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Submitted in fulfillment of the requirements for the Doctorate degree in Sciences

Option: Plant biotechnology

By: CHERFIA Radia

Theme

Research of antimicrobial potentialities of an endemic plant of the genus '*Calycotome*': phytochemical and microbiological study

Members of the jury:

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Département de Biologie et Écologie Végétale

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Thèse

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Option : Biotechnologie Végétale

Par : CHERFIA Radia

Thème

Recherche de potentialités antimicrobiennes d'une plante endémique du genre *Calycotome* : étude phytochimique et microbiologique

Devant le jury :

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Dedication

I affectionately dedicate this thesis

To my dear ***parents***

In the memory of my ***father*** *'****CHERFIA Abdelhamid****' who left us very early, may God the Almighty welcome him in a vast paradise and grant him his mercy.*

To the one who gave me meaning to my existence, by offering me a trustworthy education.

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To my dear ***brothers;*** *Abdelaziz, Farouk, and Fouad*

To my lovely ***nieces*** *and* ***nephews***

I love you

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AAE	Ascorbic Acid Equivalents
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid
$AlCl_3$	Aluminum Trichloride
Aq	Aqueous
Aq R	Aqueous Residue
ATCC	American Type Culture Collection
BHT	Butylated HydroxyToluene
$CDCl_{3\text{-}d1}$	Chloroform Deuterium
$CHCl_3$	Chloroform
CID	Collision Induced Dissociation
COSY	COrrelation Spectroscopy Y
C. spinosa	*Calycotome spinosa* (L.) Link
C. villosa	*Calycotome villosa*
D1	One Dimension
D2	Bi-Dimensions
Da	Dalton
DAD	Diode Array Detector
DMSO	Dimethylsulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC_{50}	Efficient Concentration at 50% (in which the absorbance is equal to 0.5)
ESI	ElectroSpray Ionization
EtOAc	Ethyl Acetate
EtOH	Ethanol
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalents
GIFA	Guide Illustré de la Flore Algérienne
h	Hour
HESI	Heated ElectroSpray Ionization
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence /correlation
IC_{50}	Inhibition Concentration at 50%

IP %	Inhibition Percentage
IZD	Inhibition Zone Diameter
LC	Liquid Chromatography
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
LIT	Linear Ion Trap
amu	Atomic mass unit
MHA	Mueller Hinton Agar
MHz	Megahertz
MBC	Minimal Bactericidal Concentration
MIC	Minimal Inhibitory Concentration
min	Minute
MeOH	Methanol
mL	Milliliter
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
n-BuOH	n-Butanol
NMR (1D, 2D)	Nuclear Magnetic Resonance Spectroscopy (1 Dimension, 2 Dimensions)
NOESY	Nuclear Overhauser Effect Spectroscopy Y
PDA	Potatos Dextrose Agar
PDA	PhotoDiode Array
PE	Petroleum Ether
ppm	Parts per million
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
Rt	Retention time
TAC	Total Antioxidant Capacity
TFC	Total Flavonoids Content
TPC	Total Polyphenols Content
TIC	Total Ion Chromatography
TLC	Thin Layer Chromatography
UV	UltraViolet
Y%	Yield

Résumé général en Français de la thèse

(*La version intégrale est rédigée en Anglais*)

Introduction

La nature fournit un immense trésor de produits naturels, avec des activités biologiques souvent étonnantes, utilisés pendant des siècles dans le monde entier dans la médecine traditionnelle. Le *Calycotome*, genre appartenant à la famille des Fabacées (Quezel et Santa, 1963 ; Huxley et *al*., 1992), contient un certain nombre d'espèces principalement répandues dans la région méditerranéenne (Brullo et *al*., 2013). *C. spinosa* (L.) Link, l'espèce de notre étude, est un arbuste épineux et endémique en Algérie, souvent utilisée localement dans la médecine traditionnelle, atteignant deux mètres de hauteur, trifolié avec des fleurs jaunes, se développant dans des sols siliceux et bien arrosés (Quezel et Santa, 1963 ; Talavera, 1999). Bien que cette plante soit riche en métabolites bioactifs, cependant, elle demeure peu exploitée sur le plan recherche scientifique (Quezel et Santa, 1963; Larit et *al*., 2012; Krimat et *al*., 2014). De ce point de vue, un intérêt particulier s'est développé pour élucider la structure de certains composés de cette plante, utiles pour les besoins thérapeutiques et contrôle qualité. Pour ce faire, des méthodes de pointes ont été utilisées comme l'analyse de données spectroscopiques et de spectrométrie de masse comprenant une nouvelle approche combinant les RMN-1D, RMN-2D avec LIT-ESI-MS^n.

Dans ce contexte, les principaux objectifs du présent travail sont:

- Criblage phytochimique de la plante *Calycotome spinosa*;
- Extraction des composés phénoliques ainsi leur quantification;
- Évaluation *in vitro* de l'activité anti-oxydante et antimicrobienne des extraits obtenus;
- Caractérisation chimique d'extraits bioactifs basée sur LC-ESI-MS/MS;
- Purification et identification structurale des molécules bioactives par une nouvelle approche basée sur la combinaison de LIT-ESI-MS^n avec l'analyse par RMN;
- Substances séparées ont, également, été examinées pour leurs activités biologiques *in vitro*.

1- Revue bibliographique

Depuis son existence sur terre, l'être humain reconnaissait et utilisait les plantes pour sa nutrition et pour traiter ses maladies (Pelt, 2014; Pasdeloup Grenez, 2019). Au cours de ces dernières années, les résultats des recherches conduites par les spécialistes (Médecins, biologistes, chimistes, pharmaciens, ethnologues, botanistes, agronomes, écologistes, économistes, etc.) démontrent d'une part, les effets néfastes des médicaments synthétiques à base de produits chimiques sur la santé humaine et d'autre part, l'importance et l'efficacité des plantes médicinales et des produits naturels pour le bien être de l'homme (Ekor, 2014; Verbois, 2015).

Ces produits naturels qui sont très demandés à travers le monde, nécessitent plus d'intérêt pour leur valorisation en appliquant des résultats des recherches scientifiques et des techniques appropriées de production, de protection, de conservation et d'exploitation (Sassi, 2008, Koparde, 2019).

Avec une superficie de 2 381 741 Km^2, l'Algérie est le plus grand pays riverain de la Méditerranée. Ce pays est reconnu pour sa diversité en plantes médicinales et aromatiques (Blama et Mamine, 2013 ; Foi, 2014). L'utilisation de ces plantes est un savoir-faire ancestral transmis de génération en génération chez les populations, le plus souvent, rurales (Derridj et *al*., 2009; Lakel et Zermani, 2017).

La richesse de la flore algérienne est donc incontestable, elle recèle un grand nombre d'espèces classées en fonction de leur degré de rareté : 289 espèces assez rares, 647 espèces rares, 640 espèces très rares, 35 espèces rarissimes et 168 espèces endémiques (FAO, 2012).
Les parties de la plante utilisées sont, principalement, les feuilles, les fruits, les fleurs et les racines. Pour les arbres et les arbustes, l'écorce est également utilisée pour la préparation des recettes.
Récemment, Benderradji et *al*. (2015), Benarba (2016) et Hamel et *al*. (2018) ont montré que la feuille est la partie la plus utilisée des plantes médicinales en Algérie (36-62.6 %) pour le traitement de différentes maladies, suivie des parties aériennes et des tiges (17-19.8 %), puis des graines (18 %) contre 8.2-12 % pour les racines. Tous les bulbes, les rhizomes, les écorces et les résines ont un pourcentage cumulatif de 9.4 %.

En Algérie, plusieurs modes d'utilisation de la plante ont été développés, principalement, la décoction et l'infusion (Reguieg, 2011). En effet, Benarba (2016) a constaté que la décoction était la principale méthode de préparation des plantes (49 %), d'autres sont utilisées crues (32 %) ou infusées (16 %) et macérées (3 %). En revanche, les huiles essentielles et la fumigation sont les formes les moins utilisées (Hamel et *al*., 2018).

Le genre *Calycotome*, appartient à la famille des Fabacées. Ces dernières constituent la troisième famille la plus importante du règne végétal. Les Calycotomes sont des arbustes très épineux. Le nom *Calicotome* (ou *Calycotome*), du grec Calyx et Temno qui signifient respectivement calice (kalux) et coupé, fait allusion au calice qui se rompt circulairement après la floraison (Guide illustré de la flore algérienne (GIFA), 2009). Ce genre a été proposé par Link.
La classification du genre *Calycotome* a connu de nombreux changements au fil du temps (Lattanzi, 2008), sa systématique est actuellement présentée comme suit (Guaâdaoui et *al*., 2016) :

Règne : Plantes (Plantae)

Sous-règne : Plantes vasculaires (*Tracheobionta*)

Super division : Spermaphytes (*Spermatophyta*)

Division : Angiospermes

Classe : Edudicotylédones (*Eudicots*)

Sous-classe : True Rosidae I

Ordre : *Fabales*

Famille : Fabaceae (= Papilionaceae / Leguminosae)

Sous-famille : *Faboideae* Rudd (Papilionoideae Juss)

Genre : *Calicotome*

D'après (Quèzel et Santa, 1963 ; García Murillo, 1999 ; Talavera et *al*., 1999), le tableau 1 récapitule les différentes caractéristiques de ce genre.

Tableau 1 Description botanique du genre *Calycotome* (Quèzel et Santa, 1963 ; Chikhi, 2014).

Partie	**Description**
Tiges	Sont élancées et écartées, formant des buissons qui peuvent atteindre 2 mètres de hauteur. Leurs rameaux sont verts, puis bruns en vieillissant, et se terminent en épines.
Feuilles	Sont composées de trois folioles ovales. Elles sont petites, peu nombreuses et caduques.
Fleurs	Apparaissent dès la fin de l'hiver. Elles sont jaunes, groupées et très nombreuses. La fleur dont le calice ovoïde, couronné par 5 petites dents, complètement clos dans le bourgeon et se rompant circulairement par le milieu au moment de la floraison.
Fruits	Sont des gousses, ils portent deux laines aplaties, longues de trois à quatre cm, comprimées, à suture ventrale élargie et étroitement ailées de chaque côté, contenant trois à huit graines non caroncules
Graine	À une couleur brune ou brune rougeâtre, lisse, brillante, arrondie, d'environ 3 mm de diamètre.
Racines	Portent habituellement des nodosités renfermant des bactéries permettant la fixation de l'azote atmosphérique.

Le genre *Calycotome*, originaire de la région méditerranéenne (GIFA, 2009) préfère les sols siliceux. On le trouve dans les forêts de pins maritimes, dans les forêts de chênes-lièges et dans le maquis, ce qui contribue à rendre sa pénétration difficile. *Calycotome* vit dans les montagnes proches de la côte d'Afrique du Nord et sur la rive nord de la méditerranée (à savoir le Nord de l'Algérie et de la Tunisie, et le Sud de l'Italie, de la France et de l'Espagne) (Quèzel et Santa, 1963 ; Domínguez, 1987).

Deux espèces principales de ce genre, à savoir *C. villosa* et *C. spinosa*, ont été caractérisées par Rameau et *al*. (2008) dont la description botanique est présentée dans la figure 1 :

Figure 1 Caractéristiques morphologiques différenciant les deux espèces (A) *C. spinosa* et (B) *C. villosa* (Rameau, 2008).

Sur le plan phytochimique, aucune étude n'a été rapportée sur les huiles essentielles (HE) de l'espèce *C. spinosa*. En revanche, l'espèce *C. villosa,* d'après Dessi et *al*., (2001) renfermait le 1,8-cinéole, la fenchone, le camphre, l'eugénol, le (Z) -isoeugénol, le *β*-eudesmol. Par ailleurs, le vomifoliol est le seul sesquiterpénoïde isolé des feuilles et des fleurs de *C. spinosa* (Larit et *al*., 2012). Par contre, les graines et les tiges de l'espèce *C. villosa* renfermaient un des triterpènes lupéol (Pistelli et *al*., 2003), deux stéroïdes ; *β*-sitostérol et stigmastérol avec un phényléthanoïde nommé basaléthanoïde B (Alhage et *al*., 2018).

Le seul composé majoritaire de la famille des flavones qui a été isolé de l'espèce *C. spinosa* est la chrysine (Larit et *al*., 2012), en revanche, de nombreux flavonoïdes ont été isolés de l'espèce *C. villosa* (Loy et *al*., 2001 ; Pistelli et *al*., 2003 ; El Antri et *al*., 2004a ; Antri et *al*., 2010 ; Alhage et *al*., 2018).

Concernant les acides phénoliques, un acide cinnamique a été isolé de l'espèce *C. spinosa* (Larit et *al*., 2012). Par contre, plusieurs acides phénoliques ont été détectés chez l'espèce *C. villosa* incluant l'acide quinique en quantité importante et les acides ; protocatéchuique, syringique, p-coumarique, trans-férulique et vanillique en faible quantité (Boughalleb et *al*., 2020 ; Turan et Mammadov, 2020).

Par ailleurs, aucune étude n'a rapporté l'isolement d'alcaloïdes à partir de l'espèce *C. spinosa*, cependant, de nombreux alcaloïdes ont été identifiés de l'espèce *C. villosa* notamment ; la lupinine, la calycotomine, la spartéine, l'anagyrine et la lupanine avec ses dérivés (Loy et *al*., 2001), en outre, deux alcaloïdes de tétrahydroisoquinoléine ont été obtenus à partir d'un extrait MeOH des graines de *C. villosa* Subsp. *Intermédia* (El Antri et *al*., 2004b).

Bien que plusieurs propriétés pharmacologiques des plantes du genre *Calycotome* aient été rapportées, notamment les activités anti-oxydantes, antibactériennes, antifongiques, anti-

inflammatoires, antidiabétiques, hypotensives, diurétiques, vasodilatations, cytotoxiques, larvicides et anthelminthiques cependant, peu d'études sur les activités biologiques de l'espèce *C. spinosa* ont été effectuées. La seule étude évaluant l'activité anti-oxydante de cette espèce a rapporté que l'extrait hydrométhanolique de feuilles présentait une capacité anti-oxydante importante (Krimat et *al*., 2014).

Une activité antimicrobienne significative du genre *Calycotome* a, aussi, été mise en évidence en particulier chez *C. villosa* qui développait une activité antimicrobienne (vis-à-vis) de plusieurs microorganismes en l'occurrence *S. aureus, B. lentus, E. coli, P. aeruginosa, Providencia rettgeri* et *Morganella morganii* (Loy et *al*., 2001 ; Dessi et *al*., 2001). En revanche, peu de recherches ont été consacrées à l'espèce *C. spinosa*. En effet, l'étude de Krimat et *al*. (2014) a montré que l'extrait hydrométhanolique des feuilles de l'espèce *C. spinosa* a révélé de bonnes activités antimicrobiennes contre *B. subtilis, S. aureus* et *Candida albicans*.

2- Matériel et méthodes

Le présent travail a été réalisé au niveau du Laboratoire de Mycologie, de Biotechnologie et de l'Activité Microbienne (LAMyBAM), université frères Mentouri-Constantine 1, Algérie et au Laboratoire de Chimie et Physique- Approche Multi-échelles des Milieux Complexes, Université de Lorraine- Metz- France.

La plante de cette étude a été récoltée dans la région de Constantine, au Nord- Est de l'Algérie (la forêt de Chattaba-Ain Smaraa) pendant la floraison Mars-Juin (2014-2017) (figure 2). L'identification de cette plante a été authentifiée par le Professeur Khalfallah Nadra (Département de Biologie et Ecologie Végétale, faculté des SNV- Université Frères Mentouri-Constantine 1-Algérie) où elle correspond à l'espèce *Calycotome spinosa* (L.) Link.

Figure 2 *Calycotome spinosa* (L.) Link (2016).

Après la récolte et l'identification botanique, le matériel végétal a été ensuite découpé puis séché à l'ombre et à une température ambiante pendant trois à sept jours en moyenne, puis conservées dans des sacs en papier dans un endroit sec. Enfin, les parties aériennes sèches ont été mixées au broyeur électrique pour obtenir des poudres fines.

Dans cette partie expérimentale, quatre axes ont été développés :

1. **Le premier axe** concerne le criblage phytochimique de *Calycotome spinosa* (L.) Link selon (Harborne, 1973 ; Trease et Evans, 1983 ; Sofowara, 1993 ; Raaman, 2006), l'extraction des composés phénoliques par différents solvants pour obtenir des extraits méthanoliques, aqueux et des fractions chloroformiques, acétate d'éthyle et n-butanoliques des feuilles et des fleurs. Suivi par le dosage de ces composés où les teneurs obtenues sont déterminés en utilisant les réactifs suivants : le Folin-Ciocalteu (Singleton et *al*., 1999) pour les poly-phénols totaux (PT) et le trichlorure d'aluminium ($AlCl_3$) (Kosalec et *al*., 2004) pour les flavonoïdes totaux (FT).

2. **Dans le deuxième axe**, différentes techniques d'analyses physicochimiques ont été réalisées sur certains extraits et les composés isolés pour leur caractérisation et identification ; la Chromatographie [Chromatographie Couche Mince (CCM), sur Colonne (CC) et Liquide (LC)], la Spectrométrie de Masse tandem (MS/MS) et la Résonance Magnétique Nucléaire (RMN).

3. **Dans le troisième axe**, nous nous sommes intéressés au pouvoir antioxydant des extraits, des fractions et des composés séparés. Quatre méthodes complémentaires ont été choisies : la capacité anti-oxydante totale (CAT) (Prieto et *al*., 1999) qui permet de quantifier toutes les substances de l'extrait ayant une capacité à empêcher l'oxydation. Méthode du piégeage du radical libre DPPH (2,2-diphenyl-1-picrylhydrazil) (Sànchez Moreno et *al*., 1998), la plus simple à réaliser *in vitro* où le DPPH est largement employé pour évaluer le balayage de divers produits naturels et considéré comme un composé modèle pour les radicaux libres produits dans la peroxydation lipidique. Méthode de réduction de fer (FRAP) (Ferreira et *al*., 2007), une technique indicatrice de la présence des effets réductifs de l'anion hexacyanoferate (III) $[Fe(CN)_6]3-$ à l'anion hexacyanoferate (II) $[Fe(CN)_6]4-$. Enfin, la technique reposant sur l'activité du piégeage du radical libre ABTS (acide 2, 2'-Azino-Bis 3-éthylbenzoThiazoline-6-Sulphonique) (Re *et al*., 1999) a également été effectuée.

4. **Le quatrième et le dernier axe** a été focalisé sur l'étude de l'activité antimicrobienne des extraits et des fractions obtenus et aussi des composés isolés. La technique de diffusion sur gélose (Vinod et *al.*, 2010, Traoré et *al*., 2012) et la méthode d'intégration d'extrait dans un milieu de culture (Bautista-Ban~os et *al.*, 2002) ont été utilisées pour mesurer la zone d'inhibition (ZI mm)

et le pourcentage d'inhibition (PI%), respectivement. Enfin, les données obtenues des teneurs, des activités anti-oxydantes et antimicrobiennes ont été soumises à des analyses statistiques.

3- Résultats

Le présent travail porte sur la caractérisation phytochimique d'une espèce endémique du genre *Calycotome*, la recherche de potentialités anti-oxydantes et antimicrobiennes de certains extraits, de fractions et de composés isolés, suivie par l'élucidation structurale des molécules bioactives.

L'identification botanique de la plante de notre étude réalisée par le Professeur Khalfallah Nadra a révélé que son espèce végétale était *Calycotome spinosa* (L.) Link (*C. spinosa*) (figure 3). Son identification a été basée sur ses caractéristiques morphologiques. Un spécimen de référence authentifié a été déposé à l'herbier de la Faculté des SNV de l'Université FMC1, sous le code (*C. s.* 2014).

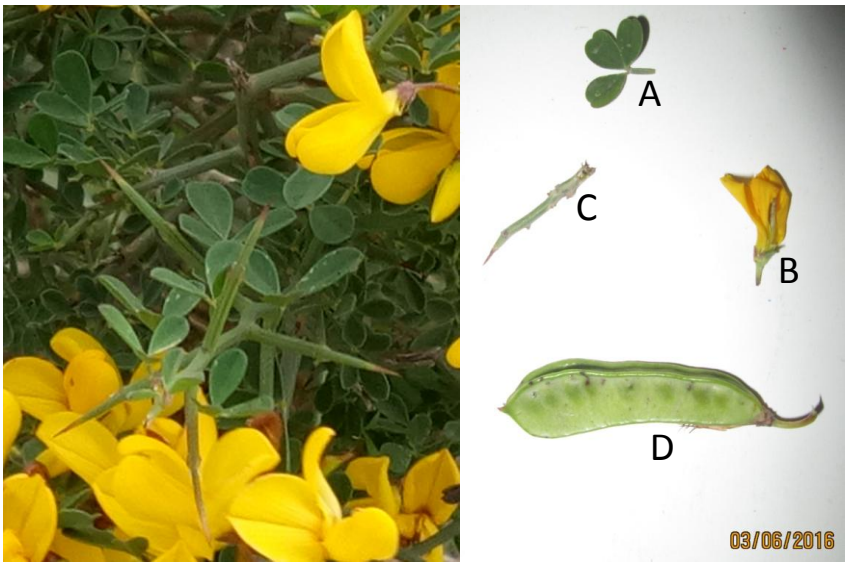


Figure 3 Différentes parties aériennes de la plante *C. spinosa.*
A : Feuille, B : Fleur, C : Epine, D : Fruit

Par ailleurs, après le séchage de chaque partie de la plante, la teneur relative en eau trouvée était de l'ordre de 43.85 ± 1.57 %, de 33.94 ± 0.8 %, de 55.43 ± 1.32 % et de 46.64 ± 2.69 % pour les feuilles, les fleurs, les tiges et les fruits, respectivement.

En effet, le criblage phytochimique effectué sur les parties aériennes de l'espèce *C. spinosa*, feuilles, fleurs, tiges et fruits a révélé la présence de différents groupes chimiques avec un degré significativement varié ; métabolites primaires comme les sucres et secondaires tels que les polyphénols, les flavonoïdes, les alcaloïdes, les tannins, les coumarines et les saponines à intérêt

thérapeutique. Les tests de recherche des acides aminés, des protéines et des huiles essentielles ont été négatifs sur ces parties.

En outre, après l'extraction, chaque extrait a été caractérisé par son rendement (R%) et sa couleur par rapport à la matière sèche. En fait, le rendement de l'extraction a révélé une différence significative entre les extraits bruts (R%; 12.60 ± 0.56 à 15.88 ± 0.53 %) et les fractions (R% ; 0.74 ± 0.02 à 9.97 ± 0.04 %) selon la partie végétale et le solvant d'extraction utilisés. Les extraits et les fractions de feuilles ont montré des valeurs élevées par rapport aux fleurs; notant que l'extrait métanolique (MeOH) avait le meilleur R% de 15.88 ± 0.53 % proche de celui de l'extrait Aqueux (Aq) de la même partie, estimé à 15.00 ± 0.35 %. Par ailleurs, les extraits MeOH et Aq de fleurs représentaient un R% élevé de 13.75 % ± 0.35 et de 12.60 ± 0.56 % ; respectivement, mais inférieur à celui des feuilles. Cependant, les fractions n-butanoliques (n-BuOH) ont exhibé des valeurs moins intéressantes avec un R % égal à 9.97 ± 0.04 et à 5.06 ± 0.08 % pour les feuilles et les fleurs, consécutivement. Pour les fractions de l'acétate d'éthyle (AcEtO) et de chloroforme ($CHCl_3$), le R % était très faible estimé à 1.74 ± 0.02 et à 1.51 ± 0.02 % pour les feuilles et de l'ordre de 0.80 ± 0.01 et de 0.74 ± 0.02 % pour les fleurs, respectivement. Les extraits et les fractions obtenus avaient des couleurs et des rendements différents.

Sur un autre volet, le dosage des composés phénoliques ; polyphénols totaux (PT) et flavonoïdes totaux (FT) a été effectué. En effet, la teneur en PT a considérablement variée dans les extraits (24.63 ± 0.35 à 98.72 ± 2.47 mg EAG / g ES) et dans les fractions (29.97 ± 1.42 à 107.75 ± 2.09 mg EAG / g ES) examinés. Par conséquent, la fraction de l'AcEtO des feuilles possédait la teneur en PT la plus élevée (107.75 ± 2.09 mg EAG / g ES) suivie de celle de l'extrait MeOH de la même partie (98.72 ± 2.47 mg EAG / g ES). Cependant, des quantités moins intéressantes ont été enregistrées, à la fois, dans les fractions $CHCl_3$ et dans les extraits Aq (41.48 ± 1.86 et 24.63 ± 0.35 mg EAG / g ES) pour les fleurs et (29.97 ± 1.42 et 32.71 ± 1.91 mg EAG / g ES) pour les feuilles, consécutivement. La teneur la plus basse en PT a été observée dans l'extrait MeOH de fleurs et estimée à 24.63 ± 0.35 mg EAG / g ES.

Parallèlement, la teneur en flavonoïdes totaux (FT) dans chaque extrait et fraction a été calculée et la teneur a varié entre 3.85 ± 0.13 et 20.87 ± 0.10 mg EQ / g ES, notant que les valeurs en FT sont inférieures à celles de PT. En effet, la quantité la plus élevée a été enregistrée dans la fraction de l'AcEtO des feuilles (20.87 ± 0.10 mg EQ / g ES) suivie des deux fractions $CHCl_3$, avec des teneurs de 19.98 ± 0.15 et de 18.44 ± 0.39 EQ / g ES pour les feuilles et les fleurs, respectivement. Par ailleurs, des quantités intéressantes égales à 17.03 ± 0.03 et 16.30 ± 0.06 mg EQ / g ES ont été

détectées dans la fraction n-BuOH de feuilles et la fraction de l'AcEtO de fleurs, consécutivement. En revanche, un faible niveau de FT a été enregistré dans la fraction n-BuOH et l'extrait Aq de fleurs, estimé à 8.20 ± 0.44 et à 6.04 ± 0.06 mg EQ / g ES, successivement. Dans le même ordre, les deux extraits MeOH ont présenté des teneurs en FT de l'ordre de 4.02 ± 0.62 et de 4.48 ± 0.16 pour les feuilles et les fleurs, consécutivement. Enfin, la valeur la moins significative de FT a été observée dans l'extrait Aq de feuilles ; 3.85 ± 0.13 mg EQ / g ES.

Le tableau 2 récapitule le R% et les teneurs en PT et en FT dans les extraits et les fractions.

Tableau 2 Rendement, Polyphénols totaux et flavonoïdes totaux dans les extraits et les fractions de *C. spinosa*.

	Rendement %		Polyphenols totaux (mg EAG /g ES)		Flavonoïds totaux (mg EQ/ g ES)	
Parties / Extraits	Feuilles	Fleurs	Feuilles	Fleurs	Feuilles	Fleurs
Methanol	15.88 ± 0.53	13.75 ± 0.35	98.72 ± 2.47	24.63 ± 0.35	4.02 ± 0.62	4.48 ± 0.16
Aqueux	15.00 ± 0.35	12.60 ± 0.57	32.71 ± 1.91	39.47 ± 0.76	3.85 ± 0.13	6.04 ± 0.06
Chloroforme	0.80 ± 0.01	0.74 ± 0.02	29.97 ± 1.42	41.48 ± 1.86	19.98 ± 0.15	18.44 ± 0.39
Acétate d'Ethyle	1.74 ± 0.02	1.51 ± 0.02	107.75 ± 2.09	64.24 ± 1.82	20.87 ± 0.10	16.30 ± 0.06
N-Butanol	9.97 ± 0.04	5.06 ± 0.08	81.45 ± 3.00	96.07 ± 2.93	17.03 ± 0.03	8.20 ± 0.44

Pour l'évaluation de l'activité anti-oxydante des extraits et des fractions, deux méthodes différentes ont été développées ; DPPH et FRAP. Dans le test DPPH, la valeur de la concentration inhibitrice à 50 % (CI_{50}) est inversement proportionnelle à la capacité anti-radicalaire d'un composé. Parallèlement, pour le pouvoir réducteur du fer (FRAP), la valeur de la concentration efficace à laquelle l'absorbance était de 0.5 (CE_{50}) est inversement proportionnelle au pouvoir réducteur mesuré. En effet, l'extrait MeOH et la fraction de l'AcEtO des feuilles étaient constamment les plus puissants avec la CI_{50} estimée à 41.04 ± 0.15 et à 45.25 ± 1.8 µg / mL pour le DPPH et la CE_{50} évaluée à 763.73 ± 0.32 et à 780.04 ± 1.36 µg / mL pour le FRAP, respectivement. Ces valeurs minimales sont proches de celles des produits de références (BHT; CI_{50}= 34.73 ± 0.23 µg / mL, AA (acide ascorbique); CE_{50}= 684.29 ± 0.02 µg / mL). Par ailleurs, les deux fractions de n-BuOH ont présenté une activité anti-oxydante remarquable, mais inférieure à celle des extraits précédents (CI_{50}= 52.8 ± 2.05 et 53.95 ± 0.19 µg / mL, CE_{50}= 831.83 ± 1.53 et 852.92 ± 0.74 µg / mL) consécutivement pour les feuilles et les fleurs. En revanche, des valeurs moins intéressantes de la CI_{50} (63.3 ± 0.12 et 54.97 ± 0.7 µg / mL) et de la CE_{50} (934.99 ± 1.44 et 976.52 ± 0.74 µg / mL) ont été enregistrées chez la fraction de l'AcEtO et l'extrait MeOH des fleurs, respectivement. Enfin, l'activité anti-oxydante la plus faible a été exercée par l'extrait Aq de feuilles avec la CI_{50} et la CE_{50} les plus élevées de l'ordre de 195.48 ± 0.81 et de 1093.88 ± 1.73 µg / mL, consécutivement. Il est à mettre en exergue que les deux méthodes utilisées dans ce travail ont abouti aux résultats confirmant l'effet antioxydant intéressant de la plante (tableau 3).

Tableau 3 CI_{50} (DPPH) et CE_{50} (FRAP) des extraits et des fractions de feuilles et de fleurs de *C. spinosa*.

	DPPH CI_{50} (µg/mL)		FRAP CE_{50} (µg /mL)	
Parties / Extraits	Feuilles	Fleurs	Feuilles	Fleurs
Methanol	41.04 ± 0.15	54.97 ± 0.7	763.73 ± 0.32	976.52 ± 0.74
Aqueux	195.48 ± 0.81	103.81 ± 0.63	1093.88 ± 1.73	864.45 ± 1.10
Chloroforme	88.10 ± 0.57	114.85 ± 0.84	954.93 ± 1.33	1065.73 ± 0.47
Acétate d'ethyle	45.25 ± 1.8	63.3 ± 0.12	780.04 ± 1.36	934.99 ± 1.44
N-Butanol	52.8 ± 2.05	53.95 ± 0.19	831.83 ± 1.53	852.92 ± 0.74
BHT	34.73 ± 0.23		/	
AA	/		684.29 ± 0.024	

L'activité anti-oxydante précédente a été suivie par l'étude de l'activité antimicrobienne. À ce niveau, l'étude a été élargie à plusieurs microorganismes où les différents extraits et fractions ont montré une activité intéressante. De ce fait, les bactéries test Gram+ étaient plus sensibles à ces extraits que les bactéries Gram- où *S. aureus* a été extrêmement sensible à l'extrait MeOH de feuilles avec une zone d'inhibition (ZI) de 20.00 ± 0.28 mm en comparaison à d'autres extraits ; la fraction de l'AcEtO des feuilles a, également, été active contre la même bactérie, *S. aureus* mais, avec une ZI moindre, estimée à 11± 0.32 mm. En outre, les bactéries ; *B. subtilis* et *S. abony* ont montré des sensibilités importantes aux même extraits précédents, MeOH et AcEtO avec des ZIs de (16.00 ± 0.50 et 13 ± 0.65 mm) et de (12 ± 0.29 et 16.00 ± 1.53 mm) respectivement. Sur un autre volet, il a été constaté que, l'extrait MeOH de feuilles, était bactéricide contre les bactéries *B. subtilis*, *S. aureus* et *S. abony* avec une concentration minimale inhibitrice (CMI) inférieure ou égale à 0.125 mg / disque et une concentration minimale bactéricide (CMB) de 1.00 mg / disque, alors que, les bactéries Gram- ; *E. coli*, *P. aeruginosa* et *K. pneumoniae* ont été moins sensibles à l'effet. Parallèlement, deux espèces fongiques phytopathogènes d'*Alternaria* testées ont montré une sensibilité à tous les extraits (MeOH et Aq) avec des pourcentages d'inhibition (PI%) variables ; *Alternaria sp.1* était la souche la plus sensible avec des PI% de 48.59 % et de 30.55 % développés respectivement par les extraits MeOH et Aq de feuilles et de 41.54 % et de 61.97 % élaborés par les extraits des fleurs, consécutivement. Cependant, aucune activité antifongique n'a été montrée contre la levure *Candida albicans* et les quatre autres isolats fongiques test ; *Penicillium sp.1*, *Penicillium sp.2*, *Aspergillus sp*. et *Rhizopus sp*.

L'extrait ayant le rendement le plus élevé et les meilleures activités a été sélectionné pour la suite du travail. En effet, l'extrait MeOH de feuilles a pu être identifié comme étant le plus actif. Cet extrait a subi un fractionnement par chromatographie sur colonne (CC) et qui abouti à l'obtention de cinq composés énumérés ; (1), (2), (3), (4) et (5).

Afin de détecter les molécules majoritaires présentes dans l'extrait MeOH choisi, ce dernier a été soumis à une combinaison de chromatographie en phase liquide, de spectrométrie de masse en

tandem équipé d'une source d'ionisation par électrospray (LC-ESI-Trap MS/MS), où les profils MS ont été analysés. Parallèlement, les composés énumérés précédemment ont, également, été analysées en utilisant une approche contenant des analyses LIT-ESI-MS^n conjointement avec l'analyse RMN pour leur identification afin de les tester biologiquement (molécules séparées).

Pour l'extrait MeOH, le piège des ions est utilisé pour examiner deux événements. Le premier est le scan *m/z* en "MS complet" et le second est la fragmentation des cinq **(5)** ions les plus intenses.
En effet, l'analyse a abouti à vingt-huit (28) composés phénoliques et un disaccharide. La majorité de ces composés phénoliques a été détectée et caractérisée pour la première fois (dans le présent travail) de l'extrait MeOH de *C. spinosa*. Tous ces composés ont été détectés grâce à leurs pics obtenus durant les 30 min d'analyse cependant, la plupart des composés les plus intéressants ont été détectés lors des premiers 15 min. Le premier pic obtenu correspond au disaccharide avec un temps de rétention de 2.1 min et les deux derniers pics désignent le dérivé de la chrysine et le kaempférol avec un temps de rétention de 14.36 et de 14.95 min, respectivement. Parmi les composés phénoliques, il y avait vingt-quatre (24) flavonoïdes, constitués le groupe principal, où la majorité se couple aux sucres pour former des flavonoïdes glycosides dans la plante, et une petite partie d'entre eux est présente sous une forme libre. En revanche, quatre acides phénoliques seulement en l'occurrence ; l'acide quinique, l'acide *p*-coumarique, l'acide caféique et l'acide sinapique-O-hexoside ont été détectés.

Le composé 1 a été découvert en tant que disaccharide et se basant sur son ion moléculaire parent à *m/z* 341 dans le spectre MS et qui a donné un ion fragment abondant à *m/z* 179 (Molina-Garcia et *al*., 2018).

Le composé 2 a un temps de rétention (tR) = 3.15 min et a été identifié comme étant acide quinique avec un ion parent à *m/z* 191 $[M-H]^-$ dans le spectre MS (Gouveia et Castilho, 2011 ; Afonso et *al*., 2017).

Le composé 3 élué à 5.51 min a produit un ion moléculaire parent à *m/z* 163 $[M-H]^-$ et un ion fragment abondant à *m/z* 119, indiquant la présence de l'acide *p*-coumarique (Spínola et *al*., 2015).

Le composé 5 avec l'ion moléculaire parent à *m/z* 179 et un ion fragment à *m/z* 135 (-44 Da), suggérant la présence de l'acide caféique (Barros et *al*., 2012).

L'analyse LC-ESI-MS a, également, montré un ion parent $[M-H]^-$ à *m/z* 431 (tR = 9.23 min) et a donné un ion fragment à *m/z* 385 ; il a été identifié comme un acide sinapique-*O*-hexoside (composé 7) (Spínola et *al*., 2015).

Le même spectre ESI-MS (en mode négatif) du même extrait présentait l'ion moléculaire de *m/z* 447 à 7.16 min, qui a un fragment de *m/z* 285, correspondant également au kaempférol. La différence de masse entre l'ion *m/z* 447 et le kaempférol peut s'expliquer par la perte d'une molécule de glucose. Alors, le composé 6 a été distingué comme le kaempférol-3-O-glucoside (Alfonso et *al.*, 2017).

En ce qui concerne le composé 23, son analyse en mode négatif a permis de détecter l'ion moléculaire avec *m/z* 533, qui possède un fragment ion *m/z* 489 (perte de -44 Da) correspondant au kaempférol-O -malonyl-hexoside, une fois qu'il se décompose pour donner un ion fragment de *m/z* 285 (perte d'une unité de glucose, -162 Da), comme avait, déjà, été décrit (Santos et *al*., 2017).

Compte tenu du modèle de fragmentation et de son élution chromatographique, le composé 29 a été attribué au kaempférol (Karar et Kuhnert, 2015).

Les composés 11, 16 et 18 ont été observés à des différents temps de rétention dans le chromatogramme, mais tous ont donné des ions $[M-H]^-$ à *m/z* 431 ; la fragmentation MS^2 des deux composés 11 et 16 a révélé un pic de base à *m/z* 311 (7.06 % et 7.81 %, respectivement) et un ion fragment moins intensif à *m/z* 341, ce qui correspond à la perte des fractions du C-glucose (Cuyckens et Claeys, 2004) ; tandis que la fragmentation MS^2 du composé 18 a donné un pic de base unique à *m/z* 269, correspondant à la perte de la fraction d'O-glucose. Sur la base de leurs ordres d'élution chromatographique et de la littérature rapportée, ces trois composés ont été identifiés comme des isomères des glucosides d'apigénine ; le composé 11 identifié comme apigénine-8-C-O-glucoside (Santos et *al*., 2017), le composé 16 étant identifié comme apigénine-6-C-glucoside (isovitexine) (Karar et Kuhnert, 2015) et le composé 18 est un apigénine-7-O-glucoside (Santos et *al*., 2017).

D'autres dérivés de l'apigénine ont, également, été trouvés dans l'extrait MeOH de *C. spinosa*, il s'agissait d'un C-glycoside en raison de la perte des fragments 90 et 30 Da, présumés être identifiés comme apigénine-C-hexoside-C-hexoside (composé 8, $[M-H]^-$ *m/z* 593) (Ferreres et *al*., 2007 ; Bouziane et *al*., 2018) ; néanmoins, il n'a pas été possible de numéroter les positions du résidu C-glycoside en raison du manque d'un composé standard pour la comparaison. De plus, 2''-O-pentoxyde-8-chexoside apigénine (composé 14 à $[M-H]^-$ *m/z* 563) a, aussi, été détectée (Barros et *al*., 2012). Par comparaison avec la littérature (Spínola et *al*., 2015), le composé 20 présentait un ion précurseur $[M-H]^-$ à *m/z* 863 et deux ions fragments à *m/z* 431 et *m/z* 269 ; il a été défini comme un autre dérivé de l'apigénine ; un dimer d'apigénine 7-O-glucoside. Le composé 25 présentait l'ion $[M-H]^-$ à *m/z* 269, son profil de fragmentation MS^2 était similaire à celui de l'apigénine. Ce composé a donné le pic de fragment *m/z* 149 de l'apigénine.

Par ailleurs, certains dérivés de lutéoline ont, pareillement, été identifiés, avec un ion fragment typique à *m/z* 285 (Spínola et *al*., 2015); lutéoline-O-hexoside (composé 12) qui présentait un ion moléculaire parent à *m/z* 447, lutéoline-O- (acétyl) hexoside (composé 19) à *m/z* 489, lutéoline-O-(diacétyl) hexoside (composé 21) avec un ion moléculaire parent à *m/z* 533, lutéoline-6,8-di-C-glucoside (composé 9) qui présentait un ion précurseur $[M-H]^-$ à *m/z* 609, et un dimer de lutéoline-O-hexoside (composé 17) à *m/z* 895. De plus, un dérivé de lutéoline est proposé pour le composé 22 élué a un tR = 13.12 min avec un ion parent à *m/z* 995 et des ions fragments à *m/z* 489 et à *m/z* 285.

Des dérivés de chrysine ont, en outre, été reconnus avec un ion fragment distinctif à *m/z* 253. Composés (4, 26 et 27) aux ions moléculaires à *m/z* 577, à *m/z* 461 et à *m/z* 877 ; consécutivement ; ont été nommés chrysine-6,8-C-diglucoside (Lin et *al*., 2013), chrysine-7-O-glucoside (Pereira et *al*., 2012) et dérivé de chrysine (Barros et *al*., 2012). Sur la base de l'analyse LC-MS et du modèle de fragmentation MS/ MS, le composé 28 a été proposé comme un isomère de la chrysine (Pereira et *al*., 2012).

Par ailleurs, les profils MS obtenus par ESI-LITMS et les spectres RMN des cinq composés isolés (1), (2), (3), (4) et (5) à partir de l'extrait MeOH ont été analysés et comparés aux standards pour faciliter leur identification moléculaire :

Le composé (1) a été trouvé sous la forme d'une poudre jaune avec un rendement de 0.56 %. Son spectre de masse, obtenu en ESI-LITMS^2 en mode négatif, présentait un pic d'ion quasi-moléculaire de rapport *m/z* 415 $[M-H]^-$ suggérant une masse moléculaire de 416 uma (calculée égale à 416.11073). Le spectre MS/MS de cet ion possédait quatre ions fils majoritaires ; à *m/z* 315 (- 100 uma), à *m/z* 313 (- 102 uma), à *m/z* 295 (- 120 uma) et *à m/z* 253 correspondant à la chrysine (- 162 uma), respectivement. Ce dernier a été fragmenté pour donner un ion de *m/z* 209, comme il l'avait déjà décrit (McNab et *al*., 2009).

Tableau 4 Déplacements chimiques en ^{1}H-RMN (400 MHz) et ^{13}C-RMN (100 MHz) du composé **(1)** dans pyridine-$_{d5}$.

N°	**δ_C**	**δ_H (m, *J* Hz)**	**N°**	**δ_C**	**δ_H (m, *J* Hz)**	**N°**	**δ_C**	**δ_H (m, *J* Hz)**
2	163.7	-	9	157.1	-	6’	126.5	8.08, d (6.5)
3	105.6	7.04, s	10	105.5	-	1’’	99.9	5.13, d (6.7)
4	182.2	-	1’	130.6	-	2’’	73.0	3.45-3.25, m
5	161.1	12.79, s	2’	126.5	8.08, d (6.5)	3’’	76.4	3.45-3.25, m
6	99.7	6.46, d (1.5)	3’	129.2	7.59, m	4’’	69.5	3.45-3.25, m
7	163.2	-	4’	132.2	7.59, m	5’’	77.2	3.45-3.25, m
8	95.0	6.86, d (1.5)	5’	129.2	7.59, m	6‘’	60.6	a: 3.70, d (10.3)/ b: 3.45-3.25, m

L'ensemble des analyses spectrales ESI-MS^n et RMN (tableau 4) précédentes du composé (1) a permis d'identifier sa correspondance à la chrysine-7-*O*-(*β*-D-glucopyranoside) (figure 4) avec une formule brute de $C_{21}H_{20}O_9$. Cette identification a été confirmée par la littérature (El Antri et *al*., 2004 ; Alhage et *al*., 2018).

Le composé (2) a été isolé sous la forme d'une poudre solide jaune pâle avec un rendement de 1.67 %. Son spectre de masse obtenu en ESI-$LITMS^2$ en mode négatif, avait un pic d'ion quasi-moléculaire *m/z* 457 $[M-H]^-$ suggérant une masse moléculaire de 458 uma (calculée égale à 458.1213). Ce pic présente 42 uma de plus que le composé (1) correspondant à la structure chimique de C_2H_2O, caractéristique de la fragmentation des flavones (Fabre *et al.*, 2001). L'ensemble de l'analyse permet d'attribuer au composé (2) la formule brute de $C_{23}H_{22}O_{10}$. Le spectre MS/MS de cet ion présentait, pareillement, quatre ions fils majoritaires ; à *m/z* 253 (- 204 uma), à *m/z* 209 (- 248 uma), à *m/z* 199 (- 258 uma) et à *m/z* 171 (- 286 uma), consécutivement. Ces analyses ont permis de constater qu'un ion fils à *m/z* 253 est commun aux deux composés (1) et (2).

Tableau 5 Déplacements chimiques en ^{1}H-RMN (400 MHz) et ^{13}C-RMN (100 MHz) du composé **(2)** dans pyridine-$_{d5}$.

N°	**δ_C**	**δ_H (m, *J* Hz)**	**N°**	**δ_C**	**δ_H (m, *J* Hz)**	**N°**	**δ_C**	**δ_H (m, *J* Hz)**
2	164.6	-	9	157.1	-	6'	126.7	7.60, d (6.7)
3	105.7	6.51, s	10	106.4	-	1''	101.5	5.0, d (7.3)
4	182.2	-	1'	101.5	-	2''	73.0	3.38, m
5	162.6	12.74, s	2'	126.7	7.60, d (6.7)	3''	75.4	3.38, m
6	100.9	6.35, d (1.6)	3'	129.5	7.3-7.4, m	4''	70.7	3.25, m
7	163.7	-	4'	132.3	7.3-7.4, m	5''	76.2	3.75, m
8	95.6	6.49, d (1.6)	5'	129.5	7.3-7.4, m	6''	171.3 64.4 21.1	a: 4.41, d (10.1) b: 4.12, d (11.8) 2.02, s

L'ensemble des données précédentes a permis d'établir la structure du composé (2) comme étant un flavonoïde glucoside et il s'agit de la Chrysin-7-*O*-*β*-D- (6 ''-acétyl) glycopyranoside (figure 4). Sur la base de ces analyses spectrales et de la littérature (Pistelli et *al*., 2003 ; El Antri et *al*., 2004 ; Alhage et *al*., 2018), sa formule moléculaire a été confirmée.

Le composé (3) a été obtenu sous forme de cristaux jaunes avec un rendement de 1.11 %. Le spectre de masse enregistré en ESI–$LITMS^2$ en mode négatif révélait un ion quasi moléculaire de *m/z* 431 (calculé égale à 431.09782) $[M-H]^-$ en accord avec la masse moléculaire de 432 uma qui corresponde à la formule brute de $C_{21}H_{20}O_{10}$. Ce composé présente donc, un atome d'oxygène supplémentaire par rapport au composé (1).

Tableau 6 Déplacements chimiques en ^{1}H-RMN (400 MHz) et ^{13}C-RMN (100 MHz) du composé **(3)** dans pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)
2	166.7	-	9	158.1	-	6’	129.5	7.85, d (8.4)
3	105.7	6.98, s	10	104.1	-	1’’	101.2	5.26, d (6.8)
4	184.0	-	1’	130.6	-	2’’	74.3	3.38, m
5	162.1	12.95, s	2’	129.5	7.85, d (8.4)	3’’	77.8	3.38, m
6	101.3	6.50, d (2)	3’	117.5	7.63, d (8.4)	4’’	71.3	3.25, m
7	164.9	-	4’	132.3	-	5’’	78.4	3.75, m
8	96.0	6.85, d (2)	5’	117.5	7.63, d (8.4)	6‘’	62.5	4.41, d (10.1) 4.12, d (11.8)

L’ensemble des analyses spectrales de la présente étude (ESIT-MS^n et RMN (tableau 6)) révèle une forte similarité de cette molécule avec les données expérimentales disponibles dans la littérature (Tan et *al*., 2010 ; Zhang et *al*., 2017), du coup, le composé (3) a été identifié comme étant ; apigenin-7-*O*-*β*-D-glycopyranoside (figure 4).

Le composé (4) se présentait sous la forme d’une poudre jaune avec un rendement de 2.22 %. Il réagit positivement au réactif de Mayer aboutissant à une précipitation jaune. Son spectre de masse obtenu en ESIT-MS en mode négatif a donné un ion moléculaire *m/z* 134 $[M-H]^-$ qui correspond à une masse de 135 uma (calculée égale à 135.06841 g/mole). Le spectre MS^2 de ce composé montrait un ion *m/z* 118 indiquant une perte de 16 uma (atome d’oxygène). Sur la base de l'analyse des spectres de masse du composé (4) et les études spectrales RMN (tableau 7) ; ^{1}H-RMN, ^{13}C- RMN, COSY, NOESY, HMBC et HSQC collectivement et avec la comparaison à des données de la littérature (Hegde et *al*., 1997), le composé (4) a été identifié comme étant le 5-hydroxyindoline (figure 4) considéré comme nouveau composé naturel avec la formule brute de C_8H_9NO. Ce résultat, obtenu dans le présent travail, est un résultat innovant car, à notre connaissance, c’est la première mise en évidence de cet alcaloïde à partir d’une source naturelle.

Tableau 7 Déplacements chimiques en ^{1}H-RMN (400 MHz) et ^{13}C-RMN (100 MHz) du composé **(4)** dans pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)
1	129.1	-
2	41.7	3.5, m
3	33.8	3.2, m
4	116.9	3.9, m
5	147.5	6.8, dd (8)
6	117.5	7.26, d (8)
7	120.4	7.2, d (2)
8	146.3	-

Le composé (5) a été obtenu sous la forme d'une poudre cristalline blanche d'un rendement de 2.78 %. Le spectre de masse obtenu en ESI-TMS^2 en mode négatif a donné un ion quasi-moléculaire *m/z* 193 $[M-H]^-$ suggérant une masse de 194 uma.

Tableau 8 Déplacements chimiques en ^{1}H-RMN (400 MHz) et ^{13}C-RMN (100 MHz) du composé **(5)** dans pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)
1	74.70	4.80, m
2	73.76	4.78, dd (9.90, 2.6)
3	85.84	4.15, dd (9.90, 9.53)
4	73.09	4.63, dd (9.53, 9.98)
5	74.20	4.76, dd (9.98, 2.6)
6	72.29	4.80, m
7	60.74	3.92, s

L'ensemble des données de la spectrométrie de masse et des spectres RMN (tableau 8), sont identiques à ceux décrits dans la littérature (Lovina et *al*., 1992, Abdoulaye et *al*., 2004 ; NIST MS, 2008 ; Raya-Gonzalez et *al*., 2008 ; De Almeida et *al*. 2012 ; Mukae et *al*., 2016). Sur la base de nos données et de leur comparaison avec les résultats publiés, la molécule (5) a été identifiée comme étant D-Pinitol (figure 4), sa formule moléculaire a, également, été déterminée et de structure $C_7H_{14}O_6$ (*m/z* 193 $[M-H]^-$). Ce résultat est une première mise en évidence de cette molécule à partir de plantes appartenant au genre *Calycotome,* objet d'étude du présent travail.

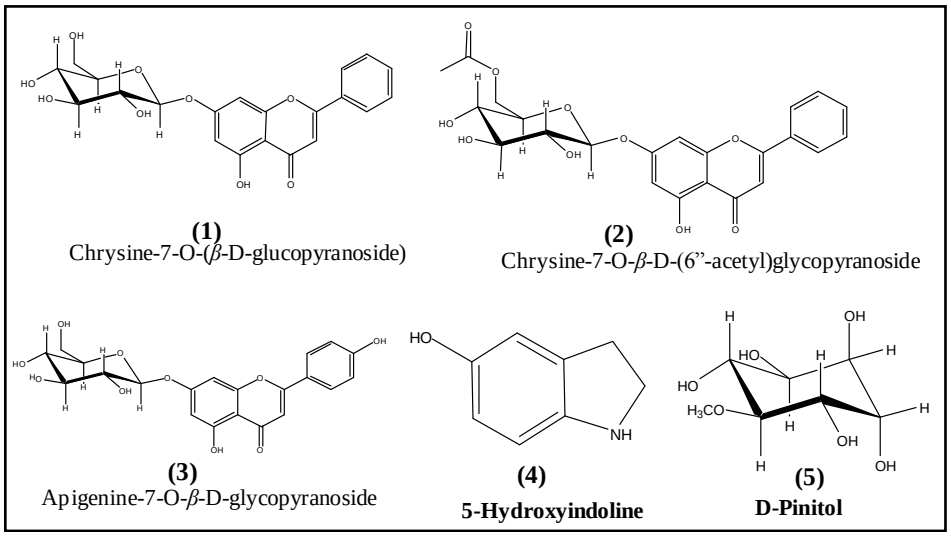


Figure 4 Structures chimiques des composés isolés **(1-5).**

Parallèlement, l'activité biologique de ces molécules a été effectuée. De ce fait, le potentiel antioxydant a été évalué par quatre méthodes différentes ; CAT, DPPH, FRAP et ABTS (tableau 9) ; en calculant soit la diminution (DPPH, ABTS) soit l'augmentation (CAT, FRAP) de l'absorbance. La meilleure activité anti-oxydante a, souvent, été montrée par le nouveau composé **(4)** (5-hydroxyindoline) dans les quatre méthodes utilisées ; (DPPH: CI_{50} <10 µg / mL; CAT = 985.54 ±

0.13 mg EAA / g ES; FRAP: CE_{50} = 344.82 ± 0.02 µg / mL; ABTS: CI_{50} = 7.8 ± 0.43 µg / mL) suivi du composé apigénine-7-O-*β*-D-glucopyranoside **(3)** qui a enregistré, également, une capacité anti-oxydante remarquable, mais inférieur à celle de la nouvelle molécule **(4).**

Tableau 9 Capacité anti-oxydante de l'extrait méthanolique des feuilles et de ses composés isolés de C. *spinosa* (L.) Link ainsi que les étalons mesurés par différentes méthodes.

Echantillon	CI_{50}/ DPPH (µg/mL)	CAT (mg EAA/g extrait)	CE_{50}/ FRAP (µg/mL)	CI_{50} ABTS (µg/mL)
Feuilles MeOH	41.04 ± 0.15	671.02 ± 0.13	763.73 ± 0.32	19.18 ± 1.3
Composé (1)	35430 ± 0.05	236.17 ± 0.17	1930.61 ± 0.17	240.42 ± 0.43
Composé (2)	34324 ± 0.12	240.94 ± 0.24	1805.46 ±0.12	247.26 ± 0.21
Composé (3)	47.36 ± 0.21	608.67 ± 0.22	814.61 ± 0.31	63.72 ± 0.64
Composé (4)	<10	985.54 ± 0.16	344.82 ± 0.02	7.8 ± 0.43
Composé (5)	37320 ± 0.34	268.69 ± 0.43	2230.08 ± 0.29	391.92 ± 0.5
BHT	34.73 ± 0.23	/	/	7.3 ± 0.5
Acide Ascorbique	/	905.95 ± 0.5	684.29 ± 0.024	/
Chaque valeur est exprimée en tant que moyenne ± écart type (n = 3); CAT = Capacité Antioxydante Totale; CI_{50} = Concentration Inhibitrice à 50 %; CE_{50} = Concentration efficace à laquelle l'absorbance était de 0.5.				

Par ailleurs, et dans l'étude microbiologique, objectif principal de la présente étude, le composé **(4)** a montré une activité antibactérienne très intéressante contre *S. aureus* (16 ± 0.5 mm), *P. aeruginosa* (9.83 ± 0.29 mm) et *S. abony* (8 ± 0.28 mm) à la concentration de [C] = 1 mg / disque. En revanche, aucune activité n'a été développée par le même composé **(4)** contre *B. subtilis*, *E. coli*, *K. pneumoniae* et *C. albicans*. Les autres composés (1), (2), (3) et (5) n'ont enregistré aucun effet sur l'ensemble des microorganismes (tableau 10).

Tableau 10 Potentiel antimicrobien *in vitro* de l'extrait MeOH de feuilles et de ses cinq composés isolés de *C. spinosa* (L.) Link

		Zone d'inhibition (mm)								
		Extrait MeOH	Composés isolés					Contrôle (+) (Standards)		Contrôle(-)
Microorganisme		**F MeOH***	**(1)***	**(2)***	**(3)***	**(4)***	**(5)***	Chloramphenicol **15 µg/disque**	Fluconazole **10 µg/disc**	DMSO
Gram +	*S.aureus*	**20± 0.28**	/	/	/	**16± 0.5**	/	40±0.07	NT	/
	B. subtilis	**16± 0.5**	/	/	/	/	/	33±0.13	NT	/
Gram -	*E. coli*	/	/	/	/	/	/	28±0.34	NT	/
	P. aeruginosa	**7.2±0.5**	/	/	/	**9.83± 0.29**	/	32±0.22	NT	/
	K. pneumoniae	/	/	/	/	/	/	30±0.17	NT	/
	A. baumannii	/	/	/	/	/	/	44±0.11	NT	/
	S. abony	**12± 0.29**	/	/	/	**8± 0.28**	/	35±0.44	NT	/
Levure	*C. albicans*	/	/	/	/	/	/	NT	35.2±0.24	/
* 1 mg/disque; NT: Non Testé										

4- Discussion

Des milliers de composés naturels divers sont produits par les plantes et beaucoup d'entre eux sont impliqués dans la défense de ces plantes. Les composés bioactifs des plantes comprend les terpénoïdes, les saponines, les composés phénoliques et phényles, les propanoïdes, les

ptérocarpanes, les stilbènes, les alcaloïdes, les glucosinolates, le cyanure d'hydrogène, l'indole et le soufre élémentaire ; le seul composé inorganique (Cooper et *al*., 1996).

La plante de notre étude a été identifiée comme étant *Calycotome spinosa* (L.) Link. La recherche bibliographique sur cette espèce a permis de constater l'absence de travaux publiés visant le screening détaillé sur la composition chimique de ses différentes parties. En revanche, certaines études considérées comme des ébauches ont introduit cette espèce en Algérie (Larit et *al*., 2012 ; Krimat et *al*., 2014). Sur un autre volet, plusieurs études sur la plante, en tant que légumineuse, ont été consacrées à sa description botanique et à son interaction avec l'environnement (Zeddam et *al*., 2007 ; Damerdji et Djedid, 2006 ; Damerdji, 2008-2009 ; Damerdji et Djedid, 2012).

En effet, le profil chimique de la plante étudiée, *C. spinosa* (L.) Link, a révélé que les composés phénoliques en l'occurrence ; les flavonoïdes, les polyphénols et les tannins sont les composés les plus abondants dans les feuilles et les fleurs, cependant, une absence totale des huiles essentielles et des protéines a été constatée. Ces résultats sont similaires à ceux révélés par Larit et *al*. (2012) qui ont travaillé sur la même espèce, notamment, sur la présence des flavonoïdes. Parallèlement, lorsque ces résultats sont comparés à ceux d'autres espèces du même genre, beaucoup de similitudes ont été constatées. En effet, nos résultats sont en accord avec ceux de Djeddi et *al*. (2015) qui ont mis en évidence la présence de plusieurs familles chimiques telles que ; les alcaloïdes, les flavonoïdes, les stérols et les triterpènes dans la partie aérienne de *Calycotome villosa* provenant de la montagne Edough (Annaba, Algérie).

Par ailleurs, nos résultats peuvent, aussi, être comparés à ceux d'Elkhamlichi et *al*. (2017) en particulier, la présence des flavonoïdes, de type flavones et flavonols, et les tannins dans les graines et l'écorce de *C. villosa* collectée d'une région marocaine. En outre, plusieurs molécules de la famille des flavonoïdes, des alcaloïdes et des polyphénols ont été séparés et identifiés à partir de *C. villosa* (Al Antri et *al*., 2004a ; Al Antri et *al*., 2004b ; Al Antri et *al*., 2004c ; Al Antri et *al*., 2010 ; Elkhamlichi et *al*., 2014 ; Turan et Mammadov, 2020) confirmant la richesse de ce genre en métabolites secondaires. À titre de comparaison, les résultats trouvés dans la présente étude sont similaires à ceux développés, dans une étude récente, par Ayoola et *al.* (2020) sur les feuilles d'un autre genre, *Desmodium adscenden.* L'étude en question a révélé, la présence des mêmes groupes de métabolites que ceux détectés dans le présent travail, à savoir ; les sucres, les polyphénols, les flavonoïdes, les tannins, les alcaloïdes et les saponines.

L'ensemble des résultats précédents montre que la présence ou l'absence de certains métabolites varient selon la partie de la plante utilisée, l'espèce et la région d'étude. En fait, les groupes

chimiques se trouvent dans toutes les parties de plantes, mais ils sont distribués différemment selon leurs rôles (Muanda, 2010 ; Pagare et *al*., 2015).

La qualité, la quantité et les activités biologiques des composés phytochimiques dépendent directement du stade de développement de la plante, de la partie de la plante et de solvants utilisés pour l'extraction et l'isolement (Senguttuvan et *al*., 2014 ; Ullah et *al*., 2017 ; Jacotet-Navarro et *al*., 2018 ; Chekroun-Bechlaghem et *al*., 2019). Les résultats obtenus dans le présent travail ont montré qu'il existe des différences significatives entre le rendement (R%), la teneur en polyphénols totaux (PT) et la teneur en flavonoïdes totaux (FT) dans les extraits bruts et les fractions de la plante étudiée. La fraction d'AcEtO des feuilles a montré un R% plus faible (1.74 ± 0.02 %), cependant, elle a enregistré les valeurs les plus élevées en PT (107.75 ± 2.09 mg EAG / g ES) et en FT (20.87 ± 0.10 mg EQ / g ES). Ces résultats sont en accord avec ceux obtenus par Turan et Mammadov (2020) travaillant sur une autre espèce du même genre (*C. villosa*), leurs résultats ont montré des teneurs en PT et en FT plus élevées dans l'extrait éthanolique (EtOH) des fleurs ; avec des teneurs en PT de 159.47 ± 0.33 mg EAG/ g et en flavonoïdes 66.21 ± 0.09 mg EQ/ g, respectivement. Ce fait, probablement liés au solvant et à la partie de la plante utilisée pour l'extraction.

En outre, dans ce travail, il a été trouvé que l'extrait MeOH de feuilles était riche en PT (98.72 ± 2.47 mg EAG/ g ES) et pauvre en FT (4.02 ± 0.62 mg EQ/ g ES), alors que son R% était élevé égal à 15.88 ± 0.53 %. Ces résultats sont moins éminents que ceux obtenus par une étude de Krimat et *al.* (2014) sur un extrait hydrométhanolique de feuilles de la même espèce où la teneur en PT estimée à 228.42 ± 8.86 mg EAG /g ES et une teneur en FT moins importante égale à 4.87 ± 0.12 mg EQ/g ES. Par ailleurs, la recherche menée par Mebirouk-Boudechiche et *al*. (2015) sur les feuilles de *C. spinosa* a abouti à des teneurs en PT et en tanins totaux de l'ordre de 119.43 et de 83.68 g équivalent acide tannique/kg MS, respectivement. Ces résultats concordent avec ceux obtenus, dans la présente étude.

Dans ce travail il a été constaté, également, qu'à l'exception de la teneur en PT de l'extrait MeOH des feuilles, les teneurs en PT et en FT des autres extraits polaires (MeOH et Aq) des deux parties de la plante ; feuilles et fleurs, étaient inférieurs à ceux de toutes les fractions ($CHCl_3$, AcEtO, n-BuOH). Alors que le R% des extraits polaires a montré des valeurs inverses. Ceci peut s'expliquer par le fait que la polarité du solvant d'extraction et la présence de nombreux autres composants chimiques que les composés phénoliques dans ces extraits polaires avaient une influence sur le contenu phytochimique et sur le rendement (Lesjak et *al*., 2011 ; Do et *al*., 2014 ; Wakeel et *al*., 2019).

La teneur en PT dans l'extrait MeOH de feuilles était élevée, proche de celle de la fraction d'AcEtO de la même partie. La tendance inverse a été observée dans le cas du FT. Cela, éventuellement, dû à la différence du solvant d'extraction. Par conséquent, le MeOH est un solvant approprié pour l'extraction de composés polyphénoliques des tissus végétaux, en raison de sa capacité à inhiber l'action de la polyphénol oxydase qui provoque l'oxydation des polyphénols, et sa facilité d'évaporation par rapport à l'eau (Yao et *al*., 2004).

Par ailleurs, et pour comparaison avec d'autres genres, les travaux de Mahmoudi et *al*. (2012) sur différentes parties de la fleur d'artichaut (*Cynaras colymus* L.) indiquent que les extraits aqueux des tiges ont une teneur en PT et en FT égale à 14.49 ± 1.51 mg EAG / g MS et à 4.18 ± 0.49 mg EQ / g MS respectivement. Ces résultats sont inférieurs à ceux des PT obtenus dans ce travail. Il est bien connu que la quantité de composés phénoliques varie en fonction des familles et des variétés de plantes (Sini et *al*., 2010 ; Belmekki et Bendimerad, 2012).

En conclusion de cette partie, il est suggéré que les différences de quantités de composés chimiques évoqués précédemment peuvent être liées aux différences de polarité entre les solvants, et par conséquent à la solubilité du soluté dans le solvant, la région et la saison de la récolte (Gomez-Caravaca et *al*., 2006 ; Jacotet-Navarro et *al*., 2018 ; Wakeel et *al*., 2019).

Les propriétés anti-oxydantes des extraits et des produits végétaux ne peuvent pas être déterminées par une seule méthode en raison de la présence d'un complexe de composés phytochimiques. Il est bien connu qu'au moins deux méthodes différentes devraient être utilisées afin d'obtenir des résultats plus fiables dans le test de l'activité anti-oxydante (Du et *al*., 2009). Pour ce faire, et afin de fournir un résultat concluant du potentiel antioxydant, des extraits bruts et des fractions de feuilles et de fleurs de *C. spinosa*, deux tests adéquats couramment utilisés ont été réalisés en l'occurrence le DPPH et le FRAP.

Le test DPPH combine l'évaluation, à la fois, de la capacité de libérer de l'hydrogène et des capacités réductrices (Cheng et *al*., 2015) des extraits et des fractions étudiées. Les résultats obtenus ont montré que les extraits et les fractions de feuilles avaient un pouvoir antioxydant plus conséquent que celui des fleurs. En effet, l'extrait MeOH de feuilles a développé l'activité anti-radicalaire la plus élevée avec un pourcentage d'inhibition (PI%) estimée à 87.92 ± 0.24 % reflétant une faible concentration inhibitrice de 50 % (CI_{50}) égale à 41.04 ± 0.15 µg / mL, cette valeur est très proche de celle du témoin positif, hydroxytoluène Butylé (BHT), qui a révélé un PI% de 89.38 ± 0.15 % et une CI_{50} estimée à 34.73 ± 0.23 µg / mL, suivi de la fraction d'AcEtO de la même partie avec une CI_{50} =

45.25 ± 1.8 µg / mL témoignant de la présence d'antioxydants potentiels dans ces constituants. En revanche, les autres extraits ne développaient pas une activité anti-oxydante satisfaisante.

Nos résultats peuvent être comparés à ceux rapportés par Krimat et *al*. (2014), où l'extrait hydrométhanolique de feuilles de *C. spinosa* a montré une potentialité anti-oxydante très importante (DPPH; CI_{50} = 29.20 ± 0.8 µg / mL) en comparaison à d'autres espèces végétales testées dans les mêmes conditions expérimentales.

En outre, une étude récente de Chikhi et *al*. (2014) sur l'huile essentielle et l'extrait éthanolique (EtOH) de la partie aérienne de *C. villosa* a révélé que la plus faible capacité de piégeage des radicaux libres a été obtenue avec l'huile essentielle (60 %), alors que, l'extrait EtOH a développé une forte activité anti-oxydante estimée à 96 %, proche l'effet de l'acide ascorbique (témoin) qui a révélé une activité égale à 98.61 %.

Par ailleurs, les résultats du présent travail corroborent, en outre, ceux d'Elkhamlichi et *al*. (2017), où ils ont démontré que l'extrait MeOH de graines de *C. villosa*, présentait une excellente activité de piégeage du radical DPPH (CI_{50} = 0.20 mg / mL) approximativement proche de la valeur du témoin positif BHT (CI_{50} = 0.19 mg / mL), suivi de l'extrait d'AcEtO avec un effet satisfaisant (CI_{50} = 0.34 mg / mL).
En outre, un travail récent d'Alhage et *al*. (2018) sur l'espèce *C. villosa* a révélé que la meilleure capacité anti-radicalaire, en utilisant le test DPPH, a été développée par l'extraits MeOH et Aq, estimée à 90 % à la concentration de 0.2 mg / mL, suivie par l'extrait de dichlorométhane de tiges avec une inhibition de 90 %, à la [C] de 1 mg / mL.

Nos résultats ont été, aussi, comparés à ceux trouvés par Boughalleb et *al*. (2020) travaillant sur *C. villosa*, où, l'activité de réduction du radical libre DPPH, par le même extrait MeOH, mais, de graines (CI_{50}) estimé à 16.5 µg / mL pour les graines collectées en 2002 et à 22.0 µg / mL pour celles collectées en 2013, montrant que le stockage des graines exerce un effet positif sur l'activité anti-oxydante.

Enfin, les résultats de Turan et Mammadov (2020) sur *C. villosa* sont, également, proches de nos résultats, où le pouvoir anti-radicalaire du DPPH le plus élevé a été révélé, mais par l'extrait EtOH de fleurs avec une CI_{50} égale à 0.6 mg / mL.

Sur un autre volet, le pouvoir antioxydant des extraits et des fractions a été testé par le pouvoir réducteur du fer (FRAP) où, le composé a la capacité à libérer des électrons (Saeed et *al*., 2012). Dans le présent travail, l'extrait MeOH et la fraction d'AcEtO des feuilles semblaient constamment

être plus efficaces, exerçant un pouvoir réducteur remarquable proche de celui de témoin positif avec une CE_{50} égale à 763.73 ± 0.32 et à 780.036 ± 1.36 µg / mL, consécutivement. Ces résultats sont en accord avec ceux d'Elkhamlichi et *al*. (2017) qui a révélé un fort pouvoir réducteur chez le même extrait MeOH, mais issu des graines et des gousses de *C. villosa*. En outre, la réduction du pouvoir antioxydant (FRAP) des extraits MeOH de graines de *C. villosa* a également été évaluée, où les graines longtemps stockées (2002) présentaient le pouvoir réducteur le plus élevé (CI_{50}=59.2 ± 0.6 µg / mL), tandis que celles de 2013 exhibaient un effet moindre (CI_{50}= 99.0 ± 0.7 µg / mL) (Boughalleb et *al*., 2020).

Il est à signaler que l'activité anti-oxydante révélée par notre étude et par d'autres études permet de mettre en exergue l'importance des plantes comme source de composés actifs. En effet, de nombreuses études ont constaté que les plantes sont une source naturelle riche en composés antioxydants et par conséquent éligible à servir comme principes actifs dans plusieurs nouveaux médicaments en particulier, les anticancéreux par le développement d'effets cytotoxiques ou cytostatiques dans les lignées cellulaires cancéreuses (Shoemaker et *al*., 2005 ; Xia et *al*., 2011). En outre, les composés phénoliques tels que ; les flavonoïdes, les acides phénoliques et les tannins, possèdent d'autres activités biologiques comme ; les activités anti-inflammatoires, anti-athérosclérotiques, qui s'avéraient liées à leur activité anti-oxydante (Chung et *al.*, 1998).

Cette activité est fortement liée à la méthode et au solvant de l'extraction. En effet, l'étude de Dessi et *al*. (2001) a révélé que l'extrait MeOH de la partie aérienne de *C. villosa,* de la région de Sardaigne, présentait une efficacité dans la prévention du processus antioxydant, ces résultats sont similaires a ceux développés par notre étude concernant *C. spinosa,* expliquant que le MeOH facilite l'extraction de flavonoïdes largement connus pour leur capacité anti-oxydante (Sreeramulu et *al*., 2013).

Par ailleurs, une corrélation modérée entre la teneur en polyphénols totaux et les propriétés anti-radicalaires testées par le test DPPH a été observée (Krimat et *al*., 2014). En effet, de nombreuses études ont montré une corrélation positive entre la teneur en différents composés phénoliques des plantes et leurs capacités anti-oxydantes (Karou et *al*., 2005 ; Lamien-Meda et *al*., 2008 ; El Hajaji et *al*., 2010 ; Belmokhtar et *al*., 2014 ; Wakeel et *al*., 2019). Car la variation de la structure chimique des composés phénoliques influence significativement, leur activité anti-oxydante différente (Tatiya et *al*., 2011).

Selon d'autres études, la capacité anti-oxydante ne dépendent pas, exclusivement, de la teneur en composés phénoliques mais, elle peut être due à d'autres phytoconstituants ou à leur effet combiné (Wong et *al*., 2006 ; Ho et *al*., 2012).

Cette constatation est nettement développée dans notre étude où, l'activité anti-oxydante des extraits, en particulier, l'extrait méthanolique, est plus significatif que celle développée par les fractions.

L'activité anti-oxydante constatée chez les différents extraits et fractions de l'espèce végétale étudiée dans ce travail en l'occurrence *Calycotome spinosa* a informé de son éventuel pouvoir réactionnel avec les cellules, de ce fait, l'idée de chercher son pouvoir antimicrobien a immergé. Dans les études subséquentes, ce pouvoir sera recherché chez les cellules transformées chez l'homme.

L'activité antimicrobienne des extraits et fractions obtenus de *Calycotome spinosa* a été, largement, recherchée chez plusieurs microorganismes organotrophes pathogènes ou opportunistes. Les résultats développés ont permis d'observer que les extraits et les fractions étudiés possédaient fréquemment un effet sur la plupart des souches microbiennes testées. Cette activité diffère selon la partie de la plante utilisée, le solvant d'extraction et la souche testée.

En effet, les extraits et les fractions de feuilles étaient, généralement, plus actifs que ceux des fleurs. Distinctement, les extraits MeOH et les fractions d'AcEtO ont, souvent, montré l'effet le plus fort par rapport aux autres extraits et fractions, en revanche, les fractions chloroformiques ($CHCl_3$) des deux parties avaient une activité moins intéressante. Le potentiel antibactérien le plus remarquable a été développé par l'extrait MeOH des feuilles dans lequel une inhibition remarquable de la croissance a été obtenue contre *S. aureus* avec une ZI de 20 ± 0.28 mm à 100 mg / mL. Cependant, cette inhibition demeure moins importante que celle révélée par l'antibiotique de référence, étant donnée qu'il s'agit d'un extrait brute (Werner et *al*., 1998 ; Sanogo et *al*., 2006). L'activité antimicrobienne de cet extrait, pourrait s'expliquer par la présence de différents composés bioactifs, notamment les flavonoïdes et les acides phénoliques.

En effet, la bactérie extrêmement sensible à l'extrait était *S. aureus*, par contre, les bactéries ; *E. coli* et *P. aeruginosa* ont révélé des sensibilités moins importantes à ce composé en considérant le diamètre d'inhibition (Ponce et *al*., 2003). Comme *S. aureus* est reconnue comme contaminant alimentaire, cet extrait, de ce fait peut désarmais servir pour la prévention (Al-Zoreky et Nakahara, 2003).

Ces résultats sont étroitement similaires à ceux trouvés par Krimat et *al*. (2014), où ils ont révélé que l'extrait hydrométhanolique de *C. spinosa* s'est avéré actif contre les bactéries ; *Bacillus sp*. et *S. aureus* avec des ZIs de 7 et 10 mm, respectivement. Le même travail a constaté l'absence d'effet de cet extrait sur *E. coli* et *P. aeruginosa*. Nos résultats sont en outre, en accord avec ceux des études antérieures menées par Loy et *al*. (2001) ; Dessi et *al*. (2001) ; Chikhi et *al*. (2014) et Djeddi et *al*. (2015) sur la plante *C. villosa*. Dans ces études, il a été trouvé que les extraits ; de dichlorométhane, de MeOH et les huiles essentielles de la partie aérienne de l'espèce évoquée, ont exercé une activité antimicrobienne contre plusieurs bactéries ; *Bacillus sp.*, *S. aureus* (ZI =10 à 20 mm), *K. pneumoniae* (ZI= 11 à 20.5 ± 2.7 mm) et *Acinetobacter sp*. (ZI= 15.7 ± 1.3 mm), et l'effet moindre a été enregistré contre *E. coli* (ZI= 12.9 ± 0.9 à 15 mm), *P. aeruginosa* (ZI= 11 à 13.1 ± 2.3 mm), *S. marcescens* (ZI= 10.2 ± 0.3 mm).

D'après l'ensemble des résultats, il est à conclure que les bactéries Gram+ testées se sont, constamment, avérées plus sensibles aux extraits et fractions étudiés que les bactéries Gram-. Cette constatation est conforme à celle dégagée par les études précédentes (Pirbalouti et *al*., 2010 Nalubega et *al*., 2011 ; Madureira et *al*., 2012 ; Sulaiman et *al*., 2013) qui ont attribué les différences observées à la variation de la composition chimique de la paroi cellulaire, de la structure des deux types de micro-organismes et à la nature des composés testés. En effet, la résistance des bactéries Gram- n'est pas surprenante, elle est en relation avec la nature de leurs membranes externes (imperméable à la plupart des agents biocides) (Faucher et Avril, 2002).

Conjointement, nos résultats ont révélé une activité antifongique intéressante des quatre extraits testés, MeOH et Aq des feuilles et des fleurs contre les deux espèces d'*Alternaria* testées, par contre, aucune inhibition n'a été développée par ces extraits contre *Candida albicans* et les quatre moisissures phytopathogènes ; *Penicillium sp.1, Penicillium sp.2, Aspergillus sp. et Rhizopus sp*. Ces résultats corroborent ceux obtenus par Loy et *al*. (2001) et Dessi et *al*., (2001), qui n'ont révélé aucune activité antifongique contre *C. albicans* de l'extrait MeOH des feuilles d'une autre espèce végétale du même genre étudié dans le présent travail en l'occurrence, *C. villosa*. Cependant, l'extrait hydrométhanolique de feuilles de la même espèce a montré une activité antifongique modérée contre la même levure, *C. albicans*, avec une ZI égale à 7 mm (Krimat et *al*., 2014 ; Barhouchi et *al*., 2017).

La compréhension des mécanismes d'action des extraits des végétaux sur les microorganismes demeure un point de revue et de recherche. En effet, plusieurs travaux (Srinivasan, 2016 ; Lawal et *al*., 2017) ont rapporté que l'activité antimicrobienne de plusieurs extraits de plantes a été étudiée, *in vitro*. De ce fait, la croissance de plusieurs souches microbiennes est inhibée par diverses

concentrations de ces extraits. Les composés phytochimiques développant une activité antimicrobienne se répartissent en plusieurs groupes à savoir ; les composés phénoliques, les terpénoïdes, les huiles essentielles, les alcaloïdes et les polypeptides (Cowan, 1999 ; Savoia, 2012).

En effet, dans le présent travail, il a été constaté que les extraits ayant des teneurs en polyphénols totaux élevées agissaient positivement contre les microorganismes. Cette corrélation significative est en accord avec certaines études (Meng et *al*., 2001 ; Berahou et *al*., 2007 ; Omojate Godstime et *al*., 2014 ; Tamokou et *al*., 2017) qui ont révélé que les polyphénols et les phénols possèdent de multiples mécanismes d'action antimicrobiens par rapport aux autres groups chimiques. Ils sont capables de former un complexe irréversible avec des acides aminés nucléophiles dans les protéines, conduisant à leur inactivation et, donc, à une perte de fonction chez les microorganismes. Par ailleurs, il a été démontré que les polyphénols et les phénols perturbent les membranes microbiennes et inactivent les enzymes microbiennes (Cowan, 1999 ; Tamokou et *al*., 2017).

Il est très opportun de signaler comme fait intéressant que, la capacité anti-oxydante et le potentiel antibactérien sont positivement corrélés avec les teneurs en polyphénols totaux et en flavonoïdes totaux (Xia et *al*., 2011 ; Bubols et *al*., 2013). Cet effet a été clairement constaté l'espèce végétale étudiée (*C. spinosa*) ce qui lui confère la possibilité d'introduction dans la thérapie et la préservation des aliments.

Comme il a été précédemment, la chromatographie sur colonne de l'extrait MeOH des feuilles a révélé cinq composés différents énumérés ; (1), (2), (3), (4) et (5). Ces composés ont été identifiés, comme étant la chrysine-7-O-β-D-glucopyranoside (1), la chrysin-7-*O-β*-D- (6"-acétyl) glycopyranoside (2), l'apigénine-7-O-β-D-glucopyranoside (3), le 5-Hydroxyindoline (4) et le D-pinitol (5). Les deux derniers composés (4) et (5) sont isolés pour la première fois à partir du genre *Calycotom*e.

L'importance de l'identification des cinq composés peut être améliorée par l'élucidation de leur activité biologique. Car dans certains cas, les composés purifiés présentaient des activités biologiques. En effet, le composé 5-hydroxyindoline (4) (mis en évidence pour la première fois dans ce travail) a exercé une activité anti-oxydante très élevée en comparaison avec celle de l'extrait MeOH, ce qui permet de conclure que le principe actif du composé a subi une concentration. En revanche, le composé (5) malgré sa pureté n'a pas donné un effet antioxydant remarquable.

Par ailleurs, et dans la potentialité antimicrobienne, objectif principal de la présente étude, le composé (4) a révélé une activité antibactérienne très intéressante contre *S. aureus*, *P. aeruginosa* et *S. abony* à la concentration de [C] = 1 mg / disque, mais inferieure à celle obtenue par l'extrait brut,

MeOH des feuilles, sur les mêmes souches et à la même concentration. Cependant, aucune activité n'a été développée par le même composé (4) contre *B. subtilis*, *E. coli*, *K. pneumoniae* et *C. albicans*. De même, aucune activité antimicrobienne n'a été mise en évidence par les autres quatre composés (1), (2), (3) et (5) sur l'ensemble des microorganismes.

Il est intéressant de noter que le composé (4), 5-Hydroxyindoline, a présenté la capacité anti-oxydante la plus élevée. Cette activité a généré, en effet, son potentiel antimicrobien. En revanche le composé (5) (D-pinitol), avait un pouvoir antioxydant moindre, et par conséquent, n'a développé aucun potentiel antimicrobien. De ce fait, de nombreuses données ont démontré que les propriétés anti-oxydantes des composés des plantes médicinales jouent un rôle primordial dans la lutte contre les microorganismes pathogènes (Atmani et *al*., 2011 ; Benhammou, 2016).

À titre de discussion des composés séparés dans cette étude, il a été constaté que :

La chrysine-7-O-*β*-D-glucopyranoside (1) est un flavone glucosidé précédemment isolé à partir de plusieurs espèces végétales appartenant à des Fabacées et à d'autres familles de plantes telles que *Sarcotheca griffithi* (Muharini et *al*., 2014), *Acaccia pennata* (Kim et *al*., 2015) et *Cytisus villosus* Pourr (Larit et *al*., 2018). Il a, également, été isolé à partir de *Calycotome spinosa* (L.) Link (Larit et *al*., 2012) et de *C. villosa* Subsp. Intermedia (El Antri et *al*., 2004 ; Charkaoui-Tangi et *al*., 2008 ; Alhage et *al*., 2018). Dans cette étude, une faible activité anti-oxydante de chrysine glucoside a été observée. Nos résultats sont en accord avec ceux d'une étude précédente qui a révélé une capacité anti-oxydante moindre de ce composé isolé d'*Adenocarpus mannii* (Ndjateu et *al*., 2014). Par ailleurs, dans le présent travail, ce composé n'a révélé aucune inhibition antimicrobienne à la concentration [C] = 1 mg / disque. Cette constatation peut être expliquée par le fait que les flavones glucosides ont, généralement, une faible influence antimicrobienne (Liu et *al*., 2010). Cet fait peut être confirmé par la présence d'un fragment de glucose dans sa structure ; où, des travaux antérieurs ont montré que la présence de nombre croissant de fragments de sucre dans une structure de composé réduit son pouvoir cytotoxique (Chen et *al*., 1995 ; Wang et *al*., 2007).

L'apigénine-7-O-*β*-D-glucopyranoside (3) est un flavone glucoside isolé à partir de différentes espèces végétales en l'occurrence ; *Asystasia gangetica* (L.) (Kanchanapoom et Ruchirawat, 2007), *Cytisus multiflorus* (Pereira et *al*., 2012), *Paeonia ostii* (Zhang et *al*., 2017) et *Platycladus orientalis* (Selim et *al*., 2019). Dans l'étude actuelle, nos résultats ont révélé que l'apigénine-7-O-*β*-D-glucopyranoside (3) possède une capacité anti-oxydante appréciable sans, pour autant, développer une activité antimicrobienne à une concentration de 1 mg/disque. Ces résultats sont en accord avec ceux révélés par (Petrus et Bhuvaneshwari, 2012) qui ont montré que les

isomères du composé (3), l'apigénine-6-C-*β*-D-glucopyranoside et l'apigénine-8-C-*β*-D-glucopyranoside, sont considérés comme des meilleurs piégeurs d'ABTS $^{\cdot +}$ et d'O_2^{-}, respectivement. De plus, les résultats du présent travail sont semblables à ceux développés par une recherche préalable dans laquelle cette molécule (l'apigénine) a montré de nombreux effets biologiques légers dans un certain nombre de systèmes mammifères, à la fois *in vitro* et *in vivo*, et qui sont, principalement, liés à son effet antioxydant (Nkhili, 2009). Cependant, nos résultats ne sont pas en accord avec des autres études qui ont montré une activité antifongique remarquable de ce composé (Qudsia et *al*., 2015 ; Hamsalakshmi et *al*., 2018) et un effet bactéricide et bactériostatique très efficace (Cushnie et *al*., 2003 ; Martini et *al*., 2004).

5-Hydroxyindoline (4) (mis en évidence pour la première fois dans ce travail) est un alcaloïde indolique. Il a, déjà, été signalé comme une molécule synthétique, mais il a été obtenu, pour la première fois, en tant que produit naturel, isolé *de C. spinosa*. En effet, ce nouveau composé a montré, comme révélé précédemment, une excellente capacité anti-oxydante et un effet antibactérien très fort. Ces résultats sont en similitude avec ceux développés, pour la première fois, à partir d'une plante *Phoebe chekiangensis* en Chine qui ont constaté une forte active contre la Schizophrénie (Hegde et *al*., 1997). Une autre étude récente sur les activités des éponges marines a, également, révélé que les alcaloïdes indoliques isolés à partir des éponges *Hyrtios erectus* et *Ircinia*, tels que l'ester méthylique d'acide 5-hydroxy-1*H*-indole-3-carboxylique et l'ester éthylique de 5-hydroxy-1*H*-indole-3-glyoxylate ; avaient plusieurs activités biologiques intéressantes (Netz et Opatz, 2015 ; Abdjul, 2016 ; Abdjul et *al*., 2018).

Le D-pinitol (5), 3-O-méthyl-D-chiro-inositol (mis en évidence pour la première fois dans ce travail des plantes du genre *Calycotome*), est un composé, en général, bioactif. Les résultats de la présente étude a révélé que le D-pinitol isolé et identifié à partir de *C. spinosa*, avec une quantié abondante, ne possède ni potentiel antimicrobien ni antioxydant appréciable. En revanche, le D-pinitol isolé de *Robinia pseudoacacia* a montré une activité antifongique contre les souches phytopathogènes ; *S. fuliginea* et *E. cichoracearum* responsables de la maladie de l'oïdium (Chen et Dai, 2014). Ce composé a, également, été isolé à partir de plusieurs plantes comme *Zygophyllum melongena* (Ganbaatar et *al*., 2016) et *Rhizophora apiculata* (Lakshmi et *al*., 2006) où son effet sur le métabolisme du glucose est bien connu, par lequel il peut agir comme un agent hypoglycémiant, où sa capacité à soulager les symptômes associés au diabète a été brevetée (Dowd et Stevens, 2002 ; Kim et *al*., 2007). En outre, le D-pinitol est considéré une molécule de tolérance osmotique associée aux stress de sécheresse et de salinité chez le soja (Silvente et *al*., 2012). De même, une étude plus approfondie de (Popp et Smirnoff, 1995) a révélé que l'accumulation de D-pinitol dans les plantes

stressées est considérée comme bénéfique pour l'adaptation au stress, la stabilisation de la membrane et l'altération osmotique. Ce composé avait, également, des activités larvicides et des effets de bio-contrôle sur les insectes (Dreyer et *al*., 1979 ; Chaubal et *al*., 2005 ; Honda et *al*., 2012), chose qui n'a pas été testée dans ce travail. Par contre, l'activité anti-oxydante et antimicrobienne de l'extrait MeOH des feuilles de *C. spinosa*, en particulier de ses composés (1), (2), (3), (4) et (5) sont rapportés ici pour la première fois où une publication a été accomplie (Cherfia et *al*., 2020).

5. Conclusion et perspectives

Au terme de cette étude, il a été conclu que le criblage phytochimique, la quantification des composés phénoliques, avec les potentiels antioxydants et antimicrobiens de certains extraits et fractions de feuilles et de fleurs de *C. spinosa* (L.) Link ont abouti à des résultats intéressants. En effet, deux nouveaux composés ; le composé 5-hydroxyindoline (**4)** comme alcaloïde indole et le D-pinitol (**5**) comme cyclitol; ont été isolés pour la première fois à partir de l'extrait MeOH de feuilles de *C. spinosa*, conjointement, avec trois flavonoïdes glucosidés bien connus ; chrysine-7-O- (β-D-glucopyranoside) (**1**), chrysine-7-O-β-D (6″-acétyl) glucopyranoside **(2)** et apigénine-7-O-β-D-glucopyranoside (**3**). Le 5-hydroxyindoline s'avérait le plus actif de l'ensemble des composés obtenus dans ce travail dans les conditions expérimentales testées. Cette molécule est caractérisée par son potentiel antibactérien très significatif contre *S. aureus*, *P. aeruginosa* et *Salmonella abony*, conjointement, avec sa capacité anti-oxydante très remarquable. À la lumière de ces résultats, il a été conclu que les parties aériennes de *C. spinosa* (L.) Link, habituellement utilisées en médecine traditionnelle en Algérie, peuvent servir comme une source naturelle de molécules bioactives possédant des capacités anti-oxydantes, antimicrobiennes et, éventuellement, d'autres activités biologiques. De ce fait, la valorisation de *C. spinosa* comme source de principes actifs à utilités thérapeutique et agroalimentaire est une voie prometteuse car, cette plante sauvage occupe des superficies importantes dans le nord Algérien et son développement ne nécessite aucun entretien particulier.

Au terme de cette étude, plusieurs résultats développés peuvent servir de point de départs à des recherches futures :

- Une attention particulière sera réservée au nouveau composé bioactif le 5-hydroxyindoline (4), qui n'a jamais été décrit dans la nature, pour une étude approfondie permettant d'élucider ses mécanismes d'action et ses vertus thérapeutiques et de prévention ;

- Ouverture des voies d'exploration et d'exploitation approfondies de *C. spinosa* pour en rechercher d'autres molécules d'intérêt.

Introduction

Introduction

Nature provides an enormous treasure of natural products, with often surprising biological activities, used for centuries around the world in traditional medicine. In fact, herbal remedies are the efficient source of secondary metabolites used in traditional and modern medicines (Patil et *al*., 2014) and have played a very important role in the development of drugs (Edeoga et *al*., 2005). Recently, herbal medicines have received considerable interest and have been used as an essential part of health care since they have, relatively, few side effects compared to modern therapeutics (Cos et *al*., 2008). Besides, they represent a universal approach for the discovery of new wound healing agents, because plants are rich in a huge number of bioactive molecules (Amri et *al*., 2017).

In Africa, more than 80% of the population uses these plants to provide health care (WHO, 2007). For many centuries, in Algeria as in all Maghreb countries, medicinal and aromatic plants were mainly used in rural areas (Reguieg, 2011).
As a part of the biodiversity study of certain plant resources in Algeria, the aromatic and medicinal plants of Northeast Algeria, little valued that day, were very interested in the past (Larit et *al*., 2012), *Calycotome* is one of these neglected plants.

Calycotome, a genus belonging to the Fabaceae family, contains a number of species, mainly widespread in the Mediterranean region, in particular in northern Africa and in Spain (Brullo et *al*., 2013) such as *C. spinosa* (L.) Link, *C. infesta* (C. Presl) Guss., and *C. villosa* (Poir.) Link (Garcia-Murillo, 1999; Aymerich, 2016). *C. spinosa* (L.) Link, the studied plant in our work, is an erect and endemic spiny shrub in Algeria that can reach two meters in height, trifoliate with yellow flowers during spring; it prefers siliceous and well-watered soils (Quezel and Santa, 1963; Talavera, 1999). Although this plant is rich in bioactive metabolites, it remains little exploited for scientific research. Furthermore, the aerial part of this genus, *Calycotome*, is traditionally used as an anti-tumoral agent as well as for the treatment of furuncle, cutaneous abscess, and chilblain in Sicilian traditional medicine (Lentini et *al*., 1997; Djeddi et *al*., 2015). Likewise, an infusion of the flowers of this plant is used by the Palestinian people for the treatment of cardiovascular and nervous system disorders (Said et *al*., 2002). The foliage of this species is, also, very rich in crude protein (33.7% dried matter (DM)), making it an excellent supplement of protein. Fortunately, this genus is excessively rich in several compounds such as phenolics, flavonoids, alkaloids and anthraquinones, and tannins (Mebirouk-Boudechiche et *al*., 2015). The same, a significant antimicrobial activity of *C. villosa* extracts against pathogenic bacterial strains; *S. aureus*, *E.*

coli, B. lentus, P. aeruginosa, P. rettgeri and *M. morganii* has been revealed (Loy et *al*., 2001).

Thus, mass spectrometry is the most selective technique for rapid qualitative determination of identified compounds as well as for the identification of unknown bioactive composites from extracts of natural products (Yang et *al*., 2006; Ye et *al*., 2007). To the best of our knowledge, phytochemical characterization of *C. spinosa* (L.) Link species, using a novel approach, has not yet been reported. Moreover, limited studies on its biological activities have been performed; particularly, marginal antioxidant activity was achieved. In fact, it is well established that free radicals react with all components of DNA, thereby damaging its bases and the deoxyribose backbone (Dizdaroglu et *al*., 2012).

From this point of view, a particular interest has developed in elucidating the structure of certain compounds of this plant, useful for therapeutic and quality control needs. For that, advanced methods were used such as spectroscopic data analysis and mass spectrometry including a new approach combining 1D-NMR, 2D-NMR with LIT-ESI-MS^n.

In this context, the key focuses of the present study are:

- Phytochemical screening of *Calycotome spinosa* plant;
- Extraction of phenolic compounds and their quantification;
- *In vitro* evaluation of the antioxidant activity of the obtained extracts;
- *In vitro* antimicrobial potential of certain extracts of *C. spinosa*;
- Chemical characterization of bioactive extracts based on LC-ESI-MS/MS;
- Purification and structural elucidation of bioactive compounds using a new approach consisting of LIT-ESI-MS^n with NMR analysis;
- Separated substances have, also, been examined for their *in vitro* biological activities.

Indeed, the first part of this thesis devoted to an in-depth bibliographic review that focused on previous scientific research. This part developed research presenting, also, several sections; starting with an overview of medicinal plants in herbal medicine and their chemical composition, followed by data on the different techniques for analyzing and characterizing plant extracts and products. Then, the use of medicinal plants in Algeria and their forms of use were, further, indicated; including the presentation of the studied plant "*Calycotome*".

In the second part "materials and methods", four axes are considered; the first axis encompasses the phytochemical screening of the plant '*C. spinosa'*, the extraction of phenolic compounds, followed by the quantification of these compounds. The second axis is reserved for the various physicochemical analysis techniques carried out on certain extracts for the objective of characterization and identification, including chromatography [Thin Layer Chromatography (TLC), Column (CC) and Liquid (LC)], Tandem Mass Spectrometry (MS/MS) and NMR analyses. In the third axis, the antioxidant activity of the extracts and the separated compounds of the studied plant is additionally evaluated. Indeed, four complementary methods are applied: total antioxidant capacity (TAC), DPPH free radical scavenging activity, ferric reducing antioxidant power (FRAP), and ABTS test. The fourth and last axis is focused on the antimicrobial activity (antibacterial and antifungal) of selected extracts and separated compounds. Finally, the attained data are subjected to statistical analyses.

The third part reveals the obtained results during this study. Followed by the fourth part "discussion", whereby, all the results are discussed by comparison with the different published researches.

Finally, the manuscript ends with a conclusion, as well as the future points and the perspectives that this work brings to both medical and food applications.

Bibliographic review

1- Bibliographic review

1.1- Medicinal plants and phytotherapy

Since their existence on earth, human beings have recognized and used plants for their nutrition as well as for treating various diseases (Pelt, 2014; Pasdeloup Grenez, 2019). In recent years, the results of many pieces of research conducted by specialists (doctors, biologists, chemists, pharmacists, ethnologists, botanists, agronomists, ecologists, economists, etc.) have helped to demonstrate, on one hand, the harmful effects of synthetic drugs derived from chemicals on human health and, on other hand, the importance and the effectiveness of medicinal plants and natural products for well-human being (Ekor, 2014; Verbois, 2015).

These natural products, which are in great demand throughout the world, require more interest for their valorization by applying the results of scientific research and the appropriate techniques of production, protection, conservation, and exploitation (Sassi, 2008; Koparde, 2019).

Traditional medicine is the sum of total knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve, or treat physical and mental illnesses (WHO, 2003).

Traditional medicine that has been accepted by other populations (outside its indigenous culture) is, often, termed complementary or alternative medicine (CAM) (WHO, 2003; Gurib-Fakim, 2006). The World Health Organization (WHO) reported that 80% of the emerging world's population relies on traditional medicine for therapy. During the past decades, the developed world has, also, witnessed an ascending trend in the utilization of CAM, particularly, herbal remedies (Chintamunnee and Mahomoodally, 2012; Ekor, 2014; WHO, 2019).

The wide use of traditional medicine in Africa, mainly medicinal plants, has been argued to be linked to cultural and economic reasons. This is why the WHO encourages African member states to support and integrate traditional medical practices into their health system (WHO, 2003; Abdullahi, 2011; Mahomoodally, 2013).

Plants, typically, contain mixtures of different phytochemicals, also known as secondary metabolites that may act separately, additively, or in synergy to improve health.

Medicinal plants are the richest bioresource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs. WHO encourages traditional/herbal remedies in national health care programs because these drugs are, easily, available at low cost, safe, and people have faith in them (Pandey and Tripathi, 2014; Saranraj et *al*., 2016).

1.1.1- Obtaining and harvesting of plants

Scientific studies have helped to define the optimal harvest time of each part of the plant (Anton, 1999; Coffelt and Nakayama, 2010):

- The roots at the vegetative time (autumn or winter);
- The aerial parts, most often at the flowering period;
- The leaves, just before flowering;
- Flowers at their full bloom see in the bud (hawthorn);
- The seeds, when they have lost most of their natural moisture.

1.1.2- Conservation of medicinal plants

Medicinal plants, rarely used in their fresh state, must be stored in good conditions. However, once harvested, the plant withers and dies; degradation processes often appear damaging the therapeutic activity of plants (Paris and Hurabielle, 1980; WHO, 2018).

These degradations, of an enzymatic nature, require the presence of water. They can be avoided by various means such as:

- Desiccation, which aims to inhibit the action of enzymes by elimination of water;
- Stabilization, which aims to destroy them (Paris and Hurabielle, 1980).

1.1.3- Nature of desiccation

To ensure good conservation, to favor the inhibition of any enzymatic activity after the harvest, to avoid the degradation of certain constituents as well as the bacterial proliferation; drying appears as a primordial procedure (Anton, 1999; Anton and Wichtl, 2003). Various drying techniques can be used:

- In the sun and in the open air for bark and roots;
- Sheltered from too bright light for flowers, to avoid a change in their appearance, and sometimes their activity (essential oils).

- With a well-chosen drying temperature, because the chemical composition can vary depending on the conditions.

1.1.4- Different extraction methods

A number of medicinal plants are still used today in the form of decoctions and infusions, but most of them have been abandoned in favor of synthetic pharmaceutical products (Van Wyk and Wink, 2018). However, current knowledge allows us to analyze these plants and often the activity recommended by our ancestors. A relationship between the chemical structure and the biological activity is then tending (Kushi et *al*., 2012).

The active ingredients in a medicinal plant are chemical agents capable of activity. The presence of these components, often, in extremely small quantities in the plant requires separations, generally, delicate (Bandaranayake, 2006).

Decoction, infusion, and maceration are the separation methods widely used for the overall extraction of active ingredients and which are followed by series of chromatographic separation to reach a pure substance of an active ingredient (Nafiu et *al*., 2017).

1.1.4.1- Infusion

Perhaps the best-known preparation is an infusion. Infusion is a method of extracting the active ingredients or the aromas from a plant by dissolving it in an initially boiling liquid, which is allowed to cool for ten to twenty minutes. The term also designates drinks prepared by this method, such as herbal teas. The solvent is not necessarily water; it can also be oil or alcohol (Nogaret-Ehrhart, 2003; Handa et *al*., 2008, Nafiu et *al*., 2017).

1.1.4.2- Decoction

A decoction is another method for extracting the active ingredients from a, generally, vegetable preparation by dissolving it in an appropriate solvent (usually water), which presupposes that these substances are not thermolabile. It is mainly applied to the hardest parts of the plant; underground like roots, bark, seeds, and woods which hardly release their active ingredients during an infusion. The decoction consists of heating the element with water, until the latter is boiling, to extract the active ingredients (Nafiu et *al*., 2017).

To make a decoction, prepare the parts of the desired plants, cut and divided if necessary, and place them in a container filled with water. The whole is brought to a boil and kept at temperature for a variable time, generally between two and fifteen minutes. In the end, let cool and filter the liquid using a strainer before using it.

The decoction allows more complete extraction of the active principles than the infusion but does not apply everywhere because the temperature modifying or degrading certain active ingredients (Nogaret-Ehrhart, 2003).

1.1.4.3- Maceration

Maceration consists of soaking the dry plant in cold water or in a suitable solvent such as alcohol for several hours. A solvent is a liquid that retains the active ingredients in the plant. It is advisable to select the well solvent according to the used plant. The advantage of maceration is generally the conservation of the active ingredients during the process (Nogaret-Ehrhart, 2003). Maceration involved soaking plant materials (crude or powdered), in a covered container, with a solvent and allowed to stand at room temperature for a period, of a minimum of three days, with frequent agitation (Handa et *al*., 2008).

1.1.4.4- Other forms of use of medicinal plants

In traditional herbal medicine systems, herbal remedies are also prepared in several ways which usually vary based upon the utilized plant, and sometimes, what condition is being treated. Some of these methods, in addition to the previous three ones (infusions (hot teas), decoctions (boiled teas), and maceration), include tinctures (alcohol and water extracts), pills (honey pills), powder, tablets, syrup, poultice, compress, juices which are detailed more fully by Nafiu et *al*. (2017).

1.1.5- Selection of extraction solvent in biological activities

The biological activities of plant compounds depend on the type of solvent used in the extraction procedure, in addition to other factors (Muhamad et *al*., 2017; Abubakar and Haque, 2020).

The choice of solvent is guided by what is intended with the final extract. Since the final product will usually contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay (Ncube et *al*., 2008; Nasir et *al*., 2015; Žlabur et *al*., 2018). However, the residual solvent should be eliminated as well as possible in the oven or at a reasonable laboratory temperature, not above 40°C. The affinity of various plant metabolites to common extraction solvents has been established.

Polyphenolic compounds, such as flavonols and most other reported bioactive compounds are, generally, soluble in polar solvents such as methanol (Ncube et *al*., 2008). It was shown, in several reports, that antimicrobials are not water-soluble and thus organic

solvent extracts have been found to be more potent (Parekh et *al*., 2006; Ncube et *al*., 2008). For example, hydrosoluble flavonoids have no antimicrobial significance meanwhile water-soluble phenolics are only important as antioxidant molecules (Yamaji et *al*., 2005; Nang et *al*., 2007; Ncube et *al*., 2008). In contrast, water-soluble molecules, including polysaccharides and polypeptides (various lectins) are commonly more effective as inhibitors of pathogen adsorption and have no real impact as antimicrobial agents (Cowan, 1999).

Indeed, solvents currently used for the investigation of the antimicrobial activity of plants include methanol, ethanol, water, dichloromethane, as well as acetone (Ncube et *al*., 2008; Abubakar and Haque, 2020). Acetone, although not a usually used solvent, has been used by a number of authors (Masoko et *al*., 2007) and has been recommended by Masoko and Eloff as a good extractant of antimicrobial phytochemicals (Masoko and Eloff, 2006). Some compounds extracted by different solvents are summarized in Tab. 1.

Tab. 1 Solvents used for active components extraction (Cowan, 1999; Ncube *et al*., 2008; Sandjo *et al*., 2014; Lim et *al*., 2019).

Solvent	**Compounds extracted**
Acetone	Phenols, flavonols
Chloroform	Terpenoids, flavonoids
Ether	Terpenoids, fatty acid, coumarins, alkaloids
Ethanol	Terpenoids, polyphenols, polyacetylenes, flavonoids, tannins, alkaloids
Methanol	Terpenoids, saponins, phenones, polyphenols, flavonoids, flavones, quassinoids, lactones, anthocyanins, tannins, alkaloids, xanthoxyllines
Water	Terpenoids, polypeptides, saponins, lectins, anthocyanins, tannins, starches

Despite the development of the chemical drug industry, there are still populations who prefer to use medicinal plants to treat various ailments (Ekor, 2014). Herbal medicine is very widespread in Algerian society, and many plants and their extracts are used in traditional therapy. The use of these plants is not specific to benign diseases, but also extends to incurable illnesses.

1.2- Plants metabolites

A metabolite is an intermediate organic compound or derived from metabolism. This term is, generally, limited to small molecules. Metabolites have various functions; including

energy, structure, signaling, stimulant, and inhibitory effects on enzymes (Ncube and Van Staden, 2015; Pott et *al*., 2019).

In plants, there are two main classes of metabolites; primary and secondary.

- **Primary metabolites**

A primary metabolite is a type of metabolites that is directly involved in the normal growth, development, and reproduction of an organism or cell. This compound, generally, has a physiological function in this organism, that is to say, an intrinsic function. The primary metabolites include amino acids, lipids, carbohydrates, and nucleic acids (Bénard, 2009; De Geyter, 2012).

- **Secondary metabolites**

The term "secondary metabolite", which was probably introduced by Albrecht Kossel in 1891 (Hartmann, 2007), is used to describe a wide range of chemical compounds in plants, which are responsible for the peripheral functions, indirectly, essential to plant life. Such as intercellular communication, defense, regulation of catalytic cycles, etc (Lattanzio et *al*., 2008, Wink, 2015)

1.2.1- Importance of secondary metabolites

Unlike primary metabolites, secondary metabolites do not directly participate in the development of the organism (the plant, typically) (Lattanzio et *al*., 2008). They participate in the relationship life of the plant, and they have very varied roles (Bartwal et *al*., 2013). They can serve as a defense (bitter or toxic secretions for predators) or on the contrary, attract certain species with beneficial roles (pollinators) (Richardson, 2015). They can also allow communication between plants, by alert messages for example, or be a part of the structure of the plant (tannins and lignin) (Das et *al*., 2013).

These secondary metabolites have very important functions for the survival and the propagation of the plants that produce them, as chemical signals, to defend their producer against herbivores and pathogens, as they participate in allelopathic responses (competition between plants for germination and growth), some provide protection against solar radiation and still, others facilitate the dispersal of pollen and seeds (Jeaun et *al*., 2005; Khare et *al*., 2020).

Secondary metabolites are also widely exploited by humans in different fields: in the culinary field as colors and flavorings, in the agricultural field as herbicides, and in the medicinal field as antibiotics, antioxidants, drugs, etc. (Bruneton, 1993; Krief, 2004).

1.2.1.1- Antimicrobial secondary metabolites and their modes of action

Plant-derived compounds of therapeutic values, mostly, belong to diverse groups of secondary metabolites (Savoia, 2012). They have a wide range of activity, according to the species, the topography, as well as the climate conditions, and may contain different categories of active principles (Arruda et *al*., 2011; Assob et *al*., 2011; Savoia, 2012).

Variations in the chemical composition modify their antimicrobial activities (Matias et *al.*, 2016). Among 109 new antibacterial drugs approved in the period 1981–2006, 69% originated from natural products, and 21% of antifungal drugs were natural derivatives or compounds mimicking natural products (Osman et *al*., 2012; Savoia, 2012). Useful antimicrobial phytochemicals belong to three main groups including phenolics, terpenoids, and alkaloids (Cowan, 1999; Tamokou et *al*., 2017). The main categories of plant antimicrobials and their modes of action will be illustrated in Tab. 2.

Tab. 2 Mode of action of phytochemical groups against microorganisms (Cowan, 1999; Omojate Godstime et *al*., 2014; Tamokou et *al*., 2017).

Chemical class	**Mechanism of action**
Alkaloids	Intercalate into cell wall and DNA
Terpenes	Membrane disruption
Phenols and polyphenols	Substrate deprivation, metal ion complexation, membrane disruption, bind to adhesins, complex with cell wall, enzymes inhibition
Flavonoids	Inactivate enzymes, complex with cell wall, bind to adhesins
Quinones	Bind to adhesins, complex with cell wall, enzymes inhibition
Tannins	Bind to proteins, enzyme inhibition, substrate deprivation, complex with cell wall, metal ion complexation
Coumarins	Interaction with eucaryotic DNA

1.2.1.2- Antioxidant activity

Nowadays, there is an increasing interest in the biology of free radicals (Lobo et *al.*, 2010). This is not only due to their role in acute phenomena such as trauma or ischemia, but also to their involvement in many unremitting pathologies associated with aging such as cancer, cardiovascular and inflammatory diseases, and degeneration of the immune system (Guinebert et *al*., 2005; Khanna et *al*., 2014).

Recently, many studies have demonstrated that intracellular reactive oxygen species (ROS), such as superoxide anion radicals, hydroxyl radical (OH), and lipid peroxides play

a significant role in the pathogenesis of acute gastric damage induced by ethanol as well as many chronic diseases including diabetes mellitus and cancers (Bierl et *al*., 2006; Aprioku, 2013). The extreme reactivity of ROS and the resultant oxidative stress provoke severe damage at the cellular level leading to cell death (Xu et *al*., 2018).

Antioxidants have been demonstrated to play important roles in ROS scavenging via several mechanisms (Parke, 1999), thereby reducing the adverse outcomes of ROS-induced injury. It is therefore not surprising that epidemiological studies have demonstrated that increased consumption of fruits and vegetables is associated with reduced risks of chronic diseases as cancers, likely due to their antioxidant-rich contents including phenolic compounds (He and Xia, 2007; Willett, 2010; Al-Shwyeh et *al*., 2015).

1.2.2- Classification of secondary metabolites

Secondary metabolites fall into many groups, but there are typically three main groups: phenolic compounds, terpenes, and alkaloids (Abderrazak and Joël, 2007) (Fig. 1). Each of these classes contains a very wide variety of compounds that have a very wide range of activities in human biology (Krief, 2004).

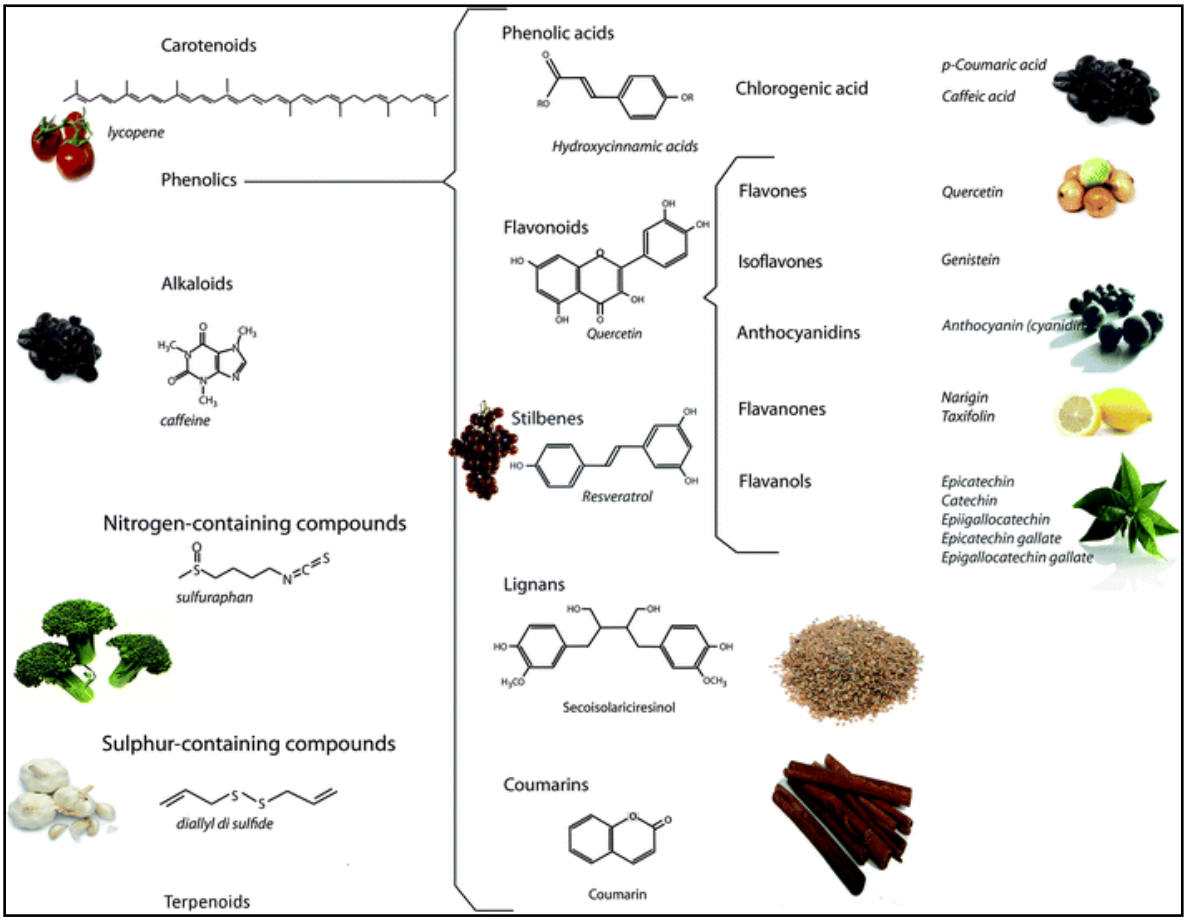


Fig. 1 Different classes of plant secondary metabolites (Nagina, 2016).

1.2.2.1- Phenolic compounds

"Phenolic compounds" or "polyphenols" are secondary metabolites of high molecular weight, widely distributed in the plant kingdom (Lin et *al*., 2016). They are aromatic molecules made up of a phenyl group (C6) and a hydroxyl (-OH) (Pereira et *al*., 2009). They are characterized, as the name suggests, by the presence of several phenolic groups associated in more or less complex structures. Phenolic acids, flavonoids, and tannins (and lignins) can be named in this family (Marchiosi et *al*., 2020). Most of these phenolic compounds are derived from aromatic amino acids: tyrosine and phenylalanine (Urquiaga and Leighton, 2000; Macheix et *al*., 2005).

- **Location of phenolic compounds in plants**

Polyphenols are secondary metabolites, widely, distributed in the plant kingdom being found in all fruits and vegetables. These compounds found in all plant parts, but with a varied quantitative distribution between the different tissues. More than 8000 phenolic structures have been identified (Urquiaga and Leighton, 2000; Waksmundzka-Hajnos and Sherma, 2011) ranging from simple molecules like phenolic acids to highly polymerized substances like tannins (Dai and Mumper, 2010).

At the cell level, phenolic compounds are mainly distributed in two compartments: the vacuoles and the wall (Ferreres et *al*., 2011). At the tissue level, the location of polyphenols is linked to their role in the plant. Even within the leaves, the distribution of compounds is variable, for example, anthocyanins and flavonoids are mainly present in the epidermis (Lister, 1994). At the level of the whole plant, it should be noted that certain compounds are only accumulated in well-defined organs. In apples, for example, phenolic compounds are involved in the coloring of the skin via anthocyanins, and in quality (relationships with bacteria, fungi, insects, UV resistance) (Julkunen-Tiitto et *al*., 2015). All phenolic compounds categories are involved in the mechanisms of resistance (Dicko et *al*., 2006). They ensure communication between cells, plants, as well as plants and animals.

- **Biosynthesis of phenolic compounds**

Polyphenols are synthesized by two biosynthetic pathways:
Shikimate pathway (Kening et *al*., 1995).
Phenylpropanoid pathway (Hoffmann et *al*., 2004).

- **Use of phenolic compounds**

Polyphenols are an integral part of human and animal food (Martin and Andriantsitohaina, 2002).

From an applied point of view, these molecules form the basis of the active ingredients found in medicinal plants, combined with their difficulty in production (Pandey et *al.*, 2009). In humans, these trace molecules play an important role in acting directly on the nutritional quality of fruits and vegetables, and their impact on the health of consumers (antioxidant effect, protective effect against the appearance of certain cancers, etc.) (Macheix et *al*, 2005; Bénard, 2009).

They are also used as additives for food, pharmaceutical, and cosmetic industries (Zillich et *al*., 2015). They are synthesized by all plants and they participate in defense reactions to different biotic stresses (pathogens, injuries, symbiosis) or abiotic (light, UV radiation, low temperature, deficiencies). Polyphenols contribute to the organoleptic quality of plant foods (color, astringency, aroma, bitterness) (Visioli et *al*., 2000).

The nature and the function of phenolic compounds accumulating in plants are variable. These defense compound groups get together different classes of compounds such as prenylated isoflavonoids, stilbenes, coumarins, flavonols, or even aurones. Other compounds have signaling functions such as salicylic acid, a signal molecule in resistance mechanisms. Injury and attack by herbivores induce the synthesis of chlorogenic acid or phenolic esters linked to cell walls, these compounds being able to act directly as defense molecules or serve as precursors to the synthesis of lignin, suberin, and other polyphenolic barriers. In addition, the quantity of anthocyanins increases sharply after cold stress or nutritional stress (Hoffmann, 2003).

Phenolic compounds play an important role in the metabolism of the plant but also can react in the interactions of plants with their biological and physical environment (Boubekri, 2014).

- **Biological activities of phenolic compounds**

Polyphenols have many biological activities *in vitro* (antibacterial, anti-carcinogenic, anti-inflammatory) related to their reducing nature and their affinity for proteins and metal ions. Polyphenols, therefore, having well-established antioxidant properties and in connection

with the inhibition of oxidation in both the food sector (lipid oxidation) and the physiological (oxidative stress).

These substances provoke a lot of interest in several fields, nutrition by their preventive character with regard to various diseases mentioned above, in cosmetology and especially in the food industries by their implications, in particular, on the flavor of food and their impact on the preservation of food products… .Thus, they could constitute an alternative to the use of synthetic food additives, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which have shown harmful effects (carcinogenic effect) (Suhaj, 2006; Morand and Milenkovic, 2014).

Phenolic compounds are more and more used in therapy (Crozier et *al*., 2010). Many studies suggest that polyphenols participate in the prevention of cardiovascular diseases (Manach et *al*., 2005). Their consumption results in a temporary increase in the antioxidant capacity of the plasma in the hours following the meal.

When they reach the arteries, they prevent the oxidation of low-density lipoproteins (LDL), which is one of the key factors in the pathophysiological process of atherosclerosis (Cheng et *al*., 2017).

Polyphenols also act by inhibiting the platelet aggregation involved in the phenomenon of thrombosis which can lead to occlusion of the arteries (Manach et *al*., 2005). They are grouped in the category of veinotonics and vasculo-protectors (Ghosh et *al*., 2009). A certain number of polyphenolic molecules are also involved in a clinical study as antiplatelet agents or hypotensive without convincing results (Martin and Andriantsitohaina, 2002). Polyphenols are associated with many physiological processes in food quality, involved when the plant is subjected to mechanical injury. The ability of a plant species to resist attacks by insects and microorganisms is often correlated with the content of phenolic compounds (Bahorun, 1997).

These compounds show antioxidant (Gomez-Caravaca et *al*., 2006; Xiuzhen et *al*., 2010), anticarcinogenic, anti-inflammatory, anti-atherogenic, antithrombotic analgesic, antibacterial, antiviral (Babar et *al*., 2007), anti-allergen, and vasodilator activities (Falleh et *al*., 2008; Hodgson, 2010).

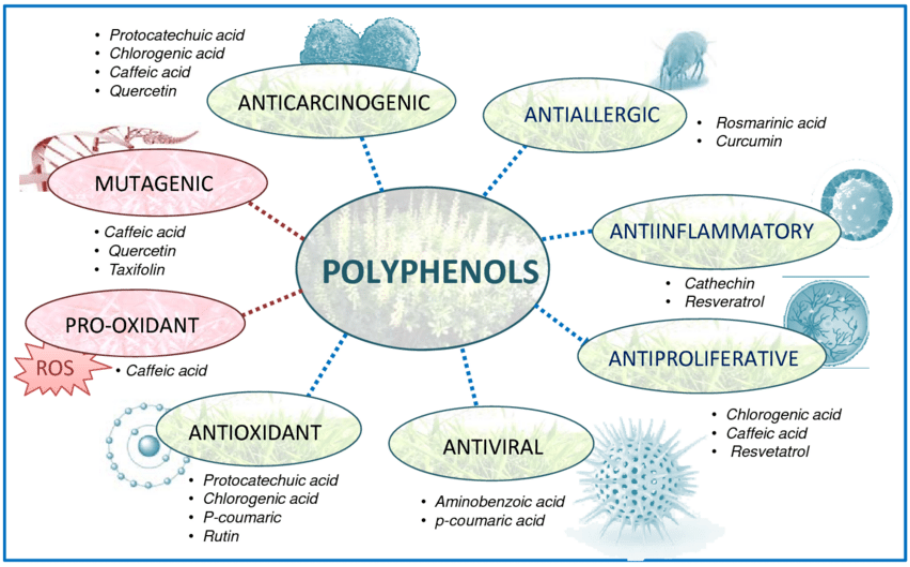


Fig. 2 Biological effects of phenolic compounds (Zitka et *al*., 2011).

- **Classification of phenolic compounds**

Polyphenols can be grouped into two main groups (Fig. 3):

Non-flavonoids, the main compounds are phenolic acids, stilbenes, lignans, lignins, and coumarins (Hoffmann, 2003).

Flavonoids, which are mainly flavones, flavanones, flavonols, isoflavones, anthocyanins, proanthocyanidins, and flavanols (Pincemail et *al*., 2007).

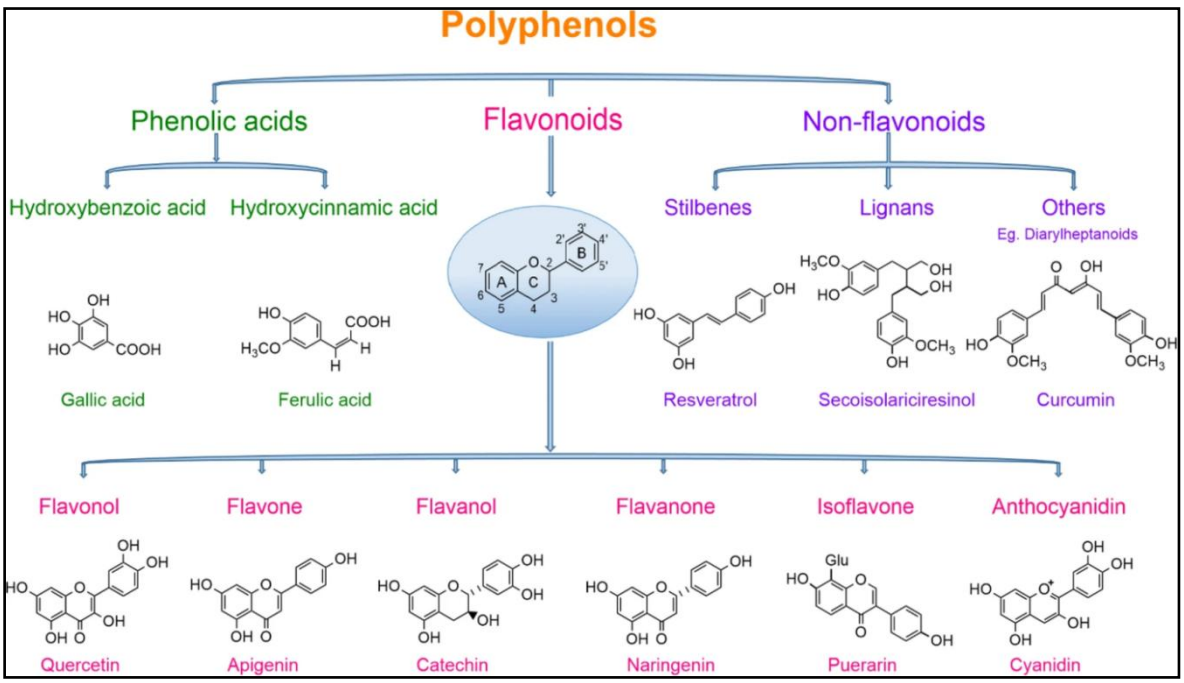


Fig. 3 Different classes of phenolic compounds (Rambaran, 2020).

Flavonoids

Flavonoids occupying a prominent place in the group of phenols, flavonoids are ubiquitous secondary metabolites of plants. It is estimated that around 2% of organic carbon photo-synthesized by plants, or some 109 tonnes per year, is converted into flavonoids (Lhuillier, 2007). They constitute a group of more than 6000 natural compounds which are almost universal in vascular plants (Panche et *al*., 2016). They constitute pigments responsible for the yellow, orange, and red colorings of different plant organs (Iwashina, 2015). More than 4,000 flavonoids have been identified in plants, and the list continues to grow. This is because of the appearance of many substitution models; the primary substituents (hydroxyl group) can themselves be substituted (glycosylated or acylated) sometimes giving very complex structures (Kumar and Pandey, 2013). The main classes of flavonoids are flavonols, flavones, flavanones, flavan-3-ols, isoflavones, and anthocyanins (Sadasivam and Thayumanavan, 2003; Chira et *al*., 2008), they vary in their structural characteristics by the functional diversity around the oxygenation of the heterocycle.

- **Etymology**

The name flavonoid would come from the term flavedo, designating the external layer of orange peel (Rivera-Cabrera et *al*., 2010), however other authors assumed that the term flavonoid was rather lent from the flavus; (flavus = yellow) (Malešev and Kuntić, 2007).

- **Chemical structure**

The flavonoids have the same basic chemical structure, they have a carbon skeleton of fifteen carbon atoms (C6-C3- C6) (Emerenciano et *al*., 2007) consisting of two aromatic rings (A) and (B) which are linked together by a C3 chain thus forming the heterocycle (C) (Oliveira et *al*., 2014), carrying free phenol, ether or glycoside functions (Narayana, 2001; Maleśev and Kuntić, 2007).

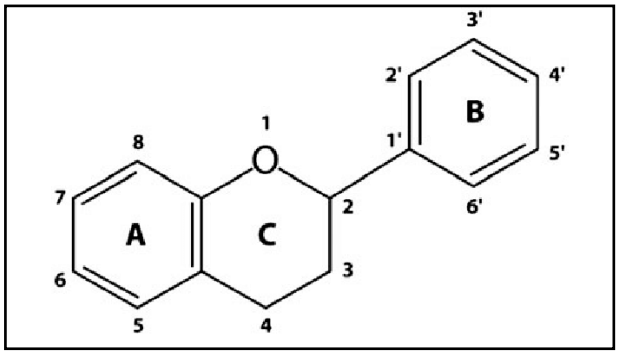


Fig. 4 Basic skeleton of flavonoids (Pal and Saha, 2013).

- **Distribution**

Flavonoids can be found in all the parts of plants. In the majority of cases, flavonoids are present in a glycosylated form in plants because glycosylation has the effect of making them less reactive and more water-soluble, allowing their storage in the vacuoles of the epidermal cells of flowers, the epidermis and the mesophyll of leaves, the parenchyma of stems and roots. Flavonoids are ubiquitous (over-answered) in photosynthetic cells and are commonly found in: fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis, and honey (Cushnie and Lamb, 2005; Le Roy et *al*., 2016).

The animal world is also concerned with flavonoids. Found for example in bees propolis (Ramos and Miranda, 2007), where bees use the antifungal and the antibacterial properties of polyphenols to sanitize their hives (Lotfy, 2006; Przybyłek and Karpiński, 2019). It should be noted that flavanones and flavones have been isolated from a marine coral and a small number of fungi (Lhuillier, 2007).

- **Biological and pharmacological effects of flavonoids**

Flavonoids are of therapeutic interest starting from the discovery of vitamin C by Szent Gyorgyi (1928), and other properties. Being ubiquitous distribution within plants, flavonoids could be at the origin of the preventive and the curative virtues of several medicinal plants (Bose et *al*., 2018). The main property initially attributed to flavonoids, is to be vasculoprotective and venotonic, because they are capable of reducing the permeability of blood capillaries and strengthening their resistance (Bruneton, 1999; Panche et *al*., 2016).

Currently, flavonoids are known for their remarkable pharmaco-biological activities such as, among others, antiviral, antimicrobial, and anticancer effects (Narayana et *al.*, 2001; Seyoum et *al.*, 2006) antiallergic, anti-inflammatory, anti-thrombotic, anti-tumor, and hepato-protectors (Middleton et *al.*, 2000). These activities are attributed in part to the antioxidant properties of these natural compounds (Panche et *al*., 2016).

1.2.2.2- Alkaloids

Alkaloids are basic nitrogen compounds that are extracted either in acidic water or in solvents like chloroform after alkalization (Jones and Kinghorn, 2006). They generally precipitate with iodometallic reagents (Dragendorff reagent) and are, very often, biologically active (Waterman et *al*., 1993). There are, indeed, molecules like quinine, drugs (cocaine), anticancer drugs (vincristine and taxol), molecules used as poisons (strychnine) and stimulants (caffeine). Most natural alkaloids are of plant origin, they are found mainly in angiosperms (Tantisewe, 1992).

- **Classification of alkaloids**

There are three central types of alkaloids (Dey et *al*., 2020):

- ✓ **True alkaloids**, which are biosynthetically derived from amino acids, and which have at least one heterocycle (example: strychnine derived from tryptophan).
- ✓ **Proto-alkaloids**, which derive from amino acids but for which nitrogen is outside the ring structures (example: colchicine).
- ✓ **Pseudo-alkaloids**, which do not derive from amino acids (example: caffeine).

- **Biological properties of alkaloids**

The main role of alkaloids is to defend the plant against mammals and insects (Nabavi et *al.*, 2020). Their mode of action depends on the plant species: some plants have alkaloids that cause neurological syndromes. These plant species have caused numerous intoxications and the death of cattle. For digital plants, digitalis causes an increase in heart contractions or even cardiac arrest depending on the dose (Hussain et *al*., 2018; Nabavi et *al.*, 2020). Thus, many alkaloids are used in pharmacies:

- Morphine is a major pain reliever.
- Codeine is used as an analgesic and cough suppressant.
- Quinine helps fight malaria.
- Atropine dilates the pupils, which facilitates ophthalmological examinations.
- Vinblastine is used in cancer chemotherapy.

Other alkaloids have more common uses such as:
- Nicotine used in the manufacture of insecticides and cigarettes.
- Cocaine is a drug with a stimulating action.

- **Antimicrobial activities of alkaloids**

Naturally occurring alkaloids are heterocyclic nitrogenous compounds; they usually display antimicrobial effects (Omulokoli et *al*., 1997). Some of them are:

Solamargine, a glycoalkaloid from the berries of *Solanum khasianum*, and other alkaloids may be useful against HIV infection (Sethi, 1983).

Halocyamine A inhibited the growth of several Gram+ bacteria, including *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, and also the yeast *Candida neoformans* with a MIC equal to 150, 50, 100, and 100 µg/ mL, respectively (Azumi et *al*., 1990). Nortopsentins A, B, and C displayed reasonable antifungal activity against *Candida albicans* (Sakemi and Sun, 1991).

Dragmacidin D introverted the growth of several Gram+ and Gram- bacteria, including *E. coli*, *B. subtilis* with MIC values of 15.6 and 3.1 µg /mL, respectively. It also inhibits the growth of several opportunistic yeasts like *Candida aeruginosa*, *C. albicans*, and *C. neoformans* at a MIC of 62.5, 15.6, and 3.9 µg /mL, correspondingly (Wright et *al*., 1992).

Reserpine showed potential antimycobacterial activity against *Mycobacterium tuberculosis*, strain H37Rv, and antioxidant activities. Reserpine displays 55% of growth inhibition of *M. tuberculosis* H37Rv (ATCC 27294) at 6.25 µg/mL concentration (Begum et *al*., 2012). Eudistomin E inhibits the growth of *E. coli* and *Penicillium atrovenetum* (Gul and Hamann, 2005).

Besides, good antibacterial and antifungal activities of alkaloids from *Epinetrum villosum* with minimal inhibitory concentration (MIC) of 31 µg /mL have been reported (Otshudi et *al*., 2005). The alkaloids from *Sida acuta* were also found to exert a good *in vitro* antibacterial activity against several pathogenic bacteria with MIC values that ranged from 16 to 400 µg /mL (Karou et *al*., 2005).

In addition, cosmoline, a bisbenzyl isoquinoline alkaloid isolated from the root bark of *E. villosum* also showed good antimicrobial activity against several microorganisms (Otshudi et *al*., 2005).

Recently, one new carbazole alkaloid, clausamine H, isolated from *Clausena anisata* leaves together with three known carbazoles, ekeberginine, girinimbine, and murrayamine A showed antibacterial properties against Gram+ and Gram- bacteria (Tatsimo et *al*., 2015).

Moreover, berberine, an isoquinoline alkaloid, was isolated from roots and stem bark of *Berberis* species, used in traditional medicine against bacterial, fungal, protozoal, and viral infections (Kim et *al*., 2002). It is active on several microorganisms, targeting RNA polymerase, gyrase, and topoisomerase IV as well as nucleic acids (Yi et *al*., 2007).

1.2.2.3- Terpenoids

Terpenes also referred to isoprenoids and their derivatives containing additional elements usually oxygen, are called terpenoids.

- **Antimicrobial activities of terpenoids**

The antimicrobial activities of some monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and their derivatives were, recently, reviewed **(**Kuete, 2013; Mahizan et *al*., 2019). The antimicrobial activity of terpenoids is rather poor compared to that of phenolics and alkaloids, though some compounds of this group were reported active against some bacteria and fungi. Herein some antimicrobial terpenoids:

Two known sesquiterpene lactones; vernolide and vernodalol, isolated from *Vernonia amygdalina* displayed good antimicrobial activities against several bacterial and fungal species (Erasto et *al*., 2006).

Besides, two clerodane diterpenoids isolated from the Cameroonian plant, *Microglossa angolensis* as well as spinasterol also displayed antifungal and antibacterial activities in a MIC range of 1.56–100 µg/mL (Tamokou et *al*., 2009).

The mechanism of action of terpenoids is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Naili et *al*., 2010; Termentzi et *al*., 2011; Mahizan et *al*., 2019).

1.3- Analysis and characterization techniques of plants metabolites

1.3.1- Spectrophotometric assays

The increase in the use of herbal preparations in the medical field, in particular, requires the development of analytical techniques allowing the quantitative determination of the active constituents (Upton et *al*., 2019). Thus, spectrophotometric assays, simple and rapid to implement, are specially adapted for the routine control of the plants. They relate to total polyphenols and total flavonoids (Woisky and Salatino, 1998). Thus, (Popova et *al*., 2004) have shown that the quantification of compounds' groups with similar chemical structures

correlates better with the observed biological activities. It, therefore, provides more information than the individual quantification of compounds. These methods are, moreover, simple to set up, offer good repeatability, and have acceptable precision.

1.3.1.1- Determinations of total polyphenols

The most commonly used method for determining the level of total polyphenols is that of Folin-Ciocalteu (Folin and Ciocalteu, 1929; Singleton and Rossi, 1965). Despite the sensitivity and the simplicity of the method, it is not, strictly specific for polyphenols. Indeed, the Folin-Ciocalteu reagent evaluates the total reducing capacity of the whole sample. It is therefore important to know globally, what are the classes of molecules present in the sample to be analyzed for a good estimate of the total polyphenols by this method.

1.3.1.2- Determination of total flavonoids

While the Folin-Ciocalteu method is commonly accepted for determining the content of total polyphenols, there is no single method for the determination of total flavonoids and several methods are proposed. Woisky and Salatino (1998) described a method in which aluminum chloride ($AlCl_3$) formed yellow complexes with flavonoids by measuring absorbance at 420 nm and using quercetin as standard. This method was then modified, and only flavones and flavonols were evaluated by complexation with $AlCl_3$ while 2,4-dinitrophenylhydrazine (DNP) was used to estimate flavanones and dihydroflavonols (Chang et *al*., 2002; Marghitas et *al*., 2009). These methods are fast; inexpensive and do not require the use of many analytical standards; they are therefore widely used in the determination of flavonoid levels in fruits, vegetables, cereals, and medicinal plants.

1.3.2- Separation techniques

1.3.2.1- Chromatographic techniques

Several chromatographic techniques are used for the analysis and the isolation of the compounds contained in plants. They can be coupled to a UV detector, most often of the diode array detector (DAD) type, but also to a mass spectrometer.

- **Thin layer chromatography**

Thin layer chromatography (TLC) is, generally, used to compare multiple plant samples, or different extracts or fractions from the same plant (Santos et *al*., 2002). Certain biological activities such as antifungal and antibacterial activities can be directly determined on TLC plates (Sawaya et *al*., 2002; Raghukumar et *al*., 2010), the compounds responsible for

these activities can be purified by preparative TLC and identified by other means. However, the use of TLC for the characterization of the plant is quite limited, since the number of constituents is often high and commercial standards not always available (Sawaya et *al*., 2011).

- **Column chromatography**

Open column chromatography (CC) was used to isolate many chemical compounds, and more recently, to isolate the triterpenes, isoflavones, benzophenones, and phenolic compounds contained in the plants (Tao et *al*., 2009; Luo et *al*., 2012; Shah et *al*., 2014; He et *al*., 2015). This makes it possible to obtain, from the complex, fractions containing a reduced number of compounds and to be able to link these to biological activities according to the bio-guided fractionation process (Ujang et *al*., 2013).

- **Gas chromatography**

Gas chromatography coupled with mass spectrometry (GC / MS) is a technique particularly suitable for the analysis of essential oils or volatile compounds contained in plants (Marriott et *al*., 2011; Al-Rubaye et *al*., 2017). But most of the time, to be able to analyze the more polar compounds that are found in alcoholic extracts, it is necessary to derive them with bis (trimethylsilyl) trifluoroacetamide (BSTFA) in order to make these compounds, which are then found in the form of more volatile mono, di and tri-trimethylsilylated compounds (Proestos et *al*., 2006; Alrabie et *al*., 2019); the GC / MS spectra banks available to facilitate the identification of the products (Horai et *al*., 2010).

- **High performance liquid chromatography**

High performance liquid chromatography (HPLC) is the most used technique for the analysis and the characterization of phenolic compounds in plant extracts (Gomez-Caravaca et *al*., 2006) because it presents a high resolution, a high reproducibility, and relatively a short analysis duration (Wollgast and Anklam, 2000). It can be used for the separation, the quantitative determination, and the identification of polyphenols. Usually used in reversed-phase, it would have three essential points: the column, the elution solvent, and the detector (Wollgast and Anklam, 2000; Stalikas, 2007).

Since plants' extracts are very often soluble in methanol, ethanol, or in hydro-alcoholic solutions, reversed-phase in HPLC has become the most used chromatographic method for the analysis of plants compounds, as shown by numerous publications in the field (Yabré et *al*., 2018; Zhang et *al*., 2018). Indeed, phenolic compounds absorbing in UV can easily

be detected by a diode array detector (DAD) in order to obtain their absorption profiles (Zhang et *al*., 2013). HPLC is also often coupled to mass spectrometry (LC / MS^n), the most common mode of ionization being electrospray ionization (ESI) (Ho et *al*., 2003).

1.3.3- Identification techniques

1.3.3.1- Direct-injection mass spectrometry

Mass spectrometry by direct injection (or infusion) can be used as a rapid screening method, capable of characterizing samples of plants from different geographic origins (and therefore of different compositions) (Rodrigues et *al*., 2007). The majority of the compounds of interest contain phenolic groups, so it is the electrospray ionization in negative mode $[ESI\text{-}MS]^-$ which gives the best answers, as shown by Liigand et *al*. (2017) in their study. Direct injection mass spectrometry is also used to determine the molecular mass of a purified compound, in particular thanks to the high resolution (HRESI-MS in the case of electrospray ionization). Another source of ionization was also used by Pietta et *al*., (2002); atmospheric pressure chemical ionization (APCI), to also compare several samples (Huang et al., 2015).

1.3.3.2- Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) is a non-destructive technique capable of giving information on the chemical structure of compounds. It is generally used for a purified compound in order to obtain its precise structure using the 1-D NMR (^1H and ^{13}C) and 2D-spectra (COSY, NOESY, HMBC, HMQC, etc.). It is essential in the case of the identification of new compounds. For the first time, Bertelli et *al.* (2012) used ^1H-NMR to identify, directly, phenolic and flavonoid compounds contained in extracts.

Nuclear magnetic resonance spectroscopy exploits the magnetic properties of atomic nuclei. This technique is widely used for structural characterization and composition analysis of natural compounds. ^1H and ^{13}C-NMR measurements can be performed to distinguish and quantify aromatics and aliphatics (Oliviero Rossi et *al*., 2018). Both one-dimensional (1D) and two-dimensional (2D) NMR experiments can be performed to characterize bio-oil (Hao et *al*., 2016). In addition to ^1H and ^{13}C-NMR, other active nuclei such as ^{31}P and ^{19}F can be used.

1.4- Aromatic and medicinal plants in Algeria

With an area of 2 381 741 km^2, Algeria is the largest country bordering the Mediterranean. This country is recognized for its diversity of medicinal and aromatic plants, as well as its

various popular uses throughout the country (Blama and Mamine, 2013; Foi, 2014). The use of these plants is an ancestral knowledge transmitted from generation to generation among populations, most often, rural (Derridj et *al*., 2009; Lakel and Zermani, 2017).

In the Hoggar (Great Desert) and in the absence of doctors, in certain isolated regions, the Tuaregs take care of themselves with medicinal and aromatic plants of which they know the secret transmitted from father to son (Reguieg, 2011). In Kabylia, when there is snow and the roads are cut, mountain people use medicinal and aromatic plants to cure themselves (fumigation of eucalyptus leaves against influenza). In the steppe during transhumance, nomads use white sagebrush (*Artemisia ludoviciana*) to fight indigestion (Fig. 5) (Mokkedem, 2004 ; Sahi, 2016).

Thymus vulgaris L., *Zingiber officinale Roscoe*, *Trigonella foenum-graecum* L., *Rosmarinus officinalis* L., and *Ruta chalepensis* L. (Fig. 5) were the most frequent species used by local healers in South-west Algeria to treat kidney, cancer, digestive, and respiratory diseases (Benarba, 2016).

The richness of the Algerian flora is therefore indisputable; it conceals a large number of species classified according to their degree of rarity: 289 fairly rare species, 647 rare species, 640 very rare species, 35 extremely rare species, and 168 endemic species (FAO, 2012).

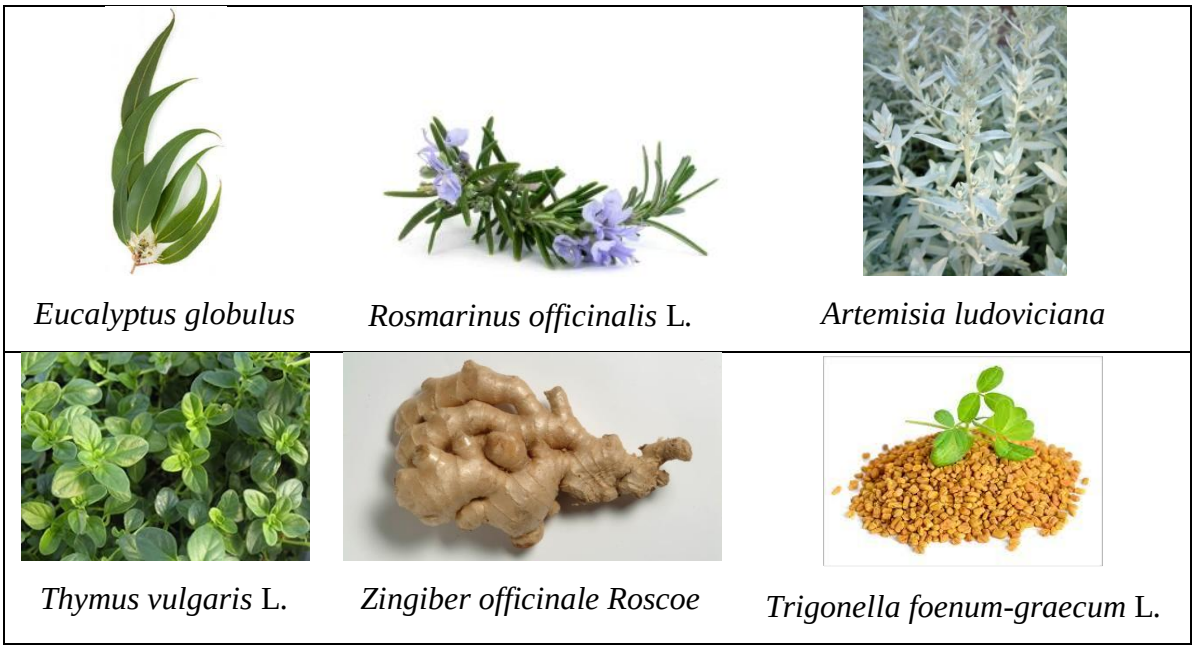


Fig. 5 Aromatic and medicinal plants mostly used in everyday life.

1.4.1- Plant parts mostly used

The parts used are mainly leaves, fruits, flowers, and roots. For trees and shrubs, the bark is also used for the preparation of recipes.

Recently, Benderradji et *al*. (2015) demonstrated that in Southeast Algeria, leaves were the most commonly used parts in the treatment of different ailments. Furthermore, the results of the surveys conducted by Benarba (2016) and Hamel et *al*. (2018) showed that the leaf is the most used part of the medicinal plants in Algeria (36-62.6%), followed by the aerial parts and stems (17-19.8%), then seeds (18%) against 8.2-12% for the roots. All bulbs, rhizomes, barks, and resins have a cumulative percentage of 9.4%.

1.4.2- Different forms of use of medicinal plants in Algeria

Several forms of employment have been identified in Algeria, mainly decoction and infusion (Reguieg, 2011). Benarba (2016) found that a decoction was the major mode of plant preparation (49%) in the South-West of Algeria. In addition, different medicinal plants are used as raw (32%), infused (16%), or macerated (3%). Besides; Oral, topical, inhalation, and nasal routes were the reported ways of administration in the study area.

Benarba (2016) has, furthermore, reported that the most herbal remedies in South-west Algeria were administered orally (80%) and out of the 83 cited plants, 45 species are administered with other ingredients such as other plants (66%) or non-plant-adjuvants (34%) such as olive oil, honey, milk, sugar, yogurt, or eggs. Honey is the adjuvant most added to different herbal remedies in the South-west of Algeria (53%).

However, the infusion is the most frequent (42.5%) in Algeria Tell, then comes the decoction with 40%, and the powder with 20% (Hamel et *al*., 2018). The majority of remedies to treat gastric, cardiovascular, and urogenital diseases are prepared mainly by infusion (Daoudi et *al*., 2015). Essential oils and fumigation are the least used forms by residents of the Edough Peninsula (Hamel et *al*., 2018).

1.4.3- Therapeutic use of plants in Algeria

Regarding the treated ailments, 35 species are reported to be used to treat more than one disease in the South-west of Algeria. According to Benarba (2016), gastrointestinal disorders were the most commonly treated ailments with medicinal plants in South-west Algeria (33.6%), followed by respiratory diseases (23%) and cardiovascular diseases (9%).

Traditionally, plant species from the Algerian Tell have also been used to treat a wide variety of symptoms. They are used as a diuretic, an astringent, an anti-inflammatory, and in the treatment of injuries, rheumatism, skin and respiratory diseases, fever, and pain (Daoudi et *al*., 2015; Hamel et *al*., 2018).

1.5- *Calycotome* genus

Calicotome (or *Calycotome*), from Greek *Calyx* and *Temno* which signify calyx (kalux) and cut respectively, alludes to the calyx which breaks circularly and seems cut after flowering (Guide Illustré de la Flore Algérienne 'GIFA', 2009). The genus *Calicotome* was created by Link.

The word "*Calicotome*" was spelled with "y" in the old flora (which is etymologically logical: the Greek "u" giving "y" in French), but in the new flora, it takes an "i"**.**

1.5.1- Systematic and Botanical description of the genus *Calycotome*

The classification of the genus *Calycotome* has experienced many changes and divisions over time (Lattanzi, 2008), the systematic of it is currently presented as the following (Guaâdaoui et *al*., 2016):

Kingdom: Plants (Plantae)
Subkingdom: Vascular Plants (Tracheobionta)
Superdivision: Spermaphytes (Spermatophyta)
Division: Angiosperms
Class: Edudicotyledons (Eudicots)
Subclass: True Rosidae I
Order: Fabales
Family: Fabaceae (= Papilionaceae / Leguminosae)
Subfamily: Faboideae Rudd (Papilionoideae Juss)
Genus: *Calicotome* Link

The genus *Calycotome* belongs to the Fabaceae family. This latter constitutes the third most important family in the plant kingdom (around 16,000 species) after Asteraceae and Orchidaceae. Calycotomes are very thorny shrubs.

According to (Quèzel and Santa, 1963; García Murillo, 1999; Talavera et *al*., 1999), the table below englobes the different characteristics of this genus:

Tab. 3 Botanical description of the genus *Calycotome* (Quèzel and Santa, 1963; Chikhi, 2014).

Plant part	Description
Stems	They are slender and spread apart, forming bushes that can reach 2 meters in height. Their branches are green, then brown as they mature, and end in thorns.
Leaves	They are composed of three oval leaflets. They are small, few, and obsolete.
Flowers	They appear at the end of winter. They are yellow, grouped, and very numerous. The flower including the ovoid calyx, crowned by 5 small teeth, completely closed in the bud and breaking circularly in the middle at the time of flowering.
Fruits	They are pods, they bear two flattened pieces of wool, three to four cm long, compressed, with enlarged ventral suture and tightly winged on each side, containing three to eight seeds, not caruncles.
Seed	It has a brown or reddish-brown color, smooth, shiny, rounded, about 3 mm in diameter.
Roots	They usually carry nodules containing bacteria allowing the fixing of atmospheric nitrogen.

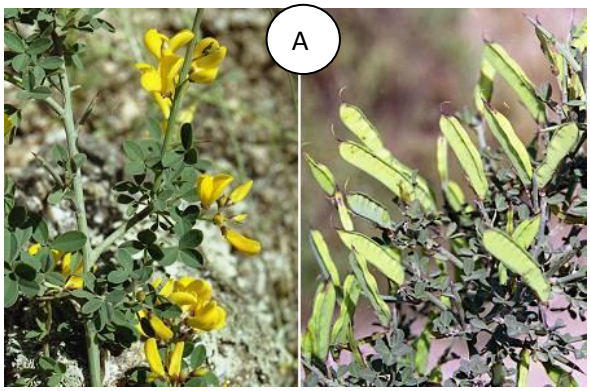
1.5.2- Origin and geographical distribution of the genus "*Calycotome* "

Calycotome genus, native to the Mediterranean region, was probably introduced in Australia as an ornamental or hedge plant (GIFA, 2009). The *Calycotome* prefers siliceous soils. When it is found in the scrubland, it is a sign of certain poverty in limestone. It is found in maritime pine forests, in the cork oak forests, and the maquis, which helps to make it difficult to penetrate. *Calycotome* lives in the mountains close to the coast in North Africa and southern Spain (namely the north of Algeria and Libya, Italy, France, and Spain) (Quèzel and Santa, 1963; Domínguez, 1987).

1.5.3- Species of the genus *Calycotome* (*C. spinosa* and *C. villosa*)

Two main Calycotomes, namely *C. villosa* and *C. spinosa*, were distinguished by Rameau et *al.* (2008) whose botanical description is shown herein Tab. 4.

Tab. 4 Morphological characteristics differentiating the two species (A) *C. spinosa* and (B) *C. villosa* (Loi et *al*., 2004; Rameau et *al.*, 2008; GIFA, 2009)

Plant		
Origin of the name	The name "spinosa" means thorns; it refers to the thorns that the plant carries on the branches	The name "villosa" refers to the hairs that cover all the plant parts. The common name also refers to these hairs
Branches	Thorny, striated, divaricate, glabrous or glabrescent	Branches and thorns more furrows and more or less pubescent
Leaves	Trifoliate (leaflets more or less folded lengthwise)	Blackening little at desiccation
Flowers	Solitary or gathered 2-4 Calyx: silky hairy, truncated/ standard hairless	More numerous (6-15)
Pods	Long, hairless, shiny, and black at maturity.	Subquadrangular, very hairy, reddish at maturity / Sutures all winged
Fruits	Seeds (4-8) yellowish-brown	Seeds
Keel	Curved slightly inwards pubescent	Wider, almost obtuse
Other names	Thorny Calycotome (spinosus)	Hairy Calicotome (villosus)
	- Argelus (vulgar name) in French - Thorny Broom or Spiny Broom in English - *Spartium spinosum* L. in Spanish - Guendoul in Arabic	- Teria (vulgar name) in Italy - *Sparzio villoso* in Italy - *Spartium villosum* in Spanish - Guendoul in Arabic

1.5.4- Phytochemistry of the genus *Calycotome*

This subsection focuses on phytochemicals isolated from the genus *Calycotome*. The chemical composition of plants varies with the location, the variety, the plant part, and the stage of maturity (Luo et *al*., 2012).

1.5.4.1- Phenolic compounds

- **Flavonoids**

Many flavonoids have been isolated from the genus *Calycotome*. Some of them are illustrated in Fig.6.

The major flavonoid constituent of *C. spinosa* was found to be Chrysin, a flavone aglycone. The plant was also reported to contain a flavone glucoside Chrysin 7-*O*-*β*-D-glucopyranoside isolated from EtOAc fraction of leaves and flowers (Larit et *al*., 2012).

However, a huge number of flavonoids were detected, isolated, and identified in *C. villosa* species as; chrysin, genistein, luteolin, apigenin, orientin, kaempferol, butein, calythropsin, and their derivatives in its different parts (Loy et *al*., 2001; Pistelli et *al*., 2003; El Antri et *al*., 2004a; El Antri et *al*., 2010, Alhage et *al*., 2018).

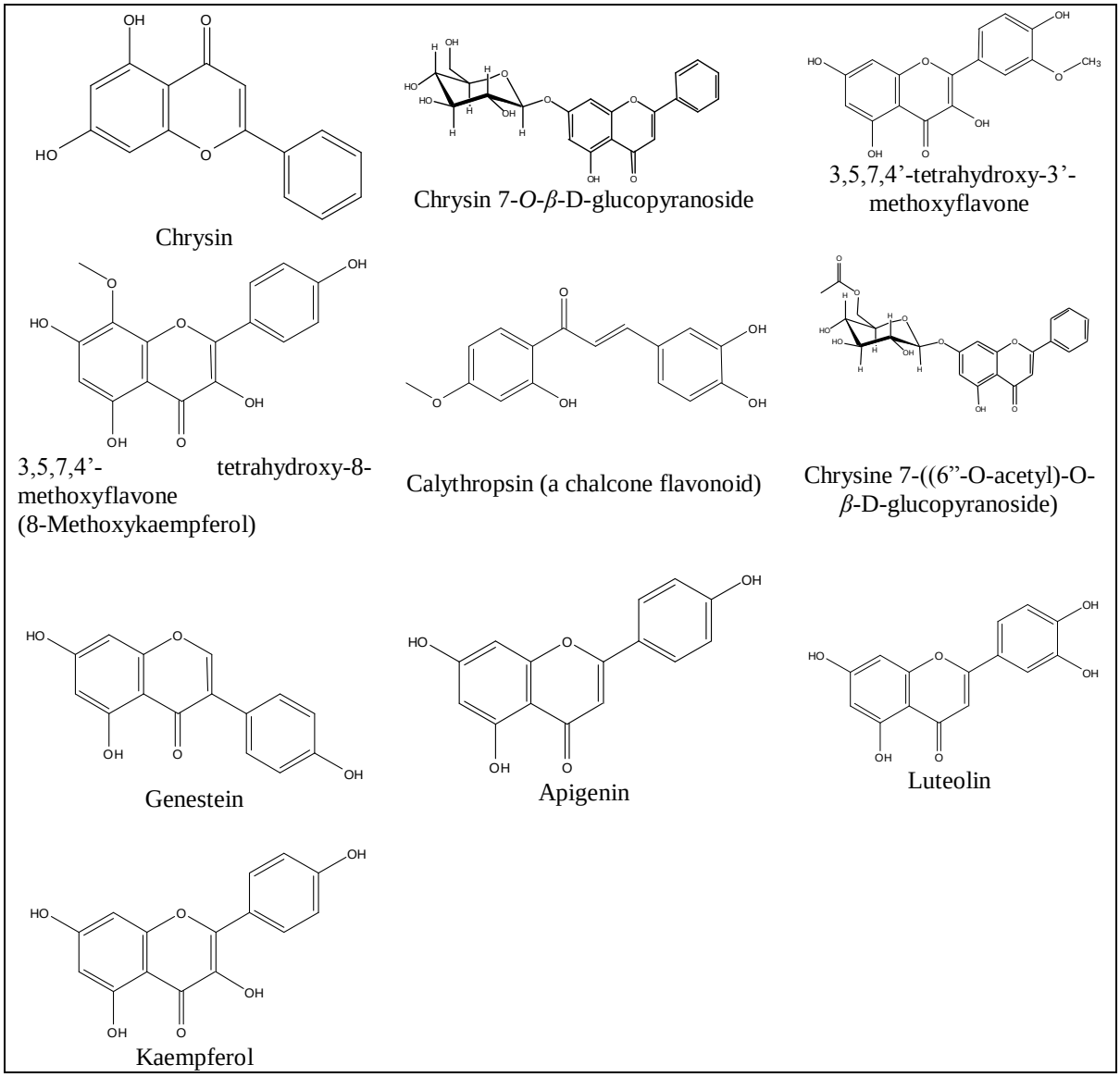


Fig. 6 Structures of some flavonoids isolated from the genus *Calycotome*.

- **Phenolic acids**

Regarding the phenolic acids of the genus *Calycotome* **(**Fig. 7), there is a cinnamic acid that was isolated from *C. spinosa* (Larit et *al*., 2012). In contrast, other phenolic acids were detected and isolated from *C. villosa* including; quinic acid that was the most abundant compound, while protocatechuic acid, syringic acid, *p*-coumaric acid, trans-ferulic acid, and vanillic acid were detected at a low amount (Boughalleb et *al*., 2020; Turan and Mammadov, 2020).

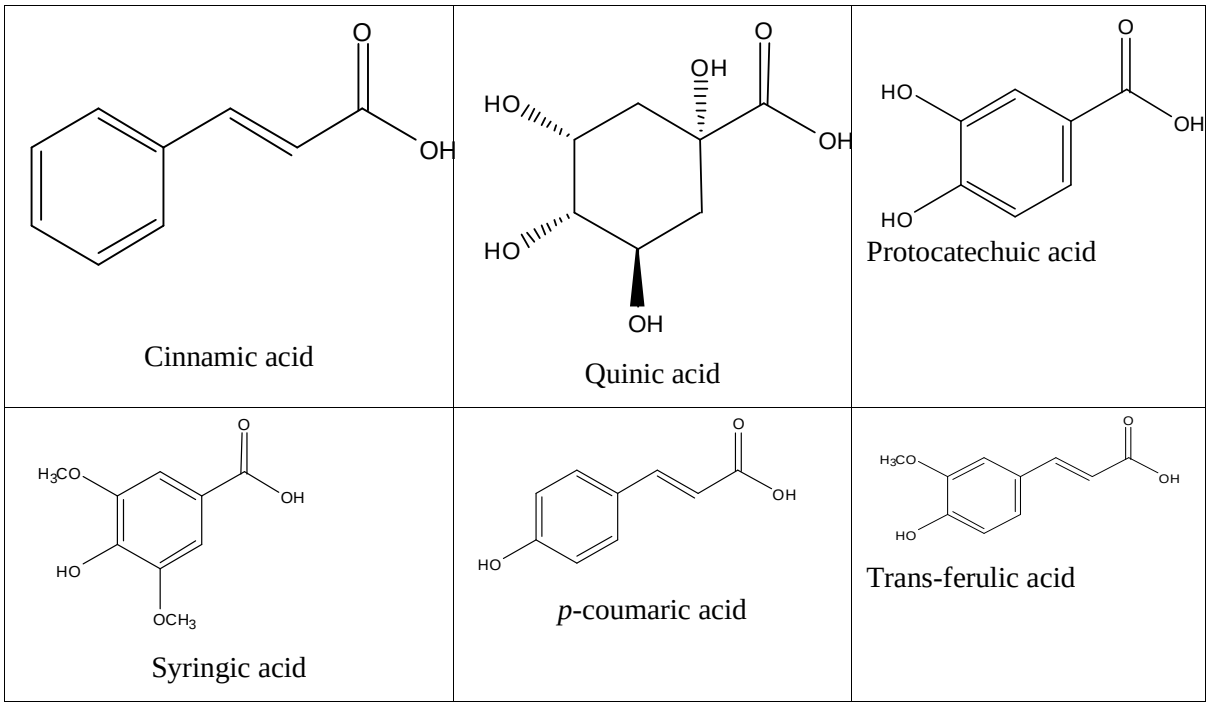


Fig. 7 Structures of some phenolic acids isolated from the genus *Calycotome*.

1.5.4.2- Alkaloids

In addition, no study has reported the isolation of alkaloids from the species *C. spinosa*, however, many alkaloids have been identified from the species *C. villosa* as; lupinine, calycotomine, sparteine, anagyrine, lupanine, and their derivatives (Loy et *al*., 2001). In addition, two tetrahydroisoquinoline alkaloids were obtained from a MeOH extract of *C. villosa* Subsp. Intermedia seeds (El Antri et *al*., 2004b)

Elkhamlichi et *al*., (2014) have, also, reported that 1-hydroxymethyl-6,7-dimethoxy-3,4-dihydroisoquinoline-N-oxide and a paraben derivative named methyl-4-hydroxybenzoate (9) have, further, been isolated from *C. villosa* Subsp. *intermedia* seeds.

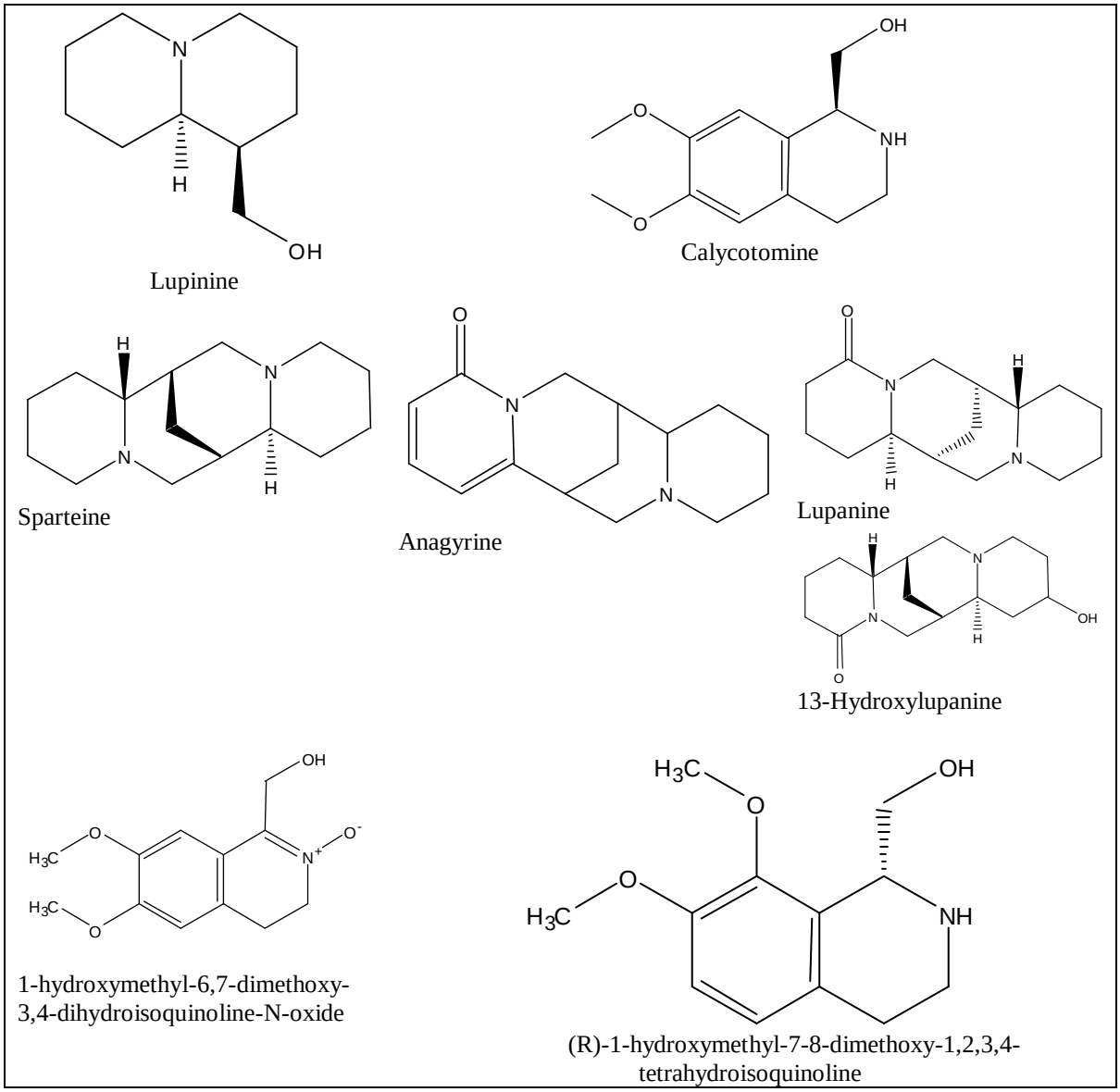


Fig. 8 Structures of selected alkaloids isolated from *Calycotome villosa*.

1.5.4.3- Essential oils and terpenoids

Phytochemically, no studies have been reported on essential oils (EO) of the species *C. spinosa*. In contrast, the essential oil of *C. villosa* species, according to Dessi et *al*. (2001) contained 1,8-cineole, fenchone, camphor, eugenol, (Z)-isoeugenol, β-eudesmol, and others.

Furthermore, the vomifoliol is the only sesquiterpenoid isolated from the leaves and the flowers of *C. spinosa* (Larit et *al*., 2012). In contrast, the seeds of the species *C. villosa* contained a lupeol as a triterpene (Pistelli et *al*., 2003), and two steroids; *β*-sitosterol and stigmasterol (Fig. 9) were isolated from *C. villosa* stems with a phenylethanoid called basalethanoid B (Alhage et *al*., 2018).

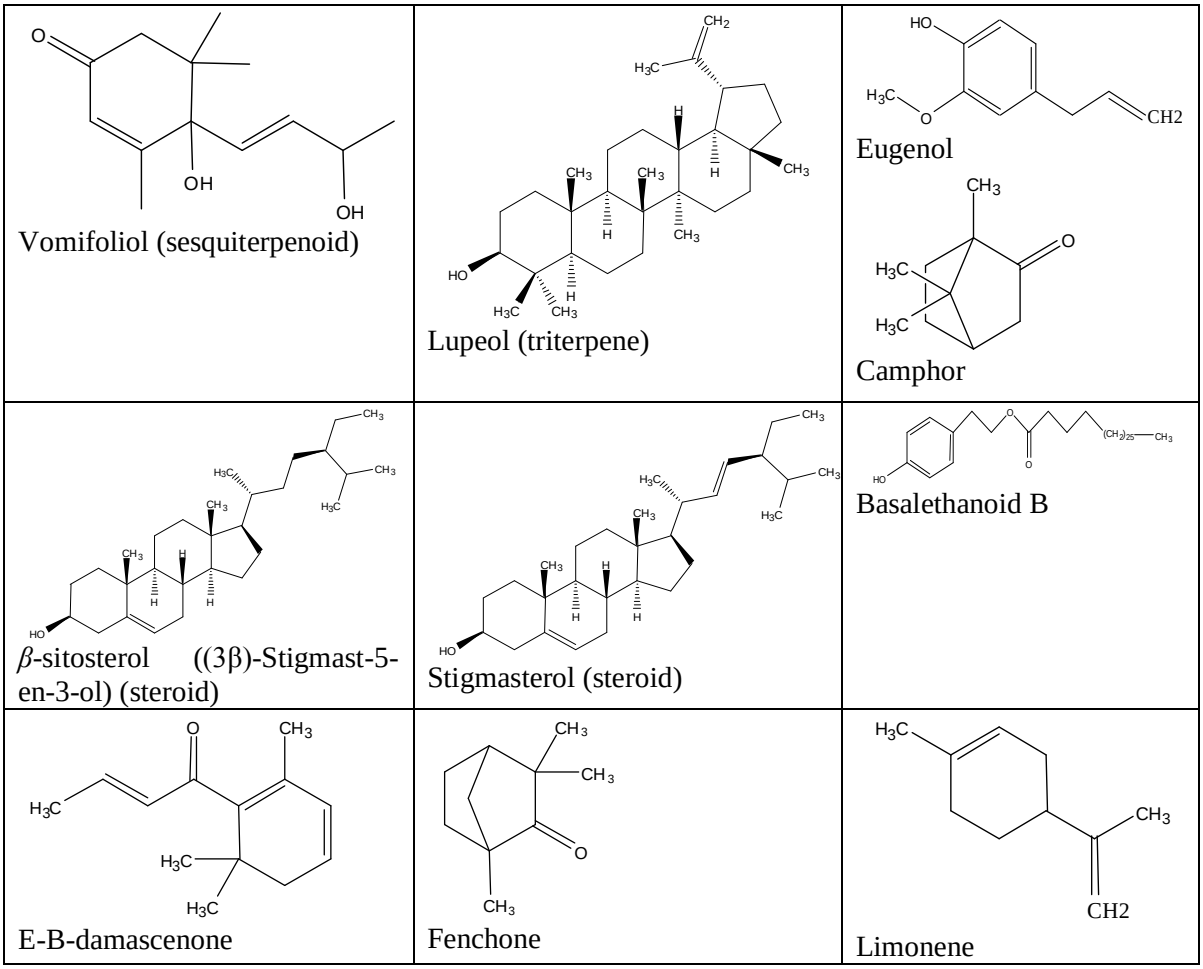


Fig. 9 Structures of some selected essential oils and terpenes isolated from the genus *Calycotome.*

1.5.4.4- Other constituents

Other phytochemical constituents (Fig. 10) were, additionally, identified in *C. villosa* different parts as; fatty alcohol (falcarinol), fatty acids (pentadecanoic acid, hexadecanoic acid, dodecanoic acid, nonanoic acid, and octadecanoic acid), coumaran (dihydrobenzofuran), and pterocarpans (Loy et *al*., 2001; Chikhi et *al*., 2014; Palu et *al*., 2020).

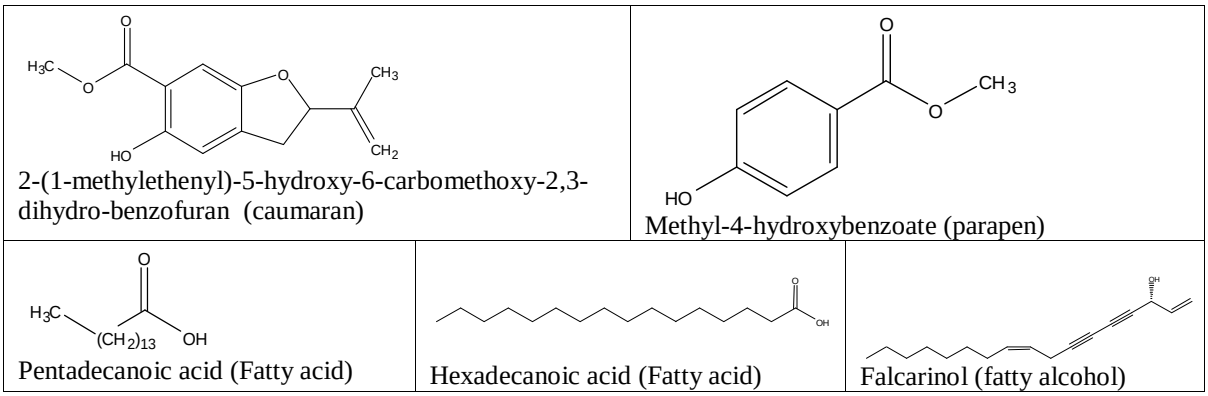


Fig. 10 Structures of some other constituents reported from the genus *Calycotome*.

1.5.5- Pharmacology of *Calycotome* genus

Although several pharmacological properties of the genus *Calycotome* have been reported including antioxidant, antibacterial, antifungal, anti-inflammatory, antidiabetic, hypotensive, diuretic, vasodilatation, cytotoxic, larvicidal, and anthelmintic activities; limited biological studies were carried out on *C. spinosa* plant.

1.5.5.1- Antioxidant activity

The only study that evaluated the antioxidant activity of *C. spinosa* species reported that the hydromethanolic extract of *C. spinosa* (L.) Link leaves exhibited an important antioxidant capacity (Krimat et *al*., 2014). However, many other antioxidant investigations were conducted on *Calycotome villosa* species. It was reported that the methanol extract of *C. villosa* from the Sardinia region exhibited efficiency in preventing the antioxidant process (Dessi et *al*., 2001). Besides, a recent study by Chikhi et *al*. (2014) on the essential oil and the ethanol extract of the aerial part of *C. villosa* showed that the ethanol extract had an excellent antioxidant activity using the DPPH test.

As well, three different antioxidant methods (TAC, DPPH, and FRAP) were used to test the antioxidant properties of *C. villosa* seeds (Elkhamlichi et *al*., 2017; Boughalleb et *al*., 2020). They have observed that the methanol and the ethyl acetate extracts have shown a remarkable antioxidant capacity, and the antioxidant capacity was slightly increased with the storage duration of seeds. A further work of Alhage et *al*. (2018) on the same species, *C. villosa*, has revealed that the best scavenging ability using the DPPH test was shown by the methanolic extract of stems.

Turan and Mammadov (2020) have, further, reported that the ethanol extract of *C. villosa* flowers exhibited higher antioxidant activity than the stem extract in all using assays; DPPH and β-carotene.

1.5.5.2- Antimicrobial activities

A significant antimicrobial activity of the genus *Calycotome* has also been carried out, in particular, in *C. villosa* species. In which various extracts (chloroform, methanol, ethanol, dichloromethane, and essential oil) from its aerial parts developed an antimicrobial activity towards several microorganisms as follows; *S. aureus*, *B. lentus*, *E. coli*, *P. aeruginosa*, *Providencia rettgeri*, *Morganella morganii*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Cryptococcus neoformans*, and *Acinetobacter sp.* (Loy et *al*., 2001; Dessi et *al*., 2001; Chikhi et *al*., 2014; Djeddi et *al*., 2015). In contrast, little researches have been

performed on the species *C. spinosa*. Indeed, a study conducted by Krimat et *al*. (2014) revealed that the hydromethanolic extract of the leaves of the species *C. spinosa* showed good antibacterial activities against *B. subtilis* and *S. aureus*, and antifungal activity against *Candida albicans*.

1.5.5.3- Other pharmacological activities

Other medicinal uses have, also, been reported for *C. villosa* (Poiret) Link as an antitumoral agent (Hartwell, 1982). Besides, it has been used for the treatment of furuncle, cutaneous abscess, and chilblain in Sicilian folk medicine (Lentini et *al*., 1997).

In Tunisia, the roots of the same plant are used traditionally as a decoction against rheumatism, and the leaves are also used to heal wounds (GIFA, 2009). In Italy, the roots of *C. villosa* are applied directly to the skin as a corn pad (Loi et *al*., 2004).
In addition to the antioxidant and the antimicrobial activities; extracts, essential oil, and isolated compounds from *C. villosa* showed diverse pharmacological activities including anti-inflammatory, antidiabetic, hypotensive, diuretic, and vasodilatation (Dessí et *al*., 2001; Chikhi et *al*., 2014; Elkhamlichi et *al*., 2017; Alhage et *al*., 2018).

Moreover, a recent report by Turan and Mammadov (2020) indicated that the ethanol extract of *C. villosa* flowers from Turkey had significant cytotoxic activity against *Artemia salina*, a larvicidal activity against *Culex pipiens*, as well as an anthelmintic activity against *Tubifex tubifex*.

1.5.6- Toxicity status of the genus *Calycotome*

A previous study (Dessi et *al*., 2001) reported that the methanol extract and the essential oil from *C. villosa* growing in Sardinia were non-toxic ($MNTD_{50}$ > 500 µg/mL). Besides, the aqueous extract of *C. villosa* (Poiret) Link seeds appeared to be non-toxic and did not produce mortality or clinically significant changes in the hematological and biochemical parameters in rats (Lyoussi et *al*., 2018).

Note: No previous study evaluated the toxicity of *C. spinosa* species.

Materials and methods

2- Materials and methods

Plants become the starting point for all scientific researches, in particular the discovery of natural molecules with very strong antioxidant and antimicrobial activities. Noting that oxidants, whatever their origins, constitute a very serious problem not only at the public health level but also at the agro-food industry one. In this context, searching for natural antioxidants and antimicrobials, as well as evaluating biological properties of different parts of the plant species *Calycotome spinosa* (L.) Link namely: leaves and flowers were interested in.

This work was carried out at the Laboratoire de Mycologie, de Biotechnologie et de l'Activité Microbienne (LAMyBAM), Frères Mentouri University -Constantine1- Algeria- and also at the Laboratoire de Chimie et Physique- Approche Multi-échelles des Milieux Complexes, Lorraine University- Metz- France.

In this experimental part, four axes were considered:

1. **The first axis** concerns the phytochemical screening, the extraction of phenolic compounds by different solvents followed by the quantification of these compounds. The obtained amounts, expressed in mg/g DE, are determined using the following reagents: Folin-Ciocalteu for total polyphenols contents (Singleton et *al*., 1999), aluminum trichloride for total flavonoids contents (Kosalec et *al*., 2004).
2. **In the second axis**, different physicochemical analyses techniques were carried out on certain extracts, fractions, and isolated compounds for their characterization and identification; Chromatography [Thin Layer (TLC), Column (CC) and Liquid (LC) Chromatography], Tandem Mass Spectrometry (MS/MS and MS^n) and Nuclear Magnetic Resonance (NMR).
3. **In the third axis**, we are interested in the antioxidant activity of the extracts, the fractions, and the separated compounds from the studied parts. Four complementary methods were used: total antioxidant capacity (CAT) (Prieto et *al*., 1999), which quantifies all the substances in the extract that have a capacity to prevent oxidation. 2, 2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging method (Sánchez Moreno et *al*., 1998; Sánchez-Moreno et *al*., 2002) which is the simplest one to carry out *in vitro*. DPPH is widely used to assess the scanning of various natural products and is considered as a model compound for the free radicals produced in lipid peroxidation. Ferric reducing antioxidant power (FRAP) (Ferreira et *al*., 2007) which is an indicative technique for the presence of reducing effects of the hexacyanoferate (III) [Fe $(CN)_6$] 3- anion to the

hexacyanoferate (II) anion [Fe $(CN)_6$] 4-. Free radical scavenging ABTS (acid 2, 2'-Azino-Bis 3-ethylbenzoThiazoline-6-Sulphonique) activity (Re et *al*., 1999) was also used.

4. **The fourth** and last axis was based on the antimicrobial activity, antibacterial and antifungal, of the obtained extracts and fractions, as well as the isolated compounds. The disc diffusion technique on agar solid medium (Vinod et *al*., 2010, Traoré et *al*., 2012) and the method of integration of extract in a culture medium (Bautista-Ban˜os et *al*., 2002) were used to measure the inhibition zones diameter (IZD mm) and the inhibition percentage (IP %), respectively. Finally, data obtained from the contents, the antioxidant, and the antimicrobial activities of different parts were subjected to statistical analyses.

2.1- Chemicals and reagents

All the deuterated solvents such as pyridine-$_{d5}$ and chloroform-$_{d1}$ used for NMR analysis; ammonium molybdate, ascorbic acid, butylated hydroxytoluene (BHT), and DPPH were from Sigma-Aldrich, whereas HPLC-grade methanol, acetonitrile, chloroform, dichloromethane, dimethyl sulfoxide were purchased from Sigma Chemicals Co. (Saint Louis, MO). The commercial standards (luteolin, luteolin 7-glucoside, kaempferol, kaempferol 3-glucoside, apigenin, *p*- coumaric acid, and caffeic acid) purchased from Merck KGaA, Darmstadt, Germany were prepared at a concentration of 10^{-5}M in methanol for analyses. All other chemicals were of analytical grade.

2.2- Plant material

2.2.1- Collection and identification of plant material

The plant material consists of the aerial parts of a plant species belonging to the genus *'Calycotome'*. This plant was collected in the region of Constantine, in the North-East of Algeria (Chattaba forest -Ain Smaraa), during the flowering period in March-June 2014-2017 (Fig. 11). The collected plant was authenticated by Professor Khalfallah Nadra (Department of Plant Biology and Ecology, Faculty of SNV- Frères Mentouri University-Constantine1- Algeria) by referring to the systematic manual of floristics (Fennane et *al*., 2007).

Fig. 11 *Calycotome spinosa* (L.) Link (2016).

2.2.2- Choice of plant

The choice of this plant was based on an ethnopharmacological survey of the population having knowledge about its use in traditional medicine.

2.2.3- Preparation of plant material

After collection, the different parts of the plant material were then cut up and dried in the shade and at room temperature (25°C) for an average of three to seven days, then stored in paper bags in a dried place. Finally, these dry parts; leaves, flowers, stems, and fruits were pulverized with an electric grinder to obtain thin powders (Fig. 12) which will be used after.

Fig. 12 Dried and crushed different parts of *C. spinosa* (L.) Link.

2.2.4- Determination of relative water content

After drying, the relative water content (RWC) was measured for each part following the formula of Pieczynski et *al*. (2013):

$$RWC\% = \left(\frac{FW-DW}{TW-DW}\right) \text{X } 100.$$

FW: fresh weight (mg); DW: dry weight (mg); TW: turgid weight (mg).

This physiological parameter was developed to have a preliminary idea about the water content and the needed quantity of a wet plant part to obtain the necessary weight in powder. This diameter changes from one year to another and from one region to another according to different factors; temperature, rain, season, and etc.

2.3- Phytochemical screening of *C. spinosa* aerial parts

Qualitative phytochemistry was based on colored reactions or precipitation by specific chemical reagents carried out on the extracts reconstituted from the lyophilized powder of each part. It was considered as the first estimate of the preliminary data on the extracts' constituents. This preliminary screening of phytochemical groups, primary and secondary metabolites, was carried out according to the common phytochemical methods described by Harborne (1973); Trease and Evans (1983); Sofowara (1993); Raaman (2006). The main ones can be mentioned: polyphenols (flavonoids, anthocyanins, and tannins), alkaloids, saponosides, steroids, coumarins, terpenes, sugars…

The results were classified into: very positive: (+++); moderately positive: (++); weakly positive: (+); negative test: (-).

The aqueous (Aq) extracts were prepared from 100 g of the powder of each plant organ placed under mechanical stirring in 1000 mL of distilled water for 24 hours at laboratory temperature. The obtained extracts were centrifuged for 20 min at 4000 rpm at room temperature and then filtered using Wattman N^{o}1 paper filter.

2.3.1- Detection of primary metabolites

2.3.1.1- *Biuret test (proteins)*

2 mL of the aqueous extract (prepared as shown previously) are added to 5-6 drops of NaOH at 5%, after stirring, 5 to 7 drops of $CuSO_4$ (2 %) are added. The appearance of pink or purple color indicates the presence of proteins.

2.3.1.2- *Fehling test (sugars)*

1 mL of the aqueous extract and 1 mL of the Fehling solution (A + B) are placed in a tube that boiled after. The appearance of the brick red precipitate indicates the presence of sugars.

2.3.2- Detection of secondary metabolites

2.3.2.1- Polyphenols

- *Ferric chloride test (phenolic compounds)*

The reaction with ferric chloride ($FeCl_3$) allowed the characterization of polyphenols. A drop of $FeCl_3$ solution (2%) was added to 2 mL of extract. The appearance of the dark blue-green coloration indicates the presence of polyphenols.

- *Tannins test*

The tannins are highlighted from 1 mL of the extract placed in a tube in the presence of a few drops of $FeCl_3$. After stirring, the color turns black-blue in the presence of gallic tannins and greenish-brown in the presence of catechic tannins.

- *Coumarins test*

The coumarins are revealed from 2 mL of extract placed in a tube contains 3 mL of NaOH (10%), after stirring, the formation of yellow color indicates the presence of coumarins.

- *Anthocyanins test*

The anthocyanins are detected by placing 5 mL of extract in a tube to which 15 mL of H_2SO_4 (10%) (Acidic medium) are added, after stirring, the mixture is added to 5 mL NH_4OH (10%) (Basic medium). The presence of anthocyanins is confirmed by a blue-purplish coloration in a basic medium.

- *Flavonoids test*

A mixture of a few drops of Mg^{+2} and drops of concentrated HCl, placed in a tube, is added to 2 mL of extract. The appearance of pink, orange, or red coloration indicates the presence of flavonoids.

Flavonoids are also highlighted by adding 4 mL of sodium hydroxide (NaOH) and an alcoholic solution of $FeCl_3$ to the extract. The appearance of yellow color indicates the presence of flavonoids.

- *Anthraquinones test*

For the detection of anthraquinones, 10 mL of the extract are added to 5 mL of NH_4OH (10%). After shaking for a few minutes, the appearance of a red ring indicates the presence of anthraquinones.

2.3.2.2- Foam test (saponosides)

For the detection of saponosides, 10 mL of aqueous extract placed in a test tube are shaken for 15 seconds and then left to stand for 15 min. A height of persistent foam, greater than 1 cm indicates the presence of saponosides.

2.3.2.3- Terpenes test (sterols and triterpenes)

The steroids are identified after the addition of 5 mL of acetic anhydride to 5 mL of a hot extract. The mixture is added to 0.5 mL of concentrated sulfuric acid. After agitation, the appearance, at the interphase, of a purple ring turning blue then green, indicates a positive reaction.

2.3.2.4- Alkaloids test

The alkaloids were detected using reagents as Mayer, Wagner, or Dragendorff.

10 mL of the extract are evaporated until a volume of 0.2 mL is obtained, to which 1.5 mL of HCl (2%) are added. After stirring the acid solution, one to two drops of Mayer, Wagner, or Dragendorff reagents are added. The appearance of a yellowish-white or brown precipitate indicates the presence of alkaloids.

2.3.2.5- Essential oils Test

The essential oils extraction was carried out by hydrodistillation in a Clevenger apparatus. Three distillations were carried out by boiling 200 g of fresh plant material with 1 L of water in a 2 L flask surmounted by a column 60 cm long connected to a refrigerant. The essential oils yield was determined in relation to the utilized matter.

2.4- Extraction and fractionation

Extraction and fractionation were first performed on the plant' parts to obtain extracts and fractions on which the bio-assays were realized.

2.4.1- Preparation of *C. spinosa* methanolic and aqueous extracts

The methanolic (MeOH) and the aqueous (Aq) extracts were obtained from 100 g of the powder of each organ placed under mechanical stirring in 1000 ml of each solvent (MeOH and distilled water) for 24 hours at laboratory temperature. This operation, maceration in

methanol and in water, was repeated three (3) times under the same conditions. The obtained extracts were centrifuged for 20 min at 4000 rpm at room temperature and then filtered using Wattman N°1 paper filter. Successful filtrates are concentrated using a rotary evaporator (Heidolph) at reduced pressure at 45 °C.

2.4.2- Fractionation "flavonoids extraction"

The fractionation protocol for the flavonoid fractions of chloroform ($CHCl_3$), ethyl acetate (EtOAc), and n-butanol (n-BuOH) was carried out according to the method of Bekkara et *al*. (1998) (Fig. 13).

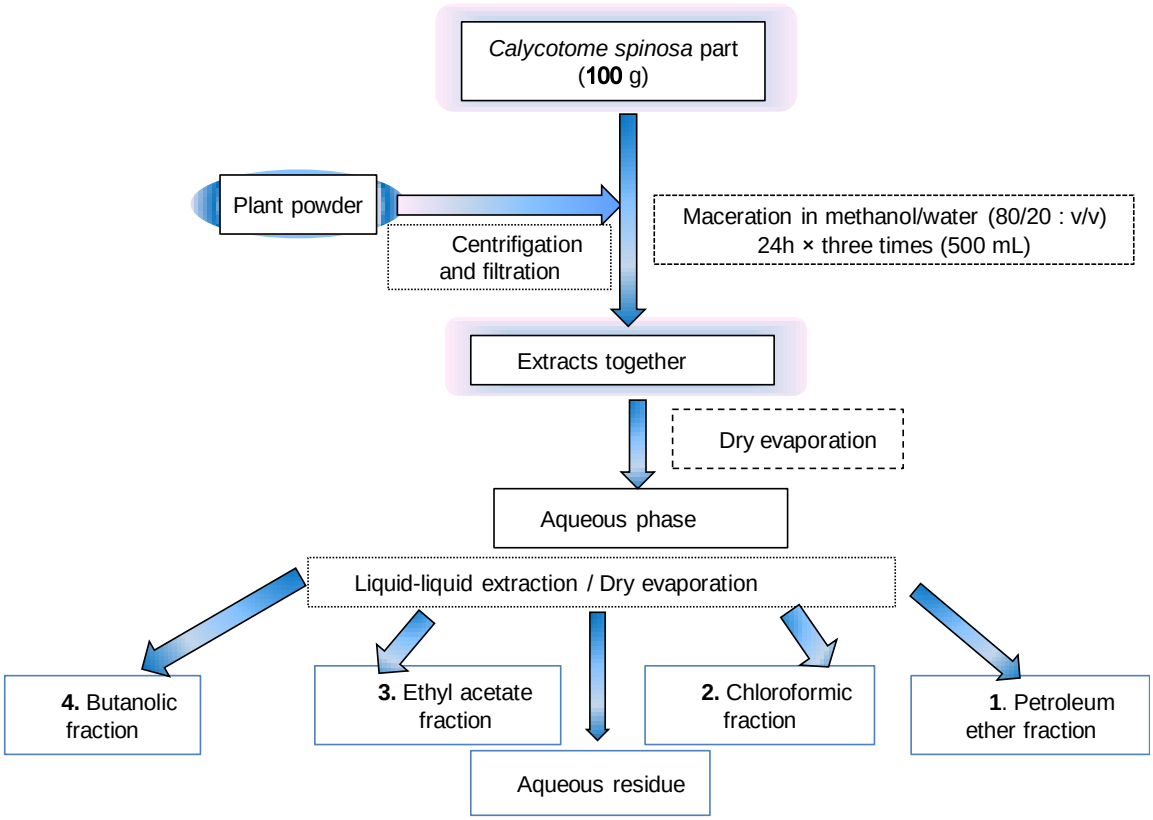


Fig. 13 Fractionation protocol for flavonoids extraction (Bekkara et *al*., 1998).

2.5- Quantitative analyses of *C. spinosa* extracts and fractions

2.5.1- Determination of extraction yield

The yield of the obtained extracts and fractions; MeOH, Aq, $CHCl_3$, EtOAc, and n-BuOH; of both parts leaves and flowers, is determined according to the formula of Falleh et *al*. (2008) as shown herein:

$$\mathbf{Y}\% = \frac{\text{Mass of extract residue}}{\text{Mass of plant powder}} \text{X}100$$

2.5.2- Determination of total polyphenols content (Folin-Ciocalteu)

Several analytical methods can be used for the quantification of total phenols. The Folin-Ciocalteu (1929) reagent analysis is the most used.

This reagent consists of a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMO_{12}O_{40}$). During oxidation, it is reduced to a mixture of blue tungsten oxide and molybdenum (Ribéreau-Gayon, 1968). The color produced, in which the maximum absorption is between 725 and 760 nm, is proportional to the amount of polyphenols present in the analyzed extract.

The determination of total polyphenols content (TPC) was carried out by an adapted method of Singleton and Rossi (1965) using Folin-ciocalteu reagent. Indeed, 200 µL of each extract are mixed with 1 mL of the Folin-Ciocalteu reagent (2M) diluted 10 times. The tubes are kept at room temperature for 5 min, and then 800 µL of sodium carbonate (Na_2CO_3 at 7.5%) are added and mixed. After the incubation of the samples for 60 min at room temperature (25 °C) in the dark, the absorbance is measured at 765 nm with a UV / Visible spectrophotometer (Schimadzu, UV-1280). The calibration curve is carried out by gallic acid as a standard, following the same steps of the assay, at different concentrations (from 6.25 to 200 µg / mL). All measurements are repeated three (3) times (Singleton et *al*., 1999). The blank is similarly prepared by replacing the 200 µL of the extract and the standard by the methanol. The results are expressed in milligrams of gallic acid equivalents / g of dried extract (mg GAE / g DE).

2.5.3- Determination of total flavonoids content ($AlCl_3$)

Flavonoids are capable of trapping free radicals by forming less reactive flavoxyl radicals, this ability can be explained by their property of donating a hydrogen atom from their hydroxyl group.

The aluminum trichloride ($AlCl_3$) method of (Chang et *al*., 2002; Kosalec et *al*., 2004) was slightly modified to quantify the total flavonoids content (TFC) in the different extracts of the plant. The $AlCl_3$ forms a very stable complex with the hydroxide groups OH of phenols. This yellow complex absorbs visible light at a wavelength of 430 nm. Phenols are estimated by UV spectroscopy, in which quercetin is used as a standard at a wavelength λ = 430 nm**.**

TFC was quantified as the following:

1 mL of each sample is added to 1 mL of aluminum chloride solution $AlCl_3$ (2%) in methanol. After shaking, the samples are incubated for 30 min at room temperature in the dark. The absorbance is measured at 430 nm using a UV/ Visible spectrophotometer (SHIMADZU UV-1280). All analyses are repeated three times. The quantification of total flavonoids is done according to a calibration curve performed by a standard flavonoid; quercetin; which is diluted in several concentrations from 0.02 to 0.2 mg/ mL. The blank is equally prepared, but by replacing the 1 mL of the extract with the methanol. The results are expressed in milligrams of quercetin equivalents/ g of dried extract (mg QE/ g DE) (Kosalec et *al*., 2004).

2.6- Qualitative analyses of *C. spinosa* extracts and fractions

2.6.1- Thin Layer Chromatography (TLC)

A thin layer chromatographic analysis was carried out with Silica gel plates $60HF_{254}$ on aluminum sheets, which constitute the stationary phase. The samples (extracts and fractions) are deposited at a reference point on these plates which are then submerged in glass vats, filled to approximately 0.5 cm with a mobile phase, which is a binary mixture of solvents. The analysis was carried out for the previously obtained extracts. To find a system giving the best separation, the extracts were tested with several elution systems. After observing the plates under a UV lamp, the elution system which gave good separation of all the obtained extracts and fractions was composed of $CHCl_3$ and MeOH in a 9/1 proportion.

2.6.2- LC-ESI-MS/MS analysis of leaves methanol extract

The analysis of polar extracts, namely MeOH and Aq extracts, of leaves and flowers has been carried out by LC-ESI-TMS/MS, but this part will only include the analysis of a single extract (the leaves MeOH extract), considering the results obtained with its yield, phenolic compounds contents, antioxidant and antibacterial activities, which will be presented. The objective of this analysis is to provide a huge number of the detected and the identified metabolites.

For LC-MS analysis, a high performance liquid chromatography system (Dionex Ultimate 3000, Dionex, France) was connected to a dual-pressure linear ion trap mass spectrometer (LTQ Velos Pro, Thermo Fisher Scientific, San Jose, CA, USA). For the separation of polyphenols from the leaves methanol extract of *C. spinosa*, a C18 reversed-phase column was used (Symmetry Shield, 4.6 × 50 mm, 3.5 µm, Waters). 20 µL of the sample were injected. The flow rate was kept to 500 $\mu L.min^{-1}$ and a constant elution gradient was applied

from 0 (5% acetonitrile / 95% methanol) to 30 min (100% acetonitrile) during the LC run. HESI (Heated Electrospray Ionization Source) interface was plugged to the ion source of the LTQ mass spectrometer, with a capillary temperature set at 300 °C, a heating source at 250 °C, a sheathed gas flow at 10.00 psi, an auxiliary gas flow at 1 L/min, and a sweep gas flow at 1 a.u. with the injection waveforms. MS system was running from 110 to 2000 *m*/*z* at MS scan rate of 9 Hz. To confirm chromatographic peak assignment, MS/MS by CID (Collision Induced Dissociation) was systematically conducted on the most intense 5 mass peaks of each mass spectrum. Furthermore, some standards (mentioned in the chemicals and reagents section) were purchased then analyzed in order to confirm the different retention times and the fragmentation spectra. Subsequently, spectrometry has also been carried out in two modes: positive and negative. *During the positive mode*, the source voltage was set at +5 kV and the source current at 100 µA. *During the negative mode*, the source voltage was set at −4 kV, the source current at 100 µA, the RF level of the S-Lens at 60%, the multi-polar RF amplifier at 800.00 (Vp-p), the multi-pole offset at 2 V, the lens voltage at 3 V, the multi-pole shift at 9 V, the second lens voltage at 15 V, the lens shift at 90 V and the multi-polar shift 1 at 20 V. The utilized method (Becker, 2014) was optimized in the negative mode for polyphenols analysis; whereby these latest molecules were well ionized.

The control of the system and the data treatment were realized using the Thermo Xcalibur software Version 2.2 (2011) (XCALI-97211 Revision D May 2011) by Thermo Fisher Scientific.

2.6.3- Column chromatography of leaves methanol extract

This technique allows the splitting of products according to their polarity. The column separation was started by choosing the best elution system (according to the result of the previous technique, TLC). The performed tests have shown that the best separation was obtained with the system ($CHCl_3$ / MeOH: 9/1). Next, the size and the diameter of the column were chosen according to the quantity of the sample to be purified (in our case the diameters of the used big column are (60 cm, 10 cm), and (15 cm, 5 cm) for the small Flash column chromatography.

Note: Among the obtained extracts in this study, only one extract (methanolic extract of leaves) was selected to continue our phytochemical analyses according to its yield, phenolic compounds contents, and biological activities.

The separation was done as the following:

A quantity of nine grams (9.0 g) of methanolic extract from *C. spinosa* leaves was subjected to a silica gel [Silica 60M (0.04-0.063)] column chromatography (60 cm x 10 cm) prepared in chloroform. The elution was carried out by polarity gradient of the chloroform/ methanol system (100: 0 to 0: 100), starting with $CHCl_3$ / MeOH: 9/1. A total of 540 test tubes were collected (25 mL for each) and analyzed with F254 (60 HF_{254}) silica gel plates using $CHCl_3$ / MeOH as the elution system. The compounds were visualized under an ultraviolet lamp at the wavelength of 254 and 366 nm for each tube. Similar tubes were pooled together to give eight (08) main fractions (fractions 1-8). TLC was performed for these eight obtained fractions using the same elution system. Fractions comprising the major spots were chosen to make another separation (small Flash column: 15 cm x 5 cm). The selected fractions were F4, F5, F6, and F8. Fraction 8 (F8) (3.2 g), which was eluted with $CHCl_3$ / MeOH (9: 1), was then deposited for other purification by a silica gel column chromatography (small Flash column) using a $CHCl_3$ / MeOH gradient system (100: 0–0: 100). The compounds were also visualized under a UV lamp at the wavelength of 254 and 366 nm. Similar tubes were, furthermore, combined to yield 10 major sub-fractions [sub-fractions F8 (1) to F8 (10)]. A successive washing by MeOH and $CHCl_3$ was carried out.

Three different pure precipitates from this fraction with diverse colors and weights were obtained; yellow compound **(3)** (100 mg), green compound **(4)** (200 mg), and white compound **(5)** (250 mg). The same procedures were applied on Fraction 5 (F5). Two pure precipitates with various colors were also found; yellow compound **(1)** (50 mg) and green compound **(2)** (150 mg).

2.7- Identification of the isolated compounds

A list of the *m/z* ratio was obtained from the LIT-ESI-MS report. An MS^2 experiment was performed simultaneously on the highest peaks by applying the following strategies:

The observed MS^2 spectrum was compared to the literature. The neutral loss observed between the parent ion and the fragments was calculated. Indeed, in the plant kingdom, a well-known neutral loss is particularly informative such as -162 for glucosidic derivatives, and the loss of 44 in negative ion mode indicates the presence of a carboxylic acid. Some compounds have been identified using the literature review on species belonging to the genus *Calycotome* and on other plants (with the notable fact that *Calycotome spinosa* is rather poorly documented in terms of secondary metabolites). In addition, certain isolated compounds, a posteriori identified in NMR, helped us to reconsider certainly abandoned spectra leading to the successful identification of certain molecules. Furthermore, in our

case, the NIST database (NIST, 2008) was mainly used to confirm the compounds' structural elucidation.

2.7.1- LIT-ESI-MS^n analysis of the isolated compounds

The five (**1-5**) isolated compounds, were dissolved to 3 mg / mL in $CHCl_3$/ MeOH (50/50: v/v). The prepared solutions were immediately diluted to a concentration of 300 µg / mL using methanol as a solvent. The five studied compounds (**1-5**) in this work were directly infused in the mass spectrometer via an ESI source in which ions are produced (HESI source from Thermo Scientific). Then, the linear ion trap (LIT) can accumulate, confine and eject ions by applying different trapping potentials on axial electrodes. The ions are maintained in the middle of the LIT with a radial field RF.

This instrument was used according to its ability to easily achieve MS^n experiments for structural characterization (Becker, 2014). In the common ion trap, the selection of the parent ion is carried out by the ejection of the other features from the linear trap. This is performed by applying RF-sweep, corresponding to the different resonance frequencies of the unwanted ions. Thus, the ions are ejected along the radial axis. Another mode of excitation is possible with the application of an auxiliary alternative-current field. This excitation leads to an increase in the kinetic energy of the ions ejected from the LIT.

The control of the system and the data treatment were realized using Thermo Xcalibur software Version 2.2 (2011) (XCALI-97211 Revision D May 2011) by Thermo Fisher Scientific.

2.7.2- Nuclear Magnetic Resonance Analysis (NMR)

The structural elucidation of the five isolated compounds was based on different NMR spectra; D1 (^{1}H and ^{13}C) and D2 (COSY, NOESY, HMBC, and HSQC) which were recorded on a Brüker Advance 400 MHz spectrometer in the NMR service of the SRCMC Laboratory at the University of Lorraine, Metz-France.

The chemical shifts are expressed in ppm, compared to the TMS (TetraMethylSilane) that taken as an internal reference. The coupling constants *J* are expressed in Hertz. The multiplicity of resonance signals is indicated by the following abbreviations: (s) singlet, (d) doublet, (dd) doublet of doublet, (t) triplet, (q) quadruplet, (qt) quintuplet, (m) multiplet. The structures of the compounds were also confirmed by comparison with the reference data from the literature. MestReNova LITE 5.2.5-5780 software was used to treat NMR spectra.

2.8- *In vitro* Biological activities

2.8.1- *In vitro* antioxidant activities

Highlighting the *in vitro* antioxidant activity of various extracts (MeOH and Aq), and fractions ($CHCl_3$, EtOAc, and n-BuOH) obtained from the leaves and flowers of the tested plant *C. spinosa;* was carried out by two distinct methods: the DPPH free radical scavenging activity and the ferric reducing antioxidant power (FRAP). Besides, the antioxidant activity of the five isolated compounds with their extract (MeOH of leaves) was performed by four different techniques; in addition to the two previous ones, the total antioxidant capacity (TAC) and the ABTS radical cation assay have been used.

2.8.1.1- Total antioxidant capacity (TAC)

- **Principle**

The total antioxidant capacity (TAC) of the samples was evaluated by the phosphomolybdenum method of Prieto et *al*. (1999). This technique is based on the reduction of molybdenum Mo (VI) present in the form of ions molybdate MoO_4^{-2} to molybdenum Mo (V) MoO_2^{+} in the presence of the extract in order to form a green phosphate / Mo (V) complex at acid pH.

- **Procedure**

A volume of 0.1 mL of each sample in methanol was mixed with 1 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were screwed and incubated at 95 °C for 90 min. After cooling, at room temperature, the absorbance of the solutions was measured at 695 nm against a blank which contains 1 mL of the reagent solution and 0.1 mL of methanol, and it was incubated under the same conditions as the samples. The total antioxidant capacity is expressed in milligrams of ascorbic acid equivalents per gram of the dried extract (mg AAE / g DE). All the experiments were repeated three times.

2.8.1.2- DPPH free radical scavenging activity

- **Principle**

DPPH is a stable free radical with violet color in solution, it has a characteristic of absorbance between 512 and 517 nm, and the violet color disappears quickly when DPPH is reduced to diphenyl picryl hydrazine by a compound with anti-radical property, thus causing discoloration. The intensity of the color is proportional to the capacity of the antioxidants

present in the medium to give protons (Sanchez-Moreno, 2002). The reaction can be summarized in the following equation:

$$DPPH^{*} + (AH)n \rightarrow DPPH\text{-}H + (A^{*})n$$

(AH) represents a compound capable of yielding hydrogen to the DPPH radical (purple) to transform it into diphenyl picryl hydrazine (yellow) (Brand-William et *al*., 1995).

If free radicals have been scavenged, DPPH color will be generated to yellow.

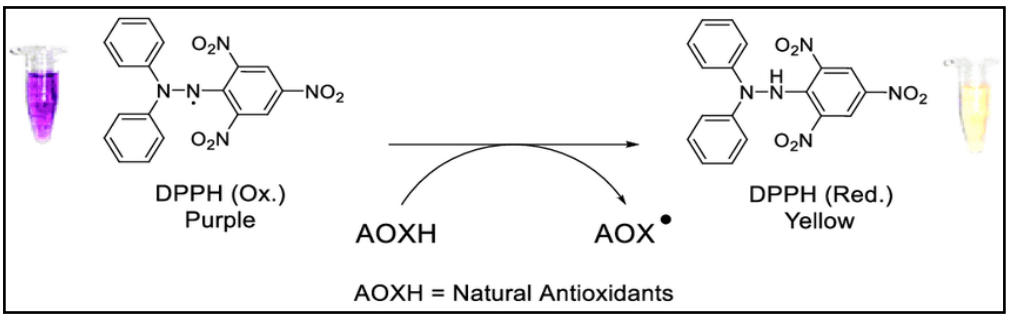


Fig. 14 Principle of DPPH free radical scavenging capacity assay (Arce-Amezquita et *al*., 2019).

- **Procedure**

The DPPH solution (2.4 mg DPPH in 100 mL MeOH) was prepared in advance (at least 1-2 hours) because its solubilization is difficult, and it does not keep for more than 4-5 days at -5°C in the dark (Popovici et *al*., 2009). From our plant extracts and the positive control BHT (Standard), different concentrations were prepared in methanol: 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 mg / mL. According to this method, a volume of 1 mL DPPH methanolic solution was added to 500 µl of both the samples and the standard at various concentrations. The blank consisted of 500 µL of methanol added to 1 ml of DPPH. The reaction mixture was stirred vigorously and left in the dark for 30 min. Shorter times have also been reported by some authors, such as 5 min (Lebeau et *al*., 2000) or 10 min (Schwarz et *al*., 2001), but in our experiments, the time of 30 min was found to be the optimum (time required for stable signals). Each test was repeated three times. The absorbances were measured at 517 nm using a spectrophotometer (SHIMADZU UV-1280) against a negative control (blank). The antioxidant capacity to scavenge the free radical is estimated as a discoloration percentage of DPPH in methanol solution. The inhibition percentage of antioxidant activity (%) was determined according to the following equation:

Antioxidant activity % = (A_{blank} - A_{sample}) x 100 / A_{blank} (Sharififar et *al*., 2007).

A_{blank}: Absorbance of blank

A_{sample}: Absorbance of the tested compound.

The IC_{50} (half maximal Inhibitory Concentration) value is the concentration of the sample that can scavenge 50% of DPPH free radical; it was calculated graphically from the inhibition rates of different concentrations of each sample. The IC_{50} value is inversely proportional to the antioxidant property of the sample. Thus, a low IC_{50} value indicates a strong antioxidant activity.

2.8.1.3- Ferric reducing antioxidant power (FRAP) assay

This test is considered as a direct, rapid, and inexpensive assay to measure the antioxidant power.

- **Principle**

The FRAP mechanism is based on electron transfer rather than hydrogen atom transfer (Prior et *al.*, 2005). The reducing power of iron (Fe^{3+}) in the extracts was determined according to the method described by Ferreira et *al.* (2007). This test is based on the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants. Its redox reaction is revealed by the transfer of the yellow color of Fe^{3+} to the blue-green color of Fe^{2+}**.** The intensity of this coloration is proportional to the reducing power. The high absorbance of an extract means its high reducing power.

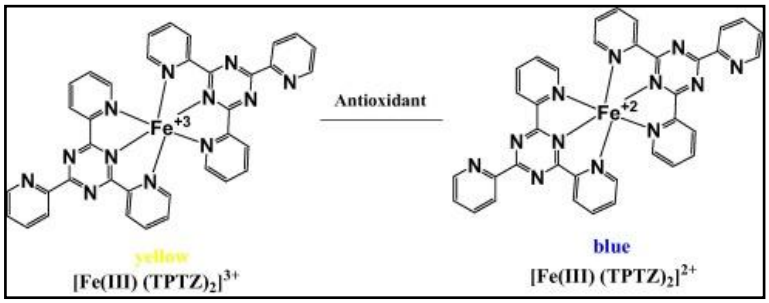


Fig. 15 Redox reaction in ferric reducing antioxidant power (FRAP) assay (Pérez-Cruz et *al.*, 2018).

- **Procedure**

A range of five dilutions (0.1, 0.2, 0.3, 0.4 and 0.5 mg / mL) was realized for all samples; extracts and standard (ascorbic acid). 2.5 mL of each sample at different concentrations diluted in methanol were mixed with 2.5 mL of the phosphate buffer solution (0.2M, pH 6.6) and 2.5 mL of potassium ferricyanide ($(K_3Fe(CN)_6)$ at 1%). The whole was stirred and incubated in a water bath at 50°C for 20 min. Then, a volume of 2.5 mL trichloroacetic acid (TCA at 10%) was added to the mixture to stop the reaction, the entire was carefully mixed and centrifuged for 10 min at 3000 rpm. 2.5 mL of distilled water and 0.5 mL of ferric chloride ($FeCl_3$ at 0.1%) were added to 2.5 mL of the supernatant. Then left to standing for 10 min. The absorbance was then measured at 700 nm using a UV-Visible

spectrophotometer (SHIMADZU UV-1280) against a blank. Ascorbic acid was used as a positive control and its absorbance was measured under the same conditions as the samples. For the whole experiment, each test was carried out in triplicate (Elmastaş et *al*., 2015) and the results were calculated by the average of the three tests.

The value of the EC_{50} (Efficient concentration equivalents to 50% of reducing power) is defined as being the concentration of the substrate that can exhibit 50% of FRAP capacity. It was also calculated graphically as IC_{50} but from the absorbances of different concentrations of each sample. A low EC_{50} value indicates a strong antioxidant capacity. The blank is similarly prepared, replacing the extract with distilled water which allows the device (spectrophotometer) to be calibrated (Singleton and Rossi, 1965).

2.8.1.4- ABTS cation radical test

- **Principle**

ABTS is also a free and stable radical. It is widely used to assess the antioxidant power of biological compounds. This radical is capable of reacting with conventional antioxidants such as phenols and thiols but also with any hydrogen or electron donor compound (Rice-Evans et *al*., 1995). This cation radical is easily formed by oxidation in the presence of potassium persulfate to give a solution colored green-blue. Adding an antioxidant to a solution of this cation radical causes the reduction of this radical and the decrease in absorbance at 734 nm. This decrease depends on the antioxidant activity of the tested compounds, but often also on the time and the concentration (Re et *al*., 1999).

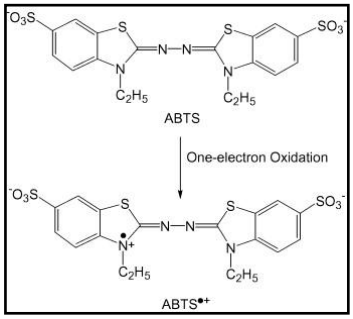


Fig. 16 ABTS structure: reduced form ABTS, radical cation form ABTS + • (Lee and Yoon, 2008).

- **Procedure**

The anti-radical activity of the tested samples and the standard was determined by a method based on the reduction of the $ABTS^{+\bullet}$ radical. The $ABTS^{+\bullet}$ radical was generated by the reaction of an $ABTS^{+\bullet}$ aqueous solution (7 mM) with $K_2S_2O_8$ (2.45 mM) in the dark for 16 h and the adjustment of the absorbance at 734 nm is 0.7 at room temperature (Re et *al*., 1999). The samples (100 µL) were added to 400 µL of ABTS and the absorbance was read at 734

nm after 10 minutes. Several concentrations of samples were prepared and the inhibition percentage (IP %) is calculated using the following formula:

IP% = [(Abs $_{Blank}$ - Abs $_{Sample}$) / Abs $_{Blank}$] x 100.

The IC_{50} value which is the required concentration of the sample to show 50% inhibition of the ABTS free radical was determined as indicated in the DPPH method. In the blank, the sample volume is replaced by the same volume of solvent. BHT was used as a standard. All of these tests have been performed three times (Erenler et *al*., 2015).

2.8.2- *In vitro* antimicrobial activities

- **Tested microorganisms**

The studied plant samples were, individually, tested against a group of thirteen (13) distinct pathogenic microorganisms; including six bacteria; two Gram+: *Bacillus subtilis* (ATCC-6633) with *Staphylococcus aureus* (ATCC-25923); and four Gram-: *Escherichia coli* (ATCC-25922), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (ATCC- 27853), and *Salmonella abony* (NCTC 6017). As well, pathogenic yeast (*Candida albicans* (ATCC 10231)) and six phytopathogenic molds isolated from durum wheat grains: *Alternaria sp.1, Alternaria sp.2, Penicillium sp.1, Penicillium sp.2, Aspergillus sp., and Rhizopus sp.* were also tested. The six bacteria and the yeast were brought from the laboratory of bacteriology - University Hospital "Ibn Badis"- Constantine, Algeria. The choice of these microorganisms was accomplished according to their distinguished bad effects on human being health; skin infections, vaginal infections, food contaminations, and etc.

- **Preparation of culture media**

Depending on the used methods and the tested microbial strains, the culture media were prepared (Appendix 2).

2.8.2.1- Antimicrobial activity "disc diffusion assay"

This test was carried out using the disc diffusion method in solid medium (NCCLS, 2004, Vinod et *al*., 2010; Traoré et *al*., 2012) for the evaluation of the antimicrobial activity of the obtained plant samples by determining the inhibition zone diameter (IZD) in mm.

- ***Preparation of discs***

Discs of 6 mm in diameter were cut from filter papers (Wattman N°1), sterilized and impregnated with the extracts and the fractions at different concentrations in DMSO 2% (Dimethyl Sulfoxide) (12.5, 25, 50, 75, 100 mg / mL), the five isolated compounds at 100

mg/ mL, the antibiotic (Chloramphenicol at 1.5 mg/ mL), the antifungal (Fluconazol at 1 mg/ mL), and the DMSO (10 µL/ disc) (Traoré et *al*., 2012).

- ***Reactivation of strains***

From the storage tubes, the bacteria and the yeast to be tested were seeded on Petri dishes containing selective media suitable for the used microbial strains. Then they incubated at 37 °C for 24 hours for the bacteria and 28 ° C for 24 to 48h for the yeast, in order to obtain young and well isolated microbial colonies.

- ***Inoculums' preparation***

After 24 hours of incubation of the bacterial and the yeast strains, one to two well-isolated and perfectly identical colonies were taken, then emulsified in a sterile tube containing saline solution 0.9%, and then shaken using the vortex. The densities of the inoculums suspensions were adjusted to obtain final concentrations match of 0.5 Mc Farland (1.2 x10^7 cells / mL) (Daouda, 2015).

- ***Efficiency test***

Using a sterile swab dipped in a standardized bacterial or yeast suspension, the entire surface of the dishes containing Mueller-Hinton medium for bacterial stains and Sabouraud dextrose agar for yeast was uniformly seeded. When the surface has dried up (around 5 min), the prepared discs were delicately deposited using flambed forceps to Bunsen burner on the medium surface previously sown with microbial strains. At the same time, antimicrobial positive controls [chloromphenicol (15 µg/ disc) for bacteria, and fluconazol (10 µg/ disc) for the yeast], as well as the DMSO (2%) were also tested. The prepared agar dishes have been kept at 4°C for 20 min in order to allow the pre-diffusion of the extracts. Then, they were incubated at 37 °C for 24 hours for bacteria and at 28 ° C for yeast (Traoré et *al*., 2012).

- ***Reading***

The activity was visually estimated by the presence or the absence of colonies, and then the inhibition zone diameter (IZD) of the studied strains was measured in millimeters after 24 hours of incubation (Jirovetz et *al*., 2003).

Determination of the Minimal Inhibitory Concentration (MIC)

The MIC was determined using the agar disc diffusion method (Billerbeck et *al*., 2002). The plant extracts' and fractions' solutions have been prepared at different concentrations in DMSO (12.5, 25, 50, 75, 100 mg / mL). These various concentrations were soaked on discs (sterile filter paper) and then the dishes were incubated for 24 h at 37 °C and at 28 °C for bacteria and yeast, respectively. The MIC values were recorded as the lowest concentrations of the compounds allowing growth inhibition. DMSO has steadily been verified for the absence of its antimicrobial activity.

2.8.2.2- Antifungal activity

- ***Sampling of durum wheat grains***

A sampling of stored durum wheat grains of the "GTADUR" variety was done from the region of Constantine - Algeria.

- ***Sorting of grains***

The brought-back grains had different characteristics; length, width, thickness, volume, weight and density, shape, and color. The sorting was accomplished according to the size, color, and appearance of the grains: any change in size, color or general appearance of the grains makes it possible to suspect their internal contamination. The choice of grains will be guided by these characteristics.

- ***Grains' surface disinfection***

Many durum wheat grains disinfected on the surface were chosen for the isolation of fungi. The treatment of these grains was performed with NaOCl solution at 2% for 1 to 2 min. After at least 3 rinses for 1-3 min in sterile distilled water, the grains were dried with sterile filter paper to be then sown (Pacin et *al*., 2002; Ghiasian et *al*., 2004).

- ***Isolation of fungi***

According to (Djossou, 2011), fungi were isolated by direct contact on the PDA medium. The dried grains were taken using forceps disinfected with ethanol and flamed, and then they were placed on the surface of the Petri dishes containing the PDA agar medium (Appendix 2) supplemented with an antibiotic from the Amoxicillin family of a reason of 5 grains per Petri dish**.** The whole was incubated at 30 ± 2 ° C for 3 to 7 days.

- ***Purification of fungal isolates***

Under aseptic conditions, daily observations were carried out using a binocular from the germination of the grains till the appearance of the mycelia. A disc of 6 mm of each fungus was transplanted using a Pasteur pipette and a sterile platinum handle in the center of the Petri dish containing a PDA medium, supplemented with the antibiotic (30 mg/ L), then incubated at 30 ° C for 6-7 days (Botton et *al*., 1990; Guiraud, 2003). To obtain pure strains, the purification must be carried out by transplanting a terminal hypha in the center of the dish containing the same medium and then incubated in the same conditions (Guiraud, 2003).

- ***Identification of fungal isolates***

The identification of the fungal genera depends on both macroscopic and microscopic examinations (Barnett and Hunter, 1972; Chabasse et *al*., 2002; Guiraud, 2003; Leyral and Vierling, 2007; Djabali and Barkat, 2013).

Macroscopic examination (cultural characteristics)

It is the study of colonies' appearance. A colony is a cluster, visible to the naked eye, made up of billions of mycelia of fungi whose size, shape, color of colonies (surface and reverse), speed of growth, and odor are the characteristics of each genus and each species. The study of the appearance of the colonies requires naked eye observation, in the presence of natural or artificial light.

Microscopic examination

Microscopic observation allows a morphological study of the cells. It includes examination in the fresh state (examination between slide and coverslip of live molds) and examination after staining with cotton blue more often on dried and fixed smears; hyphae and spores (color, size, septation).

- **Efficacy test**

Six pure phytopathogenic fungi isolated from a collection of Algerian durum wheat were chosen to test the antifungal activity of our extracts: *Alternaria sp.1, Alternaria sp.2, Penicillium sp.1, Penicillium sp.2, Aspergillus sp.,* and *Rhizopus sp.* The selected fungi were reactivated and allowed to grow using the PDA medium for 4 to 7 days at a temperature of 30 ± 2 °C before their use in the antifungal activity test. This test consists of incorporating the crude extracts "MeOH and aqueous extracts of leaves and flowers" into an agar medium. The extract to be tested is weighed in a hemolysis tube and dissolved in a solution of

DMSO. The final concentration of DMSO in the culture medium must not be greater than 2%. Inhibition of mycelial growth was determined by cutting discs of 6 mm in diameter from the edge of a young fungal culture and placing the disc in the center of a Petri dish on PDA containing 20 mg / mL of each extract (MeOH and aqueous extracts of the leaves and flowers of *C. spinosa*) (Bautista-Ban˜os et *al*., 2002). The dishes were left to incubate at 30 ± 2 C° and the experiment ended when the negative control culture (PDA without extract) completely colonized the agar surface. Radial growth was measured against two replicates per experiment. The results were expressed as an inhibition percentage (IP %) of radial growth in the media containing extracts relative to the negative control according to the formula of Bautista- Ban˜os et *al*. (2002). In addition, the efficiency of the commercial fungicide Fluconazol was evaluated using 1 g. L^{-1} in PDA medium (Chavez-Quintal et *al*., 2011). The inhibition percentage (IP %) was calculated using the following formula:

$$\mathbf{IP\% = [(C\text{-}T) / C] \times 100}.$$

C: Average diameter of the estimated mycelial growth of the fungus tested on control medium (mm).

T: Average diameter of the mycelial growth on the medium, treated with a precise concentration of product (mm).

Conservation of microorganisms

The most common and the simplest used method for preserving strains, consists of transplanting the obtained pure strains in tubes on inclined agar (NA for bacteria, Sabouraud + Actidione for yeast, and PDA + antibiotic for molds). The cultures are maintained for 24 hours at 37 °C for bacteria and 7 days at 28 °C for molds to allow maximum growth, and then they are stored at 4 °C to promote their viability and to limit the possibilities of variations (Botton et *al*., 1990; Takahashi et *al*., 2008).

2.9- Statistical analyses

All the experiments were carried out twice or three times. Data were expressed as mean ± standard deviation (SD). The values of IC_{50} and EC_{50} were calculated using Graph Pad Prism 7 software (Graph Pad Software, Inc., La Jolla, California, USA). The different correlation coefficients have been demonstrated utilizing Microsoft Excel 2007.

Results

3- Results

This work focuses on the phytochemical characterization of an endemic plant species of the genus *Calycotome*. The search for the antioxidant activity and the antimicrobial potential of certain extracts and isolated compounds, followed by the structural elucidation of bioactive compounds were, furthermore, the purposes of the present study.

3.1- Identification of the studied plant

The botanical identification of the studied plant by Professor Khalfallah Nadra revealed that its plant species was *Calycotome spinosa* (L.) Link (*C. spinosa*) (Fig.17). Its identification was based on its morphological characteristics. An authenticated voucher specimen was deposited in the herbarium of the SNV Faculty, University FMC1, under the code (C. s. 2014).

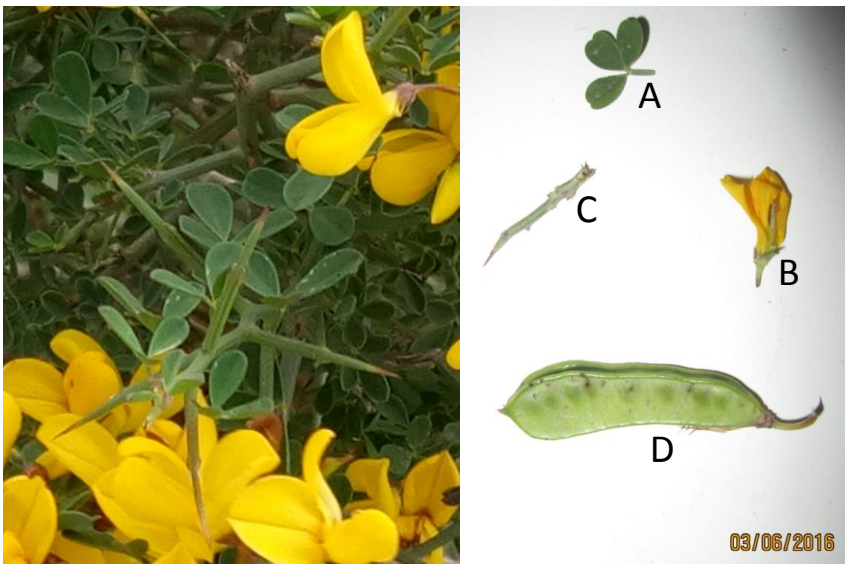


Fig. 17 Different aerial parts of *C. spinosa*.

A: Leaf, **B**: Flower, **C**: Spine, **D**: Fruit.

Besides, after each aerial part of the plant has dried, the relative water content was measured, and its value varied from one part to another; 43.85 ± 1.57%, 33.94 ± 0.8%, 55.43 ± 1.32%, and 46.64 ± 2.69% for leaves, flowers, stems, and fruits; respectively.

3.2- Phytochemical screening of *C. spinosa* aerial parts

The detection of primary and secondary metabolites in *C. spinosa* aerial parts; leaves flowers, stems, and fruits were performed using specific assays for each chemical group. The results have been confirmed, using standards and achieved either by the presence of precipitation or by the change in the color.

In this study, the phytochemical screening carried out on the different aerial parts of *C. spinosa* (Tab. 5, Appendix 1) revealed the presence of diverse phytochemical groups; primary metabolites such as sugars, and secondary ones like polyphenols, flavonoids, alkaloids, tannins, coumarins, and saponins of therapeutic interest.

Tab. 5 Preliminary phytochemical screening of *C. spinosa* aerial parts.

		Plant parts			
		Leaves	Flowers	Stems	Fruits
Phytochemical tests (compounds classes)	1. Biuret Test (Proteins)	-	-	-	-
	2. Fehling test (sugars)	++	++	++	++
	3. Ferric chloride test (phenolic compounds)	+++	++	++	+++
	4. Tannins test	+	+	+	+
	5. Coumarins	++	++	++	+
	6. Alkaline reagent test (flavonoids)	++	+++	++	+++
	7. Anthraquinones test	++	+	++	++
	8. Foam test (saponins)	++	+	++	+
	9. Sterols and triterpenes test	+	+	+	+
	10. Dragendorff ‘s test (alkaloids)	+++	+++	+++	+++
	11. Volatile oils test (essential oil)	-	-	-	-

The results of the phytochemical screening (Tab. 5) revealed the presence of different groups of secondary metabolites in the four parts of the plant, as well as the presence of carbohydrates as primary metabolites. Proteins and essential oils were, however, absent in all the aerial parts of *C. spinosa*.

Indeed, polyphenols, flavonoids, and alkaloids were the most abundant compounds in the plant, whereby they have shown significant amounts in all the parts, in particular leaves, fruits, and flowers; respectively.

3.3- Quantitative analyses of *C. spinosa* extracts and fractions

3.3.1- Determination of extraction yield

After the extraction, the yield (Y%) of methanolic (MeOH) and aqueous (Aq) extracts, as well as of chloroform ($CHCl_3$), ethyl acetate (EtOAc), and n-butanol (n-BuOH) fractions from *C. spinosa* leaves and flowers (Tab. 6, Fig. 18) was determined.

Tab. 6 Yield and color of extracts and fractions from *C. spinosa* leaves and flowers.

Plant part / Extract	Leaves		Flowers	
	Y%	Color	Y%	Color
Methanol	15.88 ± 0.53	Dark green	13.75 ± 0.35	Yellow
Aqueous	15.00 ± 0.35	Green	12.60 ± 0.57	Brown
Chloroform	0.80 ± 0.01	Green	0.74 ± 0.02	Yellow
Ethyl Acetate	1.74 ± 0.02	Yellow	1.51 ± 0.02	Yellow
N-Butanol	9.97 ± 0.04	Brown	5.06 ± 0.08	Brown

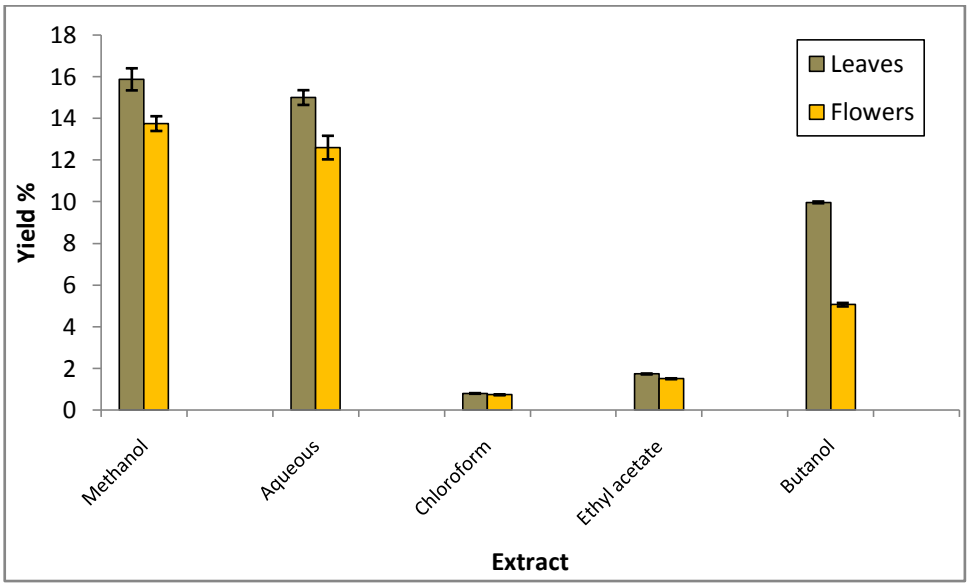


Fig. 18 Histogram showing the yield of different extracts and fractions from *C. spinosa* leaves and flowers.

In light of the results illustrated in Fig. 18 and Tab. 6, each extract was characterized by its Y % and its specific color relative to the dry matter. In fact, the Y% revealed a significant difference between the crude extracts (Y%; 12.60 ± 0.57 to 15.88 ± 0.53%) and the studied fractions (Y%; 0.74 ± 0.02 to 9.97 ± 0.04) depending on both the plant part and the extraction solvent used. Whereby, the leaf extracts and fractions showed high Y% values compared to those of the flowers; noting that the MeOH extract had the best Y% equal to 15.88 ± 0.53% close to that of the Aq extract of the same part, estimated at 15.00 ± 0.35%. Besides, the MeOH and the Aq extracts of flowers represented high Y% values of 13.75 ± 0.35% and 12.60 ± 0.57%, respectively, but lower than those of the leaves. However, the n-BuOH fractions exhibited less interesting values with a Y% equal to 9.97 ± 0.04 and to 5.06 ± 0.08% for leaves and flowers, consecutively. For the EtOAc and the $CHCl_3$ fractions, the Y% was very low estimated at 1.74 ± 0.02 and at 0.80 ± 0.01% for the

leaves, and equal to 1.51 ± 0.02 and 0.74 ± 0.02% for flowers, successively. The obtained extracts and fractions had different colors and yields.

On another aspect, the determination of extraction yield followed by the estimation of total polyphenols and total flavonoids contents. The principal reason for this choice of these chemical groups, in the fact, is that the main antioxidant and antimicrobial properties of plants are attributed to them.

3.3.2- Determination of total polyphenols content (Folin-Ciocalteu)

The total polyphenols content (TPC) estimated by the Folin-Ciocalteu colorimetric method was realized according to a linear calibration curve (y = 6.473 x + 0.071) of gallic acid, as a reference, at different concentrations. The correlation coefficient (R^2) for the standard curve exceeded 0.99 under the test conditions (Appendix 1). The obtained results of TPC are shown in Fig. 19 and Tab. 7.

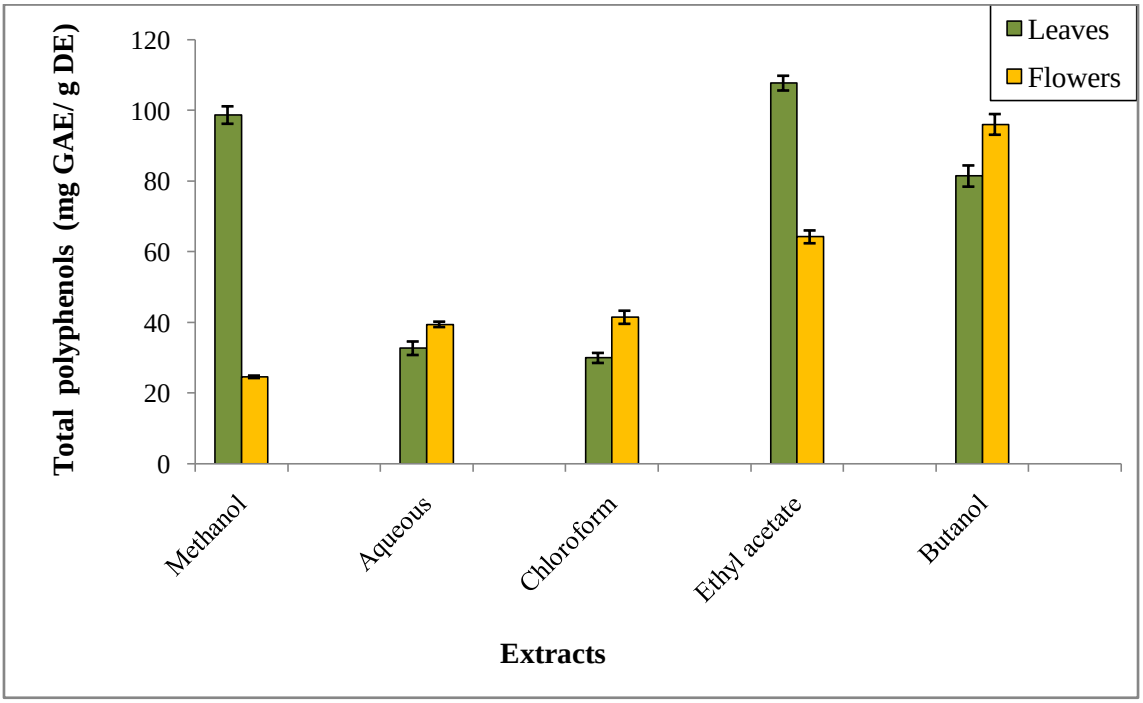


Fig. 19 Total polyphenols content in extracts and fractions from *C. spinosa* leaves and flowers. (Each value represents the average of three tests ± SD).

The amount of TPC has, considerably, varied in the examined plant extracts (24.63 ± 0.35 to 98.72 ± 2.47 mg GAE / g DE), and fractions (29.97 ± 1.42 to 107.75 ± 2.09 mg GAE / g DE). Therefore, the EtOAc fraction of the leaves had the highest TPC followed by that of the MeOH extract of the same part (107.75 ± 2.09 and 98.72 ± 2.47 mg GAE / g DE, respectively). Besides, the n-BuOH fractions of both parts, leaves and flowers, have shown

an important content (TPC= 81.45 ± 3.00 and 96.07 ± 2.93 mg GAE / g DE, consecutively). However, less interesting amounts were recorded in both the $CHCl_3$ fractions and the Aq extracts (41.48 ± 1.86 and 29.97 ± 1.42; 39.47 ± 0.76 and 32.71 ± 1.91 mg GAE / g DE) for flowers and leaves, consecutively. The lowest TPC has been observed in the MeOH extract of flowers equal to 24.63 ± 0.35 mg GAE / g DE.

3.3.3- Determination of total flavonoids content ($AlCl_3$)

At the same time, the quantification of total flavonoids content (TFC) was carried out according to the aluminum chloride method "$AlCl_3$" using the constructed calibration curve (Y= 2.918 x - 0.015) of quercetin as reference (Appendix 1). Where, the correlation coefficient exceeded 0.977. The obtained results of TFC in each extract and fraction are presented in Fig. 20 and Tab. 7.

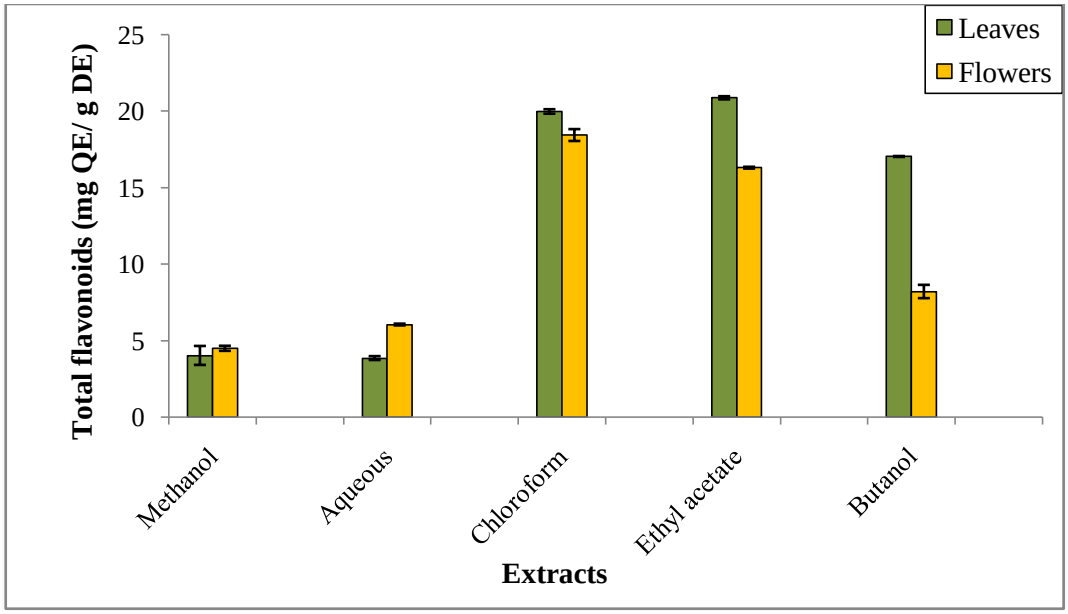


Fig. 20 Total flavonoids content in extracts and fractions from *C. spinosa* leaves and flowers. (Each value represents the average of three tests ± SD).

The amount of TFC has varied between 3.85 ± 0.13 and 20.87 ± 0.10 mg QE / g DE in the examined extracts and fractions, noting that these values of TFC are lower than those of TPC. Indeed, The highest quantity was recorded in the EtOAc fraction of leaves (20.87 ± 0.01 mg QE / g DE) followed by the two $CHCl_3$ fractions, with TFC of 19.98 ± 0.15 and of 18.44 ± 0.39 QE / g DE for leaves and flowers, consecutively.

Besides, interesting amounts equal to 17.03 ± 0.03 and to 16.30 ± 0.06 mg QE / g DE were, also, detected in the n-BuOH fraction of leaves and in the EtOAc fraction of flowers, respectively. In contrast, a low level of TFC was recorded in the n-BuOH fraction and the

Aq extract of flowers reached 08.20 ± 0.44 and 6.04 ± 0.06 mg QE / g DE, correspondingly. Likewise, the two MeOH extracts exhibited less important TFC equal to 4.02 ± 0.62 and to 4.48 ± 0.16 for leaves and flowers, respectively. Finally, the least significant value of TFC was observed in the Aq extract of leaves; 3.85 ± 0.13 mg QE / g DE.

Tab. 7 Total polyphenols and total flavonoids contents in extracts and fractions from *C. spinosa* leaves and flowers.

	Total polyphenols content (mg GAE / g DE)		Total flavonoids content (mg QE/ g DE)	
Plant parts / Extracts	Leaves	Flowers	Leaves	Flowers
Methanol	98.72 ± 2.47	24.63 ± 0.35	04.02 ± 0.62	04.48 ± 0.16
Aqueous	32.71 ± 1.91	39.47 ± 0.76	03.85 ± 0.13	06.04 ± 0.06
Chloroform	29.97 ± 1.42	41.48 ± 1.86	19.98 ± 0.15	18.44 ± 0.39
Ethyl acetate	107.75 ± 2.09	64.24 ± 1.82	20.87 ± 0.10	16.30 ± 0.06
N-Butanol	81.45 ± 3.00	96.07 ± 2.93	17.03 ± 0.03	08.20 ± 0.44

3.4- *In vitro* biological activities of *C. spinosa* extracts and fractions

All of the biological tests were performed at Laboratoire de Mycologie, de Biotechnologie et de l'Activité Microbienne (LaMyBAM), Département de Biologie Appliquée, FSNV, Université Frères Mentouri, Constantine 1, Algeria.

3.4.1- *In vitro* antioxidant activity

For the evaluation of the antioxidant activity of both extracts and fractions, two different methods have been developed; DPPH and FRAP. In the DPPH test, the value of inhibitory concentration at 50% (IC_{50}) is inversely proportional to the anti-radical capacity of a compound. At the same time, for the reducing power (FRAP), the value of the effective concentration at which the absorbance equal to 0.5 (EC_{50}) is, also, inversely proportional to the measured reducing power.

3.4.1.1- DPPH free radical scavenging activity

The anti-radical activity was estimated spectrophotometrically by following the reduction of DPPH at 517 nm.

Based on the data that represented the anti-radical power as a function of the different concentrations of the BHT (reference) and of the plant samples, their regression curves have been constructed (Appendix 1) to measure their IC_{50}.

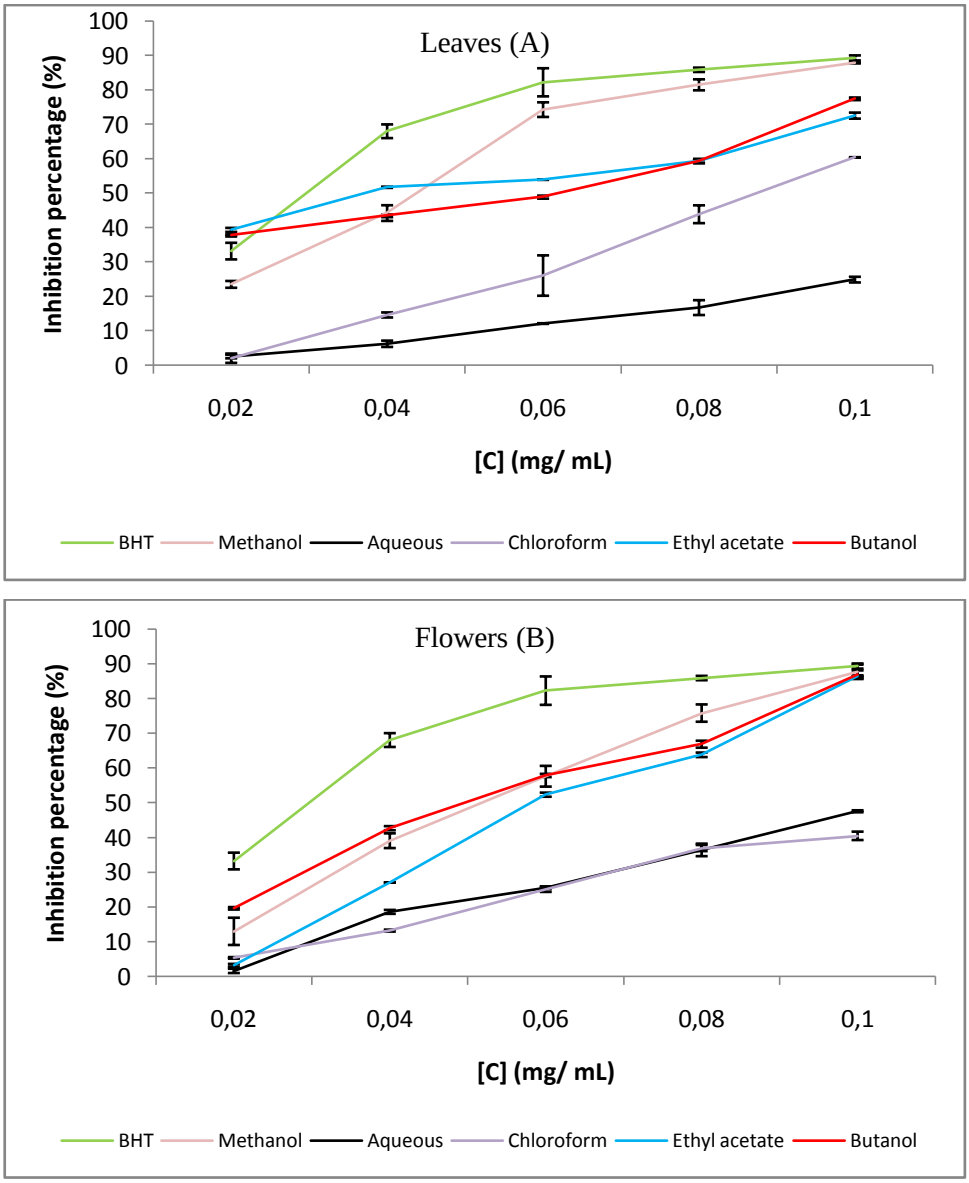


Fig. 21 DPPH radical scavenging activity (%) of BHT and *C. spinosa* extracts and fractions.

The anti-radical capacity of the plant extracts and fractions was inferior to that of the BHT that exerted the strongest activity equal to 89.38 ± 0.73% **(**Fig. 21) with the lowest IC_{50} of 34.73 ± 0.23 µg/mL.

For plant parts, the leaves samples were typically more active than those of flowers, exhibiting lower IC_{50}.

As shown in Fig. 22 and Tab. 8, the MeOH extract and the EtOAc fraction of leaves were, constantly, seemed to be the most efficient with the IC_{50} of 41.04 ± 0.15 and of 45.25 ± 1.8 µg/ mL, respectively. These lower values are close to that of the reference BHT.

Besides, the two fractions of n-BuOH exhibited a remarkable antioxidant activity, but lower than those of the two previous plant samples, with an IC_{50} of 52.8 ± 2.05 and of

53.95 ± 0.19 µg/ mL for leaves and flowers, consecutively. While, less interesting values of the IC_{50} reached 54.97 ± 0.7 and 63.3 ± 0.12 µg/ mL were, respectively, exhibited in the MeOH extract and the EtOAc fraction of flowers. Finally, the weakest antioxidant activity was exerted by the Aq extract of leaves with the highest IC_{50} value, 195.48 ± 0.81 µg/ mL.

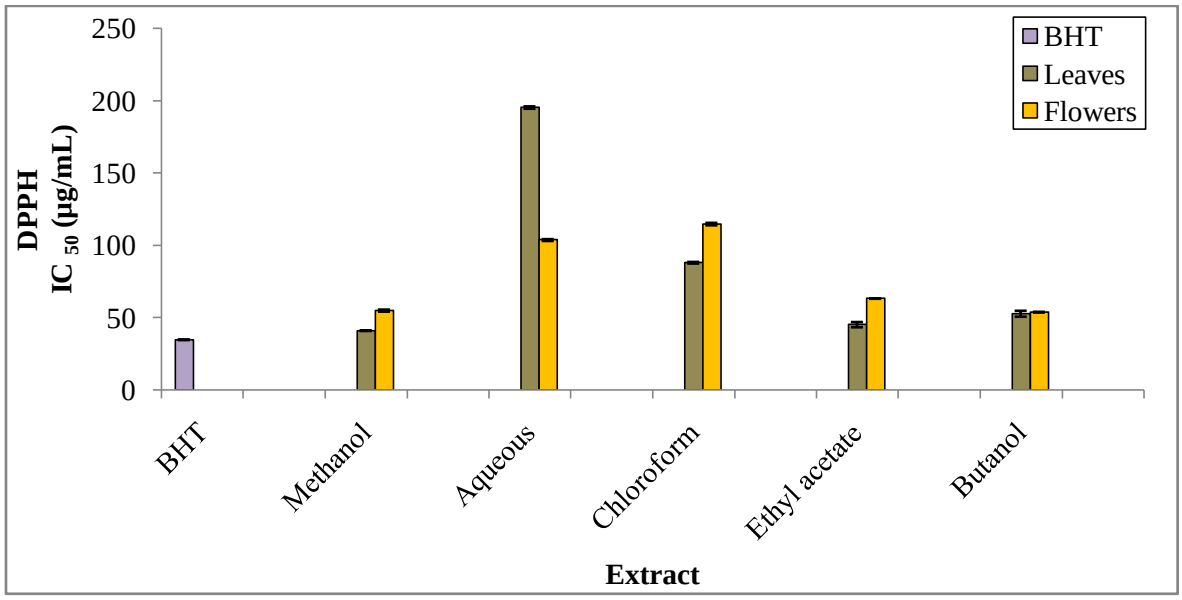


Fig. 22 Histogram representing the IC_{50} values of *C. spinosa* extracts and fractions using DPPH method. (Each value represents the average of three tests ± SD).

3.4.1.2- Ferric reducing antioxidant power (FRAP) assay

In the same way, as the previous test (DPPH), the antioxidant capacity of the examined extracts and fractions of the two parts, leaves and flowers, were also tested using the FRAP method, whereby, the ascorbic acid was used as an antioxidant reference. Based on the data that have revealed the absorbance as a function of the different concentrations of the ascorbic acid, as well as of the plant samples; their regression curves have, also, been constructed to measure their EC_{50} (Appendix 1). The results of the reducing activity (EC_{50}) are shown in Fig. 23 and Tab. 8.

Indeed, the leaves extracts and fractions, constantly, exhibited higher reducing powers compared to those of flowers, but significantly lower than that of ascorbic acid. Whereby, the MeOH extract and the EtOAc fraction of leaves were, evermore, the most powerful (EC_{50} = 763.73 ± 0.32 and 780.04 ± 1.36 µg/ mL, respectively). These significant low values are, strongly, close to that of ascorbic acid (EC_{50}= 684.29 ± 0.02 µg/ mL). Besides, the two BuOH fractions and the Aq extract of flowers, similarly, exhibited a strong power with the EC_{50} values of 831.83 ± 1.53, 852.92 ± 0.74, and 864.45 ± 1.10 µg/mL, consecutively. Conversely, less interesting values of the EC_{50}; 934.99 ± 1.44, 976.52 ±

0.74, and 954.93 ± 1.33 µg/mL have been shown in the flowers EtOAc fraction and MeOH extract, as well as in the leaves $CHCl_3$ fraction, correspondingly. The $CHCl_3$ fraction of flowers had also less important power with an EC_{50}= 1065.73 ± 0.47 µg/mL. In the end, the slightest activity was recorded in the Aq extract of leaves with the highest EC_{50} value equal to 1093.88 ± 1.73 µg/ mL. These results are very similar to those obtained in the DPPH assay.

Thus, it should be emphasized that the two used methods, DPPH and FRAP, in this work led to results confirming the interesting antioxidant effect of the plant.

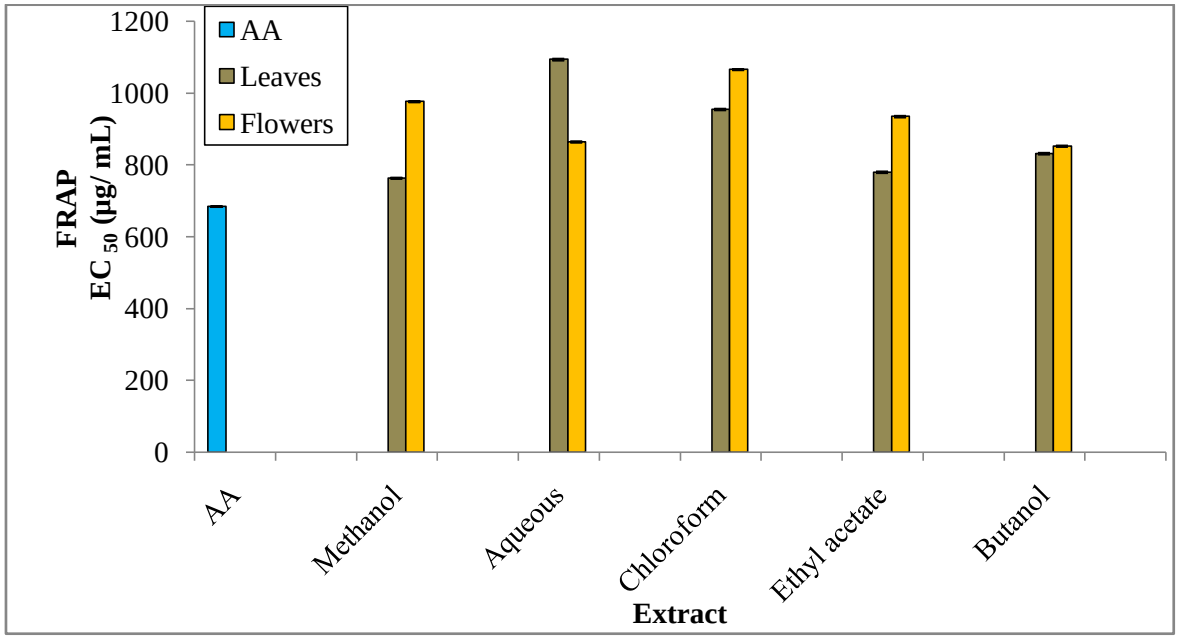


Fig. 23 Histogram representing the EC_{50} values of *C. spinosa* extracts and fractions using FRAP method. (Each value represents the average of three tests ± SD).

Tab. 8 IC_{50} and EC_{50} values of extracts and fractions from *C. spinosa* leaves and flowers using DPPH and FRAP methods.

	DPPH IC_{50} (µg/mL)		FRAP EC_{50} (µg/mL)	
Parts / Extracts	Leaves	Flowers	Leaves	Flowers
Methanol	41.04 ± 0.15	54.97 ± 0.7	763.73 ± 0.32	976.52 ± 0.74
Aqueous	195.48 ± 0.81	103.81 ± 0.63	1093.88 ± 1.73	864.45 ± 1.10
Chloroform	88.10 ± 0.57	114.85 ± 0.84	954.93 ± 1.33	1065.73 ± 0.47
Ethyl acetate	45.25 ± 1.8	63.3 ± 0.12	780.04 ± 1.36	934.99 ± 1.44
N-Butanol	52.8 ± 2.05	53.95 ± 0.19	831.83 ± 1.53	852.92 ± 0.74
BHT	34.73 ± 0.23		/	
AA	/		684.29 ± 0.02	
BHT: Butylated hydroxytoluene, AA : Ascorbic acid.				

On another side, and for the objective of linking between previous results of both the phenolic compounds contents and the antioxidant activity of the tested extracts and fractions; in the present study, an interesting positive correlation was, clearly, shown between the total polyphenols content (TPC) and the two antioxidant activities; whereby, 0.62 and 0.81 correlation coefficients have been recorded between TPC and DPPH test, as well as between TPC and FRAP assay, respectively.

3.4.2- *In vitro* antimicrobial activity

The previous antioxidant activity was followed by the study of the antimicrobial activity. At this level, this study was extended to several microorganisms where the different extracts and fractions showed interesting effects.

The antimicrobial test results of the studied plant samples against the tested microorganisms were shown in Figs. 24-36.

3.4.2.1- Antibacterial activity

In the present work, the studied extracts, MeOH and Aq, as well as the different fractions; $CHCl_3$, EtOAc, and n-BuOH from both parts of *C. spinosa;* leaves and flowers; in addition to the antibiotic were evaluated for their antibacterial effect by the disc diffusion technique against two Gram+ bacterial strains and four Gram- bacteria.

The *in vitro* antibacterial activity was translated by the appearance of inhibition zones around the impregnated discs by the studied extracts that have been dissolved in DMSO (2%) to obtain the required concentrations. The Inhibition Zone Diameter (IZD) (expressed in mm) differs from one bacterium to another, as well as from one extract to another (Figs. 24-29).

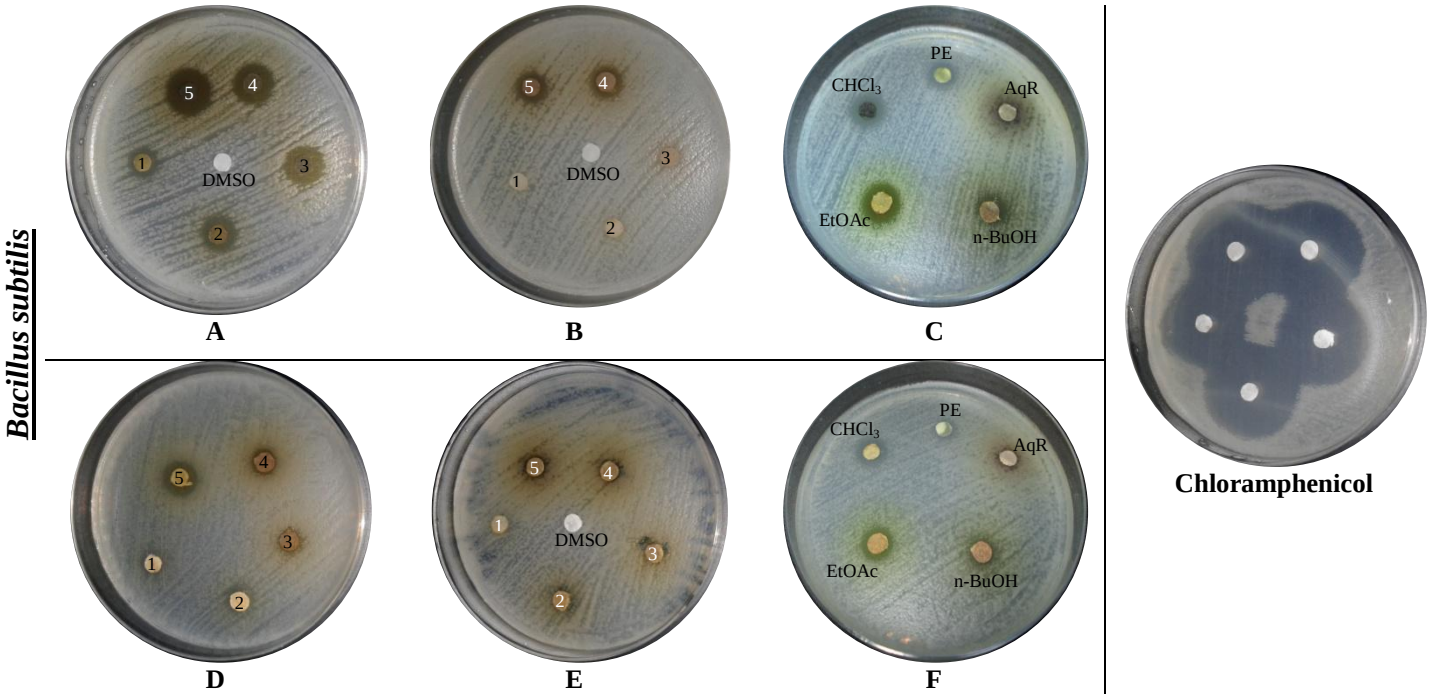


Fig. 24 Antibacterial activity of extracts and fractions from *C. spinosa* leaves and flowers against *Bacillus subtilis.*

A: Leaf MeOH, **B**: Leaf Aq, **C**: Leaf fractions, **D**: Flower MeOH, **E**: Flower Aq, **F**: Flower fractions, **1**: [C]= 0.125 mg/disc, **2**:[C]= 0.25 mg/disc, **3**:[C]= 0.5 mg/disc , **4**:[C]= 0.75 mg/disc, **5**:[C]= 1 mg/disc, **DMSO**:Dimethyl sulfoxide, **PE**: Petroleum Ether, **$CHCl_3$**:Chloroform, **EtOAc**: Ethyl acetate, **n-BuOH**: n-Butanol, **AqR**: Aqueous residue.

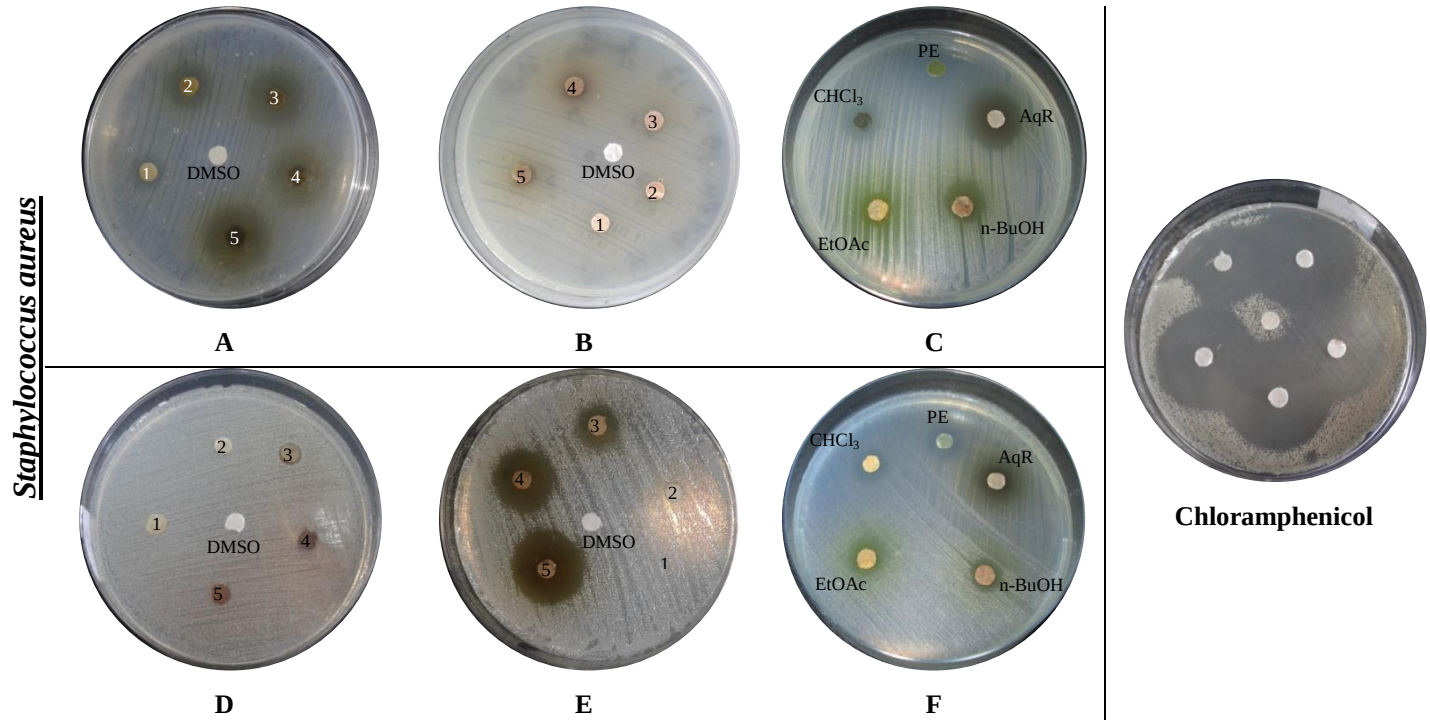


Fig. 25 Antibacterial activity of extracts and fractions from *C. spinosa* leaves and flowers against *Staphylococcus aureus.*

1 A: Leaf MeOH, **B**: Leaf Aq, **C**: Leaf fractions, **D**: Flower MeOH, **E**: Flower Aq, **F**: Flower fractions, **1**: [C]= 0.125 mg/disc, **2**:[C]= 0.25 mg/disc, **3**:[C]= 0.5 mg/disc , **4**:[C]= 0.75 mg/disc, **5**:[C]= 1 mg/disc, **DMSO**:Dimethyl sulfoxide, **PE**: Petroleum Ether, **$CHCl_3$**:Chloroform, **EtOAc**:Ethyl acetate, **n-BuOH**:n-Butanol, **AqR**: Aqueous residue.

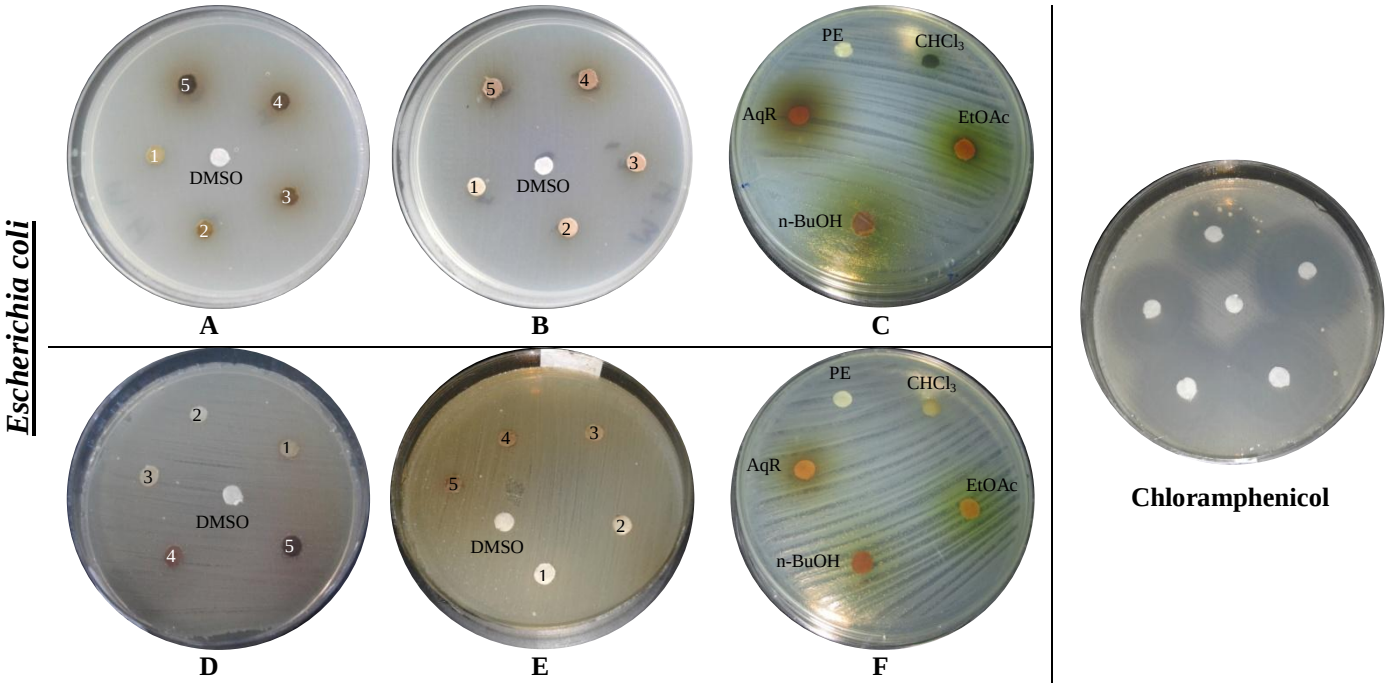


Fig. 26 Antibacterial activity of extracts and fractions from *C. spinosa* leaves and flowers against *Escherichia coli.*

1 A: Leaf MeOH, **B**: Leaf Aq, **C**: Leaf fractions, **D**: Flower MeOH, **E**: Flower Aq, **F**: Flower fractions, **1**: [C]= 0.125 mg/disc, **2**:[C]= 0.25 mg/disc, **3**:[C]= 0.5 mg/disc , **4**:[C]= 0.75 mg/disc, **5**:[C]= 1 mg/disc, **DMSO**:Dimethyl sulfoxide, **PE**: Petroleum Ether, **$CHCl_3$**:Chloroform, **EtOAc**:Ethyl acetate, **n-BuOH**:n-Butanol, **AqR**: Aqueous residue.

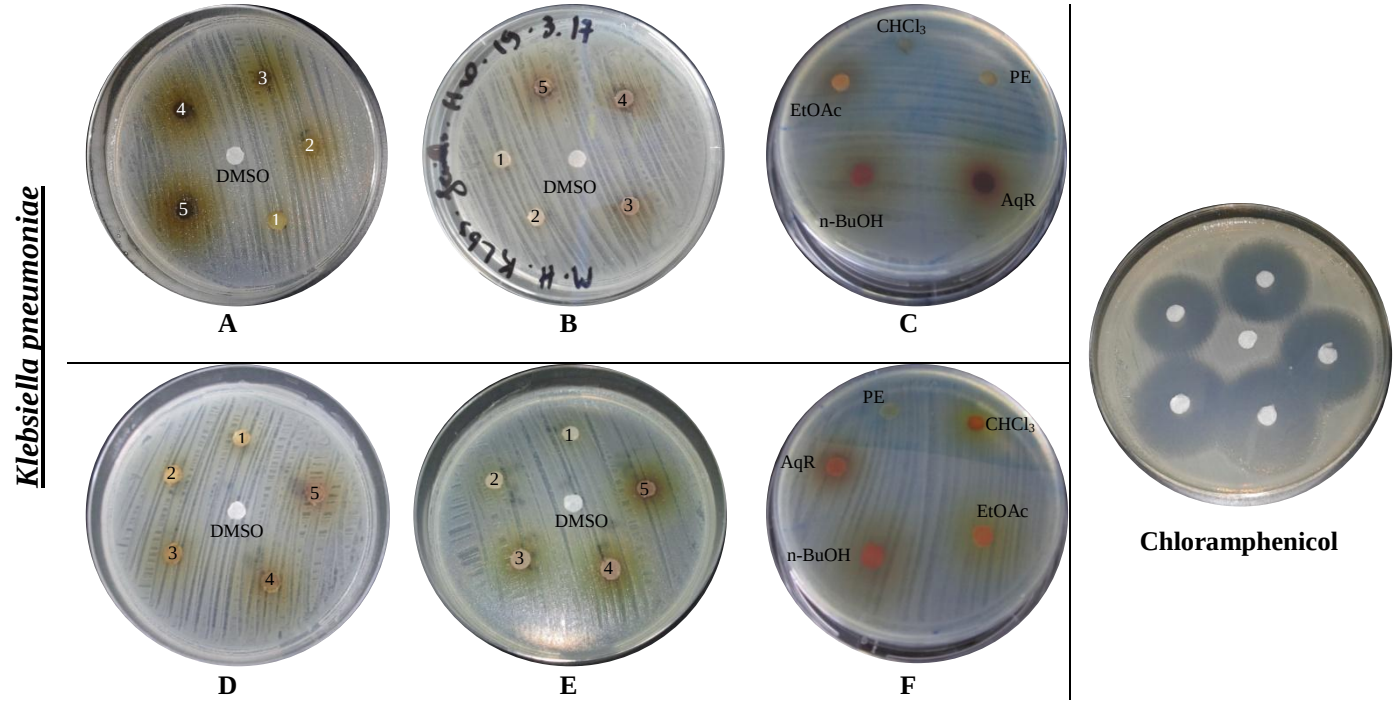


Fig. 27 Antibacterial activity of extracts and fractions from *C. spinosa* leaves and flowers against *Klebsiella pneumoniae.*

A: Leaf MeOH, **B**: Leaf Aq, **C**: Leaf fractions, **D**: Flower MeOH, **E**: Flower Aq, **F**: Flower fractions, **1**: [C]= 0.125 mg/disc, **2**:[C]= 0.25 mg/disc, **3**:[C]= 0.5 mg/disc , **4**:[C]= 0.75 mg/disc, **5**:[C]= 1 mg/disc, **DMSO**:Dimethyl sulfoxide, **PE**: Petroleum Ether, **$CHCl_3$**:Chloroform, **EtOAc**:Ethyl acetate, **n-BuOH**:n-Butanol, **AqR**: Aqueous residue.

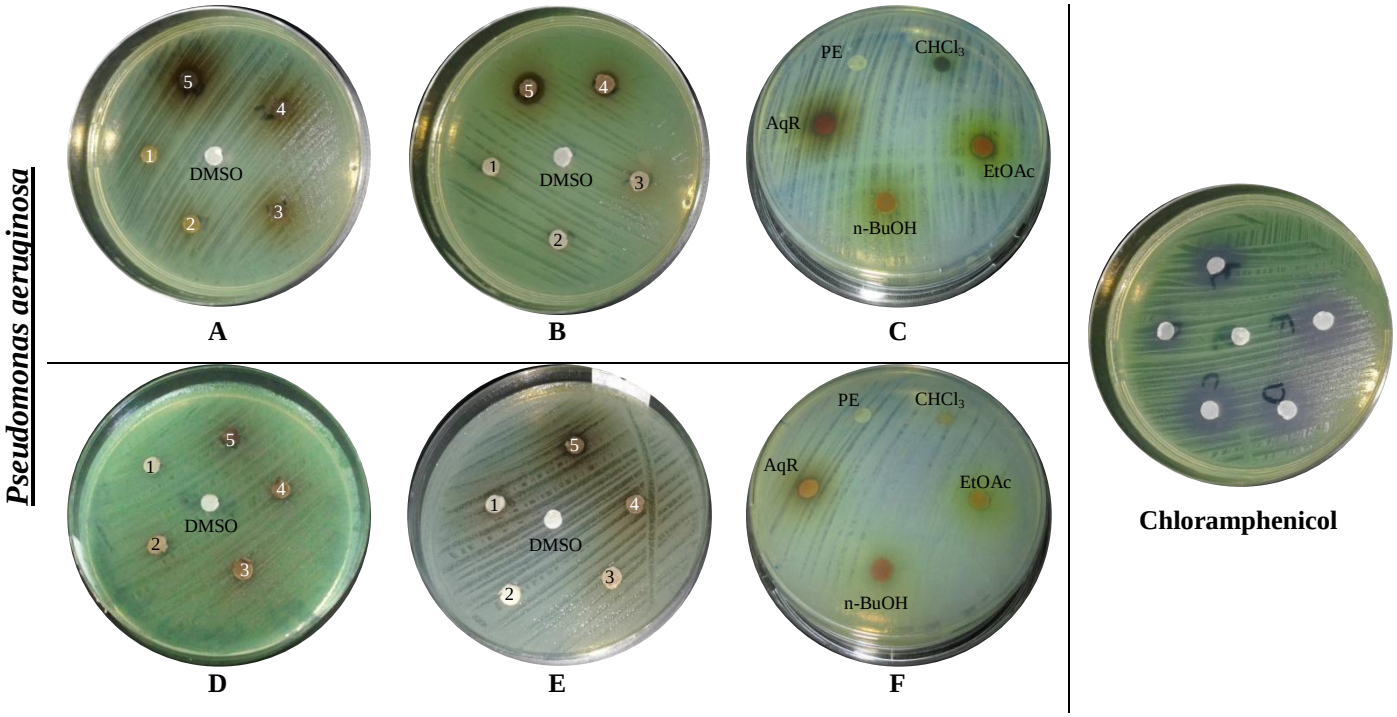


Fig. 28 Antibacterial activity of extracts and fractions from *C. spinosa* leaves and flowers against *Pseudomonas aeruginosa*.

A: Leaf MeOH, **B**: Leaf Aq, **C**: Leaf fractions, **D**: Flower MeOH, **E**: Flower Aq, **F**: Flower fractions, **1**: [C]= 0.125 mg/disc, **2**:[C]= 0.25 mg/disc, **3**:[C]= 0.5 mg/disc , **4**:[C]= 0.75 mg/disc, **5**:[C]= 1 mg/disc, **DMSO**:Dimethyl sulfoxide, **PE**: Petroleum Ether, **$CHCl_3$**:Chloroform, **EtOAc**:Ethyl acetate, **n-BuOH**:n-Butanol, **AqR**: Aqueous residue.

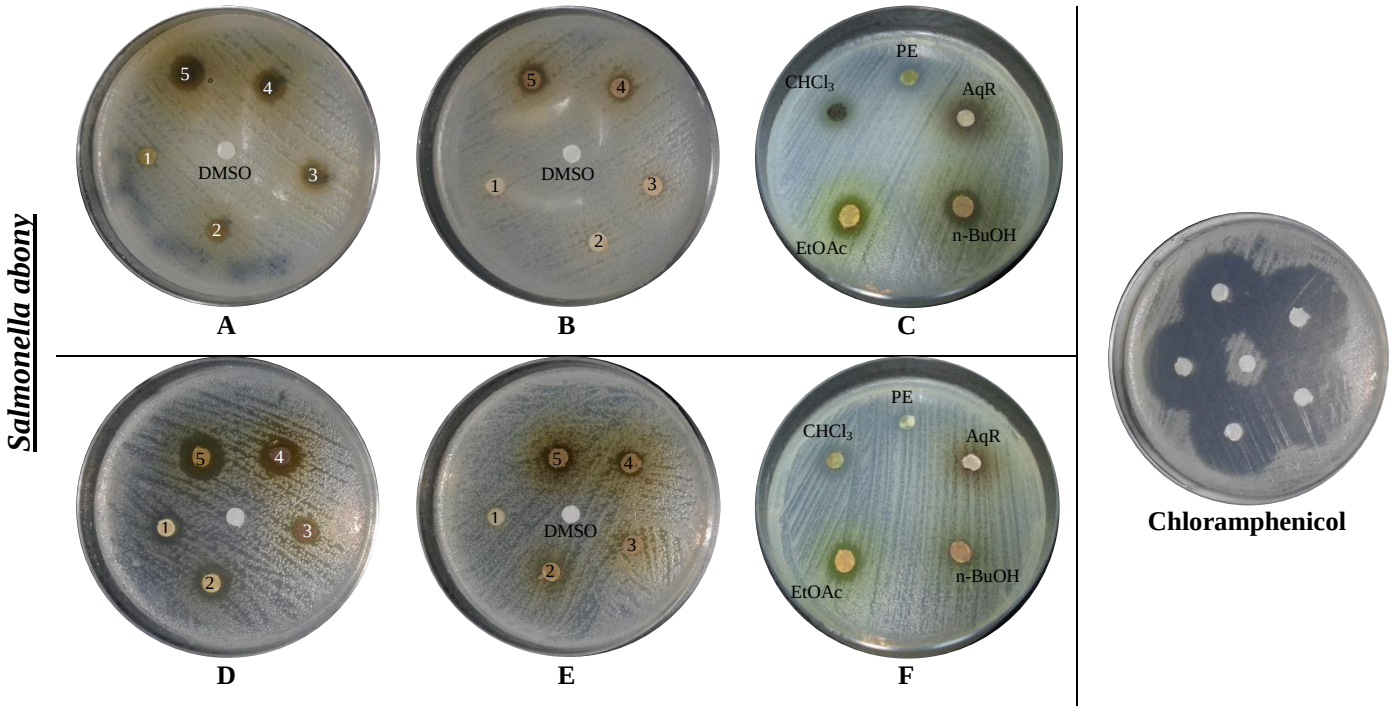


Fig. 29 Antibacterial activity of extracts and fractions from *C. spinosa* leaves and flowers against *Salmonella abony*.

A: Leaf MeOH, **B**: Leaf Aq, **C**: Leaf fractions, **D**: Flower MeOH, **E**: Flower Aq, **F**: Flower fractions, **1**: [C]= 0.125 mg/disc, **2**:[C]= 0.25 mg/disc, **3**:[C]= 0.5 mg/disc , **4**:[C]= 0.75 mg/disc, **5**:[C]= 1 mg/disc, **DMSO**:Dimethyl sulfoxide, **PE**: Petroleum Ether, **$CHCl_3$**:Chloroform, **EtOAc**:Ethyl acetate, **n-BuOH**:n-Butanol, **AqR**: Aqueous residue.

Indeed, the obtained results exhibited that the studied extracts and fractions were, frequently, active against most of the tested bacterial strains. This activity differs according to the used plant part, the extraction solvent, and the tested strain.

Tab. 9 Inhibition zones diameters of extracts and fractions from *C. spinosa* leaves and flowers against the tested microorganisms.

	Inhibition zone diameter (mm)							
	Extract (1 mg/ disc)	*B. subtilis*	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. abony*	*C. albicans*
Leaves	MeOH	16 ± 0.5^{b}	20 ± 0.28^{b}	$/^{e}$	$/^{e}$	$/^{d}$	12 ± 0.29^{b}	$/^{e}$
	Aq	11 ± 0^{b}	$/^{e}$	$/^{e}$	$/^{e}$	10 ± 0^{c}	10 ± 0^{c}	$/^{e}$
	$CHCl_3$	12 ± 0^{b}	11 ± 0^{b}	$/^{e}$	$/^{e}$	$/^{e}$	12 ± 0^{b}	$/^{e}$
	EtOAc	13 ± 0.65^{b}	11 ± 0.32^{b}	$/^{e}$	$/^{e}$	7 ± 0^{d}	16 ± 1.53^{b}	$/^{e}$
	n-BuOH	9 ± 0^{c}	11 ± 0^{b}	$/^{e}$	$/^{e}$	10 ± 0^{c}	9 ± 0^{c}	$/^{e}$
Flowers	MeOH	11.7 ± 0.03^{b}	14 ± 0.17^{b}	$/^{e}$	$/^{e}$	$/^{e}$	14.5 ± 0.2^{b}	$/^{e}$
	Aq	7.5 ± 0.07^{d}	17.1 ± 0.13^{b}	$/^{e}$	$/^{e}$	$/^{e}$	11 ± 0.87^{b}	$/^{e}$
	$CHCl_3$	11 ± 0.65^{b}	12 ± 0.76^{b}	$/^{e}$	$/^{e}$	$/^{e}$	12 ± 0.43^{b}	$/^{e}$
	EtOAc	11 ± 0.6^{b}	12 ± 0.08^{b}	$/^{e}$	$/^{e}$	$/^{e}$	12 ± 0.00^{b}	$/^{e}$
	n-BuOH	9 ± 0^{c}	9 ± 0^{c}	$/^{e}$	$/^{e}$	$/^{e}$	7 ± 0^{d}	$/^{e}$
	DMSO (10µL/ disc)	$/^{e}$	$/^{e}$	$/^{e}$	$/^{e}$	$/^{e}$	$/^{e}$	$/^{e}$
	ATB (15 µg/ disc)	33 ± 0.13^{a}	40 ± 0.07^{a}	28 ± 0.34^{a}	30 ± 0.17^{a}	32 ± 0.22^{a}	35 ± 0.44^{a}	NT
	ATF (10 µg/ disc)	NT	NT	NT	NT	NT	NT	35.2 ± 0.24^{a}
a: extremely high inhibition, b: high inhibition, c: moderate inhibition, d: low inhibition, e: no inhibition, NT: Not Tested.								

The IZD ranged from no inhibition to 20.00 ± 0.28 mm for the leaves extracts and fractions, and from no inhibition to 17.10 ± 0.13 mm for those of the flowers at the same concentration [C]=1 mg/ disc.

These values exerted, firstly, that the Gram + test bacteria were, typically, more sensitive to these extracts and fractions than the Gram- bacteria. Secondly, the leaves extracts and fractions were, generally, more active than those of flowers. Thirdly, the MeOH extracts and EtOAc fractions have, often, shown the strongest effect compared to the others. In fact, *S. aureus* was extremely sensitive to the MeOH extract of leaves with an inhibition zone (IZ) of 20.00 ± 0.28 mm in comparison with the other extracts. The EtOAc fraction of the same part was, also, active against the same bacterium, *S. aureus* but, with a lower IZ, estimated at 11 ± 0.32 mm. In addition, the bacterial strains; *B. subtilis* and *S. abony* showed significant sensitivities to the same previous extract and fraction, MeOH and EtOAc, with IZs of (16.00 ± 0.50 and 13 ± 0.65 mm) and (12 ± 0.29 and 16.00 ± 1.53 mm) respectively.

On the other hand, Aq extracts, n-BuOH, and $CHCl_3$ fractions of the two parts revealed a moderate or a less interesting antibacterial effect, with the exception of flowers Aq extract

that revealed a great inhibition towards *S. aureus* equal to 17.1 ± 0.13 mm. In the same, the least susceptible strains were, usually, the Gram- bacteria; *E. coli*, *P. aeruginosa* and *K. pneumoniae* with either no inhibition or less important IZD developed by the studied samples. The antibiotic, in particular the chloramphenicol as a positive control, had the strongest activity on all the tested bacteria compared to plant various extracts and fractions.

The minimal inhibitory concentration (MIC) of each active extract against the sensitive bacterial strains was individually measured. It ranged from ≤ 0.125 to 1.00 mg/ disc for the extracts and fractions of leaves, and from 0.125 to 1.00 mg/disc for those of flowers. The lower MIC values were revealed towards *B. subtilis, S. aureus, and S. abony* by the MeOH extracts and the EtOAc fractions of both plant parts.
On another aspect, and according to the ratio MBC/MIC, the antibacterial activity was also valued. If the ratio MBC/MIC was ≤ 4, the effect was considered as bactericidal; but if the ratio MBC/MIC > 4, the activity was defined as bacteriostatic (Levison, 2004; Benjamin et *al.*, 2012).

In fact, it was found that the MeOH extract of leaves was bactericidal against *B. subtilis*, *S. aureus*, and *S. abony* with a MIC estimated at ≤ 0.125 mg/ disc and a MBC of 1.00 mg/ disc. Besides, the leaves' EtOAc fraction was bactericidal against *S. abony*. Whereas, Gram- bacteria; *E. coli*, *P. aeruginosa*, and *K. pneumoniae* were less sensitive to the effect.
As a conclusion of this part, the most promising activity was, effectively, displayed against the Gram+ bacteria; *S. aureus* and *B. subtilis*, as well as against the Gram- bacterium; *S. abony*.

3.4.2.2- Antifungal activity

All the previous extracts and fractions from the two parts of *C. spinosa* were, further, tested against the pathogenic yeast *Candida albicans* using the disc diffusion method as the antibacterial test. However, the only polar extracts; MeOH and Aq; of both plant parts, leaves and flowers, were selected to carry out the antifungal test against six phytopathogenic fungi isolated from durum wheat grains. This latest antifungal activity was carried out with the objective of using the studied plant in agro-alimentary applications.

The antifungal effect on the mycelial growth compared to the negative control was reflected by a decrease in the mycelial growth and modifications in its macroscopic aspects or one of them.

- **Isolation, purification, and identification of phytopathogenic fungi**

Isolation of phytopathogenic fungi allowed us to have a variety of isolates with different macroscopic and microscopic aspects.

A total number of 34 purified fungal isolates were obtained from a sample of durum wheat grains cultured on three Petri dishes. Macroscopic and microscopic studies have allowed highlighting six fungal genera (Tab. 11), presented in the predominance decreasing order as follows: *Alternaria, Penicillium, Aspergillus, Rhizopus, Fusarium, and Chaetomium*. *Alternaria* is the predominant genus with a frequency of 76.47% of the isolated fungi, represented by many species. After that, the genus *Penicillium* comes representing 11.76% of all isolates grouping together four different species. The *Aspergillus,* the *Rhizopus*, the *Chaetomium,* and the *Fusarium* genera represent respectively 2.94% of all isolated molds with only one species for each (Tab. 10).

The obtained results show that there is a variation of the fungi with different aspects.

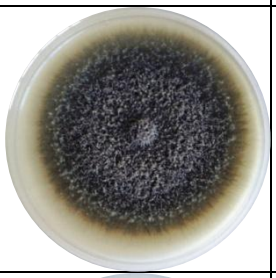
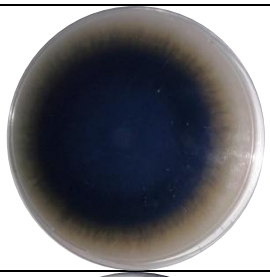
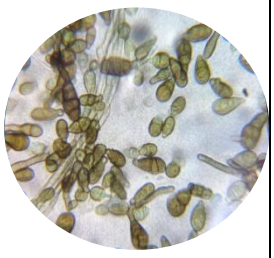
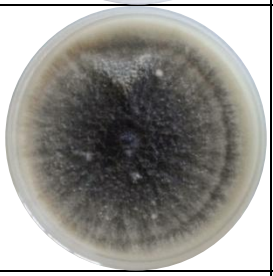
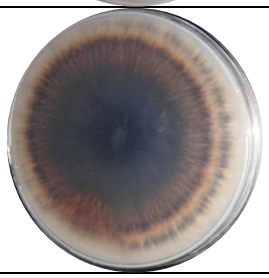
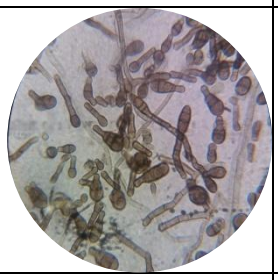
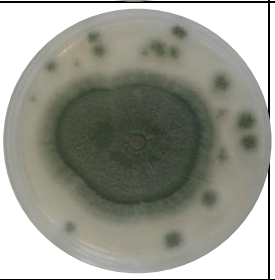
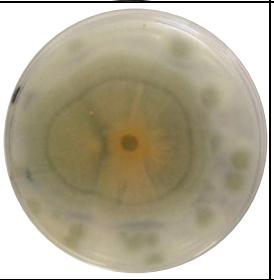
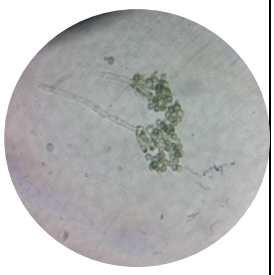
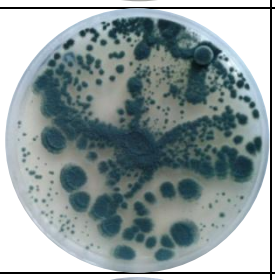
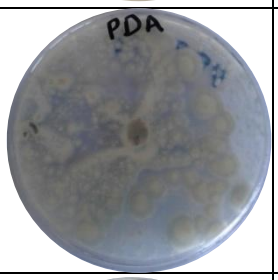
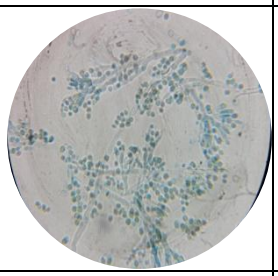
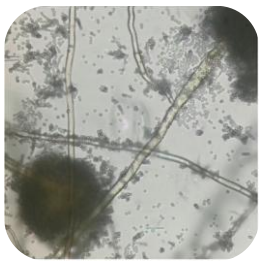
Tab. 10 Fungal isolates from a sample of durum wheat grains.

Fungal isolates	Total number of purified isolates	Appearance percentage %
Alternaria spp.	**26**	**76.47**
Alternaria sp.1	7	20.59
Alternaria sp.2	7	20.59
Alternaria sp.3	5	14.71
Alternaria sp.4	4	11.76
Other Alternaria	**3**	8.82
Penicillium spp.	**4**	**11.76**
Penicillium sp.1	1	2.94
Penicillium sp.1	1	2.94
Penicillium sp.1	1	2.94
Penicillium sp.1	1	2.94
Aspergillus sp.	**1**	**2.94**
Rhizopus sp.	**1**	**2.94**
Chaetomium sp.	**1**	**2.94**
Fusarium sp.	**1**	**2.94**
Total	**34**	**100**

To identify a fungus, it is first to recognize its genus, which is a group of organisms linked together by common characteristics (Cahagnier and Richard-Molard, 1998).
The isolates showed different characters with various colors.
A preliminary classification of fungal isolates with the same characteristics; the morphological appearance of the mycelium, the thallus color, and the growth rate; was achieved.
The six fungal isolates selected for the antifungal test are species causing deterioration of cereals, health risks (the secretion of mycotoxins), or both.

Macroscopic and microscopic characters of the selected isolates for antifungal activity are illustrated in Tab. 11.

Tab. 11 Macroscopic and microscopic characteristics of tested fungal isolates.

Fungal isolate	Macroscopic aspect		Microscopic aspect (X100)	Appearance
	Surface	Reverse		
Alternaria sp.1				This fungus grows quickly with green colonies. The reverse side is dark green to black. Under a microscope (X100), its hyphae and conidiophores are septate.
Alternaria sp.2				It grows rapidly, the colonies initially white-gray and quickly become dark. The reverse side is dark green. Under the microscope (X100), hyphae and conidiophores are also septate.
Penicillium sp.1				This fungus grows gently on the culture media used in mycology; its growth is medium with a powdery colony of pistachio green color. The reverse is white. Under a microscope, septate hyphae bearing conidiophores, phialides are arranged in a spiral at the end of conidiophores.
Penicillium sp.2				This fungus grows easily on the culture media used in mycology; its growth is fast with a powdery colony of green color. The reverse is white.
Aspergillus sp.				The growth of this fungus is a little slow 5 to 7 days, its colonies are powdery white to cream, beige to brown in color, yellow to green-brown on the back. Under a microscope we can see globular vesicles, conidia globose smooth.
Rhizopus sp.				The colonies of this strain grow very quickly and extensively, have a cottony texture with white color. Under a microscope (X100) we can see broad non-septate filaments of the conidia.

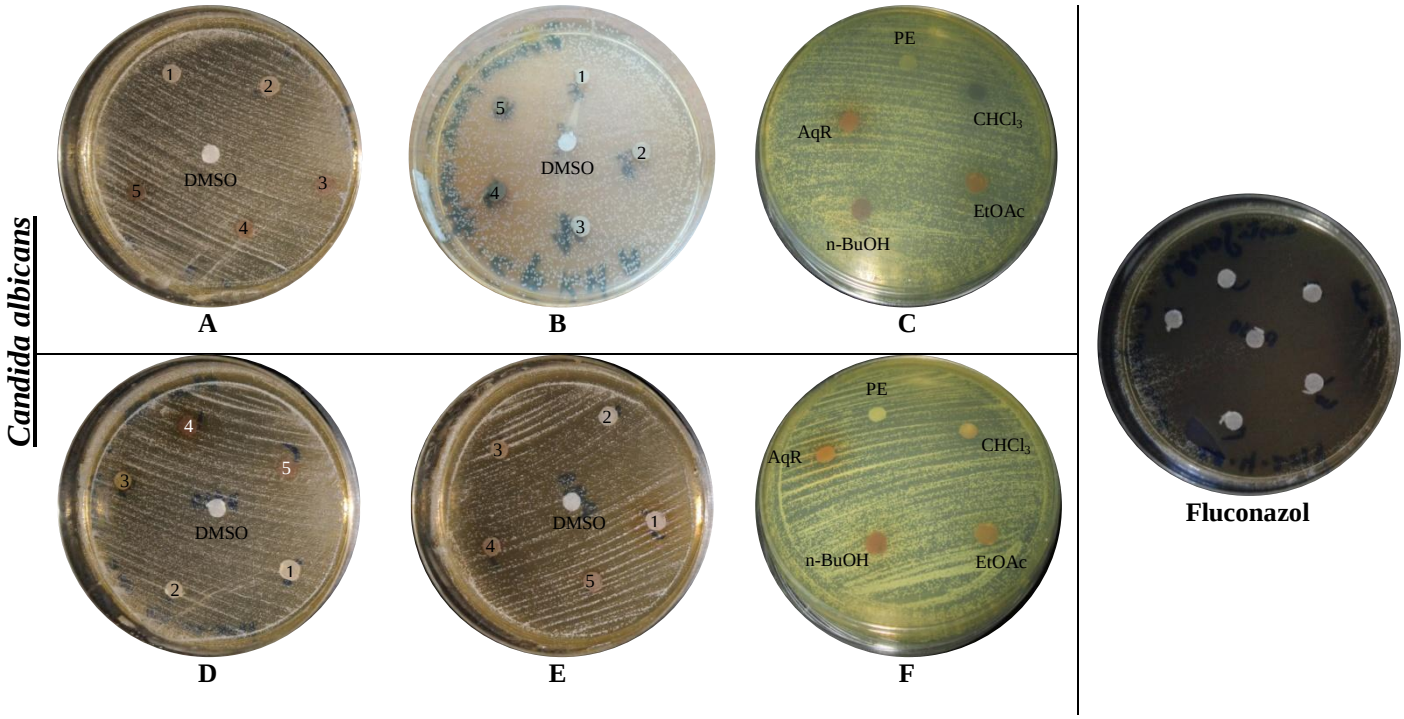


Fig. 30 Antifungal activity of extracts and fractions from *C. spinosa* leaves and flowers against *Candida albicans*.

A: Leaf MeOH, **B**: Leaf Aq, **C**: Leaf fractions, **D**: Flower MeOH, **E**: Flower Aq, **F**: Flower fractions, **1**: [C]= 0.125 mg/disc, **2**:[C]= 0.25 mg/disc, **3**:[C]= 0.5 mg/disc , **4**:[C]= 0.75 mg/disc, **5**:[C]= 1 mg/disc, **DMSO**:Dimethyl sulfoxide, **PE**: Petroleum Ether, **$CHCl_3$**:Chloroform, **EtOAc**:Ethyl acetate, **n-BuOH**:n-Butanol, **AqR**: Aqueous residue.

Indeed, the antifungal activity against *C. albicans* was realized and no inhibition zone (IZ) was observed by all *C. spinosa* extracts and fractions. However, the fluconazol, as a positive control, showed a remarkable activity with an IZ equal to 50.2 ± 0.4 mm at [C] = 10 µg/ disc (Fig.30, Appendix 2).

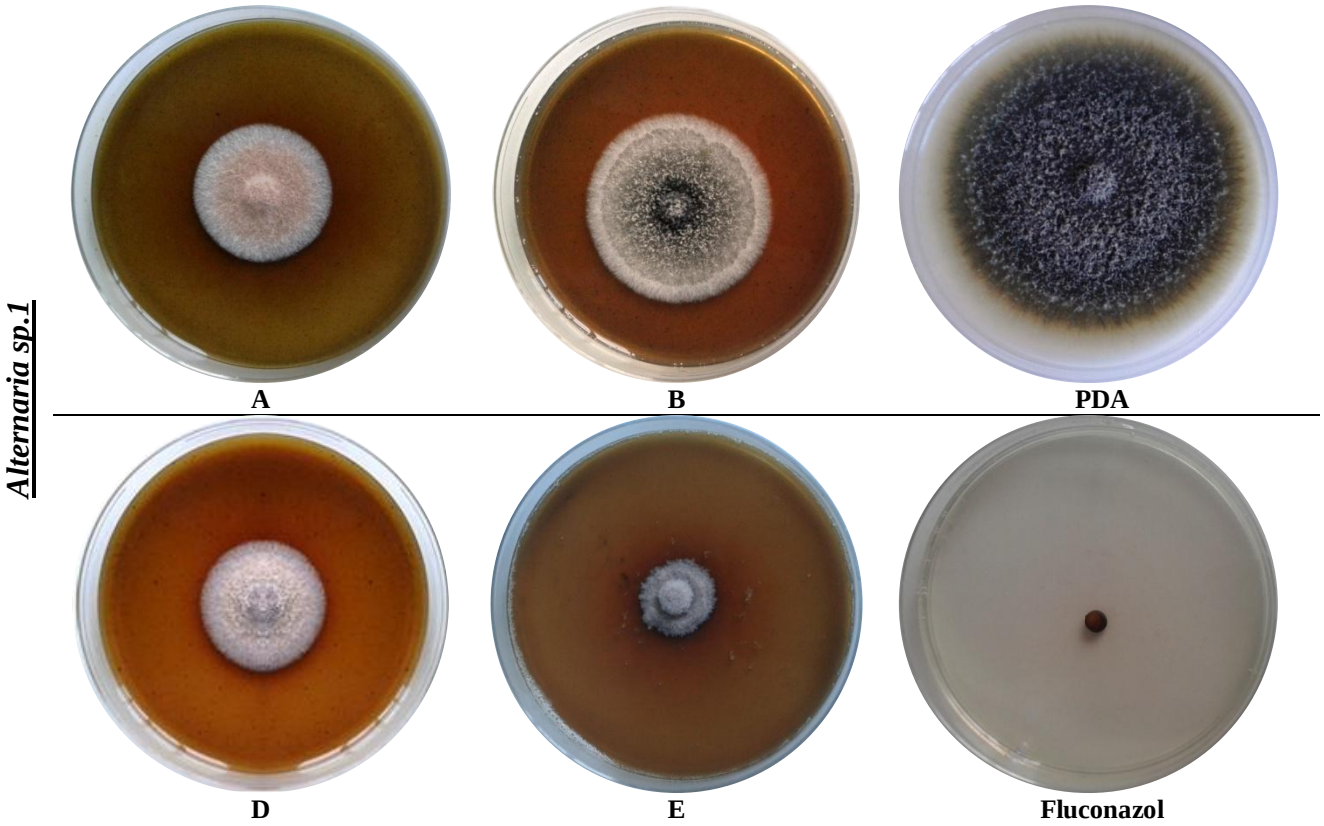


Fig. 31 Antifungal activity of extracts from *C. spinosa* leaves and flowers against *Alternaria sp.1*.
A: Leaf MeOH, **B**: Leaf Aq, **D**: Flower MeOH, **E**: Flower Aq.

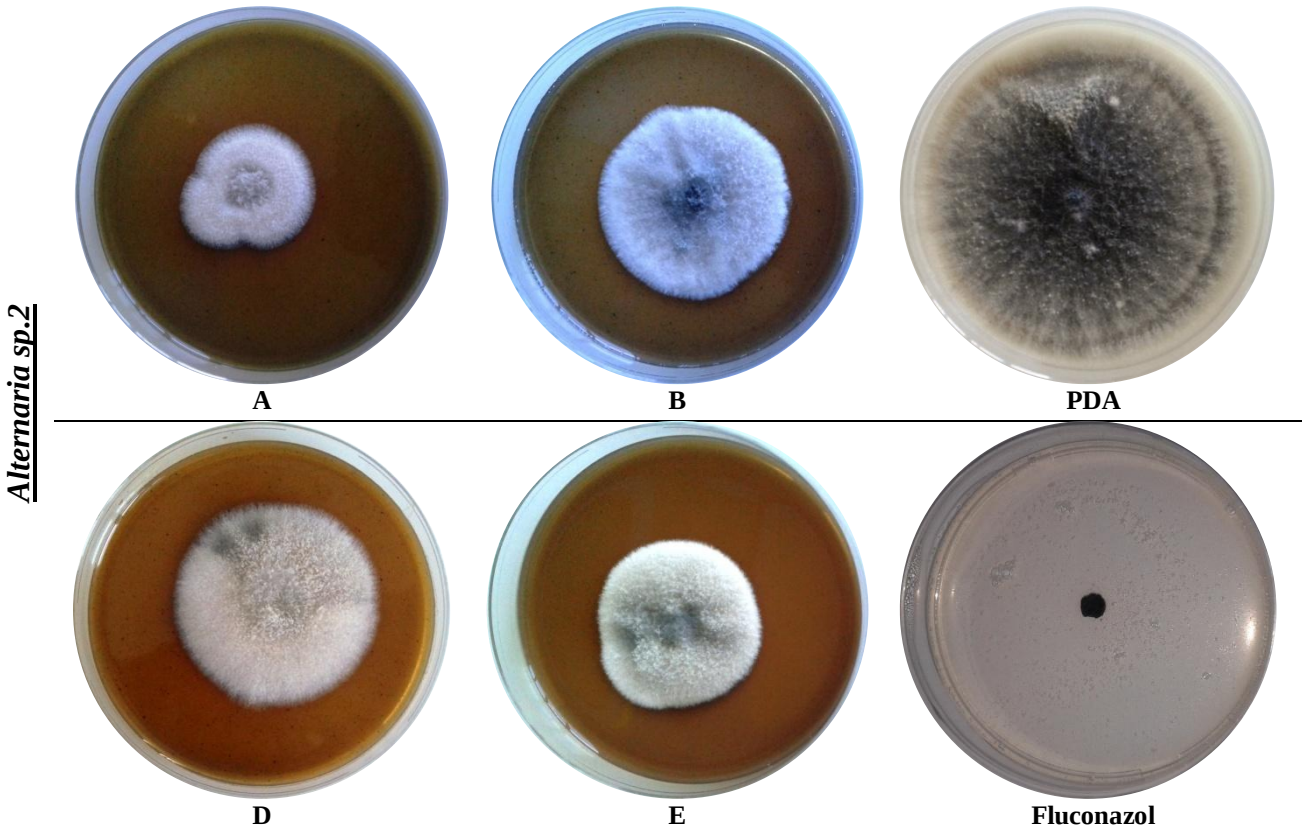


Fig.32 Antifungal activity of extracts from *C. spinosa* leaves and flowers against *Alternaria sp.2*.
A: Leaf MeOH, **B**: Leaf Aq, **D**: Flower MeOH, **E**: Flower Aq.

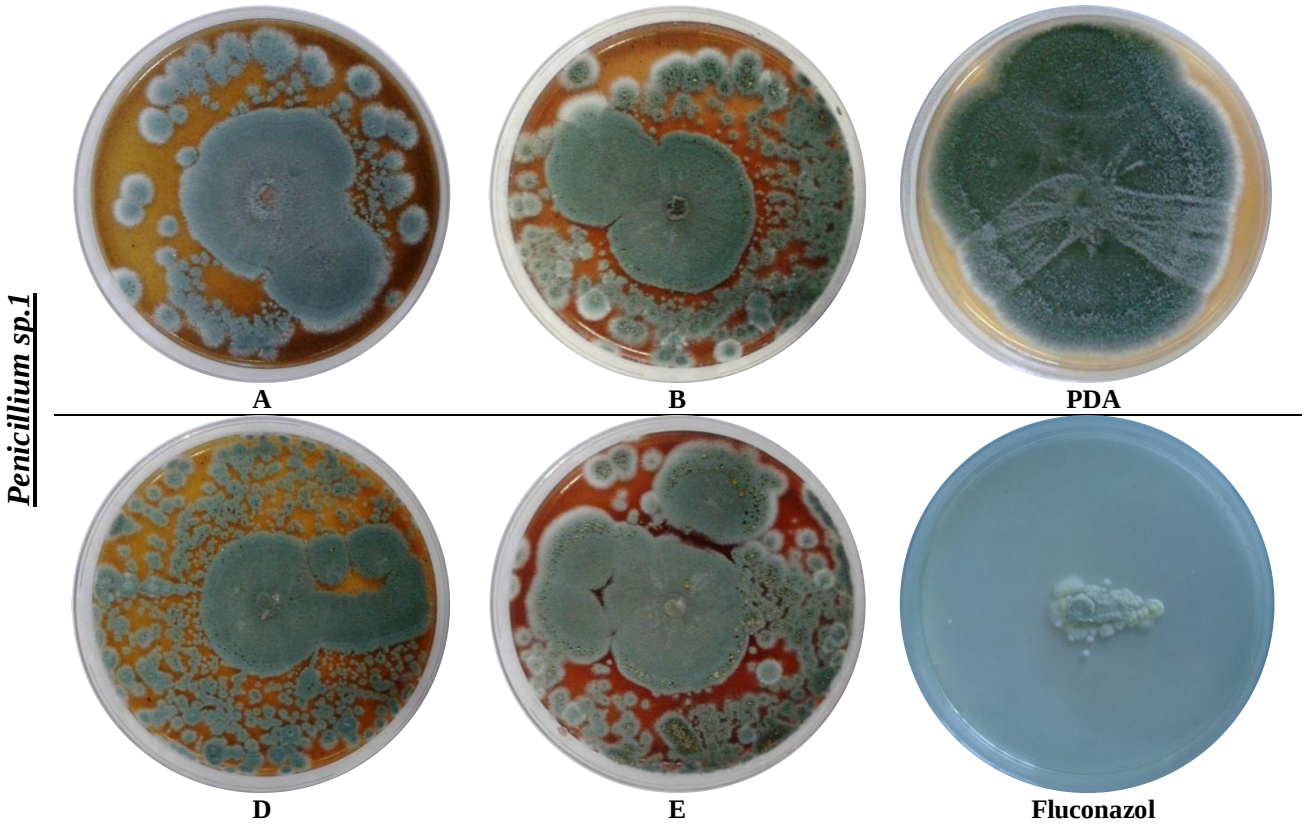


Fig. 33 Antifungal activity of extracts from *C. spinosa* leaves and flowers against *Penicillium sp.1*.
A: Leaf MeOH, **B**: Leaf Aq, **D**: Flower MeOH, **E**: Flower Aq.

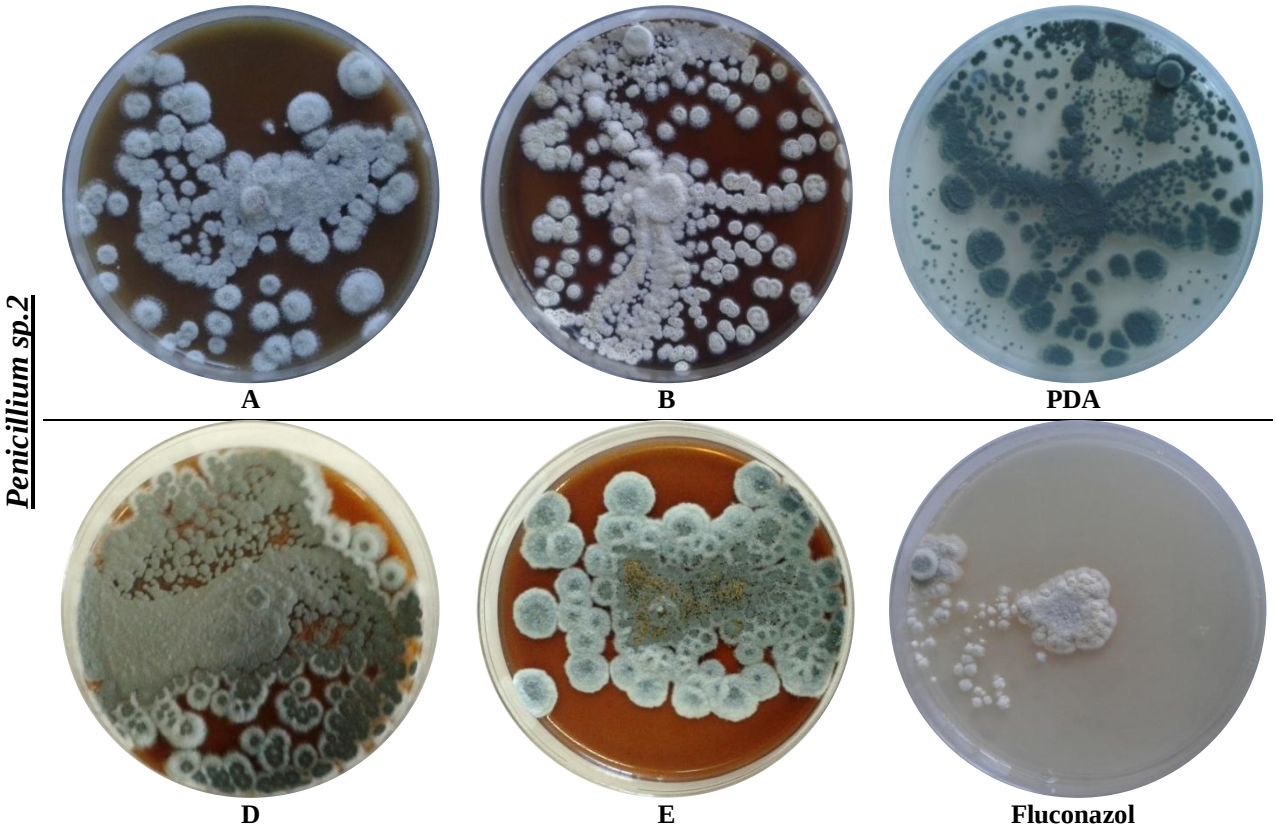


Fig. 34 Antifungal activity of extracts from *C. spinosa* leaves and flowers against *Penicillium sp2.*
A: Leaf MeOH, **B**: Leaf Aq, **D**: Flower MeOH, **E**: Flower Aq.

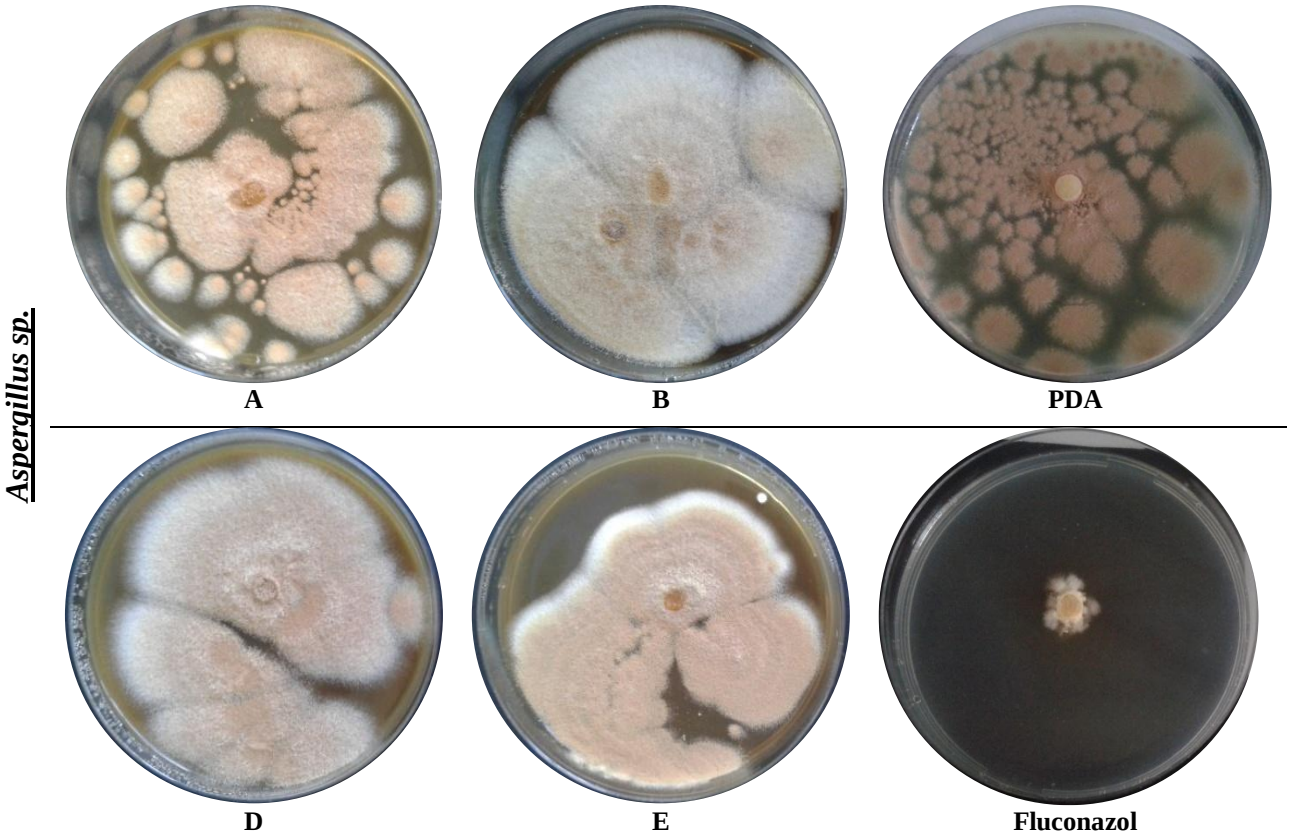


Fig. 35 Antifungal activity of extracts from *C. spinosa* leaves and flowers against *Aspergillus sp.*
A: Leaf MeOH, **B**: Leaf Aq, **D**: Flower MeOH, **E**: Flower Aq.

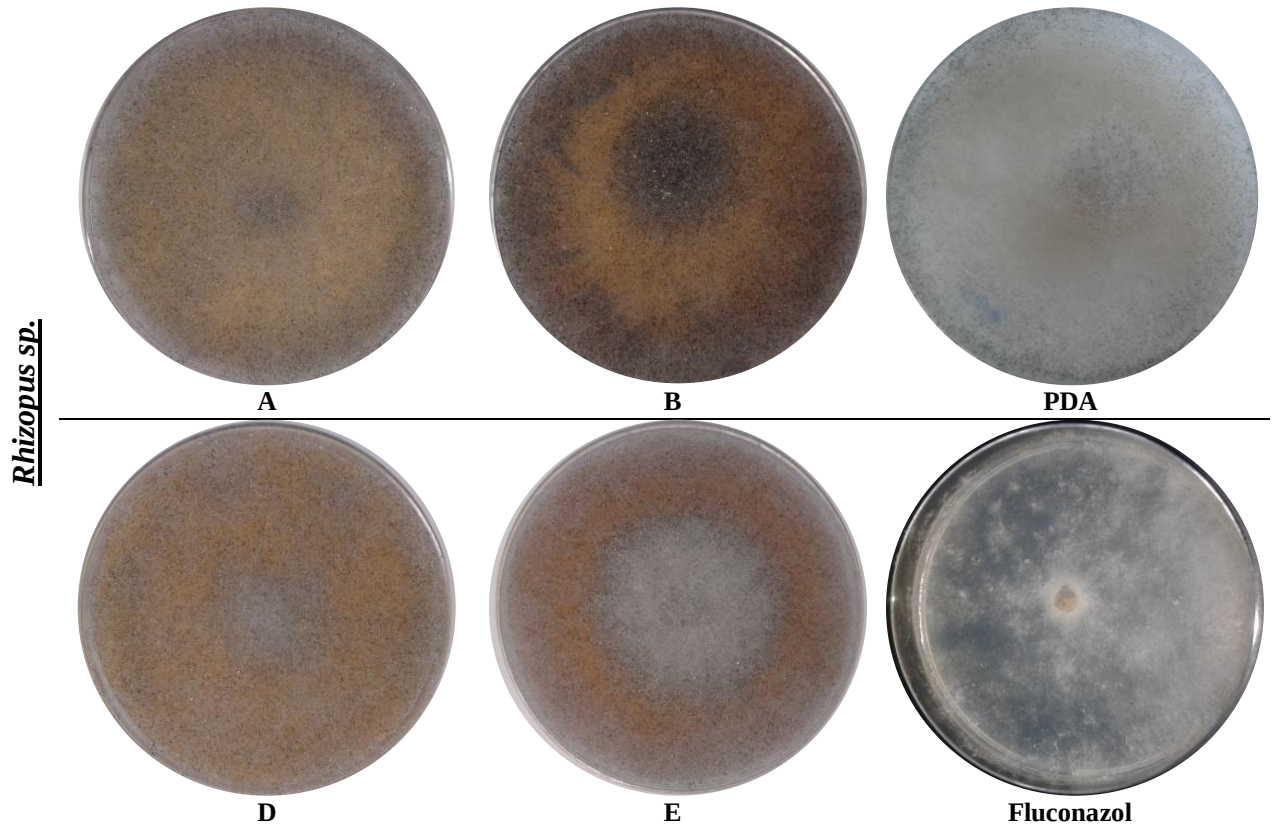


Fig. 36 Antifungal activity of extracts from *C. spinosa* leaves and flowers against *Rhizopus sp.*
A: Leaf MeOH, **B**: Leaf Aq, **D**: Flower MeOH, **E**: Flower Aq.

At the same time, the inhibition percentage (IP %) of the selected polar extracts, MeOH and Aq, on the six tested molds, varying from 0 % to 61.97 %.

It was found that all the extracts exhibited an antifungal activity at 20 mg / mL on two out of six tested fungal species. Whereby, the two tested phytopathogenic fungal species of *Alternaria* showed sensitivity to all extracts (MeOH and Aq) with varying IP %; *Alternaria sp.1* was the most susceptible fungus with an IP % of 48.59% and 30.55% developed, respectively, by MeOH and Aq extracts of leaves, and an IP% of 41.54% and 61.97% exhibited by those of flowers, consecutively. Besides, *Alternaria sp.2* was also sensitive to the tested extracts with IPs% lower than those of the previous species; 43.05% and 30.55% for MeOH and Aq extracts of leaves, as well as 26.25% and 38.75% for those of flowers; consecutively. However, no antifungal activity was shown against the other four tested phytopathogenic fungi; *Penicillium sp.1*, *Penicillium sp.2*, *Aspergillus sp.*, and *Rhizopus sp.* (Tab. 12).

Tab. 12 Inhibition percentage of polar extracts from *C. spinosa* leaves and flowers against fungal isolates.

IP %		Fungal species					
		Alternaria sp.1	*Alternaria sp.2*	*Penicillium sp.1*	*Penicillium sp.2*	*Aspergillus sp.*	*Rhizopus sp.*
Extracts (20 mg/ mL)	Leaf MeOH	48.59 ± 0.05	43.05 ± 0.6	0	0	0	0
	Leaf Aq	30.55 ± 0.34	30.55 ± 1.01	0	0	0	0
	Flower MeOH	41.54 ± 0.52	26.25 ± 0.64	0	0	0	0
	Flower Aq	61.97 ± 0.71	38.75 ± 0.43	0	0	0	0
+ control	Fluconazol (1g/L)	100 ± 0.00	100 ± 0.00	77.78 ± 0.81	66.67 ± 0.76	88.89 ± 0.45	11.11 ± 0.2

The extract having the highest extraction yield, the interesting phenolic compounds content, and the best biological activities was selected for further work. In fact, the MeOH extract of leaves could be identified as being the most active. This extract was subjected to both Liquid Chromatography Electrospray Ionization- Trap- Mass Spectrometry (LC-ESI-Trap MS/MS) analysis and fractionation by Column Chromatography (CC). This later resulted in the production of five enumerated compounds; (1), (2), (3), (4), and (5).

3.5- LC-ESI-Trap MS/MS analysis of leaves methanol extract

In order to identify the majority of molecules present in the chosen leaves MeOH extract of *C. spinosa*; the MS profiles using LC-ESI-MS/MS were analyzed.

The ion trap is configured to examine two events. The first is the *m/z* scan in "full MS" and the second is the fragmentation of the five (5) most intense ions when a threshold of intensity is exceeded. The Base Peak Chromatogram (BPC) obtained from the leaves methanol extract in negative mode is presented in Fig. 37.

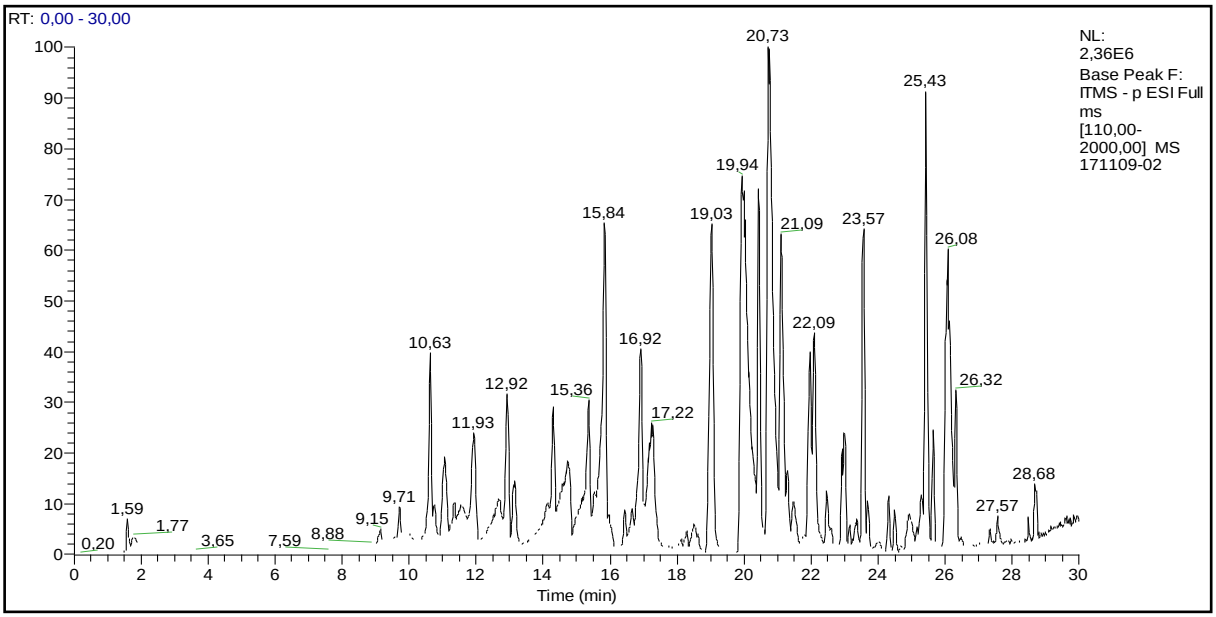


Fig. 37 LC-ESI-MS Base Peak Chromatogram (BPC) of leaves methanol extract from *C. spinosa* (L.) Link obtained with a reversed-phase C18 column in negative mode.

To elucidate the structures of chemical compounds in *C. spinosa* leaves, the methanol extract was subjected to a combination of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and fitted with an electrospray ionization source (ESI) (Tab.13).

Indeed, the profile of the MeOH extract of the leaves was analyzed by LC-ESI-MS (in negative and positive modes) in the range of *m/z* 110–2000 Da. Whereby, the initial analysis of this extract indicated that its ionization, in negative mode, revealed several peaks, corresponding to various and very high quantities of phenolic compounds compared to the positive mode, where few peaks were detected. In fact, this mode has been shown to be very effective for the ionization of polyphenols and flavonoids (Rodrigues et *al.*, 2007) that are often associated with plants' bioactive metabolites (Daayf et *al.*, 2012). Furthermore, the identification and the characterization of the compounds were carried out

by LC-ESI-MS^2 fragmentation data and by comparison with authentic standards in the literature; some of them have been confirmed using commercial references (listed in chemicals and reagents section).

Tab.13 reports some of the tentatively identified molecules with their chromatographic retention times, MS^2 fragmentation ions, assigned identities, chemical formulas, and the compounds' references; respectively. Compounds were numbered by their elution order.

In fact, the analysis yielded a total of twenty-eight (28) phenolic compounds and one disaccharide as shown in Tab. 13. The majority of phenolic compounds were detected and characterized for the first time, in the present work, from the MeOH extract of *C. spinosa*. All these identified phenolic compounds were denoted by their respective peaks obtained within 30 min, and most of them concentrated during the initial time of 15 min. The earliest two obtained peaks were corresponding to the disaccharide and the quinic acid with a retention time (Rt) of 2.15 and of 3.15 min, respectively, and the last two peaks detected the chrysin derivative and kaempferol with Rt of 14.36 and of 14.95 min, consecutively.

Among the phenolic compounds; phenolic acids and flavonoids constitute the essential components. Indeed, there were twenty-four (24) flavonoids, made up of the main group, where the majority couples with sugars to form glycosidic flavonoids in the plant, and a small part of them is present in a free form as aglycone. In contrast, only four phenolic acids, namely quinic acid, *p*-coumaric acid, caffeic acid, and sinapic acid-O-hexoside were detected (Tab. 13). The identification of flavonoids can also depend on the loss of CO and H_2O in fragment information.

Tab. 13 Characterization of compounds found in the methanolic extract from *C. spinosa* leaves by LC-ESI-MS/MS in negative mode.

N°	Rt (min)	LC-ESI-MS $[M-H]^-$ (m/z)	LC-ESI-MS/MS *m/z* (% Base Peak)	Assigned identification	Chemical Formula	References
1	2.1	341	MS^2 [341]: 179 (100), 161 (19.87), 143 (14.78), 119 (12.80), 113 (12.87)	Disaccharide	$C_{12}H_{22}O_{11}$	Molina-Garcia et *al*., 2018
2	3.15	191	MS^2 [191]: 173 (30.46), 111 (100)	Quinic acid	$C_7H_{12}O_6$	Gouveia and Castilho, 2011 ; Afonso et *al*., 2017
3	5.51	163	MS^2 [163]: 147 (11.13), 135 (12.38), 119 (100)	*p*- Coumaric acid	$C_9\ H_8\ O_3$	Karar and Kuhnert, 2015; Spínola et *al*., 2015
4	7.00	577	MS^2 [577]: 559 (11.28), 457 (100), 487 (30.4), 337 (17.91)	Chrysin-6,8-*C*-diglucoside	$C_{27}H_{30}O_{14}$	Lin et *al*., 2013
5	7.01	179	MS^2 [179]: 135 (100)	Caffeic acid	$C_9H_8O_4$	Barros et *al*., 2012
6	7.16	447	MS^2 [447]: 429 (9.06), 401 (54.6), 379 (13.78), 357 (42.35), 327 (92.51), 285 (100)	Kaempferol-3-*O*-glucoside	$C_{21}H_{20}O_{11}$	Afonso et *al*., 2017
7	9.23	431 $[M-H+HCOO^-]^{-2}$	MS^2 [431]: 385 (100), 311 (41.06), 179 (3.25)	Sinapic acid-*O*-hexoside (Formate adduct)	$C_{18}H_{24}O_{12}$	Spínola et *al*., 2015
8	9.72	593	MS^2 [593]: 503 (32.83), 473 (100), 413 (27.54), 353 (19.29)	Apigenin 6,8- di-hexose (Vicenine)=Apigenin-*C*-hexoside-C-hexoside	$C_{27}H_{30}O_{15}$	Ferreres et *al*., 2007 Bouziane et *al*., 2018
9	9.72	609	MS^2[609]: 489 (100), 429 (49.80), 357 (56.23), 327 (52.18)	Luteolin 6.8-di-*C*-hexoside (Lucenin-2)	$C_{27}H_{30}O_{16}$	Karar and Kuhnert, 2015; Spínola et *al*., 2015
10	10.73	447	MS^2 [447]: 357 (45.57), 327 (100), 285 (24.99)	Luteolin 8-*C*-galactoside or Luteolin 8-*C* glucoside (orientin)	$C_{21}H_{20}O_{11}$	Karar and Kuhnert, 2015
11	10.79	431	MS^2 [431]: 341 (7.06), 311 (100), 269 (7.69),	Apigenin 8-*C*-glucoside (vitexine)	$C_{21}H_{20}O_{10}$	Santos et *al*., 2017
12	10.95	447	MS^2[447]: 327 (92.51), 285 (100)	Luteolin-7-*O*-glucoside (Cynaroside)	$C_{21}H_{20}O_{11}$	Santos et *al*., 2017
13	11.03	593	MS^2[593]: 413 (100), 293 (10.02)	Apigenin 6,8-di-*C* glucoside (vicenin-3)	$C_{27}H_{30}O_{15}$	Karar and Kuhnert, 2015
14	11.29	563	MS^2[563]: 443 (3.73), 413 (100), 293 (8.33)	2" -*O*-Pentoxide-8-*C*-hexoside apigenin	$C_{26}H_{28}O_{14}$	Barros et *al*.,2012
15	11.32	447	MS^2[447]: 285 (100)	Tetrahydroxyisoflavone-*O*-hexoside	$C_{21}H_{20}O_{11}$	Ye et *al*., 2012
16	11.37	431	MS^2 [431]: 341 (7.81), 311 (100), 269 (6.66)	Apigenin 6-*C*-glucoside (isovitexin) or (vitexin)	$C_{21}H_{20}O_{10}$	Karar and Kuhnert, 2015
17	11.41	895	MS^2[895]: 447 (100), 285 (12.78)	Luteolin-*O*-hexoside dimmer	$C_{42}H_{40}O_{22}$	Spínola et *al*., 2015
18	11.54	431	MS^2[431]: 311 (15.97), 269 (100), 268 (11.39)	Apigenin-7-*O*-glucoside (Apigetrin)	$C_{21}H_{20}O_{10}$	Santos et *al*., 2017
19	12.40	489	MS^2 [489]: 429 (11.13), 285 (100), 327 (7.34)	Luteolin-*O*-(acetyl) hexoside	$C_{23}H_{22}O_{12}$	Spínola et *al*., 2015
20	12.71	863	MS^2[863]: 431 (100), 269 (4.32), 268(1.41)	Apigenin-*O*-hexoside dimmer	$2C_{21}H_{20}O_{10}$	Spínola et *al*., 2015
21	13.09	533	MS^2[533]: 489 (100)	Luteolin –*O*-diacetyl hexoside	$C_{24}H_{22}O_{14}$	Spínola et *al*., 2015
22	13.12	995	MS^2[995]: 489 (100), 285 (7.43)	Luteolin derivative (Luteolin-7-*O*-glucoside dimmer)	$2C_{21}H_{20}O_{11}$	-
23	13.15	533	MS^2[533]: 489 (100)	Kaempferol-*O*-malonylhexoside	$C_{24}H_{22}O_{14}$	Santos et *al*., 2017
24	13.37	473	MS^2[473]: 413 (34.24), 311 (23.39), 269 (100)	Apigenin derivative [Apigenin 8-C-(6"-acetyl) galactoside]	$C_{23}H_{22}O_{11}$	Simirgiotis et *al*., 2013
25	13.56	269	MS^2 [269]: 269 (100), 270 (24.72), 225 (6.46)	Apigenin	$C_{15}H_{10}O_5$	Pereira et *al*., 2012
26	14.06	461 $[M-H+HCOO^-]^{-2}$	MS^2 [461]: 415 (25.29), 253 (100)	Chrysin-7-*O*-glucoside (Formate adduct)	$C_{21}H_{18}O_{12}$	Pereira et *al*., 2012
27	14.39	877	MS^2 [877]: 831 (36.44), 669 (36.68), 461 (100), 415 (2.66), 253 (25.76)	Chrysin derivative	/	Barros et *al*., 2012
28	14.36	253	MS^2 [253]: 253 (100), 209 (5.65)	Chrysin isomer	$C_{15}H_{10}O_4$	Pereira et *al*., 2012
29	14.92	285	MS^2 [285]: 285 (100), 241 (6.31)	Kaempferol	$C_{15}H_{10}O_6$	Karar and Kuhnert, 2015

Compound 1 was found to be a disaccharide based on its parent molecular ion at *m*/*z* 341 in the MS spectrum that yielded an abundant fragment ion at *m*/*z* 179 (Molina-Garcia et *al*., 2018).

Compound 2 at Rt = 3.15 min has been identified as a quinic acid with a parent ion at *m*/*z* 191 $[M-H]^-$ in the MS spectrum (Gouveia and Castilho, 2011; Afonso et *al*., 2017).

Compound 3 eluted at 5.51 min produced a parent molecular ion at *m*/*z* 163 $[M-H]^-$ and an abundant fragment ion at *m*/*z* 119, indicating the presence of *p*-coumaric acid (Spínola et *al*., 2015).

Compound 5 with the parent ion at *m*/*z* 179 and the fragment ion of *m*/*z* 135 (loss of -44 Da), suggesting the presence of caffeic acid (Barros et *al*., 2012).

LC-ESI-MS analysis, also, showed a parent ion $[M-H]^-$ at *m*/*z* 431 (Rt = 9.23 min), with a fragment ion at *m*/*z* 385 (loss of -46 Da); it has been identified as a sinapic acid-*O*-hexoside (formate adduct) (compound 7) (Spínola et *al*., 2015).

The same ESI-MS spectrum (in negative mode), of the same extract, exhibited the parent molecular ion of *m*/*z* 447 at 7.16 min, which has a fragment ion of *m*/*z* 285, corresponding to kaempferol. The difference in mass between the *m*/*z* 447 ion and kaempferol can be explained by the loss of a glucose molecule. So, depending on its MS^2, compound 6 was distinguished as kaempferol-3-*O*-glucoside (Afonso et *al*., 2017).

Regarding the compound 23, its negative mode analysis made it possible to detect the parent molecular ion with *m*/*z* 533, which has a fragment ion *m*/*z* 489 (loss of -44 Da), corresponding to kaempferol-*O*-malonyl-hexoside, once it breaks down to give an ion of *m*/*z* 285 (loss of a unit of glucose, -162 Da), as it had already been described (Santos et *al*., 2017).

Based on the fragmentation pattern and the chromatographic elution of compound 29, it was assigned to kaempferol (Karar and Kuhnert, 2015).

Apigenin derivatives were, also, found in *C. spinosa* methanolic extract, indicating to be a *C*-glycoside due to the characteristic loss of 90 and 30 Da moieties, being putatively identified as apigenin-*C*-hexoside-*C*-hexoside (compound 8, $[M-H]^-$ *m*/*z* 593), as previously described (Ferreres et *al*., 2007; Bouziane et *al*., 2018); nonetheless, it was not

possible to number the *C*-glycoside residue positions owing to the deficient of a standard compound for comparison.

Besides, compounds 11, 16, and 18 observed at different retention times in the chromatogram, all of them gave a parent ion $[M-H]^-$ at *m*/*z* 431; MS^2 fragmentation of both compounds 11 and 16 yielded a base peak of *m*/*z* 311, and a less intensive fragment ion at *m*/*z* 341 (7.06% and 7.81%, respectively, of the base peak), which corresponded to the loss of moieties from the *C*-linked glucose (Cuyckens and Claeys, 2004). Whilst, MS^2 fragmentation of compound 18 yielded a single predominant base peak at *m*/*z* 269, this fragment is, further, the characteristic of apigenin, corresponding to the loss of moiety of *O*-linked glucose (431 → 269: -162 Da). Based on their chromatographic elution orders, and reported literature, these three compounds were identified as isomers of apigenin glucosides; compound 11 identified as apigenin-8-*C*-*O*-glucoside (Santos et *al*., 2017), compound 16 being identified as apigenin-6-*C*- glucoside (isovitexin) (Karar and Kuhnert, 2015), and compound 18 to apigenin-7-*O*-glucoside (apigetrin) (Fabre et *al*., 2001; Llorent-Martinez et *al*., 2015; Santos et *al*., 2017).

In addition, 2″-*O*-Pentoxide-8-*C*hexoside apigenin (compound 14 at $[M-H]^-$ *m*/*z* 563) was, also, detected (Barros et *al*., 2012).

In comparison with the literature (Spínola et *al*., 2015), compound 20 exhibited a precursor ion $[M-H]^-$ at *m*/*z* 863 and two fragment ions at *m*/*z* 431 and at *m*/*z* 269; it has tentatively been assigned as another derivative of apigenin; a dimmer of apigenin 7-*O*-glucoside.
Compound 25 exhibited $[M-H]^-$ ion at *m*/*z* 269, its MS^2 fragmentation pattern was similar to that of apigenin. Whereby, this compound gave the *m*/*z* 149 fragment peak of apigenin.

Some luteolin derivatives were, likewise, identified with typical fragment ion at *m*/*z* 285 (Spínola et *al*., 2015): luteolin-*O*-hexoside (compound 12) that displayed a parent molecular ion at *m*/*z* 447, luteolin-*O*-(acetyl) hexoside (compound 19) at *m*/*z* 489, luteolin-*O*-diacetyl hexoside (compound 21) with molecular ion at *m*/*z* 533, luteolin-6,8-di-C-glucoside (compound 9) that exhibited a precursor ion $[M-H]^-$ at *m*/*z* 609, and a dimmer of luteolin-*O*-hexoside (compound 17) at *m*/*z* 895.

In addition, a luteolin derivative is proposed for the compound 22 eluted at Rt = 13.12 min with a parent ion at *m*/*z* 995 and fragment ions at *m*/*z* 489 and at *m*/*z* 285.

Chrysin derivatives have, moreover, been recognized with a distinctive fragment ion at *m*/*z* 253. Compounds (4, 26, and 27) at molecular ions *m*/*z* 577, 461, and 877; consecutively; were putatively named chrysin-6,8-C-diglucoside (Lin et *al*., 2013), chrysin-7-*O*-glucoside (formate adduct) (Pereira et *al*., 2012), and chrysin derivative (Barros et *al*., 2012).

Based on LC-MS analysis and MS/MS fragmentation pattern, compound 28 was, also, proposed as a chrysin isomer (Pereira et *al*., 2012).

The MS^2 spectra with the chemical structures of the twenty-nine (29) identified compounds from the leaves MeOH extract of *C. spinosa* are illustrated in the following Figs. 38-44.

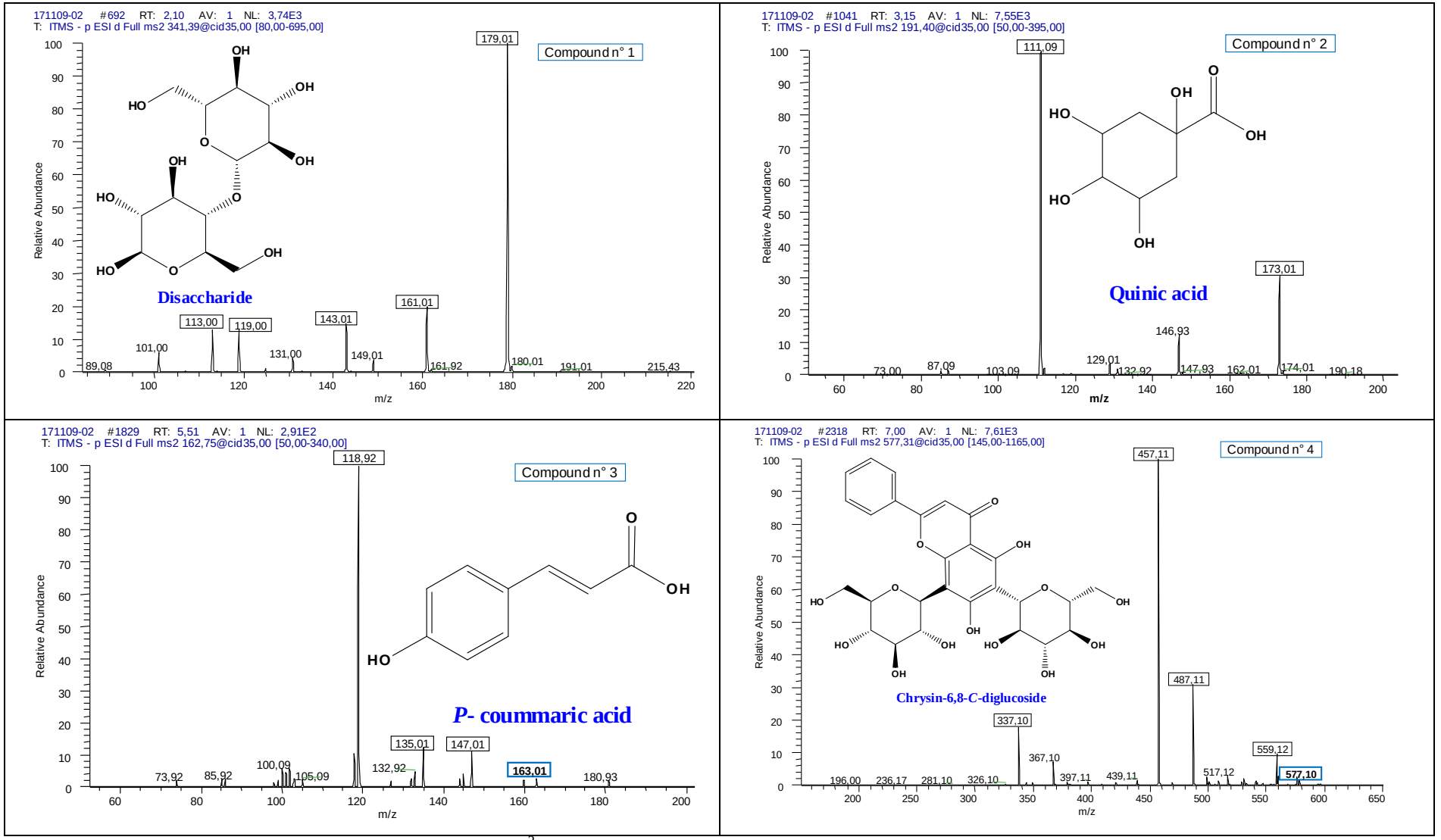


Fig. 38 MS^2 spectra of compounds 1, 2, 3, and 4 in negative mode.

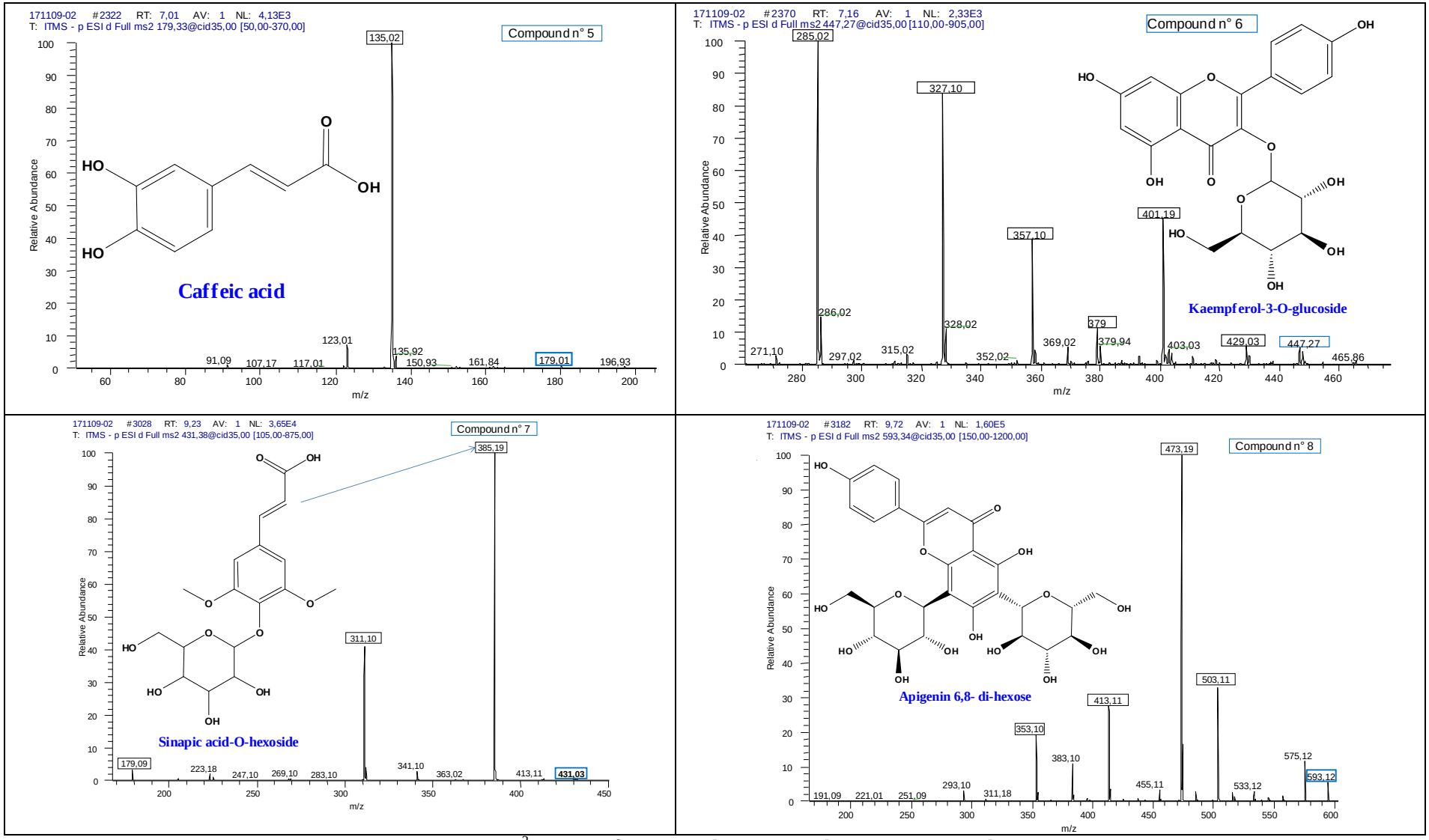


Fig. 39 MS^2 spectra of compounds 5, 6, 7, and 8 in negative mode.

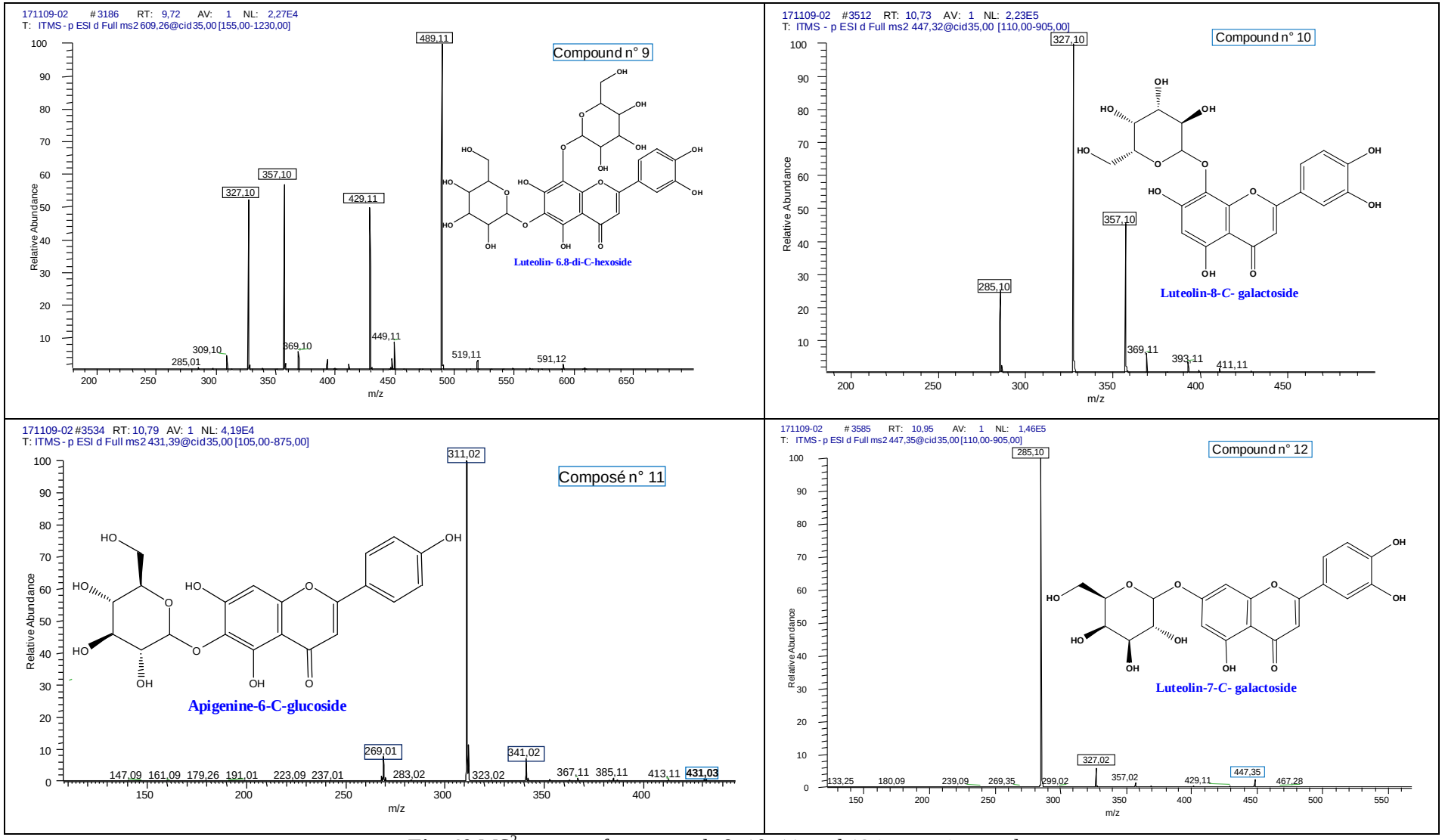


Fig. 40 MS^2 spectra of compounds 9, 10, 11, and 12 in negative mode.

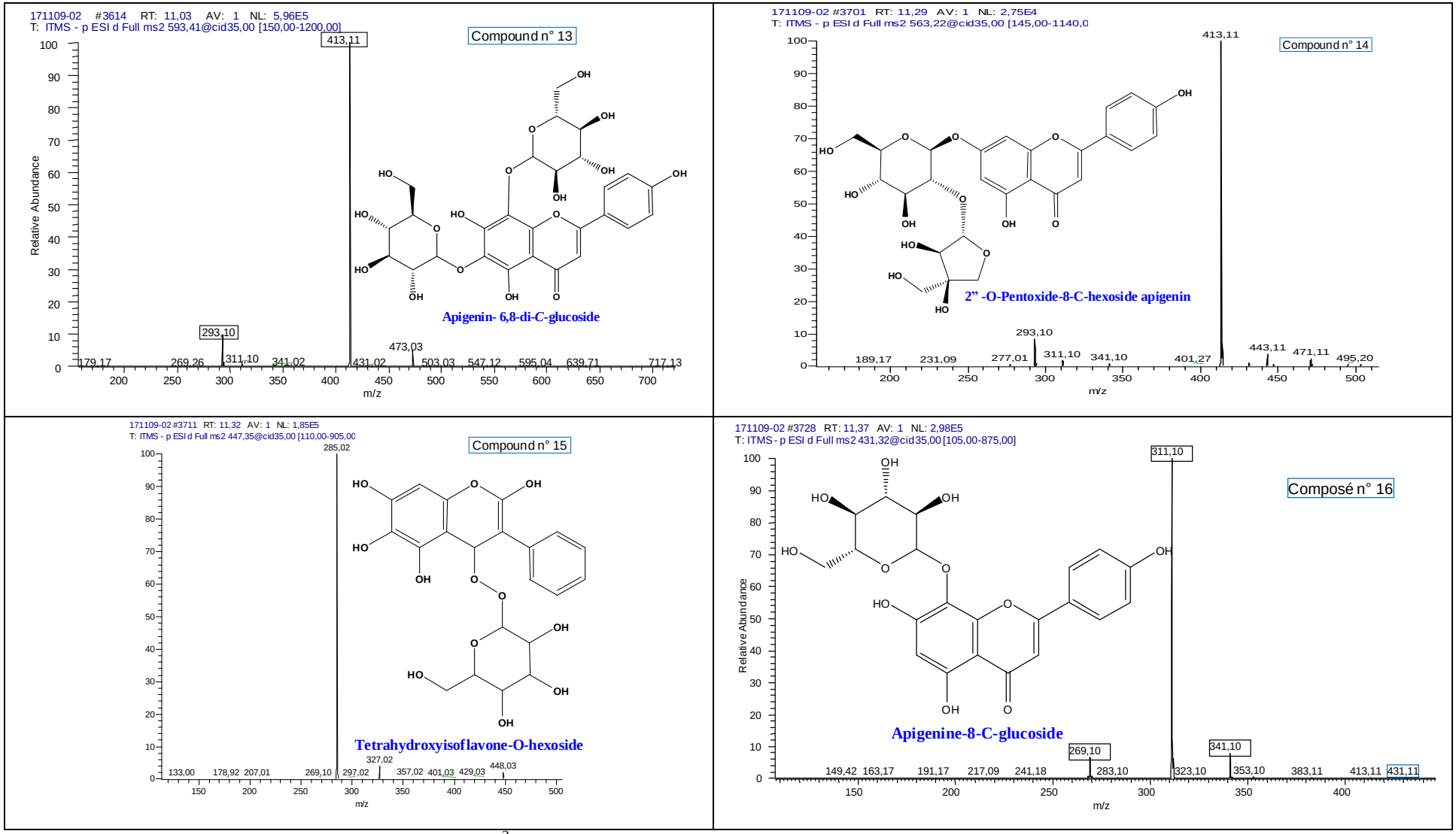


Fig. 41 MS^2 spectra of compounds 13, 14, 15, and 16 in negative mode.

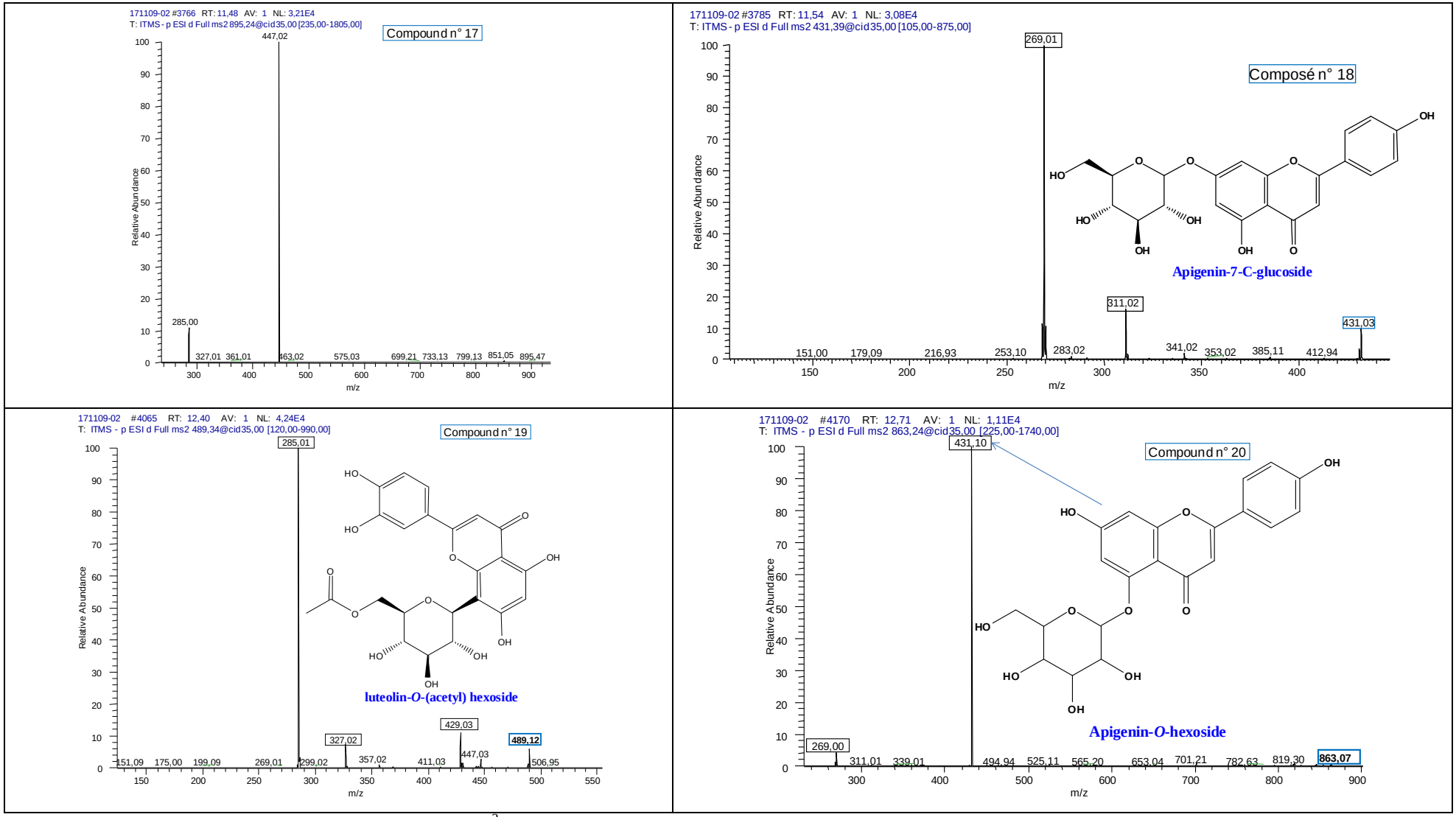


Fig. 42 MS^2 spectra of compounds 17, 18, 19, and 20 in negative mode.

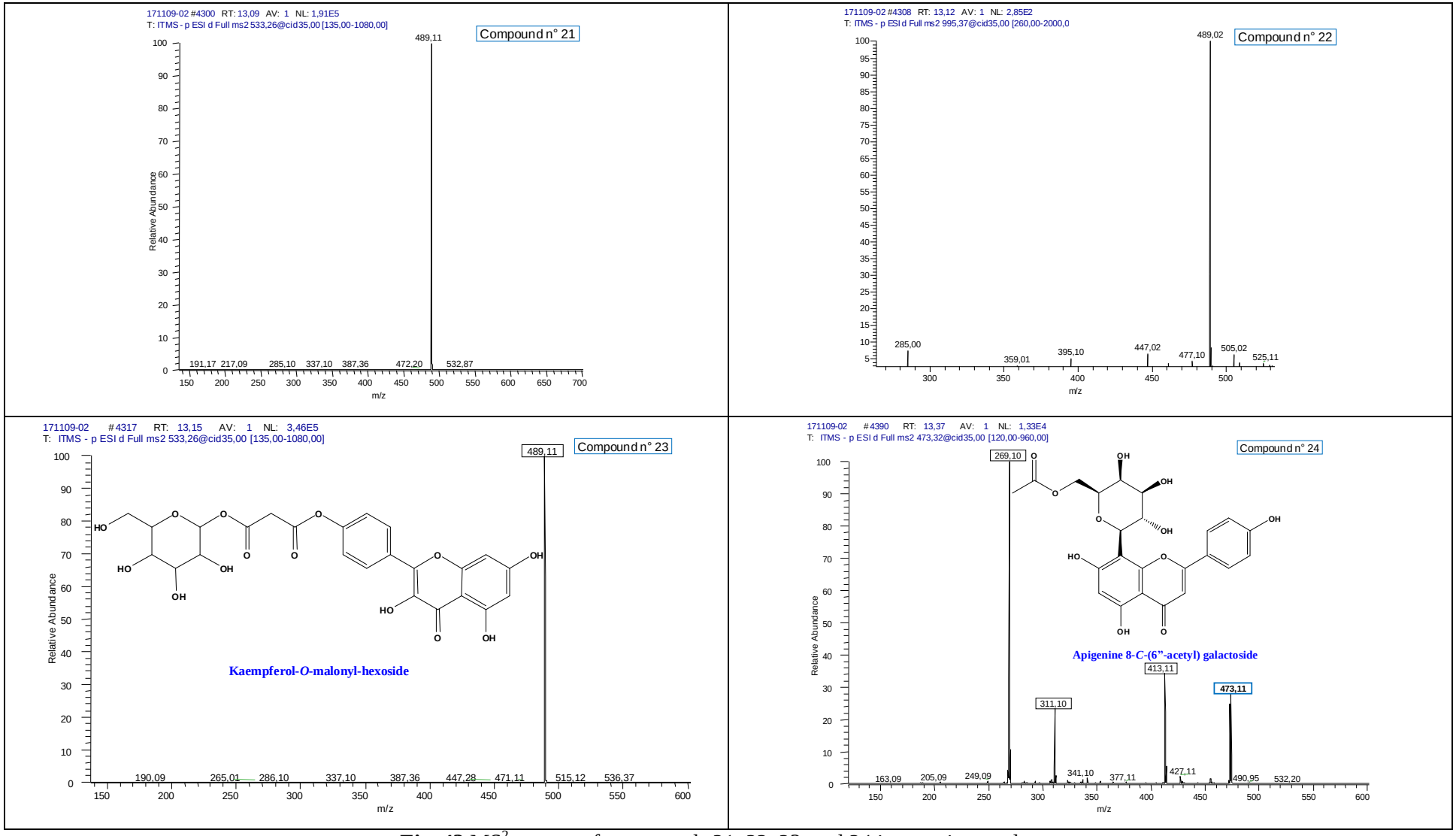


Fig. 43 MS^2 spectra of compounds 21, 22, 23, and 24 in negative mode.

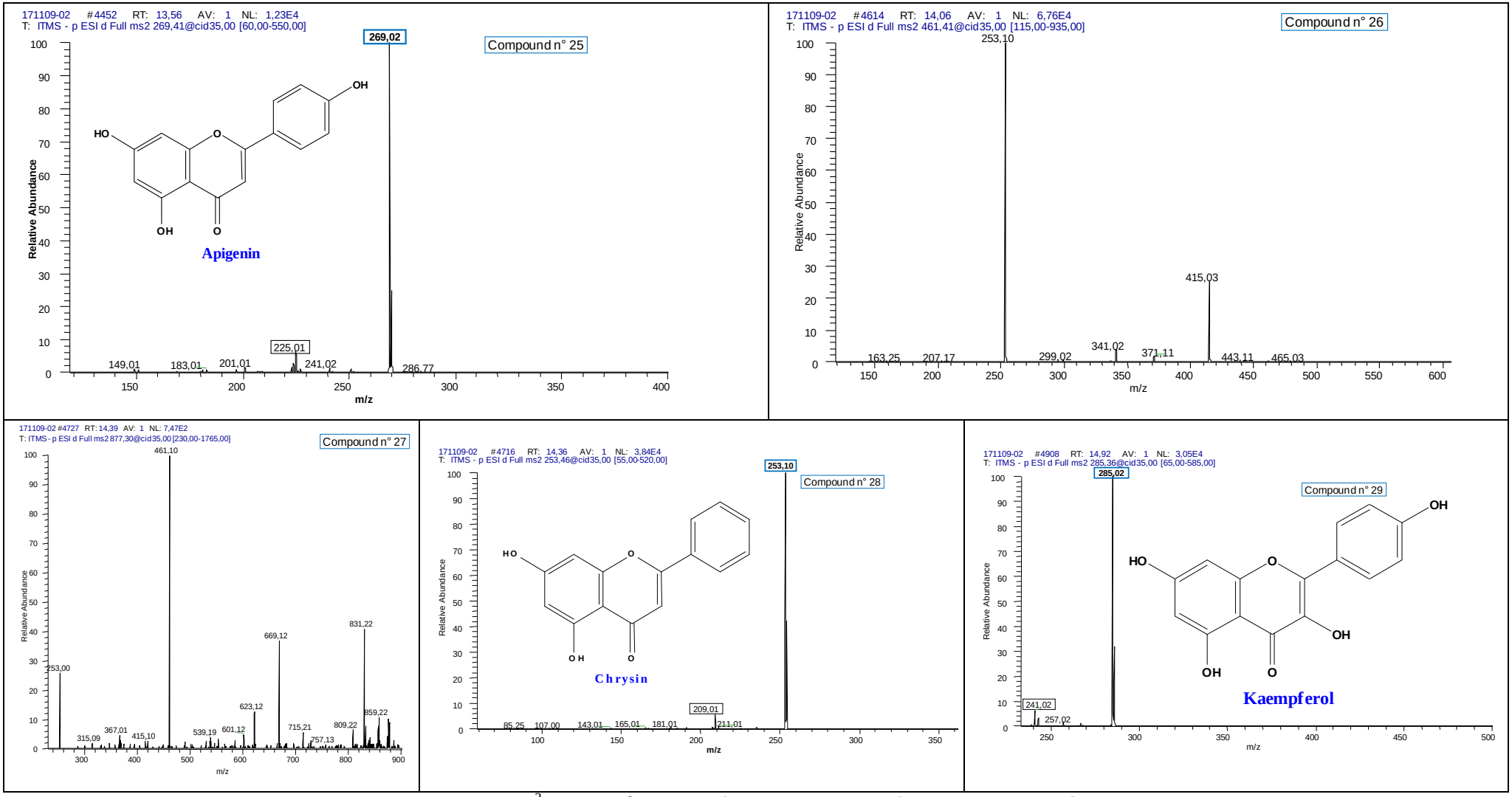


Fig. 44 MS^2 spectra of compounds 25, 26, 27, 28, and 29 in negative mode.

3.6- Column chromatography of leaves methanol extract

As we mentioned before, the same extract, MeOH extract of leaves, was subjected to fractionation by CC. The results of this part are summarized in this diagram (Fig. 45).

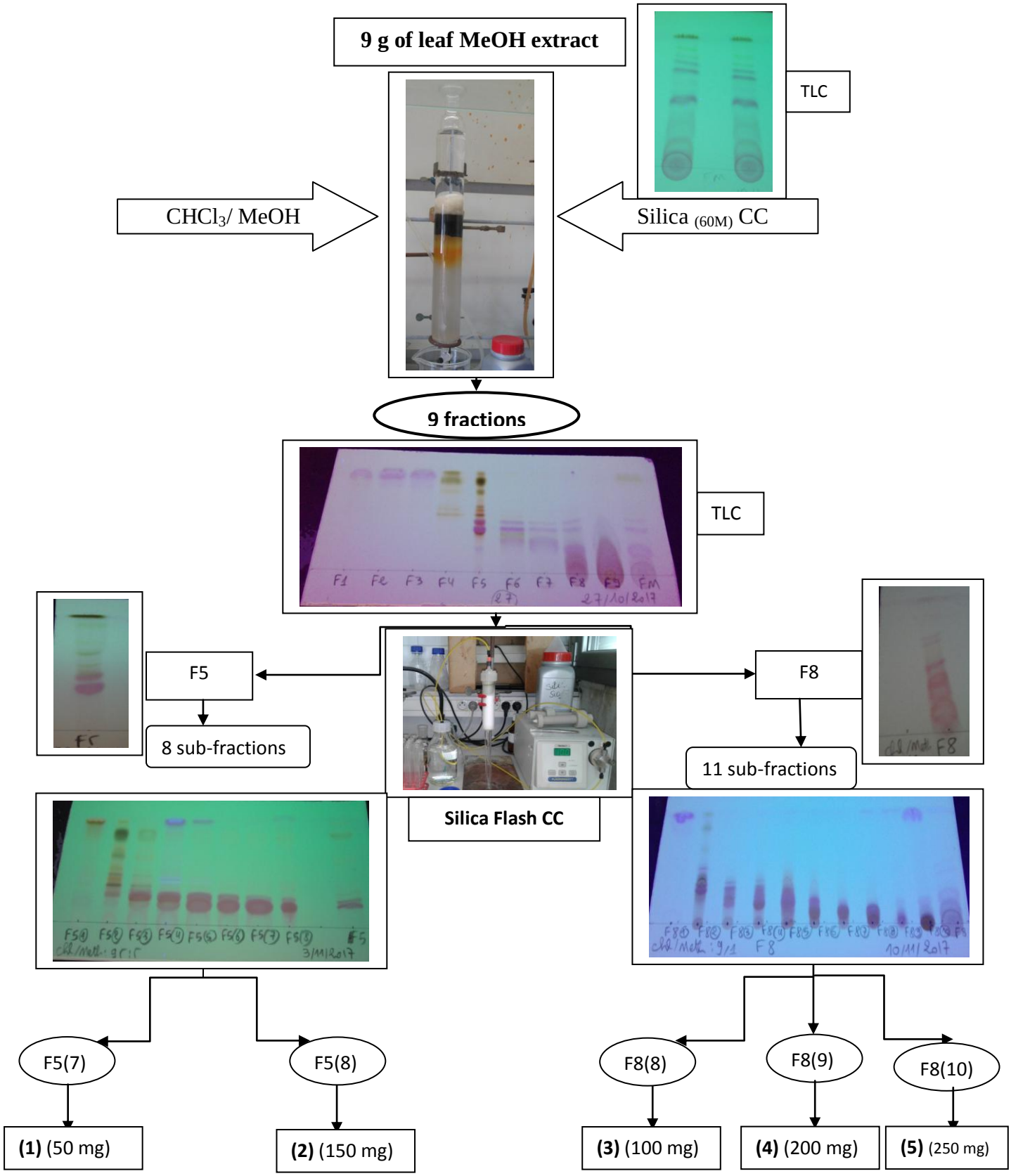


Fig. 45 Diagram of leaf MeOH extract separation by Column Chromatography.

In fact, the CC yielded five compounds; (1), (2), (3), (4), and (5) with different colors and yields. Two precipitates were separated from Fraction 5 (F5); the yellow is Compound **(1)** (50 mg) and the green is compound **(2)** (150 mg). Besides, three other precipitates were, also, isolated from Fraction 8 (F8); compound **(3)** with a yellow color (100 mg), compound **(4)** has a green color (200 mg), and the white color for the compound **(5)** (250 mg).

3.7- Identification of the isolated compounds (1-5) and their structural elucidation

The five purified compounds (**1-5)** were directly infused in the mass spectrometer via an ESI source in which ions were produced (HESI source from Thermo Scientific).
Fragmentation was operated by CID (Collision Induced Dissociation) with helium (He) gas. This type of experiment can be reproduced several times to obtain MS^n spectra.

The MS profiles by negative LIT-ESI-MS of the five (**1-5**) compounds isolated from leaves MeOH extract of *C. spinosa* were analyzed and compared to references to facilitate their molecular identification. The mass spectra were recorded between 100 and 1500 Da. Identified compounds (**1-5)** using this approach; LIT-ESI-MS^n with NMR, are described below.

3.7.1- Structural elucidation of compound (1)

Compound (**1)** was found as a yellow powder with a yield of 0.56%. Its mass spectrum, obtained in LIT-ESI-MS in negative mode made it possible to distinguish a molecular ion with *m/z* 451, which has an ion fragment *m/z* 253 corresponding to chrysin aglycone (Appendix 3), once it is fragmented to give an ion of *m /z* 209, as it had already been described (McNab et *al*., 2009). The difference in mass (-198) between the ion of *m /z* 451 and chrysin can be explained by the loss of one molecule of glucose and one molecule of H_2O.
The same ESI-MS spectrum of compound **(1)** (negative mode) showed the ion *m /z* 493, which also has the same ionic fragment of *m /z* 253, also corresponding to chrysin aglycone.
Additionally, the MS spectrum obtained in the negative mode of this compound has a quasi-molecular ion peak with a ratio *m/z* 415 $[M-H]^-$ suggesting a molecular mass of 416 amu (calculated 416.11073 g/ mol). The MS/MS spectrum of this ion had four major fragment ions at *m /z* 315 (loss of 100 amu), at *m /z* 313 (loss of 102 amu), at *m /z* 295 (loss of 120 amu), and at *m /z* **253** (loss of 162 amu), respectively (Fig. 46). This latest fragment ion, at m/z 253, also representing the chrysin aglycone with a loss of 162 mass units from the parent molecular ion suggested the glucose or the galactose moiety.

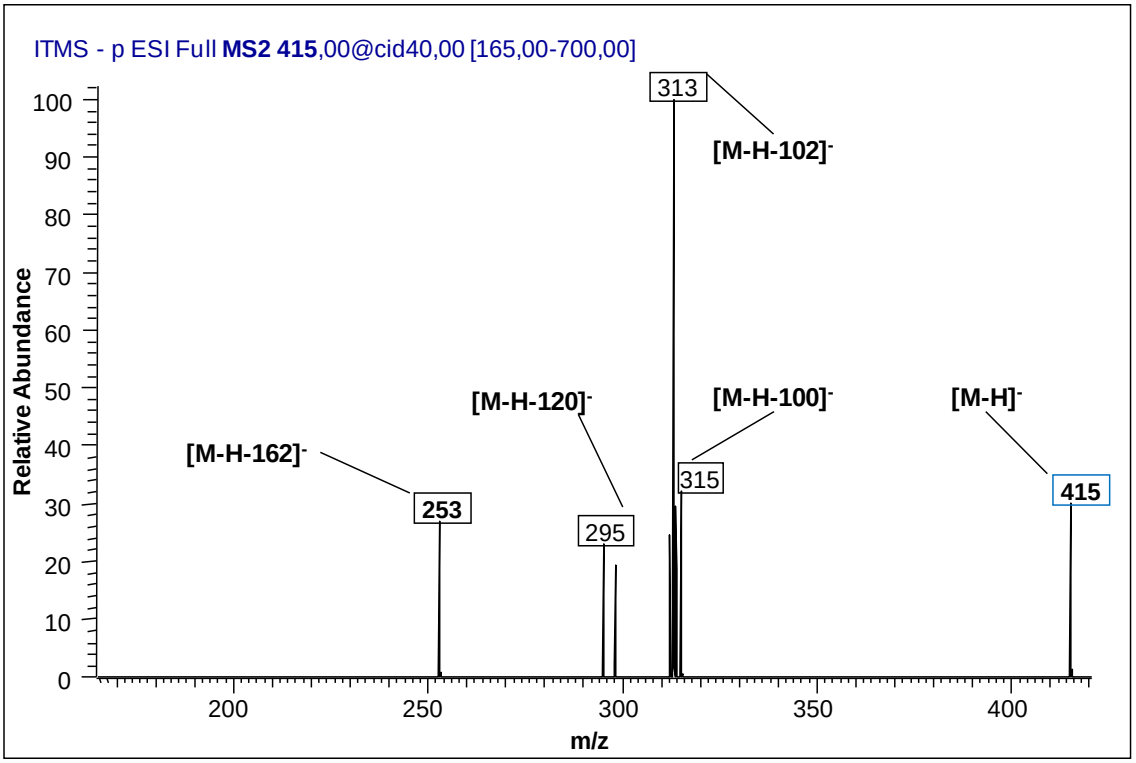


Fig. 46 MS/MS spectrum of compound **(1)** recorded in negative mode.

The **^{1}H-NMR** spectrum (Fig. 47) exhibited a flavonoid pattern, it clearly showed:

- Three signals at δ 7.04 (1H, s), 6.86 (1H, d, J=1.5), and 6.46 ppm (1H, d, J=1.5) typical of protons at C-3, C-8 and C-6, respectively, of a flavones skeleton.
- Chemical shifts of 8.08 (d, J=6.5, H2', H6'), and of 7.59 ppm (m, H3', H4', H5') suggested that it is unsubstituted in the B-ring of the flavonoid.
- An unblinded singlet signal resonating at δ 12.79 ppm was assigned to the C-5 hydroxyl.
- Other 1H-NMR shifts at δ 3.20 to 3.70 ppm suggesting the presence of a sugar moiety substitution on the hydroxyl group at C-7 that was identified as β-D-glycosyl.
- Doublet signal at δ 5.13 ppm was assigned to the anomeric proton (H-1") with a coupling constant (J = 6.7 Hz) indicating a β-configuration (Qin et al., 1993; Liu et al., 2010).
- These data confirmed that the aglycone of the compound (1) is 5, 7-dihydroxyflavone (chrysin).

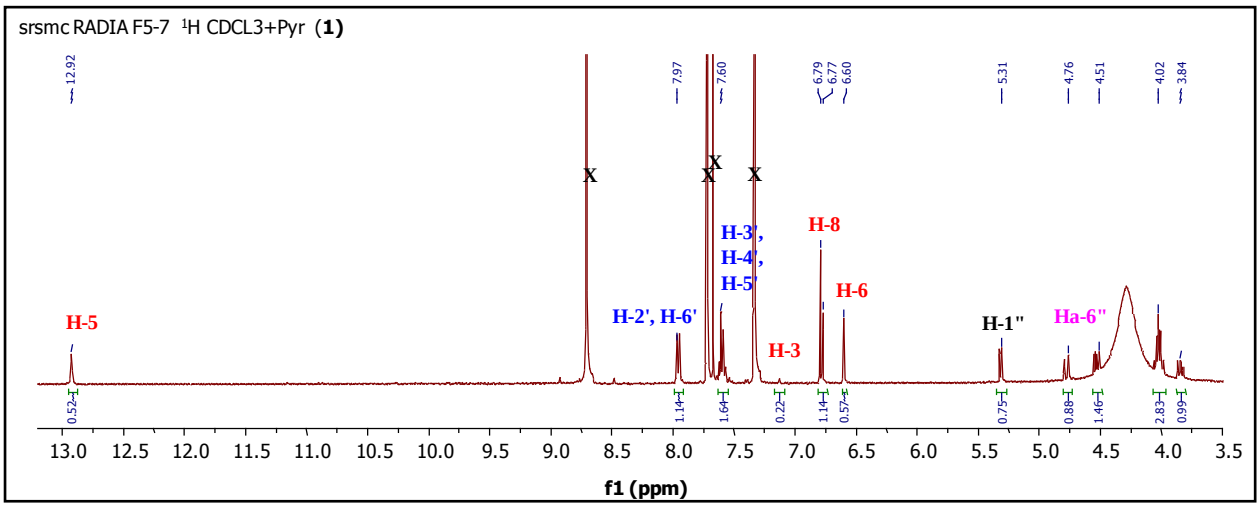


Fig. 47 ^{1}H-NMR spectrum of compound **(1)** (400 MHz, $CDCl_3$ + pyridine-$_{d5}$); **X**: solvent.

On the **^{13}C-NMR** spectrum (Fig. 48);

- The presence of 21 carbon signals was confirmed.
- The six signals of the sugar moiety were clearly observed appearing at δ_C 60.6, 69.5, 73, 76.4, 77.2, and 99.9 ppm and the anomeric carbon signal at δ_C 99.9 ppm was also assigned.
- Signals resonating at δ_C 126.5 to 132.2 ppm are corresponding to the aromatic carbons.

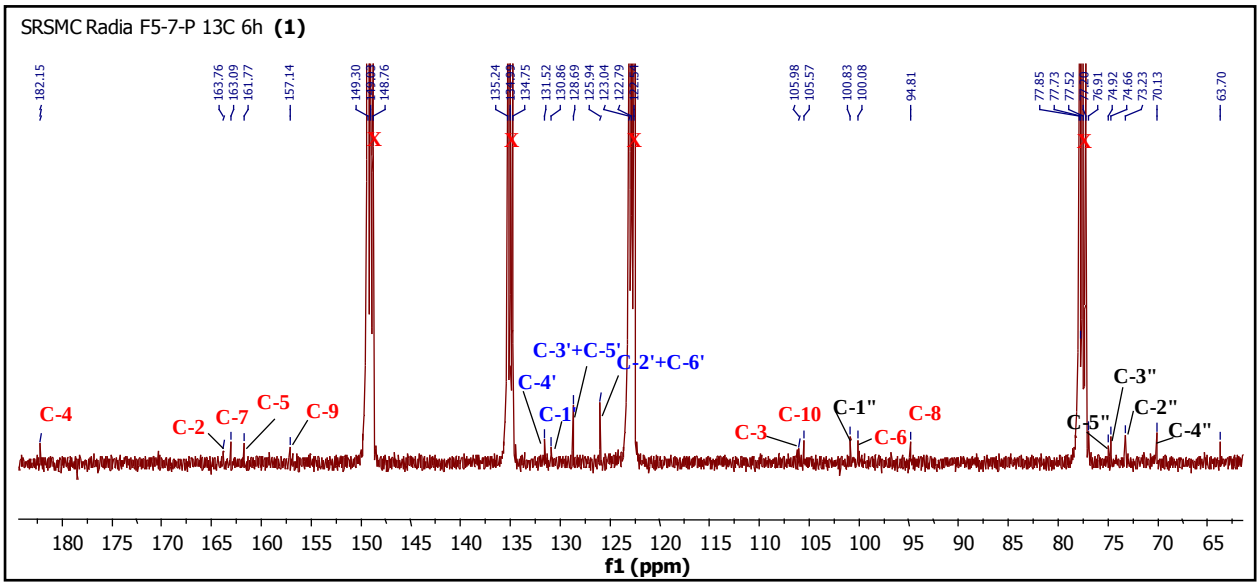


Fig. 48 ^{13}C-NMR spectrum of compound **(1)** (100 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: solvent.

Tab. 14 Chemical shifts in ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of compound **(1)** in $CDCl_3$ + pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)
2	163.7	-	9	157.1	-	6′	126.5	8.08, d (6.5)
3	105.6	7.04, s	10	105.5	-	1″	99.9	5.13, d (6.7)
4	182.2	-	1′	130.6	-	2″	73.0	3.45-3.25, m
5	161.1	12.79, s	2′	126.5	8.08, d (6.5)	3″	76.4	3.45-3.25, m
6	99.7	6.46, d (1.5)	3′	129.2	7.59, m	4″	69.5	3.45-3.25, m
7	163.2	-	4′	132.2	7.59, m	5″	77.2	3.45-3.25, m
8	95.0	6.86, d (1.5)	5′	129.2	7.59, m	6″	60.6	a: 3.70, d (10.3)/ b: 3.45-3.25, m

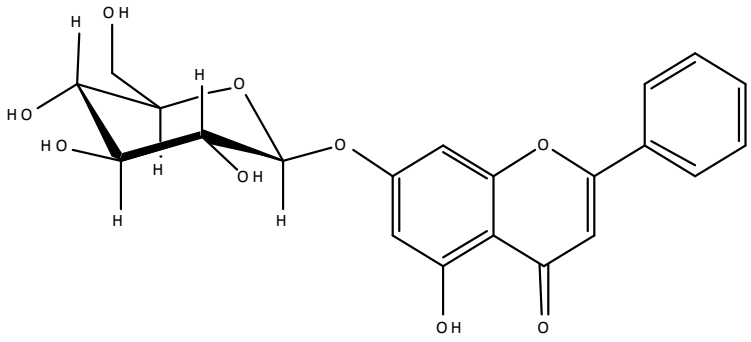
The **COSY** spectrum of compound (1) (Appendix 3) showed correlations between:

- The proton signals of H-3 at *δ* 4.15, of H-4 at *δ* 4.63, and of H-2 at *δ* 4.78 ppm;
- H-2 (*δ* 4.78) and H-1 (*δ* 4.80);
- As well as between H-4 (*δ* 4.63) and H-5 (*δ* 4.76).

Furthermore, H-5 coupled with H-6 (*δ* 4.80), which allowed to completely elucidating this compound structure.

Besides, **HMBC** correlation (Appendix 3) that was observed from H-1*″* to C-7 (δ_C 164.9) of the A-ring showed the attachment of the glucose unit at C-7. Since the chemical shift value of C-1*″* and the coupling constant of H-1*″* were suggestive of an *O*-glucosidation.

All the previous data of ESI-MS^n and of NMR spectral analyses, illustrated above, made it possible to identify its correspondence to chrysin-7-*O*-(*β*-D-glucopyranoside) with a crude formula of $C_{21}H_{20}O_9$ (Fig. 49). This structural identification of the compound (1) has been confirmed by the literature (El Antri et *al*., 2004a; Alhage et *al*., 2018).



(1)

Fig. 49 4*H*-1-Benzopyran-4-one, 7-(β-D-glucopyranosyloxy)-5-hydroxy-2-phenyl- « Chrysin-7-*O*-(*β*-D-glucopyranoside) ».

3.7.2- Structural elucidation of compound (2)

This compound **(2)** was isolated as a pale yellow solid powder with a yield of 1.67%.

Its mass spectrum, recorded in LIT-ESI-MS^2 in negative mode, indicates a quasi-molecular ion peak *m/ z* 457 $[M-H]^-$ suggesting a molecular mass of 458 amu (calculated 458.1213 g/mol). This ion has 42 amu more than the compound (1), corresponding to the chemical structure of C_2H_2O, a characteristic of the flavones' fragmentation (Fabre et *al*., 2001). This makes it possible to attribute to the compound (2) this crude formula $C_{23}H_{22}O_{10}$.

The MS/MS spectrum of this ion, similarly, exhibited four major fragment ions at *m/z* 253 (loss of 204 amu), at *m /z* 209 (loss of 248 amu), at *m /z* 199 (loss of 258 amu), and at *m /z* 171 (loss of 286 amu), consecutively (Fig. 50). This analysis has shown that the fragment ion at *m/z* 253, characteristic of the chrysin aglycone unit, and the loss of 204 mass ($C_8H_{14}O_6$) from the parent molecular ion proved the structure of the sugar moiety. This fragment ion, *m/z* 253, is common to both compounds (1) and (2).

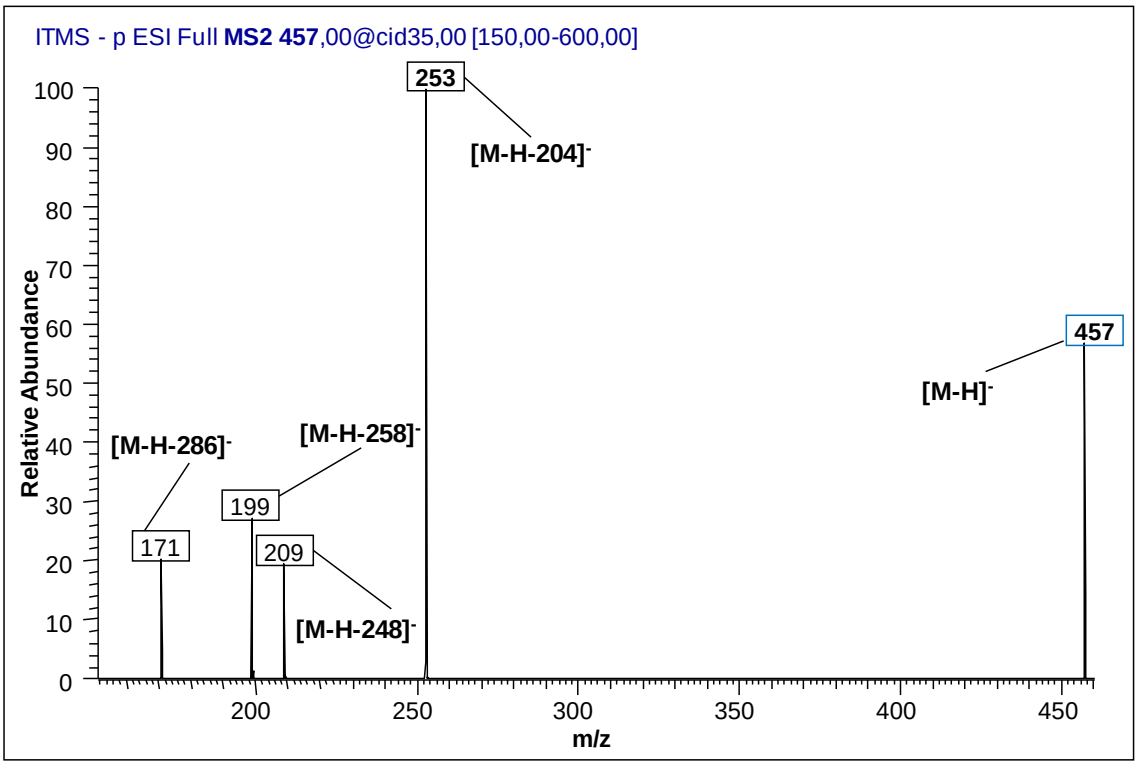


Fig. 50 MS/MS spectrum of compound **(2)** recorded in negative mode.

The **NMR** spectra of compound **(2)** were, relatively, similar to those of compound **(1)**, except for some differences in the sugar moiety. The NMR data proposed that **(2)** is a flavonoid that has also chrysin aglycone.

The study of the **^{1}H-NMR** spectrum of compound **(2)** (Fig. 51) reveals, in addition to the aromatic signals of two meta-coupled doublets at δ 6.35 and 6.49 ppm (1H, *J* = 1.6 Hz of

each) represented H-6 and H-8 respectively, and a signal at δ 6.98 ppm (1H, s) typical of the proton at C-3 of a flavone skeleton.

- The signal at δ 12.74 ppm (1H, s) is characteristic of a C-5 hydroxyl.
- Furthermore, the signals of a glycosidic moiety were visible (3.25 to 4.41 ppm), in particular, the anomeric proton, which appeared at δ_H 5.00 ppm as a doublet with a coupling constant of J = 7.3 Hz indicating a β-linkage of the sugar unit to the aglycone.
- Besides, the sugar moiety chemical shifts resembling those of compound **(1)**, a singlet signal at δ 2.02 ppm corresponds to the acetyl group on position 6".

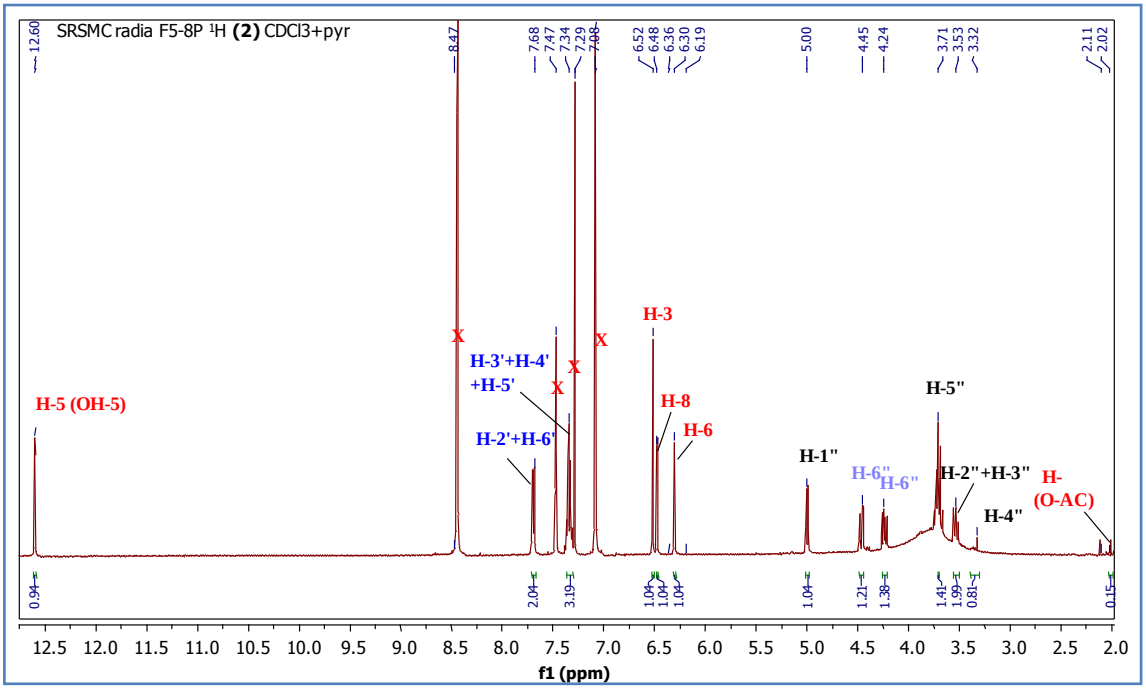


Fig. 51 ^{1}H-NMR spectrum of compound **(2)** (400 MHz, $CDCl_3$ + pyridine-$_{d5}$); **X**: solvent.

Besides, the **^{13}C-NMR** spectrum (Fig. 52) proved that compound **(2)** is, indeed, a glucoside of chrysin.

Its sugar moiety location established at C-7 based on the typical glucosylation carbon shifts that were similar to those of the compound **(1)**. However, it indicated the presence of two additional signals; the methyl signal at δ_C 21.1 ppm together with the carbonyl signal at δ_C 171.3 ppm; more than those of compound **(1)** corresponding to the acetyl group carbons. So, the presence of β-D-(6"- acetyl) glucose moiety was confirmed.

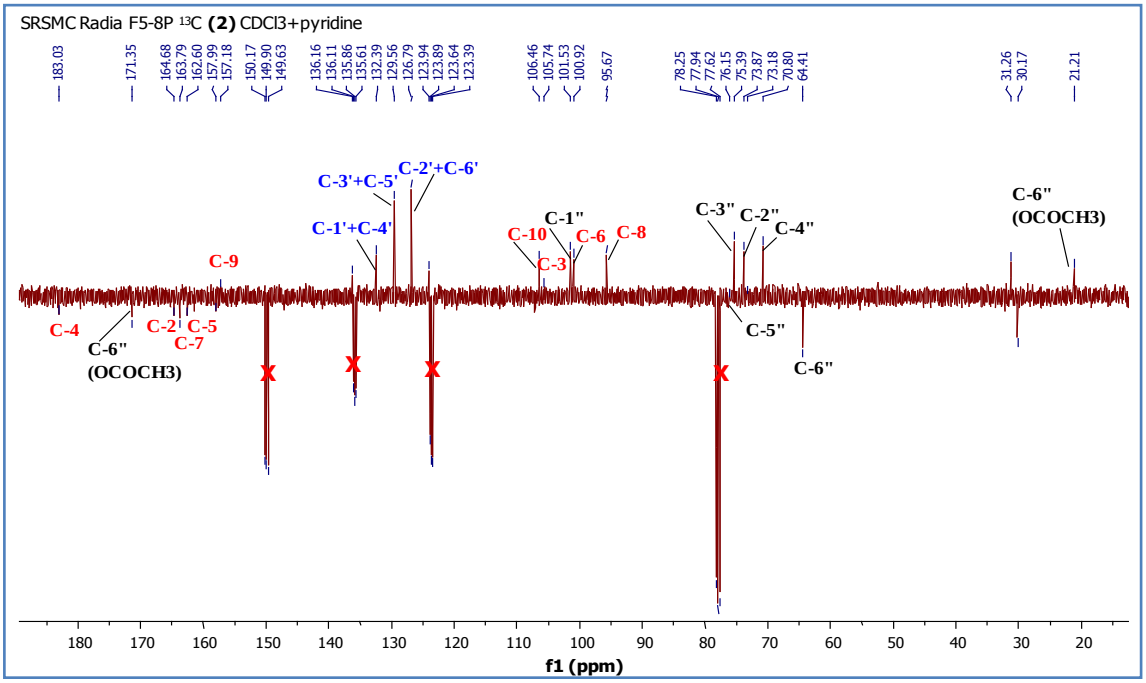


Fig. 52 ^{13}C-NMR spectrum of compound **(2)** (100 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: solvent.

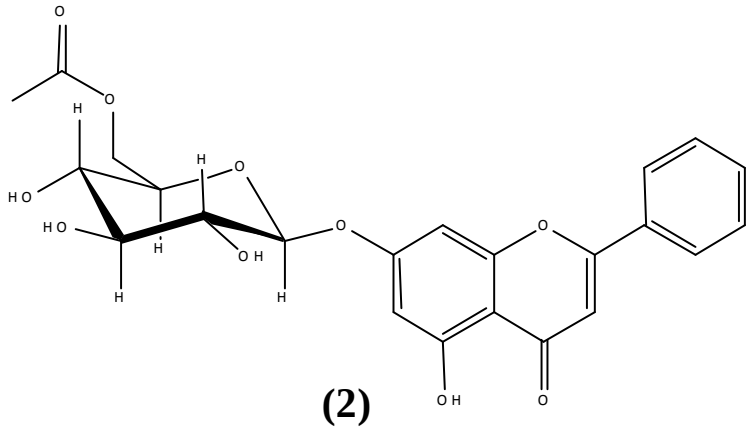
Tab. 15 Chemical shifts in ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of compound **(2)** in $CDCl_3$ + pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)
2	164.6	-	9	157.1	-	6′	126.7	7.60, d (6.7)
3	105.7	6.51, s	10	106.4	-	1′′	101.5	5.0, d (7.3)
4	182.2	-	1′	101.5	-	2′′	73.0	3.38, m
5	162.6	12.74, s	2′	126.7	7.60, d (6.7)	3′′	75.4	3.38, m
6	100.9	6.35, d (1.6)	3′	129.5	7.3-7.4, m	4′′	70.7	3.25, m
7	163.7	-	4′	132.3	7.3-7.4, m	5′′	76.2	3.75, m
8	95.6	6.49, d (1.6)	5′	129.5	7.3-7.4, m	6′′	171.3 64.4 21.1	a: 4.41, d (10.1) b: 4.12, d (11.8) 2.02, s

Furthermore, the cross peak from the anomeric proton H-1” (δ 5.0) to C-7 (δ 163.7) in the HMBC spectrum confirmed that the glycosylation of this compound takes place in the 7th position. Thus, the ^{1}H-COSY (Appendix 3) and HMBC spectra validated the above assignments.

All of the previous data allowed us to establish the structure of the compound (2) (Fig. 53). It is a glucosylated flavonoid, Chrysin-7-*O*-*β*-D-(6”-acetyl) glycopyranoside.

Based on these spectral analyses and the literature (Pistelli et *al*., 2003; El Antri et *al*., 2004a; Alhage et *al*., 2018), compound (2) molecular formula was determined as follows: ($C_{23}H_{22}O_{10}$).



(2)

Fig. 53 4*H*-1-Benzopyran-4-one, 7-[(6-*O*-acetyl-β-D-glucopyranosyl)oxy]-5-hydroxy-2-phenyl- « Chrysin-7-*O*-*β*-D-(6"-acetyl) glycopyranoside ».

3.7.3- Structural elucidation of compound (3)

Compound (**3**) was obtained as a yellowish crystallized powder with a yield of 1.11%. The MS spectrum recorded in ESI-LITMS in negative mode of this molecule showed three molecular ion peaks at *m/z* 523, at *m/z* 489, and at *m/z* 431(Appendix 3) which correspond respectively to $[M-H + 92 (2Cl + Na)]^-$, $[M -H + 58 (Na + Cl)]^-$, and $[M-H]^-$.

Besides, the mass spectrum LIT-ESI-MS^2 in negative mode revealed a quasi-molecular ion of m/z 431 (calculated 431.09782 g/ mol) $[M-H]^-$(Fig. 54) in agreement with the molecular mass 432 amu, which corresponds to the crude formula of $C_{21}H_{20}O_{10}$. Thus, this compound **(3)**, therefore, has an additional oxygen atom compared to the compound **(1).**

The MS^2 spectrum of this ion, similarly, presents three major fragment ions at *m/z* 341 (loss of 90 amu), at *m /z* 311 (loss of 120 amu), and at *m /z* 269 (loss of 162 amu), successively (Fig. 54). The fragment ion at *m/z* 269, characteristic of the apigenin aglycone unit, and the loss of 162 mass ($C_6H_{12}O_5$) from the parent molecular ion proved the structure of the glucose moiety.

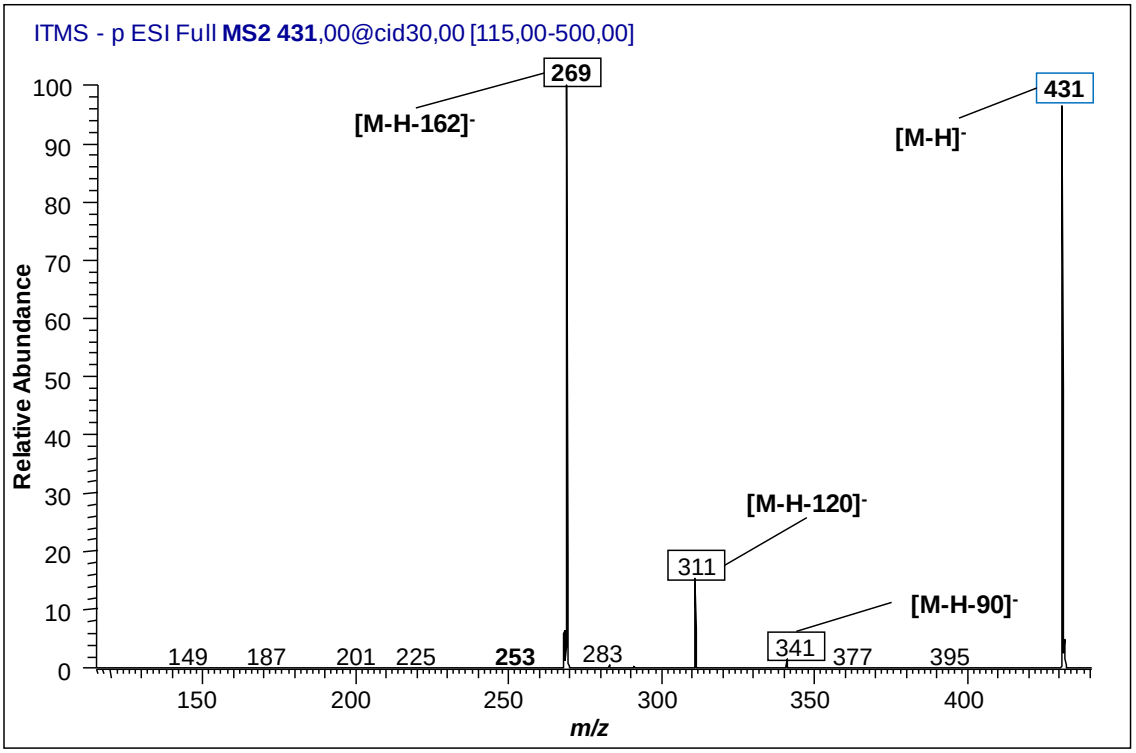


Fig. 54 MS/MS spectrum of compound **(3)** recorded in negative mode.

The presence of a flavone skeleton in the compound **(3)** could, easily, be deduced from the **^{1}H-NMR** spectrum (Fig. 55), in which it exhibited:

- Signal of an exchangeable proton at δ 12.95 (1H, s);
- Signals of aromatic protons A2B2-type at δ 7.85 (d, H2 ', H6') and 7.63 (d, H3 ', H5') on B-ring;
- Two doublets at δ 6.50 (d, H6) and 6.85 (d, H8) on the A-ring, together with an olefinic proton at δ 6.98 (s, H3) on a flavone C-ring.
- The 1H-NMR also showed signals due to a β-glucopyranosyl unit [δ 5.26 (d, J = 6.8 Hz, H1")]. The value J (6.8 Hz) of the anomeric proton indicated the β-configuration of the glucose moiety.

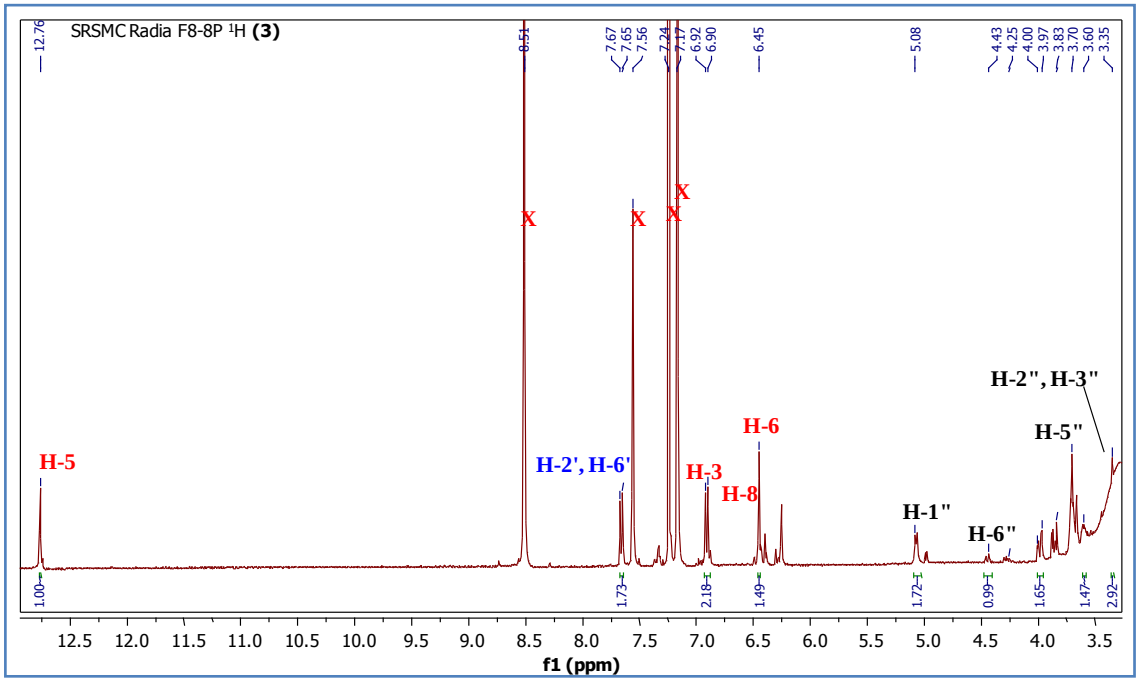


Fig. 55 ^{1}H-NMR spectrum of compound **(3)** (400 MHz, $CDCl_3$ + pyridine-$_{d5}$).

The recorded ^{13}C-NMR spectrum of compound **(3)** (Fig. 56) revealed 21 carbon signals whose aglycone chemical shifts were in good agreement with those of apigenin, and the sugar moiety signals were in high concurrence with those of *β*-D-glucosyl moiety.

The glucopyranosyl moiety attachment was deduced to be at C-7 according to the glycosylation rule.

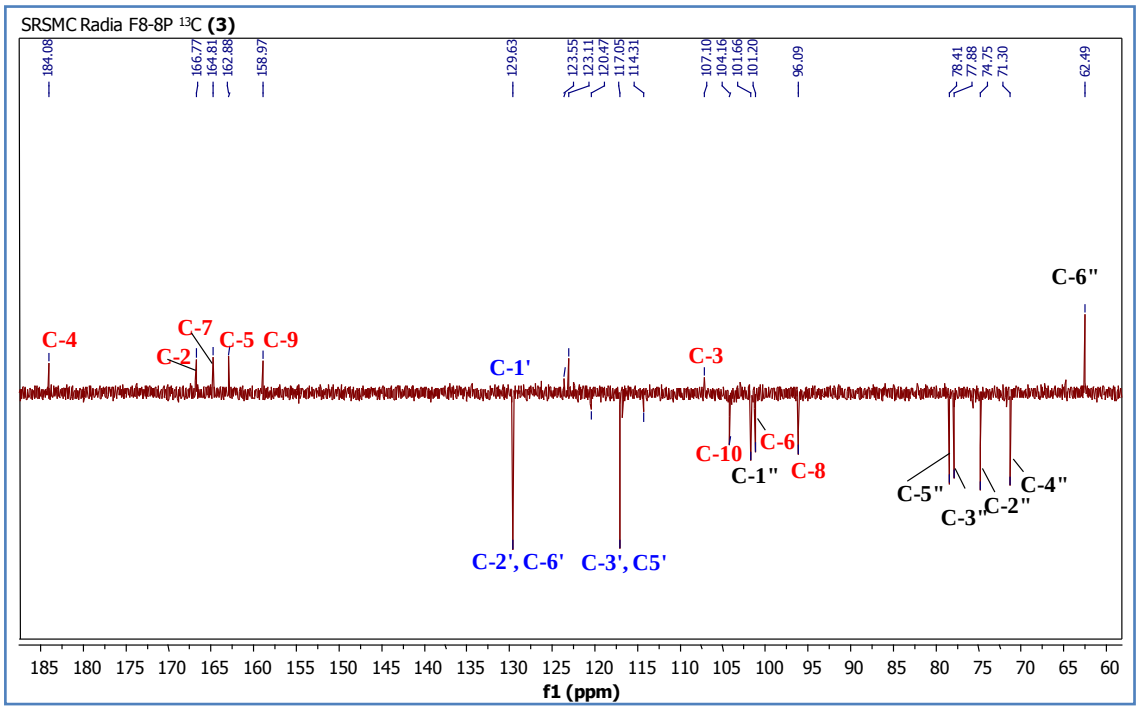


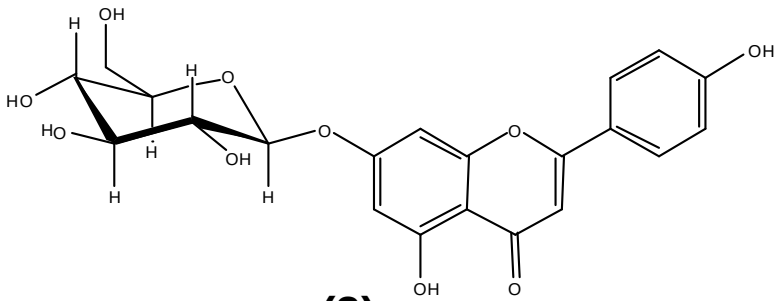
Fig. 56 ^{13}C-NMR spectrum of compound **(3)** (100 MHz, $CDCl_3$ + pyridine-$_{d5}$).

Tab. 16 Chemical shifts in ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of compound **(3)** in $CDCl_3$ + pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)	N°	δ_C	δ_H (m, *J* Hz)
2	166.7	-	9	158.1	-	6′	129.5	7.85, d (8.4)
3	105.7	6.98, s	10	104.1	-	1′′	101.2	5.26, d (6.8)
4	184.0	-	1′	130.6	-	2′′	74.3	3.38, m
5	162.1	12.95, s	2′	129.5	7.85, d (8.4)	3′′	77.8	3.38, m
6	101.3	6.50, d (2)	3′	117.5	7.63, d (8.4)	4′′	71.3	3.25, m
7	164.9	-	4′	132.3	-	5′′	78.4	3.75, m
8	96.0	6.85, d (2)	5′	117.5	7.63, d (8.4)	6′′	62.5	4.41, d (10.1) 4.12, d (11.8)

Furthermore, the **HMBC** spectrum (Appendix 3) confirmed that the anomeric proton of the glucopyranosyl moiety at δ_H 5.26 (d, H1") showed a long-range correlation with (C-7) (δ_C 164.9).

All the above spectral and physical analyses of the present study (LIT-ESI-MS^n, ^{1}H-NMR, and ^{13}C-NMR) (Fig. 57) exerted a strong similarity of this molecule to the experimental data available in the literature (Tan et *al*., 2010; Zhang et *al*., 2017). So, compound (3) was characterized as apigenin-7-O-*β*-D-glycopyranoside ($C_{21}H_{20}O_{10}$).



(3)

Fig. 57 4*H*-1-Benzopyran-4-one, 7-(β-D-glucopyranosyloxy)-5-hydroxy-2-(4-hydroxyphenyl)- « Apigenin-7-*O*-*β*-D-glycopyranoside ».

3.7.4- Structural elucidation of compound (4)

This compound (4) is in the form of a yellow powder with a yield of 2.22%. It reacts positively to Mayer's reagent displaying in yellow precipitation.

Its mass spectrum obtained in LIT-ESI-MS in the negative mode gave a molecular ion *m/z* 134 $[M-H]^-$ that corresponds to a mass of 135 amu (calculated 135.06841 g/mol). Besides, the MS/MS spectrum of this compound (Fig. 58) revealed an ion *m/z* 118, indicating a loss of 16 amu (oxygen atom).

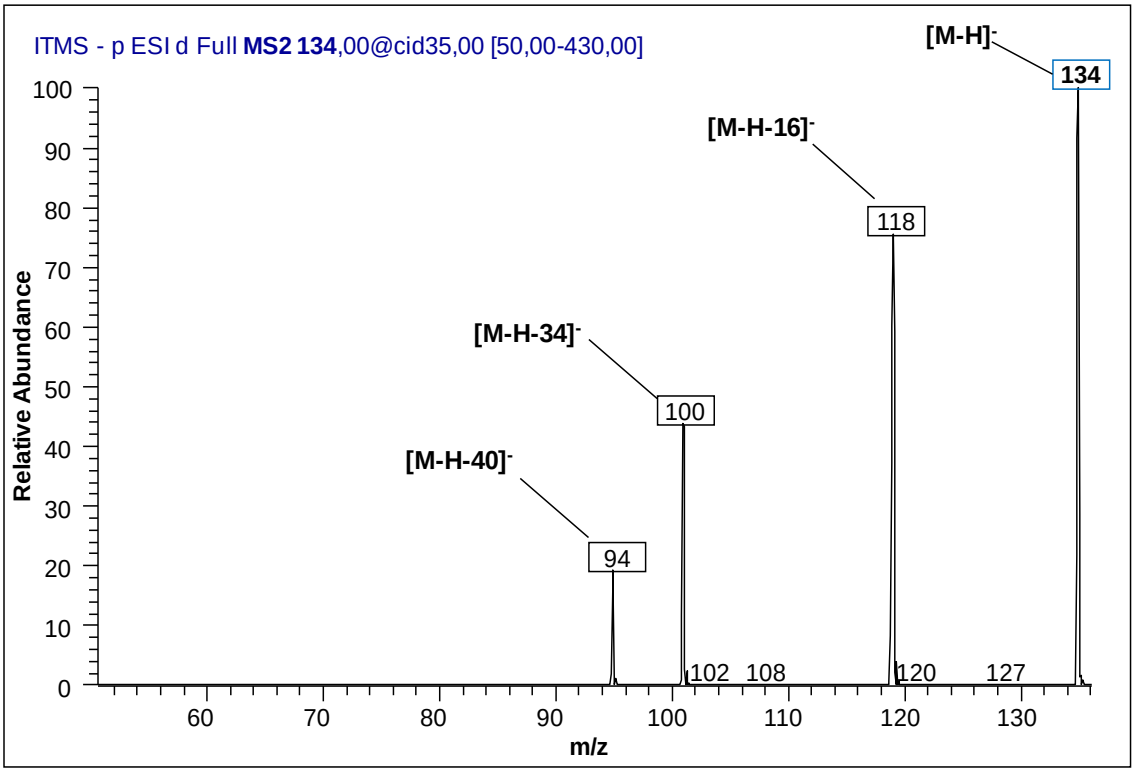


Fig. 58 MS/MS spectrum of compound **(4)** recorded in negative mode.

The NMR spectra of the compound **(4)** (Figs. 59-64), interpreted in **Tab. 17** show all the peaks corresponding to the structure.

The **^{1}H-NMR** spectrum of this composite has:

- Three multiples; two integrating for 2H and one for 1H at δ_H values 3.2, 3.5, and 3.9 ppm for hydrogens relate to carbon 3, 2, and 4; respectively.
- One doublet at δ_H 6.8 (1H) ppm due to the OH attaches to C-5.
- Two doublets integrating 1H at δ_H 7.2 and 7.26 ppm represent H-7 and H-6, in that order.

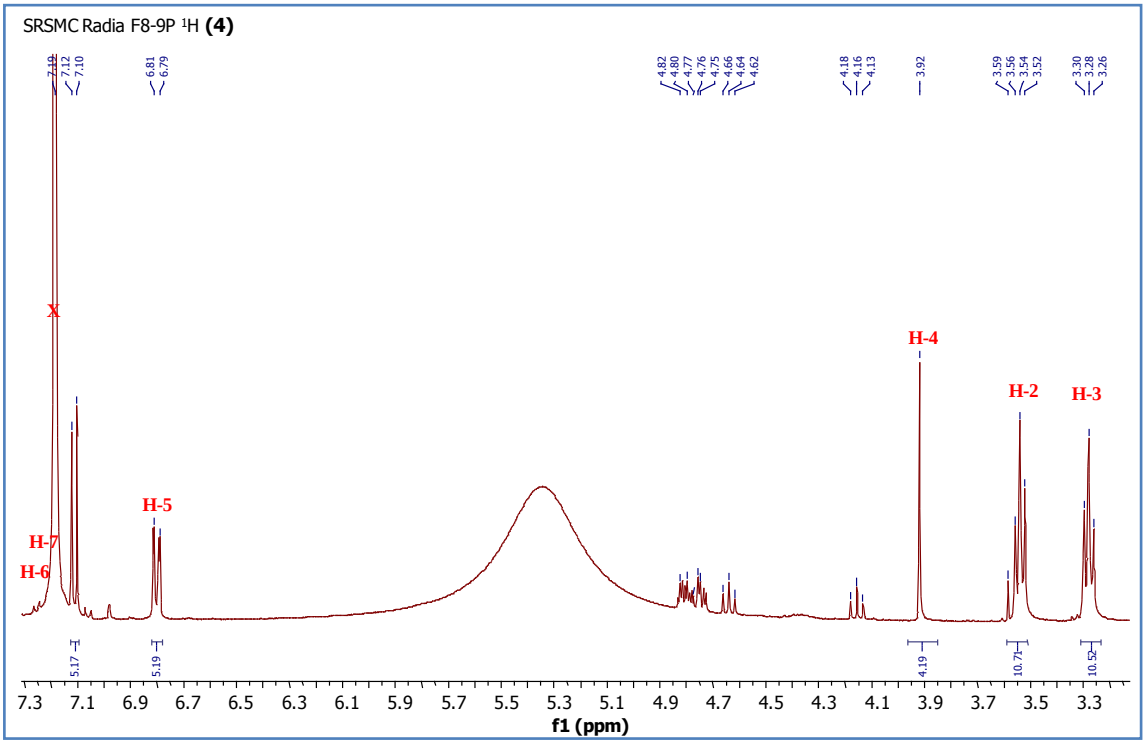


Fig. 59 ^{1}H-NMR spectrum of compound **(4)** (400 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: Solvent.

The ^{13}C-NMR spectrum (Fig. 60), on the other hand, indicates the presence of eight carbon signals with the following chemical shifts: δ_C 33.8 (C-3), 41.7 (C-2), 116.9 (C-4), 117.5 (C-6), 120.4 (C-5), 129.1 (C-1), 146.3 (C-8), 147.5 (OH).

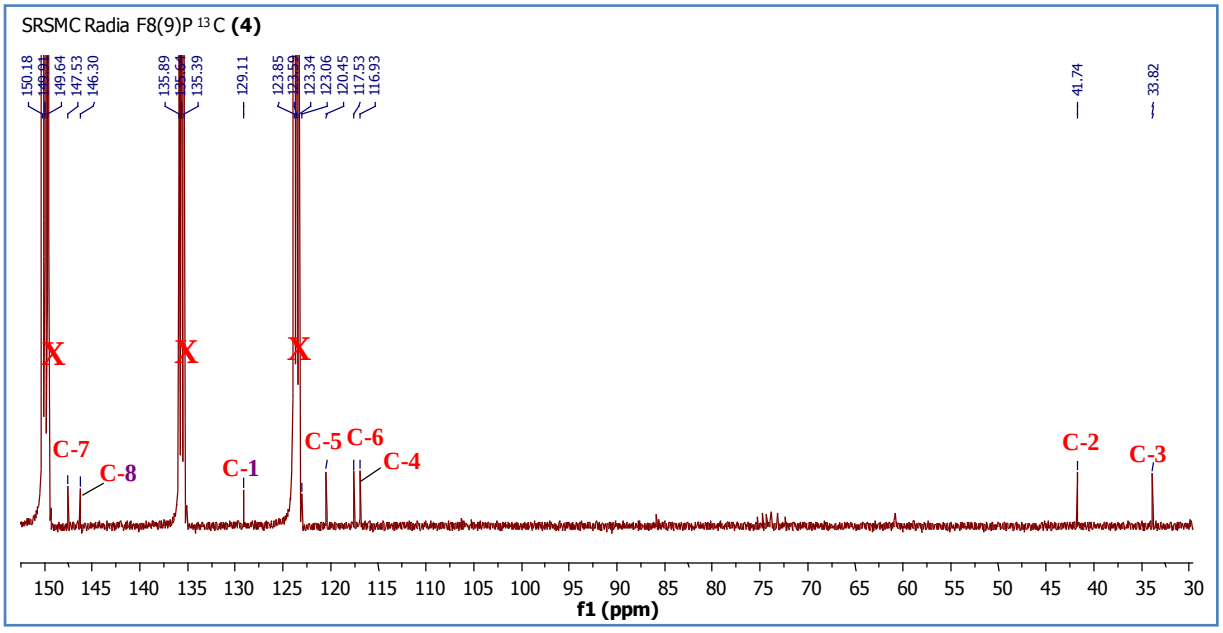


Fig. 60 ^{13}C-NMR spectrum of compound **(4)** (100 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: Solvent.

Tab. 17 Chemical shifts in ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of compound **(4)** in $CDCl_3$ + pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)
1	129.1	-
2	41.7	3.5, m
3	33.8	3.2, m
4	116.9	3.9, m
5	120.4	6.8, dd (8)
6	117.5	7.26, d (8)
7	147.5	7.2, d (2)
8	146.3	-

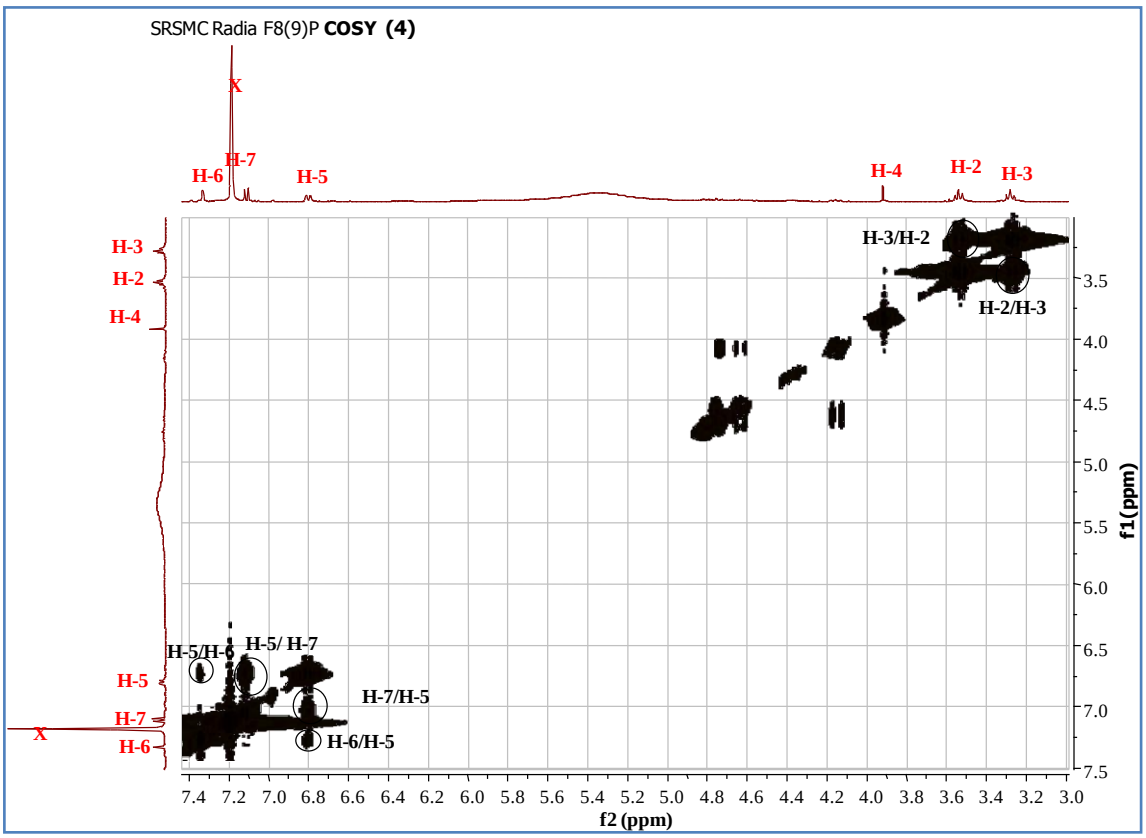


Fig. 61 COSY-NMR spectrum of compound **(4)** (400 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: Solvent.

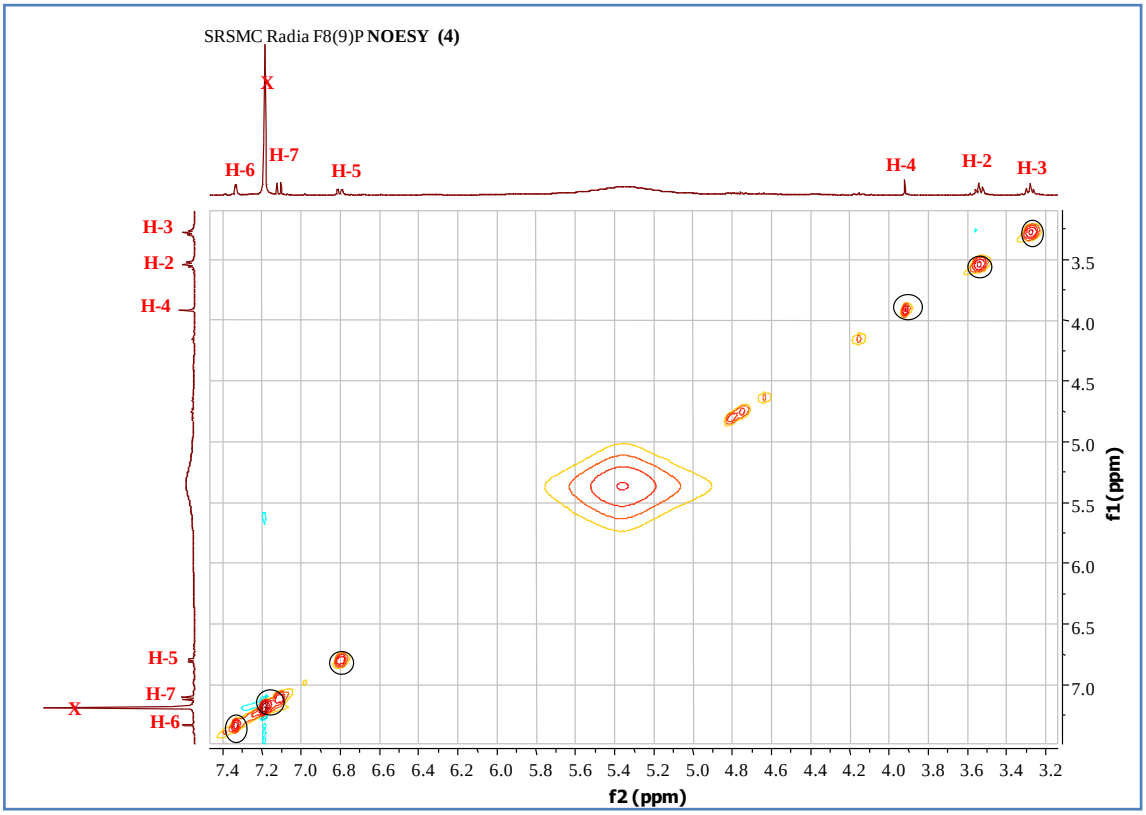


Fig. 62 NOESY-NMR spectrum of compound **(4)** (400 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: Solvent.

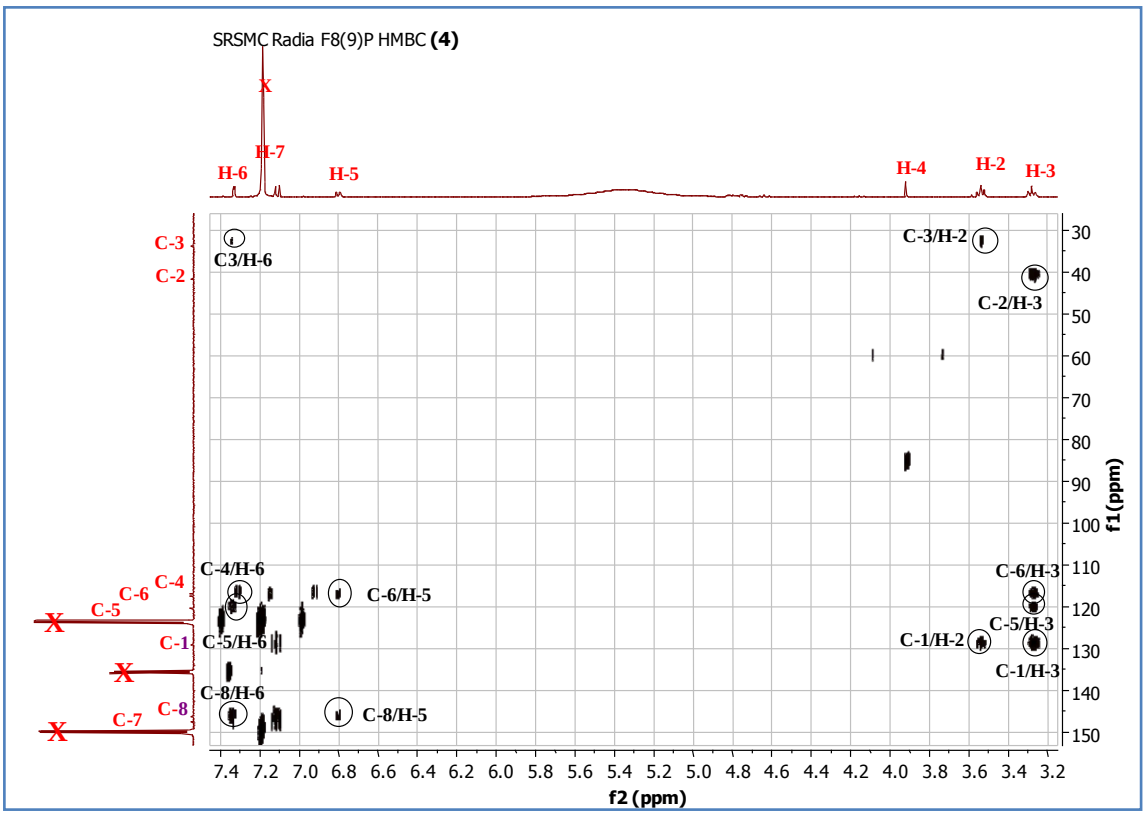


Fig. 63 HMBC-NMR spectrum of compound **(4)** (100 MHz/ 400 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: Solvent.

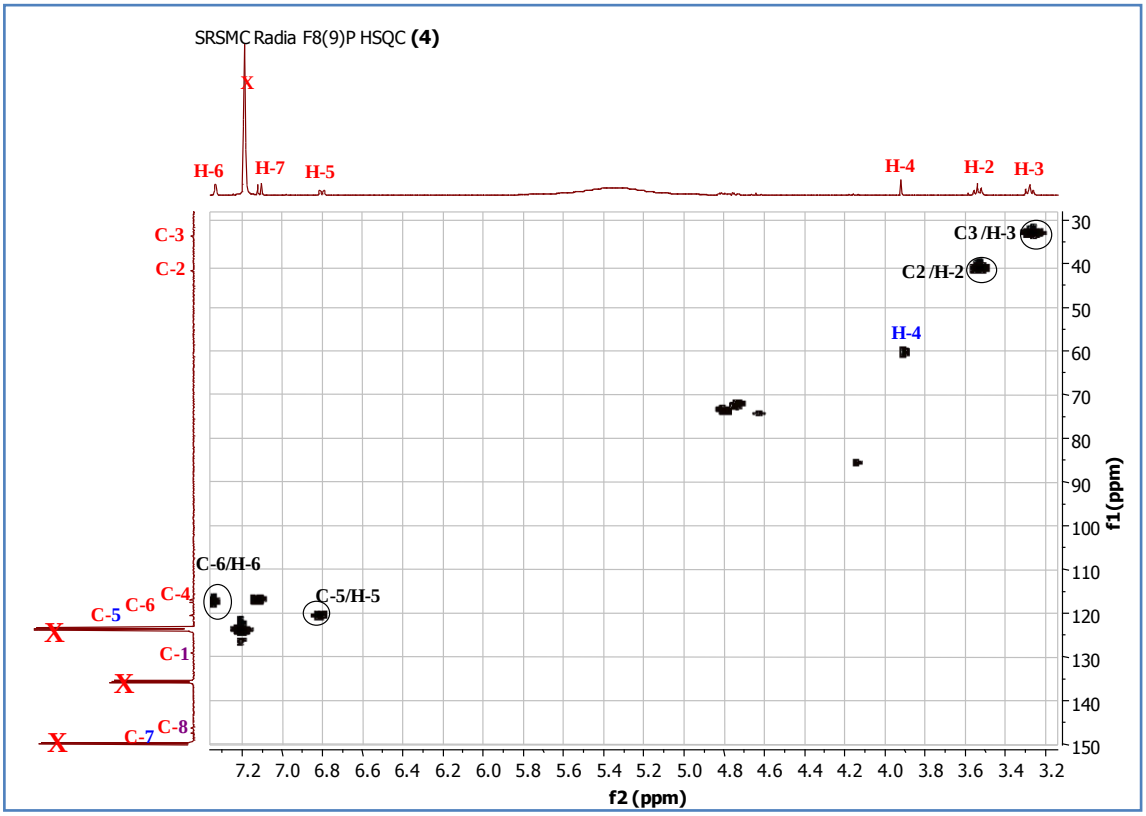
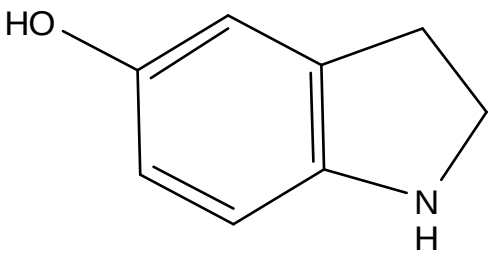


Fig. 64 HSQC-NMR spectrum of compound **(4)** (100 MHz/ 400 MHz, $CDCl_3$ + pyridine-$_{d5}$); X: Solvent.

Based on the data analysis of the mass spectra of compound (4), and its different NMR spectral studies; ^{1}H NMR, ^{13}C NMR, COSY, NOESY, HMBC, and HSQC collectively with the comparison to data from the literature (Hegde et *al.*, 1997), compound **(4)** has identified as being 5-Hydroxyindoline (Fig. 65) considered as a new natural compound with the crude formula C_8H_9NO (MW = 135 g / mol). This result, obtained in the present work, is an innovative finding because, to our knowledge, it is the first evidence of this alkaloid from a natural source.



(4)

Fig. 65 5-Hydroxyindoline.

3.7.5- Structural elucidation of compound (5)

This compound **(5)** was obtained in the form of a white crystalline powder with a yield of 2.78%. Its mass spectrum in LIT-ESI-MS^2 in negative mode gave a quasi-molecular ion *m/z* 193 $[M-H]^-$ suggesting a mass of 194 amu (Fig. 66).

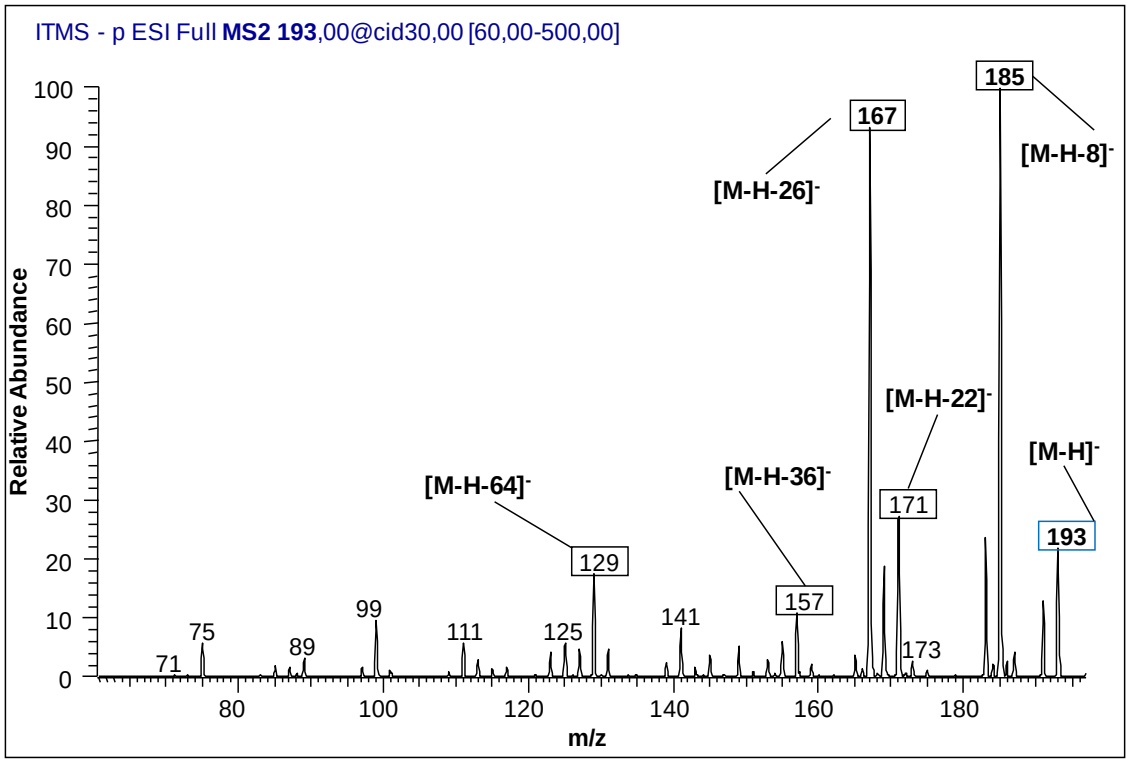


Fig. 66 MS/ MS spectrum of compound **(5)** recorded in negative mode.

In this study, the following characteristics of the compound **(5)** were found by NMR analysis:

^{1}H-NMR spectrum at 400 MHz in pyridine-$_{d5}$ (Fig. 67) showed the presence of six signals in the range of 3.92 to 4.80 ppm:

- Singlet signal integrating for 3H at δ_H 3.92 ppm assigned to the methyl (OCH_3) group.
- Four doublet doublet integrating for 1H at δ_H 4.15, 4.63, 4.76, and 4.78 ppm assigned to H-3, H-4, H-5, and H-2, respectively.
- Multiplet integrating for 2H at δ_H 4.80 ppm attributed to H-1 and H-6.

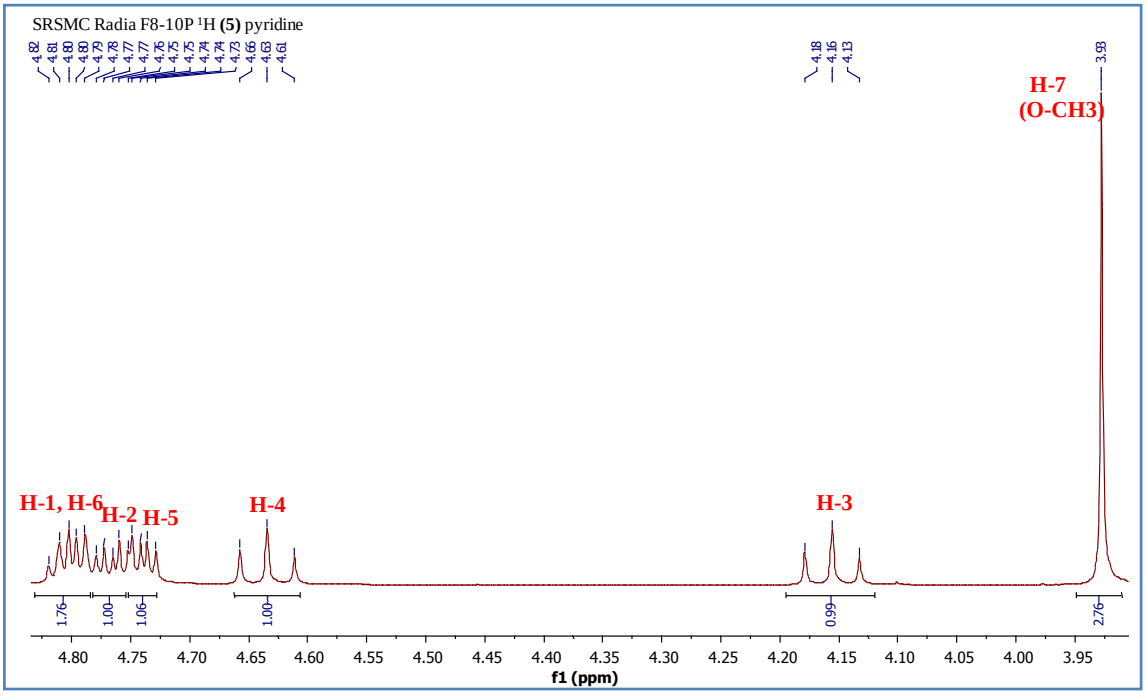


Fig. 67 ^{1}H-NMR spectrum of compound **(5)** (400 MHz, pyridine-$_{d5}$).

Besides, the **^{13}C-NMR** spectrum displayed seven carbon signals with chemical shifts within the region of heteroatom-linked carbon atoms: δ_C 60.74 (OCH3), 72.29 (C-6), 73.09 (C-4), 73.76 (C-2), 74.20 (C-5), 74.70 (C-1), and 85.84 (C-3).

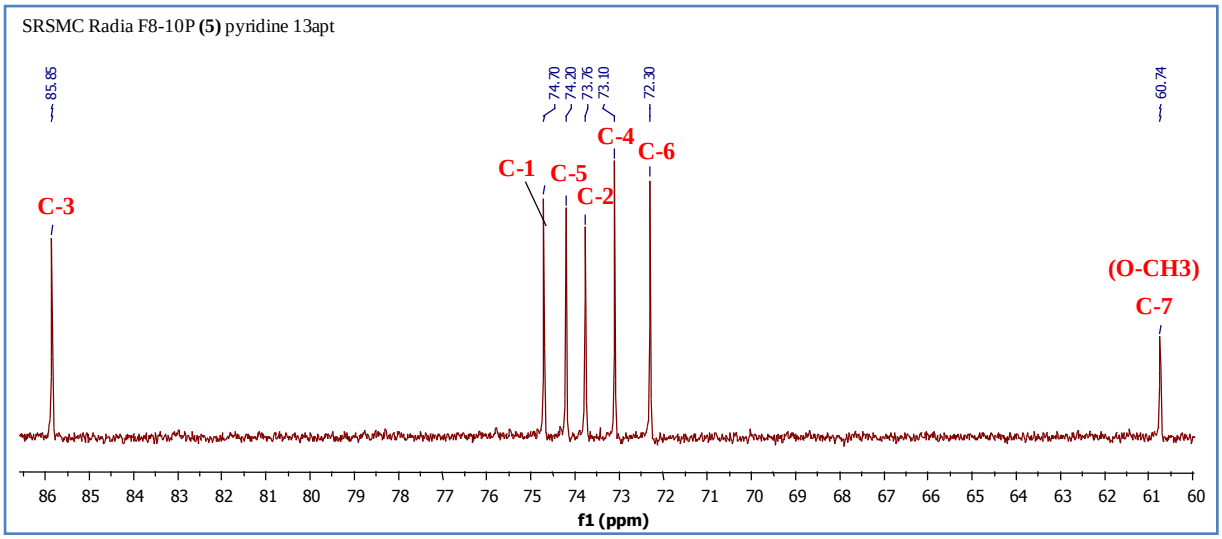


Fig. 68 ^{13}C-NMR spectrum of compound **(5)** (100 MHz, pyridine-$_{d5}$).

Tab. 18 Chemical shifts in ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of compound (5) in pyridine-$_{d5}$.

N°	δ_C	δ_H (m, *J* Hz)
1	74.70	4.80, m
2	73.76	4.78, dd (9.90, 2.6)
3	85.84	4.15, dd (9.90, 9.53)
4	73.09	4.63, dd (9.53, 9.98)
5	74.20	4.76, dd (9.98, 2.6)
6	72.29	4.80, m
7	60.74	3.92, s

In the **COSY** spectrum of compound **(5)** (Fig. 69), correlations were found between:

- The proton signals of H-3 at *δ* 4.15, of H-4 at *δ* 4.63, and of H-2 at *δ* 4.78 ppm;
- H-2 (*δ* 4.78) and H-1 (*δ* 4.80);
- As well as between H-4 (*δ* 4.63) and H-5 (*δ* 4.76).

Furthermore, H-5 coupled with H-6 (*δ* 4.80), which allowed to completely elucidating the compound **(5)** structure.

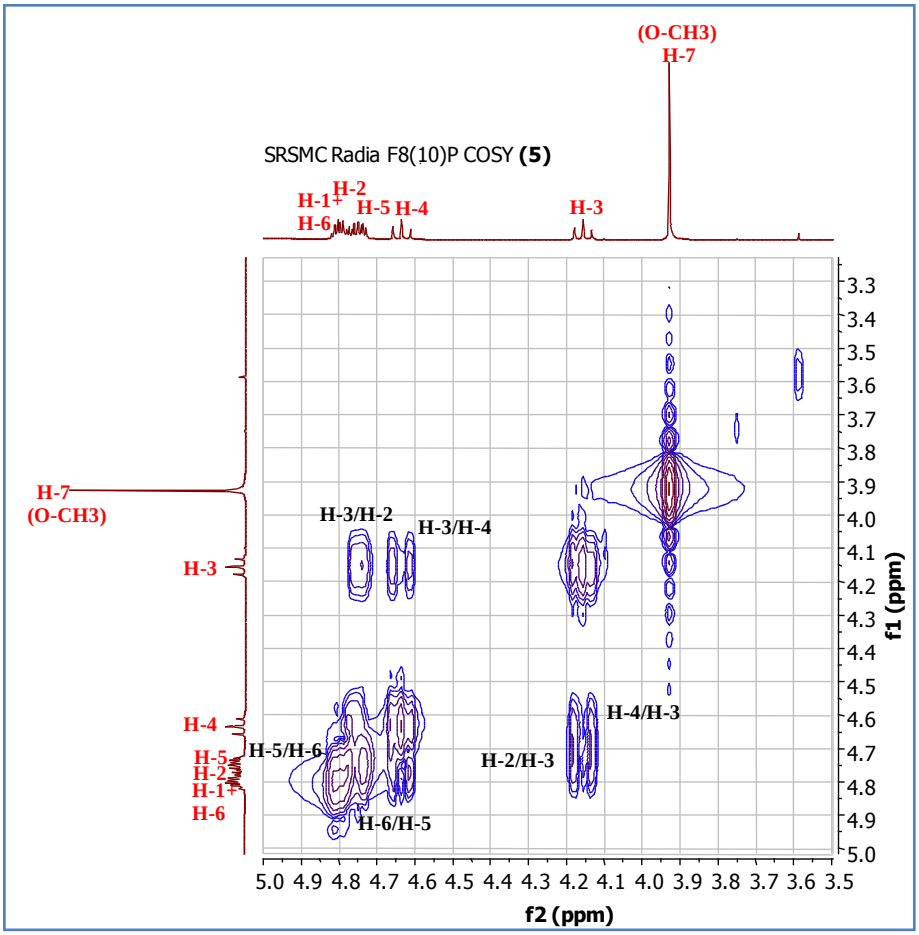


Fig 69 COSY-NMR spectrum of compound **(5)** (400 MHz, pyridine-$_{d5}$).

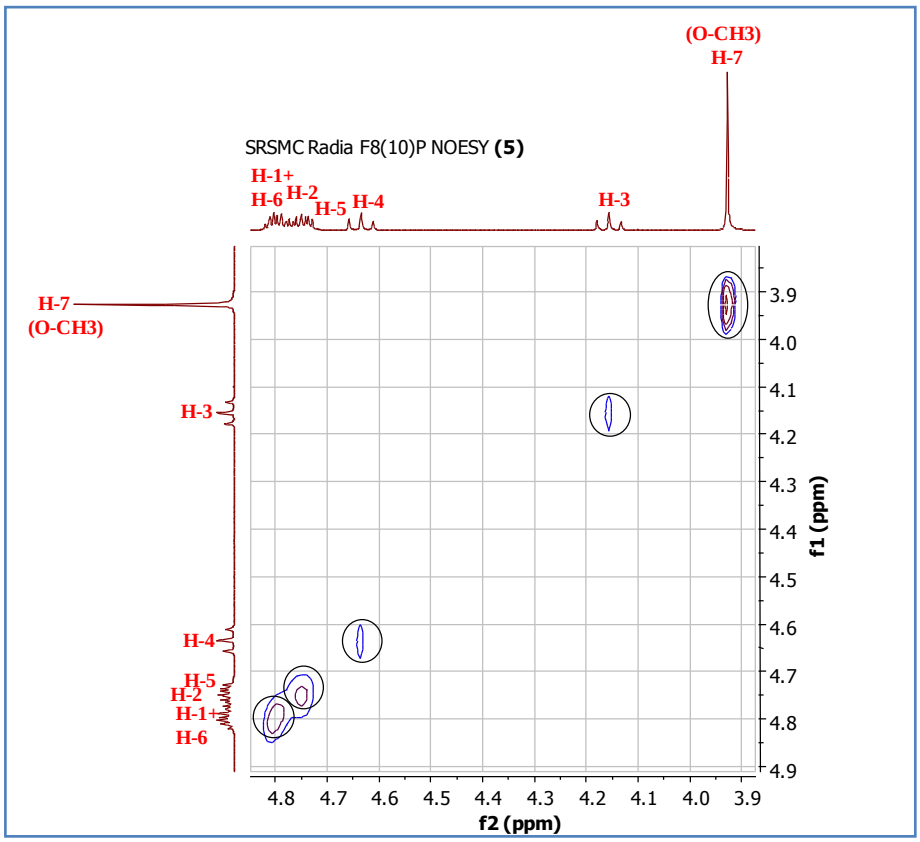


Fig. 70 NOESY-NMR spectrum of compound **(5)** (400 MHz, pyridine-$_{d5}$).

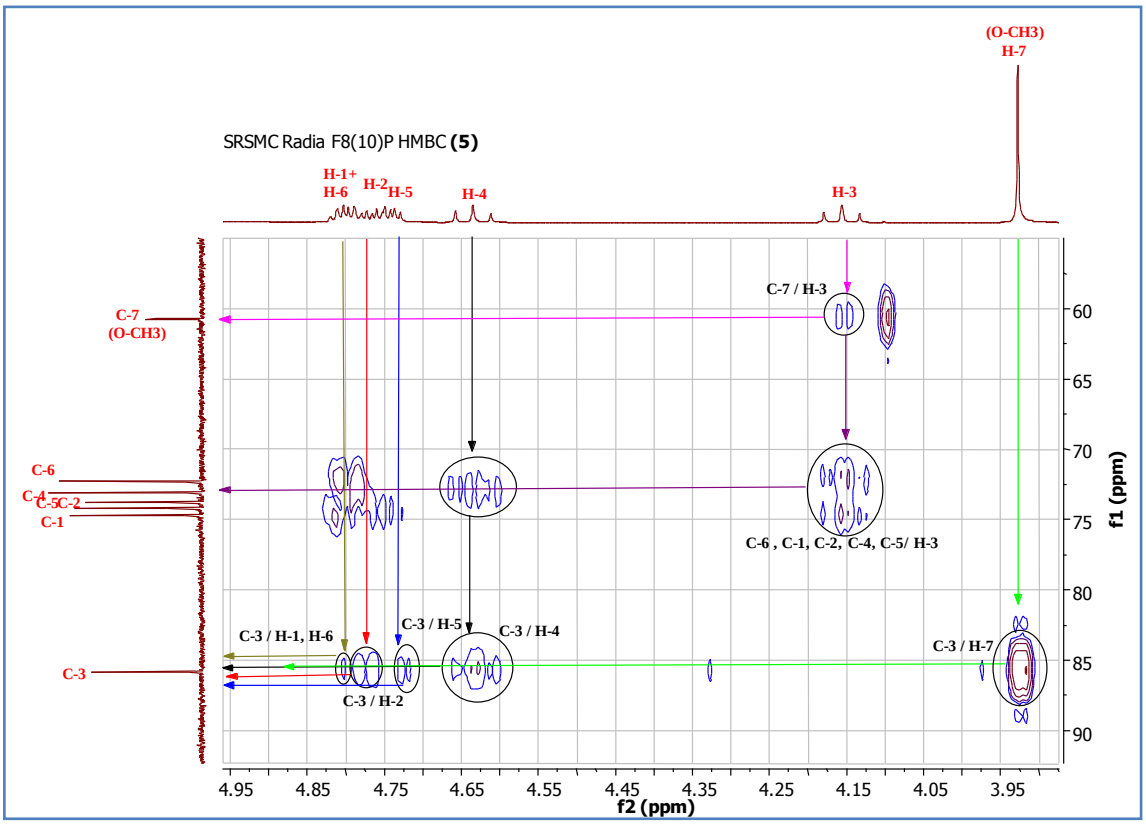


Fig. 71 HMBC-NMR spectrum of compound **(5)** (100 MHz/ 400 MHz, pyridine-$_{d5}$).

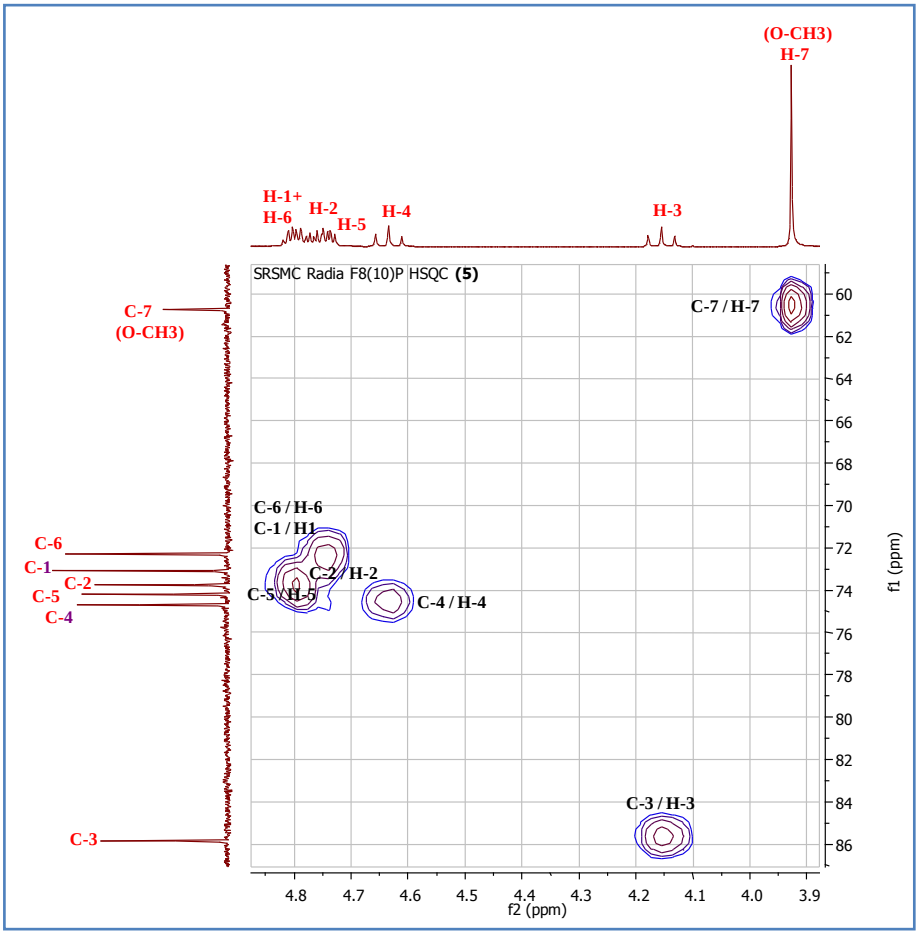
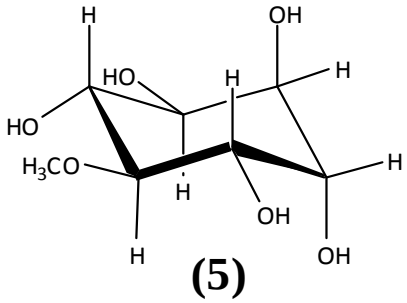


Fig. 72 HSQC-NMR spectrum of compound **(5)** (100 MHz/ 400 MHz, pyridine-$_{d5}$).

All of the above data, mass spectrometry with NMR spectra, have been identical to those described in the literature (Abdoulaye et *al*., 2004; Raya-Gonzalez et *al*., 2008; De Almeida et *al*. 2012; Mukae et *al*., 2016)**.** Furthermore, the mass spectrum of this compound fits that which has been deposited in the NIST MS library (2008). Thus, based on our data and by their comparison with the published findings, compound **(5)** has been identified as **D-Pinitol (**Fig. 73**),** its molecular formula was determined as $C_7H_{14}O_6$ (*m/z* 193 $[M-H]^-$). This result is the first established of this molecule from a plant belonging to the genus *'Calycotome'*, object of study of the present work.



(5)

Fig. 73 D-*chiro*-Inositol, 3-*O*-methyl- « **D-Pinitol** ».

3.8- *In vitro* biological activities of the isolated compounds (1-5)

At the same time, the biological activities of these compounds were carried out.

3.8.1- *In vitro* antioxidant activity of the isolated compounds (1-5)

The antioxidant potential of the five compounds (**1**–**5)**, was evaluated following four different methods; total antioxidant capacity (TAC), DPPH, FRAP, and ABTS test; by calculating either the decrease (DPPH, ABTS) or the increase (TAC, FRAP) in the absorbance.

The best activity has often been demonstrated by the compound **(4**) (5-hydroxyindoline) using the four methods, followed by the standard of each test, while the MeOH extract of leaves (the source of this molecule) and the compound (3) (Apigenin-7-*O*-*β*-D-glucopyranoside) had remarkable anti-radical potential, but less important than that of the new composite (4) (Tab.19).

3.8.1.1- Total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of the studied isolated compounds as well the leaves MeOH extract of *C. spinosa* (L.) Link is expressed as the number of equivalents of ascorbic acid from a calibration curve ($y = 0.0041\ x - 0.35$; $R^2 = 0.980$) displayed in (Appendix 1).

The total antioxidant capacities of the obtained compounds ranged from 236.17 ± 0.17 to 985.54 ± 0.16 mg AAE / g DE, with a constantly maximal performance of the compound **(4)** (985.54 ± 0.16 mg of AAE / g DE) and a minimal act of the compound **(1)** (236.17 ± 0.17 mg AAE / g DE) at the same concentration **(**Tab. 19). Compound **(3)** has also a high antioxidant capacity equal to 608.67 ± 0.22 AAE / g DE. However, less interesting capacities were detected in compounds **(2)**, and **(5)** with 240.94 ± 0.24 and 268.69 ± 0.43 AAE / g DE, respectively. The MeOH extract of leaves, also, tested by this method showed an interesting result with a TAC equal to 671.02± 0.13 mg AAE / g DE.

3.8.1.2- DPPH free radical scavenging activity

Based on the data represented the anti-radical power as a function of the different concentrations of the five compounds, the regression curves have been constructed to measure their IC_{50}.

As indicated in Tab. 19, compound (**4)** (**5-hydroxyindoline**) had the highest inhibition percentage of 73.14 ± 0.45% (at 100 µg/mL) with the lowest IC_{50} **< 10 µg/mL**, showing a scavenging activity more than that of the commercial reference BHT (DPPH; IC_{50} = 34.73 ±

0.23 µg/mL). The compound (**3)** was, also, active but less than the compound **(4)** with IC_{50} = 47.36 ± 0.21 µg/mL. However, compounds (**1), (2),** and (**5)** have revealed insignificant antioxidant capacities. A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value.

3.8.1.3- Ferric reducing antioxidant power (FRAP) assay

The regression curves of the five compounds have been constructed to calculate their EC_{50} values (Appendix 1).

As can be seen in Tab. 19, compound **(4)** has moreover developed the most significant reducing power with an EC_{50} = 344.82 ± 0.02 µg / mL more powerful than ascorbic acid as a reference (EC_{50} = 684.29 ± 0.024 µg / mL). Besides, the compound **(3)** was, also, proved to be effective with an EC_{50} of 814.61 ± 0.31 µg / mL; but significantly with lower activity than that of the new compound (4). However, compounds **(1)**, **(2)**, and **(5)** have indetermined activity. Higher reducing power is similarly related to a lower EC_{50} value.

3.8.1.4- ABTS cation radical test

The IC_{50} of the methanol crude extract and its five compounds ranged from 7.8 ± 0.43 to 391.92 ± 0.5 µg/ mL (Tab. 19**)**. It can, also, be noticed that the strongest ABTS scavenging effect among them was, usually, observed by the 5-hydroxyindoline (**4)** with the lowest IC_{50} (7.8 ± 0.43 µg/mL). While the weakest activity was detected by compound (**5)** (D-pinitol) with a higher IC_{50} = 391.92 ± 0.5 µg/mL.

Superb correlations between the four used methods were observed; they ranged from -0.84 to 0.99. DPPH and FRAP exhibited the strongest positive correlation (0.99), followed by FRAP/ABTS (0.97), then ABTS/DPPH with a high correlation coefficient equal to 0.96. However, lower negative correlations -compared to the previous- were obtained between the total antioxidant capacity test as a quantitative method and other procedures; TAC/FRAP (-0.93), TAC/DPPH (-0.92), as well as TAC/ ABTS; revealed the lowest negative correlation with a coefficient of -0.84, but always remained powerful.

Tab. 19 Antioxidant activity of the isolated compounds (1-5) from MeOH extract of *C. spinosa* leaves as well as standards measured by different assays.

Samples	**IC_{50}/ DPPH (µg/mL)**	**TAC (mg AAE/g DW)**	**EC_{50}/ FRAP (µg/mL)**	**IC_{50} ABTS (µg/mL)**
Leaf MeOH	41.04± 0.15	671.02± 0.13	763.73± 0.32	19.18± 1.3
Compound (1)	354.30± 0.05	236.17± 0.17	1930.61± 0.17	240.42± 0.43
Compound (2)	343.24± 0.12	240.94± 0.24	1805.46±0.12	247.26± 0.21
Compound (3)	47.36± 0.21	608.67± 0.22	814.61± 0.31	63.72± 0.64
Compound (4)	<10=6.02± 0.11	985.54± 0.16	344.82 ± 0.02	7.8± 0.43
Compound (5)	373.20± 0.34	268.69± 0.43	2230.08± 0.29	391.92± 0.5
BHT	34.73± 0.23	/	/	7.3± 0.5
Ascorbic acid	/	905.95± 0.5	684.29± 0.024	/
Each value is expressed as the mean ± standard deviation (n = 3); TAC = total anti-oxidant capacity; IC_{50} = inhibition concentration 50%; EC_{50} = effective concentration at which the absorbance was 0.5.				

3.8.2- *In vitro* antimicrobial activity of the isolated compounds (1-5)

In the microbiological study, the main objective of the present study, the compound (4) showed very interesting antimicrobial activity. Indeed, the antimicrobial results of the five compounds against the tested microorganisms were shown in Tab 20 and Fig.74.

Three out of seven microorganisms were sensitive to only one compound (4); the others were resistant to all tested molecules. Compound **(4),** the only active composite, had the strongest bactericidal activity against *S. aureus* with an IZD equal to 16.00 ± 0.50 mm at [C]=1 mg/ disc and a MIC of 0.25 ± 0.00 mg/ disc. It exerted also a bacteriostatic activity against both *P. aeruginosa* and *S. abony* with moderate IZDs of 9.83 ± 0.29 and of 8.00 ± 0.28 mm, respectively at the same [C], with a MIC of 0.75 mg/disc and non determined MBC (> 1 mg/disc). However, no activity was exhibited by the same compound (**4**) against *B. subtilis*, *E. coli*, *K. pneumoniae*, and *C. albicans* that were resistant to all the tested compounds. Besides, the other four composites **(1)**, **(2)**, **(3),** and **(5)** were inactive towards all the tested microorganisms and no IZDs were observed.

Tab. 20 Inhibition zones diameters of antimicrobial activity of the isolated compounds (1-5).

		Inhibition zone (mm)								
		crude extract	Isolated compounds					Control (+) (Standards)		Control (-)
Microorganism		**L MeOH ***	**(1)***	**(2)***	**(3)***	**(4)***	**(5)***	Chloramphenicol **15 µg/disc**	Fluconazol **10 µg/disc**	DMSO
Gram +	*S.aureus*	**20 ± 0.28** [b]	/ [e]	/ [e]	/ [e]	**16± 0.5** [b]	/ [e]	40 ± 0.07 [a]	NT	/ [e]
	B. subtilis	**16 ± 0.5** [b]	/ [e]	/ [e]	/ [e]	/ [e]	/ [e]	33 ± 0.13 [a]	NT	/ [e]
Gram -	*E. coli*	/ [e]	/ [e]	/ [e]	/ [e]	/ [e]	/ [e]	28 ± 0.34 [a]	NT	/ [e]
	P. aeruginosa	**7.2 ± 0.5**[d]	/ [e]	/ [e]	/ [e]	**9.83 ± 0.29**[c]	/ [e]	32 ± 0.22 [a]	NT	/ [e]
	K. pneumoniae	/ [e]	/ [e]	/ [e]	/ [e]	/ [e]	/ [e]	30 ± 0.17 [a]	NT	/ [e]
	S. abony	**12 ± 0.29** [b]	/ [e]	/ [e]	/ [e]	**8 ± 0.28**[c]	/ [e]	35 ± 0.44 [a]	NT	/ [e]
Yeast	*C. albicans*	/ [e]	/ [e]	/ [e]	/ [e]	/ [e]	/ [e]	NT	35.2 ± 0.24[a]	/ [e]
* 1 mg/disc; e: no inhibition; NT: Not Tested, L MeOH: Leaf methanol extract.										

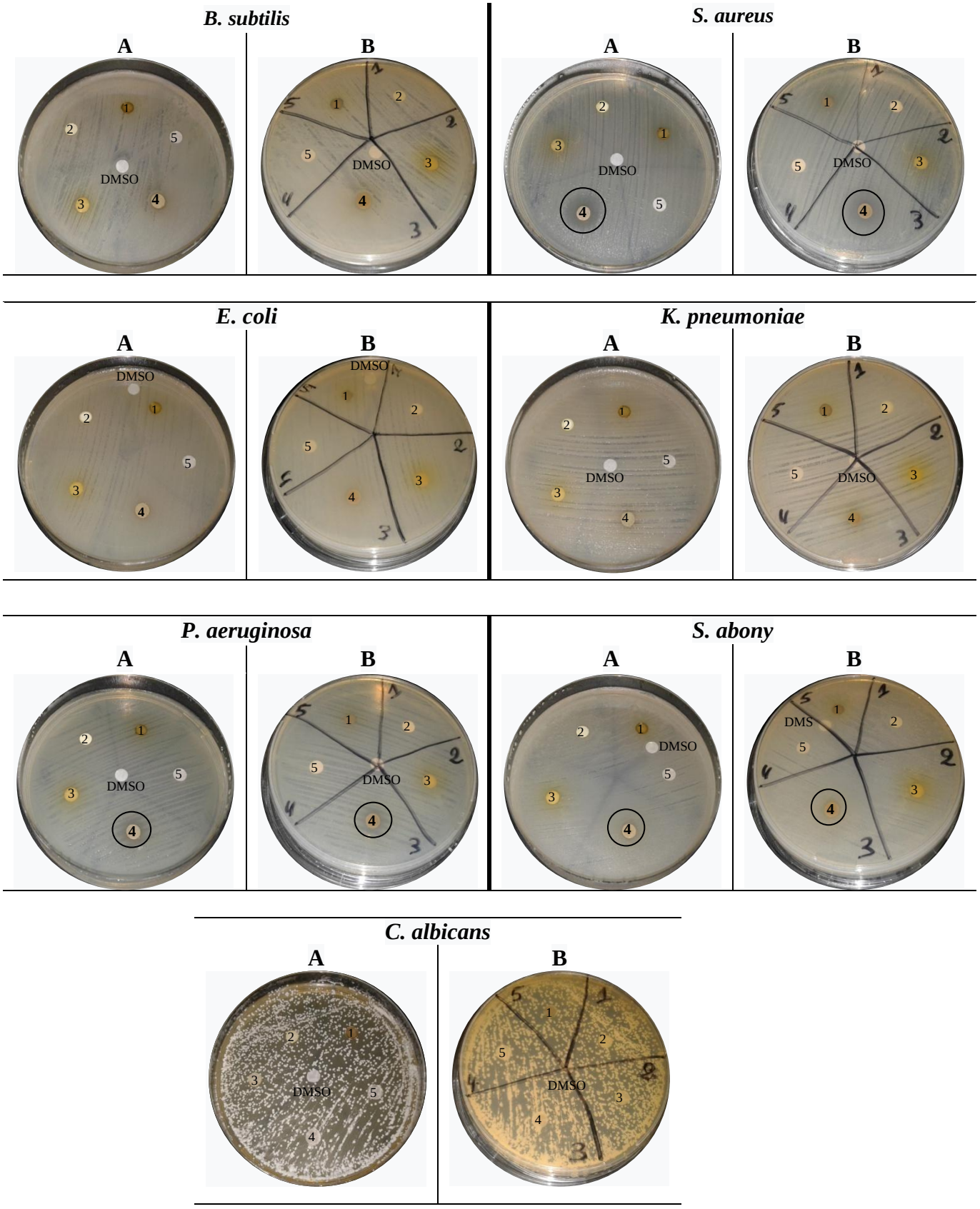


Fig. 74 *In vitro* antimicrobial activity of the isolated compounds; (**1**), (**2**), (**3**), (**4**), and (**5**).

A: surface, B: reverse.

Discussion

4- Discussion

Thousands of diverse natural products are produced by plants and many of them are involved in plant defense. The phytochemical diversity of bioactive compounds includes terpenoids, saponins, phenolics and phenyls, propanoids, pterocarpanes, stilbenes, alkaloids, glucosinolates, hydrogen cyanide, indole, and also elemental sulfur; the only inorganic compound (Cooper et *al*., 1996).

The plant of our study was identified as *Calycotome spinosa* (L.) Link (*C. spinosa*). The bibliographic research on this species revealed the absence of published works aimed at the detailed phytochemical screening of its different parts. However, some studies considered as outlines; have introduced this species in Algeria (Larit et *al*., 2012; Krimat et *al*., 2014). On another aspect, several studies on the plant, as a legume, have been devoted to its botanical description, and its interaction with the environment (Zeddam et *al*., 2007; Damerdji and Djedid, 2006; Damerdji, 2008-2009; Damerdji and Djedid, 2012).

Indeed, the chemical profile of the studied plant, *C. spinosa*, revealed that the phenolic compounds including flavonoids, polyphenols, and tannins are the most abundant compounds in its leaves and flowers; however, a complete absence of essential oils and proteins has been carried out. These results are similar to those revealed by Larit et *al*. (2012) who worked on the same species, in particular on the presence of flavonoids. At the same time, when these results are compared with those of other species of the same genus, many similarities were noted. Indeed, our results are in agreement with those of Djeddi et *al*. (2015) who demonstrated the presence of several chemical families as alkaloids, flavonoids, sterols, and triterpenes in the aerial part of *Calycotome villosa* (*C. villosa*) from the Edough mountain (Annaba, Algeria).

Moreover, our results can also be compared to those of Elkhamlichi et *al*. (2017), in particular the presence of flavonoids such as flavones and flavonols, and tannins in seeds and bark of *C. villosa*, collected from a Moroccan region. In addition, several molecules belong to the family of flavonoids, alkaloids, and polyphenols have been separated and identified from *C. villosa* (Al Antri et *al*., 2004a, Al Antri et *al*., 2004b, Al Antri et *al*., 2004c, Al Antri et *al*., 2010; Elkhamlichi et *al*., 2014, Turan and Mammadov, 2020) confirming the richness of this genus in secondary metabolites. By way of comparison, the results found in the present work are similar to those developed in a recent study by Ayoola et *al*. (2020) on leaves of another genus, *Desmodium adscenden*. The study in

question revealed the presence of the same groups of metabolites as those detected in the present work, namely; sugars, polyphenols, flavonoids, tannins, alkaloids, and saponins.

The above results showed that the presence and/or the absence of certain metabolites vary depending on the used part of the plant, the plant species, and the region of study. In fact, chemical groups are found in all parts of plants, but they are distributed differently depending on their roles (Muanda, 2010; Pagare et *al*., 2015).

The quality, the quantity, and the biological activities of the phytochemicals depend, directly, on; the plant development stage, the plant part, and the solvent used for the extraction and the isolation (Senguttuvan et *al*., 2014; Ullah et *al*., 2017; Jacotet-Navarro et *al*., 2018; Chekroun-Bechlaghem et *al*., 2019). Indeed, the obtained results in the present work have shown that there are significant differences between the yield (Y %), the total polyphenols content (TPC), and the total flavonoids content (TFC) in the crude extracts and the fractions of the studied plant. The EtOAc fraction of the leaves showed a lower Y % (1.74 ± 0.02%); however, it recorded the highest values in TPC (107.75 ± 2.09 mg GAE / g DE) and in TFC (20.87 ± 0.10 mg QE / g DE). These results are in agreement with those obtained by Turan and Mammadov (2020) working on another species of the same genus (*C. villosa*), where their results showed higher values of TPC and TFC in the ethanolic extract (EtOH) of flowers; with TPC of 159.47 ± 0.33 mg GAE / g DE and TFC of 66.21 ± 0.09 mg QE / g DE, respectively. This fact is probably related to both the solvent and the plant part used in the extraction.

Besides, in this work, it was found that the MeOH extract of leaves was rich in TPC (98.72 ± 2.47 mg GAE / g DE) and poor in TFC (4.02 ± 0.62 mg QE / g DE), while its Y% was high equal to 15.88 ± 0.53%. These results are less prominent than those obtained by a study of Krimat et *al*. (2014) on a hydromethanolic extract of leaves of the same species, where the highest TPC estimated at 228.42 ± 8.86 mg GAE / g DE and a lower TFC equal to 4.87 ± 0.12 mg QE / g DE were obtained. In addition, the research conducted by Mebirouk-Boudechiche et *al*. (2015) on the *C. spinosa* leaves revealed that the TPC and the total tannin contents values were equal to 119.43 and 83.68 g tannic acid equivalents/ kg DM, respectively. These results are consistent with those obtained in the present study.

In this investigation, it was also observed that with the exception of the TPC of the MeOH extract of the leaves, the TPC and the TFC of the other polar extracts (MeOH and Aq) of the two parts of the plant; leaves and flowers, were lower than those of all fractions

($CHCl_3$, EtOAc, n-BuOH). While the Y% of the polar extracts showed inverse values. This can be explained by the fact that the polarity of the extraction solvent and the presence of many other chemical components, more than the phenolic compounds, in these polar extracts had an influence on the phytochemical content and on the yield (Lesjak et *al.*, 2011; Do et *al.*, 2014; Wakeel et *al.*, 2019).

The TPC in the MeOH extract of leaves was high, close to that of the EtOAc fraction of the same part. The opposite trend was observed in the case of TFC. This is probably, due to the difference in the extraction solvent. Therefore, the MeOH is a suitable solvent for the extraction of polyphenolic compounds from plant tissues, due to its ability to inhibit the action of polyphenol oxidase which causes the oxidation of polyphenols, and to its ease of evaporation in comparison with water (Yao et *al*., 2004).

Moreover, and for comparison with other plant genera, the study of Mahmoudi et *al*. (2012) on different parts of the artichoke flower (*Cynaras colymus* L.) indicated that the aqueous extract of the stems has a TPC and a TFC equal to 14.49 ± 1.51 mg GAE / g DM and to 4.18 ± 0.49 mg QE / g MS, respectively. These results are lower than those of the TPC obtained in this work. It is well-known that the amount of phenolic compounds varies depending on the plants' families and varieties (Sini et *al*., 2010; Belmekki and Bendimerad, 2012).

In conclusion of this part, it is suggested that the differences in the amounts of chemical compounds mentioned above may be related to the differences in the polarity between the solvents, and therefore to the solubility of the solute in the solvent, as well to the region and the season of harvesting (Gomez-Caravaca et *al*., 2006; Jacotet-Navarro et *al*., 2018; Wakeel et *al*., 2019).

The antioxidant properties of plant extracts and products cannot be determined by a single method due to the presence of a complex of phytochemicals. It is well-known that at least two different methods should be used in order to obtain more reliable results in the test of antioxidant activity (Du et *al*., 2009). For that, and in order to provide a conclusive result of the antioxidant potential of both the crude extracts and the fractions of leaves and flowers of *C. spinosa*, two suitable tests, commonly used, were carried out namely DPPH and FRAP.

The DPPH test combines the assessment of both the capacity to release hydrogen and the reducing abilities (Cheng et *al*., 2015) of the studied extracts and fractions. The obtained results showed that the extracts and the fractions of the leaves had greater antioxidant power than that of the flowers. Indeed, the MeOH leaf extract developed the highest anti-radical activity with an inhibition percentage (IP %) estimated at 87.92 ± 0.24 % reflecting a low inhibitory concentration of 50% (IC_{50}) equal to 41.04 ± 0.15 µg / mL, this value is very close to that of the positive control, butylated hydroxytoluene (BHT), which revealed an IP% of 89.38 ± 0.15 % with an IC_{50} of 34.73 ± 0.23 µg / mL, followed by the fraction EtOAc of the same part with an IC_{50} = 45.25 ± 1.8 µg / mL confirming the presence of potent antioxidants in these constituents. On the other hand, the other extracts did not develop a satisfactory antioxidant activity.

Our findings can be compared to those reported by Krimat et *al*. (2014), where the hydromethanolic extract of *C. spinosa* leaves showed a very significant antioxidant potential (DPPH; IC_{50} = 29.20 ± 0.8 µg / mL) compared to other plant species tested under the same experimental conditions.

Moreover, a recent study carried out by Chikhi et *al*. (2014) on the essential oil and the ethanolic extract (EtOH) of the aerial part of *C. villosa* revealed that the lowest free radical scavenging capacity (DPPH) was obtained by the essential oil (60%), so that the EtOH extract developed a strong antioxidant activity estimated at 96%, close to the effect of ascorbic acid (positive reference) which revealed an activity equal to 98.61%.

Furthermore, the results of this work, further, corroborate those of Elkhamlichi et *al*. (2017), where they demonstrated that the MeOH extract of *C. villosa* seeds exhibited an excellent DPPH radical scavenging activity (IC_{50} = 0.20 mg / mL) approximately close to the value of the positive control BHT (IC_{50} = 0.19 mg / mL), followed by the EtOAc extract with a satisfactory effect (IC_{50} = 0.34 mg / mL).

In addition, a recent work conducted by Alhage et *al*. (2018) on the *C. villosa* species revealed that the best anti-radical capacity, using the DPPH test, was developed by the MeOH and the Aq extracts equal to 90% at the concentration of 0.2 mg / mL, followed by the dichloromethane extract of stems with 90% inhibition, at a concentration [C] = 1 mg / mL.

Our results were, also, comparable to those found by Boughalleb et *al*. (2020) working on *C. villosa*, where the reduction activity of the free radical DPPH, by the same MeOH

extract, but, of seeds (IC_{50}) estimated at 16.5 µg / mL for the seeds collected in 2002 and at 22.0 µg / mL for those collected in 2013, showing that seed storage has a positive effect on antioxidant activity.

Finally, our results are also close to those of Turan and Mammadov (2020) on *C. villosa*, where the highest anti-radical power of DPPH was revealed, but by the EtOH extract of flowers with an IC_{50} equal to 0.6 mg / mL.

On another aspect, the antioxidant power of the studied extracts and fractions was tested by the ferric reducing antioxidant power (FRAP) where, the compound has the capacity to release electrons (Saeed et *al*., 2012). In the present work, the MeOH extract and the EtOAc fraction of the leaves seemed to be, consistently, more effective, exerting a remarkable reducing power close to that of positive control with an EC_{50} equal to 763.73 ± 0.32 and to 780.04 ± 1.36 µg / mL, consecutively. These results are in agreement with those of Elkhamlichi et *al*. (2017) which revealed a strong reducing power in the same MeOH extract but obtained from seeds and pods of *C. villosa*. Besides, the reducing antioxidant power (FRAP) of MeOH extracts from *C. villosa* seeds was also evaluated, where the seeds stored for a long time (2002) showed the highest reducing power (IC_{50} = 59.2 ± 0.6 µg / mL), while those of 2013 revealed a less important effect (IC_{50} = 99.0 ± 0.7 µg / mL) (Boughalleb et *al*., 2020).

It should be noted that the antioxidant activity revealed by our study and by other studies, makes it possible to highlight the importance of plants as a source of bioactive compounds. Indeed, many studies have found that plants are a natural source rich in antioxidant compounds, and therefore eligible to serve as active ingredients in several new drugs in particular; anticancer drugs by the development of cytotoxic or cytostatic effects in cancerous cell lines (Shoemaker et *al*., 2005; Xia et *al*., 2011). In addition, phenolic compounds such as; flavonoids, phenolic acids, and tannins have other biological activities such as anti-inflammatory and anti-atherosclerotic activities, which have been shown to be linked to their antioxidant activity (Chung et *al*., 1998).

This activity is, strongly, related to both the method and the solvent of the extraction. Indeed, the study of Dessi et *al*. (2001) revealed that the MeOH extract of the aerial part of *C. villosa*, in Sardinia region, showed efficacy in preventing the antioxidant process, these results are similar to those developed in our study on *C. spinosa*, explaining that MeOH

facilitates the extraction of flavonoids widely known for their antioxidant capacity (Sreeramulu et *al*., 2013).

Besides, an interesting positive correlation was, clearly, shown in the present study between the total polyphenols content (TPC) and the two antioxidant activities; whereby, 0.62 and 0.81 correlation coefficients have been recorded between TPC and DPPH test, as well as between TPC and FRAP assay, consecutively. Our results are in agreement with those of Krimat et *al*. (2014), in which a moderate correlation between the total polyphenols content in the same plant and the anti-radical properties tested by the DPPH activity was observed. Indeed, many studies have, also, revealed a positive correlation between the content of different phenolic compounds in plants and their antioxidant capacities (Karou et *al*., 2005; Lamien-Meda et *al*., 2008; El Hajaji et *al*., 2010; Belmokhtar et *al*., 2014; Wakeel et *al*., 2019) due to the fact that the variation in the chemical structure of phenolic compounds, significantly, influences their different antioxidant activity (Tatiya et *al*., 2011).

According to other studies, the antioxidant capacity does not depend, exclusively, on the content of phenolic compounds but, it can be due to other phytoconstituents or to their combined effect (Wong et *al*., 2006; Ho et *al*., 2012).

This finding is, clearly, developed in our study where the antioxidant activity of the extracts, in particular the methanolic extract, is more significant than that developed by the fractions.

The antioxidant activity observed in the various extracts and fractions of the studied plant species in this work, *Calycotome spinosa*, informed of its possible reactive power with the cells, therefore, the idea of seeking its antimicrobial power submerged. In subsequent studies, this power will be sought in transformed cells in humans.

The antimicrobial activity of the obtained extracts and fractions from *Calycotome spinosa* has, widely, been investigated in several pathogenic or opportunistic organotrophic microorganisms. The developed results made it possible to observe that the studied extracts and fractions had frequently an effect on most of the tested microbial strains. This activity differs according to the used part of the plant, the extraction solvent, and the tested strain.

In fact, the leaves extracts and fractions were, generally, more active than those of the flowers. Distinctly, MeOH extracts and EtOAc fractions have, often, shown the strongest

effect compared to other extracts and fractions, in contrast, the chloroform ($CHCl_3$) fractions of both parts had less activity. The most remarkable antibacterial potential was developed by the MeOH extract from the leaves, in which a remarkable growth inhibition was obtained against *S. aureus* with an IZ of 20 ± 0.28 mm at 100 mg / mL. However; this inhibition, of the crude extract, remains less significant than that shown by the reference, antibiotic. This may be contributed to the fact that plant extracts in crude form contain little concentrations of bioactive ingredients (Werner et *al*., 1998; Sanogo et *al*., 2006; Sulaiman et *al*., 2013). The antimicrobial activity of this extract could be explained by the presence of various bioactive compounds, in particular, flavonoids and phenolic acids.

Indeed, the extremely sensitive bacterium to the mentioned extract was *S. aureus*, on the other hand, the bacterial strains; *E. coli* and *P. aeruginosa* revealed lower sensitivities to this extract considering the diameter of inhibition (Ponce et *al*., 2003). As *S. aureus* is recognized as a food contaminant, this extract can therefore be used for prevention (Al-Zoreky and Nakahara, 2003).

These results are, closely, similar to those found by Krimat et *al*. (2014), where they revealed that the hydromethanolic extract of *C. spinosa* was shown to be active against the following bacteria; *Bacillus sp.* and *S. aureus* with IZs of 7 and 10 mm, respectively. The same previous work did not find any inhibition effect of this extract on *E. coli* and *P. aeruginosa*. Our results are, moreover, in agreement with those of previous studies conducted by Loy et *al*. (2001); Dessi et *al*. (2001); Chikhi et *al*. (2014), and Djeddi et *al*. (2015) on *C. villosa*. In these studies, it was found that the extracts; dichloromethane, MeOH, and essential oils from the aerial part of the evoked species, exerted an antimicrobial activity against several pathogenic bacteria; *Bacillus sp., S. aureus* (IZ = 10 to 20 mm), *K. pneumoniae* (IZ = 11 to 20.5 ± 2.7 mm), and *Acinetobacter sp.* (IZ = 15.7 ± 1.3 mm), where the less important effect was recorded against *E. coli* (IZ = 12.9 ± 0.9 to 15 mm), *P. aeruginosa* (IZ = 11 to 13.1 ± 2.3 mm), and *S. marcescens* (IZ = 10.2 ± 0.3 mm).

Based on all the previous results, it is concluded that the tested Gram+ bacteria were constantly more sensitive to the studied extracts and fractions than the Gram- bacteria. This finding is consistent with that drawn from previous studies (Pirbalouti et *al*., 2010; Nalubega et *al*., 2011; Madureira et *al*., 2012; Sulaiman et *al*., 2013) which attributed the observed differences to the variation in the chemical composition of the cell wall, the

structure of the two types of microorganisms, and the nature of the tested compounds. Indeed, the resistance of Gram- bacteria is not surprising, it is related to the nature of their outer membranes (impermeable to most biocidal agents) (Faucher and Avril, 2002).

Collectively, our findings revealed an interesting antifungal activity of the four tested extracts, MeOH and Aq of the leaves and flowers, against the two tested species of *Alternaria*, on the other hand, no inhibition was developed by these extracts against the yeast *Candida albicans* and the four phytopathogenic fungi; *Penicillium sp.1*, *Penicillium sp.2*, *Aspergillus sp.*, and *Rhizopus sp.* These results corroborate those obtained by Loy et *al*. (2001) and Dessi et *al.*, (2001), who did not reveal any antifungal activity against *C. albicans* by the MeOH extract of the leaves of another plant species of the same studied genus in the present work, *C. villosa*. However, the hydromethanolic extract of leaves of the same species showed moderate antifungal activity against the same yeast, *C. albicans*, with an IZ equal to 7 mm (Krimat et *al*., 2014; Barhouchi et *al*., 2017).

Understanding the mechanisms of action of plant extracts on the microorganisms remains a point of review and research. Indeed, several studies (Srinivasan, 2016; Lawal et *al.*, 2017) have reported that the antimicrobial activity of several plant extracts has been studied, *in vitro*. Therefore, the growth of several microbial strains is inhibited by various concentrations of these extracts. The phytochemicals developing an antimicrobial activity fall into several groups namely; phenolic compounds, terpenoids, essential oils, alkaloids, and polypeptides (Cowan, 1999; Savoia, 2012).

Indeed, in the present study, it was observed that the extracts having high total polyphenols contents acted positively against the microorganisms. This significant correlation is in agreement with certain studies (Meng et *al*., 2001; Berahou et *al*., 2007; Omojate Godstime et *al*., 2014; Tamokou et *al*., 2017) which revealed that the polyphenols and the phenols have multiple antimicrobial mechanisms of action compared to other chemical groups. They are able to form an irreversible complex with nucleophilic amino acids in proteins, leading to their inactivation and, therefore, to their loss of function in microorganisms. Moreover, the polyphenols and the phenols have been shown to disrupt microbial membranes and inactivate microbial enzymes (Cowan, 1999; Tamokou et *al*., 2017).

It is very opportune to report as an interesting fact that, the antioxidant capacity and the antibacterial potential are positively correlated with the contents of total polyphenols and

total flavonoids (Xia et *al*., 2011; Borges-Bubols et *al*., 2013). This effect has, clearly, been seen in the studied plant species (*C. spinosa*), which makes it suitable for its use in therapy and food preservation.

As mentioned before, column chromatography of the leaves MeOH extract revealed five different compounds enumerated; (1), (2), (3), (4), and (5). These compounds have been identified as chrysin-7-O-*β*-D-glucopyranoside (1), chrysin-7-O-*β*-D-(6"-acetyl)-glycopyranoside (2), apigenin-7-O-*β*-D-glucopyranoside (3), 5-Hydroxyindoline (4), and D-pinitol (5). The last two compounds (4) and (5) are isolated for the first time from the genus *Calycotome*.

The importance of identifying the five compounds can be enhanced by elucidating their biological activities. In fact, in some cases, the purified compounds exhibited biological activities. Indeed, the compound 5-hydroxyindoline (4) (highlighted for the first time in this work) exerted a very high antioxidant activity in comparison with that of the MeOH extract, which allows concluding that the principle active compound has undergone concentration. In contrast, compound (5) despite its purity did not give a remarkable antioxidant effect.

Furthermore, and in the antimicrobial potential, the main objective of the present study, the compound (4) revealed a very interesting antibacterial activity against *S. aureus, P. aeruginosa,* and *S. abony* at the concentration of 1 mg/ disc, but lower than that obtained by the crude extract, MeOH extract from the leaves, on the same strains and at the same concentration. However, no activity was developed by the same compound (4) against *B. subtilis, E. coli, K. pneumoniae,* and *C. albicans.* Likewise, no antimicrobial activity was developed by the other four compounds (1), (2), (3), and (5) on all tested microorganisms.

Interestingly, compound (4), 5-Hydroxyindoline, exhibited the highest antioxidant capacity. This activity generated, in fact, its antimicrobial potential. In contrast, compound (5) (D-pinitol) had less antioxidant power and therefore did not develop any antimicrobial potential. As a result, numerous data have shown that the antioxidant properties of compounds in medicinal plants play an essential role in the fight against pathogenic microorganisms (Atmani et *al*., 2011; Benhammou, 2016).

As a discussion of the separated compounds in this study, it was found that:

Chrysin-7-O-*β*-D-glucopyranoside **(1)** is a glucosidic flavone previously isolated from several plant species belonging to Fabaceae and other plant families such as *Sarcotheca griffithi* (Muharini et *al*., 2014), *Acaccia pennata* (Kim et *al*., 2015), and *Cytisus villosus* Pourr (Larit et *al*., 2018). It has, also, been isolated from *Calycotome spinosa* (L.) Link (Larit et *al*., 2012), and *C. villosa* Subsp. Intermedia (El Antri et *al*., 2004a; Cherkaoui-Tangi et *al*., 2008; Alhage et *al*., 2018). In this study, a weak antioxidant activity of glucosidic chrysin was observed. Our results are in agreement with those of a previous study that revealed a lower antioxidant capacity of this compound isolated from *Adenocarpus mannii* (Ndjateu et *al*., 2014). Moreover, in the present work, this compound did not reveal any antimicrobial activity at the concentration [C] of 1 mg/ disc. This finding can be explained by the fact that flavone glucosides, generally, have a weak antimicrobial influence (Liu et *al*., 2010). This fact can be confirmed by the presence of a glucose moiety in its structure; where, previous works have shown that the presence of an increasing number of sugar fragments in a compound structure reduces its cytotoxic effectiveness (Chen et *al*., 1995; Wang et *al*., 2007).

Apigenin-7-O-*β*-D-glucopyranoside **(3)** is a glucosidic flavone isolated from different plant species as; *Asystasia gangetica* (L.) (Kanchanapoom and Ruchirawat, 2007), *Cytisus multiflorus* (Pereira et *al*., 2012), *Paeonia ostii* (Zhang et *al*., 2017), and *Platycladus orientalis* (Selim et *al*., 2019). In the current study, our results revealed that apigenin-7-O-*β*-D-glucopyranoside (3) has an appreciable antioxidant capacity without, however, developing any antimicrobial activity at [C] of 1 mg/ disc. These results are in agreement with those revealed by (Petrus and Bhuvaneshwari, 2012) who showed that the isomers of compound (3), apigenin-6-C-*β*-D-glucopyranoside, and apigenin-8-C-*β*-D-glucopyranoside, are considered to be the best scavengers of $ABTS^{\cdot+}$ and O_2^{-}, respectively. Besides, the results of the present research are similar to those developed by a previous investigation in which this molecule (apigenin) has, slightly, shown numerous biological effects in a number of mammalian systems, in both *in vitro* and *in vivo*, and that are mainly related to its antioxidant effect (Nkhili, 2009). However, our results are in disagreement with other studies which have shown a remarkable antifungal activity of this compound (Qudsia et *al*., 2015, Hamsalakshmi et *al*., 2018), as well as efficient bactericidal and bacteriostatic effects (Cushnie et *al*., 2003, Martini et *al*., 2004).

5-Hydroxyindoline **(4)** (highlighted for the first time in this work) is an indole alkaloid. It has already been reported as a synthetic molecule, but it was obtained, for the

first time, as a natural product isolated from *C. spinosa*. In fact, this new compound has shown, as previously revealed, an excellent antioxidant capacity and a very strong antibacterial effect. These results are similar to those developed, for the first time, from a plant *Phoebe chekiangensis* in China, which found a strong activity of it against Schizophrenia (Hegde et *al*., 1997). Other recent studies on the activities of marine sponges also revealed that other indole alkaloids isolated from *Hyrtios erectus* and *Ircinia* sponges, such as 5-hydroxy-1H-indole-3-carboxylic acid methyl ester and 5-hydroxy-1H-indole-3-glyoxylate ethyl ester; had several interesting biological activities (Netz and Opatz, 2015; Abdjul, 2016; Abdjul et *al*., 2018).

D-pinitol **(5)**, 3-O-methyl-D-chiro-inositol (highlighted for the first time in this work from a plant belonging to the genus *Calycotome*); it is, in general, a bioactive compound. The results of the present study revealed that the D-pinitol isolated and identified from *C. spinosa*, in abundance amount, does not have any appreciable antimicrobial or antioxidant potential. In contrast, D-pinitol isolated from *Robinia pseudoacacia* has shown antifungal activity against the following phytopathogenic strains; *S. fuliginea* and *E. cichoracearum* responsible for powdery mildew disease (Chen and Dai, 2014). This compound has also been isolated from several plants such as *Zygophyllum melongena* (Ganbaatar et *al*., 2016), and *Rhizophora apiculata* (Lakshmi et *al*., 2006) where its effect on glucose metabolism is well-known, by which it may act as a hypoglycemic agent, in which its ability to alleviate symptoms associated with diabetes has been patented (Dowd and Stevens, 2002; Kim et *al*., 2007). Besides, D-pinitol is considered as an osmotic tolerance molecule associated with drought and salinity stresses in soybeans (Silvente et *al.*, 2012). Likewise, a further study conducted by Popp and Smirnoff (1995) revealed that the accumulation of D-pinitol in stressed plants is considered to be beneficial for stress adaptation, membrane stabilization, and osmotic alteration. This compound had also larvicidal activities and bio-control effects on insects (Dreyer et *al*., 1979; Chaubal et *al*., 2005; Honda et *al*., 2012), something which has not been tested in this work.

On the other hand, the antioxidant and the antimicrobial activities of the MeOH extract from the leaves of *C. spinosa*, in particular of its compounds (1), (2), (3), (4), and (5) are reported here for the first time wherein a publication has been accomplished (Cherfia et *al*., 2020).

Conclusion and perspectives

5- Conclusion and perspectives

At the end of this study, it was concluded that the phytochemical screening, the quantification of phenolic compounds, as well as the antioxidant and the antimicrobial potentials of certain extracts and fractions from leaves and flowers of *C. spinosa* (L.) Link led to interesting results. Indeed, two new compounds; 5-hydroxyindoline **(4)** as an indole alkaloid, and D-pinitol (5) as a cyclitol; have been isolated for the first time from the MeOH extract of *C. spinosa* (L.) Link leaves, together with three well-known glucosidic flavonoids; Chrysin-7-*O*-(*β*-D-glucopyranoside) (**1**), Chrysin- 7-*O*-*β*-D-(6″-acetyl) glucopyranoside (**2**) and Apigenin-7-*O*-*β*-D-glucopyranoside (**3**). Furthermore, remarkable antioxidant capacities were recorded by the two compounds **(3)** and **(4)**. Indeed, the 5-Hydroxyindoline **(4)** was found to be the most active among all the obtained compounds in this work under the same tested experimental conditions. This molecule (4) is characterized by its very significant antibacterial potential against *S. aureus*, *P. aeruginosa*, and *Salmonella abony* together with its extremely superb antioxidant capacity. In the light of these experiments, it could also be concluded that the aerial parts of *C. spinosa* (L.) Link, usually used in traditional medicine in Algeria, can serve as a natural source of bioactive compounds with antioxidant and antimicrobial capacities; as well as the possibility of other biological activities. Therefore, the development of *C. spinosa* (L.) Link as a source of bioactive ingredients for therapeutic and agro-alimentary uses is promising way because; this wild plant occupies large areas in the north of Algeria, and its development does not require any particular maintenance.

At the end of the present work, several developed results can serve as a starting point for future research:

- Particular attention will be prearranged to the new bioactive compound, 5-hydroxyindoline (4), which has, never, been described in nature, for an in-depth study to elucidate its mechanisms of action, as well its therapeutic and preventive virtues;
- Opening of profoundly exploration and exploitation ways for *C. spinosa* to search for other molecules of interest.

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6- References

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ملخـــص

ملخـــص

الهدف الرئيسي من هذا البحث هو وصف الخواص الكيميائية النباتية والتقدير الكمي للمركبات الفينولية، بالإضافة إلى تقييم إمكانات مضادات الأكسدة ومضادات الميكروبات لبعض المستخلصات من أوراق وأزهار النبات المستوطن *Calycotome spinosa* (L.) Link. . بالإضافة إلى تنقية و معرفة الصيغة الكيميائية واختبار القدرات المضادة للأكسدة والمضادة للميكروبات للمركبات المعزولة و النشطة بيولوجيًا. نتيجة لذلك، تم الكشف عن وجود العديد من المجموعات الكيميائية المثيرة للاهتمام في هذا النبات مثل البوليفينولات والفلافونويدات والقلويدات والسكريات. تحتوي المستخلصات الميثانولية MeOH والمائية إضافة للمستخلصات الجزئية $CHCl_3$و AcEtO و n-BuOH من الأوراق والأزهار على كميات عالية من البوليفينولات الكلية مقارنة بمحتواها من المركبات الفلافونويدية. في الواقع ، أعطى المستخلص الجزئي AcEtOمن الأوراق أعلى كمية تقدر ب 107.75 ± 2.09 مغ مقابل حمض الغاليك / غ من المستخلص الجاف ، متبوعًا بمستخلص الميثانول MeOH من نفس الجزء النباتي (98.72 ± 2.47 مغ مقابل حمض الغاليك / غ من المستخلص الجاف، وعلى عكس ذلك، يحتوي مستخلص MeOH للأزهار على أقل كمية من هذه المركبات (24.63 ± 0.35 مغ مقابل حمض الغاليك / غ من المستخلص الجاف. بالإضافة إلى ذلك، أظهر نفس المستخلص الخام ونفس المستخلص الجزئي من الأوراق أقوى نشاط إزالة الجذور الحرة(DPPH ؛ IC_{50} =45.25 ± 1.8 و 41.04 ± 0.15 ميكروغرام / مل، على التوالي) ، بالإضافة إلى قوة اختزال ملحوظة (FRAP ؛ EC_{50}=763.73± 0.32 و 780.04 ± 1.36 ميكروغرام / مل ، على التوالي) ، و يرجع ذلك لمحتواها الجيد نسبيًا من المركبات الفينولية مقارنة بالمستخلصات الأخرى المختبرة. النشاط البيولوجي السابق رافقه نشاط مضاد للميكروبات. وبهذا الصدد ، أظهرت المستخلصات المختلفة نشاطًا ممتازًا مضادًا للميكروبات حيث كانت بكتيريا الاختبار جرام +Gram أكثر حساسية لهذه المستخلصات من بكتيريا جرام -Gram . حيث كانت *S. aureus* أكثر حساسية لمستخلص MeOH للأوراق بإظهارمنطقة التثبيط (ZI) 20.00 ± 0.28 مم ، على عكس البكتيريا ؛ *B. subtilis* و *S. abony* التي أظهرت حساسيات لمستخلص MeOH والمستخلص الجزئي AcEtO من الأوراق مع ZIs تساوي (16.00 ± 0.50 و 13 ± 0.65 مم) و (12 ± 0.29 ؛ 16.00 ± 1.53 مم) على التوالي. بالإضافة إلى ذلك، أظهر مستخلص MeOH من الأوراق نشاطا مضادا للجراثيم ضد بكتيريا *B. subtilis* و *S. aureus* و *S. abony* مع MIC ≥ 0.125 مغ / قرص و MBC قدره 1.00 مغ / قرص ، بينما كانت بكتيريا جرام – Gram ، *E. coli* و *P. aeruginosa*و *K. pneumoniae* أقل حساسية. بالإضافة إلى ذلك ، أظهر نوعان من الفطريات الممرضة للنبات من جنس *Alternaria* حساسية لجميع المستخلصات القطبية الخام التي تم اختبارها بنسب متفاوتة من التثبيط (٪ PI) ، و على عكس ذلك ، لم يظهر أي نشاط مضاد للفطريات ضد الخميرة *Candida albicans* والعزلات الفطرية الأربعة الأخرى المختبرة ؛ *Penicillium sp.1* و *Penicillium sp.2* و*Aspergillus sp.* و *Rhizopus sp.* . من كل النتائج السابقة، استنتج أن مستخلص MeOH من الأوراق كان عموما الأكثر نشاطا، لذلك تم اختياره لتحليل LC-ESI-MS2 وفصل الجزيئات النشطة بيولوجيًا. أدى هذا الفصل باستخدام كروماتوغرافيا العمود (CC) إلى نتيجة مثيرة للاهتمام، وهي في الواقع عرض لأول مرة لمركبين جديدين من النبات موضوع الدراسة ؛ 5-هيدروكسي إندولين **(4)** وD-Pinitol **(5)**،إلى جانب ثلاثة مركبات فلافونويد ية جلوكوزيدية معروفة؛ chrysine-7-(*β*-D-glucopyranoside chrysine-7-O-*β*-D (6″-acetyl) glucopyranosid **(2)**،**(1)** و apigenine-7-O-*β*-D-glucopyranoside **(3)** .تم تحديد الصيغ الكيميائية لهذه المركبات المعزولة من خلال تحليل البيانات الطيفية وقياس الطيف الكتلي ؛ الذي يتكون من طريقة كيميائية جديد ة تضم RMN-1D، RMN-2D مع LIT-ESI-MS^n. أظهر المركب الجديد ، - 5 Hydroxyindoline (4)، قدرة عالية جدًا على مقاومة البكتيريا *S. aureus* بـ 16± 0.5 مم ، *P. aeruginosa* (9.83±0.29 مم) و *S. abony* ب 8 ± 0.28 مم ، كما كشف عن قدرة فائقة مضادة للأكسدةDPPH :IC_{50} > 10 ميكروغرام / مل ، CAT=985.54 ± 0.13 مغ مقابل حمض الأسكوربيك /غ من المستخلص الجاف ؛ FRAP: EC_{50} = 344.82 ± 0.02 ميكروغرام / مل ؛ABTS:IC_{50}=7.8 ± 0.43 ميكروغرام / مل) ، متبوعًا بمركب apigenin-7-O-β-D-glucopyranoside (3)الذي أظهر أيضًا قوة ملحوظة مضادة للأكسدة ، ولكن أقل من المركب الجديد (4). تؤكد هذه النتائج المثيرة للاهتمام أن الجزء الهوائي لنبات Link (L.) *spinosa* .*C*، ولا سيما الأوراق ، التي تستخدم عادة في الطب التقليدي في الجزائر ، يمكن اعتباره مصدرًا طبيعيا للمواد الفعالة للغاية ذات الأنشطة المضادة للأكسدة و المضادة الميكروبات.

الكلمـات المفتاحيـة : *Calycotome spinosa* (L.) Link، $LC\text{-}ESI\text{-}MS^2$، RMN، 5-Hydroxyindoline، النشاط المضاد للميكروبات، النشاط المضاد للأكسدة.

Appendices

Appendix 1

Pictures represent the results of phytochemical screening, polyphenols content, flavonoids content, and antioxidant activities of *C. spinosa* (L.) Link

<table>
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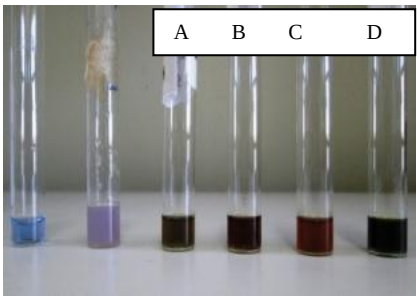
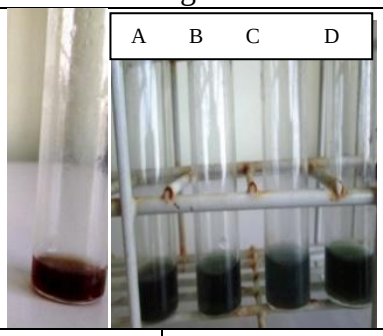
<tr><td colspan="2"> 
</td><td colspan="2"> 
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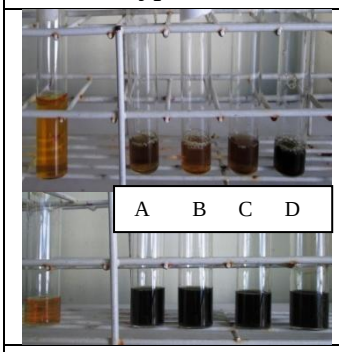
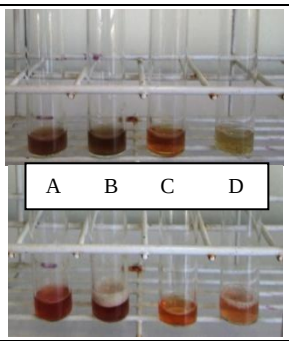
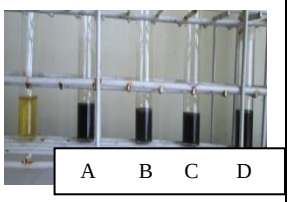
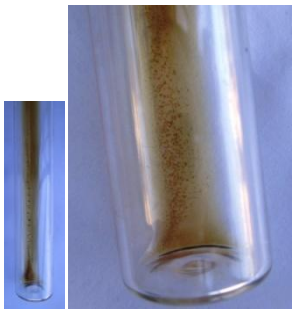
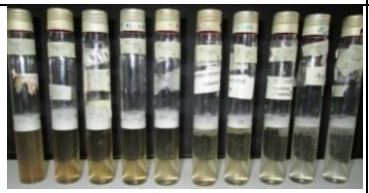
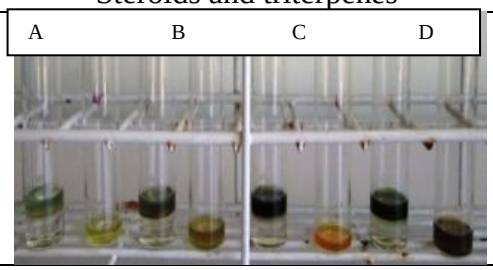
<tr><td>Polyphenols</td><td>Flavonoids</td><td>Tannins</td><td>Alkaloids</td></tr>
<tr><td> 
</td><td> 
</td><td> 
</td><td>  </td></tr>
<tr><td colspan="2">Saponins</td><td colspan="2">Steroids and triterpenes</td></tr>
<tr><td>  </td><td>  </td><td colspan="2"> 
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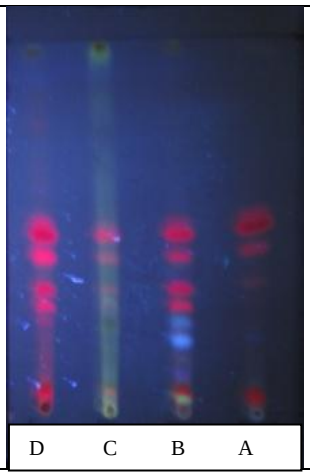
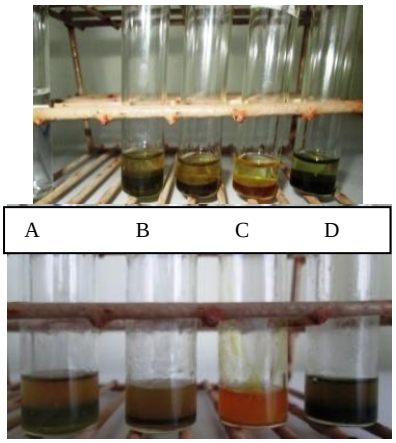
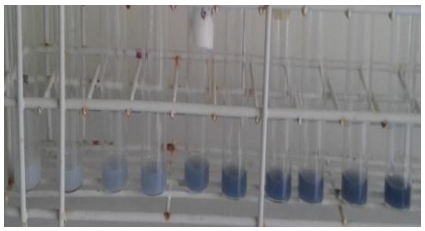
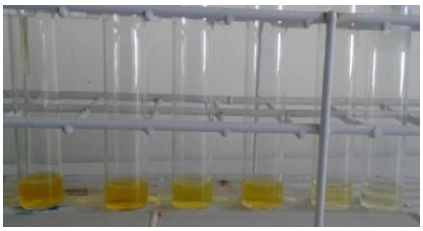
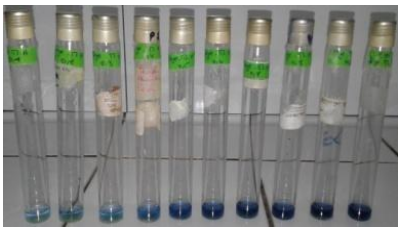
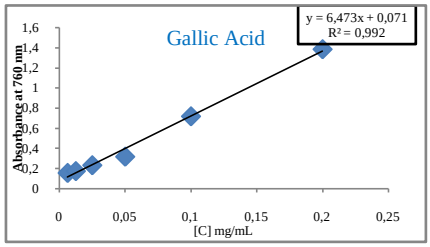
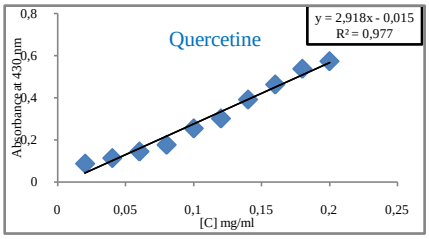
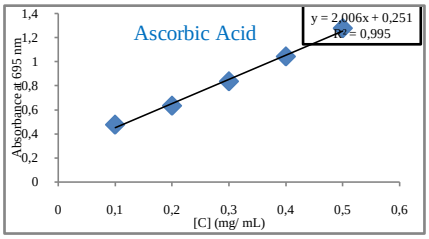
<tr><td colspan="2">Coumarins</td><td colspan="2">Anthraquinones</td></tr>
<tr><td colspan="2"> 
</td><td colspan="2"> 
</td></tr>
<tr><td colspan="4">A : Fruits, B : Stems, C : Flowers, D : Leaves</td></tr>
</table>

Calibration curves

Total Polyphenols	Total Flavonoides	Total Antioxidant Capacity
		
		

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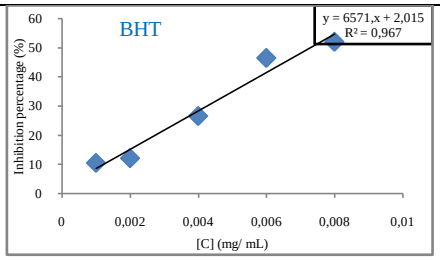
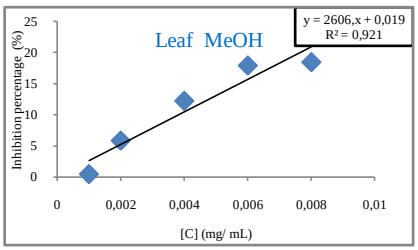
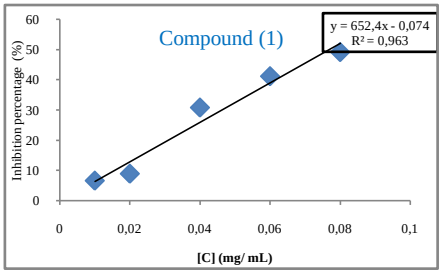
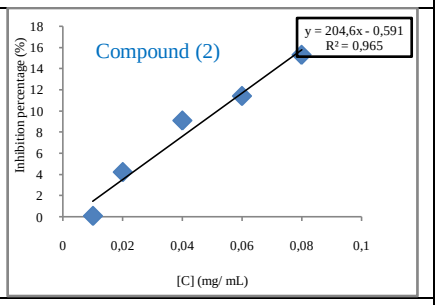
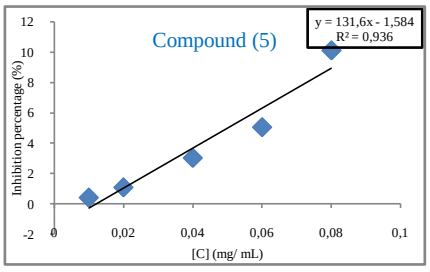


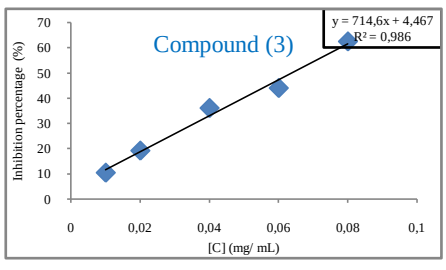
BHT Calibration curve

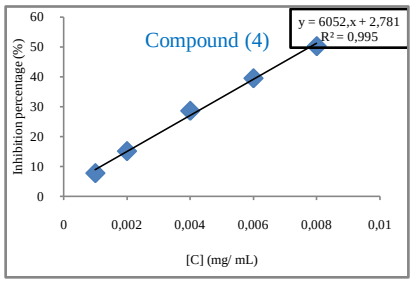




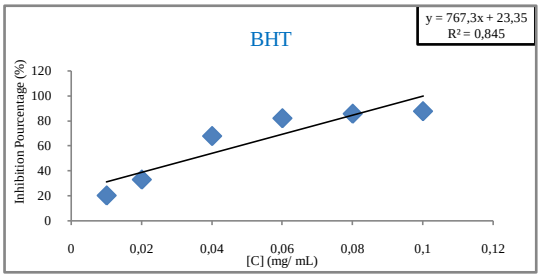




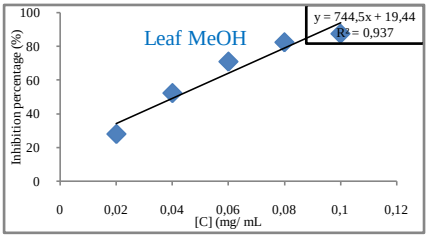


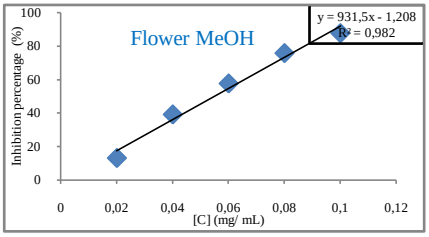


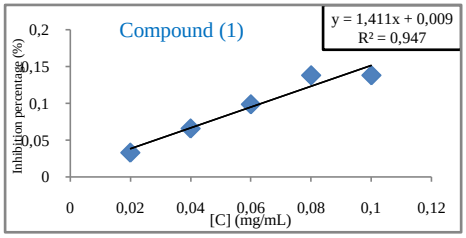
DPPH test

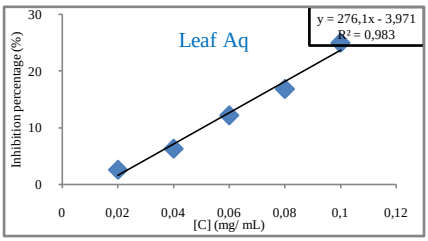


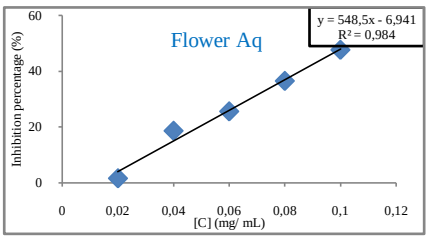
BHT calibration curve

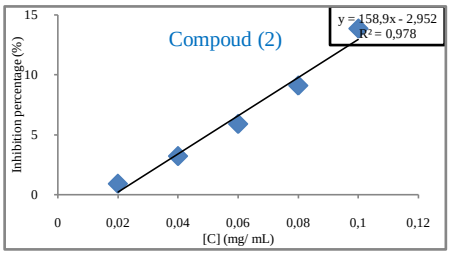


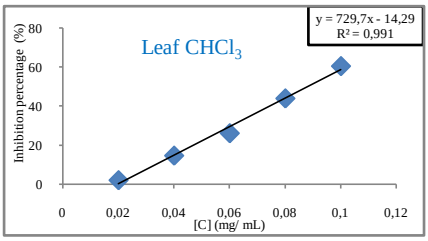


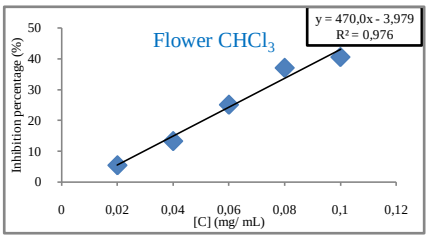


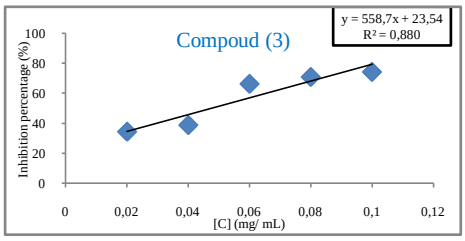


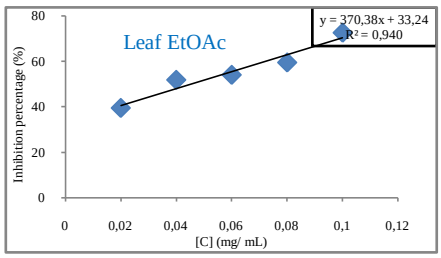


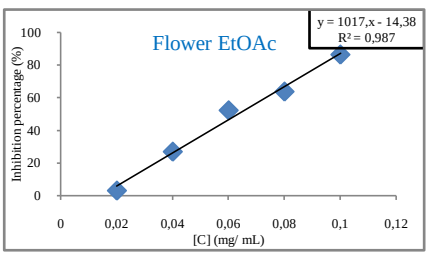


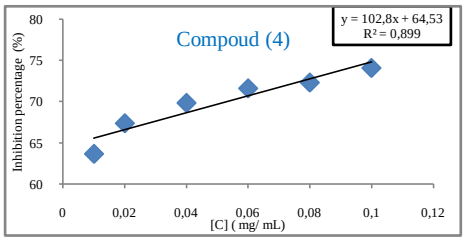


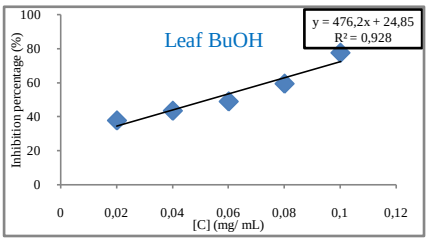


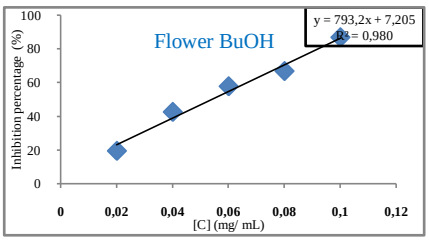


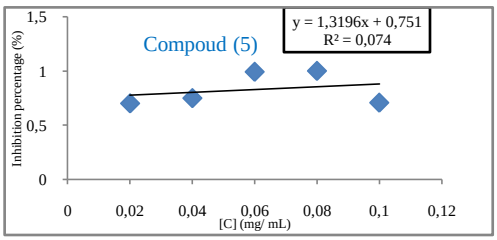




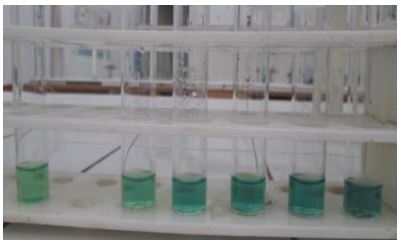


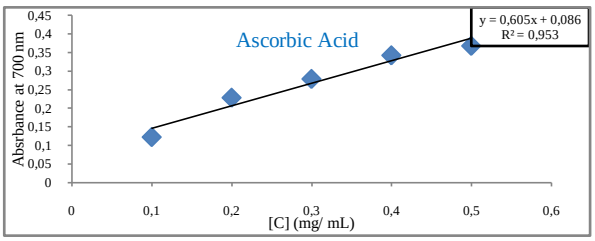




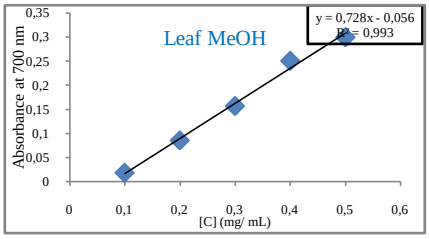


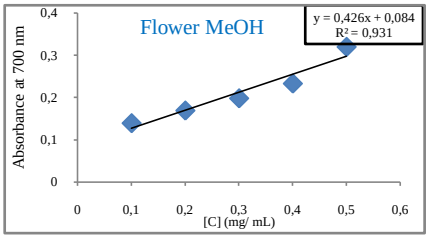
FRAP test

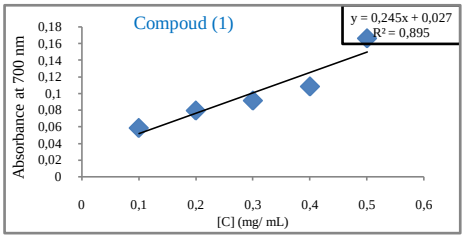


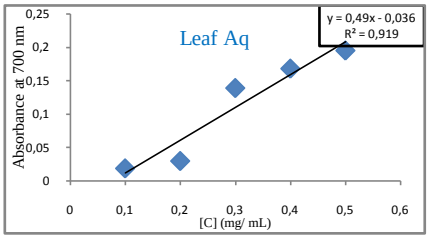


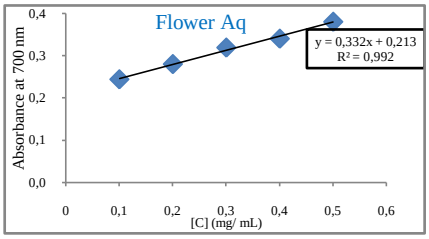
Ascorbic acid calibration curve

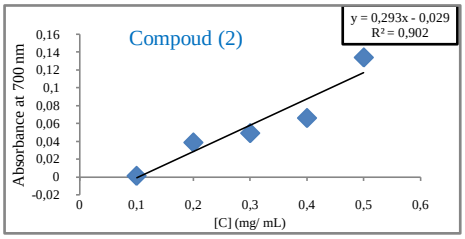


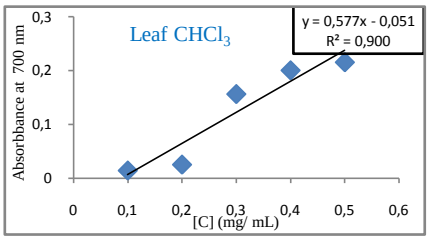


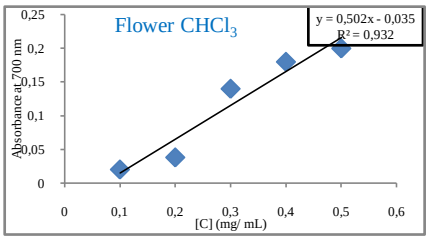


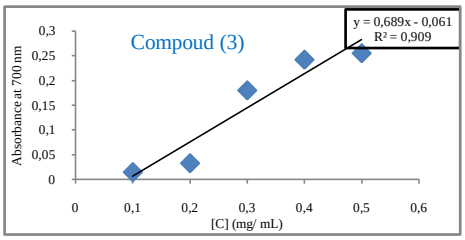


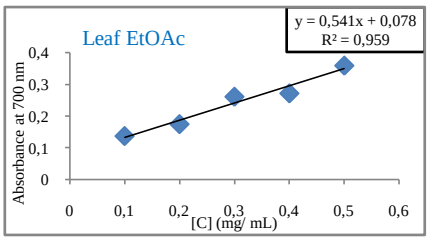


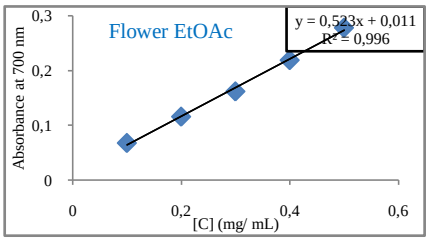


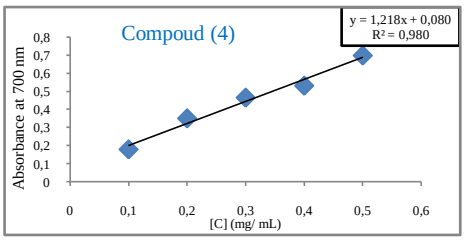


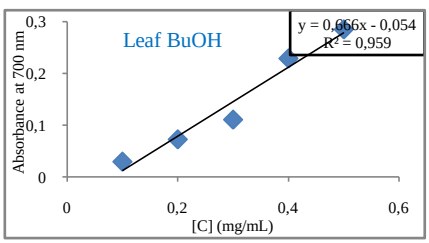


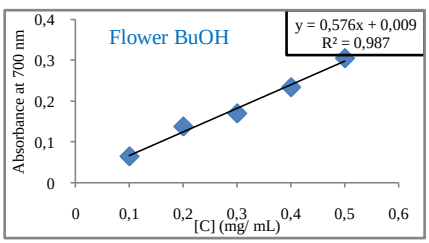


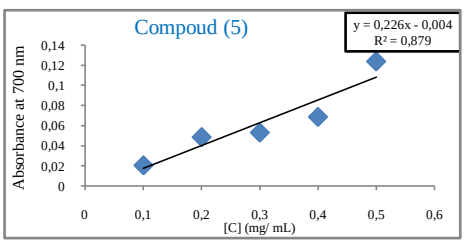












DPPH radical scavenging activity (%) of BHT and *C. spinosa* different extracts.

[C] (mg/ml)	BHT*	Chloroform extract		Ethyl acetate extract		N-Butanol extract		Methanol extract		Aqueous extract	
	30 min	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
0.02	33.25±2.40	2.14±1.35	5.372±0.21	40.06±3.19	1.47±0.17	40.81±3.06	12.17±0.41	23.59±0.98	13.01±3.92	2.61±0.50	1.63±0.62
0.04	68.07±1.99	14.71±0.73	13.22±0.27	51.80±1.33	9.64±1.88	56.12±1.75	31.23±1.23	44.30±2.27	39.10±2.11	6.33±0.91	18.62±0.56
0.06	82.29±4.08	26.15±5.86	25.02±0.65	55.63±1.22	39.41±1.76	62.68±0.06	42.29±2.17	74.35±2.12	57.65±2.98	12.21±0.09	25.56±0.39
0.08	85.92±0.62	43.96±2.59	36.99±0.87	67.61±4.01	63.82±0.64	78.81±4.43	63.11±4.76	81.56±1.59	75.84±2.49	16.85±2.15	36.45±1.80
0.1	89.37±0.15	60.48±0.056	40.50±1.20	73.31±0.40	86.47±0.23	80.62±0.5	86.88±1.23	87.92±0.73	87.79±1.99	24.97±0.82	47.57±0.283

Appendix 2

Antimicrobial activity

Composition and preparation of culture media

Medium	Ingredients	In gram/liter
Mueller Hinton Agar Medium (MH) pH=7.4± 0.2	Beef Extract	300
	Acid Hydrolysate of Casein	17.5
	Starch of corn	1.5
	Agar	17
	Distilled water	1 000 mL
Nutrient Agar Medium (NA) pH=7.2± 0.2	Peptone	10
	Beef Extract	5
	Sodium chloride	5
	Agar	15
	Distilled water	1 000 mL
Sabouraud Dextrose Agar with Chloramphenicol Medium (SAB+) pH= 6.4± 0.2	HMC Peptone *	10
	Dextrose monohydrate	40
	Chloramphenicol	0.05
	Agar	15
	* Equivalent to Peptone (Meat and Casein)	
	Distilled water	1 000 mL
Potato Dextrose Agar Medium (PDA) pH= 5.6±0.2 - Wash the unpeeled potato; - Cut into cubes in 500 ml of distilled water; - bring to a boil for 30 - 45 min; - On the other hand, melt the agar in 500 mL of distilled water; - Crush the potato, filter and add the filtrate to the agar solution; - Add glucose; - Fill the volume to 1000 mL; Sterilize by autoclaving at 121 ° C for 15 minutes (Larpent, 1997).	Potato	250
	Glucose	20
	Agar	20
	Distilled water	1 000 mL

Medium	Ingredients	Gram/Liter
Chapman Medium pH= 7.4± 0.2	Peptone from casein	10
	Beef Extract	1
	D(-)mannitol	10
	sodium chloride	75
	Phenol red	0.025
	Agar agar	15
	Distilled water	1 000 mL
Mac Conkey Agar Medium pH= 7.4± 0.2	Peptone (Pancreatic digest of gelatin)	17
	Proteose peptone (meat and casein)	3
	Lactose monohydrate	10
	Bile salts	1.5
	Sodium chloride	5
	Neutral red	0.03
	Crystal Violet	0.001
	Agar	15
	Distilled water	1 000 mL
Cetrimide Agar Base Medium (CAB) pH = 7.1± 0.2	Pancreatic Digest of Gelatin	20
	Dipotassium Sulfate	10
	Magnesium Chloride	1.4
	Cetrimide (Cetyltrimethylammonium Bromide)	0.3
	Glycerine (Glycerol)	10
	Agar	13.6
	Distilled water	1 000 mL
Tryptone-Soya-Agar Medium (TSA) pH 7.3 ± 0.2	Pancreatic Digest of Casein	15
	Peptic Digest of Soybean Meal	5
	Sodium Chloride	5
	Agar	15
	Distilled water	1 000
Hektoen Medium pH 7.6 ± 0.2	Protease peptone	12
	Yeast extract	3
	Lactose	12
	Sucrose	2
	Salicin	9
	Bile Salts mixture	9
	Sodium chloride	5
	Sodium thiosulfate	5
	Ferric ammonium citrate	1.5
	Acid fuchsin	0.1
	Bromothymol blue	0.065
	Agar	14
	Distilled water	1 000 mL

Leaves

Inhibition Zones Diameters of antimicrobial activity of extracts and fractions of *C. spinosa* leaves, antibiotic, antifungal, and DMSO.

		Inhibition zone diameter (mm)						
Extract	[C] (mg/disc)	*B. subtilis*	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. abony*	*C. albicans*
Aq	0.125 0.25 0.50 0.75 1	/ / 7±0.6 10±0.34 11±0	/ / / / /	/ / / / /	/ / / / /	/ / / 8±0.12 10±0	/ / / 10±0.32 10±0	/ / / / /
MeOH	0.125 0.25 0.50 0.75 1	10±0.67 11±0.3 12±0.21 15±0.76 16±0.5	10±0.75 13±0.34 16±0.21 18±0.5 20±0.28	/ / / / /	/ / / / /	/ / / / /	7±0.53 8±0.12 10±0.32 12±0.84 12±0.29	/ / / / /
PE	0.125 0.25 0.50 0.75 1	/ / / / 10±0	/ / / / /	/ / / / /	/ / / / /	/ / / / /	/ / / / 12±0	/ / / / /
$CHCl_3$	0.125 0.25 0.50 0.75 1	10±0.5 10±0.12 11±0.00 11±0.88 12±0	/ 9±0.00 9±0.12 10±1.01 11±0	/ / / / /	/ / / / /	/ / / / /	11±0.21 11±0.32 12±0.3 12±0.11 12 ±0	/ / / / /
EtOAc	0.125 0.25 0.50 0.75 1	10±0.11 10±0.43 10±0.21 11±0.11 13±0.65	/ / / 10±0.12 11±0.32	/ / / / /	/ / / / /	/ / / / 7±0	12±0.43 13±0.0 14±0.12 16±0.5 16±1.53	/ / / / /
n-BuOH	0.125 0.25 0.50 0.75 1	/ / 8±1.21 8±0.32 9±0	/ / / 9±0.23 11±0	/ / / 6.5±0.32 8±0	/ / / / /	/ / / 9±1.1 10±0	/ / 7±0.53 8±0.41 9±0	/ / / / /
DMSO	10µl	/	/	/	/	/	/	/
Chlrmph	15µg/disc	33±0.13	40±0.07	28±0.34	30±0.17	32±0.22	35±0.44	NT
Fluconazol	10µg/disc	NT	NT	NT	NT	NT	NT	35.2±0.24

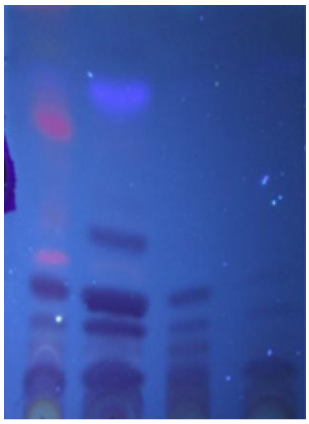
« Discs diameter 6 mm »

Flowers		Inhibition Zones Diameters of antimicrobial activity of extracts and fractions from *C. spinosa* flowers, antibiotic, antifungal, and DMSO.						
		Inhibition zone diameter (mm)						
Extract	[C] (mg/disc)	*B. subtilis*	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. abony*	*C. albicans*
Aq	0.125 0.25 0.50 0.75 1	/ / 7±0 7.2±0 7.5±0.07	/ 10±0.86 13.6±1.18 15.3±1.32 17.1±0.13	/ / / / /	/ / / / /	/ / / / /	/ / / 9±0.12 11±0.87	/ / / / /
MeOH	0.125 0.25 0.50 0.75 1	7.5±0.07 09±0 10±0 10.5±0.07 11.7±0.03	8±0.51 09±0.26 10.3±0.25 12+0.2 14±0.17	/ / / / /	/ / / / /	/ / / / /	11±0.14 12.5±0.07 12.7±0 13±0.14 14.5±0.21	/ / / / /
PE	0.125 0.25 0.50 0.75 1	/ / / / 9±0.5	/ / / / 11±0.12	/ / / / /	/ / / / /	/ / / / /	/ / / / /	/ / / / /
CHCl3	0.125 0.25 0.50 0.75 1	/ / 9±0.5 9±0.04 11±0.65	/ / 6.5±0.31 7±0.0 12±0.76	/ / / / /	/ / / / /	/ / / / /	/ / 9±0.0 11±0.71 12±0.43	/ / / / /
EtOAc	0.125 0.25 0.50 0.75 1	/ / 8±0.89 10±0.14 11±0.6	/ / 7±0.54 7.5±0.13 12±0.08	/ / / / /	/ / / / /	/ / / / /	/ / 11±1.32 11.5±1.4 12±0.00	/ / / / /
n-BuOH	0.125 0.25 0.50 0.75 1	/ / 6.5±0.12 6.5±0.32 9±0	/ / / / 9±0	/ / / / /	/ / / / /	/ / / / /	/ / / 7±0.0 7±0	/ / / / /
DMSO	10µl	/	/	/	/	/	/	/
Chlrmph	15µg/disc	33±0.13	40±0.07	28±0.34	30±0.17	32±0.22	35±0.44	NT
Fluconazol	10µg/disc	NT	NT	NT	NT	NT	NT	35.2±0.24

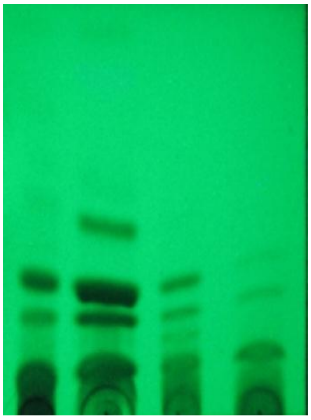
Appendix 3
Chemical analyses

Thin layer Chromatography

Leaves

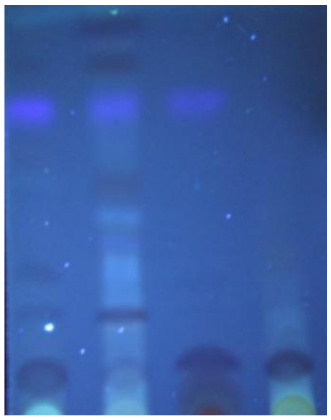


(UV-366 nm)



(UV-254 nm)

Flowers



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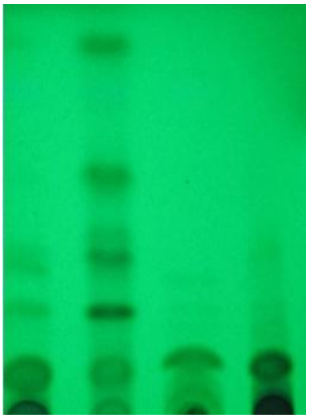


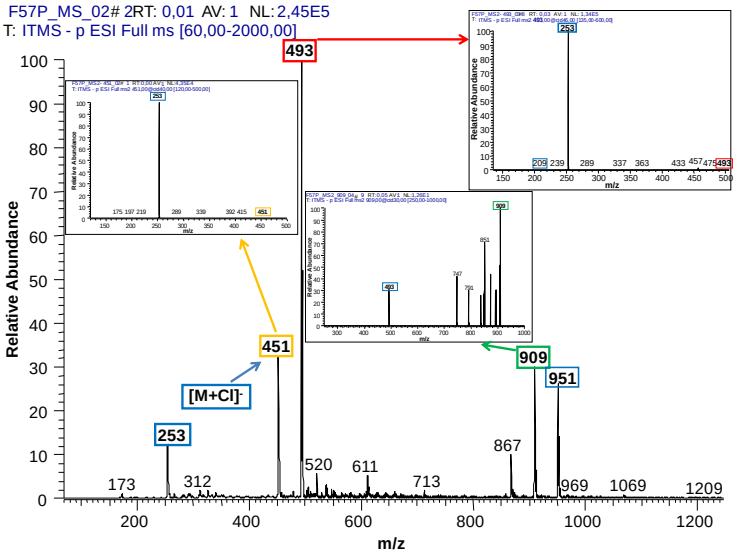
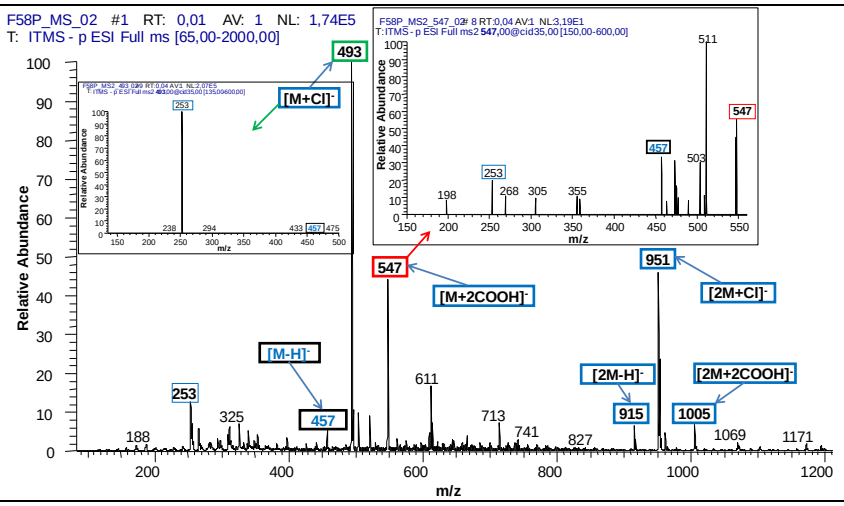
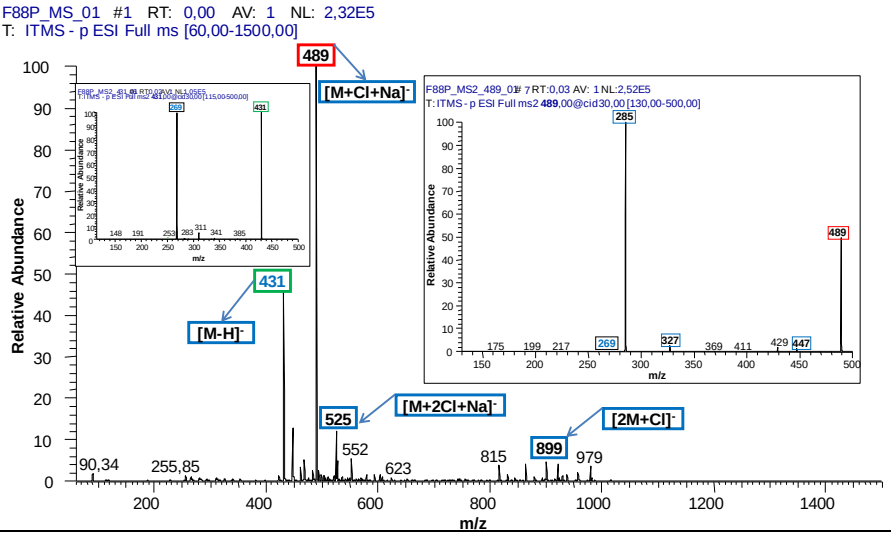
(UV-254 nm)

TLC of leaves and flowers samples at UV-366 and UV-254 nm ($CHCl_3$/MeOH: 9/1)

The Retention factor (Rf) was calculated as the following:

$$Rf = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

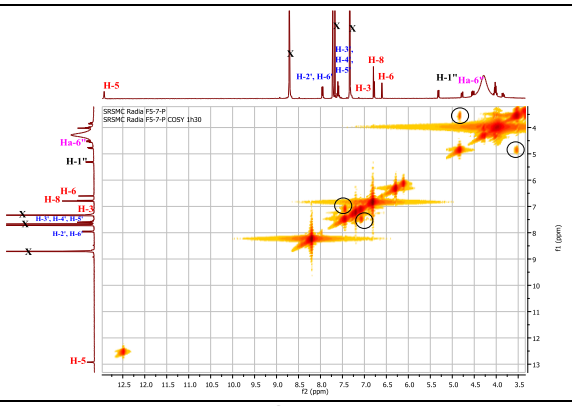
Results of Thin Layer Chromatography of both leaves and flowers samples (Tentative identification).									
Leaves					**Flowers**				
Extract	Spot	Fluorescence	Rf	flavonoids Type	Extract	Spot	Fluorescence	Rf	flavonoids Type
MeOH	1	grey	0.13	/	MeOH	1	black	0.153	/
	2	grey	0.2	/		2	grey	0.261	/
	3	brown	0.26	/		3	black	0.369	Flavones 3 OH
	4	black	0.34	Flavones 3 OH		4	violet	0.769	Flavonols 5,6,7 tri-OH free
	5	Light pink	0.39	/	$CHCl_3$	1	black	0.153	/
	6	pink	0.74	Flavones 3OH et 5OH, 4 OH		2	grey	0.261	/
$CHCl_3$	1	grey	0.144	/		3	black	0.384	Flavones 3 OH
	2	brown	0.26	/		4	grey	0.461	/
	3	black	0.33	Flavones 3 OH		5	grey	0.6	/
	4	black	0.46	/		6	violet	0.769	Flavonols 5,6,7 tri-OH free
	5	violet	0.80	Flavonols 5,6,7 tri-OH free		7	light grey	0.876	/
EtOAc	1	grey	0.144	/		8	grey	0.969	/
	2	grey	0.2	/	EtOAc	1	black	0.153	/
	3	brown	0.26	/		2	violet	0.769	Flavonols 5,6,7 tri-OH free
	4	black	0.33	Flavones 3 OH	n-BuOH	1	black	0.153	/
n-BuOH	1	black	0.16	/					
	2	grey	0.28	/					
	3	black	0.34	Flavones 3 OH					

MS spectra recorded in negative mode of isolated compounds	
	MS → MS^2
Compound (1)	
Compound (2)	
Compound (3)	

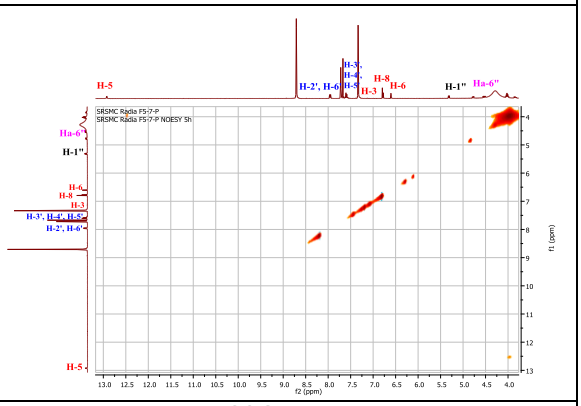
NMR 2D spectra of the isolated compounds

Compound (1)

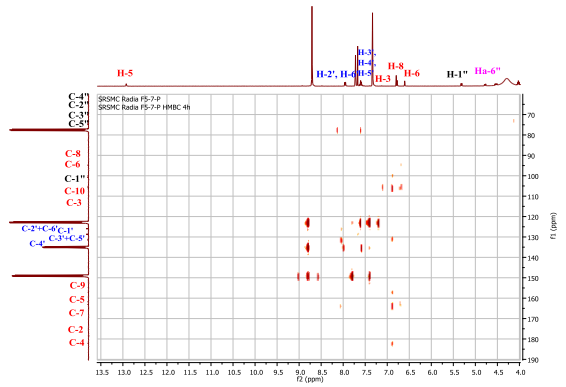
COSY-NMR



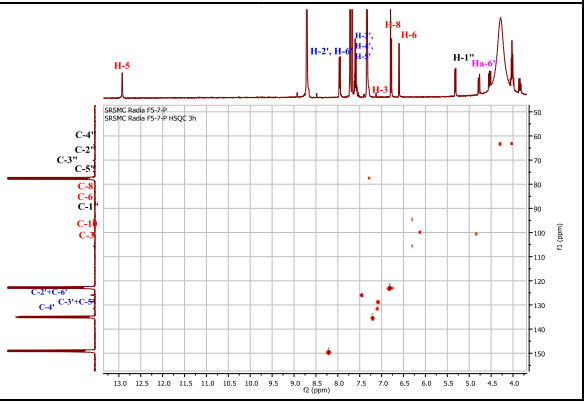
NOESY-NMR



HMBC-NMR

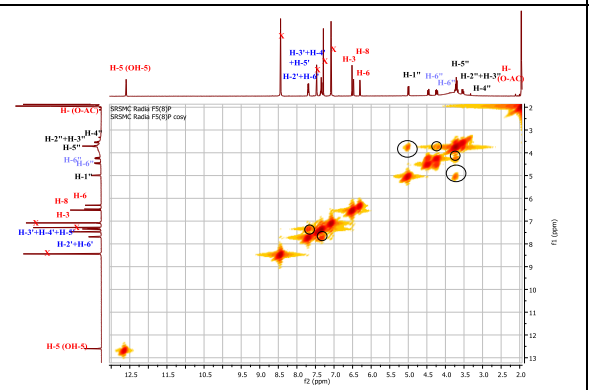


HSQC- NMR

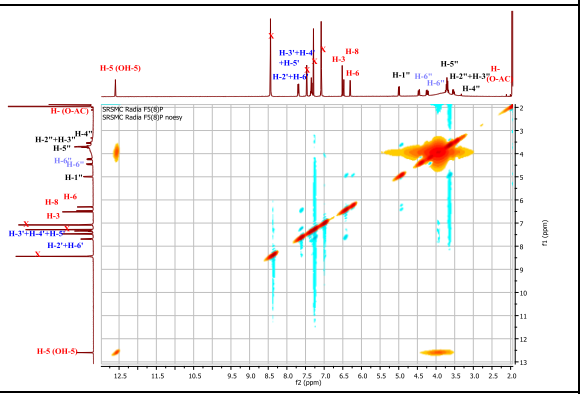


Compound (2)

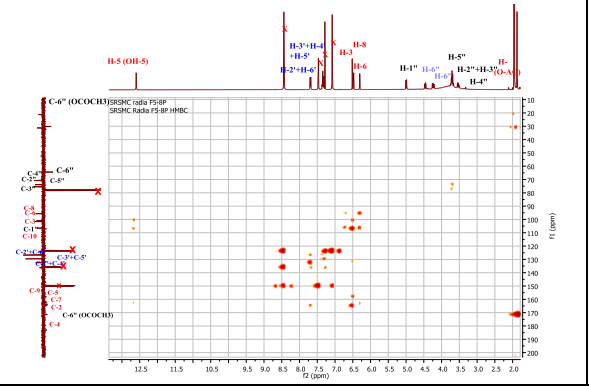
COSY-NMR



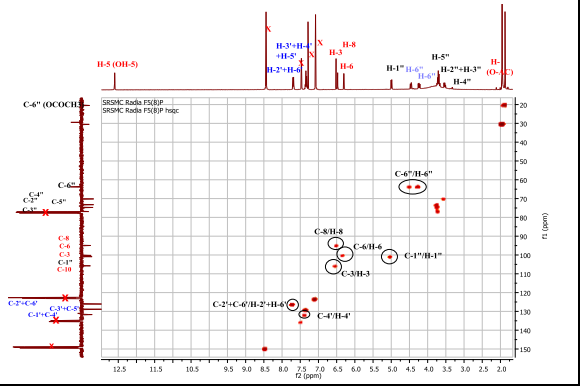
NOESY-NMR



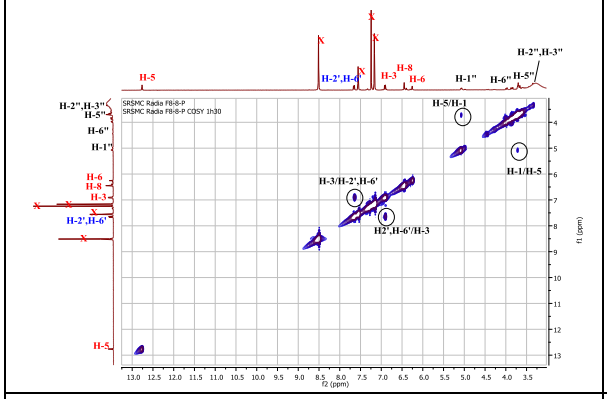
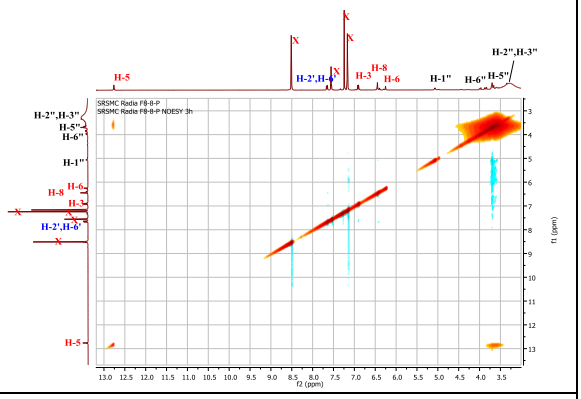
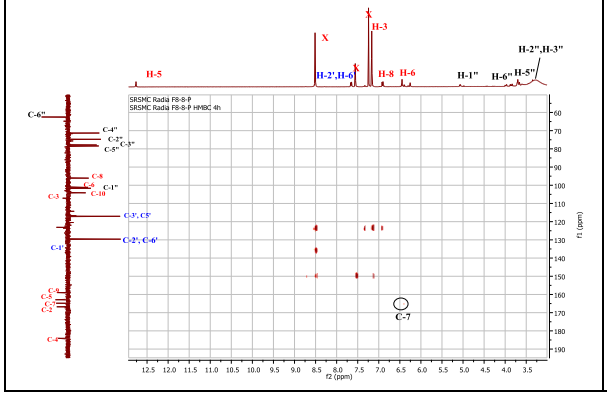
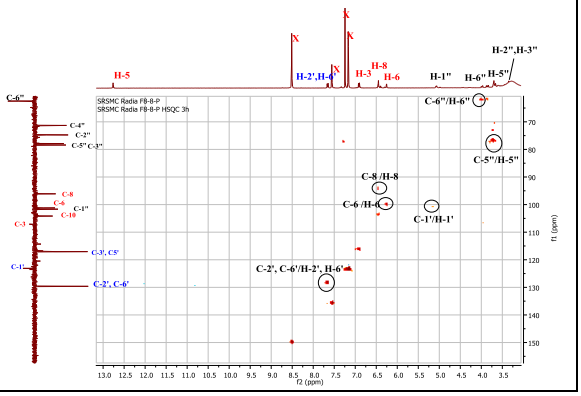
HMBC-NMR



HSQC- NMR



Compound 3

COSY-NMR	NOESY-NMR
	
HMBC-NMR	**HSQC- NMR**
	

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Thèse de Doctorat en Sciences	Option: **Biotechnologie végétale**

Thème : Recherche de potentialités antimicrobiennes d'une plante endémique du genre *Calycotome* : étude phytochimique et microbiologique

Résumé

L'objectif principal de cette recherche porte sur la caractérisation phytochimique, la quantification des composés phénoliques et l'évaluation des potentiels antioxydants et antimicrobiens de certains extraits et fractions de feuilles et de fleurs de la plante endémique *Calycotome spinosa* (L.) Link. Pour ce faire, la purification, l'identification structurale et l'investigation des capacités anti-oxydantes et antimicrobiennes des composés bioactifs isolés ont été élucidées. De ce fait, de nombreux groupes chimiques intéressants ont été détectés chez cette plante comme les polyphénols, les flavonoïdes, les alcaloïdes et les sucres. Les extraits (MeOH et aqueux) et les fractions ($CHCl_3$, AcEtO et n-BuOH) de feuilles et de fleurs révélaient des teneurs élevées en polyphénols totaux en comparaison avec leur teneur en flavonoïdes. En effet, la fraction d'AcEtO de feuilles a donné la quantité la plus élevée (107.75 ± 2.09 mg EAG / g ES), suivie de l'extrait MeOH de la même partie (98.72 ± 2.47 mg EAG / g ES), cependant, l'extrait MeOH de fleurs possédait la plus faible quantité en ces composés (24.63 ± 0.35 mg EAG / g ES). Par ailleurs, il a été montré que le même extrait et la même fraction développaient la plus forte activité de piégeage du radical libre (DPPH ; CI_{50} = 45.25 ± 1.8 et 41.04 ± 0.15 µg / mL respectivement) en plus d'un remarquable pouvoir réducteur (FRAP ; CE_{50} = 763.73 ± 0.32 et 780.04 ± 1.36 µg / mL, consécutivement), due à leur teneur, relativement, bonne en composés phénoliques par rapport aux autres extraits testés. L'activité biologique précédente a été accompagnée par l'activité antimicrobienne où les différents extraits ont montré une excellente activité antimicrobienne. De ce fait, les bactéries test Gram + étaient plus sensibles à ces extraits que les bactéries Gram- où *S. aureus* a été la plus sensible à l'extrait MeOH de feuilles avec une zone d'inhibition (ZI) de 20.00 ± 0.28 mm en comparaison à d'autres extraits, en revanche, les bactéries ; *B. subtilis* et *S. abony* ont montré des sensibilités à l'extrait MeOH et à la fraction d'AcEtO des feuilles avec des ZIs de (16.00 ± 0.50 et 13 ± 0.65 mm) et (12 ± 0.29 et 16.00 ± 1.53 mm) respectivement. L'extrait MeOH de feuilles, en outre, était bactéricide contre *B. subtilis*, *S. aureus* et *S. abony* avec une CMI ≤ 0.125 mg / disque et un CMB de 1.00 mg / disque, alors que, les bactéries Gram- ; *E. coli*, *P. aeruginosa* et *K. pneumoniae* ont été mois sensibles. Par ailleurs, deux espèces fongiques phytopathogènes d'*Alternaria* étaient sensibles à tous les extraits testés avec des pourcentages d'inhibition (PI%) variables, cependant, aucune activité antifongique n'a été montrée contre la levure *Candida albicans* et les quatre autres isolats fongiques test ; *Penicillium sp.1*, *Penicillium sp.2*, *Aspergillus sp*. et *Rhizopus sp*. De l'ensemble des résultats, il a été conclu que l'extrait MeOH de feuilles s'avérait le plus performant, par conséquent, il a été sélectionné pour l'analyse LC-ESI-MS^2 et pour la séparation des molécules bioactives. Cette séparation en utilisant la chromatographie sur colonne (CC) a abouti à un résultat très intéressant, il s'agit, en effet, de la mise en évidence, pour la première fois dans ce travail, de deux nouvelles molécules à partir de la plante, sujet d'étude ; 5-Hydroxyindoline (4) et D- Pinitol **(5)**, conjointement avec trois flavonoïdes glucosides bien connus ; chrysine-7-*O*-(*β*-D-glucopyranoside) **(1)**, chrysine-7-*O*-*β*-D-(6″ -acétyl) glucopyranoside **(2)** et apigénine-7-O-*β*-D-glucopyranoside **(3).** Les structures chimiques de ces composés ont été élucidées par l'analyse de données spectroscopiques et de spectrométrie de masse ; comprenant une nouvelle approche RMN-1D, RMN-2D avec LIT-ESI-MS^n. Le nouveau composé, 5-Hydroxyindoline **(4),** a montré un potentiel antibactérien très important contre *S. aureus* (16 ± 0.5 mm), *P. aeruginosa* (9.83 ± 0.29 mm) et *S. abony* (8 ± 0.28 mm) et a révélé une capacité anti-oxydante pertinente (DPPH: CI_{50} <10 µg / mL ; CAT = 985.54 ± 0.13 mg EAA / g ES ; FRAP: CE_{50} = 344.82 ± 0.02 µg / mL ; ABTS: CI_{50} = 7.8 ± 0.43 µg / mL), suivi du composé apigénine-7-O-*β*-D-glucopyranoside **(3)** qui a développé, également, un pouvoir antioxydant remarquable, mais inférieur à celui du nouveau composé **(4).** Ces résultats intéressants confirment que la partie aérienne de *C. spinosa* (L.) Link, notamment les feuilles, habituellement utilisée en médecine traditionnelle en Algérie, peut être considérée comme une source de substances à activités anti-oxydantes et antimicrobiennes très efficaces.

Mots clés: *Calycotome spinosa* (L.) Link, LC-ESI-MS^2, RMN, 5-Hydroxyindoline, activité antimicrobienne, activité anti-oxydante.

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Last name: **CHERFIA** First name: **Radia**	Viva Date: **06/ 01/ 2021**
Doctoral Thesis in Sciences	Option: **Plant Biotechnology**

Theme : Research of antimicrobial potentialities of an endemic plant of the genus '*Calycotome*': phytochemical and microbiological study

Abstract

The main objective of this research is the phytochemical characterization, the quantification of phenolic compounds, as well as the evaluation of the antioxidant and the antimicrobial potentials of certain extracts and fractions of leaves and flowers from the endemic plant *Calycotome spinosa* (L.) Link. Besides, the purification, the structural identification, and the investigation of the antioxidant and the antimicrobial capacities of isolated bioactive compounds have also been elucidated. As a result, many interesting chemical groups have been detected in this plant such as polyphenols, flavonoids, alkaloids, and sugars. The extracts (MeOH and aqueous) and the fractions ($CHCl_3$, EtOAc, and n-BuOH) of the leaves and the flowers have high total polyphenols contents (TPC) compared to their flavonoids contents. Indeed, the EtOAc fraction of leaves exhibited the highest amount of TPC (107.75 ± 2.09 mg GAE / g DE), followed by the MeOH extract of the same part (98.72 ± 2.47 mg GAE / g DE), however, the MeOH extract of flowers had the lowest amount of these compounds (24.63 ± 0.35 mg GAE / g DE). Besides, it was shown that the same extract and the same fraction of leaves revealed the strongest free radical scavenging activity (DPPH; IC_{50} = 45.25 ± 1.8 and 41.04 ± 0.15 µg / mL, respectively), in addition to a remarkable reducing power (FRAP; EC50 = 763.73 ± 0.32 and 780.04 ± 1.36 µg / mL, consecutively), due to their, relatively, good content in phenolic compounds compared to the other tested extracts. The previous biological activity was accompanied by antimicrobial activity. Therefore, the diverse extracts displayed excellent antimicrobial activity, where the Gram+ test bacteria were more sensitive to these extracts than the Gram- ones. Thus, *S. aureus* was more sensitive to the MeOH extract of leaves with an inhibition zone (IZ) of 20.00 ± 0.28 mm, in contrast, bacterial strains ; *B. subtilis* and *S. abony* showed sensitivities to the MeOH extract and the EtOAc fraction of leaves with IZs of (16.00 ± 0.50 and 13 ± 0.65 mm) and (12 ± 0.29 and 16.00 ± 1.53 mm), respectively. the leaves' MeOH extract was, furthermore, bactericidal against *B. subtilis*, *S. aureus* and *S. abony* with MICs ≤ 0.125 mg/ disc and a MBC of 1.00 mg/disc, whereas, Gram- bacteria; *E. coli*, *P. aeruginosa*, and *K. pneumoniae* were less susceptible. Moreover, two phytopathogenic fungal species of *Alternaria* were sensitive to all the tested extracts (MeOH and Aqueous) with variable inhibition percentages (IP %), however, no antifungal activity was observed against the yeast '*Candida albicans'* and the other four tested fungal isolates; *Penicillium sp.1*, *Penicillium sp.2*, *Aspergillus sp.*, and *Rhizopus sp.* From all these results, it was concluded that the leaves MeOH extract was the most efficient; therefore, it was selected for LC-ESI-MS^2 analysis and for the separation of bioactive molecules. This separation using column chromatography(CC) led to a very interesting result; it is in fact the highlight, for the first time, of two new molecules from the plant, subject of study; 5-hydroxyindoline **(4)** and D-Pinitol **(5)**, together with three well-known glucosidic flavonoids; Chrysin-7-*O*-(*β*-D-glucopyranoside) **(1)**, chrysin-7-*O*-*β*-D-(6″-acetyl) glucopyranoside **(2),** and apigenin-7-*O*-*β*-D-glucopyranoside **(3)**. The chemical structures of these isolated compounds have been elucidated based on spectroscopic analysis data and mass spectrometry; comprising a new approach 1D-NMR, 2D-NMR with LIT-ESI-MS^n. The new compound, 5-Hydroxyindoline **(4)**, demonstrated a very strong antibacterial potential against *S. aureus* (16 ± 0.5 mm), *P. aeruginosa* (9.83 ± 0.29 mm), and *S. abony* (8 ± 0.28 mm), as well it revealed a pertinent antioxidant capacity using four different methods (DPPH : IC_{50} < 10 µg / mL ; TAC = 985.54 ± 0.13 mg AAE / g DE ; FRAP : EC_{50} = 344.82 ± 0.02 µg / mL ; ABTS: IC_{50} = 7.8 ± 0.43 µg / mL), followed by the compound apigenin-7-*O*-*β*-D-glucopyranoside **(3)** which, also, exhibited remarkable antioxidant power, but lower than that of the new compound **(4)**. These interesting results confirm that the aerial part of *C. spinosa* (L.) Link, in particular the leaves, usually used in traditional medicine in Algeria, can be considered as a source of substances with very effective antioxidant and antimicrobial activities.

Key words: *Calycotome spinosa* (L.) Link, LC-ESI-MS^2, NMR, 5-Hydroxyindoline, antimicrobial activity, antioxidant activity.

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