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Integrated mass spectrometry approach to screening of phenolic molecules in *Hyphaene thebiaca* fruits with their antiradical activity by thin-layer chromatography

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Hyphaene thebaica is an ancient palm whose fruits are an integral part of the human diet. An approach based on high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) in the negative ion mode, evidencing phenolic molecules as the main constituents of the plant has been developed. These phenolic molecules, widely distributed in plants and considered highly beneficial for their antioxidant properties, are very difficult to separate and characterize. Their presence has been rationalized on the basis of their mass spectrometry profiles. A Thermo Surveyor HPLC system and a C18 reversed phase column are used, and the mobile phase with a mixture of acetonitrile and 0.03% formic acid (v/v) in water in the gradient elution mode at flow rate 0.4 mL/min, detected at 360 nm. Phenolic molecules are screened based on accurate mass of pseudomolecular $[M-H]^-$ ions and tandem mass spectrometry (MS/MS) data. The data obtained in our research show that *Hyphaene thebaica* fruits are a rich source of phenolic acids and flavonoids with evident of strong antioxidant.

Keywords: Doum palm, Phenolic molecules screening, Liquid chromatography, Electrospray ionization, Mass spectrometry, Free radical scavenger.

The Doum palm (*Hyphaene thebaica* (L.) Mart. = *Corypha thebaica* L. = *Cucifera thebaica* Del. = *Hyphaene sinaitica* Furrtado) is so named from the word 'Doum' which means 'permanence', in allusion to the persistence of the tree under abnormal conditions[1,2]. The fruits are edible and the thin dried brown rind is made into molasses, cakes and sweetmeats. Herb tea of Doum is popular in Egypt and believed good for hypertension[3,4]. Roots are used in the treatment of Bilharzias while the resin of the tree has demonstrated, diuretic, diaphoretic properties and also recommended for tap worm as well as against animal bites[5]. The aqueous extract of the fruits exhibited an antioxidant effect (Hsu, *et al.* 2006). Earlier investigations performed on (Doum palm) *Hyphaene thebaica* (L.) resulted in the isolation of estrone from kernel and fruits and flavonoids from fruits and leaves[6-8].

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has been recognized as a powerful analytical tool with its high sensitivity, short run time and less use of toxic organic solvents used as mobile phase compared to reversed phase stand-alone HPLC coupled with Diode-Array Detector[9-12].

As a part of our studies on the biologically active metabolites from medicinal plants, we describe herein the fingerprinting screening and hypothesis of phenolic molecules by using LC-ESI-MS/MS from the fruits of *Hyphaene thebaica* (L.).

Experimental Section

Plant material

The fruits of *Hyphaene thebaica* (L.) (Doum palm) were collected in March 2012 from Saudi Arabia Kingdom and identified by Prof. A. Hamed according to Täckholm (1974)[2].

Extraction and sample preparation

The dried fruits of *Hyphaene thebaica* (200 g) were first defatted extracted with hexane and then with 80% MeOH. The aqueous methanolic extract was concentrated under reduced pressure to give 25 g of crude extract (*ca*: 12.5% crude extract). 5 g of extract was loaded onto a C18 column (4×50 cm, 40-63µm LiChroprep, Millipore Corp, Bedford) and eluted with 40 (H-1, 3 g), 60 (H-2, 2 g) and 80% (H-3, 4g) and 100% (H-4) of methanol. 0.5 g of fraction 40% was infused with water (100 mL) for 10 min. An aliquot of the infusion was filtered through a Millex filter (0.45 nm) and analyzed by HPLC-MS.

High-performance liquid chromatography and ESI-mass spectrometry screening of phenolic fraction (H-1)

The components of the infusion were separated using Thermo Surveyor HPLC system and a C18 reversed phase (RP) column (5 µm, 2.1 mm × 150 mm; Symmetry, Waters, MA, USA). The gradient of mobile phases A (water containing 0.03% formic acid) and B (MeCN containing 0.03% formic acid) was as follows: 0 min (10% B); 20 min (30% B); 40 min (45%, B); 45 min (100% B); 50 min (100% B). The flow rate was 0.4 mL/min, and column temperature was 50°C.

The flow generated by the chromatographic system was introduced directly into the ESI source. MS analysis was performed using a LCQ Advantage Max ion trap instrument (Thermo, San Jose, CA, USA) controlled by Xcalibur 1.3 SR1 software. The ion source operated in the negative ion mode with following parameters: capillary voltage 4 V, spray voltage 4.1 kV, tube lens offset 0 V, capillary temperature 240°C, and sheath gas (N_2) flow rate 50 (arbitrary units). Ions were scanned in the *m/z* range from 150 to 2000 with three microscans and maximum injection time of 150 ms. Two scan events were set to run sequentially. The first event was a full scan to acquire ions in the selected range. The second scan event was MS/MS fragmentation experiment performed on the most intense ion from the acquired set. Normalized collision energy of 35% was used to generate fragment ions.

To obtain general ESI-MS fingerprint of the infusion, it was diluted ten times with 40% MeOH and introduced directly into the ion source of the mass spectrometer at flow rate of 5 µL/min using syringe pump. The source and the scanning parameters were essentially as described above, except that in this case data-dependent acquisition was not performed.

TLC-DPPH$^{\cdot}$ test

The analyzed fraction (H-1) was applied to Kieselgel 60 F_{254} HPTLC 10 × 10 cm, 0.25 mm chromatographic plates (Merck) by means of a micropipette with the scale. All the samples were applied band-wise (8 mm wide), with a distance of 2 mm between them, and the 10 mm distance from both the left and low edge of the plate. The plates were developed in vertical chambers pre-saturated for 15 min with mobile phase. The plates were developed to the distance of 90 mm. The 5 µL aliquot of standard solution and 10 µL of the analyzed fractions were applied onto the plates. The following mobile phase was used in the study: ACN-H_2O-$CHCl_3$-HCOOH (60:15:10:5, v/v/v/v).The plates were dried in a hood for 30 min before screening. For chemical screening vanillin-sulfuric acid reagent was applied. It was prepared by adding 1 g vanillin to 100 mL 20% sulfuric acid in methanol. TLC plates were immersed for 5 s in this reagent and then heated for 5 min at 105°C. For detection of free radical scavengers, TLC plates were immersed for 5 s in freshly prepared 0.2% (w/v) methanolic or *n*-hexane DPPH$^{\cdot}$ solution (2,2-diphenyl-1-picrylhydrazyl). After removing DPPH$^{\cdot}$ excess, plates were kept in the dark for 30 min and then scanned by means of flat bed scanner.

The scans were saved in jpg mode and further processed by means Image J 1.43u image processing program (Wayne Rasband, National Institutes of Health, USA; http://rsbweb.nih.gov/ij/). The images were processed to obtain the real chromatograms according to previously elaborated procedure[13]. In the first step the median filter function (Process/Filters/ Median) with 5 pixels width was used to remove the noise. Subsequently the baseline drift was removed by bandpass filter (Process/FFT/ Bandpass Filter). After these operations the 'Plot Lines' option was applied to generate the profile plots.

Results and Discussion

ESI-MS and ESI-MS/MS screening of phenolic fraction

Since phenols contain one or more hydroxyl and/or carboxylic acid groups. MS data were acquired in negative ionization mode. The phenolic molecules were tentatively screened on basis of the analysis from the deprotonated ions and the fragments released in MS/MS experiments. MS/MS spectra are very useful for identifying the aglycones of flavonoids, and the analysis of fragmentation patterns is highly diagnostic, allowing the elucidation of structures by mass fragmentation screening.

Data obtained from the ESI-MS analyses of the extracts of doum fruits are summarized in Table 1. As shown in Table 1, a total of 18 polyphenol molecules distributed in four major categories; hydroxycinnamic acid derivatives, hydroxybenzoic acid derivatives, flavonoids and stilbenoid type have been analyzed in the present study. Since polyphenols contain one or more hydroxyl and/or carboxylic acid groups, MS data were acquired in negative ionization mode.

The $[M-H]^-$ at *m/z* 335 was deduced as molecular ion peak for three molecules (Rt 11.60, 12.44 and

Table 1 — Characterization of the individual phenolic molecules in *Hyphaene thebaica* (L.) fruits extract using HPLC-ESI-MS/MS

Peak	R_t[a]	Compound	[M-H]$^-$	Major fragments (*m/z*)
1	11.60	X-O-Caffeoylshikimic acid ($C_{16}H_{16}O_8$)	335	317 (5)[a], 291 (40), 273 (5), 247 (5), 179 (100), 161 (90), 155 (5), 135 (40)
2	12.44	X-O-Caffeoylshikimic acid ($C_{16}H_{16}O_8$)	335	317 (5), 291 (40), 273 (5), 247 (5), 179 (100), 161 (90), 135 (40)
3	12.96	X-O-Caffeoylshikimic acid ($C_{16}H_{16}O_8$)	335	317 (5), 291 (40), 273 (5), 247 (5), 179 (100), 161 (90), 135 (40)
4	15.43	Chlorogenic acid-benzyl-O-hexoside ($C_{28}H_{32}O_{14}$)	593	575 (20), 533 (5), 503 (30), 473 (100), 413 (5), 383 (20), 353 (20), 297 (10)
5	18.42	Resveratrol ($C_{14}H_{12}O_3$)	227	209 (5), 199 (5), 185 (20), 157 (5), 121 (10), 113 (30)
6	20.04	Caffeoyl tartaric acid ($C_{13}H_{12}O_9$)	311	283 (10), 191 (5), 167 (5)
7	20.15	Quercetin-O-rutinoside ($C_{27}H_{30}O_{16}$)	609	591 (3), 465 (3), 473 (3), 343 (20), 301 (100), 287 (5), 271 (10), 255 (3), 229 (5), 179(10)
8	20.66	Quercetin-O-hexoside ($C_{21}H_{20}O_{12}$)	463	343 (100), 301 (90), 217 (90)
9	21.03	Luteolin-O-rutinoside ($C_{27}H_{30}O_{15}$)	593	447 (3), 285(100), 243 (3), 217 (3), 193 (3), 182(3)
10	21.73	Isorhamentin-O-rutinoside ($C_{28}H_{32}O_{16}$)	623	543 (5), 459 (20), 339 (10), 315 (100), 300 (20)
11	24.99	Quercetin ($C_{15}H_{10}O_7$)	299	284 (100), 271 (75), 269 (20),
12	25.20	Hydroxybenzoic acid-O-Hexoside ($C_{13}H_{16}O_8$)	299	137
13	25.51	Isorhamentin-O-methyl ($C_{17}H_{13}O_7$)	329	314(100), 135 (25)
14	27.56	Caffeoyl tartaric acid derivative	511	431 (90), 421 (60), 375 (60), 341 (100), 311(60)
15	27.61	Caffeoyl tartaric acid ($C_{13}H_{12}O_9$)	311	283 (35), 243 (5), 161(5)
16	27.75	Isorhamnetin-3-O-hexoside ($C_{22}H_{21}O_{12}$)	477	315 (20), 314 (100), 300 (20)
17	27.96	Quercetin-O-rutinoside-O-sulphate ($C_{27}H_{30}O_{16}SO_3$)	689	609 (100), 381 (3), 301 (5)
18	30.54	Quercetin-O-hexoside-O-sulphate ($C_{21}H_{20}O_{12}SO_3$)	543	463 (100), 381 (10), 301(30)

[a]The relative abundance between parenthesis.

12.94) (Fig. 1). Based on HPLC-ESI-MS^2 for each compound, results revealed the presence of the similarity fragmentation pattern. The [M-H]$^-$ ion at *m/z* 335 lost 156 Da to produce two base peaks at *m/z* 179 [M–$C_7H_8O_4$–H]$^-$ and lost 174 Da to produce the base peak at *m/z* 161 [M–$C_7H_{10}O_5$–H]$^-$. This revealed the presence of the structure of caffeic and shikimic acids. Moreover, the presence of ion peaks at *m/z* 291 [M–CO_2–H]$^-$ due to loss 44 Da and at *m/z* 247 [M–CO_2–H]$^-$ due to broken of shikimic ring and loss of –CH_2CHOH. This indicated that the two acids were linked together through ether linkage. Generally, phenolic acids produce a typical fragmentation pattern after collision induced dissociation, characterized by the loss of a CO2 (44 u) from the carboxylic acid group, providing an anion of [M–H–COO]$^-$. Comparison of this fragmentation pattern with previous reports indicated that the structures of the three peaks are isomers of X-O-caffeoyl shikimic acid[14]. These three isomers were reported previously from *Pheonix dactylifera* and it's very difficult to purify each one because their properties are close to each other[15].

The [M-H]$^-$ at *m/z* 227 (Rt 18.42) peak 5,was deduced as molecular ion peak for one molecule. Product ion at *m/z* 185 [M–CHCOH–H]$^-$ due to loss of 42 Da. The presence of ion product at *m/z* 159 [M–C_3H_3COH–H]$^-$ due to loss of 68 Da (Table 1, Figs. 1 and 2). This suggests that the molecule, of probable molecular mass of resveratrol. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a stilbenoid, a type of natural phenol. The previous screening

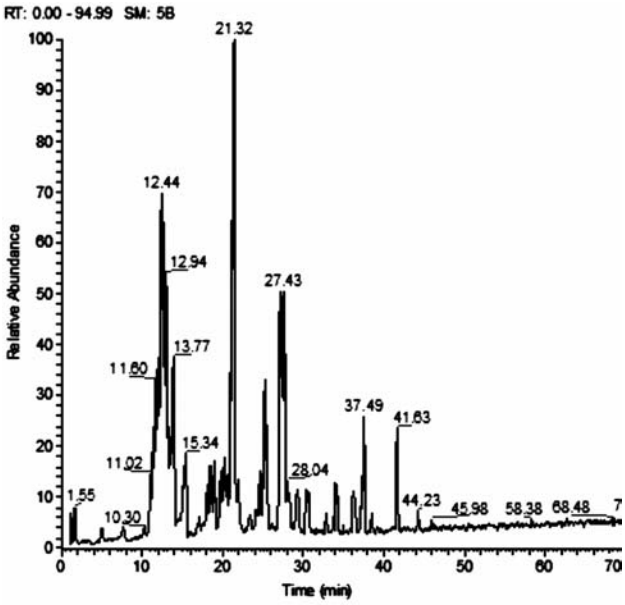

Fig. 1 — HPLC-DAD chromatogram of methanolic extract of *Hyphaene thebiaca*

showed that resveratrol might be a key ingredient in red wine that helps prevent damage to blood vessels reduces "bad" cholesterol and prevents blood clots[16,17].

A sugar conjugate of hydroxybenzoic acid eluting at 25.20 min (peak 12) showed $[M–H]^-$ ions of *m/z* 299. Accurate mass measurement suggested the molecular composition as that of hydroxybenzoic acid-O-hexoside. Subsequent MS/MS experiment revealed the loss of hexose moiety producing deprotonated 4-hydroxybenzoic acid at *m/z* 137.

Table 1 and Fig. 2 shows the $[M–H]^-$ ion at *m/z* 463 (Rt 20.66), peak 8. Fragmentation of the parent ion at *m/z* 463 the $[M – H]^-$ for peak 8 produced two daughter ions with one at *m/z* 301 corresponding to quercetin aglycone, due to loss of 162 Da to hexose moiety, revealing the compound to be quercetin hexoside. This indicates the structure related to quercetin-3-O-hexoside.

The presence of one deoxyhexose and one hexose moieties linked to a quercetin aglycone in the structure for peak 7 (Rt 20.15) was identified by the parent ion at *m/z* 609 $[M–H]^-$ (Table 1). The presence of product ion at *m/z* 463 $[M–146–H]^-$ due to cleavage of deoxyhexose and a fragment ion at *m/z* 301 due to loss of hexose unit and at *m/z* 287. Thus, this molecule has deoxyhexose and hexose moieties linked to a quercetine. It has been deduced to be Quercetin-O-rutinoside (Fig. 2).

MS profiling of peak 9 (Rt 21.03) yielded a parent ion at *m/z* 593 $[M–H]^-$ and product ions at *m/z* 447 $[M–146–H]^-$ due to cleavage of deoxyhexose, and at *m/z* 285 $[M–146–162–H]^-$ due to cleveage of hexose. The presence of product ion at *m/z* 285 was corresponding to rutinose moiety and luteolinaglycone, revealing the compound to be luteolin-7-*O*-rutinoside (Table 1 and Figs 1 and 2).

Another peak at Rt 15.43 min (4) with the same ion peak at *m/z* 593 but its fragmentation was totally different. It gave daughter ions at 575 due to loss of 18 Da (H2O), 413 due to cleavage of 162 Da (hexose) and 79 Da due to cleavage of benzyl ring. The presence of product ion at 353 may be correlated to chlorogenic acid. Unfortunately, the collesion energy could not fragment this ion product. It has been deduced chlorogenic acid-benzyl-O-hexoside.

Table 1 shows the $[M–H]^-$ two parent ions having the same molecular weight at *m/z* 311 (Rt 27.61) peak 15 and the ion at *m/z* 161 formed by the neutral loss of 150 mass units as a result of tartaric acid fission. This indicates the structure related to two isomers of Caffeoyl tartaric acid (Figs. 1 and 2). Another peak has been observed at Rt 20.04 with the same parent ion but the fragmentation pattern slightly different. This may be related to the stereochemistry of both caffiec or tartaric acids, or their linkage together.

MS profiling of peak 17 (Rt 30.54) yielded a parent ion at *m/z* 543 $[M–H]^-$ and product ions at *m/z* 463 $[M–80–H]^-$ due to cleavage of sulphate group, and at *m/z* 301 $[M–80–162–H]^-$ due to cleavage of hexose. Thus, this molecule has been deduced to be Quercetin-O-hexoside-O-sulphate (Fig. 2).

The MS/MS experiments revealed that the $[M-H]^-$ ions at *m/z* 477 was isorhamnetin-3-O-hexoside. Glycosylated flavonoids constituted the bulk of the polyphenols in the plants. Hexose and rutinose conjugates of flavonoids were most commonly observed. The MS/MS experiments revealed that the $[M-H]^-$ ions at *m/z* 477 eluting at 27.75 min was isorhamnetin-3-O-hexoside. This hexoside showed the loss of a hexose moiety (162 Da). In addition to the fragment ion at *m/z* 315 corresponding to deprotonated molecular ion of isorhamnetin, the isorhamnetin-3-O-hexoside produced a fragment ion at *m/z* 300 further confirming that the hexose derivative was that of isorhamnetin. As expected isorhamnetin-3-O-hexoside was only detected in the extracts of doum. Likewise, quercetin pentoside was assigned for peak 11 (Rt 24.99) based on the $[M – H]^-$

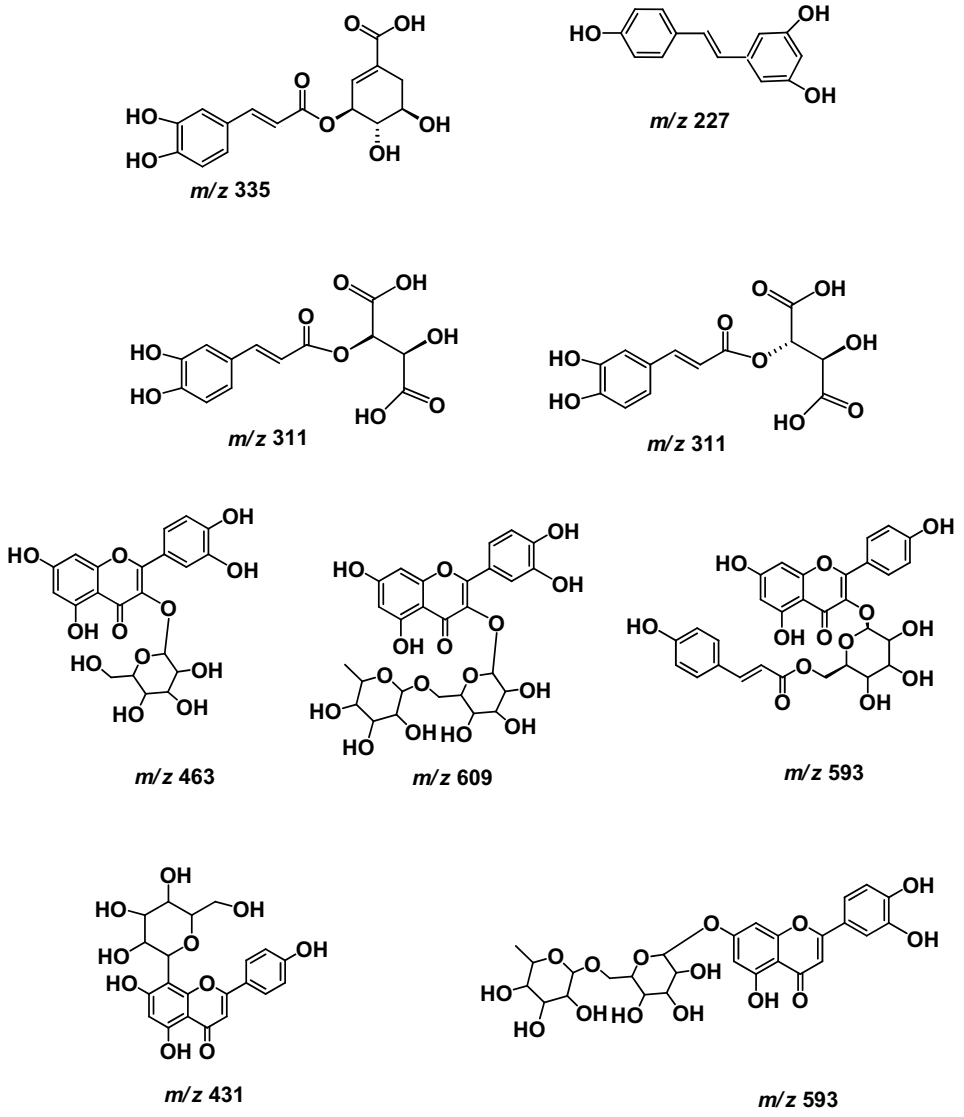

Fig. 2 — Proposed structure of the phenols screened in Doum fruits

value at *m/z* 299 and the product ion at *m/z* 284, 281, 271 and 269 representing quercetin aglycone.

MS profiling of peak 14 (Rt 27.56) yielded a parent ion at *m/z* 511[M–H]$^-$ and product ions at *m/z* 431 [M-80-H]$^-$ due to loss of sulphate or C_6H_6, at *m/z* 413 [M-80-H_2O-H]$^-$, at *m/z* 311 [M-80-H_2O-[M–H_2O–2 (HC≡C–OH)-H]$^-$ due to loss of 102 Da. The presence of ion product at *m/z* 311 indicated it may be caffeoyl tartaric acid derivative.

Table 1 showed two ion peaks (17, 18) at 27.96 and 30.54 min, respectively. Peak 17 has typical fragmentation pattern for Quercetin-O-rutinoside-O-sulphate, while peak 18 was typically to quercetin-O-hexoside-O-sulphate.

Free radicals scavenging properties of phenolic fraction obtained from *Hyphaene thebiaca* fruits

Thin-layer chromatography coupled with bio-detection was used in our study to screen free radical activity of phenolic fraction obtained from *Hyphaene thebiaca* fruits. Contrary to the publication of Pobłocka-Olech and Krauze-Baranowska, sample purification step was avoided, in our research, all the fractions and the crude extract were chromatographed without prior cleaning steps[18]. Unfortunately TLC system proposed by the aforementioned authors was unsuitable for the resolution of phenols present in *Hyphaene thebiaca* extract due to greater polyphenols' complexity contained within the analyzed samples.

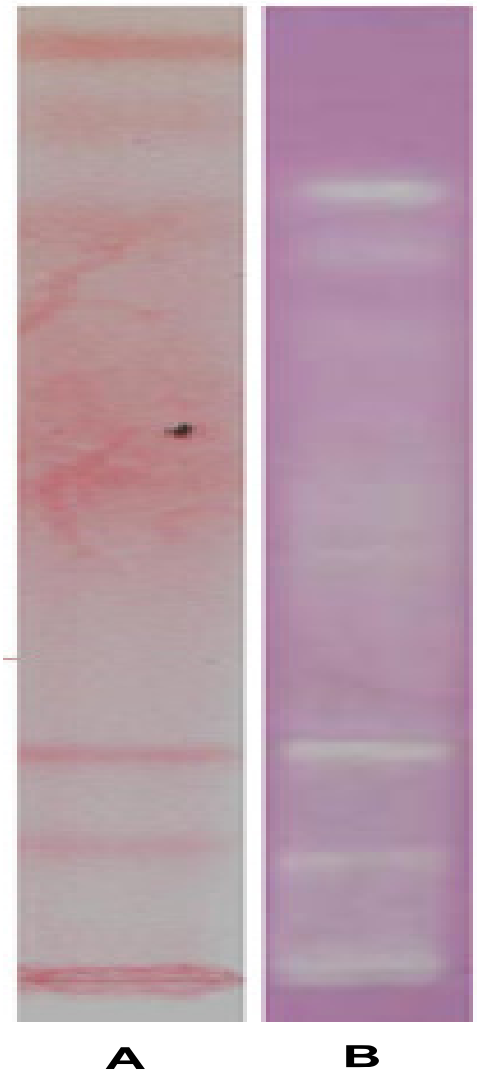

Fig. 3 — Densitograms extract from images for phenolic fraction stained with: (A) methanolic vanillin-sulphuric and (B) DPPH methanolic reagent

Therefore eluent containing water was optimized for the resolution of these substances. The analyzed samples were chromatographed with the use of the following mobile phase: ACN-H_2O-$CHCl_3$-HCOOH (60:15:10:5, v/v/v/v). It was found that eluents containing acetonitrile instead of methanol gave sharper peaks. The plates were visualized with vanillin-sulfuric acid reagent to reveal the chemical character of the molecules. With the use of this spraying reagent, rose-colored bands appeared, giving evidence that the separated molecules are phenol derivatives. The comparison of our densitograms and those presented in the paper by Pobłocka-Olech and Krauze-Baranowska, clearly shows sharper peaks were obtained with the use of aqueous eluent (Fig. 3)[13]. Therefore this TLC HILIC system may be proposed for example for quantitative, densitometric determination of phenols present in complex samples. It can be attributed to the fact, that under HILIC conditions the more polar molecules interact with water-enriched stationary phase more strongly resulting in greater adsorption on the silica gel surface.

To screen the samples for the presence of direct antioxidants the plates were dipped in a solution of a stable free radical DPPH$^{\cdot}$. The yellow bands of free radical scavengers appeared immediately after staining with DPPH $^{\cdot}$methanolic solution. Zones that turn yellow immediately after staining are categorized usually as strong antioxidants[17-19]. However the observed results depend on the adsorbent type as well as solvent used for DPPH$^{\cdot}$ dissolution. It was observed that dipping the plate, with phenols fraction in *n*-hexane DPPH$^{\cdot}$ solution caused the yellow spots to appear) later when compared with the results obtained after staining with methanolic solution. Therefore we discourage to categorize polyphenols into the groups of strong, moderate and weak antioxidants, as such classifications are always subjective and results obtained by different laboratories are difficult to be compared. Therefore our current researches are also aimed at developing a standard TLC-DPPH$^{\cdot}$ procedure.

Conclusion

Application of LC-ESI-MS/MS technique in the current study provides useful information to characterize 17 phenolic molecules in the extracts of doum. Fragments produced during CID analysis of the molecules mentioned above are the diagnostic features of these molecules which could be used to identify them in different extracts. Results of accurate mass measurements are another diagnostic feature of these molecules and proved useful to differentiate molecules with same nominal mass but dissimilar exact masses. An HPLC-MS-MS method is developed to determine various phenolic acids and flavonoids in *Hyphaene thebiaca* fruits. A total of 17 molecules, including one 3,5,4'-trihydroxy-trans-stilbene, 6 phenolic acids and 10 flavonoids, were separated by employing a Gemini C18 column and a gradient mobile phase of 0.03% formic acid and acetonitrile with flow rate at 1.0 mL/min and detection at 280 nm. Identification is carried out based on the retention behaviour as well as absorption and mass spectral

characteristics. Considerable body of evidence indicates that diet rich in these phenolic acids, flavonoid glycosides and stibline derivative may be beneficial for human health. The data obtained in our research show that *Hyphaene thebiaca* can be a good source of phenol derivatives, therefore it can be considered as a rich source of antioxidant agents.

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