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THESIS

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Presented by **:**

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Phytochemical and biological studies of Scabiosa stellata.L (Caprifoliaceae) & Sedum carealeum. Vahl (Crassulaceae)

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DEDICATION

Success does not lie in

"Results"

But in "Efforts"

"Being" the best is not so important

"Doing" the best is All what matters

I dedicate this work to

My parents,

Sisters, brothers,

Nephews,

All my family

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Abbreviations

δ	chemical shift
AcOEt	ethyl acetate
BHT	butylhydroxytoluene
CC	column chromatography
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
COSY	correlated spectroscopy
1D	one- dimensional
2D	two-dimensional
d	doublet
dd	doublet of doublet
DEPT	distortionless enhancement by polarisation transfer
DMSO	dimethylsulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EP	Petroleum ether
ESI	electro spray ionisation
g	gram
GC-MS	gas chromatochraphy- mass spectrometry
Glu	glucose
Gal	galactose
H_2O	Water
HMBC	heteronuclear multiple bonding connectivity
HSQC	heteronuclear single quantum connectivity
Hz	hertz
J	coupling constants
LC-MS	liquid chromatography- mass spectrometry
m	multiplet
m/z	mass / electric charge
mau	atomic mass unit
Me	methyl
MeOH	methanol
mg	milligram

MHz	megahertz
ml	milliliter
MS	mass spectrometry
MS/MS	mass spectroscopy / mass spectroscopy
MS^n	Multiple stage tandem MS
Na	sodium
NMR ^1H	proton nuclear magnetic resonance
NMR ^{13}C	carbon nuclear magnetic resonance
ppm	part per million
Rha	rhamnose
RP-18	reversed phase silica with C-18 functional groups
Rt	Retention time
s	singulet
S.D.	Standard Deviation
SiO_2	normal silica gel
sl	large singulet
t	triplet
TLC	thin-layer chromatography

CONTENTS

Chapter III : *Experiment procedures*

Chapter IV : *Results and discussion*

INTRODUCTION

INTRODUCTION

From the pharmacological perspective plants are a treasure, in fact they still are the main source of bioactive compounds that can be used directly in food, cosmetic and pharmaceutical industries, or can inspire the synthesis of more active derivatives.

Since the prehistoric era and until the development of synthetic drugs, plants have been the basis for nearly all medicinal therapy. Moreover, medicinal plants represent the only source of therapeutic remedies in some countries of Africa (90% population), while are considered as health promoting in developing countries.

Algeria is characterized by large variety of soils (littoral, steppe, mountains and desert), climates and biodiversity, with toxic and medicinal plants. Its flora contains thousands of species (Mediterranean, Saharan and tropical palaeo flora) that can be phytochemically studied. Indeed, in Algerian flora can be found several botanical families and many medicinal plants (Ziani et al., 2015). From the vast number of species that grow in Algeria 15% are endemic and a many are underexplored from both phytochemical and pharmacological points of view. The valorisation of the medicinal plants found in Algerian flora will be of a great contribution to the Algerian pharmaceutical industry and will certainly produce an economic impact.

Phytochemical studies may be directed towards the characterization of the essential oils or plant extracts chemical compositions. Phytochemical screening can assist taxonomic classification (Kumar and Jaafar, 2017) whilst bioassay guided studies can target and identify biologically active compounds in complex plant extracts (Harborne et al, 1998).

In this context, particularly in the continuity of our laboratory research program, this manuscript was established. And the main purpose was the phytochemical study of two Algerian plants, growing in the east of Algeria (Batna), the *Scabiosa stellata* L. and the *Sedum caeruleum* L.. Both plants are found in dry sunny grassland and rocky hillsides and belong to different families, Caprifoliaceae and Crassulaceae, respectively. Both families are recognised to have many medicinal plants (Byalt et al, 2011, Christopoulou et al, 2008) which are also rich in secondary metabolites of great biological interest, such as flavonoids and saponins (Hofmann and Bittrich. 2016, Milad. 2014). Moreover species of these families have contain various biological activities already described (Hofmann and Bittrich. 2016, Milad. 2014). *Scabiosa stellata* and *Sedum caeruleum* species were not phytochemically studied, so they are prompted to

be studied. In the hope to contribute and to enlarge the knowledge of their chemical constituents, find bioactive compounds and contribute to amplify their economic value.

This thesis is presented in four chapters as shown:

- The first chapter details with a bibliographic study of the previous biological and pharmacological properties and their secondary metabolites already described for both genera.
- The second chapter highlights some analytical methods used in this study.
- The third chapter is focused on the experimental work done as the separation and purification procedures along with the GC-MS and UHPLC-MS analyses.
- In the last chapter we expose the obtained results in particulary emphasis on the compounds full characterization, performed using modern spectroscopic methods [UV-visible, mono and bi-dimensional NMR (^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC and NOESY) and mass spectrometry]. Both of GC-MS and UHPLC-MS profiles were discussed, and we represent the biological tests as well.
- At the end some conclusions and future perspectives will be addressed.

Chapter I

Bibliographic Review

1.1. Scabiosa genus: Pharmacological properties and phytochemistry

1.1.1. Taxonomy and botanical aspects

Currently, genus *Scabiosa* belongs to the family of although in previous reports appears included in the Dipsacaceae family. Due to the morphological and molecular phylogenetic analyses Dipsacaceae is no longer recognised as a family and their species are currently placed in the family Caprifoliaceae (George and Ronald, 2013). The Caprifoliaceae family for long time ago (in 1789) was named by the French Botanist Antoine Laurent de Jussieu. Apparently, this family species are goats' favourite food so named Goats' leaf, which in Latin is *Caprifolium* and ultimately lead to Caprifoliaceae (Acharya and Mukherjee, 2017). Various fancy bushes among the 810 – 900 species included in the family increases their economic value (George and Ronald, 2013). This family, also known by the name of honeysuckle, is distributed mainly eastern in Asia and North America (Hofmann and Bittrich, 2016) and the family members are mostly shrubs and vines, have leaves opposite, simple, entire or occasionally lobed. The flowers are actinomorphic or zygomorphic, in cymes; calyx 5-4, tube fused with ovary and terminating in small imbricated or open teeth. Several members of this family possess floral or extra-floral nectars' which attract pollinators and, also have flowers that often emit a sweet heavy odour (Acharya and Mukherjee, 2017).The Caprifoliaceae comprises 857 accepted species belonging to 53 plant genera, among which is the *Scabiosa* genus (The Plant List).

The genus *Scabiosa* L. is considered a large taxonomically complex genus with more than 80 species (Carlson et al., 2012), however, accordingly to the Plant List only 62 are accepted (Figure 1). *Scabiosa* species are mainly distributed in the Mediterranean Basin, Asia and southern Africa (Carlson et al., 2012), and in Algeria can be found 11 *Scabiosa* species (Quezel and Santa, 1963).

S. atropurpurea L. *S. succisa* L. *S. tschilliensis* Grüning

Figure 1 Photos of some *Scabiosa* genus species

1.1.2. Pharmacological properties

The following scabiosa species (*Scabiosa*, *S. atropurpurea* L., *S. columbaria* L., *S. comosa* Fisch. Ex Roem. & Schult., *S. stellata* L., *S. tschilliensis* Grüning, *S. arvensis* L.[1], *S. succisa* L.[2]) are used in traditional medicine systems (Chinese Pharmacopoeia Committee, 1998; Bonet et al., 1999; Bammi and Douira, 2002; Rigat et al., 2007; Ferrer-Gallego, 2014; Kose et al., 2015; Moteetee and Kose, 2016; Gras et al., 2017). However pharmacological investigation of some species is lacking but also some taxonomic confirmations. Therefore, studies of the pharmacological activity of the genus *Scabiosa* are reduced and mainly concerned with extracts and/or compounds biological activities. Moreover, the species studied are also restricted, *S. atropurpurea* (Bussmann et al., 2010; Elhawary et al., 2011), *S. columbaria* (Vuuren and Naidoo, 2010), *S. comosa* (Ma et al., 2016), *S. tschilliensis* (Ma et al., 2016; Wang et al., 2017), *S. hymettia* Boiss. & Spruner (Christopoulou et al., 2008), *S. arenaria* Forssk.[3] (Besbes at al., 2012; Hlila et al., 2015; Hlila et al., 2016a; Hlila et al., 2016b), *S. prolifera* L.[3] (Al-Qudah et al., 2017) and recently *S. stellata* (Lehbili et al., 2018). An overview of the evaluations carried out and above mentioned, revealed that the majority are *in vitro* assessments of the antimicrobial and the antioxidant activities. Some *in vitro* cytotoxic evaluations were also reported, as well as the less common activities, such as anti-HCV (Ma et al., 2016), anti-tyrosinase (Lehbili et al., 2018) and acetylcholinesterase inhibition (Hlila et al., 2015). The *in vivo* study of the *S. atropurpurea* ethanolilhawary et al., 2011) due to the fact that, as far as we are aware is the only *in vivo* study extract antihyperglycaemic and hepatoprotective activities can be highlighted.

1.3. Secondary metabolites isolated from the genus Scabiosa L.

To understand the pharmacological activity of the genus *Scabiosa* species are essential detailed and extensive phytochemical investigations. Up to date, a wide spectrum of secondary metabolites has been isolated from *Scabiosa* genus, from which flavonoids and terpenoids can be emphasised. The names of these constituents and the plant from which they were isolated are listed in Table 1 (page 10); their structures are depicted in Figures 2-6 pages 6-10.

Although not many organic acids were found in the genus *Scabiosa*, from the ones found (Figure 2; Table1), two can be highlighted, the caffeic (**1**) and the chlorogenic (**2**) acids. Actually caffeic acid (**1**) is an important fragment of the chlorogenic acids, which are

[1] This name is a synonym of *Knautia arvenses* (L.) Coult.
[2] This name is a synonym of *Succisa pratensis* Moench.

[3] Is an unresolved name.

recognised for their important activities (Marques and Farah, 2009). It should be emphasise, however, this studies were performed long-ago so the species identification is somehow confused, for instance there are two *Scabiosa bipinnata*, the *S. bipinnata* Nyman and *S. bipinnata* K.Koch, and both are considered unresolved (The Plant List). Another species reported is *Scabiosa caucasica* M.Bieb. Which is now days considered a synonym of *Lomelosia caucasica* (M.Bieb.) Greuter & Burdet., in fact, only *Scabiosa olgae* Albov is an accepted name (The Plant List). These aspects point out that careful identification of the plant material are imperative to avoid extra phytochemical work.

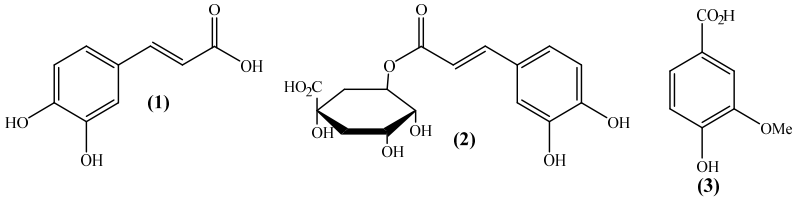


Figure 2. Structures of the organic acids isolated from the genus *Scabiosa*

Flavone derivatives are one of the most important secondary metabolites due to their biological properties (Verma and Pratap, 2010), from which anticancer (Cárdenas et al., 2006), anti-inflammatory (Moscatelli et al., 2006) and antioxidant (Beyer and Melzig, 2003) activities can be highlighted. Subsequently the occurrence of these metabolites both as aglycones and glycosides in *Scabiosa* genus (Figure 3; Table1) can explain and/or confirm the claimed medicinal properties.

The structures analysis (Figure 3) demonstrates that the flavone derivatives isolated from species of the genus *Scabiosa* are mostly derivatives of apigenin, diosmetin and luteolin, which are polyhydroxylated flavones. The other derivatives reported are flavonol type such as kaempferol and quercetin derivatives, also polyhydroxylated compounds. This fact is noteworthy due to the recognised biological properties of these polyhydroxylated flavones (Verma and Pratap, 2010).

Some works were also performed in the previous century, presenting the same problem with the species identification, but the work of Perdetzoglou *et al.* (1994) work can be emphasised not only due to the reported flavones derivatives identified but also due to the proper species identification. In their work the authors used the flavone content to establish that *Scabiosa argentea* L. and *Scabiosa tenuis* Spruner ex Boiss. are taxonomically independent species (Perdetzoglou et al., 1994).

Another interesting work is the phytochemical study of the species *S. hymettia* (Christopoulou et al., 2008) where two interesting kaempferol derivatives, the astragallin

(kaempferol 3-*O*-β-D-glucoside) (**5**) and the new natural compound kaempferol-3-*O*-[3-*O*-acetyl-6-*O*-(*E*)-*p*-coumaroyl]-β-D-glucoside (**11**), were isolated (Figure 3; Table1). Conversely, the species identification properly has not been reported in previeuos work carried out by Garaev *et al.* (2008) and Al-Qudah *et al.* (2017). Nevertheless, they have reported the isolation of some interesting flavones such as palustroside (**16**) a diosmetin derivative and also the new natural compound, the kaempferol-3-*O*-[4″,6″-di-(*E*)-*p*-coumaroyl]-β-D-galactoside (**12**) (Figure 3; Table1).

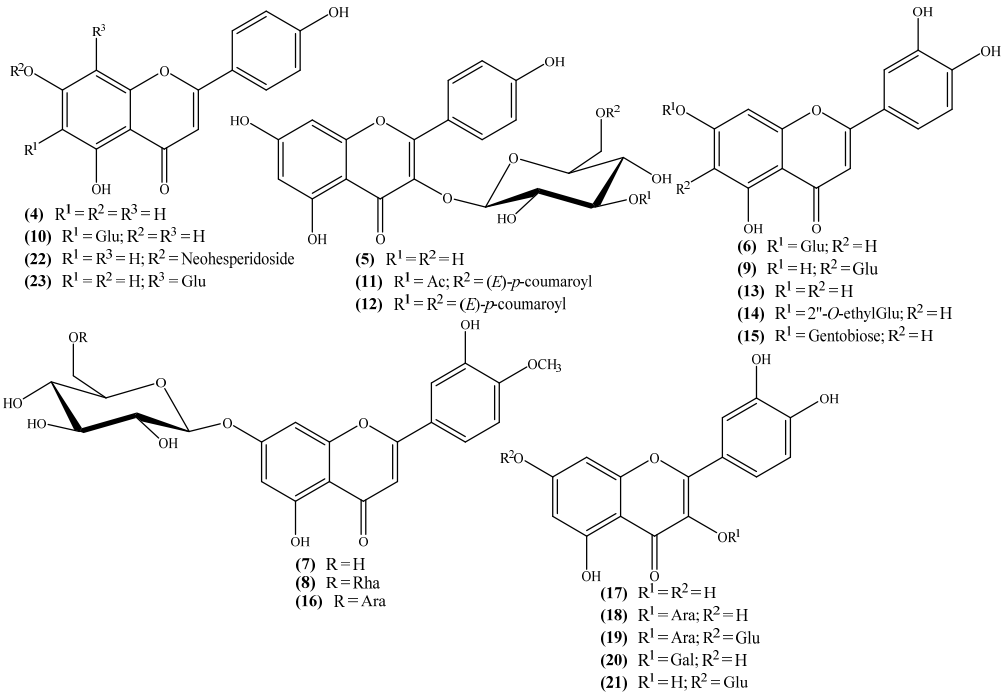


Figure 3. Flavonoids isolated from the genus *Scabiosa* (Ara=arabinose; Gal=galactose; Glu=glucose; Rha=rhamnose)

One of the largest and most diverse classes of secondary metabolites produced by plants is the terpenoids. In plants these compounds play several functions (Pichersky and Raguso, 2016) and are used by humans in several industries, from which the pharmaceutical should be accentuated (Singh and Sharma, 2015). From our survey, in the genus *Scabiosa* these metabolites are also representative although only a few species were studied. Several iridoid derivatives were isolated from *Scabiosa* species (Figure 4; Table 1) whereas the steroids are only two, β-sitosterol (**29**) and its glucoside (**30**) (Figure 4; Table 1). Nevertheless, the more reported terpenoids are the pentacyclic triterpenoids (Figure 5; Table 1).

The presence of β-sitosterol (**29**) and/or derivatives seems to be important due to their recognised biological properties and potential use in treatment of various illnesses (Saeidnia et al., 2014). Unfortunately, as above mentioned, the plant studied was not properly identified

(Al-Qudah et al., 2017) aspect that will prevent its recommendation as a source of this compound.

To iridoids are also attributed several biological activities (Ghisalberti, 1998; Tundis et al., 2008) which improves the value of the *Scabiosa* species that produce this type of compounds. The works that reported these metabolites are recent and gladly the plants are well identified allowing their recommendation for further studies, in particular the species *S. hymettia* (Christopoulou et al., 2008) and *Scabiosa variifolia* Boiss. (Papalexandrou et al., 2003), which are not reported as medicinal plants, but certainly can be a source of important bioactive compounds. In the case of *S. atropurpurea* (Polat et al., 2010) we are in the presence of a medicinal plant so these studies are always recommended to validate the plant medicinal use.

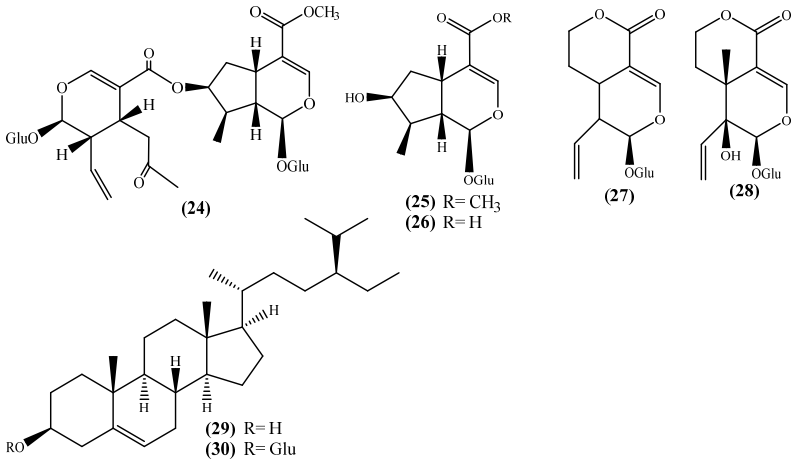


Figure 4. Iridoid and steroid derivatives isolated from the genus *Scabiosa* (Glu=glucose)

Pentacyclic triterpenoids occur naturally in many plants (Jäger et al., 2009) and from a biological perspective present a remarkable broad spectrum of pharmacological activities, from which the anti-inflammatory (Yadav et al., 2010) and the antitumor (Kamble et al., 2014; Chudzik et al., 2015) can be emphasized. The richness of the *Scabiosa* species in pentacyclic triterpenoids (Figure 5) seems to be obvious especially if we take into account the reduced number of species studied (Table 1). It is also worth mentioning the fact that almost all the isolated pentacyclic triterpenoids are saponins and this seems to be a characteristic of the genus *Scabiosa*. The main aglycones are oleanolic and pomolic acids, with glucose, xylose, rhamnose and arabinose as sugars (Figure 5).

Along with the above mentioned information about pentacyclic triterpenoids biological activities, it should be stressed the case of oleanolic acid (**32**) for which several biological activities have been reported (Sultana and Ata, 2008) being the most significant its potential

as cancer therapy drug (Shanmugam et al., 2014). Pomolic acid is less studied although there are evidences that can be an anti-HIV agent (Sultana and Ata, 2008).

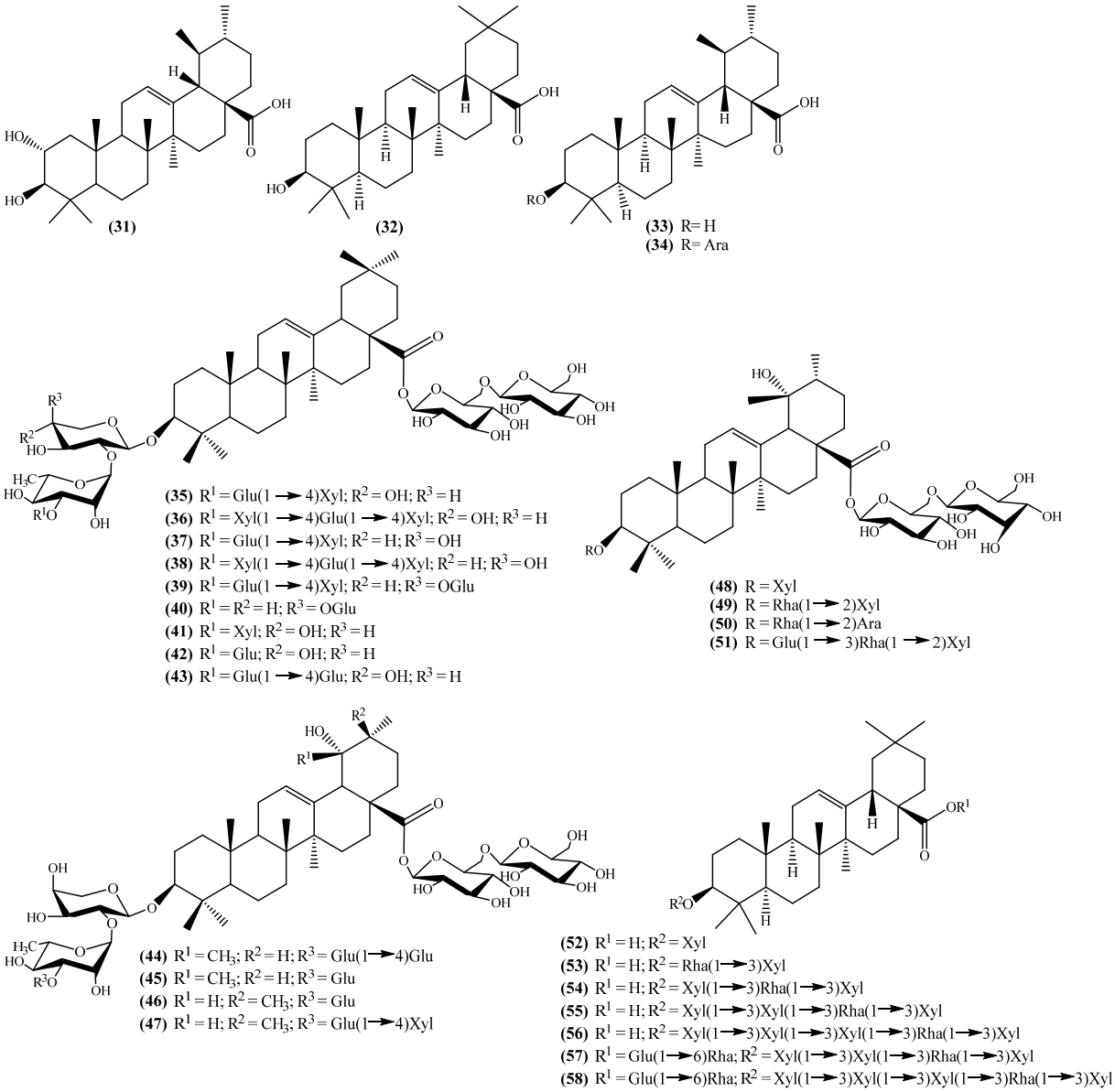


Figure 5. Pentacyclic triterpenoids isolated from the genus *Scabiosa* (Ara=arabinose; Gal=galactose; Glu=glucose; Rha=rhamnose; Xyl=xylose)

The literature survey demonstrates that *S. tschilliensis* can be a good source of these pentacyclic triterpenoids acids (Table 1) through a cleavage of the sugars moieties. Moreover, the presence of these secondary metabolites may be the reason for this plant medicinal use.

Scabiosa rotata M .Bieb. As far as we are aware, is not used in folk medicine but it is also a good source of pomolic acid (Table 1). On the other hand, *S. soongorica*[4] Schrenk can be regarded as a good source of oleanolic acid. Once again we noticed that the plants identification is vital to recommend its use.

[4] Although the authors indicate that they study the species *Scabiosa soongorica* Schrenk, we think that the current name is *Scabiosa songarica* Schrenk

Other compounds isolated from *Scabiosa* species were reported by Christopoulou *et al.* (2008) and were isolated from *S. hymettia*, stressing the high value of this species in polyphenolic content (Figure 6; Table1).

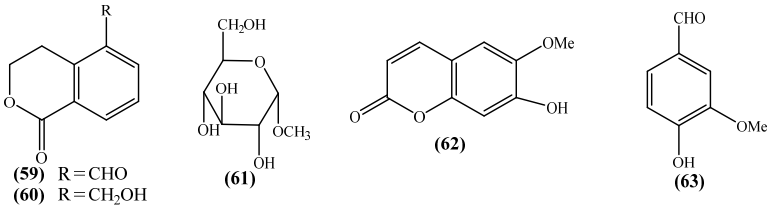


Figure 6. Other compounds isolated from the genus *Scabiosa*

Table 1. The secondary metabolites isolated from *Scabiosa* species

No.	Compound name	Source (Ref.)
Organic acids		
1	Caffeic acid	*S. bipinnata*[a] (Kuril'chenko et al., 1971) *S. olgae* (Zemtsova et al., 1972) *S. caucasica*[b] (Zemtsova and Bandyukova, 1971)
2	Chlorogenic acid	*S. bipinnata*[a] (Kuril'chenko et al., 1971) *S. olgae* (Zemtsova et al., 1972) *S. caucasica*[b] (Zemtsova and Bandyukova, 1971)
3	Vanillic acid	*S. hymettia* (Christopoulou et al., 2008)
Flavone derivatives		
4	Apigenin	*S. caucasica*[b] (Zemtsova and Bandyukova, 1971) *S. prolifera*[a] (Al-Qudah et al., 2017) *S. tenuis* (Perdetzoglou et al., 1994)
5	Astragallin	*S. hymettia* (Christopoulou et al., 2008)
6	Cynaroside	*S. caucasica*[b] (Zemtsova and Bandyukova, 1971; Garaev et al., 2008) *S. bipinnata*[a] (Kuril'chenko et al., 1971) *S. olgae* (Zemtsova et al., 1972) *S. prolifera*[a] (Al-Qudah et al., 2017) *S. tenuis* (Perdetzoglou et al., 1994) *S. argentea* (Perdetzoglou et al., 1994)
7	Diosmetin-7-*O*-β-glucoside	*S. argentea* (Perdetzoglou et al., 1994)
8	Diosmetin-7-*O*-rutinoside	*S. caucasica*[b] (Zemtsova and Bandyukova, 1971)
9	Isoorientin	*S. argentea* (Perdetzoglou et al., 1994) *S. prolifera*[a] (Al-Qudah et al., 2017)
10	Isovitexin	*S. tenuis* (Perdetzoglou et al., 1994)
11	Kaempferol-3-*O*-[3-*O*-acetyl-6-*O*-(*E*)-*p*-coumaroyl]-β-D-glucoside	*S. hymettia* (Christopoulou et al., 2008)
12	Kaempferol-3-*O*-[4″,6″-di-(*E*)-*p*-coumaroyl]-β-D-galactoside	*S. prolifera*[a] (Al-Qudah et al., 2017)
13	Luteolin	*S. tenuis* (Perdetzoglou et al., 1994)
14	Luteolin-7-*O*-(2''-*O*-ethyl-β-D-	*S. prolifera*[a] (Al-Qudah et al., 2017)

	glucoside)	
15	Luteolin-7-*O*- β-gentiobioside	*S. argentea* (Perdetzoglou et al., 1994) *S. tenuis* (Perdetzoglou et al., 1994)
16	Palustroside	*S. caucasica*[b] (Garaev et al., 2008)
17	Quercetin	*S. argentea* (Perdetzoglou et al., 1994)
18	Quercetin-3-*O*-arabinoside	*S. argentea* (Perdetzoglou et al., 1994)
19	Quercetin-3-*O*-β-L-arabinoside-7-*O*-β-D-glucoside	*S. bipinnata*[a] (Kuril'chenko et al., 1971)
20	Quercetin-3-*O*-galactoside	*S. argentea* (Perdetzoglou et al., 1994)
21	Quercimeritrin	*S. caucasica*[b] (Garaev et al., 2008)
22	Rhoifolin	*S. caucasica*[b] (Zemtsova and Bandyukova, 1971)
23	Vitexin	*S. tenuis* (Perdetzoglou et al., 1994)
Terpenoid derivatives		
24	Cantleyoside	*S. atropurpurea* (Polat et al., 2010) *S. variifolia* (Papalexandrou et al., 2003)
25	Loganin	*S. hymettia* (Christopoulou et al., 2008) *S. atropurpurea* (Polat et al., 2010) *S. variifolia* (Papalexandrou et al., 2003)
26	Loganic acid	*S. hymettia* (Christopoulou et al., 2008) *S. atropurpurea* (Polat et al., 2010) *S. variifolia* (Papalexandrou et al., 2003)
27	Sweroside	*S. atropurpurea* (Polat et al., 2010) *S. variifolia* (Papalexandrou et al., 2003)
28	Swertiamarin	*S. hymettia* (Christopoulou et al., 2008) *S. atropurpurea* (Polat et al., 2010) *S. variifolia* (Papalexandrou et al., 2003)
29	β-Sitosterol	*S. prolifera*[a] (Al-Qudah et al., 2017)
30	β-Sitosterylglucoside	*S. prolifera*[a] (Al-Qudah et al., 2017) *S. soongorica*[c] (Akimailiev et al., 1988)
31	Corosolic acid	*S. prolifera*[a] (Al-Qudah et al., 2017)
32	Oleanolic acid	*S. bipinnata*[a] (Kuril'chenko et al., 1971) *S. caucasica*[b] (Garaev et al., 2008)
33	Ursolic acid	*S. prolifera*[a] (Al-Qudah et al., 2017)
34	Ursolic acid-3-*O*-β-D-arabinoside	*S. prolifera*[a] (Al-Qudah et al., 2017)
35	Hookeroside A	*S. tschilliensis* (Zheng et al., 2004)
36	Hookeroside B	*S. tschilliensis* (Zheng et al., 2004)
37	Scabiosaponin A	*S. tschilliensis* (Zheng et al., 2004)
38	Scabiosaponin B	*S. tschilliensis* (Zheng et al., 2004)
39	Scabiosaponin C	*S. tschilliensis* (Zheng et al., 2004)
40	Scabiosaponin D	*S. tschilliensis* (Zheng et al., 2004)
41	Scabiosaponin E	*S. tschilliensis* (Zheng et al., 2004)
42	Scabiosaponin F	*S. tschilliensis* (Zheng et al., 2004)
43	Scabiosaponin G	*S. tschilliensis* (Zheng et al., 2004)
44	Scabiosaponin H	*S. tschilliensis* (Zheng et al., 2004)
45	Scabiosaponin I	*S. tschilliensis* (Zheng et al., 2004)
46	Scabiosaponin J	*S. tschilliensis* (Zheng et al., 2004)
47	Scabiosaponin K	*S. tschilliensis* (Zheng et al., 2004)
48	Scabrioside A	*S. rotata* (Baykal et al., 1998)
49	Scabrioside B	*S. rotata* (Baykal et al., 1998)
50	Scabrioside C	*S. rotata* (Baykal et al., 1998)
51	Scabrioside D	*S. rotata* (Baykal et al., 1998)
52	Songoroside A	*S. soongorica*[c] (Akimailiev et al., 1988)

53	Songoroside C	*S. soongorica*[c] (Akimailiev et al., 1976)
54	Songoroside E	*S. soongorica*[c] (Akimailiev et al., 1976)
55	Songoroside G	*S. soongorica*[c] (Akimailiev et al., 1976)
56	Songoroside I	*S. soongorica*[c] (Akimailiev et al., 1976)
57	Songoroside M	*S. soongorica*[c] (Akimailiev et al., 1976a)
58	Songoroside O	*S. soongorica*[c] (Akimailiev et al., 1976a)
Other compounds		
59	Erythrocentaurin	*S. hymettia* (Christopoulou et al., 2008)
60	5-(Hydroxymethyl)isochroman-1-one	*S. hymettia* (Christopoulou et al., 2008)
61	Methyl-α-D-glucopyranoside	*S. prolifera*[a] (Al-Qudah et al., 2017)
62	Scopoletin	*S. hymettia* (Christopoulou et al., 2008)
63	Vanillin	*S. hymettia* (Christopoulou et al., 2008)

S. = *Scabiosa*; [a]Is an unresolved name; [b]This name is a synonym of *Lomelosia caucasica* (M.Bieb.) Greuter & Burdet.; [c]This name should be *Scabiosa songarica* Schrenk;

1.1.4. Scabiosa stellata L.: an underexplored species from Algeria

Starflower pincushions is *Scabiosa stellata* L. common name, a species that gathered some attention and this year four phytochemical works were published, being two of them a result of this thesis work (Lehbili et al., 2018; Lehbili et al., 2018a; Rahmouni et al., 2018; Rahmouni et al., 2018a). *Scabiosa stellata* is an herbaceous plant forming dense small cushions (Figure 7). It is an annual plant (10-40 cm) hispid, with twigs dichotomies, solated capitulums at the top of short stalks (5-15 cm), radical leaves, toothed or incised, and bluish, radiant flowers with 5 unequal lobes (Quezel and Santa, 1963).

Scabiosa stellata grows widely in Algeria and it is used in traditional medicine to treat heel cracks (Bammi and Douira, 2002) and until this year was one of the less studied species of the genus, reason why was chosen by us.

Figure 7 Photos and classification of *Scabiosa stellata* L.

Amazingly the phytochemical study of *S. stellata* became a hot topic in the last months, some interesting activities were found, with particular relevance for the antibacterial one

(Lehbili et al., 2018), and the phytochemical studies revealed the presence of interesting secondary metabolites such as iridoids, flavonoids, saponins among others (Lehbili et al., 2018; Lehbili et al., 2018a; Rahmouni et al., 2018; Rahmouni et al., 2018a). Herein we detailed the recent and interesting work of Lehbili *et al.* (2018, 2018a) that revealed important metabolites found in the ethanolic extract of the whole plant. It is obvious that this phytochemical study allowed uncovering the chemical profile of *S. stellata* and naturally adding value to the plant due to significance of the metabolites found [compound (**12**) in Figure 3(page 7); compound (**27**) in Figure 4; compound (**33**) in Figure 5 and compounds depicted in Figure 8.

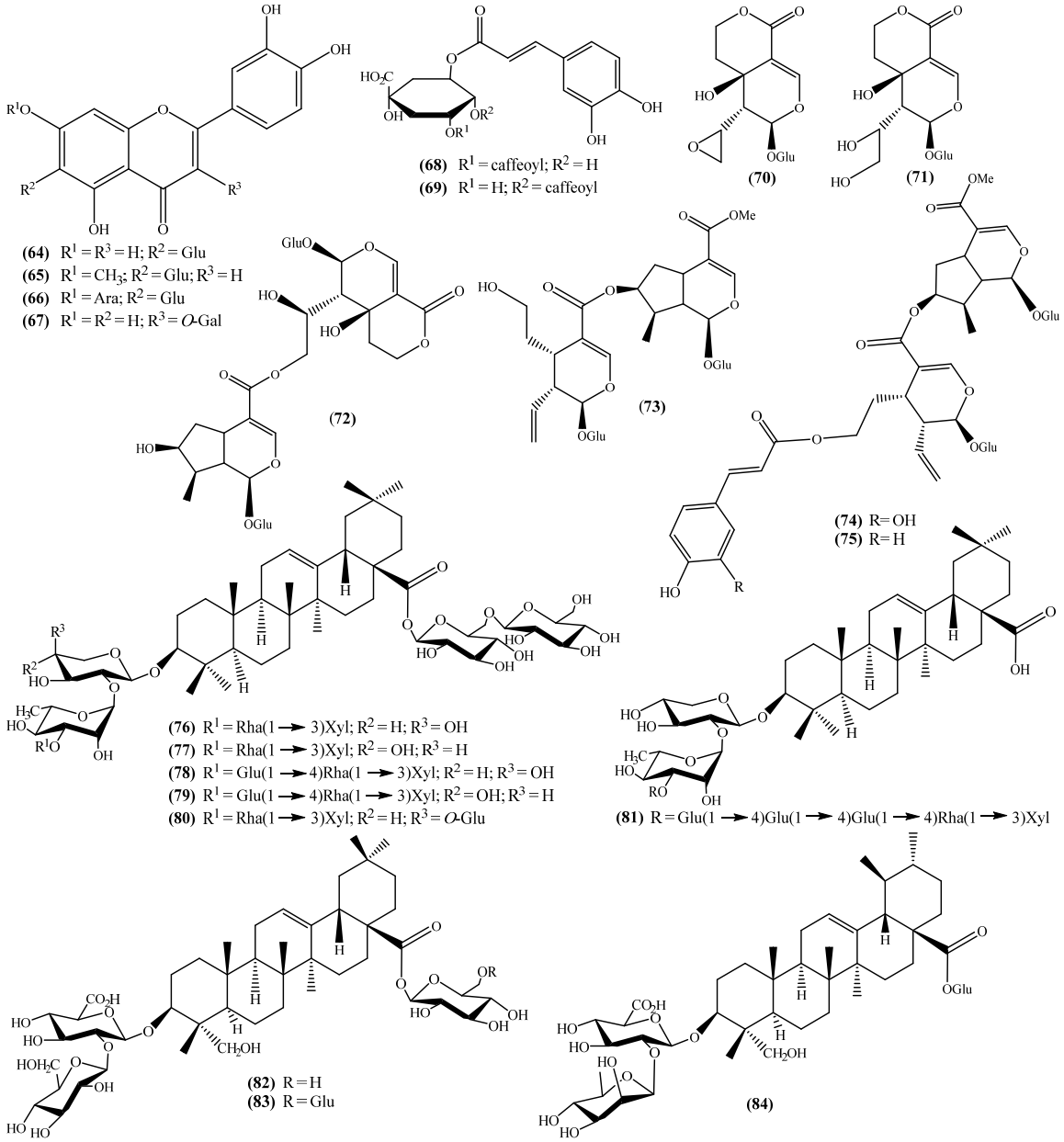


Figure 8. Secondary metabolites isolated from *S. stellata* (Ara=arabinose; Gal=galactose; Glu=glucose; Rha=rhamnose)

No doubt the most relevant aspect is the isolation of several new natural compounds such as the bis-iridoids 7-*O*-caffeoyl-sylvestroside (**74**) and 7-*O*-(*p*-coumaroyl)-sylvestroside (**75**), and the saponins named scabiostellatosides A to H (**76** to **84**). Scabiostellatosides A to E (**76** to **81**) are oleanoic acid derivatives, scabiostellatosides F and G (**82** and **83**) are hederagenin derivatives and scabiostellatoside H (**84**) is a ursolic acid derivative (Figure 8). Additionally the 7-*O*-caffeoyl-sylvestroside (**74**) showed moderate cytotoxic activity (IC_{50} 35.9 µg/mL) against brosarcoma cell lines (HT1080).

In this thesis following chapter will be discussed in detail our phytochemical study of *S. stellata* extracts as well as the lipophilic and polyphenolic profiles both disclosed during this work (Rahmouni et al., 2018; Rahmouni et al., 2018a).

1.2. *Sedum* genus: Pharmacological properties and phytochemistry

1.2.1. Morphological description

Sedum (Orpin) is the most investigated genus in *Crassulaceae* family and several smaller genera (Hart. 1991) contains around 470 succulent species generally grows in northern hemisphere, some located in the southern part of Africa and Latin America (Xu. 2015) Crassulaceae is one of largest families of flowering plants comprises 1400- 1500 species and 33 genera(Byalt. 2011; Gontcharova and Gontcharov. 2009) , Crassus = thick means the plants more or less Succulents, have Particular Photosynthetic of Metabolites (C.A.M. Metabolism for Crassulacean Acid Metabolism). Many species are used in horticulture; presented in different regions in the earth; throughout the Northern hemisphere as well as in Southern Africa and Australia with conspicuous centres of diversity in Mexico, the European and temperate Asiatic mountains. (Hart. 1997). Modern studies confirmed that the family Crassulaceae is a well circumscribed , however, identification of monophyletic groups of all ranks in the family is very difficult because of a great diversity of their morphological and cytological, they are divided into 6 subfamilies : Crassuloideae, Kalanchoideae, Cotyledonoideae, Echeverioideae, species of Kalanchoe, Crassula, Echeveria and Sedum are of substantial economic importance features Gontcharova and Gontcharov. 2009). Plants of this family are Perennial or rarely annual herbs or subshrubs with: Bisexual or unisexual yellow, purple or scarlet flowers, varying from 3- to 32-merous; sepals free or united into a tube; Leaves succulents, (Sub) sessile usually alternate and spiral, or opposite-decussate, frequently aggregate into rosettes, simple; fruits dehiscent follicles, capsular;

seeds are minute, elongated, Smallish, to 1.5-3 mm, mostly brownish; embryo is straight and endosperm ; root red tips, colored by an anthocyan pigment which is intensified by bright light (Quezel. 1963). Members of this family are not considered as important crop plants, but they are used for horticulture; many members have an unusual attractive appearance, and are quite hardy. (Milad . 2014) as there are other members useful for : rock garden, scree gardens (decorative), recommended for salad and traditional medicine (Byalt. 2011)

Sedum a genus includes annual succulent rock herbs and shrubs inhabiting mostly open, sunny and arid habitats, and in some cases moist habitats. They are distinctive in having acuminate fleshy leaves, fleshy stems and yellow, yellowish or creamy to white tiny flowers.(mahmoud et al) They store water in their leaves and give flowers in star with 5 branches (sometimes 4 or 6).

S. arce *S. album* *S. Spurium*

Figure 9: Photos of some *sedum* genus species

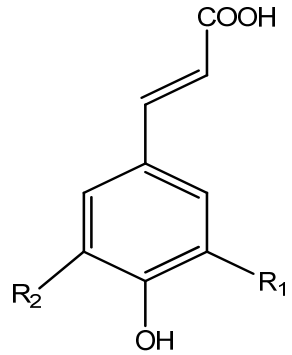
1.2.2. Pharmacological properties

for a long time Sedum plants have been incorporated into botanist's lists of cures due to the various uses as: radioprotective, antitumour, anti-inflammatory, liver protector, ...ect (Stephenson, 1994) antiscorbutic and local anesthetic, skin irritation and diphtheria; and more, several species of this genus considered as vegetable salad . For example: *Sedum sarmentosum* is a main medicinal plant used in China, it is commercially available as emollient, vulnerable, astringent, gastric and renal regulator, but too much, it causes stomach upset (Jung. 2008); *S. dendroideum* used as Gastric treatment (inflammation, ulcer), Anti-inflammatory, antinociceptive, antioxidant (Meloa. 2009) ; *S. Formosanum* is a diabetes treatment; *S. cressipes* has Emollient, vulnerable and resolving proprieties(Chou. 1976)

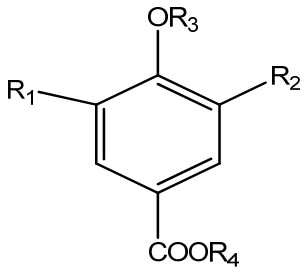
1.2.3. Secondary metabolites isolated from the genus *Sedum*.

The genus *Sedum* is part of the family crassulaceae whose representatives, due to their economic and therapeutic interest, have been the subject of numerous phytochemical studies. Nevertheless, given the number of species not yet studied, this genus is still an important source of natural products such as phenolic acids, terpenes, coumarins, flavonoids and other secondary metabolites such as megastigmans and alkaloids(Stevens. 1996; Wei. 2014).

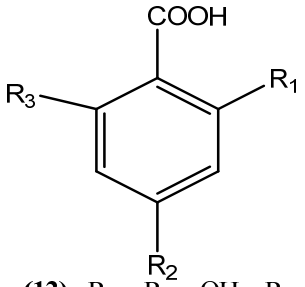
Phenolic acids constitute a family of organic molecules widely present in the plant kingdom, they are secondary metabolites, characterized by the presence of an aromatic ring bearing at least one hydroxyl and carboxylic acid groups, some of organic acids are isolated from species (14 species) of this genus(table 2); ***S. Reflexum and S. Acre*** the richest onese, with the main organic acids and the most abondante are cafiec acid, gallic acid, Syringic acid they are known with their central roles in biochemistry . The glycosylated phenolic acids are not widespread in the genus. 3,5-dimethoxybenzoic-4-O-glucoside acid (8) is the only once isolated (table 2, page 26),



(1) $R_1 = OH, R_2 = H$
(2) $R_1 = R_2 = H$
(3) $R_1 = OCH_3, R_2 = H$
(4) $R_1 = R_2 = OCH_3$



(5) $R_1 = R_2 = R_3 = R_4 = H$
(6) $R_1 = R_3 = R_4 = H$
(7) $R_1 = R_3 = R_4 = H, R_2 = OCH_3$
(8) $R_1 = R_2 = OCH_3, R_3 = H, R_4 =$ Glu
(9) $R_1 = R_2 = OH, R_3 = R_4 = H$
(10) $R_1 = R_2 = OH, R_3 = H, R_4 = CH_3$
(11) $R_1 = R_3 = H, R_3 = R_2 = OH, R_4 = CH_3$



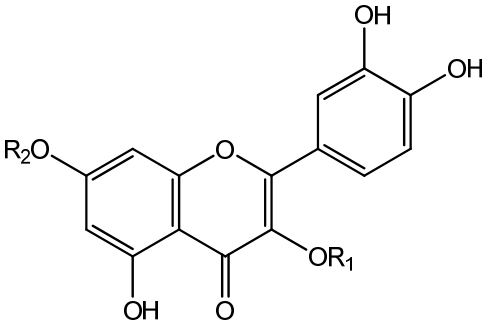
(12) $R_1 = R_2 = OH, R_2 = H$
(13) $R_1 = R_3 = H, R_2 = OH$
(14) $R_1 = R_2 = OH, R_3 = OH$
(15) $R_1 = OH, R_2 = R_3 = H$

Figure 10: Structures of isolated phenolic acids from *sedum* genus(Chawki et al., 2015)

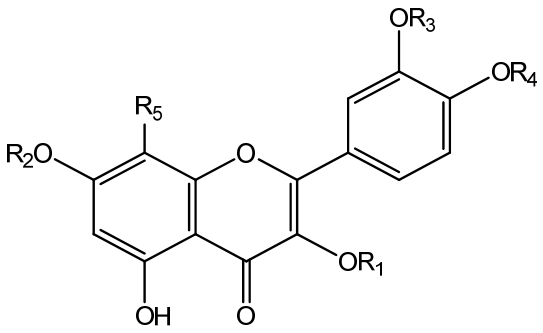
On the other hand certain reported isolated compounds showed the widely distribution of flavonoids in species of *sedum* genus (more then 90 flavnoid) table 2, being mainly present as, isoflavonoids, Flavonols , Flavons, Catechin and chalcons; the known compounds such as Quercetin, Kaempferol, Myricetin, Isorhamnetine are reported in more then 23 species. Their glycosides are the most common in these species as: Quercetin-3- O- β-D-glucoside, Kaempferol-3- O- α-L-rhamnoside, Quercetin-3- O-α-L-rhamnoside, Quercetin-3-O-α-D-rutinoside..... ect. These compounds are also doted with several biological properties

such as nti-HIV activity, tyrosinase inhibition, antirotavirus activity, antistress activity, antiobesity activity, anticholinesterase potential, antiadipogenic activity, and antiallergic activity (Xiao., 2017) whereas C-glycosyls are less abundant in this genus.

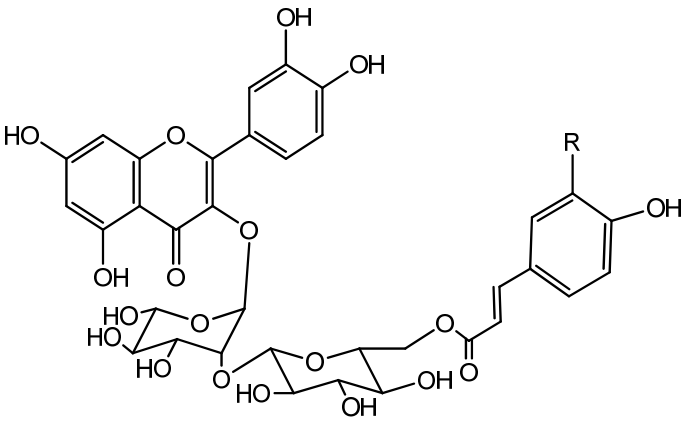
Several phytochemical studies carried out by **He** in **1997** allowed the isolation and identification of flavonoids; chalcones (**120, 121**, figure 11 , page 19) and another catechins types (table 2, page 25)



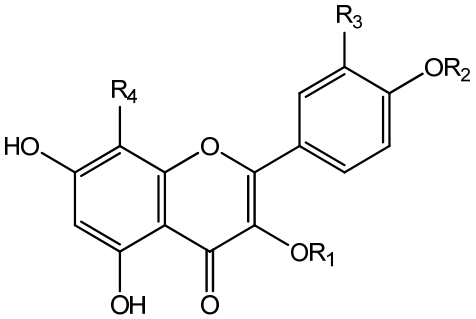
(16) $R_1 = R_2 = H$
(17) R_1 =H , R_2 = Rha
(18) R_1 = Glu , $R_2 = H$
(19) R_1 = Rha, R_2 = Glu
(20) R_1 = Rutinoside, $R_2 = H$
(21) R_1 = Galactoside, $R_2 = H$
(22) R_1 = Glu, R_2 = Rha
(23) R_1 = Rha-Glu, R_2 = Rha
(24) $R_1 = R_2$ = Glu
(25) $R_1 = R_2$ = Rha
(26) R_1 = Rha, $R_2 = H$
(27) $R_1 = R_2$ = Rha-Glu
(28) $R_1 = CH_3$, $R_2 = H$
(29) R_1 = 2''-galloylrhamnoside, $R_2 = H$
(30) R_1 = 2''-galloylglucoside, $R_2 = H$
(31) R_1 = Gala-Xyl, $R_2 = H$



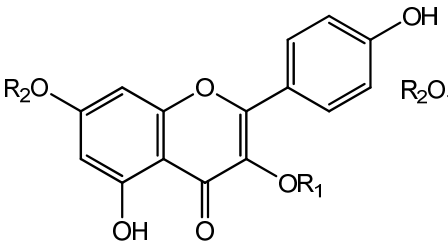
(34) $R_1 = R_2 = R_3 = R_5$ =H, R_4 = Glu
(35) $R_1 = R_4 = R_5 = R_2$ =H, R_3 = Glu
(36) $R_1 = R_2 = R_4$ =H, $R_3 = CH_3$, $R_5 = OCH_3$,
(37) R_1 = Glu, $R_2 = R_4 = H$, $R_3 = CH_3$, $R_5 = OCH_3$
(38) $R_1 = R_4 = H$, R_2 = Glu, $R_3 = CH_3$, $R_5 = OCH_3$
(39) $R_1 = R_4 = H$, R_2 = Rha, $R_3 = CH_3$, $R_5 = OCH_3$
(40) $R_1 = R_2$ = Glu, $R_3 = CH_3$, $R_5 = OCH_3$
(41) R_1 = (6''-p-coumaroyl)-glucoside, $R_2 = R_4 = H$, $R_3 = CH_3$ $R_5 = OCH_3$
(42) $R_1 = R_2 = R_3 = H$, $R_4 = H$, $R_5 = OCH_3$
(43) $R_1 = R_3 = R_4 = H$, R_2 = Rha, $R_5 = OCH_3$
(44) $R_1 = R_2$ = Glu, $R_3 = R_4 = H$, $R_5 = OCH_3$
(45) $R_1 = R_2 = R_3 = R_4 = H$, R_5 = OH
(46) $R_1 = R_2 = R_3 = R_4 = H$, R_5 = O-Glucuronide
(47) $R_1 = R_2 = R_3 = R_4 = H$, R_5 = O-Glu
(48) $R_1 = R_2 = R_3 = R_4 = H$, R_5 = O-Xyl
(49) $R_1 = R_3 = R_4 = H$, R_2 = Xyl, R_5 = OH



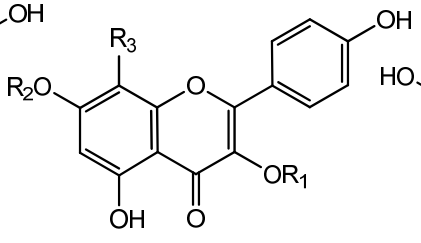
(32) R = OH
(33) R = H



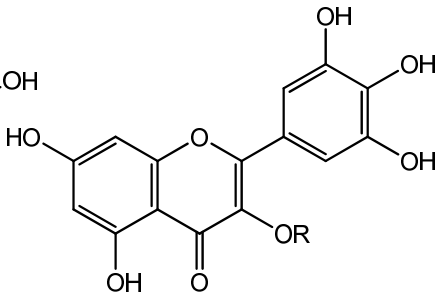
(71) $R_1 = R_3 = R_4 = H$, R_2 = Glu
(72) R_1 = Glu, $R_2 = CH_3$, $R_3 = R_4 = OCH_3$



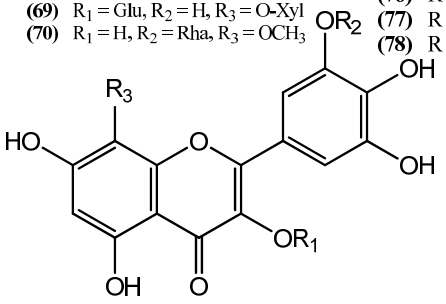
(50) $R_1 = R_2 = H$
(51) $R_1 = H$, $R_2 = Glu$
(52) $R_1 = H$, $R_2 = Rha$
(53) $R_1 = H$, $R_2 = Ara$
(54) $R_1 = Rha$, $R_2 = H$
(55) $R_1 = Glu$, $R_2 = H$
(56) $R_1 = Ara$, $R_2 = H$
(57) $R_1 = R_2 = Rha$
(58) $R_1 = R_2 = Glu$
(59) $R_1 = Rha$, $R_2 = Glu$
(60) $R_1 = Glu$, $R_2 = Rha$
(61) $R_1 = Rha(1 \rightarrow 2) Glu$, $R_2 = Glu$
(62) $R_1 = Rha(1 \rightarrow 2) Glu$, $R_2 = Rha$
(63) $R_1 = R_2 = Rha\text{-}Glu$
(64) $R_1 = Rha$, $R_2 = Rha\text{-}Glu$



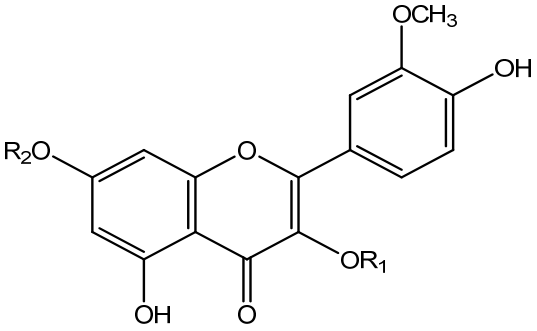
(65) $R_1 = Rha\text{-}Glu$, $R_2 = Rha$, $R_3 = OCH_3$
(66) $R_1 = R_2 = Glu$, $R_3 = OCH_3$
(67) $R_1 = R_2 = H$, $R_3 = OH$
(68) $R_1 = R_2 = H$, $R_3 = OCH_3$
(69) $R_1 = Glu$, $R_2 = H$, $R_3 = O\text{-}Xyl$
(70) $R_1 = H$, $R_2 = Rha$, $R_3 = OCH_3$



(73) R = H
(74) R = Rha
(75) R = Glu
(76) R = Gala
(77) R = Ara
(78) R = (2''-O-galloyl)rhamnoside

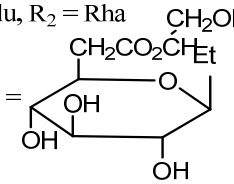


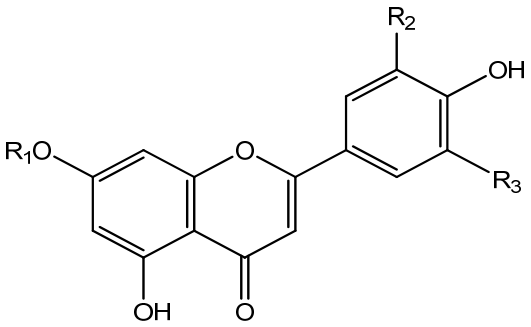
(79) $R_1 = R_2 = H$, $R_3 = OCH_3$
(80) $R_1 = R_3 = H$, $R_2 = CH_3$
(81) $R_1 = Glu$, $R_2 = H$, $R_3 = OCH_3$



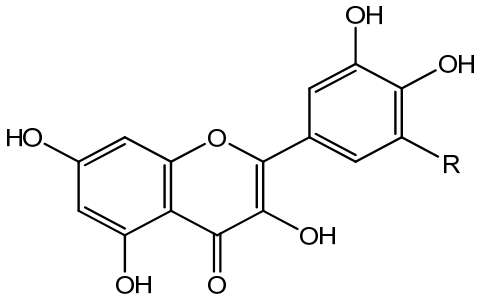
(82) $R_1 = R_2 = H$
(83) $R_1 = H$, $R_2 = Rha$
(84) $R_1 = H$, $R_2 = Glu$
(85) $R_1 = H$, $R_2 = Sophoroside$
(86) $R_1 = Rha$, $R_2 = H$
(87) $R_1 = Glu$, $R_2 = H$
(88) $R_1 = R_1 = R_2 = Glu$
(89) $R_1 = Rha$, $R_2 = Glu$
(90) $R_1 = Rha$, $R_2 = Sophoroside$
(91) $R_1 = Rha$, $R_2 = Glu\text{-}Glu$
(92) $R_1 = (2''\text{-acetyl})Glu$, $R_2 = H$
(93) $R_1 = Glu$, $R_2 = Rha$
(94) $R_1 = Rha\text{-}Glu$, $R_2 = Rha$

(95) $R_1 = Glu$, $R_2 =$





(96) $R_1 = H$, $R_2 = OCH_3$, $R_3 = OCH_3$
(97) $R_1 = Glu$, $R_2 = OCH_3$, $R_3 = OCH_3$
(98) $R_1 = Glu$, $R_2 = OH$, $R_3 = OH$
(99) $R_1 = R_3 = H$, $R_2 = OCH_3$
(100) $R_1 = GLU$, $R_2 = OCH_3$, $R_3 = H$
(101) $R_1 = RRha$, $R_2 = OCH_3$, $R_3 = H$
(102) $R_1 = H$, $R_2 = OH?$ $R_3 = H$
(103) $R_1 = Glu$, $R_2 = OH$, $R_3 = H$
(104) $R_1 = Glu$, $R_2 = R_3 = H$
(236) $R_1 = R_2 = R_3 = H$
(237) $R_2 = R_3 = H$, $R_1 = Glu$



(105) R = H
(106) R = OH

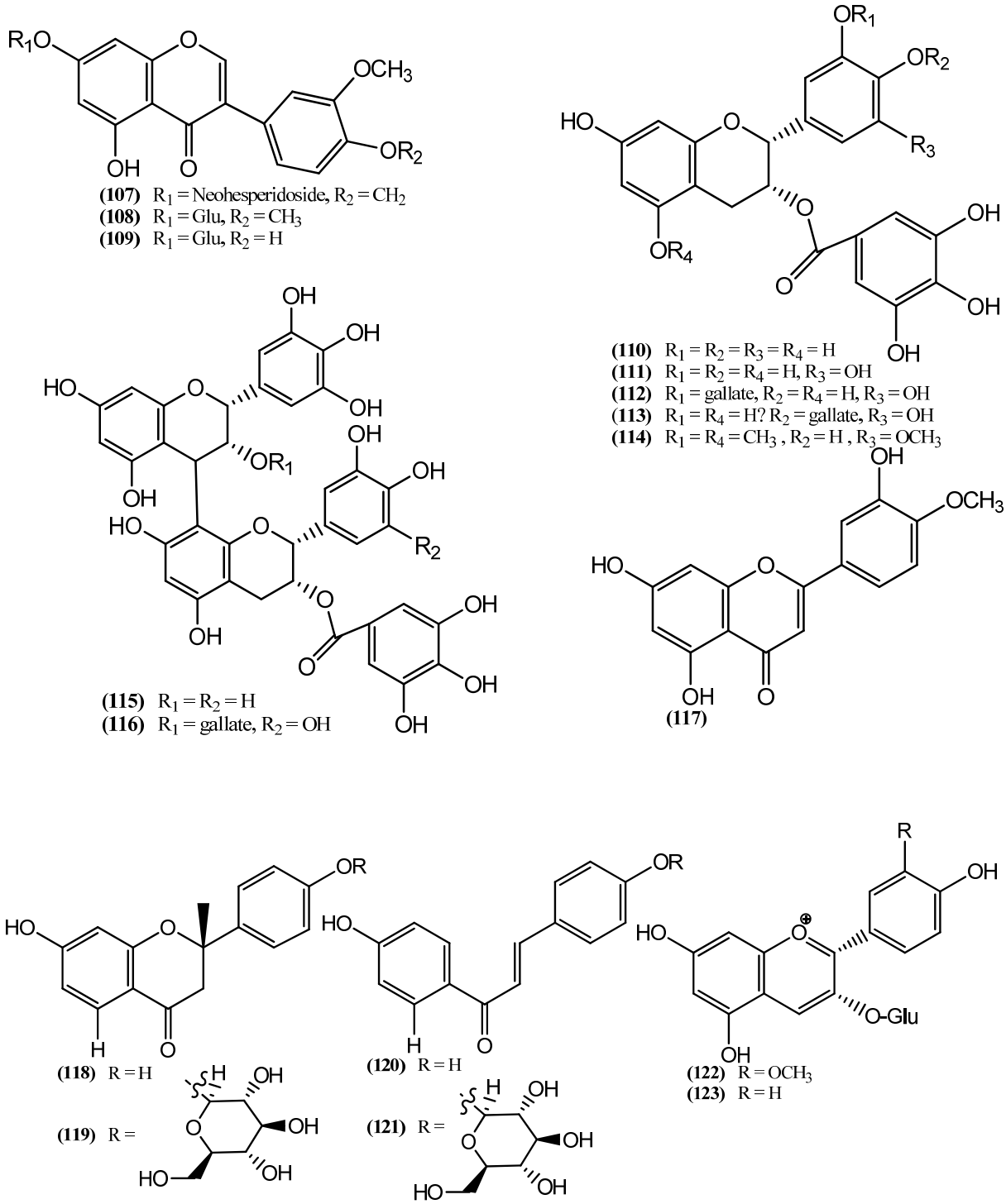
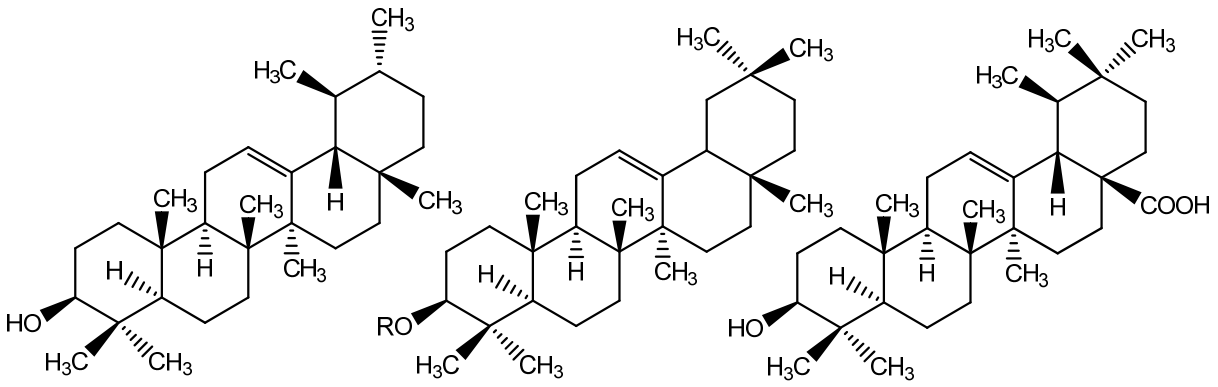


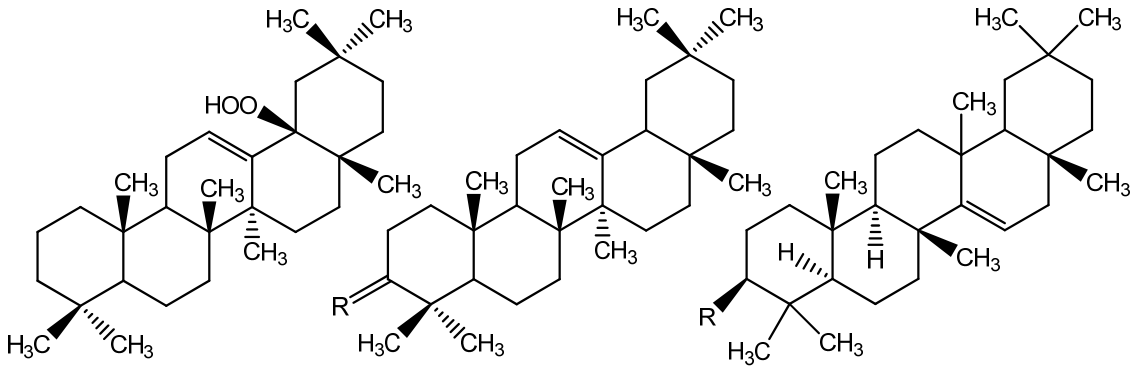
Figure 11 : Structures of isolated flavonoids from *sedum* genus (Chawki et al., 2015)

Terpenoids known as isoprenoids, this term refers to a group of substances with the skeleton of terpenes has one or more chemical functions (alcohol, aldehyde, ketone, acid, lactone, etc.). They usually have fungicidal, insecticidal properties and they attract pollinators.

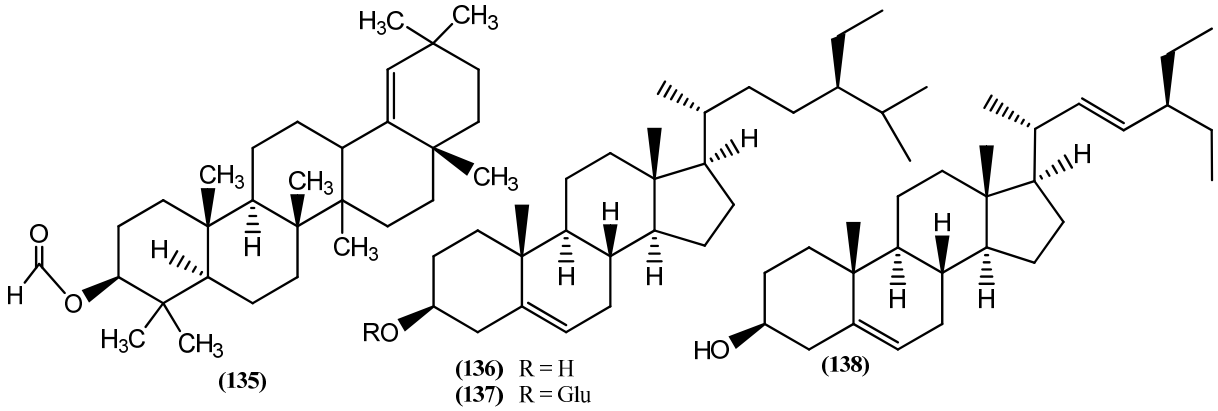
These constitute very important group of natural substances within species of *sedum* genus, The phytochemical investigations carried out, particularly on plants of this genus allowed the isolation of 19 terpenes most of them are pentacyclic triterpenes and steroid triterpenes (Table 1, figure 12). Their molecular structures have been established through spectroscopic and chemical analysis methods.



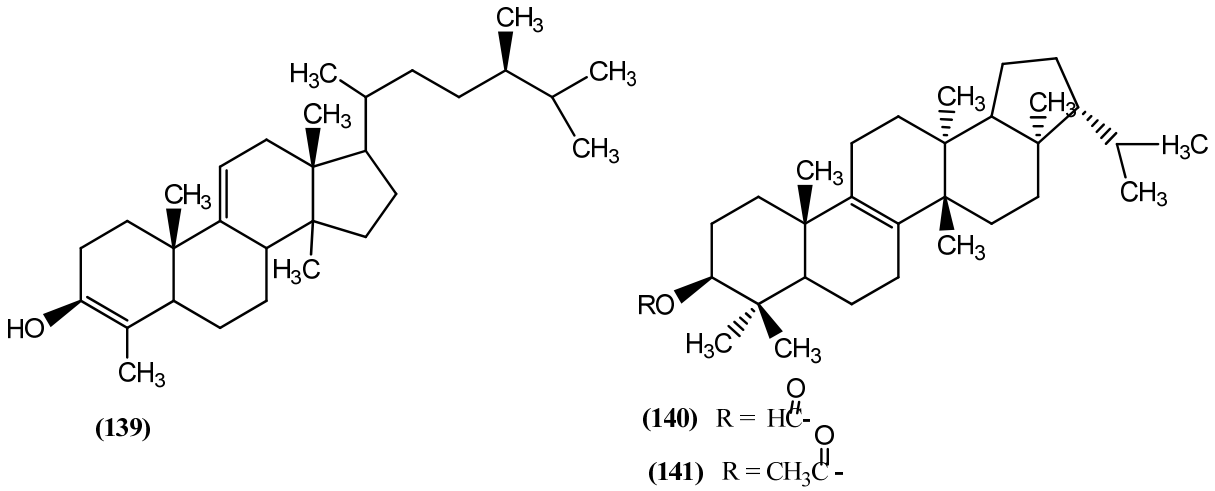
(124) **(125)** R = H **(126)** R = Ac **(127)**



(128) **(129)** R = O **(130)** R = OH, H **(131)** R = H, OH **(132)** R = OAc **(133)** R = OOCH **(134)** R = O



(135) **(136)** R = H **(137)** R = Glu **(138)**



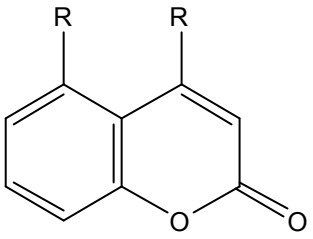
(139)

(140) R = HC-

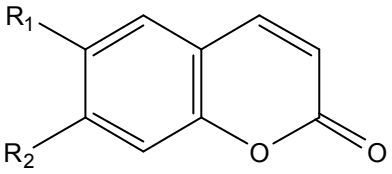
(141) R = CH_3C -

Figure 12: Structures of isolated tepenes from *sedum* genus (Chawki et al., 2015)

Coumarins are natural substances their name is derived tfrom "coumarou", name of the tonka bean called Dipterix odorataWilld, known as 1-benzopyran-2-one or 1,2-benzopyrone . Coumarins are widely distributed in the plant kingdom. They are found in all parts of the plant (roots, stems, leaves, fruits and essential oils of seeds). Several phytochemical studies have shown that isolated coumarins of the genus *Sedum* are relatively rare. We list only 7 coumarins (Table 2, Figure 13) they are of a great interest due to their pharmacological properties mainly : anti-inflammatory, anticoagulant, antitumor, diuretic, antimicrobial, analgesic and antiviral (Ford et al., 2001)



(143) R = H
(144) R = OH

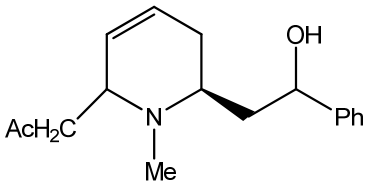


(145) $R_1 = R_2 = H$
(146) $R_1 = H$, $R_2 = OH$
(147) $R_1 = OH$, $R_2 = H$
(148) $R_1 = OCH_3$, $R_2 = OH$
(149) $R_1 = O-$, $R_2 = O-$

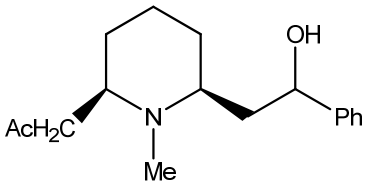
Figure 13: Structures of isolated coumarins from *sedum* genus (Chawkiet et al., 2015)

Alkaloids are very important Substances of natural origin and restricted distribution, nitrogenous structure (N included in a heterocycle) and basic character. They exist in plants as salts. Having a biosynthetic origin of an amino acid, they are endowed with a significant

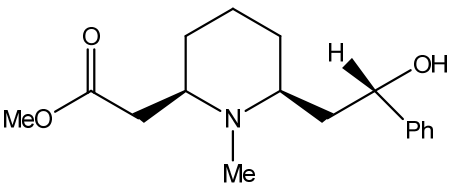
pharmacological activity.in this genus 40 alkaloids were isolated from 16 species thus It is also worth mentioning the fact that almost major isolated alkaloids in Crassulaceae family .are the main of them were present in *sedum* species especially *Sedum acre* (20 alkaloid) and this seems to be a characteristic of this species, the aerial parts of this plant are used in folk medicine (Wolbis and Krolikowska., 1988) most of these alkaloids are sedacrine, sedamine sedridine, sedinine and sedinone as described in table2 , figure 14



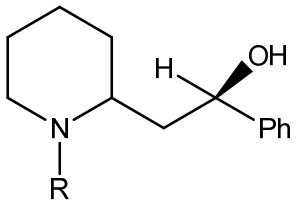
(150)



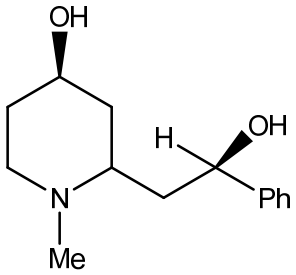
(151)



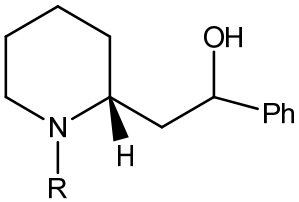
(152)



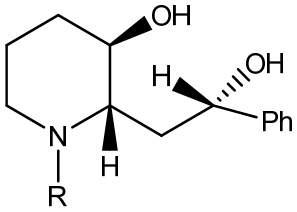
(153) R = Me
(154) R = H



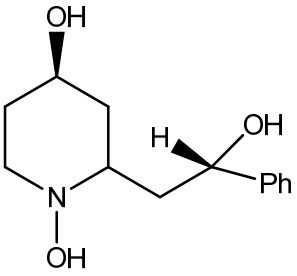
(155)



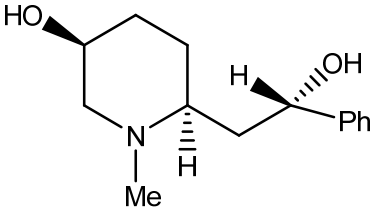
(156) R = Me
(157) R = H



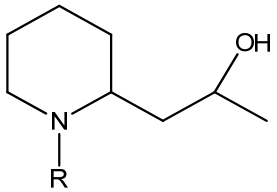
(158) R = CH_3
(159) R = H



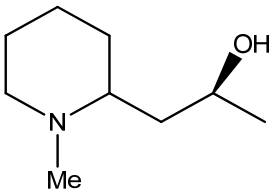
(160)



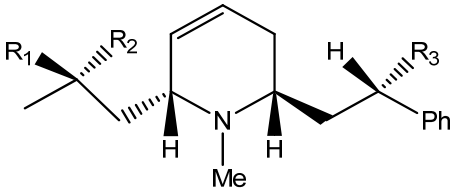
(161)



(162) R = H
(163) R = Me



(164)



(165) $R_1 = R_3 = H$
(166) $R_1 = H$, $R_2 = R_3 = OH$
(167) $R_1 = R_3 = OAc$, $R_2 = H$

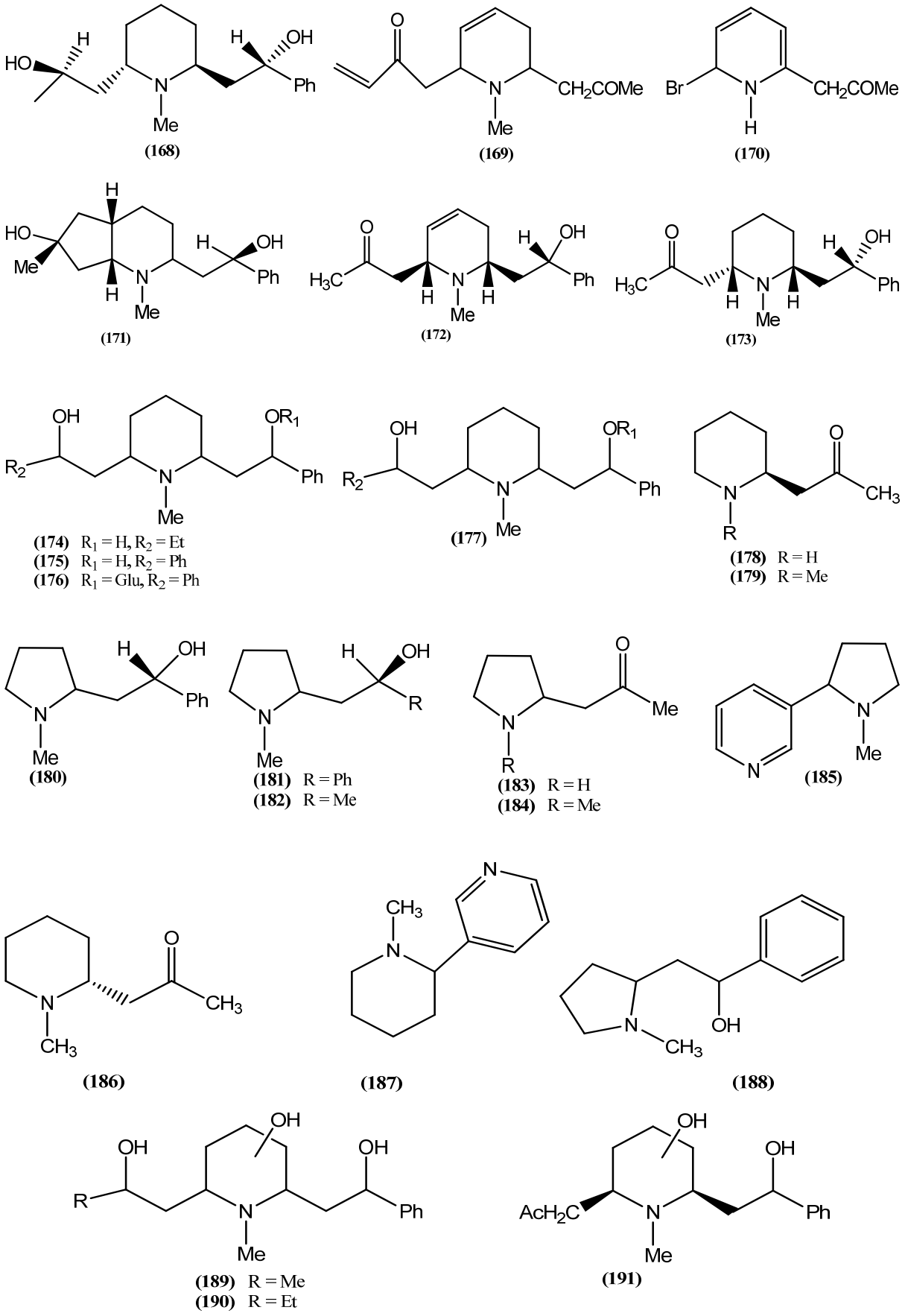
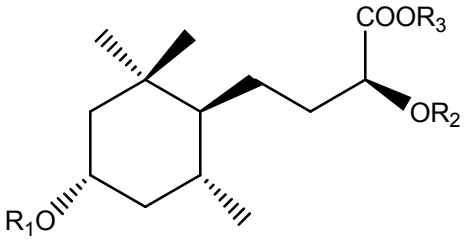
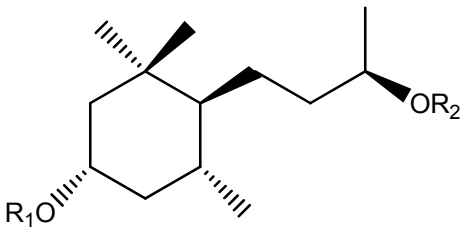


Figure 14 : Structures of isolated alkaloids from *sedum* genus (Chawki et al., 2015)

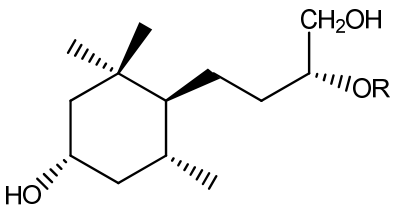
Megastigmans are degradation compounds that have 13 carbon atoms in the form of butene cyclohexene. They are produced by the cleavage of carotenoids (tetraterpenes)
33 megastamans have been isolated from the Chinese species; *Sedum sarmentosum*, which is used in traditional medicine in the treatment of hepatitis. (Table 2, Figure 15)



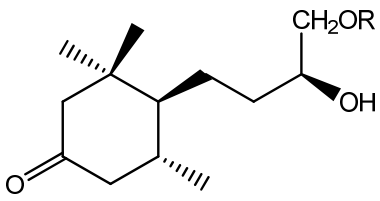
(192) $R_1 = R_2 = H$
(193) $R_1 = Glu, R_2 = R_3 = H$
(194) $R_1 = R_3 = H, R_2 = Glu$
(195) $R_1 = R_2 = H, R_3 = Glu$
(196) $R_1 = R_3 = H, R_3 = Xyl$
(197) $R_1 = R_2 = Glu, R_3 = H$
(198) $R_1 = Glu, R_2 = Xyl, R_3 = H$



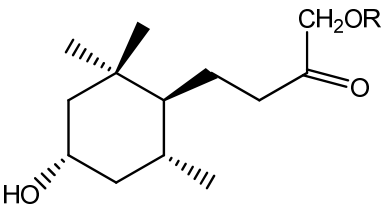
(199) $R_1 = Glu\text{-}Rha, R_2 = H$
(200) $R_1 = H, R_2 = Glc\text{-}Rha$
(201) $R_1 = H, R_2 = Glu\text{-}Api$
(202) $R_1 = H, R_2 = Glu$
(203) $R_1 = Glu, R_2 = H$
(204) $R_1 = R_2 = Glu$
(205) $R_1 = R_2 = H$



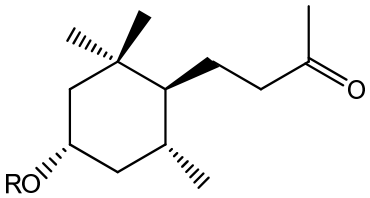
(206) R = Glu
(207) R = H



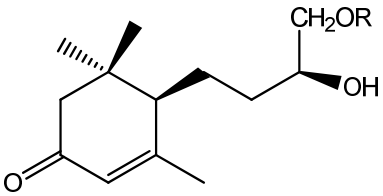
(208) R = Glu
(209) R = H



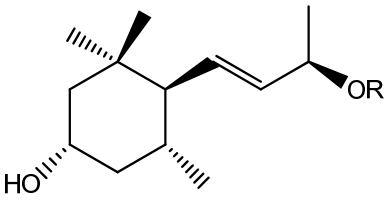
(210) R = Glu
(211) R = H



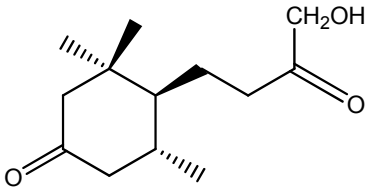
(212) R = Glu-Rha
(213) R = H



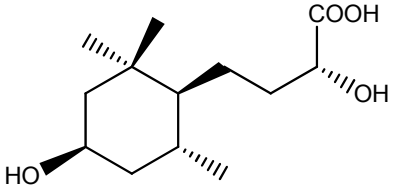
(214) R = Glu
(215) R = H



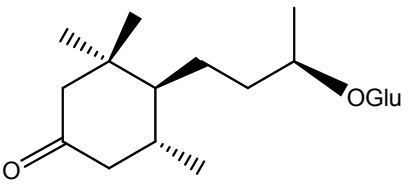
(216) R = Glu
(217) R = Glu
(218) R= H



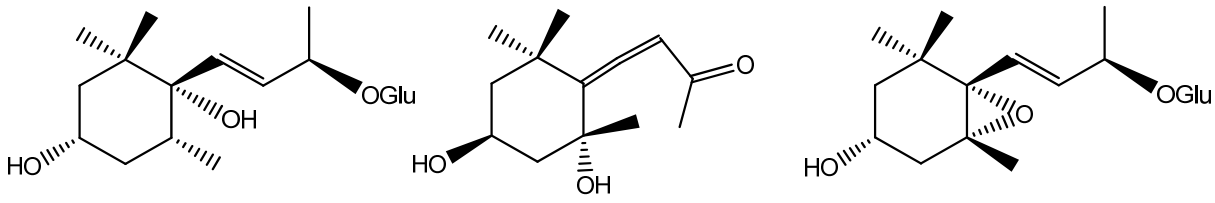
(219) R = Glu
(220) R = H



(221)



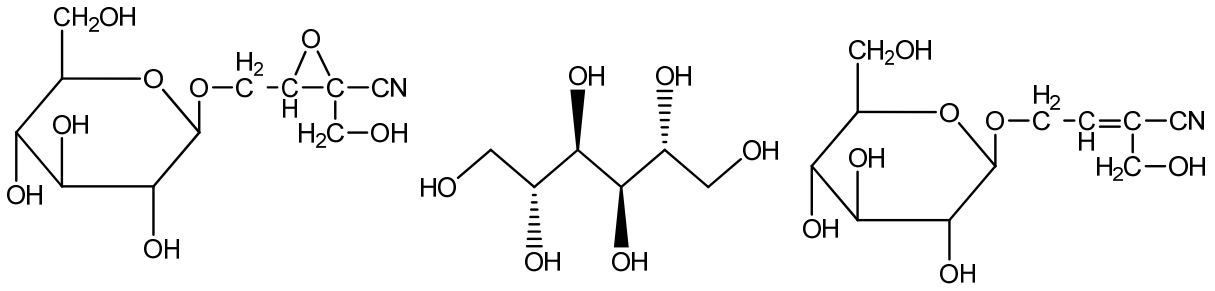
(222)



(223) **(224)** **(225)**

Figure 15. Structures of isolated megastigmans from *sedum* genus (Chawki et al., 2015)

Several phytochemical studies have been carried out on different species of the genus Sedum allowed the isolation of other compounds, such as saccharides and aliphatic hydrocarbon derivatives. Table2, Figure 16



(227) **(228)** **(226)**

CH_3-$(CH_2)_{24}$-CH_2-OH **(229)**

CH_3-$(CH_2)_{26}$-CH_2-OH **(230)**

CH_3-$(CH_2)_{28}$-CH_2-OH **(231)**

CH_3-$(CH_2)_{28}$-CH_2-OH **(232)**

CH_3-$(CH_2)_{16}$-COO-$(CH_2)_{25}$-OH **(233)**

CH_3-$(CH_2)_{16}$-COO-$(CH_2)_{27}$-OH **(234)**

CH_3-$(CH_2)_{16}$-COO-$(CH_2)_{29}$-OH **(235)**

Figure 16: Structures of other compounds isolated from *sedum* genus (chaouki. 2015)

Table 2. The secondary metabolites isolated from *Sedum* species genus

No.	Compound name	Source (Ref.)
Organic acids		
1	Caffeic acid	*S. Takesimense* (Thuong et al., 2007) *S. populifolium* (Demidenko et al., 1978) *S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987 ; Korul'kin., 1986) *S. maximum*(Wolbis., 1987) *S. aizoon* (Korul'kin., 1986; Wolbis and Olszewska, 1996) *S. ewersii(*Korul'kin, 1986) *S. hybridum(*Korul'kin . , 1986) *S. kamtchaticum (*Korul'kin., 1986) *S. purpureum(*Korul'kin., 1986) *S. telephium ((*Korul'kin., 1986)
3	Ferulic acid	*S. Takesimense* (Thuong et al., 2007)

		S. reflexum (Wolbis., 1989) *S. acre*(Wolbis.,1987 ; Korul'kin., 1986) *S. maximum*(Wolbis., 1987) *S. aizoon* (Korul'kin., 1986) *S. hybridum (*Korul'kin., 1986) *S. purpureum (* Korul'kin., 1986) *S. telephium (* Korul'kin., 1986)
9	Gallic acid	*S. Takesimense* (Thuong et al., 2007) *S. reflexum* (Wolbis. 1989) *S. acre* (Wolbis. 1987 ; Korul'kin. 1986) S. aizoon(Korul'kin., 2001; Wolbis and Olszewska. 1996) *S. ewersii* (Korul'kin., 1986) *S. hybridum(*Korul'kin. 1986) *S. kamtchaticum (* Korul'kin. 1986; Daphné. 2009) *S. middendorffianum* (Shnyakina and Murzina . 1974) *S. selskianum* (Shnyakina and Murzina . 1974) *S. pallescens* (Chou,.1986)
10	Methylgallate	*S. Takesimense* (Thuong et al., 2007) S. aizoon(Korul'ki., 1986; Wolbis and Olszewska. 1996)
2	p-coumaric Acid	*S. reflexum* (Wolbis. 1989) *S. acre* (Wolbis. 1987 ; Korul'kin. 1986) S. aizoon(Korul'kin. 1986; Wolbis and Olszewska. 1996) *S. album* (Wolbis. 1987) *S. alfredi* (Chou et al., 1986)
4	Sinapic Acid	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis. 1987 ; Korul'kin. 1986)
5	Syringic acid	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987 ; Korul'kin., 1986) *S. maximum*(Wolbis., 1987) *S. aizoon* (Korul'kin., 1986; Wolbis and Olszewska, 1996) *S. hybridum(*Korul'kin. 1986) *S. kamtchaticum* (Korul'kin. 1986; Daphné. 2009) *S. purpureum(* Korul'kin. 1986) *S. telephium (* Korul'kin. 1986)
16	Protocatechuic acid	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987 ; Korul'kin., 1986) *S. maximum*(Wolbis., 1987)
12	Gentisic acid	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987 ; Korul'kin., 1986) *S. aizoon* (Korul'kin., 1986; Wolbis and Olszewska, 1996) *S. hybridum (*Korul'kin. 1986) *S. ewersii (*Korul'kin, 1986)
7	Vanillic acid	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis et al., 1987 ; Korul'kin., 1986) *S. maximum* (Wolbis., 1987) *S. aizoon* (Korul'kin., 1986; Wolbis and Olszewska,

		1996) *S. ewersii(*Korul'kin., 1986) *S. hybridum(*Korul'kin., 1986) *S. kamtchaticum (*Korul'kin., 1986) *S. telephium (* Korul'kin. 2001) *S. album* (Wolbis., 1987)
13	p-hydroxybenzoic acid	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987 ; Korul'kin., 1986) *S. maximum* (Wolbis., 1987) *S. aizoon* (Korul'kin., 1986; Wolbis and Olszewska, 1996) *S. album* (Wolbis., 1987)
14	resorcylic acid	*S. reflexum* (Wolbis., 1989)
15	salicylic acid	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987 ; Korul'kin., 1986)
11	methylester protocatechuic acid	*S. acre* (Wolbis., 1987 ; Korul'kin., 1986)
8	3,5-dimethoxybenzoic-4-O-glucoside acid	*S. alfredi* (Chou and Men., 1986)
Flavonoids derivatives		
16	Quercetine	*S. maximum* (Gnedkov,et al., 1981) *S. acre* (Mylius., 1872*)* *S. populifolium* (Shnyakina and Murzina., 1974) *S. purpureum* (Pokotylo et al., 1974) *S. hybridum* (Gendaram et all., 2011) *S. ewersii* (Krasnov and Kondareva. 1976) *S. telephium L.*(Mulinacci et al., 1995) *S. montanum* (Stevens et al., 1994) *S. sediforme* (Niemann et al., 1976) *S. album* (Wolbis., 1989) *S. reflexum (*Wolbis., 1989b) *S. aizoon L* (Wolbis., 1996) *S. takesimense* (Thuong et al., 2007) *S. montanum* (Stevens et al., 1994) *S. ruthenicum* (Gumenyuk et al., 1976) *S. kautschaticum* (Shnyakina and Zapesochnaya, 1975) *S. kamtchaticum* (Zapesochnaya and Shnyakina. 1978) *S. selskianum* (Shnyakina and Murzina ., 1974) *S. pallescens* (Shnyakina and Murzina., 1974) *S. orchroleucum* (Niemann et al., 1976) *S. middendorffianum* (Shnyakina. 1973)
50	Kaempferol	*S. maximum* (Gnedkov,et al., 1981) *S. acre* (Mylius., 1872) *S. populifolium* (Shnyakina and Murzina., 1974) *S. purpureum* (Pokotylo et al., 1974) *S. hybridum* (Gendaram et all., 2011) *S. ewersii* (Korul'kin. 2001; Krasnov and Kondareva. 1976 ; *S. telephium L.* (Korul'kin. 2001) *S. middendorffianum* (Shnyakina. 1973) *S. selskianum* (Shnyakina and Murzina ., 1974) *S. pallescens* (Shnyakina and Murzina., 1974) *S. ruthenicum*(Gumenyuk et al., 1976)

		S. kautschaticum (Shnyakina and Zapesochnaya, 1975) *S. aizoon* (Korul'kin., 2001) *S. kamtchaticum* (Zapesochnaya and Shnyakina., 1978)
73	Myricetine	*S. maximum* (Gnedkov,et al., 1981) *S. populifolium* (Shnyakina and Murzina.1974) *S. middendorffianum* (Shnyakina. 1973) *S. selskianum* (Shnyakina and Murzina ., 1974) *S. montanum* (Stevens et al., 1994) *S. sediforme* (Sakar.et al., 1993) *S. reflexum* (Wolbis., 1989b) *S. aizoon L* (Wolbis., 1996) *S. takesimense* (Thuong et al., 2007) *S. kautschaticum*(Shnyakina and Zapesochnaya,1975) *S. sexangulare* (Wolbis and Kawalec., 1990) *S. orchroleucum* (Niemann et al., 1976)
7	Quercetin-7- O- α-L-rhamnoside	*S. maximum* (Gnedkov,et al., 1981) *S. caucasium* (Zaitsev, 1983)
18	Quercetin-3- O- β-D-glucoside	*S. maximum* (Gnedkov,et al., 1981) *S. hybridum* (Gendaram et all., 2011) *S. reflexum*(Wolbis., 1989b ; Wolbi., 1993) *S. sarmentosum* (Oh et al., 2004) *S. aizoon (*(Korul'kin., 2001) *S. ewersii* (Korul'kin,. 2001) *S. stoloniferum* (Petereit et al., 1998) *S. purpureum* (Korul'kin,. 2001) *S. telephium (*Korul'kin., 2001) *S. acre* (Wolbis., 1987)
22	Quercetin-3-O-β-D-glucosyl-7-O-α-D-rhamnoside	*S. maximum* (Wolbis., 1987) *S. purpureum* (Pokotylo et al., 1974) *S. hybridum* (Gendaram et all., 2011) *S. telephium L* .(Mulinacci et al., 1995)
10	Quercetin-3- O-α-L-rhamnoside	*S. maximum* (Wolbis.,1987) *S. sediforme*(Niemann et al.,1976) *S. album* (Wolbis., 1989) *S. reflexum (*Sakar et al., 1993) *S. ewersii* (Korul'kin,. 2001) *S. aizoon* (Korul'kin,. 2001) *S. hybridum* (Korul'kin,. 2001) *S. kamtchaticum* (Korul'kin,. 2001) *S. purpureum* (Korul'kin,. 2001) *S. telephium* (Korul'kin,. 2001)
20	(Rutine) Quercetin-3- O-α-D-rutinoside	*S. acre* (Mylius., 1872*)* *S. ruthenicum*(Gumenyuk et al., 1976) *S. kamtchaticum* (Korul'kin,. 2001) *S. ewersii* (Krasnov and Kondareva. 1976) *S. aizoon* (Korul'kin,. 2001) *S. hybridum* (Korul'kin,. 2001)
24	Quercetin-3,7-di- O- β-D-glucoside	S. acre (Wolbis., 1987)
	Quercetin-3-α-L-O-rhamnosyl-7-O-β-Dglucoside	*S. alfredi (S.formosanum)* (Chou., 1986)
21	Quercetin-3-O-β-D-galactoside	*S. populifolium* (Shnyakina and

	(Hyperoside)	Zapesochnaya.,1975) *S. kautschaticum* (Shnyakina and Zapesochnaya.,1975)
23	Quercetin-3,7-di- O- α-L-rhamnoside (Hyperoside)	*S. telephium* L (Mulinacci et al., 1995) *S. sarmentosum* (Morikawa et al., 2007)
	Quercetin-3-O-β-D-galactosido-(1-6)-O-β-Dxyloside	*S. hybridum* (Korul'kin,. 2001) *S. ewersii* (Korul'kin,. 2001)
42	8-methoxyquercetine (Corniculatusine)	*S. orchroleucum* (Niemann., 1976)
28	Quercetin-3-methoxy	*S. album* (Wolbis., 1989)
34	Quercetin-4'- O-β-D-glucoside	*S. album* (Wolbis., 1989)
	Quercetin-3-(2''-galloyl)-α-L-rhamnoside	*S. aizoon L* (Wolbis., 1996)
	Quercetin-3'- O-β-D-glucoside	*S. hybridum* (Krasnov et al., 1976
33	Quercetin-3-O-α-(6'''-p-coumaroylglucosyl-β-1,2-rhamnoside)	*S. sarmentosum* (Oh et al., 2004)
32	Quercetin-3-O-α-(6'''-caffeoylglucosyl-β-1,2-rhamnoside)	*S. sarmentosum* (Oh et al., 2004)
45	Gossypetine (8-hydroxyquercetine)	*S. aizoon L* (Lin., 2014) *S. kautschaticum* (Shnyakina and Zapesochnaya, 1975). *S. populifolium* (Shnyakina and Zapesochnaya.,1975) *S. album* (Wolbis., 1989) *S. ewersii* (Korul'kin,. 2001) *S. hybridum (*Gendaram, 2011)
46	Gossypetine-8-glucuronide	*S. album* (Wolbis., 1989)
47	Gossypetine-8-O-β-D-glucoside (Gossypine)	*S. populifolium* (Shnyakina and Zapesochnaya.,1975) *S. kautschaticum (*Shnyakina and Zapesochnaya, 1975)
48	Gossypetine-8-O-xyloside	*S. takesimense (*Thuong. 2007**)**
49	Gossypetine-7-O-xyloside	*S. ewersii* (Korul'kin,. 2001) *S. hybridum* (Korul'kin,. 2001)
78	Gallomyricitrine	*S. middendorfianum* (Shnyakina., 1979) *S. sichoteuse* (Shnyakina., 1979) *S. selskianum* (Shnyakina., 1979) *S. kamtschaticum* (Shnyakina., 1979)
23	Quercetin-3-α-D-O-neohesperidosyl-7-α-D-Orhamnoside	*S. telephium L* .(Mulinacci et al., 1995) *S. sarmentosum* (Oh et al., 2004)
52	Kaempferol-7- O- α-L-rhamnoside	*S. maximum* (Wolbis. 1987) *S. ewersii* (Krasnov and Kondareva., 1976) *S. caucasium* (Zaitsev et al., 1983) *S. pallescens* (Shnyakina and Murzina.,1974)
54	Kaempferol-3- O- α-L-rhamnoside	*S. hybridum* (Korul'kin,. 2001) *S. ewersii* (Korul'kin,. 2001) *S. telephium (*Korul'kin,. 2001) *S. purpureum* (Korul'kin,. 2001) *S. sediforme* (Sakar. Et al.,1993) *S. stoloniferum* (Petereit et al., 1998) *S. maximum* (Wolbis. 1987) *S. acre* (Korul'kin,. 2001)

51	Kaempferol-7- O- β-D-glucoside	*S. purpureum* (Pokotylo et al., 1974) *S. sarmentosum* (Oh et al., 2004)
55	Kaempferol-3- O-β-D-glucoside	*S. ruthenicum* (Gumenyuk et al., 1976)
53	Kaempferol-7-O-arabinoside	*S. populifolium* (Demidenko and Krasnov. 1978)
54	Kaempferol-3-O-arabinoside (Astagalin)	*S. sarmentosum* (Oh et al., 2004)
58	Kaempferol-3,7-di-O-β-D-glucoside	*S. cressipes* (Khetwal et al., 1988)
57	Kaempferol-3,7-di-O-α-L-rhamnoside (Kaempferitrine)	*S. maximum* (Wolbis. 1987) *S. telephium L* (Mulinacci et al., 1995) *S. pallescens* (Shnyakina et al., 1974) *S. dendroideum* (De Melo et al., 2005) *S. hybridum* (Korul'kin,. 2001)
59	Kaempferol-3-O-α-L-rhamnoside-7-O-β-Dglucoside	*S. purpureum* (Pokotylo et al., 1974) *S. hybridum* (Korul'kin,. 2001)
60	Kaempferol-3-O-β-D-glucosyl-7-O-α-Lrhamnoside	*S. maximum* (Wolbis. 1987) *S. telephium L (*Mulinacci et al., 1995) *S. dendroideum* (De Melo et al., 2005)
62	Kaempferol-3-O-α-D-rhamnosylglucoside-7-α-L-O-rhamnoside	*S. dendroideum* (De Melo et al., 2005) *S. telephium* (Mulinacci et al., 1995)
64	Grosvenosine	*S. sarmentosum* (Oh et al., 2004)
61	Kaempferol-3-O-α-L-rhamnosylglucoside-7-β-D-O-glucoside	*S. dendroideum* (De Melo et al., 2005)
63	Kaempferol-3,7-di-O-α-L-rhamnosylglucoside	*S. hybridum* (Korul'kin,. 2001)
65	Kaempferol-8-methoxy-3-O-α-Lrhamnosylglucoside-7- O- α-L-rhamnoside	*S. sexangulare* (Wolbis and Kawalec., 1990)
67	Kaempferol-8-hydroxy(Herbacetine	*S. album* (Wolbis., 1989)
68	Kaempferol-8-methoxy (Sexangularitine)	*S. sexangulare* (Wolbis and Kawalec., 1990) *S. acre* (Combier at al., 1968)
71	Kaempferol-4'-O- β-L-glucoside	*S. ewersii* (Krasnov and Kondareva. 1976)
43	Corniculatusine-7-O-α-L-rhamnoside	*S. sexangulare* (Wolbis and Kawalec., 1990)
44	Corniculatusin-3,7- di-O-β-D-glucoside	*S. alfredi (S. formosanum)* (Men., 1986)
42	8-methoxyquercetine (Corniculatusine)	*S. orchroleucum* (Wolbis., 1989) *S. montanum* (Wolbis., 1989) *S. sediforme* (Wolbis., 1989) *S. sexangulare* (Wolbis and Kawalec., 1990)
74	Myricetine-3-O-α-L-rhamnoside (Myricitrine)	*S. aizoon L* (Wolbis., 1996) *S. populifolium* (Demidenko and Krasnov. 1978) *S. kautschaticum*(Demidenko and Krasnov. 1978) *S. sediforme* (Sakar et al. 1993) *S. reflexum* (Wolbis., 1989b) *S. montanum* (Stevens et al., 1994) *S. maximum* (Gnedkov,et al., 1981)
76	Myricetine-3-O-galactoside	*S. populifolium* (Demidenko and Krasnov. 1978) *S. kautschaticum* (Demidenko and Krasnov. 1978)
79	8-methoxymyricetine	*S. sexangulare* (Wolbis and Kawalec., 1990)
75	Myricetine-3- O- β-D-glucoside (Isomyricitrine)	*S. maximum* (Wolbis. 1987) *S. populifolium* (Demidenko and Krasnov. 1978) *S. reflexum* (Wolbis., 1989b)

		S. kautschaticum (Demidenko and Krasnov. 1978)
77	Myricetine-3-O-arabinoside	*S. reflexum* (Wolbis., 1989b) *S. montanum* (Stevens et al., 1994)
80	3'-methoxymyricetine (Laricitrine)	*S. cressipes* (Khetwal et al., 1988)
81	Myricetine-8-methoxy-3- O- β-D-glucoside	*S. sexangulare* (Wolbis and Kawalec., 1990)
69	Rhodalidin	*S. takesimense* (Thuong. 2007**)**
102	Lutéolin	*S. sarmentosum* (He and Wang., 1997) *S. takesimense* (Thuong et al., 2007)
103	Lutéoline-7- O- β-D-glucoside	*S. alfredi (S. formosanum)*(Men., 1986) *S. sarmentosum*(He and Wang., 1997) *S. takesimense* (Thuong et al., 2007)
104	Apigenine-7- O- β-D-glucoside	*S. sarmentosum* (He and Wang., 1997)
96	Tricine	*S. sarmentosum* (He and Wang., 1997)
97	Tricine-7- O- β-D-glucoside	*S. sarmentosum* (He and Wang., 1997)
98	Tricetine-7- O- β-D-glucoside	*S. alfredi (S. formosanum)* (Men., 1986)
36	Limocetrine (sedoflorigenin)	*S. acre* (Malterud and Nordal., 1991) *S. sexangulare* (Wolbis and Kawalec., 1990) *S. sarmentosum* (He and Wang., 1997) *S. reflexum (*Wolbis., 1989b)
38	Limocitrine-7- O-β-D-glucoside	*S. acre* (Wolbis , and Krolikowska,. 1988)
37	Limocitrine-3-O-β-D-glucoside	*S. acre* (Malterud and Nordal., 1991) *S. sexangulare* (Wolbis and Kawalec., 1990) *S. sediforme* (Niemann et al., 1976) *S. reflexum (*Wolbis., 1989b) *S. alfredi (S. ormosanum)* *S. sarmentosum* (He and Wang., 1997)
39	Limocitrine-7-O-α-L-rhamnoside	*S. sexangulare* (Wolbis and Kawalec., 1990)
41	Limocitrine-3-O-(6''-O-p-coumaryl)glucoside	*S. Alfredi (S.formosanum)*
40	Limocitrine-3,7-di- O-β-D-glucoside	*S. acre* (Wolbis , and Krolikowska,. 1988) *S. slfredi (S.formosanum)* *S. Sarmentosum* (He and Wang., 1997)
70	Sexangularitine-7-α-L-rhamnoside	*S. sexangulare* (Wolbis and Kawalec., 1990)
72	Isorhamnetine-8-methoxy-3-O-β-D-glucoside	*S. acre* (Mylius., 1972)
82	Isorhamnetine	*S. maximum* (Gnedkov,et al., 1981) *S. acre* (Krolikowska, 1966) *S. album (*Wolbis., 1989) *S. ruthenicum* (Gumenyuk et al., 1976) *S. Forsterianum* (Niemann et al., 1976) *S. Pruinatum* (Niemann et al., 1976) *S.Tenuifolium* (Niemann et al., 1976)
84	Isorhamnetine-7- O β-D-glucoside	*S. album* (Wolbis., 1989) *S. sarmentosum* (He and Wang., 1997) *S. acre* (Mylius., 1972)
83	Isorhamnetine-3- O- α-L-rhamnoside	*S. album* (Wolbis., 1989)
88	Isorhamnetine-3,7-di- O- β-D-glucoside	*S. acre* (Mylius., 1972) *S. sarmentosum* (He and Wang., 1997) *S. alfredi (S. Formosanum)* (Men., 1986)
91	Isorhamnetine-3-α-L-O-rhamnosyl-7-O-β-D-diglucoside	*S. album* (Wolbis., 1989)
90	Isorhamnetine-3-O-α-L-rhamnosyl-	*S. album* (Wolbis., 1989)

	7-Osophoroside	
56	Isorhamnetine-7- O- α-L-rhamnoside	*S. caucasium* (Zaitsev et al., 1983)
85	Isorhamnetine-7-O-sophoroside	*S. album* (Wolbis., 1987)
87	Isorhamnetine-3- O- β-D-glucoside	*S. maximum* (Gnedkov,et al., 1981)
92	Isorhamnetine-3-(2"-acetyl)glucoside	*S. acre* (Wolbis , and Krolikowska,. 1988)
93	Isorhamnetine-3-O-β-D-glucoside-7-O-α-Lrhamnoside	*S. sarmentosum* (He and Wang., 1997)
94	Isorhamnetine-3-O-α-L-rhamnosylglucoside-7-O- α-L-rhamnoside	*S. sarmentosum* (He and Wang., 1997)
87	Isorhamnetine-3-O- β-D-glucoside	*S. acre* (Krolikowska., 1972)
99	Chrysoeriole	*S. lineare* (Li and Zuo., 1991)
100	Chrysoeriole-7- O- β-D-glucoside	*S. alfredi (S. formosanum)*
101	Chrysoeriole-7- O- β-L-rhamnoside	*S. alfredi (S. formosanum)*
105	(+)-Taxifoline	*S. reflexum* (Wolbi., 1993)
106	(+)-dihydromyricetine	*S. reflexum (*Wolbi., 1993)
110	(-)-Epicatechin-3-O-gallate	*S. sediforme* (Sakar Et al., 1993) *S. Stoloniferum* (Petereit et al., 1998)
111	(-)-Epigallocatechin-3-O-gallate (Prodelphinidine)	*S. sediforme* (Sakar Et al., 1993) *S. Stoloniferum* (Petereit et al., 1998) *S. litoreum (*Sakar et al,. 1997)
112	(-)-Epigallocatechin-3,3'-di-O-gallate	*S. Stoloniferum* (Petereit et al., 1998)
113	(-)-Epigallocatechin-3,4'-di-O-gallate	*S. Stoloniferum* (Petereit et al., 1998)
114	Epigallocatechin-3-O-gallate-(4—8)-epigallocatechin-3-O-gallate	*S. Stoloniferum* (Petereit et al., 1998)
115	Epigallocatechine(4—8)-epicatechine-3-Ogallate	*S. litoreum (*Sakar et al,. 1997)
107	5-hydroxy-3',4'-dimethoxy-isoflavone-7-Oneohesperidoside	*S. alfredi (S. formosanum)* (Chou et al.,1986)
108	5-hydroxy-3',4'-dimethoxy-isoflavone-7-O-β-Dglucoside	*S. alfredi (S. formosanum)* (Chou et al.,1986)
109	3'-methoxyorobol-7-β-D-glucoside	*S. lineare* (Li and Zuo., 1991)
117	Pratenseine	*S. lineare* (Li and Zuo., 1991)
118	Liquiritigenine	*S. sarmentosum* (He et al., 1997)
120	Isoliquiritigenine	*S. sarmentosum* (He et al., 1997)
119	Liquiritine	*S. sarmentosum* (He et al., 1997)
121	Isoliquiritine	*S. sarmentosum* (He et al., 1997)
123	Peonidine-3-O-β-D-glucoside	*s. aizoon* (Korul'kin,. 2001) *S. hybridum* (Korul'kin,. 2001) *S. purpureum* (Korul'kin,. 2001) *S. telephium* (Korul'kin., 2001)
122	Pelargonidine-3-O-β-glucoside	*S. ewersii* (Korul'kin,. 2001) *S. hybridum* (Korul'kin,. 2001) *S. purpureum* (Korul'kin,. 2001)
66	Herbacetine-8-methoxy-3,7-di- O-β-D-glucoside	*S. sarmentosum* (Morikawaet al., 2007)
114	2R,3R)-7,4'-dihydroxy-5,3',5'-trimethoxy flavan-3-O-gallate	*S. sediforme* (Sakar. 1993)

Terpenoid derivatives		
124	α-amyrine	*S. morganianum* (Wollenweber et al., 1999)
125	β-amyrine	*S. morganianum* (Wollenweber et al., 1999)
126	β-amyrin acétate	*S. morganianum* (Wollenweber et al., 1999)
127	Oleanolic acid	*S. aizoon* (Krasnov and Petrova.,1970)
136	β-sitosterol	*S. aizoon* (Krasnov and Petrova.,1970) *S. aixoon* (Wei-lin et al., 2008) *S. sarmentosum* (He et al., 1997) *S. lineare (*Sakar., et al 1997 *S. hybridum (*De Melo et al., 2005)
128	Sarmentolin (18β-hydroxyperoxyolean-12-en-3-one)	*S. sarmentosum* (Wollenweber et al., 1999)
129	δ-amyrone	*S. sarmentosum* (He et al., 1998)
130	3-epi-δ-amyrine	*S. sarmentosum* (He et al., 1998)
131	δ-amyrine	*S. sarmentosum* (He et al., 1998)
132	Taraxerylacetate	*S. forsterianum (*Stevens et al., 1994 b) *S. amplexicaule (*Stevens et al., 1994 b) *S. montanum (*Stevens et al., 1994 b) *S. erectum (*Stevens et al., 1994 b) *S. orientale (*Stevens et al., 1994 b) *S. sediforme (*Stevens et al., 1994 b)
137	Daucosterol	*S. sarmentosum* (He et al., 1997 b)
139	Sarmentosterol	*S. sarmentosum* (He et al., 1997)
	3,6-stigmas-4-en-3,6-diol	*S. sarmentosum* (He et al., 1997)
138	Stigmasterol	*S. formosanum (*Stevens et al., 1994)
140	Fern-8-en-3-ylformate	*S. forsterianum (*Stevens et al., 1994 b) *S. amplexicaule (*Stevens et al., 1994 b)
141	Fer-8-en-3-ylacetate	*S. forsterianum (*Stevens et al., 1994 b)
133	Taraxerylformate	*S. forsterianum (*Stevens et al., 1994 b)
134	Taraxerone	*S. montanum (*Stevens et al., 1994 b) *S. erectum (*Stevens et al., 1994 b) *S. orientale (*Stevens et al., 1994 b) *S. ochroleucum (*Stevens et al., 1994 b) *S. pruinatum (*Stevens et al., 1994 b)
135	Germanicylformate	*S. pruinatum (*Stevens et al., 1994 b) *S. montanum (*Stevens et al., 1994 b) *S. erectum (*Stevens et al., 1994 b) *S. sediforme (*Stevens et al., 1994 b)
Coumarins		
143	Coumarin	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987) *S. album* (Wolbis., 1987) *S. maximum* (Wolbis., 1987) *S. hybridum* (Korul'kin., 2001) *S. purpureum* (Korul'kin., 2001) *S. telephium* (Korul'kin., 2001)
145	Esculetin	*S. reflexum* (Wolbis., 1989) *S. acre* (Wolbis., 1987) *S. album* (Wolbis., 1987) *S. maximum* (Wolbis., 1987) *S. selskianum* (Shnyakina and Zapesochnaya.,1975) *S. kautshaticum* (Shnyakina and Zapesochnaya., 1975) *S. caucasium* (Zaitsev et al., 1983)

		S. populifolium (Demidenko et al., 1978) *S. hybridum* (Korul'kin., 2001)
144	4,5-dihydroxycoumarin	*S. ewersii* (Korul'kin., 2001) *S. hybridum* (Korul'kin., 2001) *S. kamtchaticum* (Korul'kin., 2001)
146	Umbelliferon	*S. reflexum* (Wolbis., 1989) *S. ewersii* (Krasnov et al.,1976) *S. populifolium* (Demidenko et al., 1978)
148	Scopoletin	*S. album*(Wolbis., 1987)
147	7-dihydroxycoumarin	*S. ewersii* (Korul'kin., 2001) *S. hybridum* (Korul'kin., 2001) *S. purpureum* (Korul'kin., 2001) *S. telephium* (Korul'kin., 2001) *S. acre* (Wolbis., 1987) *S. aizoon* (Korul'kin., 2001)
149	6,7-dioxycoumarin	*S. ewersii* (Krasnov et al.,1976)
Alkaloids		
150	Sedacrin	*S. lydium* (Diak and Kohlmunzer., 1981) *S. acre* (He et all., 1997)
151	Sedinon	*S. acre* (He et all., 1997) *S. lydium* (Diak and Kohlmunzer., 1981) *S. oryzifolium* (Kim et al., 1996)
152	Sederin	*S. acre* (He et all., 1997)
153	Sedamin	*S. acre* (Gulubov and Bozhkova II., 1972) *S. lydium* (Diak and Kohlmunzer., 1981) *S. maximum* (Logar et al.,1974) *S. aizoon* (Krasnov et al.,1976) *S. purpureum* (Krasnov et al.,1976) *S. hybridum* (Krasnov et al.,1976) *S. oryzifolium* (Kim et al., 1996)
154	Norsedamin	*S. acre* (Hootelé et al., 1985)
155	(+)-4-hydroxysedamin	*S. acre* (He et all., 1997)
156	Allosedamin	*S. acre* (Ibebeke-Bomangwa and Hootelé., 1987) *S. oryzifolium* (Kim et al., 1996)
157	Norallosedamin	*S. acre* (Hootelé et al., 1985) *S. oryzifolium* (Kim et al., 1996)
158	(-)-3-hydroxyallosedamin	*S. acre* (Ibebeke-Bomangwa and Hootelé., 1987)
159	(-)-3-hydroxynorallosedamin	*S. acre* (Ibebeke-Bomangwa and Hootelé., 1987)
160	4-hydroxyallosedamin	*S. acre* (He et all., 1997)
161	(-)-5-hydroxysedamin	*S. acre* (Hootelé et al., 1985)
162	Sedridin	*S. acre* (Gulubov and Bozhkova II., 1972) *S. maximum* (Logar et al.,1974) *S. oryzifolium* (Kim et al., 1996) *S. japonicum* (Kim et al., 1996) *S. polytrichoides* (Kim et al., 1996)
165	Sedinin	*S. acre* (Krasnov et al.,1976) *S. aizoon* (Krasnov et al.,1976) *S. purpureum* (Krasnov et al.,1976) *S. hybridum* (Krasnov et al.,1976)) *S. maximum* (Logar et al.,1974)
166	8-episedinin	*S. acre* (He et all., 1997)
167	Diacetyledinin	*S. acre* (Colauand and Hootelé.,1984)
168	Dihydrosedinin	*S. acre* (He et all., 1997)
169	Sedien	*S. acre* (Maksimovic et al., 1990)

170	Sedienedion	*S. acre* (Maksimovic et al., 1990)
171	Sedacryptin	*S. acre* (He et all., 1997)
172	2-episedacrin	*S. acre* (He et all., 1997)
173	2-episedinon	*S. acre* (Colauand and Hootelé.,1984)
174	Lelobanidin	*S. acre* (Francis et al., 1977)
175	Lobelanidin	*S. acre* (Francis et al., 1977)
176	Lobelanidine glucoside	*S. acre* (Piccinni-Leopardi et al., 1987)
177	8-propyl-10-phenyl-lobeliolon	*S. acre* (Francis et al., 1977)
185	Nicotin	*S. acre* (Gulubov et al., 1972) *S. album* (Gill et al., 1979) *S. carpaticum* (Gill et al., 1979) *S. telephium* (Gill et al., 1979) *S. oppositifolium* (Gill et al., 1979) *S. pallidum* (Gill et al., 1979) *S. populifolium* (Gill et al., 1979)
187	N-methylanabasin	*S. acre* (Van der Wal et al., 1981).
189	Hydroxysedinol	*S. acre* (Francis et al., 1977)
190	Hydroxylelobanidine	*S. acre* (Francis et al., 1977)
191	Hydroxysedinone	*S. acre* (He et all., 1997)
178	Pelletierin	*S. sarmentosum* (Kim et al., 1996) *S. oryzifolium* (Kim et al., 1996) *S. japonicum* (Kim et al., 1996) *S. lepidopodium* (Kim et al., 1996) *S. morrisonensis* (Kim et al., 1996) *S. polytrichoides* (Kim et al., 1996) *S. bulbiferum* (Kim et al., 1996)
179	N-methylpelletierine	*S. lepidopodium* (Kim et al., 1996) *S. morrisonensis* (Kim et al., 1996) *S. polytrichoides* (Kim et al., 1996) *S. sarmentosum* (Beyerman et al., 1972) *S. oryzifolium* (Kim et al., 1996)
	Pyrrolallosedamine	*S. oryzifolium* (Kim et al., 1996)
	Pyrrolsedamine	*S. oryzifolium* (Kim et al., 1996) *S. polytrichoides* (Kim et al., 1996)
	Hygroline	*S. oryzifolium* (Kim et al., 1996)
	Methylisopelletierine	*S. aizoon* (Krasnov et al.,1976) *S. purpureum* (Krasnov et al.,1976) *S. hybridum* (Krasnov et al.,1976)
	1-phenyl-2(2-N-methylpyrrolidyl)ethanol	*S. oryzifolium* (Kim et al., 1996)
	N-methylallosedridine	*S. sarmentosum* (Beyerman et al., 1972) *S. polytrichoides* (Kim et al., 1996)
Megastigmans		
192	Sarmentol A	*S. sarmentosum* (Yoshikawa et al. 2007)
193	Sedumoside A1	
194	Sedumoside A2	
195	Sedumoside A3	
196	Sedumoside A4	*S. sarmentosum* (Ninomiya et al., 2007)
197	Sedumoside A5	
198	Sedumoside A6	
199	Sedumoside E1	*S. sarmentosum* (Shnyakina and Zapesochnaya., 1975)
200	Sedumoside E2	
201	Sedumoside E3	
206	Sedumoside B	

208	Sedumoside C	*S. sarmentosum* (Yoshikawa et al. 2007)
210	Sedumoside D	
212	Sedumoside G	*S. sarmentosum* (Shnyakina and Zapesochnaya., 1975)
214	Sedumoside H	*S. sarmentosum* (Yoshikawa et al. 2007)
216	Sedumoside F1	*S. sarmentosum* (Shnyakina and Zapesochnaya., 1975)
217	Sedumoside F2	
219	Sedumoside I	*S. sarmentosum* (Ninomiya et al., 2007)
207	Sarmentol B	
209	Sarmentol C	
211	Sarmentol D	
213	Sarmentol G	*S. sarmentosum* (Shnyakina and Zapesochnaya., 1975)
215	Sarmentol H	*S. sarmentosum* (Ninomiya et al., 2007)
218	Sarmentol F	*S. sarmentosum* (Shnyakina and Zapesochnaya., 1975)
220	Sarmentol I	*S. sarmentosum* (Yoshikawa et al. 2007)
221	Sarmentoic acid	
202	Myrsinionoside D	*S. sarmentosum* (Ninomiya et al., 2007)
203	Alangionoside J	
204	Platanionoside D	
225	3-Hydroxy-5,6-epoxy-β-ionol-9-O-β-D-glucoside	
222	Myrsinionoside A	
223	Alangionoside A	
224	Staphylionoside D	
205	(3S,5R,6S,9R)-Megastigman-3,9-diol	*S. sarmentosum* (Shnyakina and Zapesochnaya., 1975)
Other compounds		
226	Sarmentosine	*S. sarmentosum* (Li et al., 1981) *S. stenopetalum* (Nishidaet al., 1995) *S. augustifolium* (Li. Et al., 1981)
229	Hexacosanol	*S. formosanum* (Wang et al., 1976)
230	Octacosanol	*S. formosanum* (Wang et al., 1976)
231	Triacontanol	*S. formosanum* (Wang et al., 1976)
233	Hexacosanylstearate	*S. formosanum* (Wang et al., 1976)
234	Octacosanylstearate	*S. formosanum* (Wang et al., 1976)
228	Mannitol	*S. formosanum* (Wang et al., 1976)
227	Sarmentosine epoxide (4-β-D-glacopyranosyloxy-2,3-epoxy-2-hydroxymethylbutyronitrile)	*S. cepaea* (Nahrstedt et al., 1982)
232	Tritriacontane	*S. lineare* (Sakar et al., 1997)
235	Triacontanylstearate	*S. formosanum* (Wang et al., 1976)

1.2.4. *Sedum careuleum* L.: a potential source of therapeutic agents

Sedum caeruleum Vahl has synonymys names: *Oreosedum caeruleum* (L.) , *Sedum azureum Desf* and *Sedum heptapetalum Poir* . An annual plant 5-15 cm., Glabrescent, with slender root; stem upright or ascending, often branching from the base; leaves sparse, oblong in club, very obtuse, glabrous; blue flowers, with pedicels longer than the flower, forming a broad panicle, very ramose, a little pubescent-glandular; 6-7 sepals; 6-7 petals, lanceolate, 2-3 times longer than the calyx; 10-15 stamens; carpels erect, oval, terminated by style as long as they are. Flowering April-June. Rocks of Corsica, where it is quite abundant: Sardinia, Italy, Sicily; Malta; Tunisia and Algeria. (Quzel., 1962)

Figure16. Photos and classification of *Sedum carealeum Vahl*

sedum caereleum mainly known in horticulture to cover soil. The only phytochemical and boilogcal studies on the plant has been done by C. Bensouici (2015) who described the presence of terpenes (ursolic acid, daucosterol, -sitosterol-3-O-b-D-galactopyranoside) , flavonoids (apigenin, apigetrin, apiin) and antioxidant, anticholinesterase, antibacterial activities .

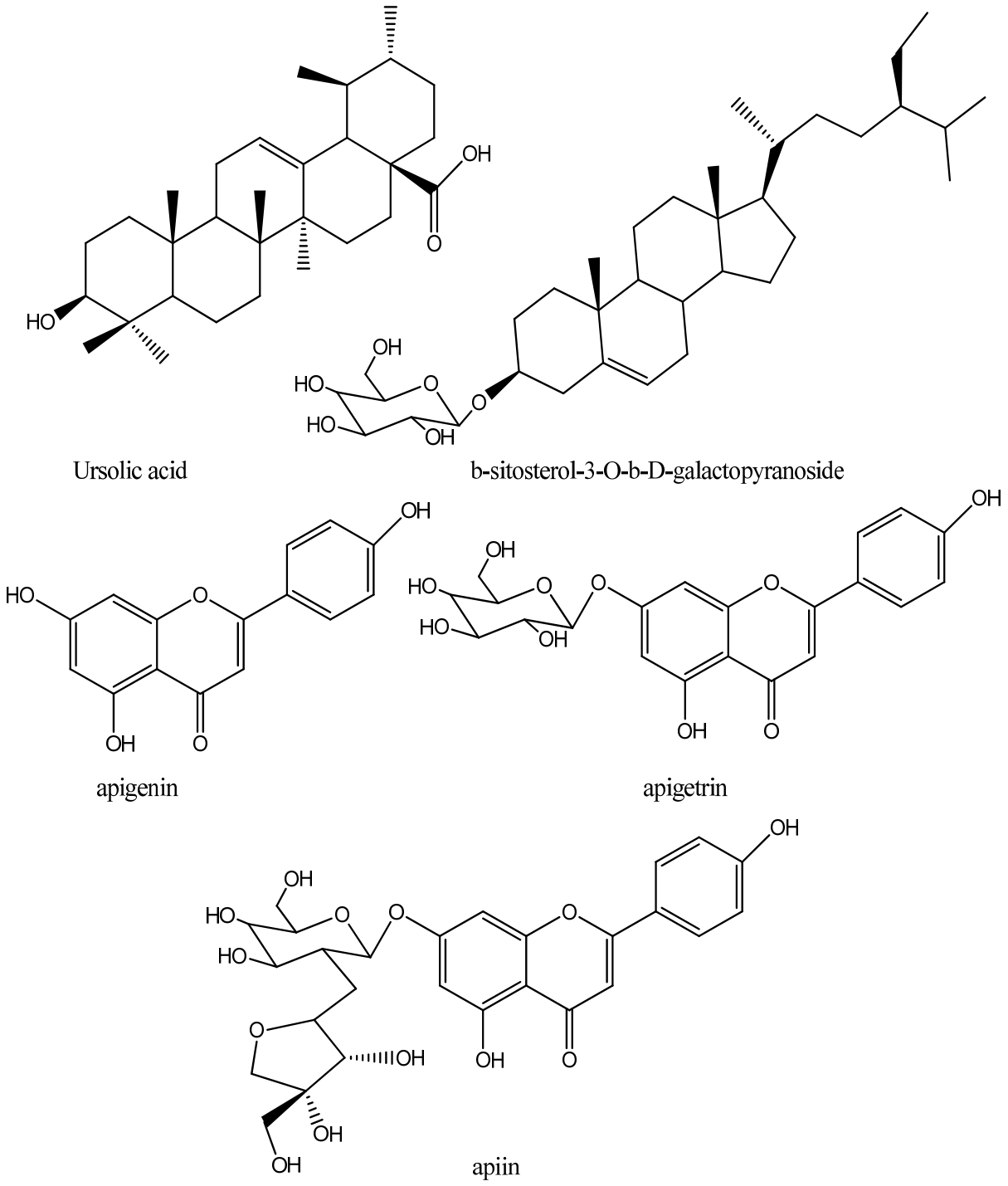


Figure 17. Secondary metabolites isolated from *S. carealeum Vahl*

Chapter II

Analytic Methods

Once the compounds are isolated and need to be identified in order to give them a biological meaning. The identification of discriminant signals is probably the most complex and time-consuming task metabolomic analyzes. It most often requires the use of several analytical techniques combination, such as NMR, MS, UV, IR, LC-MS and GC-MS.(Bruno et al., 2018) At present the commun methods used of all these methods, the most used at present are NMR, GC-MS and LC-MS. **(Dias et al., 2016)** As it is known the components differ in size, nature of their functional groupings, volatility and their polarity, so until now, there is no universal analytical method interested in all these molecules. Each analytical method has its advantages and its limitations. The choice of the analytical method used for acquisition of fingerprints metabolism therefore depends on the expected results and possible metabolic pathways of interest, and is a compromise between speed, selectivity and sensitivity. (**Bradshaw., 2006)**

2.1.Mass spectrometry(MS)

Mass spectrometry is an analytical technique for detecting and identifying molecules (solid, liquid or gaseous) through measuring their mass. In addition, it makes it possible to characterize the chemical structure of the molecules by fragmenting them and to carry out quantitative analyzes. It plays today an important role in the studies of environmental pollution, organic and pharmaceutical chemistry - biochemistry (peptides, proteins, ...) - medical: analysis, detection - geology, archeology and doping due to its sensitivity, selectivity and ability to make rapid quantitative analyzes (Hofmann et al., 1999) ; The molecules are often separated by chromatography before introducing into the ion source. The analytical principle of all mass spectrometric techniques includes three steps: ionization of an analytical molecule, separation in an electromagnetic field and detection in an ion detector (figure 2.1)

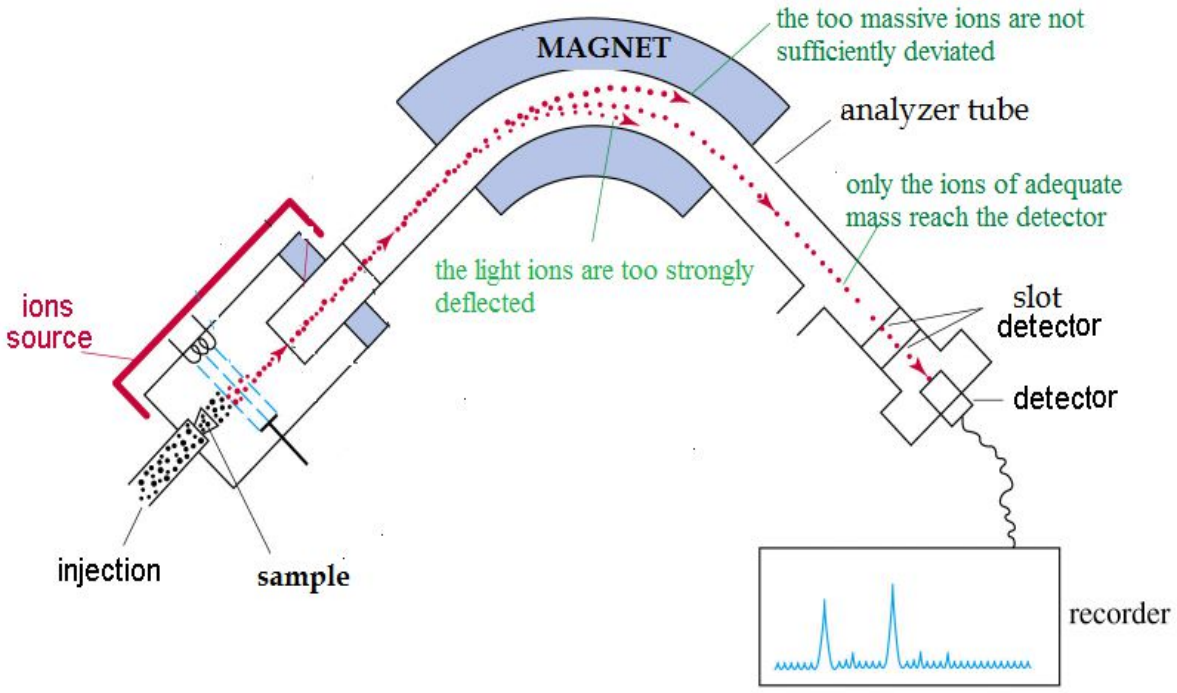


Figure 2.1. Composition of mass spectrometer

So a mass spectrometer consists five main units (Fig.1):

1. sample introduction system,
2. Ions source or ionization chamber. Many ionization methods exists, the most widespread one is the electronic impact.
3. Analyzer for the separation of ions according to their mass and charg (m/z).
4. Detector that detects the ions coming out of the analyzer.
5. Recorder which is a computer set of data processing transforms information received by the detector to a mass spectrum.

New strategies have been developed for studies of many natural products and molecules, as well as spectrometers equipped with different sources and analyzers possessing their own performance for specific applications.

2.1.2 Main sources of ionization

Different sources of ionization existing and giving access to various types; ionization of soft ionization allow to the conservation of molecular precursor and hard ionization gives access to fragments of molecular precursor. The choice of the source of ionization important according to the application desired and supported on the different criteria;

2.1.2.1 Matrix assisted laser desorption / ionization- mass spectrometry(MALDI)

Introduced in 1985 by (Karas et al)have been followed by several applications to biological compounds , after thirty years following the introducing it was developed to analyze the molecules of high molecular mass (Karas et al., 1985). It consists in mixing of a sample to be co-crystallized on a saturated matrix(solution of small organic molecules) on a target (generally a conductive and chemically inert metal support). Than the irradiation of this mixture (sample- matrix) , allows to generate ions in the gas phase and these guide ions to analyzer by an electric field. More, matrix capable of absorbing at the same wavelength as the laser, will cause the accumulation of a large amount of energy in the condensed phase by electronic excitation of the molecules of the matrix (Même., 2010, Hillenkamp and Katalinić., 2007) Figure 2.2.

The choice of the matrix is important because it absorbs the energy of the laser beam in order to protect sample and also helps the desorption to the transfer of the gas phase and ions of sample, the Most used are the derivatives of cinnamic and benzoic acids. (Dreisewerd., 2003).

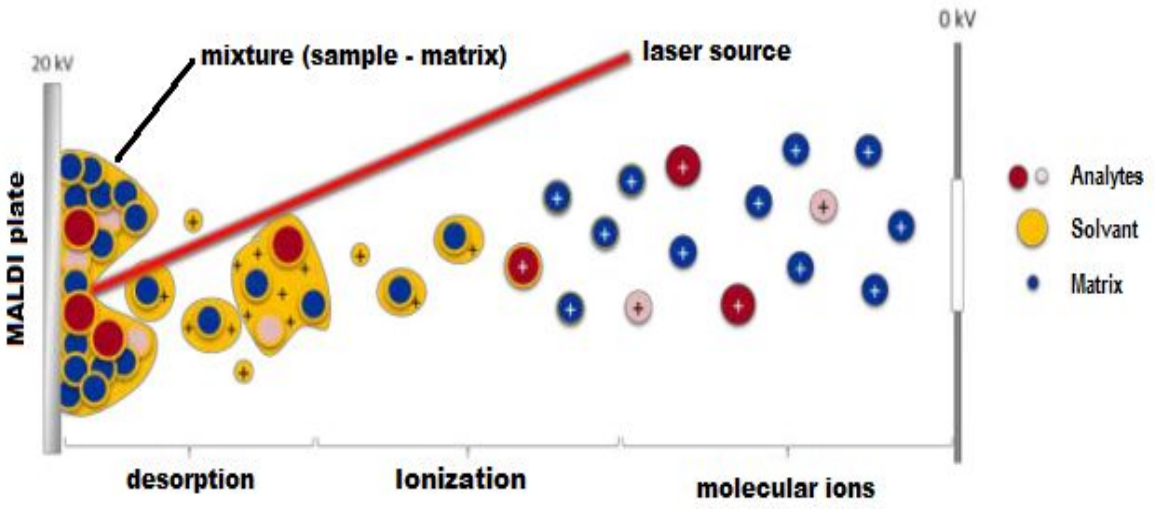


Figure. 2.2 schematic principle of desorption / ionization of molecules by MALDI source (Même., 2010)

2.1.1.3 Electrospray sources (ESI: Electrospray Ionisation)

This technique, developed by J. B. Fenn in the mid-1980s, is used in mass spectrometry to produce ions from compounds in solution. It is a good coupled to liquid separations, its soft ionization mode allows to the conservation of molecules without fragmentation and its

capacity to generate multicherge ions makes it possible to analyze compounds of high molecular weight, thus this type of ionization is adapted to the study of a large variety of compounds in which polar and nonvolatile compounds. (Banerjee and Mazumdar., 2012), Figure 2.3

the ionization process by ESI ms takes place at atmospheric pressure and comprises several steps;

- ✓ the formation of the charged droplets.
- ✓ the Coulomb fusion of droplets loaded in fine droplets.(lead to formation of a daughter droplet containing only ion)
- ✓ the emission of ions in the gas phase.

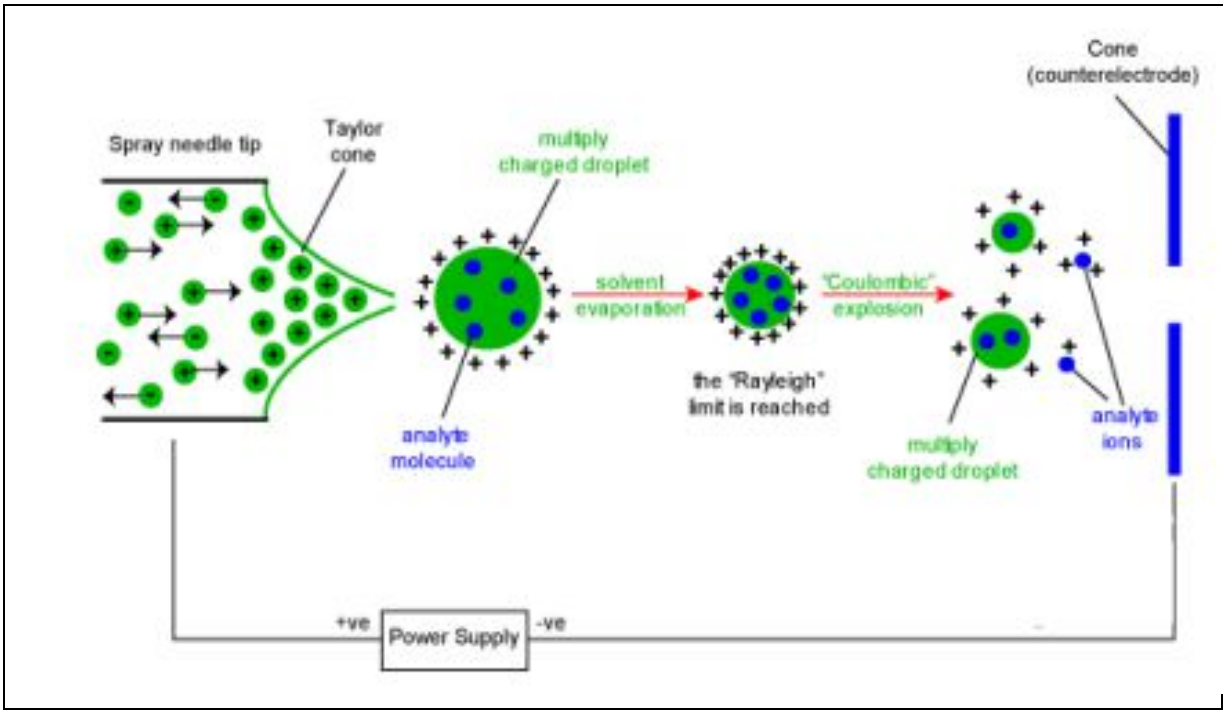


Figure 2.3 Schema of the mechanisms of ion formation

2.1.3 Analyzers

Works for separation and determination of m/z ratio. As there are wide variety of sources, there are many analyzers that are classified in two categories:

2.1.3.1 Ion trap analyzers

can be used to produce fragmentation sequential (MS). Indeed, by correctly selecting the applied voltage values of the axial modulation but also the frequencies applied on the caps and the amplitude of the alternating voltage, it is possible to keep in the trap only those ions having a given m / z ratio

2.1.3.2 Ion beam analyzers

Like quadripol(Q) and time of flight (TOF)

2.1.4 Detectors

used to measure the number of electrons and amplify the signal for achieve good sensitivity. The most used is an electron multiplier. Then the signal is recorded and a mass spectrum is produced. The most used :

- ✓ Faraday Cup (Direct Current Detection)
- ✓ Postacceleration (Daly Knob) Detector
- ✓ Electron Multiplier Notes
- ✓ Photomultiplier Detector Notes
- ✓ Continuous Dynode Electron multiplier
- ✓ Microchannel Plate Detector
- ✓ Array Detector
- ✓ Multichannel Plate (MCP) Detector
- ✓ Conversion Dynode (Klimeš., 2009)

2.2. Gas chromatography coupled with mass spectrometer (GC-MS)

Is a coupling technique between a gas chromatography and a mass spectrometer, was pioneered in the 1950's by Fred W. McLafferty and Roland S. Gohlke (Gohlke and McLafferty., 1993) for the purpose of separating and identifying the composition of complex organic mixtures provided that they are capable of volatilization at tolerated temperatures by this technique and until now it still called selective mass detector,.The identification of compounds is based on the fragmentation of organic molecules by transmitted to the analytes with electronic ionization under defined operating conditions. The mass spectrum produced as a function of the mass / charge ratio of the compounds is either manually interpreted or compared to the data bases **(NIST, ... ect)** which contain more than 100,000 compounds.

Main applications:

This GC / MS technique makes it possible to precisely identify and / or quantify numerous substances present in very small quantities, even in traces. GC-MS applications include:

- The identification and quantification of essential oils and plant extracts
- the dosage of drugs or narcotics,
- the environmental analysis

- Forensic medicine and the identification of all unknown substances even in the form of traces.

However, GC-MS analysis requires thermostable and volatility of analytes. Thus, it is often necessary to perform a chemical modification step (derivation) in order to make polar and little volatile compounds to volatile ones. This sample treatment is enough binding and may lead to degradation reactions, while at the same time the quantitative aspect, the equilibrium of the reaction being able to be modified in certain samples. Else On the other hand, GC – MS (figure 2.4) coupling is often based on the use of electronic impact sources rarely allow to obtain the mass of the intact compound but only to observe the ions of its fragmentation. The absence of the molecular ion makes it difficult to identify compounds unknown or not described in the databases. All these reasons led to development of separation techniques in the liquid phase for metabolomic approaches. (Stančin., 2018)

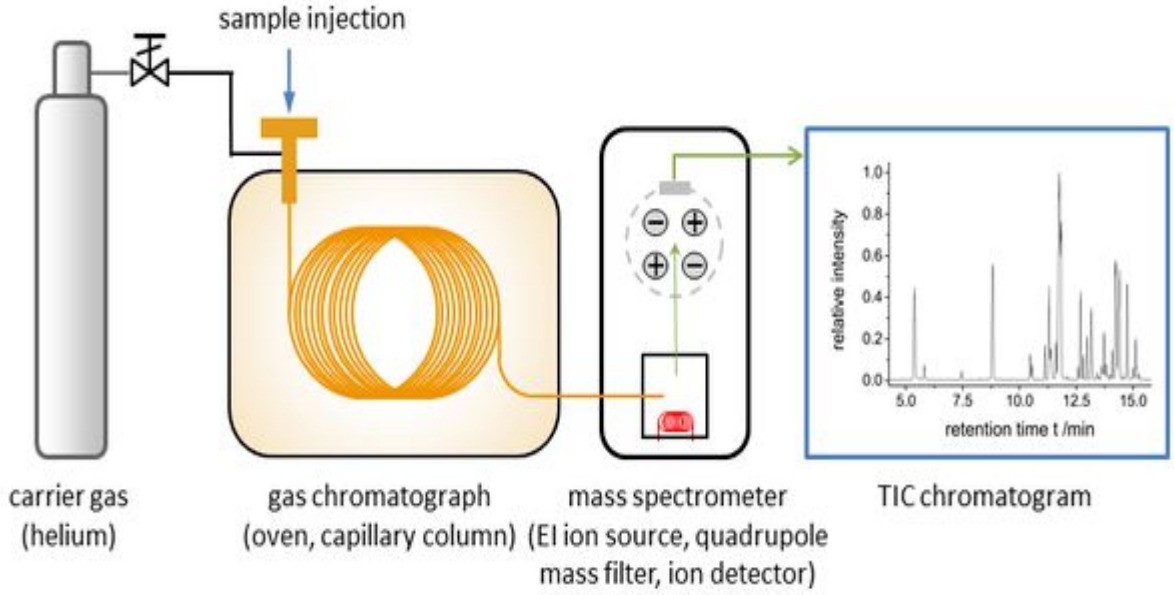


Figure 2.4 Gas chromatography coupled with mass spectrometer (GC-MS) **(http://www.skz.de)**

2.3. Liquid chromatography (LC)

Liquid chromatography is based on the principle of separation of solvent-driven compounds (mobile phase) in a tube called chromatographic column contain stationary phase, three of columns exist; normal, filled column (contain porous "granules") and capillary column (covered inside a thin film) To separate a mixture injected at the entrance of the column where it is diluted in the mobile phase which leads to through the column. If the stationary

phase has been well chosen (reversed phase C18 is the most commonly used), the constituents of the mixture, called generally solutes, are unequally retained during the crossing of the column. From this phenomenon called retention it follows that the constituents of the injected mixture are all move less quickly than the mobile phase and their moving speeds are different. They are thus eluted from the column one after the other and therefore separated.(Saito et al., 2004; Harris., 2007).

2.3.1 Liquid chromatography coupled with mass spectrometer (LC-MS)

1958 is the beginning of modern liquid chromatography where the coupling of liquid chromatography to mass spectrometry (LC-MS) was appeared later than those with gas chromatography because of the technological obstacles related to their realization, MS) available as standard on commercial equipment as early as 1968 - see Chromatographic Couplings with Mass Spectrometry.(Pitt., 2009)

In LC / MS, the initial datas et consists of a set of chromatograms (1 for each sample). For each chromatogram, representing the intensity of the total ion current depending on the retention time, there is actually a third dimension that corresponds to the resolution in m / z of ionic species detected in each spectrum.

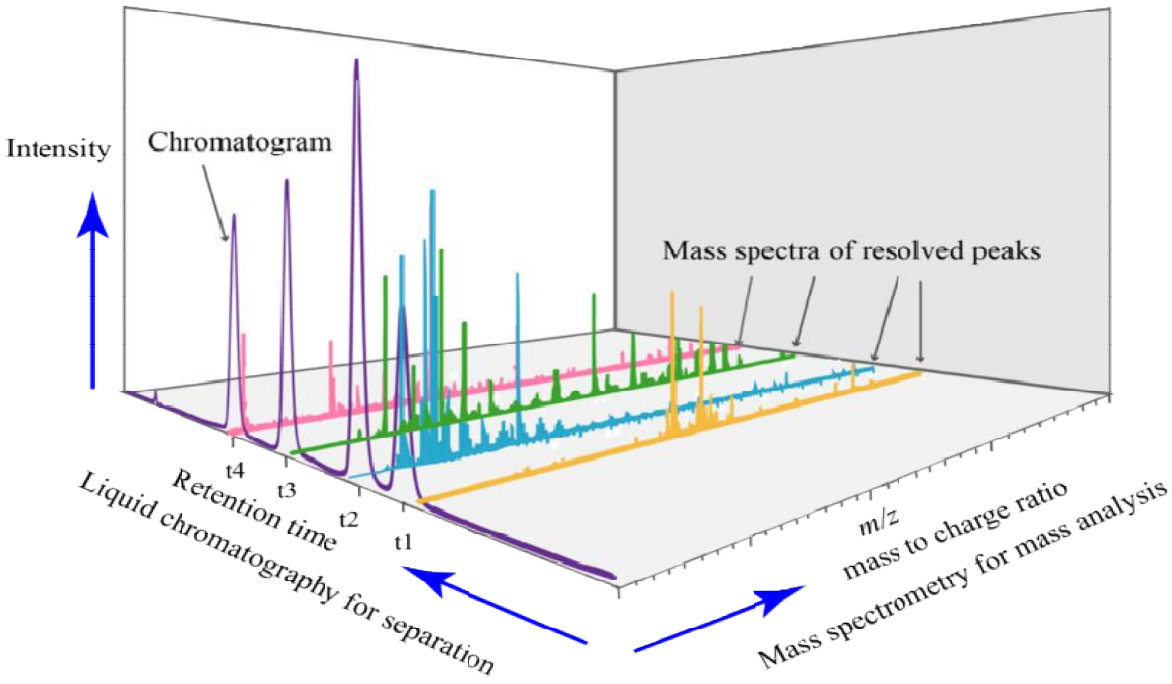


Figure 2.5 Schematic representation of chromatogram (3D)obtained by LC-MS
www.wikivisually.com

Compared to NMR, the data preprocessing step is made even more complex by the presence of a coupled separation technique, a large amount of backgroundnoise, artifacts and redundancy in the data. Thus, the separative technique introduces offsets temporal peaks corresponding to variations in retention time. In addition, background noise can be variable according to the different areas of the chromatogram and the shape of the peaks change by a peak to the other. The preprocessing of these data will consist in extracting the peaks (m / z t torque intensity) in each sample and then realign in the domains chromatographic and spectral. This preprocessing step is described in some publications

2.3.2 UHPLC-MS

Couplings between liquid chromatography and mass spectrometry (LC-MS) appeared later than those with gas chromatography because of the technological obstacles related to their realization, the main limitation being the need to evaporate the mobile chromatographic phase in order to pass the analytes in the gas phase before entering the mass spectrometer. These problems have been solved with the development of atmospheric pressure ionization methods such as electrospray **(**Bowers and Sanaullah., 1996)

Recently, metabolomic analyzes have been performed using Ultra-High Pressure Liquid Chromatography (UHPLC) systems (fig. 2.6) These systems represent an alternative to capillary columns with the use of columns with particle diameters of less than 2 μm (Plumb et al., 2004)

The basic principles of chromatography can be explained by the van Deemter curve describes the relationship between the linear velocity (flow) and the height equivalent to a theoretical plateau or HEPT, which is directly related to the efficiency of the column

Liquid chromatography coupled with mass spectrometry is a method of analysis that combines the performance of liquid chromatography and mass spectrometry to precisely identify and / or quantify many substances.((Van Deemter et al., 1956)

LC-MS uses an UPLC system, but as the mobile phases of the liquid leave the column, the sample is vaporized as microdroplets. These evaporate rapidly and release ionized molecules of the analyte which are then separated in mass spectrometry Interest of LC- MS coupling

- Separation of a mixture to obtain an identification of most constituents
- Have the highest sensitivity possible
- To be universal, that is to say to detect all the eluted substances
- Provide as much structural data as possible

- Being selective (identification of a targeted constituent)
- Allow quantitative analysis Allow quantitative analysis.

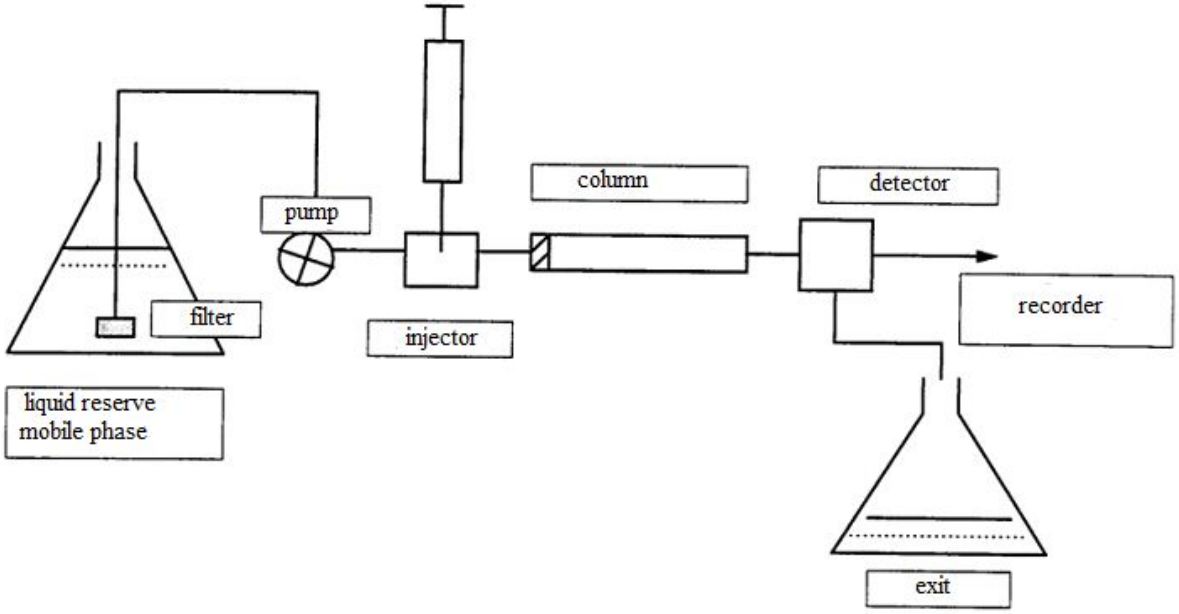


Figure 2.6. UHPLC ingredients

2.3.3 RP-HPLC

Conventional RP-HPLC is often insufficient to achieve medium separations biological complex. The use of capillary columns and monoliths to improve the resolution has been reported. Indeed, the inside diameter (between 200 and 320mm) and the size of filling particles of these columns decreasing, this leads to an improvement in the resolution chromatographic. The co-elutions of the metabolites are thus reduced and the suppression effects Ionization may be attenuated (Saito et al., 2004). The increase of the signal / noise ratio obtained also causes an increase in sensitivity. In this latest years , the use of a capillary column increases the number of detected peaks which allows to improve the separation between the different groups of samples studied, in other words, the improvement of the classification of samples in groups.

2.4. NMR

NMR is one of premier methods of analysis have been successfully applied to the study of the metabolome (Gowda et al., 2008). This method is based on the existence of transitions between the two states atomic nuclei with a non-zero magnetic moment (^{1}H $^{13}C^{15}N$ or ^{31}P) that exist when placed in an intense magnetic field (Meusinger and Chippendale., 2014) NMR presents a number of advantages: it is non-destructive, fast, simple to implement, robust, it generates spectra rich in direct structural information and

whose signals undergo little offset during the entire analysis.It based also on measuring the absorption of a radiofrequency by an atomic nucleus in a strong magnetic fieldThe atomic nucleus resonates at a specific frequency(Wolter et al., 2003). NMR provides numerous structural information relating to non-covalent edifices, however, NMR spectra are becoming increasingly difficult to interpret as the complexity of this type of edifices grows. in addition, this technique does not include the analysis of buildings containing paramagnetic ions, such as copper II or cobalt II or analysis of edifices with a molecular weight greater than 40 kDa(Vila et al., 1997). If the universal presence of hydrogen in biomolecules makes proton NMR (^{1}H NMR) a method of choice, most metabolites each having a large number of signals, signal assignment may be delicate. Indeed, the NMR spectra obtained during the analysis of biofluids contain the resonances of hundreds of metabolites, a number of which overlap forming multiplets indistinguishable. A second dimension of NMR (^{13}C for example) or methods chromatographic data can be coupled to the NMR ^{1}H. Using a second dimension was considered by Dumas et al. (Dumas et al., 2002)A method using NMR^{1}H-^{13}C HMBC (Heteronuclear Multiple Bonding Connectivity) was thus considered. 2D NMR generates for one even composed several signals that are highly correlated. A model using the formation of dendrograms has been put in place. It opposes two types of correlations. Strong correlations Positive correspond to the structural relations between the signals, they are therefore characteristic signals belonging to the same compound. Positive or negative correlations less may explain a physiological link between these weakly correlated signals (Dumas et al., 2002; Cloarec et al., 2005). Finally the characterization of unknown molecules is delicate with this technique alone since it does not gives no information on the molecular mass of the compounds. Recent advances technology has improved its sensitivity through the development of angle rotation Magic and cryoprobes(Spraul et al., 2003; Wang et al. 2003; Keun et al., 2002)

Chapter III

Experimental procedures

3.1. Material and methods

3.1.1 Separation purification, analysis and identification technique

A/ In this study GCMS analyses were performed using a Shimadzu Gas Chromatograph QP2010 Ultra equipped with Autosampler AOC-20i, Ion source: electronic impact High-performance Quadrupole Mass Filter. Separation of compounds was carried out in a DB-5 J&W capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness) using helium as the carrier gas (35 $cm.s^{-1}$). The chromatographic conditions were as follows: start time at 6.5 min; initial temperature, 90 °C for 4 min; temperature rate, 16 °C min^{-1} up to 180 °C, followed by temperature rate, 6 °C min^{-1} up to 250 °C; followed by temperature rate, 3 °C min^{-1} up to 300 °C which was maintained for 5 min.; injector temperature, 320 °C; transfer-line temperature, 300 °C; split ratio, 1:50. The mass spectrometer was operated in the electron impact (EI) mode with energy of 70 eV, and data were collected at a rate of 1 $scan.s^{-1}$ over a range of m/z 33-750. The ion source was kept at 250 °C.

B/ UHPLC-MS was performed using a Thermo Scientific Ultimate 3000RSLC (Dionex) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer. The column used was a Thermo Scientific hypersil gold column (Part nº 25002-102130; Dim 1000 mm x 2.1 mm; Lot 14913; SN 10518298) with a part size of 1.9 μm and its temperature was maintained at 30 °C. The mobile phase was composed of (B) acetonitrile and (A) 0.1% formic acid in water (v/v), both degassed and filtered before use. The flow rate was 0.2 mL / min. The elution gradient was 5% (solvent A) for 14 min, 40% (solvent A) over 2 min, 100% (solvent A) over 7 min and the re-equilibration of the column with 5% of solvent A for 10 min. The injection volume was 2 μL. UV-vis spectral data were gathered in a range of 250 to 500 nm and the chromatographic profiles were documented at 280 nm. The mass spectrometer used was an LTQ XL linear ion trap 2D equipped with an orthogonal electrospray ion source (ESI). The equipment was operated in negative-ion mode with electrospray ionization source of 5.00 kV and ESI capillarity temperature of 275 °C. The full scan covered a mass range of 50 to 2000 *m/z*. Collision-induced dissociation MS/MS and MS^n experiments were simultaneously acquired for precursor ions.

C/ NMR spectra {^1H, ^{13}C, HSQC, HMBC [71 ms (7 Hz)], COSY} were measured in $CDCl_3$, on a Bruker Avance 300 (300.13 MHz for ^1H and 75.47 MHz for ^{13}C) or Bruker Avance 500 with crioprobe (500.13 MHz for ^1H and 125.76 MHz for ^{13}C) spectrometers and using TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (*J*) in Hz.

D/ **CC** Column chromatography was performed with silica gel 60 (Merck Kieselgel, 70–230 mesh) and Sephadex LH-20

F/ Preparative thin layer chromatography (prep. TLC) was performed on glass plates (20920 cm) precoated with Merck silica gel 60 GF254(0.5 mm thickness) and activated at 100–110 °C for 12 h.

3.1.2 Reagents and solvents used

- The reagents used in the extraction (Hexane and EtOH) were analytically pure. The solvents used in the fractionation and purification (DCM, EthAc , *n*-butanol, MeOH) were previously distilled with exception of $CHCl_3$.
- For the characterization of the compounds by NMR, deuterated chloroform and methanol (CDCl, CD_3OD).
- In the derivatization of the samples, for GC-MS analysis, pyridine, bis (trimethylsilyl) trifluoroacetamide (BSTFA) (99%) and trimethylsilyl chloride (TMSCI) (99%).
- The reegents; palmitic (99%), linoleic (99%) and gallic acids, 1-pentadecanol (99%), 1-eicosanol (98%) and cholesterol (99%), tetradecane (99%), hexadecane (99.5%), tetracosane (99%), octadecane (99%), citric (>99.5%), ursolic (98%), oleanolic (99%), linoleic (≥99%), palmitic (≥99%) acids, sorbitol (99%),D-mannitol (98%),D-(+)-galactose (>99%),D-(+)-mannose (>99%), D-(+)-talose (>99%), D-(+)-xylose (>99%), D-(-)-arabinose (>99%), D-(-)-ribose (>99.5%), D-fructose (99%), sucrose (>99%), maltose (>98%), cellobiose (>98%), b-sitosterol (98%), 5-cholesten-3b-ol (99%), stigmasterol (97%), campesterol (95%), lupeol (99%), glycerol (>99%), myo-inositol (99%), tetradecanol (98%) and 1-palmitoylglycerol (99%) were the pure compounds used as references in GCMS analyses.
- Tetracosan (99%) was used as the internal standard in GC-MS analyses.
- The phenolic standards benzoic acid, 4-hydroxy-3-methoxybenxoic acid, gallic acid, catechin, rosmarinic acid, isorhamnetin, kaempferol, luteolin and quercetin, 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxycinnamic acid (p-Coumaric acid) ferulic acid and chlorogenic acid were used as standars in LC-MS analyses .

3.2 Plant material

Fresh whole plants of *Scabiosa stellata* L. was collected in June 2015 from Algeria (Belezma National Park, Batna, **35° 35′ 41.52″ N, 5° 56′ 13.75″ E**) .A voucher specimen was identified by Dr. Bachir Oudjehih professor of Agronomic Institute, University of Batna under the reference number **VAREN/SS/2013/123.**

Fresh plant materiel of *Sedum caeruleum*. was collected on June 2014 from Algeria (Belezma National Park, Batna, **35° 35′ 41.52″ N, 5° 56′ 13.75″ E**) A voucher specimen was identified by Dr. Bachir Oudjehih professor of Agronomic Institute, University of Batna under the reference number **VAREN/SS/2013/124.**

Image 3.1 Locations of collected plants

3.3 Chemical studies

In the Natural products field scientists try to extract and characterize the secondary metabolites produced by plants, giving more attention to the ones used in traditional medicine. To carry out this thesis work were chosen two species *Scabiosa stellata* L. and *Sedum caeruleum* L., mainly due to its abundant distribution in Algeria and aiming to find economical value for both. Extraction is a crucial step not only because it is necessary to extract as much as possible chemical components to establish the lipophilic (GC-MS) and polyphenolic (UHPLC-MS) profiles but also without perform chemical transformations. In this regard the work was structured in several steps (Fig 3.1) which include the above mentioned profiles but also phytochemical purifications and if possible biological evaluations.

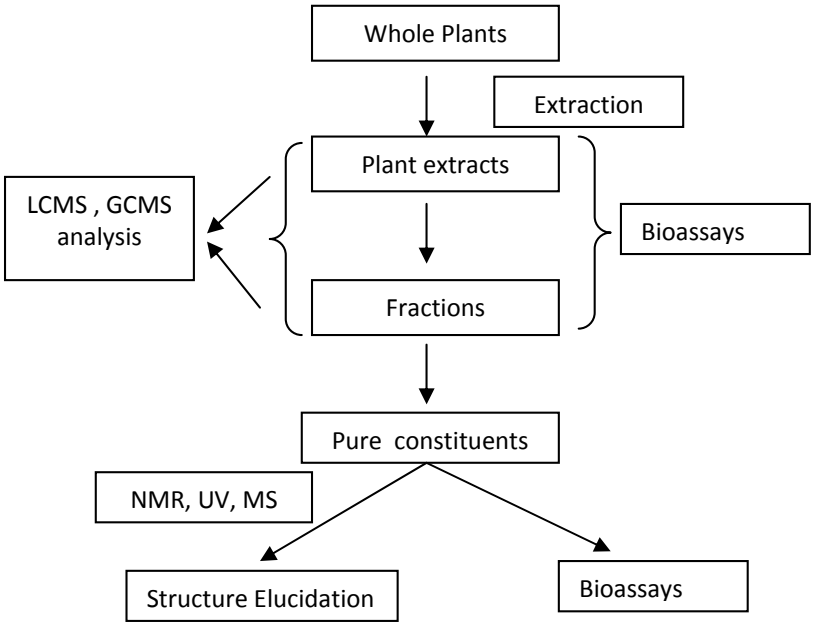


Figure 3.1 Scheme of the work carried out in this dissertation

3.3.1 Extraction and treatment of the plant material

Scabiosa stellata all plant was dried during 72 hours at room temperature and the dried material (500 g) were crushed and extracted; firstly with pro-analysis grade hexane at room temperature. The extract was filtred and concentrated in vacuo to obtain 4.95g of hexane extract. The mec was extracted with ethanol using sohxlet to obtain 88.60g of ethanol extract, which was a sequential dissolved using different solvents with increasing polarities. In this way three fractions were obtained; dichloromethane fraction (DCMF), ethyl acetate fraction (EAF) and *n*-butanol fraction (*n*-BF), respectively 10.90, 4.70 and 45.80 g (fig 3.2).

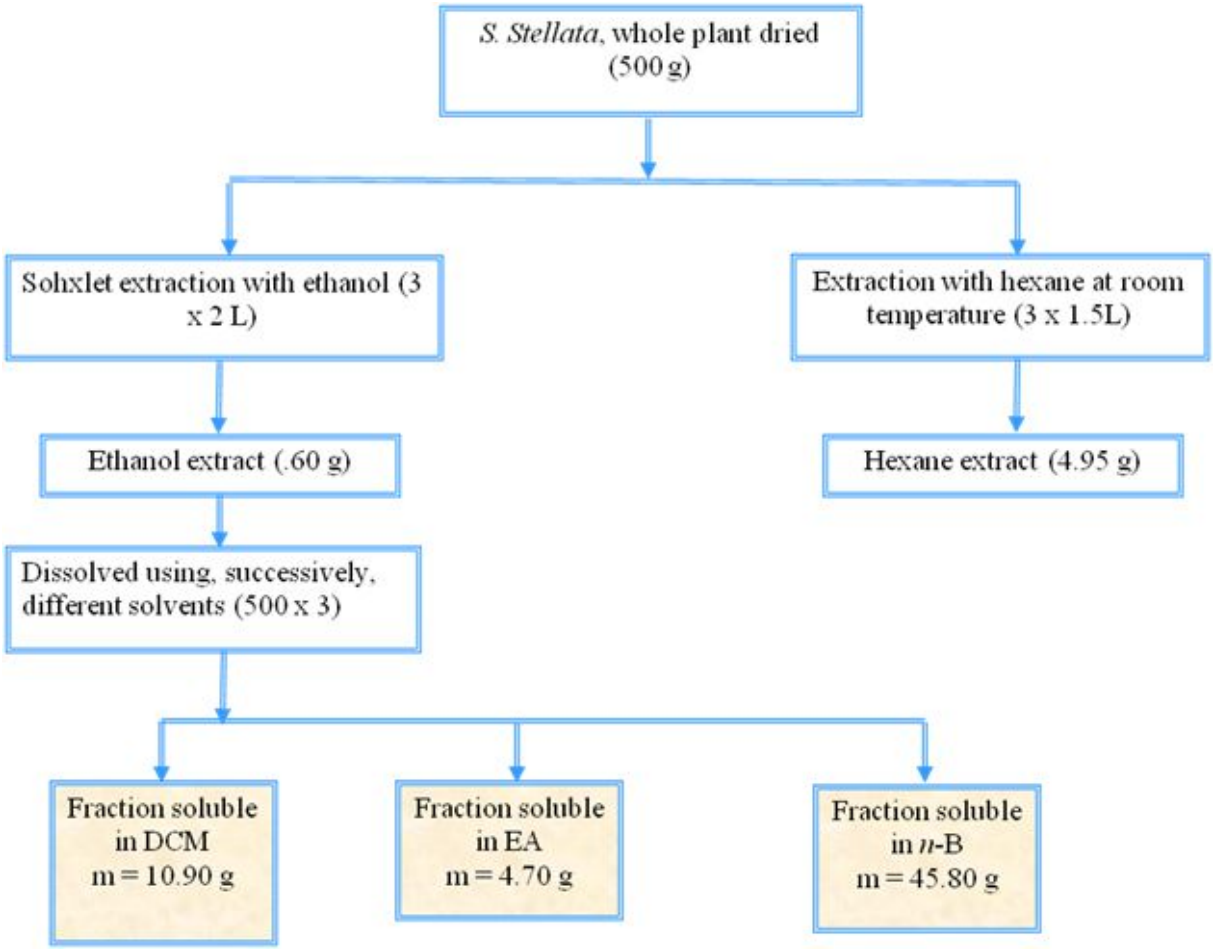


Figure 3.2 Extractive process of the dried plant material and treatment of the ethanolic crude extract (*S.stellata)*

485 g dried whole plant of *Sedum carealeum* was powdered and extracted in the same procedure extractive of *S.stellata* species (The extractive procedure of *Sedum carealeum* is schematized in the figure 3.3) which led to 12.05 g of hexane extract, 3.2 g of DCMF, 10g of EAF and 48.1 g of *n*-BF

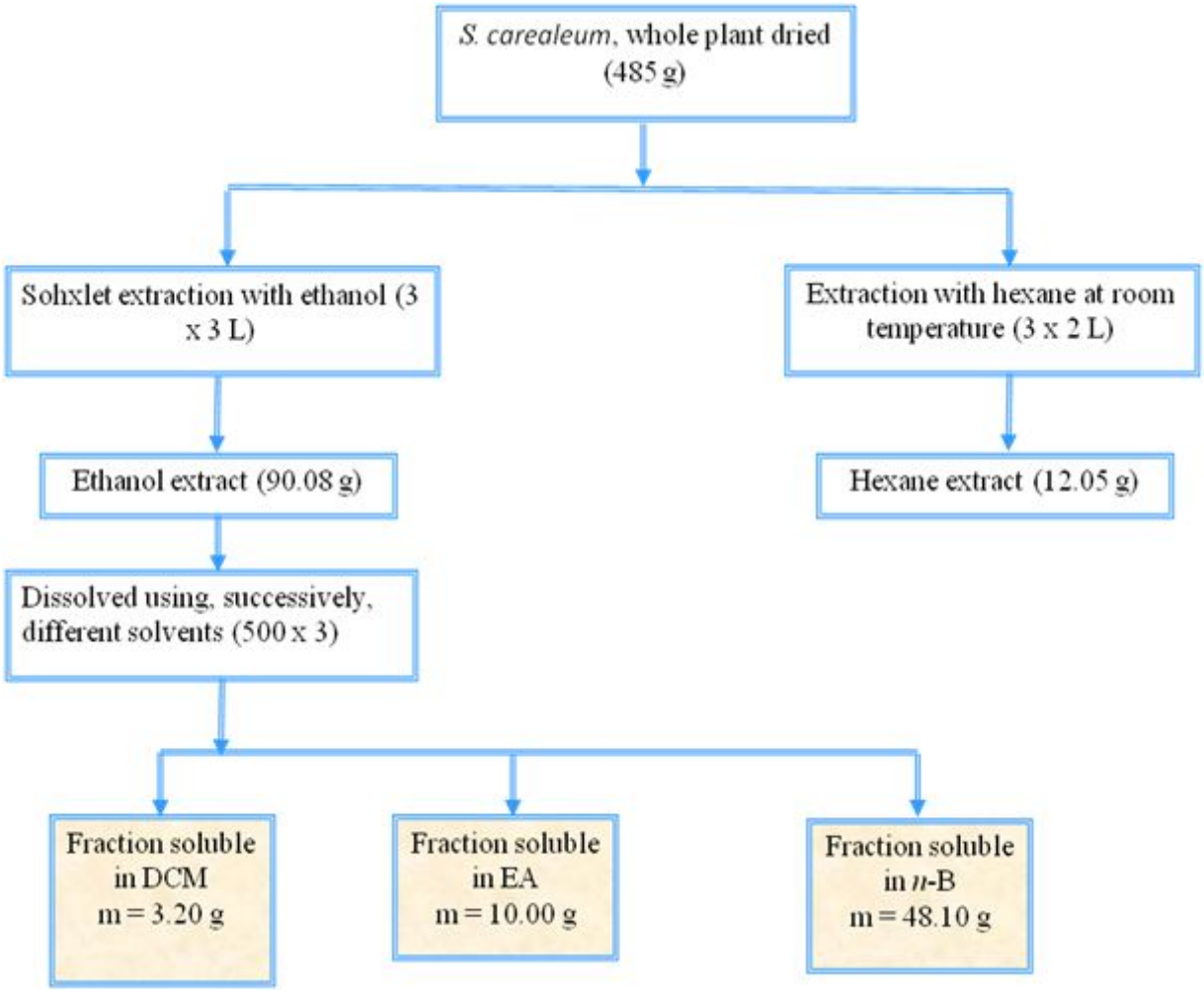


Figure 3.3 Extractive process of the dried plant material and treatment of the ethanolic crude extract (*S.carealeum)*

3.3.2 Isolation and purification of the non-polar extracts

2.5 g of hexane extract (***S. Stellata***) was subjected to column chromatography using a step gradient elution of *n*-hexane: ethyl acetate (100:0 to 0:100) and ethyl acetate: methanol (100:0 to 0:100) to afford several fractions.

Fraction 4 (200 mg) was chromatographed over a Sephadex LH20 column eluted with methanol and yielded pure compounds **1** (stearic acid, 3.0 mg) and **2** (1,3-*O*-dilinoleoyl-2-*O*-palmitoyl glycerol, 2.0 mg), respectively.

Fraction 5(60 mg) was chromatographed under the same conditions and yielded pure compounds **3** (1-*O*-linolenoyl-2-O-linoleoyl-3-*O* oleoyl glycerol, 6.0 mg) and **4** (ursolic acid, 2.5 mg), respectively.

Fraction 6(12 mg) was washed with acetone and gave compound **5**(stigmasterol, 3.5 mg) (Fig 3.4).

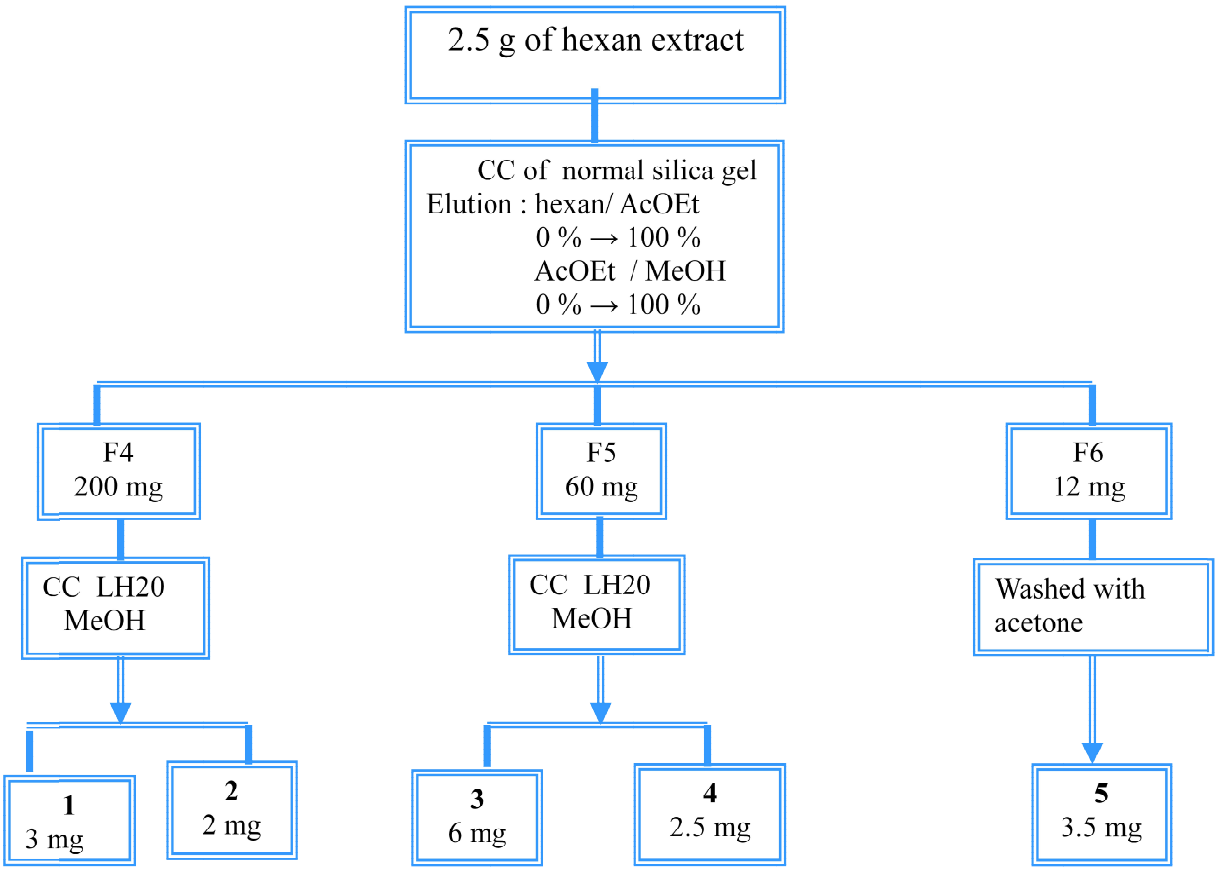


Figure 3.4 Fractionation and purification of *S. stellata* hexane extract

1.5 g of Hexan extract (***S. Caeruleum)*** was chromatographed on CC of silica gel, is carried out with a gradient of elution hexane / AcOEt (100:0 to 0:100), eighte fractions were obtained.

Fraction 3(250mg) was subjected on prep. TLC with hexane / AcOEt (95:05) elution yielded a pure compound **P1** (5mg).

Fraction 4 (10mg) was seprated by CC using sephadex LH20 lead to compound **P2**(5mg).

Fraction 6 (250mg) was injected on CC of normal silica gel eluted with hexane / AcOEt (100:0 to 0:100 gave several sub-fractions, sub-fraction 5 was passed on CC of LH20 to obtain pure compound **P3** (3mg)**.**

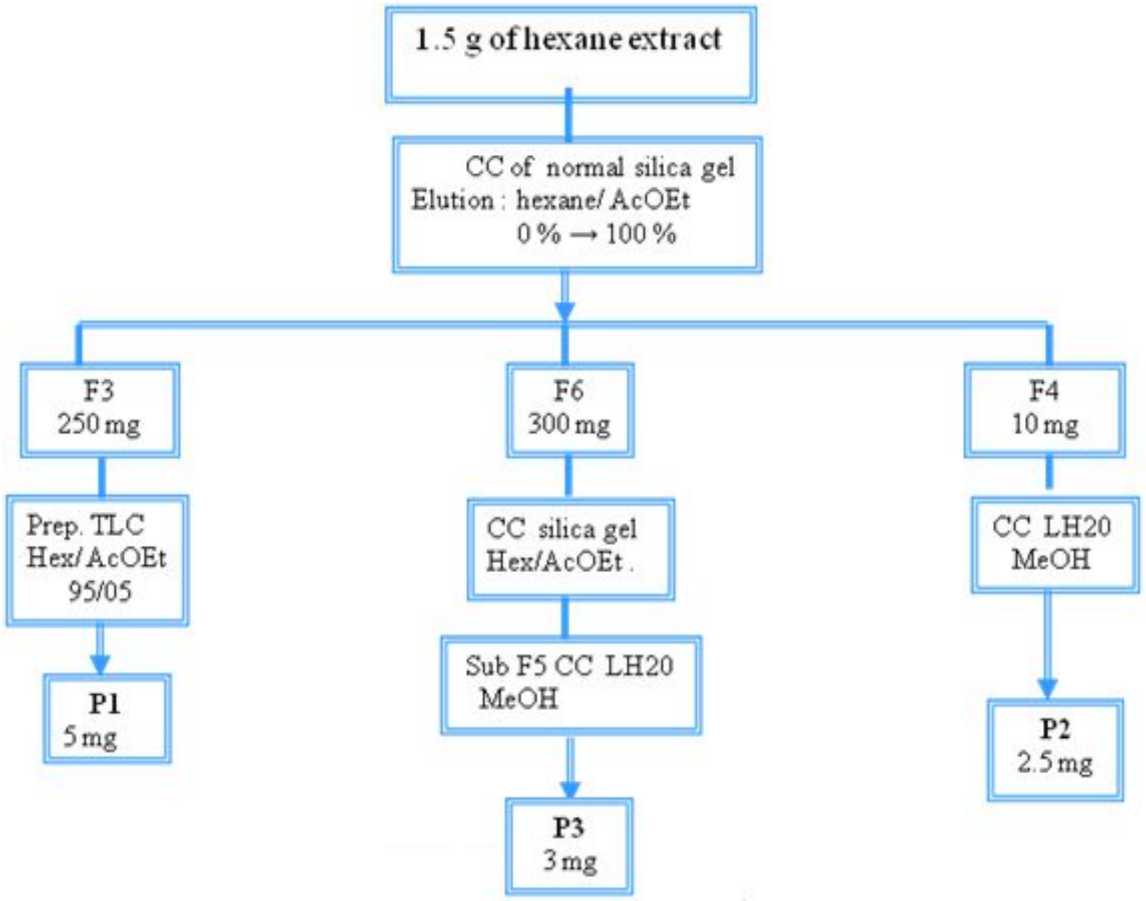


Figure 3.5 Fractionation and purification of *S.caeruleum* hexane extract

3.3.3 Isolation and purification of the polar extracts

EAF of *S.stellata* (2 g) was chrmatographed over silica gel column with a gradient elution starting with hexane/ethyl acetate (100:0 to 0:100) and followed by ethyl acetate/methanol e (100:0 to 0:100) elution.

Several sub-fractions were obtained, sub-fraction 3(27 mg) was isolated as pure compound **C1** (27 mg**)**.

From the less polar sub-fractions was separated over thin-layer chromatography and using hexane/ethyl acetate (85:15) to give compound **C2** (4 mg).

Sub-fraction 15 (20 mg) was purified over sephadex LH20 column and eluted with methanol, allowed to the isolation of compounds **C3** (6mg) and **C4** (7mg) **(Fig. 3.6)**

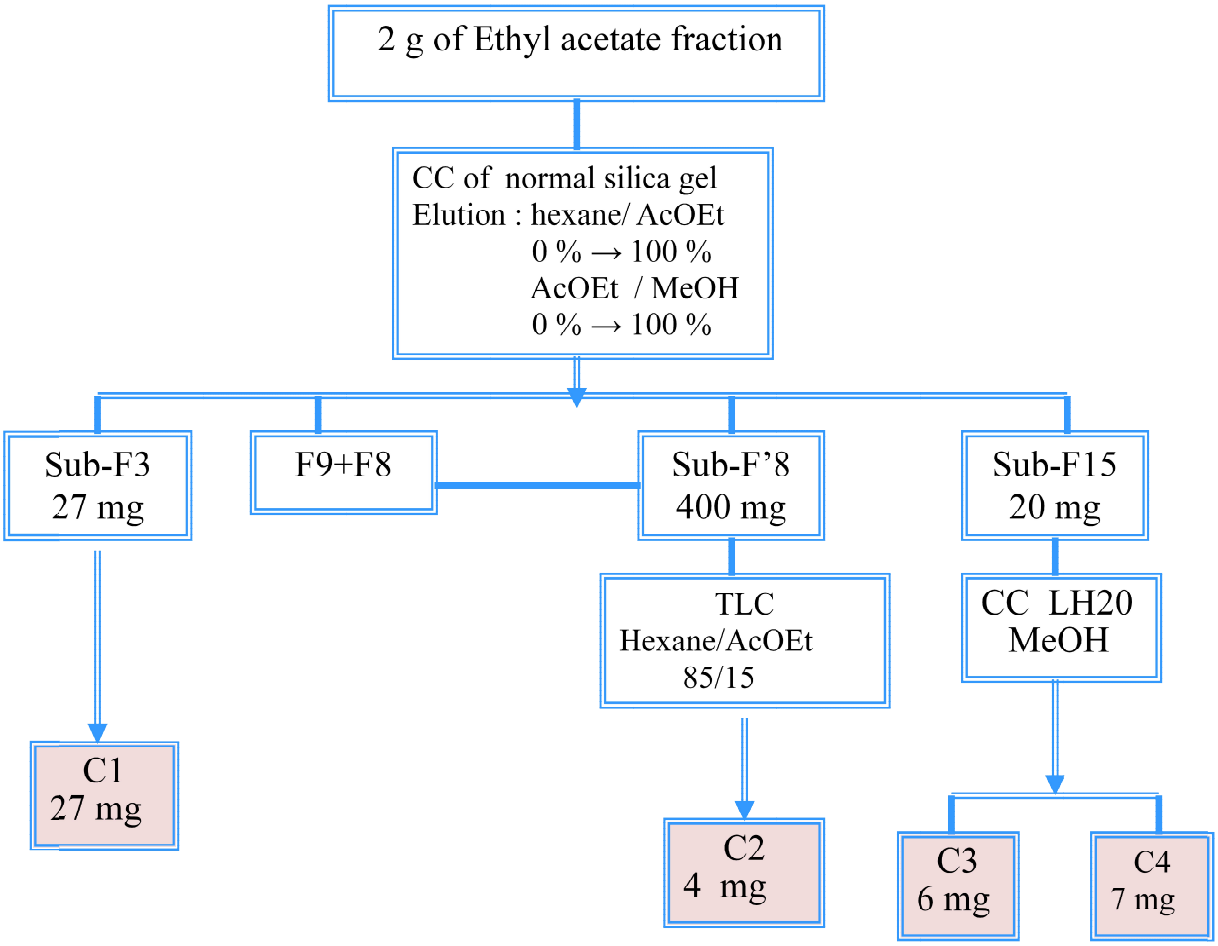


Figure 3.6 fractionation and purification of ethyl acetate fraction *(S.stellata)*

EAF of *S. Caeruleum* (4.25g) was separated over column chromatography using silica gel and a gradient elution with starting with hexane/ethyl acetate (100:0 to 0:100) and followed by ethyl acetate/methanol (100:0 to 0:100). Fourteen sub-fractions were abtained.

Sub-fraction 5 (20 mg) was subjected to CC using sephadex LH20 eluted by methanol, leading to compound **P4** (4mg).

Sub-fraction 8 (500 mg) was subjected to prep. TLC eluted with hexane / AcOEt (85:15) yielded compounds **P 5** (7.5 mg) and **P6** (3.5 mg) as pure.

Sub-fraction 9 (12 mg) was seprated over CC using sephadex LH20 leading to compound **P7** (2 mg).

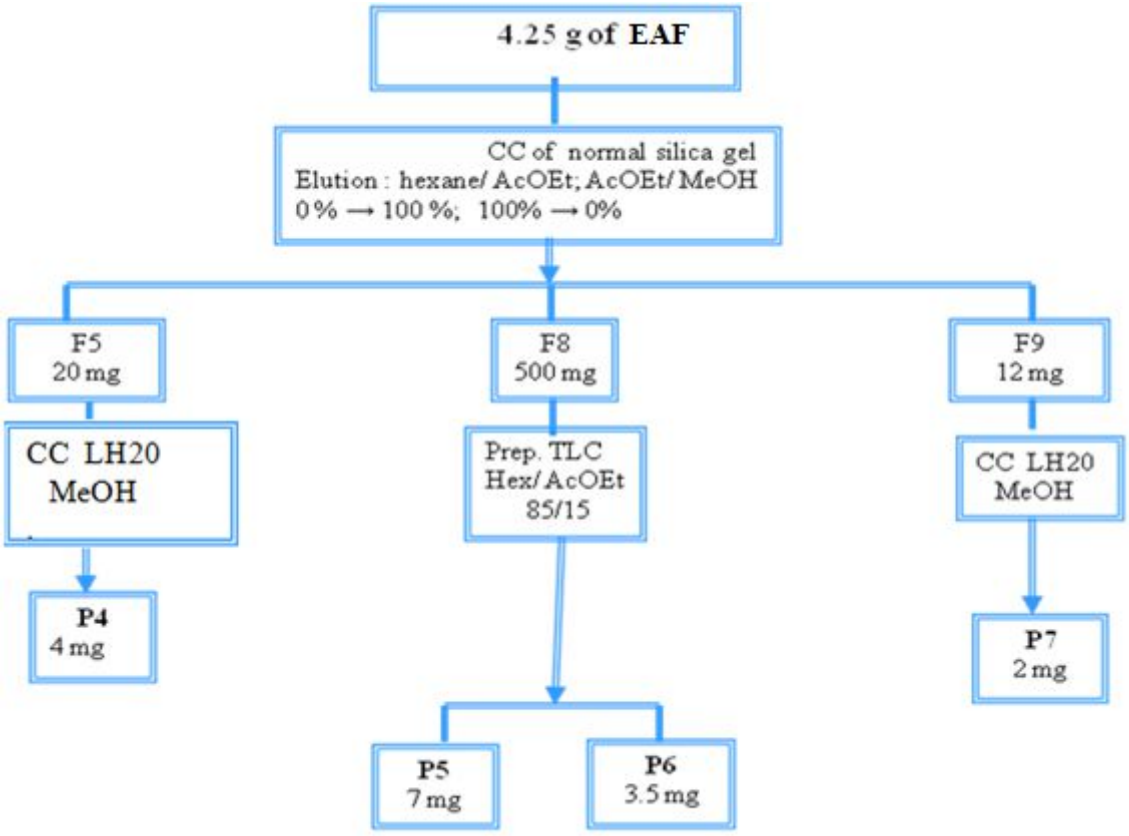


Figure 3.7 Fractionation and purification of EAF (*S.caeruleum)*

n-BF of S. Stellata (7 g) was chrmatographed over column silica gel and a gradient elution with chloroform/methanol (100:0 to 0:100).

Twenty five sub-fractions were obtained, their purification yielded several phenolic compounds. Sub-fractions (F1-F4) after their purification over sephadex LH20 column eluted with chloroform/methanol (50:50), Compounds; **C5** (4mg), **C6**(2mg), **C7**(5mg) and **C8**(2mg) were obtained.

Sub-fraction 12 (550 mg) was rechromatographed over a silica gel column eluted with a gradient elution of dichloromethane/methanol (100:0 to 0:100) to give compound **C9**(3 mg).

Sub-fraction 14 (10 mg) after purification over sephadex LH20 column with methanol to yeild compound **C10** (4 mg).

Purification of Sub-Fractions eleven and fifteen allowed respectively to obtain compound **C11** (14 mg) and **C12** (7 mg). Finally sub-fraction twenty gave pure compound **C13** (2 mg).

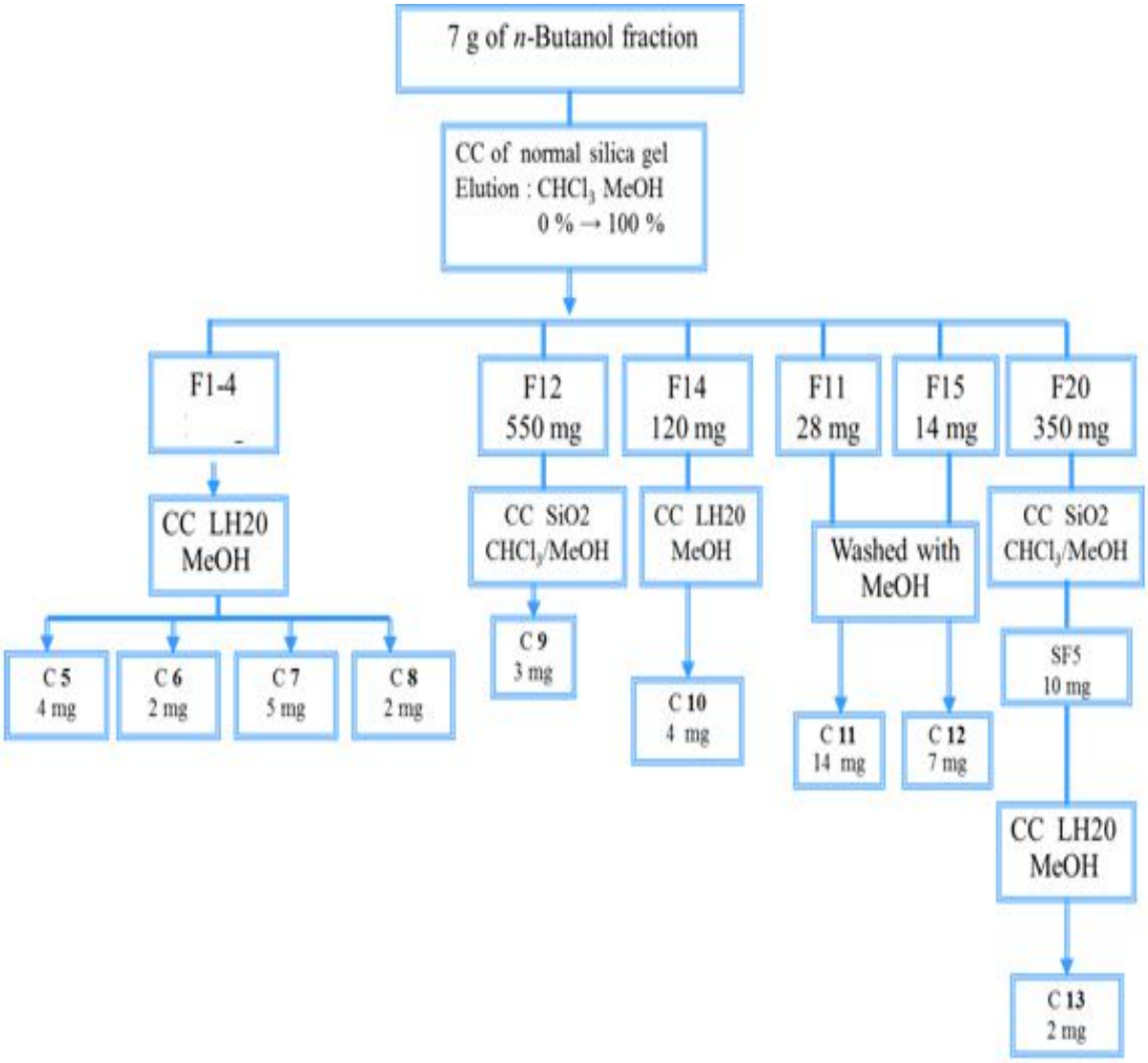


Figure 3.8 Fractionation and purification of *n*-butanol fraction (*S. Stellata*)

3.4. Gas chromatography-Mass spectrometry (GC-MS) analysis

Before GC-MS analysis, four aliquots of the hexane extract (20 mg) were silylated accordingly to a known method and usually used in our department (Freire et al., 2002). Each sample was dissolved in 1000 µL of dichloromethane and 200 µL of internal standard (IS) solution (tetracosane), 250 µL of pyridine, 250 µL of BSTFA and 50 µL of TMSCl were added. The mixture was maintained at 70 °C for 30 min being the hydroxyl and/or carboxyl groups of the present compounds converted into trimethylsilyl (TMS) ethers and/or esters, respectively. Afterwards were injected into the GC-MS apparatus. The quantity of silylation agents (BSTFA and TMSCl) was sufficient to ensure the silylation of all hydroxyl groups present in the samples compounds. All the compounds possessing hydroxyl groups are

transformed, using the same procedure, into the correspondents TMS derivatives and no compounds with free hydroxyl groups were detected.

From total ion chromatogram the peaks were identified by comparing their mass spectra with the mass spectral libraries (NIST 14 Mass Spectral and Wiley Registry™ of Mass Spectral Data), with MS spectra and MS fragmentation pattern published in the literature (AOCS Lipid Library, 2017; Golm Metabolome Database, 2017; Suttiarporn et al., 2015; Vilela et al., 2014; Hrabovski et al., 2012; Füzfai et al., 2008; Razboršek et al., 2008; Oliveira et al., 2006; Kitson et al., 1991; Petersson, 1969;), by comparing the retention times and mass spectra data of the standard compounds injected in the same chromatographic conditions. Moreover, when possible the retention index relative to n-alkanes (C_5 - C_{36}) was compared with published data retention indexes.

For quantification purposes, four independent replicates of each sample were submitted to silylation procedure and each one injected in triplicate. The internal standard method was applied and the amount of metabolites present was achieved from the calibration curves obtained with the most closed pure standard compounds available or its TMS derivatives (if they have hydroxyl groups). All the injected samples and standards solutions contain a fixed quantity of internal standard (tetracosane). The calibration curves were obtained by injection of five known concentrations (5 μg/mL to 1.5 mg/mL) and the detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves represented in table 1 (LOD = 3standard deviation/slope and LOQ = 10standard deviation/slope). Values of correlation coefficients confirmed linearity of the calibration plots (Table 3.1). The concentrations of the standards were chosen in order to guarantee the quantification of each compound in the samples by intrapolation in the calibration curve. The results were expressed in mg of compound / kg of dried plant, as mean ± standard deviation of four independent analyses.

Four independent replicates of each sample were analysed and each aliquot was injected in duplicated. The presented results are the average of the concordant values obtained for each sample (less than 5% variation between injections of the same aliquot and between aliquots of the same sample) and expressed as mean values ± standard deviation (MV ± SD). One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test were performed using the GraphPad Prism version 7 for Windows (Graphpad Software, Inc.) to compare the results of each independent replicates. A *p*-value lower than 0.0001 was considered statistically significant in all analyses

Table. 3.1. Linearity (y = mx + b, where y corresponds to the standard peak area / internal standard peak area ratio and x corresponds to the mass of standard/mass of internal standard ratio), LOD and LOQ of pure compounds used as reference

Standard compound	Slope (m)[§]	Intercept (b)[§]	R^2	LOD[§§]	LOQ[§§]
Methyl palmitate	0.1983	-0.0017	0.9992	13	43
Palmitic acid	0.2143	0	0.9944	15	50
Linoleic acid	0.1994	-0.0003	0.9975	12	40
Myo-inositol	7.1931	-0.0109	0.9952	5	17
1-Palmitoylglycerol	7.2283	-0.0009	0.9975	3	10
Tetradecan-1-ol	7.2366	-0.0037	0.9937	3	10
Octadecane	2.0283	-0.0448	0.9985	8	27
Triacontane	2.0154	-0.0311	0.9991	10	33
Mannose	4.1401	-0.0801	0.9998	3	10
Maltose	4.1380	-0.1126	0.9999	5	17
β-Sitosterol	2.5254	-0.0033	0.9983	12	40
α-Tocopherol	2.4738	-0.0028	0.9993	5	17
Ursolic acid	2.6034	-0.0207	0.9996	10	33

[§]in area counts/mg
[§§]in μg/mL

3.5 Ultra-high-performance chromatography coupled to photodiode-array detection and electrospray ionization/ion trap mass spectrometry (UHPLC-DAD-ESI/MSn) analysis

For the UHPLC-MS analysis, 50 mg of each extract were dissolved in 5 mL of methanol (final concentration 10 mg/mL) and the resulting solutions were filtered through a 0.2 mL nylon membrane (Whatman). Three independent analyses were carried out for reproducibility.

The identification of individual phenolic compounds in the UHPLC analysis was achieved by comparison of their retention times, UV-Vis spectra and MS^n spectra data with those of the closest available reference standards and data reported in the literature. In addition, the structure of some phenolic components was further confirmed by NMR analysis after their purification.

The semi quantification of the main individual phenolic compounds in the extract was performed by peak integration at 260 nm, through the external standard method, using the most close reference compounds available. The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves represented in table 2.2 (LOD = 3 standard deviation/slope and LOQ = 10 standard deviation/slope). The calibration curves were obtained by injection of five known concentrations with variable ranges and the concentrations of the standards were chosen in order to guarantee the quantification of each compound in the samples by intrapolation in the calibration curve. Values of correlation coefficients confirmed linearity of the calibration plots (Table 3.2). The results were expressed in mg of compound / g of dried extract, as mean ± standard deviation of four independent analyses.

Results were expressed as mean ± standard deviation of three independent assays and analyzed through unpaired Student's test or ANOVA combined with Tukey's test (Graph Pad Prism 5). P values of less than 5% ($p < 0.05$) were considered to be significant.

Table 3.2 Linearity, LOD and LOQ of pure compounds used as reference

Standard compound	Range concentration[§]	Slope (m)[§§]	Intercept (b)[§§]	R^2	LOD[§]	LOQ[§]
Benzoic acid	0.5-500	16748	111	1.0000	12	40
Gallic acid	0.5-500	557	-728	0.9988	11	37
Catechin	0.5-250	142	-58	0.9997	8	27
Caffeic acid	0.5-550	618	-70	0.9998	14	47
p-Coumaric acid	0.5-550	716	122	0.9990	15	50
Ferulic acid	0.5-500	1633	6	0.9993	10	33
Rosmarinic acid	0.5-250	706	1228	0.9976	7	23
Chlorogenic acid	0.5-250	659	-8	0.9989	9	30
Isorhamnetin	0.5-100	629	-2316	0.9991	3	10
Kaempferol	0.5-175	792	-76	0.9969	5	17
Luteolin	0.5-100	354	-221	1.0000	3	10
Quercetin	0.5-175	317	-3	0.9992	4	13

[§]in μg/mL
[§§]in area counts/mg

Chapter IV

Results and discussion

4.1 Identification of the compounds isolated from *Scabiosa stellata*

The compounds isolated from *S. stellata* were fully characterised using NMR spectroscopy, mono and bidimensional (1H, 13C, DEPT, HSQC, COSY, HMBC). Mass spectrometry (ESI-MS) was also performed not for structural characterization purposes but to confirm the presence of the molecular ion peak. However, it should be noticed here that the mass analysis was performed after the NMR analysis, so compounds were dissolved in the deuterated solvents, reason why the mass spectra are not clean. Whenever possible, comparison with literature data was also used. In the following sections of this chapter the most important features that allowed the structure elucidation of all compounds will be discussed.

4.1.1 Structural characterisation of compound 1

Compound 1 was isolated as a white powder, its ESI-MS mass spectrum (Fig.4.1), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 591, which corresponds to $[2M+Na]^+$, suggests a molecular mass is 284 mau.

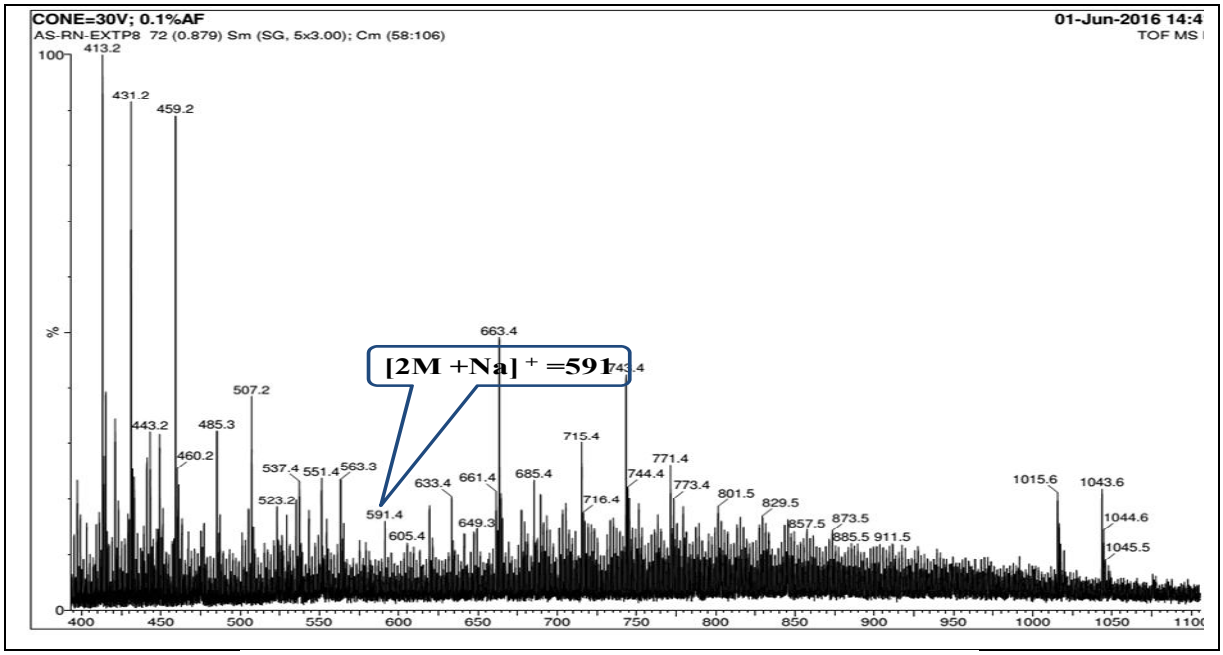


Figure. 4.1 ESI-MS mass spectrum of the compound 1

The ^{1}H NMR spectrum of this compound indicates the absence of aromatic and olefinic signals (Fig.4.2) and the existence of signals at:

Peak appears at δ 2.35 ppm (2H, t, *J* 7.6 Hz) as a triplet can be attributed to α-carbonyl protons;

δ 1.63 ppm is a multiplet that also corresponds to 2H which seem to be coupled with the α-carbonyl ones;

In the range of δ 1.25-1.30 ppm a maltiplet confirming the presence of several aliphatic CH_2 protons.

Finally, the peak appear at δ 0.88 ppm as triplet (*J* 7.1 Hz) confirming the presence of terminal CH_3 group.

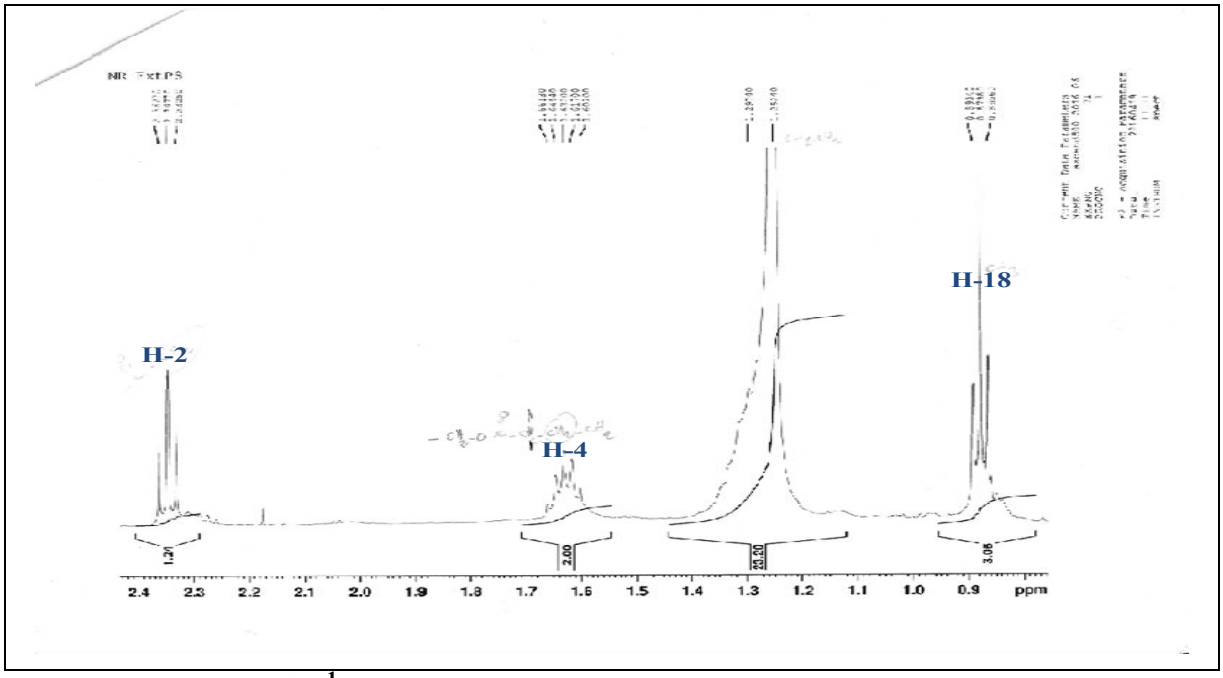


Figure. 4.2 ^{1}H NMR Spectrum of compound 1($CDCl_3$, 500 MHz)

According to ^{13}C NMR spectrum (Fig.4.3) we can detecte important signals at:

- δ 178.98 ppm, attributed to carbonyl group;
- δ 33.88 ppm, due to the α-carbonyl carbon resonance;
- δ 14.15 ppm, due to the CH_3 terminal group.
- $(CH_2)_n$ groups appear at δ 22.72-29.72 ppm.

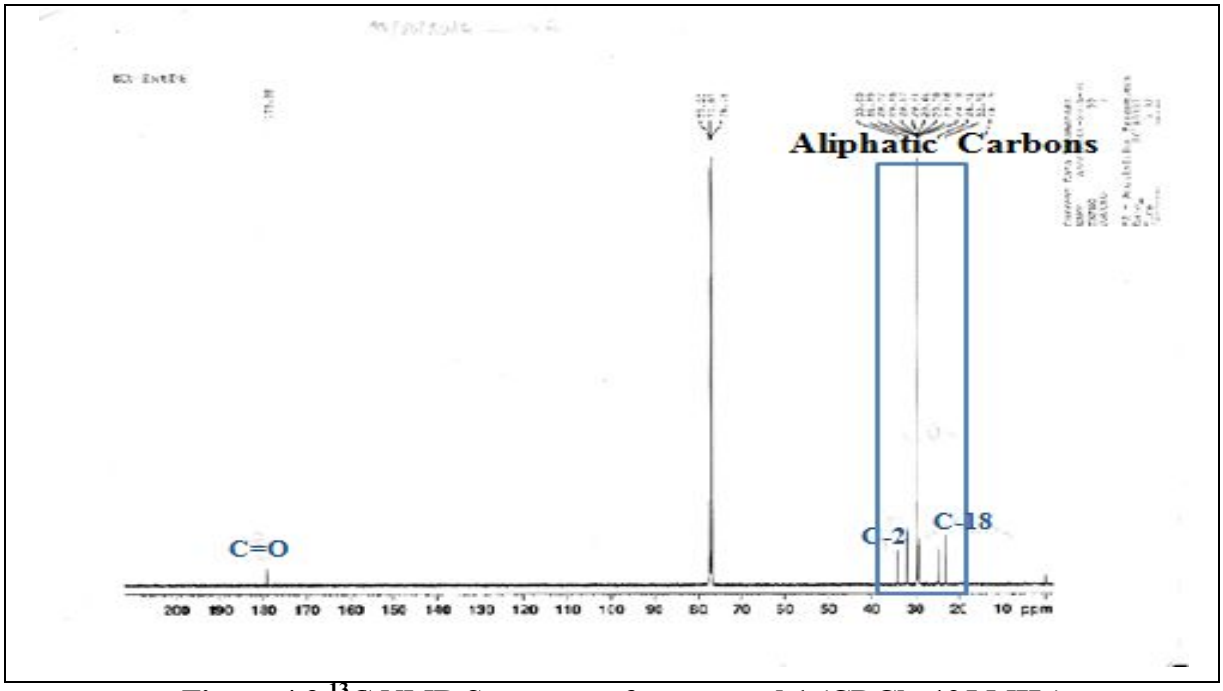


Figure.4.3 ^{13}C NMR Spectrum of compound 1 ($CDCl_3$, 125 MHz)

These data make it possible to suggest the membership of compound 1 to the class of saturated fatty acids with molecular mass 284 mau.

COSY spectrum analysis (Fig.4.4) of compound 1 showed the correlations between proton H-3(1.63 ppm) and two protons H-2 at 2.35ppm , H-3 and H-4 at 1.25-1.30 ppm which confirm the ^{1}H- NMR data and helpes in the assignment of the carbon chemical shifts. Howover the carbon assignment accomblished with HSQC spectrum analysis

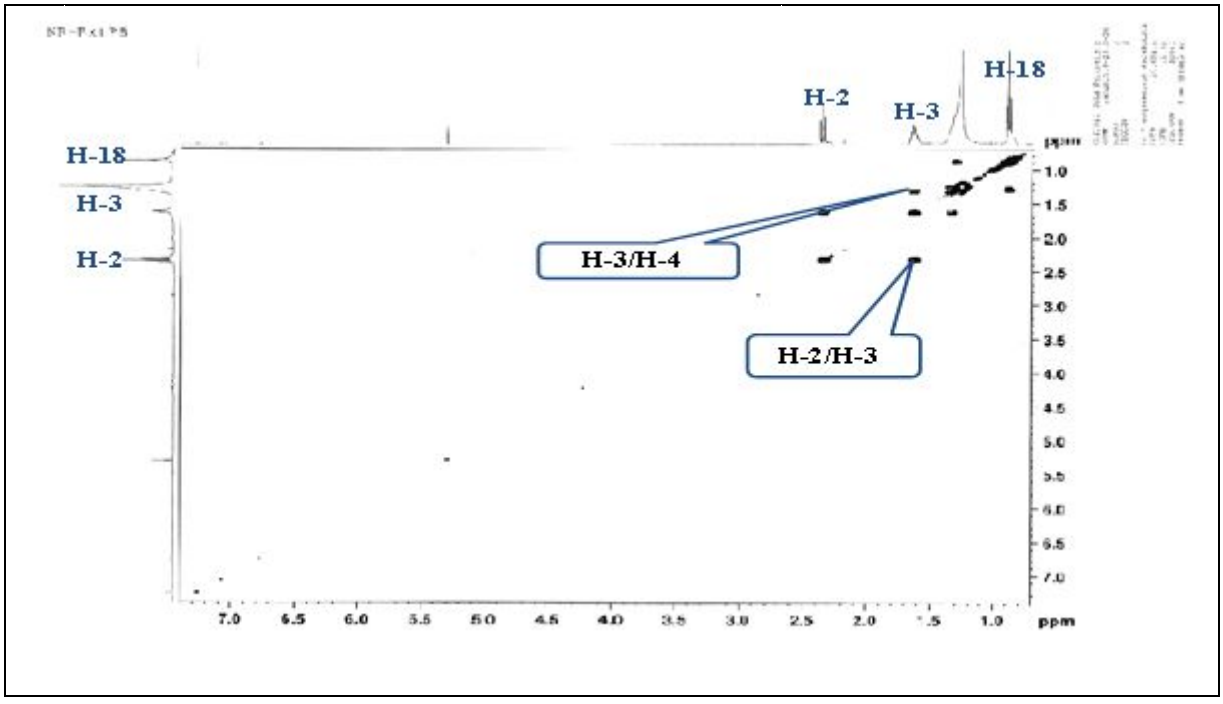


Figure. 4.4 COSY Spectrum of compound 1 ($CDCl_3$, 500 MHz)

HSQC spectrum analysis, showed the correlations between carbons and protons which are directly attached are shown in Fig.4.5 and the chemical shift values are presented in Table 1.

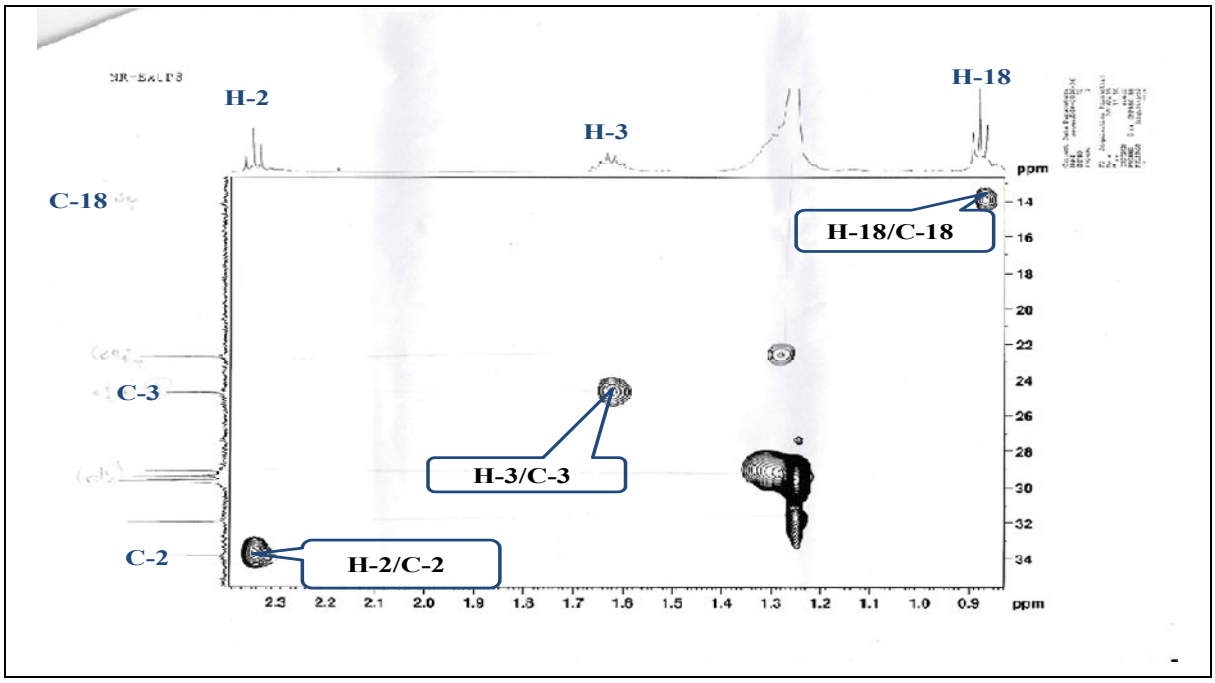


Figure. 4.5 HSQC Spectrum of compound ($CDCl_3$, 500 MHz)

Based on the above mentioned data, which is summerized in table 1, and also comparing with literature data, it was possible to suggest compound 1 as a stearic acid (Fig.4.6).

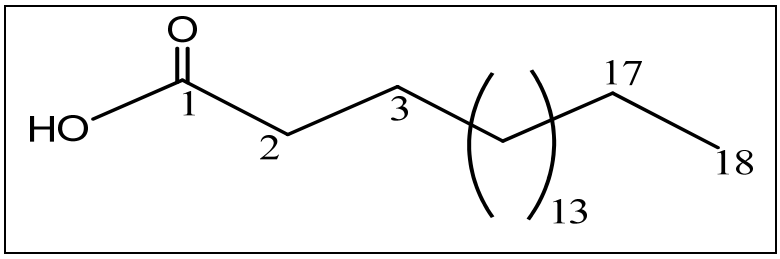


Figure. 4.6 Stearic acid

Table 4.1. Shows ^{1}H and ^{13}C NMR Chemical shifts of compound 1 recorded in $CDCl_3$ *(500 MHz)*

Postion	δ ppm	δ ppm
1	-	178.98
2	2.35 (t; *J* 7.6 Hz)	33.88
3	1.63 (m)	24.71
4-17	1.25 -1.30 (m)	29.08-29.72
18	0.88 (t; *J* 7.1 Hz)	14.30

4.2 Structural characterisation of compound 2

Compound 2 was obtained as a colourless oil, its ESI-MS mass spectrum (Fig.4.7), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 877, which corresponds to $[M+Na]^+$, suggests a molecular mass 854 mau.

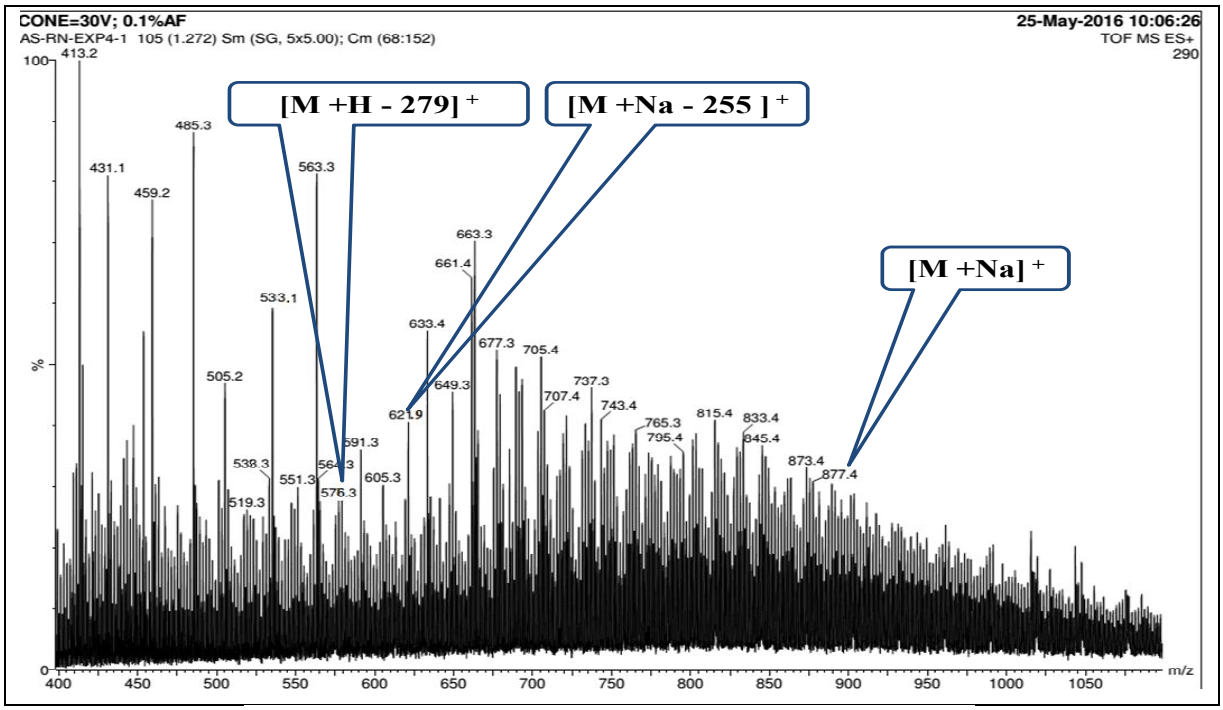


Figure 4.7 ESI-MS mass spectrum of the compound 2

^{1}H and NMR data analysis shows that both the ^{1}H and ^{13}C spectra do not display signals in the characteristic zones of aromatic protons and carbons while characteristic peaks of unsaturated fatty acid are present. For example, ^{1}H NMR spectrum (Fig. 4.8) reveals the following peaks:

- at δ 0.83-0.90 ppm attributed to methyl groups;
- olefinic protons appear at δ 5.2-5.5 ppm;
- allylic and bis-allylic methylenes appear at δ 1.98 to 2.12 and 2.73 to 2.80 ppm;
- signals at δ 4.14 ppm (2H, dd, *J* 5.9 and 11.9 Hz), 4.29 ppm (2H, dd, *J* 4.3 and 11.9 Hz) and 5.28-5.33 ppm (1H, m), characteristic of the triglyceride H-1, H-3 and H-2 protons (Fig.4.9);
- Signals at δ 2.34 ppm (t; *J* 1.7 Hz) and 2.29 ppm (t; *J* 1.8 Hz) are protons of acyl moieties in triacylglycerols.

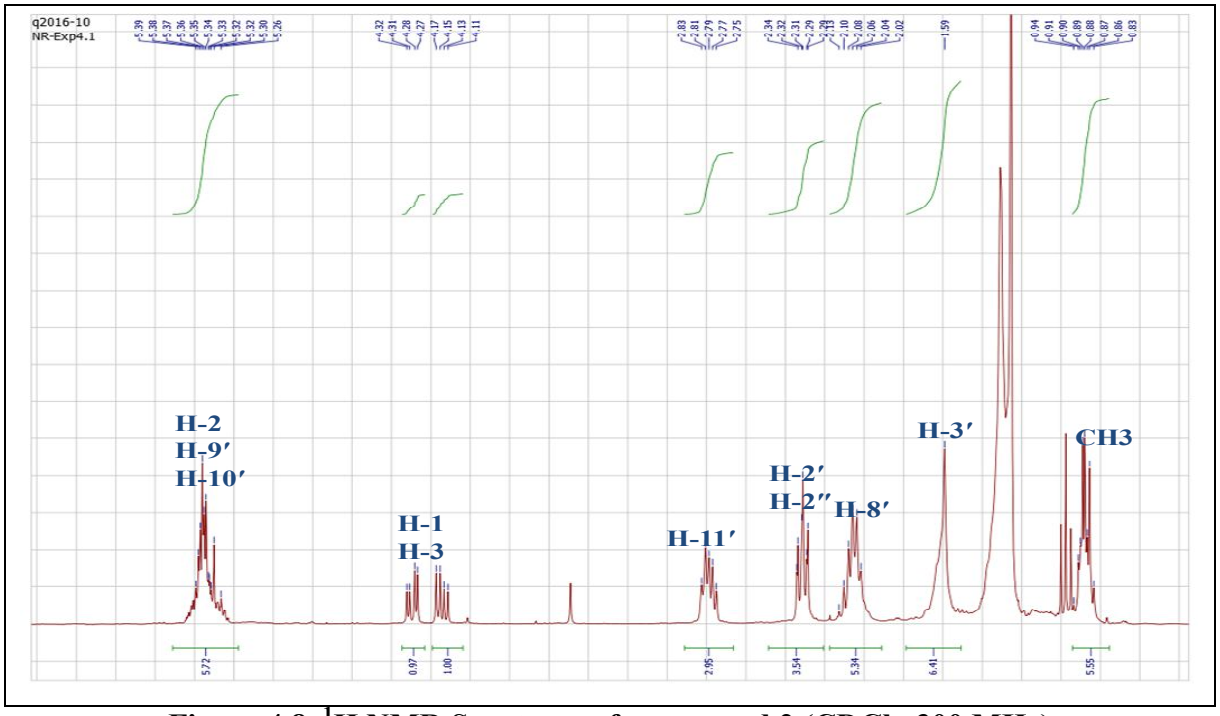


Figure 4.8. ^{1}H NMR Spectrum of compound 2 ($CDCl_3$, 300 MHz)

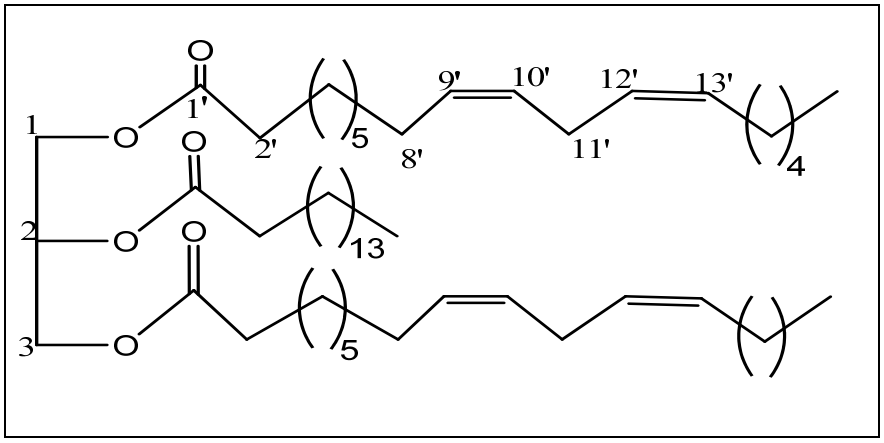


Figure. 4.9 1,3-O-dilinoleoyl-2-O-palmitoyl glycerol

On the other hand the analysis of the ^{13}C NMR spectrum (Fig. 4.10) allowed the detection of important signals such as:

- alkoxycarbonyl carbons at δ 172.9 and 173.3 ppm;
- Olefinic carbons at δ 127.1-132.0 ppm;
- CHOR and CH_2OR carbons at δ 68.9 and 62.1 ppm;
- Aliphatic long chains at δ 13.7-34.2 ppm.

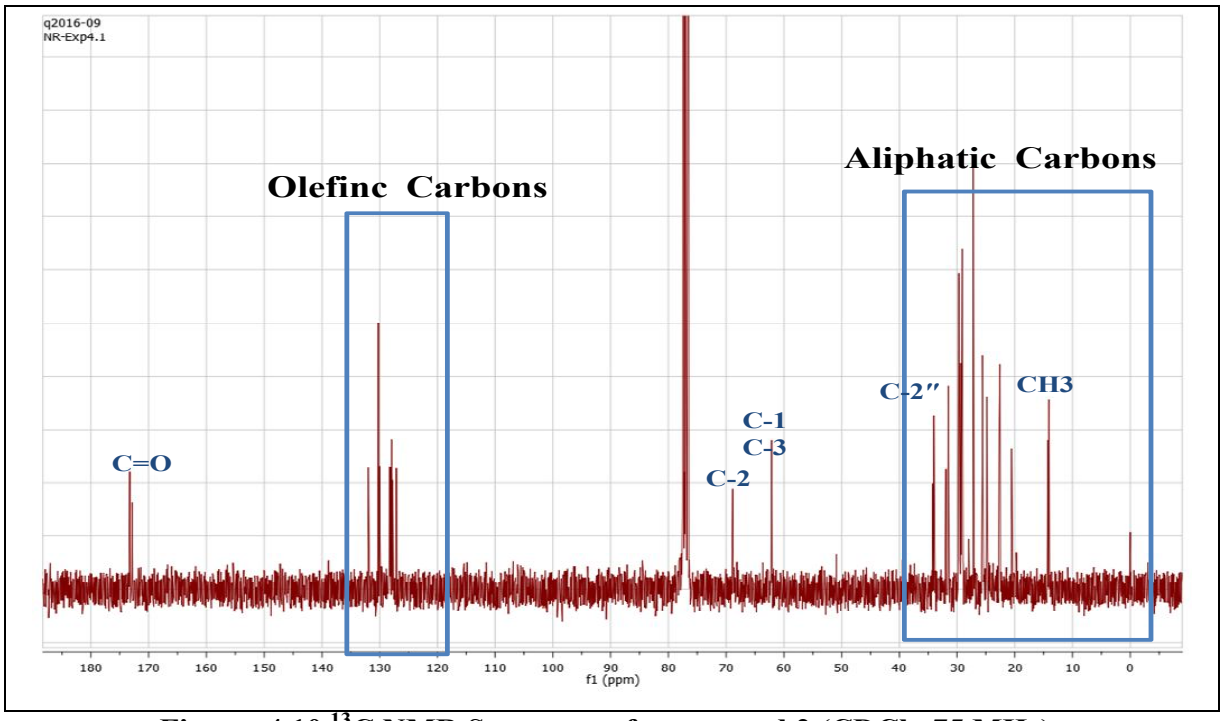


Figure. 4.10 ^{13}C NMR Spectrum of compound 2 ($CDCl_3$, 75 MHz).

Based on the presented data structure of compound 2 can established as a symmetrical triglyceride. Using the correlations detected in the bidimensional experiments, HSQC, COSY and HMBC (Fig. 4.11, 4.12 and 4.13) help to assign both of carbons and protons atoms as it is shown in table 2. And these data are consistent with the proposed structure, which is illustrated in figure 4.9.

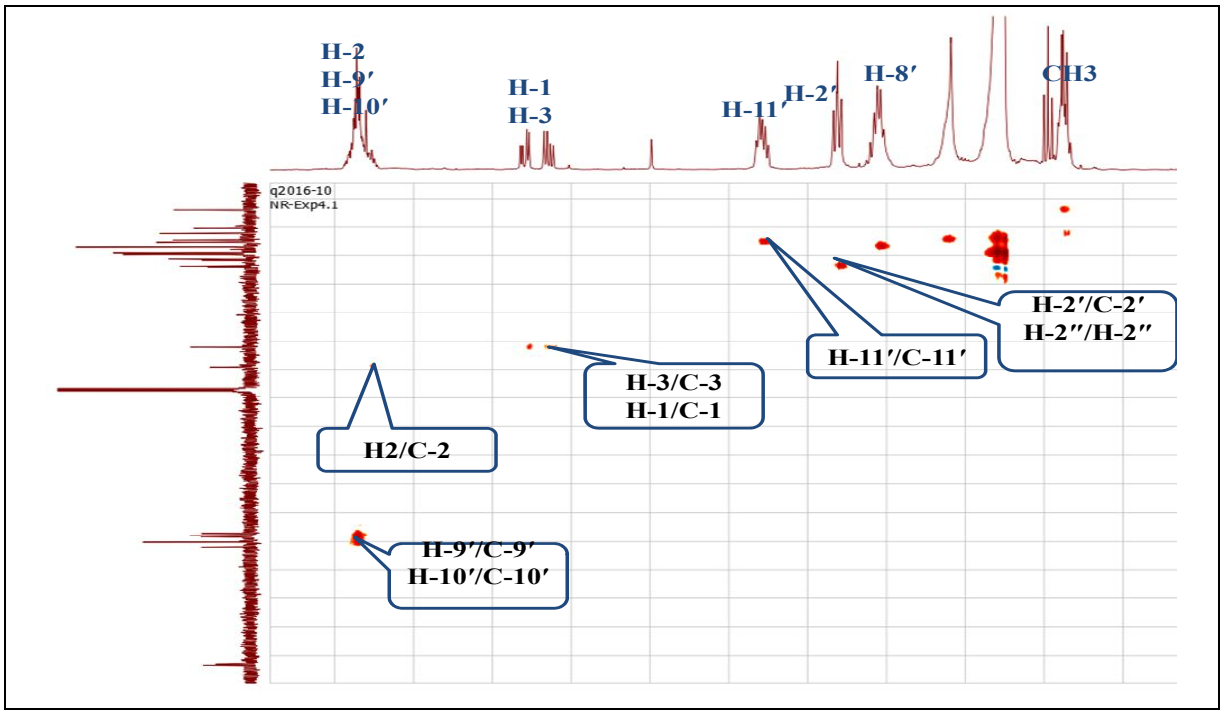


Figure 4.11. HSQC Spectrum of compound 2 ($CDCl_3$, 300 MHz)

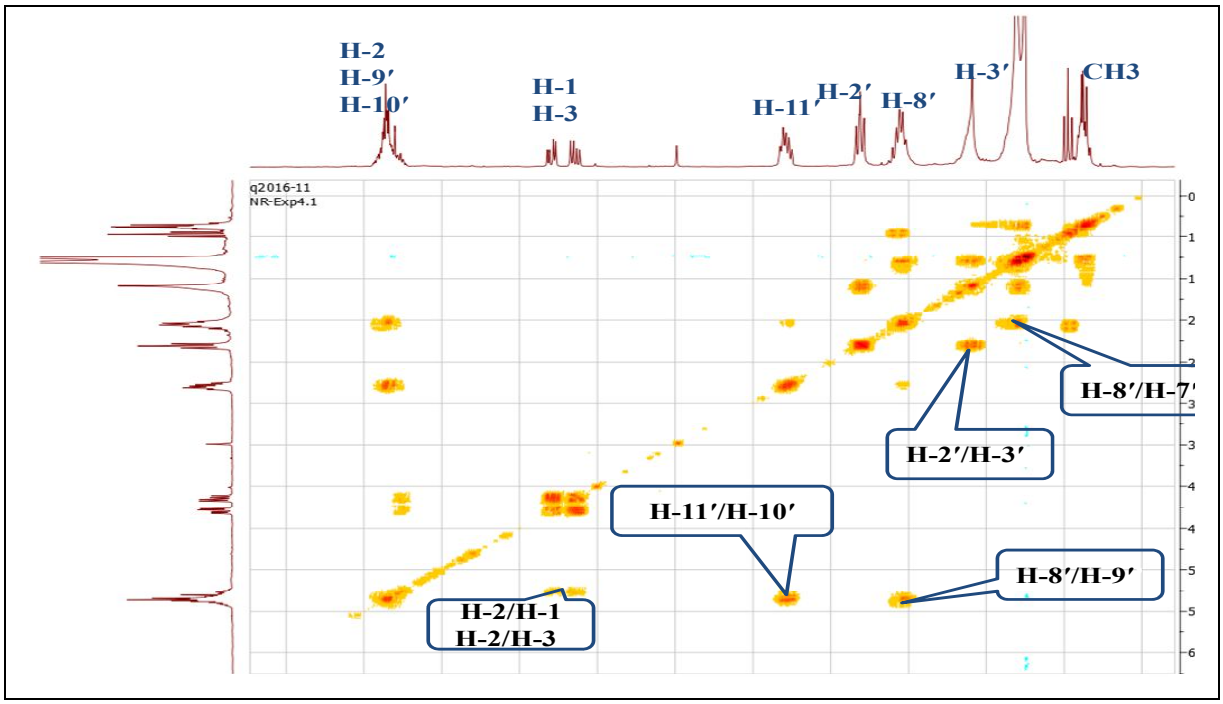


Figure 4.12. COSY Spectrum of compound 2 ($CDCl_3$, 300 MHz)

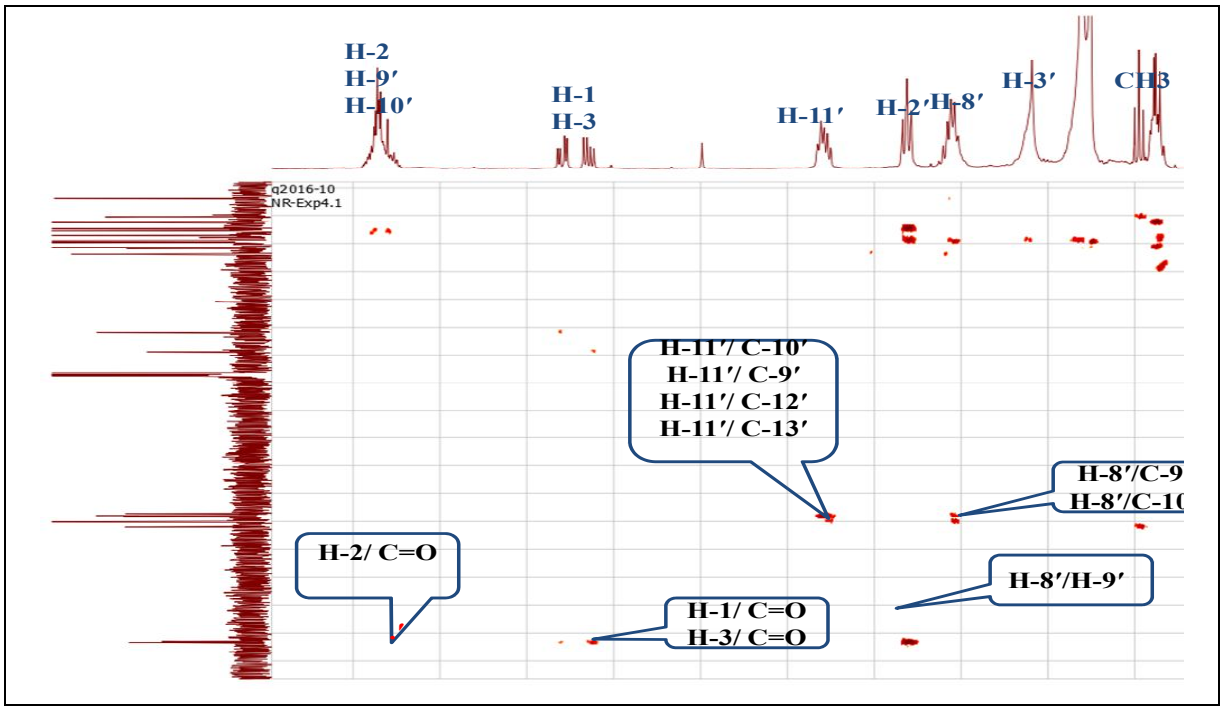


Figure 4.13. HMBC Spectrum of compound 2 ($CDCl_3$, 300 MHz)

Table 4.2. shows ^{1}H and ^{13}C NMR Chemical shifts of compound 2, recorded in $CDCl_3$*(300 MHz)*

Position		
	δ_H (ppm)	δ_C (ppm)
1,3	4.14 (dd; *J* 5.9, 11.9 Hz) 4.29 (dd; *J* 4.3, 11.9 Hz)	62.1
2	5.28-5.33 (m)	68.9
2’	2.34 (t; *J* 1.7Hz)	34.19*
2’’	2.29 (t; *J* 1.8 Hz)	34.03*
3’	1.54-1.67 (m)	24.8
5’	1.24-1.4 (m)	29.1
8’	1.98-2.12 (m)	27.2
9’ / 10’	5.34-5.40 (m)	127.1 / 131.3
11’	2.73-2.80 (m)	25.5
C=O	-	172.9 - 173.3

4.1.3 Structural characterisation of compound 3

Compound 3 was obtained as a colourless oil, its ESI-MS mass spectrum (Fig. 4.14), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 879, which corresponds to $[M+H]^+$, suggests a molecular mass 878 mau.

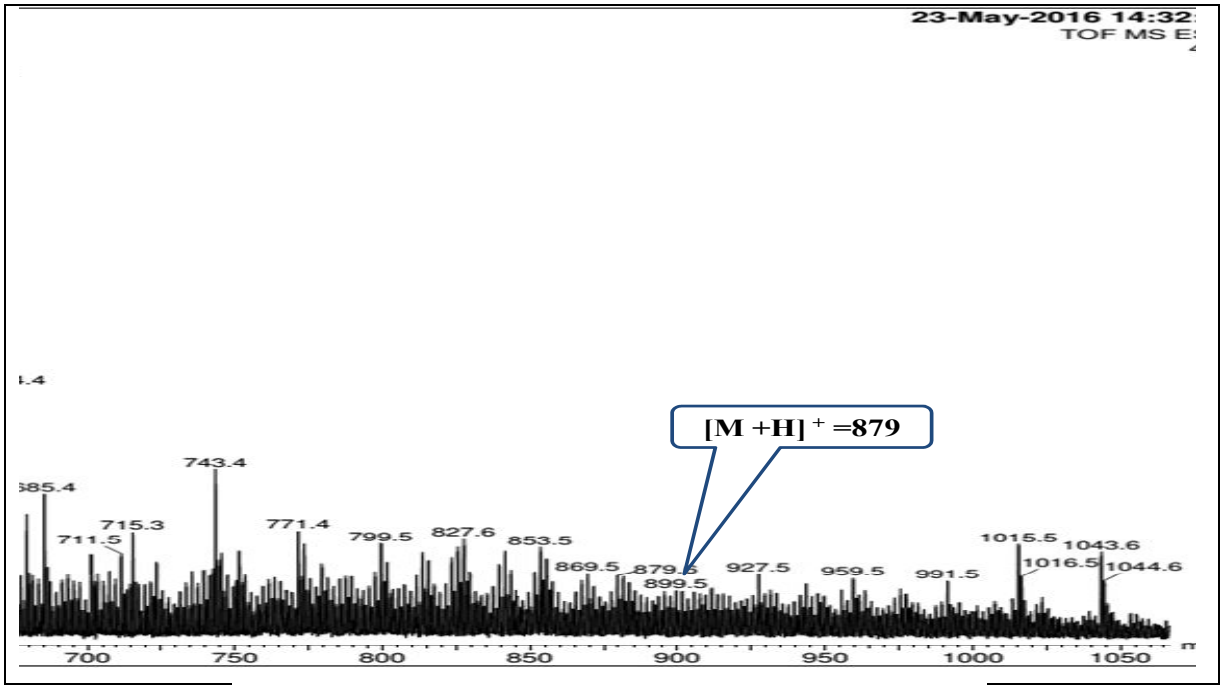


Figure. 4.14 ESI-MS mass spectrum of the compound 3

The comparison of the ^{1}H NMR spectrum (Fig. 4.15) of compound 3 with the spectrum of compound 2 (Fig. 4.8) shows a great structural similarity and suggest that this compound 3 is also a triglyceride. In fact, from the ^{1}H NMR spectrum can be highlight the following signals:

- CH_3 groups at δ 0.83-0.90 ppm and 0.97 ppm (3H, t, *J* 7.5 Hz);
- olefinic protons at δ 5.28 to 5.42 ppm;
- allylic and bis-allylic methylenes at δ 2.02-2.10 ppm and 2.75-2.82 ppm;
- the characteristic signal at δ 2.31 ppm (td, *J* 3.1 and 16.3 Hz), due to protons of Triacylglycerols acyl moieties;
- three signals at δ 4.13 ppm (2H, dd, *J* 4.9 and 11.9 Hz), 4.28 ppm (2H, dd, *J* 4.3, 11.9 Hz) and 5.24-5.30 ppm (1H, m), characteristic of the triglyceride H-1, H-3 and H-2 protons.

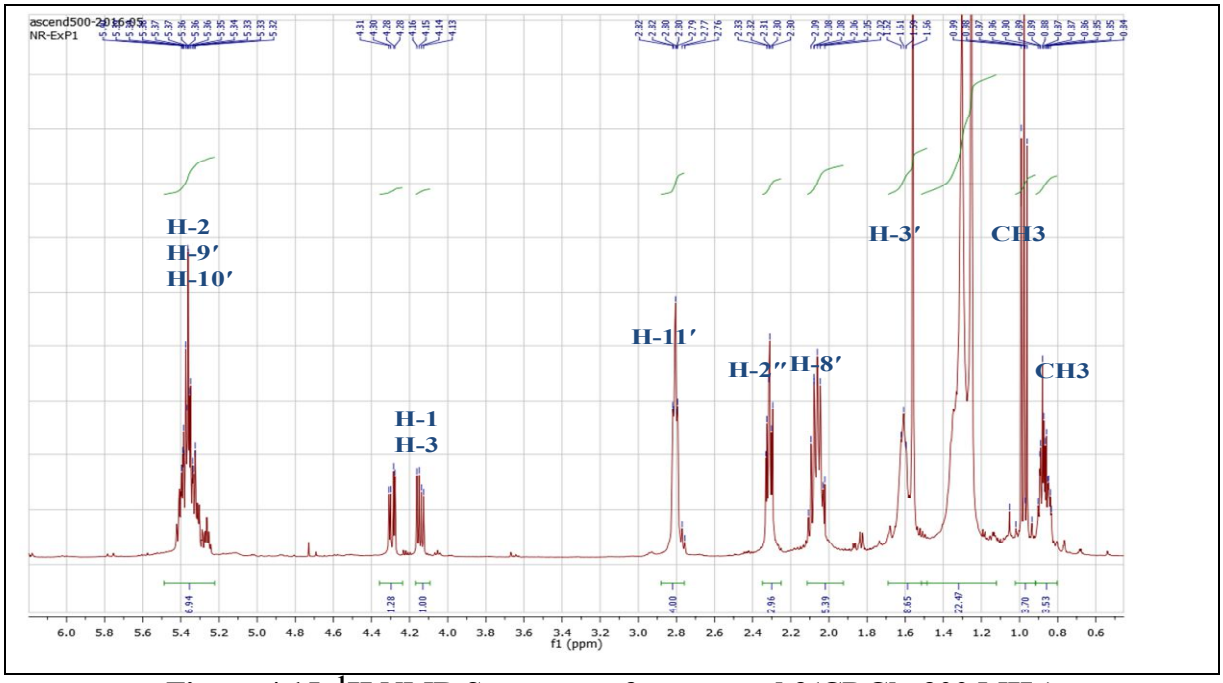


Figure 4.15. ^{1}H NMR Spectrum of compound 3($CDCl_3$, 300 MHz)

^{13}C NMR spectrum (Fig. 4.16) shows the signals attributable to:

- Olefinic carbons resonating at δ127. 1-132.0 ppm;
- oxymethine carbons at δ 62.1 (C-3, C-1) and 68.9 (C-2) ppm;
- Aliphatic carbons at δ 22.8-34.2 ppm.

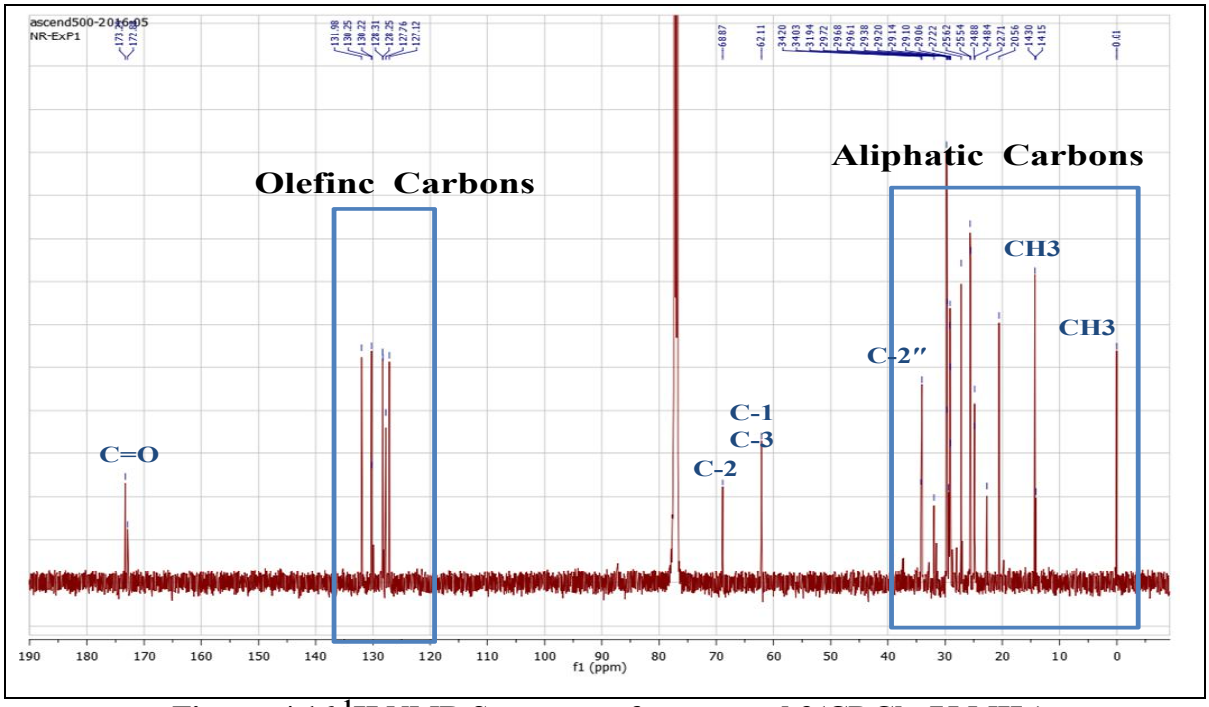


Figure. 4.16 ^{1}H NMR Spectrum of compound 3($CDCl_3$, 75 MHz)

All these spectral data, which are summarised in table 3, make it possible to attribute the structure depicted in figure 17 to the compound 3. Moreover, this 1-*O*-linolenoyl-2-*O*-linoleoyl-3-*O*-oleoyl glycerol was isolated for the first time in *Sacabiosa* genus.

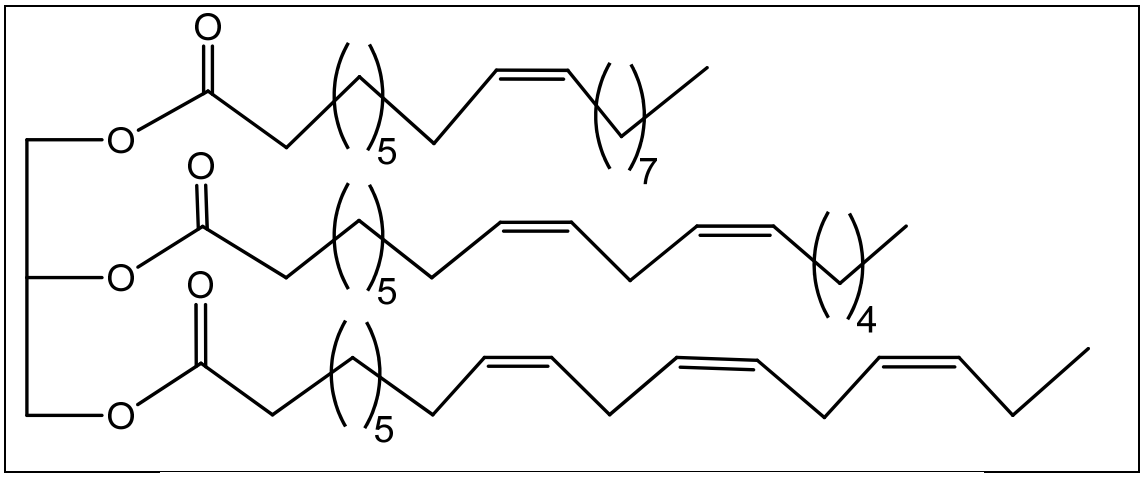


Figure. 4.17 1-O-linolenoyl-2-O-linoleoyl-3-O-oleoyl glycerol

Table 4.3. ^{1}H and ^{13}C NMR Chemical shifts of compound 3, recorded in $CDCl_3$*(300 MHz)*

§Position		
	δ_H (ppm)	δ_C (ppm)
1,3	4.13 (dd; *J* 4.9, 11.9 Hz) 4.28 (dd; *J* 4.3, 11.9 Hz)	62.1
2	5.24-5.30 (m)	68.9
2’	2.31 (td; *J* 3.1, 16.3 Hz)	34.0 / 34.2
3’	1.55-1.63 (m)	24.8/24.9
5’	1.23-1.34 (m)	29.0 / 32.0
8’	2.02-2.10 (m)	27.2
9’ / 10’	5.28-5.42 (m)	127.1 / 132.0
11’	2.75-2.82 (m)	25.5 / 25.6
C=O	-	172.9 - 173.3

4.1.4 Structural characterization of compound 4

Compound 4 was obtained as a white powder, its ESI-MS mass spectrum (Fig. 4.18), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 479, which corresponds to $[M+H]^+$, suggests a molecular mass 456 mau.

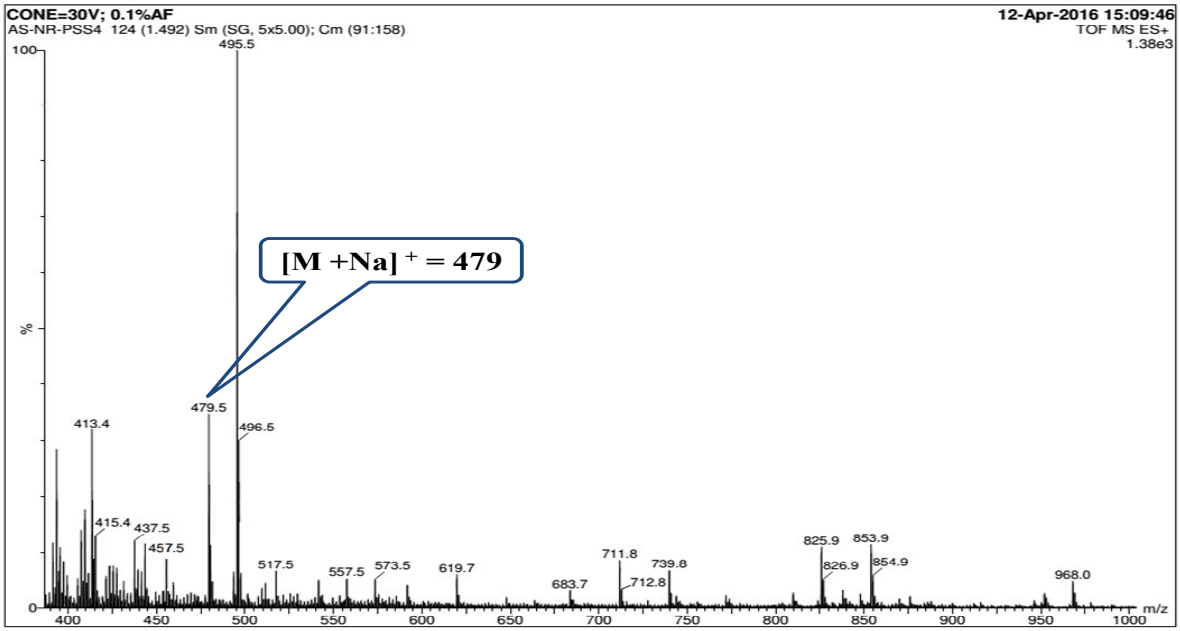


Figure. 4.18 ESI-MS mass spectrum of the compound 4

Analysis of the ^{1}H NMR spectrum of compound 4 (fig. 4.19) recorded in ($CDCl_3$+ CD_3OD) shows,

- Five signals corresponding to seven methyl groups of a pentacyclic triterpene skeleton resonating at δ 0.78 ppm (3H, s), 0.84 ppm (3H, s) , 0.88 ppm (3H, d, J= 6.4 Hz), 1.1ppm (3H, s) and 0.97 ppm (9H, m);
- Triplet at δ 5.24 ppm (H; *J* = 3.5 Hz) attributable to H-12 of a pentacyclic triterpene;
- Doublet of doublets at δ 3.17 ppm (1H; *J* = 10.5; 5.6 Hz) attributable to the proton oxymethine H-3;
- Doublet at δ 2.20 ppm (1H; J= 10.6 Hz) attributable to H-18.

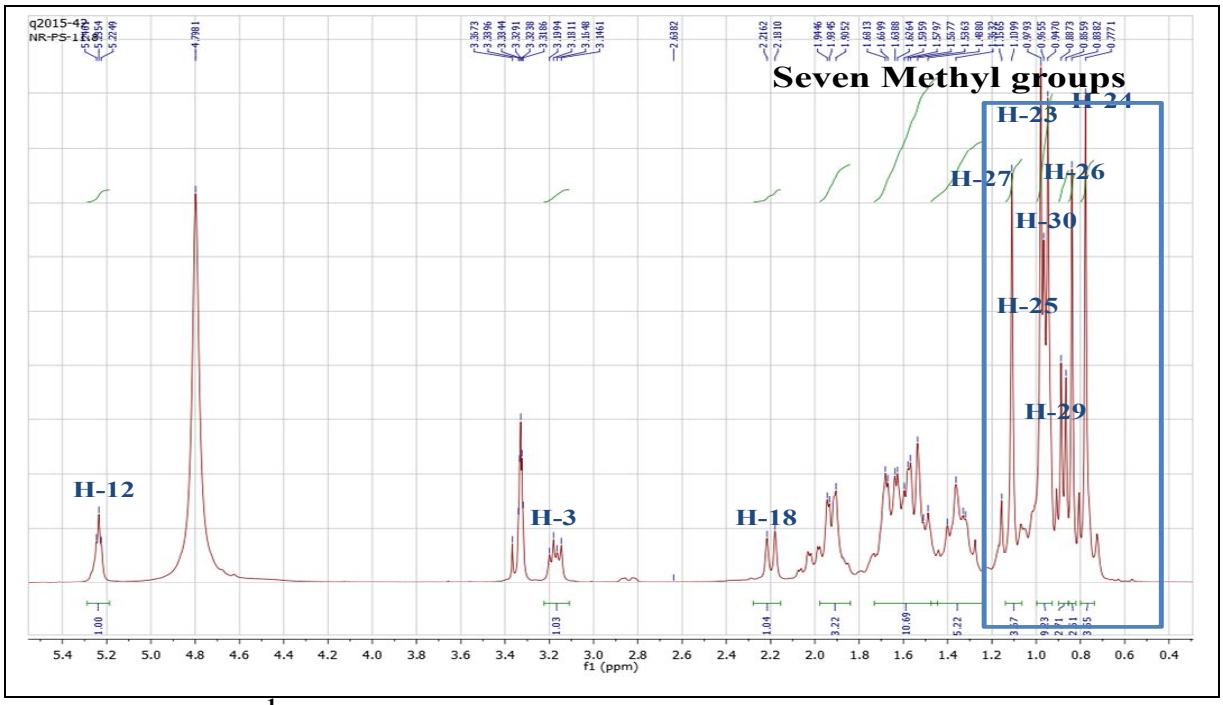


Figure. 4.19 ^{1}H NMR Spectrum of compound 4($CDCl_3$ + CD_3OD, 300 MHz)

^{13}C NMR spectrum (Fig. 4.20) reveals the presence of 30 signals distributed as following:

- Seven methyls carbons;
- Two signals appearing at δ126.37 ppm and 139.06 ppm correspond to the carbons Ethylenic C-12 and C-13 respectively;
- A signal of a carbon corresponding to the carbonyl group of an acid fonction at δ 181.35 ppm attributable to C-28;
- A signal of a carbon oxymethine at δ 77.68 ppm;
- Six quaternary carbons;
- CH_2 and CH carbons are detected.

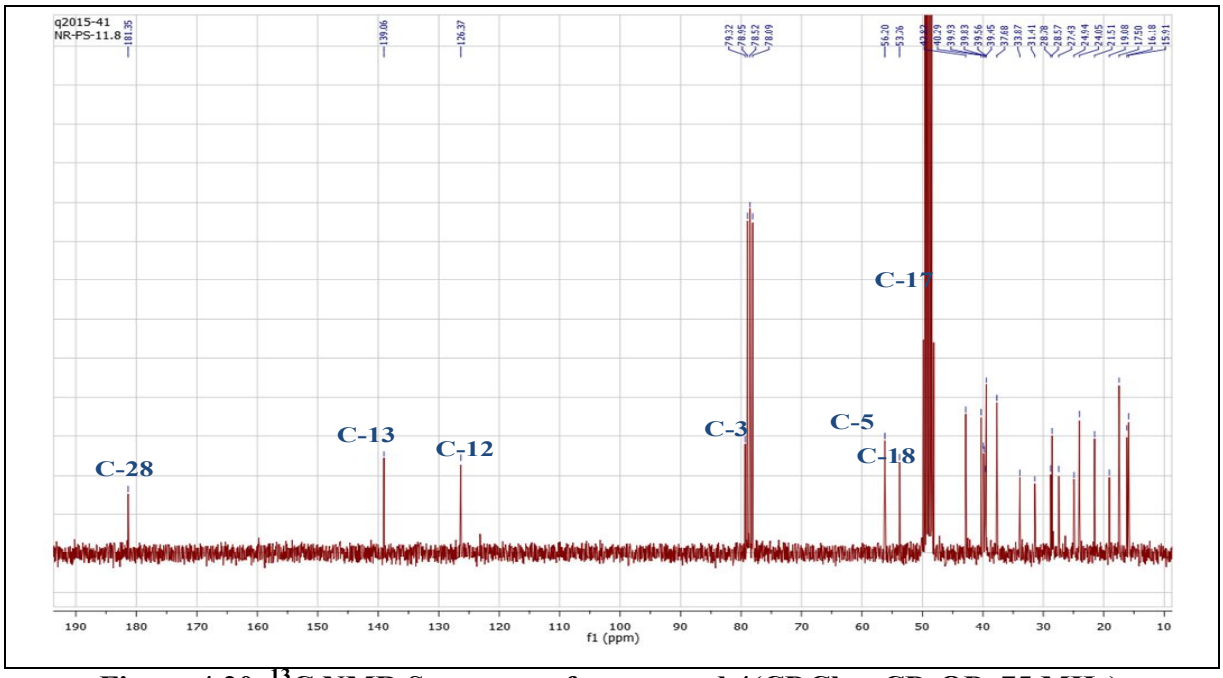


Figure 4.20. ^{13}C NMR Spectrum of compound 4($CDCl_3$ + CD_3OD, 75 MHz)

The COSY H-H experiment (Fig. 4.21) shows correlation that allows assigning the chemical shifts of several protons as follow:

- from the proton H-3 already assigned, this experiment makes it possible to identify the H-2 (1.57 ppm) which correlates with proton H-1 resonant at δ (m; 1.05 ppm; 1.60 ppm);
- H-12 correlate with H-11 resonant at δ (m; 1.92 ppm);
- H -11 also shows correlation spot with H-9(m, 1.55 ppm);
- H-18 correlate with H-19 at δ 1.34 ppm.

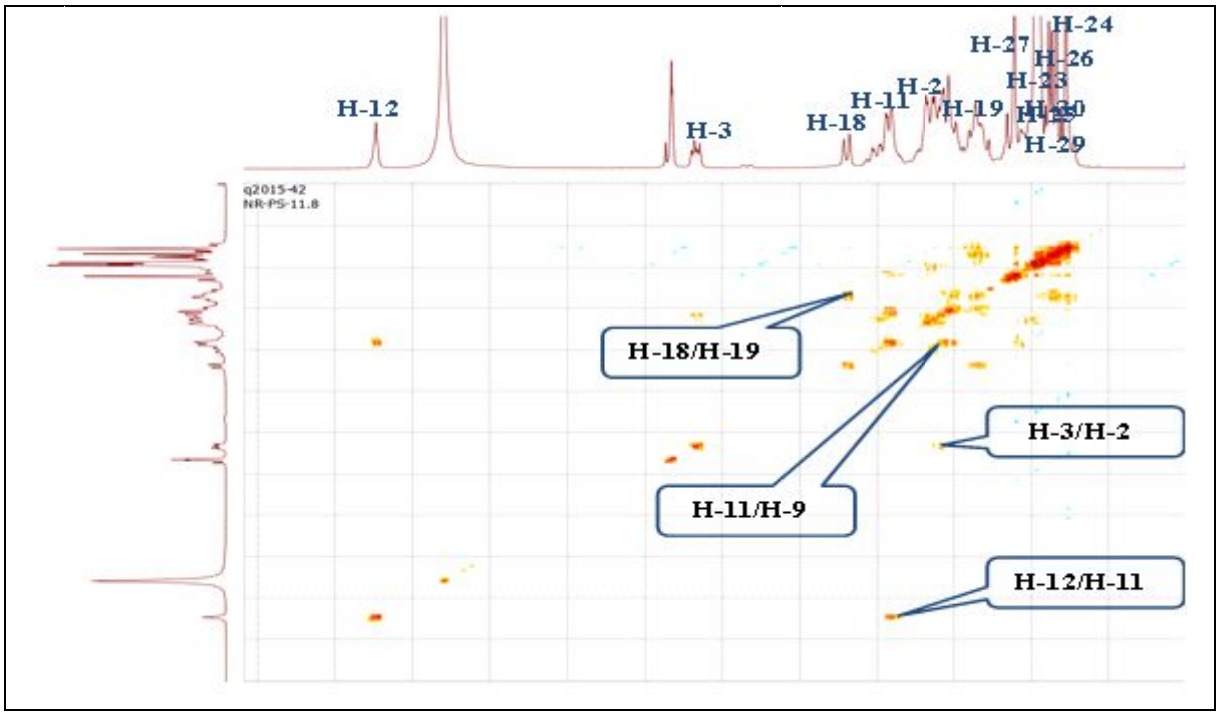


Figure. 4.21 COSY spectrum of compound 4 ($CDCl_3$ + CD_3OD, 300 MHz)

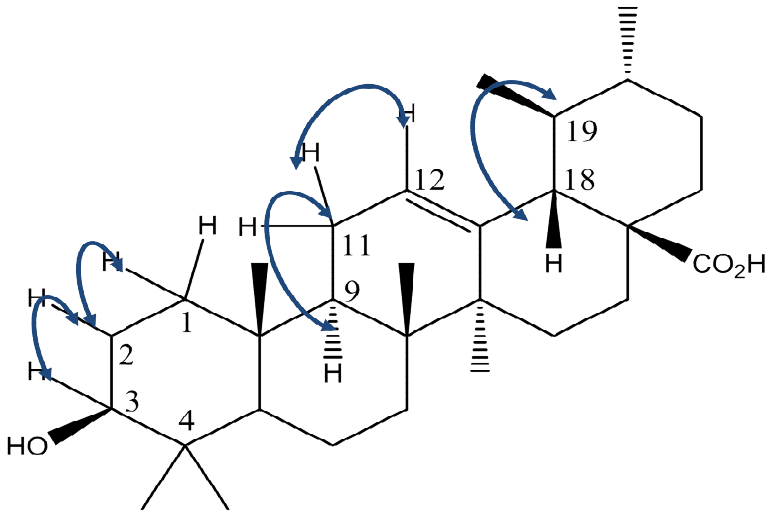


Figure. 4.22 COSY correlations

From these ^1H assignments; the carbons beared them can be deduced by HSQC experiment (Fig. 4.23), as: (C-1, C-2, C-3, C-9, C-11, C-12, C-18 and C-19) (Table. 4.4).

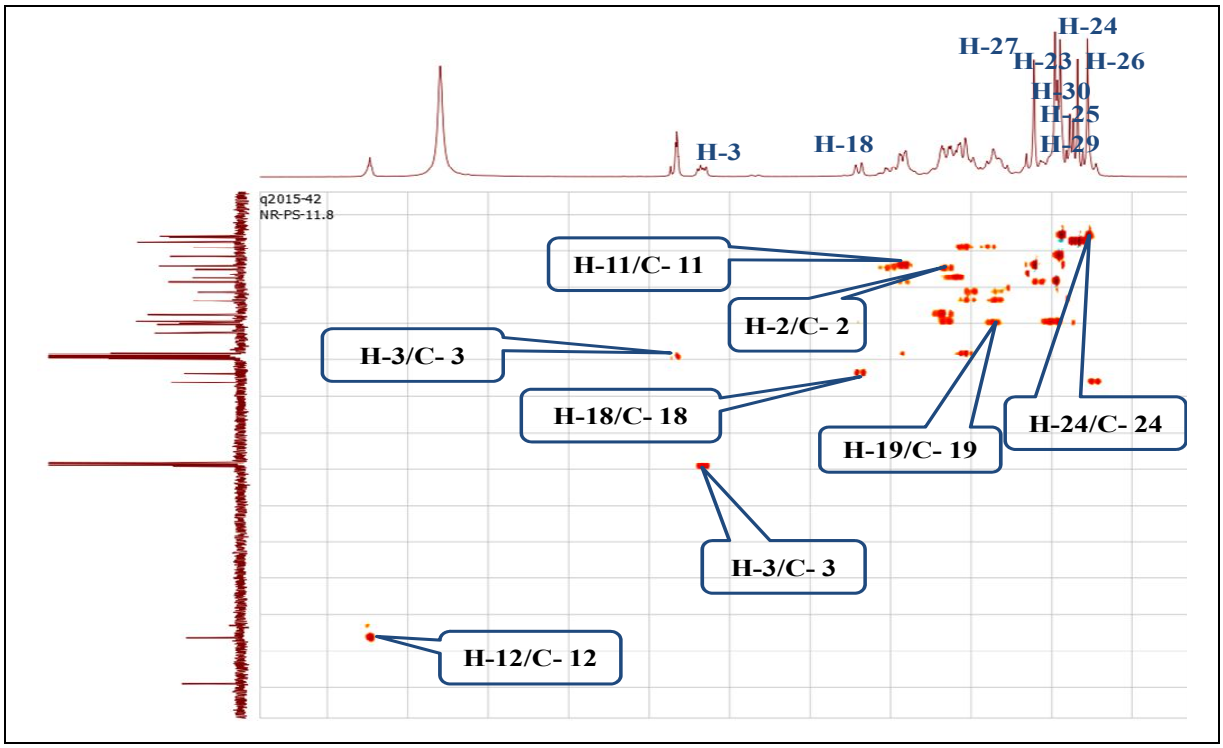


Figure. 4.23 HSQC Spectrum of compound 4($CDCl_3$ + CD_3OD, 300 MHz)

HMBC experiment (Fig. 4.24) shows the correlations between:

- The proton oxymethine H-3 and four carbons C-2, C-4, C-23 and C-24 resonate at δ 27.43, 39.83, 28.57 and 17.49 ppm respectively.

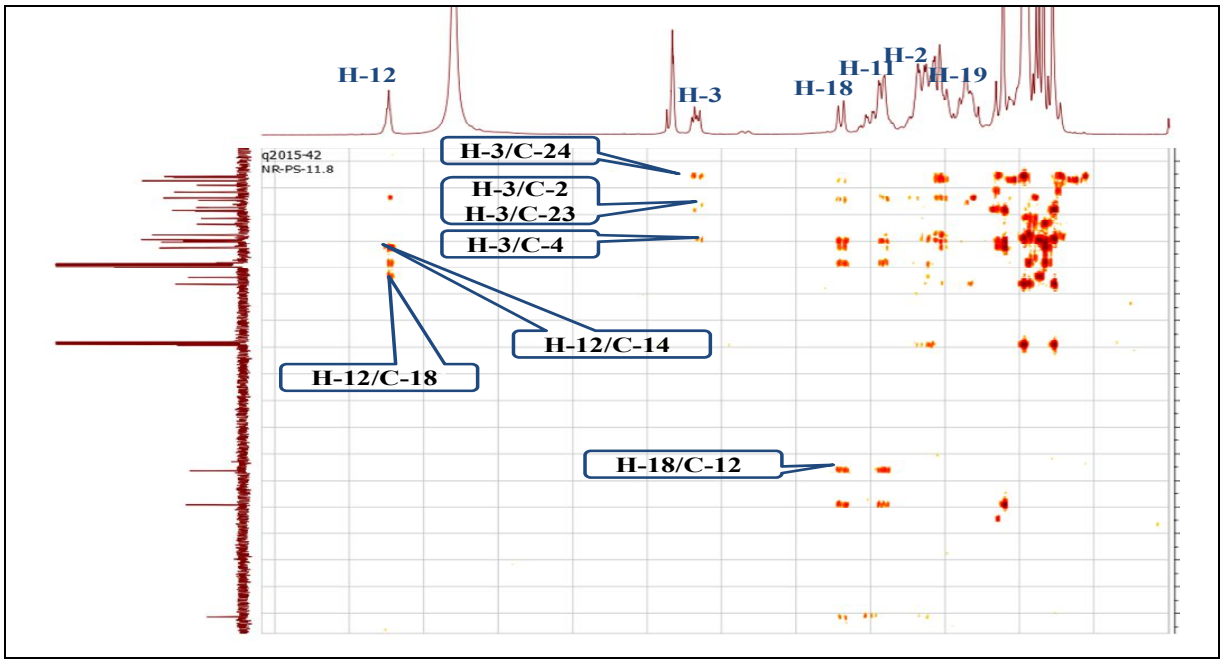


Figure 24. HMBC Spectrum of compound 4($CDCl_3$ + CD_3OD, 300 MHz)

The proton H-5 is located at δ 0.72 ppm (m) following the correlations that present in HMBC

(fig. 4.24) with a quaternary carbon at δ 37.63 ppm (C-10), already the carbons C-23 and C-24 and another methyl carbon at δ 15.9 ppm (C-25) are identified

Based on these data analysis (^{1}H, ^{13}C, HSQC, HMBC and COSY) and comparison with literature (Amzad and Zhari., 2010), the compound 4 is identified as **ursolic acid (fig. 4.25)**

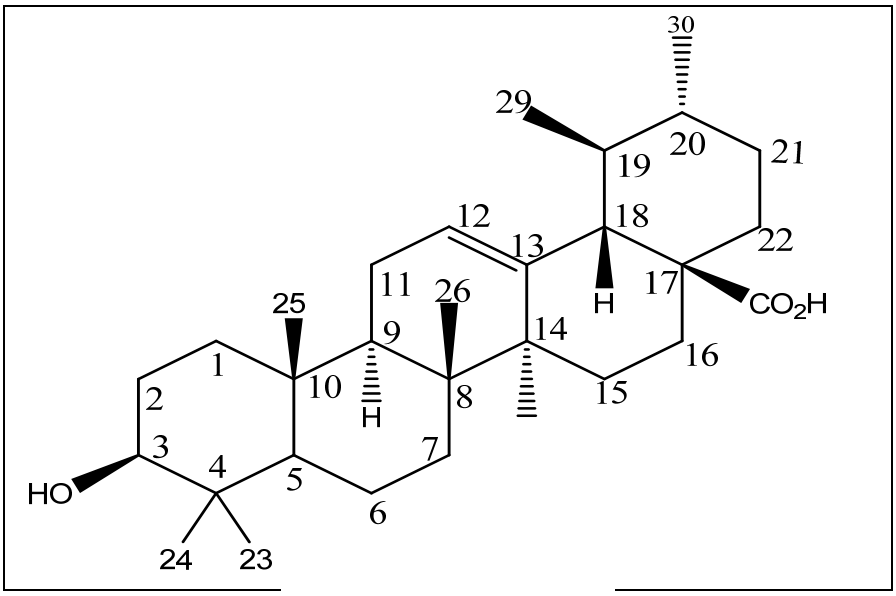


Figure. 4.25 Ursolic acid

Table 4.4. ^{1}H and ^{13}C NMR Chemical shifts values of compound 3, recorded in $CDCl_3$*(300 MHz)*

C_{number}	δ_C (ppm)	δ_H (ppm)
1	39.45	α 1.60(m) β 1.05(m)
2	27.43	1.57(m)
3	77.68	3.16(dd; J 10.5, 5.6 Hz)
4	39.83	-
5	56.20	0.72(m)
6	19.08	α 1.50(m) β 1.37(m)
7	19.08	α 1.50(m) β 1.37(m)
8	40.20	-
9	48.14	1.55(m)

10	37.63	-
11	24.11	1.92(m)
12	126.37	5.23(t, *J* 3.6 Hz)
13	139.03	-
14	42.90	-
15	28.78	α 1.05(m) β 1.87(m)
16	24.94	2.02 td, *J* 3.8; 13.8 Hz)
17	48.14	-
18	53.76	2.20(d, *J* 10.6 Hz)
19	39.93	1.34 (m)
20	39.83	0.92 (m)
21	31.41	α 1.33(m) β 1.49(m)
22	37.8	1.63 (m)
23	28.57	0.98(s)
24	17.42	0.78 (s)
25	15.91	0.95 (s)
26	17.49	0.84(s)
27	24.02	1.1 (s)
28	181.35	-
29	72.3	0.88 (d; J 6.4Hz)
30	21.51	0.97 (m)

1.5. Structural characterisation of Compound 5

Compound 5 was isolated as white crystals soluble in chloroform, its mass experiment was carried out using ESI-MS in positive mode (Figure 4.26). The mass spectrum of this compound presented an ion quasi-molecular at m/z 413 which suggested the molecular mass 412 mau corresponding to the molecular formula $C_{29}H_{48}O$.

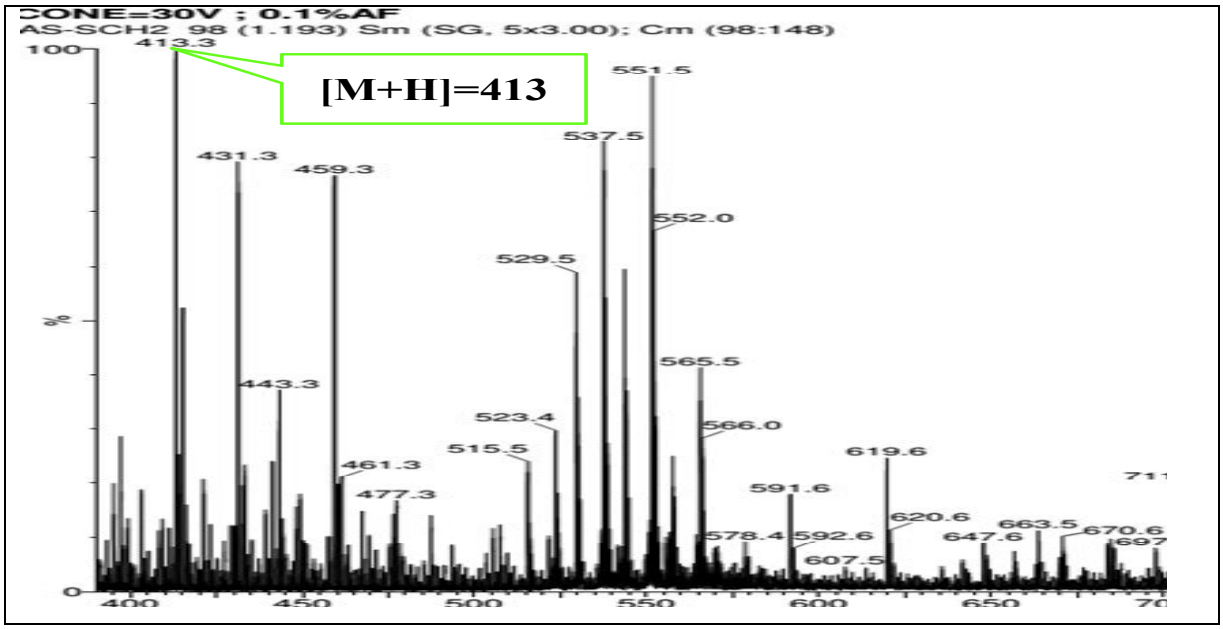


Figure 4.26 ESI-MS mass spectrum of the compound 5

^{1}H-NMR spectrum (Fig. 4.27) showed the following singals:

- Multiplets, doublets and singlets with high intensity peaks at δ 0.60-1.03 ppm, indicating the presence of six methyl groups;
- Multiplet at δ 3.53 ppm corresponding to a proton oxymethine;
- Large doublet at δ 5.35 ppm (*J*= 5.1 Hz) and two resonant signals in the form of a doublet of doublets at δ 5.01(dd, *J* = 15.1, 8.7 Hz) and 5.14 ppm (dd, *J* = 15.1, 8.7 Hz), characteristics of three olefinic protons.

From these observations, a steroidal skeleton can be attributed to compound 5.

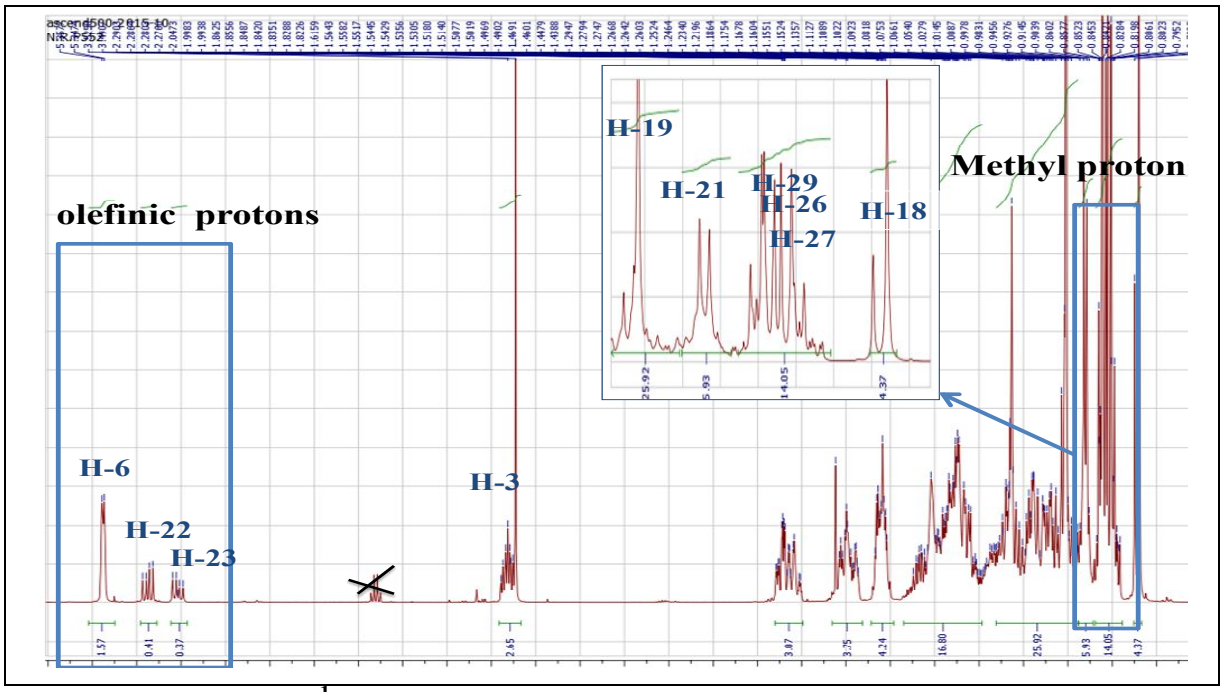


Figure. 4.27 ^{1}H NMR Spectrum of compound 5 ($CDCl_3$ + CD_3OD, 500 MHz)

According to the data obtained from ^{13}C analysis (fig. 4.28) of compounds 5 which shows:

- Six signals can be easily attributed to methyl groups occurring at 11.9 to 19.8 ppm;
- Four signals at δ 121.7, 140.7, 138.3 and 129.3 ppm corresponding to four carbons (CH and quaternary) of alkene carbons and finally a signal marked at δ 71.8 ppm indicating the presence of a methoxy group (CH_3-O-).

All these signals are characteristic of sterol skeleton.

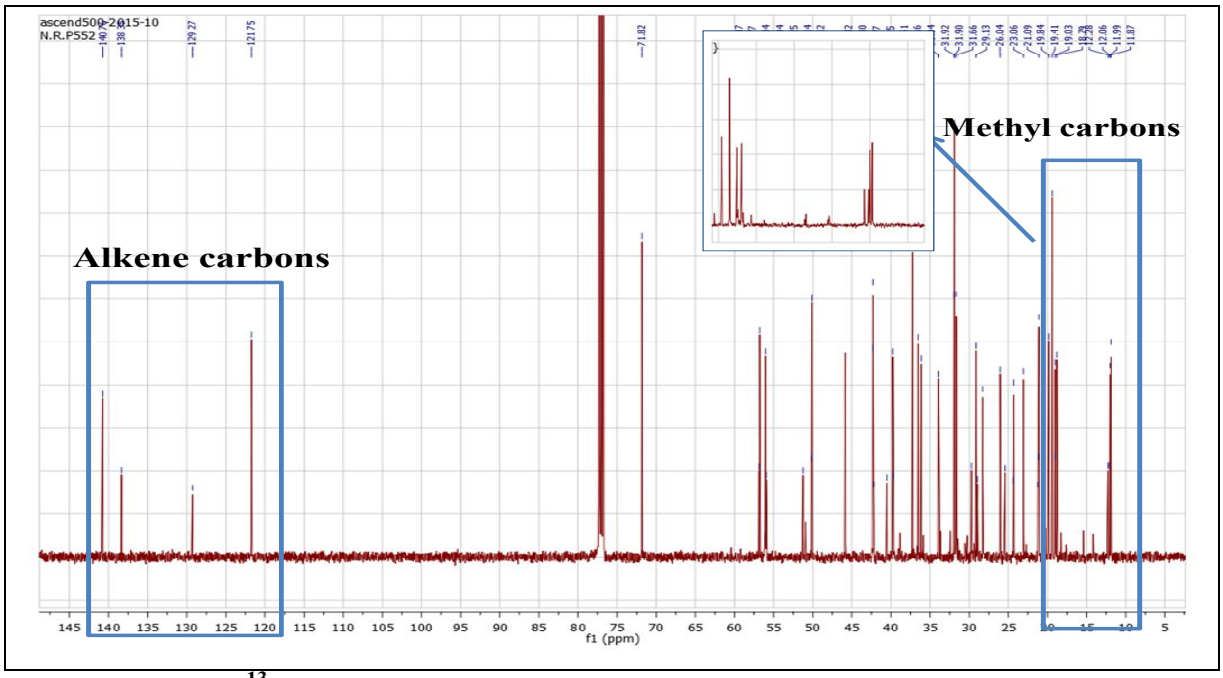


Figure 28.^{13}C NMR Spectrum of compound 5 ($CDCl_3$ + CD_3OD, 125 MHz)

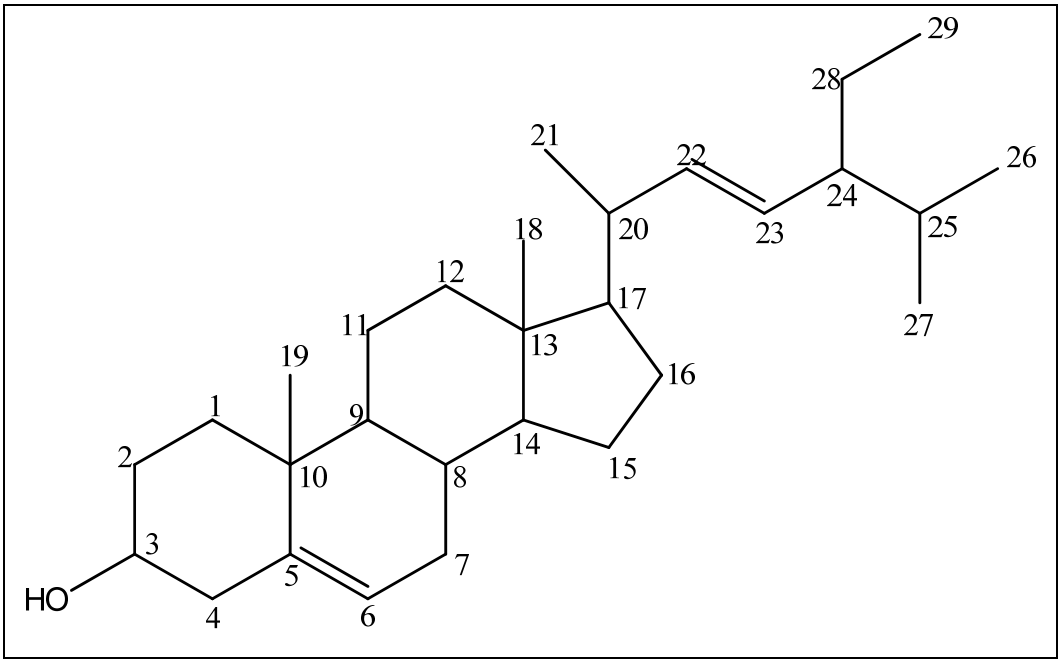


Figure. 29 Skeleton of a sterol

The proton-proton correlation experiment COSY H-H (Fig. 4.30) shows the following correlations:

- H-22 and H-23 appearing respectively at δ 5.01 ppm (dd, *J* = 15.1, 8.7 Hz) and δ 5.14 ppm (dd, *J* = 15.1, 8.7 Hz);
- H-23 and H-24 appearing at δ 1.51 ppm (m);
- H-6 and H-7 appearing respectively at δ 5.35 ppm and δ 1.98ppm (m);
- H-7 and H-8 appears at 1.47 ppm;
- H-3 (3.53 ppm) with H-4 and H-2 protons appearing respectively at δ 2.27(m) and 1.84 ppm.

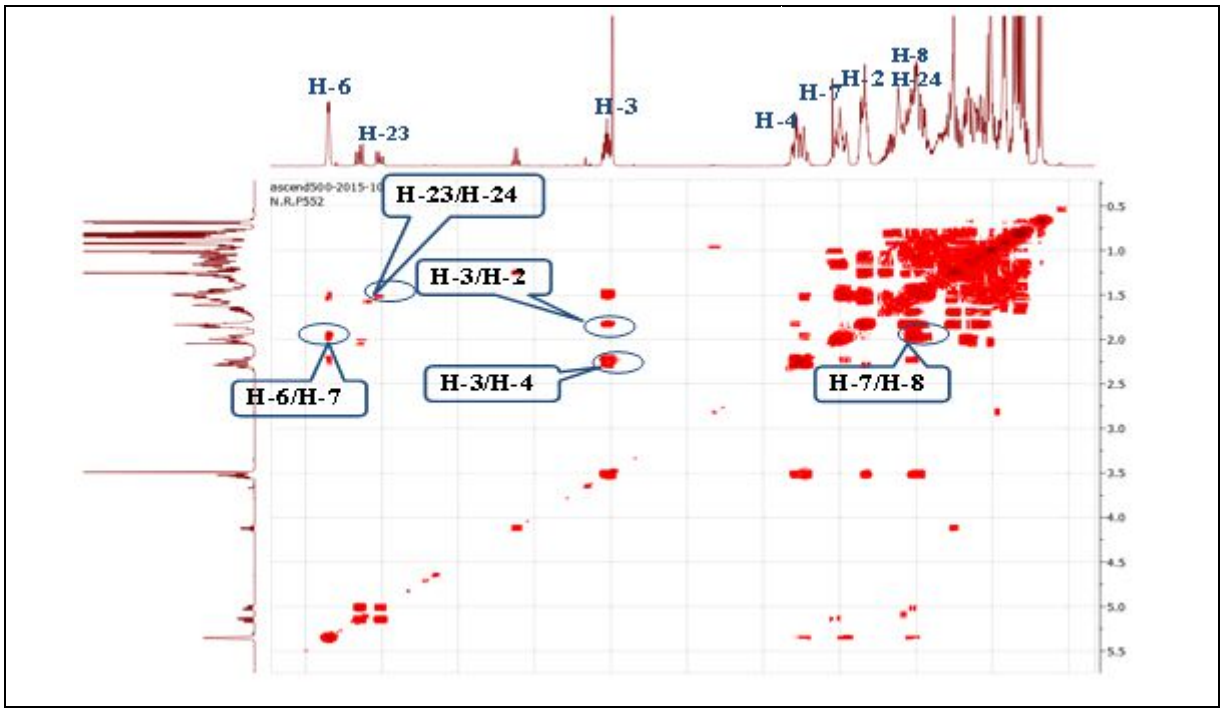


Figure. 4.30 COSY Spectrum of compound 5 ($CDCl_3$ + CD_3OD, 500 MHz)

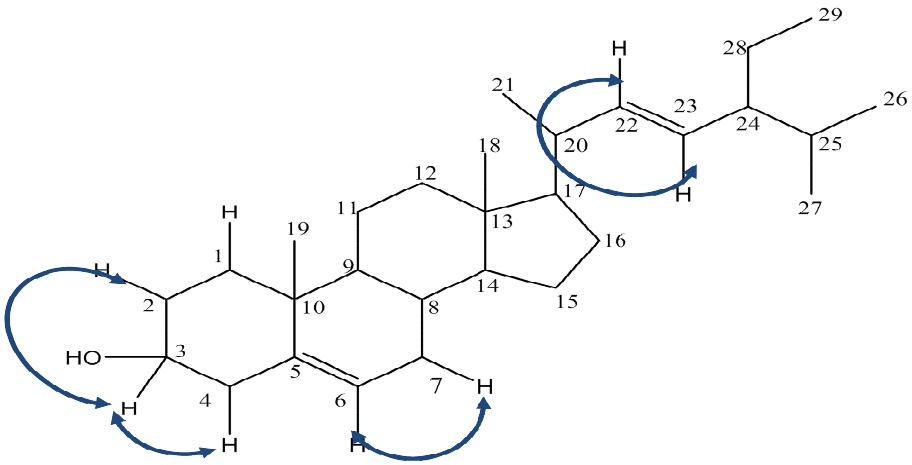


Figure. 4.31 COSY correlations

The HSQC carbon-proton correlation experiment (Fig. 4.32) allowed us to attribute protons to their carbons. Indeed we observe the couplings between:

- H-3 (3.53 ppm) and its carbon C-3 at δ 71.82 ppm;
- H-6 (5.35 ppm) and its carbon C-6 at δ 121.7 ppm;
- H-29 (0.81 ppm) and its carbon C-29 at δ 12.28 ppm;
- H-18 (0.68 ppm)and its carbon C-18 at δ 12.06 ppm;
- H-22 (5.01 ppm) and its carbon C-22 at δ 138.3 ppm;
- H-23 (5.14 ppm) and its carbon C-23 at δ 129.3 ppm;
- H-24 (1.51ppm) and its carbon C-24 at δ 51.2 ppm;
- H-8(1.47 ppm) and its carbon C-8 at δ 31.82 ppm;
- H-2 (1.84 ppm) and its carbon C-2 at δ 31.66 ppm;
- H-7 (1.50 ppm) and its carbon C-7 at δ 31.92 ppm.

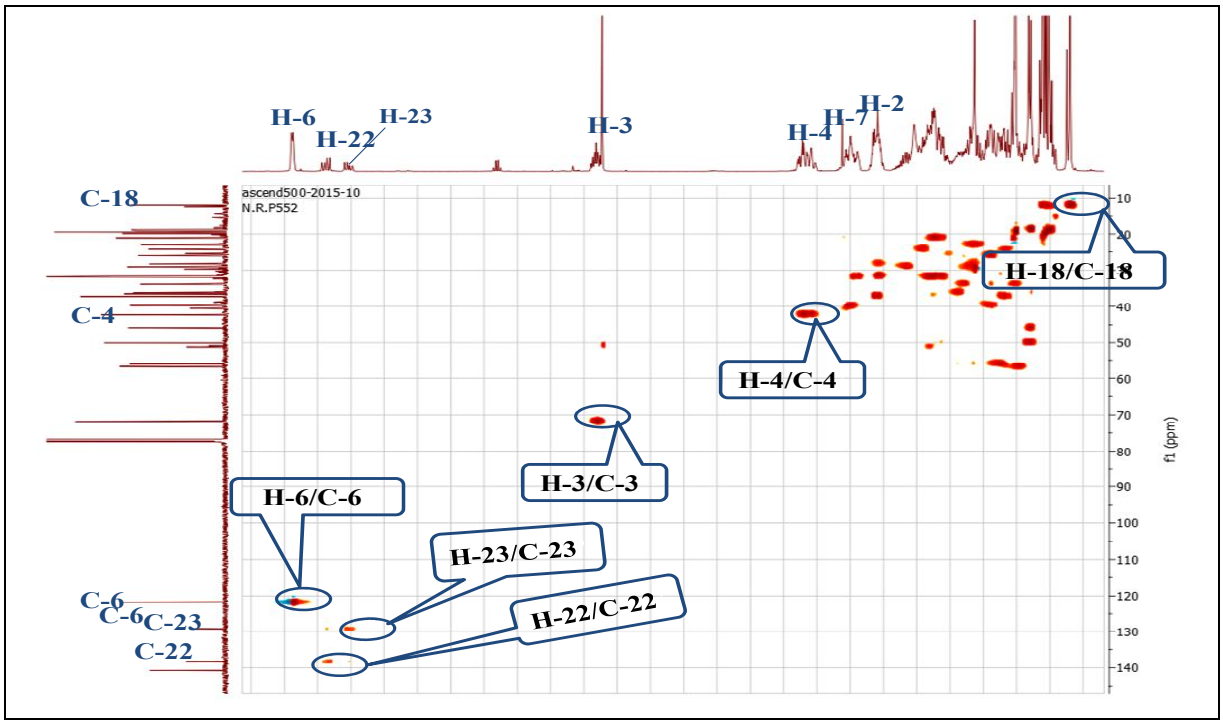


Figure. 4.32 HSQC Spectrum of compound 5 ($CDCl_3$ + CD_3OD, 500 MHz)

For more confirmations, the HMBC experiment shows long-distance two- and three-links carbon- proton correlations as it is clear in figures 4.33, 4.34, 4.35 and 4.36.

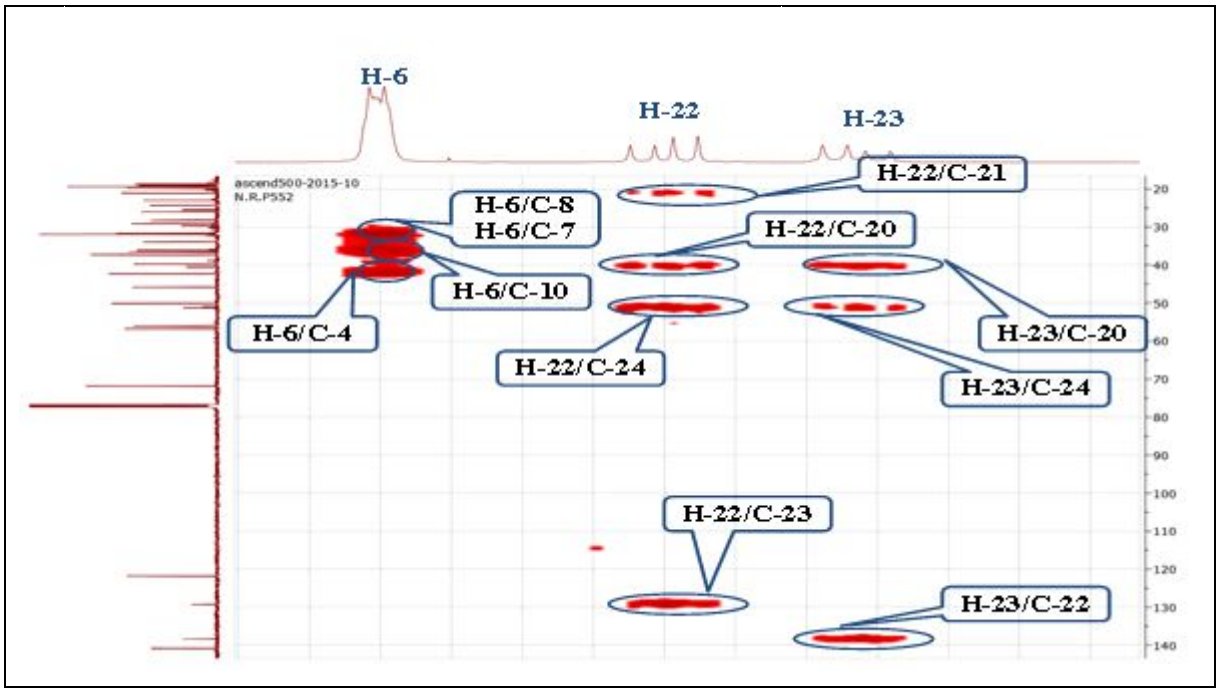


Figure. 4.33 HMBC Spectrum of compound 5 ($CDCl_3 + CD_3OD$, 500 MHz)

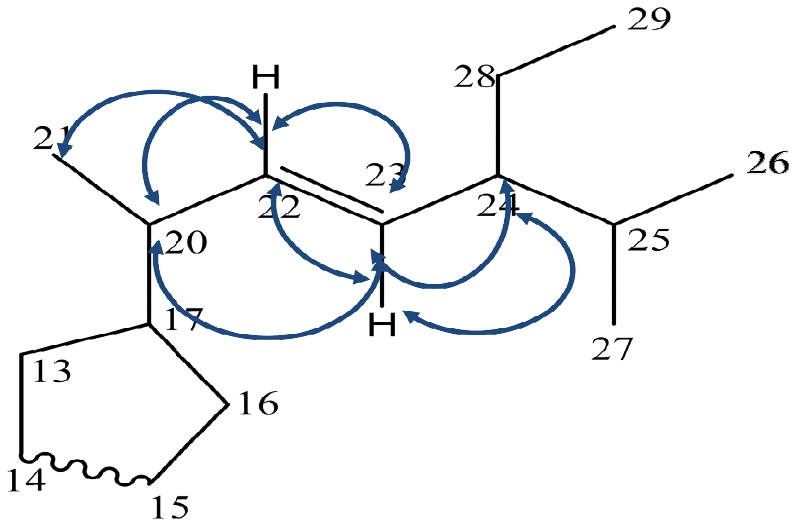


Figure. 4.34 HMBC correlations of compound 5

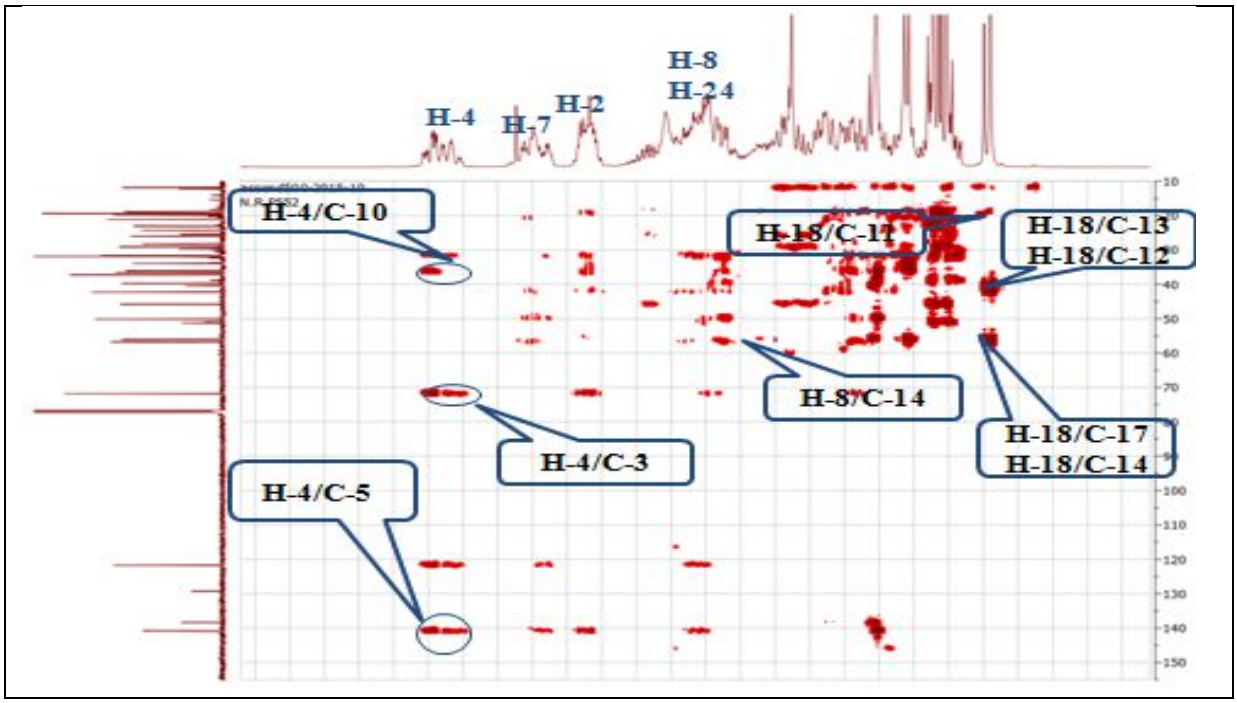


Figure. 4.35 HMBC Spectrum of compound 5 ($CDCl_3 + CD_3OD$, 500 MHz)

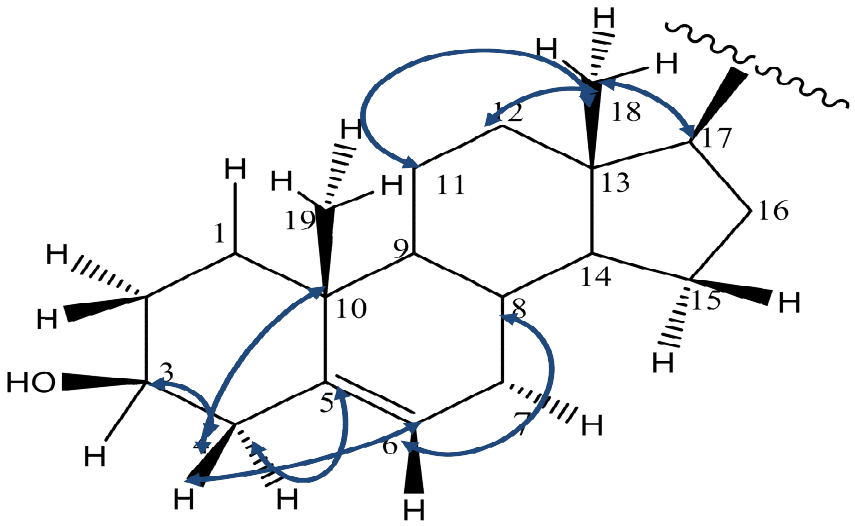


Figure. 4.36 HMBC Correlations of compound 5

Based on the above mentioned data, which is more detailed in table 4.5, and also comparing with literature (Pierre and Moses., 2015) data, was possible to establish that compound 1 is a Stigmasterol. According to the literature (Pierre and Moses., 2015) β-sitosterol and Stigmasterol are always in a mixture form in which may have maximum portion of stigmasterol. It is very difficult to obtain Stigmasterol in pure state.

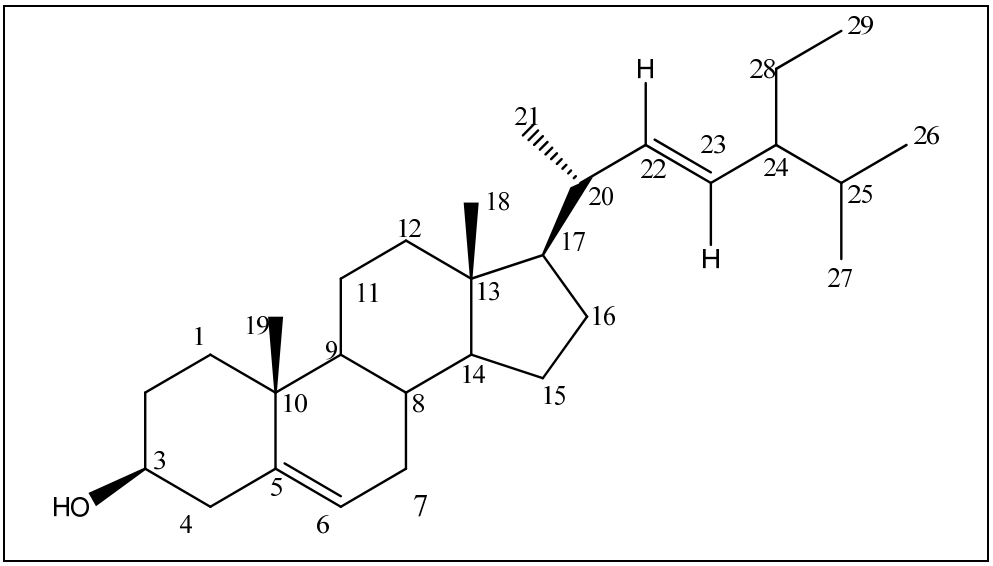


Figure. 4.37 stigmasterol

Table 4.5. ^{1}H and ^{13}C NMR Chemical shifts values of compound 5, recorded in $CDCl_3$ *(300 MHz)*

C_{number}	δ_H (ppm)	δ_C (ppm)
1	1.10(m) 1.86(m)	37.25
2	1.84(m)	31.66
3	3.53(m)	71.82
4	2.27(m)	42.32
5	-	140.76
6	5.35 (d, *J* 5.1Hz)	71.82
7	1.50 1.96	31.92
8	1.47(m)	31.92
9	0.93	50.12
10	-	36.52
11	1.49	21.07
12	1.17 2.10	39.72
13	-	42.30
14	1.04(m)	56.77
15	1.27 1.48	56.77
16	1.25 1.58	28.95
17	1.20	55.83

18	0.68(s)	12.06
19	1.01(s)	19.38
20	2.00	40.52
21	0.92(d, *J* 6.6Hz)	21.17
22	5.14 (dd, *J* 15.1, 8.7 Hz)	138.35
23	5.01(dd, *J* 15.1, 8.7 Hz)	129.27
24	1.51(m)	51.24
25	1.48(m)	31.9
26	0.84(d; *J* 6.4Hz)	21.09
27	0.79(d; *J* 6.4Hz)	19.02
28	1.12(m) 1.44(m)	25.41
29	0.81(t; *J* 7.1)Hz	0.88 (d; J 6.4Hz)

4.1.6. Structural characterisation of Compound C3

Compound C3 was obtained as a yellow amorphous solid, its ESI-MS mass spectrum (Fig. 4.38), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 383, which corresponds to $[2M+Na]^+$, suggests a molecular mass 180 mau.

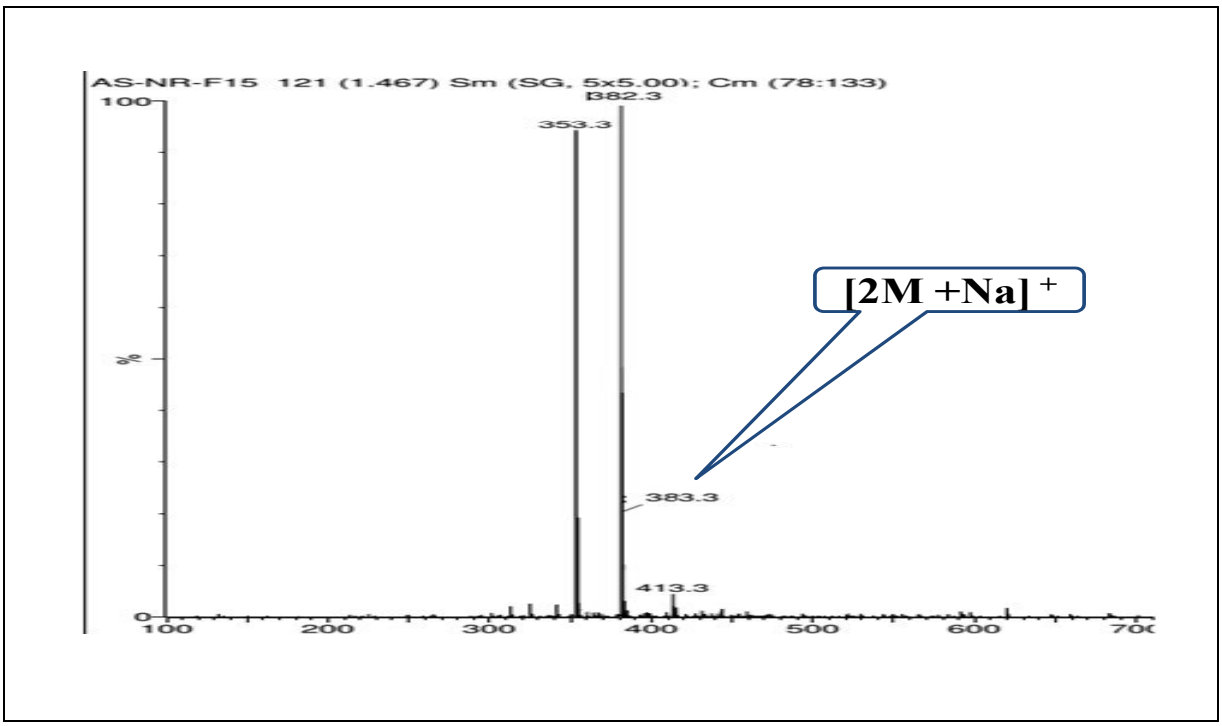


Figure. 4.38 ESI-MS mass spectrum of the compound C3

Analysis of ^{1}H NMR spectrum (fig. 4.39) shows the following peaks:

- Doublet with 1H of integration at δ 7.17 ppm (J = 2.1 Hz) attributed to H-2;
- Doublet of doublet with 1H of integration at δ 7.04 ppm (J_{H6-H2} = 2.1, J_{H6-H5} 8.2 Hz);
- Doublet with 1H of integration at δ 6.88 ppm (J = 8.2 Hz);

Are characteristic peaks of an aromatic compound tri-substituted benzene ring.

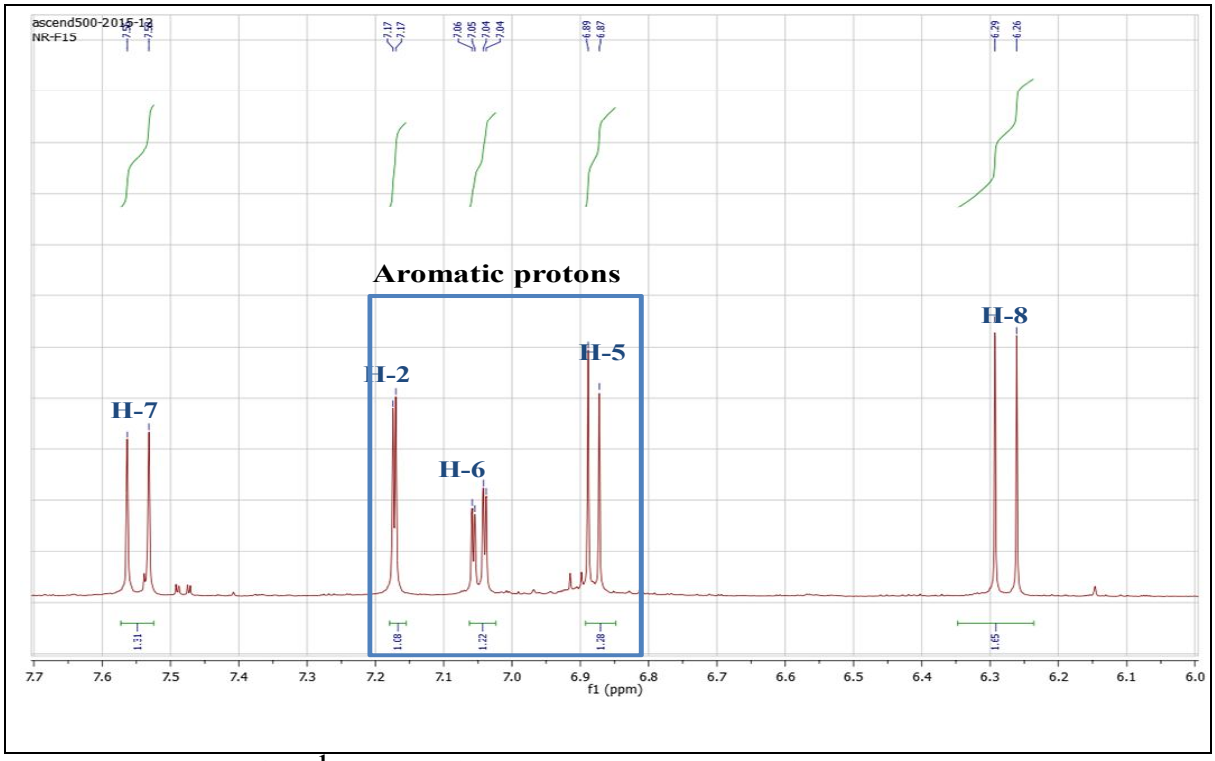


Figure. 4.39 ^{1}H NMR Spectrum of compound C3 ($CDCl_3$, 500 MHz)

- Doublet at δ 7.55 ppm (J, 15.7 Hz) and 6.28 ppm (J, 15.7 Hz) shows the presence of ethylenic protons (CH=CH) protons of trans configuration.

The ^{13}C NMR spectrum (fig. 4.40) and HSQC (fig. 4.41) of compound C3 show the presence of nine carbon atoms:

- Carbonyl of an acid function at δ 168.05 ppm;
- Three quaternary carbons one at δ 127.70 ppm and the others are oxygenated appears at δ 146.25 and 148.62 ppm;
- Five methine (CH) groups including two ethylenic at δ (145.80 and 115.76 ppm) and three aromatic at δ (115.06, 116.30 and 122.39 ppm).

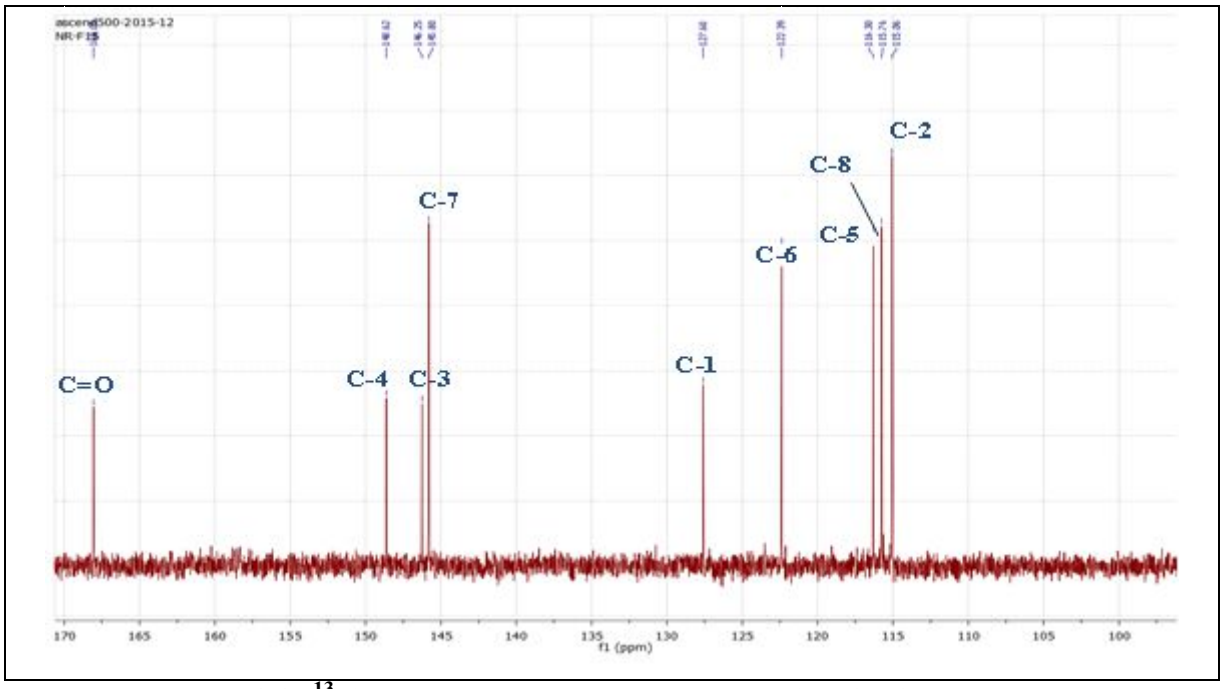


Figure. 4.40 ^{13}C NMR Spectrum of compound C3 ($CDCl_3$, 125 MHz)

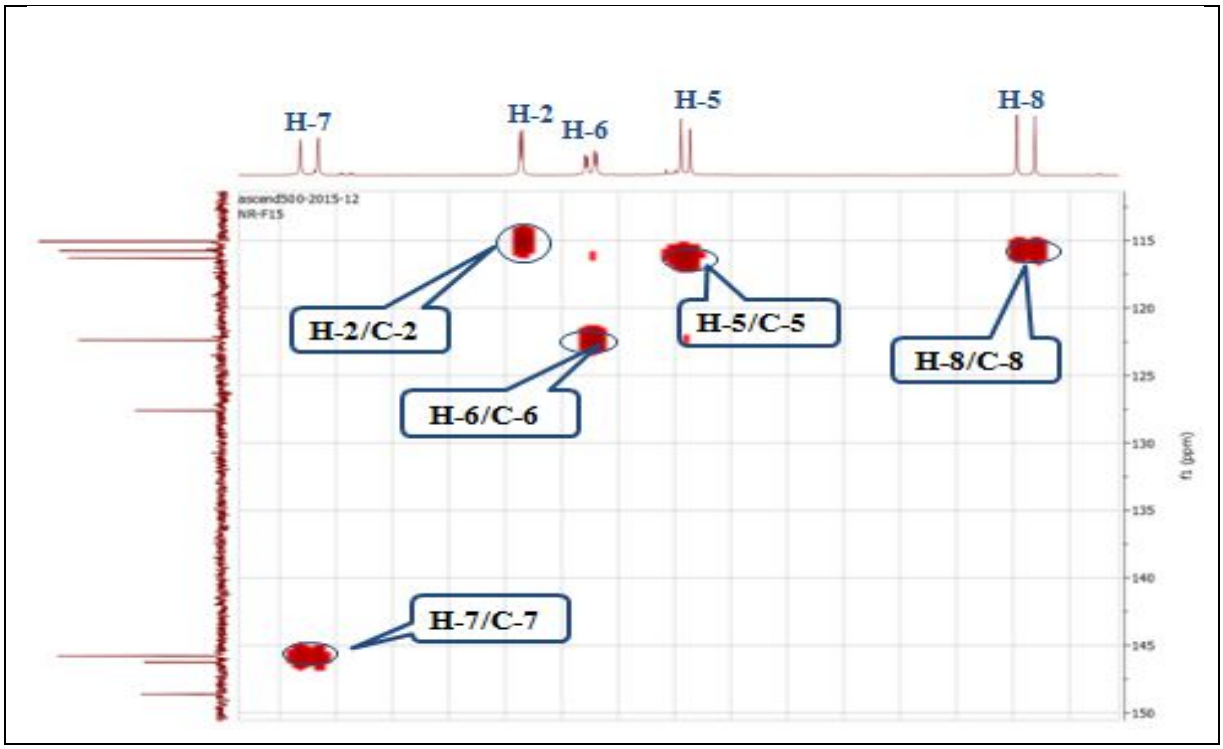


Figure. 4.41 HSQC Spectrum of compound C3 ($CDCl_3$, 500 MHz)

The structure is confirmed by HMBC spectrum analysis (fig. 4.42) which shows correlations between protons and carbons in long distance:

- H-7 gives signals correlated to carbons C-1, C-6, C-8 and C-9;
- H-8 gives a signals correlated to carbons C-1 and C-9.

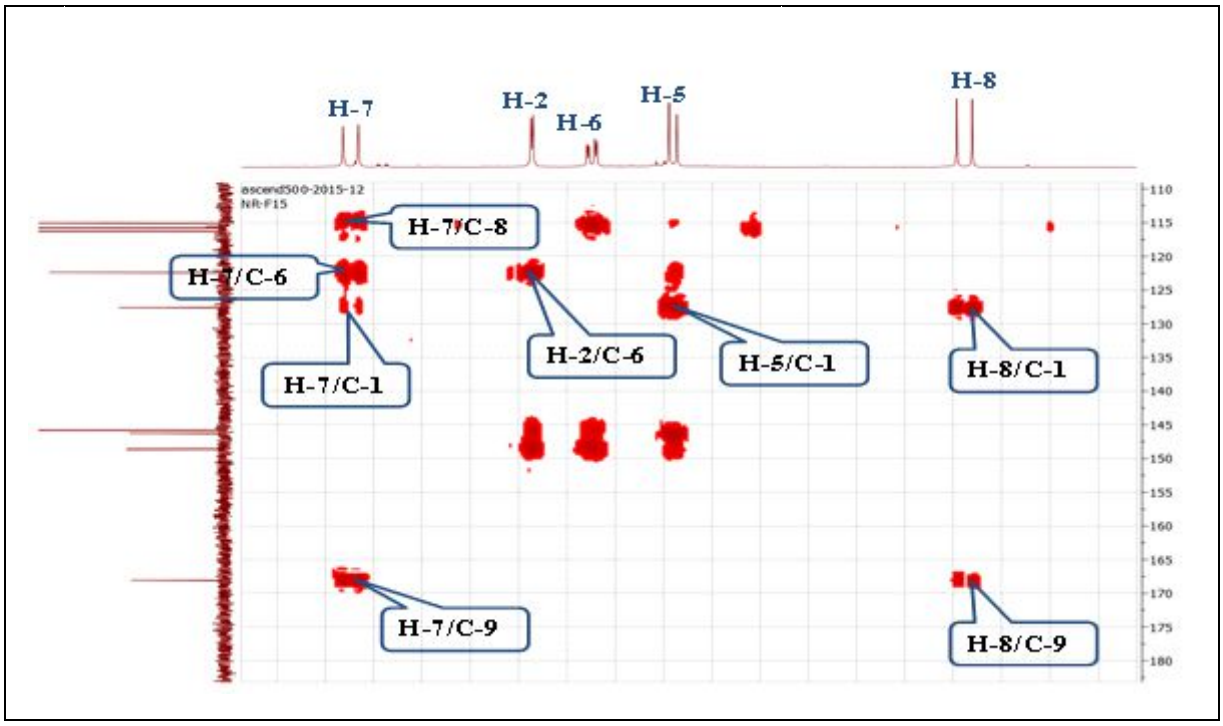


Figure. 4.42 HMBS Spectrum of compound C3 ($CDCl_3$, 500 MHz)

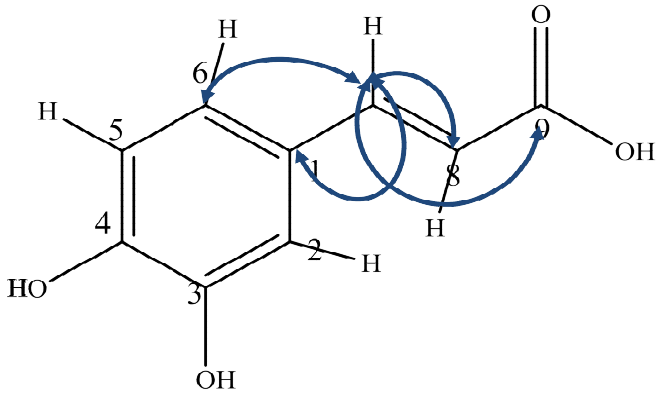


Figure. 4.43 HMBC correclations

Data collected from (^{1}H, ^{13}C, HSQC, COSY and HMBC) epectra analysis are in favor of **caffeic acid** structure hence compound 3 can be established as shown in figure. 4.44

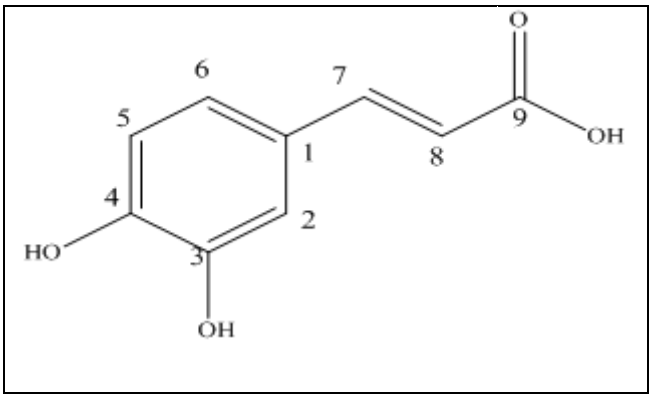


Figure. 4.44 Caffeic acid

Table 4.6. ^{1}H and ^{13}C NMR Chemical shifts of compound C3, recorded in $CDCl_3$ *(500 MHz)*

Position	δ_H *ppm*	δ_C ppm
1	-	127.60
2	7.17 (d; *J* 2.1 Hz)	115.06
3	-	146.25
4	-	148.62
5	6.88 (d; *J* 8.2 Hz)	116.30
6	7.04 (dd; *J* 8.2, 2.1 Hz)	122.39
7	7.55 (d; *J* 15.9 Hz)	145.80
8	6.28 (d; *J* 15.9 Hz)	115.76
9	-	168.05

4.1.7 Structural characterisation of Compound C4

Compound C4 was obtained as a yellowish amorphous solid, its ESI-MS mass spectrum (Fig. 4.45), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 247, which corresponds to $[M+H]^+$, suggests a molecular mass 246 mau.

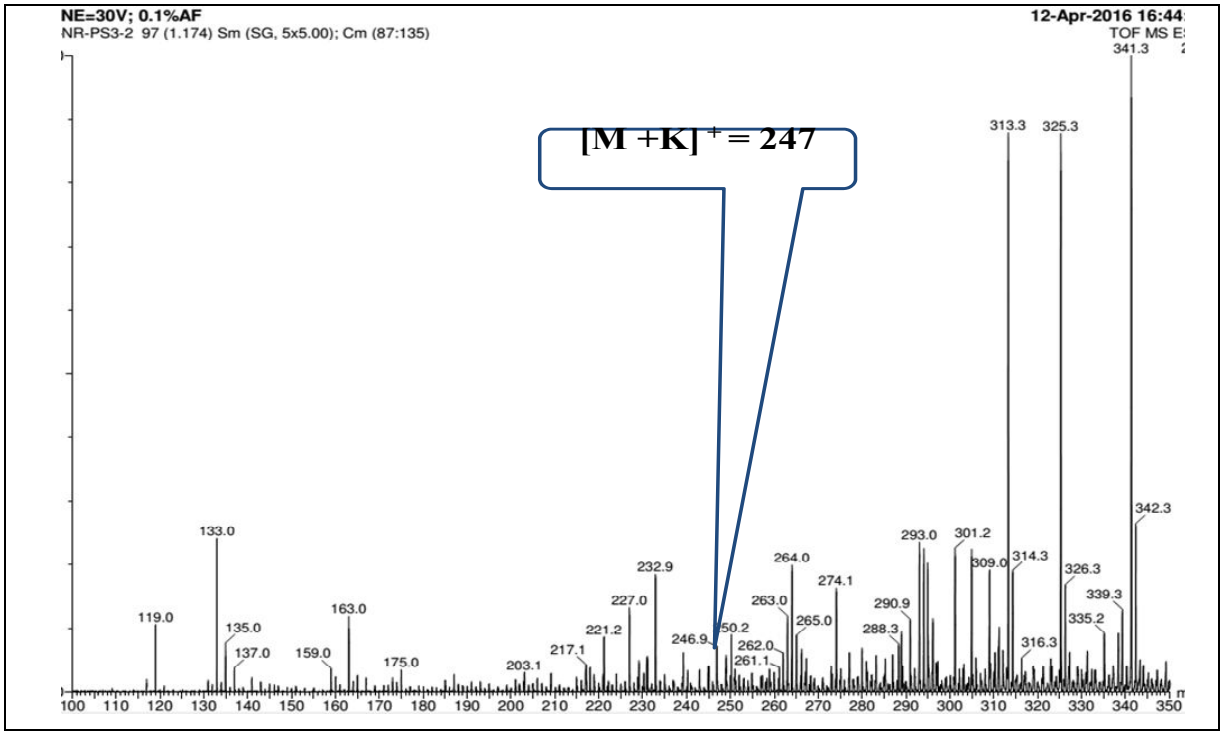


Figure. 4.45 ESI-MS mass spectrum of the compound C4

Analysis of ^{1}H NMR spectrum of compound C4 recorded in CD_3OD (fig. 4.46) that it has a very similar structure of compound C3. The only difference is the existence of two additional signals corresponding to 3-H, 2-H at δ 1.33 ppm (t, *J*= 7.1 Hz); 4.23 ppm (dd , *J*= 7.2 , 14.3 Hz)

The remaining atoms assignments are still similar;

- Four doublets of integration 1H at δ 7.04 ppm (*J* = 2.0 Hz), 6.79 ppm (*J*= 8.2 Hz), 7.55ppm (*J*=15.9 Hz) and 6.27 ppm (*J*= 15.9 Hz) attributable respectively to H-2, H-5, H-7 and H-8;
- Doublet of doublet of integration 1H at δ 6.96 ppm (*J* = 2.0, 8.2 Hz) attributable to H-6.

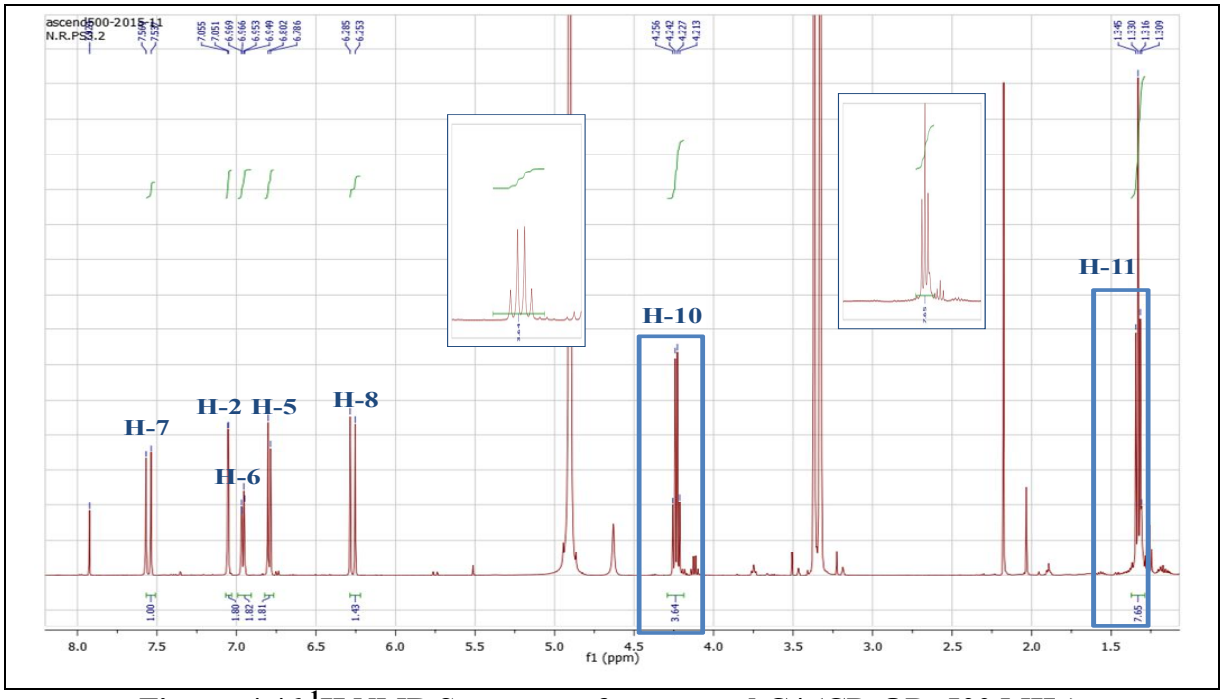


Figure. 4.46 ^{1}H NMR Spectrum of compound C4 (CD_3OD, 500 MHz)

Analysis of ^{13}C NMR spectrum (fig. 4.47) and HSQC spectrum (fig. 4.48) indicate chemical shift of all molecule's carbons

C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-9, C-10 and C-11 (respectively; 127.70, 115.05, 146.74, 149.56, 116.47, 122.89, 146.83, 115.22, 169.33, 61.41 and 14.63 ppm)

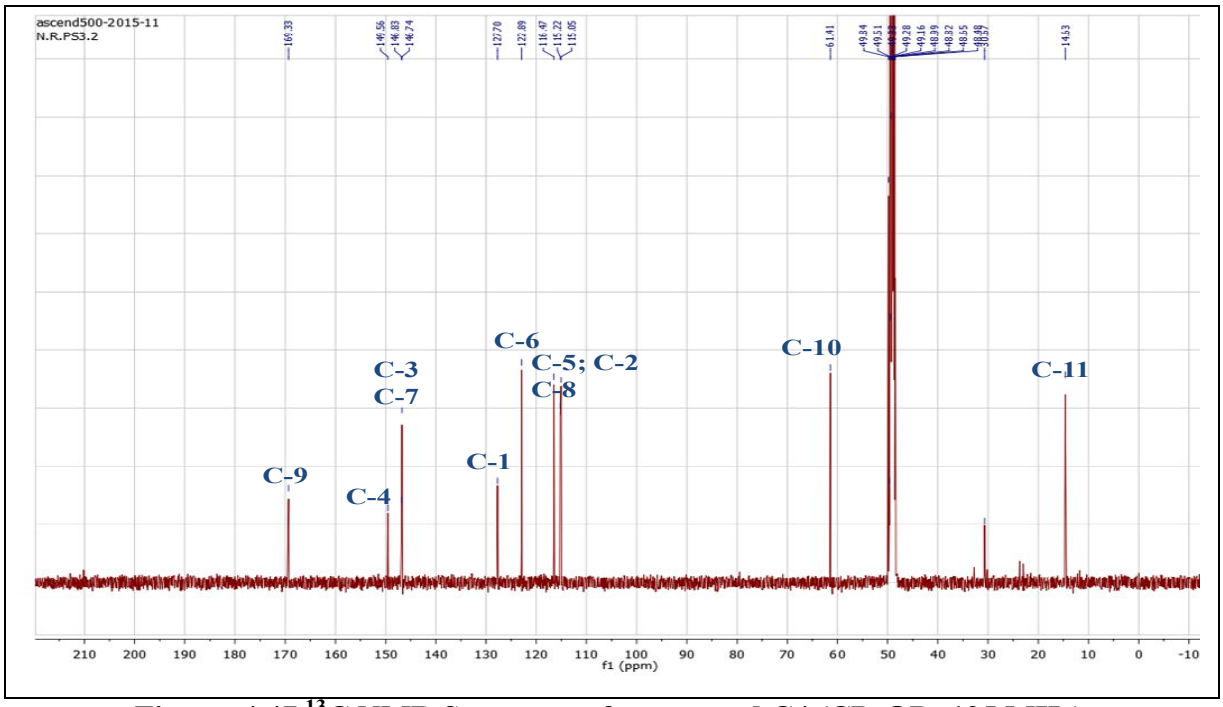


Figure. 4.47 ^{13}C NMR Spectrum of compound C4 (CD_3OD, 125 MHz)

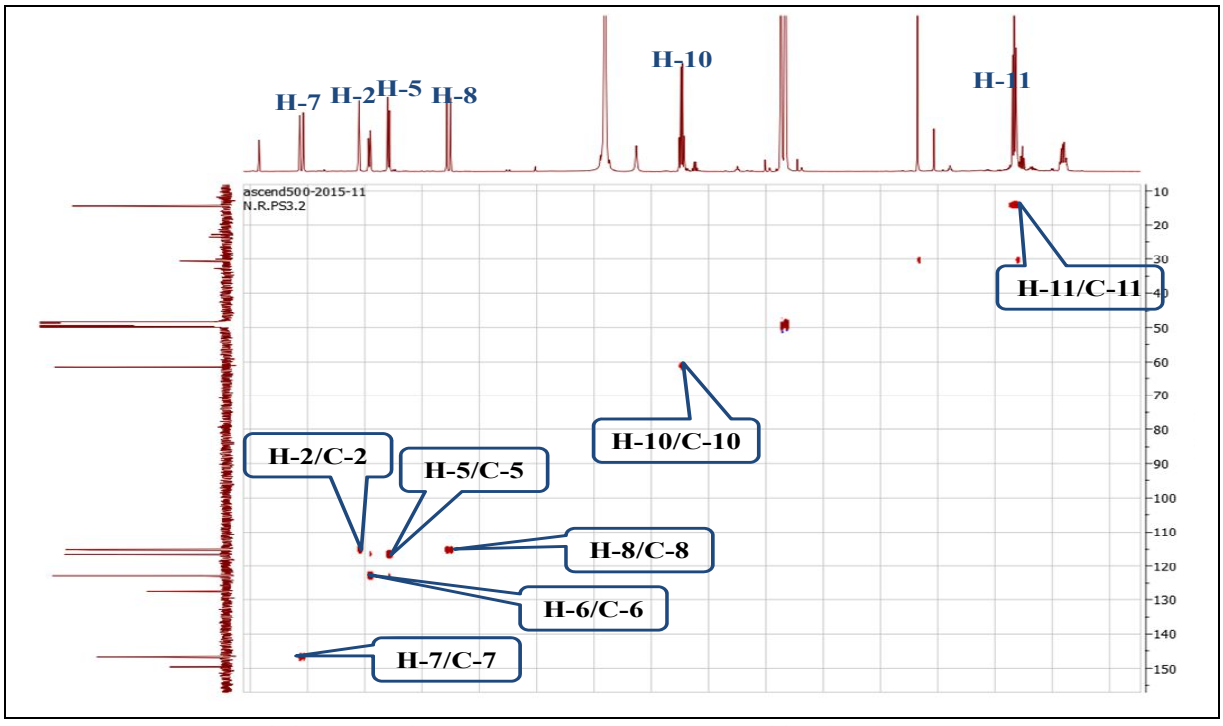


Figure. 4.48 HSQC Spectrum of compound C4 (CD_3OD, 500 MHz)

HMBC experiment (Fig. 4.49) shows correlations: H-11 with C-10 and H-10 with C-9 which confirms the structure of the compound C4 as **ethyl caffeate** (fig. 4.50).

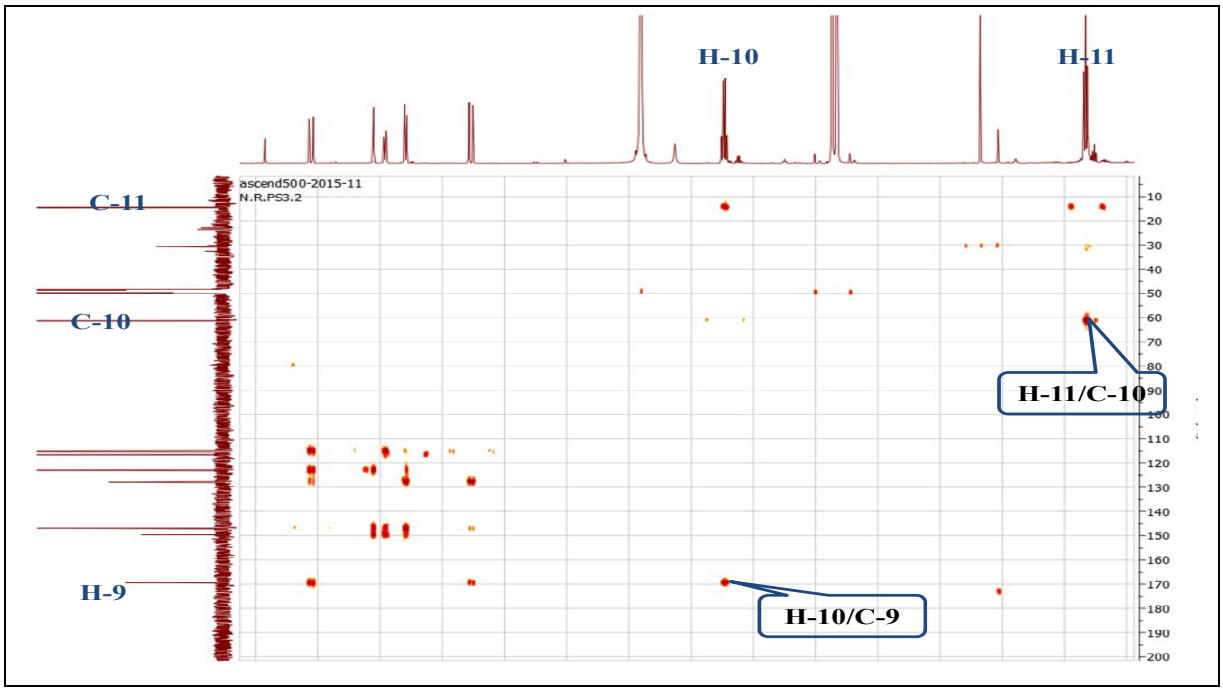


Figure. 4.49 HMBC Spectrum of compound C4 (CD_3OD, 500 MHz)

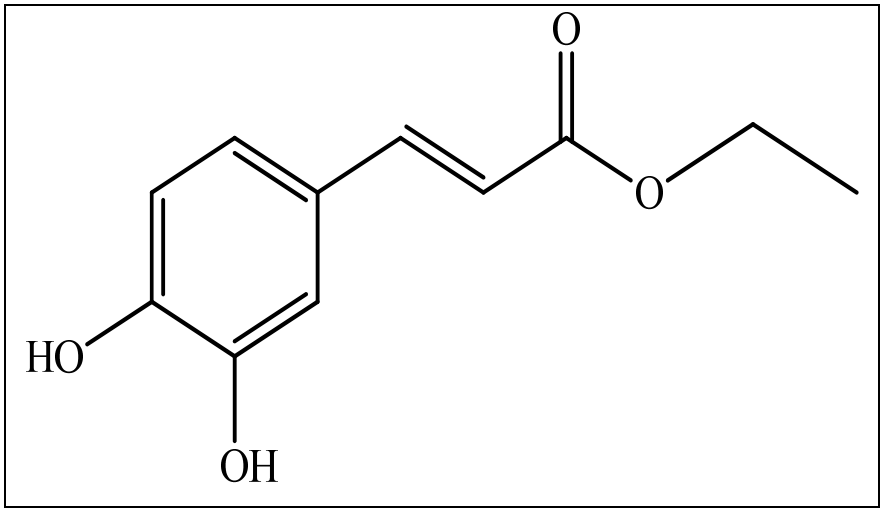


Figure. 4.50 Ethyl caffeate

Table 4.7. ^{1}H and ^{13}C NMR Chemical shifts of compound C4, recorded in CD_3OD *(500 MHz)*

Postion	δ_H *ppm*	δ_C *ppm*
1	-	127.70
2	7.04 (d; *J* 2.0 Hz)	115.05
3	-	146.74

4	-	149.56
5	6.79 (d; *J* 8.2 Hz)	116.47
6	6.96 (dd; *J* 8.2, 2.0 Hz)	122.89
7	7.55 (d; *J* 15.9 Hz)	146.83
8	6.28 (d; *J* 15.9 Hz)	115.22
9	-	169.33
10	4.23 (dd; *J* 7.2, 14.3 Hz)	61.41
11	1.33 (t; *J* 7.1 Hz)	14.63

4.1.8. Structural characterisation of Compound C2

Compound C2 was obtained as a yellowish amorphous solid, its ESI-MS mass spectrum (Fig. 4.51), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 271, which corresponds to $[M+H]^+$, suggests a molecular mass 270 mau.

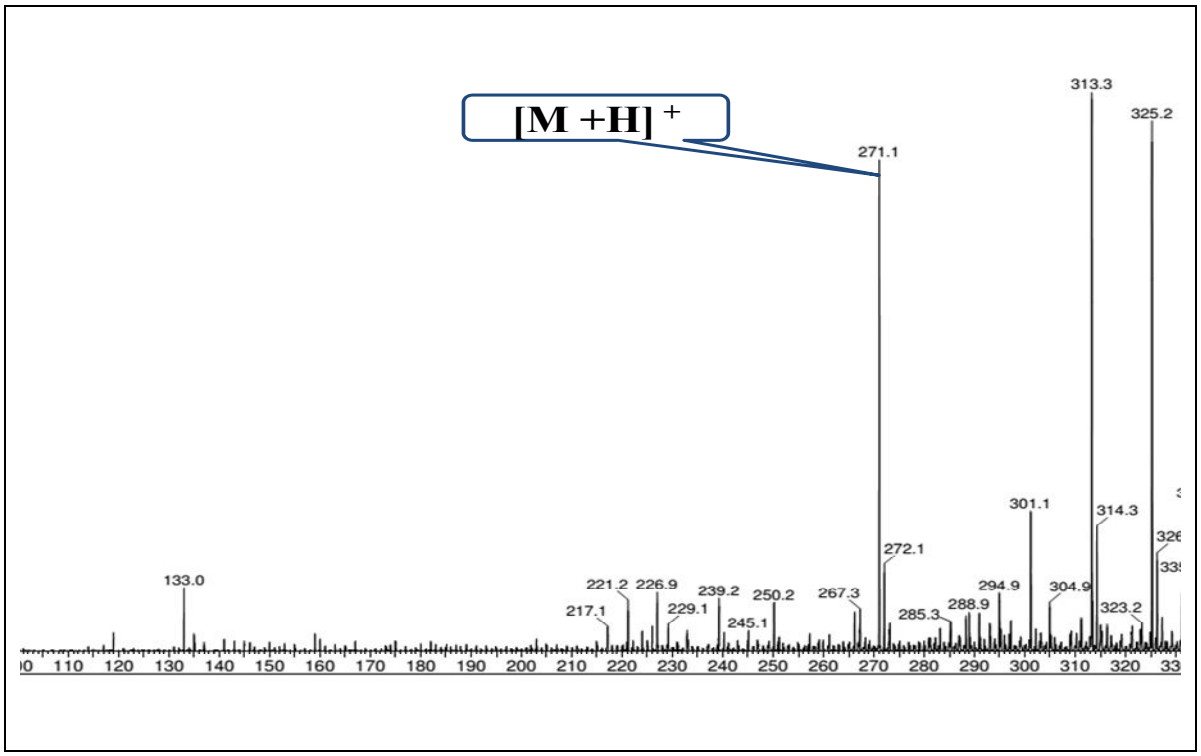


Figure. 4.51 ESI-MS mass spectrum of the compound C2

^{1}H NMR spectrum analysis (fig. 4.52), of compound C2 indicates:

- Two doublet with 2H integration of each at δ 7.87 ppm (J = 8.8 Hz) and 6.95 ppm (J = 8.9 Hz) characteristic of aromatic ring B; para bi-substituted, attributed to H-6′/H-2′ and H-3′/H-5′.
- Two doublet with 1H integration at δ 6.24 ppm (J = 2.1 Hz) and 6.49 ppm (J = 2.1 Hz) characteristic of aromatic ring A; meta bi-substituted, attributed to H-6 and H-8
- Singlet with 1H integration at δ 6.62 ppm corresponds to H-3

All these data analysis are characteristic of an apigenin.

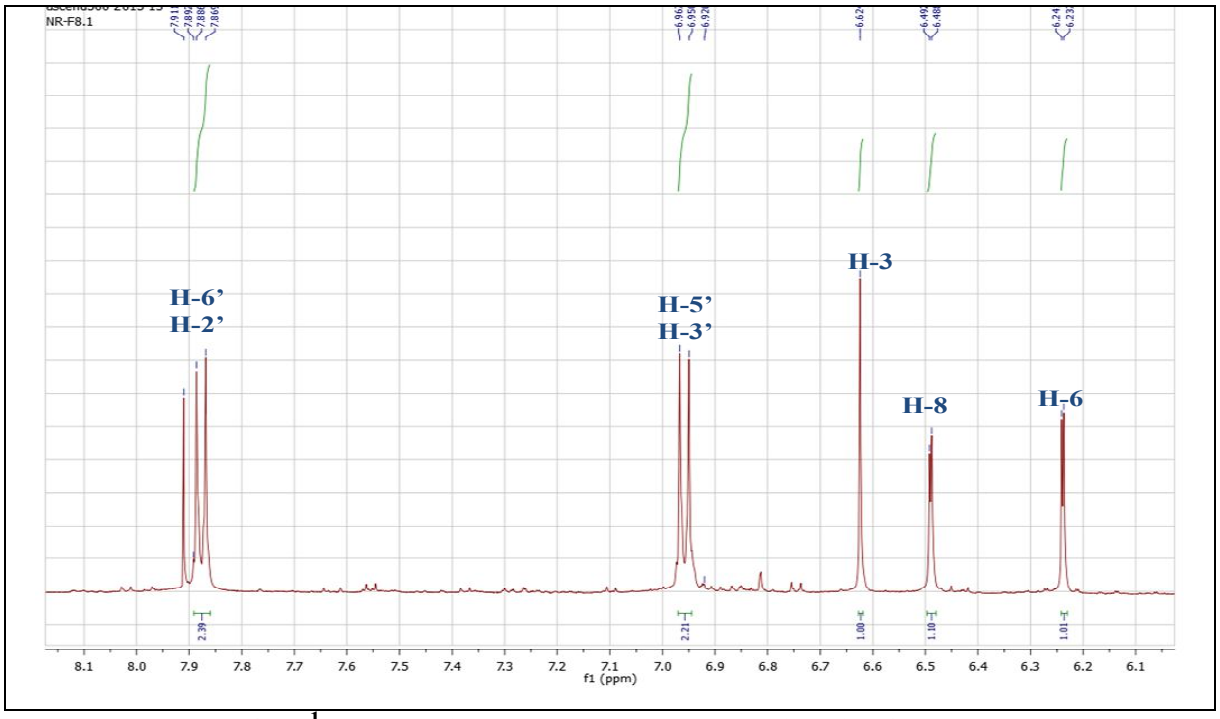


Figure. 4.52 ^{1}H NMR Spectrum of compound C2 (CD_3OD, 500 MHz)

From the HSQC spectrum analysis, showed the correlation between carbons and protons which are directly attached are shown in Figure 4.53 and the chemical shift values are presented in Table 4.8.

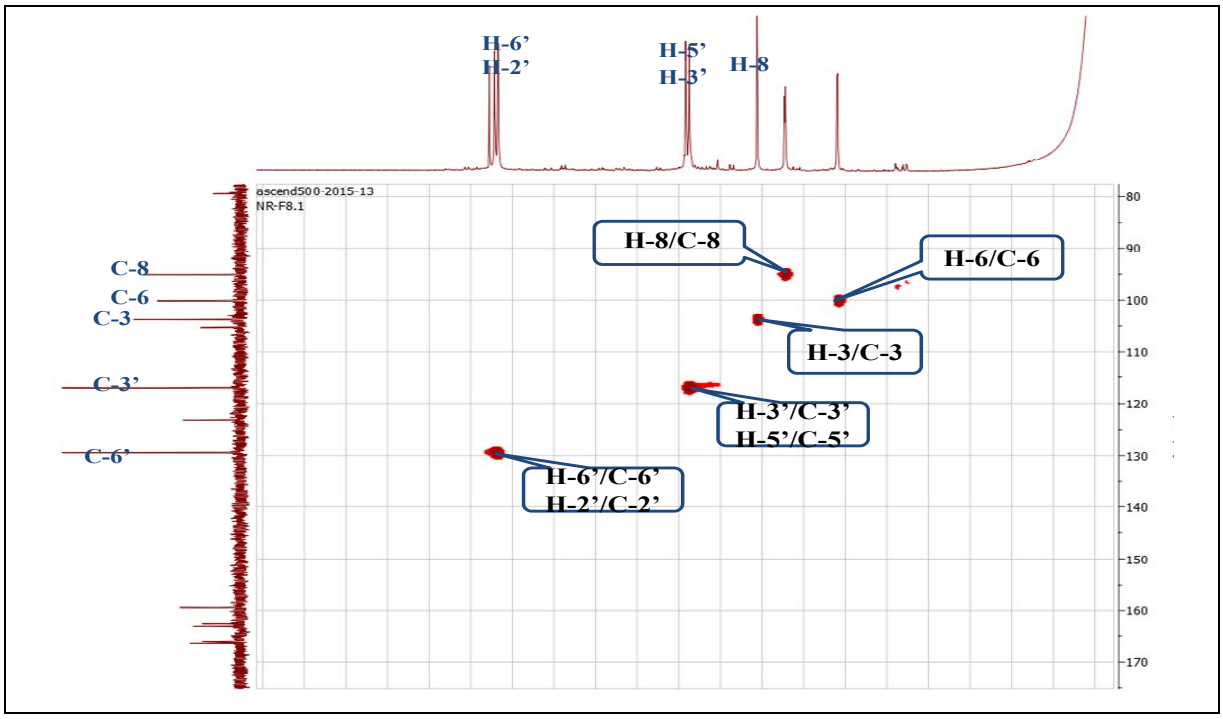


Figure. 4.53 HSQC Spectrum of compound C2 (CD_3OD, 500 MHz)

HMBC spectrum (fig. 4.54) helps for confirmation structural of the compound overall shows the connectivity between rings A, B and C by the observed correlations between:

- H-3 (δ 6.62ppm) and C-1′(δ 123.21 ppm), C-4(δ 183.92ppm), C-2(δ 166.22 ppm) Which confirms the flavone type.

The spectrum displayed correlations between:

- H-2'/ H-6′ (δ 7.87 ppm) and three quaternary carbons C-1' (δ 127.10 ppm), C-4′(δ 162.71 ppm), C-2(δ 166.22 ppm);
- H-3'/H-5′ (δ 6.73 ppm) and two quaternary carbons C-4′(δ 162.71 ppm), C-1' (δ 127.10 ppm); Confirm the para system of the ring B
- H-8 (δ 6.49 ppm) and three quaternary carbons C-9 (δ 159.04), C-7 (δ 166.00 ppm), C-10 (105.27 ppm)
- H-6 (δ 6.24 ppm) and quaternary carbons C-7 (δ 166.00 ppm), C-5(δ 163.15 ppm), C-10 (105.27 ppm)

Confirm the meta system of ring A of flavones.

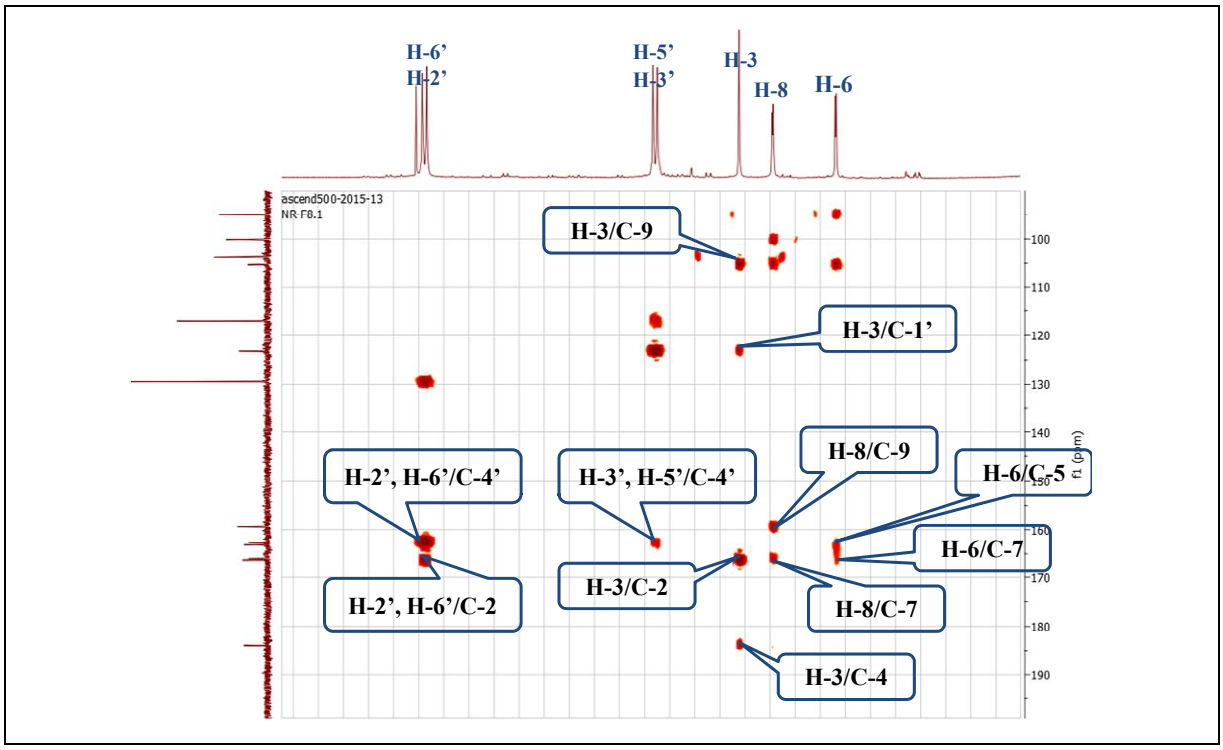


Figure. 4.54 HMBC Spectrum of compound C2 (CD_3OD, 500 MHz)

All these spectral in coincidence with literature (Chaturvedula and Prakash. 2013), were found to be identical to those of a trihydroxy flavone structure known as Apigenin (fig. 4.55) or 5,7,4'-trihydroxyflavone (The spectroscopic data are collected in the table. 4.8)

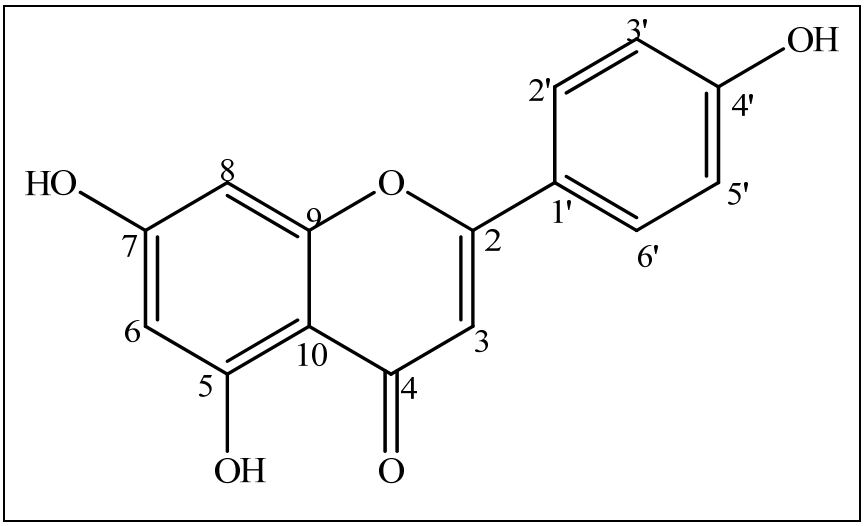


Figure. 4.55 Apigenin

5,7,4'-trihydroxyflavone

Table 4.8. ^{1}H and ^{13}C NMR Chemical shifts of compound C2, recorded in CD_3OD *(500 MHz)*

Postion	***δ_H ppm***	***δ_C ppm***
2	-	166.33
3	6.62(s)	103.78
4	-	183.92
5	-	163.15
6	6.24(d; *J* 2.1 Hz)	100.14
7	-	166.00
8	6.49 (d; *J* 2.1 Hz)	95.06
9	-	159.04
10	-	105.27
1′	-	123.21
2′	7.87 (d; *J* 8.8 Hz)	129.47
3′	6.95 (d; *J* 8.9 Hz)	117.03
4′	-	162.71
5′	6.95 (d; *J* 8.9 Hz)	117.03
6′	7.87 (d; *J* 8.8 Hz)	129.47

4.1.9 Structural characterisation of Compound C5

This Compound C5 was obtained as a yellowish amorphous solid, its ESI-MS mass spectrum (Fig. 4.56), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 287, which corresponds to $[M+H]^+$, suggests a molecular mass 286 mau.

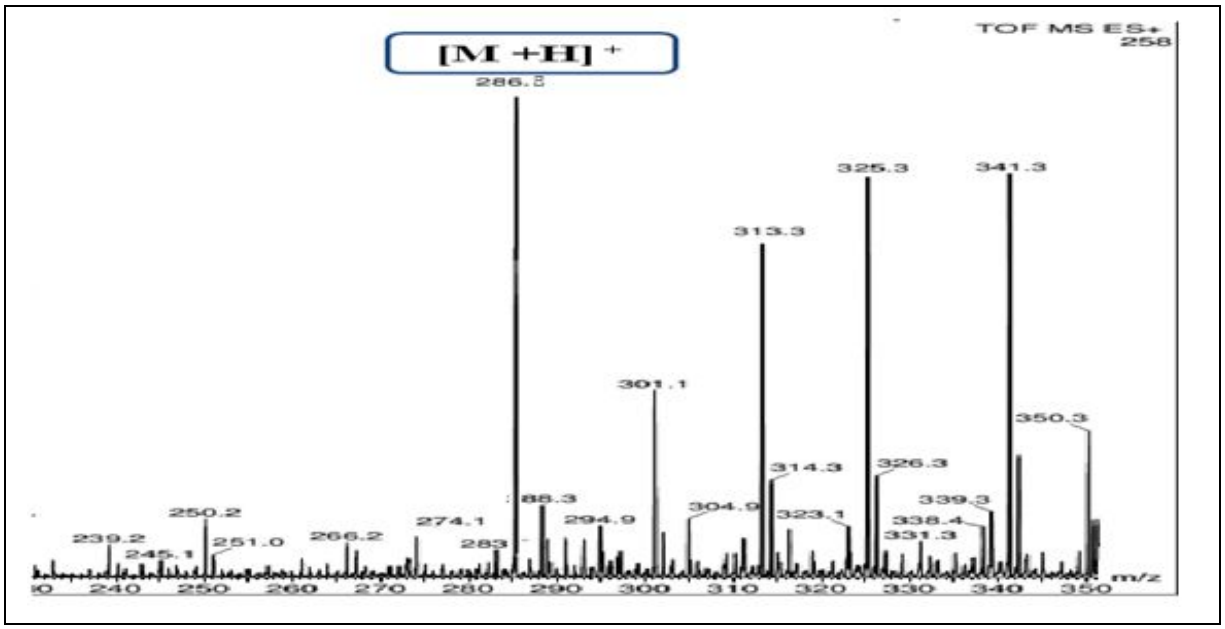


Figure. 4.56 ESI-MS mass spectrum of the compound C5

^{1}H NMR spectrum analysis (fig. 4.57) of the compound C5 shows five main signals:

- Two signals at δ 6.94 ppm (d, J = 8 Hz) with 1H integration attributed to H-5’ and at δ 7.39 ppm (m) with 2H integration assigned to H-2' and H-6' of cycle B of flovonoid structure ;
- Signal at δ 6.47 ppm appears as doublet with (J= 2.0 Hz) attributed to H-8.
- At δ 6.23 and 6.47 ppm we reveal Two doublets with J= 2.0 Hz; typical of meta coupling protons H-6 and H-8 of cycle A.
- Singlet at δ 6.64 ppm (H-3).All these data analysis are characteristic of luteolin

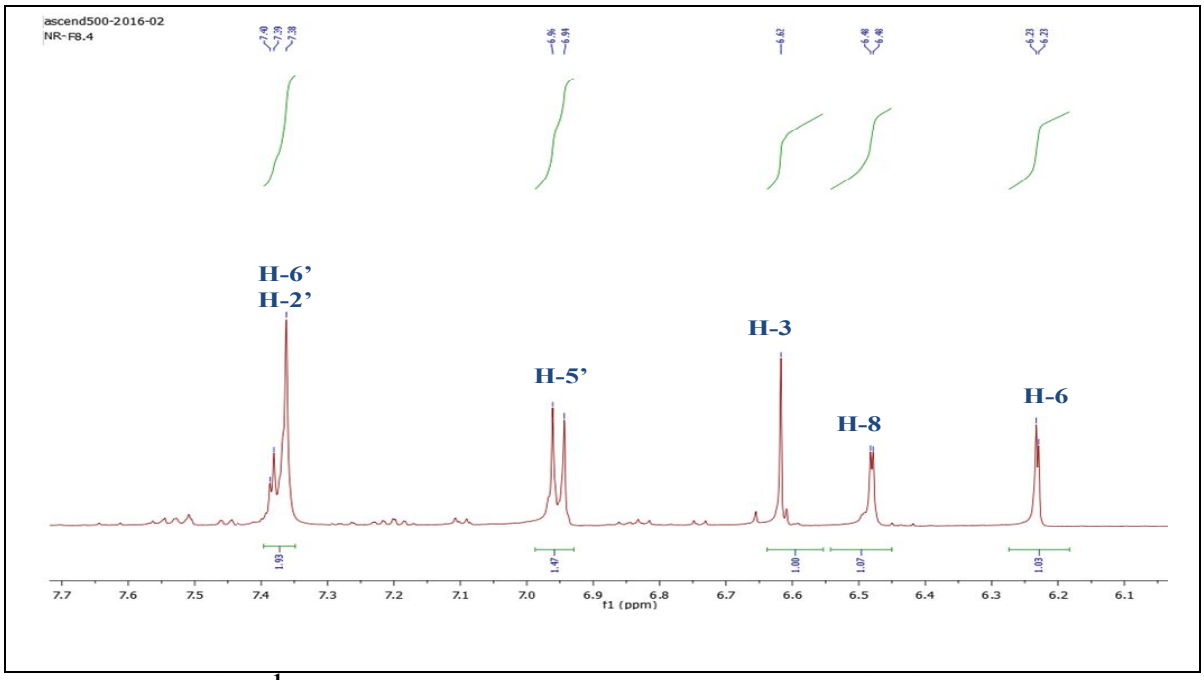


Figure. 4.57 ^{1}H NMR Spectrum of compound C5 (DMSO (d_6), 500 MHz

^{13}C NMR spectrum analysis of compound C5 (fig. 4.58) presents fifteen distinct signals; nine quaternary carbons of which one is carbonyl group (δ 181.56 ppm) and six aromatic CH carbons (table.4.9).

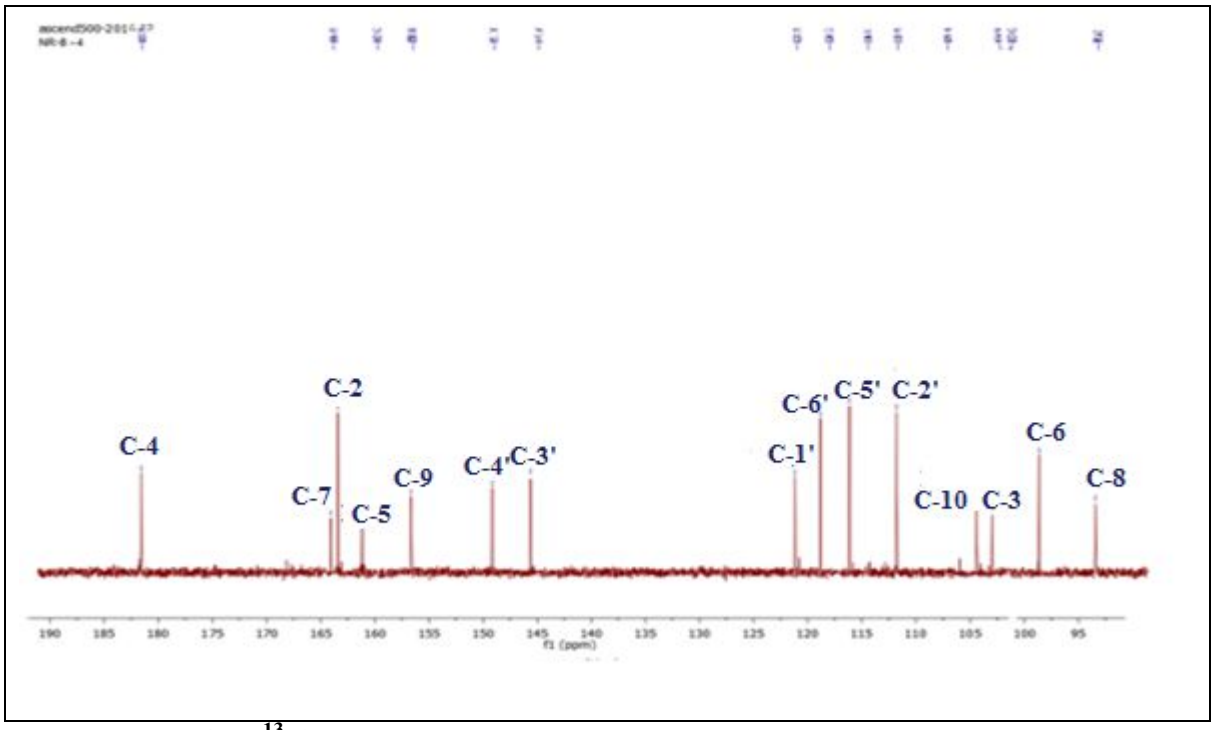


Figure. 4.58 ^{13}C NMR Spectrum of compound C5 (DMSO (d_6), 125 MHz)

All the spectroscopic data are shown in Table. 4.9, as well as the comparison with the data of the literature (Chaturvedula and Prakash. 2013), make it possible to identify the compound C5 as 3',4',5,7-Tetrahydroxyflavone or luteolin.(fig. 4.59)

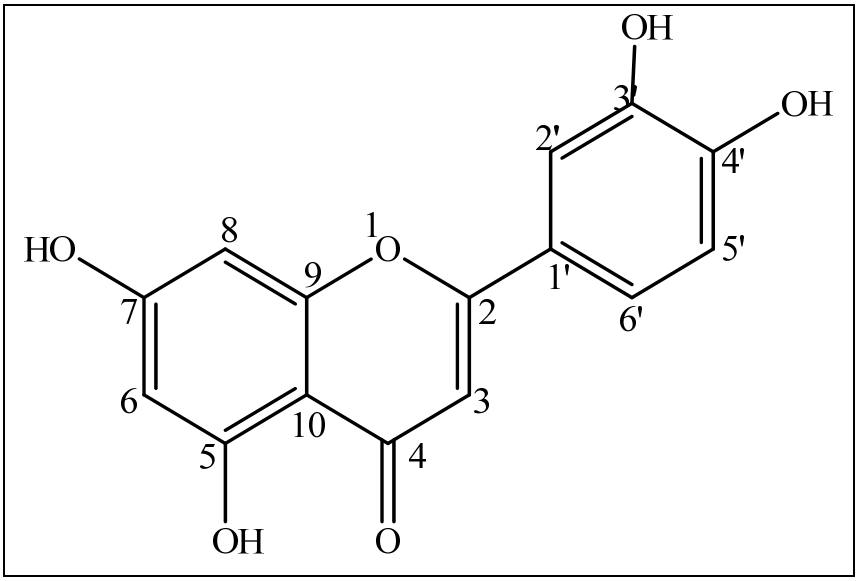


Figure. 4.59 Luteolin

3',4',5,7-Tetrahydroxyflavone

Table. 4.9 ^{1}H and ^{13}C NMR Chemical shifts of compound C5, recorded in DMSO d_6 *(300 MHz)*

Postion	δ_H ***ppm***	δ_C ***ppm***
2	-	164.31
3	6.64 (S)	102.94
4	-	181.76
5	-	161.57
6	6.24 (d; *J* 2.0Hz)	98.89
7	-	163.87
8	6.47 (d; *J* 2.0Hz)	93.88
9	-	157.36
10	-	103.80
1′	-	121.61
2′	7.39(m)	113.45
3′	-	145.82
4′	-	149.73
5′	6.94 (d, *J* = 8 Hz)	116.08
6′	7.39(m)	119.06

4.1.10. Structural characterization of Compound C6

This Compound C6 was isolated as a yellowish powder, its ESI-MS mass spectrum (Fig. 4.60), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 471, which corresponds to $[M+Na]^+$, suggests a molecular mass 448 mau.

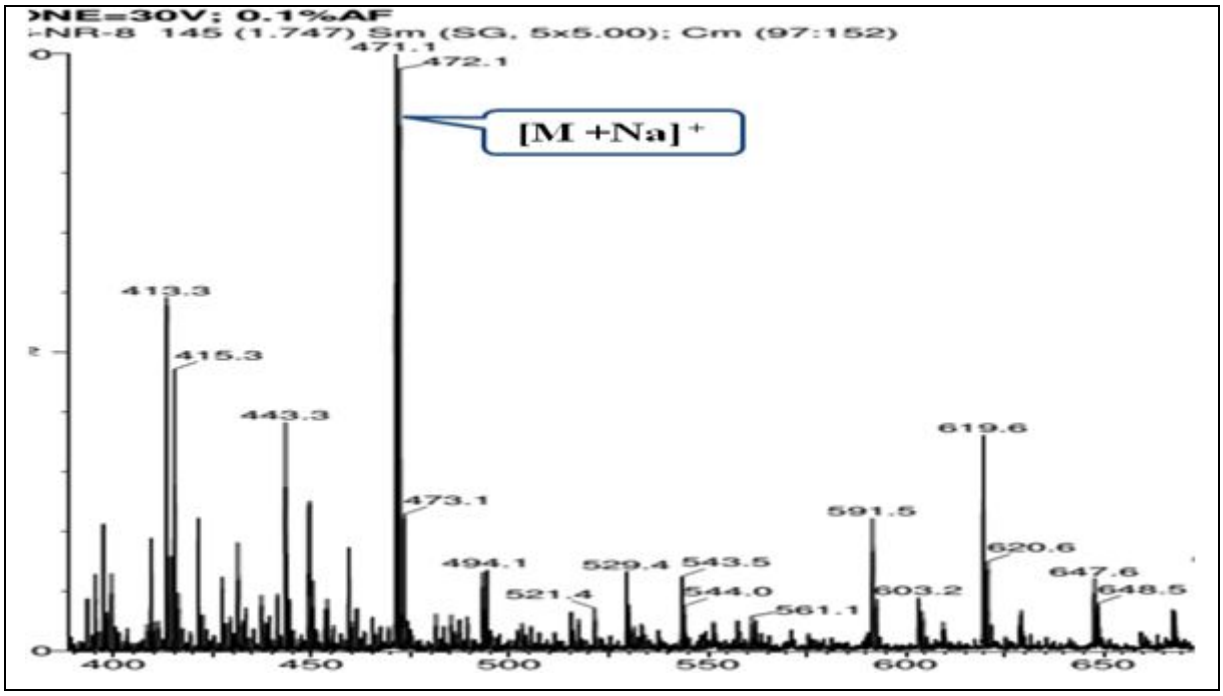


Figure. 4.60 ESI-MS mass spectrum of the compound C6

The study of ^{1}H (fig. 4.61) spectrum analysis as well as their comparison with the spectrum of preceding compounds, in particular compound C5 and C6 confirms that it is a derivative of lutoelin with an additional signals in the average of δ 3.40-4.25 ppm characteristic of osidic protons, the doublet at δ 5.48 ppm (*J* 7.7Hz) refers to the presence of anomeric proton H-1″ which shows axial-axial coupling with H-2″ (*J* 9.2 Hz) indicating β configuration of the sugar unit type glucose.

Also this group is revealed in ^{13}C NMR spectrum analysis (fig. 4.62) by the presence of signals at δ 75.34, 72.44, 71.69, 82.55 and 80.23 ppm characteristic of -CHOH carbons of glucose unit.

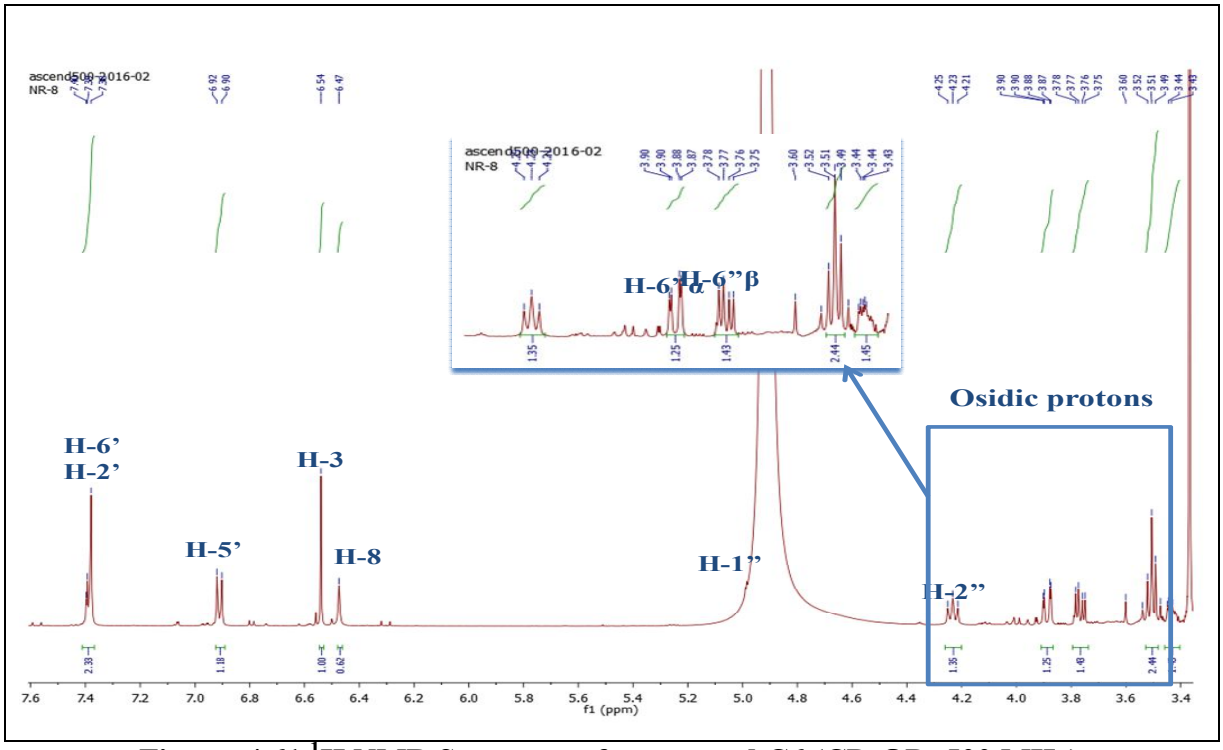


Figure. 4.61 ^{1}H NMR Spectrum of compound C6 (CD_3OD, 500 MHz)

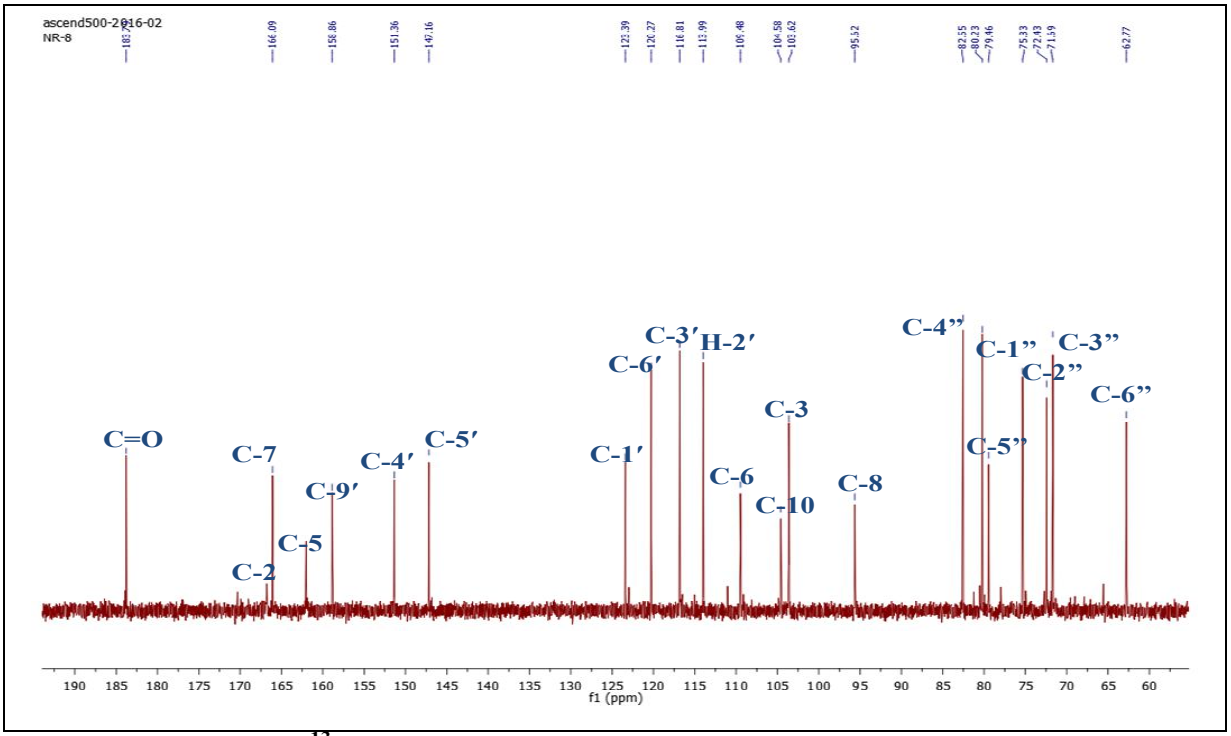


Figure. 4.62 ^{13}C NMR Spectrum of compound C6 (CD_3OD, 500 MHz)

Analysis of HSQC (fig. 4.64), COSY (fig. 4.63) spectra also make it possible to assign the protons and carbons of the sugar. Indeed; C-1 ", C-2", C-3 ", C-4", C-5″, and C-6″ resonant at δ 75.34, 72.44, 71.69, 82.55, 80.23 and 62.77 ppm; respectively.

These data fit with glucose sugar unit.

and their protons H-1″, H-2″, H-3″, H-4″, H-5″, H-6″ α and H-6″β resonant at δ 5.48 ppm (d, 7.7 Hz); in concordance with a β-cofiguration of glucose unit. At δ 4.23 ppm (t, *J*= 9.2 Hz), 3.50 ppm (m), 3.43 ppm (m), 3.50 ppm (m), 3.88 ppm (dd, 2.2, 12.0 Hz) and 3.76 ppm (dd, 5.2, 12.1 Hz).

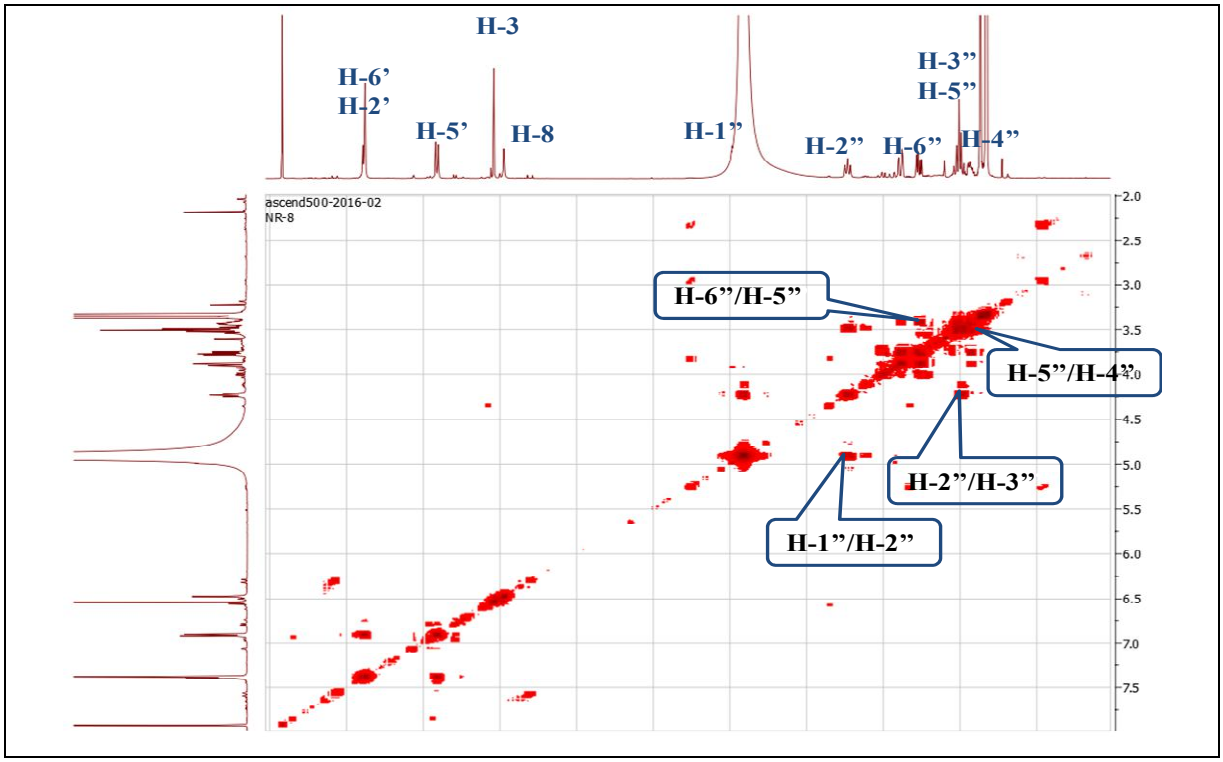


Figure. 4.63 COSY Spectrum of compound C6 (CD_3OD, 500 MHz)

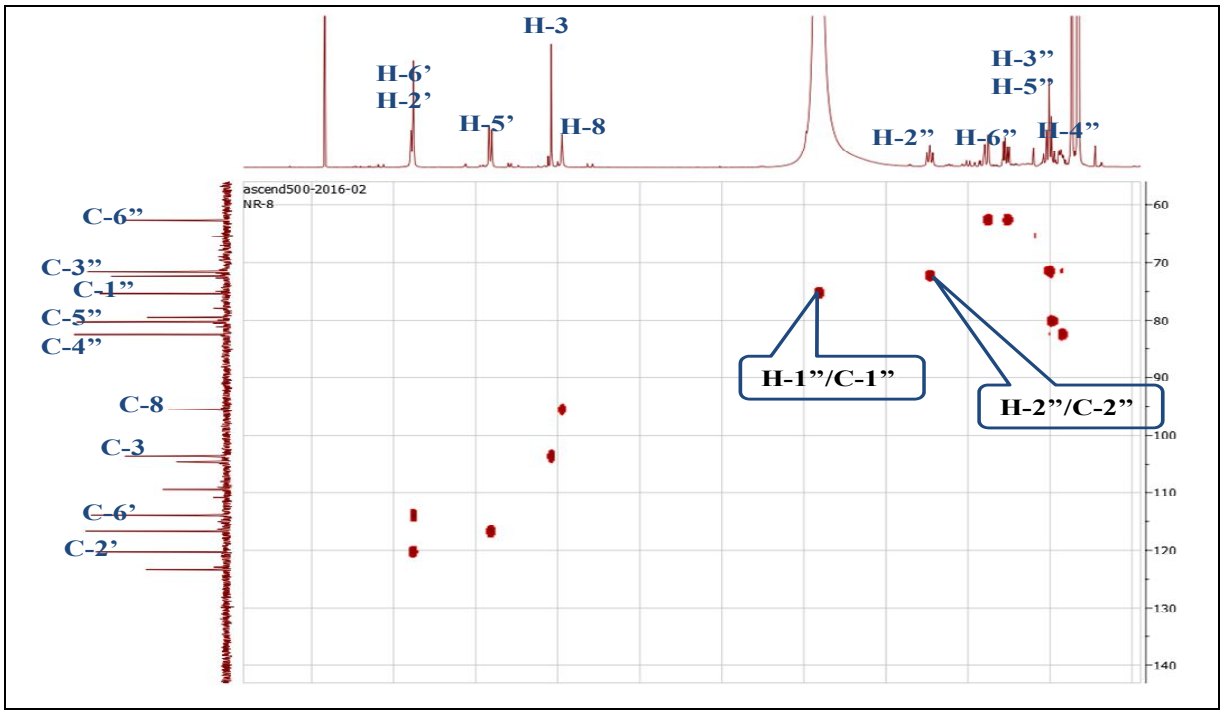


Figure. 4.64 HSQC Spectrum of compound C6 (CD_3OD, 500 MHz)

HMBC correlation gives correlation (fig. 4.65) between anomeric proton H-1" (δ 5.48 ppm) of glucose and quanternary carbon at δ 109.48 ppm corresponding to C-6, which confirms the link of glucose with lutoelin in position 6 .

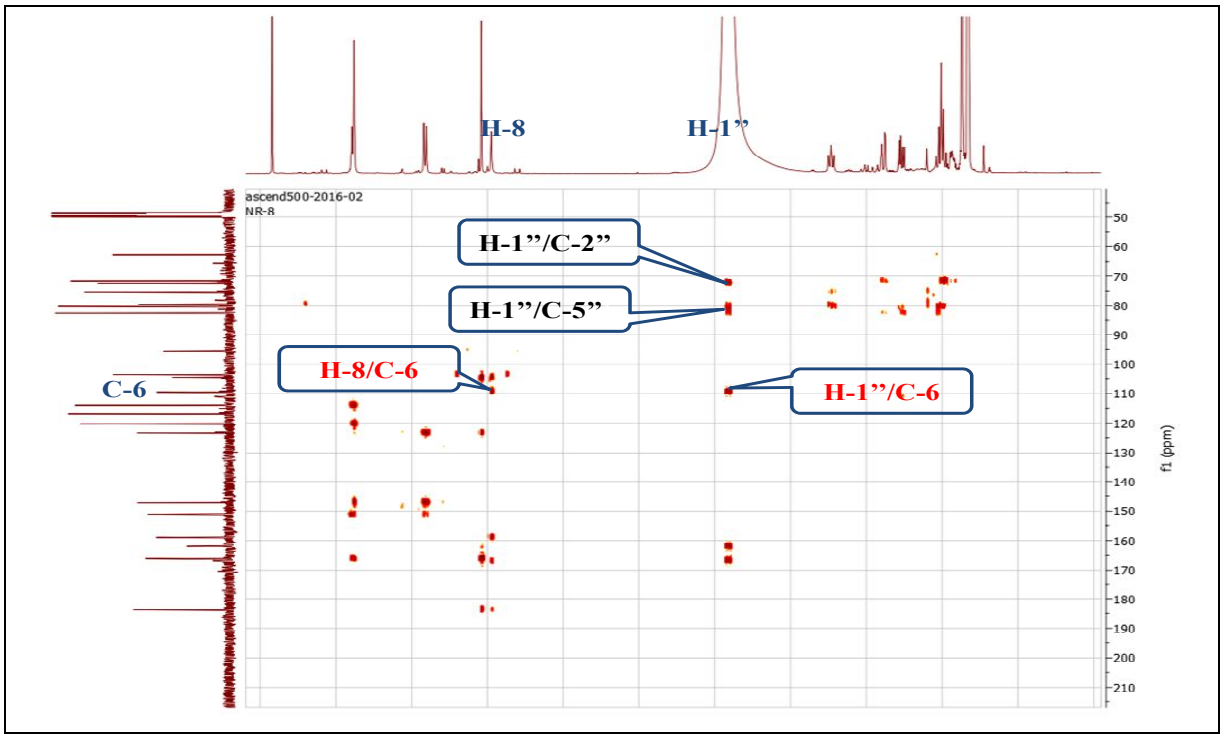


Figure. 4.65 HMBC Spectrum of compound C6 (CD_3OD, 500 MHz)

The obtained data from ^{1}H, ^{13}C, COSY, HSQC and HMBC analysis led to the final structure of compound C6 which is Lutoelin-C-6 Glucose or isoorientin (fig. 4.66).

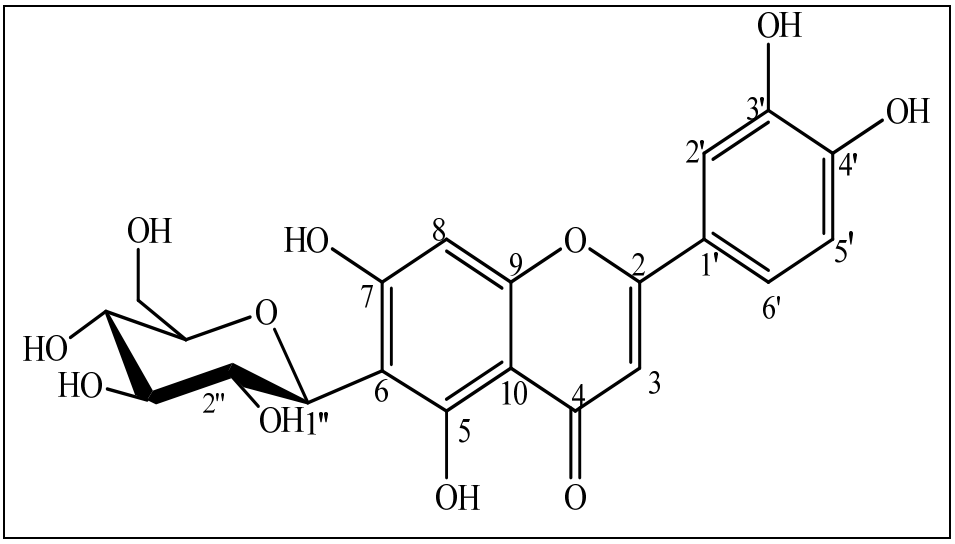


Figure. 4.66 Isoorientin

Table 4.10. ^{1}H and ^{13}C NMR Chemical shifts of compound C6, recorded in CD_3OD *(500 MHz)*

Postion	***δ_H ppm***	***δ_C ppm***
2	-	166.87
3	6.54 (S)	103.62
4	-	183.79
5	-	162.02
6	-	109.48
7	-	166.09
8	6.47 (S)	95.63
9	-	158.81
10	-	104.51
1′	-	123.39
2′	7.38(m)	113.99
3′	-	147.15
4′	-	151.36
5′	6.91(d; J 8.9 Hz)	116.81

6′	7.37(m)	120.27
1″	5.48(d; *J* 7.7 Hz)	75.34
2″	4.23(t; *J* 9.2 Hz)	72.44
3″	3.50(m)	71.69
4″	3.43(m)	82.55
5″	3.50(m)	80.23
6″	α 3.88(dd; *J* 2.2, 12.0 Hz) β 3.76(dd; *J* 5.2, 12.1 Hz)	62.77

4.1.11. Structural characterisation of Compound C8

This Compound C8 was isolated as a yellowish powder, its ESI-MS mass spectrum (Fig. 4.67), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 617, which corresponds to $[M+Na]^+$, suggests a molecular mass 594 mau.

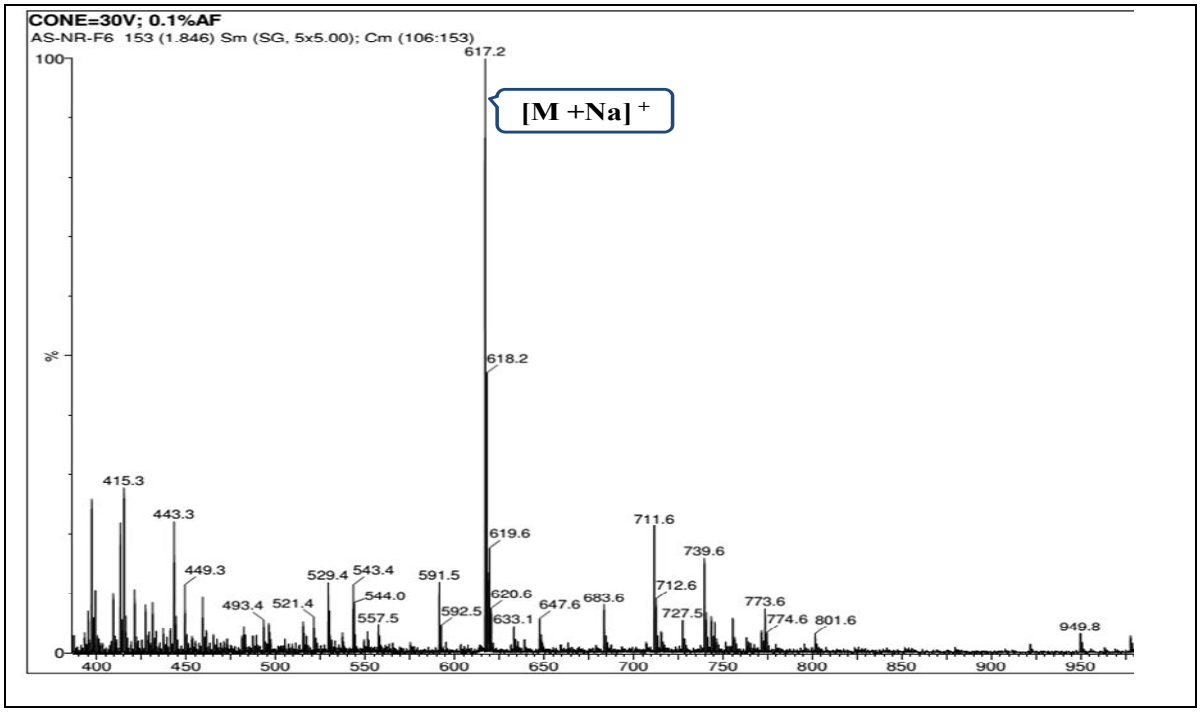


Figure. 4.67 ESI-MS mass spectrum of the compound C8

^{1}H NMR spectrum of the compound C8 recorded in CD_3OD (fig. 4.68) shows signals of several aromatic protons resonating between δ 6.00-8.10 ppm and osidic protons between δ 3.40 -5.30 ppm as following :

- Two doublets at δ 6.15 ppm (*J* = 2.0 Hz) and 6.34 ppm (*J* =2.0 Hz) attributable to H-6 and H-8 have coupling constant J= 2.0 Hz of meta system belong to ring A of

kaempferol skeleton. Other two doublets appear at δ 8.00 ppm (*J* =8.9 Hz) attributable to 6.85 ppm (*J* =8.9 Hz) characteristic of a para-substituted aromatic ring B of kaempferol skeleton attributable to H-3′/H-5′ and H-2′/H-6′;

- Two doublets signals appear with 2H integration at δ 7.33 ppm *(J* =8.6 Hz) and 6.83 ppm *(J* =8.7 Hz) characteristic of a para-substituted aromatic ring (fig. 4.69), correspondent to H-2‴/ H-6‴ and H-3‴/ H-5‴ protons, respectively;
- The two doublets which appear at δ 7.41 ppm (*J* =16.0 Hz) and 6.08 ppm *(J* =16.0 Hz) characteristic of olefinic protons in trans position (coupling constant is of order J = 16.0 Hz);
- A doublet at δ5.25 ppm (*J* = 7.5Hz) attributed to an anomeric proton H-1″ with *J* = 7.5Hz. which is in concordance with β-configuration

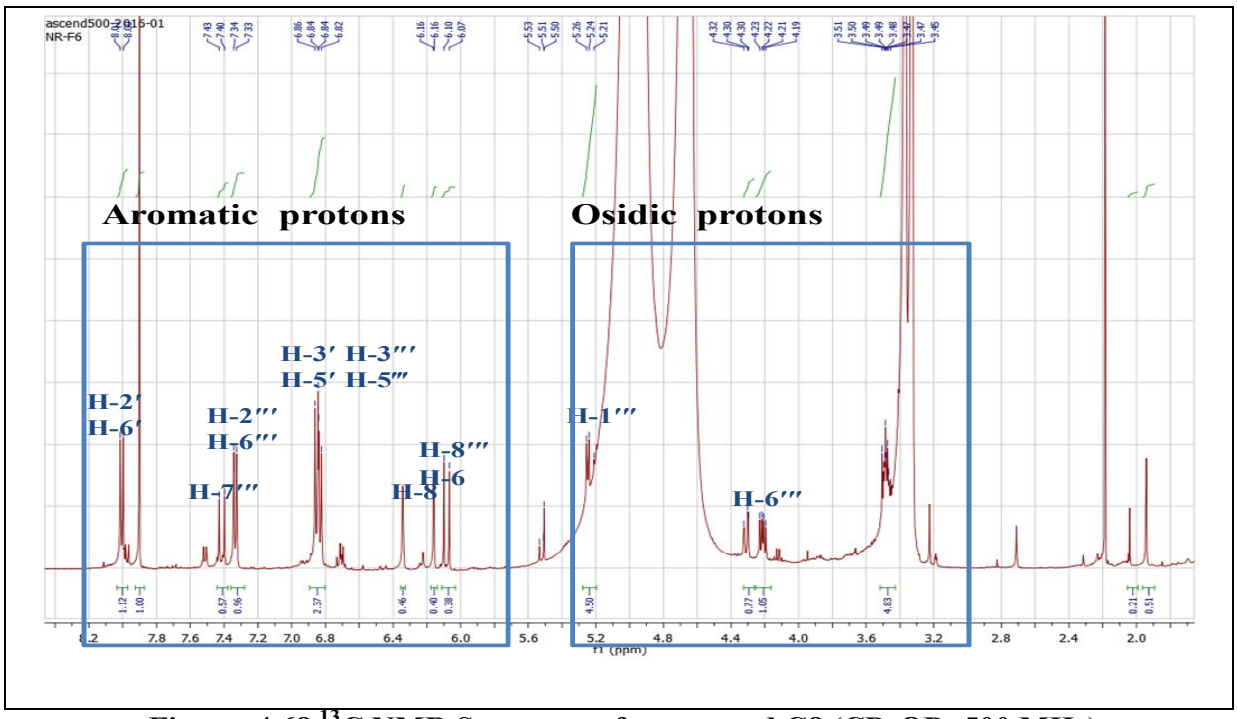


Figure. 4.68 ^{13}C NMR Spectrum of compound C8 (CD_3OD, 500 MHz)

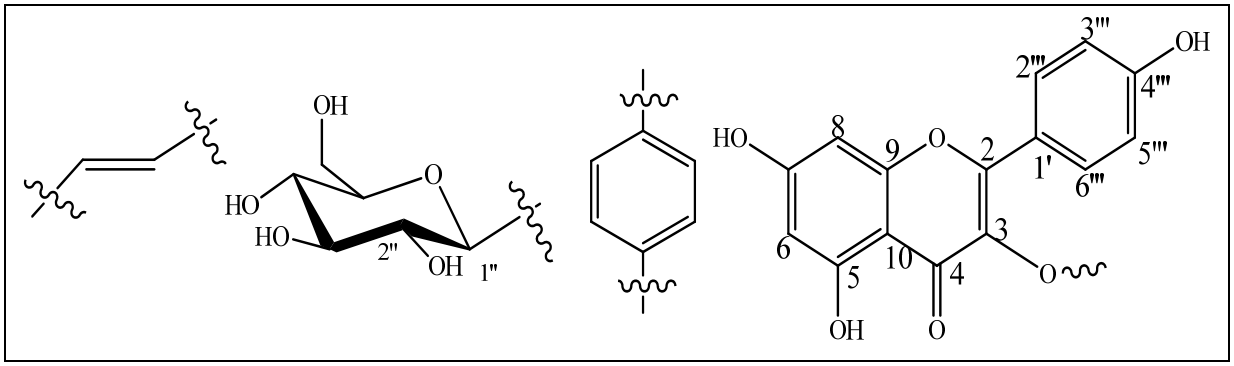


Figure. 4.69 olefinic, para-substituted aromatic ring and kaempferol skeletons

COSY experiment (fig. 4.70) confirms the assignment of other protons of kaempferol skeleton and para-substituted aromatic ring it reveals many spots of correltion between the neighboring protons, also confirms assignment of sugar's protons which resonant at δ 3.46-3.50(m) attributed to H-2″, H-3″and H-5″, 3.35-3.37(m) attributed to H- 4″, 4.20(dd, J= 6.7; 11.8) and 4.31(dd, J= 2.2;11.8Hz) attributed to H-6″α and H-6″β

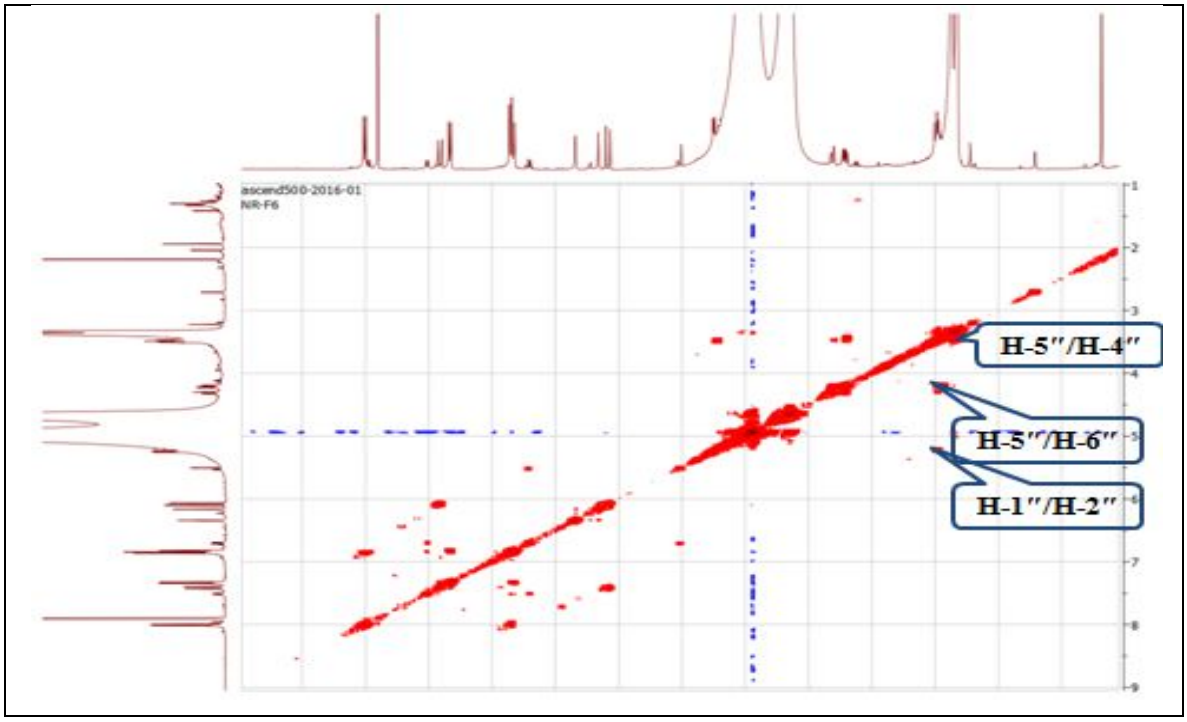


Figure. 4.70 COSY Spectrum of compound C8 (CD_3OD, 500 MHz)

The analyses of ^{13}C NMR spectrum (fig. 4.71) as well as the HSQC spectrum (fig. 4.72) make it possible to locate:

- C-2 '/C-6'; C-3'/C-5' and C-6, C-8 carbons of the flavonic rings at δ (131.2, 116.07, 100.11 and 94.90 ppm), respectively;
- C-1 ", C-2", C-3 ", C-4", C-5 "and C-6" carbons of sugar at δ 103.74; 75.72; 77.90; 71.70; 75.64 and 64.26 ppm, respectively. Combaining these locations or assignments and ^{1}H NMR data confirm the type of sugar as β-glucopyranosyl. Moreover, ^{13}C NMR spectrum (fig. 4.71) shows the presence of carbonyl (C = O) group of conjugated ester at δ 168.86 ppm;

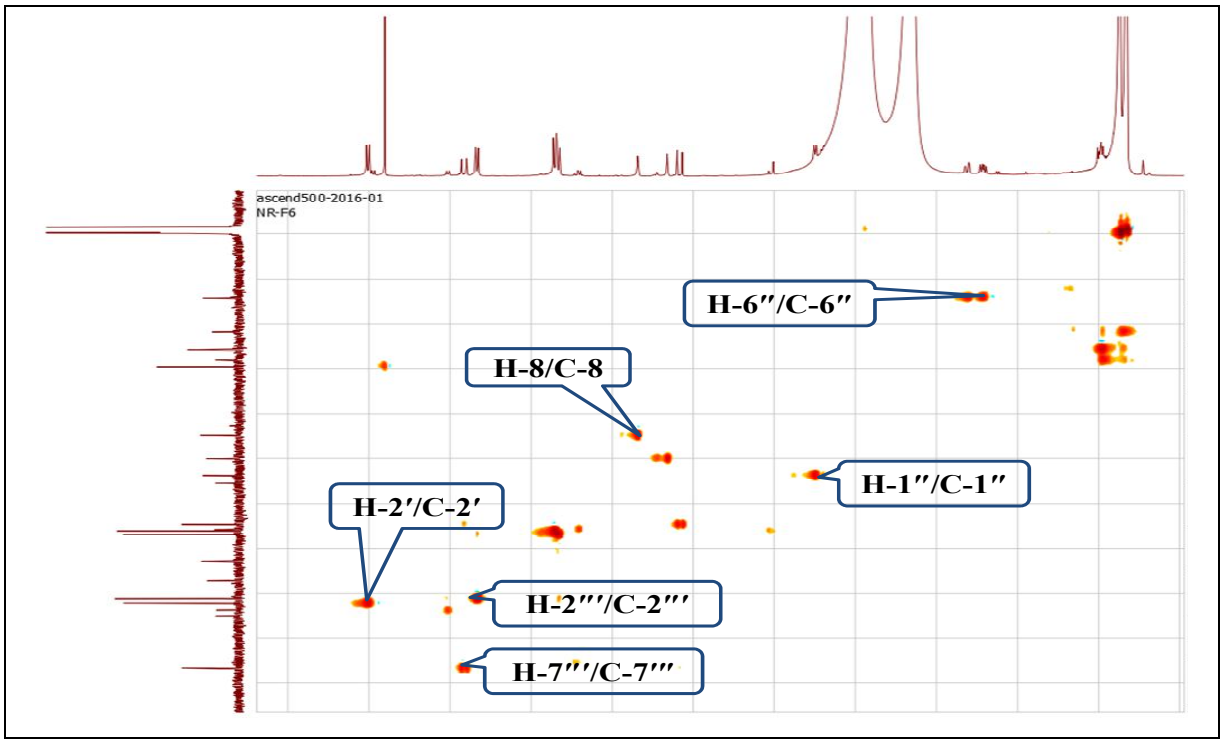


Figure. 4.72 HSQC Spectrum of compound C8 (CD_3OD, 500 MHz)

The HSQC spectrum (fig. 4.72) can also be used to assign CH carbons signals at δ 114.62 and 146.62 ppm and aromatic ring C-2 ‴/C-6 ‴ and C-3 ‴/ C-5 ‴ at δ (132.18; 116,82 ppm).

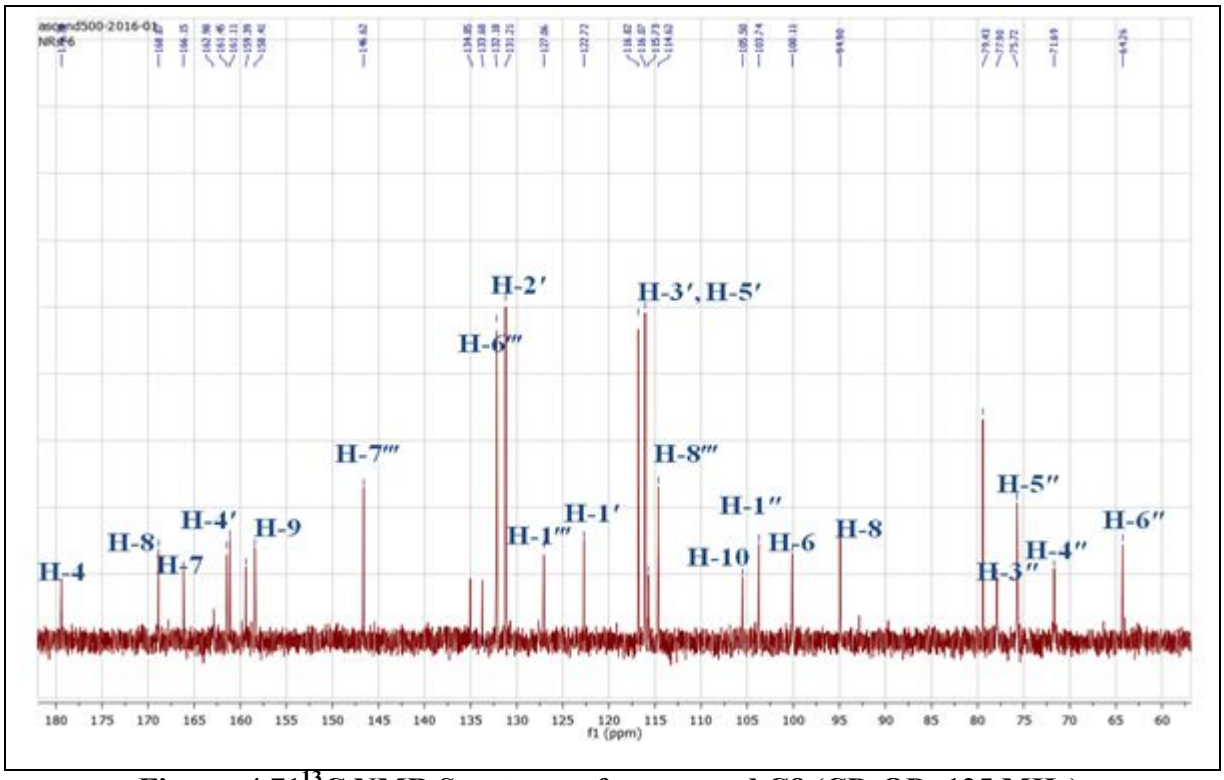


Figure. 4.71 ^{13}C NMR Spectrum of compound C8 (CD_3OD, 125 MHz)

HMBC spectrum analysis (fig. 4.73) shows the connectivity of kaempferol skeleton and and β-D-glucopyranosyl in position 3 from the noticed correlation between proton H-1″ and carbon C-3); the correlations between C-9‴ and H-7‴ also H-8‴ with C-1‴ gives p-coumaroyle group which linked with β-D-glucopyranosyl in position 6 due to observation of correlation between proton H-6″ and carbon C-9‴.

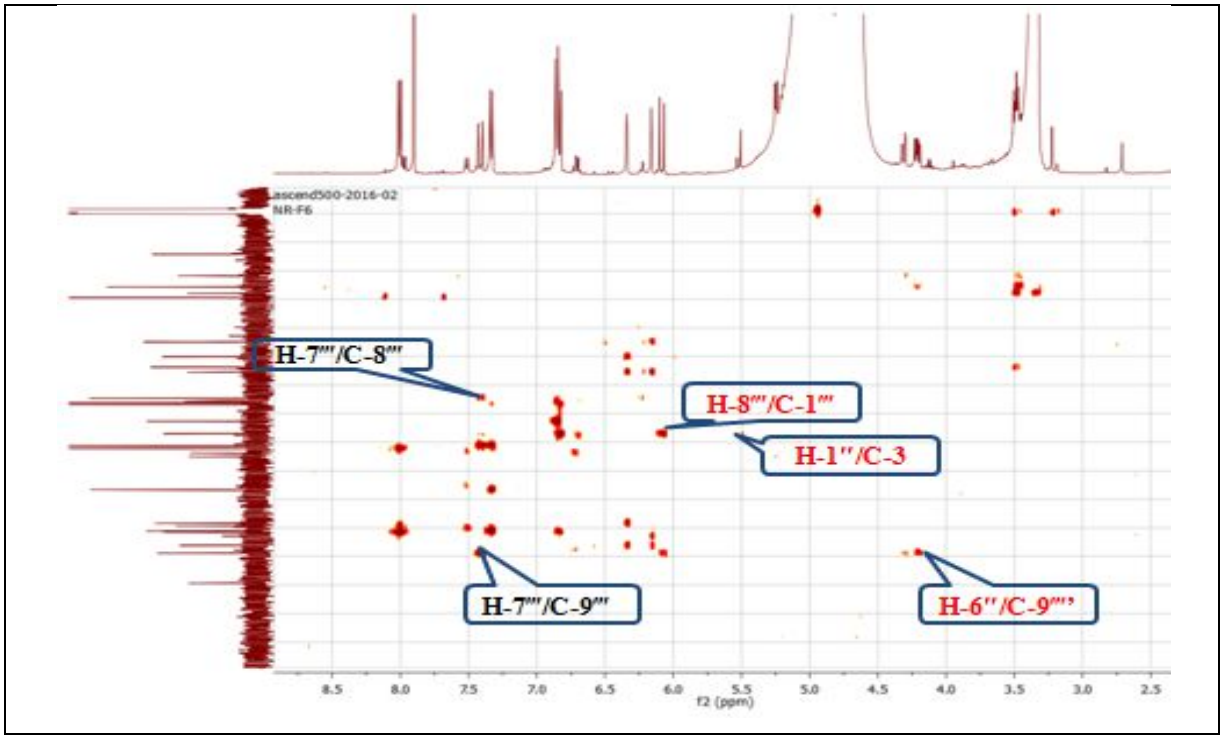


Figure. 4.73 HMBC Spectrum of compound C8 (CD_3OD, 500 MHz)

All of these spectral data as well as the comparison with the data found in the literature allow us to determine the structure of compound C8 as a kaempferol-3-O- [6 "-O- (E) -p-coumaroyl] -β-D-glucopyranoside called Tiliroside(fig. 4.74)

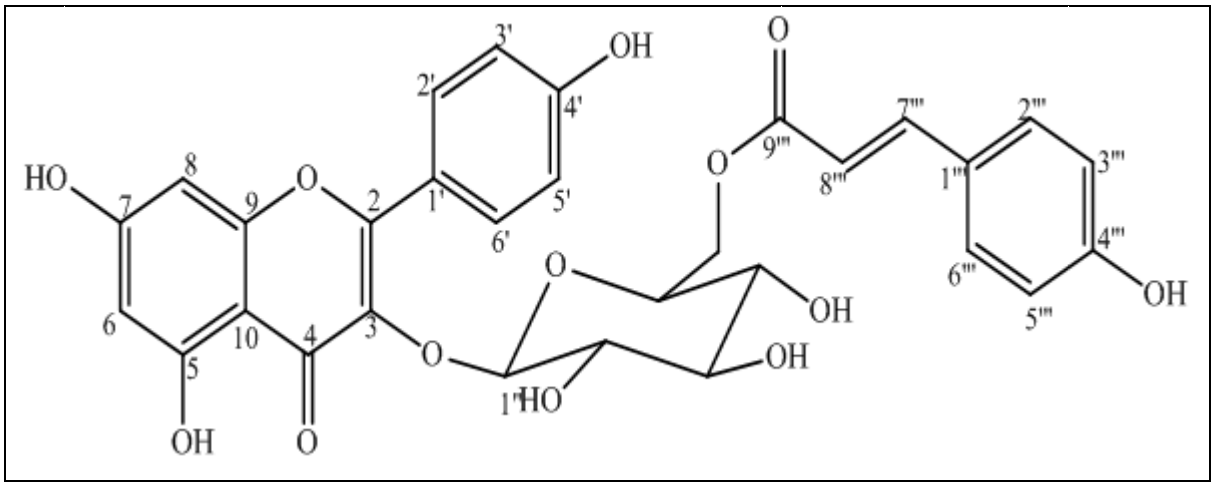


Figure. 4.74 kaempferol-3-O-[6"-O-(E)-p-coumaroyl]-β-D-glucopyranoside Tiliroside

Table 4.11. ^{1}H and ^{13}C NMR Chemical shifts of compound C8, recorded in CD_3OD*(500 MHz)*

Postion	***δ_H ppm***	***δ_C ppm***
2	-	159.39
3	-	135.03
4	-	179.40
5	-	162.87
6	6.15 (d; *J* 2.1Hz)	100.11
7	-	166.15
8	6.34 (d; *J* 2.0Hz)	94.90
9	-	158.41
10	-	105.50
1′	-	122.72
2′	8.00(d; J 8.9 Hz)	131.20
3′	6.85(d; J 8.9 Hz)	116.07
4′	-	161.45
5′	6.85(d; J 8.4 Hz)	116.07
6′	8.00(d; J 8.9 Hz)	131.20
Glucose 1″	5.25(d; *J* 7.5 Hz)	103.74
2″	3.46-3.50(m)	75.72
3″	3.46-3.50(m)	77.90
4″	3.35-3.37(m)	71.70
5″	3.46-3.50(m)	75.64
6″	α 4.20(dd; *J* 6.7, 11.7 Hz) β 4.31(dd; *J* 2.2, 11.8 Hz)	64.26
p-coumaroyl 1‴	-	127.55
2‴	7.33 (d; *J* 8.6 Hz)	132.18
3‴	6.83 (d; *J* 8.7 Hz)	116.82
4‴	-	161.11
5‴	6.83(d; *J* 8.7 Hz)	116.82

6‴	7.33(d; *J* 8.6 Hz)	132.18
7‴	7.41(d; *J* 15.9 Hz)	146.62
8‴	6.08(d; *J* 15.9 Hz)	114.62
9‴	-	168.8

4.1.12 Structural characterisation of Compound C10

This Compound was isolated as a white crystals soluble in water, its ESI-MS mass spectrum (Fig. 4.75), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 231, which corresponds to $[M+Na]^+$, suggests a molecular mass 208 mau.

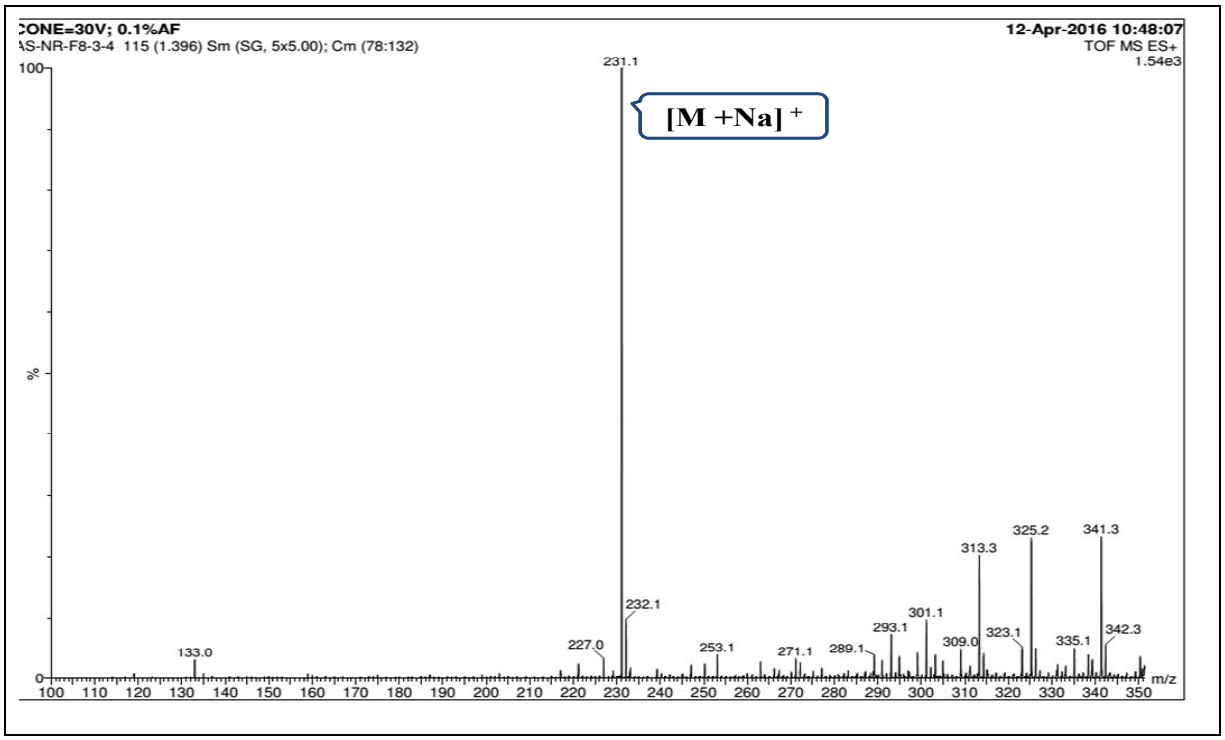


Figure 4.75 ESI-MS mass spectrum of the compound C10

^{1}H NMR spectrum (Fig. 4.76) indicates the presence of several signals, of integration 1H and 2H, resonating in the zone characteristic of the oside (3.1 to 4.50 ppm), and methyl (CH_3) group at δ 1.17 ppm (t, J= 7.05Hz) Also the ^{13}C NMR spectrum (Fig. 4.77) shows 8 signals; four of them are representing CHOH carbons at δ 75.08, 71.73,77.90 and 78.08ppm; two groups of CH_2.OH at δ 62.18 and 66.18 ppm; and CH_3 group at δ 15.44 ppm highlights the presence of a hexose unit.

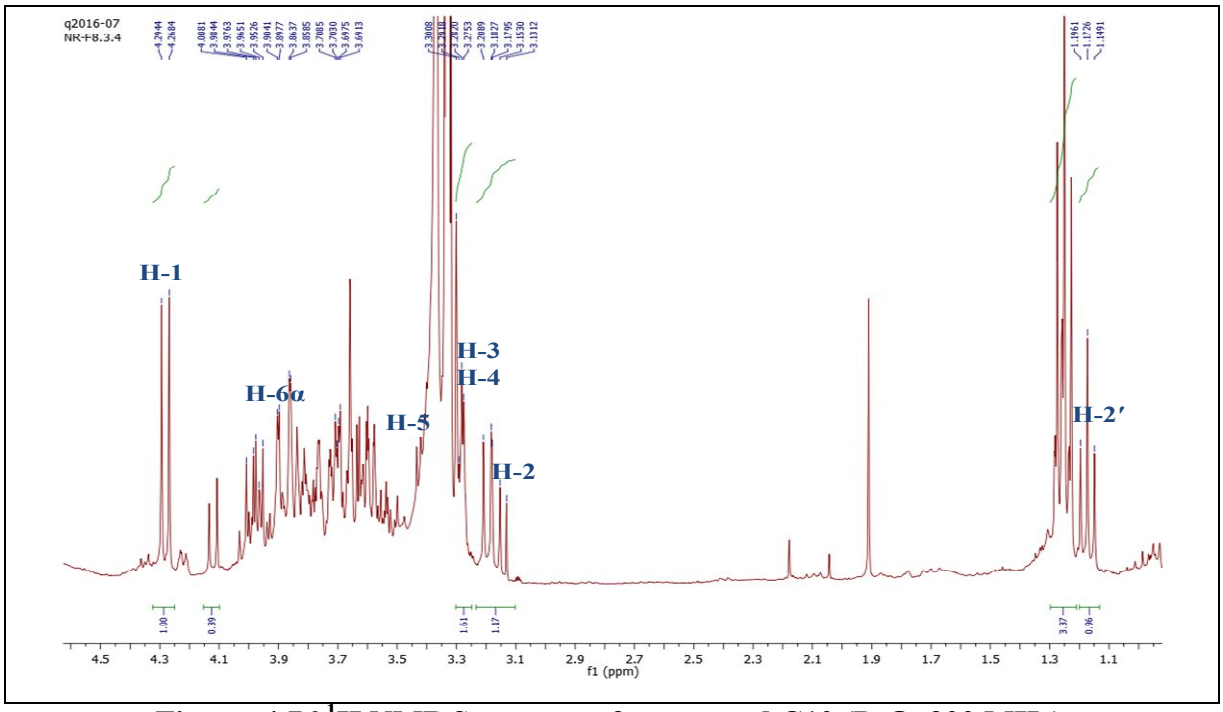


Figure. 4.76 ^{1}H NMR Spectrum of compound C10 (D_2O, 300 MHz)

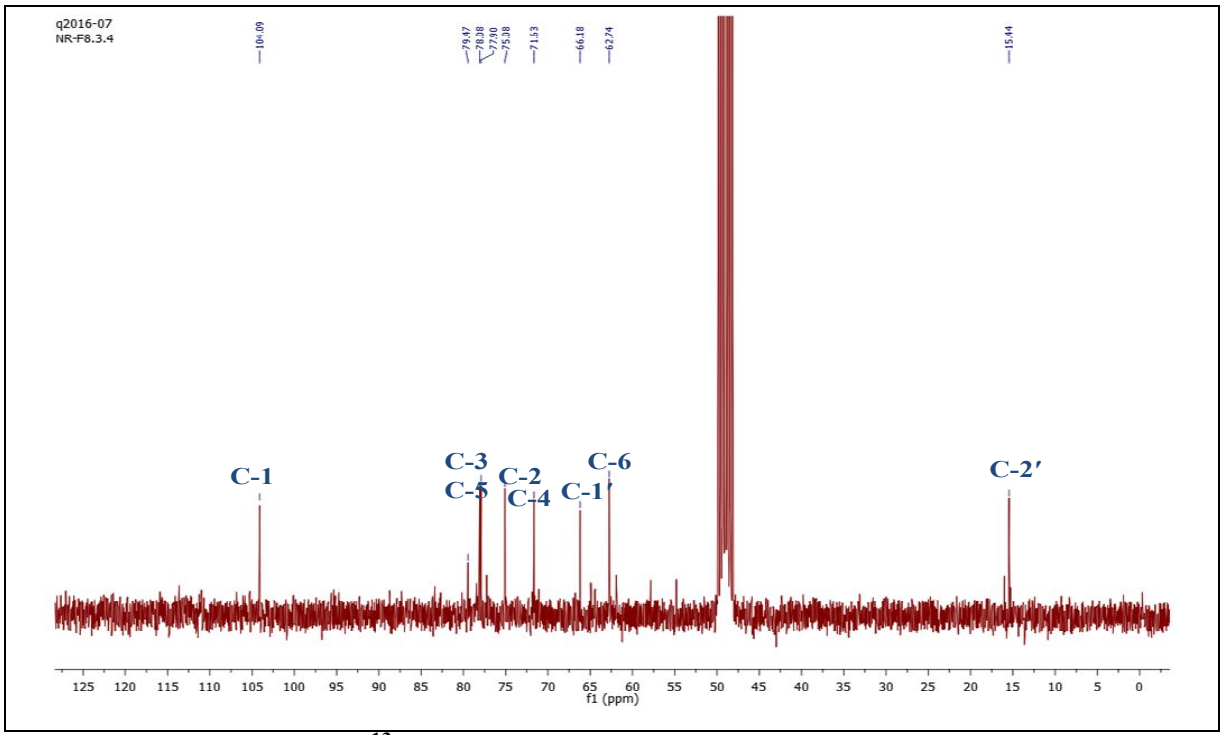


Figure. 4.77 ^{13}C Spectrum of compound C10 (D_2O, 75 MHz)

The identification of hexose protons (H-2, H-3, H-4, H-5, H-6α and H-6β) are deduced from the COSY H-H experimrnt (Fig. 4.78) Which resonant at δ 3.18 ppm (dd, J= 7.9; 15.5 Hz), 3.29 ppm (m), 3.27 ppm (m), 3.30 ppm (m), 3.90 ppm (dd, J= 1.9; 11.8 Hz) and 3.70 ppm (dd, J= 5.1; 11.8 Hz). The HSQC spectrum (fig. 4.78) indicates correlation between each carbon with the corresponding proton as follow; C-1(104.09 ppm), C-2(75.08 ppm), C-3(77.90 ppm), C-4 (71.63 ppm), C-5(78.08 ppm) and C-6(62.18 ppm). The proton at δ 4.27 ppm (d, J= 7.8 Hz) is attributed to anomeric proton H-1. These chemical shifts are characteristics of hexose; β-D-Glucose type, linked with $OCH_2.CH_3$ group confirmed by HMBC experiment (fig. 4.80) which shows correlation between H-1 and C-2′.

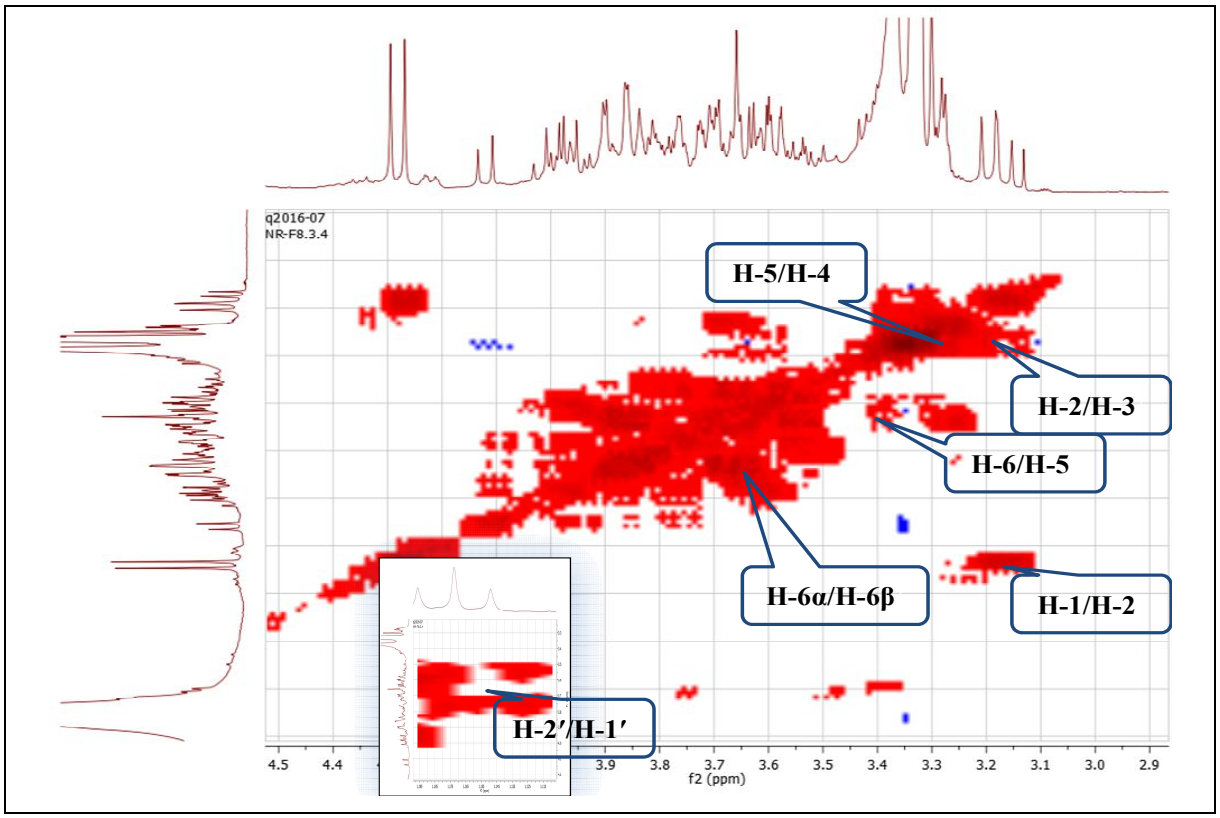


Figure. 4.77 COSY Spectrum of compound C10 (D_2O, 300 MHz)

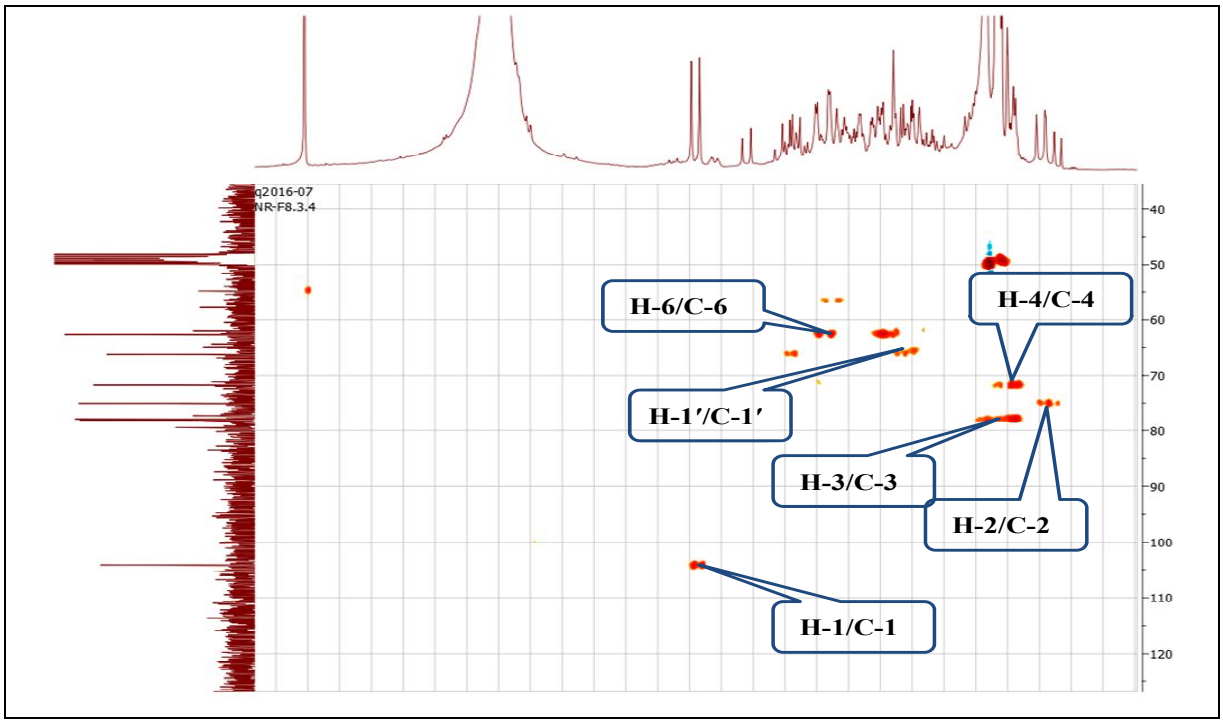


Figure. 4.79 HSQC Spectrum of compound C10 (D_2O, 300 MHz)

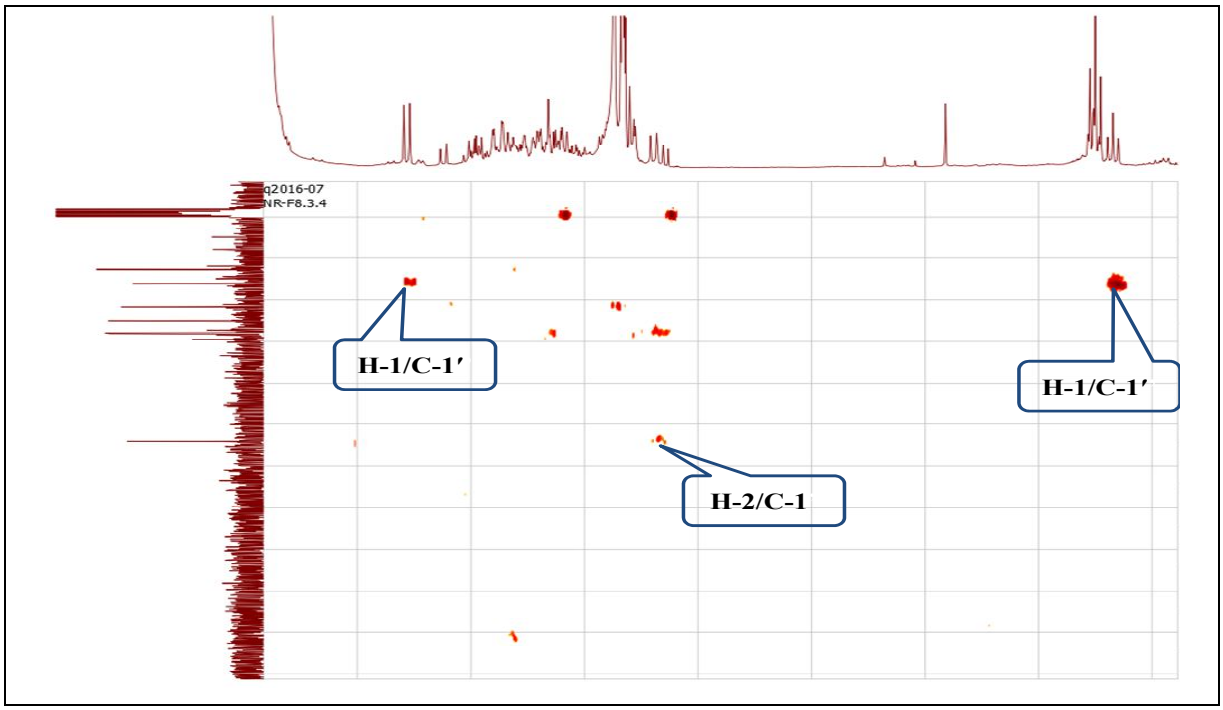


Figure. 4.80 HMBC Spectrum of compound C10 (D_2O, 300 MHz)

All the spectral data of the compound C10 were identified by comparison with literature (Teague et al., 2004) for **1-O-ethyl-β-D-glucopyranoside**

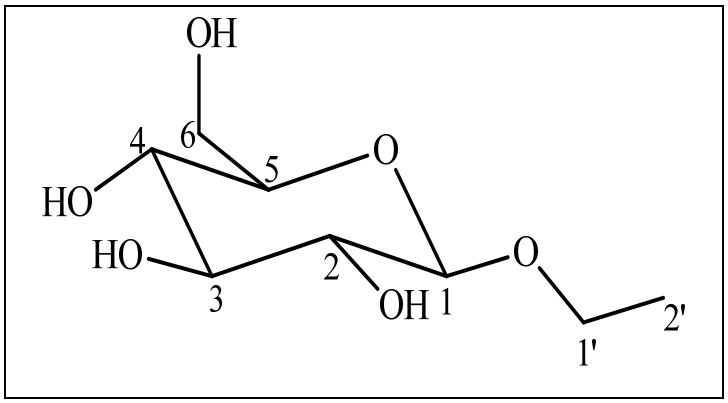


Figure. 81 1-O-ethyl-β-D-glucopyranoside

Table. 4.12 ^{1}H and ^{13}C NMR Chemical shifts of compound C10 recorded in D_2O *(300 MHz)*

Postion	δ_H *ppm*	δ_C *ppm*
1	4.27(d; *J* 7.8 Hz)	104.09
2	3.18 (dd; *J* 7.9, 15.5 Hz)	75.08
3	3.29(m)	71.63
4	3.27(m),	77.90
5	3.30(m),	78.08
6	3.90(dd, *J*1.9; 11.8 Hz) 3.70(dd, *J*5.1; 11.8 Hz)	62.18
1′	3.62 (q, *J* 7.1 Hz)	66.18
2′	1.17 (t, *J* 7.1 Hz)	15.44

4.1.13 Structural characterisation of Compound C12

This Compound was isolated as a white crystals soluble in water, its ESI-MS mass spectrum (Fig. 4.82), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 365, which corresponds to $[M+Na]^+$, suggests a molecular mass 342 mau.

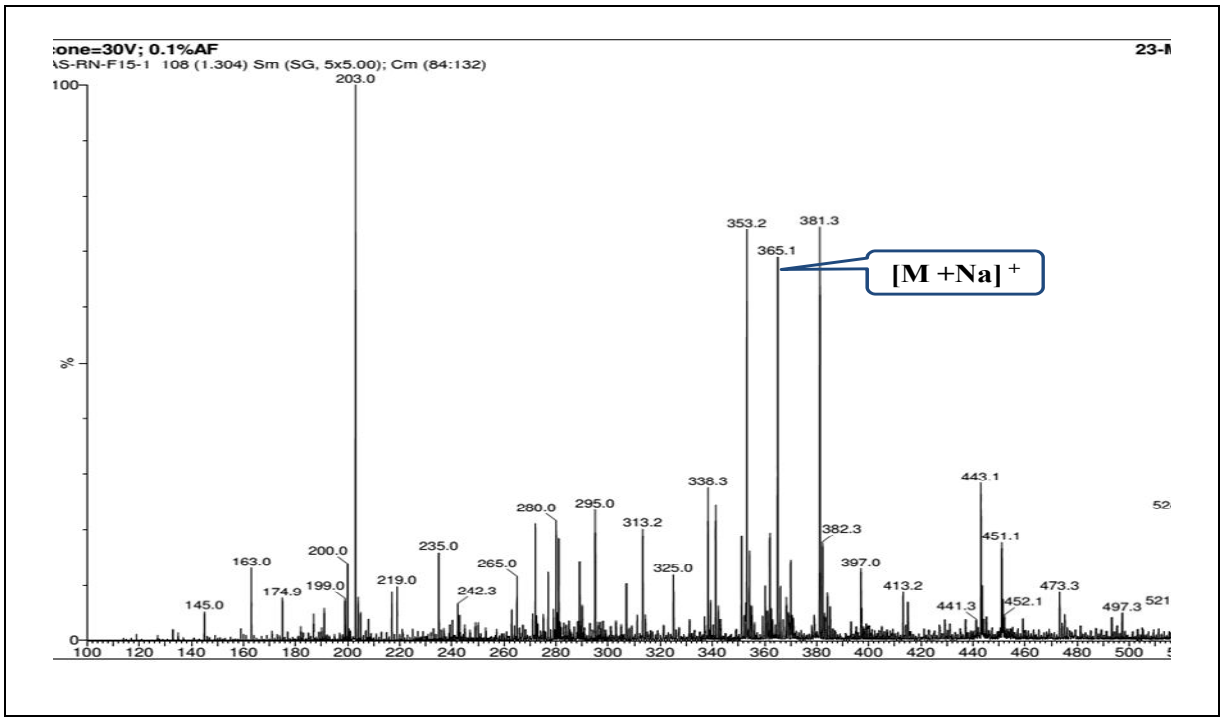


Figure 4.82 . ESI-MS mass spectrum of the compound C12

^{1}H and ^{13}C NMR spectrum analysis (Fig. 4.83 and 4.84) of compound C12 indicates signals in the average area 3.00-5.30 ppm corresponding the osidic protons.The comparison of these spectrum with those of compound C10, clearly shows the presence of one glucose unit, its anomeric proton appears at δ 5.27 ppm (d, J = 3.8 Hz, H-1, 92.09). This value (*J* = 3.8 Hz) fits well with the α configuration, another osidic unit is supposed to be a fructofuranose by the presence of two CH_2OH carbons at δ 61.22 and 62.27 ppm and quaternary carbon atom at δ C 103.58 ppm attributed to anomeric proton which will be confirmed by COSY, HSQC and HMBC analysis.

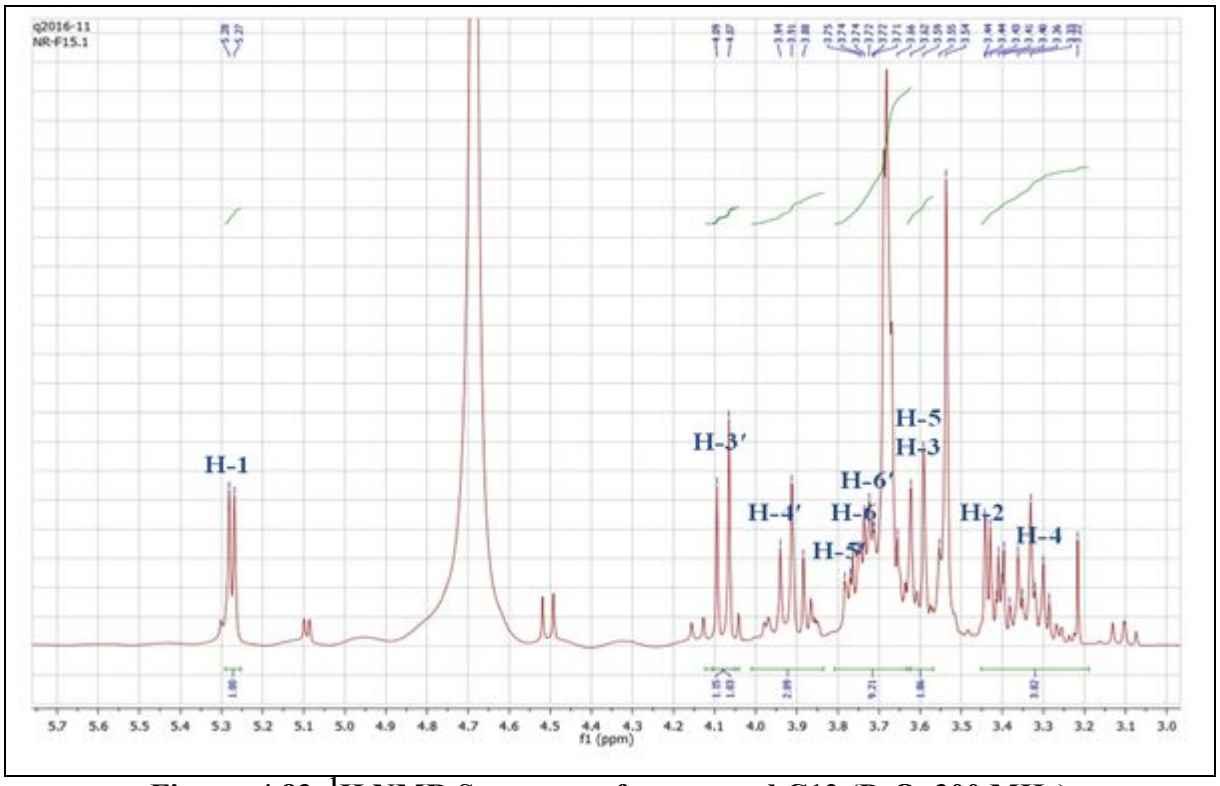


Figure. 4.83 ^{1}H NMR Spectrum of compound C12 (D_2O, 300 MHz)

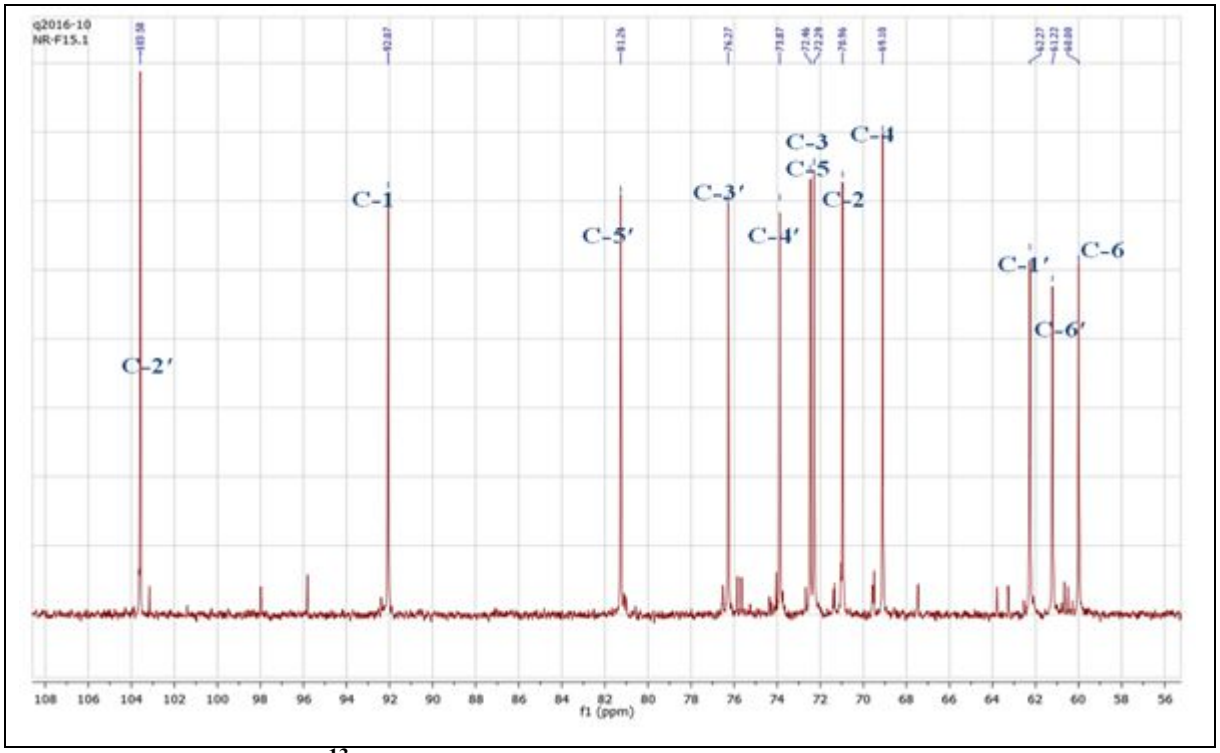


Figure. 4.84 ^{13}C NMR Spectrum of compound C12 (D_2O, 75 MHz)

COSY H-H (Fig. 4.86) and HSQC (Fig. 4.85) experiments of this compound make it possible to attribute the protons and carbons of each saccharide unit as its showd in table. 4.13. The comparison of these data and literature (Lyantagaye. 2013), indicates the presence of the α-D glucose and β-fructofuranose units in this compound.

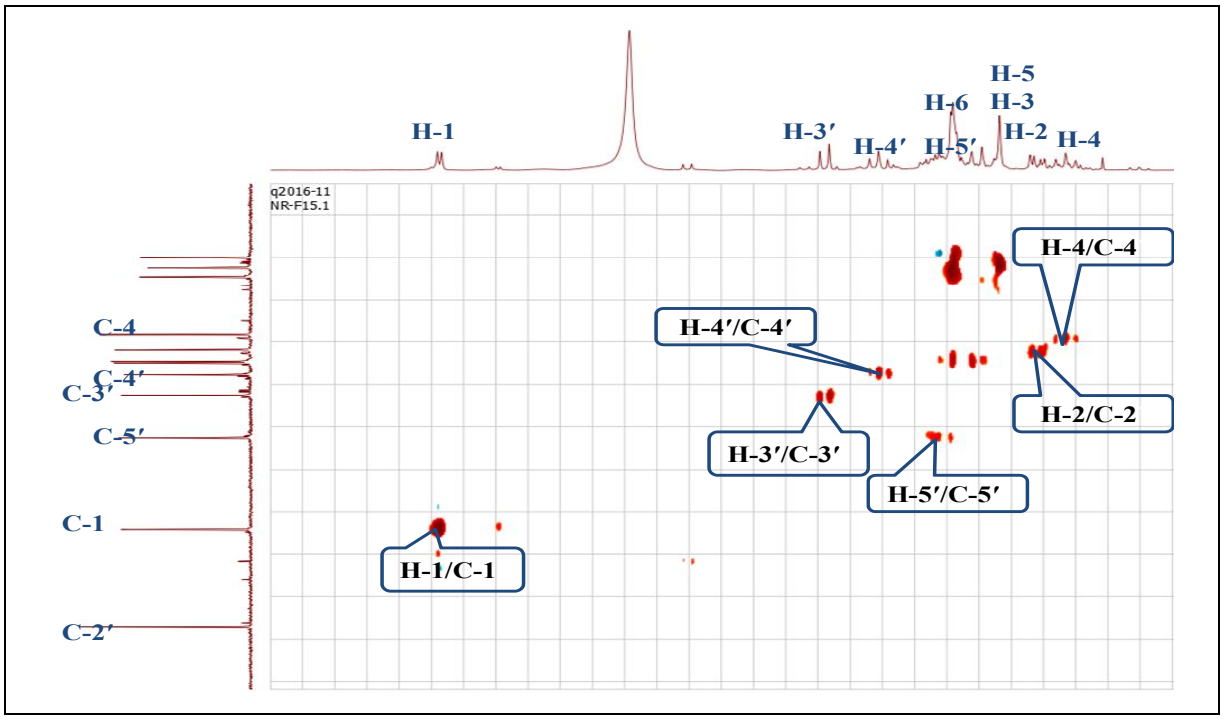


Figure. 4.85 HSQC Spectrum of compound C12 (D_2O, 300 MHz)

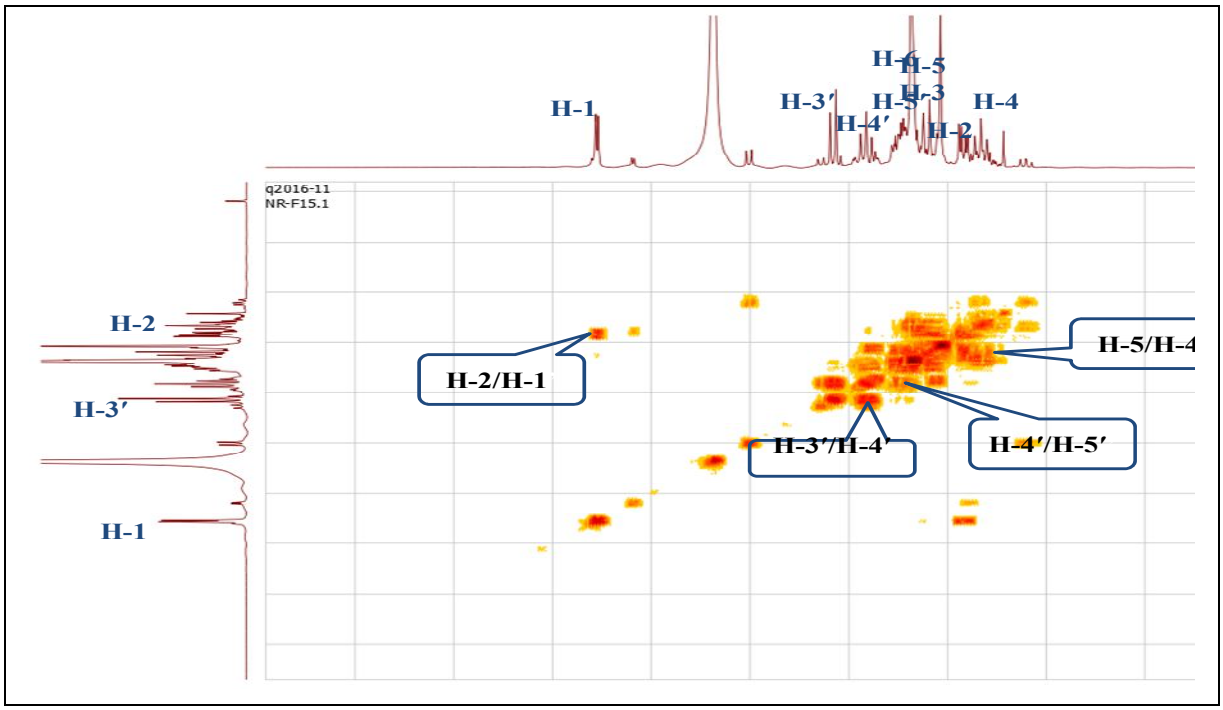


Figure. 4.86 COSY Spectrum of compound C12 (D_2O, 300 MHz)

The junctions of these disaccharide units are performed by HMBC spectrum (fig. 4.87) indicating the presence of correlation between the H-1 proton (5.27 ppm) of the glucose and the carbon C-2' (103.58 ppm) of fructofuranose unit.

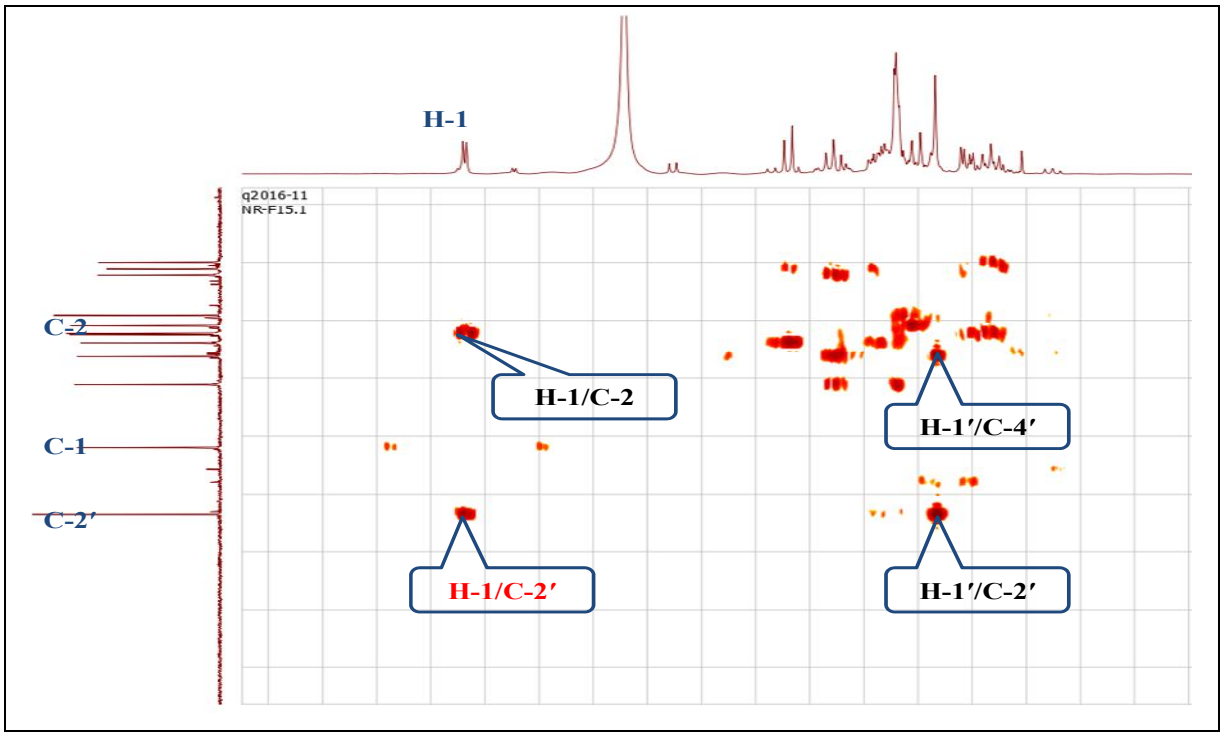


Figure. 4.87 HMBC Spectrum of compound C12 (D_2O, 300 MHz)

These observations of ^{1}H NMR, ^{13}C , HSQC, COSY, HMBC, ESI-MS mass spectrum and literature comparison (Lyantagaye. 2013) indicate that the compound C12 is a disaccharide known as sucrose or β-D-fructofuranosyl-(2'→1)-α-D-glucopyranoside.(fig. 88) The chemical shifts of the protons and carbons of the compound are shown in Table. 4.13.

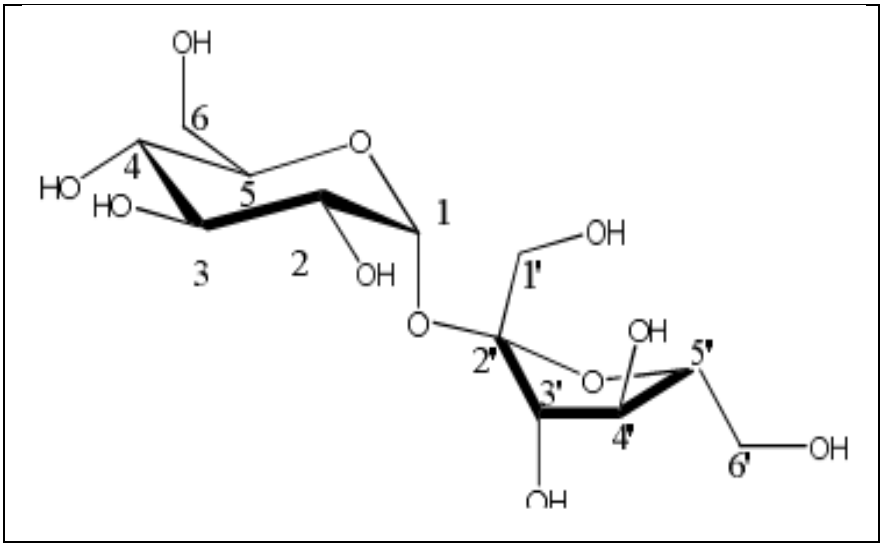


Figure. 4.88 β-D-fructofuranosyl-(2'→1)-α-D-glucopyranoside

Sucrose

Table. 4.13 1H and ^{13}C NMR Chemical shifts of compound C12 recorded in D_2O *(300 MHz)*

Postion	***δ_H ppm***	***δ_C ppm***
Glucose 1	5.25(d; *J* 3.8 Hz)	92.07
2	3.44(dd; *J* 3.8, 9.8.0Hz)	70.07
3	3.60 (m)	72.46
4	3.38(dd; *J* 3.3, 9.2.0Hz)	69.04
5	3.63(m)	72.29
6	α 3.55(m) β 3.68(m)	60.00
Fructofuranose **1′**	α 3.52(m) β 3.67(m)	62.27
2′	-	103.58
3′	4.07 (d; *J* 9.0 Hz)	76.23
4′	3.91(t; *J* 16.8 Hz)	73.87
5′	3.75(m)	81.26
6′	3.79(m)	61.22

4.1.14. Structural characterisation of Compound C11

Compound C11 was obtained as a white pouder soluble in DMSO, its ESI-MS mass spectrum (Fig. 4.89), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 203, which corresponds to $[M+Na]^+$, suggests a molecular mass 180 mau.

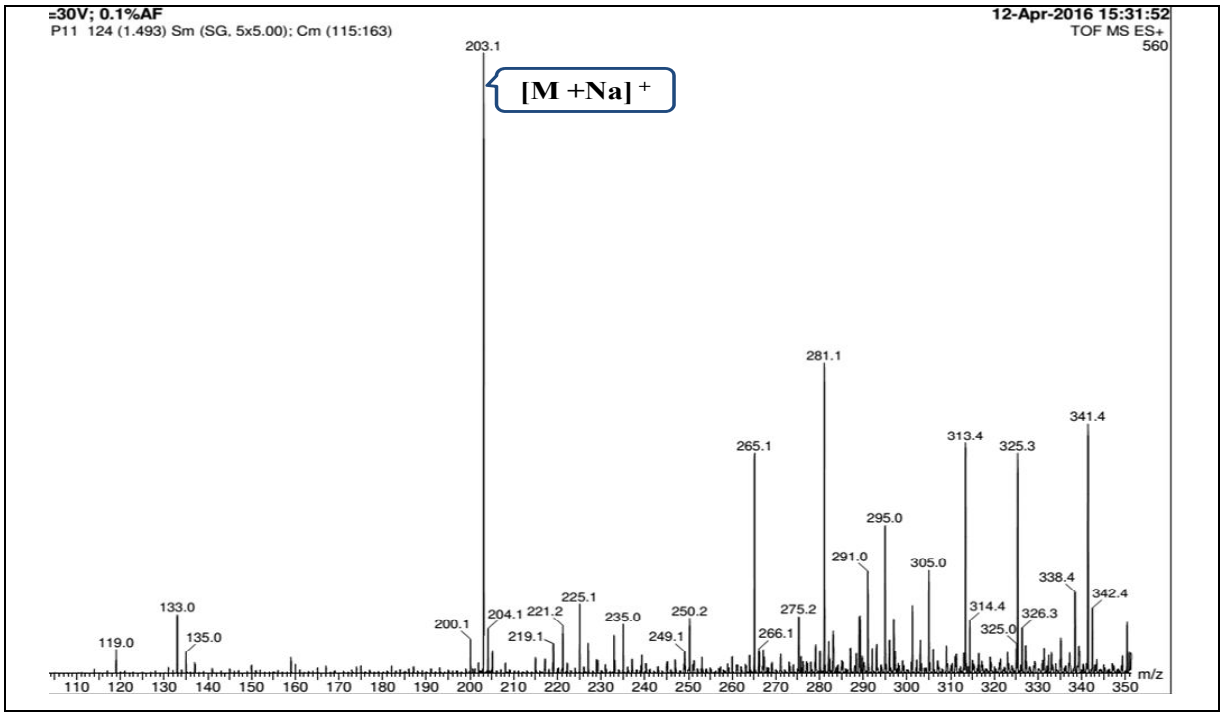


Figure 4.89 ESI-MS mass spectrum of the compound C11

Analysis of ^{1}H NMR spectrum of compound C11 recorded at 300 MHz in DMSO-d6 (fig. 4.90) showing several signals ranging from δ 3.00 to 4.00 ppm, provides information on the hydroxylated nature of the molecule. Indeed, it is observed:

- Three triplet signals; two with 1H integration at δ 3.94 ppm (t, J = 2.9 Hz)and 3.16 ppm (t, J= 9.2Hz) and one with 2H integration at δ 3.51 ppm (t, J= 9.2 Hz), assigned for H-2, H-5 and H-4/H-6;
- One doublet of doublets signal at δ 3.41 ppm (dd, J = 2.9, 9.9 Hz) assigned for H-1/H-3.

^{13}C spectrum analysis (fig. 4.91) indicates also the presence of six signals which are CHOH (oxymethine) of osidic group.

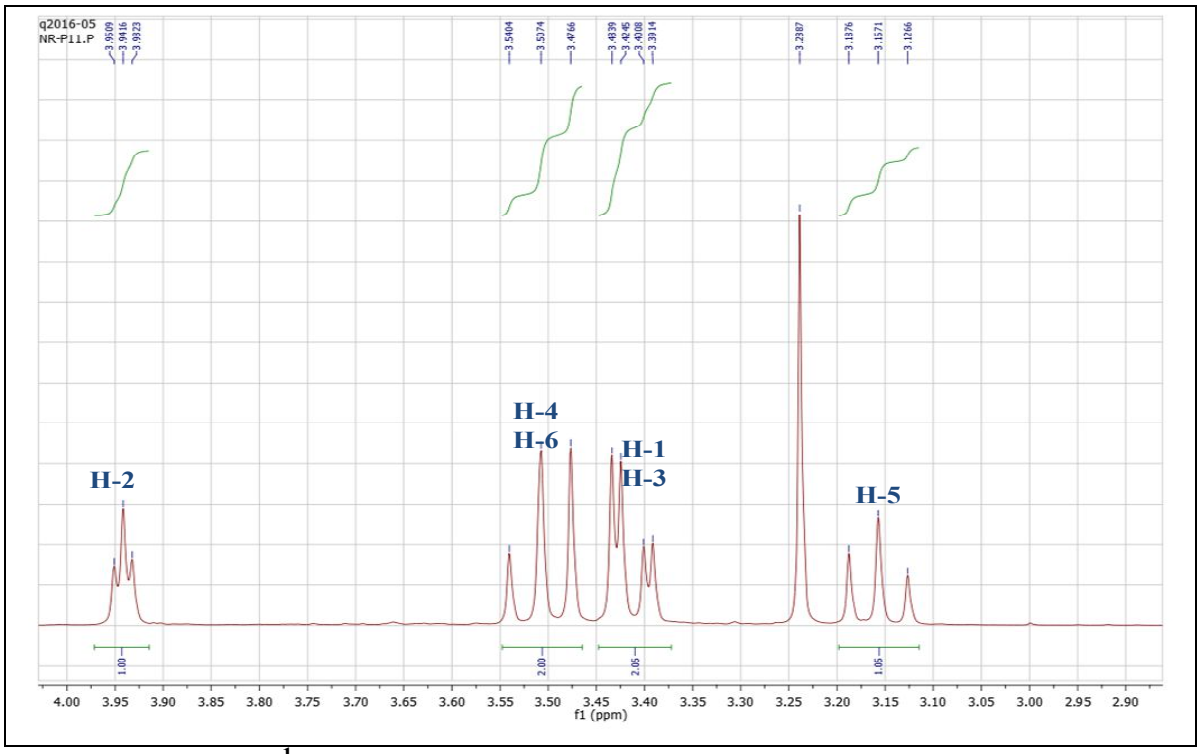


Figure. 4.90 ^{1}H NMR Spectrum of compound C11 (DMSO(d_6), 300 MHz)

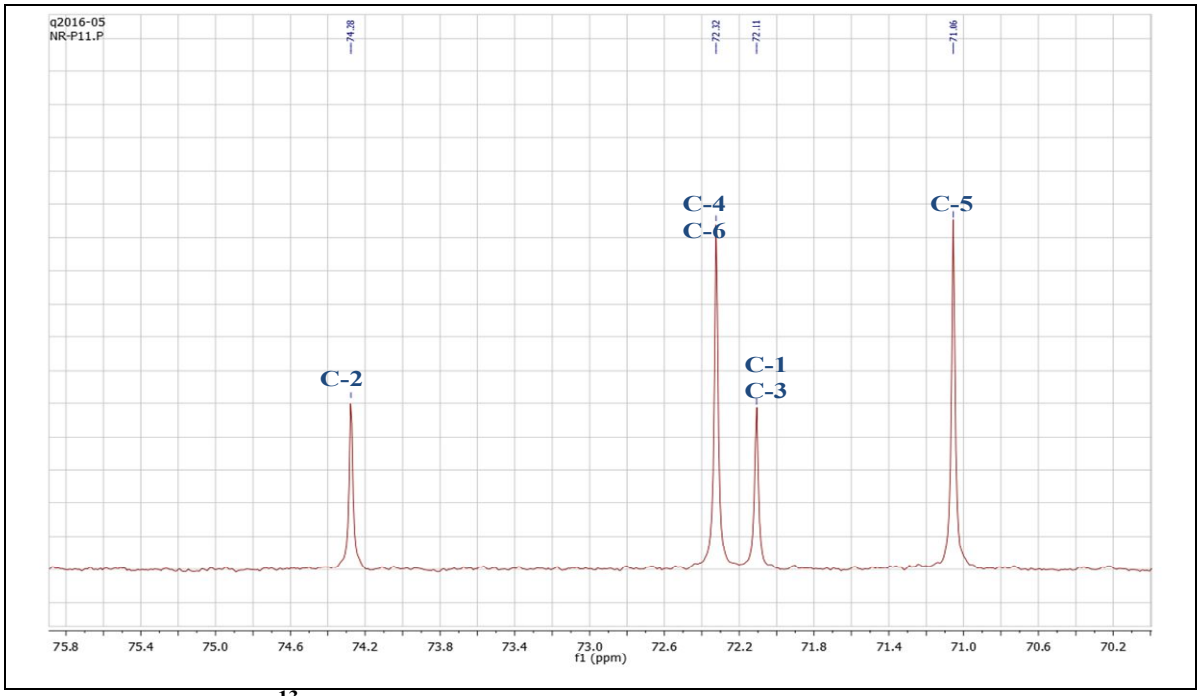


Figure. 4.91 ^{13}C NMR Spectrum of compound C11 (DMSO (d_6), 75 MHz)

HSQC (Fig. 4.92.) and COSY H-H (Fig. 4.93) spectra make it possible to attribute the protons and carbons of compound C11 and their chemical shift values are presented in table 4.14.

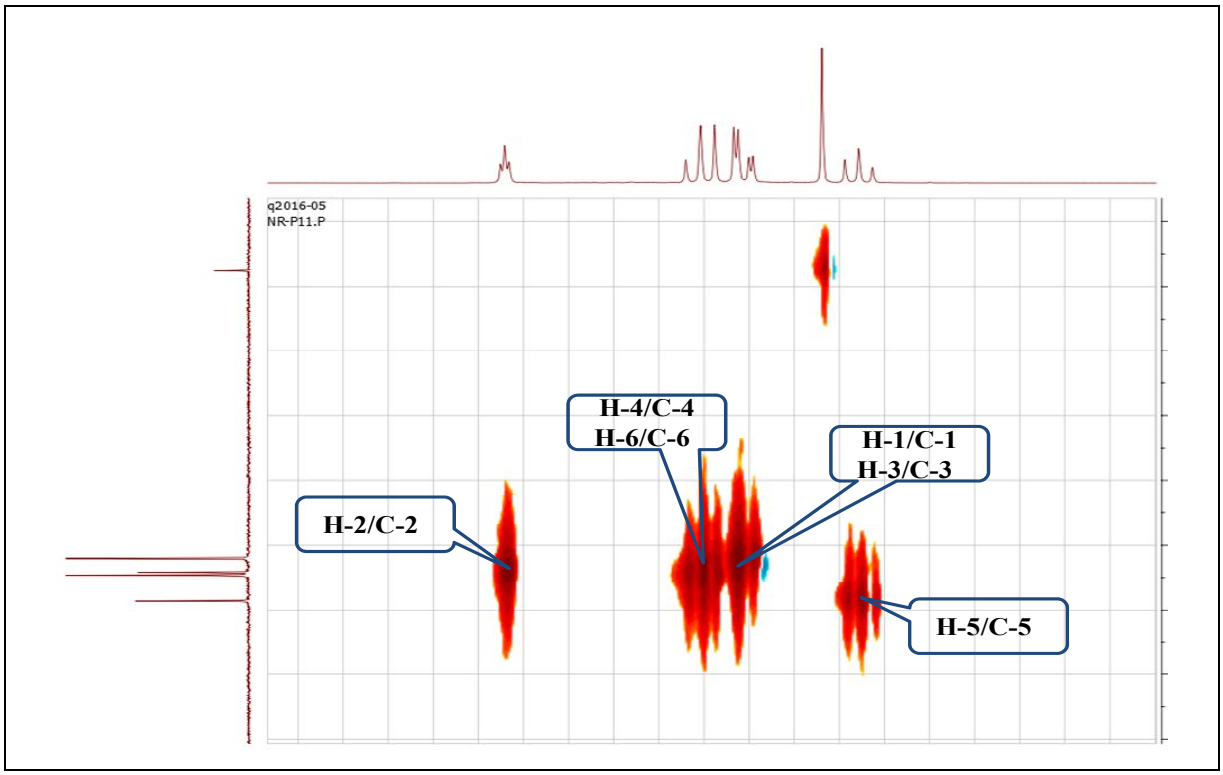


Figure. 4.92 HSQC Spectrum of compound C11 (DMSO (d_6), 300 MHz)

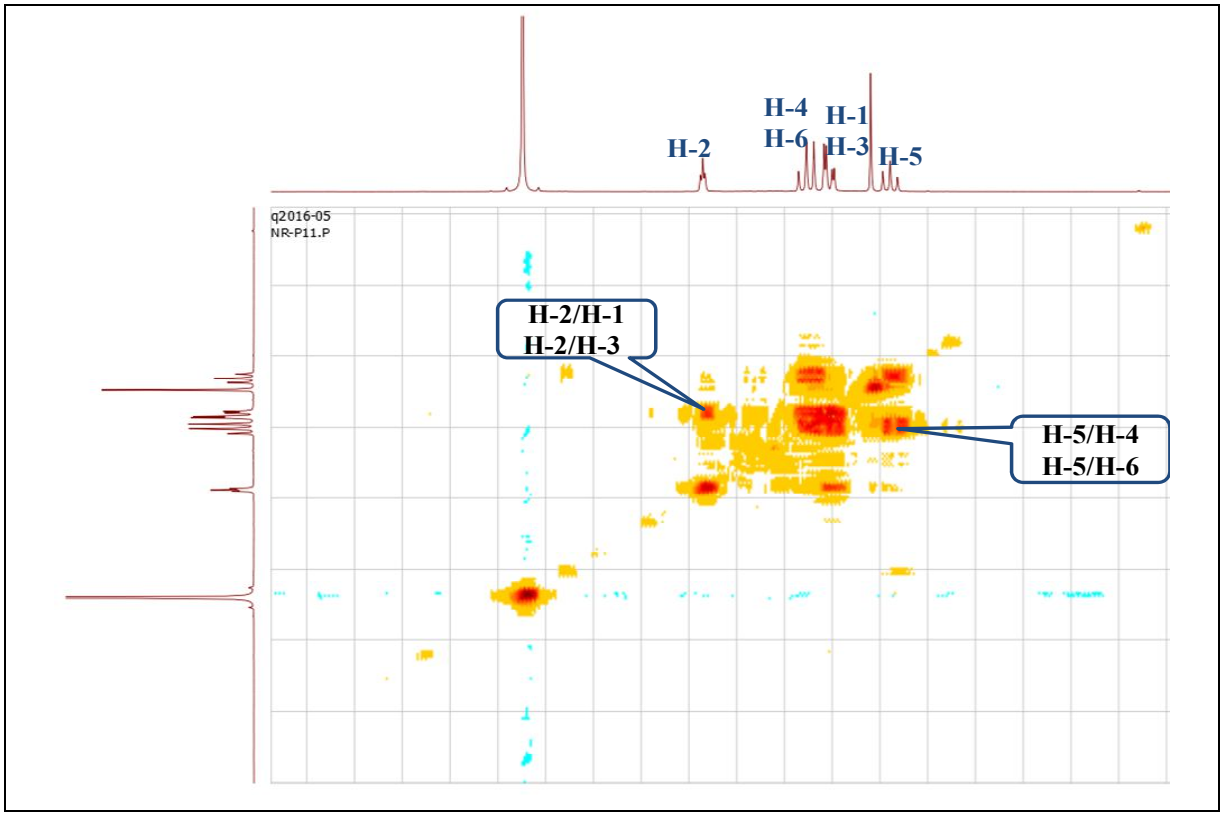


Figure. 4.93 COSY Spectrum of compound C11 (DMSO (d_6), 300 MHz)

From the exposed data (MS/ESI, NMR 1H, NMR 13C, COSY and HSQC) in addition to the comparison with literature data (Rebecca et al., 2012) proved to be identical to those of a sugar known as a **myo-inositol** (Fig. 4.94)

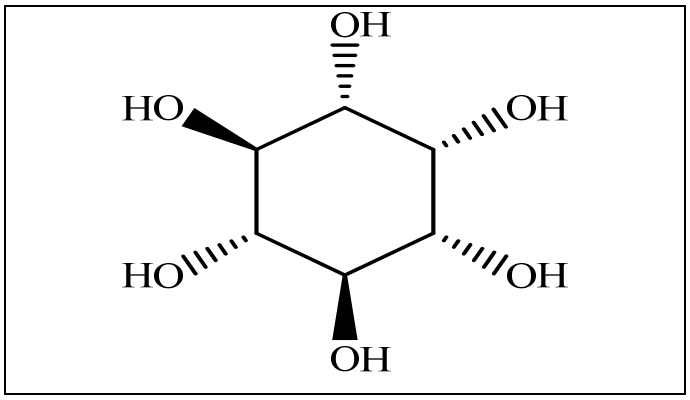


Figure. 4.94 Myo-inositol

Table. 4.14 Chemical shifts NMR ^{1}H and NMR ^{13}C of compound C11 recorded in D_2O (300 MHz)

Postion	***δ_H ppm***	***δ_C ppm***
1	3.41 (dd; *J* 2.9, 9.9 Hz)	92.07
2	3.94(t; *J* 2.9 Hz)	70.07
3	3.41 (dd; *J* 2.9, 9.9 Hz)	72.46
4	3.51(t; *J* 9.2 Hz)	69.04
5	3.16(t; *J* 9.2Hz)	72.29
6	3.51(t; *J* 9.2 Hz)	60.00

4.1.15 Structural characterisation of Compound C13

Compound C13 was obtained as yellowish powder, its ESI-MS mass spectrum (Fig. 4.95), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 793, which corresponds to $[M+Na]^+$, suggests a molecular mass 770 mau.

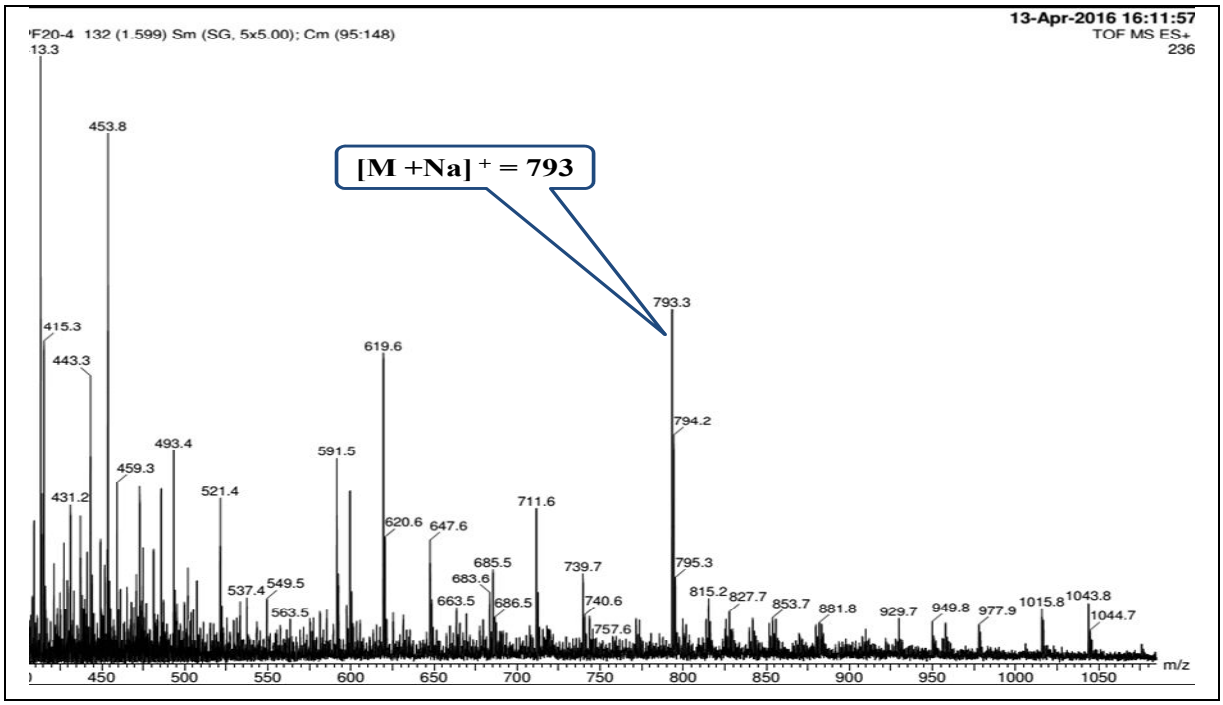


Figure. 4.95 ESI-MS mass spectrum of the compound C13

Structural analysis of ^{1}H NMR (Fig. 4.96.A, 4.96.B), COSY H-H (Fig. 4.97) spectrum of the compound C13 reveals:

- Three aromatic protons at δ 7.96 ppm (d, J = 2.1Hz), 6.94 ppm (d, J = 8.4 Hz) and 7.60 ppm (dd, J = 8.4; 2.1 Hz), thier coupling constants indicates the presence of an ABX system with three protons characteristic of 1 ', 3', 4'-trisubstituted aromatic ring, which is the ring B of flavonol. These protons are respectively assigned to H-2 ', H-5' and H-6 ';
- Two signals resonante at δ 6.21 ppm (d, J= 2.1Hz) and 6.41ppm (d, J= 2.1Hz) with 1H integration. The value of the coupling constants between these protons (J = 2.1 Hz) indicates that the coupling is Meta type. According to the COSY spectrum these two protons belong to the same spin system, corresponding to the H-6 and H-8 protons of the flavonol ring A;
- The sugar region showed three signals at δ 5.75 ppm(d, J = 7.7 Hz), 4.56 ppm (d, J = 1.5) Hz) and 5.21 ppm (d, J = 1.3 Hz), which are characteristics of anomeric protons, indicate the presence of three hexoses. Signals resonante in the average 3.30 - 4.50 ppm attributable to protons of less than three sugars;
- The two doublets at δ 1.08 ppm (d, J = 6.2 Hz) and 0.93 ppm (d, J = 6.2 Hz), typical of methyl groups, confirm the existence of two α- rhamnoses units of, the signal at δ 5.75(d, J = 7.7 Hz) ppm, confirms the presence of β-glucose unit.

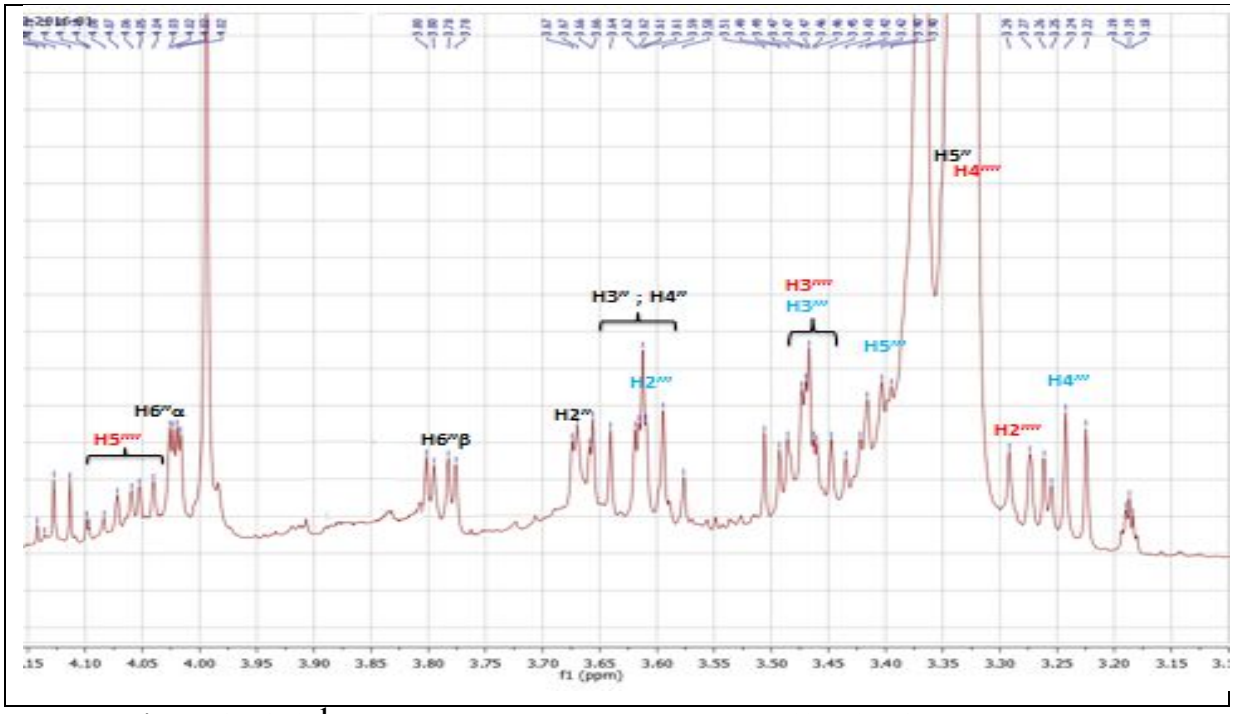


Figure.4.96. A **^{1}H NMR Spectrum of compound C13 CD_3OD, 500 MHz)**

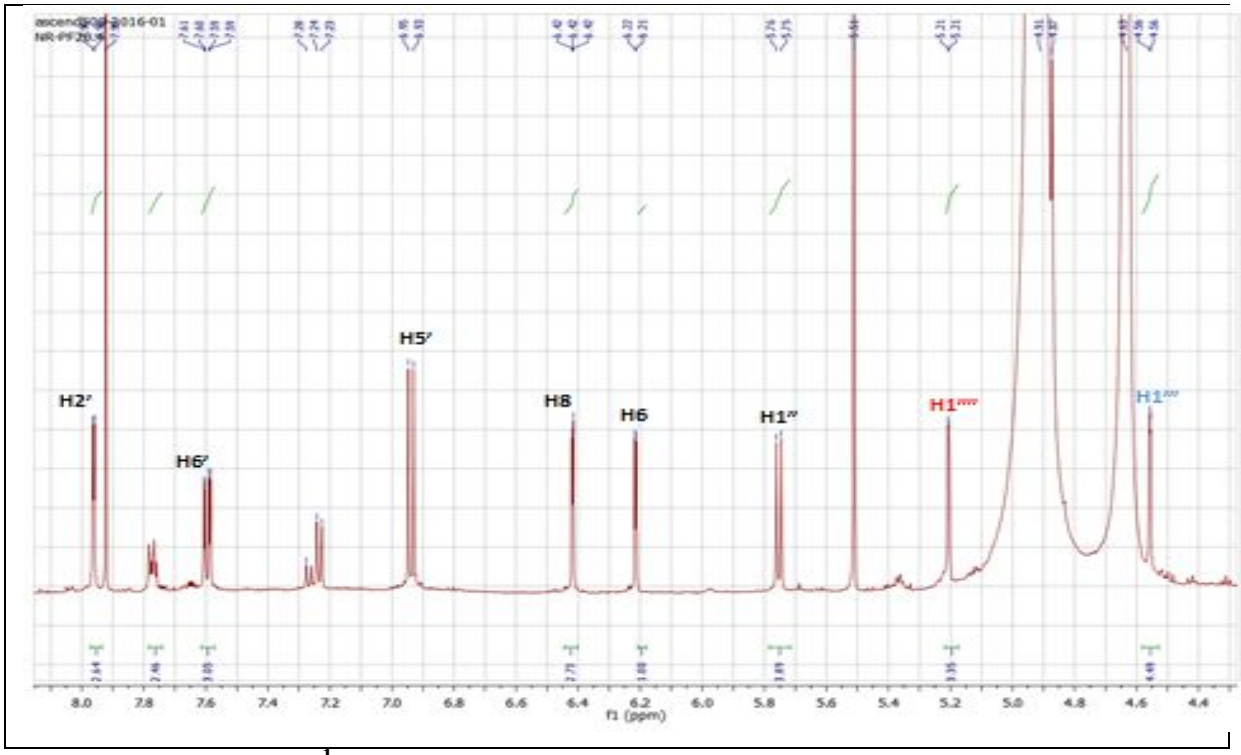


Figure. 4.97.B **^{1}H NMR Spectrum of compound C13 CD_3OD, 500 MHz)**

^{13}C NMR spectrum of compound C13 (Fig. 4.97), allows the observation of 30 signals corresponding to 30 carbon atoms which are the following:

- Carbonyl at δC 179.2 corresponding to the C-4;
- Oxygenated aromatic carbons resonating between (133 and 165) ppm;
- Seventeen CHOH , $HOCH_2$ groups resonante at δ 68-103 ppm zone corresponding to the carbons of three hexoses units;
- Methoxyl (OCH3) group detected at δ 57.00 ppm;
- Eight CH aromatic carbons detected at δ 93-123 ppm.

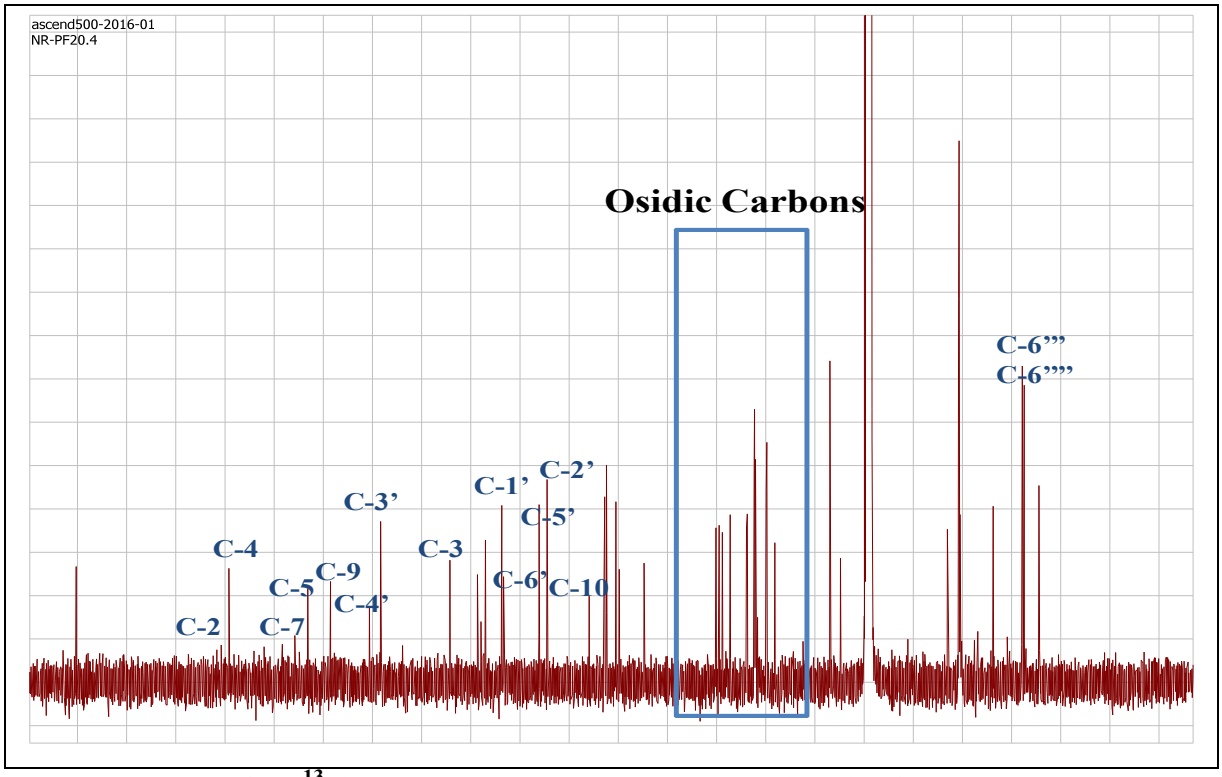


Figure. 4.97 ^{13}C NMR Spectrum of compound C13 CD_3OD, 125 MHz)

The combined analysis of HMBC and HSQC spectrum (Fig. 4.99, 4.98) allowed to confirm the structure of compound C13:

- H-2' and H-5' correlate in HMBC with 2 quaternary carbons at δ 149.5 and 122.1ppm attributable to C-4' and C-1'. The chemical shift of C-5' (δ 114.8 ppm) is determined by HSQC experiment,
- Two aromatic protons H-2' and H-6' of cycle B correlate in HSQC experiment with their carbon resonant at δ 114.5 (C-2') and 123.7 (C-6') ppm, and correlate in HMBC experiment with three oxygenated quaternary carbon C-2 (δ 158.4 ppm), C-3′ (148.4 ppm) and C-4′ (150.6 ppm);

- H-6 and H-8 of cycle A already identified in HSQC with their carbon at δ 99.81 and 94.8, also correlate in HMBC in 2J with an oxygenated aromatic carbon at δ 165.8 (C-7) and 3J with a quaternary aromatic carbon at δ 105.9 (C-10). C-5 (δC 163.2) and C-9 (δC 158,6) are identified by the following correlations presented in 2J with protons H-6 and H-8 respectively;
- From the anomeric protons H-1", H-1‴, and H-1⁗ spectral analysis COSY, allows to identify 21 protons attesting to the presence of three hexoses.

According to the preceding data; Compound C13 is C-3, C-5, C-7, C-1′, C3′ substituted flavonol; it can be tamarixetin.

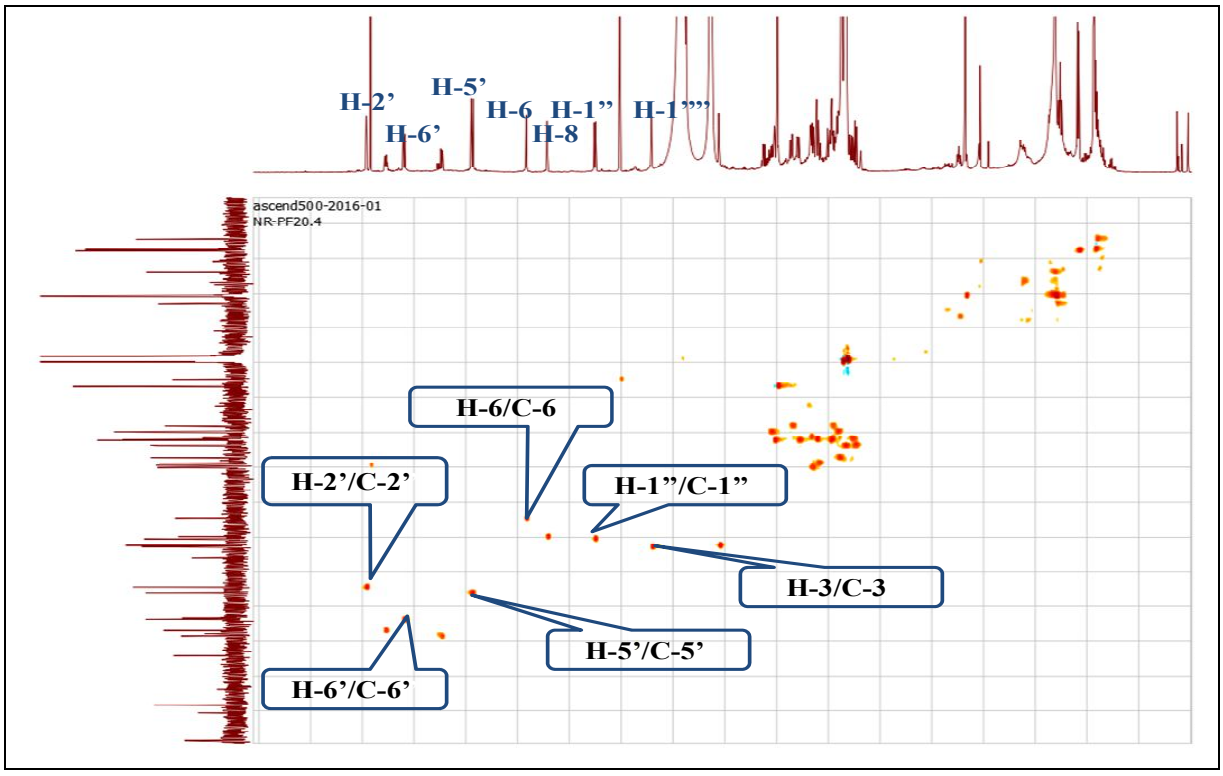


Figure. 4.98 HSQC Spectrum of compound C13 CD_3OD, 500 MHz)

From HMBC analysis spectrum (Fig. 4.99) can be noticed the correlation between:

- The anomeric proton of the glucose unit and the tamarixetin's carbon C-3(134.3 ppm),
- The anomeric protons of rhamnose units H-1‴ and H-1⁗ with C-6″ (72.4 ppm) and C-2″ (80.5 ppm). of glucose unit

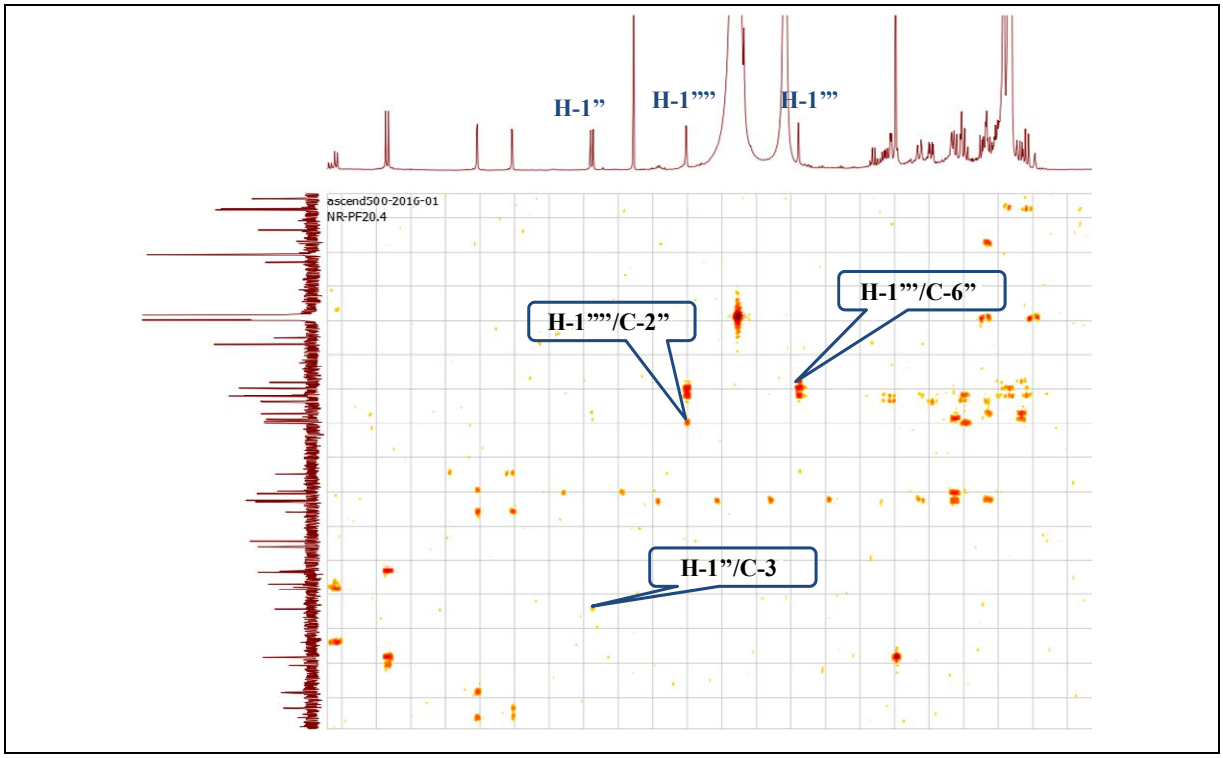


Figure. 4.99 HMBC Spectrum of compound C13 CD_3OD, 500 MHz

All these spectral data, which are summarised in table 4.15, make it possible to attribute the structure depicted in figure 4.100 to the compound C13. Moreover, **tamarixetin 3-α-L-rhamnosyl-(1→2)[α-L-rhamnosyl-(1→6)]β-D-glucoside]** was isolated for the first time in *Scabiosa* genus and.

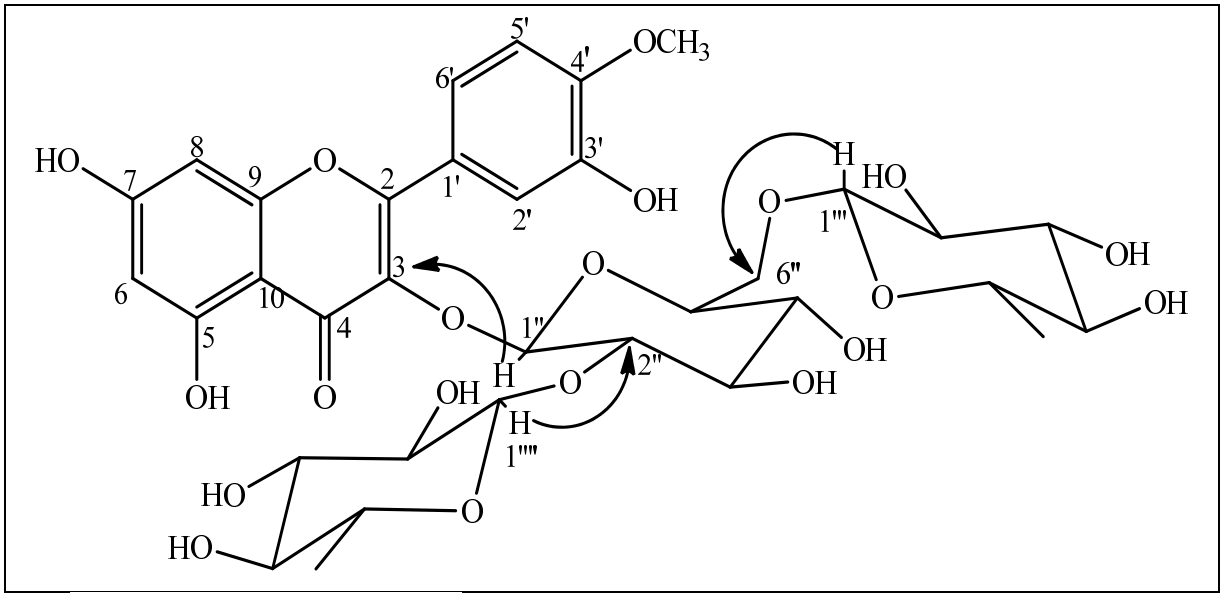


Figure. 4.100 Tamarixetin 3-β-L-rhamnosyl-(1→2)[β-L-rhamnosyl-(1→6)]β-D-glucoside]

Table. 4.15 Chemical ^{1}H and ^{13}C NMR Chemical shifts of compound C13, recorded in CD_3OD*(500 MHz)*

C_{number}	δ_C (ppm)	δ_H (ppm)
2	158.4	-
3	134.3	-
4	179.2	-
5	163.2	-
6	99.81	6.21 (d, *J* 2.1 Hz)
7	165.8	-
8	94.8	6.41 (d, *J* 2.1 Hz)
9	158.6	-
10	105.9	-
1’	123.3	-
2’	114.5	7.96 (d, *J* 2.1 Hz)
3’	148.4	-
4’	150.6	-
5’	116.1	6.94 (d, *J* 8.4Hz)
6’	123.7	7.60 (dd, *J* 2.1; 8.4 Hz)
glucose 1’’	100.5	5.75 (d, *J* 7.7 Hz)
2’’	80.1	3.67 (dd, *J* 2.2; 7.7 Hz)
3’’	78.8	3.58-3.64 (m)
4’’	73.9	3.58-3.64 (m)
5’’	77.2	3.32-3.35 (m)
6’’	72.4	3.79 (dd, *J* 3.4; 9.5 Hz)
		4.02 (dd, *J* 1.6; 3.4 Hz)
rhamnose 1’’’	102.8	4.56 (d, *J* 1.5Hz)
2’’’	72.1	3.61-3.62 (m)
3’’’	72.3	3.45-3.49 (m)
4’’’	73.8	3.22-3.27 (m)
5’’’	69.8	3.38-3.43 (m)
6’’’	17.8	1.08 (d, *J* 6.2 Hz)
rhamnose 1’’’’	102.5	5.21 (d, *J* 1.3 Hz)
2’’’’	72.0	3.26-3.29 (m)

3’’’’	72.3	3.45-3.49 (m)
4’’’’	73.9	3.30-3.33 (m)
5’’’’	69.9	4.06 (dq, *J* 9.8; 6.2 Hz)
6’’’’	17.4	0.93 (d, *J* 6.2
OCH_3	57.0	3.98 (s)

4.1.16. Structural characterization of Compound C9

Compound C9 was obtained as yellowish powder, its ESI-MS mass spectrum (Fig. 4.101), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 539, which corresponds to $[M+Na]^+$, suggests a molecular mass 516 mau.

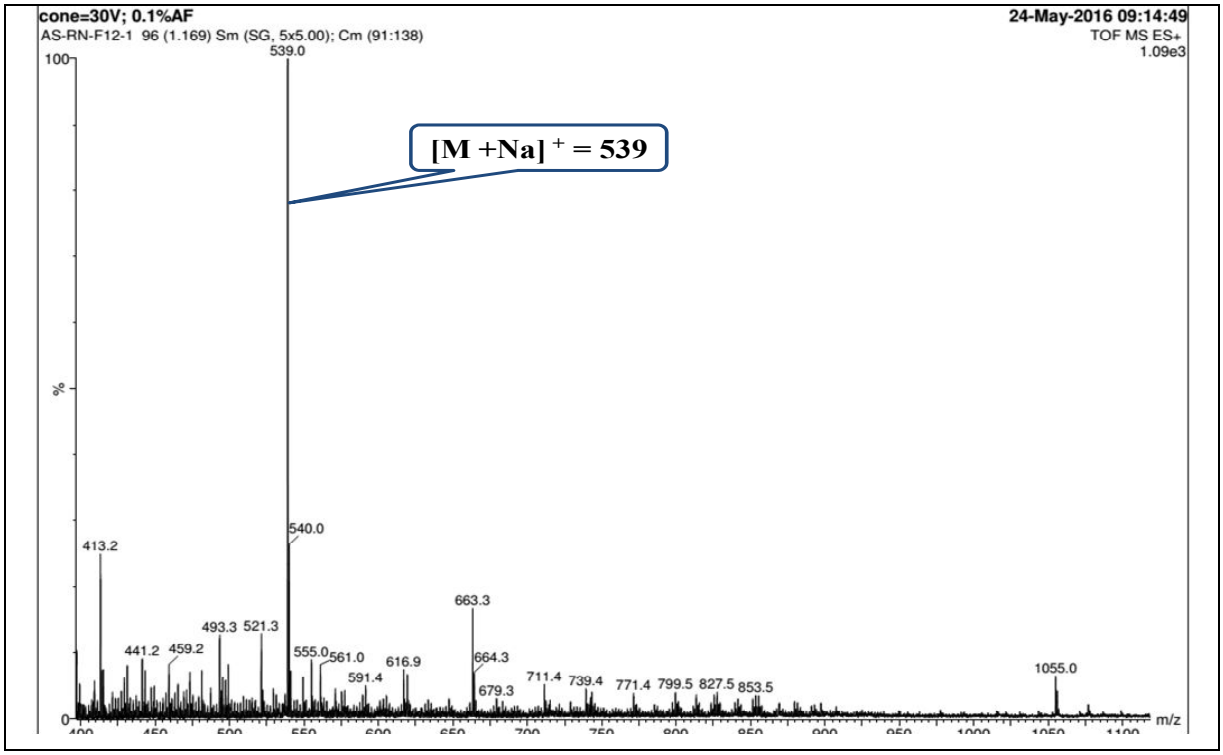


Figure 4.100 ESI-MS mass spectrum of the compound C9

^{1}H NMR and COSY spectrum analysis (Fig. 4.101 and 4.102) confirms the existence of characteristic signals of two caffeoyl acids in compound C9 as following:

- Four olefinic protons at δ 6.28(d, J = 15.7 Hz), 7.59(d, J = 15.9 Hz), 6.21(d, J = 15.9 Hz) and 7.52 (d, J = 15.7 Hz) ppm are respectively attributed to the protons 2′, 3′, 2″ and 3″. The coupling constant between these protons (J = 15.9 Hz) indicates trans position.
- Aromatic protons of two rings first one their protons resonante at δ 7.07(1H, d, J = 1.6 Hz), 6.91 (2H, m)attributed to H-5′, H-9′ and doublet of doublets at δ6.75(2H, dd,

J = 2.5, 8.2 Hz), assigned to H-8′ which confirms its ortho-coupling with H-5′ and meta-coupling with H-9′;

- Protons of second ring; H-8″ resonante at δ 6.75(dd, J = 2.5, 8.2 Hz), which confirms its ortho-coupling with H-5″(1H, d, J= 1.5 Hz)and meta-coupling with H-9″(m);

On the other hand the presence of:

- Two large doublet at δ 2.29(dl, 15.3 Hz) and 2.02(dl, 15.4Hz) ppm and two multiplets resonante at δ 2.18(m) and 2.1(m) ppm assigned to H-6α, H-6β, H-2α and H-2β using COSY analysis;
- Doublet at δ 4.32 (d, 3.5 Hz), the signals at δ 5.12(dd, 2.9, 10.2) and 5.70 (m) attributed to H-3, H-4 and H-5;

Attest to sepose the presence of quinic acid unit.

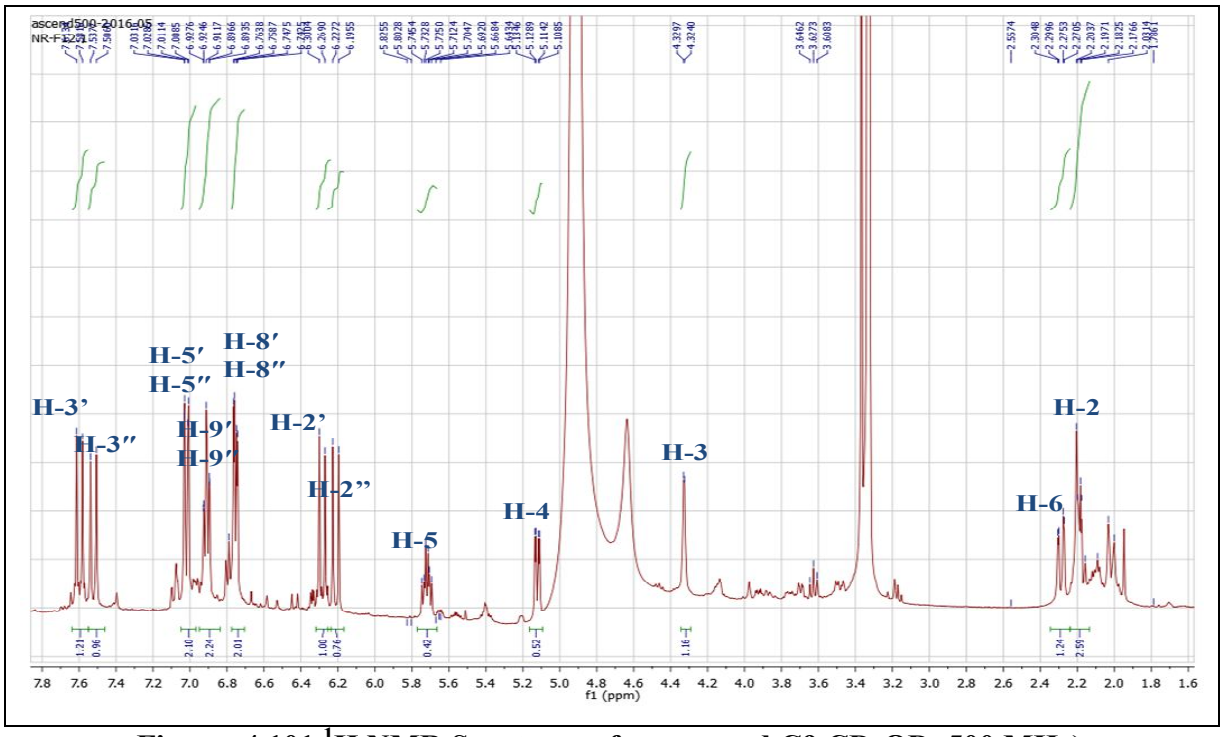


Figure. 4.101 ^{1}H NMR Spectrum of compound C9 CD_3OD, 500 MHz)

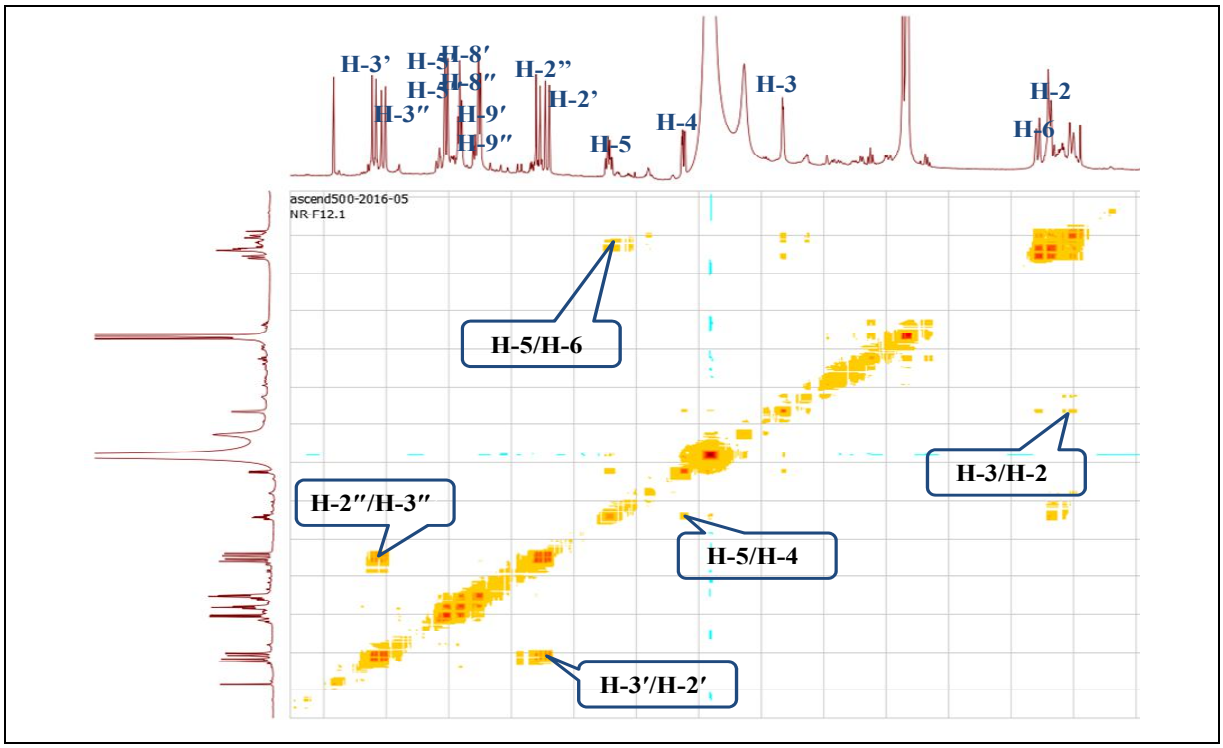


Figure. 4.102 COSY Spectrum of compound C9 (CD_3OD, 500 MHz)

^{13}C NMR and HSQC (Fig. 4.103, 4.104) experiments show 24 signals corresponding to 26 carbons, including 10 quaternary carbons (by HSQC spectrum), which are distributed as following:

- Three carbonyls group at 168.71, 168.61and 180.49 ppm (acids function according to the chemical shift value) attributable to C-1′, C-1″ and C-7 using HSQC spectrum;
- Olefinic carbons 114.93, 147.53, 114.89 and 147.34 ppm attribute to 2′, 3′, 2″and 3″
- Aromatic carbons resonante at δ 122-150 ppm;
- The carbons at δ 38-78 ppm confirm the presence of quinic acid.

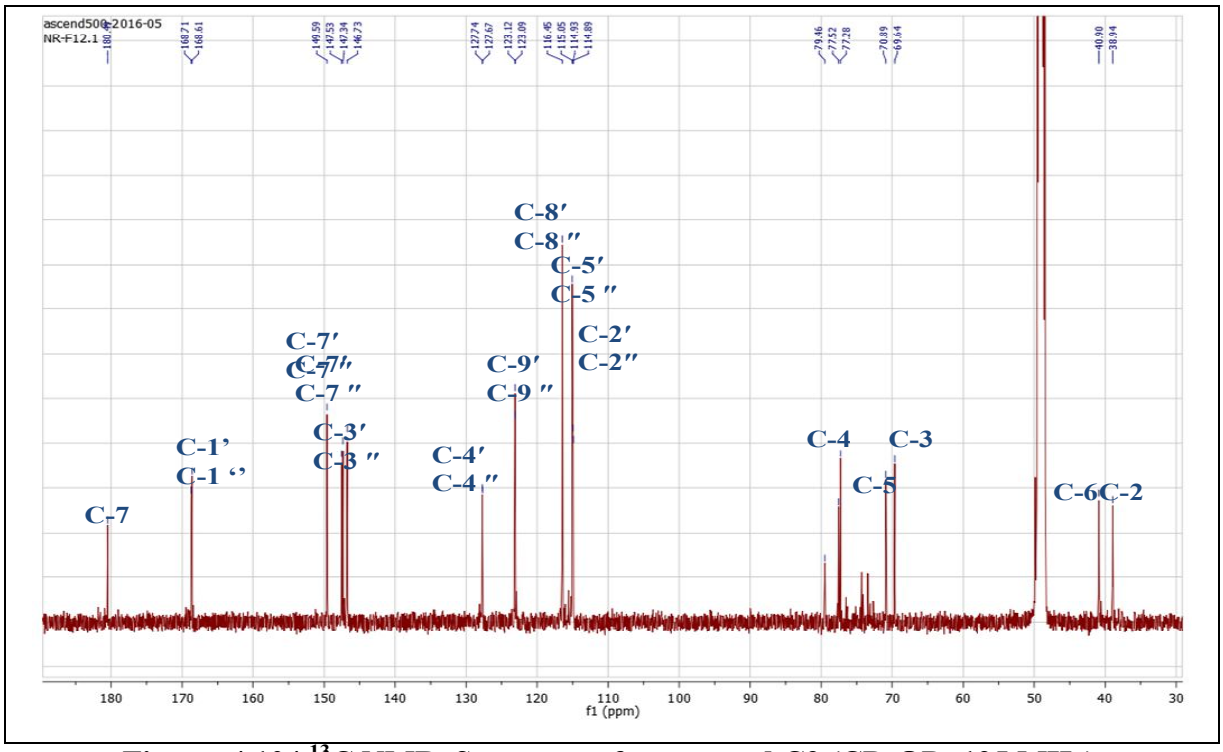


Figure. 4.104 ^{13}C NMR Spectrum of compound C9 (CD_3OD, 125 MHz)

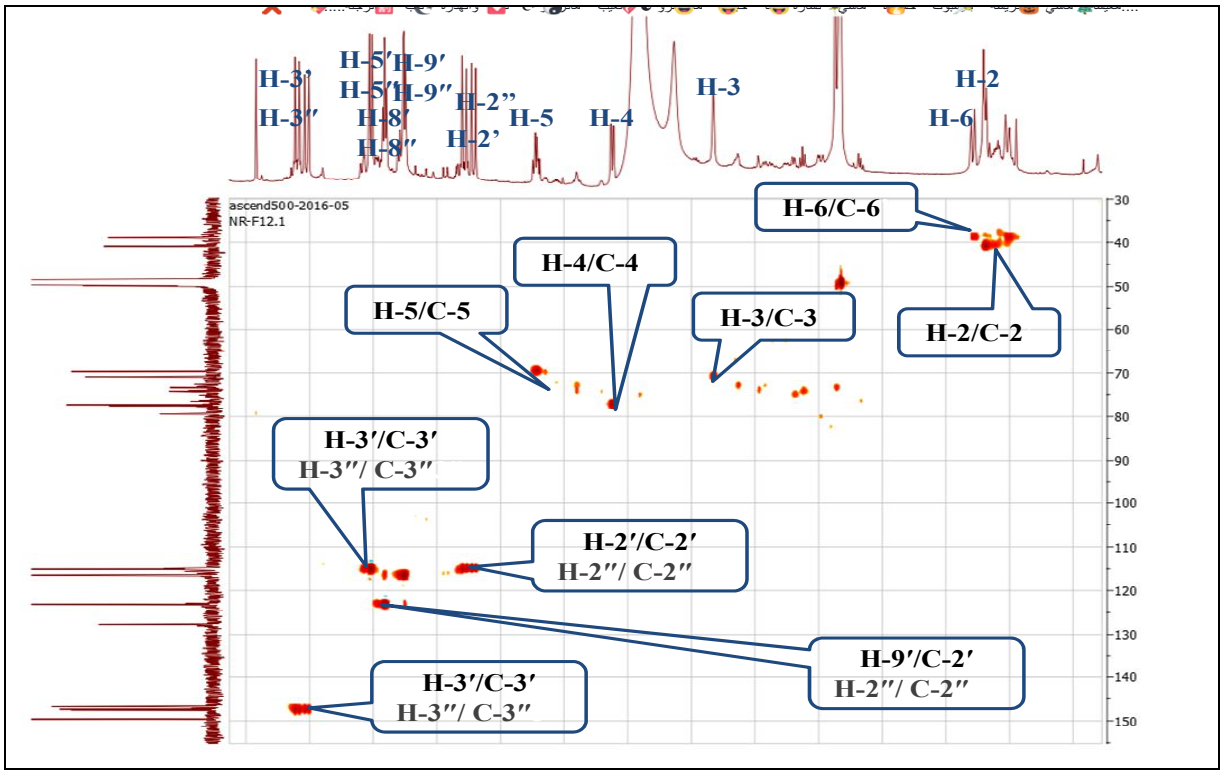


Figure. 4.103 HSQC Spectrum of compound C9 (CD_3OD, 500 MHz)

The HMBC experiment (Fig. 104) allows assigning the other carbons, also indicates the connectivities of caffeoyl acids units in C-4 and C-5 due to the correlations between C-1′ (168.71 ppm); C-1″ (168.61ppm) and H-4; H-5, respectively.

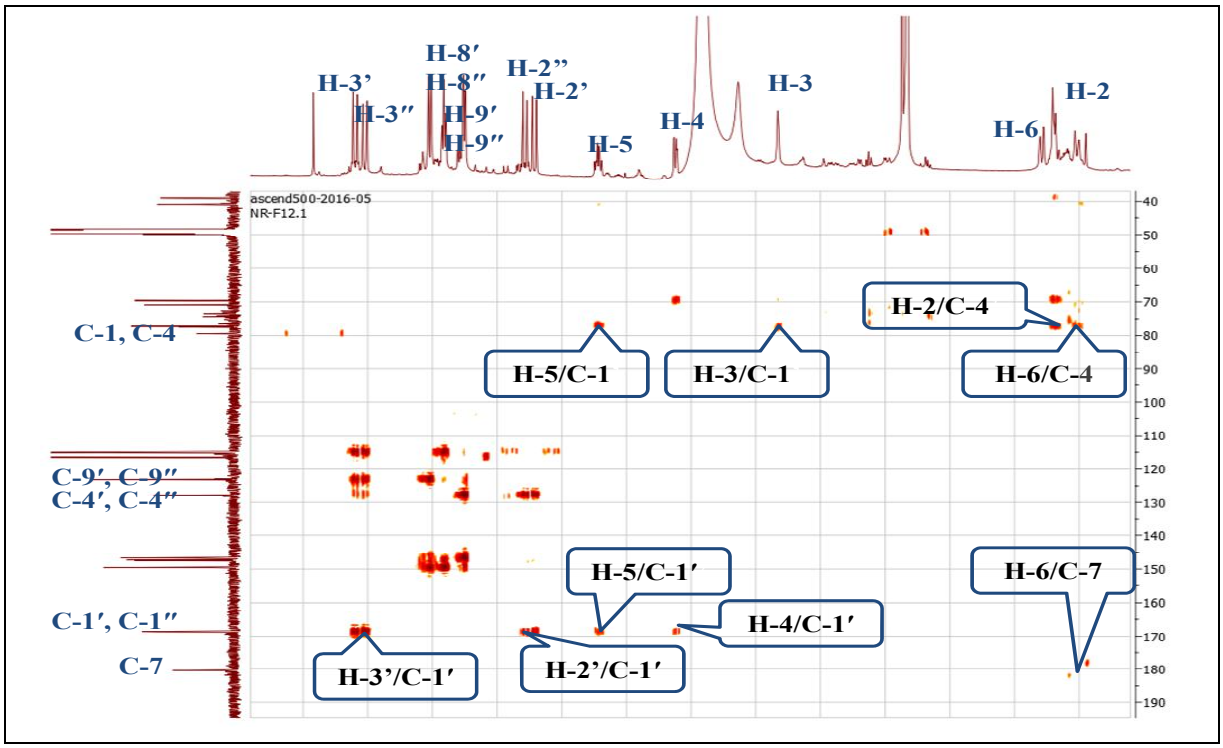


Figure. 4.104 HMBC Spectrum of compound C9 (CD_3OD, 500 MHz)

The proposed structure for compound C9 is **4,5-O- dicaffeoylquinic acid** (fig. 4.105), was established by comparison of spectroscopic data (ESI-MS, ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC) with literature data (Nicola et al., 2015).

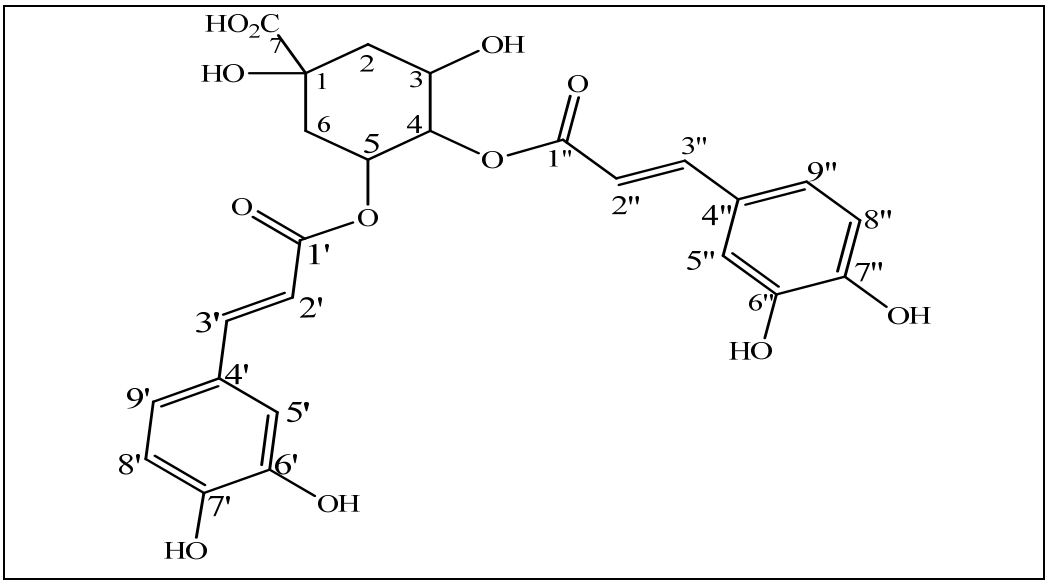


Figure. 4.105 4,5-O-dicaffeoylquinic acid

Table.4.16 Chemical ^{1}H and ^{13}C NMR Chemical shifts of compound C13, recorded in CD_3OD*(500 MHz)*

Postion	***δ_H ppm***	***δ_C ppm***
1	-	77.52
2	2.18(m), 2.10 (m)	38.94
3	4.32(d, J 3.5 Hz)	69.64
4	5.12(dd; *J* 2.9, 10.2 Hz)	77.28
5	7.5(m)	70.89
6	2.29 (dl) 2.02(dl)	40.91
7	-	180.49
1′	-	168.71
2′	6.28(d; *J* 15.7 Hz)	114.92
3′	7.59(d; *J* 15.9 Hz)	147.53
4′	-	127.74
5′	7.07(d; J 1.6 Hz)	115.22
6′	-	146.79
7′	-	149.58
8′	6.75(dd; *J* 2.5, 8.2 Hz)	116.45
9′	6.91(m)	123.12
1″	-	168.61
2″	6.21 (d; *J* 15.9 Hz)	114.89
3″	7.52 (d; *J* 15.9 Hz)	147.34
4″	-	127.67
5″	67.01 (d; *J* 1.5 Hz)	115.05
6″	-	146.73
7″	-	149.5
8″	6.75(dd; *J* 2.5, 8.2 Hz)	116.45
9″	6.91 (d; *J* 1.6 Hz)	123.09

4.2. Identification of the compounds isolated from *sedum carealeum*

4.2.1. Structural characterisation of Compound P4

Compound P4 was obtained as brown oil, its ESI-MS mass spectrum (Fig. 4.106), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 355, which corresponds to $[2M+Na]^+$, suggests a molecular mass 166 uma.

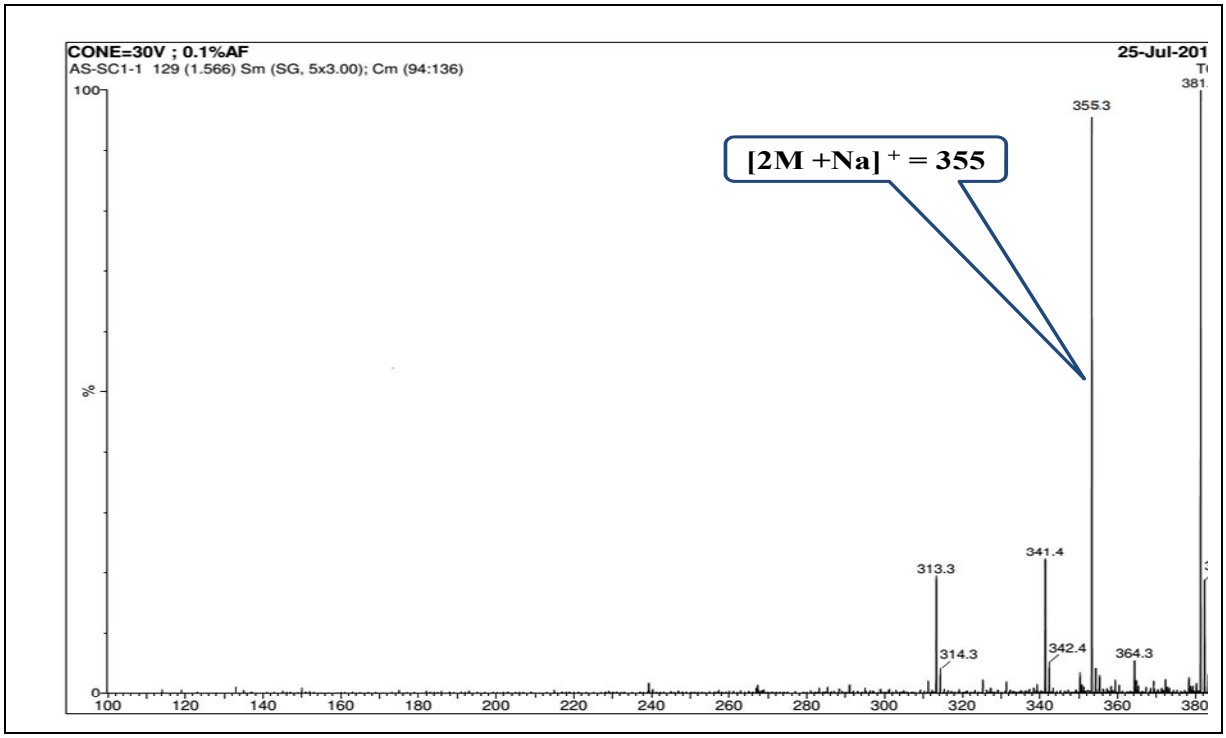


Figure 4.106 ESI-MS mass spectrum of the compound P4

Analysis of ^{1}H NMR spectrum (Fig. 4.107) shows the following signals:

- Two Doublet of doublets resonante at δ 7.96(2H, dd, J= 4.8, 14.3 Hz) and 6.85(2H, dd, J= 4.8, 14.3 Hz) ppm;
- An doublet of doublets at δ 4.34(2H, dd, *J* 7.2,14.3Hz) ppm attribuated to OCH_2 proton ;
- Triplet resonante at δ 1.38 (3H, t, *J* 7.2) ppm;

These signals characterized of an aromatic compound; benzene ring para di-substituted.

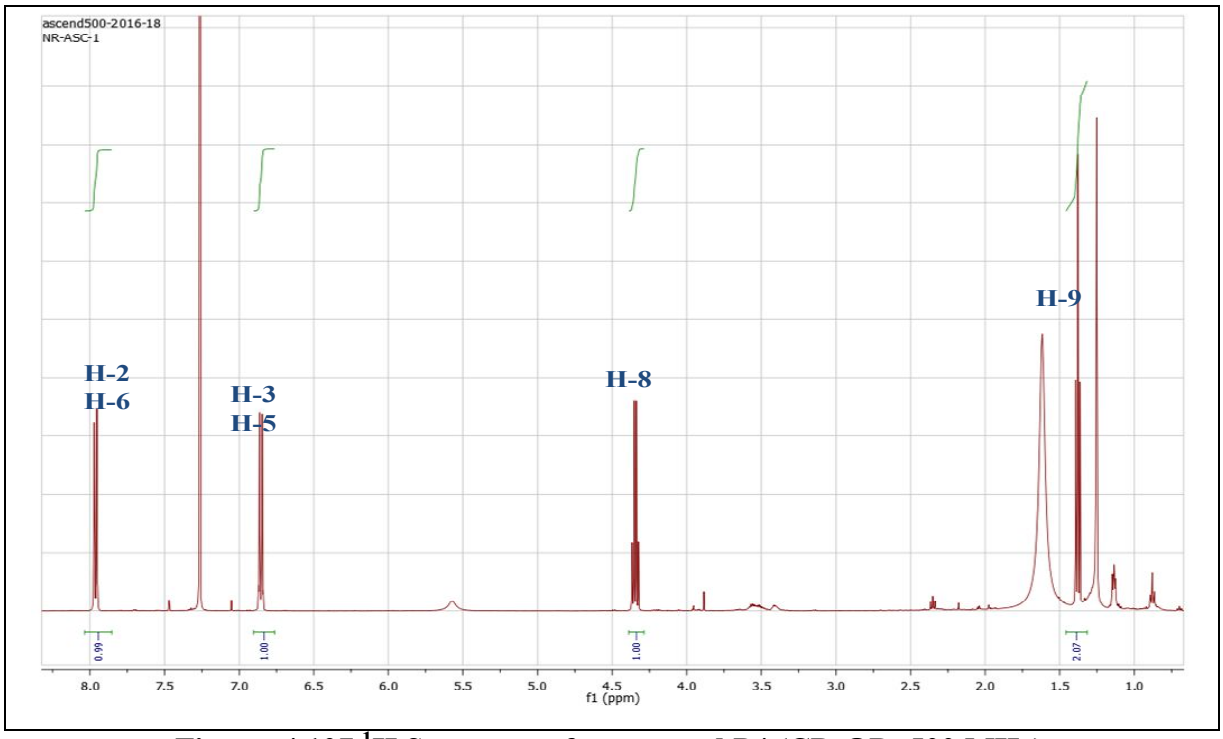


Figure. 4.107 ^{1}H Spectrum of compound P4 (CD_3OD, 500 MHz)

^{13}C NMR spectrum analysis (Fig. 4.108) confirms the presence of :

- R -OCH_2 and CH_3 carbons resonante at δ 60.72; 14.38ppm;
- aromatic CH carbons at δ (131.87and 115.10 ppm);
- Quaternary carbons at δ 123.24, 159.54 ppm (oxygenated) and acid or ester function at δ 166.33 ppm.

All these cumulative data indicate compound P4 is a para-substituted benzyl by ester and hydroxyl groups.

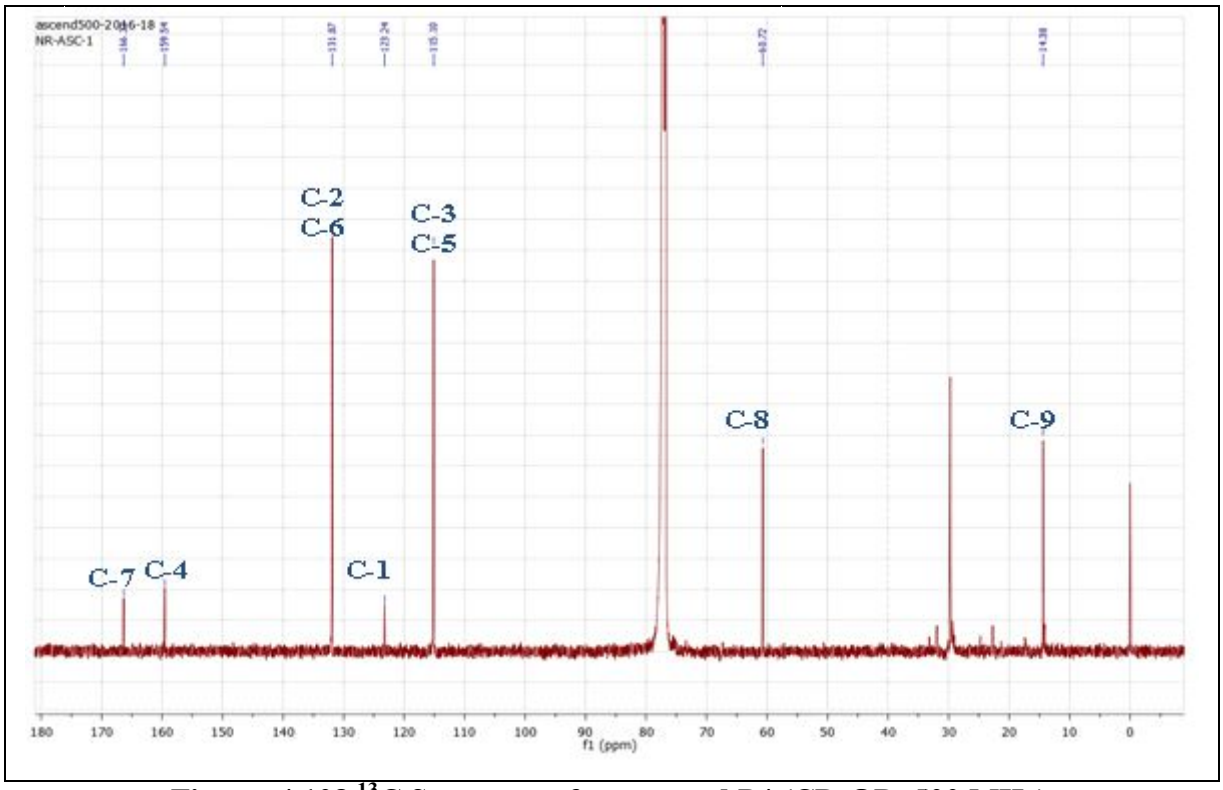


Figure. 4.108 ^{13}C Spectrum of compound P4 (CD_3OD, 500 MHz)

^{1}H and ^{13}C NMR, COSY and HSQC (fig. 4.109) analysis lead to the primary structure and it is confirmed by HMBC spectrum which shows correlations between the carbon C-7(166.33ppm) and two protons H-2(7.96ppm) , H-8(4.34 ppm).(fig. 4.110)

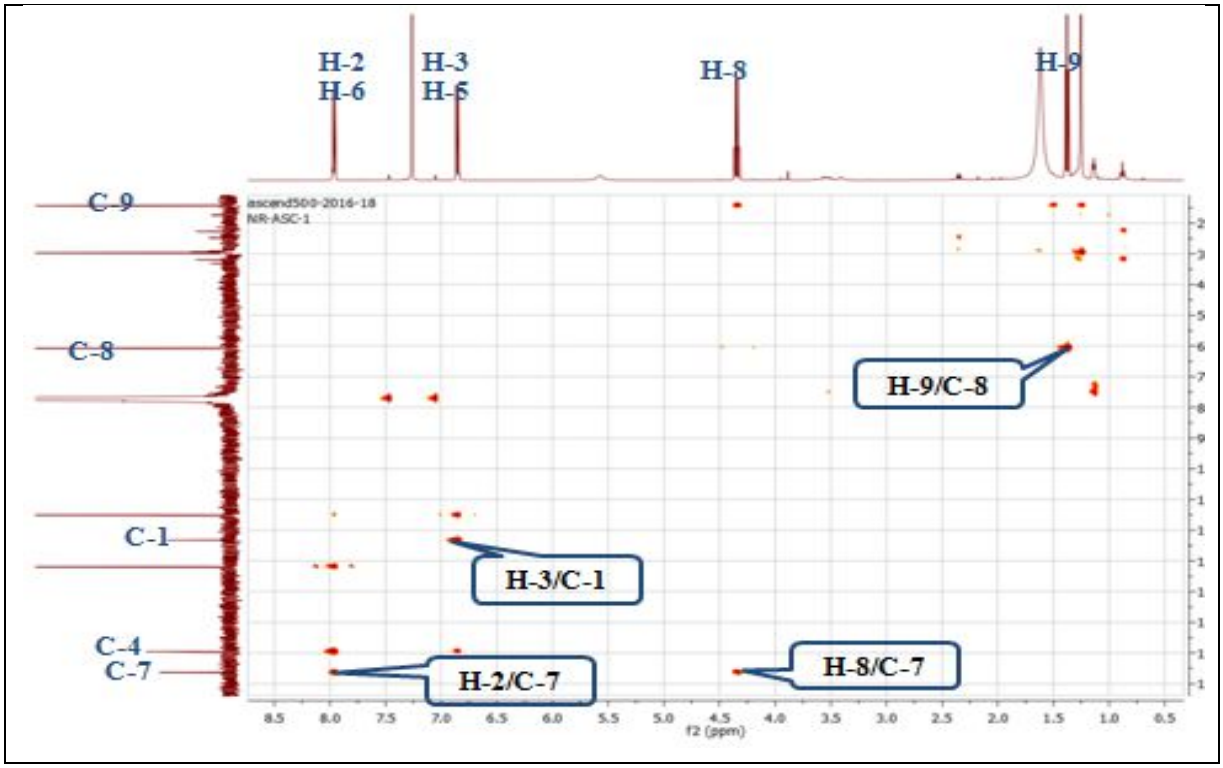


Figure. 4.109 HMBC Spectrum of compound P4 (CD_3OD, 500 MHz)

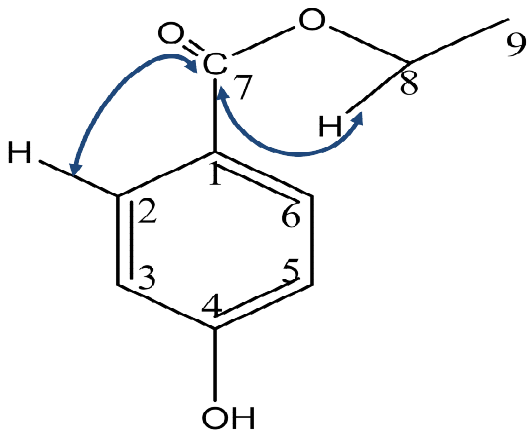


Figure. 4.110. Interesting correlation of HMBC spectrum analysis

Based on the above mentioned data, which is more detailed in table 4.17, and also comparing with literature data, was possible to establish that compound P4 is **4-hydroxy ethylbenzoate**

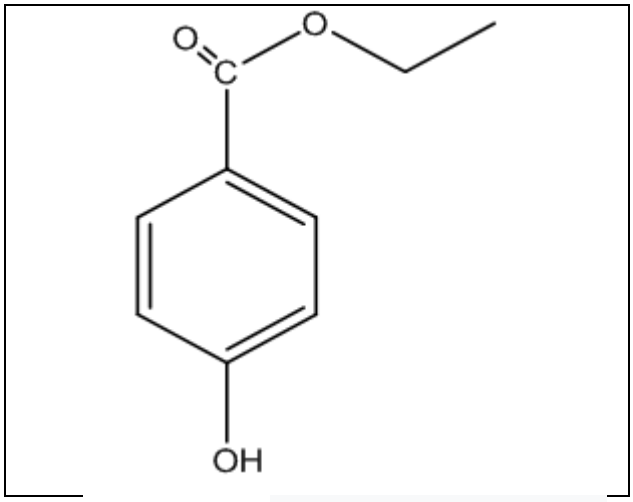


Figure. 4.111 4-hydroxy ethylbenzoate

Table. 4.17. Chemical ^{1}H and ^{13}C NMR Chemical shifts of compound P4, recorded in CDCl*(500 MHz)*

Postion	δ_H *ppm*	δ_C ppm
1	-	123.24
2	7.96 (dd; *J* 4.8, 14.3 Hz)	131.87
3	6.85 (dd; *J* 4.8, 14.3 Hz)	115.10
4	-	159.54
5	6.85 (dd; *J* 4.8, 14.3 Hz)	115.10
6	7.96 (dd; J 4.8, 14.3 Hz)	131.87

7	-	166.33
8	4.34 (dd; J 7.2, 14.3 Hz)	60.72
9	1.38 (t; 7.2Hz)	14.38

4.2.2. Structural characterisation of Compound P5

Compound P5 was obtained as brown oil and its ESI-MS mass spectrum (Fig. 4.112), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 315, which corresponds to $[2M+K]^+$ and suggests a molecular mass equal to 138 uma.

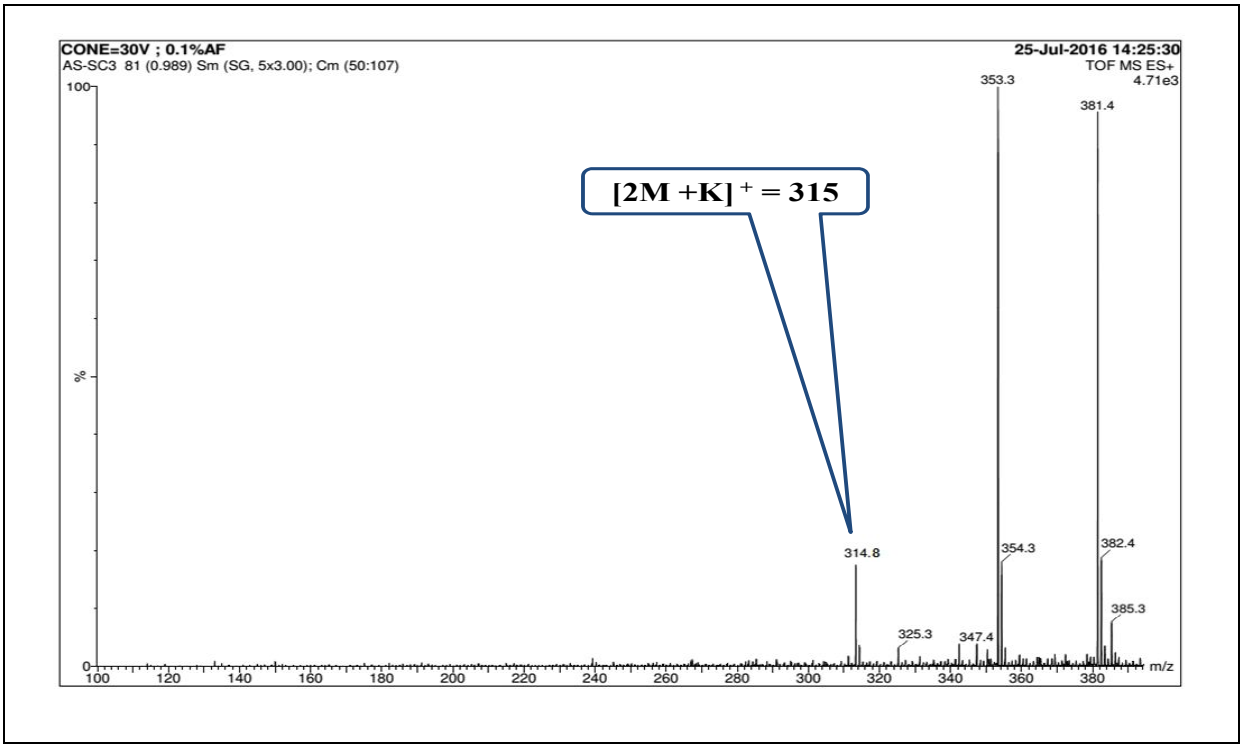


Figure 4.112 ESI-MS mass spectrum of the compound P5

^{1}H NMR spectrum (Fig. 4.113) of compound P5 has a different appearance comparing to the compound P4 already have been identified. Indeed, it shows only two signals as a doublet with 2H integration at δ 7.84(2H, d, *J*= 8.7 Hz) and 6.77 (2H, d, 8.7 J= Hz) ppm characteristic of aromatic ring para bi-substituted.

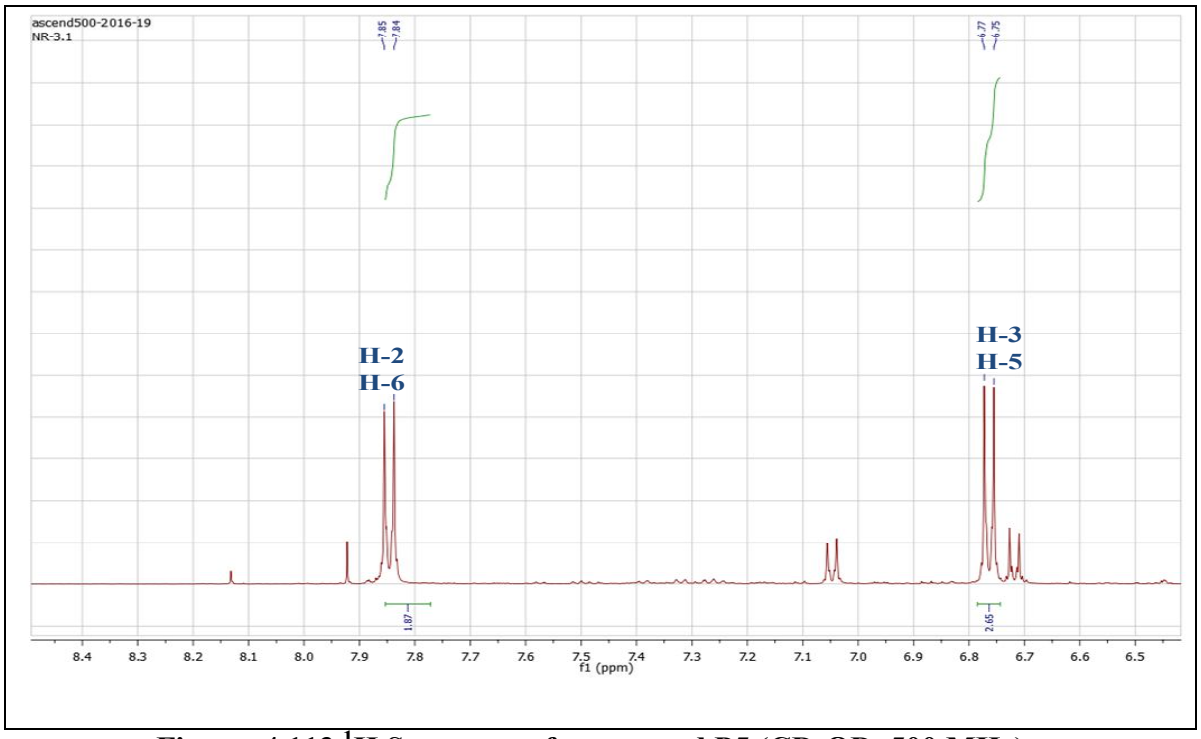


Figure. 4.113 ^{1}H Spectrum of compound P5 (CD_3OD, 500 MHz)

In ^{13}C NMR analysis spectrum (Fig. 4.114), the following signals are observed:

- 128.63 (C-1), 161.34 (C-4) and 173.94 (C-7) ppm are quaternary carbons, also signals at δ 132.44(C-2/ C-6) and 115.30 (C-3/C-5)ppm.

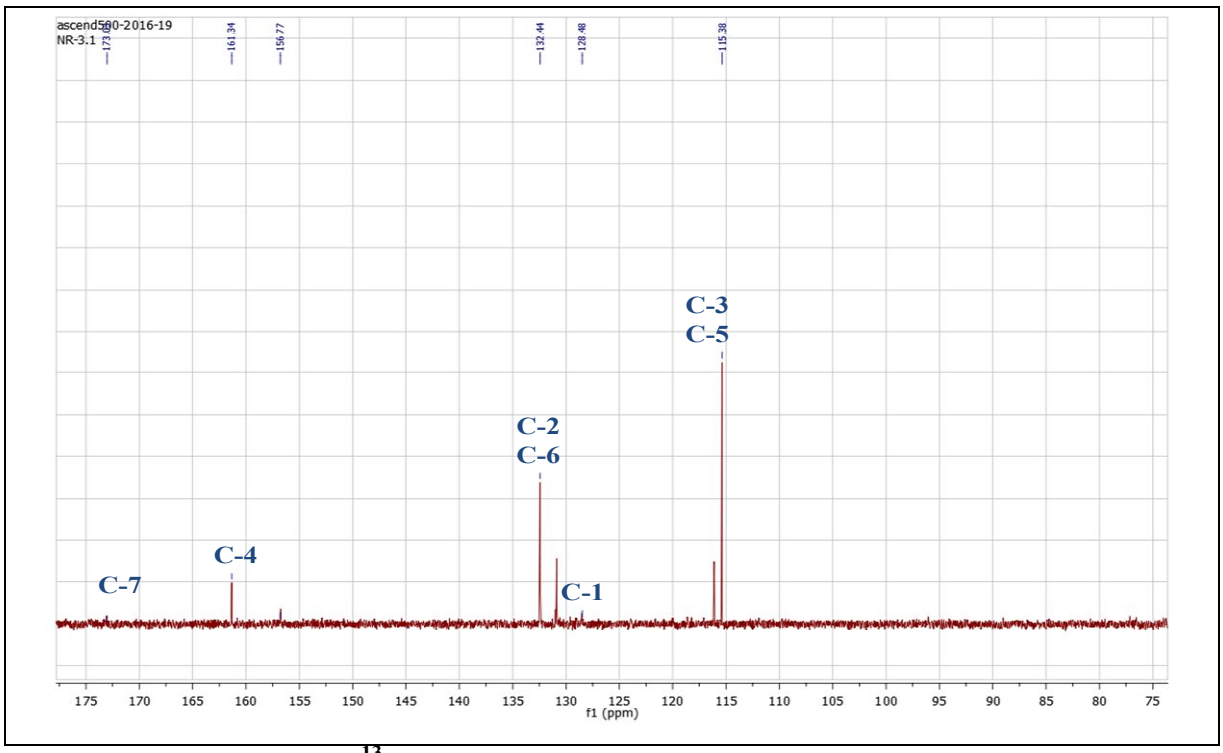


Figure. 4.114 ^{13}C Spectrum of compound P5 (CD_3OD, 125MHz)

From the data exposed previously, the compound P5 has been elucidated as 4-**hydroxybezoic acid**

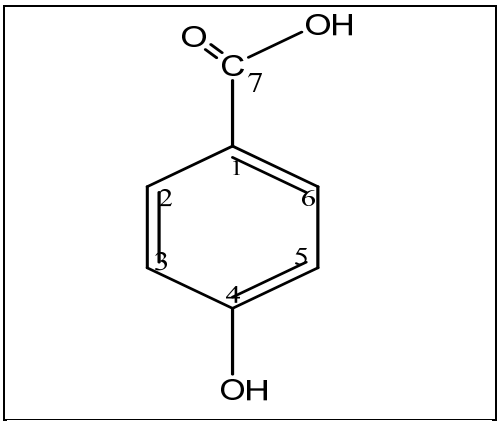


Figure. 4.115 4-Hydroxybezoic acid

Table. 4.18 Chemical ^{1}H and ^{13}C NMR Chemical shifts of compound P5, recorded in $CDOD_3$*(500 MHz)*

Postion	δ_H *ppm*	δ_C ppm
1	-	128.63
2	7.96 (d; *J* 8.7Hz)	132.44
3	6.85 (d; *J* 8.7Hz)	115.30
4	-	161.34
5	6.85 (d; *J* 8.7Hz)	115.30
6	7.96 (d; *J* 8.7Hz))	132.44
7	-	1173.94

4.2.3 Structural characterisation of Compound P6

This compound isolated in the form of yellowish amorphous solid.

NMR spectra data ^{1}H, ^{13}C and ESI-MS of this compound confirm its structure and indicate that it is apegenin (fig. 4.116). This compound was already isolated and identified (page 99; fig 45, 46, 47, 48).

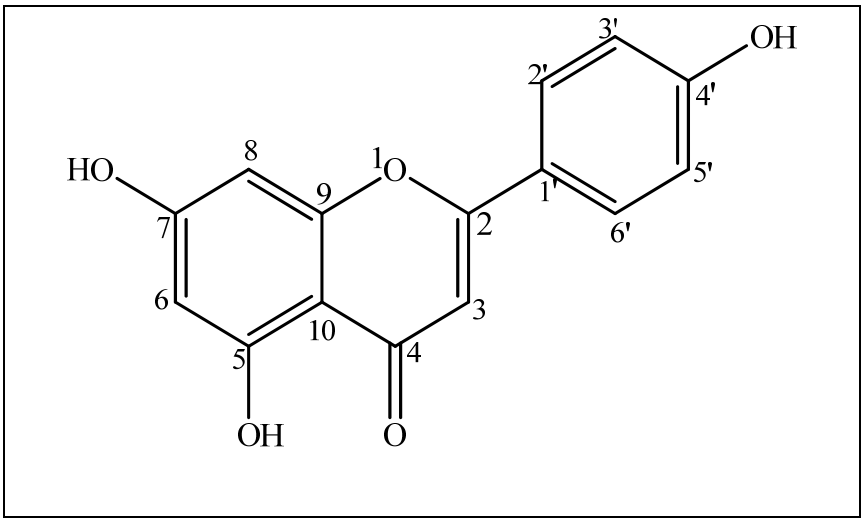


Figure. 4.116 Apegenin

4.2.4. Structural characterisation of Compound P7

Compound P7 was obtained as yellowish amorphous solid, its ESI-MS mass spectrum (Fig. 4.117), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 303, which corresponds to $[M+H]^+$, suggests a molecular mass 302 mau.

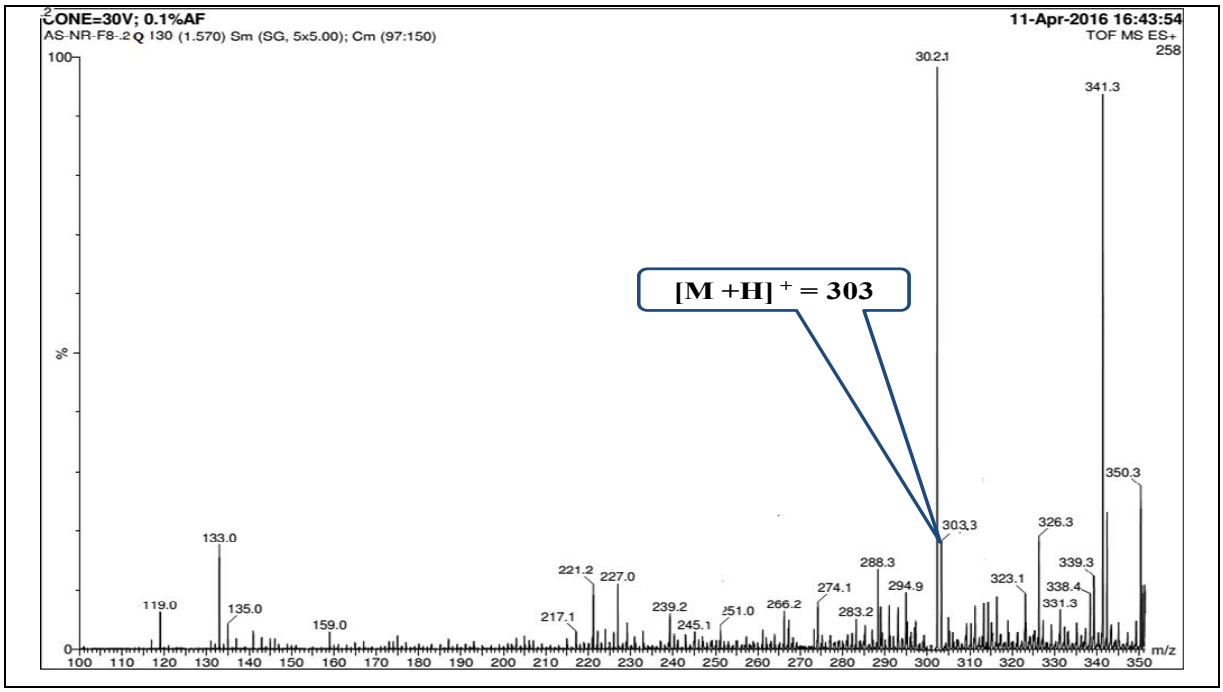


Figure 4.117 ESI-MS mass spectrum of the compound P7

On the ^{1}H NMR spectrum (Fig. 4.118) of compound P7, shows the following signals:

- Two aromatic protons in the form of two doublets resonante at δ 6.41 and 6.20 (J = 1.9 Hz) ppm, typical of a Meta coupling between H-8 and H-6 of ring A;

- Three aromatic protons at δ 7.75 (d, J = 1.91 Hz), 7.65 (dd, J = 8.5, 1.9 Hz) and 6.9 (d, J = 8.5 Hz) ppm; are assigned to H-2 ', H-6' and H-5' of ring B.

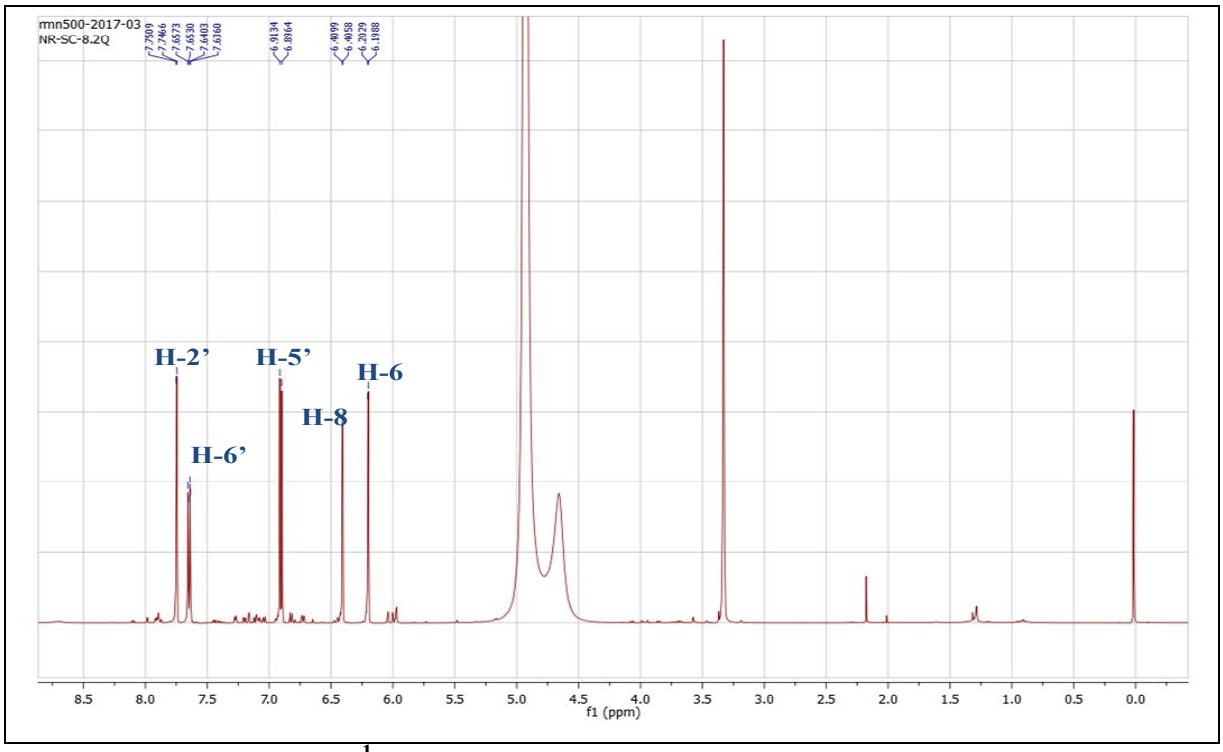


Figure. 4.118 ^{1}H Spectrum of compound P7 (CD_3OD, 500 MHz)

^{13}C NMR spectrum of compound (Fig. 4.119) presents fifteen signals. Among these, ten quaternary carbons detected including a carbonyl group (δ 177.31 ppm) and a C-3 oxygenated carbon characteristic of a flavonol (δ 137.21 ppm), and five aromatic CH carbons.

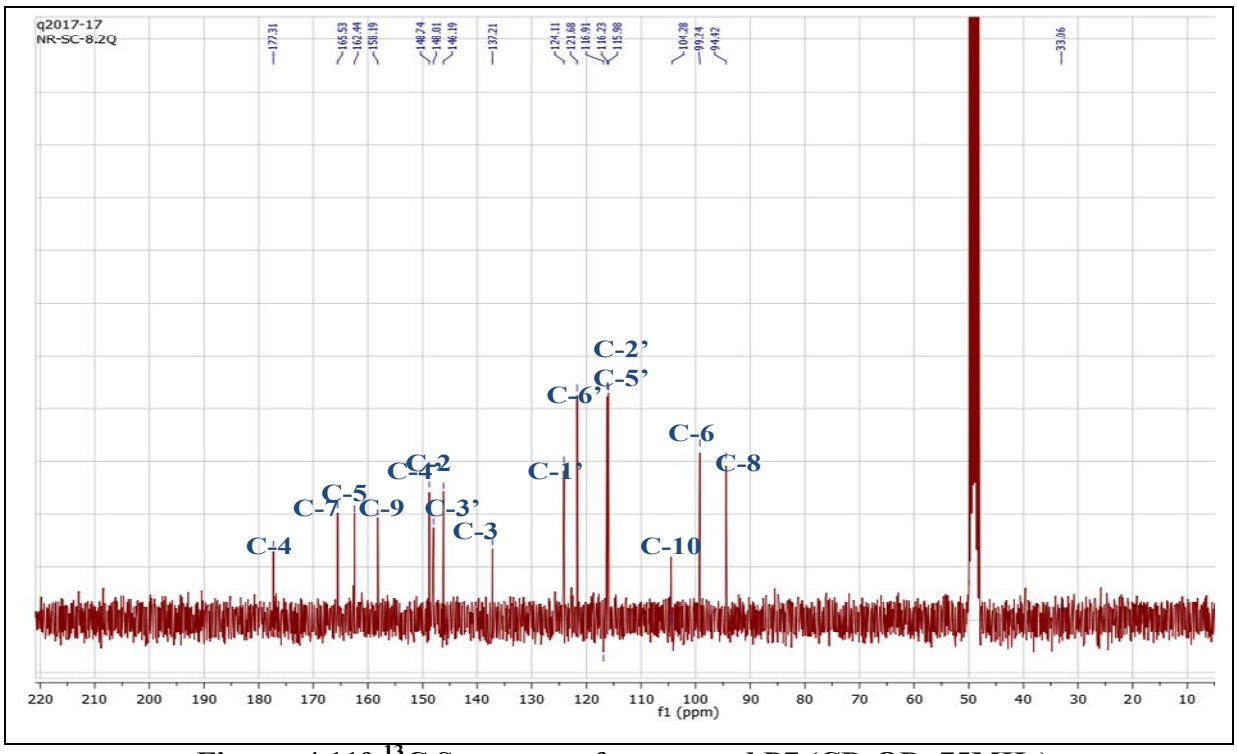


Figure. 4.119 ^{13}C Spectrum of compound P7 (CD_3OD, 75MHz)

All of these data as well as the comparison with the data of the literature (Huang et al., 2013) compound P6 is identified as 5,7,3 ', 4'-tetrahydroxyflavonol or quercetin.

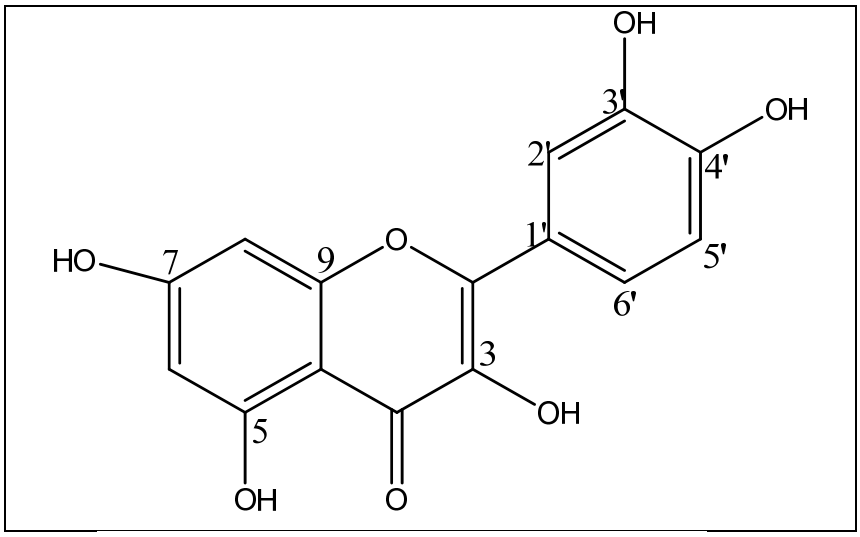


Figure. 4.120 5,7,3 ', 4'-tetrahydroxyflavonol

Quercetin

Table. 4.19 Chemical ^{1}H and ^{13}C NMR Chemical shifts values of compound P7, recorded in $CDOD_3$*(500 MHz).*

C_{number}	δ_C (ppm)	δ_H (ppm)
2	148.01	-
3	137.21	-
4	177.31	-
5	162.44	-
6	99.24	6.21 (d, *J* 1.9 Hz)
7	165.53	-
8	94.42	6.40 (d, *J* 1.9 Hz)
9	158.19	-
10	104.20	-
1’	124.11	-
2’	115.98	7.75 (d, *J* 1.91 Hz)
3’	146.19	-
4’	148.7	-
5’	116.23	6.9 (d, J 8.5 Hz).
6’	121.68	7.65 (dd, J 8.5, 1.9 Hz)

4.2.5 Structural characterisation of Compound P1

this Compound was obtained as brown oil, its ESI-MS mass spectrum (Fig. 4.117), recorded in positive mode, shows the presence of a pseudomolecular ion peak at m/z 567, which corresponds to $[2M+Na]^+$ and suggests a molecular mass, 272 mau.

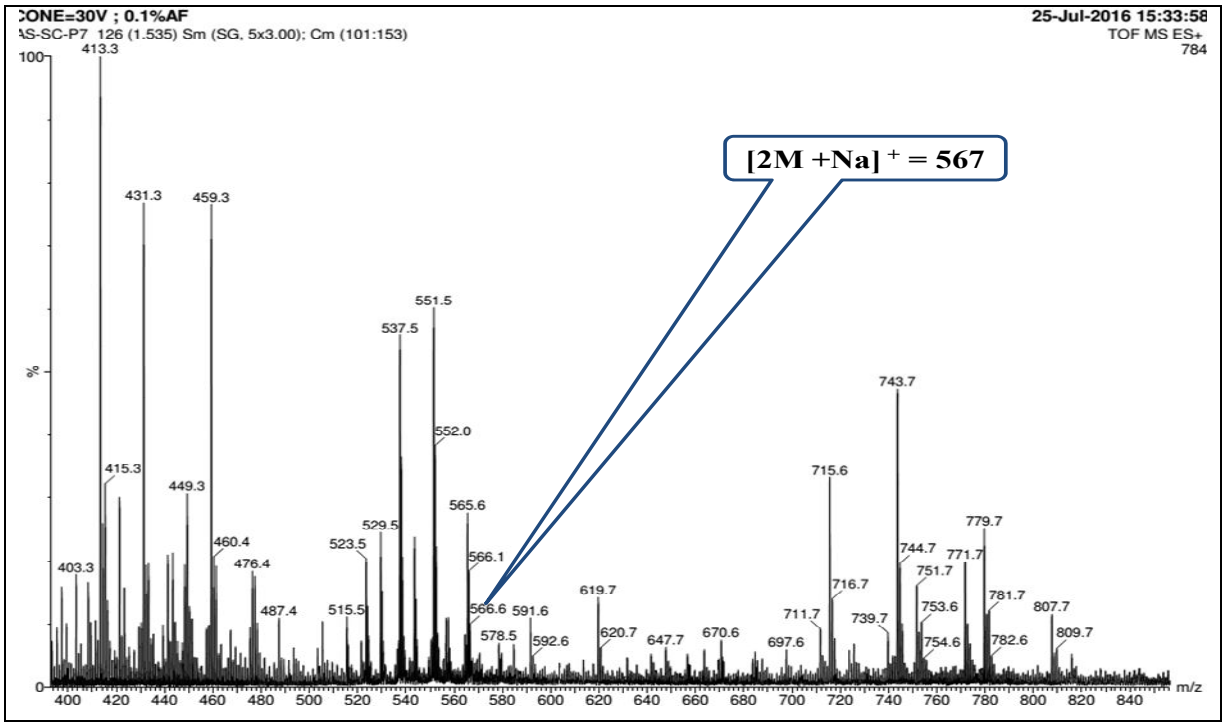


Figure .121 ESI-MS mass spectrum of the compound P1

^{1}H NMR spectrum (Fig. 4.122) of compound P1 indicates the absence of aromatic signals only four signals were appeared:

- Multiplet resonant at δ 2.34(2H, m) ppm correspondants to acyl moiety;
- signals in the area ranging from 1.25 to 1.30 (m) ppm correspondants to CH_2 groups;
- CH-OH proton at δ 4.05 ppm (1H, t, J= 12.1 Hz);
- Triplet signal with integration of 3H at δ 0.86 ppm (J= 7.1 Hz) indicating the existence of terminal methyl group.

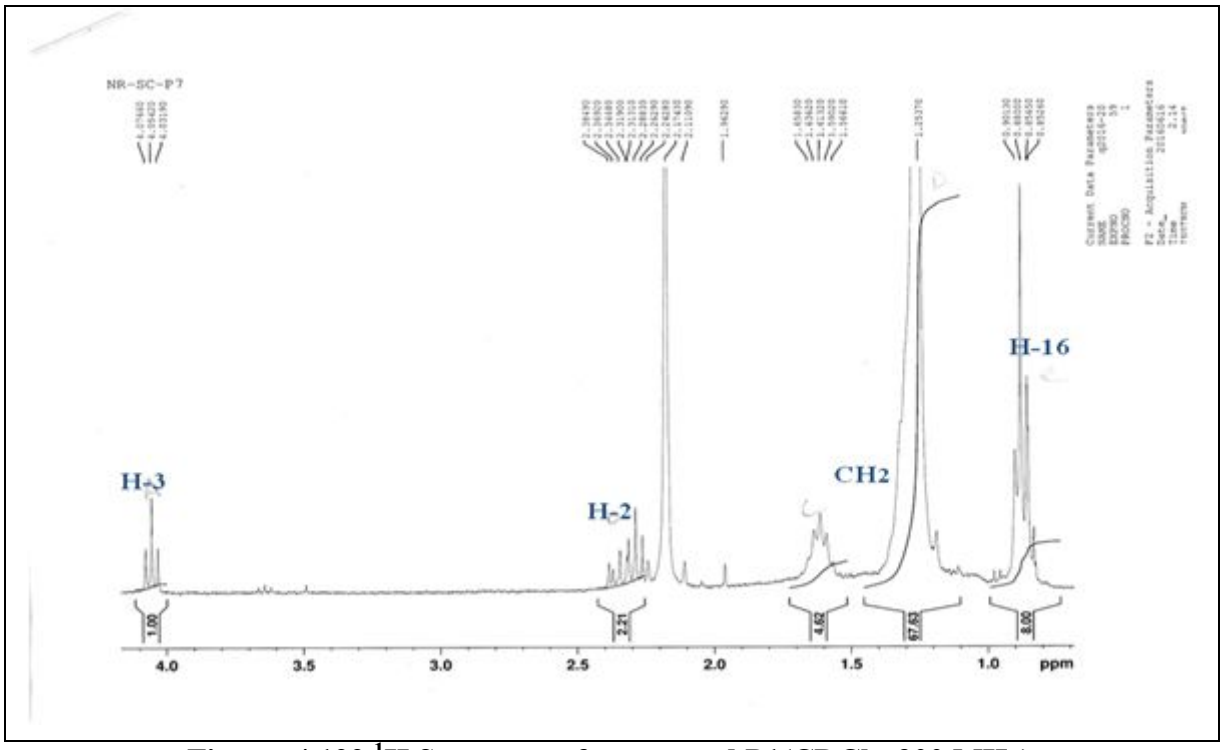


Figure. 4.122 ^{1}H Spectrum of compound P1($CDCl_3$, 300 MHz)

^{13}C NMR spectrum of the compound P1(Fig. 4.123) reveals the presence of the signals:

Aliphatic carbons between 14.12 and 31.93 ppm;

O=C-$\underline{CH_2}$ carbon at δ 34.42ppm;

oxygenated carbon at δ 64.41 ppm and carbonyl function at δ 174.07ppm.

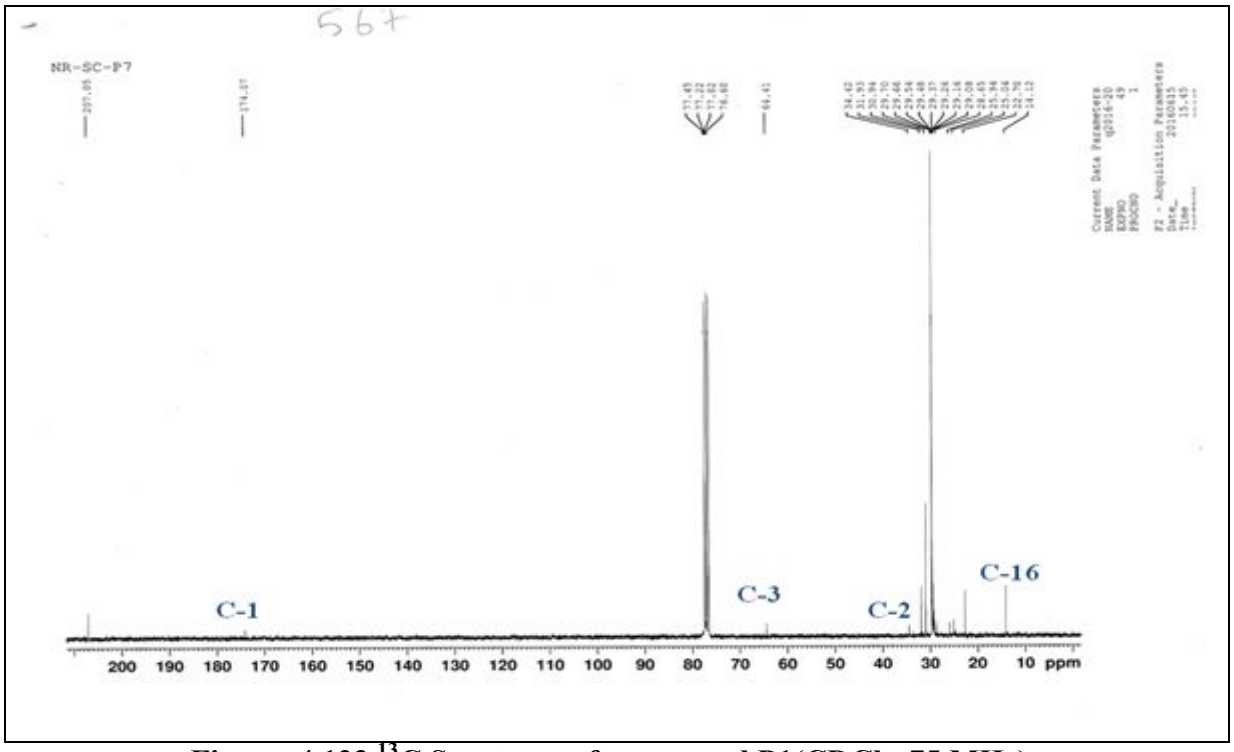


Figure. 4.123 ^{13}C Spectrum of compound P1($CDCl_3$, 75 MHz)

Compound P1 has been established on the basis of HMBC spectrum analysis (Fig. 4.124), according to the correlations spots between carbonyl (C-1) and Protons at δ 4.05(H-3) and 2.34 ppm (H-2) .

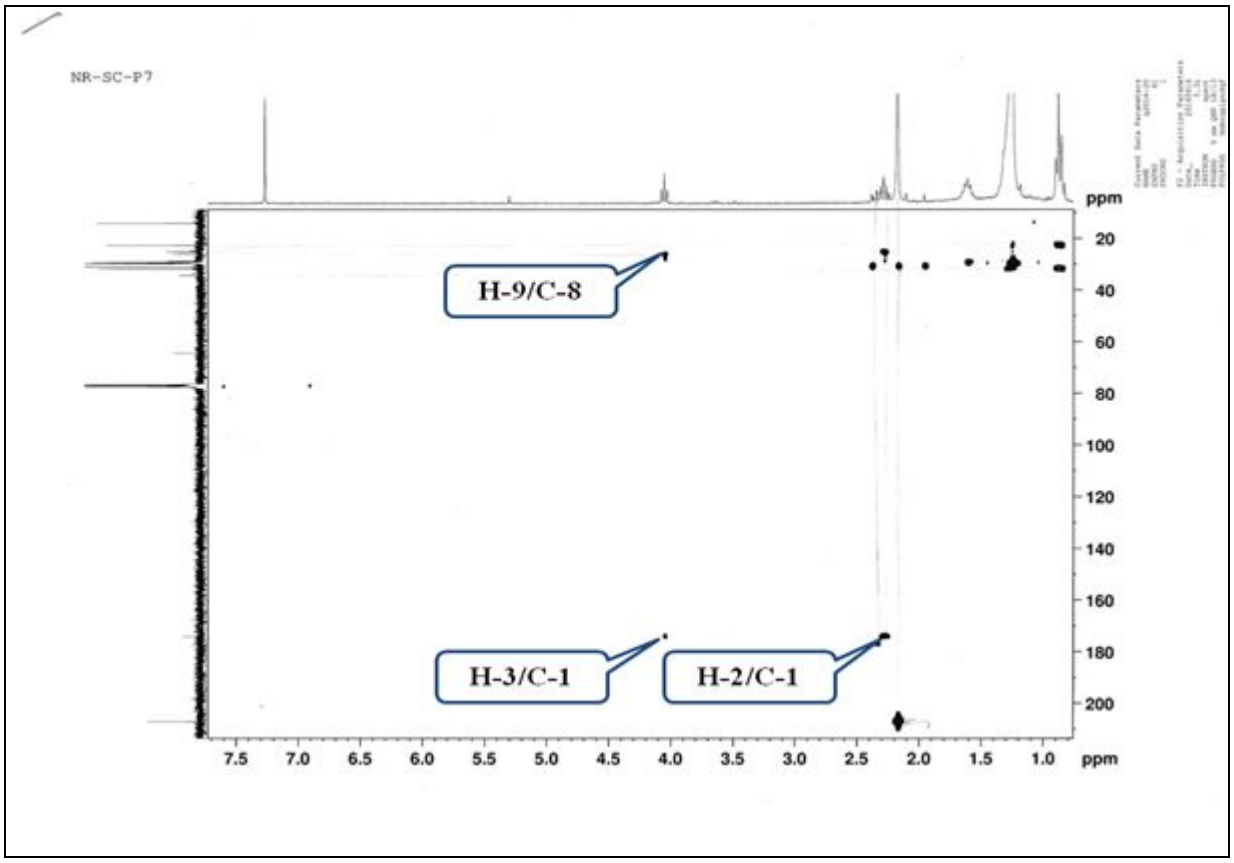


Figure. 4.124 HMBC Spectrum of compound P1 ($CDCl_3$, 300 MHz)

All these spectral data (^1H, ^{13}C, COSY, HSQC and HMBC) have lead to identify compound P1 as a **3-hedroxyhexadecanoic acid**

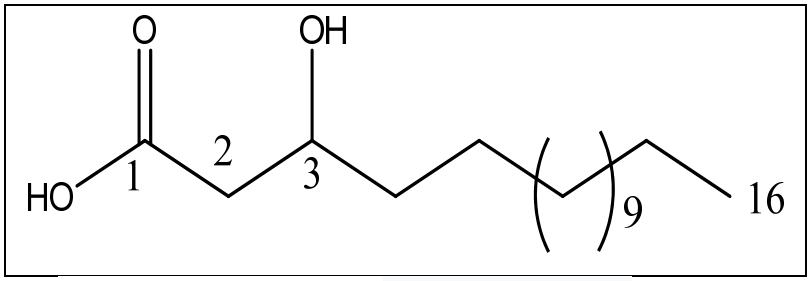


Figure. 4.125 3-hedroxyhexadecanoic acid

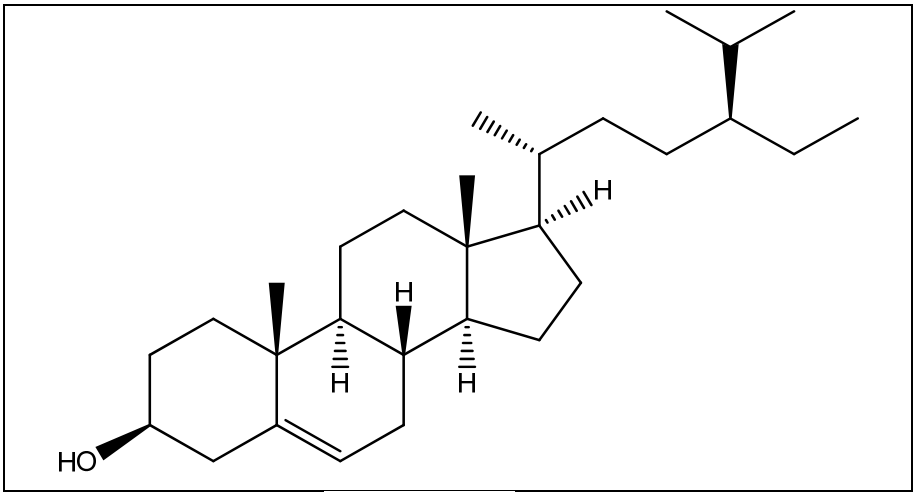
Table. 4.20 Chemical ^{1}H and ^{13}C NMR Chemical shifts of compound, recorded in **$CDCl_3$** *(300 MHz)*

Postion	δ_H *ppm*	δ_C ppm
1	-	174.07
2	2.34(m)	34.42
3	4.05(t; *J* 12.1 Hz)	64.41
4-15	1.25-1.65	22.70-31.93
16	0.86(t, J= 7.1 Hz)	14.12

4.2.6 Structural characterisation of Compound P2

This compound is isolated as a white powder soluble in methanol.

The comparison of spectral data of compound P2 with literature (Khatun et al., 2012) shows a great structural similarity and confirms that this compound is a β-sitosterol, which is considered a common secondary metabolite in all plants of vegetable kingdom. Several main studies on the pharmacological properties of β-sitosterol have been done and showed a wide variety of properties as antinheoplastic, anti-inflammatory and anti-pyretic (Dae-Sup et al., 2004).

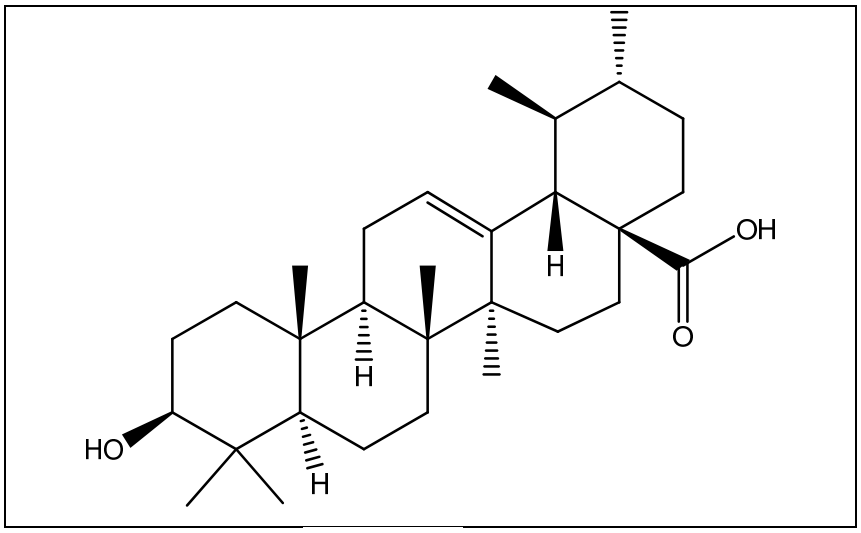


Beta-sitosterol

4.2.7 Structural characterisation of Compound P3

This compound isolated in the form of white powder soluble on $CHCl_3$.

^1H, ^{13}C NMR spectrum data and ESI-MS of this compound confirm its structure and elucidation as ursolic acid. This compound was already isolated and identified in this chapter (pages 77-82 figures 4.18-4.25)



Ursolic acid

4.3 Identification and quantification of phenolic compounds in the S. stellata ethanolic extract

The screening of the ethanolic extract of *S. stellata* by UHPLC-DAD-ESI/MS^n revealed an UV chromatogram, recorded at 305 nm (Fig. 4.3.1/A), presenting two major peaks eluted at 6.66 and 10.14 min and several other minor peaks. From the twenty five phenolic compounds identified, nine are chlorogenic acid derivatives and thirteen are flavonoid derivatives (data of the retention time, maximum wavelength, molecular ions species and fragments are presented in Table 4.3.1), and represent, respectively, 30% and 56% of the total phenolic amount. The fact that these types of compounds are recognized as antioxidants (Rice-Evans and Packer, 1998; Marques and Farah, 2009) is consistent with the above mentioned antioxidant activity.

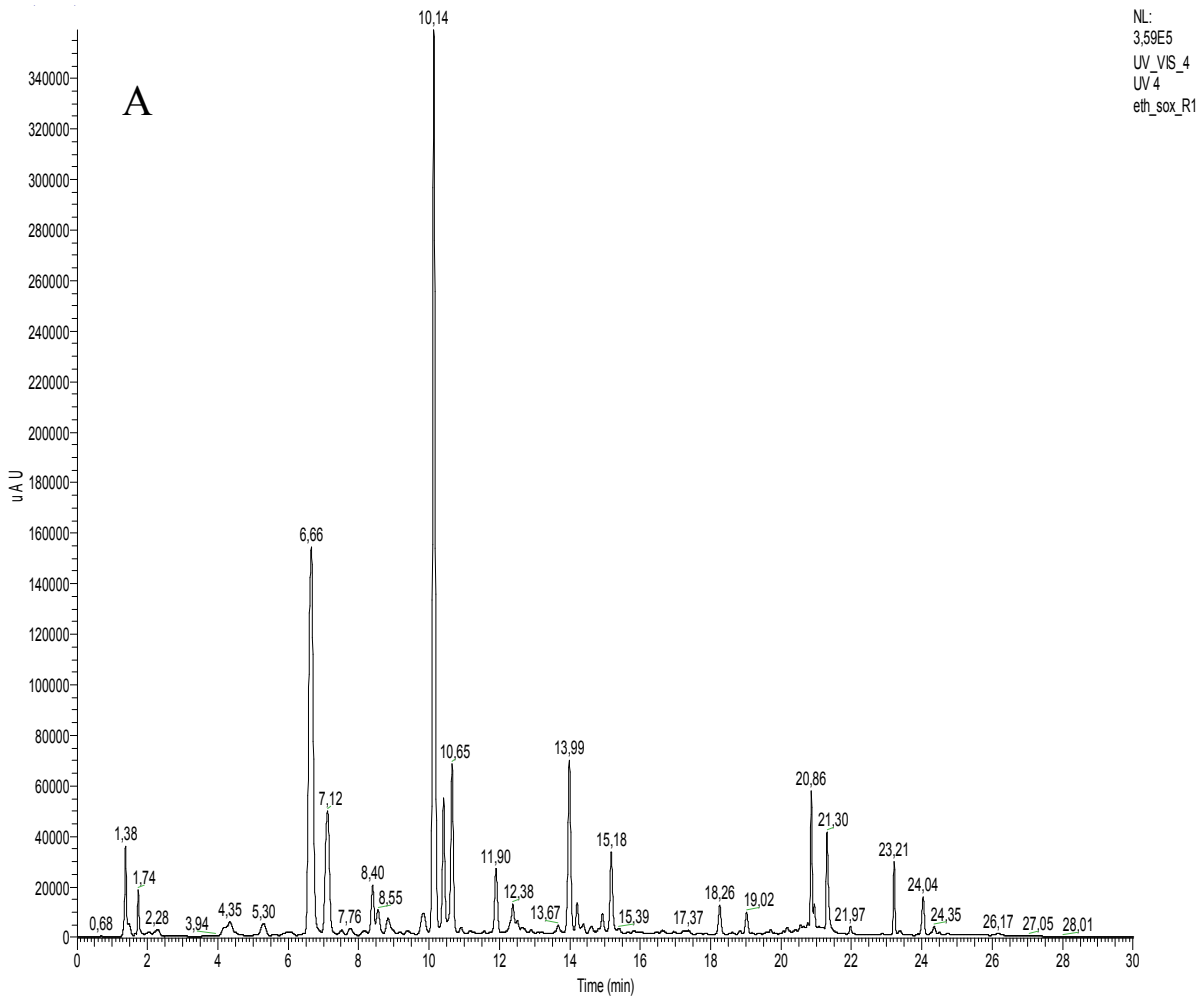


Figure 4.3.1. A) UHPLC chromatogram of *S. Stellata* ethanolic extract recorded at 305 nm

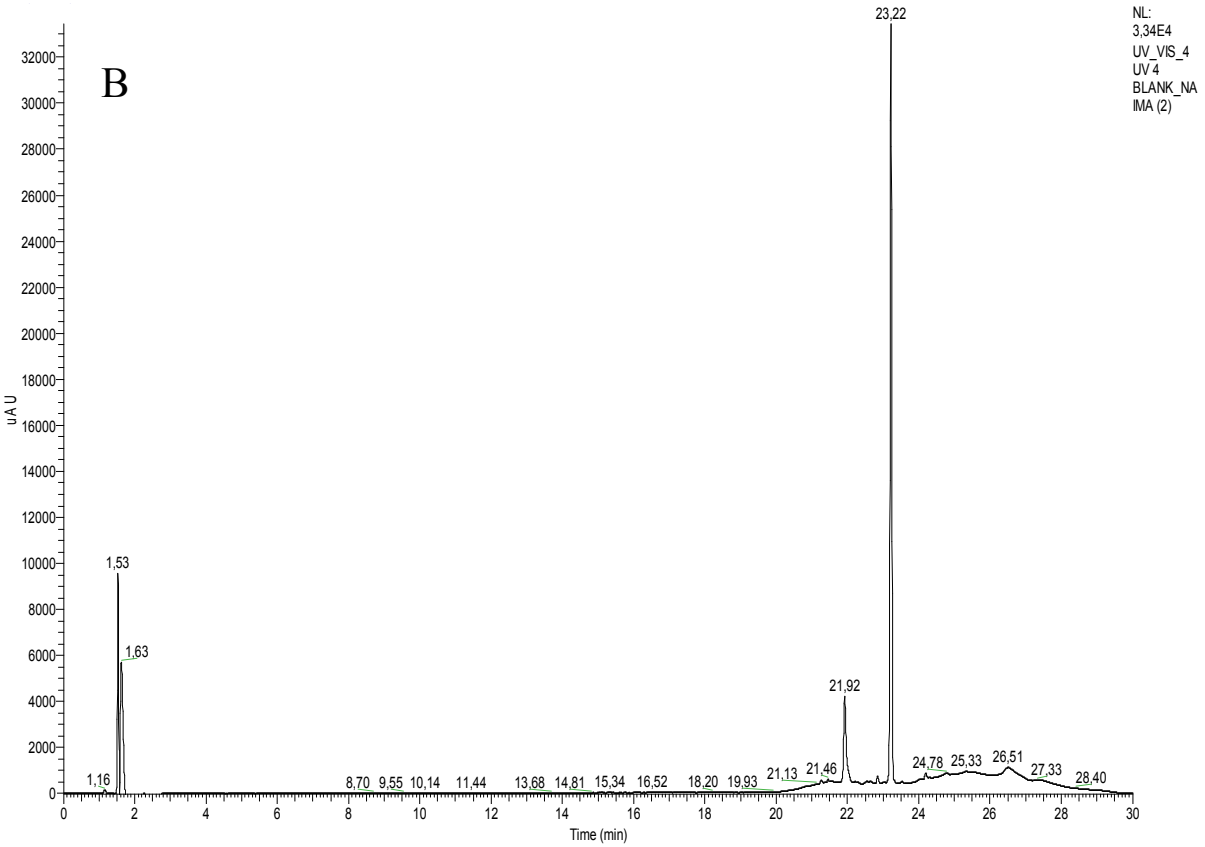


Figure 4.3.1. B) UHPLC chromatogram of solvents used recorded at 305 nm.

The nine chlorogenic acids herein identified as constituents of the *S. stellata* ethanolic extract were mainly identified through their pseudomolecolar ions ($[M-H]^-$) and MS^n fragments. The literature data is rich in these acids MS data due to their ubiquitously occurrence in plants The (Stalmach et al., 2006; Bastos et al., 2007; Plazonić et al., 2009; Gouveia and Castilho., 2011; Catarino et al., 2015; Martins et al., 2016; Bessada et al., 2016;)

consequently peaks at 4.35, 6.66, 7.12 and 8.55 min with $[M-H]^-$ at m/z 353 were identified as 1-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 3-*O*-caffeoylquinic and 5-*O*-caffeoylquinic acids, respectively. The 4-*O*-caffeoylquinic acid is easily distinguished from the others due to its characteristic MS^2 base peak at m/z 173(fig 4.3.2), whereas in the others MS^2 fragment ions the bases peak is at m/z 191. 3-*O*-Caffeoylquinic acid could be assigned to be the peak at 7.12 min due to the absence of the fragment ion at m/z 135 in the MS^2 spectra characteristic in the others caffeoylquinic acids (fig 4.3.3),.

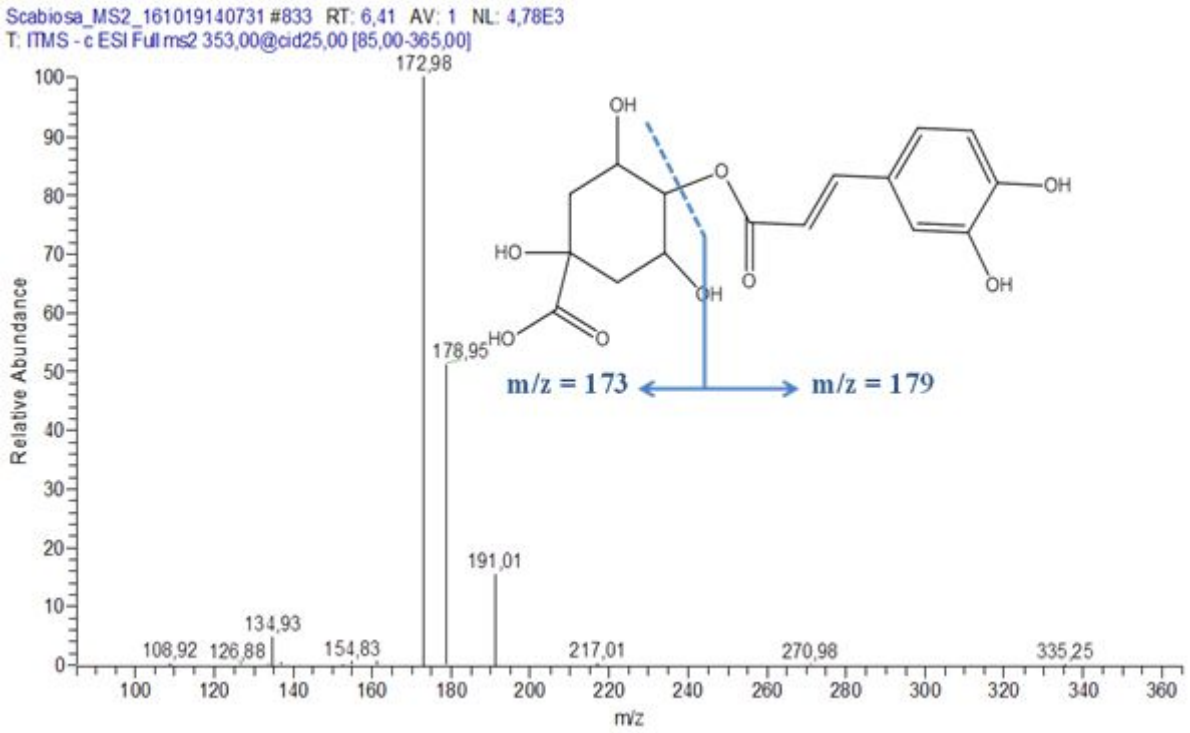


Figure. 4.3.2. Spectrum of MS^2 at Rt 4.35 (4-*O*-caffeoylquinic)

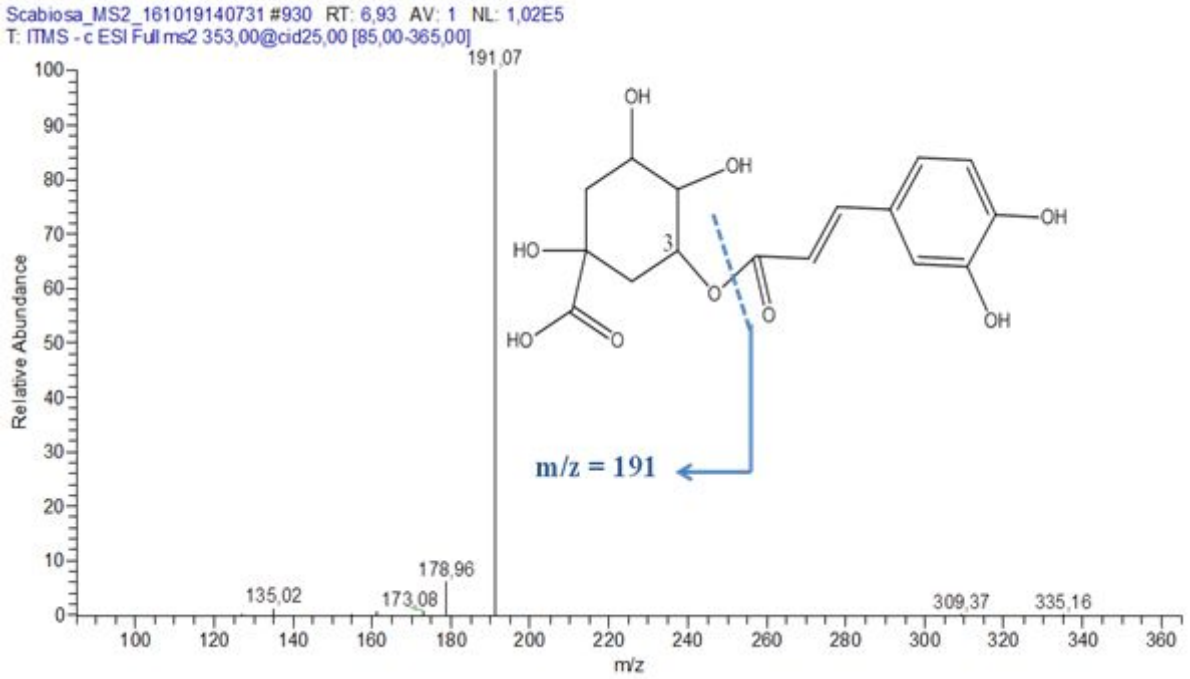
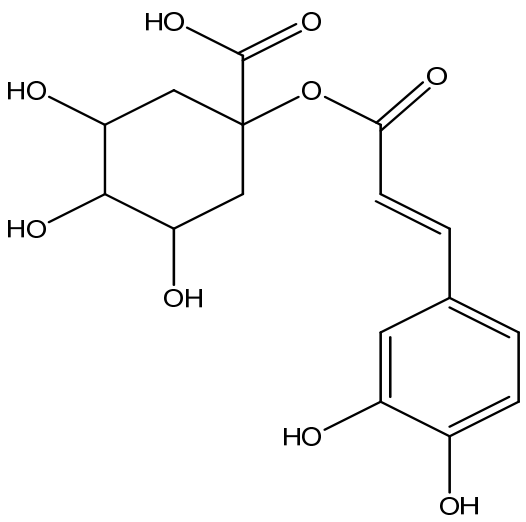
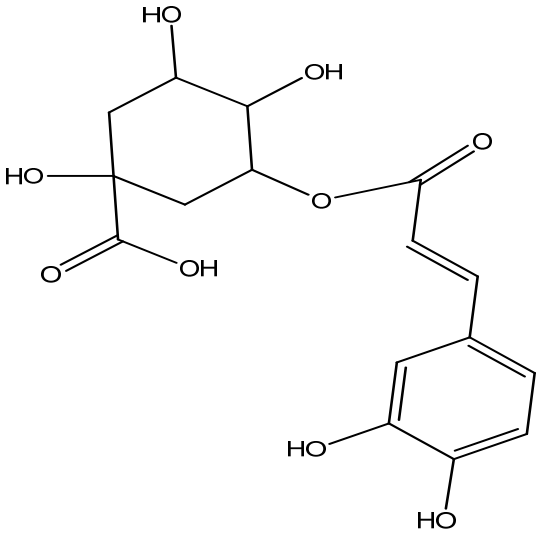


Figure 4.3.3. Spectrum of MS^2 at Rt 7.12 (3-*O*-caffeoylquinic)

Finally the other two derivatives assignment was possible with the MS^3 spectrum.



1-*O*-caffeoylquinic



5-*O*-caffeoylquinic

The other two monosubstituted quinic acids, peaks at 8.83 and 9.83 min were identified, respectively as 5-*O*-*p*-coumaroylquinic acid and 5-*O*-*F*eruloylquinic acid due to their $[M-H]^-$ at m/z 337 and m/z 367. Furthermore the base peak in MS^2 at m/z 191 allowed us to distinguish the 5-*O*-*p*-coumaroylquinic acid from the other possible isomers (Plazonić et al., 2009).

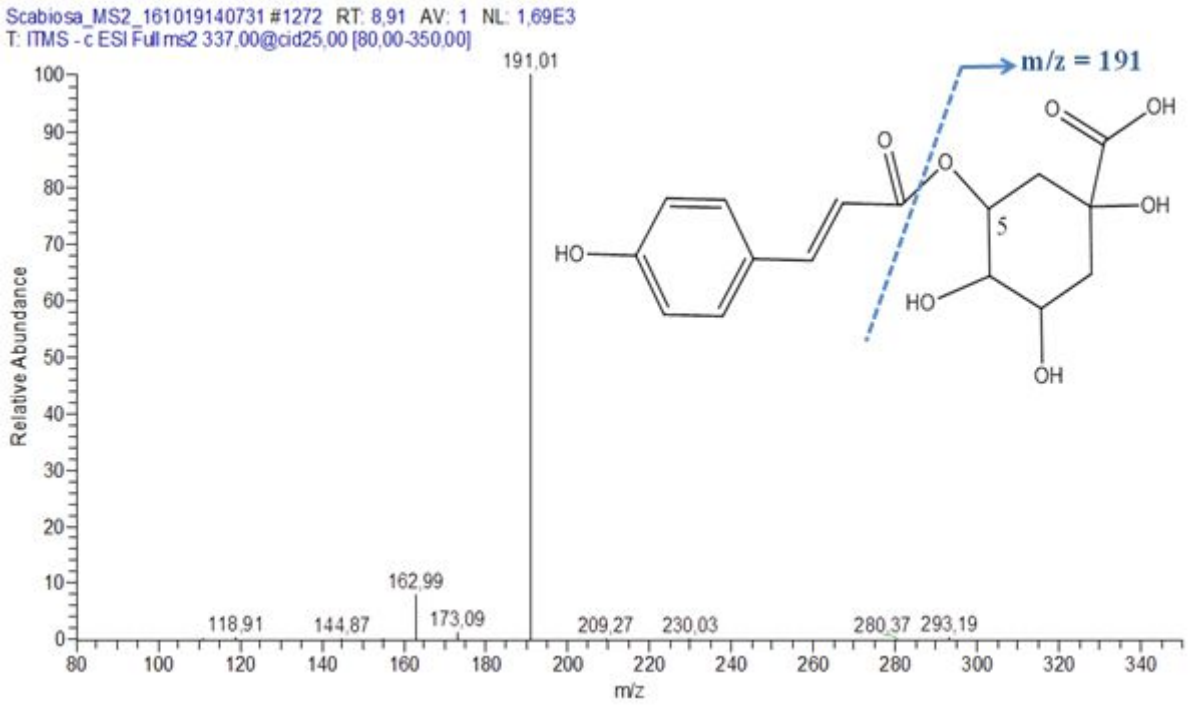


Figure. 4.3.4 Spectrum of MS^2 at Rt 8.83 (5-*O*-*p*-coumaroylquinic acid)

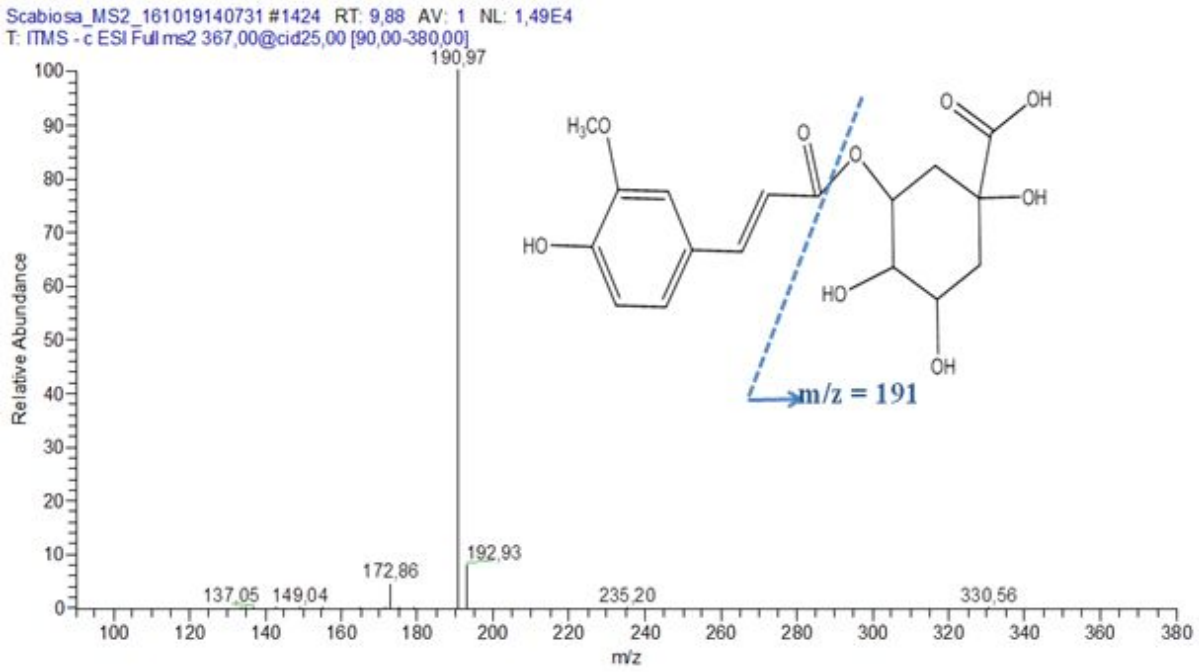


Figure. 4.3.5 Spectrum of MS^2 at Rt 9.83 (5-*O*-*F*eruloylquinic acid)

The other chlorogenic acids found are disubstituted quinic acids and correspond to the peaks at 13.99, 14.38 and 15.18 min, all with $[M-H]^-$ at m/z 515 and MS^2 at m/z 353

Peaks at 14.38 and 15.18 min could be assigned, respectively to 3,4-*O*-dicaffeoylquinic and 3,5-*O*-dicaffeoylquinic acids, due to the base peaks at the MS^3 spectra (respectively m/z 173 and m/z 191). In the case of peak at 13.99 min the phytochemical study of the extract allowed the isolation and full characterisation of 4,5-*O*-dicaffeoylquinic acid (isochlorogenic acid C9) ,which could be used as standard and confirm the identification of this peak. It is interesting to notice that *S. stellata* areal parts are rich in these important metabolites particularly in 4-*O*-caffeoylquinic acid, which accounts for 46% of the total chlorogenic acids content (Table 4.3.1).

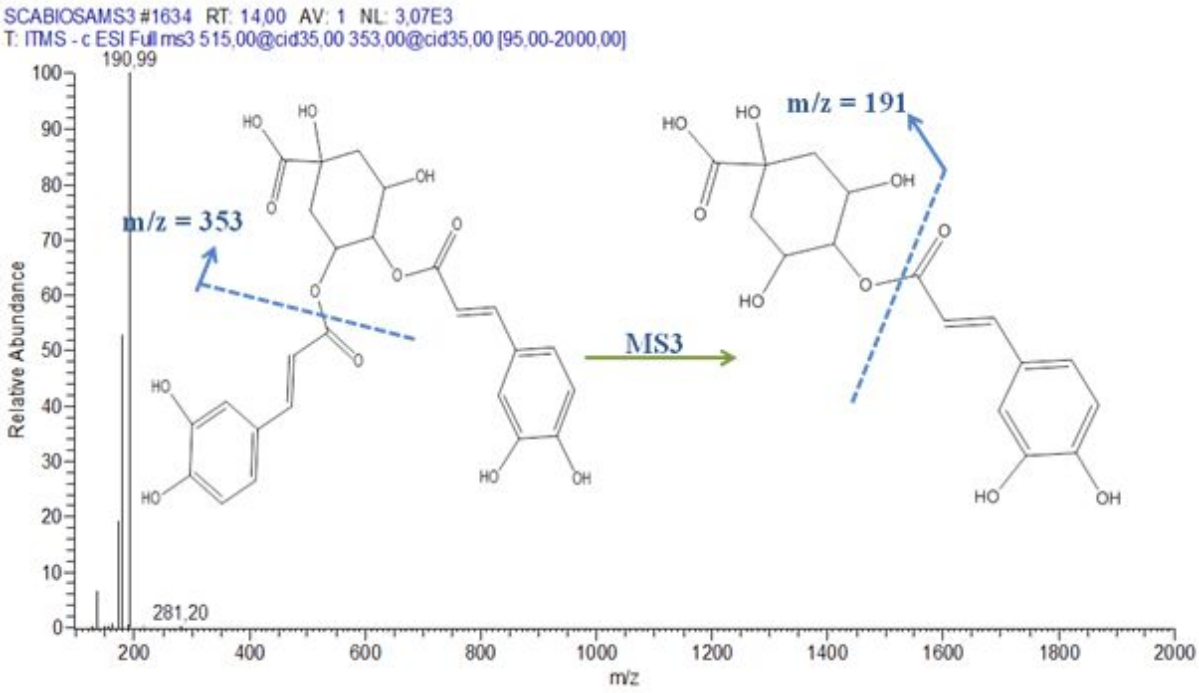


Figure. 4.3.6 Spectrum of MS^3 at Rt 14.38 (3,4-*O*-dicaffeoylquinic)

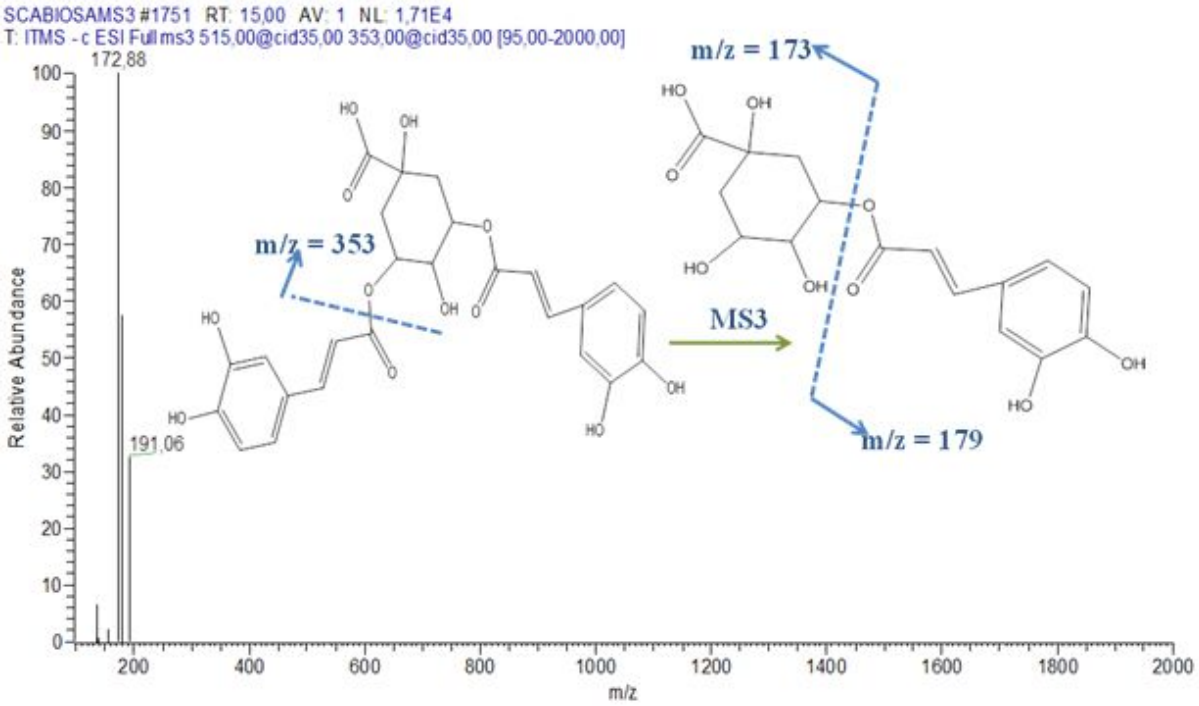


Figure. 4.3.7 Spectrum of MS^3 at Rt 15.18 (3,5-*O*-dicaffeoylquinic acids)

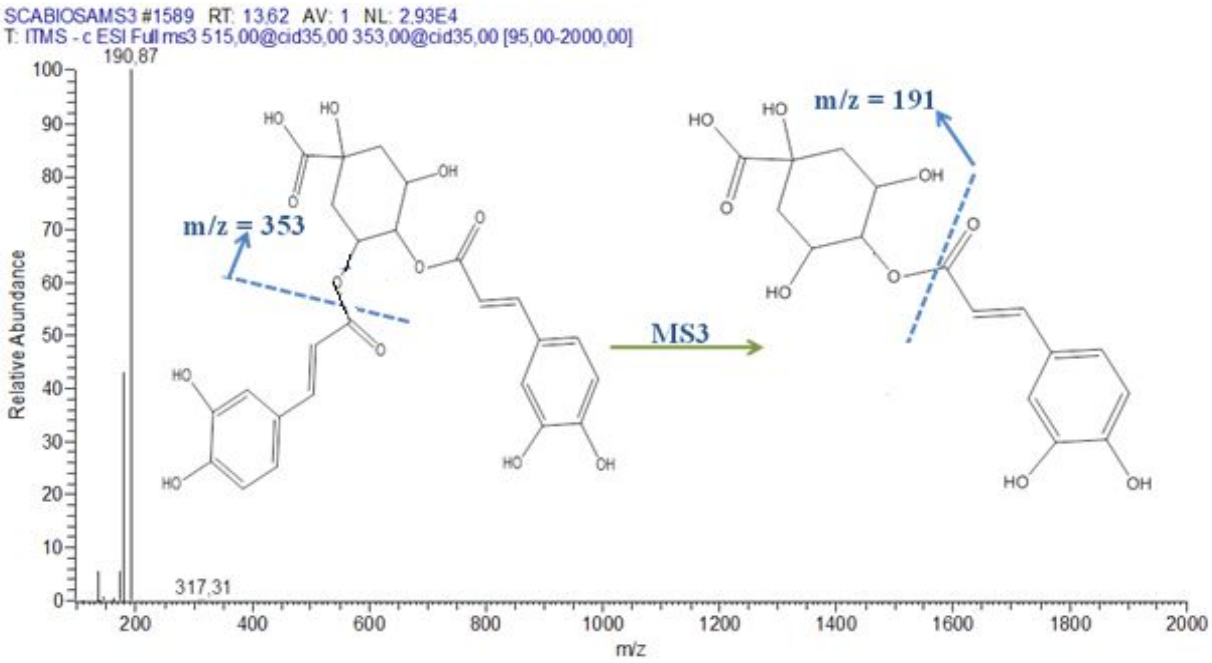


Figure. 4.3.8 Spectrum of MS^3 at Rt 13.99 (4,5-*O*-dicaffeoylquinic acid)

The ethanolic extract profile of *S. stellata* shows the presence of another important family of secondary metabolites, the flavonoids for which the literature is also rich in MS data (Ferreres et al., 2007; Ferreres et al., 2008; Ferreres et al., 2011; Pereira et al., 2012; Brito et al., 2014; Barros et al., 2013; Martins et al., 2016; Bessada et al., 2016; Ferreres et al., 2017).

The detailed analysis of the characteristic MS^n fragment ions (Table. 4.1.3) allowed the identification of several flavonoid glycosides, which in fact represent 56% of the total phenolic amount. The main aglycones are flavone type (apigenin, diosmetin and luteolin) and flavonol type (kaempferol, quercetin and tamarixetin). The majority of the identified compounds is common secondary metabolites and was previously reported. Moreover the results herein reported are identical to the ones reported in the literature (Pereira et al., 2012; Brito et al., 2014; Barros et al., 2013; Martins et al., 2016). In all cases the ion fragment with m/z value of the key aglycone is the base peak of MS^2 or MS^3 (Table 4.1.3), confirming the identification. Luteolin glycosides are the major constituents and account for 74% of the total flavonoid content (Table 4.1.3).

Moreover, luteolin-6-*C*-glucoside, which elutes at 10.14 min is the major constituent of the *S. stellata* ethanolic extract (34%), which have m/z 447 and MS^2 327 (-120) loss of C- hexose, the structure confirmed by $MS^3$and literature

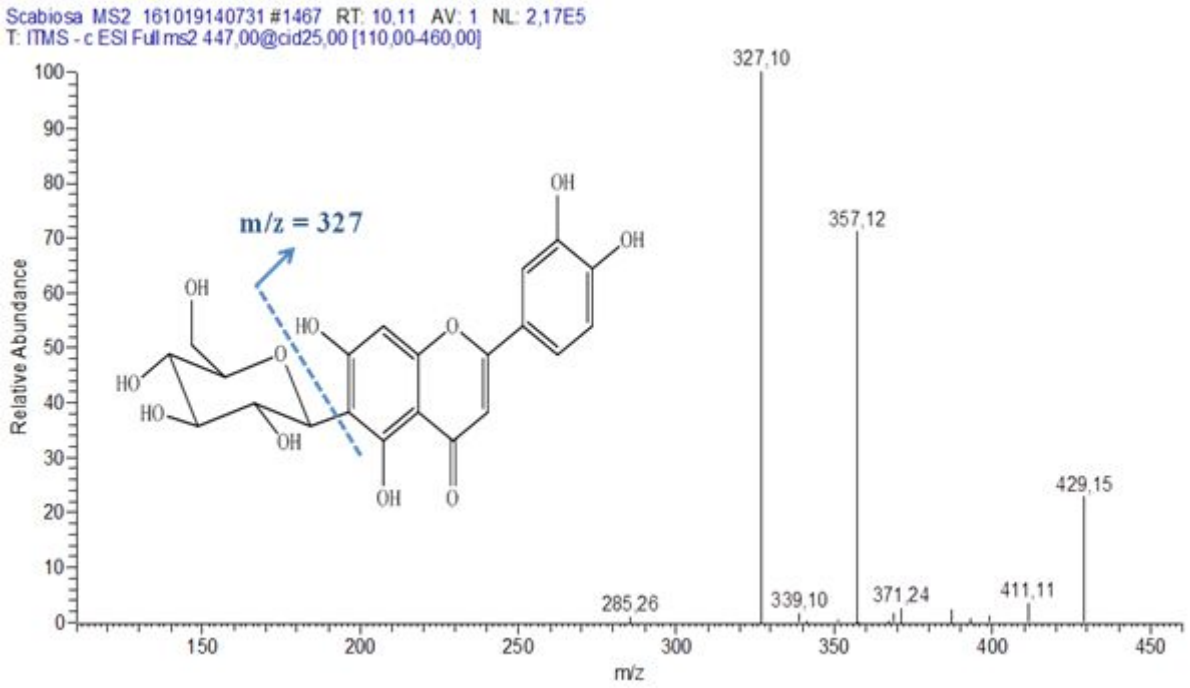


Figure. 4.3.9. Spectrum of MS^2 at Rt 10.14 (luteolin-6-*C*-glucoside)

Tamarixetin glycosides are less reported and in the *S. stellata* ethanolic extract were found three examples. The peak eluted at 14.93 min correspond to extremely small amount of tamarixetin-*O*,*O*-dihexoside, the $[M-H]^-$ at m/z 639 and the base peak at m/z 315 in MS^2 suggest it. (fig. 4.3.10)

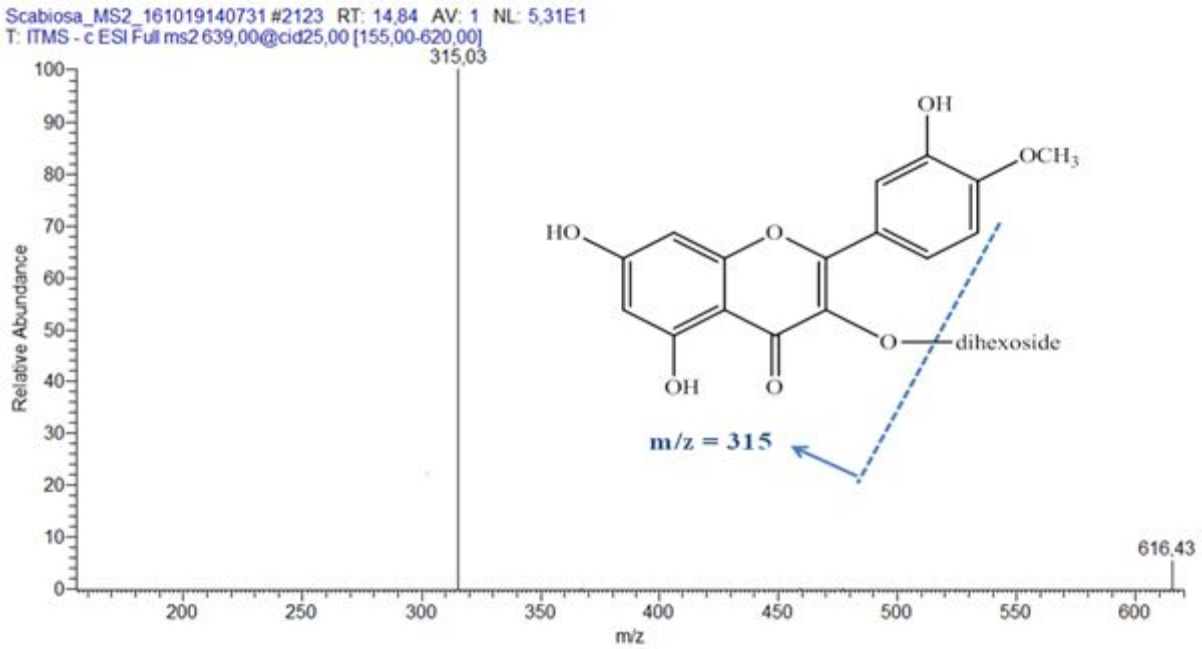


Figure. 4.3.10 Spectra of MS^2 at Rt 14.93 (tamarixetin-*O*,*O*-dihexoside)

Besides the peak eluted at 20.86 have similar absorption in the UV-Vis region and also the base peak at m/z 315 in MS^2 confirming that should be a tamarixetin derivative (Table 3.1) and in this case in a considerable amount (nearly 7%).

The careful analysis of the chromatogram (Fig. 4.3.1/A) allowed to detect a peak eluted at 20.94 min with $[M-H]^-$ at m/z 769 and the base peak at m/z 315 in MS^2, due to its proximity with the peak at 20.86 min the quantification was not possible (Table 4.1.3). However the similarity of this peak data and the above mentioned ones suggests that it is also a tamarixetin derivative. The phytochemical study allowed the isolation and characterization of flavonoids and their glycosides . which confirmed the above discussed identification, being the most noticeable case the isolation and full characterization of tamarixetin 3-β-L-rhamnosyl-(1→2)[β-L-rhamnosyl-(1→6)]β-D-glucoside] C**13** . This compound allowed the confirmation of the above mentioned identification of the peak eluted at 20.94 min and the other tamarixetin derivatives.

Table 4.3.1 Identification of UHPLC/DAD/ESI-MS^n data, and quantification of the most relevant fractions from the ethanolic extract of *S. Stellata*.

Rt (min)	λ_{max}	Molecular ion $[M-H]^-$ (m/z)	Main fragment ESI^-MS^2; (MS^3) (m/z)	mg/g dried extract	Compound
1.38	191, 267	387	341, 369; (179, 143, 161)	8.24 ± 0.03	α-1-Caffeoylglucose derivative
1.74	193, 202	128	85, 109	4.30 ± 0.02	Cyanuric acid
4.35	204, 324	353	**191**, 179, 135; (173, 127, 109)	nq	1-*O*-Caffeoylquinic acid
5.30	211, 278, 323	223	205, 115, 143, 159	0.26 ± 0.01	Sinapic acid
6.66	217, 298, 325	353	191, 179, **173**, 135; (111, 93)	26.41 ± 0.30	4-*O*-Caffeoylquinic acid
7.12	216, 299, 325	353	**191**, 179; (173, 127, 85)	8.93 ± 0.12	3-*O*-caffeoylquinic acid
8.40	206, 269, 348	609	489, 447, (357, 327, **285**)	1.84 ± 0.03	Luteolin-6-*C*-glucoside-7-*O*-glucoside
8.55	199, 214, 270, 304	353	**191**, 179, 135; (173, 127, 85)	1.47 ± 0.01	5-*O*-Caffeoylquinic acid
8.83	220, 274, 310	337	**191**, 173; (127, 110, 93)	tr	*5-O-p-Coumaroylquinic acid*
9.83	230, 326	367	**191**; (173, 85)	0.97 ± 0.02	5-*O*-Feruloylquinic acid
10.14	209, 269, 350	447	429, 357, 327; (309, 297, **285**)	66.31 ± 0.30	Isoorientin (luteolin-6-*C*-glucoside)
10.40	211, 269, 350	579	561, 447, 357, 327; (309, 297, **285**)	9.78 ± 0.26	Luteolin-2”-*O*-pentosyl-6-*C*-hexoside
10.65	211, 270, 346	461	371, 341, 313; (**299**, 231)	13.97 ± 0.11	Diosmetin-6(or 8)-*C*-glucoside
11.90	225, 270, 338	563	443, 431; (311, 283, **269**)	2.82 ± 0.01	Apigenin-2’’-*O*-pentosyl-8-*C*-glucoside
12.38	232, 256,	463	**301**; (268, 179, 151)	0.97 ± 0.04	Quercetin-3-*O*-glucoside

	353				
13.99	220, 241, 327	515	**353**; (191, 173)	16.03 ± 0.03	4,5-*O*-Dicaffeoylquinic acid
14.21	237, 267, 337	609	489, 369; (298, **285**, 231)	1.23 ± 0.01	Lucenin 2 (luteolin-6,8-di-*C*-glucoside)
14.38	242, 326	515	**353**, 335; (173,111)	tr	3,4-*O*-Dicaffeoylquinic acid
14.93	240, 268, 314	639	616, **315**	tr	Tamarixetin-*O,O*-dihexoside
15.18	242, 326	515	**353**; (191, 171, 127)	3.74 ± 0.02	3,5-*O*-Dicaffeoylquinic acid
18.26	239, 270, 351	613	489, 447, 429; (369, 309, **285**)	0.43 ± 0.02	Luteolin-6-*C*-glucoside derivative
19.02	243, 267, 314	593	447, **285**	0.37±0.02	Kaempferol-3-*O*-rutinoside
20.86	237, 267, 314	635	477, **315**	14.49 ± 0.02	Tamarixetin derivative
20.94	237, 267, 313	769	623, 477, **315**	nq	Tamarixetin glycoside
21.30	243, 269, 313	739	593, 447, **285**	10.85 ± 0.01	Kaempferol-3-*O*-rutinoside derivative
				57.55 ± 0.11[#]	Total chlorogenic acids
				108.20 ± 0.17[#]	Total flavonoids

nq – not quantified
tr – traces
[#]obtained by propagation

The phytochemical study allowed the isolation of caffeic acid and its derivatives as well as several glucosides, confirming the presence of other caffeic acid derivatives in the extract. Some of the isolated compounds were not detected by UHPLC-DAD-ESI/MS^n, such as compound C**1**, probably because the conditions used are not suitable for these compounds. In the case of the ethyl derivatives isolated (compounds C**4** and C**10**) the cleavage of the ethyl group can occur. Furthermore we suspect that these ethyl derivatives are not natural compounds but transformations occurred during the extraction with ethanol.

4.4 Gas chromatography-Mass spectrometry (GC-MS) analysis

4.4.1 Chemical composition of the S. stellata lipophilic extract

The detailed analysis by GC-MS allowed the identification and quantification data (expressed as mg of compound per kg of dry plant) of the *S. stellata* lipophilic extract is summarized in Table 4.4.1.

Compounds were identified by:

- comparison of the MS of their TMS derivatives with the GC-MS spectral library;
- comparison with spectra found in the literature (NIST14.lib and WILEY229.LIB);
- comparison with pure silylated standards;
- Interpretation of pattern fragmentation MS spectrum.

Sometimes the chromatogram peaks can be identified their quantification cannot be separated, as in peak 5 and 4 so the amount 210. 84 mg kg^{-1} is referred to both compounds.

The identified compounds could be distributed in five groups, representative of similar chemical families, namely carboxylic acids and esters, which represent 87.1% of all identified compounds, followed by alkanes (7.9%), terpenes (3.4%), sugars (1.3%) and finally alcohols (0.3%) (Fig. 4.4.2). To the best of our knowledge there are no studies of *Scabiosa* genus lipophilic profile, only the GC-MS analysis of essential oils was reported and for *S. arenaria* (Besbes et al. 2012) and *S. flavida* Boiss. & Hausskn. (Javidnia et al., 2006).

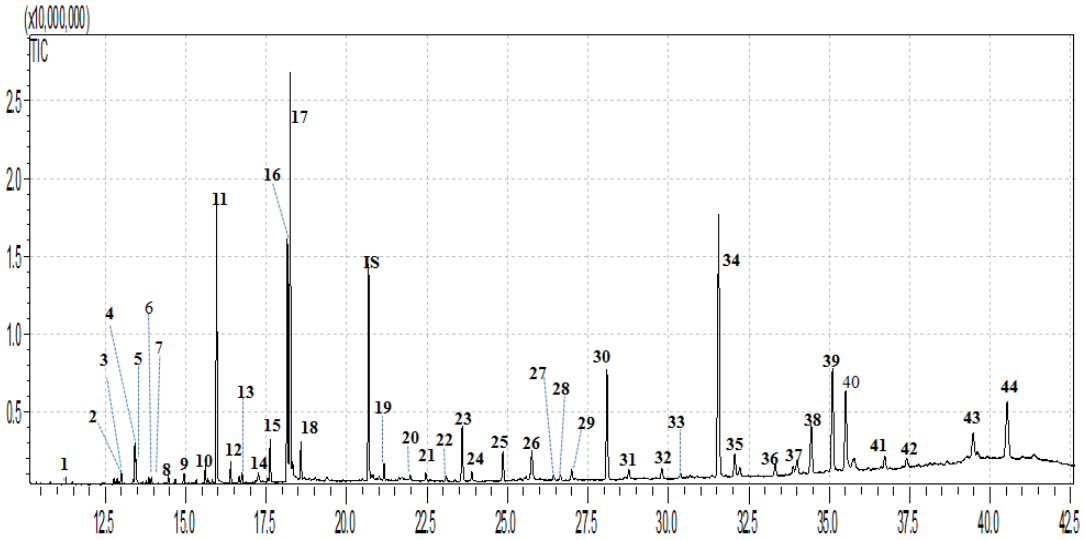


Figure 4.4.1 GC-MS profile of the *S. stellata* lipophilic extract. Peak identification as in Table 4.4.1. IS - tetracosane (internal standard).

Consequently the results comparison is problematic. However, there are indications that species from *Cephalaria* genus, which is included in the same family of the *Scabiosa* genus (formerly the Dipsacaceae and currently Caprifoliaceae), present this high values in long chain carboxylic acids (Kirmizigül et al., 2012). A detailed analysis of the long chain carboxylic acid amount (Table 4.4.1) shows that several saturated acids are present whereas the unsaturated ones are only three (linoleic, linolenic and hexadecatrienoic acids), being the unsaturated nearly twice (5413.07 mg/kg) the amount of the saturated ones (2902.30 mg/kg). The most abundant fatty acids are linolenic (40.8% of the fatty acids amount), linoleic (22.5%) and palmitic (24.7%), being the first two unsaturated ones. It is common knowledge that polyunsaturated fatty acids are essential to human development and health. Moreover they are being associated to decrease the risk of cancer and/or cardiovascular illnesses (Chen et al., 2013) and, due to their potential antioxidant and anti-inflammatory activities, they are also studied as potential therapeutic agents for neurological disorders (Lei et al. 2016). It should be highlighted that these results showed a very interesting ω-6/ω-3 fatty acids ratio (ω-6/ω-3 = 0.55) in the plant, since this ratio is associated with decrease of cardiovascular, cancer, inflammatory and autoimmune diseases risk (Simopoulos, 2008).

Table 4.4.1 Lipophilic components of the the *S. stellata* hexane extract (mg of compound / kg of dried plant)

Peak	Rt (min)	Compound name	MV ± SD
Carboxylic acids and esters			
1	11.28	Dodecanoic acid ($C_{12}H_{24}O_2$)	34.26 ± 0.81
3	12.99	Isocitric acid ($C_6H_8O_7$)	95.13 ± 1.67
4	13.43	Quinic acid ($C_7H_{12}O_6$)	210.84 ± 3.20
5	13.46	Myristic acid ($C_{14}H_{28}O_2$)	210.84 ± 3.20
8	14.47	Methyl palmitate ($C_{17}H_{34}O_2$)	46.31 ± 1.35
10	15.61	Hexadecatrienoic acid ($C_{16}H_{26}O_2$)	146.67 ± 0.99
11	15.96	Palmitic acid ($C_{16}H_{32}O_2$)	2052.38 ± 5.80
13	16.75	Methyl linolenate ($C_{19}H_{32}O_2$)	69.31 ± 1.23
14	17.55	Ethyl 9,12-octadecadienoate	34.27 ± 0.50
16	18.16	($C_{20}H_{36}O_2$)	1874.90 ± 4.02
17	18.25	Linoleic acid ($C_{18}H_{32}O_2$)	3391.50 ± 17.37
18	18.58	Linolenic acid ($C_{18}H_{30}O_2$)	287.67 ± 14.02
19	21.17	Stearic acid ($C_{18}H_{36}O_2$)	149.44 ± 4.95
24	23.90	Eicosanoic acid ($C_{20}H_{40}O_2$)	93.33 ± 3.46
29	27.00	Behenic acid ($C_{22}H_{44}O_2$)	109.45 ± 3.21
33	30.39	Lignoceric acid ($C_{24}H_{48}O_2$)	57.33 ± 0.71
42	37.42	Hexacosanoic acid ($C_{26}H_{52}O_2$)	152.70 ± 6.27
		Triacontanoic acid ($C_{30}H_{60}O_2$)	
			Total = 8805.49 mg kg^{-1}
Alkanes			

20	21.98	Pentacosane ($C_{25}H_{52}$)	13.15 ± 0.29
25	24.86	Heptacosane ($C_{27}H_{56}$)	40.40 ± 2.17
27	26.44	Octacosane ($C_{28}H_{58}$)	15.90 ± 0.35
30	28.10	Nonacosane ($C_{29}H_{60}$)	141.19 ± 7.01
32	29.80	Triacontane ($C_{30}H_{62}$)	23.13 ± 0.74
34	31.57	Hentriacontane ($C_{31}H_{64}$)	370.11 ± 22.54
36	33.33	Dotriacontane ($C_{32}H_{66}$)	26.21 ± 1.20
39	35.11	Tritriacontane ($C_{33}H_{68}$)	163.57 ± 7.34
			Total = 793.66 mg kg^{-1}
		Alcohols	
12	16.40	Inositol ($C_6H_{12}O_6$)	17.09 ± 0.36
15	17.63	Phytol ($C_{20}H_{40}O$)	9.63 ± 0.27
31	28.78	Hexacosan-1-ol ($C_{26}H_{54}O$)	3.64 ± 0.26
22	23.10	1-Monopalmitine ($C_{32}H_{38}O_6$)	1.55 ± 0.05
			Total = 31.91 mg kg^{-1}
		Sugars	
2	12.77	D-Fructofuranose ($C_{20}H_{40}O$)	13.52 ± 0.16
6	13.85	D-Mannopiranose ($C_{20}H_{40}O$)	13.52 ± 0.22
7	13.94	D-Galactopyranoside ($C_{20}H_{40}O$)	13.88 ± 0.28
9	14.95	D-Talopyranose ($C_{20}H_{40}O$)	14.73 ± 0.27
23	23.60	Sucrose ($C_{20}H_{40}O$)	40.72 ± 1.70
26	25.76	3-α-Monnobiose ($C_{20}H_{40}O$)	30.80 ± 0.76
			Total = 127.17 mg kg^{-1}
		Terpenes	
28	26.65	Squalene ($C_{28}H_{50}$)	4.20 ± 0.17
35	32.23	α-Tocopherol ($C_{28}H_{48}O_2$)	10.41 ± 0.04
37	34.02	Campesterol ($C_{28}H_{48}O$)	18.38 ± 0.78
38	34.44	Stigmasterol ($C_{29}H_{48}O$)	54.33 ± 1.39
40	35.52	β-Sitosterol ($C_{29}H_{50}O$)	110.63 ± 0.79
41	36.73	Lupeol ($C_{30}H_{50}O$)	17.16 ± 0.20
43	39.49	Ursolic acid ($C_{30}H_{48}O_3$)	42.83 ± 0.30
44	40.54	Oleanolic acid ($C_{30}H_{48}O_3$)	88.02 ± 0.79
			Total = 345.96 mg kg^{-1}

The presence of alkanes in plants is well predictable and used in chemotaxonomy (Bush et al., 2013), so their identification in the *S. stellata* lipophilic extract expected. This group of compounds is the second more abundant (7.9%) (Fig. 2) and the C_{30} to C_{33} chain lengths represent the most abundant ones (73.4% of the alkanes amount) (Table 4.4.1), which is in concordance with the fact that *S. stellata* herein study origin is Algeria.

Finally, another important group, although less representative, is the terpenes (3.4%) (Fig. 4.4.2). The sterol content represents 53.0% of the terpenes amount and β-sitosterol is the most abundant one (32.0% of the terpenes amount). There are evidences that plant sterols intake promote health benefits mainly reducing the level of cholesterol in blood (Quílez et al., 2003), being another indication of the nutritional value of *S. stellata*. The content in triterpenic acids

(ursolic acid and oleanolic acid) although not very high (37.8% of the terpenes amount and 1.3% of all identified compounds) (Table 4.4.1) is also noteworthy due to the fact that they are recognised as biologically active metabolites (Szakiel et al., 2012), namely as antioxidant and anti-aging agents (Bourgeois et al., 2016).

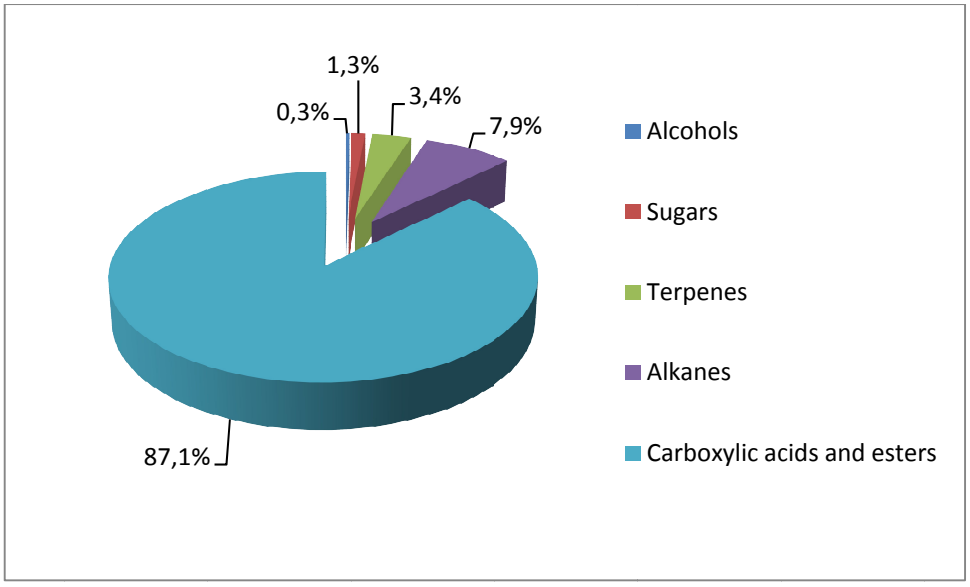


Figure. 4.4.2 Percentage of the five groups in which the identified compounds were allocated

4.5. Evaluation of antioxidant activity:

Overview :

In biological systems, an antioxidant is a substance that, in low concentration compared to the quantity of oxidizable substances such as reactive oxygen (ROS), significantly delays or prevents the oxidation of substrates like lipids, proteins, DNAs and carbohydrates. It intercept the free radicals before they react with the substrate (Tiwari et al., 2004), Thus it can neutralize free radicals (free radicals are very reactive and rapidly attack molecules in neighboring cells, the damage caused is inevitable and probably treated by repair) processes and increase shelf life by delaying the process of lipid peroxidation, which is one of the main reasons for the deterioration of food and pharmaceutical products during treatment and storage (Halliwell et a.,l 1997)

Biological antioxidants are divided into four classes according to Prior et al. (2005):

- The compounds with a low molecular weight (tocopherols, ascorbic acid, β-carotene, glutathione, ...).
- Some hormones (estrogen, angiotensin, melatonin, etc.)
- Enzymes (example, superoxide dismutase, glutathione peroxidise).
- Large molecules (albumin, catalase, ferritin, other proteins).

the antioxidant capacity of the molecules can be evaluated either *in vivo* or *in vitro* on living organisms, using tests that mimic physiological phenomen, to evaluate the antioxidant activity in natural extract, different methods have been developed, these methods implicate mixtures of oxidizing species, such as radicals or oxidized metal complexes with a sample that contains antioxidants that can inhibit genesis of radicals. these antioxidants can proceed according to two major mechanisms either by hydrogen atom transfer or by electron transfer. Table (Prior et al., 2005).

In this work the antioxidant activity of the extracts of species was evaluated in vitro by three different methods. The FRAP , DPPH radical trapping method and ABTS assay

Table 4.5.1. In vitro Antioxidant Capacity Assays (huang et al., 2005)

Methods	***Equation of mechanism***	***mechanism***
- FRAP (Ferric ion Reducing Antioxidant Power). - TEAC (Trolox equivalent antioxidant capacity)	$M(n)+ AH \longrightarrow AH^{+\cdot} + M(n-1)$	Electron transfer reaction

- Total phenolic content		
- ORAC (oxygen radical absorbance capacity) - TRAP (total radical trapping antioxidant parameter)	$AH + X^{\cdot} \rightarrow XH + A^{+\cdot}$	Hydrogen atom transfer
- DPPH scavenging - ABTS scavenging	In this case the reactions are very complexe follow one of two mechanisms according to the structure of the antioxidants or the nature of the reaction medium	Based on both of the two mechanisms

4.5.1 DPPH radical-scavenging assay

Principle: DPPH (α, α-diphenyl-β-picrylhydrazyl) radical is a widely used method to evaluate the free radical scavenging ability of natural compounds. This assay is based on the measurement of the scavenging ability of antioxidant toward the stable radical and turning the colour of the solution from purple to yellow ((Brand-Williams et al. 1995). DPPH• shows a strong absorption maximum at 517 nm (purple)

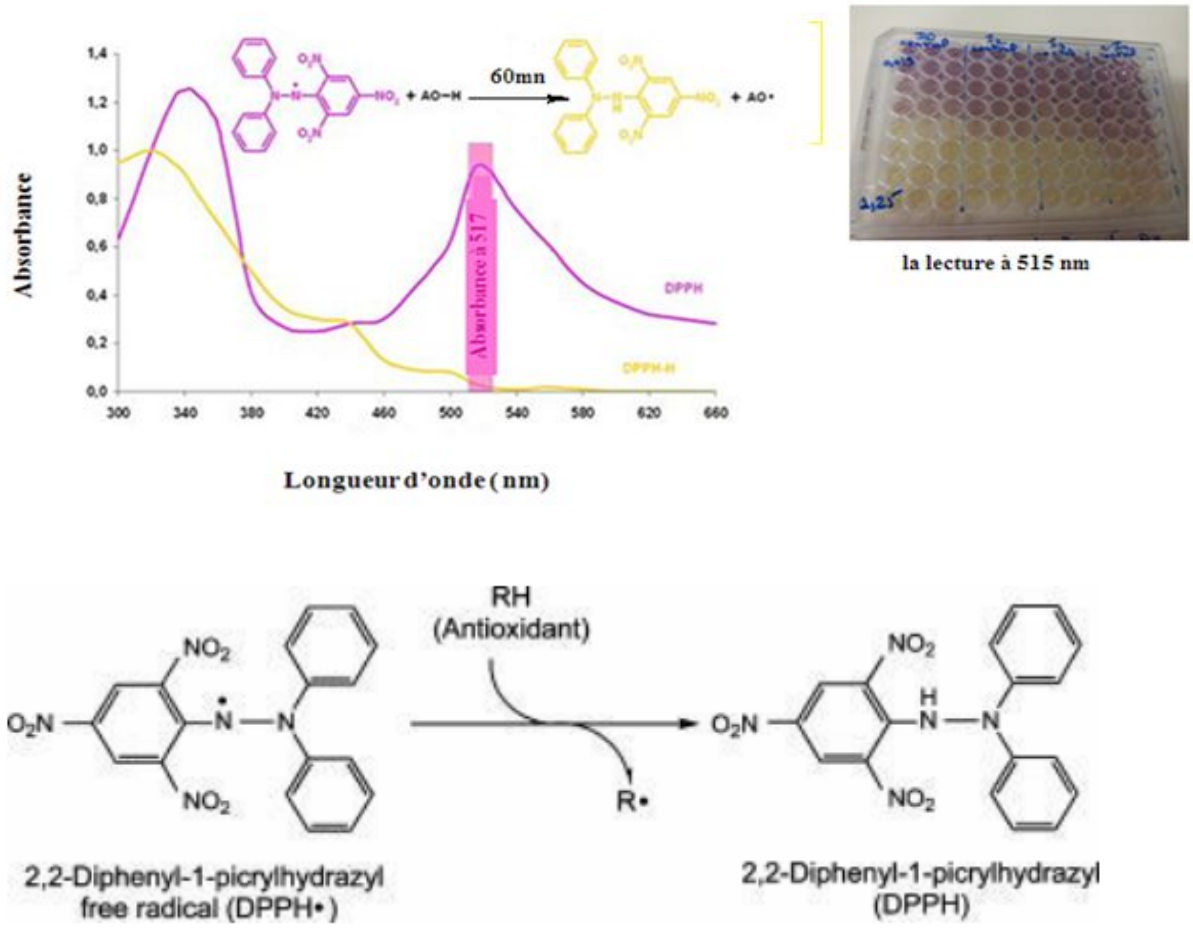


Figure 4.5.1: DPPH• free radical conversion to DPPH by anti-oxidant compound.

The radical-scavenging activity was carried out following a previously reported procedure (Pereira et al., 2013), with some modifications. The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the fractions (50 µL) and 250 µL of a methanolic solution containing DPPH (0.2 mM). The micro-plates were then placed in the dark and at room temperature for 30 min. The absorbance was measured at 517 nm using a micro-plate reader (model EAR 400, Labsystems Multiksan MS) with reference to a control without extract. The radical-scavenging activity was calculated as a percentage of DPPH discoloration using the equation: DPPH scavenging effect % = [(A0-A1)/A0)] *100 where A0 is the absorbance of the control reaction and A1 is the absorbance of the test fraction. Based on graphic values of percentage of DPPH inhibition *vs.* fraction concentration, the IC_{50} of each extract was estimated (Table 4.5.2). Ascorbic acid was used as the reference.

For the calculation of IC 50 we use the reaction:

$$IC50 = \frac{(x2 - x1)}{(y2 - y1)}(50 - y_1) + x_1$$

4.5.2 Ferric ion Reducing Antioxidant Power (or iron (III) to iron (II) reducing activity

***Principle*:** Antioxidant compounds cause the reduction of ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form because of their reductive capabilities. Prussian blue colored complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm . In this assay, yellow color of the test solution changes to green or blue color depending on the reducing power of antioxidant samples. A higher absorbance indicates a higher ferric reducing power (text extracted from Ravi Kiran et al., J Bioanal Biomed 2012, 4:4).

$K_3Fe(CN)_6$ + Reductive antioxidant=$Fe(CN)_6^{-4}$

$Fe(CN)_6^{-4} + FeCl_3 \rightarrow Fe_4[Fe(CN)_6]_3$ (Prussian blue)

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action.

4.5.3 ABTS scavenging :

Principle: The pre-formed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) is generated by oxidation of ABTS with potassium persulfate ($K_2S_2O_8$). The oxidation of ABTS gives the solution a dark green coloration which suffers decolourization when in the presence of hydrogen-donating antioxidants, such as flavonoids, hydroxycinnamates, carotenoids, which cause the reduction of $ABTS^{\cdot+}$.(Re et al., *Free radical biology & medicine*, 29:9/10:1231-1237, 1999)

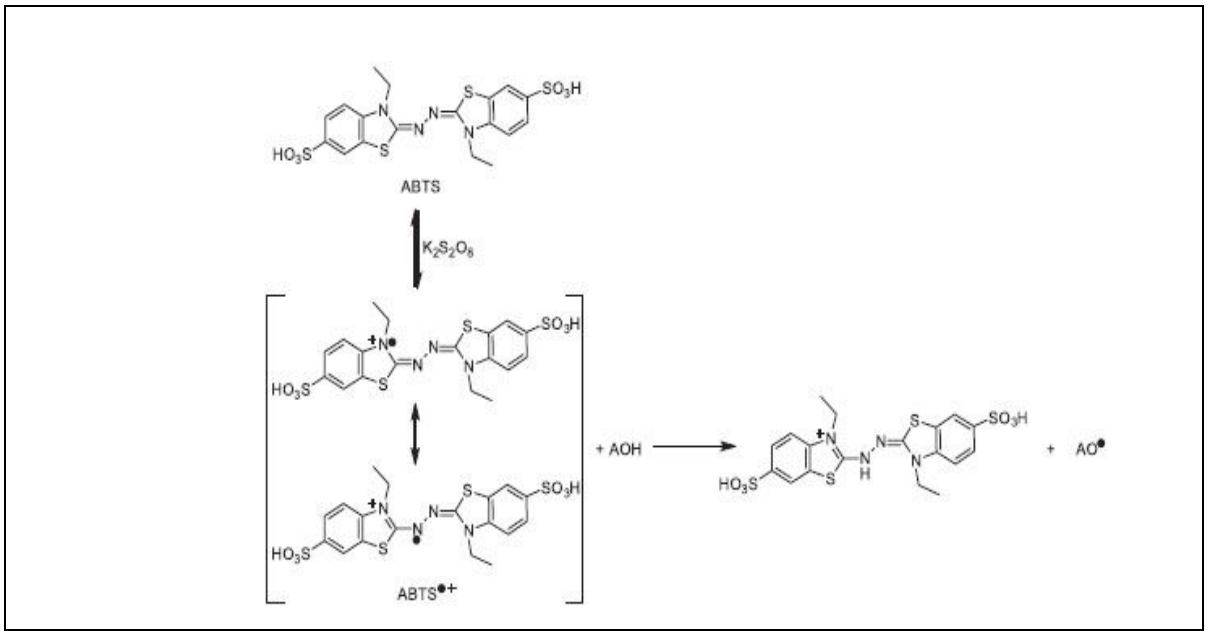


Figure 4.5.2 conversion of ABTS by anti-oxidant compound.

The ABTS assay was carried out following a previously reported procedure (Re et al., 1999), with some modifications. A volume of 250µL of ABTS solution (7mM) was mixed with 50 µL of the plant extract fractions at various concentrations. The reaction mixture was left in the dark during 20min, and its absorbance was recorded at 734 nm. As for the antiradical activity, ABTS scavenging ability was expressed as IC_{50} (µg/mL) as shown in table 2. Trolox was used as the reference.

4.5.4 Determination of total polyphenol

Determination of total polyphenols contents by Folin-Ciocalteu method allowed to compare the efficiency of extraction solvents (Butanol and ethyl acetate) to solubilize and to extract these compounds and to determine also the contents of foliar organs by these metbolites. The results showed that n-butanol fraction showed the highest contents of phenolic compounds (Table 4.5.2). They are in the order of 11.86 mg EAG g-1MS. For the extraction with ethyl acetate, the contents are two times and a half lowers in the order of 4.75 mg EAG g-1MS

Table 4.5.2 Extraction yields and antioxidant capacity of *S. stellata* ethanolic extract fractions

Fraction	Mass[a]	Total phenolic content[b]	DPPH (IC_{50})[c]	ABTS assay (IC_{50})[c]	Reducing power (EC_{50})[c]
DCMF	12.3	< 1.00	> 250	> 250	> 250
EAF	5.3	4.74 ± 0.01*	71.82 ± 0.04*	40.41 ± 0.02*	202.41 ± 0.10*
n-BF	51.7	11.86 ± 0.05*	64.46 ± 0.01*	27.87 ± 0.01*#	161.11 ± 0.08*#
Reference	-	-	8.21 ± 0.03[d]	12.07 ± 0.04[e]	18.03 ± 0.01[f]

Data represent the mean values ± SD of three independent assays performed in triplicate (N = 3).
[a]% of dry weight
[b]mg GA/g DF
[c]µg/mL
[d]Reference used was ascorbic acid

[c]Reference used was trolox
[f]Reference used was BHA
[*]Statistically significant different with respect to the reference (Tukey's test), $p < 0.05$.
[#]Statistically significant different with respect to EAF (unpaired Student's *t*-test), $p < 0.05$.

4.5.5 Results and descussion:

Aiming to establish the phenolic profile of *S. stellata* an ethanolic extract was obtained and fractioned with dichloromethane fraction (DCMF), ethyl acetate fraction (EAF) and *n*-butanol fraction (*n*-BF). The antioxidant potential of these fractions was estimated by three *in vitro* assays and the results are shown in (table 4.5.2.) DPPH and ABTS assays measure the abilities of the fractions to scavenge free radicals while reducing power assay evaluate their ability to reduce Fe^{3+} to Fe^{2+}. All the same these assays can indicate the fraction antioxidant capacity and. The gathered data allowed inferring some general conclusions regarding the antioxidant activity of each fraction. The DCMF has no significant activity which is in accordance with its low content in phenolic compounds. The *n*-BF is the more active one in all the assays which is also in harmony with its higher content in phenolic compounds. Although the overall results are considerably higher than the reference it seems that *S. stellata* possess antioxidant metabolites that can be further evaluated.

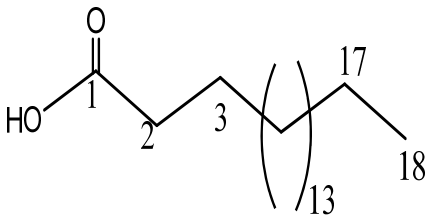
Considering that this species biological activity should be more related with the phenolic content, a previous determination the fractions total phenolic content was performed as well as their antioxidant activity (Table 4.5.2). These determinations allowed to confirm that the fraction obtained with dichloromethane, although more representative than the one obtained with ethyl acetate (more than two times higher), is less rich in phenolic compounds. This low content in phenolic compounds also explains why IC_{50} and EC_{50} values were not found for this fraction antioxidant assays (Table 4.5.2). The antioxidant potential of these fractions was estimated by three in vitro assays and the results are shown in table 5.2. DPPH and ABTS assays measure the abilities of the fractions to scavenge free radicals while reducing power assay evaluate their ability to reduce Fe^{3+} to Fe^{2+}, in all cases it is recognized that the phenolic compounds are responsible for these antioxidants abilities So, the gathered data allowed inferring some general conclusions regarding the antioxidant activity of each fraction. The DCMF has no significant activity, which is in accordance with its low content in phenolic compounds. The *n*-BF is the more active one in all the assays, which is also in harmony with its higher content in phenolic compounds. Although the overall results are considerably higher than the used references it seems that *S. stellata* possess antioxidant metabolites that can be further evaluated. The only data, concerning antioxidant activity, reported so far involves the DPPH assay and the results herein reported are similar. These bioassays' guide

us to perform the phytochemical study of the EAF and *n*-BF fractions. The total phenolic content of both fractions (Table 4.5.2),

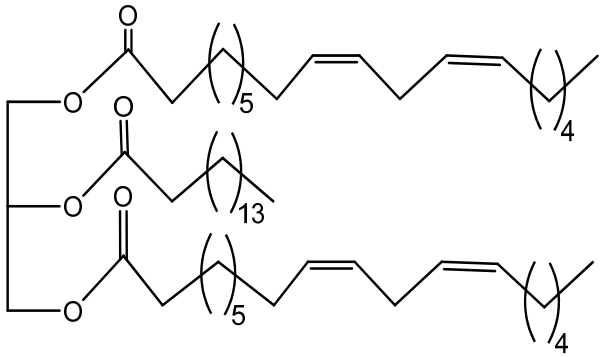
Conclusion

The present work describes the chemical investigation of two Algerian plants called *scabiosa stellata* and *sedum carealeum*. This study carried out on the hexane, ethyl acetate and *n*-butanol fractions of the whole plants which were evaluated by using separation, analyses and biological methods. Leading to the isolation and identification of eighteen compounds from *S. Stellata* and seven compounds from second one . The characterised compounds belong to four classes of secondary metabolites: flavonoids, triterpenes, faty acids and phenolic acids. They are as following:

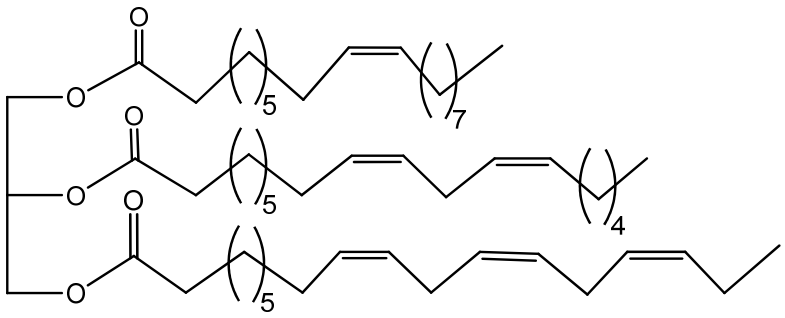
- **From *S. Stellata* :**



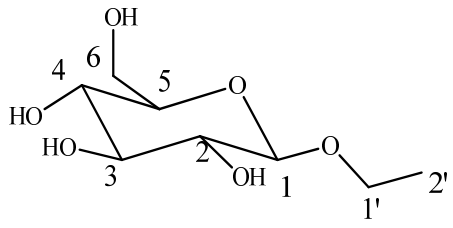
Stearic acid



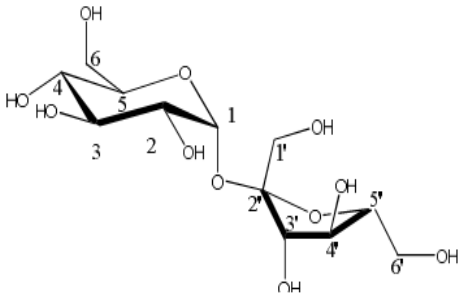
1,3-O-dilinoleoyl-2-O-palmitoyl glycerol



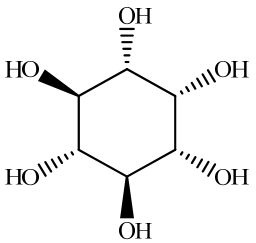
1-O-linolenoyl-2-O-linoleoyl-3-O-oleoyl glycerol



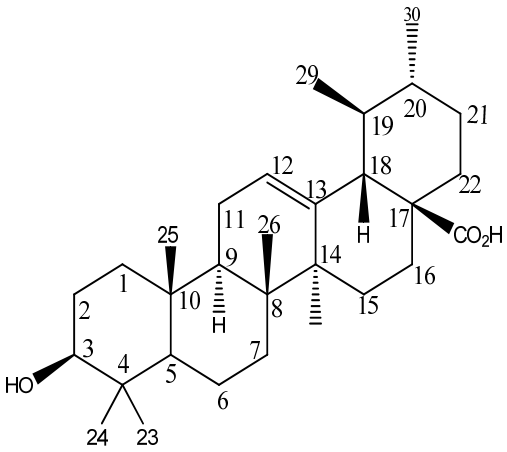
1-O-ethyl-β-D-glucopyranoside



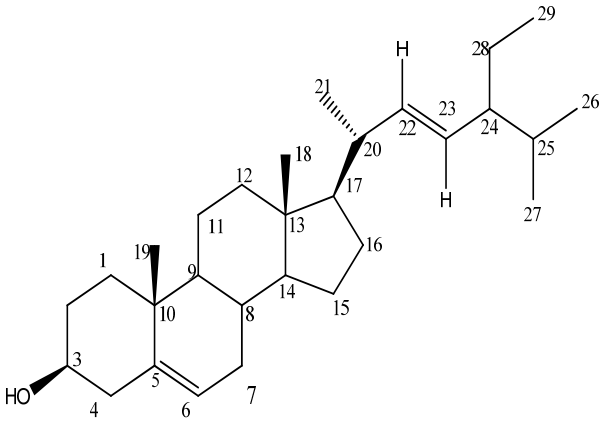
Sucrose



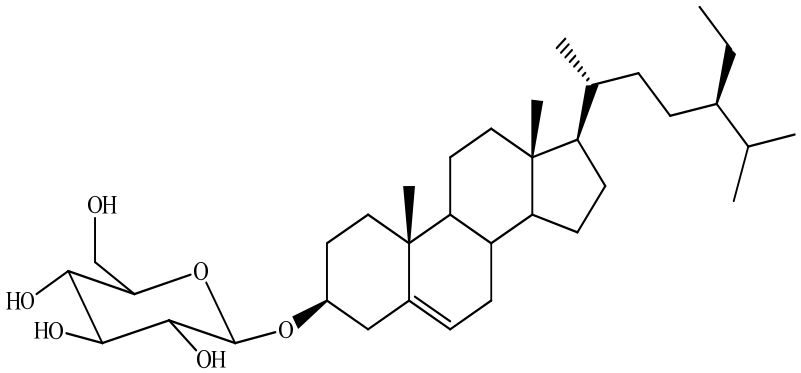
Myo-inositol



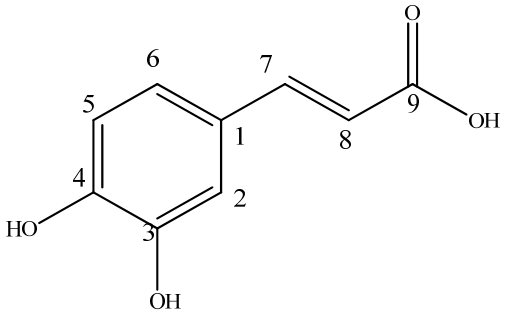
Ursolic acid



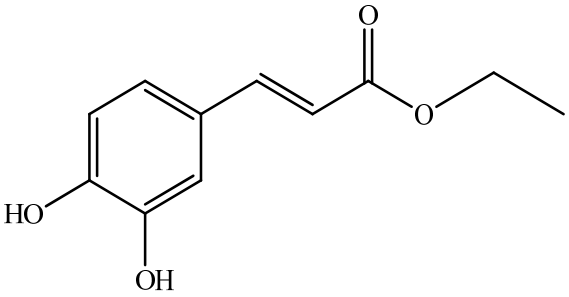
Stigmasterol



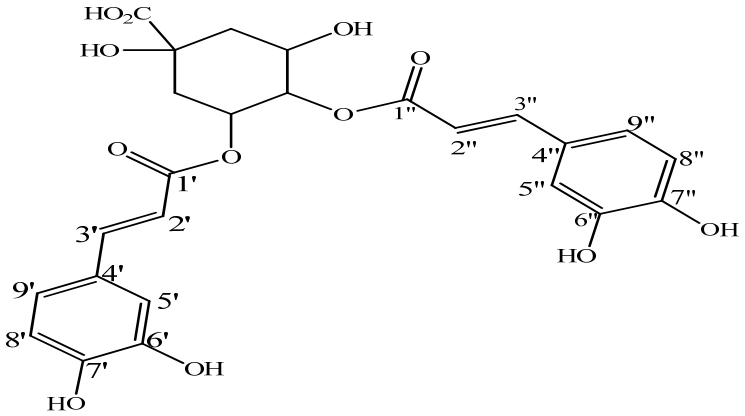
Beta-sitosterol glycose



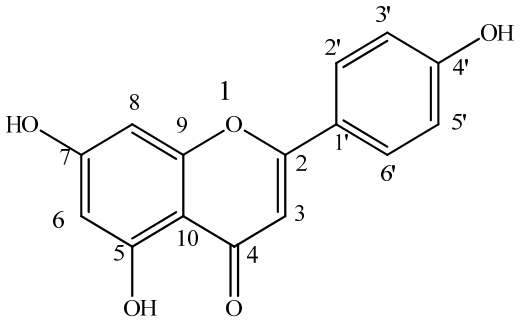
Caffeic acid



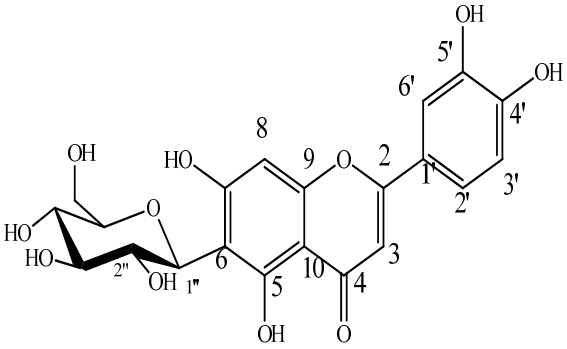
Ethyl caffeate



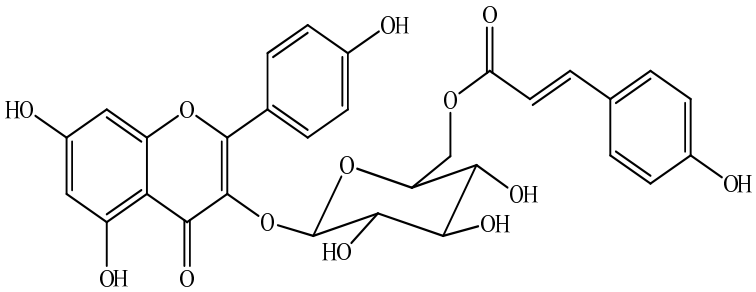
4,5-O-dicaffeoylquinic acid.



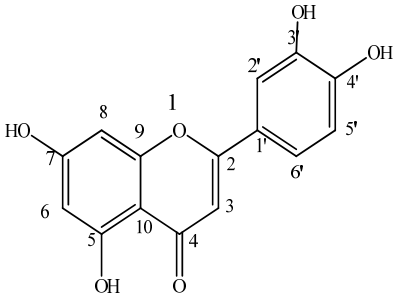
Apigenin



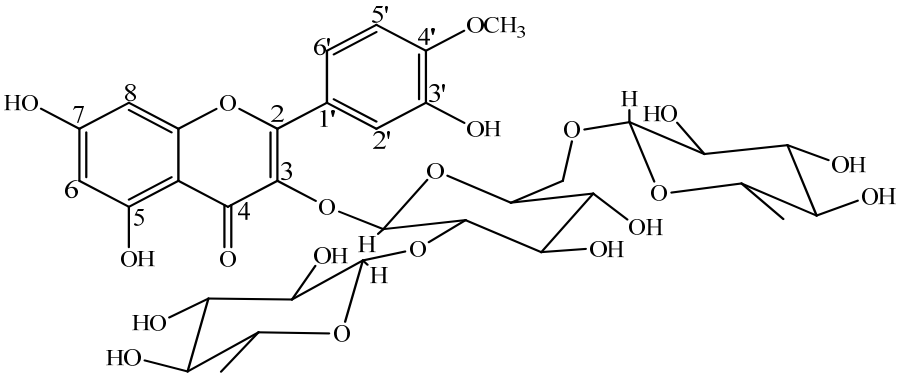
Isoorientin

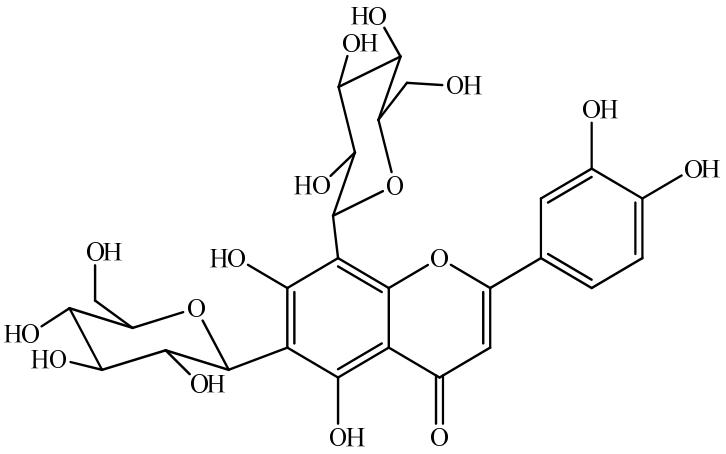


Tiliroside



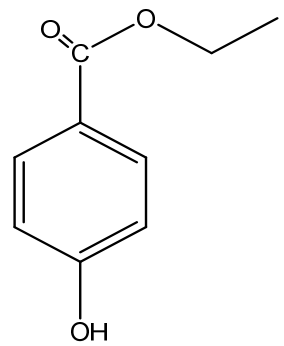
luteolin



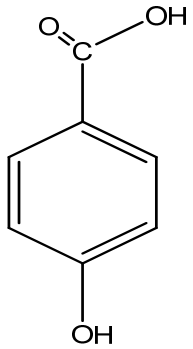


Lucenin 2

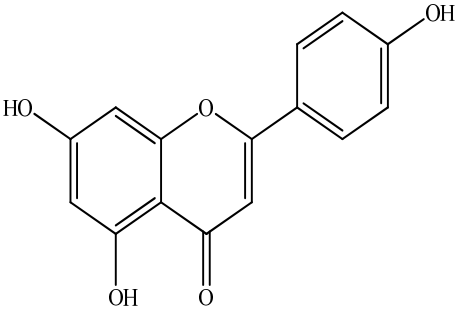
- **From *S. carealeum***



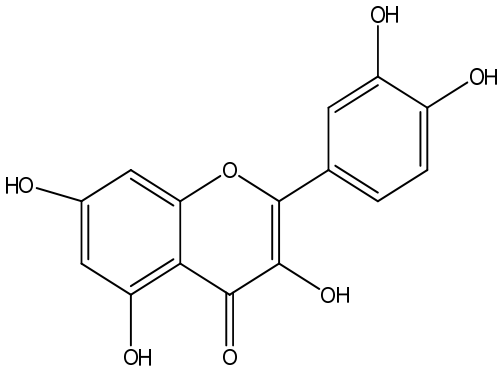
4-ethylhydroxybenzoat



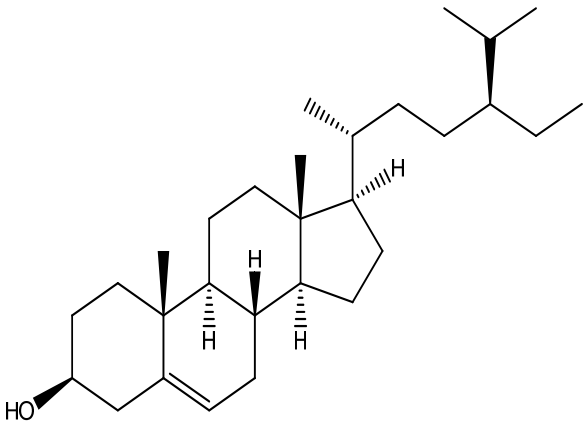
Hydroxybezoic acid



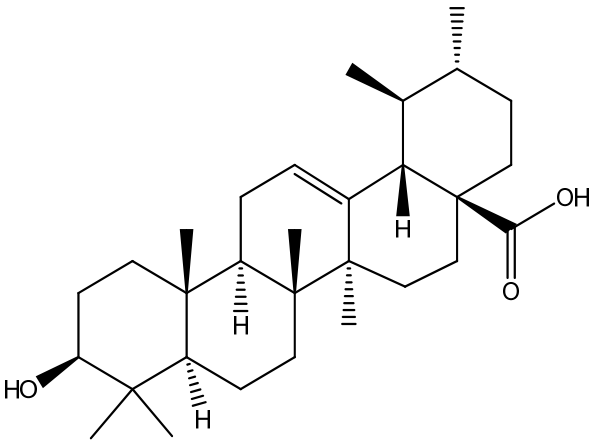
Apegenin



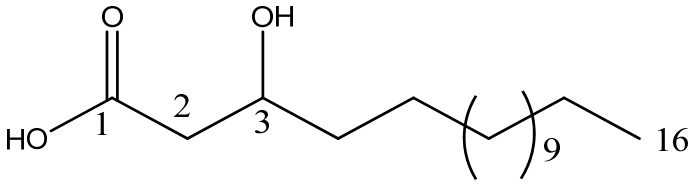
Quercetin



Beta-sitosterol



Ursolic acid



3-hedroxyhexadecanoic acid

Analysis with modern tools such as GC-MS and LC-MS^n, gives the necessary informations in a short time using a small quantities of plant material and their identification based on mass spectral characteristics. In this study GCMS, LC-MS^n were used for non polar(hexane) and polar(ethanol) extarcts, respectively.

GC-MS allows to identify about 43 non- polair metabolites belonging to several important families. Fatty acids are the most abundant; particularly linolenic (x-3), linoleic (x-6) and palmitic acids.

LC-MS^n allows to the identify about 25 compounds (phenolic ones) which are important, such as chlorogenic acids and flavonoids. Chlorogenic acids and flavonoids comprised more than 80% of the compounds found, luteolin-6-C-glucoside and 4-O-caffeoylquinic acid are the major compounds which explains the recently reported antioxidant activity of this plant extract.

Therefore, the characterization herein reported will provide information about the *S. stellata* benefits to individuals and may contribute to increase its consumption, through infusions and/or condiments.

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Papres

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ORIGINAL PAPER

Lipophilic composition of *Scabiosa stellata* L.: an underexploited plant from Batna (Algeria)

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Abstract *Scabiosa stellata* L. is the less-studied plant of the species belonging to the genus *Scabiosa*, but the fact that several *Scabiosa* species are used in traditional medicine is an incentive to study this species. The profile of the *S. stellata* hexane extract was established by gas chromatography–mass spectrometry and allowed to conclude that fatty acids and their derivatives (87%) are the major chemical families of this extract. From the identified components, linoleic (19%), palmitic (20%) and linolenic (34%) acids are the major ones. Additionally, triterpenoids, namely β-sitosterol, stigmasterol, oleanolic and ursolic acids were also found. Simultaneously, the phytochemical study of this extract allowed the isolation and full characterization using 1D and 2D NMR experiments (^{1}H and ^{13}C NMR, HSQC, COSY, HMBC) of interesting metabolites such as 1,3-*O*-dilinoleoyl-2-*O*-palmitoyl glycerol and 1-*O*-linolenoyl-2-*O*-linoleoyl-3-*O*-oleoyl glycerol. This first assessment of *S. stellata* less polar constituents allowed the identification of several important compounds among which, ursolic and stearic acids, 1,3-*O*-dilinoleoyl-2-*O*-palmitoyl glycerol and 1-*O*-linolenoyl-2-*O*-linoleoyl-3-*O*-oleoyl glycerol, were found, for the first time in *Scabiosa* genus. The results reported, index of atherogenicity (AI = 0.55), index of thrombogenicity (TI = 0.23) and the fatty acid ratio (ω-6/ω-3 = 0.55), point out the nutritional value of *S. stellata*.

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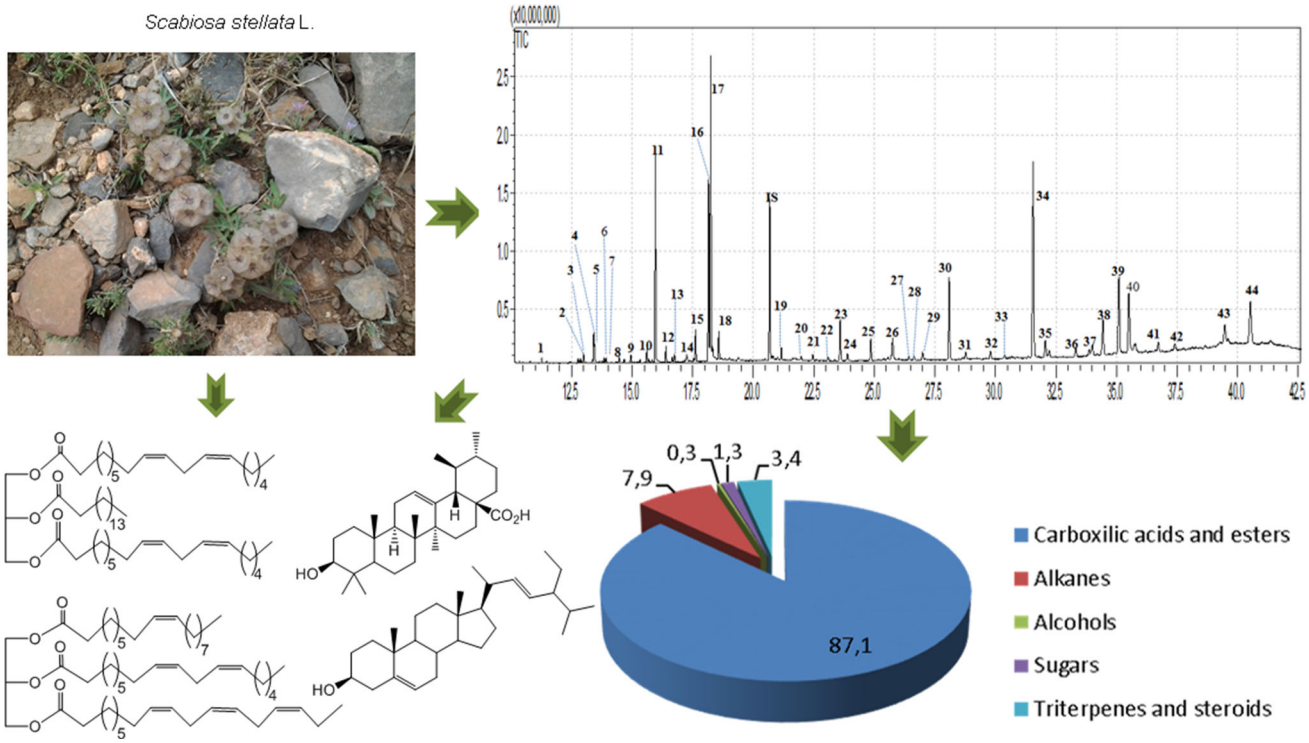
Graphical abstract



Keywords *Scabiosa stellata* L. · GC–MS profile · Fatty acids · Sterols · Terpenes · Nutritional indexes

Introduction

Lately, the natural product research area is devoted to highlight the chemical composition of several plants used by our ancestors to treat diseases. More and more instrumental techniques, which give prompt information, are the main choice for profiling complexes mixtures (Rutkowska et al. 2013). Among these techniques, gas chromatography–mass spectrometry (GC–MS) is the most selected to obtain the lipophilic profile of a plant and in doing so the medicinal and or nutritional value of that species can be recognised (Angelova and Schmauder 1999).

Scabiosa stellata L. is a plant found in dry sunny grassland and rocky hillsides belonging to *Scabiosa* genus considered the most significant member of *Dipsacaceae* family[1] (Verlaque 1982) and distributed in Southern Africa, Europe and Asia (Carlson et al. 2012; Jasiewicz 1976). Several *Scabiosa* species are reported to be found in Algeria and used in traditional medicine (Quezel and Santa 1963), however, only a few are accepted by the taxonomists, (namely *S. arenaria* Forssk., *S. argentea* L., *S. atropurpurea* L., *S. columbaria* L., *S. semipapposa* Salzm. ex DC. and *S. stellata* L.), the majority are unresolved or synonym and their inclusion in the genus *Scabiosa* is controversy. Nevertheless, *S. stellata* is one of the *Scabiosa* genus species that can be found in Algeria.

Even if there are no phytochemical reports about *S. stellata,* the fact that other *Scabiosa* species have been studied (although not their less-polar constituents) and showed evidences that species from this genus can produce biologically active molecules are an incentive to study this species. For instance *S. arenaria* and *S. hymettia* Boiss. et Spruner produce compounds that presented interesting antimicrobial properties (Besbes et al. 2012; Christopoulou et al. 2008). Recent reports indicate that compounds isolated from *S. arenaria* exhibit antioxidant and acetylcholinesterase inhibitory properties (Hlila et al. 2015a) and are able to inhibit the enzyme α-glucosidase usually associated to diabetes type 2 (Hlila et al. 2015b). Moreover, the biological evaluation of the extracts of two *Scabiosa* species used in Chinese herbal medicine, *S. comosa* Fisch. ex Roem. et Schult and *S. tschiliensis* Grüning, demonstrate anti-HCV and radical scavenging activities (Ma et al. 2015).

In this context, the present study aims to increase the depth of knowledge about *S. stellata,* in particularly establish its GC–MS profile. Simultaneously, a phytochemical study of its hexane extract was also performed

[1] Although in the articles, the authors indicate that the *Scabiosa* genus belong to the *Dipsacaceae* family, accordingly to The Plant List (http://www.theplantlist.org), this genus species is currently placed in the family *Caprifoliaceae*.

and interesting metabolites were isolated and fully characterised using several NMR experiments. So the hexane extract was obtained and studied. With this study, we hope to add significance to a plant that grows wild and currently has no economic or scientific value.

Experimental

Chemicals

Column chromatography (CC) was performed with silica gel 60 (Merck Kieselgel, 70–230 mesh) and Sephadex LH-20. Preparative thin layer chromatography (prep. TLC) was performed on glass plates (20 × 20 cm) precoated with Merck silica gel 60 GF_{254} (0.5 mm thickness) and activated at 100–110 °C for 12 h. Merck silica gel 60 GF_{254} plates were used for TLC. Spots were detected in TLC under UV light (λ 254 and 366 nm). Solvents were purchased from Panreac and Acros Organics and were of analytical grade or bi-distilled commercial solvents. Other chemicals such as ursolic acid (98%), oleanolic acid (98%), palmitic acid (99%), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (99%), trimethylsilyl chloride (TMSCl) (99%), tetradecane (99%), hexadecane (99.5%), tetracosane (99%), octadecane (99%), citric (> 99.5%), ursolic (98%), oleanolic (99%), linoleic (≥ 99%) and palmitic (≥ 99%) acids, sorbitol (99%), D-mannitol (98%), D-(+)-galactose (> 99%), D-(+)-mannose (> 99%), D-(+)-talose (> 99%), D-(+)-xylose (> 99%), D-(−)-arabinose (> 99%), D-(−)-ribose (> 99.5%), D-fructose (99%), sucrose (> 99%), maltose (> 98%), cellobiose (> 98%), β-sitosterol (98%), 5-c-holesten-3β-ol (99%), stigmasterol (97%), campesterol (95%), lupeol (99%), glycerol (> 99%), myo-inositol (99%), tetradecanol (98%) and 1-palmitoylglycerol (> 99%) were purchased from Sigma-Aldrich. α- And δ-tocopherol (98%) were purchased from Merck, while pure alkanes and *n*-paraffin mixtures (C_5-C_8, C_7-C_{10}, C_{10}-C_{16}, C_{18}-C_{24}, C_{24}-C_{36}, C_{25}-C_{35}) were supplied by Supelco Inc..

Sample preparation

Specimens of *Scabiosa stellata* L. were collected in the Belezma National Park (Batna, Algeria; 35°35′ 41.52″ N, 5° 56′13.75″ E). Flowering aerial parts of *S. stellata* were collected from its natural habitat in June 2015. A voucher specimen was identified by Dr. Bachir Oudjehih, professor of Agronomic Institute, University of Batna (Algeria) and deposited in the Herbarium under the reference number VAREN/SS/2013/123.

Dried powdered of *S. stellata* whole plant (200 g) was extracted with hexane pro-analysis grade (500 mL, three times) at room temperature for 3 days using an overhead stirrer. The mixture was filtered and the hexane was removed using a rotary vacuum evaporator. The resulting extract was dried, yielding 2.67 g of hexane extract.

Before GC–MS analysis, four aliquots of the hexane extract (20 mg) were silylated accordingly to a known method and usually used in our department (Freire et al. 2002). Each sample was dissolved in 1000 µL of dichloromethane and 200 µL of internal standard (IS) solution (tetracosane), 250 µL of pyridine, 250 µL of BSTFA and 50 µL of TMSCl were added. The mixture was maintained at 70 °C for 30 min being the hydroxyl and/or carboxyl groups of the present compounds converted into trimethylsilyl (TMS) ethers and/or esters, respectively. Afterwards were injected into the GC–MS apparatus. The quantity of silylation agents (BSTFA and TMSCl) was sufficient to ensure the silylation of all hydroxyl groups present in the sample compounds. All the compounds possessing hydroxyl groups are transformed, using the same procedure, into the correspondent TMS derivatives and no compounds with free hydroxyl groups were detected.

Gas chromatography–Mass spectrometry (GC–MS) analysis

GC–MS analyses were performed using a Shimadzu Gas Chromatograph QP2010 Ultra equipped with Autosampler AOC-20i, Ion source: electronic impact High-performance Quadrupole Mass Filter. Separation of compounds was carried out in a DB-5J&W capillary column (30 m × 0.25 mm inner diameter, 0.25 µm film thickness) using helium as the carrier gas (35 cm s^{-1}). The chromatographic conditions were as follows: start time at 6.5 min; initial temperature, 90 °C for 4 min; temperature rate, 16 °C min^{-1} up to 180 °C, followed by temperature rate, 6 °C min^{-1} up to 250 °C; followed by temperature rate, 3 °C min^{-1} up to 300 °C which was maintained for 5 min.; injector temperature, 320 °C; transfer-line temperature, 300 °C; split ratio, 1:50. The mass spectrometer was operated in the electron impact (EI) mode with energy of 70 eV, and data were collected at a rate of 1 scan s^{-1} over a range of *m*/*z* 33–750. The ion source was kept at 250 °C.

From total ion chromatogram, the peaks were identified by comparing their mass spectra with the mass spectral libraries (NIST 14 Mass Spectral and Wiley Registry™ of Mass Spectral Data), with MS spectra and MS fragmentation pattern published in the literature (AOCS Lipid Library 2017; Golm Metabolome Database 2017; Suttiarporn et al. 2015; Vilela et al. 2014; Hrabovski et al. 2012; Füzfai et al. 2008; Razboršek et al. 2008; Oliveira et al. 2006; Kitson et al. 1991; Petersson 1969), by comparing the retention times and mass spectra data of the standard

compounds injected in the same chromatographic conditions. Moreover, when possible the retention index relative to *n*-alkanes (C_5-C_{36}) was compared with published data retention indexes.

For quantification purposes, four independent replicates of each sample were submitted to silylation procedure and each one injected in triplicate. The internal standard method was applied and the amount of metabolites present was achieved from the calibration curves obtained with the most closed pure standard compounds available or its TMS derivatives (if they have hydroxyl groups). All the injected samples and standard solutions contain a fixed quantity of internal standard (tetracosane). The calibration curves were obtained by injection of five known concentrations (5 μg mL^{-1} to 1.5 mg mL^{-1}) and the detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves represented in Table 1 (LOD = 3 standard deviation/slope and LOQ = 10 standard deviation/slope). Values of correlation coefficients confirmed linearity of the calibration plots (Table 1). The concentrations of the standards were chosen to guarantee the quantification of each compound in the samples by intrapolation in the calibration curve. The results were expressed in mg of compound/kg of dried plant, as mean ± standard deviation of four independent analyses.

Statistics

Four independent replicates of the extract were analyzed; and each one of these replicates were injected twice. The presented results are the average of the concordant values obtained for each sample (less than 5% variation between injections of the same aliquot and between aliquots of the same sample) and expressed as mean values ± standard deviation (MV ± SD). One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test were performed using the GraphPad Prism version 7 for Windows (Graphpad Software, Inc.) to compare the results of each independent replicates. A p value lower than 0.0001 was considered statistically significant in all analyses.

Compound purification and characterization

The remaining part of the hexane extract of *S. stellata* was subjected to column chromatography using a step gradient elution of *n*-hexane:ethyl acetate (100:0 to 0:100) and ethyl acetate:methanol (100:0 to 0:100) to afford several fractions. Fraction 4 was chromatographed over a Sephadex LH20 column eluted with methanol and yielded pure compounds **C1** (stearic acid, 3.0 mg) and **C2** (1,3-*O*-dilinoleoyl-2-*O*-palmitoyl glycerol, 2.0 mg), respectively. Fraction 5 was chromatographed under the same conditions and yielded pure compounds **C3** (1-*O*-linolenoyl-2-*O*-linoleoyl-3-*O*-oleoyl glycerol, 6.0 mg) and **C4** (ursolic acid, 2.5 mg), respectively. Fraction 6 was washed with acetone and gave compound **C5** (stigmasterol, 3.5 mg).

NMR spectra {^{1}H, ^{13}C, *g*HSQC, *g*HMBC, *g*COSY} were measured in $CDCl_3$ [samples (2-6 mg) were dissolved in $CDCl_3$ (~ 0.5 mL) and transferred to 5 mm NMR tubes] on a Bruker Avance 300 (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C) spectrometer or Bruker Avance 500 with crioprobe (500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C) and using TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (J) in Hz. 2D *g*COSY spectrum was recorded with 200 transients over 256 increments (zero filled to 1 K) and 2 K data points with spectral widths of 4500 Hz. The recycle delay was 1 s. The data were processed in the absolute

Table 1 Linearity ($y = mx + b$, where y corresponds to the standard peak area/internal standard peak area ratio and x corresponds to the mass of standard/mass of internal standard ratio), LOD and LOQ of pure compounds used as reference

Standard compound	Slope (m)[a]	Intercept (b)[a]	R^2	LOD[b]	LOQ[b]
Methyl palmitate	0.1983	− 0.0017	0.9992	13	43
Palmitic acid	0.2143	0	0.9944	15	50
Linoleic acid	0.1994	− 0.0003	0.9975	12	40
Myo-inositol	7.1931	− 0.0109	0.9952	5	17
1-Palmitoylglycerol	7.2283	− 0.0009	0.9975	3	10
Tetradecan-1-ol	7.2366	− 0.0037	0.9937	3	10
Octadecane	2.0283	− 0.0448	0.9985	8	27
Triacontane	2.0154	− 0.0311	0.9991	10	33
Mannose	4.1401	− 0.0801	0.9998	3	10
Maltose	4.1380	− 0.1126	0.9999	5	17
β-Sitosterol	2.5254	− 0.0033	0.9983	12	40
α-Tocopherol	2.4738	− 0.0028	0.9993	5	17
Ursolic acid	2.6034	− 0.0207	0.9996	10	33

[a]In area counts mg^{-1}

[b]In μg/mL

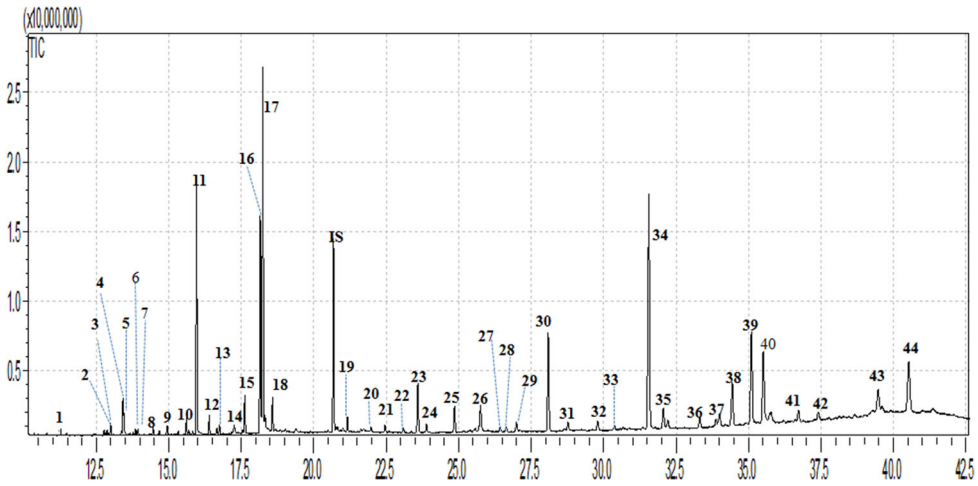


Fig. 1 Total ion chromatogram (TIC) of the *S. stellata* aerial parts hexane extract. Peak identification as in Table 2. IS tetracosane (internal standard); Peak 21-is due to silylation reagent

value mode. The phase-sensitive 1H-detected ($^{1}H,^{13}C$) *g*HSQC spectrum was recorded with 200 transients over 312 increments (zero filled to 1 K) and 2 K data points with spectral widths of 4500 Hz in F2 and 18,000 Hz in F1. The recycle delay was 1.5 s. A cosine multiplication was applied in both dimensions. The delays were adjusted according to a coupling constant $^{1}J_{(CH)}$ of 149 Hz. The *g*HMBC spectrum was recorded with 200 transients over 312 increments (zero filled to 1 K) and 2 K data points with spectral widths of 5500 Hz in F2 and 23,000 Hz in F1. The recycle delay was 1.5 s. A sine multiplication was applied in both dimensions. The low-pass J-filter of the experiment was adjusted for an average coupling constant $^{1}J_{(CH)}$ of 149 Hz and the long-range delay utilized to excite the heteronuclear multiple quantum coherence was optimized for 7 Hz.

The MS spectra were obtained using ESI^{+} with a Micromass Q-Tof 2^{TM} mass spectrometer (Manchester, UK). The ESI^{+}-HRMS were obtained using a MicroTof spectrometer with Apollo II with ESI interface, using a voltage of 4500 V applied to the needle, and a counter voltage between 100 and 150 V applied to the capillary.

1,3-*O*-dilinoleoyl-2-*O*-palmitoyl glycerol (**C2**): Colourless Oil; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data in Table 3; ESI^{+}-MS *m/z*: 877 $[M + Na]^{+}$.

1-*O*-linolenoyl-2-*O*-linoleoyl-3-*O*-oleoyl glycerol (**C3**): Colourless Oil; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data in Table 3; ESI^{+}-MS *m/z*: 879 $[M + H]^{+}$.

Results and discussion

As far as we are aware, this is the first study addressing the identification and quantification of *S. stellata* hexane extract constituents. The hexane extract of *S. stellata* all plant is around 1% of the dried plant. This seems to be a lower value but it is common when the plant is previously dried and the extraction is performed at room temperature and also it is a common value obtained with other plant hexane extracts. The GC–MS analysis of the hexane extract (chromatogram in Fig. 1 given as example) allowed the identification and quantification of 43 compounds (Table 2), which mainly belong to carboxylic acids and ester derivative families. Other compounds belonging to families such as alkanes, terpenes, alcohols and sugars could also be detected and quantified as minor constituents. Naturally, the hydroxylated and carboxylated compounds were detected as TMS derivatives. The identification of each peak (Table 2) was mainly performed by comparing the retention times and mass spectra with pure standards injected in same conditions and by comparing with the GC–MS spectral library and/or with MS data found in the literature.

Chemical composition of the *S. stellata* hexane extract

The detailed analysis by GC–MS allowed the identification and quantification data (expressed as mg of compound per kg of dry plant) of the *S. stellata* hexane extract that is summarized in Table 2. The identified compounds could be distributed in five groups, representative of similar chemical families, namely carboxylic acids and esters, which represent 87.1% of all identified compounds, followed by alkanes (7.9%), terpenes (3.4%), sugars (1.3%) and alcohols (0.3%) (Table 2). To the best of our knowledge, there are no lipophilic profiles of *Scabiosa* species, only the GC–MS analysis of essential oils from *S. arenaria* (Besbes et al. 2012) and *S. flavida* Boiss. & Hausskn. (Javidnia et al. 2006) were reported. Consequently the results comparison is problematic. However, there are indications that species from *Cephalaria* genus, which is included in the same

Table 2 Identified compounds on the hexane extract of *S. stellata* aerial parts (mg of compound/kg of dried plant)

Peak	Rt (min)	RI^A	RI^B	Identified compounds[c]	MV ± SD
Carboxylic acids and esters					
3	12.99	1909	1900	Isocitric acid ($C_6H_8O_7$)[c,d,e]	95.13 ± 1.67
4	13.43	1845	–	Quinic acid ($C_7H_{12}O_6$)[c,d,e]	210.84 ± 3.20[D]
8	14.47	1878	1878	Methyl palmitate ($C_{17}H_{34}O_2$)[a,c]	46.31 ± 1.35
13	16.75	1824	1824	Methyl linolenate ($C_{19}H_{32}O_2$)[a,c]	69.31 ± 1.23
14	17.55	2100	2093	Ethyl 9,12-octadecadienoate ($C_{20}H_{36}O_2$)[c,d,e]	34.27 ± 0.50
Saturated fatty acids					
1	11.28	1592	1590	Dodecanoic acid ($C_{12}H_{24}O_2$)[c,d,e]	34.26 ± 0.81
5	13.46	1779	1788	Myristic acid ($C_{14}H_{28}O_2$)[c,d,e]	210.84 ± 3.20[D]
11	15.96	1987	1987	Palmitic acid ($C_{16}H_{32}O_2$)[b,c]	2052.38 ± 5.80
18	18.58	2183	2186	Stearic acid ($C_{18}H_{36}O_2$)[c,d,e]	287.67 ± 14.02
19	21.17	2380	2385	Eicosanoic acid ($C_{20}H_{40}O_2$)[c,d,e]	149.44 ± 4.95
24	23.90	2573	2584	Behenic acid ($C_{22}H_{44}O_2$)[c,d,e]	93.33 ± 3.46
29	27.00	2781	2782	Lignoceric acid ($C_{24}H_{48}O_2$)[c,d,e]	109.45 ± 3.21
33	30.39	2983	2981	Hexacosanoic acid ($C_{26}H_{52}O_2$)[c,d,e]	57.33 ± 0.71
42	37.42	3431	–	Triacontanoic acid ($C_{30}H_{60}O_2$)[c,d,e]	152.70 ± 6.27
Unsaturated fatty acids					
10	15.61	2205	2201	Hexadecatrienoic acid ($C_{16}H_{26}O_2$)[c,d,e]	146.67 ± 0.99
16	18.16	2202	2202	Linoleic acid ($C_{18}H_{32}O_2$)[b,c]	1874.90 ± 4.02
17	18.25	2210	2210	Linolenic acid ($C_{18}H_{30}O_2$)[c,d,e]	3391.50 ± 17.37
Total = 8805.49 mg kg^{-1}					
Ratios and indexes of fatty acids					
				ω-6/ω-3	0.55
				AI	0.55
				TI	0.23
Alkanes					
20	21.98			Pentacosane ($C_{25}H_{52}$)[a,c]	13.15 ± 0.29
25	24.86			Heptacosane ($C_{27}H_{56}$)[a,c]	40.40 ± 2.17
27	26.44			Octacosane ($C_{28}H_{58}$)[a,c]	15.90 ± 0.35
30	28.10			Nonacosane ($C_{29}H_{60}$)[a,c]	141.19 ± 7.01
32	29.80			Triacontane ($C_{30}H_{62}$)[a,c]	23.13 ± 0.74
34	31.57			Hentriacontane ($C_{31}H_{64}$)[a,c]	370.11 ± 22.54
36	33.33			Dotriacontane ($C_{32}H_{66}$)[a,c]	26.21 ± 1.20
39	35.11			Tritriacontane ($C_{33}H_{68}$)[a,c]	163.57 ± 7.34
Total = 793.66 mg kg^{-1}					
Alcohols					
12	16.40	2187	2194	Inositol ($C_6H_{12}O_6$)[b,c,d]	17.09 ± 0.36
15	17.63	2086	2086	Phytol ($C_{20}H_{40}O$)[E,c,d,e]	9.63 ± 0.27
31	28.78	3100	3103	Hexacosan-1-ol ($C_{26}H_{54}O$)[c,d,e]	3.64 ± 0.26
22	23.10	2584	2581	1-Palmitoylglycerol ($C_{32}H_{38}O_6$)[b]	1.55 ± 0.05
Total = 31.91 mg kg^{-1}					
Sugars					
2	12.77	2030	2029	D-Fructose ($C_6H_{12}O_6$)[b,c]	13.52 ± 0.16
6	13.85	2031	2037	D-Mannose ($C_6H_{12}O_6$)[b,c]	13.52 ± 0.22
7	13.94	2036	2057	D-Galactose ($C_6H_{12}O_6$)[b,c]	13.88 ± 0.28
9	14.95	2030	2037	D-Talose ($C_6H_{12}O_6$)[b,c]	14.73 ± 0.27
23	23.60	3551	3552	Sucrose ($C_{12}H_{22}O_{11}$)[b,c]	40.72 ± 1.70
26	25.76	3560	3560	3-α-Mannobiose ($C_{12}H_{22}O_{11}$)[c,d,e]	30.80 ± 0.76

Table 2 continued

Peak	Rt (min)	RI^A	RI^B	Identified compounds[c]	MV ± SD
Total = 127.17 mg kg^{-1}					
Triterpenes and steroids					
28	26.65	2910	2914	Squalene ($C_{28}H_{50}$)[c,d,e]	4.20 ± 0.17
35	32.23	3227	3226	α-Tocopherol ($C_{28}H_{48}O_2$)[b,c]	10.41 ± 0.04
37	34.02	2685	2689	Campesterol ($C_{28}H_{48}O$)[b,c]	18.38 ± 0.78
38	34.44	2796	2797	Stigmasterol ($C_{29}H_{48}O$)[b,c]	54.33 ± 1.39
40	35.52	2789	2789	β-Sitosterol ($C_{29}H_{50}O$)[b,c]	110.63 ± 0.79
41	36.73	2845	2848	Lupeol ($C_{30}H_{50}O$)[b,c]	17.16 ± 0.20
43	39.49	3679	–	Ursolic acid ($C_{30}H_{48}O_3$)[b,c]	42.83 ± 0.30
44	40.54	3650	–	Oleanolic acid ($C_{30}H_{48}O_3$)[b,c]	88.02 ± 0.79
Total = 345.96 mg kg^{-1}					

SD standard deviation, *ω-6/ω-3* ratio (total of omega 6 acids/total of omega 3 acids), *AI* atherogenicity index, *TI* thrombogenicity index

[A]Retention index relative to n-alkanes (C_5–C_{36}) on DB5. [B]NIST 14 mass spectral data retention index. [C]All the compounds possessing hydroxyl groups are identified as the correspondent TMS derivatives. [D]Although the chromatogram peaks can be identified, their quantification cannot be separated, so this amount is referred to both compounds. [E]Although this compound is a terpene, its quantity was obtained through the alcohol calibration curve. Compounds were identified by: [a]comparison with pure standards or synthesized ones, [b]comparison with pure silylated standards, [c]comparison with the GC–MS spectral libraries NIST14.lib and WILEY229.LIB, [d]comparison with spectra found in the literature, [e]interpretation of pattern fragmentation MS spectrum

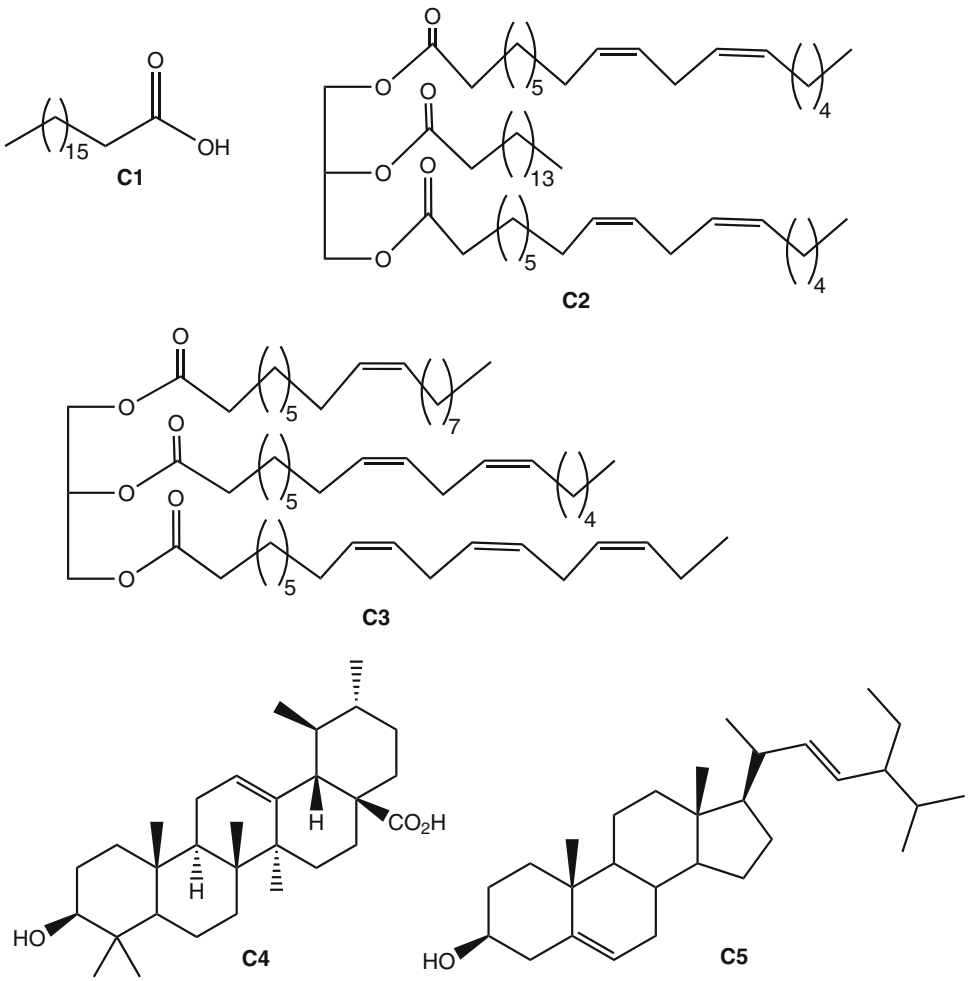


Fig. 2 Chemical structures of the compounds isolated from *S. stellata* lipophilic extract

Table 3 ^{1}H and ^{13}C NMR chemical shift values of the most significant signals for compounds **C2** and **C3**, recorded in $CDCl_3$ (500 MHz)

C^{a}_{number}	Compound C2		Compound C3	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δH (ppm)
C-1,3	62.1	4.14 (dd; *J* 5.9, 11.9 Hz)	62.1	4.13 (dd; *J* 5.9, 11.9 Hz)
		4.29 (dd; *J* 4.3, 11.9 Hz)		4.28 (dd; *J* 4.3, 11.9 Hz)
C-2	68.9	5.28–5.33 (m)	68.9	5.24–5.30 (m)
C-2′	34.19[b]	2.34 (*t*; *J* 1.7, 7.5 Hz)	34.0/34.2	2.31 (td; *J* 3.1, 16.3 Hz)
C-2″	34.03[b]	2.29 (*t*; *J* 1.8, 7.7 Hz)	–	–
C-3′	24.8	1.54–1.67 (m)	24.8/24. 9	1.55–1.63 (m)
C-5′	29.1	1.24–1.4 (m)	29.0/32.0	1.23–1.34 (m)
C-8′	27.2	1.98–2.12 (m)	27.2	2.02–2.10 (m)
C-9′/C-10′	127.1/131.3	5.34–5.40 (m)	127.1/132.0	5.28–5.42 (m)
C-11′	25.5	2.73–2.80 (m)	25.5/25.6	2.75–2.82 (m)
C=O	172.9–173.3	–	172.9–173.3	–

[a]Accordingly Fig. 3

[b]These can be interchanged

family of the *Scabiosa* genus (formerly the *Dipsacaceae* and currently *Caprifoliaceae*), present this high content in long-chain carboxylic acids (Kirmizigül et al. 2012).

A detailed analysis of the long-chain carboxylic acid amount (Table 2) shows that several saturated acids are present whereas the unsaturated ones are only three (linoleic, linolenic and hexadecatrienoic acids), being the unsaturated nearly twice (5413.07 mg kg^{-1}) the amount of the saturated ones (2902.30 mg kg^{-1}). The most abundant fatty acids are linolenic (40.8% of the total fatty acids amount), linoleic (22.5%) and palmitic (24.7%), being the first two unsaturated ones. Naturally, in these calculations, methyl palmitate and methyl linolenate (Table 2) were not considered.

It is common knowledge that polyunsaturated fatty acids are essential to human development and health. Moreover, they are being associated to decrease the risk of cancer and/or cardiovascular illnesses (Chen et al. 2013) and, due to their potential antioxidant and anti-inflammatory activities; they are also studied as potential therapeutic agents for neurological disorders (Lei et al. 2016). It should be highlighted that these results showed a very interesting ω-6/ω-3 fatty acid ratio (ω-6/ω-3 = 0.55) in the plant, since this ratio is associated with decrease of cardiovascular, cancer, inflammatory and autoimmune disease risk (Simopoulos 2008). Furthermore, the index of atherogenicity (AI) and the index of thrombogenicity (TI), respectively, AI = 0.55 and TI = 0.23, are also indicative of this plant nutritional value (Ulbricht & Southgate, 1991).

The presence of alkanes in plants is well predictable and used in chemotaxonomy (Bush & McInerney 2013), so their identification in the *S. stellata* hexane extract was expected. This group of compounds is the second more abundant (7.9%) and the C_{30}-C_{33} chain lengths represent the most abundant ones (73.4% of the alkane amount) (Table 2). Their identification was possible due to the injection in the same conditions of the standards that were used in our retention index calculations and their presence is in concordance with the fact that *S. stellata* herein study origin is Algeria and consequently subjected to the characteristic local environmental conditions (Bush and McInerney 2013).

Finally, another important group, although less representative, is the triterpenes and steroids (3.4%). The sterol content represents 53.0% of the total triterpenes and steroid amount and β-sitosterol is the most abundant one (32.0% of the total triterpenes and steroids amount). There are evidences that plant sterol intake promote health benefits mainly reducing the level of cholesterol in blood (Quílez et al. 2003), being another indication of the nutritional value of *S. stellata*. The content in triterpenic acids (ursolic and oleanolic acids) although not very high (37.8% of the terpenes amount and 1.3% of all identified compounds) (Table 2) is also noteworthy, due to the fact that their recognised biological properties (Szakiel et al. 2012), namely as antioxidant and anti-aging agents (Bourgeois et al. 2016).

Structural characterization of the isolated compounds

To confirm the identification of some compounds, the hexane extract was fractionated and each fraction purified by preparative chromatographic techniques (see experimental section), affording five compounds (Fig. 2). Compounds **C1**, **C4** and **C5** were subsequently injected in the

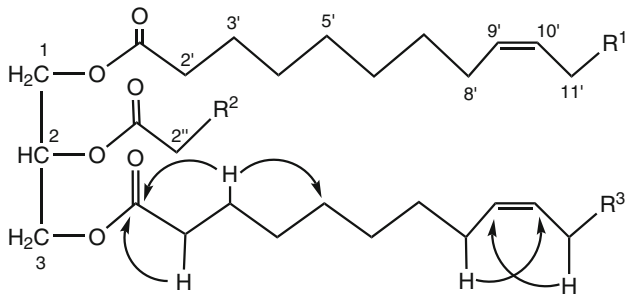


Fig. 3 Used carbon identification and main HMBC connectivities for compound **C2**

GC–MS to confirm their previous identification. These compounds, although reported herein for the first time in *Scabiosa* genus, are known and their structures were confirmed using data available in the literature, especially in the most complicated cases of ursolic acid **C4** and stigmasterol **C5** (Seebacher et al. 2003).

Less predictable was the isolation of compounds **C2** and **C3** (Fig. 2), also reported for the first time in the *Scabiosa* genus, for which the structures were proposed based on exhaustive analysis of their 1D and 2D NMR spectra (^{1}H, ^{13}C, DEPT, COSY, NOESY, HSQC e HMBC) (Table 3) and MS data (see data in Materials and methods section). Compounds **C2** and **C3** are triacylglycerol-type compounds and in both cases the mass is consistent with the proposed structures (Fig. 2). Compound **C2** is symmetrical and consequently NMR spectra are less complicated. The most important features in NMR spectra of these compounds are the carbon and proton signals of the glycerol moiety and the presence of typical carbonyl signals in the ^{13}C NMR spectra. The presence of characteristic vinyl carbon and proton signals is consistent with the presence of two *O*-linoleoyl groups (Figs. 2 and 3). In the case of compound **C3**, the NMR signals (both ^{1}H and ^{13}C) are consistent with an asymmetric structure and the presence of more vinylic systems as proposed in structure depicted in Fig. 2.

Conclusions

The present study is the first assessment of *S. stellata* less-polar constituents and allowed the identification of 43 metabolites belonging to several important families. Fatty acids are the most abundant; particularly linolenic (ω-3), linoleic (ω-6) and palmitic acids, but important sterols and triterpenic acids, such as β-sitosterol, stigmasterol, oleanolic and ursolic acids, were also found. Phytochemical investigation led to the isolation, among others, of two curious triacylglycerol-type compounds that were fully characterised using several 1D and 2D NMR experiments. Furthermore, ursolic and stearic acids, 1,3-*O*-dilinoleoyl-2-*O*-palmitoyl glycerol and 1-*O*-linolenoyl-2-*O*-linoleoyl-3-*O*-oleoyl glycerol, herein reported were found for the first time in the genus *Scabiosa*. The present research provides a comprehensive GC–MS profile of *S. stellata* and allowed to evaluate its nutritional value through important indexes such as ω-6/ω-3 fatty acids ratio, atherogenicity and thrombogenicity, and in doing so highlights its nutritional value.

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Scabiosa stellata L. Phenolic Content Clarifies Its Antioxidant Activity

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Abstract: The phenolic profile of *Scabiosa stellata* L., a species used in Moroccan traditional medicine, is disclosed. To obtain that profile the species extract was analyzed by ultra-high-performance chromatography coupled to photodiode-array detection and electrospray ionization/ion trap mass spectrometry (UHPLC-DAD-ESI/MS^n). Twenty-five phenolic compounds were identified from which isoorientin and 4-*O*-caffeoylquinic acid can be highlighted because they are the major ones. The antioxidant activity was significantly controlled by the fraction type, with the *n*-butanol fraction showing the highest antioxidant activity (FRS_{50} = 64.46 μg/mL in the DPPH assay, FRS_{50} = 27.87 μg/mL in the ABTS assay and EC_{50} = 161.11 μg/mL in the reducing power assay). A phytochemical study of the *n*-butanol fraction was performed, and some important flavone glycosides were isolated. Among them the tamarixetin derivatives—the less common ones—can be emphasized. This phytochemical study and polyphenolic profile can be correlated with *S. stellata* extracts in vitro antioxidant activity. Moreover, it can be regarded as an evidence of its medicinal use and can incentivize its consumption.

Keywords: *Scabiosa stellata* L.; phenolic profile; antioxidant activity; UHPLC-MS; NMR; flavone glycosides

1. Introduction

It has been recognized that two-thirds of the word's plant species have medicinal value. Botanical preparations used in folk medicine for multiple purposes are being increasingly studied. Furthermore, in a society concerned with health and nutrition, these natural sources are emerging as a strong alternative in pharmaceutical and nutritional fields [1–4].

The genus *Scabiosa*, mainly distributed in Southern Africa, Europe and Asia [5,6], is the most significant member of the family Caprifoliaceae and, accordingly to the Plant List data base, incorporating 72 accepted species [7], from which 11 grow wildly in Algeria and have been used in folk medicine [8]. (This genus species are currently placed in the family Caprifoliaceae; however in former publications the genus was placed in the Dipsacaceae family).

Regarding *Scabiosa stellata* L., and as far as we are aware of, only three studies involving its chemical composition were recently reported [9–11], its medicinal use in traditional medicine was also reported [12] but has not yet been validated. These previous phytochemical studies revealed the presence of important secondary metabolites, such as fatty acids and triterpenoids [9,11], among which several are new triterpenoid saponins [11]. Furthermore, new bis-iridoids and three known flavonoids

were also described [10]. Important biological activities were recently reported [10], among which the antioxidant activity of a 70% EtOH extract (In the table of the original manuscript, is indicated that the extract evaluated is a 70% MeOH. However, we think it was a mistake because in the experimental section is indicated a 70% EtOH)a fact that is important because increasing evidence has shown that antioxidant activity of plants is often related to its individual phenolic compounds [13–15]. The innovative character of studying *S. stellata*, a species whose chemical profile is not entirely established, is also relevant due to its high dissemination in the Algerian territory. With the aim of correlating the *S. stellata* phytochemicals with the antioxidant response of its extracts, a bio-guide phytochemical study was performed. In addition, as far as we know, this is the first study to report a detailed characterization in phenolic compounds of *S. stellata*, and point out to the nutritional value of this species.

2. Results and Discussion

Aiming to establish the phenolic profile of *S. stellata*, an ethanolic extract was obtained and fractioned in dichloromethane fraction (DCMF), ethyl acetate fraction (EAF) and *n*-butanol fraction (*n*-BF). Considering that this species' biological activity [10] should be more related to the phenolic content, a previous determination of fractions total phenolic content was performed as well as their antioxidant activity (Table 1). These determinations allowed confirmation that the fraction obtained with dichloromethane, although more representative than the one obtained with ethyl acetate (more than two times higher), is less rich in phenolic compounds. This low content in phenolic compounds also explains why FRS_{50} (free radical scavenge) and EC_{50} values were not found for this fraction of antioxidant assays (Table 1). The antioxidant potential of these fractions was estimated by three in vitro assays and the results are shown in Table 1. DPPH and ABTS assays measure the abilities of the fractions to scavenge free radicals while reducing power assay evaluate their ability to reduce Fe^{3+} to Fe^{2+}, in all cases it is recognized that the phenolic compounds are responsible for these antioxidants abilities [16]. Therefore, the gathered data allowed inferring some general conclusions regarding the antioxidant activity of each fraction. The DCMF has no significant activity, which is in accordance with its low content in phenolic compounds. The *n*-BF is the more active one in all the assays, which is also in harmony with its higher content in phenolic compounds. However, the overall results are considerably higher than the used references it seems that *S. stellata* possess antioxidant metabolites that can be further evaluated. Concerning antioxidant activity of this species, the only data reported so far involves the DPPH assay [10]. The herein reported results are similar to the previous ones, moreover the two extra assays herein performed also corroborate that antioxidant active metabolites can be produced by this species. Furthermore, there is evidence that other *Scabiosa* species extracts have antioxidant active [17,18]. These bioassays guide us to perform the phytochemical study of the EAF and *n*-BF fractions. The total phenolic content of both fractions (Table 1) explains the fact that more phenolic compounds were isolated from the *n*-BF fraction (Figure 1 and experimental section).

Table 1. Extraction yields and antioxidant capacity of *S. stellata* ethanolic extract fractions.

Fraction	Mass [a]	Total Phenolic Content [b]	DPPH (FRS_{50}) [c]	ABTS Assay (FRS_{50}) [c]	Reducing Power (EC_{50}) [c]
DCMF	12.3	<1.00	>250	>250	>50
EAF	5.3	4.74 ± 0.01 *	71.82 ± 0.04 *	40.41 ± 0.02 *	202.41 ± 0.10 *
n-BF	51.7	11.86 ± 0.05 *	64.46 ± 0.01 *	27.87 ± 0.01 *,#	161.11 ± 0.08 *,#
Reference	-	-	8.21 ± 0.03 [d]	12.07 ± 0.04 [e]	18.03 ± 0.01 [f]

Table Data represent the mean values ± SD of three independent assays performed in triplicate ($n = 3$). [a] % of dry weight. [b] mg GA/g DF. [c] μg/mL. [d] Reference used was ascorbic acid. [e] Reference used was trolox. [f] Reference used was BHA. * Statistically significant different with respect to the reference (Tukey's test), $p < 0.05$. # Statistically significant different with respect to EAF (unpaired Student's *t*-test), $p < 0.05$.

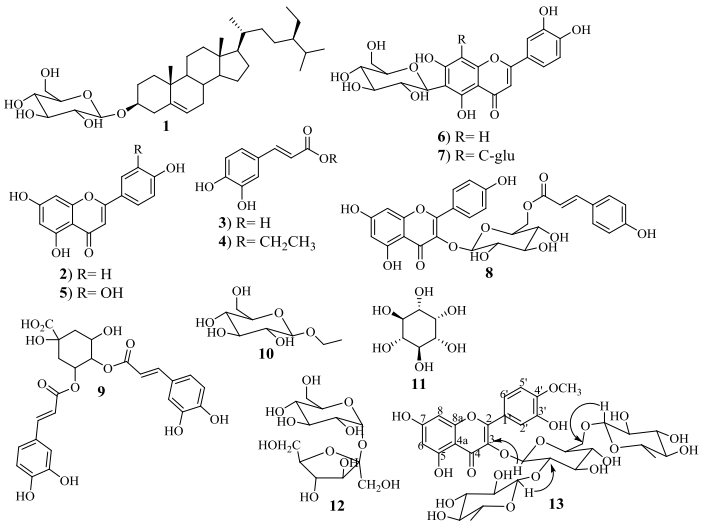

Figure 1. Structures of isolated compounds: β-sitosterol-β-D-glucoside **1**, apiginin **2**, caffeic acid **3**, ethyl caffeate **4**, luteolin **5**, isoorientin **6**, lucenin 2 **7**, tiliroside **8**, 4,5-*O*-dicaffeoylquinic acid **9**, 1-*O*-ethyl-β-D-glucoside **10**, myo-inositol **11**, β-D-fructofuranosyl-(2→1)-α-D-glucoside **12** and tamarixetin 3-β-L-rhamnosyl-(1→2)[β-L-rhamnosyl-(1→6)]β-D-glucoside] **13**.

Another significant result that can be noticed from the data in Table 1 is the fact that these solvents only extracted 69.3% of the original ethanolic extract. To obtain a more accurate phenolic profile of *S. stellata*, the ethanolic extract was screened by UHPLC-DAD-ESI/MS^n analysis and the UV chromatogram, recorded at 305 nm (Figure 2). A careful analysis of the chromatogram revealed two major peaks eluted at 6.66 and 10.14 min and several other minor peaks (Figure 2). Twenty-five phenolic compounds could be identified and, from those, nine are chlorogenic acid derivatives and thirteen are flavonoid derivatives (data of the retention time, maximum wavelength, molecular ions species and fragments are presented in Table 2), and represent, respectively, 30% and 56% of the total phenolic amount. The fact that these types of compounds are recognized as antioxidants [16,19] is consistent with the recently reported [10] and above-mentioned antioxidant activity.

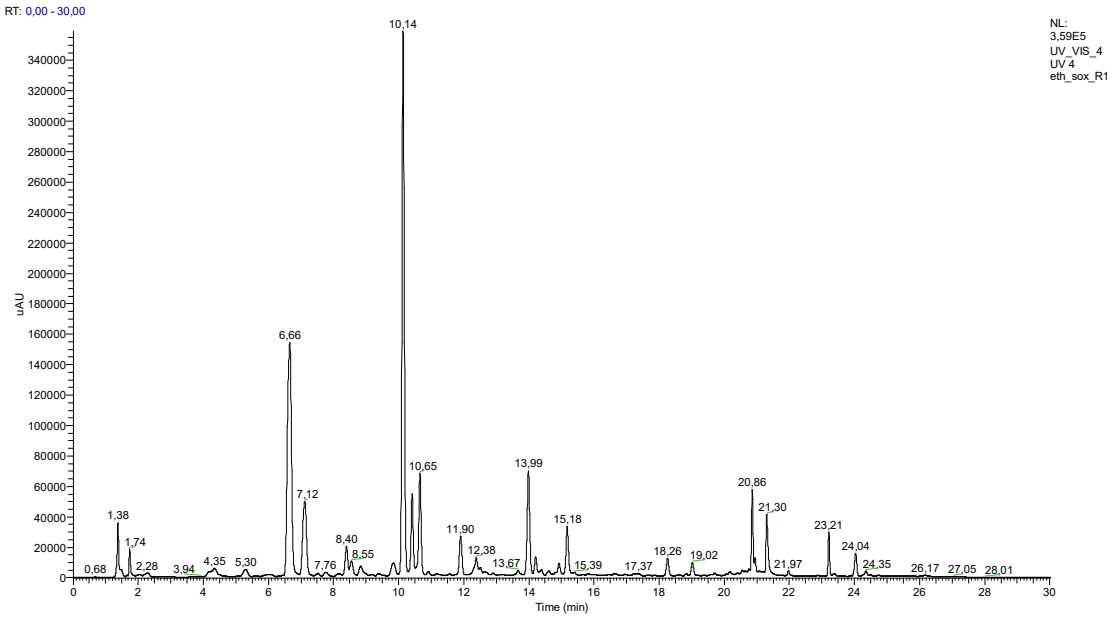

Figure 2. UHPLC chromatogram of *S. stellata* ethanolic extract recorded at 305 nm.

Table 2. Identification of UHPLC/DAD/ESI-MS^n data, and quantification of the most relevant compounds from the ethanolic extract of *S. stellata* (Retention time (Rt), wavelength of maximum absorption in the UV-Vis region (λmax), pseudomolecular and MS^n fragment ions, quantification (mean ± SD) and identification of the phenolic compounds).

Rt (min)	λmax	$[M-H]^-$ (*m*/*z*) ♦	ESI-MS^2; (MS^3) (*m*/*z*) ♠	Quantity ♣	Compound
1.38	191, 267	387	341, 369; (179, 143, 161)	8.24 ± 0.03	1-Caffeoylglucose derivative [b]
1.74	193, 202	128	85, 109	4.30 ± 0.02	Cyanuric acid [a]
4.35	204, 324	353	191, 179, 135; (173, 127, 109)	nq	1-*O*-Caffeoylquinic acid [c]
5.30	211, 278, 323	223	205, 115, 143, 159	0.26 ± 0.01	Sinapic acid [a]
6.66	217, 298, 325	353	191, 179, 173, 135; (111, 93)	26.41 ± 0.30	4-*O*-Caffeoylquinic acid [c]
7.12	216, 299, 325	353	191, 179; (173, 127, 85)	8.93 ± 0.12	3-*O*-Caffeoylquinic acid [a]
8.40	206, 269, 348	609	489, 447, (357, 327, 285)	1.84 ± 0.03	Luteolin-6-*C*-glucoside-7-*O*-glucoside [b]
8.55	199, 214, 270, 304	353	191, 179, 135; (173, 127, 85)	1.47 ± 0.01	5-*O*-Caffeoylquinic acid [c]
8.83	220, 274, 310	337	191, 173; (127, 110, 93)	tr	5-*O*-*p*-Coumaroylquinic acid [c]
9.83	230, 326	367	191; (173, 85)	0.97 ± 0.02	5-*O*-Feruloylquinic acid [c]
10.14	209, 269, 350	447	429, 357, 327; (309, 297, 285)	66.31 ± 0.30	Isoorientin (luteolin-6-*C*-glucoside) [a]
10.40	211, 269, 350	579	561, 447, 357, 327; (309, 297, 285)	9.78 ± 0.26	Luteolin-2″-*O*-pentosyl-6-*C*-hexoside [b]
10.65	211, 270, 346	461	371, 341, 313; (299, 231)	13.97 ± 0.11	Diosmetin-6(or 8)-*C*-glucoside [b]
11.90	225, 270, 338	563	443, 431; (311, 283, 269)	2.82 ± 0.01	Apigenin-2″-*O*-pentosyl-8-*C*-glucoside [b]
12.38	232, 256, 353	463	301; (268, 179, 151)	0.97 ± 0.04	Quercetin-3-*O*-glucoside (hyperoside) [b]
13.99	220, 241, 327	515	353; (191, 173)	16.03 ± 0.03	4,5-*O*-Dicaffeoylquinic acid [a]
14.21	237, 267, 337	609	489, 369; (298, 285, 231)	1.23 ± 0.01	Lucenin 2 (luteolin-6,8-di-*C*-glucoside) [b]
14.38	242, 326	515	353, 335; (173,111)	tr	3,4-*O*-Dicaffeoylquinic acid [c]
14.93	240, 268, 314	639	616, 315	tr	Tamarixetin-*O*,*O*-dihexoside [b]
15.18	242, 326	515	353; (191, 171, 127)	3.74 ± 0.02	3,5-O-Dicaffeoylquinic acid [c]
18.26	239, 270, 351	613	489, 447, 429; (369, 309, 285)	0.43 ± 0.02	Luteolin-6-*C*-glucoside derivative [b]
19.02	243, 267, 314	593	447, 285	0.37±0.02	Tiliroside [b]
20.86	237, 267, 314	635	477, 315	14.49 ± 0.02	Tamarixetin derivative [b]
20.94	237, 267, 313	769	623, 477, 315	nq	Tamarixetin glycoside [a]
21.30	243, 269, 313	739	593, 447, 285	10.85 ± 0.01	Kaempferol-3-*O*-rutinoside derivative [b]
				57.55 ± 0.11 ♥	Total chlorogenic acids
				108.20 ± 0.17 ♥	Total flavonoids

♦ molecular ion; ♠ main fragments; ♣ mg of compound/g dried extract; nq—not quantified; tr—traces; ♥ obtained by propagation. Compounds were identified by [a] comparison with pure standards, commercially available or isolated; [b] comparison with pure aglycone and literature data; [c] comparison with pure cinnamic acid derivative and literature data.

The nine chlorogenic acids herein identified as constituents of the *S. stellata* ethanolic extract were mainly identified through their pseudomolecular ions ($[M-H]^-$) and MS^n fragments. In the case of 4,5-*O*-dicaffeoylquinic acid (isochlorogenic acid C) **9** (Figure 1; Table 2 Rt = 13.99 min), which was isolated and characterized, the confirmation was also obtained by the injection of the pure compound. In fact, the literature is rich in these acids' MS data due to their ubiquitous occurrence in plants [15,20–29] and consequently, peaks at 4.35, 6.66, 7.12 and 8.55 min with $[M-H]^-$ at *m*/*z* 353 were identified as 1-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 3-*O*-caffeoylquinic and 5-*O*-caffeoylquinic acids, respectively. The 4-*O*-caffeoylquinic acid is easily distinguished from the others due to its characteristic MS^2 base peak at *m*/*z* 173, whereas in the other derivatives the base peak in MS^2 is at *m*/*z* 191 (Table 2). On the other hand, 3-*O*-caffeoylquinic acid could be assigned to be the peak at 7.12 min due to the absence of the fragment ion at *m*/*z* 135 in the MS^2 spectra characteristic in the others caffeoylquinic acids (Table 2). The other two monosubstituted quinic acids, peaks at 8.83 and 9.83 min, were identified respectively as 5-*O*-*p*-coumaroylquinic acid and 5-*O*-feruloylquinic acid due to their $[M-H]^-$ at *m*/*z* 337 and *m*/*z* 367 (Table 2). Furthermore, the base peak in MS^2 at *m*/*z* 191 allowed us to distinguish the 5-*O*-*p*-coumaroylquinic acid from the other possible isomers [22]. The analysis of the MS^3 spectra allowed other assignments and confirmation of the abovementioned data.

Disubstituted quinic acids were another type of chlorogenic acids found in the extract, and correspond to the peaks at 13.99, 14.38 and 15.18 min, all with $[M - H]^-$ at m/z 515 (Table 2). Peaks at 14.38 and 15.18 min could be assigned, respectively to 3,4-*O*-dicaffeoylquinic and 3,5-*O*-dicaffeoylquinic acids, due to the base peak in MS^3 spectra (respectively m/z 173 and m/z 191). In the case of peak at 13.99 min the phytochemical study of the extract allowed the isolation and full characterization of 4,5-*O*-dicaffeoylquinic acid **9** (Figure 1), which could be used as standard and confirm the identification of this peak. It is interesting to notice that *S. stellata* areal parts are rich in these important metabolites [19], particularly in 4-*O*-caffeoylquinic acid, which accounts for 46% of the total chlorogenic acids content (Table 2). Most of these compounds are herein reported for the first time in this species and the earlier reported presence of 3,5-*O*-dicaffeoylquinic acids and 4,5-*O*-dicaffeoylquinic acid [10] is also confirmed.

The phytochemical study allowed the isolation of caffeic acid and its derivatives as well as several glucosides (Figure 1), confirming the presence of other caffeic acid derivatives in the extract. Some of the isolated compounds were not detected by UHPLC-DAD-ESI/MS^n, from which compound **1** (Figure 1) should be highlighted. The compound was afterwards injected, and it could be detected at another wave length (250 nm) but not the one used to perform the analysis herein discussed. The same was verified in the case of the ethyl derivatives isolated (compounds **4** and **10**, Figure 1). Additionally, we cannot confirm that they are natural derivatives; in fact, they can be formed during the extraction with ethanol.

The ethanolic extract profile of *S. stellata* shows the presence of another important family of secondary metabolites, the flavonoids, for which literature is also rich in MS data [24,25,30–36] and the presence of isoorientin, hyperoside and swertiajaponin in *S. stellata* extract was recently reported [10]. The detailed analysis of the characteristic MS^n fragment ions (Table 2), as well as the phytochemical study, allowed the identification of several flavonoid derivatives, which in fact represent 56% of the total phenolic amount. The previous reported isoorientin and hyperoside [10] were also found together with several others that are herein reported for the first time in this species. The main aglycones found are flavone (apigenin, diosmetin and luteolin) and flavanol (kaempferol, quercetin and tamarixetin) types. Most of the identified compounds are known; in fact, they are secondary metabolites ubiquitous in nature. Moreover, the results herein reported are identical to the ones reported in the literature [24,32–35]. In all cases the ion fragment with m/z value of the key aglycone is the base peak of MS^2 or MS^3 (Table 2), making easier their identification. Luteolin glycosides are the major constituents and account for 74% of the total flavonoid content (Table 2). Moreover, luteolin-6-*C*-glucoside, which elutes at 10.14 min is the major constituent of the *S. stellata* ethanolic extract (34%).

Tamarixetin glycosides are less reported in the literature and in the *S. stellata* extracts, as far as we are aware, this is the first report on its occurrence. Three derivatives were detected, the peaks eluted at 14.93, 20.86 and 20.94 min. The peak eluted at 14.93 min correspond to extremely small amount of tamarixetin-*O*,*O*-dihexoside, the $[M - H]^-$ at m/z 639 and the base peak at m/z 315 in MS^2 suggest it. The peak eluted at 20.86 has similar absorption in the UV-Vis region and the base peak at m/z 315 in MS^2 confirming that it should be a tamarixetin derivative (Table 2) and in this case in a considerable amount (nearly 7%). A careful analysis of the chromatogram (Figure 2) allowed detection of the peak eluted at 20.94 min with $[M - H]^-$ at m/z 769 and the base peak at m/z 315 in MS^2; due to its proximity with the peak at 20.86 min, the quantification was not possible (Table 2). However, the similarity of this peak data and the above-mentioned ones suggests that it is also a tamarixetin derivative. The phytochemical study allowed the isolation and characterization of several flavonoids and their glycosides (Figure 1) which confirmed the above-discussed identification. In the case of the peak eluted at 20.94 min, the confirmation was also established because this tamarixetin glycoside **13** (Figure 1) was isolated and fully characterized. At first glance, this compound seems to be an unusual tamarixetin derivative, whose occurrence in nature was recently reported and not in the *Scabiosa* genus [37]. Therefore, to confirm the identification its characterization was meticulous and compared

with the previous reported data and accordingly the compound was identified as being the tamarixetin 3-β-L-rhamnosyl-(1→2)β-L-rhamnosyl-(1→6)]β-D-glucoside] **13** (Figure 1). First, the aglycone nucleus was assigned due to the characteristic signals in the aromatic region at δ 6.21 and 6.41 ppm, correspond, respectively to the resonance of protons H-6 and H-8. The signal shape, doublets with a coupling constant J = 2.1 Hz (characteristic of a *meta* position coupling), is consistent with the tamarixetin substitution pattern (Figure 1). Moreover, the ring B substitution pattern was established due to the singlet at δ 3.98 ppm confirming the presence of a methoxy group, the two doublets at δ 7.96 ppm (J = 2.1 Hz) and δ 6.94 ppm (J = 8.4 Hz) respectively to the resonance of protons H-2′ and H-5′ and a double doublet at δ 7.60 ppm (J = 8.4 and 2.1 Hz) assigned to H-6′, which confirms its *ortho*-coupling with H-5′ and meta-coupling with H-2′.

The presence of the ^{13}C-NMR characteristic signals (Table S1) also proves that the aglycone is tamarixetin; moreover, these results are in accordance with the previous data [37]. The glycoside residue was also established, with the presence of three doublets at δ 5.75, 5.21 and 4.56 ppm, characteristic signals of anomeric protons, indicative of the three hexoses. The two doublets at δ 1.08 and 0.93 ppm, typical of methyl groups, confirm that two are rhamnoses and the signal at δ 5.75 ppm confirms the presence of glucose. In the HMBC can be noticed, among others, the correlation between the anomeric proton of the glucose unit and the tamarixetin carbon C-3, the correlation between the anomeric protons of one of the rhamnose unit with the glucose carbon C-6 and the other with the glucose carbon C-2 (Figure 1). The obtained data confirm not only the glycosylic residue but also its linkage to the aglycone moiety as depicted in Figure 1.

Tiliroside **8** (Figure 1) is another important flavonoid found for the first time in this genus. It is a kaempferol derivative, the kaempferol 3-*O*-β-D-glucopyranoside-6-*p*-coumaryl ester, for which biological significance was reported [38], and was isolated but also found in the UHPLC-MS profile. It is the peak eluted at 19.02 min (Table 2).

3. Materials and Methods

3.1. Chemicals

Pure compounds were used as standards to elucidate the identification of the phytochemicals and to elaborate the calibration curves following the external standard method. Moreover, the isolated compounds were also used as standards. The phenolic standards benzoic acid, cyanuric acid, 4-hydroxy-3-methoxybenxoic acid, gallic acid, sinapic acid, catechin, rosmarinic acid, isorhamnetin, kaempferol, luteolin, diosmetin and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The phenolic standards 3,4-dihydroxycinnamic acid (caffeic acid) and 4-hydroxycinnamic acid (p-coumaric acid) were purchased from Acros Organics (Geel, Belgium). The phenolic standards ferulic acid and chlorogenic acid were purchased from Extrasynthese (Genay Cedex, France). Solvents were purchased from Panreac and Acros Organics and were of HPLC purity, analytical grade, or bi-distilled commercial solvents. Chromatographic purifications were performed using silica gel 60 (70–230 mesh, Merck Kieselgel, Kenilworth, NJ, USA), Sephadex LH-20 and Merck silica gel 60 GF254. Iron(II) sulphate, potassium hexacyanoferrate(III), iron(III) chloride, butylated hydroxyanisole (BHA), ascorbic acid, Folin & Ciocalteu's phenol reagent, trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution (ABTS solution) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Plant Collection and Extract Preparation

The whole plant of *Scabiosa stellata* L. was collected in June 2015 from Batna [the specimens of *S. stellata* were collected in the Belezma National Park (Batna, Algeria; 35°35′41.52″ N, 5°56′13.75″ E)]. A voucher specimen was identified by Dr. Bachir Oudjehih professor of Agronomic Institute, University of Batna under the reference number VAREN/SS/2013/123.

Dried-air powdered of *S. stellata* all plant (500 g) was macerated firstly with *n*-hexane to eliminate fatty acids and other lipophilic components, then was extracted with ethanol (6 L, 2 days cycles and three times) using sohxlet. The mixture was filtered, and the combined extracts were concentrated under vacuum giving 88.6 g. Then, the extract was sequential dissolved by different solvents (increasing polarities). After filtration and evaporation 10.9 g of dichloromethane fraction (DCMF), 4.7 g of ethyl acetate fraction (EAF) and 45.8 g of *n*-butanol fraction (*n*-BF) were obtained.

3.3. UHPLC-DAD-ESI/MS^n

For the UHPLC-MS analysis, 50 mg of each extract were dissolved in 5 mL of methanol (final concentration 10 mg/mL) and the resulting solutions were filtered through a 0.2 mL nylon membrane (Whatman, Maidstone, UK). Three independent analyses were carried out for reproducibility. This technique was performed using a Thermo Scientific Ultimate 3000RSLC (Dionex, Sunnyvale, CA, USA) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer. The column used was a Thermo Scientific hypersil gold column (Part n° 25002-102130; Dim 100 mm × 2.1 mm; Lot 14913; SN 10518298) with a part size of 1.9 µm and its temperature was maintained at 30 °C. The mobile phase was composed of (B) acetonitrile and (A) 0.1% formic acid in water (*v*/*v*), both degassed and filtered before use. The flow rate was 0.2 mL/min. The elution gradient was 5% (solvent A) for 14 min, 40% (solvent A) over 2 min, 100% (solvent A) over 7 min and the re-equilibration of the column with 5% of solvent A for 10 min. The injection volume was 2 µL. UV-vis spectral data were gathered in a range of 250 to 500 nm and the chromatographic profiles were documented at 280 nm. The mass spectrometer used was an LTQ XL linear ion trap 2D equipped with an orthogonal electrospray ion source (ESI). The equipment was operated in negative-ion mode with electrospray ionization source of 5.00 kV and ESI capillarity temperature of 275 °C. The full scan covered a mass range of 50 to 2000 *m*/*z*. Collision-induced dissociation MS/MS and MS^n experiments were simultaneously acquired for precursor ions.

3.4. Phytochemical Analysis

EAF was separated over column chromatography using silica gel and a gradient elution starting with hexane/ethyl acetate (100:0 to 0:100) and followed by ethyl acetate/methanol (100:0 to 0:100). Several fractions were obtained and compound **1** (Figure 1) was isolated pure. Compound **2** (Figure 1) was isolated from the less polar fractions by thin-layer chromatography and using hexane/ethyl acetate (85:15) as eluent. More polar fractions were purified by column chromatography using sephadex LH20 and eluted with methanol and allowed the isolation of compounds **3** and **4** (Figure 1). The detailed scheme is available in Supplementary Material (Figure S1).

n-BF was separated over column chromatography using silica gel and a gradient elution with chloroform/methanol (100:0 to 50:50). Twenty-five fractions were obtained and from their purification several other phenolic compounds were obtained. For example, from the first four fractions and by purification with column chromatography using sephadex LH20 and eluted with chloroform/methanol mixtures were obtained the pure compounds **5**, **6**, **7** and **8** (Figure 1). From fraction twelve was obtained after column chromatography using silica gel and eluting with a gradient of dichloromethane/methanol (100:0 to 0:100) compound **9** (Figure 1). From fraction fourteen and using a sephadex LH20 column chromatography was obtained compound **10** (Figure 1). Fractions eleven and fifteen allowed respectively the isolation of compound **11** and **12** (Figure 1). Finally, fraction twenty gave the pure compound **13** (Figure 1). The detailed scheme is available in Supplementary Material (Figure S2).

3.5. Identification and Quantification of the Phenolic Compounds

The quantification of the total phenolic content was carried out through Folin-Ciocalteu method [15] with some modifications. In a 96 well-microplate, 15 µL of a solution of each fraction was added to 60 µL of milliQ water and 15 µL of Folin-Ciocalteu reagent. After 5 min, 150 µL of a 7%

Na_2CO_3 solution was added and the mixture was homogenized and incubated in the dark at 30 °C for 60 min. The absorbance was measured at 700 nm and the total phenolic content was expressed as a function of a linear calibration curve performed by a standard (gallic acid at different concentrations, 0.001 to 0.01 µg/mL). These contents are expressed in mg of gallic acid (GA) per gram of dry plant (DF) extract (mg GA.g^{-1} DP) and presented in Table 2.

The identification of individual phenolic compounds in the UHPLC analysis was achieved by comparison of their retention times, UV-Vis spectra, and MS^n spectra data with those of the closest available reference standards and data reported in the literature. In addition, the structure of some phenolic components was further confirmed by NMR analysis after their purification. The NMR spectra {^{1}H, ^{13}C, HSQC, HMBC [71 ms (7 Hz)], COSY} were measured in $CDCl_3$, on a Bruker Avance 300 (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C) or Bruker Avance 500 with crioprobe (500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C) spectrometers and using TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (*J*) in Hz.

The semi quantification of the main individual phenolic compounds in the extract was performed by peak integration at 260 nm, through the external standard method, using the closest reference compounds available. The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves represented in Table S2 (LOD = 3 standard deviation/slope and LOQ = 10 standard deviation/slope). The calibration curves were obtained by injection of five known concentrations with variable ranges (Table S2) and the concentrations of the standards were chosen to guarantee the quantification of each compound in the samples by intrapolation in the calibration curve. Values of correlation coefficients confirmed linearity of the calibration plots (Table S2). The results were expressed in mg of compound/g of dried extract, as mean ± standard deviation of four independent analyses.

3.6. Evaluation of Antioxidant Activity

3.6.1. DPPH Radical-Scavenging Assay

The radical-scavenging activity was carried out following a previously reported procedure [39], with some modifications. The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the fractions (50 µL) and 250 µL of a methanolic solution containing DPPH (0.2 mM). The microplates were then placed in the dark and at room temperature for 30 min. The absorbance was measured at 517 nm using a microplate reader (model EAR 400, Labsystems Multiksan MS) with reference to a control without extract. The radical-scavenging activity was calculated as a percentage of DPPH discoloration using the equation: DPPH scavenging effect % = $[(A0 - A1)/A0)] \times 100$ where A0 is the absorbance of the control reaction and A1 is the absorbance of the test fraction. Based on graphic values of the inhibition percentage of DPPH vs. fraction concentration, the FRS_{50} of each extract was estimated (Table 2). Ascorbic acid was used as the reference.

3.6.2. ABTS Assay

The ABTS assay was carried out following a previously reported procedure [40], with some modifications. A volume of 250 µL of ABTS solution (7 mM) was mixed with 50 µL of the plant extract fractions at various concentrations. The reaction mixture was left in the dark during 20 min, and its absorbance was recorded at 734 nm. As for the antiradical activity, ABTS scavenging ability was expressed as FRS_{50} (µg/mL) as shown in Table 2. Trolox was used as the reference.

3.6.3. Reducing Power

The reducing power was determined according to a method described before [39]. This method consists to mixing 200 µL of the fraction at different concentrations with 200 µL of phosphate buffer (0.2 mM, pH 6.6) and 200 µL of a solution of $K_3Fe(CN)_6$ (1%). The obtained mixture was incubated for 20 min at 50 °C. Then 200 µL of TCA (10%) was added followed by vigorous stirring. Thereafter, 75 µL

of each solution obtained was put in a well of the microplate and 75 µL of distilled water and 15 µL of $FeCl_3$ (0.1%) were adjoined. Absorption reading was done at 690 nm (white is the extraction buffer). Based on the study of the change in absorbance as a function of the sample concentration, the results obtained to calculate the effective concentration (EC_{50}, mg/mL; Table 2) which is the concentration of the corresponding sample at an absorbance of 0.5. BHA was used as the reference.

3.7. Statistical Analysis

Results were expressed as mean ± standard deviation of three independent assays and analyzed through unpaired Student's test or ANOVA combined with Tukey's test (Graph Pad Prism 5). P values of less than 5% ($p < 0.05$) were considered to be significant.

4. Conclusions

Using UHPLC-DAD-ESI/MS^n, the knowledge of the *S. stellata* phenolic profile was successfully extended, for the first time establishing its content in important secondary metabolites, such as chlorogenic acids and flavonoids. Chlorogenic acids and flavonoids comprised more than 80% of the compounds found, which explains the recently reported antioxidant activity of this plant extract. Among the phenolic compounds herein reported, some for the first time in both the genus and species, both luteolin-6-*C*-glucoside and 4-*O*-caffeoylquinic acid can be highlighted because they are the major compounds. However, tamarixetin derivatives can also be emphasized, due to their detection for the first time in *Scabiosa* genus, in particular the tamarixetin 3-β-L-rhamnosyl-(1→2)[β-L-rhamnosyl-(1→6)]β-D-glucoside] whose structure confirmation requires more experiments. Furthermore, the presence of tiliroside also should be highlighted due to its importance in other plants. Therefore, the characterization herein reported will provide information about the *S. stellata* benefits to individuals and may contribute to increase its consumption, through infusions and/or condiments.

Supplementary Materials: Table S1: Selection of the most significant chemical shift values found in the ^{1}H- and ^{13}C-NMR spectra (recorded in MeOH and 500 MHz) of the compound **13**, Table S2: Linearity (y = mx + b, where y corresponds to the standard peak area and x corresponds to the mass of standard), LOD and LOQ of pure compounds used as reference.

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Sample Availability: Not available.

Abstract

This present work concerns the phytochemical and biological study of two Algerian plants *Scabiosa stelatta.*L and *Sedum carealeum vahl*. Chromatographic separations and purifications of these plants led to the isolation and identification of 18(*S. Stellata)* and 7 compounds(S. *carealeum)*. data.GC/MS analyses of hexane extract of *S. stellata* allowed the identification of 43 metabolites belonging to several important families, fatty acids and their derivatives (87%) are the major ones, wherever 19 metabolites were identified and alkenes (60%) is the major family followed by 30% of fatty acids in hexane extract. *S. stellata* showed a good activity antioxidant with: DPPH, ABTS and Reducing Power tests. Compared with the other tested extracts, the n-butanol extract showed the highest activity. Using UHPLC-DAD-ESI/MS^n, the knowledge of the *S. stellata* phenolic profile was successfully extended, for the first time establishing its content in important secondary metabolites, such as chlorogenic acids and flavonoids which comprised more than 80% of the compounds found, which explains the recently reported antioxidant activity of this plant extracts.

Key words: fatty acids, GC-MS, LC-MS, phenolic profile, Scabiosa and Sedum.

Résumé

Ce travail concerne l'étude phytochimique et biologique de deux plantes algériennes: *Scabiosa stelatta.*L et *Sedum carealeum* vahl. Les séparations chromatographiques et les purifications des extraits de ces plantes ont permis d'isoler et d'identifier 18 (S. stellata) et 7 composés (S. carealeum).Les analyses CG / SM de l'extrait d'hexane S. stellata ont permis l'identification de 43 métabolites appartenant à plusieurs familles importantes, les acides gras et leurs dérivés (87%) sont les principaux , 19 métabolites ont été identifiés : les alcènes (60%) suivi par 30% d'acides gras dans l'extrait d'hexane de la deuxieme plante. *S. stellata* a montré une bonne activité antioxydante par les testes :DPPH, ABTS et FRAP. Comparé aux autres extraits testés, l'extrait de n-butanol a montré l'activité la plus élevée. En utilisant UHPLC-DAD-ESI / MS^n, la connaissance du profil phénolique de *S. stellata* a été étendue avec succès, en établissant pour la première fois son contenu dans des métabolites secondaires importants, tels que les acides chlorogéniques et les flavonoïdes. Les acides chlorogéniques et les flavonoïdes constituaient plus de 80% des composés trouvés, ce qui explique l'activité antioxydante récemment rapportée de cet extrait végétal.

Mots clés: acides gras, GC-MS, LC-MS, profil phénolique, Scabiosa et Sedum.

ملخـــــــــــــــص

يتعلق هذا العمل بالدراسة النباتية والبيولوجية للنباتين الجزائريين Scabiosa stelatta.L وSedum carealeum العزل الكرومتوغرافي لمستخلصات هذان النباتين أدت الى عزل (*S.stellata*)18 و (*S.carealuem*)7 مركبات . تم تحديد البنية الجزئية للمركبات المعزولة بالاعتماد على تقنية NMR 1D , NMR 2D, بينت التحاليل باستخدام GC-MS على مستخلص الهكسان للنوع *S.stellata* بتحديد 43 مركب تنتمي إلى عدة عائلات مهمة من بينها الأحماض الدهنية ومشتقاتها (87 ٪) بينما تم تحديد 19 مركب و الاكنات %60) هي العائلة الابرز تليها 30% من الاحماض الدهنية من مستخلص الهكسان الخاص بنبة S.caealeum , اظهرت نبتة *s.stellata* نشاط جيد مضاد للاكسدة ياستخذام طرق DPPH , ABTS و FRAP و بالمقارنة مع المستخلصات الاخرى المدروسة اظهر مستخلص *n*-butanol اعلى نشاط , باستخدام UHPLC-DAD-ESI / MS^n تم توسيع معرفة المحتوى الفينولي*ل S.stellata* و لاول مرة, و قد كشفت الدراسة على احتواءها على مركبات ثانوية مهمة، مثل أحماض الكلوروجينيك وفلافونيدات و هي تمثل أكثر من 80٪ من المركبات الموجودة ، وهذا ما يفسر النشاط المضاد للأكسدة الذي تم الاقرارعنه مؤخرًا لهذا المستخلص النباتي.

الكلمات المفتاحية: الأحماض الدهنية ، GC-MS، LC-MS، المحتوى الفينولي ، Scabiosa و Sedum

Abstract

This present work concerns the phytochemical and biological study of two Algerian plants *Scabiosa stelatta.*L and *Sedum carealeum vahl*. Chromatographic separations and purifications of these plants led to the isolation and identification of 18(*S. stellata)* and 3 compounds(S. *carealeum)*. data.GC/MS analyses of hexane extract *S. stellata* allowed the identification of 43 metabolites belonging to several important families, fatty acids and their derivatives (87%) are the major ones, wherever 19 metabolites were identified and alkenes (60%) is the major family followed by 30% of fatty acids in hexane extract of second plant. *S. stellata* showed a good activity antioxidant with: DPPH, ABTS and Reducing Power tests. Compared with the other tested extracts, the n-butanol extract showed the highest activity. Using UHPLC-DAD-ESI/MS^n, the knowledge of the *S. stellata* phenolic profile was successfully extended, for the first time establishing its content in important secondary metabolites, such as chlorogenic acids and flavonoids. Chlorogenic acids and flavonoids comprised more than 80% of the compounds found, which explains the recently reported antioxidant activity of this plant extract.

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