Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(5):169-176

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Characterization of Some Flavonoids in *Phoenix dactylifera L* Using HPLC-MS-MS

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Abstract

The spathes of Phoenix dactylifera L (aerial parts at flowering Stage) are used in traditional medicine and are also exploited commercially as herbal drugs for the treatment of diabetes mellitus. the present study investigates the chemical composition of the spathes. Method of coupling high-performance liquid chromatography (HPLC) with electrospray ionization mass spectrometry (MS) was optimized for the separation and identification of flavonoid. Fragmentation behavior of flavonoid was investigated using The MS, MS^n and UV data together with HPLC retention time (Rt) of flavonoids allowed structural. Spectral data for all peaks were recorded in the range of 200-700 nm. Some flavonoids including three quercetin derivatives (peak8;9;10 of retention time 15.1, 16.2 and 16.8 respectively), one orient in derivative(peak 12 of Rt 17.6 min) and one flavanone derivative (peaks 13 of Rt 18.2 min) were identified in negative and positive modes using full scan mass measurements and MS^n fragmentations. All flavonoid derivatives former indicated in flavonic extract of diethyl ether.

Key words: *Phoenix dactylifera L*, Spaths, flavonoids, HPLC-MS/MS analysis.

INTRODUCTION

In recent years, research efforts are under-way on the possibilities of utilization of natural source of bioactive compounds for the dietary management of certain chronic diseases such as diabetes, obesity, cardiovascular diseases, cancer etc.[01]

Recently, herbal medicines have received great interest and have been used as an important part of health care in the anti-viral treatment field since they have relatively few side-effects compared to modern therapeutics [1,2]

Date fruits are rich in phenolic compounds possessing antioxidant activity. The pollen grains of date palm have been used in Egyptian local and in some locations in Arabia practices to improve women's fertility, date pits are roasted and used in lieu of coffee as a hot beverage[03]

Some flavonoids are more selective towards cancer cells and may have the potential for reducing side-effects compared with other anticancer drugs[01], The fruit pulp is rich in phytochemicals like phenolics, sterols, carotenoids, anthocyanins, procyanidins, and Flavonoids, some studies have shown that flavonoids, polyphenols and tannins are able to altering proliferation of cancer cell.[01]

The importance of the date in human nutrition comes from its rich composition in carbohydrate (sugars such as fructose, glucose, and sucrose; dietary fiber), salts and minerals (calcium, magnesium, phosphorus, potassium, iron, zinc, copper, manganese, and selenium), vitamins (A, A1, B, B1, B2, B3, B5, B6, and C), fatty acids, amino acids

and protein (Elleuch M ;and all 2008).Studies have shown that dates have potent anthocyanin, carotenoid, and phenolic compounds (mainly cinnamic acids) and flavonoids (flavones, flavonols and flavanones), which like most other fruits, have antioxidant properties (Biglari F and all 2009) [04]

Flavonoids are one of the most important groups of bioactive compounds in plants, which exist in the free aglycones and the glycoside forms showing a diverse structure and a broad range of biological activities. Flavonoids include several classes of compounds with similar structure having a C6-C3-C6 flavone skeleton.(de Rijke, E and all 2006),(Naczk, M and all 2004)[05]

Morover, interact with various enzymatic systems. Their inhibition of the enzymes cyclooxygenase and lipooxygenase results in a decrease of platelet activation and aggregation, protection against cardiovascular diseases, cancer chemoprevention and their anti-inflammatory activity (Yao, L.H and all 2006),(Al-Fayez, M and all 2006) Many other biological activities are attributed to flavonoids and phenolic acids: antiviral, antimicrobial, antihepatotoxic, antiosteoporotic, antiulcer, immunomodulatory, antiproliferative and apoptotic activity[05]

The inflorescence of date palm tree, in its early stages of growth is enclosed in a hard covering / envelope known as spathe which splits open as the flowers reach maturation. The spathes are removed during pollination and insemination of date. Spathe is commonly called and has a specific fragrance particularly when it is fresh and is utilized in large scale production of Tarooneh hydrodistilled water in Fars province. This water contains volatile components and is widely consumed as a beverage to improve heart functioning in local and traditional health practice. It also possesses analgesic and anti inflammatory effects [04]

EXPERIMENTAL SECTION

2.1.Plant Material

The spathes of *Phoenix dactylifera L* used for this study were collected from Touggourt in the southern Algerian in March and Avril 2012 at flowering Stage. free of physical damage and injury from insects and fungal infection, were selected and used for all experiments. Upon arrival at the laboratory [02]

Polarity is an important consideration here. Less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols) are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol–water mixtures. Glycosides have increased water solubility and aqueous alcoholic solutions are suitable. The bulk of extractions of flavonoid-containing material are still performed by simple direct solvent extraction[6,1]

2.2.Extraction

fifteengrams (15 g) of dried male spathe were placed in a soxhlet apparatus with methanol. Extraction was performed with 200 ml of an appropriate solvent for (4-5) h. After extraction, a rotary vacuum evaporator at 40 °C was used in order to remove solvent. In this experiment six solvents of different polarity were used: water, methanol, petroleum ether, diethyl ether, diethyl Acetate, n-butanol.[7,8]

*2.3.*HPLCand Mass Spectrometric Analysis

Nowadays, the main concerns about natural medicinal products are effectiveness, safety and the quality of the herbal drugs (Calixto, J. Band all 2000), (Mosihuzzaman, M and all 2008). Consequently, it is essential to identify and measure all the bioactive constituents of medicinal plants in order to ensure the biological research reliability and repeatability as well as to ensure enhancing the quality control over the pharmacological bene fits and/or hazardous. HPLC–MS plays a prominent role as an analytical tool for detecting and identifying pharmacologically active metabolites and/or reactive metabolites (V and all 2009), (Xing, J and all 2007). When compared to other detection methods, MS not just allows determining natural compounds chemical structure with known and unknown structures, but also offers excellent sensitivity to low amount of samples within relatively short analysis time as well as plays an important role in screening flavonoids and other phenolics (Gouveia, S.C.; Castilho, P.C ;2010),(Tiberti, L.A and all 2007)[09].

In the last few years, several mixtures of phenolics have been analyzed with advanced LC-MS/MS equipment (Kang, H.J and all 2011) (Steinmann, D.; Ganzera, M 2011) including various publications dealing with the on-line HPLC-MS detection of flavonoids, phenolic acids and alkaloids in citrus fruits (Liu, E.-H and all 2013),(García-Salas, P and all 2013) The advantage of HPLC-MS is its sensitivity and selectivity, and the ability to use tandem mass spectrometry for the observation of aglycones and diagnostic fragments[10]

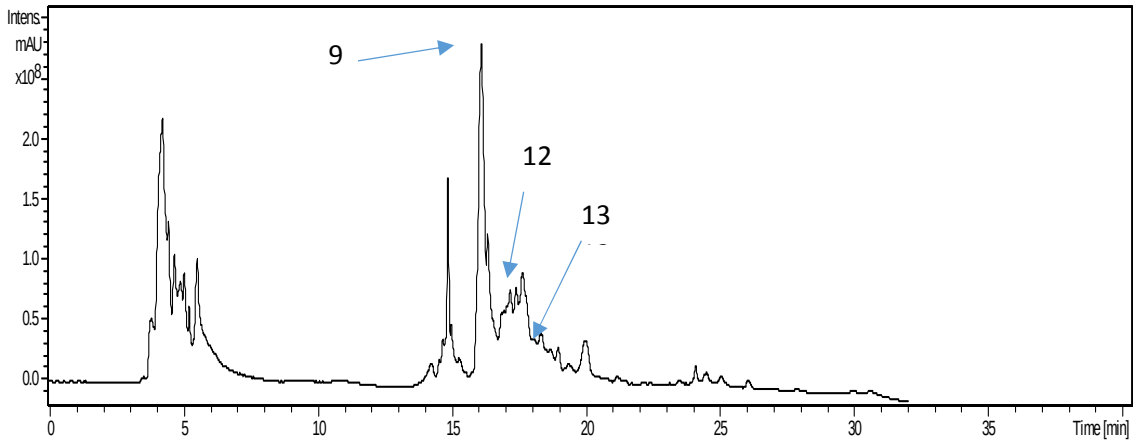
RESULTS AND DISCUSSION

In this study, a total of 13 compounds were characterized. Five flavonoid derivatives of them were unambiguously identified by comparing retention times (*TR*), UV and MS data with those of the compounds previously identified . The possible structures of another some peaks in the chromatogram were tentatively characterized on the basis of literature data. The HPLC-DAD chromatograms and total ion chromatograms (TIC) in negative and positive mode of the extracts of *Phoenix dactylifera L* are shown in Figure 1.

Figure 01 shows ionic current of the 13 natural flavonoid investigated by LC-MS. The LC conditions permitted a good separation of these compounds and were optimized for further separations of crude plant extracts containing flavonoid derivatives in 30 min.

Preliminary analysis of UV-vis spectra obtained for the peaks gave a first indication of the family of phenolic compounds [10]

Figure 01. HPLC chromatogram of flavonoid extract (diethyl ether) of spathes of *Phoenix dactylifera L*.Peak identities are numbered in Table 1,2 and Table 3



3.1.Identification of flavonoid aglycones (Quercetin derivatives)

Some detected ions (9,8 and 10) have been previously reported and their fragmentations are summarized in Table 1.

Table 1.Detection wavelength (λ), retention time (*T R*),HPLC-MS^n *m/z*

Peak	t R (min)	λ (nm)	MS	MS ^{2}	Proposed flavonoids derivative
9	16.2	230 280	$[M-H]^+$ 382.9,366.9,350.9,**327.0**,308.9,**299.9**,276.9, **272,254.9,246,236.9,227,212**,199,**180,134**	218.9,236.9	Quercetin
10	16.8		$[M-H]^+$ 366,350.9,**299,272,275,254.9,236.9,213**	348.9,332.9,315,280, 252.9,235	
8	15.1		[M-H]- 404.8,388.8,**327.1**,293,308,299,**282.9**,**272**,268.9,**248.8,227, 213, ,165,150,147,137**	182.9,165	
compounds previously identified : Quercetin-hexoside m/z [300.9]:178.8,150.8, 106.9, 120.8, 272.9, 228.9, 256.8; Quercetin-3-O-rhamnoside m/z [300.9]:178.8,150.8, 106.9, 120.8, 272.9, 228.9, 256.8; Quercetinuronic acid m/z [300.9]:178.8,150.8, 106.9, 272.9, 228.9, 256.9, 192.8, 168.8 [05] Quercetin m/z 301,179, 151 ;Quercetin 7-O-glucoside-3-O-rutinoside m/z 463, 301,179, 151 ;Rutin m/z 301,179, 151 [10] Rutin m/z 300,271,179 ,151 ; quercetin m/z 273,255,151,133 [02] Quercetin-O-hexoside 301, 273, 179, 151 ; quercetin-3-O-β-D-galactopyranoside 301, 273, 179, 151 [09] Quercetin ; quercetin-3-O-galactoside ; quercetin-3-O-glucoside ; quercetin-3-O-glucuronide ; rutin (querc-3-O-rutinoside) 300.0284 ;151.0036 [11] Quercetin m/z 303,285,275,247,257,229,153,149,165,137 [12] Quercetin 3-O-galactoside m/z 301.0342, 300.0270, 273.0054,178.9968, 151.0038 [13]				

In this fraction, a total of 13 compounds were characterized. Three of them were unambiguously identified by comparing λ_{max} (nm) and MS data with those of the flavonoids previously identified:

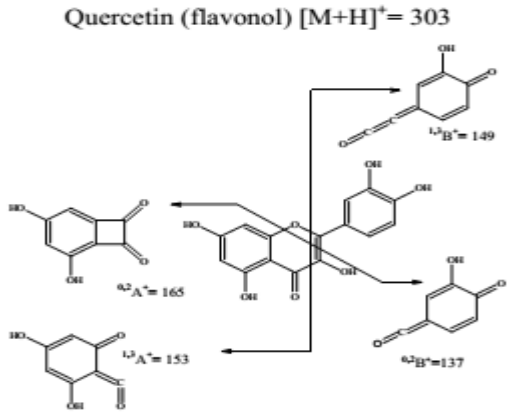
Chromatogram peak areas on256nm for quercetin-3-*O*-galactoside;256,356 nm for Quercetinhexoside;256,362 nm for quercetin-3-O-rhamnoside[05]

- 266, 356nm for quercetin-O-(O-galloyl)-hexoside; 254, 362nm for quercetin-O-hexoside[09]
- 256,372 nm for Standard compound (quercetin)[12]

Peaks 9,10 and 8 were identified as quercetin derivatives by comparing their retention time and MS/MS2 data with those of the flavonoids previously identified. In the positive ion mode, these two compounds showed common fragment ions at m/z 165 ($[^{0,2}A]^+$), 153 ($[^{1,3}A]^+$), 137 ($[^{0,2}A\ CO]^+$for quercetin, and Diagnostic fragment ions ($[M + H^+ H_2O - 2CO]^+$) and ($[M - H - H_2O - 2CO]^-$) were also observed at m/z 229,227 respectively for quercetin. [12]

The MS spectra of the $[M - H]^-$ of compounds 9 (m/z 382.9) that showed fragment ions characteristic of a protonated flavonoid aglycone. formed from the loss of a deoxyhexose ($[M - H\ 146]^-$) suggested that these compounds were flavonoid O-monoglycosides.

Scheme 1. The observed retrocyclization cleavages of quercetin



The basic fragments produced by C-ring fission of quercetin are presented in Scheme 1. In particular, the C-ring fission of fisetin results in $0,2B^+$ and $1,3A^+$ fragments that possess equal m/z=137 peaks, as well as $0,2A^+$ and $1,3B^+$ ones with m/z=149. Therefore, instead of four characteristic fragments, like in the case of quercetin and kaempferol, only two were observed (m/z=137 and m/z=149). Flavonol did not exhibit the $1,3B^+$fragment as its formation is associated with the presence of B-ring *o*- or *p*-hydroxy-substitution. This was also confirmed by the mass spectrum of galangin(Hughes, J.R and all 2001)[12]

Major diagnostic fragments of flavonoid aglycone identification are those involving the cleavage of two C-C bonds of the C-ring giving two fragment ions which provide information about the number and type of substituents in A- and B- rings. These fragment ions are designated according to the nomenclature proposed by Ma et al (Ma, Y.L 1997). For free aglyconei,jA and i,jB labels refer to the fragments containing intact A- and B-rings, respectively, in which the superscripts i and j indicate the C-ring bonds that have been broken.[05]

The structures of these compounds were proposed based on UV maxima (Table 1) as well as fragmentation pattern using ESI-MS-MS experiments (key aglycone fragments of 179 and 151 for quercetin and its methyl derivatives. In addition, the loss of 162 Daltons was indicative of hexose (glucose or galactose, the most common sugars found in flavonoids) the loss of 146 Daltons was indicative of rhamnose, the loss of 133 Daltons was indicative of pentose (xylose or arabinose, the most common pentoses found in natural products), and the loss of 308 Daltons indicative of compounds having the disaccharide structure rutinose or neohesperidose linked thorough an -*O*-glycosidic bond [10]

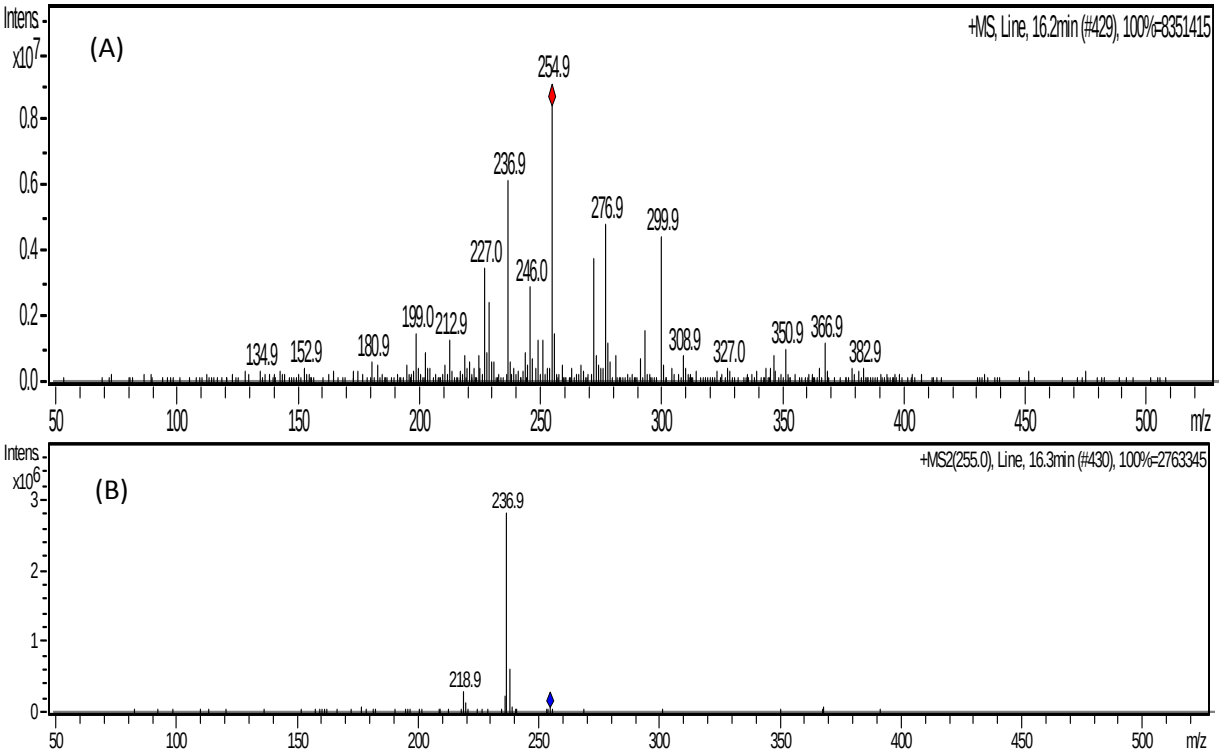


Figure 02:LC-MS/MS spectra obtained of compound peak 9,MS (A), MS^2(B)

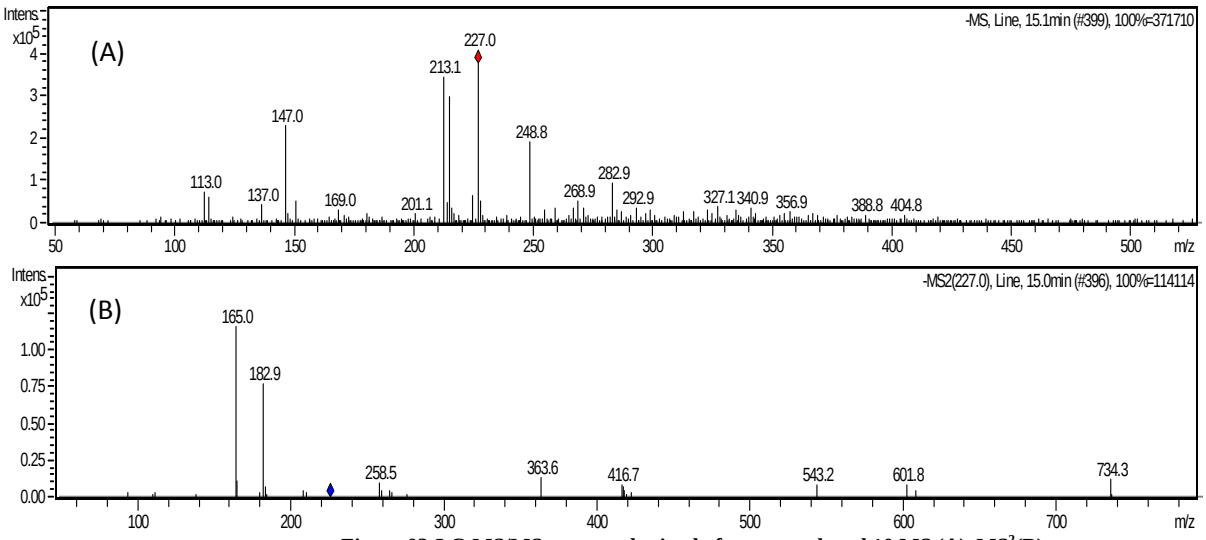
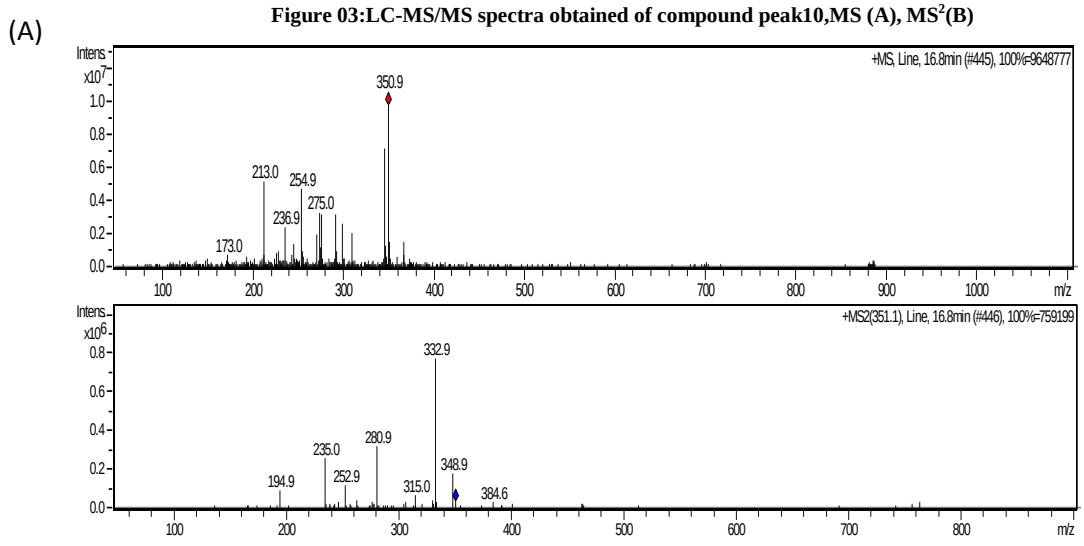


Figure 03:LC-MS/MS spectra obtained of compound peak10,MS (A), MS^2(B)



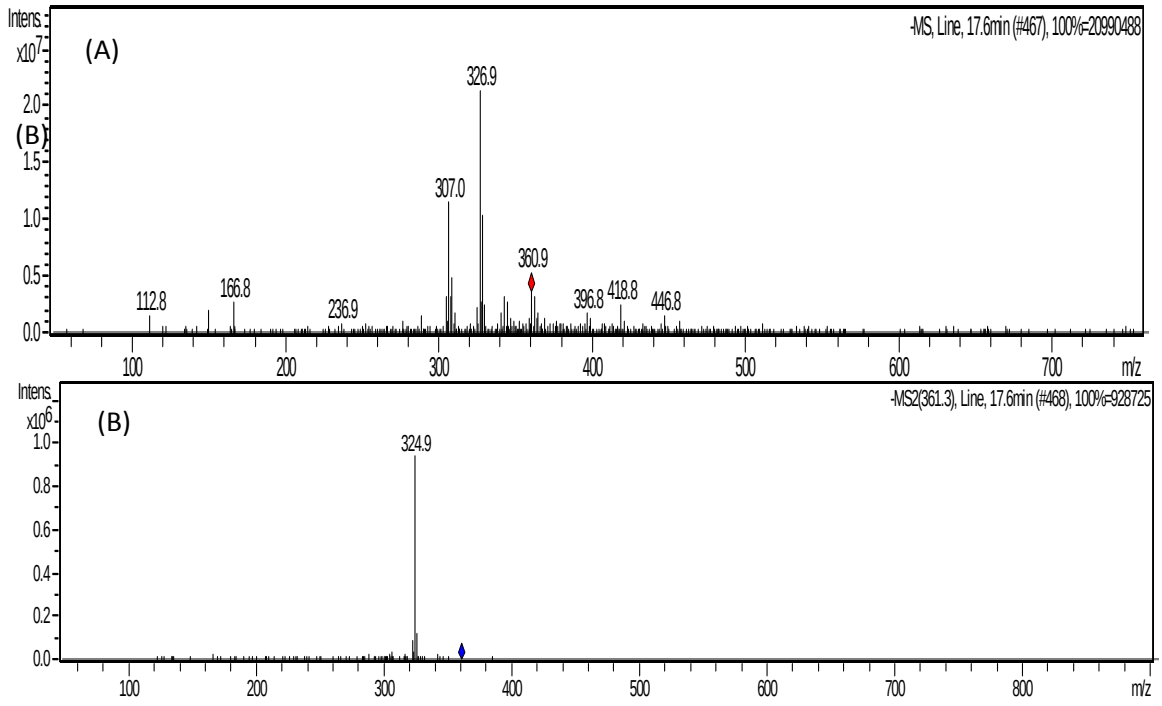


Figure 04:, LC-MS/MS spectra obtained of compound peak 8,MS (A), MS^2(B)

3.2. Identification of Luteolin glycoside derivative (Orientin derivatives)

Table 2.Detection wavelength (λ), retention time (*T R*), HPLC-MS^n *m/z*

Peak	t R (min)	λ (nm)	MS	MS^2	Proposed flavonoids derivative
12	17.6	325	[M-H]- **446.8**,418.8,396.8,**360.9**,**326.9**,307.0,236.9,166.8,112.8	324.9	Orientin
compounds previously identified : luteolin 8-C-b-D-glucopiranoside (orientin) m/z 447,357,326.9 [14] luteolin-(7-O-glucopyranosil)-8-C-glucopyranoside (orientin-7-O-glucoside) m/z 447, 357, 327, Luteolin-di-glycoside derivative m/z 447, 357, 327 [15] Orientin m/z 447, 357, 327,285 [16]					

The fragment at *m/z* 447 showed the characteristic orientin MS ions at *m/z* 357 (M−H−90), and 327 (M−H−120). The compound was tentatively assigned as luteolin-(7-*O*-glucopyranosil)-8-*C*-glucopyranoside (orientin-7-*O*-glucoside,) [14]

The product ion spectra of the ion m/z 447 of orientin differs in relative abundance of the m/z 357 (loss of 90 u) and m/z 327 (loss of 120 u).[16]

Peak12 ($[M-H]^-$ions at *m/z* 447) was tentatively identified as luteolin 8-*C*-Glucoside [10]

The MS spectra of the $[M - H]^-$ of compounds 12 (m/z 446.8) that showed fragment ions characteristic of a protonated flavonoid . formed from the loss of a deoxyhexose ($[M - H\ 146]^-$) suggested that these compounds were flavonoid O-monoglycosides

Peaks **12** with a $[M-H]^-$ ion at *m/z* 447 were assigned according to UV and mass spectral data (Table 2) of this compound is consistent with the proposed flavonoid structure, Luteolin-di-glycoside derivative

Scheme 2: The observed retrocyclization cleavages of orientin

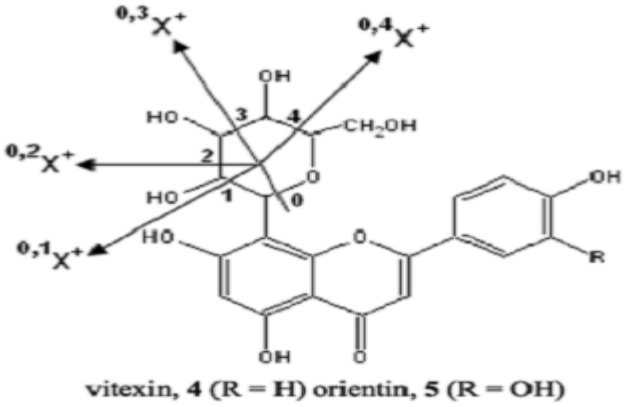


Figure 05:LC-MS/MS spectra obtained of compound peak 12,MS (A), MS^2(B)

3.3. Identification of chalcone or flavone aglycone

Table 3. Detection wavelength (λ), retention time (*T R*), HPLC-MS^n *m/z*

Peak	t R (min)	λ (nm)	MS	MS ^{2}	Proposed flavonoids derivative
13	18.2	260 325	[M-H]$^-$ 574.4,460.9,416.8,380.8,344.9,309, 284.9,268.9,243.1,223,208,150.9,112.9	344.8,308.9,280.8 ,238.8,208.8,178.9 ,136.8	chalcone or flavone aglycone
compounds previously identified : [M - H]- ions of dihydrochalcones m/z 301 : 283,268, 225,152 [17] [M - H]- ions of flavanones; 283,268,151 ; 285,270, 243, 226,175, 151,136, 1[18] [M−H]− ions of chalcones ; 283,268, 241, 226, 239, 179,153. [17]					

The absorption bands were preliminary identified as chalcone or flavone aglycone according to maxima absorption bands at about 290 and 340 nm. and the others a dihydrochalcone or flavanone aglycone (absorption bands at 230 and 290 nm)[17]

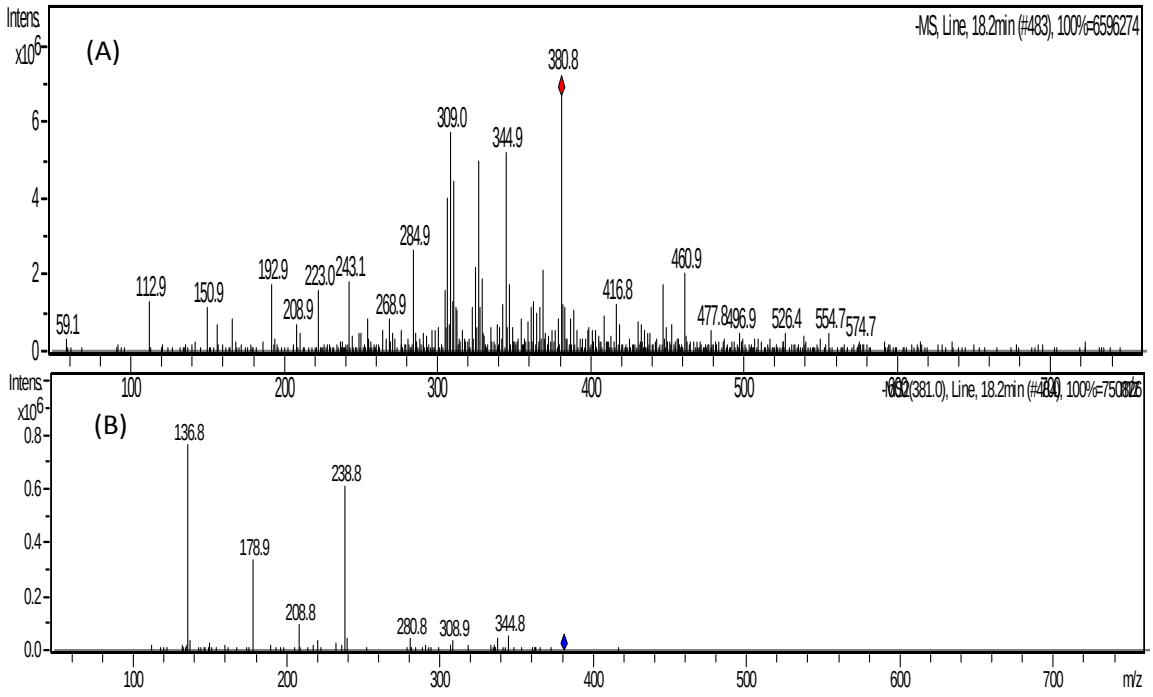


Figure 06:LC-MS/MS spectra obtained of compound peak 13,MS (A), MS^2(B)

CONCLUSION

LC/MS/MS analysis of diethyl ether fractions from the aerial parts of *Phoenix dactylifera L* (spathes) to the identification of five flavonoids derivative. Quercetin, orientin and flavanone are identified for the first time in genus *Phoenix dactylifera L*.

Acknowledgments
The authors wish to thank LA. Segni and OU. Mohamed Didi.(University kasdimerbah- Ouargla- Algeria) for their skillful technical assistance.

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