Identification and Quantification of the Major Constituents in Egyptian Carob Extract by Liquid Chromatography–Electrospray Ionization-Tandem Mass Spectrometry

Asmaa Ibrahim Owis, El-Motaz Bellah El-Naggar

Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt. Department of Pharmacognosy, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt.

ABSTRACT

Background: Carob - Ceratonia siliqua L., commonly known as St John’s-bread or locust bean, family Fabaceae - is one of the most useful native Mediterranean trees. There is no data about the chromatography methods performed by high performance liquid chromatography (HPLC) for determining polyphenols in Egyptian carob pods. Objective: To establish a sensitive and specific liquid chromatography–electrospray ionization (ESI)-tandem mass spectrometry (MS²) methodology for the identification of the major constituents in Egyptian carob extract.

Materials and Methods: HPLC with diode array detector and ESI-mass spectrometry (MS) was developed for the identification and quantification of phenolic acids, flavonoid glycosides, and aglycones in the methanolic extract of Egyptian C. siliqua. The MS and MS² data together with HPLC retention time of phenolic components allowed structural characterization of these compounds. Peak integration of ions in the MS scans had been used in the quantification technique. Results: A total of 36 compounds were tentatively identified. Twenty-six compounds were identified in the negative mode corresponding to 85.4% of plant dry weight, while ten compounds were identified in the positive mode representing 16.1% of plant dry weight, with the prevalence of flavonoids (75.4% of plant dry weight) predominantly represented by two methylapigenin-O-pentoside isomers (20.9 and 13.7% of plant dry weight). Conclusion: The identification of various compounds present in carob pods opens a new door to an increased understanding of the different health benefits brought about by the consumption of carob and its products.

Key words: Carob pods, Ceratonia siliqua L., liquid chromatography–electrospray ionization-tandem mass spectrometry, methylapigenin-O-pentoside

SUMMARY

• This research proposed a good example for the rapid identification of major constituents in complex systems such as herbs using sensitive, accurate and specific method coupling HPLC with DAD and MS, which facilitate the clarification of phytochemical composition of herbal medicine for better understanding of their nature and biological activities.

INTRODUCTION

With gaining popularity of herbal remedies worldwide, the need for assuring safety and efficacy of these products increases as well. By nature they are complex matrices, comprising a multitude of compounds, which are prone to variation due to environmental factors and manufacturing conditions. Furthermore, many traditional preparations compose of multiple herbs, so that only highly selective, sensitive, and versatile analytical techniques will be suitable for quality control purposes. By hyphenating high performance liquid chromatography (HPLC) and mass spectrometry (LC-MS) these high demands are fulfilled, providing the user with a multitude of technical options and applications. Carob - Ceratonia siliqua L., commonly known as St John’s-bread or locust bean, family Fabaceae - is one of the most useful native Mediterranean trees. In producing countries, the pods have traditionally been used as animal and human food, and currently, the main use is the seed for gum extraction. In some countries, e.g. Egypt, carob syrup is a popular drink obtained by extracting carob kibbles with water. Carob powder consists of 46% sugar, 7% protein, and small amounts of numerous minerals and vitamins and is thus quite nutritious. Apart from carbohydrates, high amounts of dietary fiber and polyphenols are characteristic of this Mediterranean food. Dietary fiber itself or a diet rich in dietary fiber is known to exert a variety of physiological effects, including improved digestion and attenuation of blood cholesterol and...
glucose levels. Dietary fiber probably also has chemopreventive potential against certain cancers, in particular, those of the gastrointestinal tract. Carob contains additional health-promoting constituents such as phytochemicals, among which the polyphenols which can exhibit antioxidative, antimutagenic, anticarcinogenic, antiproliferative, or antiestrogenic activity. Polyphenols are a very heterogeneous group that includes simple phenolic acids, cinnamic acid and its derivatives, flavonoids, isoflavones, lignans, anthocyanins, and tannins. Therefore, when included in the daily diet, these compounds may play an important role in the prevention and reduction of cancer and heart disease.[3]

There is few data about the chromatography methods performed by HPLC for determining polyphenols in carob pods which revealed the presence of condensed tannins (proanthocyanidins), composed of flavan-3-ol groups and their galloyl esters, gallic acid, (+)-catechin, (-)-epicatechingallate, (-)-epigallocatechingallate, and quercetin glycosides.[8,9] Much less interest was devoted to Egyptian carob. From this standpoint, the purpose of this research was to identify and quantify phenolic constituents present in the Egyptian carob pods. A sensitive, accurate, and specific method coupling HPLC with diode array detector (DAD) and electrospray ionization (ESI) MS was developed for the identification and quantification of polyphenolic acids, flavonoid glycosides, and aglycones in the methanolic extract of Egyptian Cerbera siliqua. The subsequent structure characterization was carried out by tandem mass spectrometry (MS^n) method. Fragmentation behavior of investigated compounds was determined using ion trap MS in both negative and positive mode. Negative ion mode was used because previous studies suggested that negative mode was more sensitive than positive mode.[7] Positive ion mode was also applied leading to the identification of fewer constituents. The fragmentation pattern of each compound offers the ability to identify the related unknown compounds. The MS and MS^n data together with HPLC retention time (T_r) of phenolic components allowed structural characterization of these compounds.

The lack of reference standards for the phenolic constituents makes it necessary to calculate the amounts present according to the peak area. Peak integration of ions in the MS scans had been used in the quantification technique for decades by analytical chemists because theoretically the peak intensity of any ion is proportional to its abundance.[8,9] The area inscribed by the peak is proportional to the amount of components separated in the chromatographic system. The relative percentage of each constituent was determined from the relative peak area and expressed as percent of dry plant weight.

**MATERIALS AND METHODS**

**Chromatographic system and mass spectrometry conditions**

Analyses were performed using a Hewlett-Packard 1100 (Waldbronn, Germany) composed of a quaternary pump with an online degasser, a thermostat column compartment, a photo DAD, an autosampler, and an Agilent 1100 Chem Station software. The HPLC separation was performed on Eclipse XDB C18 column (50 mm × 2.1 mm, 1.8 μm, Agilent Company, USA). Mobile phase consisted of two solvents, (A) methanol and (B) 0.2% formic acid. Separation of compounds was carried out with gradient elution profile: 0 min, A: B 10:90; 36 min, A: B 100:0; and 40 min, A: B 100:0. Chromatography was performed at 30°C with a flow rate of 0.2 ml/ min. Ultraviolet (UV) traces were measured at 290, 254, and 350 nm, and UV spectra (DAD) were recorded between 190 and 900 nm. The HPLC–MS system consisted of ESI interfaced Bruker Daltonik Esquire-LC ion trap MS (Bremen, Germany) and an Agilent HP1100 HPLC system equipped with an autosampler and a UV-visible absorbance detector. The ionization parameters were as follows: Positive ion mode; capillary voltage 4000 V, end plate voltage ~500 V; nebulizing gas of nitrogen at 35.0 p.s.i.; and drying gas of 10 l/min nitrogen at 350°C. Mass analyzer scanned from 15 to 1000 u. The MS–MS spectra were recorded in auto-MS–MS mode. The fragmentation amplitude was set to 1.0 V. MS2 data were acquired in both negative and positive mode.

**Extract preparation**

Carob pods were collected from the region of Giza, Egypt, 2014 and kindly authenticated by Prof. Dr. Abd Al Haleem A. Mohamed (Plant Taxonomy and Flora Research Department, Ministry of Agriculture, Giza, Egypt) for whom the authors are thankful. Voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt. The plant material was air washed into powder. One gram of accurately weight carob powder was extracted with 20 mL of methanol at 60°C, on a sonicator for 30 min, three times. Filtrated extracts were combined, evaporated to dryness under reduced pressure, dispersed in 15 mL methanol, passed through 0.45 μm filters, and stored at 4°C.

**RESULTS**

The use of LC/MS allowed the identification and quantification of 36 compounds in the Egyptian carob pods methanolic extract. Peaks assigned were based on their T_r and MS fragmentation patterns. The T_r, ESI-MS^n data and relative percentage of each constituent expressed as percent of dry plant weight were summarized in [Tables 1 and 2] in both negative and positive modes, respectively. The chemical structures of some fragments were presented in [Figure 1].

**DISCUSSION**

**Structure characterization of major constituents by tandem mass spectrometry**

**Structure characterization of the hydroxycinnamoyl derivatives by tandem mass spectrometry**

Two hydroxycinnamoyl derivatives were observed, especially caffeic acid derivatives, that were tentatively identified according to their characteristic ion at m/z 179 ([caffeic acid-H]^−) observed in their MS^n spectra. Peak 1, 25 presented a deprotonated fragment at m/z 341 u corresponding to [hexosyl caffeic acid moiety-H]^− and similar fragmentation pattern, with the loss of 162 u (hexosyl moiety), yielding a peak at m/z 179 u [caffeic acid-H]^−, being assigned as hexosyl caffeic acid derivatives.[10]

**Structure characterization of the flavonoids by tandem mass spectrometry**

Mass spectrometric methods can be used to obtain information on the carbohydrate sequence and the aglycone. Flavonoid aglycones are structurally diverse group of natural products. The most important variations in their structure are in the level of oxygenation (hydroxyl or methoxyl groups) and the point of attachment of ring B (flavonoids and isoflavonoids). When MS^n experiments are performed on instruments with ion trap analyzers, it is possible to perform tandem experiments many times (MS^n) on sequential product ions. From the mass spectra of flavonoid glycosides using MS^n, we can obtain molecular mass, structure of the aglycone (pattern of hydroxylation on aglycone, point of attachment of ring B on ring C), information about acylation of sugar hydroxyl groups, possible methylation or sulphonation of aglycone hydroxyl(s), number of sugar rings, their configuration, and in some cases placement of glycosidic bonds. In structure characterization, we first judged if the flavonoid glycoside is a C-glycosylated or O-glycosylated. The carbon-carbon bond of C-glycosyl flavonoids is resistant to rupture and in C-glycosides mainly the fragmentation of the sugar unit is observed. Losses of 120 and...
HPLC: High-performance liquid chromatography; ESI: Electrospray ionization; MS: Mass spectrometry; \( T_R \): Retention time; LC-MS: Liquid chromatography - mass spectrometry

### Table 1: Compounds identified in the Egyptian carob extract by LC–MS\(^n\) in negative-ion mode

| Peak number | Phenolic compounds                        | \( T_R \) (min) | Area fraction percentage | \([M-H]\) (m/z) | 
|-------------|------------------------------------------|-----------------|--------------------------|----------------| 
| 1           | Hexosycaffic acid derivatives            | 0.9             | 7.9                      | 379.6, 341.0, 216.6, 178.7 | [10] |
| 2           | Monogalloyl dihexose                     | 2.2             | 2.2                      | 493.4, 330.8, 312.9, 282.8, 270.8, 240.8, 210.8, 168.7 | [11,12] |
| 3           | Methylvanigenin-O-pentoside              | 4.3             | 13.7                     | 417.8, 380.9, 328.8, 292.8 | [13] |
| 4           | Methylvanigenin-O-pentoside              | 4.8             | 20.9                     | 417.7, 380.8, 328.8, 292.8, 248.8, 130.7 | [13] |
| 5           | Luteolin-O-hexose                       | 6.4             | 9.2                      | 448.0, 410.9, 340.9, 322.9, 284.7 | [14] |
| 6           | Luteolin-O-hexose                       | 6.7             | 10.6                     | 448.0, 410.9, 340.9, 322.9, 284.7 | [14] |
| 7           | Digalloyl hexose derivative              | 8.6             | 0.4                      | 595.8                      | [3,15] |
| 8           | Digalloyl hexose derivative              | 9.1             | 1                       | 595.7, 538.9, 482.8, 442.9, 330.7, 312.8, 192.8, 168.7 | [3,15] |
| 9           | Digalloyl hexose                        | 9.5             | 0.7                      | 483.8, 422.8, 364.0, 330.8, 312.7, 270.8, 230.8, 210.7, 192.8, 168.8, 150.8 | [3,15] |
| 10          | Quercetin-O-pentoside                    | 10.0            | 1.9                      | 432.6, 415.6, 394.8, 374.5, 328.9, 312.4, 292.8, 149.7, 130.7 | [3-5] |
| 11          | Epigallocatechin gallate                 | 10.9            | 0.6                      | 458.4                      | [5] |
| 12          | Trigalloyl hexose                        | 12.2            | 3.3                      | 635.5                      | [3,15] |
| 13          | Gallic acid derivative                   | 14.0            | 1.5                      | 402.2                      | [3,15] |
| 14          | Quercetin-O-hexose                       | 14.2            | 0.1                      | 464.1                      | [3-5] |
| 15          | O-Pentosyl-C-hexosyl-epigene             | 15.2            | 2.4                      | 564.6                      | [16] |
| 16          | Tetragalloyl hexose                      | 15.5            | 0.7                      | 782.7                      | [3,15] |
| 17          | Myricetin-O-deoxyhexose                  | 15.7            | traces                   | 463.2                      | [3-5] |
| 18          | Myricetin-O-hexose                       | 15.8            | traces                   | 478.9, 460.9, 439.0, 378.6, 340.9, 316.7, 270.8, 259.6, 242.8, 191.7 | [3-5,15] |
| 19          | Quercetin-O-hexose                       | 16.4            | 0.8                      | 463.2                      | [3-5] |
| 20          | Gallic acid derivative                   | 17.7            | 0.3                      | 534.7                      | [3,15] |
| 21          | Epicatechin                              | 18.4            | 1                        | 289.0                      | [17] |
| 22          | Catechin                                | 18.7            | 0.1                      | 289.0, 271.0, 186.9, 178.9, 171.0, 163.0, 152.9, 145.0, 123.0 | [17] |
| 23          | Quercetin-O-deoxyhexose                  | 19.3            | 4.1                      | 448.3                      | [3,4] |
| 24          | Luteolin                                | 22.1            | 0.1                      | 285.1                      | [3-5,15] |
| 25          | Hexosycaffic acid derivatives            | 23.9            | 0.9                      | 548.4                      | [10] |
| 26          | Apigenin-O-glucuronide                   | 44.0            | traces                   | 443.6                      | [18] |

### Table 2: Compounds identified in the Egyptian carob extract by LC–MS\(^n\) in positive-ion mode

| Peak number | Phenolic compounds                        | \( T_R \) (min) | Area fraction percentage | \([M+H]^+\) (m/z) | 
|-------------|------------------------------------------|-----------------|--------------------------|----------------| 
| 1           | Syringic acid derivative                  | 1.2             | 6.2                      | 232.9                      | [3,15] |
| 2           | Naringenin-O-hexose                       | 6.4             | 4                        | 435.1                      | [19] |
| 3           | Quercetin-O-pentoside                     | 6.9             | 3.9                      | 435.1, 413.7, 375.2, 342.2, 303.2, 290.7, 274.0, 256.0, 213.0, 202.9, 183.0, 166.9 | [3,15] |
| 4           | Quercetin                                | 19.3            | 0.9                      | 302.9                      | [3,15] |
| 5           | Kaempferol                               | 22.6            | 0.3                      | 287.3                      | [3,15] |
| 6           | Apigenin                                 | 24.3            | Trace                     | 271.2                      | [3,15] |
| 7           | Theobromine                              | 26.7            | Trace                     | 180.8                      | [3-5] |
| 8           | Chrysoeriol                              | 33.2            | 0.5                      | 301.3                      | [3] |
| 9           | Myricetin                                | 34.7            | Trace                     | 317.4                      | [3,15] |
| 10          | Tricetin dimethyl ether                  | 44.1            | 0.3                      | 331.8                      | [3,15] |

HPLC: High-performance liquid chromatography; ESI: Electrospray ionization; MS: Mass spectrometry; \( T_R \): Retention time; LC-MS: Liquid chromatography - mass spectrometry

90 u were observed, corresponding to cross-ring cleavages in the sugar unit. Fragmentation pathway of O-glycosylated flavonoids start with the cleavage of the glycosidic bonds and elimination of the sugar moieties with charge retention on aglycone. In compounds containing two or more sugars to the same aglycone core, ions arising from the cleavage of the glycosidic bonds between sugar units are weak. Although the aglycone and the glycane were all identifed d, the accurate structure of the flavonoids glycoside could not be always determined because identity and the site of connection of monosaccharide cannot be determined by LC-MS. The structures of compounds were finally identified by comparison with the literature.[22-24] Major diagnostic fragments of flavonoid aglycone identitication 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., 1997. For free aglycone, \(^1\)A and \(^2\)B 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. The possible fragmentation patterns and ion nomenclature of flavonoid glycosides are illustrated on quercetin-O-hexose in Figure 2. For flavonoid glycosides, the classical
nomenclature is proposed by Domon and Castello for glycoconjugates to
denote major fragments: \( X, Y, Z \) represent the ions still containing the
glycone, where \( j \) is the number of inter glycosidic bond broken (counted
from the glycone), and \( k \) and \( l \) denote the cleavage within the
carbohydrate rings.\(^{[7,24-27]}\) Fragmentations of quercetin-O-hexoside and
quercetin-O-deoxyhexoside identif i d in plant extract are shown in
Figures 3 and 4, respectively.

For flavonoid aglycone and its glycosides in the negative mode, the spectra
showed both the deprotonated molecule \([M + H]^-\) of the glycoside and the
ion corresponding to the deprotonated aglycone \([A + H]^-\). The presence
of protonated aglycone \([A + H]^+\) at \(m/z\) 273 demonstrated the presence of
the naringenin, \(m/z\) 301 represented chrysoeriol, and \(m/z\) 331 indicated
tricetin dimethyl ether.\(^{[3-5,13-19]}\)

**Structure characterization of phenolic acids by tandem mass
spectrometry**

In hydrolysable tannins, hydroxyphenolic acids such as gallic acid
can be esterified by D-glucose, yielding gallotannins. Thus, anion of
gallic acid \((m/z\ 169)\) was often found in MS analyses of tannins. Loss of
\((m/z\ 152)\) indicated the presence of galloyl moiety. Moreover, the
presence of syringic acid derivatives was indicated by the presence
of \((m/z\ 198)\).\(^{[3,5,11,12]}\)

**Standard-free relative quantification by peak area
of liquid chromatography and mass spectrometry**

The use of LC/MS allowed the identif i cation and quantif i cation of 36
compounds in the Egyptian carob pods methanolic extract. Twenty-six
compounds were identif i ed in the negative mode corresponding to 85.4% of
plant dry weight [Table 1], while ten compounds were identif i ed in
the positive mode representing 16.1% of plant dry weight [Table 2], with
the prevalence of flavonoids (75.4% of plant dry weight) predominantly
represented by two methylapigenin-O-pentoside isomers (20.9 and
13.7% of plant dry weight) and luteolin-O-hexoside (10.6% of plant
dry weight). On the other hand, phenolic acids (17.3% of plant dry
weight) were present in various forms mainly syringic acid derivative
and trigalloyl hexose (representing 6.2 and 3.3% of plant dry weight, respectively). Two hydroxycinnamoyl derivatives were detected in relatively appreciable amount (8.8% of plant dry weight) and identify d as hexosylcaffeic acid derivatives (7.9 and 0.9% of plant dry weight). Figure 5 illustrates a graphical representation of major constituents in carob pods methanolic extract.

CONCLUSION

The present data shows that LC/ESI-MS/MS is a useful tool for characterization and quantitation of different phenolic compounds present in Egyptian carob pods, which is performed for the first time. The results of this study clarifies that carob pods not only has a high content of phenolic antioxidants, comparable to other Mediterranean foods such as olives but also contains a rich variety of individual components from several classes: flavonoids including; free flavones (0.9% of plant dry weight), glycosylated flavones (56.8% of plant dry weight), free flavonols (2.9% of plant dry weight), glycosylated flavonols (10.8% of plant dry weight), and glycosylated flavanones (4.0% of plant dry weight); in addition to the presence of hydroxycinnamoyl derivatives (caffeic acid derivatives; 8.8% of plant dry weight) and phenolic acid derivatives (gallic acid derivatives; 11.1% of plant dry weight and syringic acid derivatives; 6.2% of plant dry weight).

To date, carob consumption has been based on the pleasant taste of the drink, especially in the Mediterranean region. The identification of various compounds present in carob pods opens a new door to an increased understanding of the different health benefits brought about by the consumption of popular carob and its products.

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Conflicts of interest

There are no conflicts of interest.

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ABOUT AUTHOR

Asmaa Ibrahim Owis, is Lecturer at the Department of Pharmacognosy,
Faculty of Pharmacy, Beni-Suef University, Beni-Suef. The research of interest
is in the area of pharmacognosy, phytochemistry and natural products research.
Additionally, she is the Deputy Director for Quality Assurance Unit, Faculty
of Pharmacy, Beni-Suef University, Beni-Suef. Moreover, she is the Principle
Investigator and Co-PI for two research project, (worth 40 000 Egyptian pounds,
each) from Development Unit of Scientific Research, Beni-Suef University, Beni-
Suef, Egypt.