C. albicans ATCC 10231

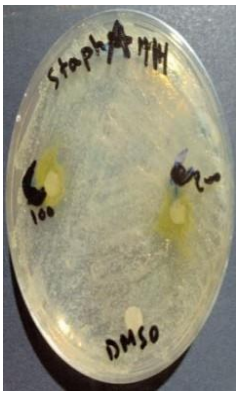
E. coli ATCC 8739

E. coli ATCC 25922

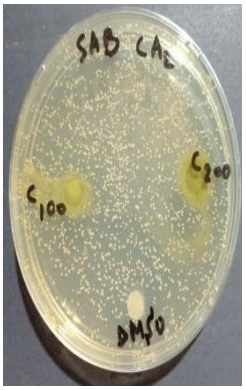
C. albicans ATCC 10237



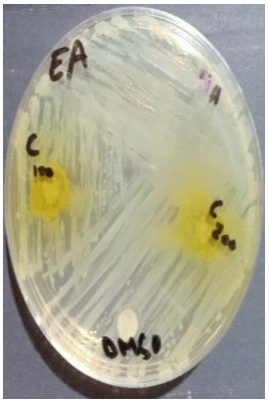
P. aeruginosa ATCC 9027



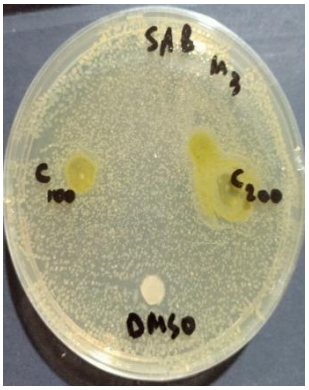
S. aureus (MRSA) ATCC 34300



C. albicans ATCC 10231

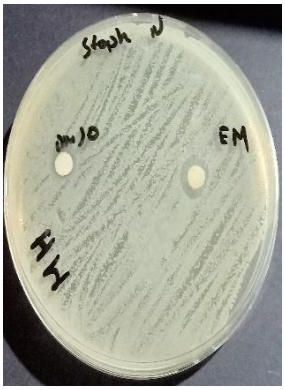


E. coli ATCC 8739

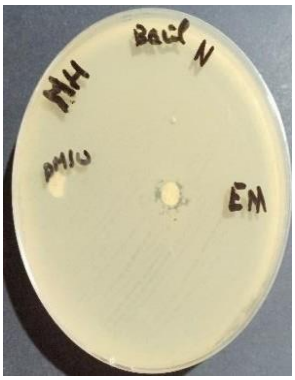


C. albicans ATCC 10237

Antibacterial activity of *C. candidissimum* methanolic extract against the tested strains , diameter of inhibition zone (mm) for the concentration range of 50 mg/mL, 100 mg/mL and 200 mg/mL

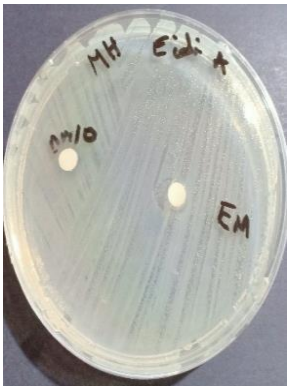


S. aureus ATCC 6538



B. cereus ATCC 14579

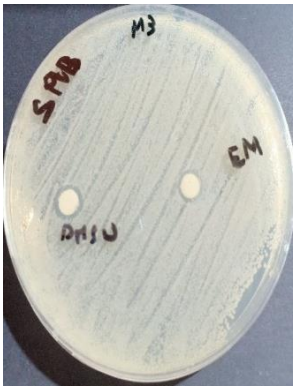
S. aureus (MRSA) ATCC 34300



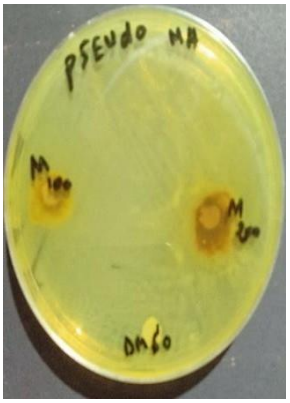
E. coli ATCC 8739

C. albicans ATCC 10231

E. coli ATCC 25922



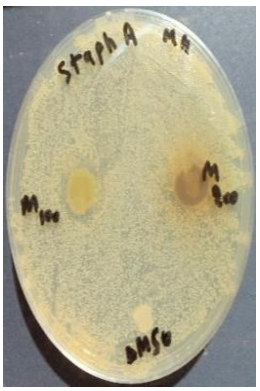
C. albicans ATCC 10237



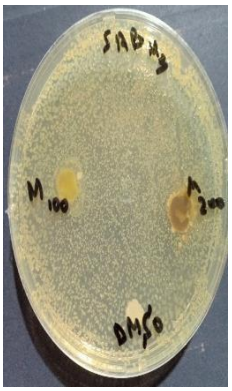
P. aeruginosa ATCC 9027

C. albicans ATCC 10231

E. coli ATCC 8739



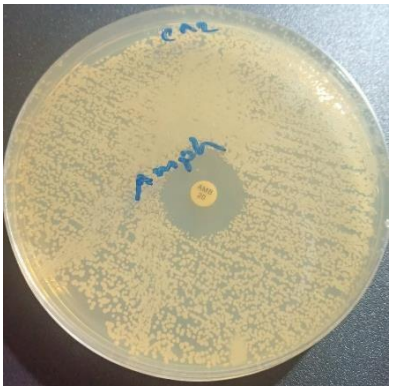
S. aureus (MRSA) ATCC 34300



C. albicans ATCC 10237

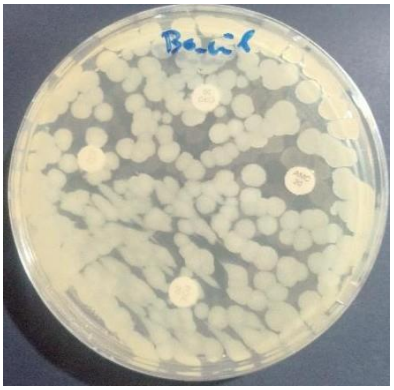
Antimicrobial activity of *M. inodora* methanolic extracts against the tested strains , diameter of inhibition zone (mm) for the concentration range of 50 mg/mL, 100 mg/mL and 200 mg/mL

Appendix VI: Antibacterial activity of antibiotics (Amoxicillin (AMC) 30 µg, Cefazolin (CZ) 30 µg, Ceftriaxon (CRO) 30µg, Cefoxitin (FOX) 30µg and Amphotericin B (AMB) 20µg) against the tested strains



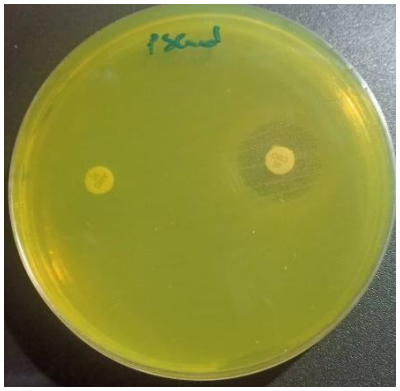
C. albicans ATCC 10231

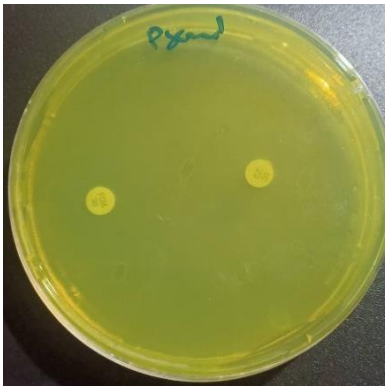
C. albicans ATCC 10237



B. cereus ATCC 14579

E. coli ATCC 25922



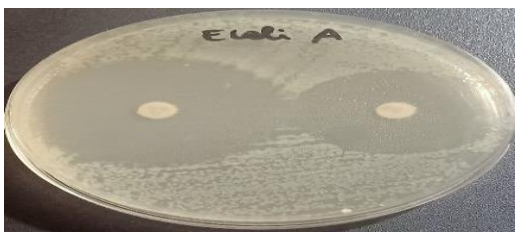


P. aeruginosa ATCC 9027

S. aureus ATCC 6538

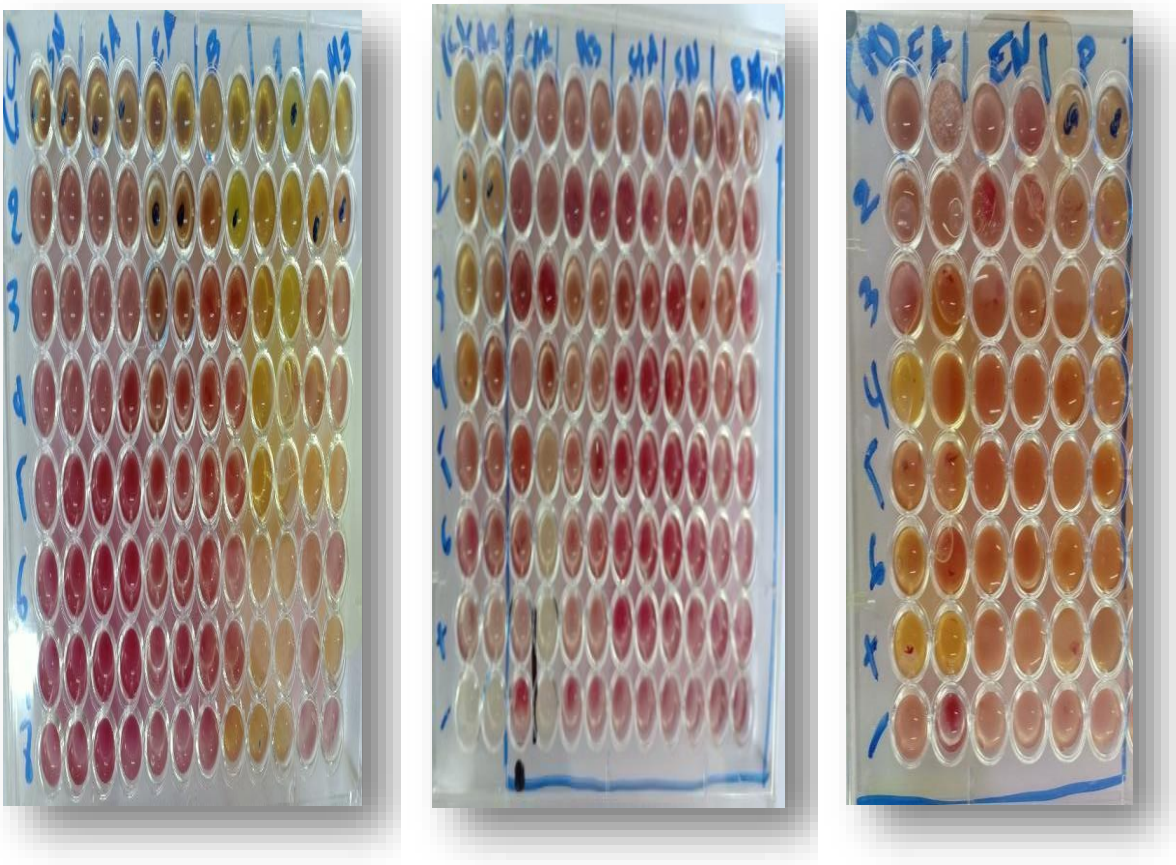
S. aureus (MRSA) ATCC 34300

E. coli ATCC 8739



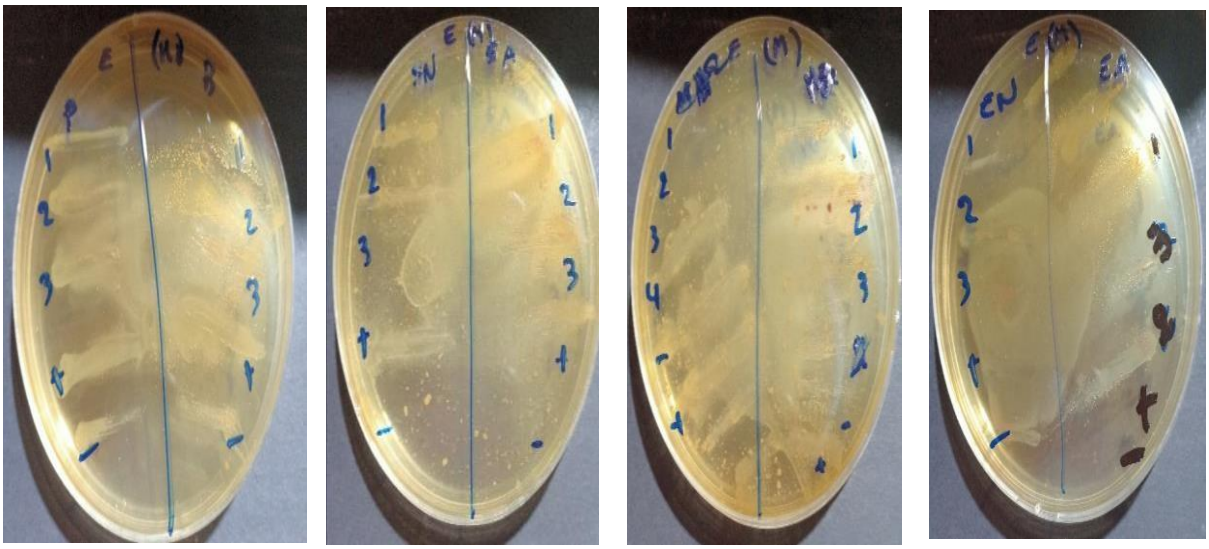
E. coli ATCC 8739

Appendix VII: Determination of Minimum Inhibitory Concentration (MIC) of *M. inodora* and *C. candidissimum* methanolic extracts against the tested microbial strains



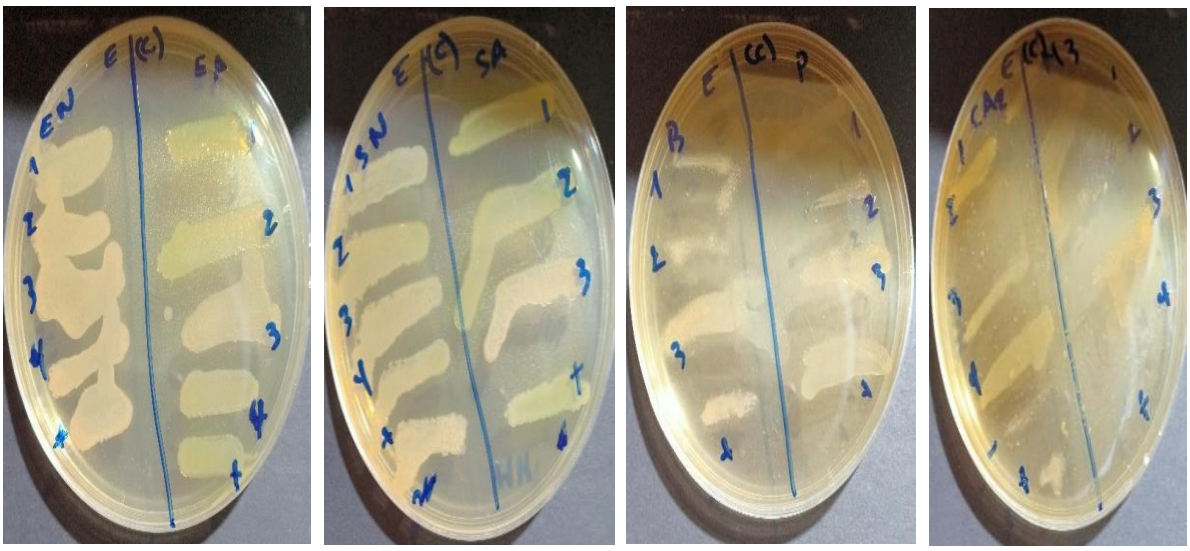
Minimum Inhibitory Concentration (MIC) of *M. inodora* and *C. candidissimum* methanolic extracts against the tested microbial strains

Appendix VIII: Determination of Minimal bactericidal concentration (MBC) and Minimal fungicidal concentration (MFC) of *M. inodora* and *C. candidissimum* methanolic extracts against the tested microbial strains



P. aeruginosa ATCC 9027	*B. cereus* ATCC 14579	*S. aureus* ATCC 6538	*S. aureus* (MRSA) ATCC 34300	*C. albicans* ATCC 10231	*C. albicans* ATCC 10237	*E. coli* ATCC 25922	*E. coli* ATCC 8739

Minimal bactericidal concentration (MBC) and Minimal fungicidal concentration (MFC) of *M. inodora* methanolic extract



E. coli ATCC 25922	*E. coli* ATCC 8739	*S. aureus* ATCC 6538	*S. aureus* (MRSA) ATCC 34300	*B. cereus* ATCC 14579	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*C. albicans* ATCC 10237

Minimal bactericidal concentration (MBC) and Minimal fungicidal concentration (MFC) of *C. candidissimum* methanolic extract

PUBLICATIONS

LIST OF PUBLICATIONS

- **Nacéra Bouriah**, Hamdi Bendif, Gregorio Peron, Mohamed Djamel Miara, Stefano Dall'Acqua, Guido Flamini, Filippo Maggi : Composition and profiling of essential oil, volatile and crude extract constituents of *Micromeria inodora* growing in western Algeria, *Journal of Pharmaceutical and Biomedical Analysis*, 2021, Volume 195,113856.
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- Bendif Hamdi, Gregorio Peron, Mohamed Djamel Miara, **Nacéra Bouriah**, Guido Flamini, Filippo Maggi, Stefania Sut & Stefano Dall'Acqua (2022): Phytochemical analysis of *Clinopodium candidissimum* (Munby) Kuntze growing in Algeria by an integrated HS-SPME-GCMS, NMR and HPLC-DAD-MSn approach: valorisation of an endemic natural source of bioactive compounds, *Natural Product Research*, DOI: 10.1080/14786419.2022.2104272

Conferences

National Communications

- **BOURIAH N.**, Bendif, H., Miara, M.D., Benslama, A. and Maggi, F. "Chemical composition of Essential Oils of *Micromeria inodora* (Desf.) Benth. Growing in the West of Algeria", national "webinar" seminar on Bio-resources: Nutrition, Health and Environment; 17 and 18 May 2021, Mohamed Boudiaf University of M'Sila, Sciences Faculty, Department of Microbiology and Biochemistry, Posted communication.
- **BOURIAH N.**, Bendif, H., Miara, M.D., "HS-SPME/GC-MS analysis of volatile constituents of *Calamintha candidissima* (Munby.) Briq. growing in Algeria", 1st National Seminar on Sustainable Agriculture and Biodiversity 11 and 12 May 2022 via Google meet, 20 August 1955 Skikda University, Department of Agronomic Sciences, Laboratory of Optimization of Agricultural Production in Sub humid Zones (LOPAZS), communication posted by video conference.

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Composition and profiling of essential oil, volatile and crude extract constituents of *Micromeria inodora* growing in western Algeria

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The chemical constituents of the aerial parts of *Micromeria inodora* (Desf.) Benth. (Lamiaceae) collected in western Algeria, an aromatic shrub traditionally consumed as an herbal remedy, have been characterized. Secondary metabolites were extracted using sequential extractions with solvents at different polarity, and they were determined using an integrated nuclear magnetic resonance (NMR) and high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) approach. The composition of the essential oil obtained by hydrodistillation was characterized by gas chromatography-mass spectrometry (GC–MS), and the aroma profile was analysed by head-space solid phase micro extraction (HS-SPME) coupled with GC–MS. NMR and LC–MS^n analyses revealed the presence of aglyconic and glycosylated flavonoids, phenylpropanoid derivatives and triterpenoid acids, mainly in the methanol, dichloromethane and *n*-hexane extracts. The analysis of the essential oil allowed to identify 66 components, mainly oxygenated mono- and sesquiterpenes (38.2 % and 32.0 %, respectively) and sesquiterpene hydrocarbons (10.8 %). The aroma profile revealed by HS-SPME-GC–MS was characterized by 45 volatile compounds, among which oxygenated monoterpenes appeared to be the most abundant (65.8 %), followed by sesquiterpene hydrocarbons (16.4 %) and monoterpene hydrocarbons (10.6 %). Overall, these data indicate that *M. inodora* could be a potential source of antioxidants and bioactive compounds, and they represent a starting point for further research on this plant species.

1. Introduction

Aromatic plants belonging to the Lamiaceae family have always been of great interest because of their pleasant smell and their medicinal properties. Many Lamiaceae species are used worldwide in the folk medicine and/or as culinary herbs. According to current knowledge, the genus *Micromeria* comprises about 70 species [1], among which some are endemic. The genus *Micromeria* encompasses sub-shrubs with upright and very branched stems, 10–50 cm high, a glabrescent calyx and a 4-mm pink corolla, small leaves (2−3 mm), sessile, acicular, with rolled edges, tomentose below [2]. On the basis of their morphological characteristics and their phylogenetic relationships, species of *Micromeria* are grouped into three sections [3]: *Cymularia*, *Eumicromeria* and *Pseudomelissa*.

Micromeria species are well known aromatic species containing appreciable amounts of essential oil. Its relative content has been used as one of the characters for the classification of this genus in the subfamily Nepetoideae [4]. *M. inodora* (Desf.) Benth. is an odourless plant that lives on rocky soil and lime stones in the western Mediterranean region, particularly located in the center of Algeria (Algerois sector) and the Balearic Islands [5]. Among its synonyms, the following should be cited: *Satureja fontanesii* Briq., *Micromeria barceloi* Willk., *Satureja barceloi* (Willk.) Pau, and *Thymus inodorus* Desf.

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Micromeria species, popularly known as Ï azirahmeur; are used in Algeria as herbal teas and replacement for mint in traditional medicine [2,6]. They also have anaesthetic, antirheumatic, antiseptic, abortifacient, and stimulant properties. In addition, they are useful for the treatment of colds and heart diseases [6,7]. Other *Micromeria* species are used in the folk medicine of many countries: in Spain, *M. graeca* and *M. biflora* are employed for gastro-intestinal disorders; in Turkey, *M. fruticosa* is used against headaches; in the Canary Islands *M. herpyllomorpha* and *M. varia* are claimed to have hair tonic properties; in Israel, the decoction is used for inflamed eyes baths or to treat abdominal pains and hypertension. The most widespread uses of *Micromeria* species are against headaches, wounds colds and skin infections [8].

The essential oil composition of several *Micromeria* species has been previously reported by other authors [8–28]. Regarding *M. inodora,* previous studies have concerned its volatile fraction [6], nevertheless, to our knowledge, no study has been conducted on the constituents of its crude extract.

To further contribute to the phytochemistry of this scarcely known species, in this work we present an exhaustive characterization of the aroma profile and the non-volatile constituents of the polar and apolar extracts of *M. inodora* aerial parts by means of different techniques, namely gas chromatography-mass spectrometry (GC–MS) analysis of the hydrodistilled essential oil, head-space solid phase micro-extraction (HS-SPME) coupled with GC–MS for the volatile fraction and an integrated NMR and high-performance liquid chromatography with mass spectrometry detection (HPLC-MS) approach for the characterization of the crude extracts.

2. Materials and methods

2.1. Plant material

M. inodora aereal parts (Fig. 1) were collected from individuals spontaneously growing in the region of Lions Mountain, Oran (North-West Algeria, 200 m a.s.l., N 35.798917, W 0.489923) in March 2016. Botanical determination was performed by Dr. Miara using available literature [2], and an herbarium specimen was deposited in the Herbarium Universitatis Camerinensis, University of Camerino, Italy, under the codex CAME 27739, and available online on the anArchive system (http://www.anarchive.it/anArchive/index.jsp). Before extraction, the plant material was dried in the shadow for 7 days at room temperature.

2.2. Essential oil extraction by hydrodistillation

M. inodora aerial parts (100 g) were cut into small pieces and submitted to hydro-distillation for 3 h in a Clevenger type apparatus, using 2 L of deionized water. A yellow essential oil was collected and dried over anhydrous Na_2SO_4. It was stored into dark sealed vials at 4 °C before further investigations.

2.3. GC–MS analysis of volatiles

For GC-EI-MS (Gas chromatography coupled with electron ionization mass spectrometry detection) analysis a Varian CP-3800 gas-chromatograph equipped with a capillary DB-5 column (30 m × 0.25 mm and 0.25 µm coating thickness) coupled with a Varian Saturn 2000 mass detector were used. The analytical conditions were as follows: injector 220 °C, and transfer line 240 °C; oven temperature programmed from 60 to 240 °C at 3 °C/min (gradient length: 60 min); carrier gas helium at 1 mL/min; 0.2 µl of a 10 % *n*-hexane solution of the essential oil were injected in the split mode (split ratio 1:30) and using the following acquisition parameters: full scan; scan range: 35–300 *m/z*; scan time: 1.0 s; threshold: 1 count. The constituents were identified by comparison of the retention times with those of authentic samples, comparing their linear retention indices (l.r.i.) relative to the series of *n*-hydrocarbons, and on computer matching against commercial (NIST 14 and ADAMS) and home-made libraries built up from pure substances.

2.4. HS-SPME/GC–MS analysis

To sampling the headspace, Supelco SPME (Solid Phase Micro-Extraction) polydimethylsiloxane (PDMS, 100 µm) devices were used. After 30 min of equilibration time, the fibre was exposed to the headspace for further 30 min, then the fibre was withdrawn into the needle and transferred to the injector of the GC–MS system, operating in the same conditions as above, apart from a splitless injection technique.

2.5. Preparation of extracts for NMR and HPLC-MS^n analyses

Sample preparation was performed according to previously published methods [29,30]. Briefly, after grinding 3 *g* of the aerial parts with a blender, sonication of 500 mg of powder was done with 50 mL of methanol for 10 min. After centrifugation, the supernatant was removed, and the residue was extracted in the same way with another 50 mL of the same solvent for two more times. The supernatants were then collected in a round-bottom flask, and the extract concentrated under reduced pressure at 30 °C using a rotary evaporator, obtaining 93.10 mg of crude extract (yield 18.62 % w/w). The extracts were stored in dark glass vials at −20 °C before chemical characterization. 500 mg of the residue was dissolved in methanol-water (1:9) (50 mL) and sonicated. Then, the solution was partitioned with *n*-hexane (20 mL for three times), dichloromethane (DCM; 20 mL for three times) and ethyl acetate (EA; 20 mL for three times). The solutions were dried under vacuum and the residues re-dissolved in deuterated methanol for NMR analysis.

2.6. NMR analysis

NMR data were obtained on a Bruker Avance III 400 MHz spectrometer using standard pulse sequences. ^{1}H-NMR was obtained for MeOH, DCM, EA and *n*-hexane fractions.

2.7. HPLC–MS^n analysis of methanol extract from stems and leaves

The phenolic constituents in the methanolic extract of the aerial parts of *M. inodora* were tentatively identified by comparison of their HPLC-MS^n fragmentation patterns with literature data and with freely available web MS databases (Metlin: https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage; MoNA: https://mona.fiehnlab.ucdavis.edu/). The sample was dissolved in methanol at a concentration of 5 mg/mL and filtered through a 0.45 µm Millipore filter. The HPLC-MS system consisted of a 212 Varian binary pump equipped with a Varian Prostar 430 autosampler, coupled to a Varian MS500 mass detector (Ion Trap), operating in both negative and positive Electrospray Ionisation (ESI) modes. The separations were obtained on an Agilent Eclipse plus C-18 column (2.1 ×150 mm, 3.5 µm). The mobile phases were composed of acetonitrile (A) and water (B), both containing 0.1 % formic acid. The gradient was as follows: 0 min, 10 % A; 20 min, 54 % of A; 23 min, 100 % of A, and isocratic up to 32 min. Re-equilibration time was 8 min. Flow rate was 0.2 mL/min. ESI parameters were: needle voltage, ± 4500 V; capillary voltage, 70 V; RF loading, 100 %; nebulizing gas pressure, 20 psi (nitrogen); drying gas pressure, 15 psi; drying gas temperature, 350 °C. Mass range was 50 −2000 Da. Fragmentation patterns of eluted compounds were obtained

Fig. 1. Morphological aspects of *Micromeria inodora* (Desf.) Benth.

using the turbo detection data scanning (TDDS) function of the instrument, setting n = 3 levels of fragmentation.

3. Results and discussion

3.1. Essential oil analysis

Hydrodistillation of the aerial parts of *M. inodora* gave 0.1 % oil yield on a dry weight basis. This value is consistent with that reported for the same species growing in Algeria [6]. Its composition is reported in Table 1. Summarizing, 66 components were identified, while in a previous study on a cumulative sample composed of 24 pooled essential oils from *M. inodora* collected from different areas of Algeria [6], 83 constituents were described. Overall, the essential oil obtained in our study was characterized by the oxygenated mono- and sesquiterpenes fractions, accounting for 38.2 and 32.0 %, respectively, followed by sesquiterpene hydrocarbons (10.8 %). On the contrary, monoterpene hydrocarbons were scarcely represented in the oil (0.7 %); non terpene aldehydes and alkanes reached 1.6 and 2.4 %, respectively. This finding is in good agreement with those reported in [6] on Algerian populations of *M. inodora*, which were dominated by oxygenated compounds (78.1 %). In detail, in this work oxygenated sesquiterpenes (46.9 %) were higher than oxygenated monoterpenes (30.4 %).

Overall, in the present study the main components were the monoterpenes α-terpinyl acetate (29.1 %) and camphor (7.0 %), together with the sesquiterpene *cis*-14-*nor*-muurol-5-en-4-one (13.8 %). Other minor monoterpene components were α-pinene (0.2 %), limonene (0.3 %) and borneol (0.2 %). Finally, other representative sesquiterpenes were caryophyllene oxide and α-cadinol (3.1 and 2.6 %, respectively). In the previous study, Benomari et al. [6] reported a different profile, rich of *trans*-sesquisabinene hydrate (20.9 %), α-terpinyl acetate (19.8 %), globulol (4.9 %), caryophyllene oxide (4.3 %), β-bisabolol (2.9 %) and *trans*-7-*epi*-sesquisabinene hydrate (2.6 %). Variations between our results and the essential oil composition reported in [6] could be due mainly to the different collections sites of the plant specimens and to seasonal variations related to the different harvesting times.

3.2. Headspace analysis of aroma profile

The SPME has the advantage to be a fast, simple and non-destructive method of pre-concentration that can be performed without solvents, with detection limits that can reach parts per billion (ppb) levels for some compounds. Using this technique, the volatiles spontaneously emitted by the powdered aerial parts of *M. inodora* were studied. The 45 volatile compounds identified are reported in Table 2. Although not directly comparable, these numbers were significantly lower than those reported in the essential oil studied (Table 1). Overall, the powder of *M. inodora* was dominated by the monoterpene fraction. Oxygenated ones were the main group, with 65.8 % of the total composition, followed by sesquiterpene hydrocarbons (16.4 %) and monoterpene hydrocarbons (10.6 %). Among volatiles, the main emitted monoterpenes were camphor (23.8 %), α-terpinyl acetate (21.3 %) and 1,8-cineole (10.2 %). Other components were present in smaller amounts, such as β-caryophyllene (3.1 %). From a qualitative standpoint, the aroma profile detected by SPME was overlapping that of essential oil for the monoterpene fraction but with a higher selectivity (76.4 *vs.* 49.0 % for monoterpenes in headspace and essential oil, respectively). On the other hand, the PDMS fibre revealed to be less sensitive for the less volatile sesquiterpenes (17.1 *vs.* 42.8 %, respectively).

3.3. NMR analysis of crude M. inodora extracts

The ^{1}H-NMR analysis of the chemical composition of *M. inodora* aerial parts was performed on extracts obtained from plant material using solvents at different polarity. The extracts, after solvent removal, were dissolved in deuterated water.

The spectrum of the methanol extract (Fig. 2) shows signals that can be assigned to different classes of secondary metabolites. In the aromatic region, signals ascribable to hydroxycinnamic derivatives could be observed. The signals at **δ** 7.03 (s), 6.95 (d; J =7.0 Hz) and **δ** 6.85 (d; J = 7.0) correspond to the position H-2, H-5 and H-6 of 1,3,4-trisubstitued aromatic ring, respectively, while those at **δ** 7.39 (d; 15.97 Hz) and 6.16 (d; 15.97 Hz) can be ascribed to the *trans* double bond. The same region of the spectrum shows signals assignable to flavonoid portions. Signals at **δ** 6.76–6.47 are ascribable to H-6 and H-8 of flavonol glycosides. Other signals in the region **δ** 4.50–5.50 can be ascribed to anomeric proton signals, while crowded signals in the region **δ** 3.00–4.00 are supporting the presence of sugar residues. The aliphatic region shows clear signals ascribable to methyl groups (both secondary and quaternary), as well as superimposed multiplets suggesting the presence of CH and CH_2 that can indicate the presence of triterpenoids.

NMR of the *n*-hexane extract shows mainly signals attributable to fatty acids and lipids (Fig. 3). Singlets at **δ** 0.851 can be assigned to terminal methyl groups, while the signals at **δ** 1.25–1.35 can be related to methylene groups from the aliphatic chain of fatty acids. Broad signal at **δ** 5.10–5.30 support the presence of unsaturated fatty acids. Methylene vicinal to olefins are revealed at **δ** 1.99 and inter-olefins signals can be assigned at **δ** 2.75, while protons at **δ** 2.28 and 1.52 could be assigned to α and β carbonyl hydrogens,

Table 1
Chemical composition of the essential oil from *Micromeria inodora* aerial part growing in Algeria.

N.	Component[a]	RI exp.[b]	RI Lit.[c]	%[d]	ID[e]
1	α-pinene	941	939	0.2	Co-I, RI,MS
2	camphene	948	946	0.1	Co-I, RI,MS
3	β-pinene	981	979	tr[f]	Co-I, RI,MS
4	2,3-dehydro-1,8-cineole	992	991	0.3	RI,MS
5	*p*-cymene	1028	1026	0.1	Co-I, RI,MS
6	limonene	1032	1031	0.3	Co-I, RI,MS
7	1,8-cineole	1034	1033	0.3	Co-I, RI,MS
8	*p*-mentha-3,8-diene	1074	1072	tr	RI,MS
9	*p*-cymenene	1091	1090	tr	RI,MS
10	nonanal	1104	1102	1.1	RI,MS
11	camphor	1145	1144	7.0	Co-I, RI,MS
12	borneol	1168	1166	0.2	Co-I, RI,MS
13	terpinen-4-ol	1179	1177	0.1	Co-I, RI,MS
14	α-terpineol	1191	1189	0.3	Co-I, RI,MS
15	*n*-decanal	1206	1203	0.4	Co-I, RI,MS
16	*trans*-carveol	1218	1217	0.2	Co-I, RI,MS
17	pulegone	1239	1237	0.4	RI,MS
18	carvone	1244	1243	0.2	Co-I, RI,MS
19	1-decanol	1272	1270	0.1	RI,MS
20	bornyl acetate	1287	1286	0.1	Co-I, RI,MS
21	(2*E*,4*E*)-decadienal	1316	1315	tr	RI,MS
22	α-terpinyl acetate	1352	1350	29.1	RI,MS
23	(2*E*)-undecenal	1368	1366	tr	RI,MS
24	α-copaene	1377	1376	0.1	Co-I, RI,MS
25	β-bourbonene	1385	1386	tr	RI,MS
26	(*E*)-caryophyllene	1419	1419	1.3	Co-I, RI,MS
27	*cis*-muurola-3,5-diene	1448	1447	0.2	RI,MS
28	α-humulene	1455	1455	0.1	Co-I, RI,MS
29	*allo*-aromadendrene	1462	1460	2.4	RI,MS
30	*cis*-cadina-1(6),4-diene	1464	1462	1.3	RI,MS
31	9-*epi*-(*E*)-caryophyllene	1468	1467	1.3	RI,MS
32	germacrene D	1482	1483	0.3	RI,MS
33	β-selinene	1487	1487	0.5	RI,MS
34	*trans*-γ-cadinene	1514	1513	0.2	RI,MS
35	*trans*-calamenene	1523	1524	1.8	RI,MS
36	δ-cadinene	1524	1524	0.3	RI,MS
37	α-cadinene	1538	1537	0.1	RI,MS
38	α-calacorene	1543	1544	0.2	RI,MS
39	*cis*-muurol-5-en-4-β-ol	1549	1550	0.1	RI,MS
40	1-*nor*-bourbonanone	1563	1561	0.2	RI,MS
41	spathulenol	1577	1576	1.2	RI,MS
42	caryophyllene oxide	1582	1583	3.1	Co-I, RI,MS
43	globulol	1584	1584	2.6	RI,MS
44	1,10-di-*epi*-cubenol	1615	1614	4.4	RI,MS
45	α-cadinol	1652	1653	2.6	RI,MS
46	*cis*-calamenen-10-ol	1661	1660	0.5	RI,MS
47	*trans*-calamenen-10-ol	1670	1668	0.3	RI,MS
48	cadalene	1675	1675	0.7	RI,MS
49	*cis*-14-*nor*-muurol-5-en-4-one	1684	1682	13.8	RI,MS
50	*epi*-α-bisabolol	1685	1686	2.3	RI,MS
51	α-bisabolol	1686	1687	0.5	Co-I, RI,MS
52	*n*-heptadecane	1700	1700	0.1	Co-I, RI,MS
53	pentadecanal	1716	1715	0.1	RI,MS
54	aristolone	1763	1762	0.4	RI,MS
55	*n*-octadecane	1800	1800	0.2	Co-I, RI,MS
56	hexahydrofarnesyl acetone	1845	1845	1.4	RI,MS
57	*n*-nonadecane	1900	1900	0.7	Co-I, RI,MS
58	*n*-hexadecanoic acid	1969	1972	1.2	Co-I, RI,MS
59	manooloxide	1990	1989	0.7	RI,MS
60	*n*-eicosane	2000	2000	0.2	Co-I, RI,MS
61	*n*-heneicosane	2100	2100	0.9	Co-I, RI,MS
62	*n*-docosane	2200	2200	0.1	Co-I, RI,MS
63	*n*-pentacosane	2500	2500	tr	Co-I, RI,MS
64	*n*-heptacosane	2700	2700	tr	Co-I, RI,MS
65	*n*-nonacosane	2900	2900	0.1	Co-I, RI,MS
66	*n*-hentriacontane	3100	3100	0.1	Co-I, RI,MS
	Total identified (%)			89.1	
	Essential oil content (%, *v/w*)			0.1	
	Monoterpene hydrocarbons			0.7	
	Oxygenated monoterpenes			38.2	
	Sesquiterpene hydrocarbons			10.8	
	Oxygenated sesquiterpenes			32.0	
	Non-terpene alkanes			2.4	
	Non-terpene aldehydes			1.6	
	Others			3.4	

[a] Compounds are listed in order of their elution from a HP-5MS column.
[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{32} alkanes.
[c] Linear retention index taken from Adams and NIST 14.
[d] Relative percentage values are means of three determinations with a RSD% in all cases below 19 %.
[e] Identification methods: Co-I, based on comparison of RT, RI and MS with those of analytical standard; MS, based on comparison with WILEY, ADAMS, FFNSC2 and NIST 08 MS libraries; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC 2 and NIST 08.
[f] Tr, % below 0.1 %.

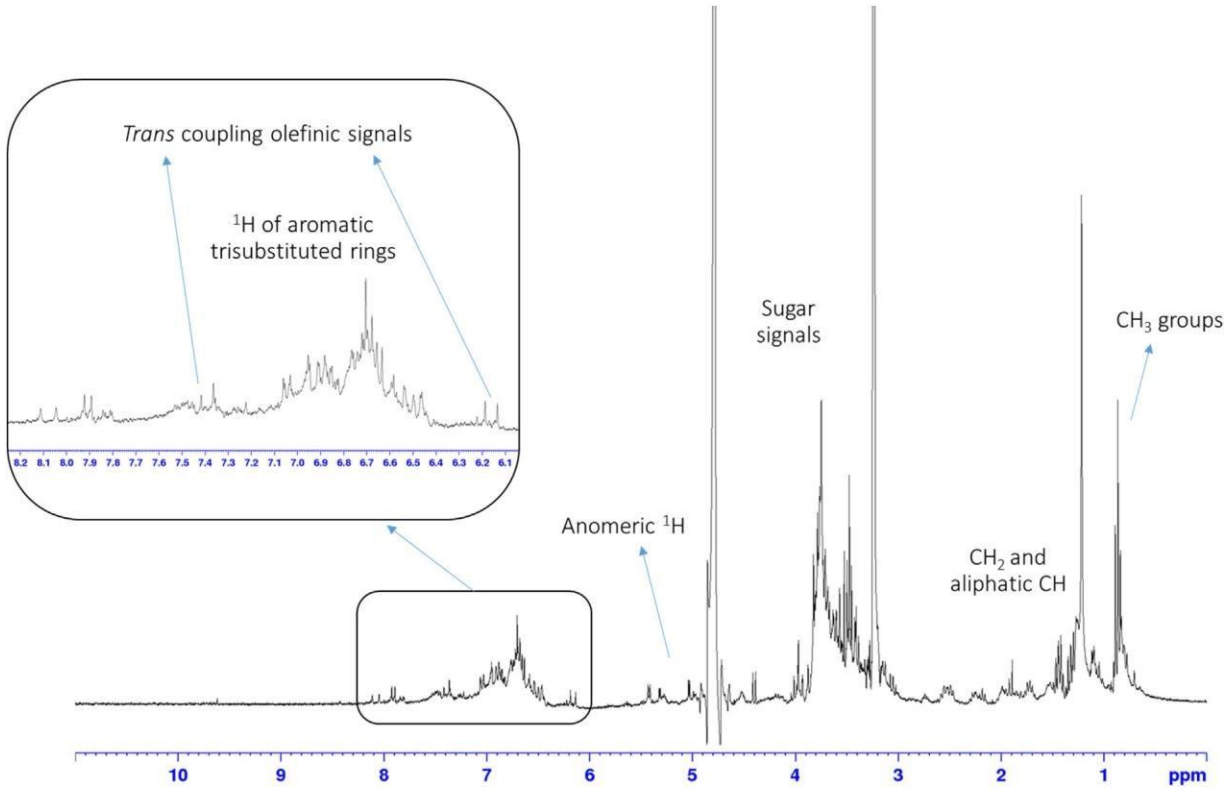

Fig. 2. ^{1}H-NMR spectrum of the MeOH fraction.

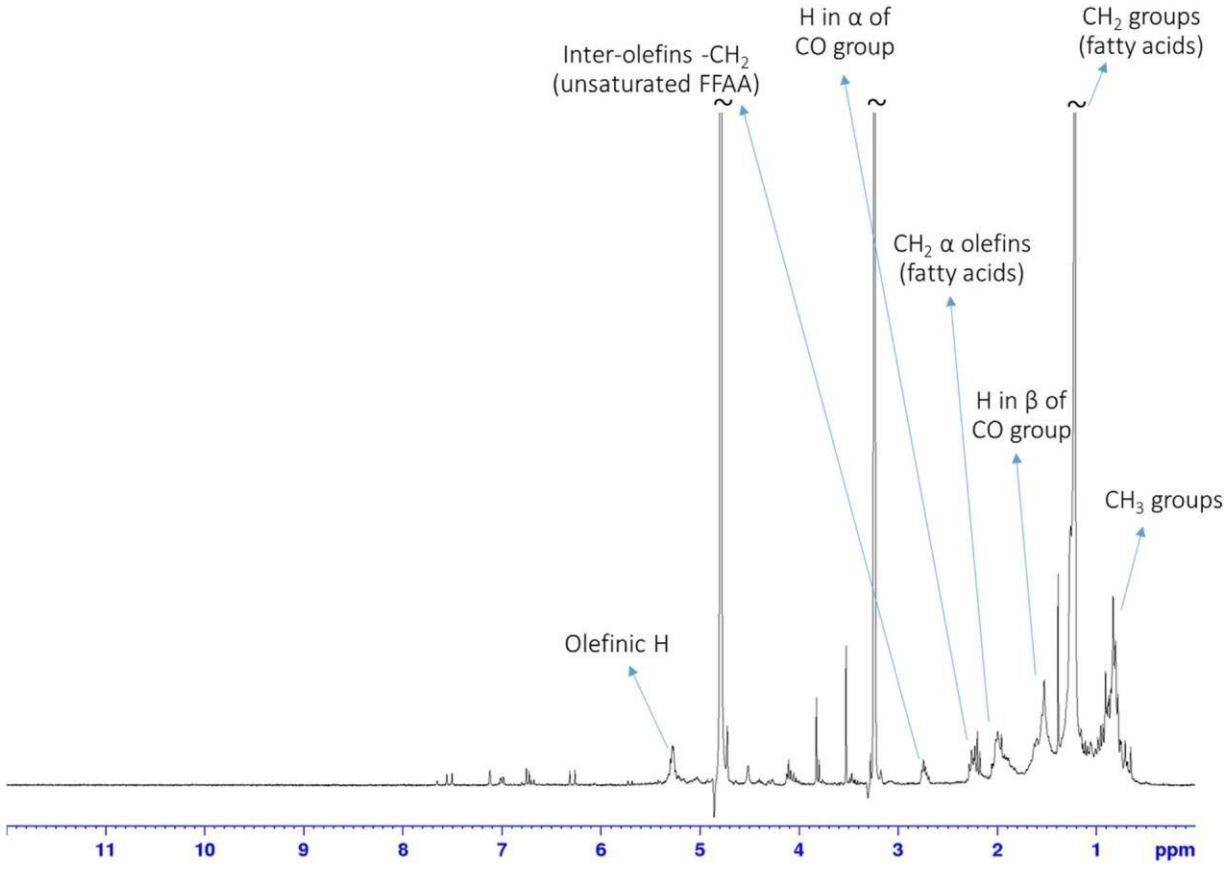

Fig. 3. ^{1}H-NMR spectrum of the *n*-hexane fraction.

Table 2
Aroma profile (percentages of identified compounds) of *Micromeria inodora* aerial parts obtained using SPME GC–MS.

No.	Constituents[a]	RI exp.[b]	RI Lit.[c]	%[d]
1	α-pinene	941	939	2.7
2	camphene	948	946	2.9
3	β-pinene	981	979	2.1
4	2,3-dehydro-1,8-cineole	992	991	2.7
5	α-phellandrene	1006	1005	1.0
6	*p*-cymene	1028	1026	0.9
7	1,8-cineole	1034	1033	10.2
8	γ-terpinene	1063	1061	0.1
9	*cis*-linalool oxide (furanoid)	1076	1074	0.3
10	terpinolene	1090	1088	0.9
11	nonanal	1104	1102	1.5
12	α-campholenal	1128	1126	0.4
13	nopinone	1139	1138	0.5
14	camphor	1145	1144	23.8
15	pinocarvone	1164	1162	0.6
16	4-terpineol	1179	1177	0.9
17	myrtenol	1195	1193	1.3
18	verbenone	1207	1204	0.7
19	pulegone	1239	1237	1.1
20	thymoquinone	1252	1252	0.3
21	linalyl acetate	1259	1257	0.3
22	(*E*)-anethole	1284	1283	1.4
23	bornyl acetate	1287	1286	1.4
24	2-hydroxy-5-methylacetophenone	1315	1316	1.3
25	α-terpinyl acetate	1352	1350	21.3
26	α-copaene	1377	1376	0.8
27	β-bourbonene	1385	1386	0.3
28	β-elemene	1392	1391	0.1
29	cyperene	1399	1398	0.3
30	α-gurjunene	1410	1409	1.4
31	β-caryophyllene	1419	1419	3.1
32	β-copaene	1431	1432	0.1
33	α-humulene	1455	1455	1.0
34	*allo*-aromadendrene	1462	1460	3.0
35	*cis*-muurola-4(14),5-diene	1463	1460	0.7
36	β-chamigrene	1476	1475	0.6
37	γ-muurolene	1479	1480	0.4
38	germacrene D	1482	1483	1.6
39	β-selinene	1487	1487	0.4
40	*cis*-β-guaiene	1491	1490	0.2
41	α-selinene	1495	1494	0.8
42	*n*-pentadecane	1500	1500	0.4
43	γ-cadinene	1514	1513	0.5
45	δ-cadinene	1524	1524	1.1
	caryophyllene oxide	1582	1583	0.7
	Total identified (%)			98.1
	Monoterpene hydrocarbons			10.6
	Oxygenated monoterpenes			65.8
	Sesquiterpene hydrocarbons			16.4
	Oxygenated sesquiterpenes			0.7
	Phenylpropanoids			1.4
	Non-terpene derivatives			3.2

respectively. Minimal signals ascribable to phenolic compounds could be observed at **δ** 6.00–7.00.

NMR spectra of DCM and EA extracts appear similar to each other, and they support the presence of triterpenes. Characteristic signals are observable, due to the removal of the lipid signals that were extracted in the *n*-hexane fraction (Fig. 4). Signals at **δ** 5.15 (m) and 5.27 (m) are ascribed to olefinic protons, and singlets in the range **δ** 0.70–1.20 are assignable to triterpenes' methyl signals [31]. Multiplets in the region between **δ** 2.25—1.12 are ascribable to methylene groups and aliphatic CH, while the doublet at **δ** 0.88 ppm (J = 6.90 Hz) is attributable to a secondary methyl group.

3.4. HPLC–MS^n analysis of secondary metabolites from M. inodora methanol extract

The HPLC-MS^n analysis of the *M. inodora* methanol extract allowed to tentatively identify thirty-eight constituents, by comparison of MS/MS spectra with literature. An exemplificative chromatogram is reported in Fig. 5. Among the constituents, 19 were identified as flavonoids. The most representative compounds of this class were luteolin derivatives, followed by quercetin, kaempferol and apigenin derivatives (Table 3). *Micromeria* species have already been reported as source of flavonoid and glucosidic conjugates, being apigenin and luteolin derivatives the most abundant ones [32,33]. Flavonoids have been reported as the responsible for several pharmacological activities attributed to many medicinal plants, notably antioxidant, antimicrobial, and enzyme inhibitory [34], hence *M. inodora* from Algeria could represent a novel evaluable source of these bioactives.

Other nine constituents were tentatively identified as phenolic acids (Table 3). Rosmarinic acid is a secondary metabolite widely encountered in nature, and has been already detected as a main component of several *Micromeria* species [33,35,36]. Furthermore, syringic acid hexoside, lithospermic acid, sagerinic acid and yunnaneic acid F have been identified in the leaves of *M. fruticosa* [37].

Table 3
Identification of phenolic constituents from the methanolic extract of *Micromeria inodora* by HPLC-MS^n.

Molecular ion (*m/z*)	Ionization mode	MS2	MS3	R.T. (min)	Tentative identification	Chemical class	Reference
269	–	**225** 224 183 151 117		21.1	Apigenin	Flavonoids	[38]
283	–	268	241 240 211	26.3	Calycosin	Flavonoids	[38]
287	+	269 241 161 **153** 135	127 **111** 97 85	13.6	Luteolin	Flavonoids	[37]
299	–	284	257 256 227	21.6	Gliricidin	Flavonoids	[38]
301	+	**286** 257 229	258	14.8	Chrysoeriol	Flavonoids	[37]
301	+	**286** 258 229 167	258	14.6	Diosmetin	Flavonoids	[37]
343	–	328 **313**	**298** 285	25.4	Dihydroxy-trimethoxyflavone	Flavonoids	[37]
359	+	**326** 298	298	27.2	Hydroxy-tetramethoxyflavone	Flavonoids	[37]
389	+	374 373 **359** 341 328	343 331 **316**	29.1	Erianthin	Flavonoids	Metlin*
447	–	285	267 241 217 199 **175** 151	13.7	Luteolin 7-O-glucoside	Flavonoids	[39]
449	–	**287** 269	**151** 135	12.4	Dihydroluteolin hexoside	Flavonoids	[40]
449	–	**287** 269	**151** 135	13.1	Dihydrokaempferol hexoside	Flavonoids	[40]
449	–	287	**151** 135	13.4	Eriodictyol-7-O-glucoside	Flavonoids	[39]
461	–	285	267 241 217 199 **175** 151	14.4	Kaempferol-3-O-glucuronide	Flavonoids	[41]
477	–	301	255	13.6	Quercetin-7-O-glucuronide	Flavonoids	[42]
551	+	303	285 **257** 229	14.9	Quercetin malonyl-hexoside	Flavonoids	[37]
577	–	269	225 151	15.1	Apigenin-7-O-rutinoside	Flavonoids	[43]
593	–	285	241 199 175 151	12.5	Luteolin-7-O-rutinoside	Flavonoids	[43]
639	–	**315** 301		15.8	Isorhamnetin-3-O-di-glucoside	Flavonoids	[44]
359	–	197 179 **161** 133	133	16.5	Syringic acid hexoside	Phenolic acids	[37]
369	–	223 197 179 **161** 133	133	16.4	Rosmarinic acid	Phenolic acids	[45]
537	–	493 383 313 **295**		14.2	Lithospermic acid	Phenolic acids	[37]
597	–	295 **197** 179		14.3	Yunnaneic acid F	Phenolic acids	[37]
637	–	461 **443**		18.9	Leucoseptoside A	Phenolic acids	[45]
718	–	**359** 341 295		14.0	Sagerinic acid	Phenolic acids	[37]
735	–	537		16.7	Danshensu derivative	Phenolic acids	[46]
759	–	561		14.5	Danshensu derivative	Phenolic acids	[46]
777	–	579		15.8	Danshensu derivative	Phenolic acids	[46]
149	+	**121** 93 65		12.8	Anethole	Terpenoids	Metlin**
471	–	**451** 427 425 383		29.5	Masilinic acid or Corosolic acid I	Terpenoids	[37]
493	–	**359** 313 295		18.5	Salvianolic acid A	Terpenoids	[47]
717	–	**519** 359 357	339 321	17.9	Salvianolic acid B/E/L	Terpenoids	[37]
104	+	60		2.5	Choline	Others	Metlin***
191	–	**173** 155 137		2.6	Quinic acid	Others	[48]
305	–	225		12.6	Methyl-hydroxyjasmonate sulphate	Others	[37]
311	–	293 275 235 **22**3 183		26.9	15,16-dihydroxy-9,12-octadecadienoic acid	Others	[49]
327	–	**229** 211		20.2	Oxiranedioctanoic acid	Others	[37]
329	–	311 293 **229** 211	211 167	21.3	Trihydroxyoctadeca−10(E)-dienoic acid	Others	[37]
371	–	**249** 231	231 175 157 113	13.9	Deacetylasperuloside	Others	[37]
377	–	**341** 215	**179** 161 143	2.6	Sucrose	Others	[39]
387	–	225 **207** 163		12.7	Tuberonic acid hexoside I	Others	[37]

*Metlin ID : 51429. **Metlin ID : 43903. ***Metlin ID : 56.

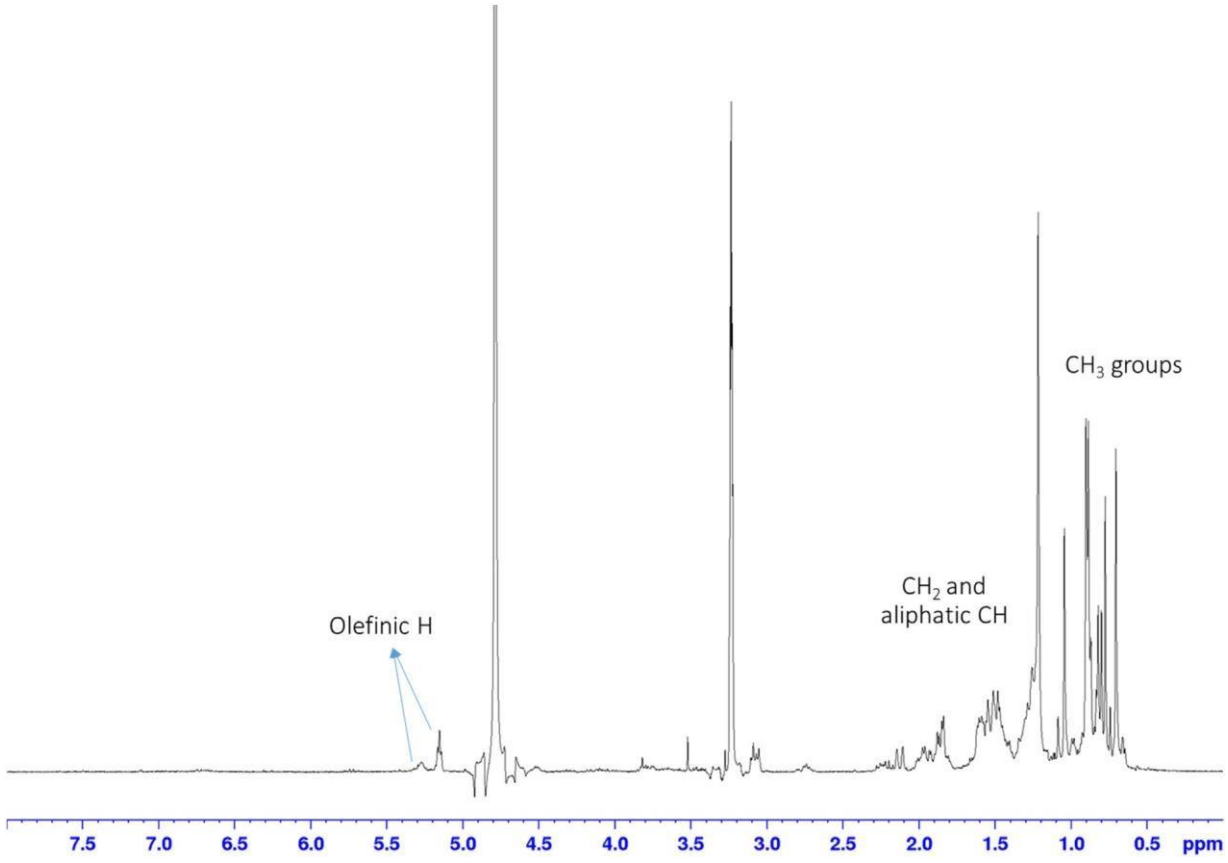

Fig. 4. ^{1}H-NMR spectrum of the DCM fraction.

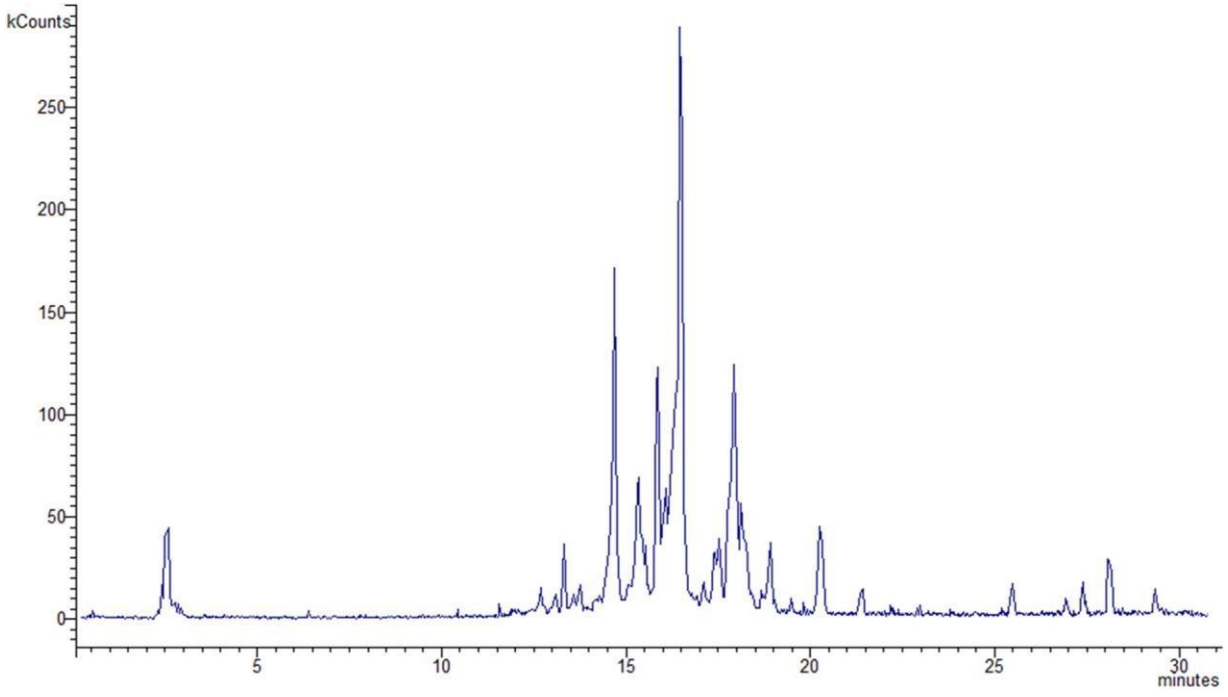

Fig. 5. Intensity of base peak chromatogram obtained from the HPLC-MS^{n} analysis of *Micromeria inodora* methanolic extract in ESI(-).

Among the terpenoids identified in the methanolic extract of *M. inodora*, two [M−H]$^{-}$ ions with *m/z* 471 and 717 have been already reported in *M. fruticosa*, but the authors could not precisely identify them by means of LC–MS data. We tentatively identify the two ions as maslinic acid or corosolic acid I (*m/z* 471) and salvianolic acid B, E or L (*m/z* 717), respectively, comparing the MS^{n} spectra with available literature [37].

Among the remaining compounds detected in *M. inodora*, two were identified as derivatives of jasmonic acid, namely methyl-hydroxyjasmonate sulphate and tuberonic acid hexoside I, while other three were fatty acids, namely 15,16- dihydroxy-9,12-octadecadienoic acid, oxiranedioctanoic acid, and trihydroxyoctadeca-10(E)-dienoic acid.

4. Conclusions

Although other characterization studies of *M. inodora* have been already published, to the best of our knowledge this is the first work reporting an extensive characterization of both volatile and polar constituents of the same species from Algeria using a multi-technique approach. GC–MS analyses of volatiles extracted using either hydrodistillation or HS-SPME yielded different results, being the two extraction methods not comparable. The analysis of the essential oil obtained by hydrodistillation allowed to identify 66 constituents, among which oxygenated mono- and sesquiterpenes were the most abundant (38.2 % and 32.0 %, respectively). Main components were the monoterpenes α-terpinyl acetate (29.1 %) and camphor (7.0 %), and the sesquiterpene *cis*-14-*nor*-muurol-5-en-4-one (13.8 %). On the contrary, the characterization of the volatile extract obtained by HS-SPME allowed to identify 45 constituents, among which camphor (23.8 %), α-terpinyl acetate (21.3 %) and 1,8-cineole (10.2 %) were the most representative. Explorative ^{1}H-NMR analyses of methanolic extract of *M. inodora* showed the presence of glycosidic derivatives of phenylpropanoids, flavonoids and triterpenes. Multi-stage HPLC-MS analysis of the same extract allowed to tentatively identify 41 of these compounds, mainly glycosylated and non-glycosylated flavonoids (19 compounds) and phenolic acids (9 compounds). Overall, the results reported in this work represent a starting point for further research on this plant species, and could help in the isolation of bioactive compounds for further pharmacological applications.

CRediT authorship contribution statement

Nacéra Bouriah: Investigation, Writing - original draft. **Hamdi Bendif:** Conceptualization, Software, Funding acquisition, Writing - original draft. **Gregorio Peron:** Methodology, Software, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization, Supervision, Writing - original draft. **Mohamed Djamel Miara:** Validation, Data curation. **Stefano Dall'Acqua:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing - review & editing, Supervision, Project administration. **Guido Flamini:** Methodology, Validation, Formal analysis, Investigation, Resources, Project administration, Writing - original draft. **Filippo Maggi:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Short Communication

Phytochemical analysis of *Clinopodium candidissimum* (Munby) Kuntze growing in Algeria by an integrated HS-SPME-GC-MS, NMR and HPLC-DAD-MS^n approach: valorisation of an endemic natural source of bioactive compounds

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Abstract

Clinopodium candidissimum (Munby) Kuntze (Lamiaceae) is used in traditional medicine and as a food condiment in Algeria, where it is known as Zaater cheleuh and Nabta elbida. Here, we report the comprehensive characterization of non-volatile polar constituents extracted from *C. candidissimum* aerial parts (a mixture of inflorescences, stems and leaves), and their aroma profile. Qualitative ^{1}H-NMR and quali-quantitative HPLC-MS^{n} analyses of fractions obtained with solvents at different polarity revealed the presence of aglyconic and glycosylated flavonoids (3.1%), phenylpropanoids (3.6%), gallic acid derivatives (0.76%), and triterpenoids (0.62%), among the others. On the other hand, HS-SPME-GC-MS allowed to identify 38 volatile constituents, among which the oxygenated monoterpenes pulegone (44.8%), piperitenone (6.6%), isopulegone (5.8%) and neo-menthol (3.8%), and the sesquiterpene hydrocarbons germacrene D (16.2%) and bicyclogermacrene (3.0%) were the most abundant. Overall, results indicate that *C. candidissimum* represents an endemic natural source of antioxidants and bioactive compounds, and they will be useful for further studies on this species.

Keywords: *Clinopodium candidissimum* (Munby) Kuntze ; Phytoconstituents; Secondary metabolites; HS-SPME-GC-MS; NMR; HPLC-DAD-MS^{n}.

SUPPLEMENTARY MATERIAL

Experimental Section

Plant Material

A mixture of inflorescences, stems and leaves of *C. candidissimum* was collected from individuals spontaneously growing in region of Djebel Murdjadjo, Oran (Northweastern Algeria, 400 m above sea level, GPS coordinates: Latitude: N 35.701930, Longitude 0.721408W) in March 2016. Botanical determination was performed by Dr. Miara using available literature (Quézel & Santa 1962) and a voucher specimen was deposited in the Herbarium Universitatis Camerinensis, University of Camerino (Italy), under the code "CAME 27740". Before undergoing extraction, plant material was washed in running water and dried at room temperature for 7 days, avoiding direct exposition to sunlight.

Extraction of aerial parts using solvents at different polarities

The extraction of *C. candidissimum* aerial parts was performed according to a previously published protocol (Dall'Acqua et al. 2010; Bendif et al. 2017). Briefly, 50 g of dried plant material were ground with a blender, suspended in 150 mL of methanol and extracted at r.t. for 15 min, by using an ultrasound bath. Suspended plant material was removed by centrifugation at 4,000 rpm for 10 min, and supernatant was collected in a round-bottom flask. The residue was extracted with other 50 mL of methanol in ultrasound bath for 15 min, and the same process was repeated twice. Supernatants were dried using a rotary evaporator at 35 °C to constant weight, in order to obtain a dried concentrated extract. This latter was stored in amber glass vials at −20 °C until analysis. The yield of the extraction was 8.8% w/w.

300 mg of the dried powdered extract were used for NMR and HPLC-DAD-MS^n analyses. The remaining extract was partitioned with solvents at different polarity, in order to obtain purified fractions for NMR analysis. The powder was suspended in 50 mL of a methanol/water (1:9) mixture and dissolved by sonication for 10 min. The obtained solution was then partitioned using 20 mL of hexane in a separation funnel, and, after removal of the organic phase, it was partitioned with 20 mL of dichloromethane (DCM), 20 mL of ethyl acetate (EA), and 20 mL of butanol. For each solvent, extraction was repeated in triplicate. Finally, the obtained fractions were dried using a rotary evaporator and dried residues were stored at -20 °C until analysis.

Explorative NMR analysis

Dried powdered extracts were dissolved in deuterated methanol and submitted to NMR analysis. NMR analyses were performed on a Bruker Avance III 400 MHz spectrometer, using standard pulse sequences. ^{1}H-NMR spectra were acquired for methanol, hexane, DCM, EA and butanol extracts.

HPLC-DAD-MS^{n} analysis of secondary metabolites in the methanol extract

Secondary metabolites in methanol extract of *C. candidissimum* vegetative parts were tentatively identified by HPLC-DAD-MS^{n}, comparing the fragmentation patterns with literature data and with standard compounds, when available. Before analysis, the dried extract was dissolved in methanol at a concentration of 5 mg/mL, and the solution was filtered through a 0.45 µm Millipore filter. The HPLC-DAD-MS^{n} method used was the same as reported in (Bendif et al. 2017; Peron et al. 2019). Briefly, a chromatographic system composed by an Agilent 1260 binary pump equipped with autosampler and DAD and coupled with a Varian 500 Ion Trap mass detector (MS) was used.

MS was equipped with an Electrospray Ionisation (ESI) source, operating in negative ion mode. To achieve the separation of secondary metabolites, an Agilent Eclipse plus C_{18} column (2.1 × 150 mm, 3.5 µm) was used as stationary phase, while a mixture of acetonitrile (A) and 0.1% formic acid in water (B) was used as mobile phase. The elution gradient was as follows: 0 min, 10% A; 20 min, 54% A; 23 min, 100% A, and isocratic up to 32 min. Re-equilibration time was 8 min. Flow rate was 0.2 mL/min. ESI parameters were: needle voltage, 4.5 kV; capillary voltage, 70 V; RF loading, 100%; nebulizing gas pressure, 20 psi (nitrogen); drying gas pressure, 15 psi; drying gas temperature, 350 °C. Data were acquired in the *m/z* range 50–2000. The turbo detection data scanning (TDDS®) function was used to acquire data about the fragmentation patterns of eluted compounds, setting n = 3 levels of fragmentation.

For quantitative purposes, DAD was used. Flavonoids, phenolic acids and gallic acid derivatives were quantified using linear calibration curves obtained by analysing 0.5-100 µg/mL solutions of rutin, chlorogenic acid and gallic acid, respectively. Calibration curves were, respectively: $y = 51.5x$-183.22 ($R^2 = 0.998$), $y = 90.54x$-32.84 ($R^2 = 0.999$), and $y = 109.72x$-68.33 ($R^2 = 0.999$). Limit of detection (LOD) for rutin, chlorogenic acid and gallic acid were 0.05, 0.02 and 0.02 µg/mL, respectively, while limits of quantification (LOQ) were 0.15, 0.06 and 0.06 µg/mL, respectively.

On the other hand, MS detector was used to achieve triterpenes quantification. The linear calibration curve was obtained by analysing 0.5-100 µg/mL solutions of ursolic acid, and its equation was $y = 651.2x$ - 26.9 ($R^2 = 0.998$). LOD and LOQ for ursolic acid were 0.15 and 0.5 µg/mL, respectively.

HS-SPME-GC-MS analysis

For HS-SPME, the technique described by (Ascrizzi et al. 2017) was applied. A Supelco Solid Phase Micro-extraction (SPME) device coated with poly-dimethylsiloxane (PDMS, 100 µm) was employed to sample the headspace of a mixture of ground stems, leaves, and flowers. Plant material was inserted into a 4 mL glass vial for 1 h at r.t., to equilibrate. Then, the SPME fibre was used to sample the headspace for 30 min. GC-MS analyses were achieved as described in (Bendif et al. 2017) using a Varian CP-3800 gas-chromatograph coupled to a Varian Saturn 2000 mass spectrometer equipped a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm). Chromatographic conditions: injector and transfer line temperatures 220 and 240 °C, respectively; oven temperature programmed from 60 to 240 °C at 3 °C /min; carrier gas (helium, 1 mL/min); splitless injection. Identification was performed by comparing retention times with those of reference pure compounds, comparing their linear retention indices (LRI) relative to the C_6– C_{28} series of *n*-hydrocarbons, and by matching their mass spectra against commercial (Adams 2007; NIST 2014) and home-made libraries built up from pure substances.

SUPPLEMENTARY FIGURES

Figure S1. Vegetative parts of wild *Clinopodium candidissimum* (Munby) Kuntze

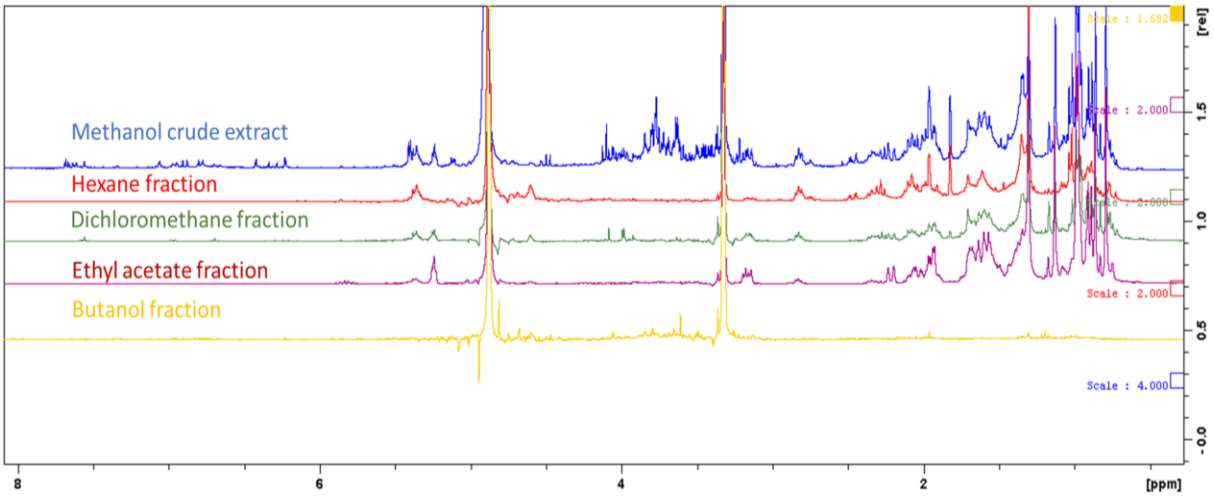

Figure S2. Superimposition of ^{1}H-NMR spectra obtained from the analysis of *C. candidissimum* methanol crude extract (blue trace), and fractions obtained from the liquid/liquid partition of methanol crude extract with solvents at different polarity, namely hexane (red trace), dichloromethane (green trace), ethyl acetate (yellow trace), and butanol (violet trace).

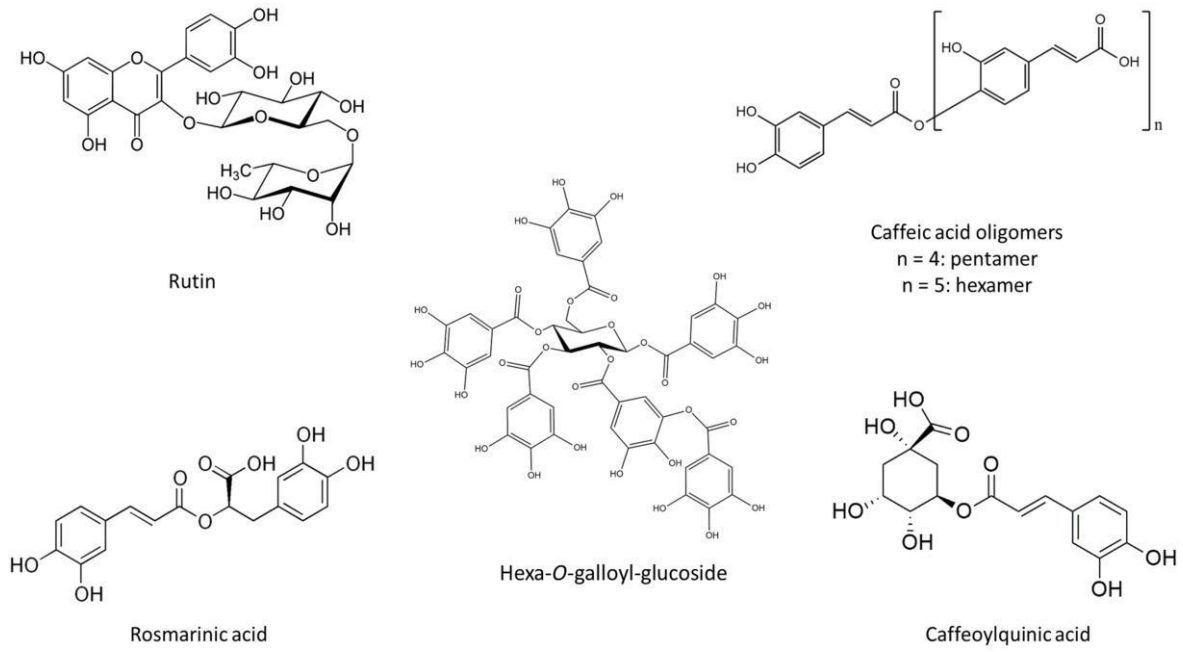

Figure S3. Chemical structures of the most abundant polar constituents identified in the methanol crude extract of *C. candidissimum* aerial parts by HPLC-MS^{n} analysis. In the Figure, the exemplificative structure of 3-O-caffeoylquinic acid isomer is reported.

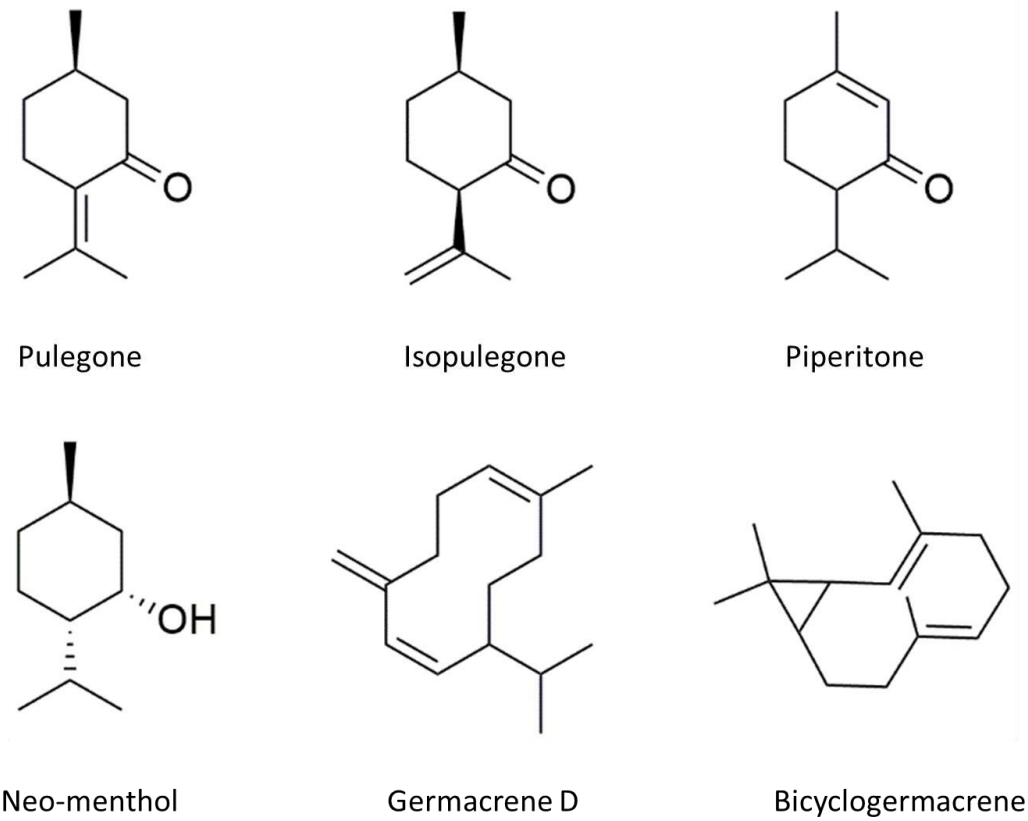

Figure S4. Chemical structures of the main volatile constituents of *C. candidissimum* aerial parts, extracted and characterized by HS-SPME-GC-MS analysis.

SUPPLEMENTARY TABLES

Table S1. Main signals observed in the ^{1}H-NMR spectra of *C. candidissimum* extracts obtained using solvents at different polarities. Tentative assignments of identified compounds to specific chemical classes are also reported.

Extract	**Compound(s)**	**^{1}H-NMR Resonance (ppm, multiplicity)**	**Assignment**
MeOH	Rutin	7.65 d	H-6’
		7.67 d	H-2’
		6.96 dd	H-5’
		6.38 d	H-8
		6.22 d	H-6
		5.11 d	Anomeric H of glucose
		4.53	Anomeric H of rhamnose
DCM, EA	Ursolic acid	5.15 brs	H -12
		3.17 dd	H-3
		0.82 d	H-29
		0.89 d	H-30
		0.84 s	H -24
		0.76	H-26
		0.89 s	H-23
		1.05	H-25
		1.26	H-27
		2.35 m	H-18
Hexane, DCM	FFAAs	5.37 m	Olefine H of unsaturated FFAA
		2.27 t	CH_2 of FFAA vicinal to CO, or vicinal to sp2 C
		1.28 m	CH_2 of FFAA aliphatic chains
		0.85 t	Terminal CH_3 of FFAA

DCM: dichloromethane; EA: ethyl acetate; FFAA: fatty acid

Table S2. Identification of phenolic constituents from the crude methanol extract of *C. candidissimum* by HPLC-MS^n in negative ion mode [ESI-].

Chemical class	$[M-H]^-$	R.T. (min)	Main MS2 fragments	Main MS3 fragments	Tentative identification	mg/g*	Ref.
Flavonoids	283	29.12	268	240 239 211	Acacetin	0.07	(Pacifico et al. 2015)
	305	16.48	225	224 207 182 181 165 163 135 133 207	Gallocatechin**	1.26	(Ozarowski et al. 2013)
	343	28.31	328 313	298 285 270 215	5,6-dihydroxy-3',4',7-trimethoxy flavone	0.27	(Salih et al. 2017)
	373	28.87	358 343	328 300	Neobaicalein	0.08	(Zhang et al. 2008)
	447	29.34	279	261 259 233	2″-Hydroxy-2″-(3″,4″,5″-trihydroxylphenyl) ethyl-3-(3-(5′,7′-dihydroxychromone)-3-hydroxybutanoate	0.29	(Pacifico et al. 2018)
	463	19.81	301	271 255 179 151	Hyperoside**	1.72	(Pacifico et al. 2015)
	489	20.95	327 285 255	255 227 211	Kaempferol-3-*O*-acetyl-hexoside	0.69	(Mekam et al. 2019)
	505	20.09	463 337 301	271 255 179 151	Quercetin acetyl-hexoside	1.23	(Barros et al. 2012)
	577	27.33	431 299 269 225	-	Apigenin 7-*O*-neohesperidoside	0.07	(Brito et al. 2014)
	593	17.06	503 473 383 353	353 325 297	6,8-*C*-dihexosylapigenin	1.28	(Pacifico et al. 2015)
	607	20.18	563 299	284	Diosmetin 7-*O*-neohesperidoside	2.21	(Brito et al.

							2014)
	609	18.98	301	286 257 242 199	Rutin**	19.36	-
	623	20.43	315 300	271 255	Isorhamnetin-3-*O*-rutinoside	1.51	(Li et al. 2016)
	755	17.75	315 300	271 255	Isorhamnetin-3-*O*-[2-*O*-*β*-xylopyranosyl-6-*O*-*α*-rhamnopyranosyl]-*β*-glucopyranoside	0.97	(Lou et al. 2001)
					Total flavonoids	**31.00**	
Phenolic acids	163	21.74	163 161 131 119	-	*p*-Coumaric acid	0.57	(Pereira et al. 2015)
	353	15.78	191 179 191 173 135	111 93	Caffeoylquinic acid isomer	6.84	(Pacifico et al. 2015)
	353	16.36	191 179 191 173 135	111 93	Caffeoylquinic acid isomer	3.22	(Pacifico et al. 2015)
	359	20.82	223 197 179 161	133	Rosmarinic acid**	5.03	(Pacifico et al. 2015)
	555	18.54	359 357 179 161	-	Salvianolic acid K	0.86	(Ziani et al. 2019)
	717	20.64	555 519	357 339 295 247 163	Salvianolic acid B	4.51	(Pacifico et al. 2015)
	895.5	20.02	851 794 697 555 357 339	-	Caffeic acid pentamer	8.39	(Pacifico et al. 2015)
	1075	20.52	913 877 715 555 519	-	Caffeic acid hexamer	6.63	(Pacifico et al. 2015)
					Total phenolic acids	**36.07**	

Gallic acid derivative	1091	18.49	939 787	-	Hexa-*O*-galloyl-glucoside	7.56	(Xiang et al. 2019)
					Total gallic derivatives	**7.56**	
Triterpenes	471	22.02	453 407	-	Hydroxyursolic acid [#]	2.11	(Li et al. 2017)
	455	23.61	407	-	Ursolic acid**[#]	4.05	(Sun et al. 2019)
					Total triterpenes	**6.16**	
Other	327	24.63	291 229 211 171	211 209	Oxo-dihydroxy-octadecenoic acid	NQ	(Llorent-Martínez et al. 2015)
	329	24.04	311 293 229 211 171	211 209	Trihydroxyoctadecenoic acid	NQ	(Llorent-Martínez et al. 2015)
	387	17.28	225 207 179 163	-	12-*O*-hexosyljasmonate	NQ	(Pacifico et al. 2015)
	377	2.63	341	179 161 119 89	Disaccharide (HCl adduct)	NQ	(Jiménez-López et al. 2017)
	539	2.57	503	383 341 323 221	Maltotriose	NQ	(Jin et al. 2018)

*Flavonoids amounts are expressed as rutin equivalents; phenolic acids amounts are expressed as chlorogenic acid equivalents; amounts of gallic derivatives are expressed as gallic acid equivalents; triterpenes amounts are expressed as ursolic acid equivalents.

**Compounds identified on the basis of comparison with standard available in the lab.

Confirmed by NMR data.

NQ: not quantified.

Table S3. Aroma profile of *C. candidissimum* aerial parts obtained by HS-SPME-GC-MS analysis.

N	Constituents	l.r.i.	Lit. RI[b]	%
1.	β-pinene	982	980	0.3
2.	p-cymene	1028	1027	0.4
3.	Limonene	1032	1031	1.1
4.	Linalool	1101	1099	0.1
5.	Camphor	1145	1144	1.0
6.	Menthone	1155	1153	0.6
7.	Menthofuran	1165	1164	0.3
8.	neo-menthol	1167	1166	3.8
9.	Isopulegone	1174	1175	5.8
10.	α-terpineol	1191	1190	1.4
11.	Decanal	1206	1204	0.2
12.	Pulegone	1239	1237	44.8
13.	Piperitone	1254	1253	0.5
14.	Isopiperitenone	1271	1272	0.1
15.	(E)-anethole	1284	1285	0.4
16.	p-cymen-7-ol	1290	1291	0.5
17.	Carvacrol	1301	1301	0.1
18.	iso-dihydrocarveol acetate	1327	1329	0.2
19.	δ-elemene	1340	1339	0.7
20.	Piperitenone	1342	1343	6.6
21.	piperitenone oxide	1365	1363	0.4

22.	α-copaene	1377	1377	0.6
23.	β-bourbonene	1385	1384	0.8
24.	β-cubebene	1391	1390	0.4
25.	β-elemene	1393	1391	1.0
26.	(Z)-jasmone	1395	1394	1.5
27.	α-gurjunene	1410	1409	0.2
28.	β-ylangene	1420	1421	1.6
29.	β-copaene	1430	1432	0.8
30.	Aromadendrene	1440	1441	0.3
31.	Alloaromadendrene	1462	1460	0.5
32.	cis-muurola-4(14),5-diene	1463	1461	0.3
33.	γ-muurolene	1479	1477	0.3
34.	germacrene D	1481	1480	16.2
35.	Bicyclogermacrene	1496	1494	3.0
36.	trans-γ-cadinene	1514	1513	0.2
37.	δ-cadinene	1524	1524	0.5
38.	Spathulenol	1577	1578	1.2
	Monoterpene hydrocarbons			1.8
	Oxygenated monoterpenes			66.2
	Sesquiterpene hydrocarbons			27.4
	Oxygenated sesquiterpenes			1.2
	Phenylpropanoids			0.4
	Non-terpene derivatives			1.7
	Total identified			98.7

[a] LRI relative to C_6-C_{28} n-alkanes on the DB-5 column. [b] LRI taken from (Adams & Sparkman 2007; NIST 2014)

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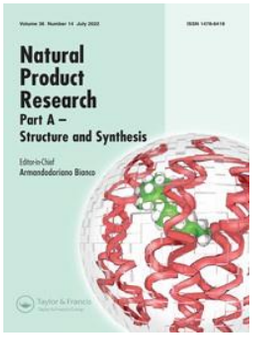

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Phytochemical analysis of *Clinopodium candidissimum* (Munby) Kuntze growing in Algeria by an integrated HS-SPME-GC-MS, NMR and HPLC-DAD-MS^n approach: valorisation of an endemic natural source of bioactive compounds

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SHORT COMMUNICATION

Phytochemical analysis of *Clinopodium candidissimum* (Munby) Kuntze growing in Algeria by an integrated HS-SPME-GC-MS, NMR and HPLC-DAD-MS^n approach: valorisation of an endemic natural source of bioactive compounds

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ABSTRACT

Clinopodium candidissimum (Munby) Kuntze (Lamiaceae) is used in traditional medicine and as a food condiment in Algeria, where it is known as Zaater cheleuh and Nabta elbida. Here, we report the comprehensive characterisation of non-volatile polar constituents extracted from *C. candidissimum* aerial parts (a mixture of inflorescences, stems and leaves), and their aroma profile. Qualitative ^{1}H-NMR and quali-quantitative HPLC-MS^n analyses of fractions obtained with solvents at different polarity revealed the presence of aglyconic and glycosylated flavonoids (3.1%), phenylpropanoids (3.6%), gallic acid derivatives (0.76%), and triterpenoids (0.62%), among the others. On the other hand, HS-SPME-GC-MS allowed to identify 38 volatile constituents, among which the oxygenated monoterpenes pulegone (44.8%), piperitenone (6.6%), isopulegone (5.8%) and neo-menthol (3.8%), and the sesquiterpene hydrocarbons germacrene D (16.2%) and bicyclogermacrene (3.0%) were the most abundant. Overall, results indicate that *C. candidissimum* represents an endemic natural source of antioxidants and bioactive compounds, and they will be useful for further studies on this species.

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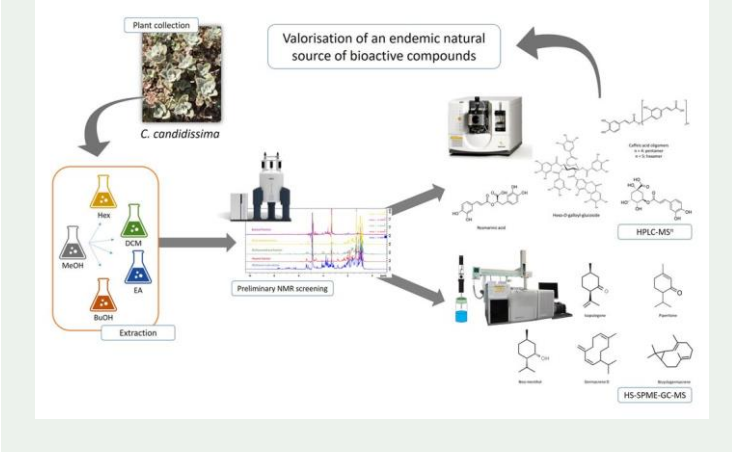

1. Introduction

Clinopodium candidissimum (Munby) Kuntze (Figure S1) is a strictly endemic species of Algeria (Quézel and Santa 1962; Melnikov 2017). This plant has been originally described in Algeria (Oran province) by Mynby as *Melissa candidissima* Munby, but later it has been reported with several synonyms, in particular *Calamintha candidissima* (Munby) Benth. and *Satureja candidissima* (Munby) Briq. (Engler et al. 1897; GBIF Secretariat 2021). Despite the fact that Dobignard and Chatelain retain *Calamintha candidissima* (Munby) Benth in their synonymic indexes of the flora of North Africa (Dobignard and Chatelain 2012), several works (Govaerts 1999; Govaerts 2003; Melnikov 2016; Melnikov 2017) retain rather *Clinopodium candidissimum* (Munby) Kuntze as an accepted name. This plant grows spontaneously between oleanders and rocky lawns in and around Oran, and it is known to the population as Zaater cheleuh and Nabta elbida (Quézel and Santa 1962). The richness of its essential oil confers to *C. candidissimum* several biological properties such as antimicrobial, insecticide, larvicide and herbicide, spasmolytic, anti-inflammatory and analgesic. This plant is effective in treating flu and infections, and as a dressing for the healing of burns and wounds. It has a warming effect and is recommended in case of respiratory tract infections (bronchitis) (Chevallier and Larousse 2001). It is used in cooking to flavour sauces or to prepare a traditional Algerian dish from the region of Sid safi, called 'Rfiss'. However, to the best of our knowledge, the chemical composition of *C. candidissimum* has been scarcely investigated up to now. In order to promote Algerian medicinal and edible plants, in this work we aimed at characterising the volatile composition and the non-volatile polar constituents of *C. candidissimum* aerial parts, using different extractive and analytical approaches. A headspace solid phase micro-extraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) method was used for the characterisation of volatiles, while integrated NMR and HPLC-DAD-MS^n approaches allowed the identification and quantification of polar constituents.

2. Results and discussion

2.1. Explorative NMR analysis

The NMR approach was used for a preliminary evaluation of the composition of *C. candidissimum* aerial parts fractions. To assess the presence of different classes of phytoconstituents, extraction of *C. candidissimum* aerial parts was performed using methanol, and this crude extract was then fractionated with solvents at different polarity, i.e., hexane, dichloromethane (DCM), ethyl acetate (EA) and butanol. The superimposition of the ^{1}H-NMR spectra obtained from the analyses of these fractions is reported in Figure S2.

Several signals in the spectrum of methanol crude extract are ascribable to fatty acids, sugars as well as phenolic constituents. A large number of signals in the more shielded part of the spectrum support the presence of aliphatic compounds, thus suggesting the presence of a hydrocarburic portion of constituents ascribable to terpenoids. Furthermore, several signals are clearly visible in the aromatic part of the spectrum, suggesting the presence of aromatic derivatives and phenolics. Among these, signals ascribable to rutin are observed: namely, the aromatic signals ascribable to the catechol ring and the H-6/H-8, as well as those of the anomeric proton of glucopyranosyl and rhamnopyranosyl units (Table S1). These are in good agreement with the literature, and with spectra of rutin acquired for comparison purposes (Napolitano et al. 2012).

Intense aliphatic signals supporting the presence of fatty acids as well as a series of sharp signals indicating the presence of quaternary methyl moieties of terpenoids are observed in the spectrum of the hexane fraction. These observations suggest a large presence of terpenoids. The spectrum of the DCM fraction presents a similar shape as hexane's one, but with a clearer aliphatic region (d 0.90-2.00). This latter shows several sharp signals suggesting the presence of quaternary methyl groups ascribable to terpenes, such as triterpenes (Sut et al. 2018). However, compared to hexane fraction, a lower amount of signals ascribable to fatty acid moieties is observed (Figure S2 and Table S1). The spectrum of the EA fraction showed the presence of triterpenoids that mostly can resume the structure of ursolic acid, due to the presence of six quaternary and one secondary methyl groups, the deshielded methylene linked to a hydroxyl group, and the olefinic signal. These assignments are in good agreement with 2a-hydroxyursolic acid (Choi et al. 1991). The spectrum of the butanol fraction did not show significant signals.

On the basis of these data, we decided to proceed with HPLC-DAD-MS^{n} for the analysis of phenolics and triterpenoids, and with HS-SPME-GC-MS for the analysis of lower molecular weight (i.e., volatile) terpenoids.

2.2. HPLC-DAD-MS^{n} analysis of secondary metabolites in the crude methanol extract

HPLC-DAD-MS^{n} analysis of *C. candidissimum* aerial parts (a mixture of inflorescences, stems and leaves) was performed on the methanol crude extract, since it was the one containing the broadest range of phytochemicals, from phenolics to terpenes. HPLC-

DAD-MS^n allowed to identify 30 secondary metabolites, whose details are reported in Table S2. By using the HPLC-DAD technique, total contents of flavonoids, phenolic acids and gallic acid derivatives were assessed at 3.1%, 3.6% and 0.76% of whole extract, respectively. The total phenolic content was comparable to that already reported for *C. vulgaris* (Khan et al. 2018). Rutin resulted as the most abundant phenolic compound among those identified, followed by several caffeic acid oligomers, caffeoylquinic acid isomers and the gallic acid derivative hexa-*O*-galloyl-glucoside. Several phenolic constituents identified in *C. candidissimum* have been already reported by other authors in other *Calamintha* spp. For instance, the glycosylated flavonoids hyperoside and 6,8-*C*-dihexosylapigenin, and the phenolic acids chlorogenic acid, rosmarinic acid and salvianolic acid B have been detected in the leaves of *C. nepeta* from Italy (Pacifico et al. 2015).

Regarding triterpenes, ursolic acids and its hydroxylated derivative were identified, and their amounts reached the 0.62% of the whole crude extract. Previously published phytochemical data on Calamintha genus have shown that ursolic acid and its derivatives are common constituents within this genus (Khodja et al. 2018). Conversely, our LC-MS results show that triterpenes are only minor constituents of *C. candidissimum* crude extract, although abundant signals attributable to the same class of phytochemicals were observed in the NMR data. These contrasting results suggest that further in depth studies are required for a more comprehensive elucidation of the triterpenes content of this plant. Chemical structures of the most abundant polar constituents identified in *C. candidissimum* are reported in Figure S3.

2.3. HS-SPME-GC-MS analysis

The analysis on the mixture of ground stems, leaves and flowers allowed to characterise 98.7% of the total volatiles emission and to identify 38 constituents (Table S3). The composition of the volatile bouquet was dominated by oxygenated monoterpenes (66.2%), which included pulegone (44.8%), piperitenone (6.6%), isopulegone (5.8%) and *neo*-menthol (3.8%). Sesquiterpene hydrocarbons were found as the second major group of chemicals (27.4%), including germacrene D (16.2%) and bicyclogermacrene (3.0%). Chemical structures of the main volatiles identified in *C. candidissimum* are reported in Figure S4. No previous studies are present in the literature about the spontaneous volatile emission of this species. Even if the SPME results cannot be directly compared with essential oil composition, these results partially reflect already published data regarding the chemical composition of essential oils from the aerial parts of C. *candidissimum,* since several studies have indicated pulegone and menthone as the two major components. For instance, the constituents identified in *C. candidissimum* essential oil by Benyoucef et *al*. (Benyoucef et al. 2020) were principally oxygenated monoterpenes, represented by pulegone (70.4%), menthol (5.2%) and isomenthone (4.5%). *Satureja calamintha* subsp. *nepeta* from Portugal has isomenthone, 1,8-cineole and *trans*-isopulegone as dominant components (Marongiu et al. 2010). Finally, in a study involving wild *C. nepeta* from Corsica, Baldovini *et al*. identified three chemotypes, whose essential oils were characterised by menthone/pulegone,

pulegone, and piperitone and piperitenone oxides as main components, respectively (Baldovini et al. 2000).

Within the *Satureja* genus, a great variability of the volatile profile has been reported, although carvacrol, thymol, p-cymene, borneol, b-caryophyllene, germacrene D, and caryophyllene oxide have been indicated as major compounds (Azaz et al. 2002). Differences between our results and those previously published may be due to the different analytical techniques used, the part of the plant studied, the age of the plant, the period of the vegetative cycle, or even to ecological and genetic factors.

3. Experimental

Included in supplemental file.

4. Conclusion

Our findings show that endemic species *Clinopodium candidissimum* (Munby) Kuntze from Algeria contains valuable components endowed with possible biological activities, including antioxidant and volatile compounds, which could find wide practical application in various areas, such as formulation and production of food additives and healthy supplements. Overall, the results of this investigation represent a starting point for further research on this plant species. These findings could be useful for other research: in particular, the combination of traditional knowledge with currently available research tools may open up new perspectives for drug discovery from natural sources.

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