Dissertation

Submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

Of

The Ruperto-Carola University of Heidelberg, Germany

For the degree of

Doctor of Natural Sciences

Presented by

Ahmad Tahrani, M. Sc. Pharmacy

Born in: Damascus Suburb, Syria

Mass Spectrometric Approaches in Profiling and Monitoring Bioreactivity of Polyphenols In Medicinal Plants

Referees: **Prof. Dr. Michael Wink**

Prof. Dr. Jürgen Reichling

Publications:

1. **Hamdan, D., El-Readi M., Tahrani, A., Herrmann, F., Kaufmann, D., Farrag, N., El-Shazly, A., Wink, M.:** Chemical composition and biological activity of *Citrus jambhiri* Lush. Food Chemistry 127, 394-403 (2011).

2. **Herrmann, F., Hamoud, R., Sporer, F., Tahrani, A., Wink, M.:** Carlina Ocide - A Natural Polyacetylene from *Carlina acaulis* (Asteraceae) with Potent Antritrypanosomal and Antimicrobial Properties, Planta Medica, June 15, DOI http://dx.doi.org/10.1055/s-0031-1279984 (2011).

3. **Mamadalieva, N. Z., El-Readi, M. Z., Janibekov, A. A., Tahrani, A., and Wink, M.:** Phytoecdysteroids of *Silene guntensis* and their *in vitro* Cyototxic and Antioxidant Activity. Zeitschrift für Naturforschung 66c, 215-224 (2011).

4. **Efferth, T., Herrmann, F., Tahrani, A., and Wink, M.:** Cytotoxic activity towards cancer cells of arteanuine B, artemisitene, scopoletin and 1, 8-cineole derived from *Artemisia annua* L. in comparison to artemisinin. Phytomedicine 18, 959-969 (2011).

5. **Hamdan, D., El-Readi, Z., Tahrani, A., Herrmann, F., Kaufmann, D., Farrag, N., E.-Shazly, A., and Wink, M.:** Secondary Metabolites of Ponderosa Lemon (*Citrus pyriformis*) and their Antioxidant, Anti-Inflammatory, and Cytotoxic Activities. Zeitschrift für Naturforschung 66c, 385-393 (2011).

6. **Mamadalieva, N., Z., Herrmann, F., El-Readi, Z., Tahrani, A., Hamoud, R., Egamberdieva, D., Azimova, S., S., Wink, M.:** Flavonoids in *Scutellaria immaculata* and S. *ramosisima* (Lamiaceae) and their biological activity. Journal of Pharmacy and Pharmacology, DOI 10.1111/j.2042-7158.2011.01336.x (2011).

7. **F. Herrmann, F. Sporer, A. Tahrani, M. Wink:** Antitrypanosomal properties of *Panax ginseng* CA Meyer – new possibilities for a remarkable traditional drug! Phytotherapy Research. (Submitted).

8. **Mohamed L. Ashour, Mahmoud Z. El-Readi, Ahmad Tahrani and Michael Wink.** A novel cytotoxic aryltetraline lactone from *Bupleurum marginatum* (Apiaceae). (Submitted).

Acknowledgment:

First of all, I would like to express my deepest gratitude to my first supervisor; Prof. Dr. Michael Wink, for his embracement and patience throughout the period of my staying at IPMB/Biology department, and special thanks to Prof. Dr. Jürgen Reichling for his kindly support as a second supervisor. Also many thanks go to Prof. Dr. Gert Fricker, for introducing me to IPMB/Heidelberg.

I would like to express my highly respect to the following persons for many useful discussions in the field of "Mass Spectrometry"; Prof. Dr. Wolf Dieter Lehmann from DKFZ/Heidelberg, Dr. Jürgen H. Gross from OCI/Heidelberg, Dr. Thomas Ruppert from ZMBH/Heidelberg and to all members of Dr. Johannes Lechner's working group at BZH/Heidelberg.

All IPMB members and especially the lovely family at Biology department I would like to thank you all for the wonderful times.

Last but not least, I would like to express my sincere thankfulness to the families Beutel and Van Massow, to Heidelberg and all Heidelberger for being my second warm home.

And to all, who contributed directly and indirectly to the achievement of this work, those who stand behind the scene, thanks a lot.

At the end I dedicate everything to my parents; Salwa and Hikmat.

Abstract:

Flavonoids are one of the most abundant secondary metabolites (SM) in the nature. They possess a wide range of the biological activity. This work comprises of two chapters, in which the application of mass spectrometry (MS) in fields of phytochemistry and secondary metabolites bioactivity were emphasized.

The first chapter covered the advantages of the hyphenated techniques of LC-MS in profiling flavonoids in some known medicinal plants; *Bupleurum marginatum, Camellia sinensis, Citrus jambhiri*, and *Scutellaria immaculata,* as well as *Scutellaria ramosisima*. LC-MS proved to be a *par excellence* technique providing many attractive features in the profiling of medicinal plant extracts and in the identification of new bioactive polyphenols.

The second chapter covered the applications of ESI-MS in monitoring non-covalent interactions between some polyphenols with different peptides. flavonoid glycosides show an ability to build non-covalent complexes with angiotensin (I) through ionic bonds. The stability of the formed complexes is dependent on the number of sugar residues contributing in the structure of the flavonoid glycoside. On the other hand, flavonoid aglycones exhibit disability to form stable complexes with angiotensin(I). Co-planarity of flavonoid aglycones makes them relatively inflexible and less complaint in forming ionic bonds with biomolecules. One exception is taxifolin, whereas the missing **π** system at $\mathbf{C_2}$-$\mathbf{C_3}$ of ring **C** grants taxifolin more flexibility in comparison to other studied glycones.

Moreover, the quantity of the interacted molecules of the tested flavonoid glycoside will increase, as the number of lysine residues in the targeted peptide increases. Complexes such as 2:1, 3:1 and even 8:1 polyphenol:peptide have been detected. The polyphenol:peptide ratio increases proportionally with the number of the phenolic groups incorporated with the chemical structure of the tested flavonoid, e.g.; EGCG> rutin> hyperoside> scutellarin.

On the other hand, flavonoid aglycones can interact with the backbone amides forming hydrogen bonds, whereas flavonoid glycosides cannot build hydrogen bonds with the backbone amides of insulin. Nevertheless, spiraeoside; 4'-O-glucoside of quercetin, can form hydrogen bonds. Cleavage of the sugar bridge at 4' position occurs spontaneously, and the related aglycone; i.e. quercetin, will be free to undergo the non-covalent interaction.

Zusammenfassung:

Flavonoide sind eine der am häufigsten vorkommenden sekundären Pflanzenstoffe (SM) in der Natur und besitzen eine Menge attraktiver medizinischen Eigenschaften. Diese Arbeit besteht aus zwei Kapiteln, in denen die Anwendungen der Massenspektrometrie (MS) in den Bereichen von Phytochemie und Bioaktivität sekundärer Pflanzenstoffe eingesetzt wurden.

Das erste Kapitel umfasst die Vorteile einer Kopplung-Techniken der LC-MS in der Profilierung von Flavonoiden in manchen bekannten Heilpflanzen; *Bupleurum marginatum*, *Camellia sinensis*, *Citrus jambhiri*, *Scutellaria immaculata*, sowie *Scutellaria ramosisima*.
LC-MS erwies sich als eine *„par excellence"* Technik und bietet viele attraktive Eigenschaften bezüglich der Profilierung von Pflanzenextrakten und die Identifizierung von neuen bioaktiven Polyphenolen.

Das zweite Kapitel umfasst die Anwendungen von ESI-MS in der Beobachtung von nicht-kovalenten Wechselwirkungen einiger Polyphenole mit verschiedenen Peptiden. Flavonoidglykoside besitzen die Fähigkeit, nicht-kovalente Komplexe mit Angiotensin (I) durch ionische Wechselwirkung bilden zu können. Die Stabilität der gebildeten Komplexe ist abhängig von der Anzahl der Zuckerreste, die sich in der Struktur des Flavonoidglykosids befinden. Flavonoidaglykone sind unfähig, stabile Komplexe mit Angiotensin (I) zu bilden. Ihre Planarität macht Flavonoidaglykone weniger flexibel und ist verantwortlich für die schwache ionische Bindung mit Biomolekülen. Eine Ausnahme ist Taxifolin, dessen fehlendes **π**-System bei $\mathbf{C_2}$-$\mathbf{C_3}$ von Ring **C** mehr Flexibilität gewährt, im Vergleich zu anderen untersuchten Flavonoidaglykone.

Die Anzahl der an Komplex-Bildung Moleküle von Flavonoidglykoside nimmt zu, je mehr der Lysin-Reste in der gezielten Peptid sind. Komplexe wie 2:1, 3:1 und sogar 8:1 Polyphenol:Peptid wurden detektiert. Außerdem, die Polyphenol:Peptid Werte erhöhen sich parallel zur zunehmenden Anzahl der phenolischen Gruppen der chemikalischen Struktur.

Auf der anderen Seite, Flavonoidaglykone; in Widerspruch zu ihren Glykosiden, besitzen die Fähigkeit mit Biomolekülen Wasserstoffbrücken zu bilden. Jedenfalls spiraeoside; ein 4'-O-glukosid von Quercetin, kann diese Wasserstoffbrücken bilden. Die Spaltung der glykosidischen Brücke an 4'-Position kann spontan passieren und daher das Aglykon; Quercetin, steht frei um der Wasserstoffbrücken zu unterliegen.

Preface

Medicinal plants and their secondary metabolites (SM) are still providing a fundamental source for bioactive agents in our modern medicine. Moreover, the global interest in medicinal plants is rapidly growing, as a considerable number of the flora in the world is still unexplored. Therefore, researchers were confronted with the necessity to develop new methods and technologies, which are more effective and reliable for the identification, isolation and evaluation of novel chemical entities. One of these methods is Mass spectrometry (MS), which proved to be unavoidable in many scientific inquiries.

One of the most abundant secondary metabolites in nature are polyphenols. They provide an enormous field of interest for many scientific disciplines. The aim of this work is to highlight the impact of MS techniques in medicinal plant researches. This work consists of two chapters, in which the electrospray mass spectrometry (ESI-MS) has been introduced as a powerful tool in the field of drug discovery. In the first chapter the benefits of ESI-MS in the area of phytochemistry, as an analytical tool coupled with high performance liquid chromatography (HPLC), have been presented. LC-MS has been employed successfully in profiling polyphenols in some medicinal plants. Compared with many traditional methods, LC-MS techniques encompass number of features offering many advantages in one single injection, such as; (i) resolving complex samples, (ii) high selectivity in controlling co-eluents, and (iii) possibility to elucidate chemical structures. The second chapter focused on ESI-MS as an alone-standing technique in studying non-covalent interactions between some polyphenols and biomolecules. As a “soft” ionization technique ESI-MS proved to be constructive in studying interactions between the different molecules. These techniques demonstrate versatile attractive advantages, such as; (i) requirement of small quantities of material, (ii) adaptability to high throughput screening (HTS), and (iii) flexibility in experimental designation. This pledges for a continuous implementation of ESI-MS in monitoring non-covalent complexes as an essential platform in modern drug discovery.

Table of Contents

Chapter one

Polyphenols profiling in some medicinal plants using LC-ESI-MS

1.1. Introduction

1.2. Material and methods

Chapter two

ESI-MS in monitoring the bio-reactivity of polyphenols

2.1. Introduction

2.2. Material and Methods

Chapter one

Profiling of Polyphenol Compounds in some Medicinal Plants Using LC-MS

1.1. Introduction:

1.1.1. Secondary metabolites and phytotherapy:

As an evolutionary response plants were obliged to produce and store a wide range of organic molecules. These substances are usually termed as secondary metabolites (SM). Some of these compounds are involved in the survival of the plants as a defense mechanism against natural enemies. Many SM could actively interact with targets in the human body inducing a bioactivity of interest (Wink, 2008c; Wink, 2008a).

Since the early history human beings recognized the curative properties of plants in their surrounding environment and developed different preparations for healing purposes. These experiences form what so-called ethno-medicine, and the practices were then documented through the centuries to build up the traditional medicine.

By modernization of analytical chemistry techniques in the last century the heritage of traditional medicine is then moved from the stage of practices documentation to a methodological and conceptual reorientation to shape up our modern medicine. Since then many phytochemical studies have been carried out to isolate and characterize new SM of biological interest (Harborne, 1998; Wink, 1999). An estimation of isolated naturally occurred secondary metabolites is presented in Table (1.1).

Most of the plant species in the world have not been yet subjected to phytochemical studied for possible biological active constituents. On the other hand, the majority of SM compounds that are identified in medicinal plants show a pleiotropic ability to interact with several targets (multi-target SM) (Wink, 2008a). Therefore, traditional medicine offers promising solutions to face the global increasing demands for new therapeutic agents (Balandrin *et al.*, 1985; Newman *et al.*, 2000). One of the most universal and widespread secondary metabolites in plant kingdom are flavonoids (Daayf and Lattanzio, 2008). The aim of this chapter is to profile and characterize number of bioactive flavonoids in some common medicinal plants by implementation a *state of art* of analytical instruments, namely, High performance liquid chromatography coupled with electrospray mass spectrometry (LC- MS).

Table (1.1) Numbers of known secondary metabolites from higher plants, Wink (2008)(Wink, 2008b)

Type of secondary metabolite	Approximate number of structures
With nitrogen	
Amines	100
Alkaloids	21 000
Nonprotein amino acids	700
Cyanogenic glucosides	60
Glucosinolates	100
Alkamides	150
Lectins, peptides	2000
Without nitrogen	
Monoterpenes	2500
Sesquiterpenes	5000
Diterpenes	2500
Triterpenes, steroids, saponins	5000
Tetraterpenes	500
Polyketides	750
Polyacetylenes, fatty acids	1500
Flavonoids, tannins	4000
Phenylpropanoids, coumarins	2000

1.1.2. Flavonoids:

1.1.2.1. The biological impact of the flavonoids:

Flavonoids represent one of the most familiar and widespread group of substances in plant's kingdom (Daayf and Lattanzio, 2008), and have been proved to possess an immense biological impact on the health of humans. They exert lipid-lowering properties and thus protective effects on the cardiovascular system (Curin and Andriantsitohaina, 2005; Pakalapati *et al.*, 2009), and play an active role as immune-modulators (Deng *et al.*, 2010), anti-inflammatory (Figueirinha *et al.*, 2010), anti-tumor (Ito *et al.*, 1999) and antimicrobial(Weisse *et al.*, 1995) agents. Moreover, isoflavonoids showed encouraging bioactivity in hormonal replacement therapies (HRT) (Pakalapati *et al.*, 2009). Further works

confirmed the ability of chatechins to scavenge radicals and eradicate oxidative stress, thus recommended them as a supportive remedy in anti-aging (Abbas and Wink, 2009), anti-neurodegenerative (Abbas and Wink, 2010) and cancer diseases (Henning *et al.*, 2010). The biological activity of flavonoids are a result of the diversity in the structural confirmations of these compounds, which made them enjoy an tremendous interest in different fields of science such as ecology, biotechnology, and medicine (Hattenschwiler and Vitousek, 2000; Wink, 2010).

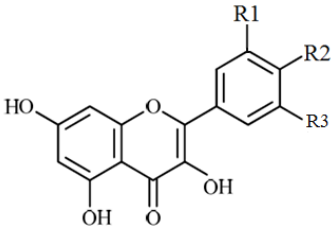
1.1.2.2. Chemical structure and classification of flavonoids:

It is estimated that more than 4000 naturally occurring flavonoids have been identified form higher plants (Wink, 2008b). According to their chemical structure flavonoids could be divided into four key groups, namely, major flavonoids, neoflavonoids, isoflavonoids, and stilbinoids (Baxter and Harborne, 1999). These major groups are then divided into different subgroups as shown in figure (1.1) (Ververidis *et al.*, 2007).

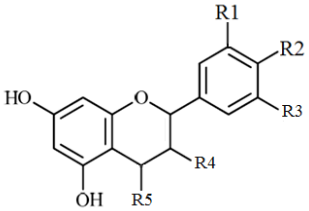
1.1.3.Profiling SM using high performance liquid chromatography (HPLC):

Nowadays, high performance liquid chromatography (HPLC) techniques are the most widely used methods of analysis in the field of phytochemistry. They have been successfully employed in the discovery and identification of new biologically active secondary metabolites (Waksmundzka-Hajnos and Sherma, 2011). HPLC methodologies depend on the concepts of liquid-solid partition, whereas, the analytes are separated according to the differences in their affinity toward a stationary solid phase (column), which is considered the heart of a HPLC system. The analytes will be first retained in the column and then migrate under the force of the mobile phase. This will result in the separation between the different constituents (analytes) of an extract at different elution times, or what so-called retention time (RT).

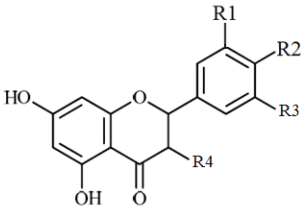
MAJOR FLAVONOIDS



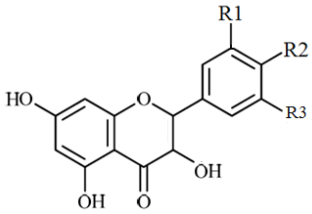
Flavonols



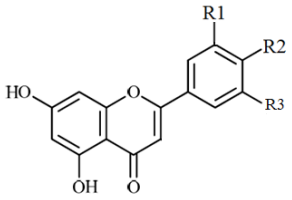
Flavanols



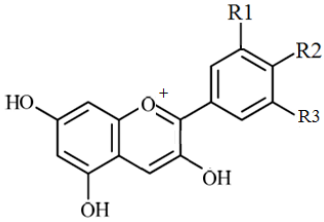
Flavanones



Flavanolols

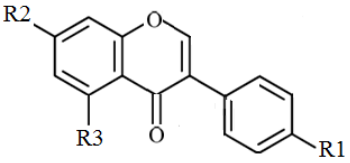


Flavones

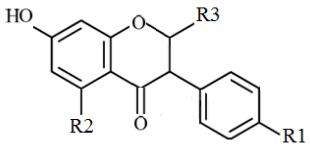


Anthocyanidins

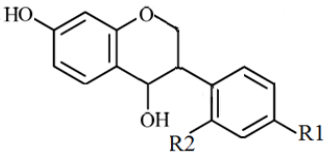
ISOFLAVONOIDS



Isoflavones

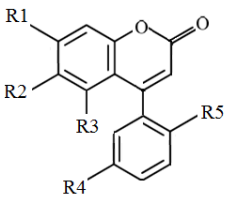


Isoflavanones

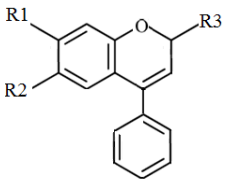


Isoflavanols

NEOFLAVONOIDS

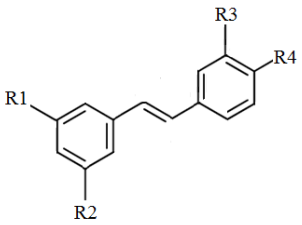


4-Arylcoumarines



Neoflavenes

STILBENOIDS



Stilbenes

Fig. (1.1) Chemical structures of key groups and subgroups of flavonoids.

There are two major concepts which are employed considering HPLC; normal-phase (NP) and reversed-phase (RP). This makes HPLC a tool *par excellence* for its capacity to carry out separation of compounds at wide range of polarities (McMaster, 2007).

1.1.3.1. Normal-phase HPLC:

The normal-phase chromatography (NP-HPLC) is the chromatographic mode which was discovered by Tswett in 1903. In this mode the column is packed with polar stationary phase. In general, NP-HPLC employs an inorganic adsorbent like silica as a polar stationary phase, and non-aqueous solvents for retention modulation. Analytes which are retained in the stationary phase will elute as the mobile phase polarity increases. The NP-HPLC mode was successfully employed in the field of natural product analysis, such as carbohydrates. Also compounds that may decompose in aqueous milieus are usually analyze using NP-HPLC (Snyder *et al.*, 1988; Meyer, 2010). On the other hand, NP-HPLC proved to be useful to control many natural compounds, e.g. phenolic acids, stevioside a sweetener which is extracted from the leaves of *Stevia rebaudiana*, and plumbagin a naphthoquinone derivative found in *Plumbago zeylanica* L. (Gupta *et al.*, 1993; Tateo *et al.*, 1999). Nevertheless, when taking in account the analysis of natural products, then reversed-phase chromatography (RP-HPLC) is often the method of choice, since that NP-HPLC bares many operational difficulties.

1.1.3.2. Reversed-phase HPLC:

More than 25 years are gone when Horvath and Melander estimated that the majority of all analytical separation can be carried out using reversed-phase chromatography (RP-HPLC). In general the analyses in this mode are performed using non-polar stationary phase; i.e. a modified normal phase polar silica, hence, the name reversed-phase (Kazakevich and LoBrutto, 2007). The modification is achieved through covalent bonding of an organic carbon chains to the silica column bed. Octadecylsilane modification or C_{18} columns are the most used kind of reversed-phase stationary phases in the analytical chemistry (Kirkland, 2004).

Figure (1.2) shows the differences in the chemical nature of the column package that is used to pack HPLC columns in both phases, i.e. normal phase, and reversed phase. Unlike the NP chromatography the analytes in this mode will be adsorbed to the non-polar stationary phase and then start to elute as the mobile phase polarity decreases.

Usually this mobile phase is buffered to prevent the interference of ionic interactions during the elution process, since that the separation should be a function of partition between stationary and mobile phase. Ionic interactions as a method of separation is observed in Ion-exchange chromatography (IC) which mainly applied in protein analyses (Small, 1989). There are many other concepts concerning chromatography, but that all lay beyond the objective of this work.

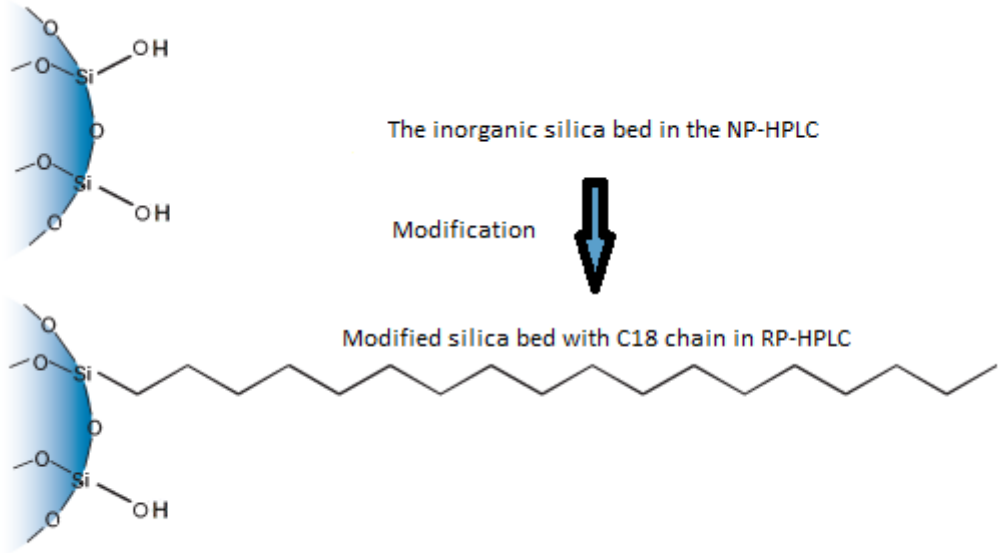


Fig. (1.2) Illustration of inorganic silica bed (Left) used to pack the HPLC columns in both modes; the normal-phase (NP) (Top) and reversed-phase (RP) (Bottom).

RP-HPLC was extensively used in the identification of highly bioactive secondary metabolites from higher plants, such as, mahanine and mahanimbine alkaloids from *Murraya koenigii* (Pandit *et al.*, 2011), ginkgolide A, ginkgolide B, and bilobalide, terpene lactones in *Ginkgo biloba* (Mesbah *et al.*, 2005), some triterpene saponins from *Chenopodium quinoa* (Kuljanabhagavad *et al.*, 2008), some active phenylpropanoids with anti-oxidative properties from *Allium sativum* L. (Ichikawa *et al.*, 2003), and more.

In the case of flavonoids RP-HPLC is still the most utilized technique all over the world. There is a huge body of literatures that reported the detection and characterization of flavonoids from different medicinal plants. For flavonoid profiling in plants extracts RP-HPLC is a straightforward technique that usually does not require complex preliminary sample treatment. In general, when preceding RP-HPLC run, the more polar compounds eluted first, i.e. flavonoid glycosides, then the less polar aglycones will follow.

1.1.3.3. Detection in HPLC:

Detector is the "Eye" for HPLC system. After the separation is accomplished, detection of eluted compounds is achieved depending on the physiochemical properties of these compounds. There are many techniques coupled with HPLC systems, such as UV/VIS and fluorescence spectroscopy, evaporative light scattering detector (ELSD), refractive index, electrochemical, conductivity detectors, and mass spectrometry.

In the field of natural product characterization and identification the UV/Vis detectors have been widely used on a routinely basis in the laboratories all over the world. UV/Vis detectors offer many advantages; they are generally affordable and have a simple instrumental design, amenable to many sorts of method development, and considered as a non-destructive technique, whereas the analyzed sample could be kept for further experimental procedures. Nevertheless, the growing interest in the identification and characterization of new bioactive SM led to the necessity to employ more sensitive and complicated techniques. That was the time when mass spectrometry coupled with HPLC became an inevitable technique in phytochemistry and rapidly adapted as the work-bench in the profiling plants secondary metabolites.

1.1.4. Hyphenated techniques, liquid chromatography mass spectrometry (LC-MS):

At the early stages of the invention of mass spectrometry, the application of such techniques were limited to mass determination and purity of a given sample (Griffiths, 2008). Later on by mid 1980s new MS machines were introduce paving the way to the development of hyphenated techniques, such as liquid chromatography mass spectrometry LC-MS.

By the invention of atmospheric pressure ionization (API) techniques in late 1960s it was then possible to transfer the studied analytes from liquid into gas phase in a form of charged molecules detectable by means of MS analyzers (Clegg and Dole, 1971). Later in 1980s electrospray ionization mass spectrometry (ESI-MS) was invented (Fenn *et al.*, 1989). ESI-MS instrumental design allows the last to be coupled to HLPC systems. Gradually, analysts started to approve the LC-MS technique as chief platform for researches in the field of analytical chemistry.

The mobile phase eluting out of the HPLC system has high pressure comparing to the manner of MS machine, this will distort the high-vacuumed mass spectrometer interface and the MS analysis. The API ionization interfaces were designed to overcome such problem be employing inert nebulizer and drying gas such as N_2. Furthermore, a pressure reducer consist of electrical lenses is utilized to steer analytes from the atmospheric pressure area to the vacuumed MS analyzer (McMaster, 2005). Hence, these MS techniques are called atmospheric pressure ionization or (API). Refer to the figure (2.3), page (69).

MS techniques offer a lot of advantages over other conventional ones. Although being a destructive method, MS in general does not require large amounts of sample, it is a sensitive technique and used to detect impurities at low concentrations (Pelaez *et al.*, 2002). LC-MS techniques provide the analysts not only with the retention time (RT) values, but also with the molecular mass as shown in figure (1.3). Moreover analyzers such as quadrupoles assign the analysis process with more flexibility. It possible by means of what so-called reconstructed ion chromatogram (RIC) to plot a chromatogram obtained from signals belongs; (i) even to a chosen m/z value, (ii) or to a series of values of mass spectra recorded as a function of retention time. This option can be valuable in; (i) fishing out a specific signal of desired analytes from a signal-rich MS spectrum, (ii) studying the existence of isomers, (iii) resolving possible co-eluting analyst in LC-MS runs, and/or (vi) providing more clear LC-MS chromatograms of compounds of interest.

One more striking feature provided by the MS machines is the tandem mass spectrometry (MS^n), which facilitate structural elucidation studies (Busch, 2010). Tandem MS is formed when MS machine consists of a series of analyzers. In this case the analyst can set the first analyzer in the MS machine in order to isolate a specific molecule through what so-called selected ion monitoring (SIM). Afterward this molecule will be guided into a second section and exposed to collision energy that results in the fragmentation of this molecule. The third analyzer will then detect the originated fragments through a function called multiple reaction monitoring (MRM). The fragmentation pattern will then facilitate the structure elucidation of the chosen molecule.

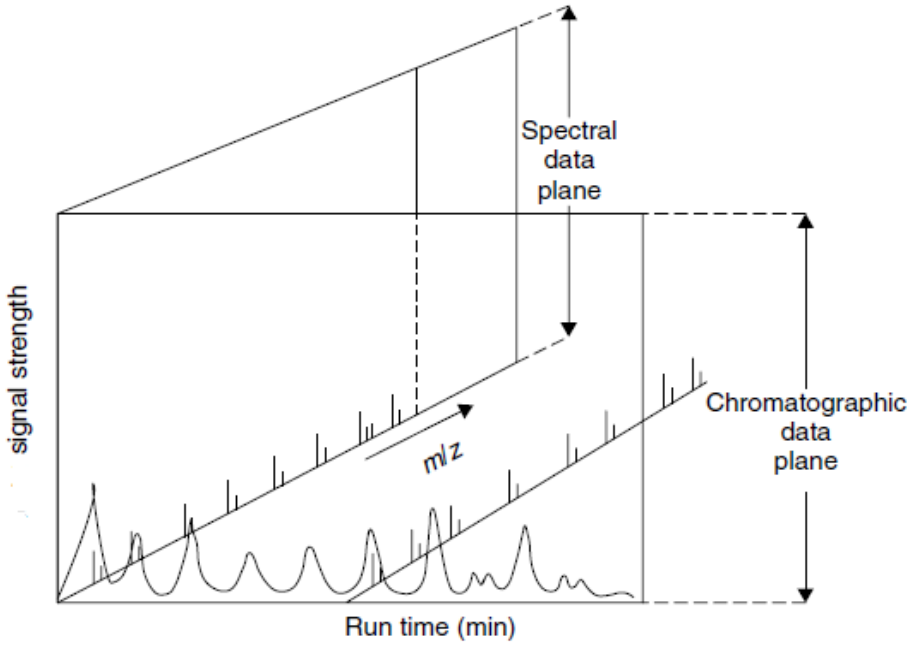


Fig. (1.3) Data obtained from LC-MS could be described as a two dimensional kind of data. It is possible to record the values of retention time for each detected analyte and simultaneously to obtain information about the molecule mass of this analyte (McMaster, 2005).

These days LC-MS is successfully established in the characterization and identification of many bioactive secondary metabolites in medicinal plants, such as the identification of iridoid glycosides in of *Scrophularia nodosa* (Wink *et al.*, 2007), analysis of triterpene glycosides in *Cimicifuga racemosa* (He *et al.*, 2000), characterization of ginsenosides in the root and leaves *Panax quinquefolium* L. (Ligor *et al.*, 2005), fingerprinting of *Psoralea corylifolia* L. (Zhao *et al.*, 2005), and studying phenylpropanoid glycosides in *Tynanthus panurensis* (Plaza *et al.*, 2005).

1.2. Material and methods:

1.2.1. Chemicals and reagents:

For HPLC experiment the following solutions were used: Chromanorm-water, acetonitrile and formic acid (HPLC grades from VWR International GmbH, Bruchsal, Germany). Whereas ammonium acetate salt (Riedel-de Haën, Seelze, Germany), glacial acetic acid (J.T.Baker, Holland) and ammonium hydroxide solution (Fluka, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) were used to prepare the buffered mobile phase and to adjust pH of solutions.

For sample preparation the following solvents (analytical grade) were used: methanol, ethanol, dichloromethane, chloroform, petroleum ether, and ethyl acetate purchased from (Merck, Darmstadt, Germany). Dimethylsulfoxide (DMSO) was obtained from (J.T.Baker, Holland).

Authentic compounds such; rutin, eriocitrin, neoeriocitrin, diosmetin 6-C-glucoside, narirutin, naringin, hesperidin, neohesperidin, limonin, nomilin, and palmitic acid were obtained from university of Heidelberg, IPMB/Biology, Germany. Epigallocatechin gallate (EGCG) was purchased from (sigma-Aldrich Chemie GmbH, Schnelldorf, Germany), and scutellarin from Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany.

1.2.2. Sample preparation:

For sample preparation; a Büchi rotary evaporator system (Büchi, Flawil, Switzerland) consist of Rotavapour R-200, heating bath B- 490, and a B-172 vacuum system, as well as a freeze dryer Alpa IL-6 (Christ, United Kingdom) provided with vacuum concentration centrifuge Univapo 150 H (Uniequip, Planegg, Germany), and a vacuum pumps Edwards 3 (Edwards, Crawley, England) were used. Prior to any LC-MS sample injection, all samples are exposed to BiofugePico centrifuge (Heraeus, Hanau, Germany) at speed of 13000X for 30 seconds to get rid of any suspended particles.

1.2.3. High-performance liquid chromatography (HPLC):

The chromatographic separation carried out by HPLC system consists of:

(L-6200 A) Merck-Hitachi pump (Hitachi, Tokyo, Japan), a (ERC α) degasser (ERC, Japan), and a reversed phase (RP-C_{18}) LiChroCART column [250 X 4 id. mm, 5 µm)] (Merck, Darmstadt, Germany).

1.2.4. Electrospray ionization mass spectrometry (ESI-MS):

MS analyses were performed using a Quattro II system (VG, England) with electrospray ionization (ESI) interface, and a triple-quad quadrupole analyzer, supported with Masslynx V4.0 program from data analysis. An Edwards 28 vacuum pump (Edwards, Crawley, England) was used to generate the high vacuum, and NG7 nitrogen (Burger, Bern, Switzerland) was used to provide the nebulizer and drying gas N_2.

1.3. Polyphenols profiling of some medicinal plants:

1.3.1. *Bupleurum marginatum*:

(This work has been done in cooperation with Dr. M. Ashour from Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany)

1.3.1.1. Introduction:

Bupleurum genus, which belongs to the Apiaceae family or Umbelliferae, grows in southern and southwestern part of China (Ashour *et al.*, 2009). Also known as Chinese thorough wax *Bupleurum* is widely used in the traditional Chinese medicine (TCM) under the name of "Chaihu" (Krapp and Longe, 2001), and officially listed in the Chinese and Japanese Pharmacopoeias in addition to the WHO monographs of the commonly used medicinal plants of China and Korea (Kim and Song, 2011).

Bupleurum marginatum is a perennial, rarely annual or biennial, herb that requires plenty of sun to flourish. The shrubs are bushy and can normally reach height up till one meter. The leaves are long with sickle-shaped. They have generally yellow flowers that appear in summer time. The root Radix bupleuri, which is the used part in the medicinal practices, is pale red with slightly bitter and spicy taste (DerMarderosian and Beutler, 2008).

Based on the practice of TCM, radix bupleuri has been used successfully for more than 2000 years either alone or in combination with other herbs for the treatment of common cold (Van Wyk and Wink, 2004), inflammation (Just *et al.*, 1998; Zu *et al.*, 2007), cancer and fever associated with malaria (Wu, 2005). Also Bupleuri extracts have been reported to have protective properties in treatment of chronic hepatitis (Motoo and Sawabu, 1994; Chiang *et al.*, 2003). Another study delivered on hot-water extract of B. falcatum confirmed an inhibitory activity against ulcerogenesis (Yamada *et al.*, 1991; Matsumoto *et al.*, 2008).

A number of phytochemical studies reported more than 100 compounds (saikosaponins, phenylpropanoids, lignans, coumarins, flavonoids, and sterols) to be isolated from different species of *Bupleurum* genus (Sanchez-Contreras *et al.*, 1998; 2002).

In addition, minor components including, polysaccharides and few alkaloids have been also mentioned in several studies.

LC-ESI/MS was used effectively to profile several saponins in Radix bupleuri, mainly saikosaponins which are reported to be responsible for the broad therapeutic effects of *B. falcatum* (Zhao *et al.*, 2011). Also some sulfated saponins have been isolated from methanolic extract of the aerial parts of *B. rigidum* (Sanchez-Contreras *et al.*, 1998).

Nevertheless, techniques like GC and GC-MS were also successfully employed to identify the occurrence of diverse essential oil, such as α-pinene, β-phellandrene, and various monoterpenes, which have been found in *B. fruticosum* (Martin *et al.*, 1993; Liu *et al.*, 2009), on the other hand the presence of phenylpropanoids was confirmed in other studies (Pistelli *et al.*, 1995; Massanet *et al.*, 1997). Meanwhile some groups were successful in isolating polyacetylenes from the dichloromethane extract of *B. longiradiatum* (Huang *et al.*, 2009), while others could identify some lignans and polyacetylenes from hexane extract of the aerial parts of *B. acutifolium* (Barrero *et al.*, 1999).

Many polyphenols have been isolated from genus *Bupleurum*, and they mostly belong to flavonoids family, such as kaempferol, isorhamnetin or quercetin. Also some other aglycones like apigenin, acacetin, chrysin, luteolin and tamarixetin have been reported (Barrero *et al.*, 1998; Pistelli *et al.*, 2005; Zhang *et al.*, 2007). Flavonoid glycosides are also isolated, whereas rutin is the most common flavonoid. In addition, narcissin, a flavonoid glycoside of isorhamnetin, were found in *B. flavum* and *B. fruticosum* (Pistelli *et al.*, 2005; Pan, 2006).

1.3.1.2. Sample preparation:

The plant material was obtained from Yunnan providence, China, and the DNA barcoding and morphological identification were done at the Botanical Garden, University of Heidelberg. The dried material of aerial parts of *B. marginatum* was grounded and then extracted using maceration with methanol. After filtration the residue was recovered from the methanol phase using rotatory evaporator under reduced pressure. The recovered residue was then reconstituted in DMSO and kept in -20^{o}C.

1.3.1.3. LC-MS parameters:

The chromatographic separation of the methanol extract was carried out by HPLC using a reversed phase C-18 (RP C-18) column. The mobile phase consisted of solvent A; water–formic acid (99.5: 0.5, v/v) and solvent B; acetonitrile. The HPLC binary pump with a flow rate of 1 mL/min was programmed to run the mobile phase as the following:

0–60 min, gradient from 0–50% B; 60– 70 min, gradient from 50–100% B; 70–73 min, isocratic at 100% B; 73–75 min, gradient from 100–0% B; and 75–80 min, isocratic at 100% A.

MS analysis was performed in both negative and positive mode under the following conditions:

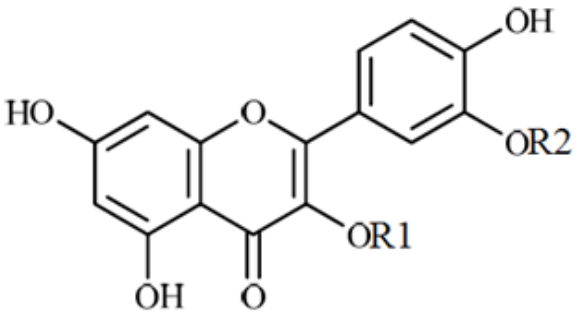
Table (1.2) Tuning parameter of ESI source at both positive and negative mode:

Tuning Parameters	Positive mode ESI(+)	Negative mode ESI(-)
Capillary (kVolts)	3,5	3,00
Cone (Volts)	25	30
Extracting (Volts)	5	5
RF Lens (Volts)	0,2	0,2
Source Temperature	120	120
Dynamic range m/z	200-800	200-800

1.3.1.4. LC-MS profiling of Polyphenols in *Bupleurum marginatum*:

Five different polyphenols, figure (1.4), were identified in the methanol extract of the aerial parts of *B. marginatum* using LC-ESI/MS. Figure (1.5) displays the chromatogram analysis of the methanol extract at the negative mode. The reconstructed MS spectra from the chromatogram revealed the deprotonated molecular-ions $[M-H]^-$ of five flavonoids with the following m/z values; 609, 463, 623, 301, and 315 belong to rutin, isoquercitrin, narcissin, quercetin, and isorhamnetin, respectively. On the other hand figure (1.6) displays the chromatogram analysis at the positive mode, and the reconstructed MS spectra revealed only four compounds; rutin, narcissin, quercetin, and isorhamnetin.

The protonated molecular-ions $[M+H]^+$ of these polyphenol had the m/z values of 611, 625, 303, and 317, respectively. Only isoquercitrin was not detectable under the positive ionization mode. Table (1.3) shows the identified compounds with the values of the detected molecular-ions at both negative and positive ionization modes.



	R1	R2
Quercetin	H	H
Isoquercetrin	glucose	H
Rutin	rutinose	H
Isorhamnetin	H	CH3
Narcissin	rutinose	CH3

Fig.(1.4) The chemical structure of the flavonoids isolated and identified in the methanol extract of *Bupleurum marginatum* aerial parts.

The MS data that obtained from the negative ionization mode revealed that the methanol extract contains small amounts of isoquercitrin. Meanwhile the MS data obtained from the positive ionization mode showed no clear signal of isoquercitrin. Thus, ESI at the negative ionization mode is more sensitive in detecting polyphenols and has lower limits of detection. Meanwhile, many flavonoids show low sensitivity in the positive mode of analysis. This could be explained by the fact that polyphenols possess the tendency to form phenolate ions; therefore the formation of a deprotonated molecular-ion is favorable over the protonated ones. Figure (1.7) represents the molecular-ions of isoquercitrin obtained from combined data of LC-MS at the relevent retention time in the negative ionization mode.

Table (1.3) Identified compounds with the values of the detected molecular-ions at both negative and positive ionization modes

Compound	Molecular weight	$[M-H]^-$	$[M+H]^+$
Rutin	610	609	611
Isoquercitrin	464	463	465
Narcissin	625	623	625
Quercetin	302	301	303
Isorhamnetin	316	315	317

The LC-MS analyses reveal that the methanol extract of the aerial parts of B. marginatum is rich of quercetin. The molecular-ions of quercetin obtained from combining the data of the chromatograms are shown in figures (1.8) and (1.9). Quercetin is reported to be the most abundant flavonoid aglycone in the *Bupleurum* genus (Pan, 2006). The bioactivity of the methanol extract as an anti-inflammatory by the inhibition of COX-2 and LOX enzymes could be due to quercetin (Rathee *et al.*, 2009).

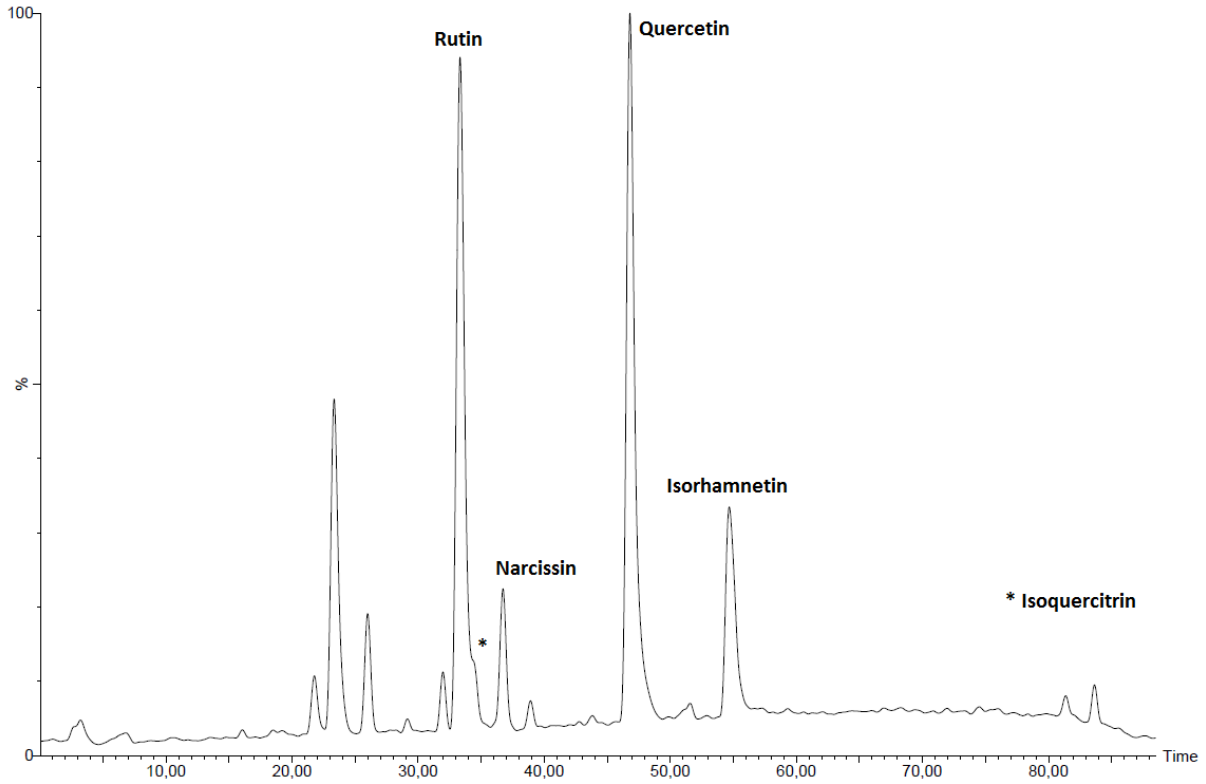


Fig. (1.5) LC-ESI/MS chromatogram of the methanol extract of *Bupleurum marginatum* aerial part at the negative ionization mode.

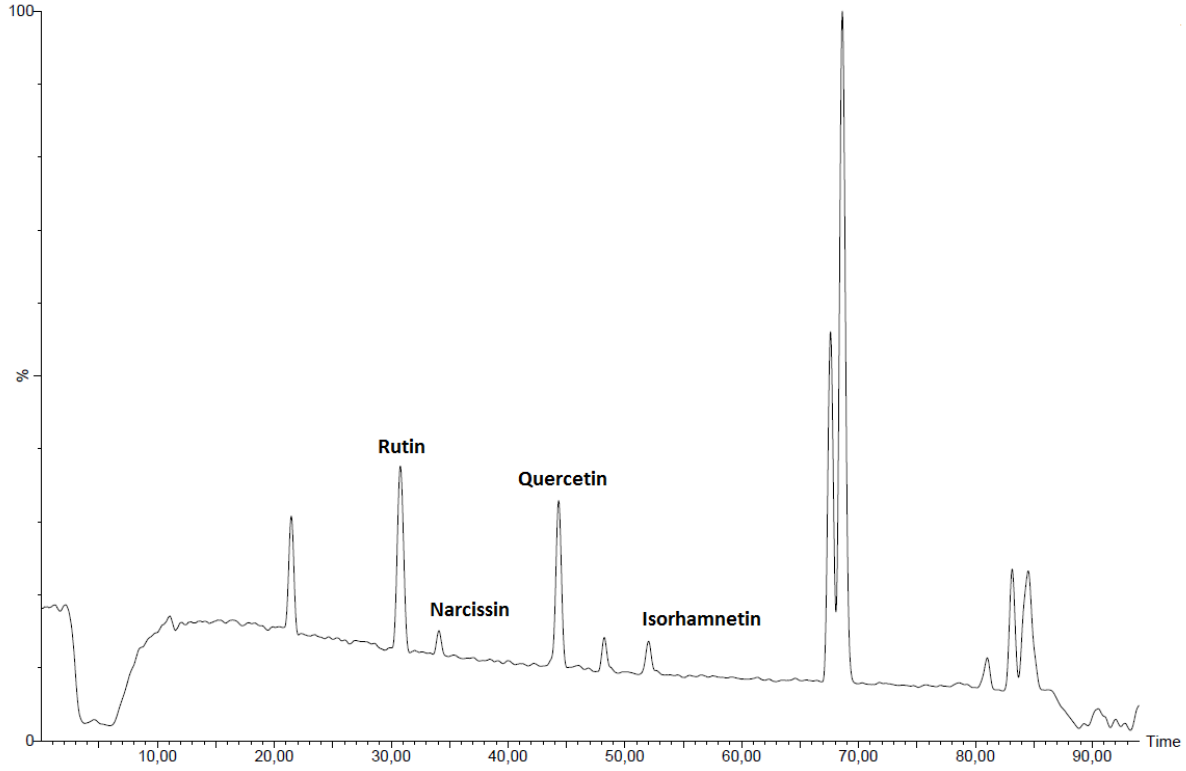


Fig. (1.6) LC-ESI/MS chromatogram of the methanol extract of *Bupleurum marginatum* aerial part at the positive ionization mode.

Another flavonoid found abundantly in the methanol extract was rutin. The molecular-ions obtained for rutin at both negative and positive ion mode are shown in figure (1.10) and figure (1.11), respectively. Rutin, a quercetin-3-O-rutinoside, is reported in earlier studies to be found in the *Bupleurum* genus (Pistelli *et al.*, 2005; Zhang *et al.*, 2007). Like many of flavonoid compounds, rutin has a wide range of biological activity. For example, its effect as anti-hemorrhagic in the treatment of venous insufficiency (Christie *et al.*, 2001). At the positive ionization mode rutin tends to lose the sugar residues in two steps, thus it is possible to detect at the positive mode the intact rutin, one glucopyranosyl fragment, and the quercetin aglycone figure (1.11). Whereas, the negative ionization mode is a softer mode and only the intact rutin is detectable figure (1.10).

Isorhamnetin is an O-methylated flavonol and also was reported to be found in the *Bupleurum* genus. Figures (1.12) and (1.13) represent the molecular-ions obtained from combined data of LC-MS, at both negative and positive modes respectively. Another flavonoid glycoside of rutinoside found in the methanol extract of the aerial parts of *B. marginatum* is narcissin. Narcissin is isorhamnetin-3-rutinoside and found in both B. *flavum* and B. *fruticosum* (Pistelli *et al.*, 2005; Pan, 2006). MS spectra of narcissin at both negative and positive ionization modes are presented in figures (1.14) and (1.15), respectively.

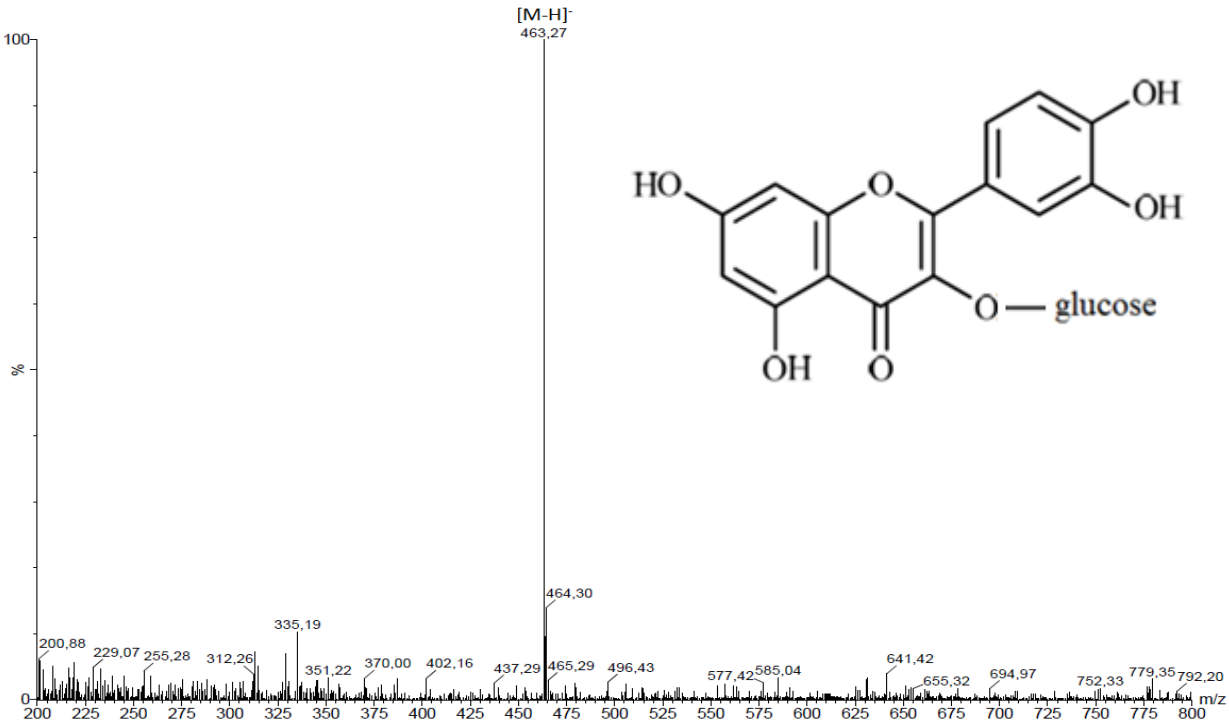


Fig. (1.7) molecular-ion of isoquercitrin at the MS negative mode. Isoquercitrin with a molecular weight of 464 will lose a proton to bear a negative charge and m/z value of 463.

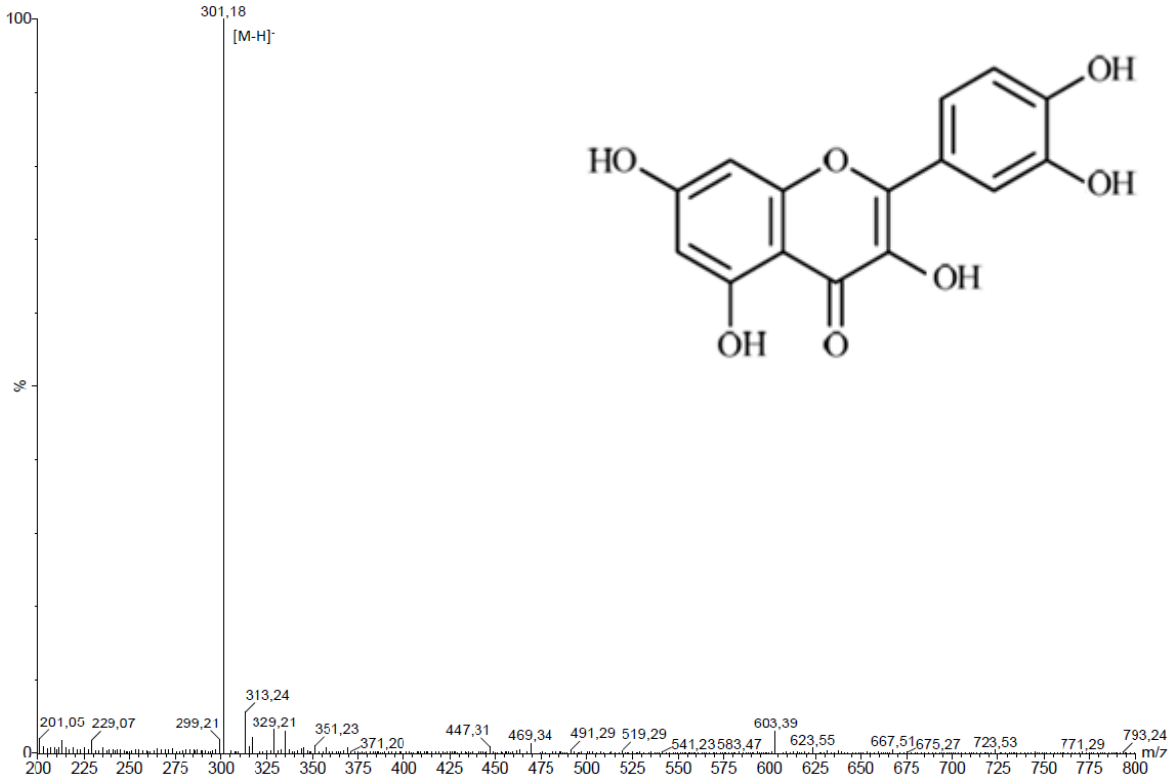


Fig.(1.8) molecular-ion of quercetin at the MS negative mode. Quercetin with a molecular weight of 302 will lose a proton to bear a negative charge and m/z value of 301.

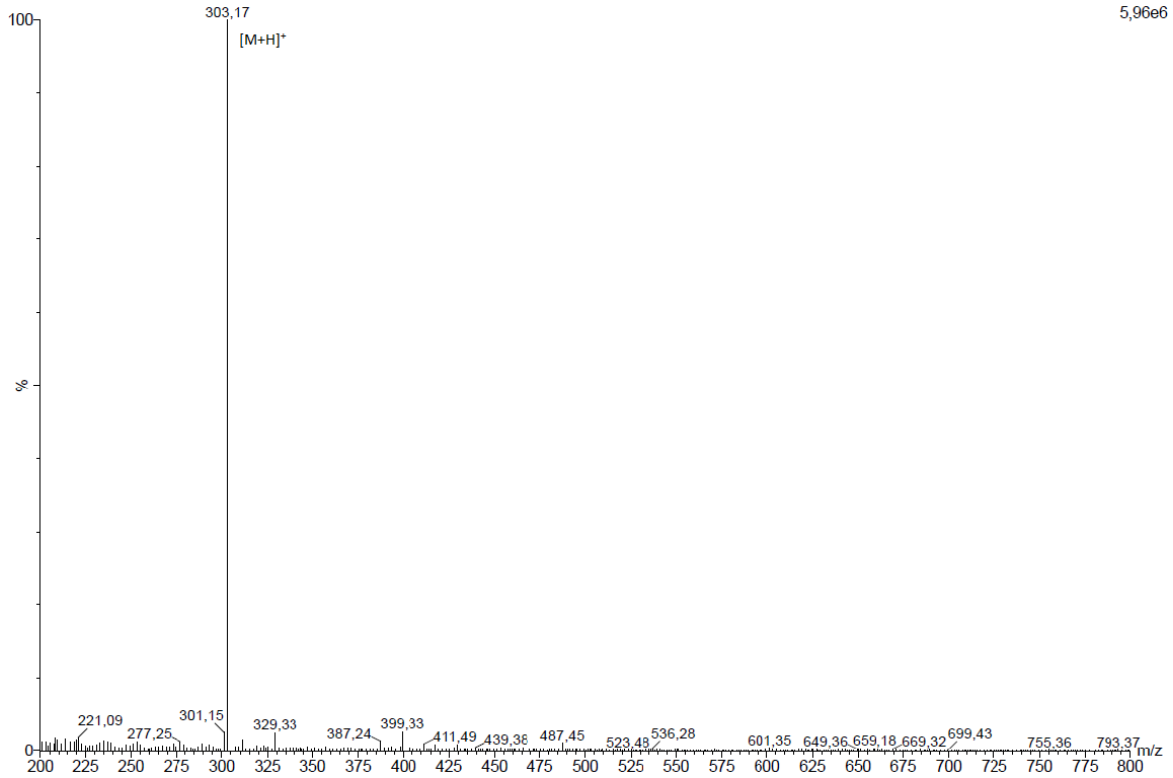


Fig.(1.9) molecular-ion of quercetin at the MS positive mode. In this mode quercetin will be protonated to bear a positive charge and a mass of 303 that is one atomic mass unite greater than its molecular weight 302.

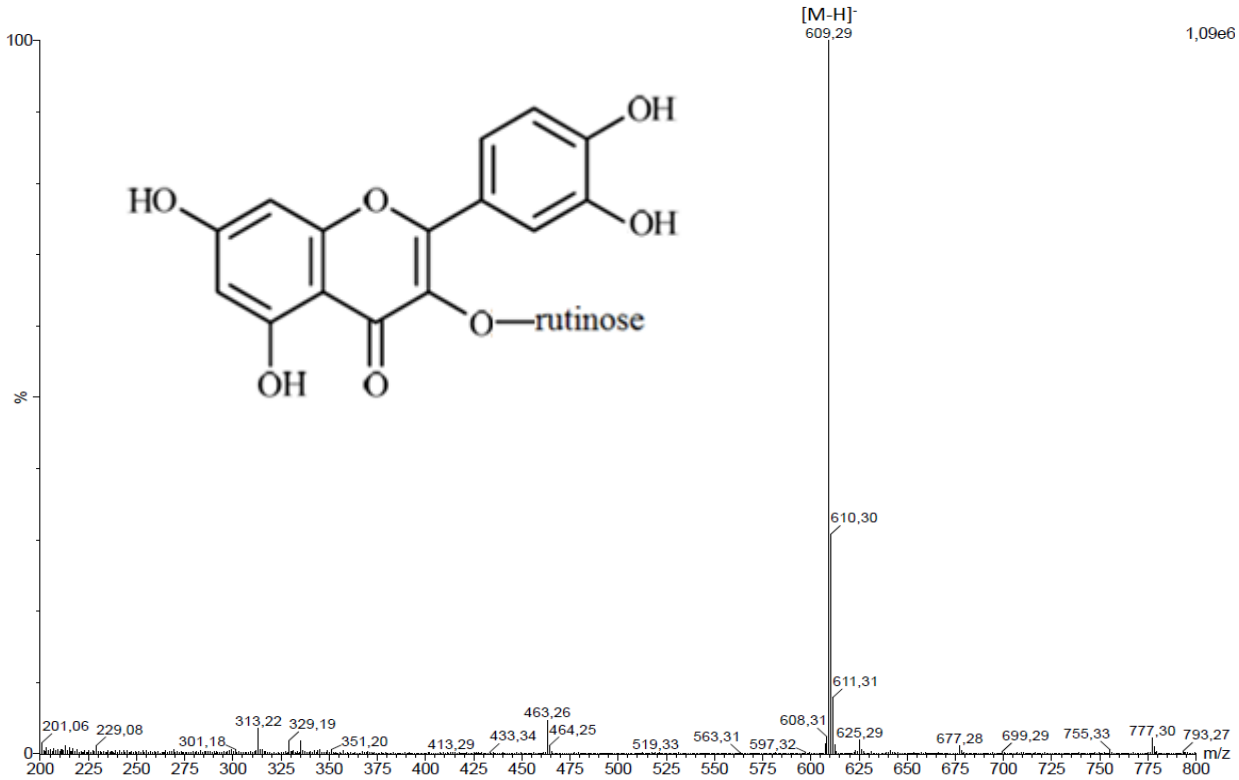


Fig.(1.10) molecular-ion of rutin at the negative mode. Rutin with a molecular weight of 610 will lose a proton to bear a negative charge and m/z value of 609.

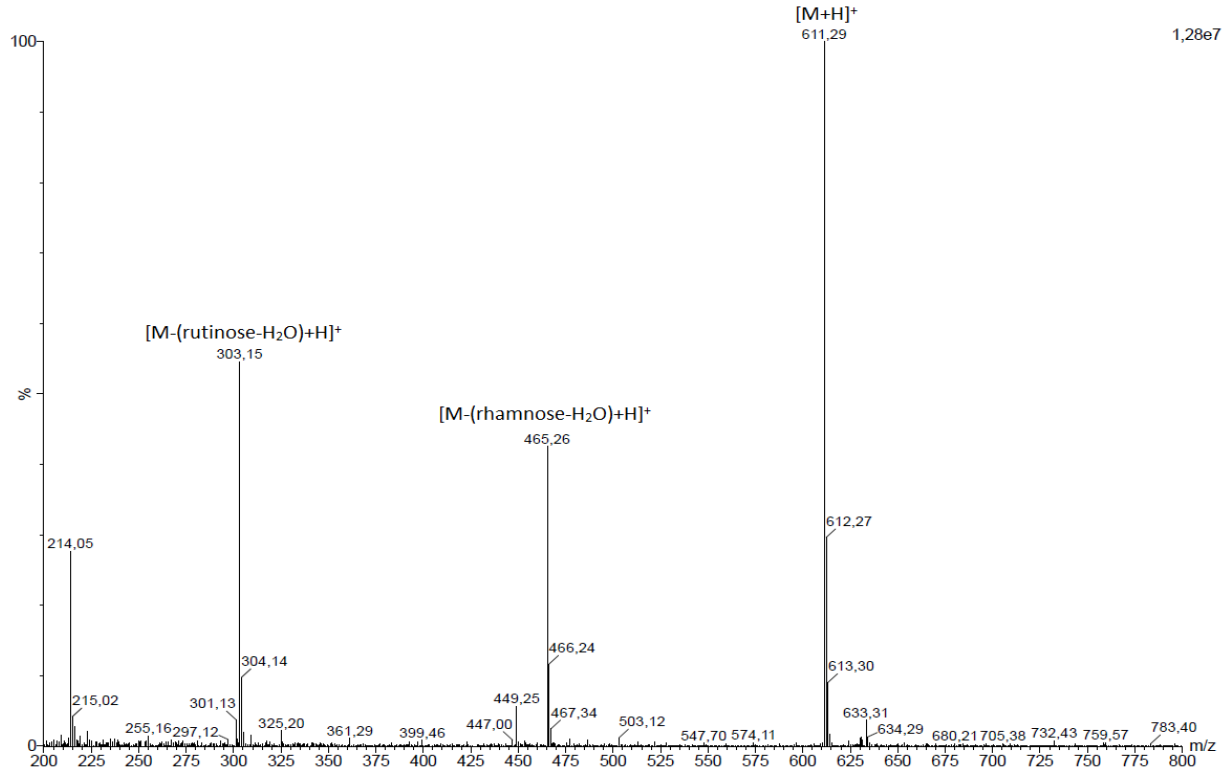


Fig.(1.11) MS(+) spectrum of rutin. The sugar bridges will cleave leading to form of two fragments, one is a mono-sugar residue, and other is the aglycone quercetin.

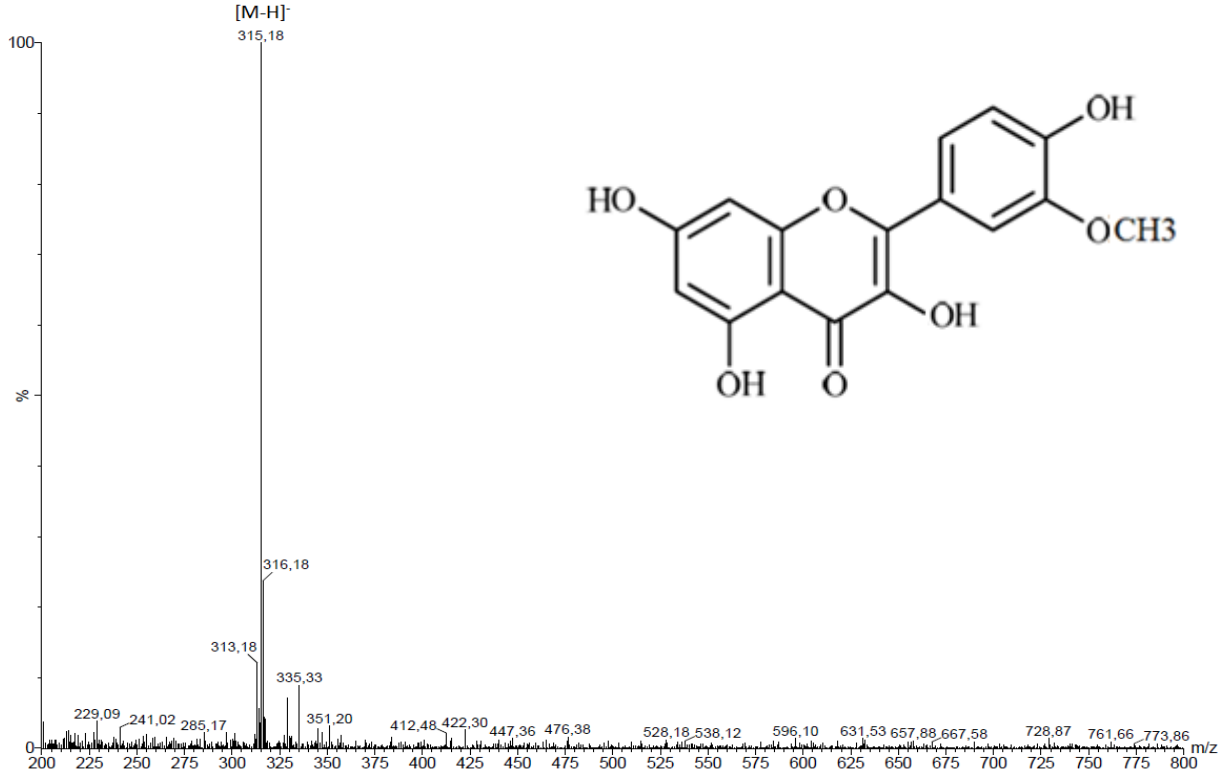


Fig.(1.12) molecular-ion of isorhamnetin at MS(-). Isorhamnetin with a molecular weight of 316 will lose a proton to bear a negative charge and m/z value of 315.

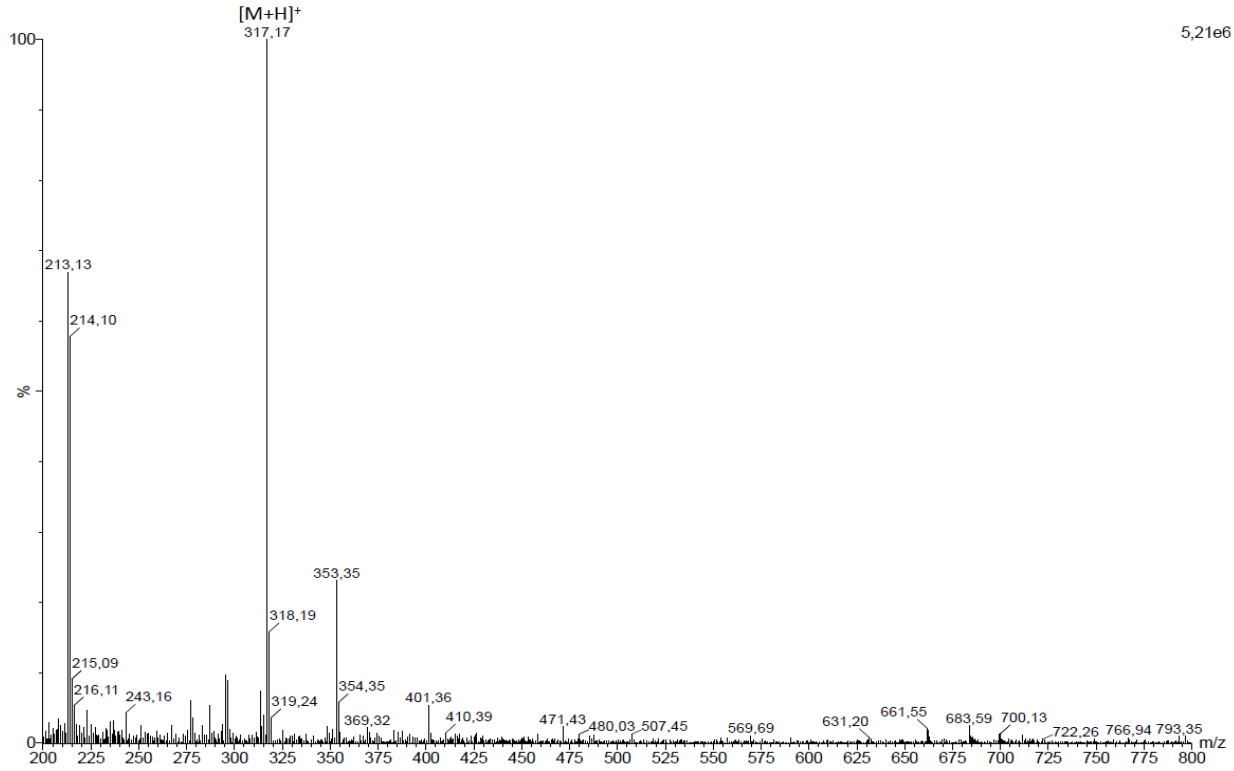


Fig.(1.13) MS(+) spectrum of isorhamnetin. The molecular weight of isorhamnetin is 316 g/mole. In this mode it will gain a proton to have m/z value of 317.

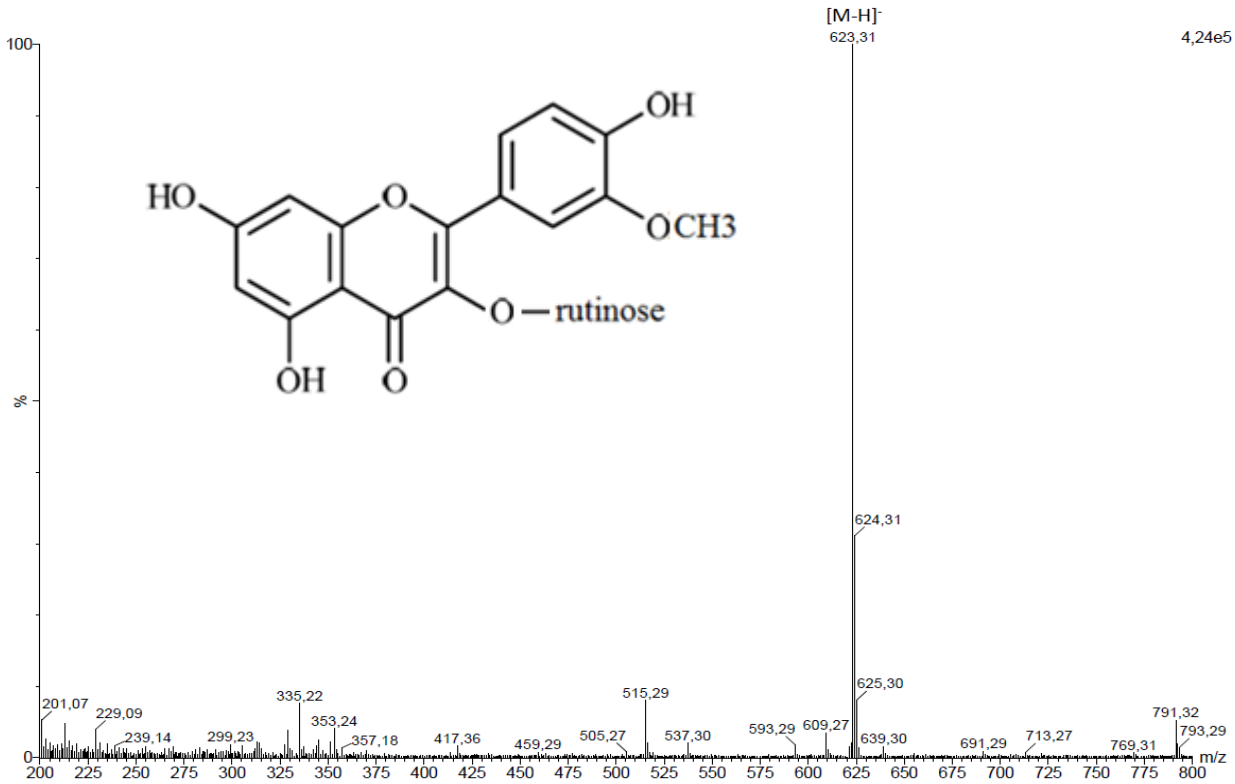


Fig. (1.14) MS(-) spectrum of narcissin. The molecular weight of narcissin is 624 g/mole. In the negative mode narcissin will be deprotonated to show m/z value of 623.

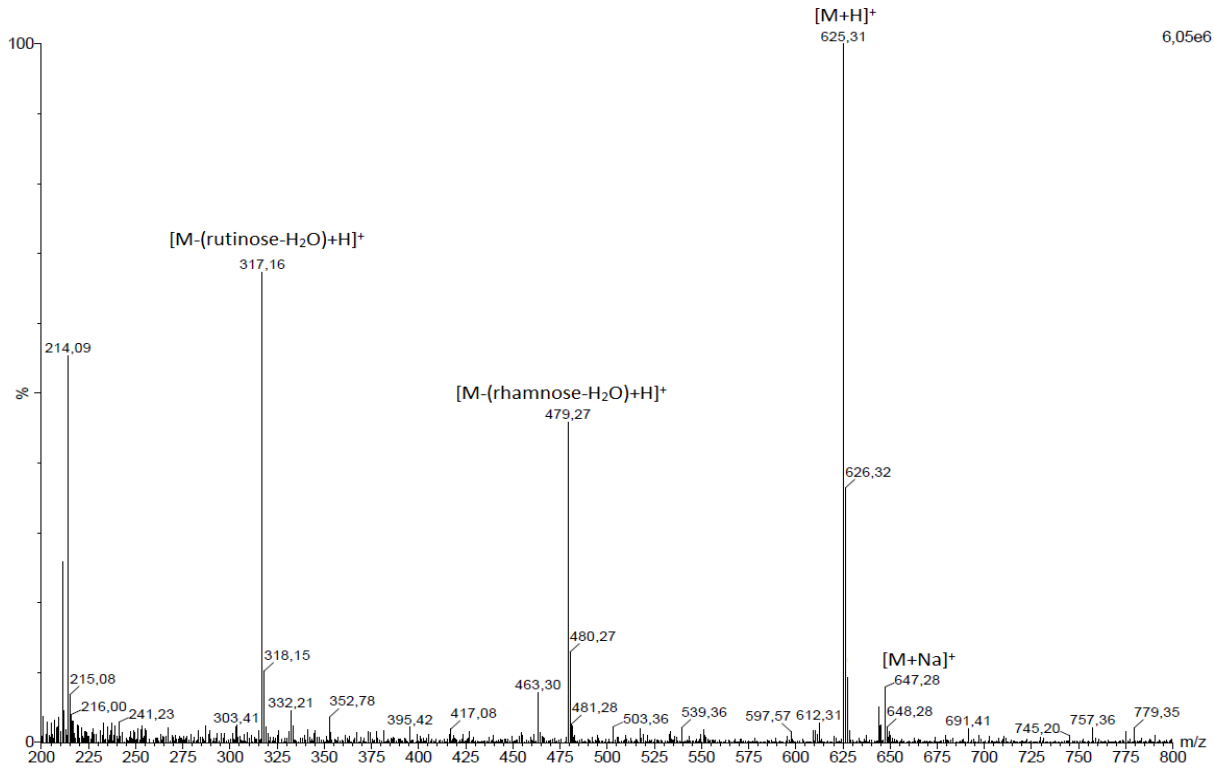


Fig.(1.15) MS(+) spectrum of narcissin. The sugar bridges will cleave leading to the formation of two fragments, one is a mono-sugar residue, and other is the aglycone isorhamnetin.

In general phenolic compounds are important secondary metabolites that present in genus *Bupleurum*. These compounds have a wide range of biological activity. Because of the huge system of phenolic groups they are able interact to different bio-targets (e.g. lipoxygenases and cyclooxygenases enzymes which are targets of anti-inflammatory drugs) through non-covalent bonds leading to functional changes, thus, inducing therapeutically effects (Wink, 2008). Furthermore, polyphenols are known to have anti-oxidant and radical scavenging properties, thus, they play a role in the prevention against cell- and DNA-damaging diseases like cancer, atherosclerosis, and neurodegenerative ones.

1.3.2. *Scutellaria immaculata* and *Scutellaria ramosissima*:

(This work has been done in cooperation with Dr. N. Mamadalieva from Institute of the Chemistry of Plant Substances AS RUz, Tashkent, Uzbekistan)

1.3.2.1. Introduction:

Skullcap is a name that refers to many plants of genus *Scutellaria*. Belongs to the family Lamiaceae, *Scutellaria* is a perennial herb native to North America and eastern Asia. In herbal medicine the name skullcap refers to *Scutellaria lateriflora* and the used parts are leaves, flowers, and stems (Fundukian, 2009).

The genus *Scutellaria* contains more than 360 species, many of them have medicinal impact on humans, and widely used in TCM. Different secondary metabolites have been isolated from *Scutellaria* species such as essential oils, iridoids, diterpenes, triterpenes, alkaloids, phytosterols, polysaccharides, as well as polyphenols.

Scutellaria species are reported to show bioactivity in treatment of neurological disorders, and inflammatory diseases. Other species are proved to have anti-viral and anti-bacterial properties (Parajuli *et al.*, 2009). In other studies *Scutellaria baicalensis* demonstrates an anti-trypanosomal activity (Yabu *et al.*, 1998; Schinella *et al.*, 2002).

Flavonoids which are abundant in *Scutellaria* genus are responsible for many pharmacological effects. The most commonly reported flavonoids from this genus are baicalein, baicalin, wogonin and wogonoside. Despite that these compounds relatively exhibit low cytotoxicity, but they still show an anti-tumor effects due to their properties in scavenging oxygen radicals, reducing NF-kappaB activity, and inhibiting several genes, which are important for the regulation of cell cycle (Li-Weber, 2009). Baicalein and baicalin also have a protective activity preventing tissue damage caused by reactive oxygen species (ROS) (Gao *et al.*, 1999). Also it has been reported that Baicalein inhibits HIV-1 reverse transcriptase (Li *et al.*, 2000).

1.3.2.2. Sample preparation:

The aerial parts and roots of *S. ramosissima* and *S. immaculata* were collected from Tashkent and Namangan region of Uzbekistan, respectively, at flowering stage. The plant material (root and aerial parts) was dried at room temperature before grounded to a fine powder with. Three different extracts were papered by using the following solvents; water, methanol, and chloroform, by over-night maceration of 100 g of plant material with 500 ml of solvent. The solvents were evaporated in a rotary vacuum evaporator at 40 °C. The extracts were then kept in +4 ^{o}C for further use.

1.3.2.3. LC-MS parameters:

The final concentration of all samples was 20 µg in 1 mL methanol. Separation was carried out using a RP-C18 end-capped column. The mobile phase consisted of A: water HPLC grade with 0.5 % formic acid, B: acetonitrile.

The mobile phase was delivered into the column in a gradient manner as the following:

1. for methanol and chloroform fractions: 0 % to 50 % B in 50 min., then to 100 % in 5 min.
2. for water fraction: from 0 % to 25 % in 50 min., then to 100 % in 5 min.

To improve the MS analysis a mechanical T splitter is build prior to the MS machine allowing only 10% of the eluted mobile phase to enter the ionization interface.

MS analyses were set using ESI interface in negative mode of ionization under the following parameters: Drying and nebulizing gas (N_2). Capillary temperature 120 °C. Capillary voltage, 3.00 kV. Lens voltage, 0.5 kV. Cone voltage 30 V. Full scan mode in mass range m/z 200-1000.

1.3.2.4. LC-MS profiling of Polyphenols in *S. immaculata* and *S. ramosissima*:

Scutellaria species are known to be rich of flavonoids and they have a lot of important curative applications in the traditional medicine in China and Japan (Sonoda *et al.*, 2004).

Flavonoids in the *Scutellaria* species demonstrate many biological activities. It is reported that flavonoids in *Scutellaria* have an anti-inflammatory activities (Liaw *et al.*, 1999), and also

able to inhibit cell proliferation and induce apoptosis in several cancer cells (Chan *et al.*, 2000).

The LC-ESI/MS spectra reflect that *S. immaculata* and *S. ramosissima* species are rich with of different kind of flavonoid compounds. Table (1.4) summarizes the flavonoids which are identified in *S. immaculata* and *S. ramosissima* extracts using LC-ESI/MS at negative ionization mode.

Many of these flavonoids, namely, chrysin-7-O-glucuronide, scutellarein-7-O-glucoside, apigenin-7-O-glucoside, baicalein-7-O-glucoside, norwogonin-7-O-glucoside, oroxyloside, wogonoside, immaculoside, 5,2'-dihydroxy-6,7,6'-trimethoxyflavanone, 5,2'-dihydroxy-6,7,8,6'-tetramethoxyflavanone, chrysin, wogonin, apigenin, isoscutellarein, scutellarein, cosmosiin (apigenin-7-O-β-D-glucopyranoside), and wogonin-7-O-β-D-glucopyranoside have been already reported in *S. immaculata* (Yuldashev *et al.*, 1992; Malikov and Yuldashev, 2002; Yuldashev and Karimov, 2005).

On the other hand studies carried out on *S. ramosissima* have accounted the following flavonoids; chrysin 7-O-β-D-glucuronide, 2(S)-2',5,7-trihydroxyflavanone 7-O-(Me-β-D-glucopyranosiduronate), 2(S)-2',5,7-trihydroxyflavanone7-O-(Et-β-D-glucopyranosiduronate), 5,2'-dihydroxy-7-O-β-D-glucopyranosylflavone, rivularin, 5,2'-dihydroxy 7-O-β-D-glucopyranosylflavanone, oroxylin A, wogonin, norwogonin, 5,2',6'-trihydroxy-6,7,8-trimethoxyflavone, and 5,6-dihydroxy-7,8-dimethoxyflavone (Yuldashev *et al.*, 1992; Yuldashev *et al.*, 1994; Yuldashev *et al.*, 1995). Meanwhile others were identified for the first time in our labs. Figures (1.16-1.18) show the LC-MS chromatograms of studied samples obtained from *S. immaculata* and *S. ramosissima*.

Table (1.4) Identification of flavonoids in *Scutellaria immaculata* and *Scutellaria ramosissima* by LC-MS at the negative ionization mode

Peak	$[M-H]^-_{m/z}$	Compounds
1	345	Unknown
2	431	Unknown
3	341	Unknown
4	547	Chrysin-6-arabinosyl-8-C-glucoside
5	623	Isorhamnetin-7-O-rha-glu
6	623	Rhamnetin-7-O-rha-glu
7	637	Unknown
8	461	Scutellarin
9	445	Baicalin
10	345	5,7,2`,5`-Tetrahydroxy-8,6`-dimethoxy flavone
11	445	Oroxylin A-7-*O*-glucoside
12	447	5,6,7-Trihydroxy flavanone(dihydroxybaicalein)-7-*O*-glucuronide
13	445	Norwogonin-7-*O*-glucuronide
14+15	429+459	Chrysin-7-*O*-glucuronide + oroxylin A-7-*O*-glucuronide
16	459	Wogonin-7-*O*-glucuronide
17	431	Unknown
18	329	Unknown
19	327	Unknown
20	359	Unknown
21+22	269 + 299	Norwogonin + 5,7,3-trihydroxy-4`-methoxyflavone
23+24	269 + 299	Baicalein +5,7,4`-trihydroxy-8-methoxyflavone
25	283	Wogonin
26	253	5,7-Dihydroxyflavone (chrysin)
27	343	5,2`-Dihydroxy-6,7,8-trimethoxyflavone

The LC-MS analyses confirmed that *S. immaculata* and *S. ramosissima* extracts contain a plentiful of flavonoids with a structural variety. Therefore, the *Scutellaria* genus enjoys a wide spectrum of biological activity. The number and the position of phenolic groups as well as other functional groups determine the nature of flavonoid bioactivity. The bioactivity can vary from being anti-oxidative (Bors *et al.*, 1990; Rice-Evans *et al.*, 1996), anti-inflammatory, anti-proliferative, and/or enzyme modulative (Agullo *et al.*, 1996; Agullo *et al.*, 1997; Gamet-Payrastre *et al.*, 1999; Sato *et al.*, 2002). Figure (1.19) illustrates the different flavonoids and their structural variety that is found in *S. immaculata* and *S. ramosissima* extracts, which explain the wide spectrum of biological activity of *Scutellaria* genus.

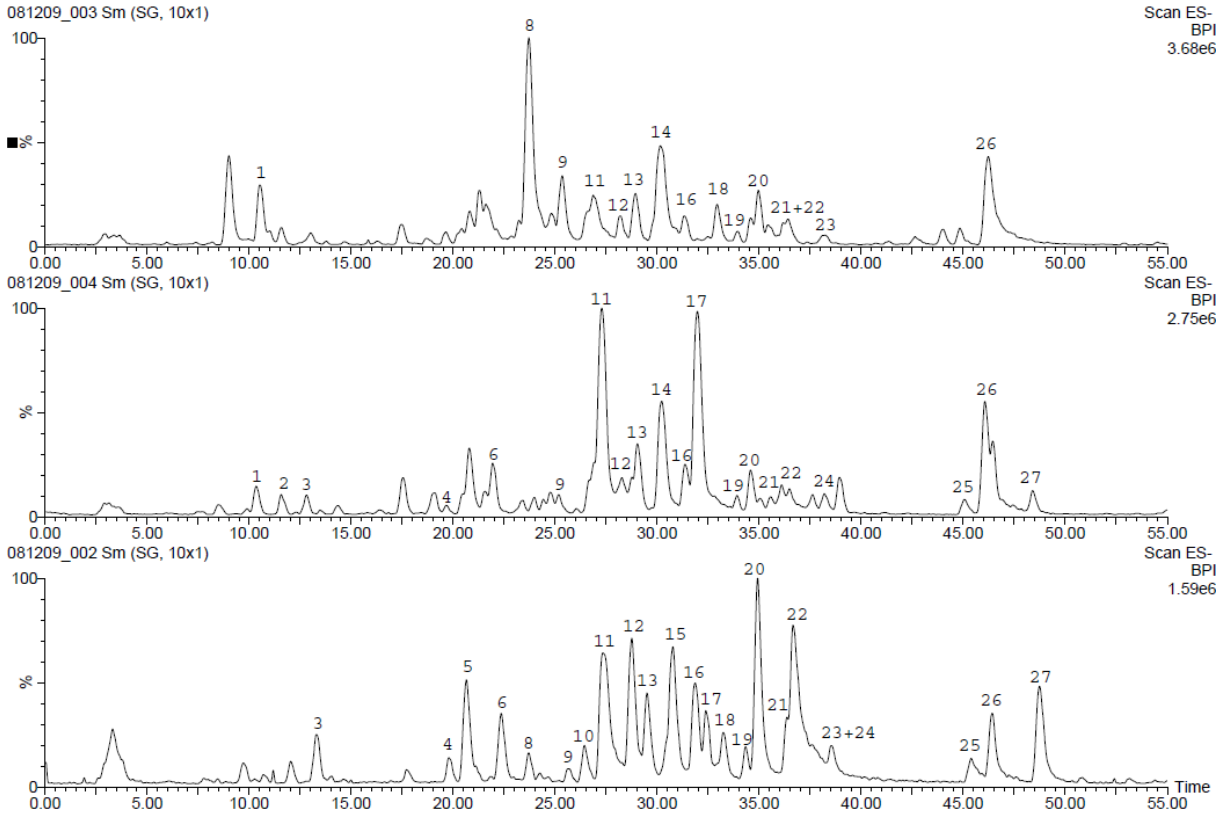


Fig.(1.16) LC-MS of methanol extracts. **a.** *S. immaculata* aerial parts. **b.** *S. ramosissima* aerial parts. **c.** *S. ramosissima* roots.

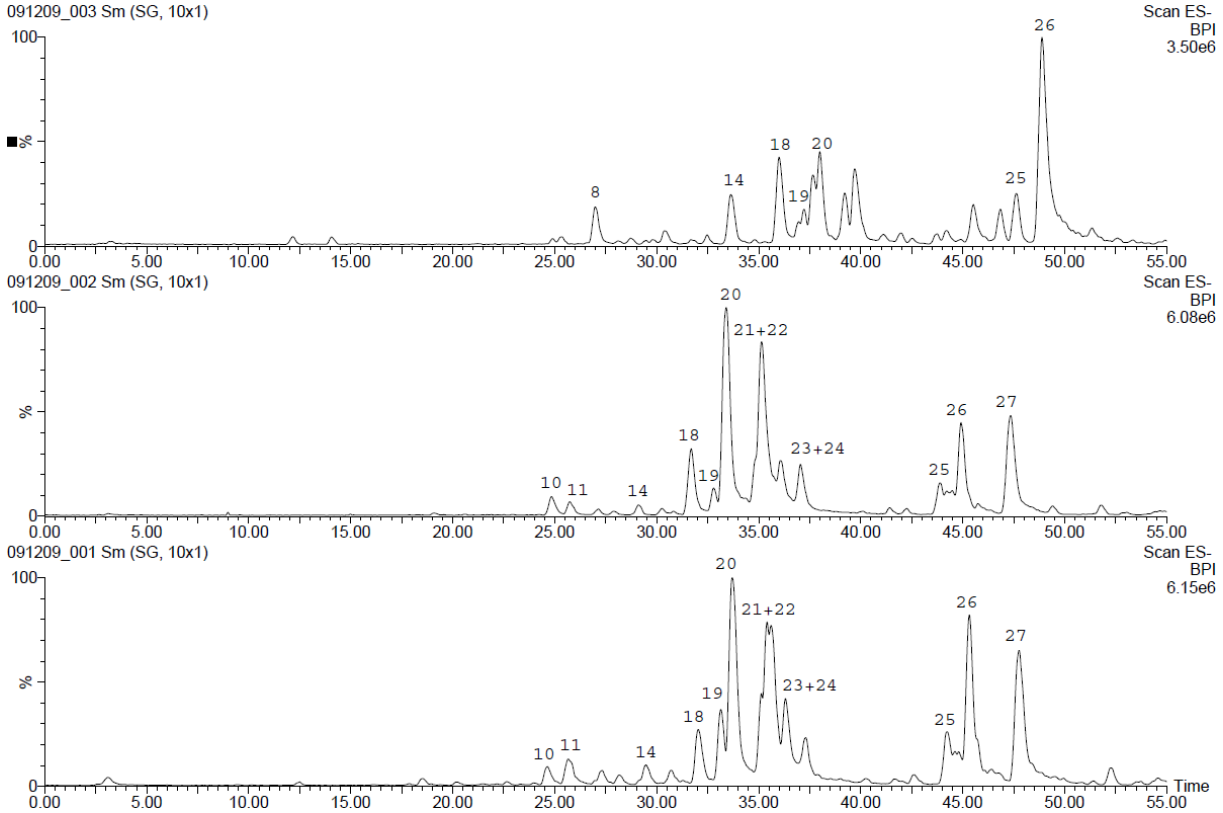


Fig.(1.17) LC-MS of chloroform extracts. **a.** *S. immaculata* aerial parts. **b.** *S. ramosissima* roots. **c.** *S. ramosissima* aerial parts.

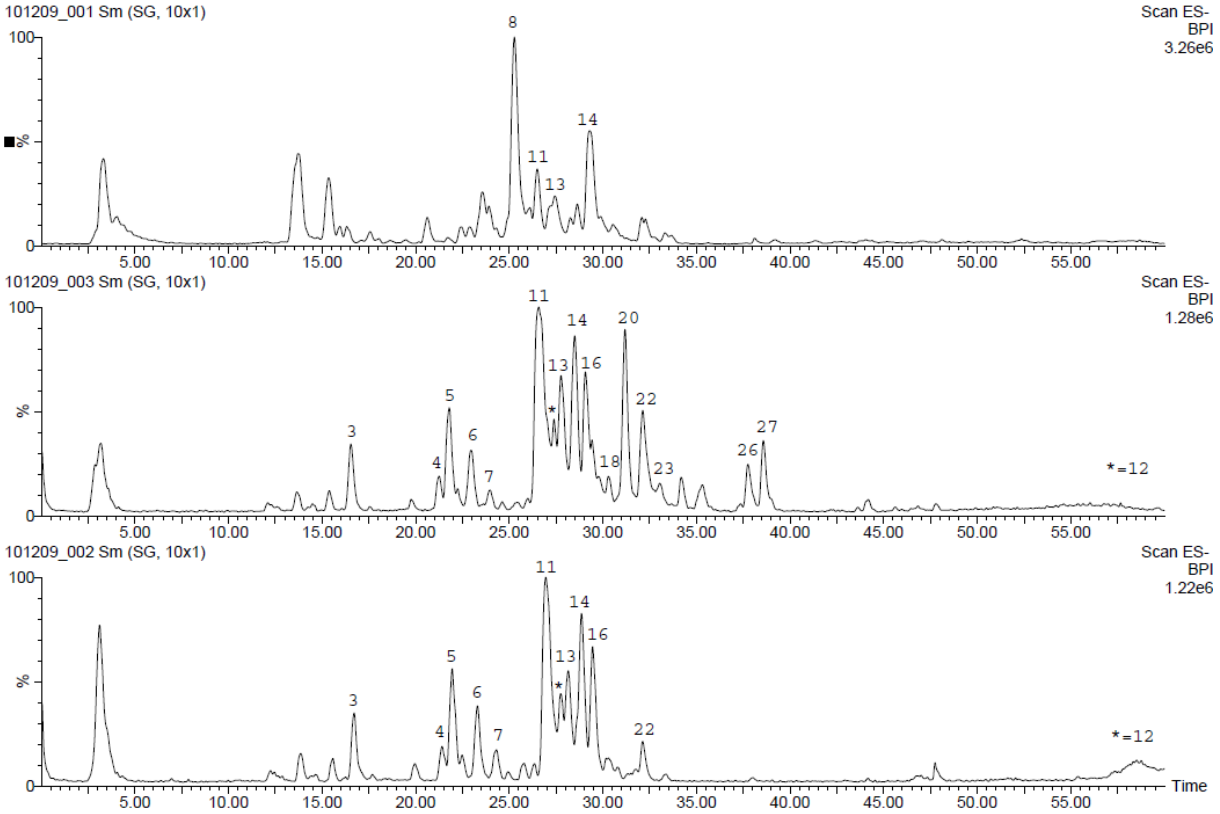
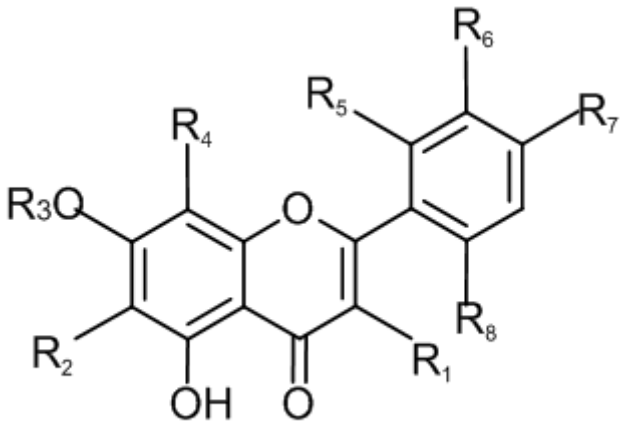


Fig.(1.18) LC-MS of water extracts. **a.** *S. immaculata* aerial parts. **b**. *S. ramosissima* roots.
c. *S. ramosissima* aerial parts



	Substance	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
4	Chrysin-6-arabinosyl-8-C-glucoside	H	Ara	H	Glu	H	H	H	H
5	Isorhamnetin-7-O-rha-glu	OH	H	Rha-Glu	H	H	OCH_3	OH	H
6	Rhamnetin-7-O-rha-glu	Rha-Glu	H	OCH_3	H	H	OH	OH	H
8	Scutellarin	H	OH	Glu acid	H	H	H	OH	H
9	Baicalin	H	OH	Glu acid	H	H	H	H	H
10	5,7,2`,5`-Tetrahydroxy-8,6`-dimethoxy flavones	H	H	H	OCH_3	OCH_3	OH	H	OH
11	Oroxylin A-7-O-glucoside	H	OCH_3	Glu	H	H	H	H	H
13	Norwogonin-7-O-glucuronide	H	H	Glu acid	OH	H	H	H	H
14	Chrysin-7-O-glucuronide	H	H	Glu acid	H	H	H	H	H
15	Oroxylin A-7-O-glucuronide	H	OCH_3	Glu acid	H	H	H	H	H
16	Wogonin-7-O-glucuronide	H	H	Glu acid	OCH_3	H	H	H	H
21	Norwogonin	H	H	H	OH	H	H	H	H
22	5,7,3-Trihydroxy-4`-methoxyflavone	OH	H	H	H	H	H	OCH_3	H
23	Baicalein	H	OH	H	H	H	H	H	H
24	5,7,4`-Trihydroxy-8-methoxyflavone	H	H	H	OCH_3	H	H	OH	H
25	Wogonin	H	H	H	OCH_3	H	H	H	H
26	Chrysin	H	H	H	H	H	H	H	H
27	5,2`-Dihydroxy-6,7,8-trimethoxyflavone	H	OCH_3	OCH_3	OCH_3	OH	H	H	H
28	Apigenin	H	H	H	H	H	H	OH	H
29	Apigenin-7-*O*-glucoside	H	H	Glu	H	H	H	OH	H
30	Cynaroside	H	H	Glu	H	H	OH	OH	H

Fig. (1.19) chemical structures of flavonoids that were identified using LC-MS at the negative ionization mode.

1.3.3. *Citrus jambhiri*:

(This work has been done in cooperation with Dr. D. Hamdan from department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt)

1.3.3.1. Introduction:

Citrus fruits, belonging to *Citrus* genus of family Rutaceae, occupy the top of the list in fruits industry. The nutritional and medicinal benefits of *Citrus* fruits have been recognized very early in the history. Many studies have demonstrated the medicinal benefits of genus *Citrus* such as cholesterol-lowering, cardio-protective, anti-carcinogenic, anti-viral, anti-bacterial and anti-fungal effects (Miller *et al.*, 2004; Manners, 2007; Tripoli *et al.*, 2007).

As well as being an important source of ascorbic acid (Vitamin C.) *Citrus* fruits are also significantly rich of bioactive secondary metabolites, e.g. carotenoids, flavonoids, limonoids, essential oils, alkaloids. Significant amounts of highly oxygenated triterpenoid compounds called limonoids have been reported (Ladaniya, 2008). Also coumarins have been isolated and detected from some *Citrus* species, such as aurapten, bergapten, and psoralin (Sulistyowati *et al.*, 1990; Ogawa *et al.*, 2000).

Among the known secondary metabolites that found in genus *Citrus,* flavonoids attracted a significant interest in the field of phytochemistry for their potential ecological, biological and chemotaxonomic impacts (Manners, 2007; Tripoli *et al.*, 2007; Ladaniya, 2008). *Citrus* fruits and *Citrus* juices are one of most universal dietary sources of phenolic compounds (Ting and Rouseff, 1986; Aherne and O'Brien, 2002; Erlund, 2004). The most considerable harvested *Citrus* fruits in the world are oranges, mandarins, grapefruits, lemons, bergamots and limes(Gattuso *et al.*, 2007).

1.3.3.2. Sample preparation:

The fresh peel of *Citrus jambhiri* Lush was extracted three times with 80% aqueous methanol. The methanol extracts were then filtered and concentrated under vacuum. The residue were reconstituted in water and partitioned against light petroleum, chloroform and

ethyl acetate. The samples was then evaporated under vacuum and kept at -20^{o}C for further analyses. Prior to LC-MS analyses a stock solution from 10 mg ethyl acetate fraction is dissolved in 1 mL DMSO, afterward 100 µL from the stock solution was diluted with 500 µL 50% aqueous acetonitrile containing 2% formic acid.

Available authentic reference compounds such as hesperidin, neohesperidin, naringin, and rutin were dissolved in DMSO and the stock solutions (10 mg/mL) were stored at 4^{o} C. Prior to injection in LC-MS system, the stock solutions were diluted with 50% aqueous acetonitrile containing 2% formic acid.

1.3.3.3. LC-MS parameters:

The chromatographic separation of the ethyl acetate fraction was achieved using a reversed phase C-18 column (RP C-18) and a mobile phase consisted of solvent A; water–formic acid (99.5: 0.5, v/v) and solvent B; acetonitrile.

The elution was carried out at a flow rate of 1 Ml/min. as the following: 0–60 min, gradient from 0–25% B; 60–62.5 min, gradient from 25–50% B; 62.5–70 min, isocratic at 50% B; 70–77 min, gradient from 50–100% B; and 77–87 min, isocratic at 100% B.

To improve the MS analysis a mechanical T splitter is build prior to the MS machine allowing only 10% of the eluted mobile phase to enter the ionization interface.
The MS analysis was performed under the following parameters: acquisition mode, ESI negative; nebulizer gas, N_2, 0.25 l/min; capillary, 3.00 kV, HV lens –0.50 kV; cone, – 35 V; source temp., 120^{o} C; and mass scan range 200–800 m/z.

1.3.3.4. LC-MS profiling of Polyphenols in *Citrus jambhiri Lush*:

The identification of the flavonoid compounds after HPLC separation is usually carried out using a photo diode array detector (PDA). After the invention of MS machines with atmospheric pressure ionization techniques (API), it was then possible to couple the HPLC

with MS and to determine the molecular mass of the detected compound, adding an extra dimension to the analytical procedure and facilitating the identification processes (Seeram *et al.*, 2006; Zhou *et al.*, 2006).

Liquid chromatography coupled with mass spectrometry, or so-called LC/MS, proved to be highly beneficial in the characterization and identification of flavonoids related compounds especially in genus *Citrus* (Careri *et al.*, 1999; Dugo *et al.*, 2005; Gattuso *et al.*, 2007; Shi *et al.*, 2007). Figure (1.20) shows the LC-ESI/MS at the negative ionization mode of the ethyl acetate fraction.

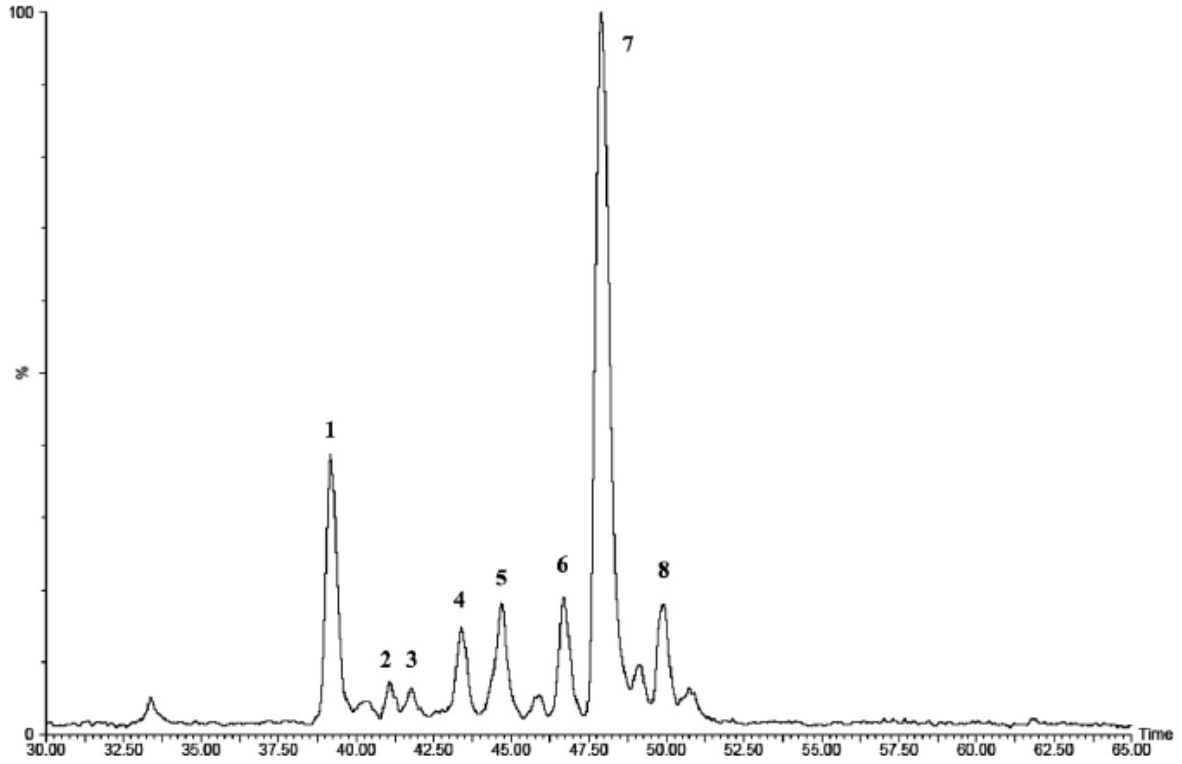
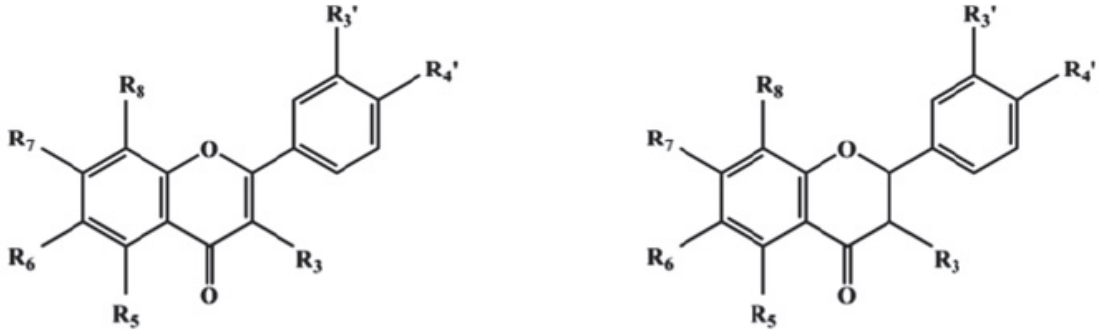


Fig. (1.20) LC–ESI/MS (negative ion mode) of the ethyl acetate fraction from the peel of *Citrus jambhiri*. Eriocitrin (1), neoeriocitrin (2), rutin (3), diosmetin 6-C-glucoside (4), narirutin (5), naringin (6), hesperidin (7), and neohesperidin (8).

A total of eight flavonoid glycosides figure (1.21); eriocitrin, neoeriocitrin, rutin, diosmetin 6-C-glucoside, narirutin, naringin, hesperidin and neohesperidin were identified. Table (1.5) summarizes the m/z values and the relative retention time (RRT) of the detected flavonoids in the ethyl acetate fraction of *Citrus jambhiri* peels.

The relative retention time was calculated in relation to hesperidin. The identification is achieved by the comparison with some authentic standards, and also with the help of literature reviews (Cappiello *et al.*, 1999; Cuyckens *et al.*, 2000).



	Compound	R_3	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
1	Diosmetin 6-C-glucoside	H	OH	Glu	OH	H	OH	OH
2	Hesperidin	H	OH	H	Rut	H	OH	OCH_3
3	Neohesperidin	H	OH	H	Neo	H	OH	OCH_3
4	Narirutin	H	OH	H	Rut	H	H	OH
5	Naringin	H	OH	H	Neo	H	H	OH
6	Eriocitrin	H	OH	H	Rut	H	OH	OH
7	Neoeriocitrin	H	OH	H	Neo	H	OH	OH
8	Rutin	ORut	OH	H	OH	H	OH	OH

Rut = rutinoside
Neo = neohesperidoside

Fig.(1.21) Chemical structure of the identified flavonoid glycosides in the ethyl acetate fraction of *Citrus jambhiri* peel.

LC-MS is a soft ionization technique and applied to obtain the total ion account (TIC) chromatograms and the related MS spectra of each peak. Figures (1.22-1.29) represent the spectra which are obtained from chromatogram shown in chromatogram figure (1.20), and reflect the MS data of each peak in addition to the molecular-ion mass of each phenolic compound separated using the HPLC method.

Table (1.5) the identification of the flavonoids in the ethyl acetate fraction of *Citrus jambhiri* peels using LC-ESI/MS at the negative ionization mode:

	Compound	Observed RRT	Reported RRT	$[M-H]^-$	MW
1	**Eriocitrin**	1.26	1.25	595/287	596
2	**Neoeriocitrin**	1.33	1.32	595	596
3	**Rutin**	1.36	1.40	609/301	610
4	**Diosmetin 6-C-glucoside**	1.41	1.42	461	462
5	**Narirutin**	1.45	1.45	579/271	580
6	**Naringin**	1.52	1.53	579	580
7	**Hesperidin**	1.56	1.56	609/301	610
8	**Neohesperidin**	1.63	1.67	609	610

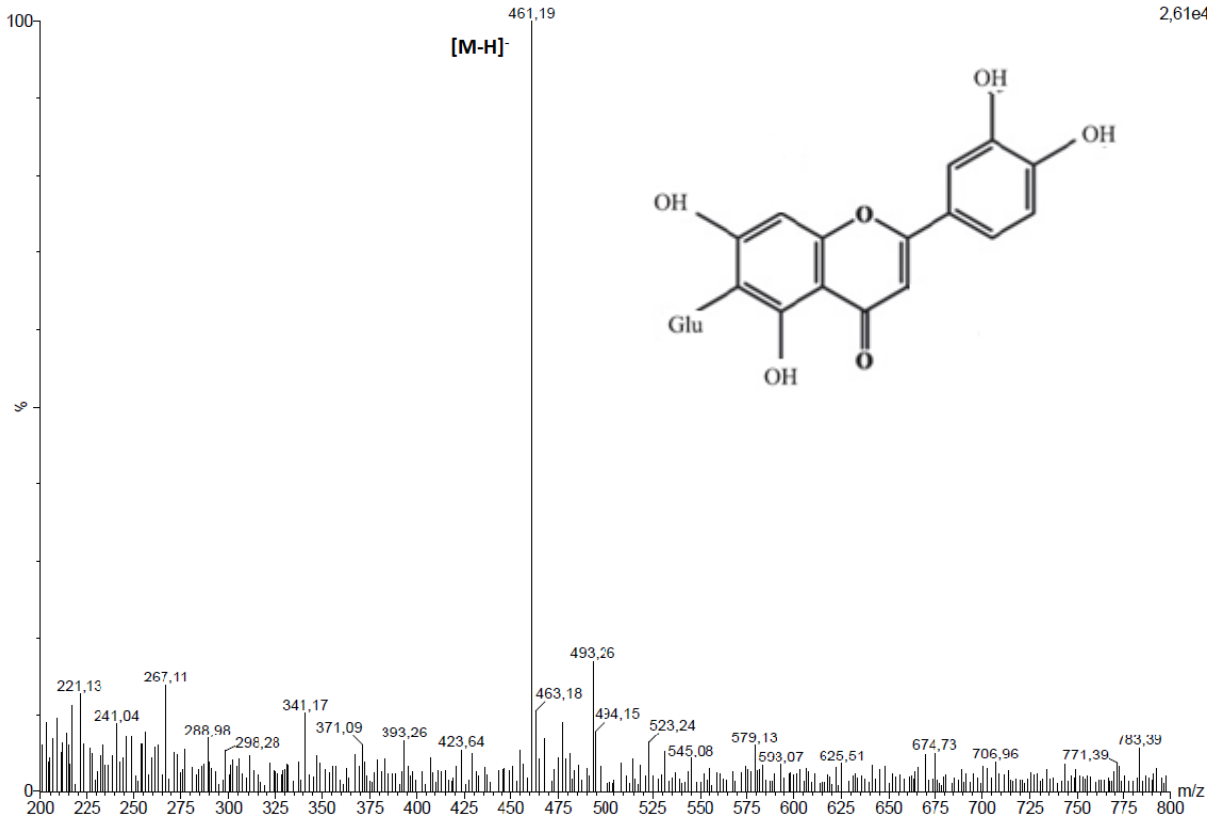


Fig. (1.22) ESI-MS(-) spectrum of the deprotonated diosmetin 6-C-glucoside.

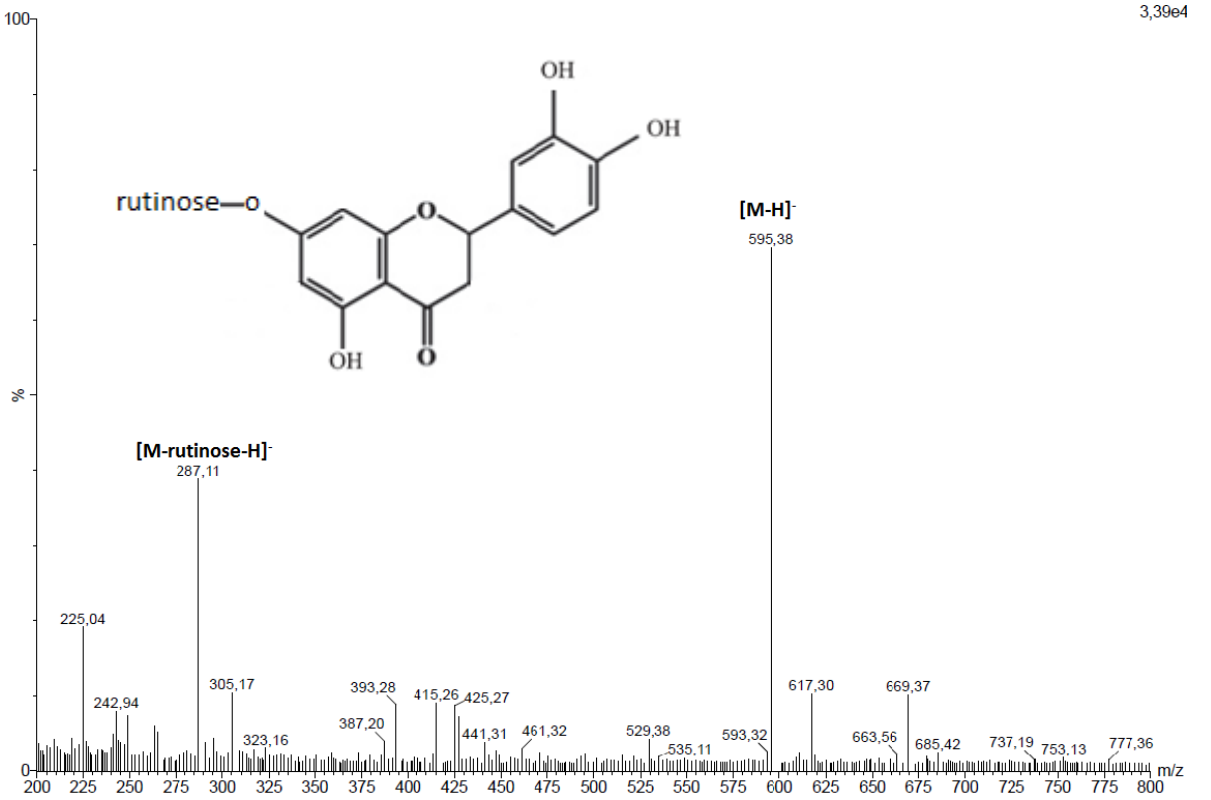


Fig. (1.23) ESI-MS(-) spectrum of eriocitrin. Eriocitrin with a molecular weight of 596 will lose a proton to bear a negative charge and m/z value of 595. A signal at m/z 287 represents the deprotonated aglycone eriodictiol.

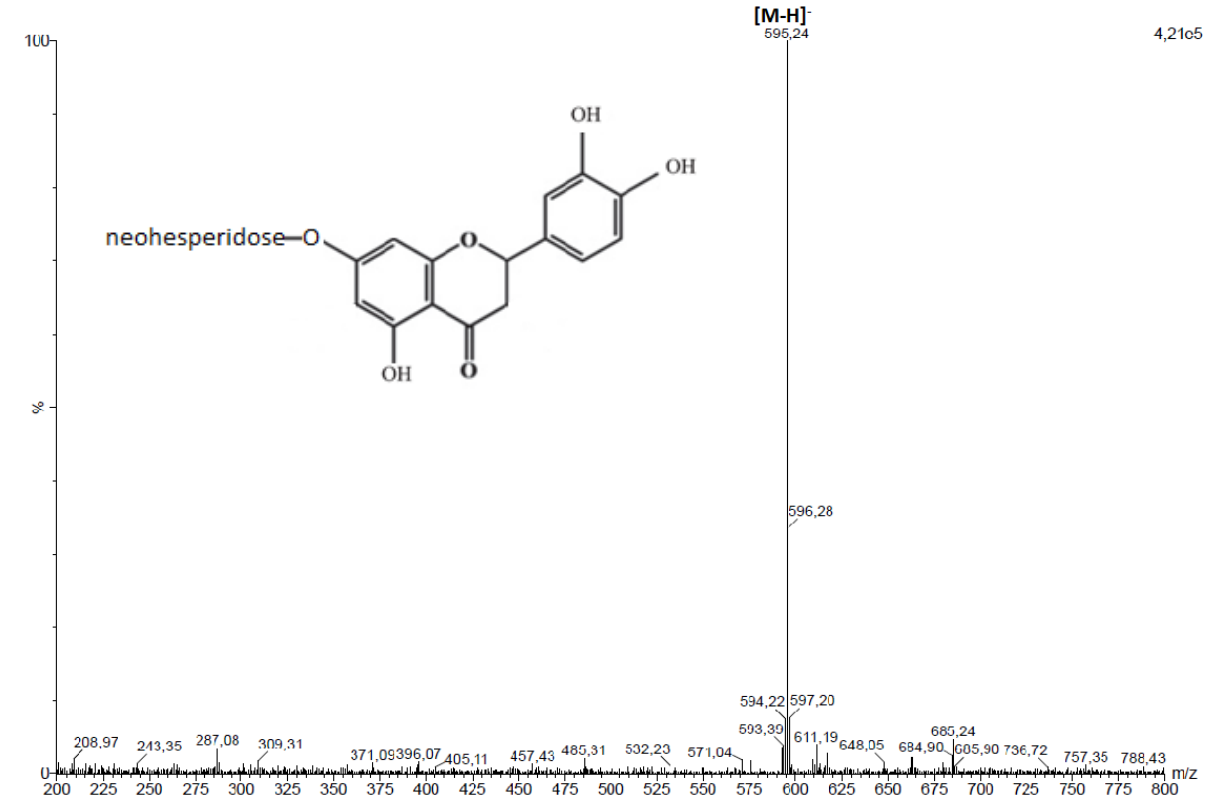


Fig. (1.24) ESI-MS(-) spectrum of neoeriocitrin. Neoeriocitirn has the same molecular weight of eriocitrin, but the MS spectrum does not show any aglycone signal.

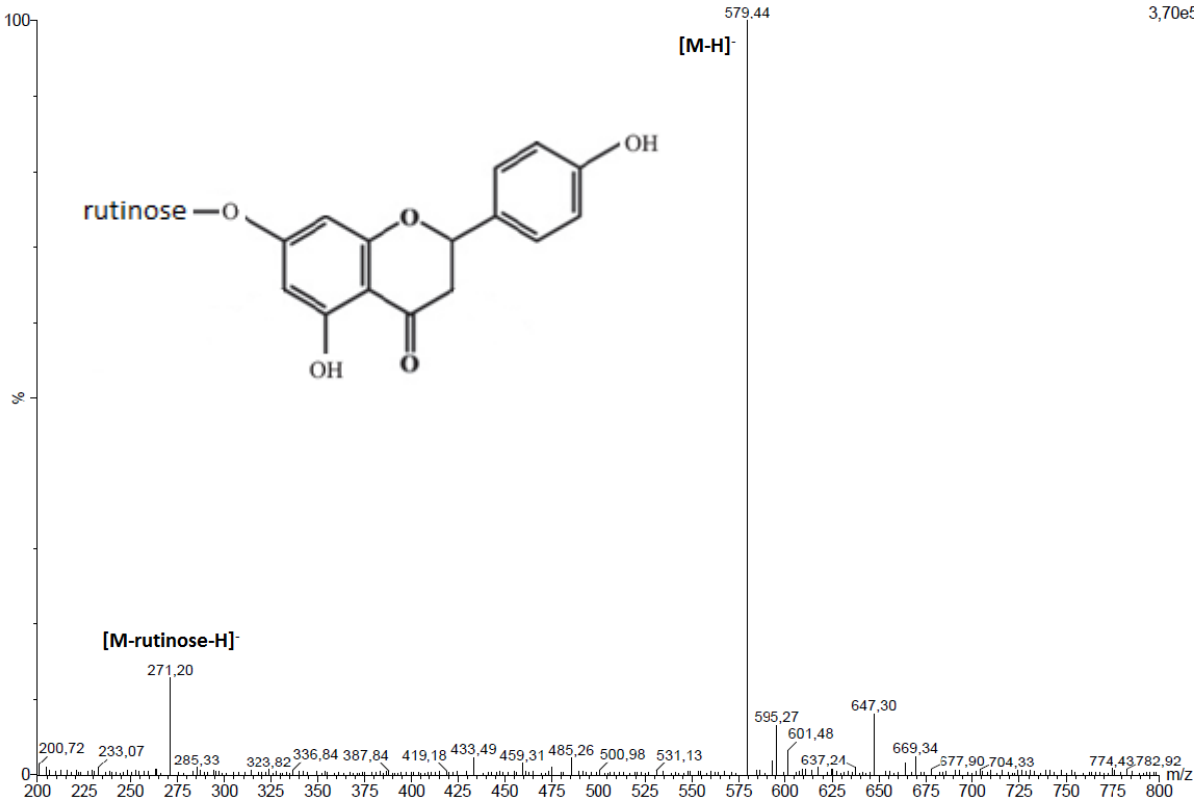


Fig. (1.25) ESI-MS(-) spectrum of narirutin. The spectrum shows, beside the signal of the intact molecule, a signal belong to the free aglycone naringenin.

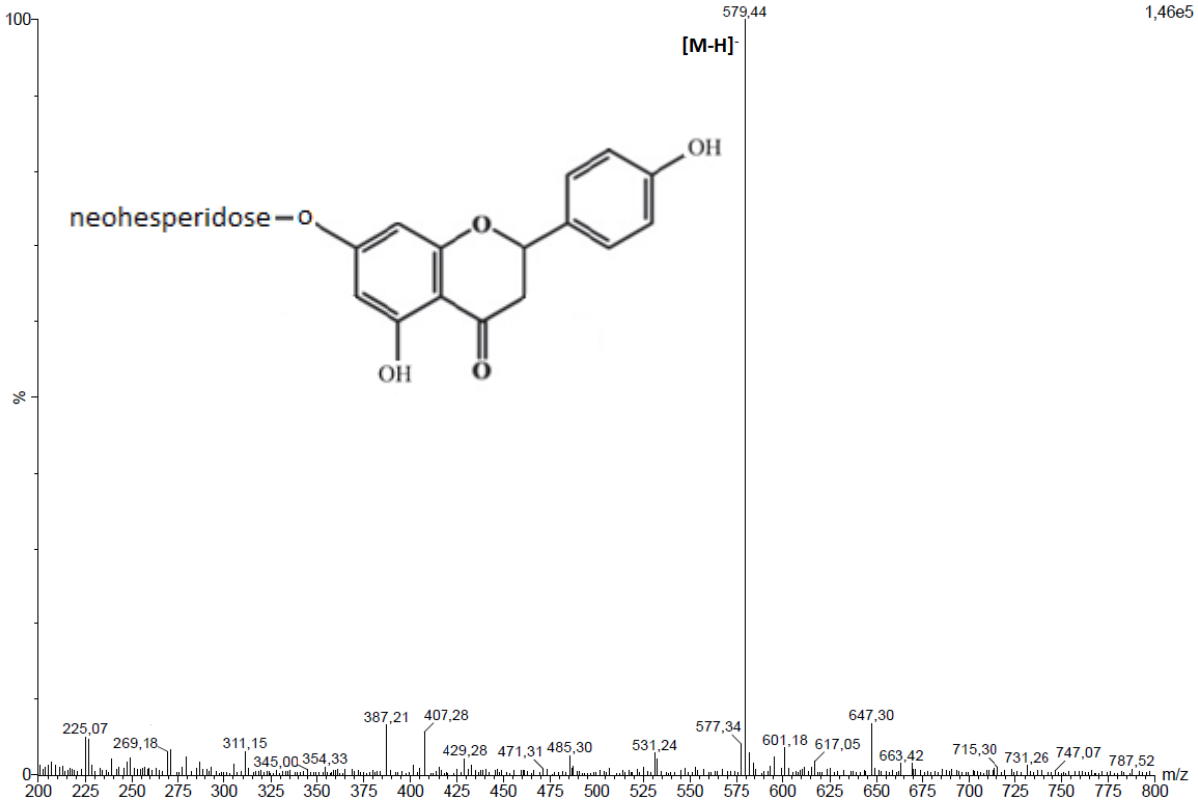


Fig. (1.26) ESI-MS(-) of naringin. Contrarily to narirutin the naringin spectrum does not show any signal related to the aglycone naringenin.

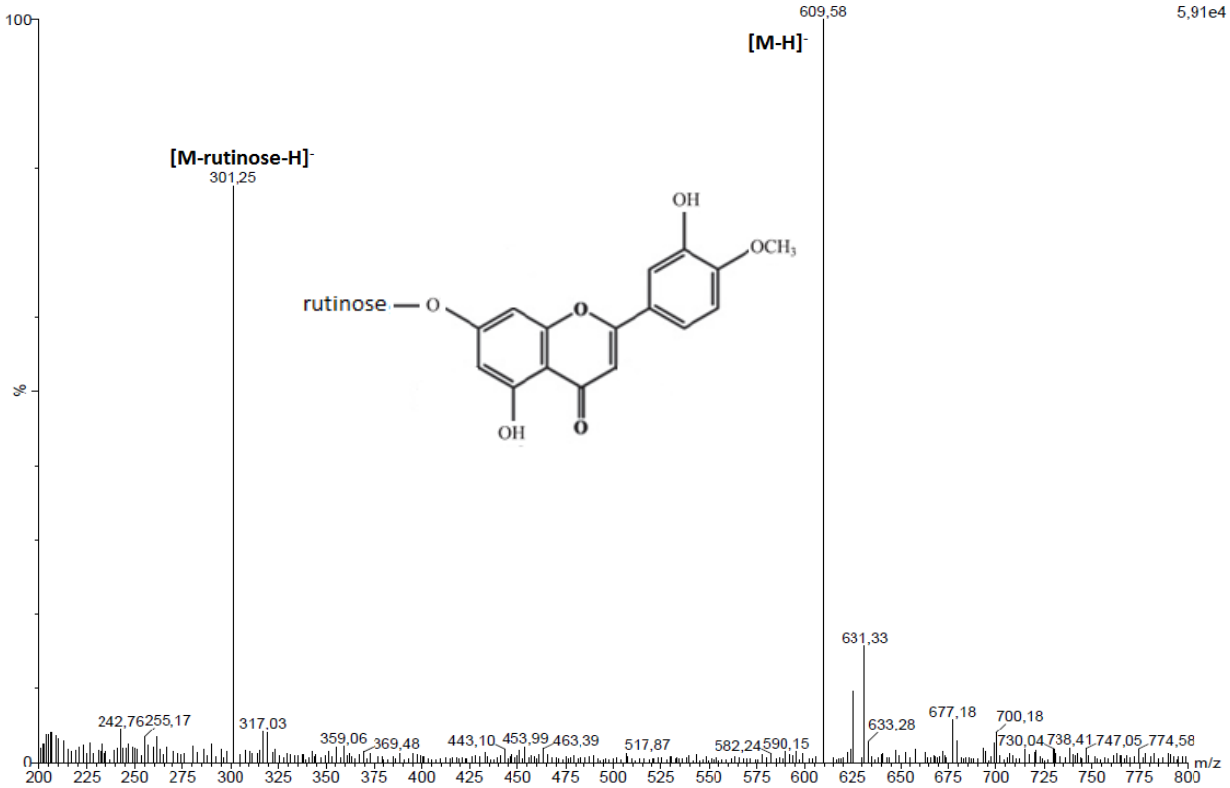


Fig. (1.27) ESI-MS(-) spectrum of hesperidin. In addition to the molecular-ion of the intact compound a strong signal belongs to the aglycone hesperetin can be seen in the spectrum.

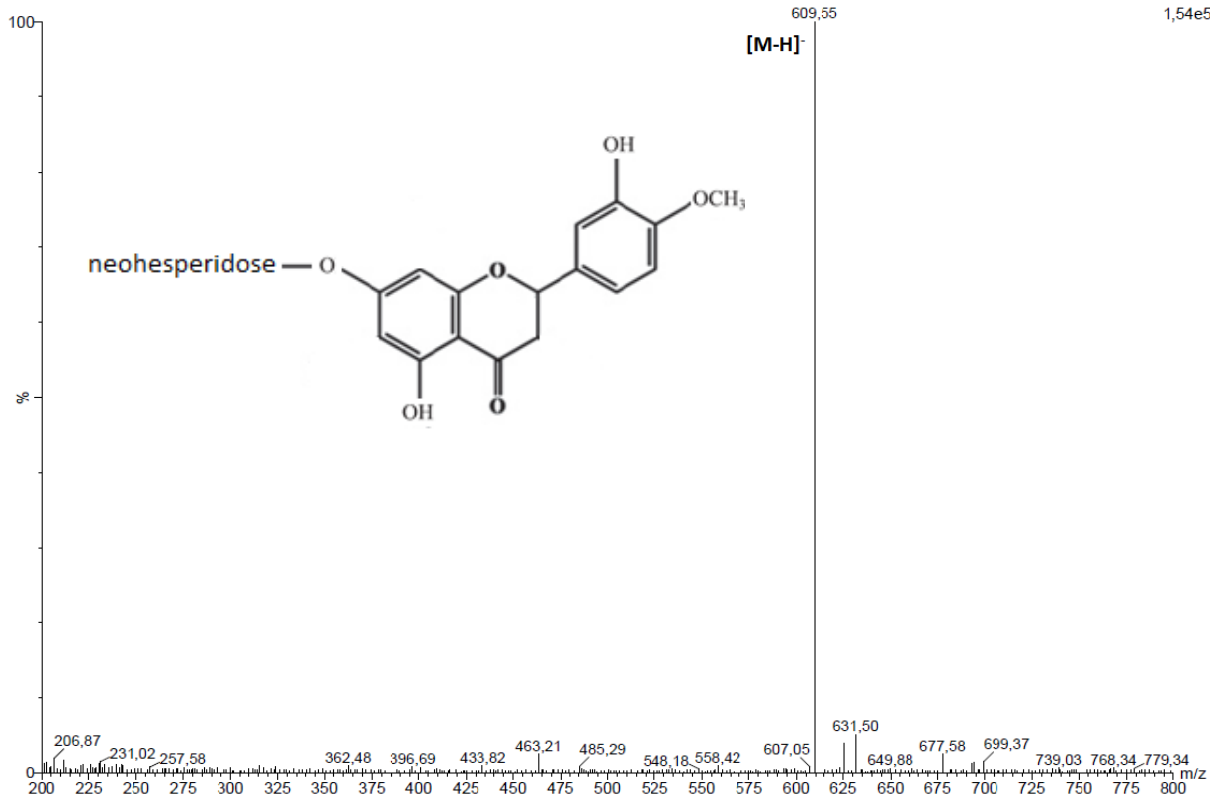


Fig. (1.28) molecular-ion of the intact neohesperidin (MW=610) under the experimental condition of LC-MS at the negative ionization mode.

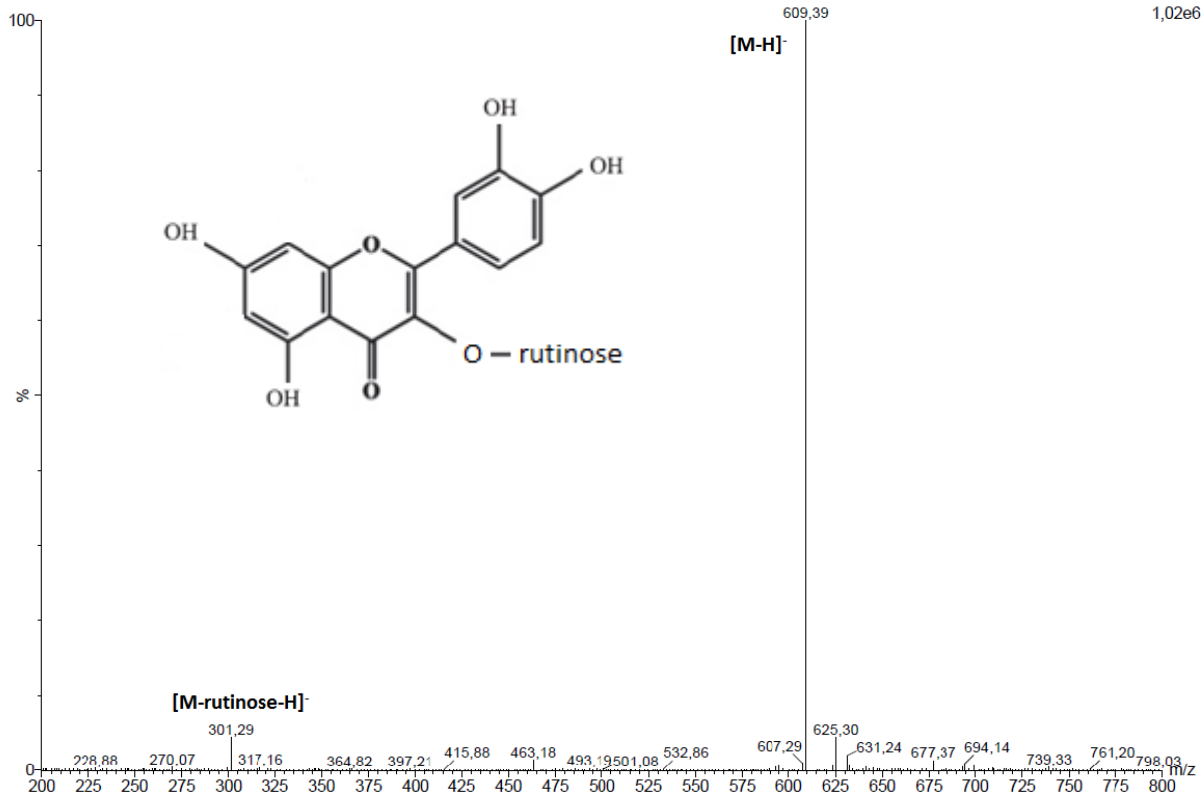


Fig. (1.29) ESI-MS(-) spectrum of rutin. The molecular-ion m/z 609 represent the deprotonated rutin MW=610. A small signal related to the aglycone quercetin m/z 301.

The MS spectra of all rutinosides (i.e. eriocitrin, rutin, narirutin and hesperidin) reveal, in addition to the molecular ion of intact molecules, signals related to the aglycone ions, while the neohesperidosides (neoeriocitrin, naringin, neohesperidin) show only the molecular ions. This findings agree with previous reports, which confirm that rutinosides tend to fragment easier than neohesperidosides (Cuyckens *et al.*, 2000).

Moreover tandem HPLC-MS/MS came as a technique to occupy a vital position considering qualitative analysis as well as structural characterization of polyphenolics in many common fruit crops (Hughes *et al.*, 2001; Sanchez-Rabaneda *et al.*, 2003a; Sanchez-Rabaneda *et al.*, 2003b; Sanchez-Rabaneda *et al.*, 2004). For example, a molecular ion with m/z value of 595 is obtained from the MS data combined from the chromatogram of the ethyl acetate fraction, when applying tandem MS/MS a fragment ion at m/z 287 represent the aglycone of eriocitrin and a neutral lost of the sugar.

1.3.4. *Camellia sinensis:*

(This work has been done in cooperation with Dr. S. Abbas from Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany)

1.3.4.1. Introduction:

Camellia sinensis belongs to theaceae family and originally grows in eastern Asia. It is a large shrub with evergreen leaves. The leaves are dark green, and the flowers are white with distinctive aroma (Chevallier, 2001). The green leaves of *Camellia sinensis* are used for more than 4000 years to prepare infusions, specially what so-called green tea (Weisburger, 1997).

In the Chinese traditional medicine the green tea has been used as a treatment of many ailments. Intensive investigations on of green tea infusions found that polyphenols, which are abundantly present in tea, may be the mostly responsible of the curative effects of green tea (DerMarderosian and Beutler, 2008).

The main compounds that are reported from *Camellia sinensis* leaves are tannis, and different kind of polyphenols mainly the following catechins; epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), epicatechin (EC), and catechin. (Graham, 1992). EGCG accounts for about 50–60% of total catechins in green tea (Weisburger, 1997). For the phytochemical studies of catechins in green tea HPLC was employed successfully and also coupled technique of HPLC with mass spectrometric has been performed (Khokhar *et al.*, 1997; Dalluge *et al.*, 1998).

In general the polyphenols are active antioxidant compounds, thus, it is likely that these polyphenols protect against ROS-mediated diseases such as cardiovascular and cancer (Hollman *et al.*, 1999). Also since that polyphenols can scavenge free radicals that mediate in the cell damage and DNA mutations; therefore, green tea infusions could play a supportive role in the curing age-related diseases (Wink and Abbas, 2009; Wink and Abbas, 2010).

1.3.4.2. Sample preparation:

60 g of dried Japanese green tea leaves that were grounded and suspended into 2 L of distilled water, then incubated overnight in water bath at 40 °C. The water extract was then filtered and freeze-dried. Dissolved in distilled water was used to reconstitute the lyophilized samples prior to any experiment.

1.3.4.3. LC-MS parameters:

The chromatographic separation of the water extract of *C. sinensis* green leaves was carried out by HPLC using a reversed phase C-18 (RP C-18) column. The mobile phase of the HPLC system was operated under gradient condition with 0.5% aqueous acetic acid (A) and acetonitrile (B), and flow rate 1 ml/min at room temperature as following: 0–60 min 0–40% B, 60–70 min 40–100% B. The acquisition of data using the MS machine was performed in both negative and positive modes with the following parameters:

Table (1.6) Tuning parameter of the electrospray ionization source at both positive and negative mode:

Tuning Parameters	Positives mode ESI(+)	Negative mode ESI(-)
Capillary (kVolts)	3,5	3,00
Cone (Volts)	25	30
Extracting (Volts)	5	5
RF Lens (Volts)	0,2	0,2
Source Temperature	120	120
Dynamic range m/z	200-800	200-800

1.3.4.4. LC-MS profiling of catechins in the water extract of *C. sinensis* green leaves:

Chromatographic techniques as a method of choice for the analysis of catechins in C. *sinensis* green leaves have been already reported (Dalluge *et al.*, 1998; Rusak *et al.*, 2008). Also others have mentioned LC-MS methods to be advantageous for the analysis of naturally occurred catechins, such that in *Acacia catechu* (Shen *et al.*, 2006), or in *Jatropha macrantha*

stems (Benavides *et al.*, 2006). The LC-MS analysis of the water extract of *C. sinensis* green leaves under the experimental conditions allowed the identification of six major Flavan-3-ols; catechin (C), epigallocatechin (EC), gallocatechin (GC), epicatechin gallate (ECG), and epigallocatechin gallate epicatechin (EGCG). Table (1.7) summarize the obtained molecular-ions of the identified catechins in the water extract of *C. sinensis* green leaves in both negative and positive ionization modes. In the negative ion mode both ECG and EGCG compounds expel the galloyl group (a loss of m/z 152) resulting in the formation of EC and EGC as shown in figures (1.34) and (1.35), respectively.

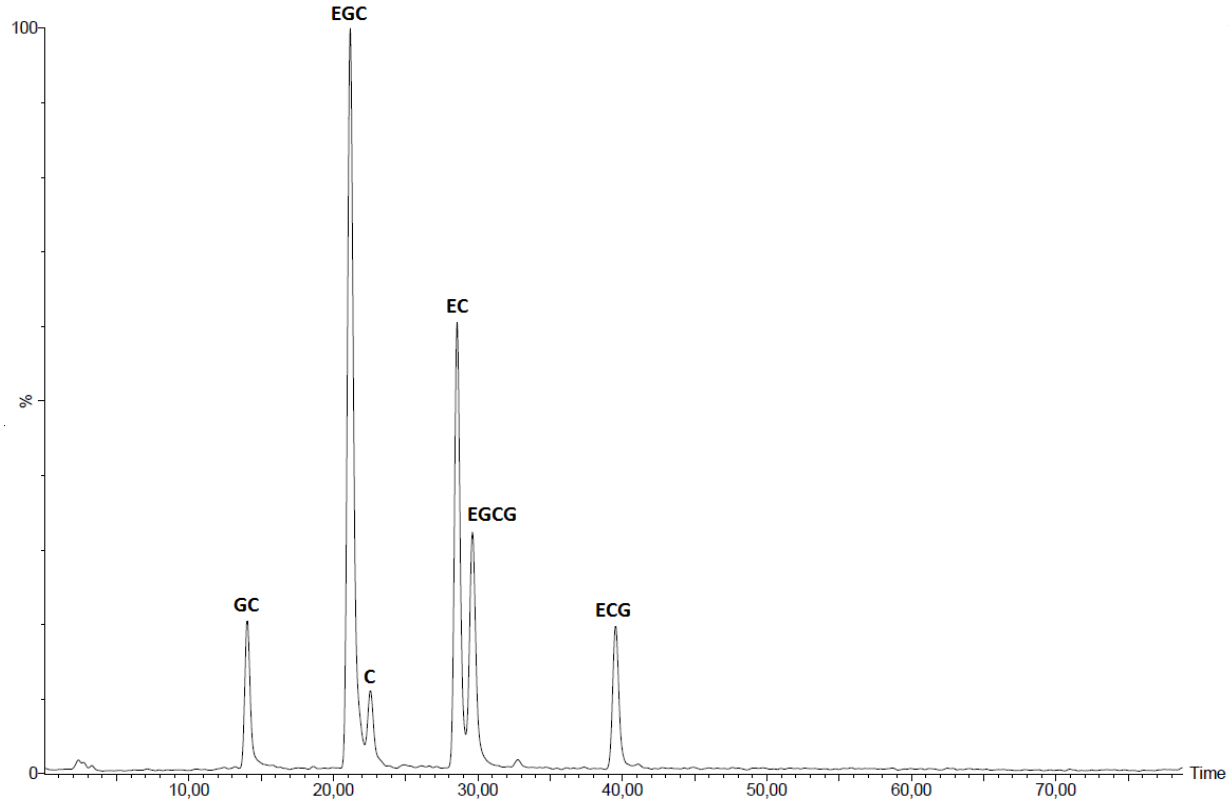


Fig.(1.30) Reconstructed ion chromatogram (RIC) obtained from the LC-MS in the negative ionization mode ESI (-) of the aqueous extract of *C. sinensis* green leaves.

Figures (1.30) and (1.31) represent the reconstructed ion chromatogram (RIC) of the LC-MS of *C. sinensis* green leaves water extract in both negative and positive ionization mode. This LC-MS findings agree with a previous work carried out with standard catechins in green tea samples (Miketova *et al.*, 1998).

The RIC option considered one of the most valuable aspects that provided when using MS techniques. This option assists the analysts to gain clearer chromatograms of compounds of

interest and to subtract unwanted signals. When comparing the results obtained from the LC-MS at the two different ionization modes it was clear, that ion intensities of catechins derivatives were more distinctive when using the negative mode. Thus, it is LC-MS methods employing ESI(-) are favorable to that employ the ESI(+). Nevertheless, the RIC option allows the refining of the ESI(+) to obtain the desired chromatogram figure (1.31).

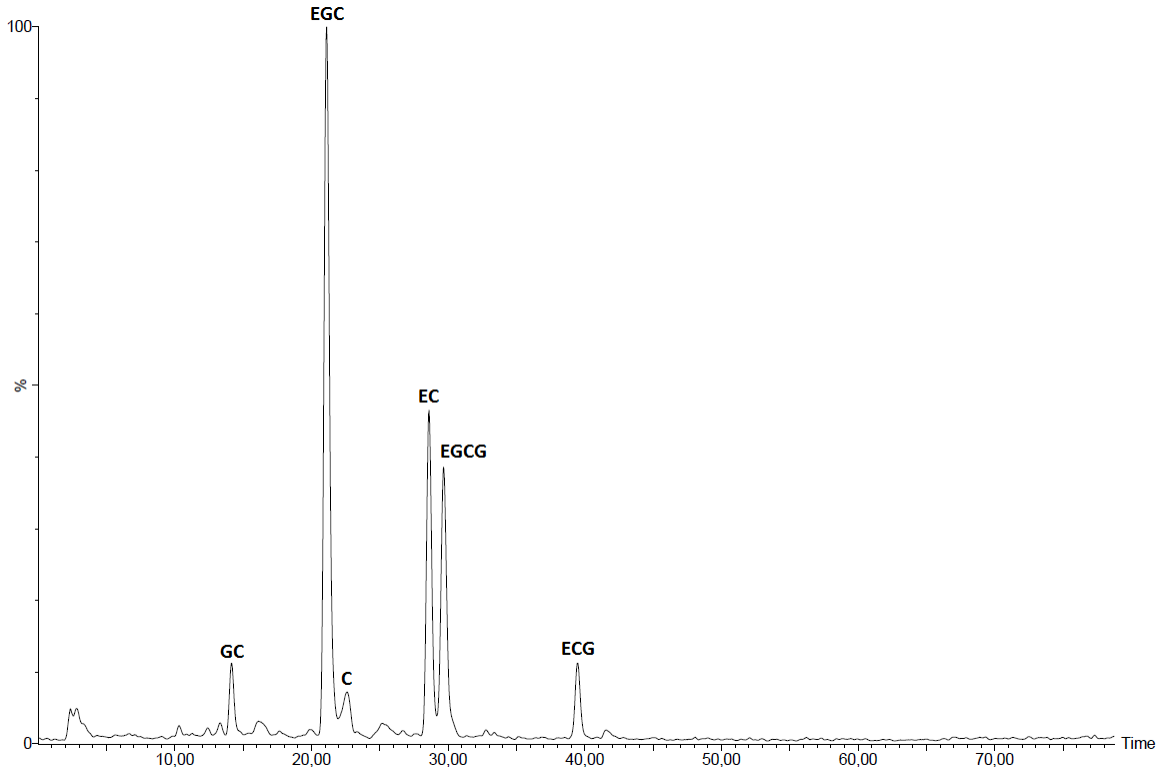


Fig.(1.31) Reconstructed ion chromatogram (RIC) obtained from the LC-MS in the positive ionization mode ESI (+) of the aqueous extract of *C. sinensis* green leaves.

The MS spectra obtained of combing data form chromatographic analysis, along with retention time data; provide clear information about the molecular weight of the identified catechins. Figures (32-36) represent the MS spectra with the characteristic molecular-ions of the identified compounds at both negative and positive ionization modes.

Both gallocatechin and epigallocatechin have the same molecular weight and show the same m/z values, figure (1.32) and table (1.7). Thus, it is not possible to distinguish between the both compounds only by relying on the MS data. Nevertheless, chromatographic data signified by retention time values can solve such problem considering the catechins. Base on the stereochamical structure the gallocatechin has 2R:3S configuration (Both B ring at the C_2

and the OH group at C_3 are situated in two different stereo level), therefore would elute earlier than epigallocatechin with the 2R:3R configuration (Koupai-Abyazani, 1991). The same conclusion can be drawn when comparing both catechin and epicatechin. The MS data of epicatechin is shown in figure (1.33).

Table (1.7) molecular-ions of the identified catechins in the water extract of *C. sinensis* green leaves in both negative and positive ionization modes and some significant fragments

Compounds	**Abbreviation**	**MW**	**$[M-H]^-$**	**$[M+H]^+$**	**Significant frag.**
Gallocatechin	GC	306	305	307	-
Epigallocatechin	EGC	306	305	307	-
Catechin	C	290	289	-	-
Epicatechin	EC	290	289	291	-
Epigallocatechin gallate	EGCG	458	457	459	m/z 305; EGC
Epicatechin gallate	ECG	442	441	443	m/z 289; EC

Moreover, the MS spectra obtained from LC-MS of ECG; figure (1.34), as well as EGCG; figure (1.35) unveil two signals under the experimental parameters of ESI(-) mode. One belongs to the intact molecule and the other is a fragment that results from the lost of galloyl group. This spontaneous fragmentation leads to the formation of EC; represented with a signal of m/z 289, that results from ECG, meanwhile EGCG MS spectrum shows a signal at m/z 305 that belongs to formation of EGC.

Small amounts of catechin are detected in the water extract of the *C. sinensis* green leave. Nevertheless, catechin could be detected using LC-MS. At the ESI(-) experimental conditions catechins (MW 290) loses one proton and the molecular-ion of $[M-H]^-$ with the m/z value of 289 is formed figure (1.36). Whereas, at the ESI(+) mode no obvious signal was observed.

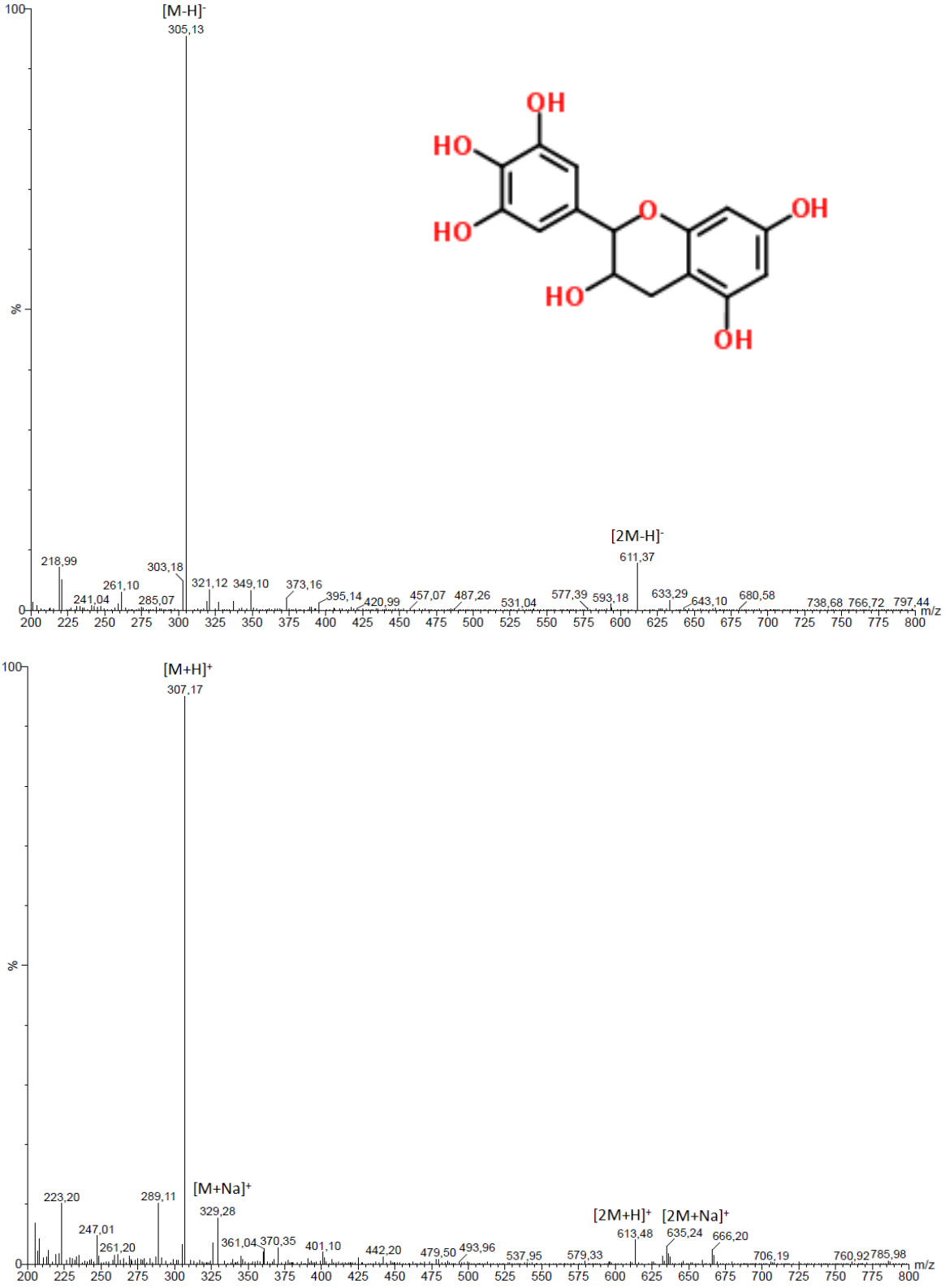


Fig.(1.32) MS spectra of GC. The upper pat shows the spectrum at ESI(-) conditions, while the lower one is the MS data obtained at the ESI(+) mode.

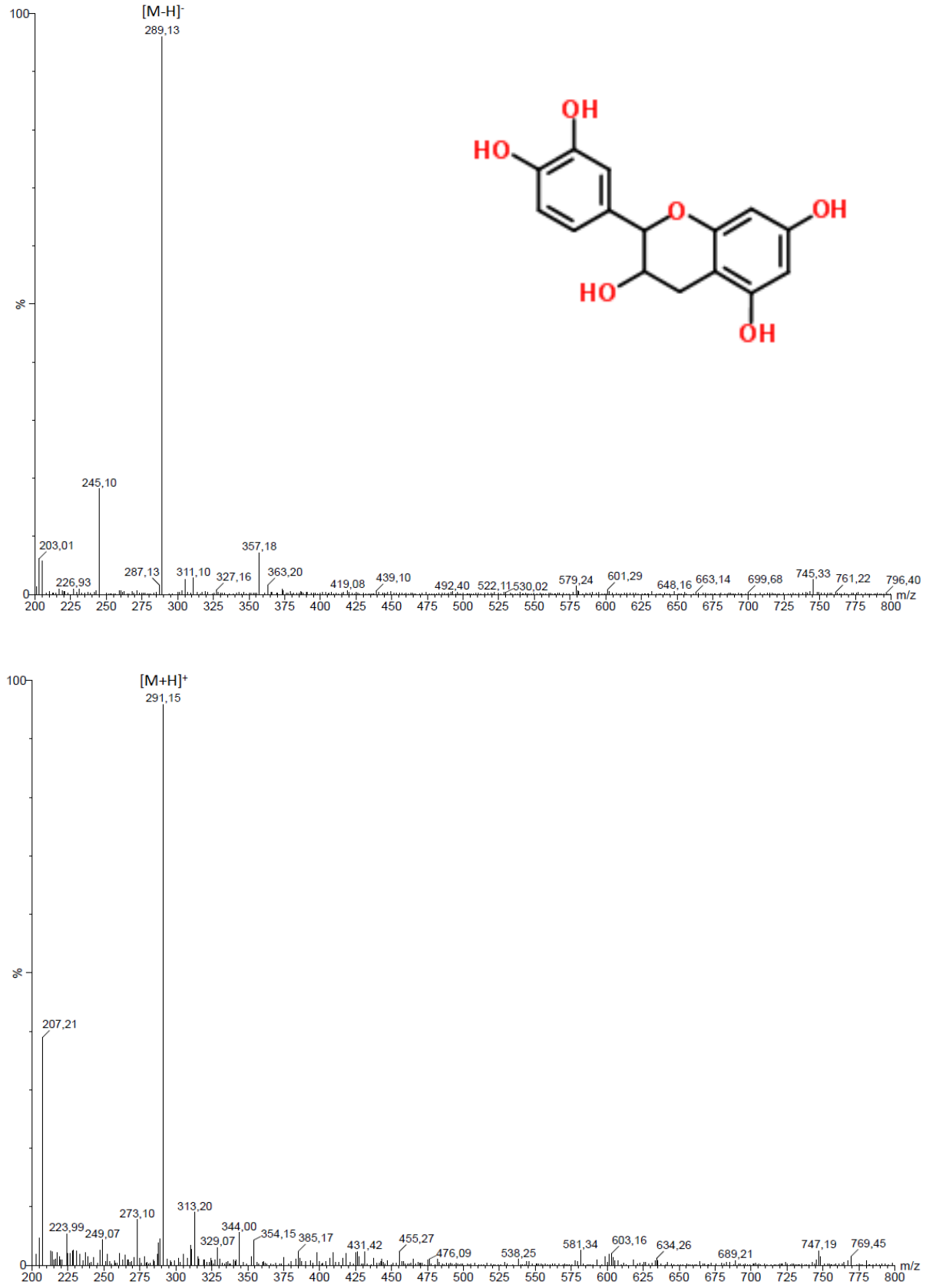


Fig.(1.33) MS spectra of EC. The upper pat shows the spectrum at ESI(-) conditions, while the lower one is the MS data obtained at the ESI(+) mode.

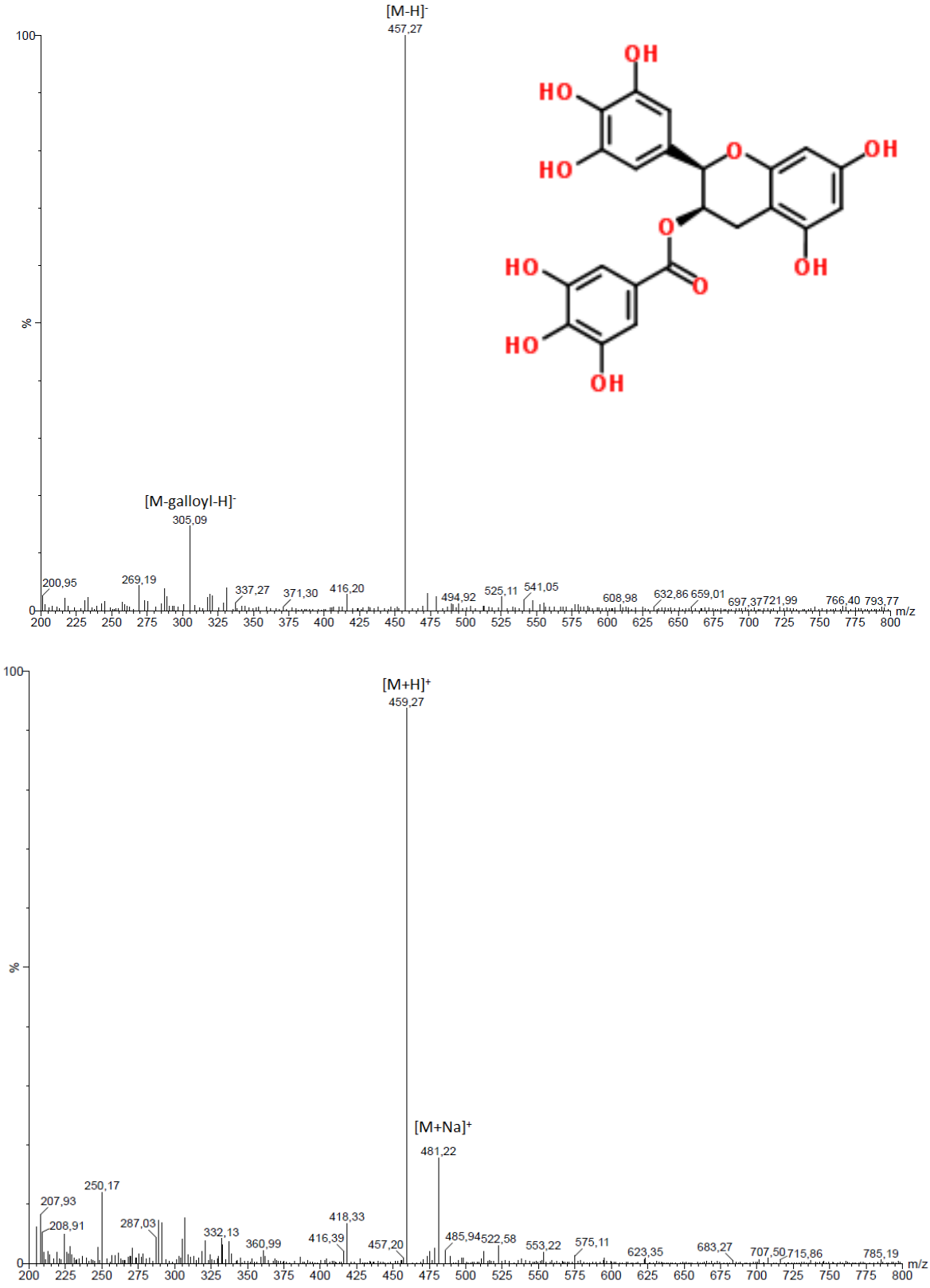


Fig.(1.34) MS spectra of EGCG. The upper pat shows the spectrum at ESI(-) conditions, while the lower one is the MS data obtained at the ESI(+) mode. ESI(-) spectra shows a signal with m/z 305 correspond to the EGC as a result that some EGCG molecules will lose a galloyl group during ionization.

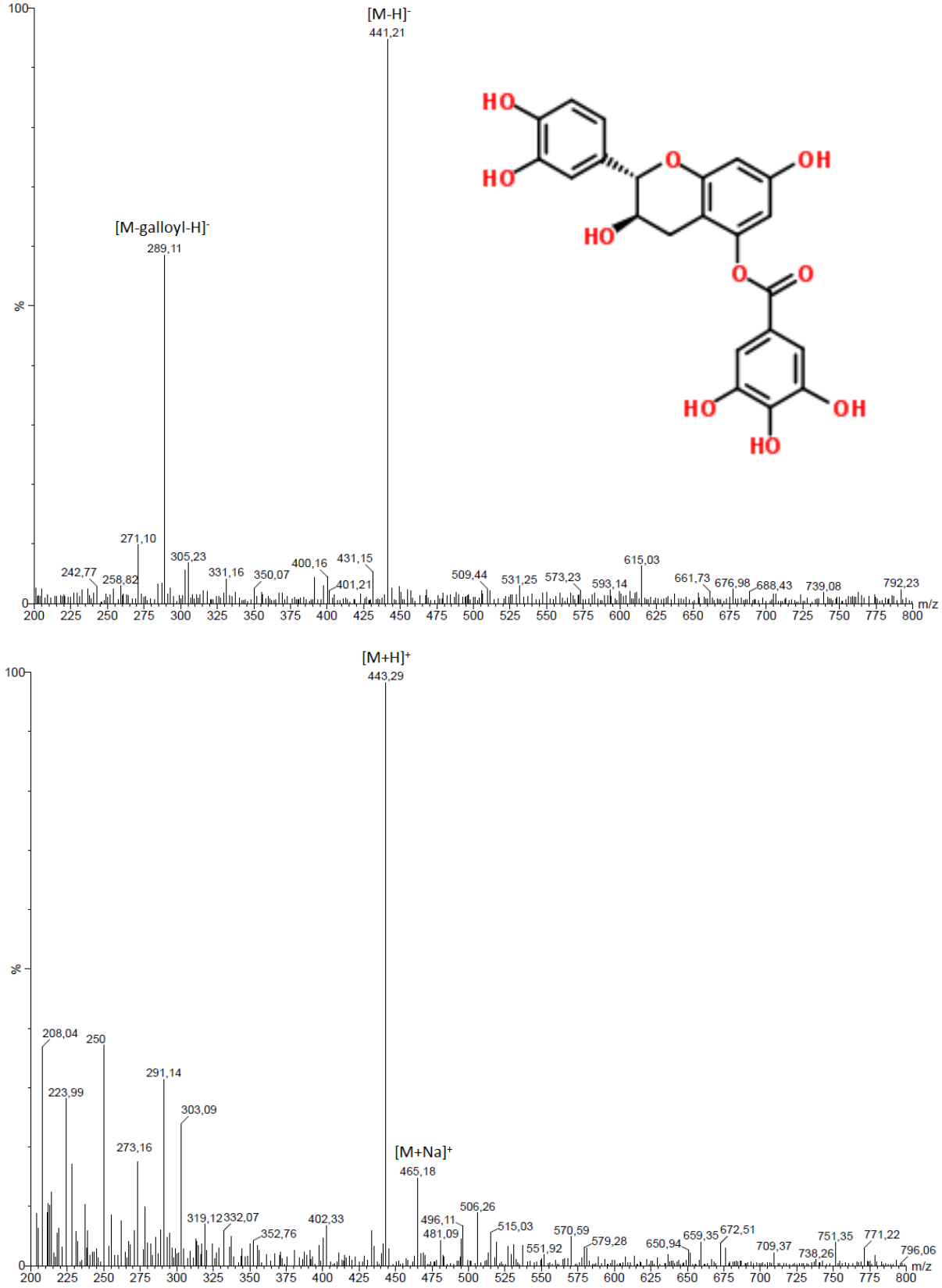


Fig.(1.35) MS spectra of ECG. The upper part shows the spectrum at ESI(-) conditions, while the lower one is the MS data obtained at the ESI(+) mode. Also some ECG molecules will lose galloyl group during the ionization in the negative mode ESI(-), leading to the formation of EC; a signal of m/z 289 confirm the formation of EC.

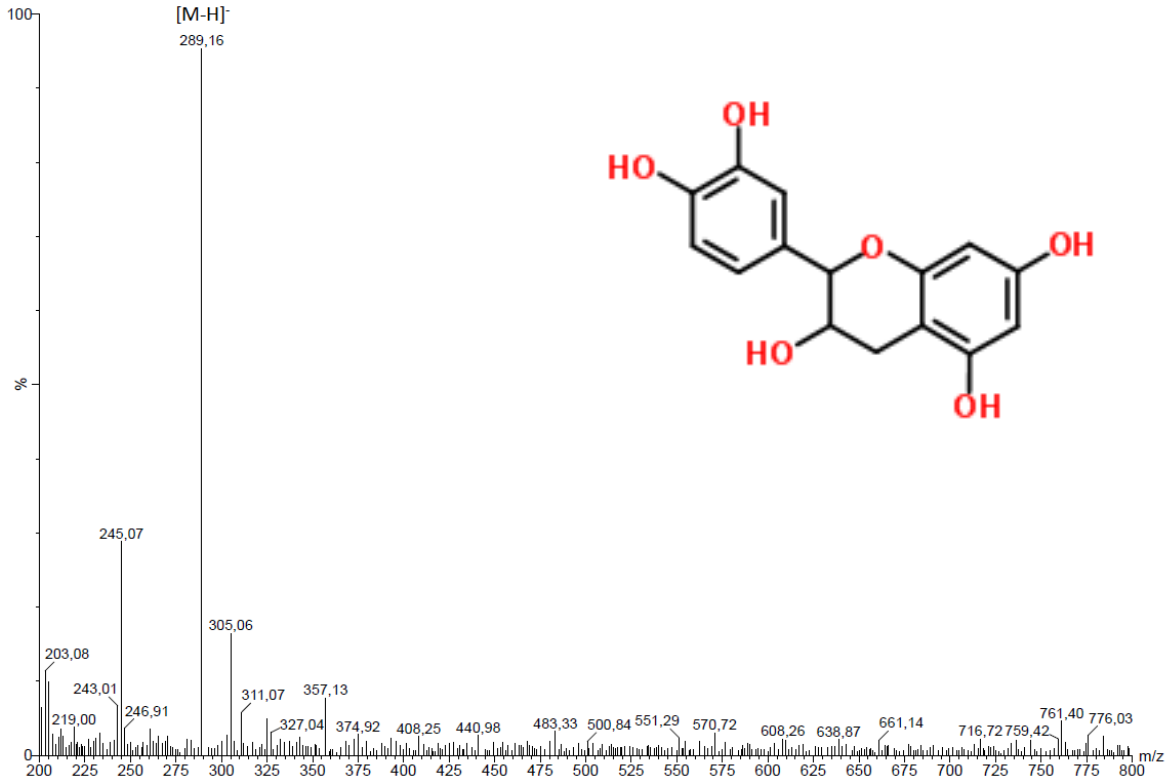


Fig.(1.36) MS spectrum of catechin (C) obtained from the LC-MS at the negative mode ESI(-). Since that the positive ion mode is less sensitive than negative mode, then a clear MS signals from the ESI(+) mode could not be obtained.

In conclusion the LC-MS analysis of the water extract of *C. sinensis* green leaves offers a lot of worthy information considering the photochemical profiling of the catechins. Since that the consumption of the *C. sinensis* green leaves is increasing dramatically, analyst are urged to achieve a fast and reliable techniques to control the green tea products. LC-MS techniques can fulfill such growing needs in the global market. The LC-MS analysis of catechins content in *C. sinensis* extracts can also improve our understanding of the ecological factors that may affect the quality of the *C. sinensis* crops. Thus, help us to optimize processes such as harvesting, therefore, improving the quality of productions.

1.4. Conclusion:

Plants secondary metabolites SM still occupy an important position as a main resource of biologically active compounds and offering many solutions to encounter the excessive global demands for new therapeutic agents. To fulfill these demands new analytical techniques have been introduced in the last thirty years shaping the modern medicine. One of the most successful analytical techniques is the high performance liquid chromatography HPLC.

Since the presentation of HPLC and many phytochemical studies have been carried out to isolate and characterize new SM compounds of biological interest. Later on, and in the 1980s after the invention of the electrospray ionization mass spectrometry (ESI-MS), it was possible to couple the HPLC with the MS machines leading to the birth of a new powerful technique that is becoming unavoidable in every phytochemical study. LC-MS techniques offer tremendous benefits over other traditional ones, such as higher sensitivity, requirement of relatively small amounts of sample, and are effectiveness in detecting impurities. In addition to the retention time (RT) and molecular mass values, LC-MS can provide the analysts with a lot of helpful information considering structure elucidation.

An important field of studies, in which LC-MS techniques are intensively employed, is the phytochemical studies of polyphenolic compounds and especially flavonoids. Polyphenolic compounds are one of the most abundant SM in the nature. A lot of investigations show the wide range of the bioactivity of these compounds. They are highly active antioxidant and can scavenge free radical; therefore, are successfully used as a supportive remedy in many diseases that involved in cell and DNA damaging, like cardiovascular diseases and cancer.

On the other hand, polyphenols can interact with biomolecules through non-covalent forces. Such interactions could affect the 3D structure of the biomolecule and lead to some desirable bioactivity. Moreover, polyphenols can interact with biomolecules at different levels and with several targets (multi-target drug), this aspect provides promising features, e.g., the development of anti-microbial activity against the Methicillin-resistant Staphylococcus aureus (MRSA) minimizing the probability of resistance development.

LC-MS was successfully employed to study the flavonoids content of extract obtained from different medicinal plants;

Bupleurum marginatum is used since a long time in the rational Chinese medicine (TCM). The root, radix *Bupleuri*, had many bioactivities in healing wounds and ameliorating fever associated with malaria. Also it shows protective properties in chronic hepatitis. The LC-MS flavonoids profiling of methanol extract of *B. marginatum* roots indicates that the roots rich of bioactive flavonoids; quercetin and isorhamnetin, as well as flavonoids glycoside; rutin, narcissi, and isoquercitrin.

Camellia sinensis Another plant that is used in the TCM. The green leaves of *C. sinensis* are used to prepare green tea. This infusion is one of the most consumed beverages on the globe. A rich source of catechins green tea shows many healthy benefits considering age-related diseases. The catechins, especially EGCG, are strong antioxidant compounds. They can capture radical and interrupt their cell-damaging processes. LC-MS techniques offered many attractive aspects and are employed intensively to control and improve the green tea production.

LC-MS techniques are also utilized in the chemotaxonomy and profiling of flavonoids in the *Citrus*. *Citrus* fruits are involved in one of the most flourishing food industry all over the world. The need to control the quality of the huge production led to the employment of LC-MS techniques which are the method of choice in many labs. *Citrus jambhiri* belong to belongs *Citrus* are wildly cultivated and consumed. The LC-MS investigation has shown that the peel of the *Citrus jambhiri* is rich of many flavonoid glycosides. Some of these flavonoid glycosides can play a role in the treatment of cancer as they demonstrate biological advantages concerning the multidrug resistance (MDR) phenomenon.

Another example for the application of LC-MS in the phytochemistry is the profiling of flavonoids in the genus *Scutellaria* (known with Skullcap). The genus *Scutellaria* are widely used in TCM and contain a variety of secondary metabolites such as essential oils, iridoids, diterpenes, triterpenoids, alkaloids, phytosterols, polysaccharides, as well as polyphenols. The genus *Scutellaria* exhibit many bioactivity in the treatment of neurological disorders and

proved to have anti-viral, anti-bacterial, and anti-trypanosomal activities. Flavonoids are abundant in *Scutellaria* genus and responsible of many pharmacological effects such as anti-tumor effects and the inhibition of HIV-1 reverse transcriptase. The most commonly reported flavonoids are baicalein, baicalin, wogonin and wogonoside.

Although that the field of phytochemistry witnessed intensive activities in the last few years, but there are still many of the medicinal plants are not yet subjected to analytical studies. Nevertheless, LC-MS as a *par excellence* technique is providing promising solutions in the profiling of plants extracts, and in the identification of new bioactive compounds. On the other hand LC-MS could pave the way to understand one of the most attractive aspects of plant extracts, namely, the activity of multi-component mixtures in the phytomedicine.

Medicinal plants in China : a selection of 150 commonly used species. In: Editor, (ed)^(eds). Book Medicinal plants in China : a selection of 150 commonly used species. Manila : W.H.O., Regional Office for the Western Pacific, 1989 (1997 [printing])

(2002) Traditional herbal medicines for modern times. In: Editor, (ed)^(eds). Book Traditional herbal medicines for modern times. London: Taylor & Francis

Abbas S, Wink M (2009) Epigallocatechin gallate from green tea (Camellia sinensis) increases lifespan and stress resistance in Caenorhabditis elegans. *Planta Medica* **75**: 216-221.

Abbas S, Wink M (2010) Epigallocatechin gallate inhibits beta amyloid oligomerization in Caenorhabditis elegans and affects the daf-2/insulin-like signaling pathway. *Phytomedicine* **17**: 902-909.

Agullo G, Gamet-Payrastre L, Fernandez Y, Anciaux N, Demigne C, Remesy C (1996) Comparative effects of flavonoids on the growth, viability and metabolism of a colonic adenocarcinoma cell line (HT29 cells). *Cancer Lett* **105**: 61-70.

Agullo G, GametPayrastre L, Manenti S, Viala C, Remesy C, Chap H, Payrastre B (1997) Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. *Biochemical Pharmacology* **53**: 1649-1657.

Aherne SA, O'Brien NM (2002) Dietary flavonols: Chemistry, food content, and metabolism. *Nutrition* **18**: 75-81.

Ashour ML, El-Readi M, Youns M, Mulyaningsih S, Sporer F, Efferth T, Wink M (2009) Chemical composition and biological activity of the essential oil obtained from *Bupleurum* marginatum (Apiaceae). *Journal of Pharmacy and Pharmacology* **61**: 1079-1087.

Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH (1985) Natural plant chemicals: sources of industrial and medicinal materials. *Science* **228**: 1154-1160.

Barrero AF, Haidour A, Munoz-Dorado M, Akssira M, Sedqui A, Mansour I (1998) Polyacetylenes, terpenoids and flavonoids from *Bupleurum* spinosum. *Phytochemistry* **48**: 1237-1240.

Barrero AF, Herrador MM, Akssira M, Arteaga P, Romera JL (1999) Lignans and polyacetylenes from *Bupleurum* acutifolium. *J Nat Prod* **62**: 946-948.

Baxter H, Harborne JB (1999) The handbook of the natural flavonoids. In: Editor, (ed)^(eds). Book The handbook of the natural flavonoids. Chichester ; New York: John Wiley

Benavides A, Montoro P, Bassarello C, Piacente S, Pizza C (2006) Catechin derivatives in Jatropha macrantha stems: characterisation and LC/ESI/MS/MS quali-quantitative analysis. *J Pharm Biomed Anal* **40**: 639-647.

Bors W, Heller W, Michel C, Saran M (1990) Flavonoids as Antioxidants - Determination of Radical-Scavenging Efficiencies. *Methods in Enzymology* **186**: 343-355.

Busch KL (2010) Mass Spectrometry-Mass Spectrometry Retrospective. *Spectroscopy* **25**: 14-+.

Cappiello A, Famiglini G, Mangani F, Careri M, Lombardi P, Mucchino C (1999) Liquid chromatographic-mass spectrometric determination of phenolic compounds using a capillary-scale particle beam interface. *Journal of Chromatography A* **855**: 515-527.

Careri M, Elviri L, Mangia A (1999) Validation of a liquid chromatography ionspray mass spectrometry method for the analysis of flavanones, flavones and flavonols. *Rapid Commun Mass Spectrom* **13**: 2399-2405.

Chan FL, Choi HL, Chen ZY, Chan PS, Huang Y (2000) Induction of apoptosis in prostate cancer cell lines by a flavonoid, baicalin. *Cancer Lett* **160**: 219-228.

Chevallier A (2001) Encyclopedia of medicinal plants. In: Editor, (ed)^(eds). Book Encyclopedia of medicinal plants. 2nd ed. edn. London: Dorling Kindersley

Chiang LC, Ng LT, Liu LT, Shieh DE, Lin CC (2003) Cytotoxicity and anti-hepatitis B virus activities of saikosaponins from *Bupleurum* species. *Planta Medica* **69**: 705-709.

Christie S, Walker AF, Lewith GT (2001) Flavonoids--a new direction for the treatment of fluid retention? *Phytother Res* **15**: 467-475.

Clegg GA, Dole M (1971) Molecular beams of macroions. 3. Zein and polyvinylpyrrolidone. *Biopolymers* **10**: 821-826.

Curin Y, Andriantsitohaina R (2005) Polyphenols as potential therapeutical agents against cardiovascular diseases. *Pharmacological Reports* **57**: 97-107.

Cuyckens F, Ma YL, Pocsfalvi G, Claeys M (2000) Tandem mass spectral strategies for the structural characterization of flavonoid glycosides. *Analusis* **28**: 888-895A.

Daayf F, Lattanzio V (2008) Recent advances in polyphenol research. In: Editor, (ed)^(eds). Book Recent advances in polyphenol research. Chicester: Wiley-Blackwell

Dalluge JJ, Nelson BC, Thomas JB, Sander LC (1998) Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography. *Journal of Chromatography A* **793**: 265-274.

Deng QL, Xu J, Yu B, He J, Zhang KY, Ding XM, Chen DW (2010) Effect of dietary tea polyphenols on growth performance and cell-mediated immune response of post-weaning piglets under oxidative stress. *Archives of Animal Nutrition* **64**: 12-21.

DerMarderosian A, Beutler JA (2008) The review of natural products : the most complete source of natural product information. In: Editor, (ed)^(eds). Book The review of natural products : the most complete source of natural product information. 5th ed. edn. Saint Louis, Mo.: Wolters Kluwer Health ; [London : Lippincott Williams & Wilkins Europe, distributor]

Dugo P, Lo Presti M, Ohman M, Fazio A, Dugo G, Mondello L (2005) Determination of flavonoids in citrus juices by micro-HPLC-ESI/MS. *Journal of Separation Science* **28**: 1149-1156.

Erlund I (2004) Review of the flavonoids quercetin, hesperetin naringenin. Dietary sources, bioactivities, and epidemiology. *Nutrition Research* **24**: 851-874.

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989) Electrospray Ionization for Mass-Spectrometry of Large Biomolecules. *Science* **246**: 64-71.

Figueirinha A, Cruz MT, Francisco V, Lopes MC, Batista MT (2010) Anti-Inflammatory Activity of Cymbopogon citratus Leaf Infusion in Lipopolysaccharide-Stimulated Dendritic Cells: Contribution of the Polyphenols. *Journal of Medicinal Food* **13**: 681-690.

Fundukian LJ (2009) The Gale encyclopedia of alternative medicine. In: Editor, (ed)^(eds). Book The Gale encyclopedia of alternative medicine. 3rd edn. Detroit: Gale, Cengage Learning

Gamet-Payrastre L, Manenti S, Gratacap MP, Tulliez J, Chap H, Payrastre B (1999) Flavonoids and the inhibition of PKC and PI 3-kinase. *General Pharmacology-the Vascular System* **32**: 279-286.

Gao ZH, Huang KX, Yang XL, Xu HB (1999) Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria* baicalensis Georgi. *Biochimica Et Biophysica Acta-General Subjects* **1472**: 643-650.

Gattuso G, Barreca D, Gargiulli C, Leuzzi U, Caristi C (2007) Flavonoid composition of Citrus juices. *Molecules* **12**: 1641-1673.

Graham HN (1992) Green Tea Composition, Consumption, and Polyphenol Chemistry. *Preventive Medicine* **21**: 334-350.

Griffiths J (2008) A brief history of mass spectrometry. *Anal Chem* **80**: 5678-5683.

Gupta MM, Verma RK, Uniyal GC, Jain SP (1993) Determination of Plumbagin by Normal-Phase High-Performance Liquid-Chromatography. *Journal of Chromatography* **637**: 209-212.

Harborne JB (1998) Phytochemical methods : a guide to modern techniques of plant analysis. In: Editor, (ed)^(eds). Book Phytochemical methods : a guide to modern techniques of plant analysis. 3rd edn. London ; New York: Chapman and Hall

Hattenschwiler S, Vitousek PM (2000) The role of polyphenols in terrestrial ecosystem nutrient cycling. *Trends Ecol Evol* **15**: 238-243.

He K, Zheng B, Kim CH, Rogers L, Zheng Q (2000) Direct analysis and identification of triterpene glycosides by LC/MS in black cohosh, Cimicifuga racemosa, and in several commercially available black cohosh products. *Planta Medica* **66**: 635-640.

Henning SM, Wang PW, Aronson WJ, Huang M, Zhang YJ, Lee RP, Heber D (2010) Green Tea Polyphenols and Metabolites in Prostatectomy Tissue: Implications for Cancer Prevention. *Cancer Prevention Research* **3**: 985-993.

Hollman PCH, Feskens EJM, Katan MB (1999) Tea flavonols in cardiovascular disease and cancer epidemiology. *Proceedings of the Society for Experimental Biology and Medicine* **220**: 198-202.

Huang HQ, Zhang X, Shen YH, Su J, Liu XH, Tian JM, Lin S, Shan L, Zhang WD (2009) Polyacetylenes from *Bupleurum* longiradiatum. *J Nat Prod* **72**: 2153-2157.

Hughes RJ, Croley TR, Metcalfe CD, March RE (2001) A tandem mass spectrometric study of selected characteristic flavonoids. *International Journal of Mass Spectrometry* **210**: 371-385.

Ichikawa M, Ryu K, Yoshida J, Ide N, Kodera Y, Sasaoka T, Rosen RT (2003) Identification of six phenylpropanoids from garlic skin as major antioxidants. *Journal of Agricultural and Food Chemistry* **51**: 7313-7317.

Ito H, Miyake M, Nishitani E, Mori K, Hatano T, Okuda T, Konoshima T, Takasaki M, Kozuka M, Mukainaka T, Tokuda H, Nishino H, Yoshida T (1999) Anti-tumor promoting activity of polyphenols from Cowania mexicana and Coleogyne *ramosissima*. *Cancer Lett* **143**: 5-13.

Just MJ, Recio MC, Giner RM, Cuellar MJ, Manez S, Bilia AR, Rios JL (1998) Anti-inflammatory activity of unusual lupane saponins from *Bupleurum* fruticescens. *Planta Medica* **64**: 404-407.

Kazakevich Y, LoBrutto R (2007) HPLC for pharmaceutical scientists. In: Editor, (ed)^(eds). Book HPLC for pharmaceutical scientists. Hoboken, N.J.: Wiley-Interscience ; Chichester : John Wiley [distributor]

Khokhar S, Venema D, Hollman PCH, Dekker M, Jongen W (1997) A RP-HPLC method for the determination of tea catechins. *Cancer Letters* **114**: 171-172.

Kim H, Song MJ (2011) Analysis and recordings of orally transmitted knowledge about medicinal plants in the southern mountainous region of Korea. *J Ethnopharmacol* **134**: 676-696.

Kirkland JJ (2004) Development of some stationary phases for reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* **1060**: 9-21.

Krapp KM, Longe JL (2001) The Gale encyclopedia of alternative medicine. In: Editor, (ed)^(eds). Book The Gale encyclopedia of alternative medicine. Detroit ; London: Gale Group

Kuljanabhagavad T, Thongphasuk P, Chamulitrat W, Wink M (2008) Triterpene saponins from Chenopodium quinoa Willd. *Phytochemistry* **69**: 1919-1926.

Ladaniya M (2008) Citrus fruit : biology, technology and evaluation. In: Editor, (ed)^(eds). Book Citrus fruit : biology, technology and evaluation. London: Academic

Li-Weber M (2009) New therapeutic aspects of flavones: the anticancer properties of *Scutellaria* and its main active constituents Wogonin, Baicalein and Baicalin. *Cancer Treat Rev* **35**: 57-68.

Li BQ, Fu T, Yao DY, Mikovits JA, Ruscetti FW, Wang JM (2000) Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry. *Biochemical and Biophysical Research Communications* **276**: 534-538.

Liaw J, Gau YY, Chao YC (1999) Effect of baicalin on tracheal permeability in ovalbumin (OA)-sensitized guinea pigs. *Pharm Res* **16**: 1653-1657.

Ligor T, Ludwiczuk A, Wolski T, Buszewski B (2005) Isolation and determination of ginsenosides in American ginseng leaves and root extracts by LC-MS. *Anal Bioanal Chem* **383**: 1098-1105.

Liu K, Lota ML, Casanova J, Tomi F (2009) The essential oil of *Bupleurum* fruticosum L. from Corsica: a comprehensive study. *Chem Biodivers* **6**: 2244-2254.

Malikov VM, Yuldashev MP (2002) Phenolic compounds of plants of the *Scutellaria* L. genus. distribution, structure, and properties. *Chemistry of Natural Compounds* **38**: 358-406.

Manners GD (2007) Citrus limonoids: analysis, bioactivity, and biomedical prospects. *J Agric Food Chem* **55**: 8285-8294.

Martin S, Padilla E, Ocete MA, Galvez J, Jimenez J, Zarzuelo A (1993) Anti-inflammatory activity of the essential oil of *Bupleurum* fruticescens. *Planta Medica* **59**: 533-536.

Massanet GM, Guerra FM, Jorge ZD, Casalvazquez LG (1997) Phenylpropanoids from *Bupleurum* fruticosum. *Phytochemistry* **44**: 173-177.

Matsumoto T, Moriya M, Sakurai MH, Kiyohara H, Tabuchi Y, Yamada H (2008) Stimulatory effect of a pectic polysaccharide from a medicinal herb, the roots of *Bupleurum* falcatum L., on G-CSF secretion from intestinal epithelial cells. *Int Immunopharmacol* **8**: 581-588.

McMaster MC (2005) LC/MS : a practical user's guide. In: Editor, (ed)^(eds). Book LC/MS : a practical user's guide. Hoboken, N.J.: John Wiley

McMaster MC (2007) HPLC, a practical user's guide. In: Editor, (ed)^(eds). Book HPLC, a practical user's guide. 2nd ed. edn. Hoboken, N.J.: Wiley-Interscience

Mesbah MK, Khalifa SI, El-Gindy A, Tawfik KA (2005) HPLC determination of certain flavonoids and terpene lactones in selected Ginkgo biloba L. phytopharmaceuticals. *Farmaco* **60**: 583-590.

Meyer V (2010) Practical high-performance liquid chromatography. In: Editor, (ed)^(eds). Book Practical high-performance liquid chromatography. 5th edn. Chichester, U.K.: Wiley

Miketova P, Schram KH, Whitney JL, Kerns EH, Valcic S, Timmermann BN, Volk KJ (1998) Mass spectrometry of selected components of biological interest in green tea extracts. *Journal of Natural Products* **61**: 461-467.

Miller EG, Porter JL, Binnie WH, Guo IY, Hasegawa S (2004) Further studies on the anticancer activity of citrus limonoids. *Journal of Agricultural and Food Chemistry* **52**: 4908-4912.

Motoo Y, Sawabu N (1994) Antitumor effects of saikosaponins, baicalin and baicalein on human hepatoma cell lines. *Cancer Lett* **86**: 91-95.

Newman DJ, Cragg GM, Snader KM (2000) The influence of natural products upon drug discovery. *Natural Product Reports* **17**: 215-234.

Ogawa K, Kawasaki A, Yoshida T, Nesumi H, Nakano M, Ikoma Y, Yano M (2000) Evaluation of auraptene content in citrus fruits and their products. *Journal of Agricultural and Food Chemistry* **48**: 1763-1769.

Pakalapati G, Li L, Gretz N, Koch E, Wink M (2009) Influence of red clover (Trifolium pratense) isoflavones on gene and protein expression profiles in liver of ovariectomized rats. *Phytomedicine* **16**: 845-855.

Pan S-L (2006) *Bupleurum* species : scientific evaluation and clinical applications. In: Editor, (ed)^(eds). Book *Bupleurum* species : scientific evaluation and clinical applications. Boca Raton: CRC/Taylor & Francis

Pandit S, Kumar M, Ponnusankar S, Pal BC, Mukherjee PK (2011) RP-HPLC-DAD for simultaneous estimation of mahanine and mahanimbine in Murraya koenigii. *Biomed Chromatogr*

Parajuli P, Joshee N, Rimando AM, Mittall S, Yadav AK (2009) In vitro Antitumor Mechanisms of Various *Scutellaria* Extracts and Constituent Flavonoids. *Planta Medica* **75**: 41-48.

Pelaez MV, Costa-Fernandez JM, Sanz-Medel A (2002) Critical comparison between quadrupole and time-of-flight inductively coupled plasma mass spectrometers for isotope ratio measurements in elemental speciation. *Journal of Analytical Atomic Spectrometry* **17**: 950-957.

Pistelli L, Bilia AR, Bertoli A, Morelli I, Marsili A (1995) Phenylpropanoids from *Bupleurum*-Fruticosum. *Journal of Natural Products-Lloydia* **58**: 112-116.

Pistelli L, Noccioli C, Giachi I, Dimitrova B, Gevrenova R, Morelli I, Potenza D (2005) Lupane-triterpenes from *Bupleurum* flavum. *Nat Prod Res* **19**: 783-788.

Plaza A, Montoro P, Benavides A, Pizza C, Piacente S (2005) Phenylpropanoid glycosides from Tynanthus panurensis: characterization and LC-MS quantitative analysis. *J Agric Food Chem* **53**: 2853-2858.

Rathee P, Chaudhary H, Rathee S, Rathee D, Kumar V, Kohli K (2009) Mechanism of action of flavonoids as anti-inflammatory agents: a review. *Inflamm Allergy Drug Targets* **8**: 229-235.

Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* **20**: 933-956.

Rusak G, Komes D, Likic S, Horzic D, Kovac M (2008) Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. *Food Chemistry* **110**: 852-858.

Sanchez-Contreras S, Diaz-Lanza AM, Matellano LF, Bernabe M, Ollivier E, Balansard G, Faure R (1998) A sulfated saponin from *Bupleurum* rigidum. *J Nat Prod* **61**: 1383-1385.

Sanchez-Rabaneda F, Jauregui O, Casals I, Andres-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventos RM (2003a) Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (Theobroma cacao). *J Mass Spectrom* **38**: 35-42.

Sanchez-Rabaneda F, Jauregui O, Lamuela-Raventos RM, Bastida J, Viladomat F, Codina C (2003b) Identification of phenolic compounds in artichoke waste by high-performance liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1008**: 57-72.

Sanchez-Rabaneda F, Jauregui O, Lamuela-Raventos RM, Viladomat F, Bastida J, Codina C (2004) Qualitative analysis of phenolic compounds in apple pomace using liquid chromatography coupled to mass spectrometry in tandem mode. *Rapid Commun Mass Spectrom* **18**: 553-563.

Sato T, Koike L, Miyata Y, Hirata M, Mimaki Y, Sashida Y, Yano M, Ito A (2002) Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and-9 in human fibrosarcoma HT-1080 cells. *Cancer Research* **62**: 1025-1029.

Schinella GR, Tournier HA, Prieto JM, Rios JL, Buschiazzo H, Zaidenberg A (2002) Inhibition of Trypanosoma cruzi growth by medical plant extracts. *Fitoterapia* **73**: 569-575.

Seeram NP, Lee R, Scheuller HS, Heber D (2006) Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionization mass spectroscopy. *Food Chemistry* **97**: 1-11.

Shen D, Wu Q, Wang M, Yang Y, Lavoie EJ, Simon JE (2006) Determination of the predominant catechins in Acacia catechu by liquid chromatography/electrospray ionization-mass spectrometry. *Journal of Agricultural and Food Chemistry* **54**: 3219-3224.

Shi P, He Q, Song Y, Qu H, Cheng Y (2007) Characterization and identification of isomeric flavonoid O-diglycosides from genus Citrus in negative electrospray ionization by ion trap mass spectrometry and time-of-flight mass spectrometry. *Anal Chim Acta* **598**: 110-118.

Small H (1989) Ion chromatography. In: Editor, (ed)^(eds). Book Ion chromatography. New York: Plenum Press

Snyder LR, Glajch JL, Kirkland JJ (1988) Practical HPLC method development. In: Editor, (ed)^(eds). Book Practical HPLC method development. New York: J. Wiley

Sonoda M, Nishiyama T, Matsukawa Y, Moriyasu M (2004) Cytotoxic activities of flavonoids from two *Scutellaria* plants in Chinese medicine. *J Ethnopharmacol* **91**: 65-68.

Sulistyowati L, Keane PJ, Anderson JW (1990) Accumulation of the Phytoalexin, 6,7-Dimethoxycoumarin, in Roots and Stems of Citrus Seedlings Following Inoculation with Phytophthora-Citrophthora. *Physiological and Molecular Plant Pathology* **37**: 451-461.

Tateo F, Sanchez MLE, Bononi M, Lubian E (1999) Stevioside content of Stevia rebaudiana (Bertoni) Bertoni grown in East Paraguay. *Italian Journal of Food Science* **11**: 265-269.

Ting SV, Rouseff RL (1986) Citrus fruits and their products : analysis, technology. In: Editor, (ed)^(eds). Book Citrus fruits and their products : analysis, technology. New York: Dekker

Tripoli E, La Guardia M, Giammanco S, Di Majo D, Giammanco M (2007) Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chemistry* **104**: 466-479.

Van Wyk B-E, Wink M (2004) Medicinal plants of the world : an illustrated scientific guide to important medicinal plants and their uses. In: Editor, (ed)^(eds). Book Medicinal plants of the world : an illustrated scientific guide to important medicinal plants and their uses. Portland, Or. ; Cambridge: Timber

Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N (2007) Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes. *Biotechnol J* **2**: 1235-1249.

Waksmundzka-Hajnos M, Sherma J (2011) High performance liquid chromatography in phytochemical analysis. In: Editor, (ed)^(eds). Book High performance liquid chromatography in phytochemical analysis. Boca Raton, Fla.: CRC ; London : Taylor & Francis [distributor]

Weisburger JH (1997) Tea and health: A historical perspective. *Cancer Letters* **114**: 315-317.

Weisse ME, Eberly B, Person DA (1995) Wine as a digestive aid: comparative antimicrobial effects of bismuth salicylate and red and white wine. *BMJ* **311**: 1657-1660.

Wink M (1999) Biochemistry of plant secondary metabolism. Book Biochemistry of plant secondary metabolism. Sheffield Academic Press ; CRC Press, Sheffield, England.

Wink M, Sesterhenn K, Distl M (2007) Occurrence of iridoid glycosides in in vitro cultures and intact plants of Scrophularia nodosa L. *Plant Cell Reports* **26**: 365-371.

Wink M (2008a) Evolutionary Advantage and Molecular Modes of Action of Multi- Component Mixtures Used in Phytomedicine. *Current Drug Metabolism* **9**: 996-1009.

Wink M. Evolution of Secondary Plant Metabolism. Life Sciences. Chichester: John Wiley & Sons; 2008b

Wink M (2008c) Plant secondary metabolism: Diversity, function and its evolution. *Natural Product Communications* **3**: 1205-1216.

Wink M, Abbas S (2009) Epigallocatechin Gallate from Green Tea (Camellia sinensis) Increases Lifespan and Stress Resistance in Caenorhabditis elegans. *Planta Medica* **75**: 216-221.

Wink M (2010) Functions and biotechnology of plant secondary metabolites. In: Editor, (ed)^(eds). Book Functions and biotechnology of plant secondary metabolites. 2nd ed. edn. Oxford: Wiley-Blackwell

Wink M, Abbas S (2010) Epigallocatechin gallate inhibits beta amyloid oligomerization in Caenorhabditis elegans and affects the daf-2/insulin-like signaling pathway. *Phytomedicine* **17**: 902-909.

Wu J-N (2005) An illustrated Chinese materia medica. In: Editor, (ed)^(eds). Book An illustrated Chinese materia medica. New York ; Oxford: Oxford University Press

Yabu Y, Nose M, Koide T, Ohta N, Ogihara Y (1998) Antitrypanosomal effects of traditional Chinese herbal medicines on bloodstream forms of Trypanosoma brucei rhodesiense in vitro. *Southeast Asian J Trop Med Public Health* **29**: 599-604.

Yamada H, Sun XB, Matsumoto T, Ra KS, Hirano M, Kiyohara H (1991) Purification of anti-ulcer polysaccharides from the roots of *Bupleurum* falcatum. *Planta Medica* **57**: 555-559.

Yuldashev MP, Batirov EK, Malikov VM (1992) Flavonoids of Epigeal Part of *Scutellaria-Ramosissima*. *Khimiya Prirodnykh Soedinenii* 178-182.

Yuldashev MP, Batirov EK, Nigmatullaev A, Malikov VM (1994) Structure of 2 New Flavonoids from *Scutellaria-Ramosissima*. *Khimiya Prirodnykh Soedinenii* 355-359.

Yuldashev MP, Batirov EK, Malikov VM (1995) New Flavonglycoside from *Scutellaria-Ramosissima*. *Khimiya Prirodnykh Soedinenii* 317-318.

Yuldashev MP, Karimov A (2005) Flavonoids of *Scutellaria immaculata* roots. *Chemistry of Natural Compounds* **41**: 32-34.

Zhang TT, Zhou JS, Wang QA (2007) Flavonoids from aerial part of Blupleurum chinense DC. *Biochemical Systematics and Ecology* **35**: 801-804.

Zhao L, Huang C, Shan Z, Xiang B, Mei L (2005) Fingerprint analysis of Psoralea corylifolia L. by HPLC and LC-MS. *J Chromatogr B Analyt Technol Biomed Life Sci* **821**: 67-74.

Zhao LC, Li W, He YF, Li XH, Wang Z, Liu WC, Zheng YN, Liang J (2011) Fast and sensitive LC-DAD-ESI/MS method for analysis of saikosaponins c, a, and d from the roots of *Bupleurum* Falcatum (Sandaochaihu). *Molecules* **16**: 1533-1543.

Zhou DY, Xu Q, Xue XY, Zhang FF, Liang XM (2006) Identification of O-diglycosyl flavanones in Fructus aurantii by liquid chromatography with electrospray ionization and collision-induced dissociation mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **42**: 441-448.

Zu N, Li P, Li N, Choy P, Gong Y (2007) Mechanism of saikosaponin-d in the regulation of rat mesangial cell proliferation and synthesis of extracellular matrix proteins. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire* **85**: 169-174.

Chapter two

ESI-MS in Monitoring the Bio-reactivity Of polyphenols

2.1. Introduction:

2.1.1. Evolutionary advantages of plants secondary metabolites:

Plants secondary metabolites (SM) are involved in many vital aspects of plants biology and mainly play a key role as a protection mechanism against natural enemies such as herbivores, bacteria, fungi and viruses (Wink, 2003). The utilization of SM in curing diseases have a long history in what so-called traditional medicine and still considered as a major source of new therapeutic agents in the modern medicine.

In order to implement their defensive role, plants evolved by building up variety of secondary metabolites that can actively interact with crucial targets; such as functional and structural proteins, biomembrane, and nucleic acids. Consequently, most SM appear to show pleiotropic effects against many targets (Wink, 2008a). Figure (2.1) illustrates the major bio-targets of microorganisms that SM may interfere with.

In general, secondary metabolites interact with targets inducing changes in the conformation of structural biomolecules preventing them to achieve their assigned functionalities (Wink, 2005). These interactions could be divided into two major categories from the physicochemical point of view; covalent and non-covalent interactions (Wink, 2008b).

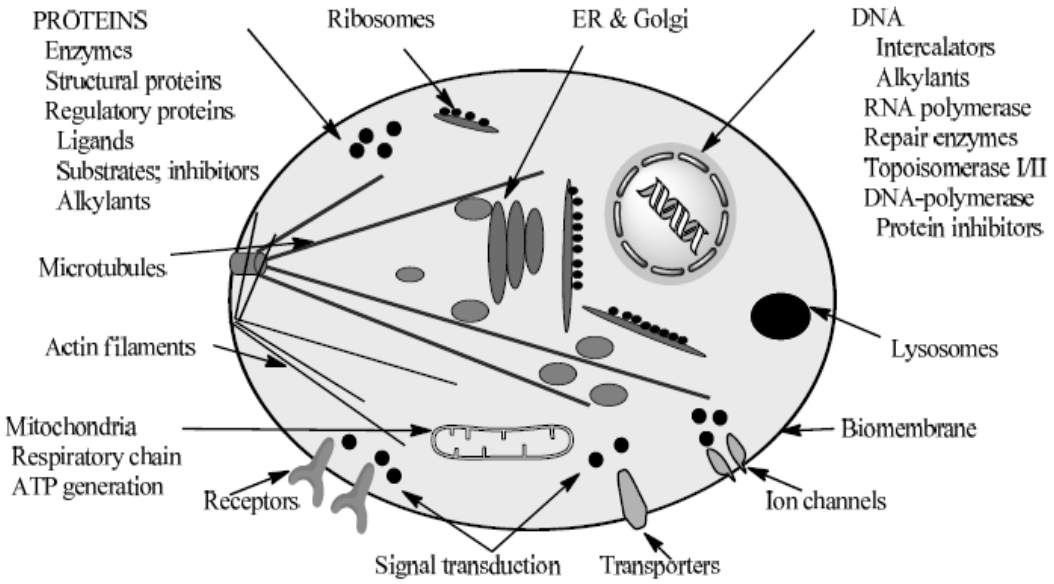


Fig.(2.1) Important molecular targets in microorganisms (Wink, 2008b).

2.1.2. Polyphenols and non-covalent interactions:

One of the most abundant secondary metabolites in nature is the polyphenol derivatives, e.g. flavonoids, stilbenes, anthocyanins and catechins.

The structure of polyphenols based on phenolic hydroxyl functions, these are able to bind to biomolecule via non-covalent bonds; i.e., hydrogen bridges and hydrophobic interactions, but mostly by means of ionic forces, whereas under physiological conditions phenolic hydroxyl groups dissociate forming negatively charged phenolate ions, which in return are able to interact with positively charged centers in biomolecules, such as amino acid residues (lysine, arginine, histidine) in proteins figure (2.2).

Since polyphenols possess plenty of phenolic hydroxyl functions, thus a single polyphenol will bind at several sites on a single biomolecule or even with several biomolecules at the same time. This will make the non-covalent complex stable enough to impair or slow down the bioactivity of the targeted biomolecule (Wink, 2008b).

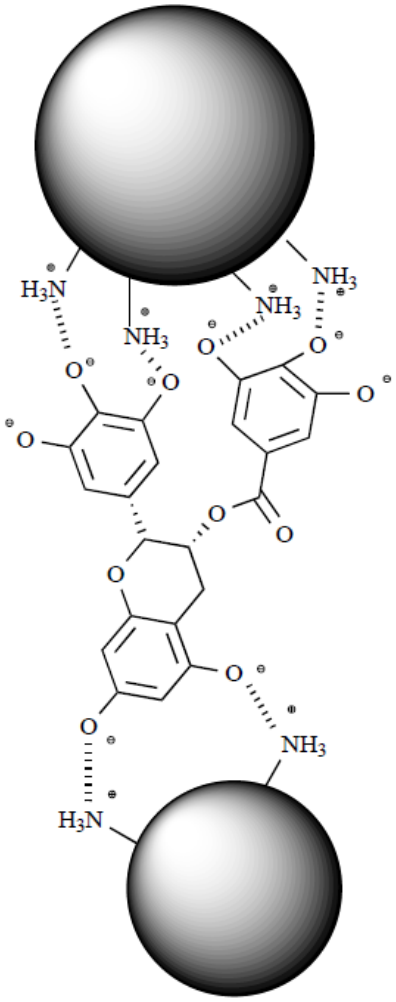


Fig.(2.2) The ionic interactions between a polyphenolic compound and a biomolecule (Wink, 2008b).

2.1.3. Employing non-covalent interactions in drug discovery:

To approve a compound as drug candidate it should be first confirmed, whether this candidate is capable to interact with a biomolecule to induce a desirable bioactivity or not. Monitoring non-covalent interactions between biomolecules and drug candidates is considered as one of the most substantial approaches in the approval of new therapeutic agents.

Non-covalent forces and interactions play a vital role in living cells. On one hand, they maintaining the conformational structure of the biomolecules (Frieden, 1975). On the other hand, the nonstop formation and dissociation of non-covalent complexes ensure many biological functions including molecular recognition, which is one of the most important fields of drug development. The curative purposes of a drug candidate (ligand) will be then achieved when the ligand shows a capability to interact with a biomolecule, inducing either distortion of the 3D structure of the targeted molecule, or masking active sites that leads even to the alteration of the bioactivity or the interruption of key pathways (Hofstadler and Sannes-Lowery, 2006).

Many techniques have been developed to monitor non-covalent interactions and served in the determination of new therapeutic agents. Since the invention of soft ionization mass spectrometry, analysts started to employ these technologies in the field of drug discovery

2.1.4. Techniques used in monitoring non-covalent interactions:

Generally it is not easy to monitor complexes formed through non-covalent interactions, due to the ephemeral nature of such complexes. Though, several well-established techniques have been developed in the past for that purpose, such as circular dichroism spectroscopy (CD), isothermal titration calorimeter (lTC) (Hensley, 1996), nuclear magnetic resonance (NMR) (Zuiderweg, 2002), Surface Plasmon resonance spectroscopy (SPR) (Szabo *et al.*, 1995), and X-ray crystallography (Palmer and Niwa, 2003a). In the last few years technique electrospray ionization mass spectrometry (ESI-MS) has emerged to be an essential tool to study non-covalent interactions is (Veenstra, 1999b; Veenstra, 1999a). ESI-MS has several

advantages over many traditional methods, this advantages according to McLafferty are: specificity, sensitivity, and speed (Loo, 1997).

2.1.4.1. Isothermal titration calorimetry (Wadso, 1997; Pierce *et al.*, 1999; Roselin *et al.*, 2010):

Isothermal titration calorimetry (ITC) is a physical approach used to study bindings between molecules in solution by measuring the heat of reaction that develops during the binding interaction. The experiment starts when a ligand is titrated into a measurement cell that contains a target molecule and the temperature differences are assessed in comparison to a reference cell. ITC methods allow analysts to determine thermodynamic parameters in a direct way. When compared to MS approaches ITC shows two major disadvantages; (i) ITC is a time consuming method with a low productivity (Mathur *et al.*, 2007). (ii) Requires relatively high mount of samples (Baranauskiene *et al.*, 2009).

2.1.4.2. Circular dichroism spectroscopy:

CD spectroscopy is widely employed to study secondary structure of proteins, as well as the conformation of peptides (Woody, 1995). The concept of this method is based on Beer-Lambert law by monitoring the differences in the absorption of a linear polarized light traveling through an optically active substance.

This method attracted the interest of many researchers, whereas high sample amounts are not required. Moreover, the design of CD spectroscopy experiments shows flexibility in the determination of crucial variables such as pH and temperature (Wallace and Janes, 2003).

CD spectroscopy is proven to be an effective technique in studying non-covalent interaction (Palivec *et al.*, 2005; Rodger *et al.*, 2005). Nevertheless, MS techniques still can provide researchers with higher specificity considering structural information.

2.1.4.3. Nuclear Magnetic Resonance Spectroscopy:

Nuclear magnetic resonance (NMR) spectroscopy is a fundamental technique used to resolve chemical structures. It has been also employed to study non-covalent interactions in solution

(Zuiderweg, 2002). Although NMR spectroscopy is considered as "non-destructive" approach, but a high amount of sample are required to produce desired images (Loo, 1997; Smith *et al.*, 1997). Moreover, it is not applicable at higher molecular masses up to 30 kDa (Daniel *et al.*, 2002; Chalmers *et al.*, 2006), and measurements are susceptible to sample impurities. On the other hand, MS are known to be "destructive" techniques, even though smaller amount of samples are required and experiments can be processed for higher molecular masses. In addition, MS techniques are robust and impurities effects can be easily subtracted from obtained data.

2.1.4.4. Surface Plasmon Resonance:

Surface plasmon resonance (SPR) spectroscopy is routinely used to investigate non-covalent interactions (Hensley, 1996). It is based on monitoring changes in optical properties of target molecule in the presence of tested compounds. The target is immobilized on a surface layer and a stream of mobile fluid phase containing the tested compound flows through an interaction channel. Once the interaction takes place between the tested compound and the immobilized target, changes in the optical properties of the target will be registered and later on binding constant could be calculated. Since this method requires immobilization of one of the interacting partners, a possible artificial non-covalent interactions many occur causing false interpretation (Mathur *et al.*, 2007).

2.1.4.5. X-ray chrystallography:

X-ray chrystallography is a widely employed technique to determine structural conformation of biomolecules. The concept of X-ray chrystallography based on resolving X-ray diffraction patterns that occurred from scattered X-ray beam after striking the crystallized biomolecule.

Since late 1940s and X-ray crystallography is employed for providing useful information about non-covalent interactions (Palmer and Niwa, 2003a). Nevertheless, the quality of formed crystals plays a critical role in the manner of the X-ray diffraction and consequently it can impair the resolution (Palmer and Niwa, 2003b; Chalmers *et al.*, 2006).

2.1.4.6. Mass Spectrometry:

Mass spectrometry (MS) is increasingly gaining the interest of researchers in many fields beyond its basic application as analytical tool for the determination of molecular masses. The main concept of MS techniques is base on the early works of J. J. Thomson, who managed to measure what so-called charge-to-mass ratio (e/m) of electron.

Later MS machines have employed the mass-to-charge ratio (m/z) concept (Griffiths, 2008), where studied molecules are first ionized, then transferred into a vacuum, and later detected and measured in response to their trajectories when they are exposed in the vacuum to electric and/or magnetic fields (Fenn *et al.*, 1989b).

At the early stages the applications of MS machines were limited to molecular mass determination and later in the detection of impurities (Griffiths, 2008), but as the mid 80s approached "softer" ionization techniques were invented allowing analysts for the first time to probe masses at the range of biomolecules (Baytekin *et al.*, 2006; Griffiths, 2008). Later in the 90s different reports mentioned that non-covalent complexes can be detected using soft ionization mass spectrometry. Gradually, researchers became more aware of the quite significant influence of MS techniques as an ideal device in probing fields of structural biology and molecular recognition based on non-covalent chemistry (Loo, 2000).

Nowadays there are two soft ionization techniques that are proven to be method of choice for ionizing proteins and peptides, namely, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) (Glish and Vachet, 2003; Griffiths, 2008)., and these techniques have attracted the awareness of biologists and pharmacologists as useful tools in the field of drug discovery (Glish and Vachet, 2003; Hofstadler and Sannes-Lowery, 2006).

The MALDI sources are mainly found within the area of proteomics, but also they were reported to be employed to monitor interactions in the solid phase (Wortmann *et al.*, 2007; Poetsch *et al.*, 2008). On the other hand, ESI sources were successfully utilized to study interactions in solutions and increasingly emerged to be an essential tools in studying non-covalent interactions in a way that simulate an interaction taking place in biological milieu (Veenstra, 1999b; Veenstra, 1999a).

Although considered as complementary techniques MS approaches have many several advantages over other traditional ones, some of these attractive advantages according to McLafferty are: specificity, sensitivity, and speed (Loo, 1997). Table (2.1) summarizes some of the disadvantages of traditional methods which have been overcome by MS techniques.

2.1.5. Electrospary Ionization Mass Spectrometry (ESI-MS):

Late 60s Dole and colleagues had made the way free to the invention of atmospheric pressure ionization (API) mass spectrometry. It was then possible to generate charged nonvolatile solutes from solution into gas form and transfer them directly to the mass analyzer (Clegg and Dole, 1971). By 80s the effort of Fenn and collaborators resulted into the invention of new generation of MS techniques, namely, the electrospray ionization mass spectrometry (ESI-MS) (Fenn *et al.*, 1989b). The electrospray mechanism proved to be suitable to preserve complexes formed out of weak molecular interactions (Cole, 2003). Consequently, researchers exploited this concept and employed ESI-MS to gain a deeper insight in the nature of interactions present in living systems.

Table (2.1) the disadvantages of some classical methods used in monitoring non-covalent interactions bypassed when using MS techniques:

Technique	Disadvantages
ITC	• Elaborative and time consuming • Low productivity
CD Spectroscopy	• Low sensitivity
NMR	• High samples amount • Mass rage restriction
SPR	• Ligands and/or targets need modification • Relatively expensive
X-ray crystallography	• Requires multi-milligram quantities • Difficulties in attaining optimal crystals

In the last few years a considerable body of literatures focused on ESI-MS as a technique to examine non-covalent interactions, e.g., studying the effect of metal ions on the folding of protein Kinase C (Shindo *et al.*, 2003), investigate the importance of Protein-Protein and Protein-RNA interaction in drug development (Klebe *et al.*, 2009), exploring the interactions between DNA and polyphenols in anti-cancer and anti-bacterial field of studies (Liu *et al.*,

2009), probing the role of melatonin in Alzheimer's disease (Bazoti *et al.*, 2005), and applications in the field of gene therapy (He *et al.*, 2011).

2.1.6. The electrospray ionization source:

The main concept of electrospray ionization methods based originally on both Zeleny's works about the effects of electrical fields on liquid surfaces, and Taylor observations considering "free jet" phenomenon. In 1968 Dole and co-workers reported the possibility of using electrospray to generate ions of macromolecules. Then in 1988 Fenn and colleagues conducted the first reliable experimental results on a large molecules (Wilm and Mann, 1994).

Figure (2.3) illustrates the ESI source. Sample solution is introduced into the ESI source, through a capillary with inert metal surfaces, either directly using syringe pump or by means of LC system. A pressurized inert gas (Nebulizing gas) turns the sample solution into small droplets (Spray form) inside the ionization space, this all occurs under atmospheric pressure. A high voltage at the metal capillary tip is generated leads to the formation of charged droplets. The droplets are pushed from the capillary tip towards a counter electrode (Extracting Cone). Assisted by inert heated gas (Drying gas) the evaporation (desolvation) of the solvent occurs as the droplets traverse the space between the capillary tip and the extracting cone. The highly charged droplets shrink gradually as the solvent evaporates and the charged molecules turn into their gas phase state. These charged molecules, or what so-called molecular ions, are driven by means of the extracting cone from the ionization chamber under atmospheric pressure towards the mass analyzer under high vacuum. Afterward, a set of electrical lenses transfer the ions to the analyzer focusing them to obtain a precise measurements.

Many types of MS analyzers have been developed to fulfill the versatile needs of the analysts, but quadrupole (Q) analyzers are the most used analyzers in combination with electrospray ionization sources.

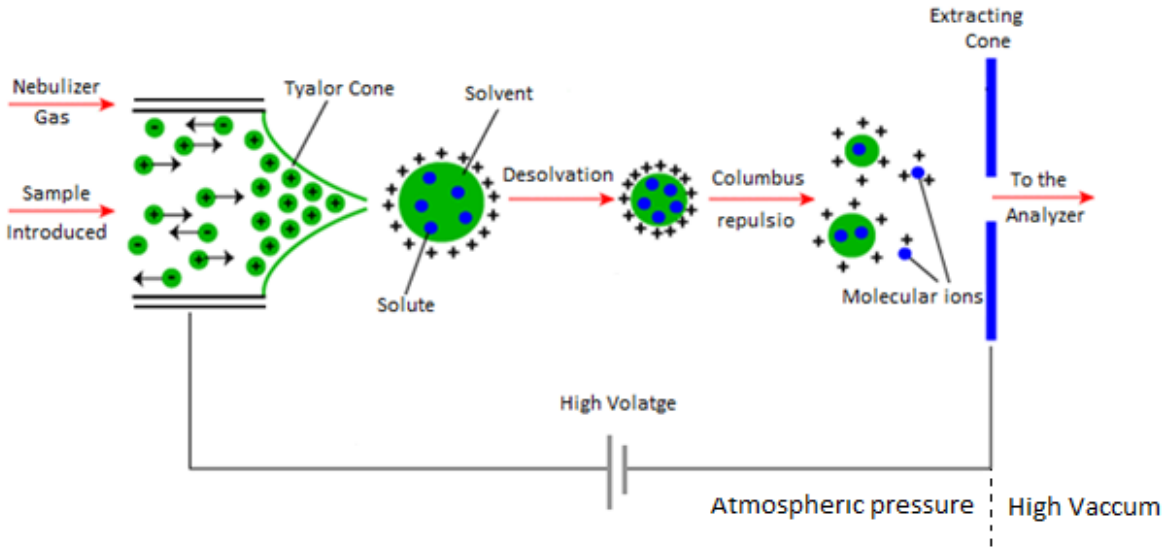


Figure (2.3) an illustration of electrospray ionization source. (Obtained from original scheme, University of Bristol, School of Chemistry).

2.1.6.1. Quadrupole analyzer (Q):

A quadrupole mass analyzer consists of four parallel metal electrodes. These electrodes are organized in a square configuration, and an electromagnetic field is generated in between. The molecular ions cross the quadrupole parallel to the electrodes in the center of the square configuration. Only ions of a certain mass-to-charge ratio (m/z) that can oscillate in the electromagnetic field will reach the ion counter, others will have unstable trajectories, will ramp into the electrodes and will be neutralized. Figure (2.4) illustrate the quadrupole analyzer and the two possible trajectories that ions may follow under the generated effects of the electromagnetic field.

This kind of analyzer allows; even (i) the selection of one molecular ion with a particular m/z by fixing the force of the electromagnetic field, this process called selected ion monitoring (SIM), or (ii) the application of multiple reactions monitoring (MRM) scan mode, in which a variable electromagnetic field is applied permitting the detection of different molecular ions with different m/z values. The first procedure permits the studying of the chemical structure and the purity of a chosen compound, meanwhile the second procedure is applied to study samples containing two analytes and more. One of the most important features of quadrupole analyzer is being robust, and the ability to provide pretty good sensitivity at low limits of detection (Pelaez *et al.*, 2002).

A triple-quad MS is a mass spectrometry instrument provided with three quadrupole analyzers lined up, or what so-called tandem mass spectrometry. The first (Q1) and third (Q3) quadrupoles act as analyzers, while the middle one (q2) is designed to work as a fragmentation chamber. A selected molecular ion (parent ion) at Q1 in the SIM mode will be steered into the q2 to undergo a fragmentation process assist with collision energy. The formed fragments (daughter ions) will pass to Q3 and analyzed using the MRM scan mode. Tandem mass spectrometry was implemented intensively to work out structural elucidation studies (Busch, 2010).

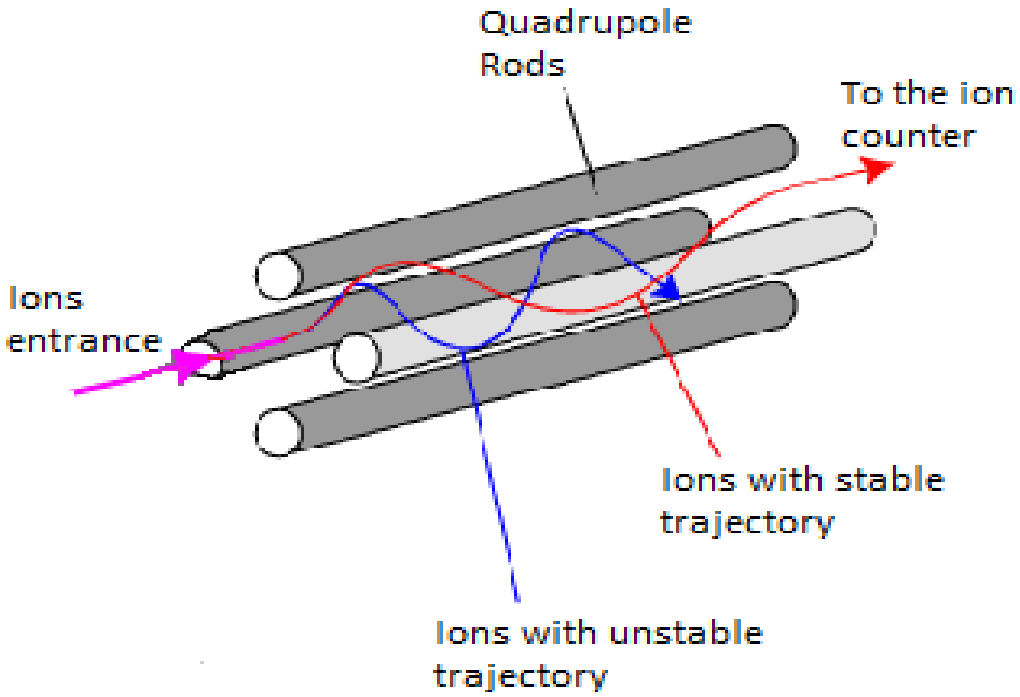


Fig. (2.4) Depiction of quadrupole analyzer. After ions enter quadrupole electromagnetic field ion resonate through the quadrupole to the analyzer, meanwhile others are unstable will be neutralized at the electrode surfaces. (Obtained from original scheme, University of Bristol, School of Chemistry).

2.1.7. Monitoring non-covalent interactions using ESI-MS:

Three main approaches are applied to find out whether a drug undergoes a desirable interaction with a targeted biomolecule or not. (i) Non-covalent mass spectrometry; the drug-biomolecule complexes remain intact while transferred from the sample solution to the MS detector. Therefore, the formed complexes are directly monitored and furthermore tandem MS can be used to assay these complexes. (ii) Hydrogen/deuterium mass spectrometry; whereas the interactions are indirectly gauged through observing the shifts in

hydrogen/deuterium exchanging rates. (iii) Condensed-phase separation techniques; are indirect techniques based of chromatographic methods to prepare the sample and separate the complexes to be monitored with MS as a supplementary detector for each individual non-covalent complex.

2.1.7.1. Non-covalent mass spectrometry:

As a gentle ionization technique ESI-MS can maintain the non-covalent complexes intact and being directly detected (Loo, 2000). Formation of adducts and aggregates have been reported in the early the works of investigating biomolecules using ESI techniques (Whitehouse *et al.*, 1985; Smith *et al.*, 1990). These adducts and aggregates are nothing but kind of non-covalently weak-bounded complexes that reflect even a self-assembly phenomena, or an interaction between biomolecules and ions that may exist in lab wares and equipments. These observations has been utilized to investigate the Heme-globin complex formation (Katta and Chait, 1991) and further in probing a wide variety of macromolecular host-guest interactions (Ganem *et al.*, 1991). These early paved the way for analysts to embrace ESI-MS in studying non-covalent interactions as a powerful tool in drug discovery at different domains (Drummond *et al.*, 1993; Tang *et al.*, 1994; Greig *et al.*, 1995; Cheng *et al.*, 1996).

In this approach complexes are formed in solution by the addition of desired ligand to buffered target at required pH. Samples are then infused directly in the MS machine through the ESI source using syringe pump, and complexes are monitored by direct evaluation of MS spectra. By means of this approach the effect of many variables on the formation of the non-covalent complexes can be monitored instantly, such as pH, solvent, temperature, and incubation time. Moreover, it is a straightforward approach in detecting sample adulterants, biomolecule degradation, artifacts and self-assembly complexes.

2.1.7.2. Hydrogen/Deuterium MS:

The hydrogen/deuterium exchange (HDX) phenomenon was successfully used to study the structural changes of proteins (Woodward *et al.*, 1982). This approach was successfully combined with many, such as infrared, ultraviolet absorption spectroscopy and NMR (Smith *et al.*, 1997). Later on, and after the intervention of MS, the hydrogen/deuterium exchange

was rapidly applied with mass spectrometry to obtain a high-resolution technique in the field of proteins structure, dynamics, and functions (Garcia *et al.*, 2004). When compared to earlier techniques, MS techniques possess many attractive features, such as sensitivity, ability to analyze larger molecule (Hoofnagle *et al.*, 2003), and amenability to high throughput screening.

The hydrogen/deuterium exchange is a chemical interaction, in which covalently bounded hydrogen atoms will be exchanged with deuterium atoms when the surrounding is offering a plenty of deuterium atoms. In HDX approaches only hydrogen atoms that are located on the amide linkage (also known as backbone amines) can be assayed, since that the other exchangeable hydrogen atoms possess fast exchange rates that make it inaccessible to any known detectors (Smith *et al.*, 1997). Since that deuterium atom is a one mass heavier than hydrogen atom, this extra one mass unit from each exchangeable hydrogen atom will be incorporated into the biomolecule increasing its total mass. These changes in mass value can be easily calculated using MS machines and with the measurement of HDX rates it is possible to, draw conclusions about dynamics of biomolecules and monitoring their interactions with tested ligands.

2.1.7.3. Condensed-phase Separation techniques:

In this category “Spin Column” is the most common applied method. The method utilizes the principles of size exclusion chromatography in a form of short chromatographic columns (Dunayevskiy *et al.*, 1997). Sample, which consists of the target (protein) and a candidate, is usually loaded on top of short gel packed chromatographic column (GPC) and exposed to centrifugation. The eluted buffer will then contain both of free unbounded target and ligand-target complexes, while free unbounded ligands would be retained in the GPC spin column. The collected elutes from different experimental settings are then denatured to win back bounded ligands, afterward these ligands are subjected to mass spectrometric measurement to find out which of tested ligand was capable to bind targeted biomolecule (Siegel, 2009).

2.1.8. Why studying non-covalent interactions of the polyphenols?

Polyphenolic compounds are considered one of the most widespread secondary metabolites (SM) in the kingdom of higher plants. They are extremely important components in human diets, and possess a versatile of pharmacological activities. Polyphenols exert hypolipidemic properties, thus they have protective effects on the cardiovascular system (Curin and Andriantsitohaina, 2005; Pakalapati *et al.*, 2009), they play an active role as immune-modulators (Deng *et al.*, 2010), anti-inflammatory (Figueirinha *et al.*, 2010), anti-tumor (Ito *et al.*, 1999) and antimicrobial(Weisse *et al.*, 1995) agents. Moreover, some polyphenols (Isoflavonoids) proved to be effective in the hormonal replacement therapy (HRT) (Pakalapati *et al.*, 2009). Some other polyphenols, namely catechins, are proved to be highly active as radicals scavengers and oxidative stress eradicators, thus been recommended as a supportive anti-aging remedy (Abbas and Wink, 2009), anti-neurodegenerative (Abbas and Wink, 2010) and cancer (Henning *et al.*, 2010) therapies.

The bioactivity of polyphenols as antioxidant and radical scavengers has been extensively reported. Less attention has been drawn to the reactivity of polyphenols towards biomolecules. It is becoming more obvious that the mechanisms of action of polyphenols go beyond the suppression of oxidative stress (Scalbert *et al.*, 2005). Through non-covalent forces polyphenols are able to modulate the 3D structure of proteins leading even to the obstruction or the slowing down of the protein bioactivity (Wink, 2008b). In the last few years a considerable body of literatures focused on ESI-MS as a technique to investigate the non-covalent interaction of polyphenols with DNA, as potential anti-cancer and anti-bacterial therapeutic agents (Liu *et al.*, 2009). Meanwhile, applications of ESI-MS in monitoring non-covalent interactions of polyphenols with proteins have been less mentioned.

2.2. Materials and methods:

2.2.1. Chemical reagents:

Hydroxyflavone, kaempferol, rhamnetin, quercetin, myricetin, taxifolin, procyanidin B2, procyanidin C1, L-epicatechin, rutin, quercitrin, spiraeoside, hyperoside, and scutellarin were obtained from Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany.

Epigallocatechin gallate (EGCG), angiotensin (I), insulin, as well as deuterium oxide were purchased from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany.

Angiotensin (I) was reconstituted in an aqueous solution of 20% acetonitrile to obtain a final concentration of 1mM and kept at -20^{o}C, whereas stock solutions of polyphenols were prepared in methanol (J.T.Baker) and reserved in 4^{o}C. Figures (2.5a), (2.5b) and (2.5c) illustrate the chemical structures of the polyphenols utilized in this study. Chromanorm-water, acetonitrile and formic acid (HPLC grades from VWR International GmbH, Bruchsal, Germany) were used throughout all experiments.

For mass spectrometric analysis, ammonium acetate salt (Riedel-de Haën, Seelze, Germany), glacial acetic acid (J.T.Baker) and ammonium hydroxide solution (Fluka, sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) were used to prepare the buffered solution and adjust pH of measured samples.

D-(+)-glucose (*Roth Carl* GmbH, Karlsruhe, Germany) a monosaccharide and stachyose (sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) a tetrasaccharide, were both tested to find out whether the sugar residues in the polyphenol glycosides play any role in the non-covalent interactions.

To gain a deeper scope into how amino acids sequence affect the formation of the non-covalent complexes, four synthesized oligopeptides were used. Table (2.2) summarize the sequence of the four oligopeptides and shows their mono-isotopic mass value. The stock solutions of 1mM were prepared using 30% aqueous acetonitrile and kept at -20^{o}C.

Table (2.2) four synthesized oligopeptides were used to study the structure-interaction relationship. The oligopeptides synthesis was performed by Dr. D. Sarko, in the research group of PD Dr. Walter Mier, at Radiopharmazeutisches Labor, Nuklearmedizin, Kopfklinik, Heidelberg, Germany:

Peptide code	Number of lysine residues	The mon-isotopic mass in Dalton
P1	2	2310
P2	4	2340
P3	8	2401
P4	8	2401

P1	Leu-Leu-Leu-**Lys**-Leu-Leu-Leu-Leu-Leu-Leu- Leu-Leu-Leu-Leu-Leu-Leu-**Lys**-Leu-Leu-Leu
P2	Leu-Leu-Leu-**Lys**-Leu-Leu-Leu-**Lys**-Leu-Leu-Leu-Leu-**Lys**-Leu-Leu-Leu-**Lys**-Leu-Leu-Leu
P3	Leu-Leu-**Lys**-Leu-**Lys**-Leu-**Lys**-Leu-**Lys**-Leu-Leu-**Lys**-Leu-**Lys**-Leu-**Lys**-Leu-**Lys**-Leu-Leu
P4	Leu-Leu-**Lys**-**Lys**-**Lys**-**Lys**-Leu-Leu-Leu-Leu-Leu-Leu-Leu-Leu-Leu-**Lys**-**Lys**-**Lys**-**Lys**-Leu-Leu

2.2.2. Mass spectrometry instrumentation:

Mass spectrometry experiments were performed using Micromass VG Quttro II machine equipped with ESI source (*paragraph 2.1.5.*) and quadrupole analyzer (*paragraph 2.1.5.1.*). Samples were directly infused into the source region at a rate of 10 µL/min using syringe pump (Razel; Fisher Scientific, France), and data were acquired in positive full scan mode.
Datas processing were performed using MassLynx V. 4.0. and all registered m/z values were calculated based on the mono-isotopic mass value.

Samples were analyzed under the following parameters: Positive ionization mode, quadrupole analyzer in scan option (MRM) at dynamic range 400-1500 m/z, drying and nebulizing gas N_2, source temperature 120^{o}C and Cone voltage: 30 kV.

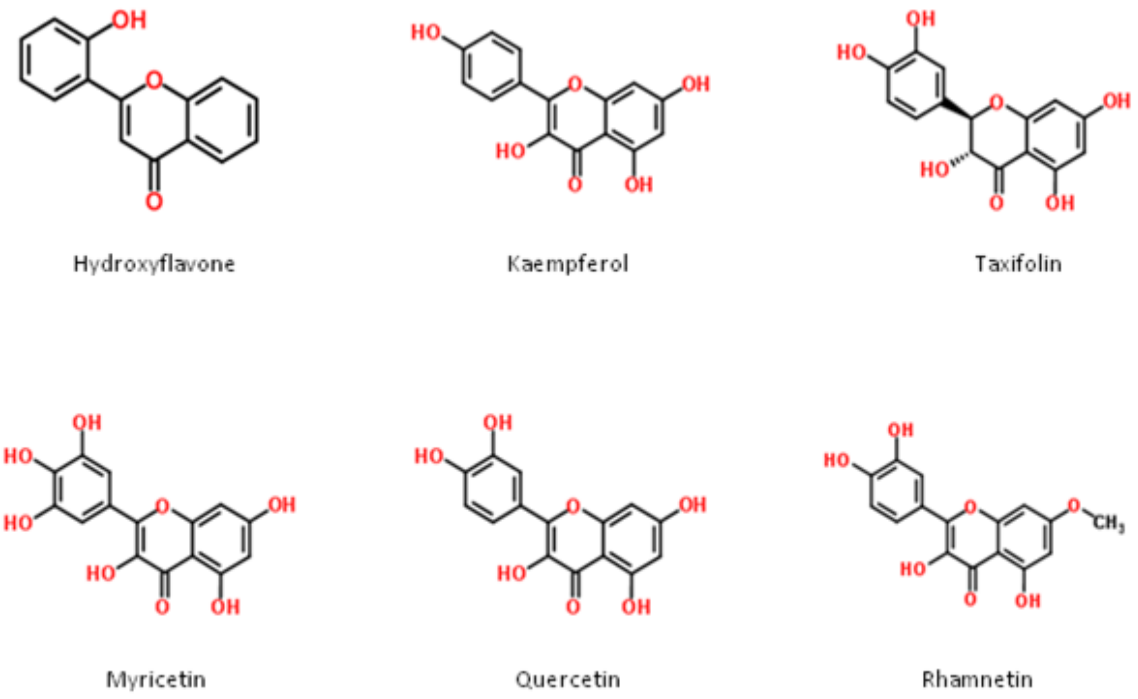


Figure (2.5a) chemical structures of flavonoid aglycones.

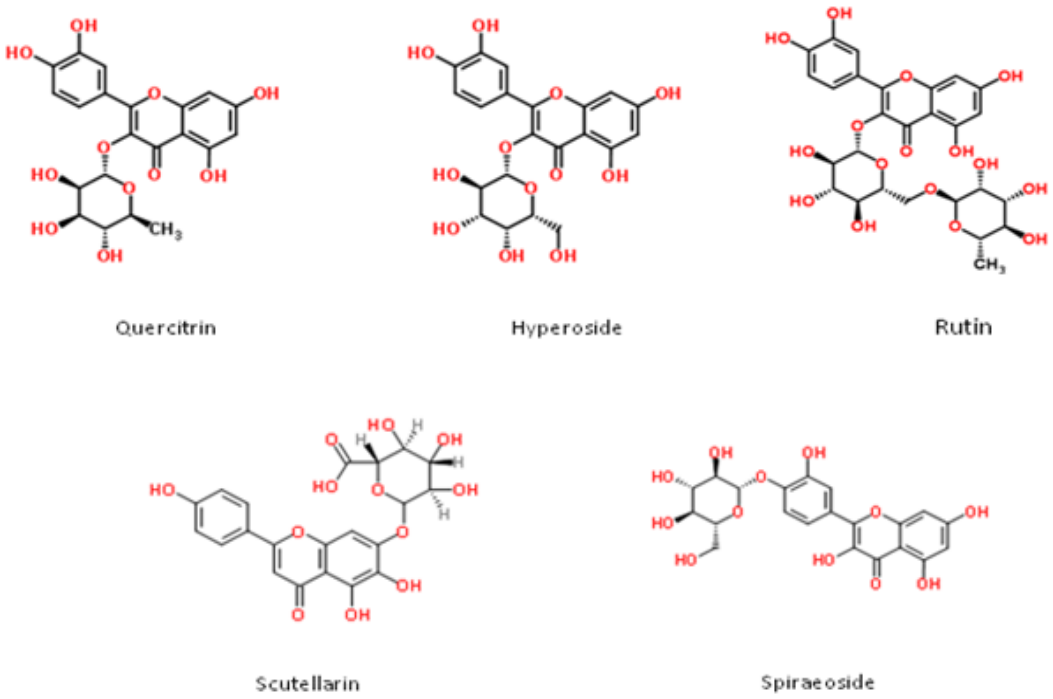


Figure (2.5b) chemical structures of flavonoid glycosides.

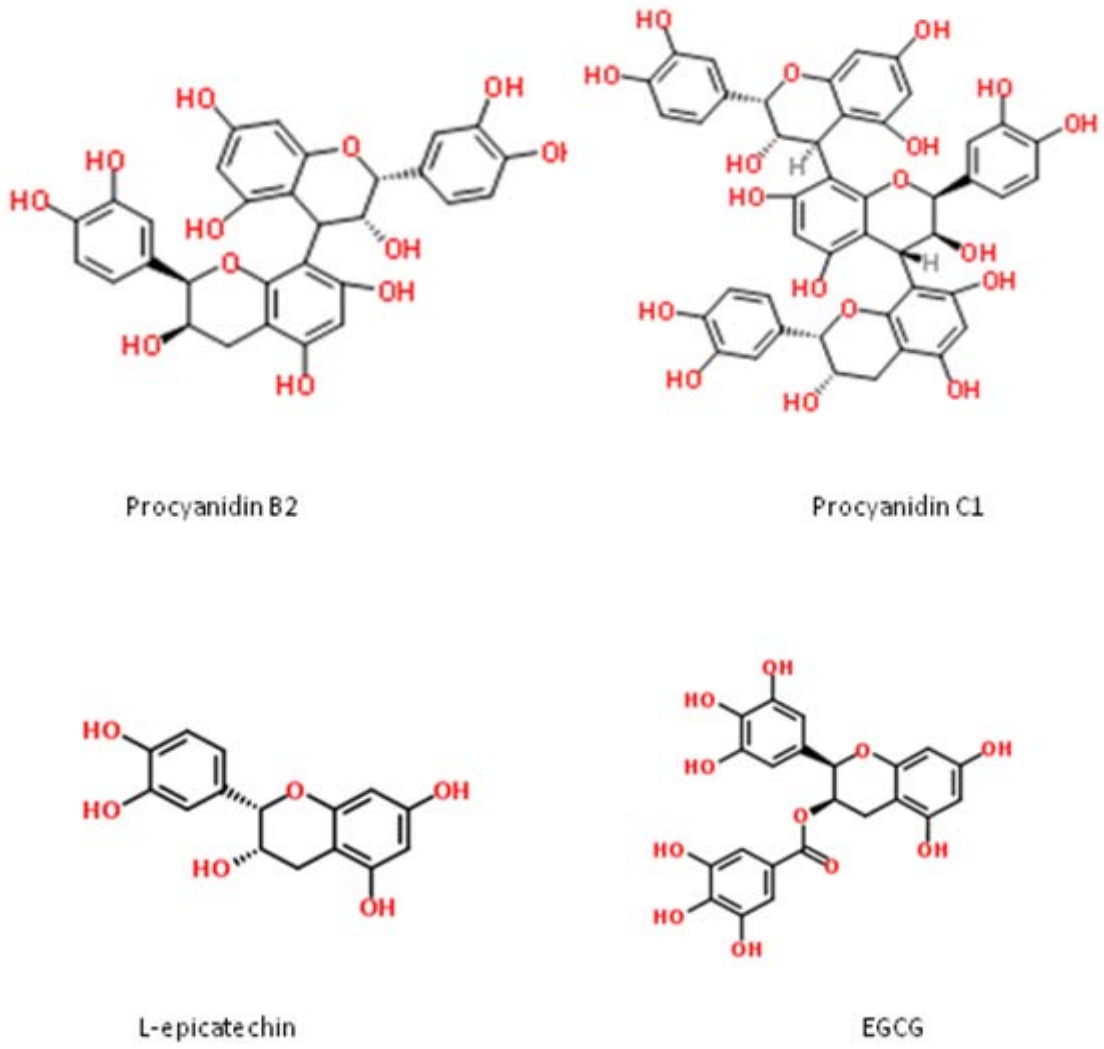


Figure (2.5c) chemical structures of procyanidins and catechins .

2.2.3. ESI-MS in monitoring the non-covalent interactions:

To investigate the non-covalent interactions by means of ESI-MS two methods were employed. (i) Non-covalent mass spectrometry (*paragraph 3.2.1.*), where both target and ligand are mixed and a direct detection of the whole complexes are feasible, (ii) hydrogen/deuterium exchange (*paragraph 3.2.2.*). The monitoring of the non-covalent interactions is assessed indirectly after.

2.2.3.1. Non-covalent mass spectrometry:

Polyphenols at different molar ratios were mixed with angiotensin (I). The final concentration of angiotensin (I) was 8 µM, and polyphenols were mixed with angiotensin (I) at four different concentrations (8, 16, 32 and 80 µM). Samples were buffered using ammonium acetate, and left to equilibrate at room temperature for 5 minutes. To monitor the relationship between peptide sequence and complexes formation, the peptides shown in table (2.2) were mixed with the polyphenols at fixed pH value of 7.0 in a ratio of 1:10 peptide to a polyphenol.

On the other hand, and since ionic interactions between polyphenols and biomolecules are dependent on the formation of phenolate ions, thus, the effect of pH alteration is monitored, where data were acquired for the samples at different points of pH values. For this purpose ammonium acetate buffer was used adjusted by adding glacial acetic acid or ammonium hydroxide solutions and the following equation is applied to evaluate the relative intensity ($Int._{complex}$) of the formed complex in comparison to the unbounded target:

$$\mathbf{Int._{complex} = (TIC / \Sigma\ TIC^{*})}$$

Where is **TIC** represents the total ion count number of ligand-target complex and **TIC*** represents the total ion count number for ions which related to unbounded target.

2.2.3.2. Hydrogen/Deuterium Exchange mass spectrometry:

HDX mass spectrometry is proven to be a very attractive method to study the formation of hydrogen bonds between biomolecules. The experiment was done according to Zhu et al.(Zhu *et al.*, 2004), whereas ice-cold, 20% acetonitrile solution containing formic acid at pH 2.5 was used instead of HCl solution to quench the reaction. Hence, the desalting step using the small RP C-18 column mentioned by Zhu is not any more necessary.

The experiment starts by allowing the targeted biomolecule to equilibrate with different concentrations of polyphenols at pH value of 7.0.The protocol starts with a high D/H ratio by adding deuterium to initiate the exchange of hydrogen with deuterium. As soon as the

exchange reaches a point near to equilibrium, the exchange is quenched by adding ice-cold solution of 20% aqueous acetonitrile containing 2% formic acid, which decreases the pH to nearby 2.5. The non-covalent interactions of the ligands with the biomolecule are then calculated using the following equation:

$$\mathbf{\Delta D = |M_{obs} - M_{lig}|}$$

Where ΔD is the calculated difference of deuterium uptake number, M_{obs} is observed mass of deuterized biomolecule in the absence of ligand and M_{lig} is the observed mass in the presence of ligand. Calculations are done based on mono-isotopic mass number.

2.3.1. The ionic interaction between angiotensin (I) and polyphenols:

2.3.1.1. MS spectrum of angiotensin (I):

Angiotensin (I) consists of 10 amino acids with the following sequence: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu. The mono-isotopic mass of the anhydrous free base of angiotensin (I) is 1295.68 Dalton. Since nitrogen atoms in amino acid residues could be theoretically protonated, thus, peptides and proteins tend to formed a multiply charged molecular ions such as $(M+nH)^{+n}$ when using the ESI-MS at the positive ionization mode (Fenn *et al.*, 1989). Fig (2.6) demonstrates the MS spectrum of 8 µM angiotensin (I) in ammonium acetate buffer solution at pH 7.0 under ESI-MS experimental conditions. Two major peaks at m/z 649 and m/z 433 were detected, representing double and triple charged molecular ions, respectively. A peak with low intensity was detected at m/z 1297 representing the single charged angiotensin (I). The signal at m/z 649.35 peak is the most intensive one. Thus, it is expected, that any formed complex between angiotensin (I) and polyphenols will probably belong to doubled charged molecular ions species. Table (2.3) show the different detected signal species obtained from angiotensin when (I) using ESI-MS.

The calculation of the mass value of angiotensin (I) from the obtained m/z signals can be expressed by following:

$$\mathbf{m/z = (MW + nH+)/n}$$

Where; m/z is the mass-to-charge ratio obtained from the spectrum.
MW represents the molecular mass of the sample.
Value n represents the number of charges of detected ions, and H the mass of a proton (approximately 1.008 Dalton).

The vale n can be calculated by assuming that any two nearby m/z signals differ by one charge. For example, if a signal at m/z 649 have "n" value of charges, then the ions at m/z 433 should have "n+1" charges, and the above equation can be used for both ions:

$$649 = (MW + nH^+)/n$$

$$433 = [MW + (n+1)\ H^+]\ /(n+1)$$

By solving the two equations the unknown can be identified. In this case n=2, and by inserting the value of n back into the upper equation we got:

$$649 = (MW + nH^+)\ n$$

$$649 \times 2 = MW + (2 \times 1.008)$$

$$MW = 1298 - 2.016$$

$$MW = 1295.99\ Da$$

The calculated value of 1295.99 Da. obtained from experimental data is in a good agreement with the theoretical mass of 1295.68 Dalton with mass error of 0.015%, which is accepted when using ESI sources.

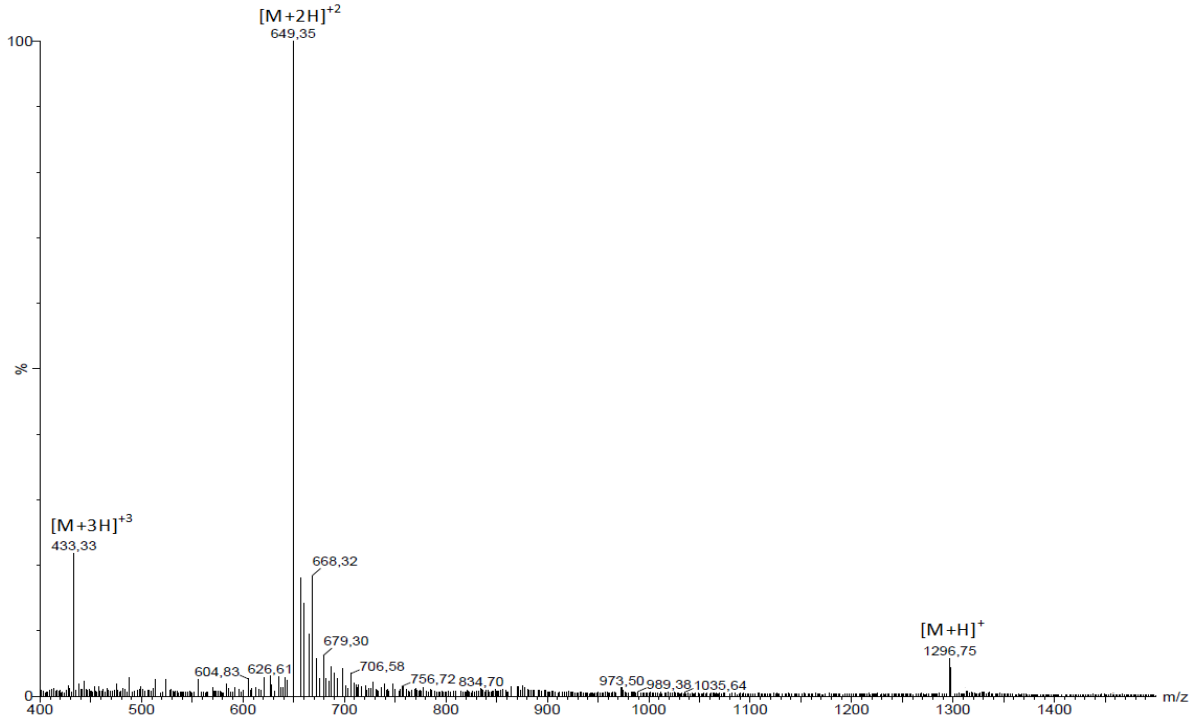


Fig. (2.6) ESI-MS (+) of 8 µM angiotensin (I) in ammonium acetate at pH 7.

Table (2.3) Detected peaks of angiotensin (I) at ESI-MS experimental conditions:

Signal species	m/z observed	m/z Calculated
$[M+H]^{+1}$	1297	1296.69
$[M+2H]^{+2}$	649	648.85
$[M+3H]^{+3}$	433	432.90

2.3.1.2. The interaction of angiotensin (I) with polyphenols:

Acquired data showed that rutin; a flavonoid glycoside of quercetin that has two sugar residues, is able to form 1:1 ligand-target complexes with angiotensin (I). A detected peak at m/z 954 represents the mono-isotopic double charged molecular ion complex of rutin and angiotensin (I). Figure (2.7) shows the MS spectrum of angiotensin (I) with rutin at molar ratio of 10 to1 rutin to angiotensin (I) in ammonium acetate buffer solution at pH 7.0. The detected signal at m/z 954 in the spectrum belongs to the doubled charged complex. Therefore, the observed M_{obs} are calculated as following:

$$m/z = (M_{obs} + nH+)/n$$

$$954 = (M_{obs} + 2 \text{ X } 1.008)/2$$

$$M_{obs} = 1905.98$$

$\mathbf{M_{obs}}$ represents the mono-isotopic deconvulated mass of the complex observed using ESI mass spectrometry. Since that the mono-isotopic mass of rutin is 610.15 Dalton and mono-isotopic mass angiotensin (I) equals 1295.68 Dalton. Thus, the 1:1 rutin-angiotensin (I) complex should have the theoretical mass value of 1905.83 Da. Compared with the observed one $\mathbf{M_{obs,}}$ we can confirm that the value 1905.98 stands for 1:1 rutin-angiotensin (I) complex with an good agreement at mass error of approximately 0.016%.

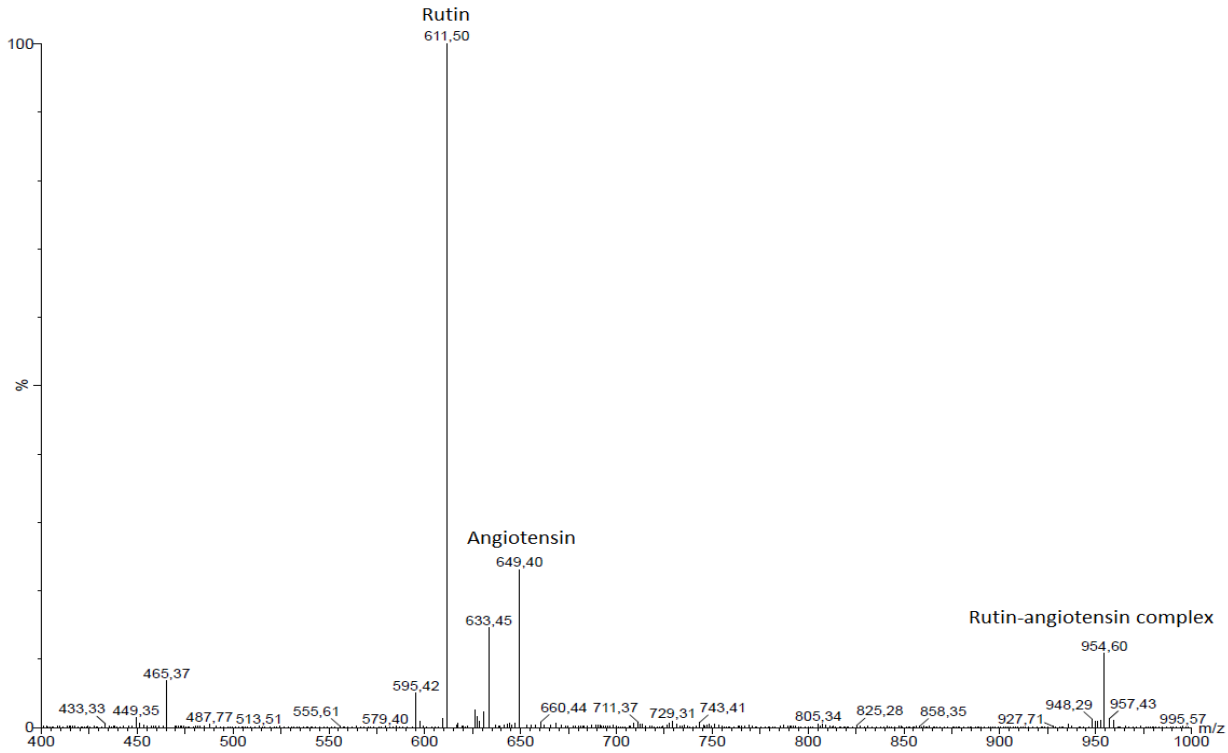


Fig. (2.7) ESI-MS (+) of angiotensin (I) and rutin at molar ration 1:10 in ammonium acetate at pH 7. The signal at m/z 954 represents 1:1 rutin-angiotensin complex. Signal at m/z 611 belongs to single-charged molecular-ion of rutin and m/z 694 is the molecular-ion of angiotensin bearing two protons.

MS spectra obtained from the different tested polyphenols showed only 1:1 binding stoichiometries complexes with angiotensin (I), and all observed and detected signals were belonged to double-charged molecular ions species molecular ions, e.g. $[M+2H]^{+2}$.

In comparison to rutin both hyperoside and spiraeoside could form a complex with angiotensin (I); figures (2.8) and (2.9). Both have the same molecular weight of 464 g/mole, and showed a signal at m/z 881 which is related to 1:1 binding stoichiometries. On the other hand, the spectrum of angiotensin (I) and quercetin; the aglycon counterpart of rutin and both hyperoside and spiraeoside, revealed no signals related to any kind of complexes figure (2.10).

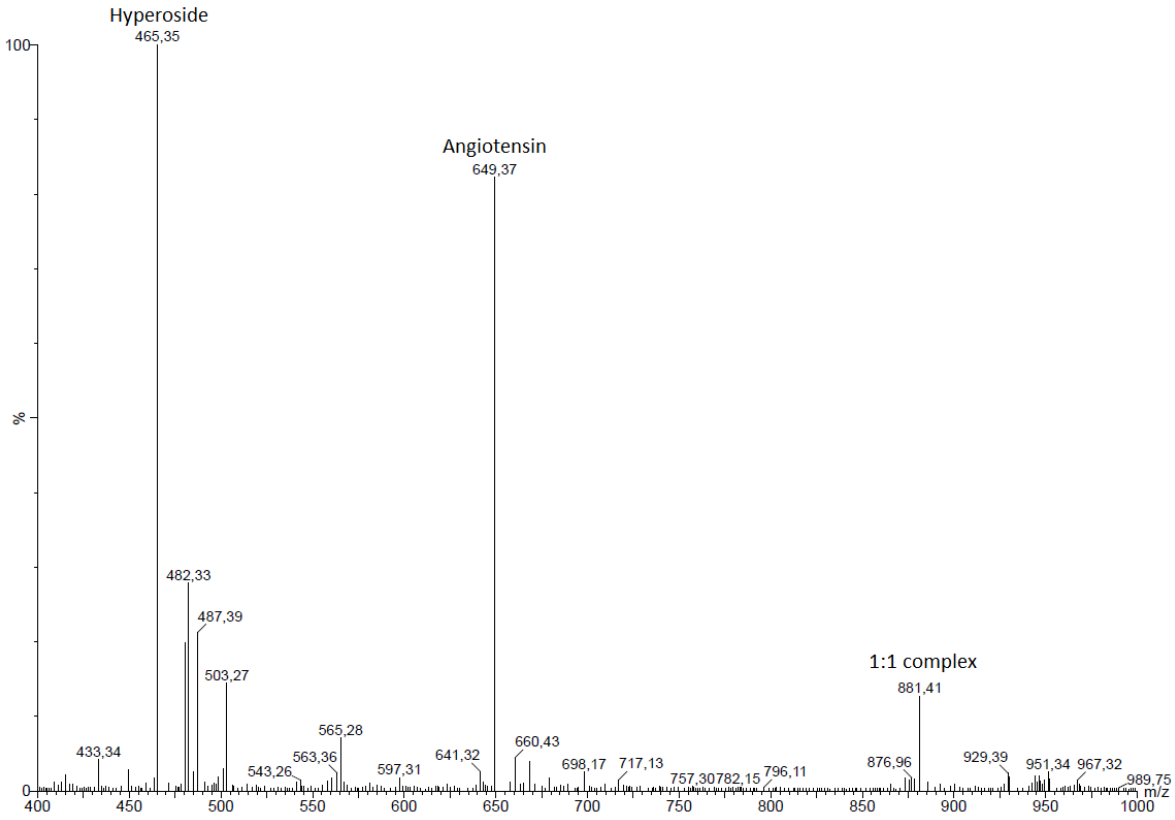


Fig.(2.8) ESI-MS (+) of angiotensin (I) and hyperoside at molar ration 1:10 in ammonium acetate at pH 7. The signal at m/z 881 represents 1:1 hyperoside-angiotensin complex. Signal at m/z 465 belongs to single-charged molecular-ion of hyperoside and m/z 694 is the molecular-ion of angiotensin bearing two protons.

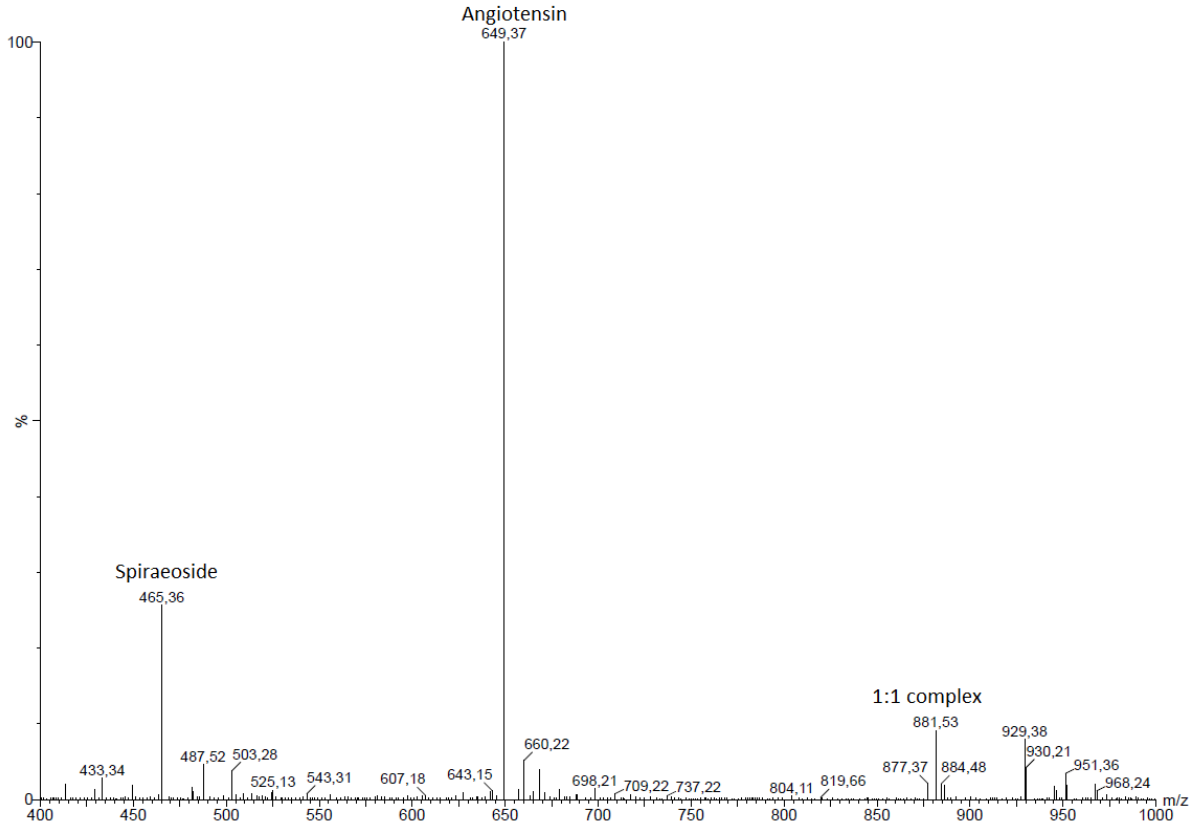


Fig.(2.9) ESI-MS (+) of angiotensin (I) and spiraeoside at molar ration 1:10 in ammonium acetate at pH 7.

Again by applying the above mentioned equation we can calculate the observed mass using m/z values obtained from MS spectra and compare it with the theoretical one:

$$m/z = (M_{obs} + nH+)/n$$

$$881 = (M_{obs} + 2 \text{ X } 1.008)/2$$

$$M_{obs} = 1759.98$$

M_{obs} represents the mono-isotopic deconvulated mass of the complex. The mono-isotopic mass of hyperoside is 464.09 Dalton and of angiotensin (I) equals to 1295.68 Dalton. Therefore, the 1:1 rutin-angiotensin (I) complex should have the theoretical mass value of 1759.77 Da. Compared with the observed one above, we can conclude that the value 1759.98 stands for 1:1 hyperoside-angiotensin (I) complex with a good agreement at mass error of approximately 0.012%.

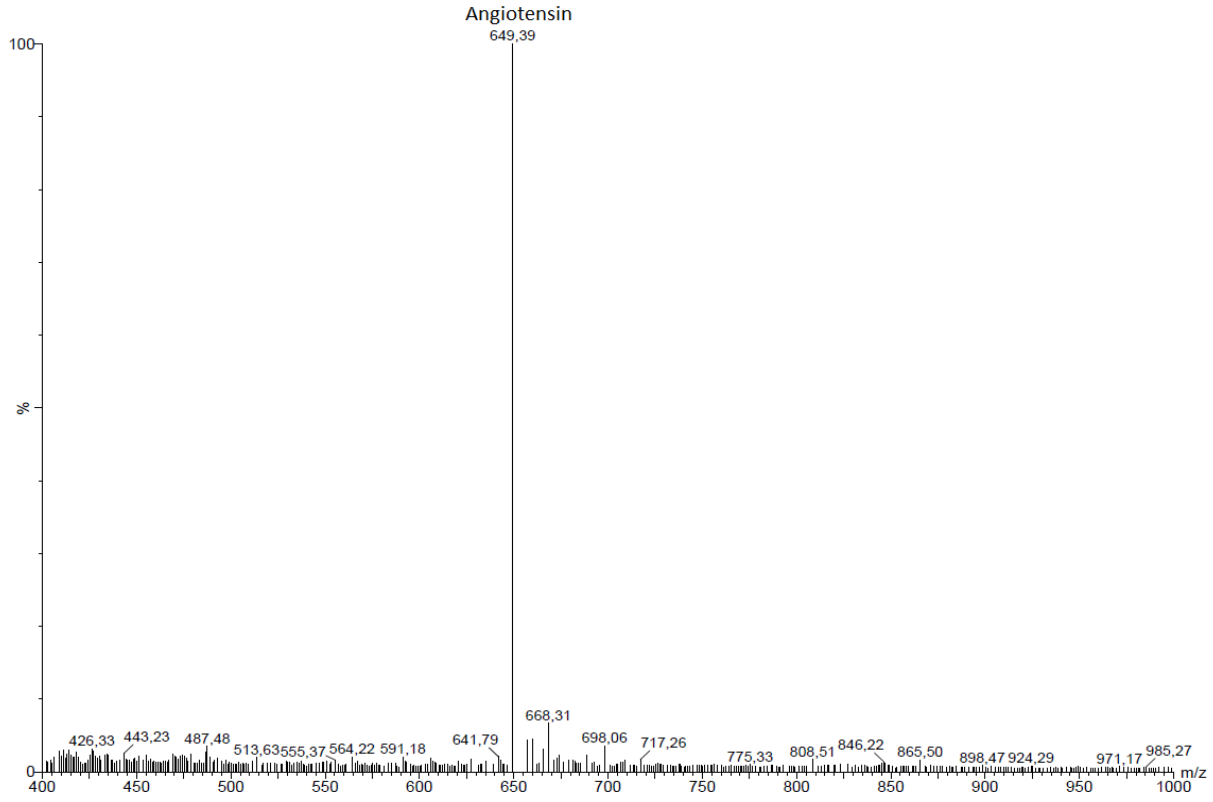


Fig.(2.10) ESI-MS (+) of angiotensin (I) and quercetin at molar ration 1:10 in ammonium acetate at pH 7. Signal at m/z 694 belongs to a doubled charged molecular-ion of angiotensin. No signal belongs to complex of quercetin-angiotensin could be detected. The presumed complex of 1:1 quercetin-angiotensin should show a signal at m/z 800.

In the case of quercetin; the mono-isotopic mass of quercetin is 302.04 Dalton and the mono-isotopic mass of angiotensin (I) equals to 1295.68 Dalton. Thus, the 1:1 complex of quercetin-angiotensin(I) should have the theoretical mass value of 1597.72 Dalton. Thus, a signal at approximately m/z 800 should be observed. From figure (2.10) we found out, that no complex is formed between the quercetin and angiotensin (I).

From the above findings we could conclude, that the sugar residue in the flavonoid glycoside is involved in the formation of the complex with angiotensin (I). To explore how the sugar residue is evolved in the formation of the complexes, two sugar compounds; D-(+)-glucose a monosaccharide, and stachyose a tetrasaccharide, were tested with angiotensin (I). The spectra of both sugar parts revealed no signals related to any kind of interactions. In consequence, it becomes clear that the formation of such complexes with angiotensin (I) is not directly taking place at level of sugar residues, but still dependent on the degree of glycolysation.

In general, all flavonoid glycosides used in this study proved to be capable to interact with angiotensin (I). Whereas, MS spectra obtained from aglycones – except that of taxifolin – unveil no signals in contribution to any kind of complex, referring to even no interaction, or weak bonding under experimental conditions figures (2.11) and (2.12). Only taxifolin, or dihydroquercetin, belongs to flavanolols could interact to angiotensin (I). The peculiarity of taxifolin chemical structure is the missing **π** system at $\mathbf{C_2}$-$\mathbf{C_3}$ at the ring **C**.

Since that polyphenols can dissociate under biological conditions forming negatively charged phenolate ions, that leads to the building of ionic bonds (Wink, 2008), thus, the increasing in phenol groups will result in a stronger interaction between both polyphenol and biomolecule. This explain how EGCG, which is rich of phenol groups, is able to form a stronger complexes with angiotensin (I) in comparison to other flavonoid glycosides as shown in figure (2.13). Table (4) summarizes m/z values, which related to detected ligand-target complexes of 1:1 binding stoichiometries in comparison to their theoretical calculated m/z.

These observations regarding the ionic interactions between angiotensin (I) and polyphenols could be explained on the basis of the physiochemical properties of the tested compounds, especially what concerns flexibility and hydrophobicity. In the aqueous solutions the polar amino acid residues in angiotensin (I) tend to bound outwards forming a polar surface rich of positively charged amino acids. On the other hand, the formed phenolate groups will target the positively charged amino acid residues in angiotensin (I) building strong and stable ionic bridges (Wink, 2008). Since these interactions are taking place at the polar surfaces, thus, compounds with higher polar surface area (PSA) will be able to form more stable complexes. Flavonoid glycosides, which have in general PSA values that are approximately twice greater than of their aglycone counterparts, will bind to angiotensin (I) forming stable complexes.

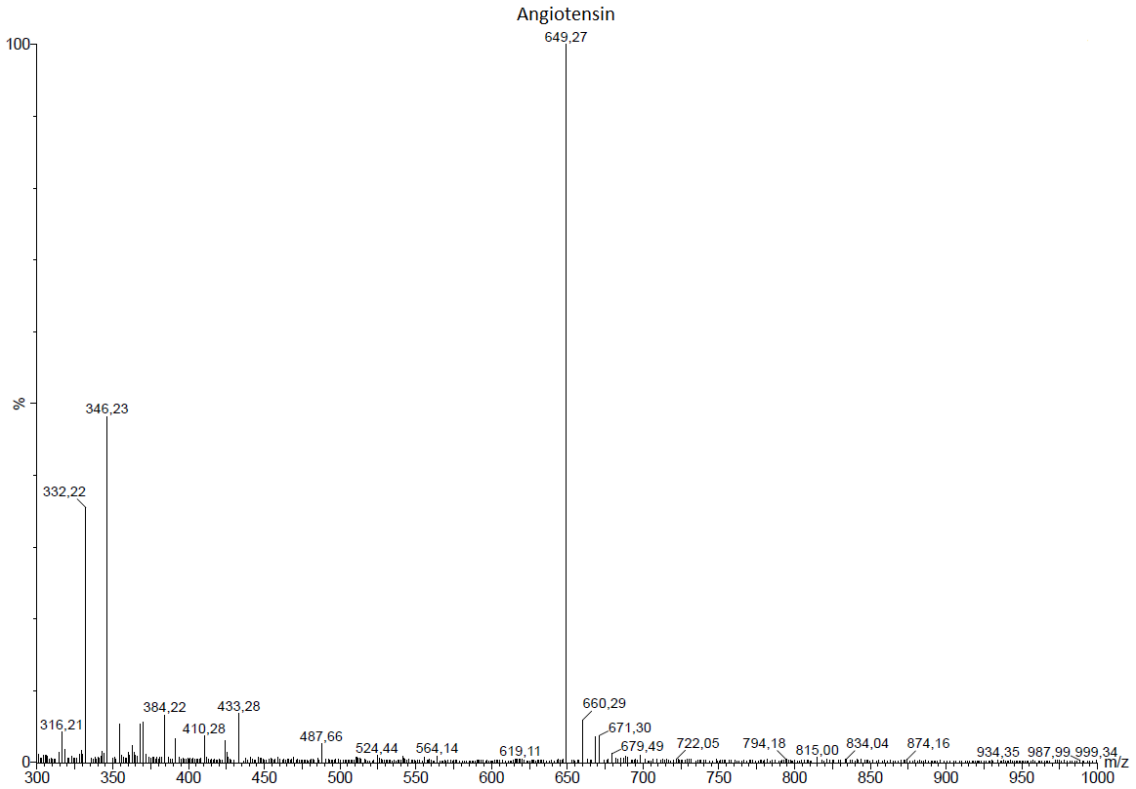


Fig.(2.11) ESI-MS (+) of angiotensin (I) and rhamnetin at molar ration 1:10 in ammonium acetate at pH 7. Signal at m/z 694 belongs to a doubled charged molecular-ion of angiotensin. No signal belongs to complex of rhamnetin-angiotensin could be detected. The presumed complex of 1:1 rhamnetin-angiotensin should show a signal at m/z 807.

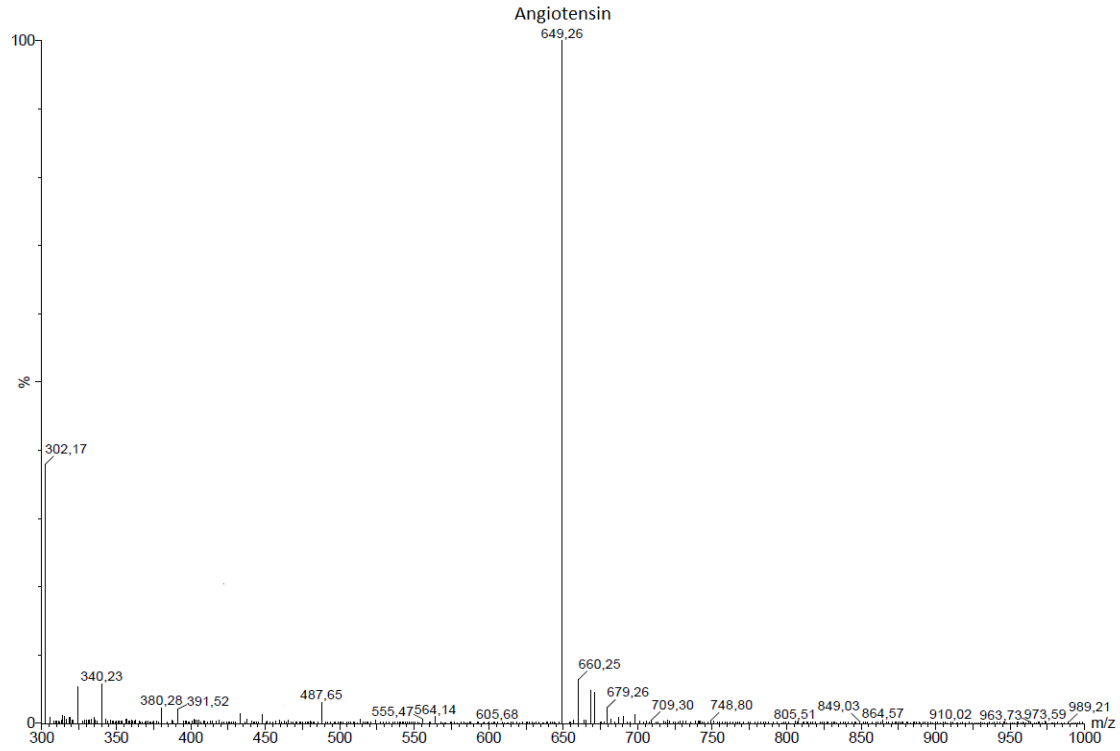


Fig.(2.12) ESI-MS (+) of angiotensin (I) and kaempferol at molar ration 1:10 in ammonium acetate at pH 7. No signal belongs to rhamnetin-angiotensin complex could be detected.

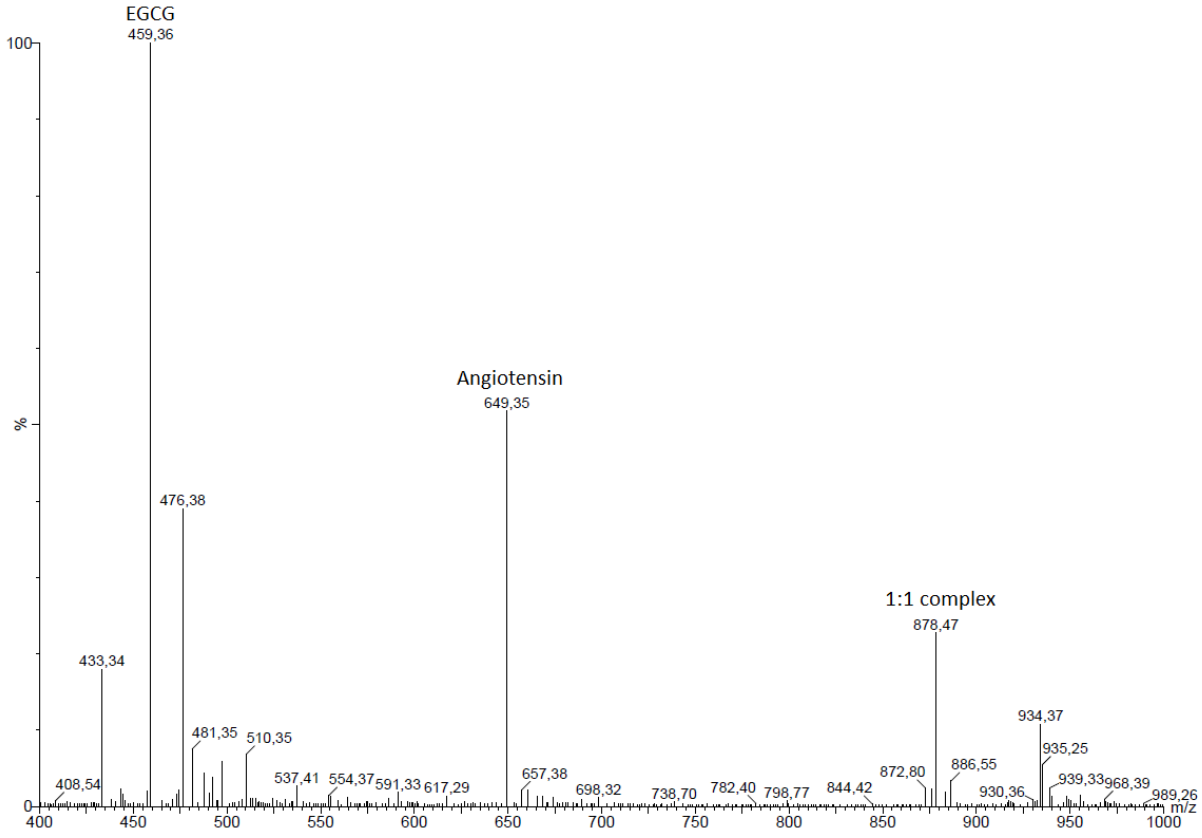


Fig.(2.13) ESI-MS (+) of angiotensin (I) and EGCG at molar ration 1:10 in ammonium acetate at pH 7. Signal at m/z 694 belongs to a doubled charged molecular-ion of angiotensin. An intensive signal at m/z 878 represent the complex of 1:1 EGCG-angiotensin(I).

Meanwhile, flavonoid aglycones will tend to interact with angiotensin (I) at the non-polar sites by means of hydrogen bridges and hydrophobic bonds. These kind of non-covalent forces are much weaker than the ionic ones, and the formed bonds of that kind cannot stay intact under the ionization conditions. This view is supported by the results obtained from H/D mass spectrometry experiment (paragraph 2.3.3.).

Although taxifolin has PSA value close to that of the above tested flavonoid aglycones. However, result obtained from MS spectrum of taxifolin proves the ability of taxifolin to form ionic bonds with angiotensin (I). In this case the missing **π** bond at **C_2-C_3** of ring **C** granted taxifolin more flexibility. For that reason, taxifolin is less prone to the structural fluctuations of the biomolecule, and are able to form stable complexes with angiotensin (I) under experimental conditions.

Table (2.4) summarizes m/z value related to detected ligand-target complexes compared to their theoretical calculated m/z values and the error in the mass differences:

Compound	Monoisotopic mass	m/z observed	m/z calculated	Error %
Angiotensin	1295.68	649	648.85	0.015
Flavonoid glycosides				
Rutin	610.15	954	953.92	0.008
Spiraeoside	464.09	881	880.89	0.012
Hyperoside	464.09	881	880.89	0.012
Aglycones				
Quercetin	302.04	No	799.87	-
Rhamnetin	316.06	No	806.88	-
Kaempferol	286.04	No	791.87	-
Taxifolin	304.06	801	800.88	0.015
Catechins				
EGCG	458.08	878	877.88	0.013

2.3.1.3. Effect of degree of glycolysation on ligand affinity:

The interpretation of the ESI-MS spectra obtained from studying flavonoid glycosides and their aglycone partners emphasize the role of sugar residues in the formation of the complexes. To study the relative affinity between the different flavonoid glycosides a competition experiment is performed.

Two flavonoid glycosides are mixed simultaneously with angiotensin (I) at 10:10:1 molar ratio, respectively and the competition coefficient was calculated by means of the following equation:

$$\mathbf{Comp._{coef.} = TIC_A/TIC_B}$$

Whereas competition coefficient is calculated from the total ion count (TIC) number of formed complex with substance A, in comparison to total ion count number of the formed complex with substance B. When comparing rutin; a disaccharide flavonoid, with hyperoside; a quercetin-3-O-galactoside, and spiraeoside; a quercetin-4'-O-glucoside, a higher affinity toward angiotensin (I) was observed in favor to rutin with a competition coefficient value of 1.7, figures (2.14a) and (2.14b).

Generally, phenolate ions interact with the positively charged amino acids residues groups and form ionic bonds. Whereas the OH groups of the sugar rests will form weak hydrogen bonds. In conclusion, the stability of the formed complex between the angiotensin (I) and flavonoid glycoside will be greater as the number of sugar rests increase.

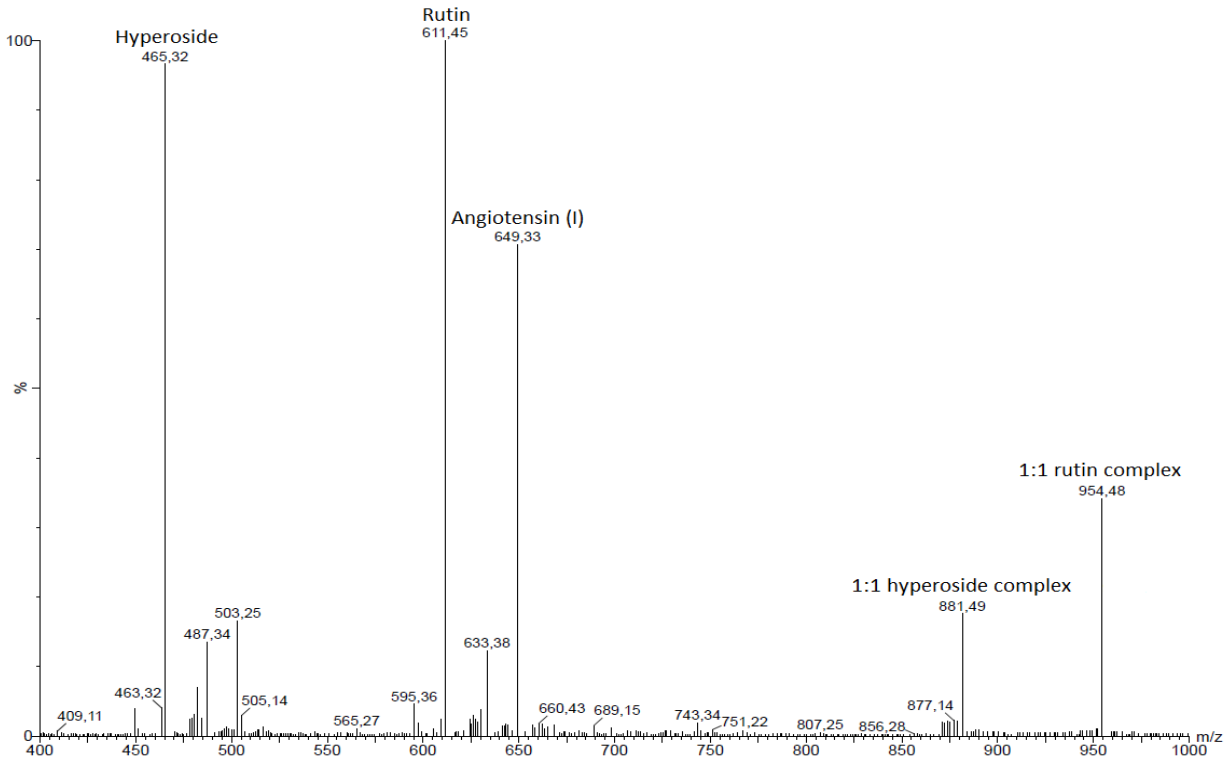


Fig. (2.14a) ESI-MS (+) of rutin and hyperoside in presence of angiotensin (I). Rutin shows higher affinity toward angiotensin(I) in comparison to hyperoside. The signal at m/z 954 is more intensive than the one at m/z 881.

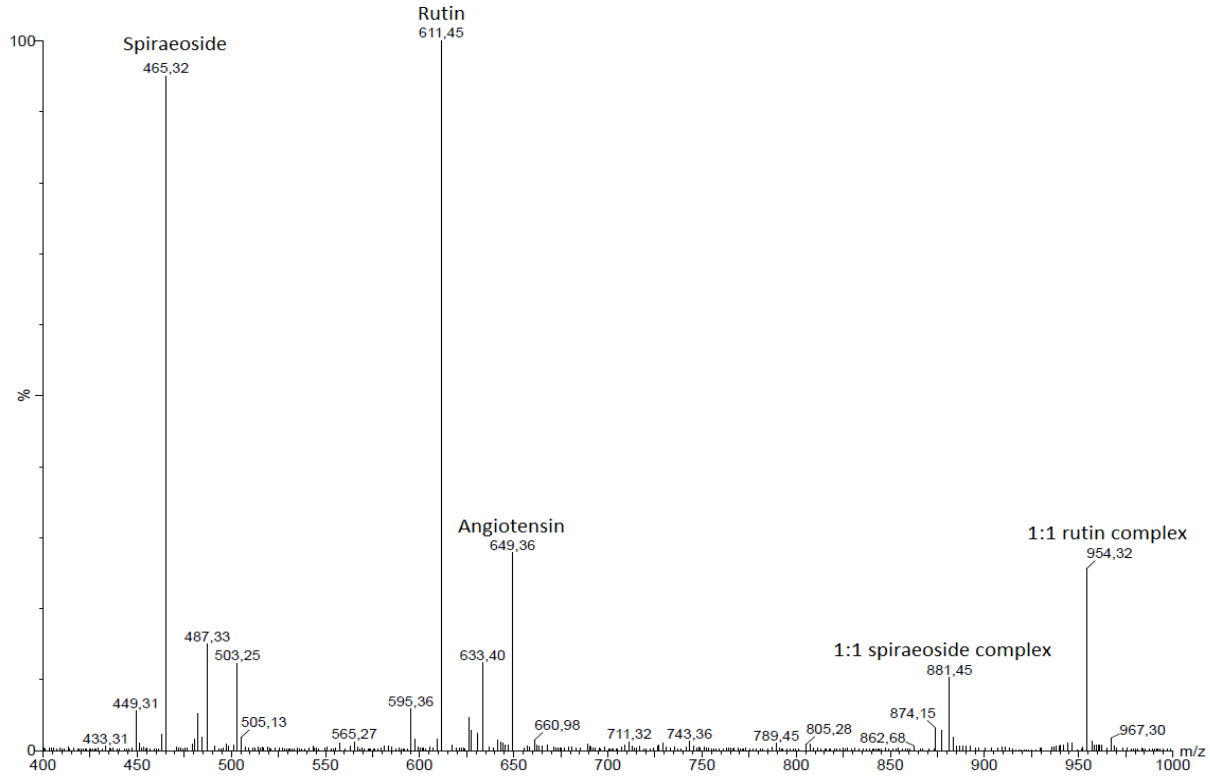


Fig. (2.14b) ESI-MS (+) of rutin and spiraeoside in presence of angiotensin (I). Rutin shows higher affinity toward angiotensin(I) in comparison to spiraeoside. The signal at m/z 954 is more intensive than the one at m/z 881. This proves that the degree of glycolysation affects the formation of the non-covalent complex between the biomolecule and the flavonoid glycoside.

2.3.1.4. Effect of ligand concentration on signal intensity:

To monitor the influence of ligand concentration on the signal intensity of formed complexes, polyphenols at different concentration (8, 16, 32 and 80 µM) were added to 8 µM of angiotensin (I).

The following equation is used to evaluate the relative binding affinity of the ligand to angiotensin (I):

$$\textbf{Contribution\%} = (TIC_{complex} / \Sigma\, TIC_{target}) \times 100$$

Where $\mathbf{TIC_{complex}}$ represents the total ion count number of ligand-angiotensin (I) complex and $\mathbf{TIC_{target}}$ represents the total ion count number which related to angiotensin (I) at the different charge-states.

Obtained spectra from rutin, figure (2.15), and hyperoside, figure (2.16), demonstrate that non-covalent complexes could be first detected at a molar ratio of 2:1 polyphenol to angiotensin (I). Moreover, the acquired data confirmed that the higher the ligand concentration is, the more intensive are the signal related to the complexes. Nevertheless, and because of self-assembly phenomenon no signals related to polyphenol-angiotensin (I) complex could be detected when polyphenols concentration exceed 80 µM.

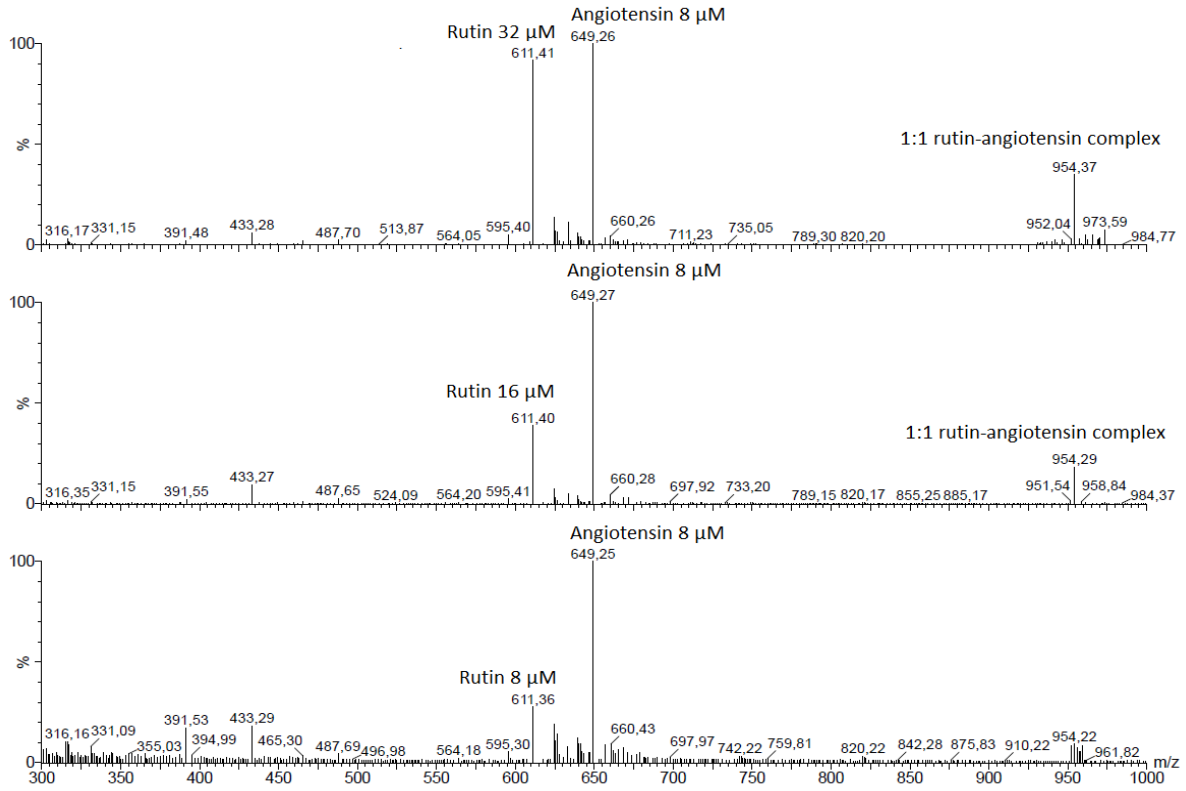


Fig. (2.15) ESI-MS(+) of rutin and angiotensin. The signal at m/z 954 represents rutin-angiotensin(I) complex and the intensity of this signal increases with the concentration of rutin from 8 (bottom) to 32 µM (top).

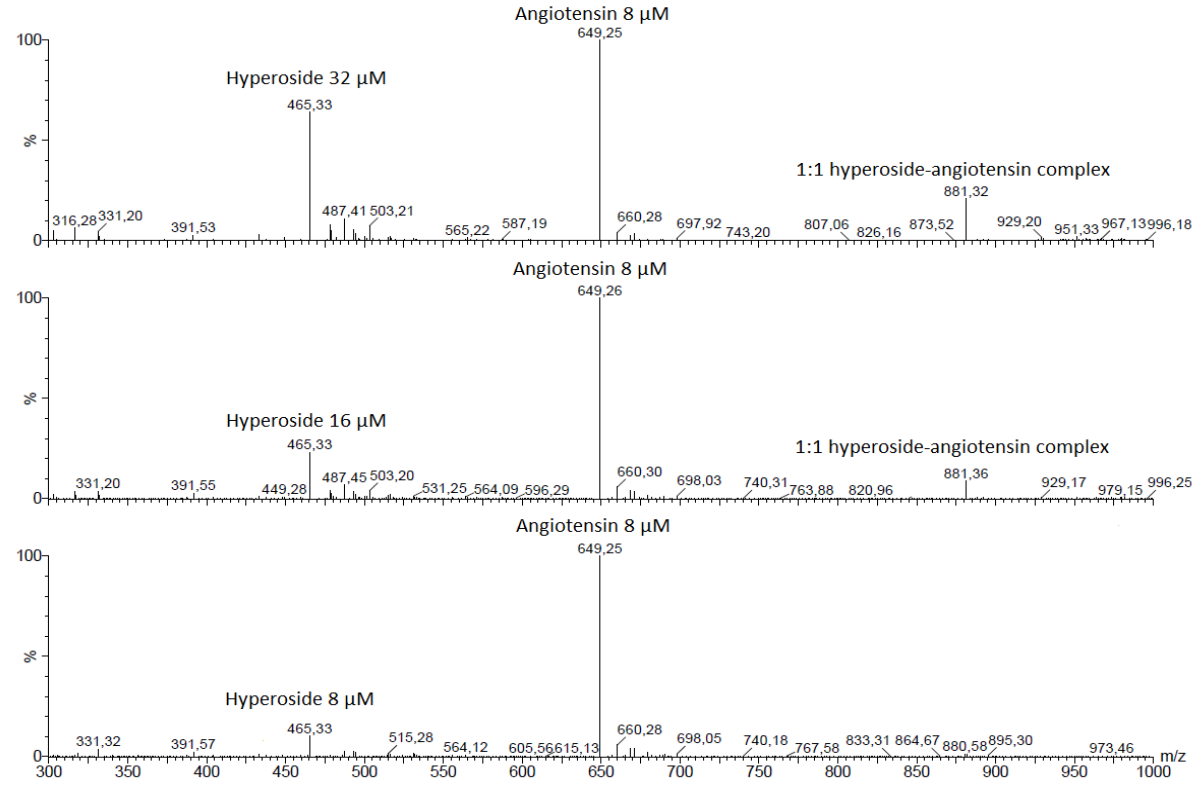


Fig. (2.16) ESI-MS(+) of hyperoside and angiotensin. The signal at m/z 881 represents hyperoside-angiotensin(I) complex. Signal intensity increases with the concentration of hyperoside from 8 (bottom) to 32 µM (top).

2.3.1.5. Effect of pH on complex formation:

Since that polyphenols tend to form more phenolate ions in basic milieu, therefore the intensity of the signal related to the complexes should increase in a direct proportional manner to the increasing of pH values. To monitor this effects angiotensin (I) was mixed with rutin at two different molar ratios 1:5 and 1:10 and data were acquired under different pH values by using ammonium acetate buffer. Following equation was applied to calculate the signal intensities result from the interaction between rutin and angiotensin(I):

$$\mathbf{Int._{complex} = (TIC / \Sigma\ TIC^{*})}$$

Where is **TIC** represents the total ion count number of rutin-angiotensin complex and **TIC*** represents the total ion count number of ions related to angiotensin (I) at the different charge-states. Results are summarized in figure (2.17).

Figure (2.17) illustrate the MS spectra of rutin mixed with angiotensin at molar ratio of 1:10, whereas the intensity of the signals related to 1:1 rutin-angiotensin complex are monitored in relation to pH values. Results are evaluated using the upper equation and summarized in figure (2.18). Signal intensity of rutin-angiotensin(I) complex increases comparatively to pH values to reach the highest level at pH values around 7 (black bars), then decreases again slightly at higher pH points. At lower molar ratio of 1:5 angiotensin to rutin the pH point, at which the signal intensity of complex reaches its highest level, is shifted to reach values near to 8 (grey bars). The explanation of the decrease in the interaction at higher pH values can be based on self-assembly phenomenon, since that the clustering of polyphenols molecules would also increase at high pH value. For the same reason the optimum pH to obtain the best affinity between rutin and angiotensin (I) is shifted from 7 to 8 when lower concentration of rutin are used.

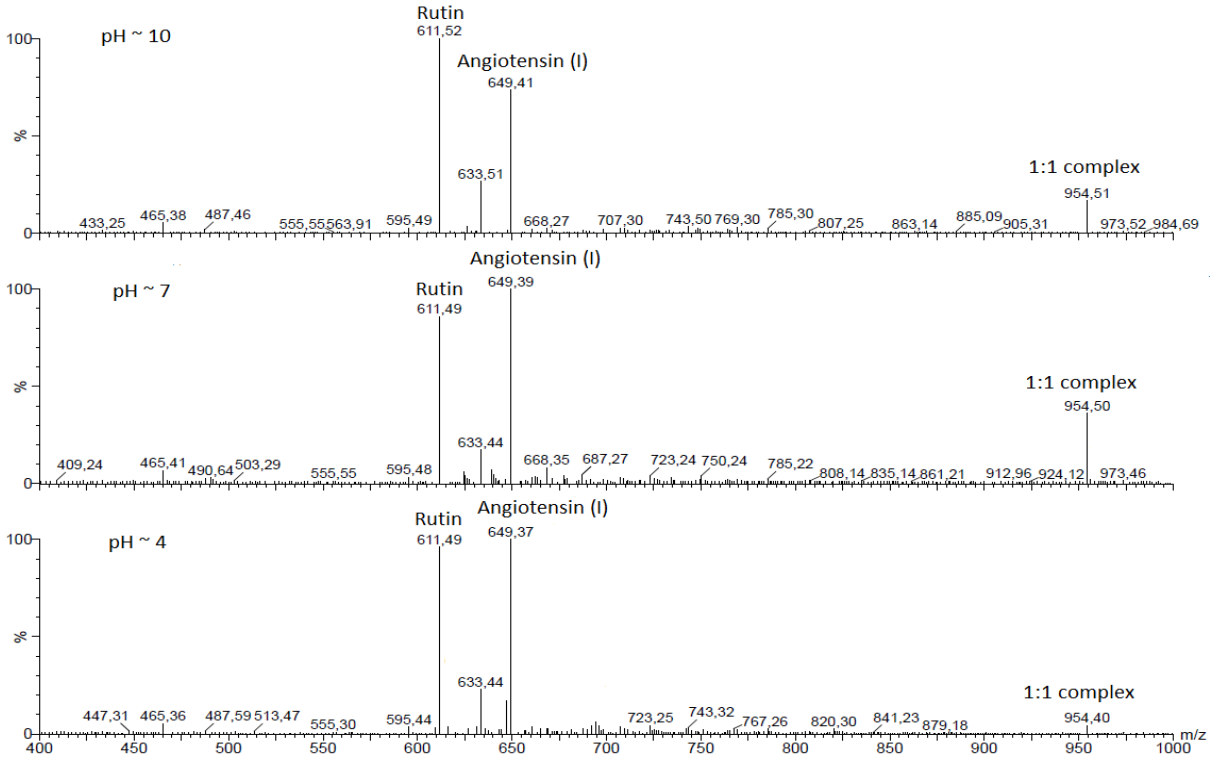


Fig.(2.17) ESI-MS(+) of rutin and angiotensin (I) at different pH points. The intensity of the MS signals related to 1:1 rutin-angiotensin(I) complexes are assessed in relation to pH values. Signal intensity of complex increases starting from pH values near 4 till reaching points near 7, before it drops again as pH reaches values around 10.

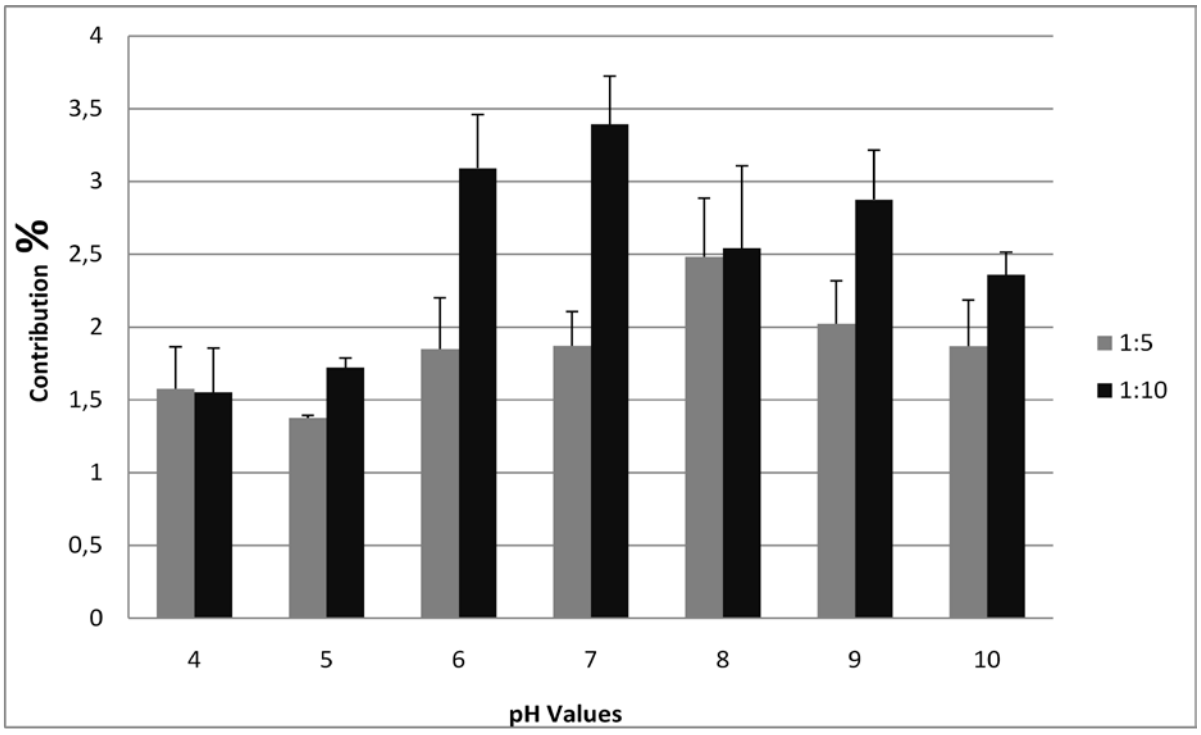


Fig. (2.18) The relationship between pH values and rutin-angiotensin(I) complex formation. Signal intensity of rutin-angiotensin(I) complex increases in a direct manner to the increase of pH values till it reaches points around 7 and 8 to drop again at higher pH levels because of phenol self-assembly phenomenon.

2.3.2. Peptide sequence-dependence of complex formation:

A Protein comprises in its structure usually several positively charged amino acid residues, e.g. lysine, arginine, and histidine. Since polyphenols possess the ability to build negatively charged phenolate ions, therefore, these positively charged amino acid residues would be targeted by the formed phenolate ions and consequently ionic bond could occur. Hence, the increase of such positively charged amino acid residues would have significant effects on the nature of the formed complexes. As mentioned pervious (paragraph 2.3.1.2.), the different tested polyphenols could only demonstrate 1:1 binding stoichiometries with angiotensin (I). Therefore, higher binding stoichiometries are expected to be observed, when the targeted peptide comprises enough positively charged amino acid residues.

To investigate the effect of the number of positively charged amino acid residues on the nature of formed complexes, three peptides containing increasing number of lysine residues were employed. Table (2.2) shows the synthesized, whereas peptides P1, P2, and P3 contained 2, 4, and 8 lysine residues, respectively. Moreover, a fourth peptide, P4, was employed to monitor the relationship between the formation of complexes and the sequence of the lysine residues. P4 is designed to have the same number of lysine residues as peptide P3, only that lysine residues were built in a different order. Table (2.5) summarizes how the polyphenol-peptide binding stoichiometries increases in correspond to the increasing number of lysine residues.

The MS spectra obtained using non-covalent ESI-MS (+) technique reflected a clear relationship between the number of lysine residues in the target peptide, as well as the kind of formed non-covalent complexes. In comparison to the results obtained when using angiotensin (I), new stoichiometries other than 1:1 ligand-target complexes were observed to be formed between the studied polyphenols and the synthesized peptides. Ligand-target binding stoichiometries such as 2:1, 3:1 and higher were registered. Meanwhile, no substantial differences have been monitored when comparing MS spectra gained from both P3 and P4, reflecting no significant relationship between the kind of formed complexes and the order of the lysine residues, but on the other hand, a possible relationship could be expected between the order of the lysine residues in the peptide and the stability of the formed complexes.

Table (2.5) the relationship between the numbers of formed complexes to the number of lysine residues in the targeted peptides for the tested polyphenols

Compounds	(polyphenol: peptide) Ratio		
	P1 (2 lysine residue)	**P2** (4 lysine residue)	**P3** (8 lysine residue)
Rutin	1:1	3:1	6:1
Quercitrin	1:1	3:1	4:1
Spiraeoside	1:1	2:1	4:1
Hyperoside	1:1	3:1	5:1
Scutellarin	1:1	2:1	2:1
EGCG	2:1	4:1	8:1
Procyanidin B2	2:1	3:1	7:1
Procyanidin C1	2:1	3:1	5:1

When testing P1 with Rutin complexes of 1:1 have been observed in the MS spectra, meanwhile P2 formed 3:1 complexes. With the most lysine-rich peptide P3, rutin built complexes of 6:1. Quercitirn, spiraeoside and hyperoside share with rutin the same flavonoid aglycone core, i.e. quercetin, therefore, they can build as many ionic bonds as rutin does. Consequently, these flavonoid glycosides reacted with the synthesized peptides in a similar manner to rutin. Nevertheless, and since they possess less sugar rests, the complexes they build are less stable, hence, the lower intensity in the MS signals of the formed complexes. Scutellarin comprises fewer phenolic groups than rutin. This explain the low binding stoichiometries, e.g. 2:1 scutellarin-P3, reflecting a lower affinity of scutellarin toward the studied peptides in comparison to rutin. On the other hand, EGCG possess much more phenolic groups than rutin. When interacting with peptide P3, EGCG could form 8:1 EGCG-P3 complexes, while rutin could form only 6:1 complexes with the same peptide. For the same reason procyanidin B2 could show higher binding stoichiometries when compared to rutin, and reacted with the above mentioned peptides nearly in a similar manner to EGCG. Meanwhile, procyanidin C1 possesses more phenolic groups compared to each EGCG and procyanidin B1, yet results obtained from procyanidin C1 reflect a lower affinity toward the

tested peptides. This controversy could be explained through the complexity of the chemical structure of procyanidin C1.

2.3.3. Hydrogen/Deuterium Mass Spectrometry (HDX-MS):

H/D mass spectrometry is a well established technique to study the formation of hydrogen bonds. Insulin was used as a target to study the relationship between the structure of polyphenols and the formation of the hydrogen bridges. Insulin was incubated with different polyphenols in D_2O solution. After the hydrogen/deuterium exchange reach a point near to equilibrium the sample was directly injected in the MS source and the m/z values were registered. The shifts in the mass values were then calculated using the following equation:

$$\Delta D = |M_{obs} - M_{lig}|$$

Where ΔD is the calculated difference of deuterium uptake number, M_{obs} is observed mass of insulin after incubation in D_2O in the absence of a ligand and M_{lig} is the observed mass of insulin when incubated with a ligand in medium rich of deuterium.

2.3.3.1. Insulin before and after deuteration:

Insulin is a hormone consists of 52 amino acids with mono-isotopic mass of 5803.65 Dalton. The positive mode ESI-MS (+) spectrum of insulin reveals series of multiple charged molecular ions in range of +4 to +6, figure (2.18) represents the ESI-MS spectra of insulin dissolved in ammonium acetate buffer at pH 7. When insulin is mixed with deuterium water and left to equilibrate at room temperature for approximately 3 hours a shift in the mass values of the different states of charge is observed. The shift reflects an increase of an averagely 43 amu (atomic mass unite) of insulin, caused by the exchanging of 43 protons with averagely 43 deuterons. Table (2.6) shows the different ion-species of insulin before and after exchange. Figure (2.19) represents the ESI-MS (+) spectrum of insulin after incubation with D_2O water for approximately three hours.

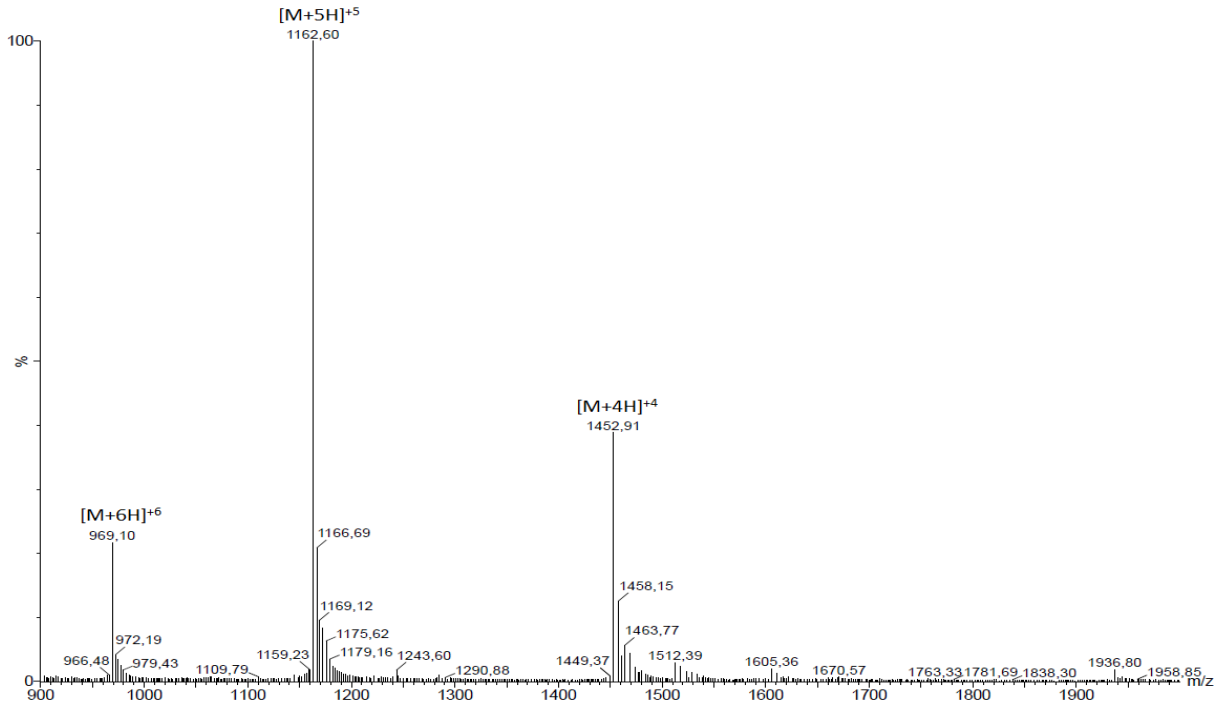


Fig.(2.18) ESI-MS(+) of insulin in ammonium acetate buffer at pH 7. Insulin has the mono-isotopic mass of 5803.65 Dalton and shows under the positive mode of electrospray ionization MS three main signals that belong to the ion species of 4, 5, and 6.

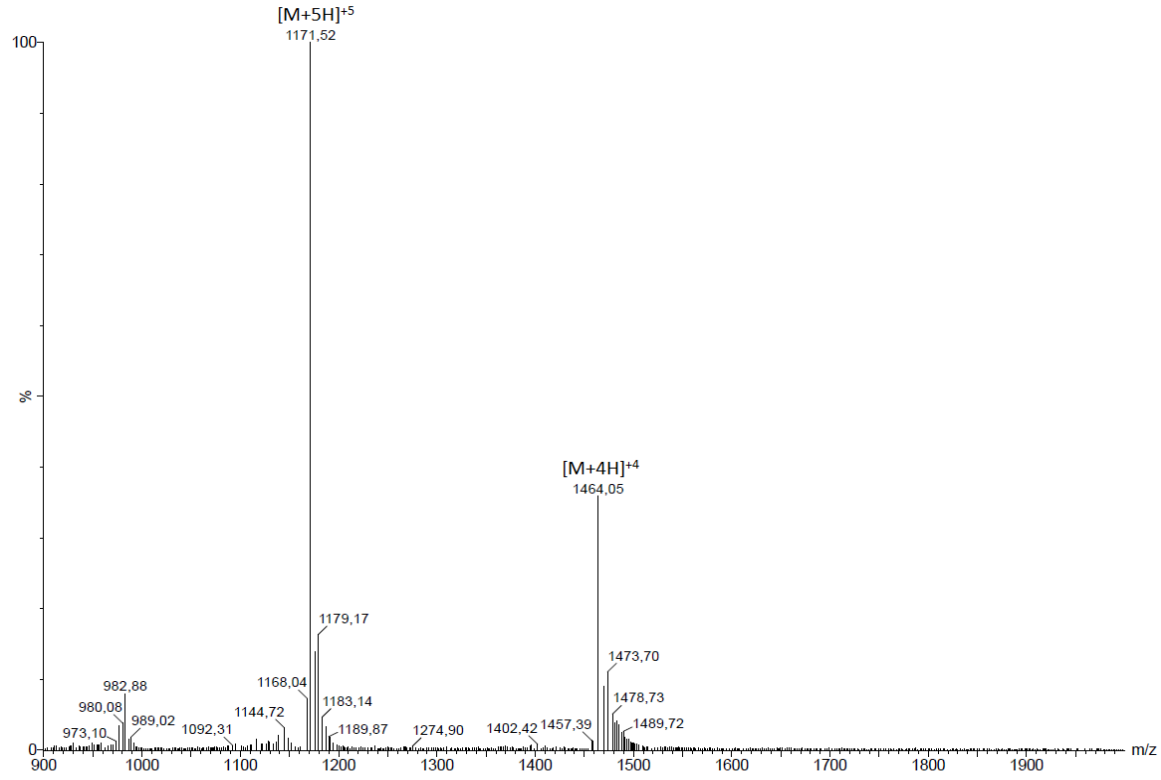


Fig.(2.19) ESI-MS(+) of insulin after three hours of incubation in D_2O water. A shift in the masses of each ion species can be observed. These shifts reflect an exchange of nearly 43 hydrogen atoms with deuterium.

Table (2.6) Observed signals insulin as a function of charge state before and after deuteration

Charge state	m/z before deuteration	m/z after deuteration	Average increase in mass units
+6	969.05	976.44	7.39
+5	1162.48	1171.35	8.87
+4	1453.06	1464.05	10,99

2.3.3.2. Non-covalent interactions between insulin and polyphenols (studying the formation of hydrogen bonds):

When a ligand interacts with the backbone amides forming hydrogen bonds, the backbone amide groups are then masked by the interacted molecule, therefore, the accessibility of the solvent, namely D_2O water will be impeded. This leads to the prevention of the hydrogen/deuterium exchange to take place at the masked sites and consequently to the decrease in deuterium up-take of the targeted biomolecule. MS spectrum of insulin in the absence of any ligand shows an H/D exchange rate of 43 amu (atomic mass units), fig. (2.19). In the presence of flavonoid aglycones a decrease in the deuterium uptake have been registered, which confirms, that the tested flavonoid aglycones are able to build hydrogen bond, causing a decrease in deuterium up-take in range around 3 to 9 amu. Figures (2.20) and (2.21) demonstrate H/D exchange of insulin before and after adding quercetin and myricetin, respectively. The top part of figure (2.20) represents the MS spectrum of insulin in the absence of quercetin. The most intensive signal is m/z 1170.66 and belongs to the $[M+5H]^{+5}$ molecular-ion specie. This represents a mass of approximately 5848.4 Dalton. When adding quercetin the $[M+5H]^{+5}$ molecular-ion specie shifts backward showing a value of m/z 1169.30 as seen in the bottom part of figure (2.20).

The calculated mass from this molecular-ion will be Dalton 5841.5 Dalton. Applying the equation we can assess the shift in the deuterium up-take:

$$\Delta D = |M_{obs} - M_{lig}|$$

$$\Delta D = |5848.4 - 5841.5|$$

$$\Delta D = 6.9$$

The value ΔD = 6.9 confirms the capability of quercetin to interact with insulin backbone amides through hydrogen bonds. The hydrogen bridges caused the masking of nearly 6.9 exchangeable hydrogen atoms. Due to the fact that myricetin has more phenolic groups than quercetin; refer to figure (2.5), therefore, the observed shift in the deuterium up-take was greater and a value of ΔD = 9.02 have been calculated. Whereas, the smallest shift belongs to rhamnetin, since that rhamnetin has the least OH groups.

On the other hand, when incubating flavonoid glycosides with insulin, no significant changes in deuterium uptake could be observed in the obtained MS spectra. Figure (2.22) confirms how rutin is unable to build hydrogen bond with the backbone amides of insulin. Nevertheless, spiraeoside; a 4'-O-glucoside of quercetin, was able to reduce deuterium up-take number causing an average shift of approximately 6 mass units as shown in figure (2.23). Table (2.7) summarizes the changes of insulin deuterium-uptake in relationship to the tested polyphenols. Bold numbers represent ligand prevention of H/D exchange; thus, reflect ability of that ligand to build hydrogen bounds.

Insulin which is pretty a huge biomolecule has a globular shape. In aqueous solutions the non-polar amino acid residues are imbedded in the core to minimize the interaction with the aqueous solvent. These non-polar groups are called the backbone amides. In the absence of a ligand, the backbone amides are accessible to solvent molecules and will undergo the H/D exchange reaction. The average mass of insulin increases in order of one mass unit for each exchangeable hydrogen atom. In the presence of an active ligand, a number of non-covalent bonds will be formed with some backbone amides preventing them from being involved in the H/D exchange reaction.

Table (2.7) the changes of insulin deuterium-uptake in the absence and presence of the ligand (polyphenol). Number in Bold reflect significant mass shift, therefore formation of hydrogen bond.

Ligand	Average deuterium up-take		Mass shift ΔD
	In absence of ligand	In presence of ligand	
Flavonoid aglycones			
Quercetin	44.67	37.97	**6.70**
Myricetin	43.82	34.80	**9.02**
Taxifolin	44.19	39.65	**6.54**
Rhamnetin	44.56	41.12	**3.44**
Flavonoid glycosides			
Rutin	43.59	43.10	0.49
Spiraeoside	43.27	37.47	**5.80**
Hyperoside	43.65	43.20	0.45
Scutellarin	44.43	43.81	0.62

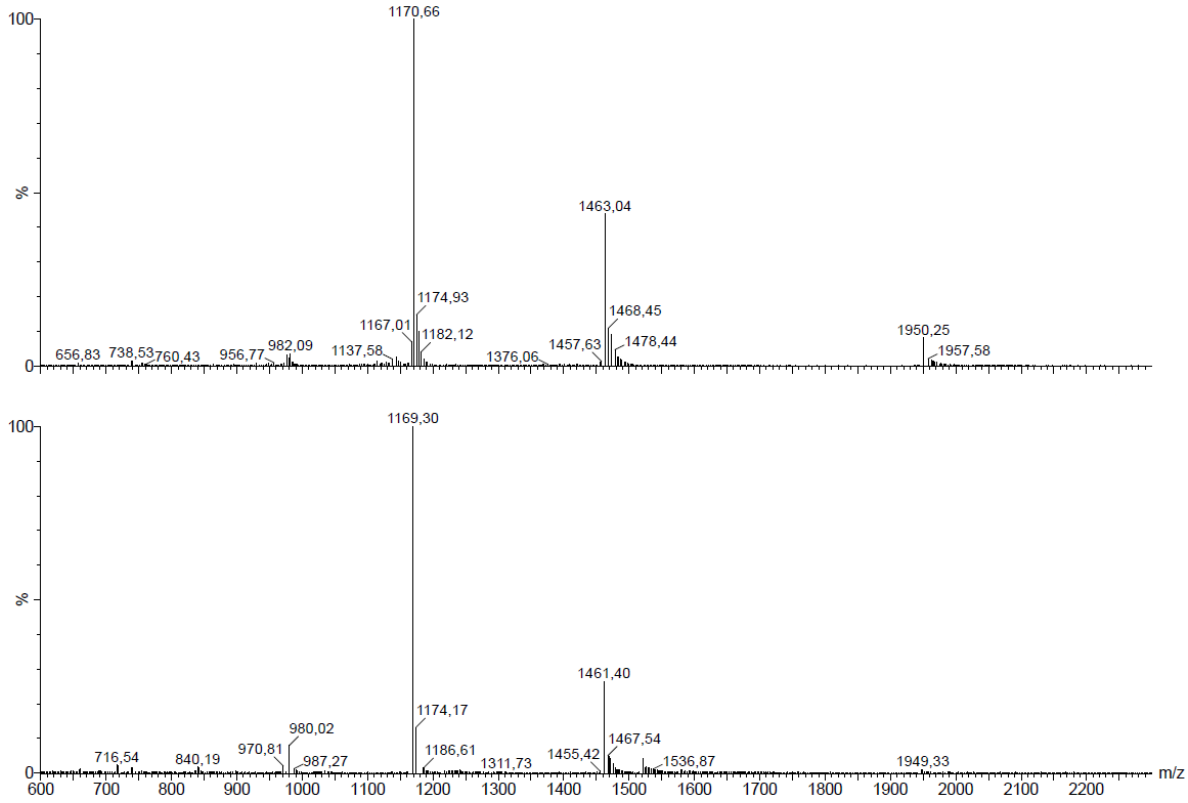


Fig.(2.20) ESI-MS(+) of insulin after been incubated in D_2O water for three hours (top). The deuterium uptake is decreased when insulin incubated with quercetin (bottom).

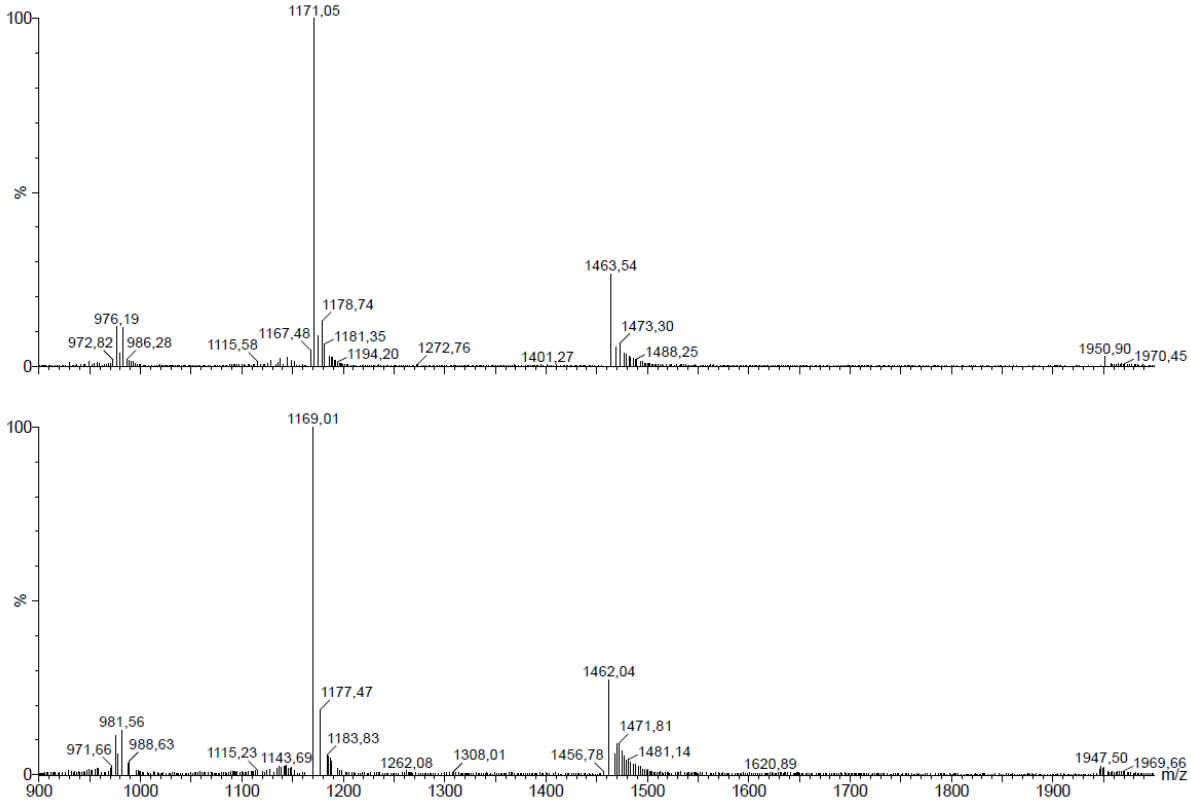


Fig.(2.21) ESI-MS(+) of insulin after been incubated in D_2O water for three hours (top). The deuterium uptake decreases when insulin incubated in the presence of myricetin (bottom).

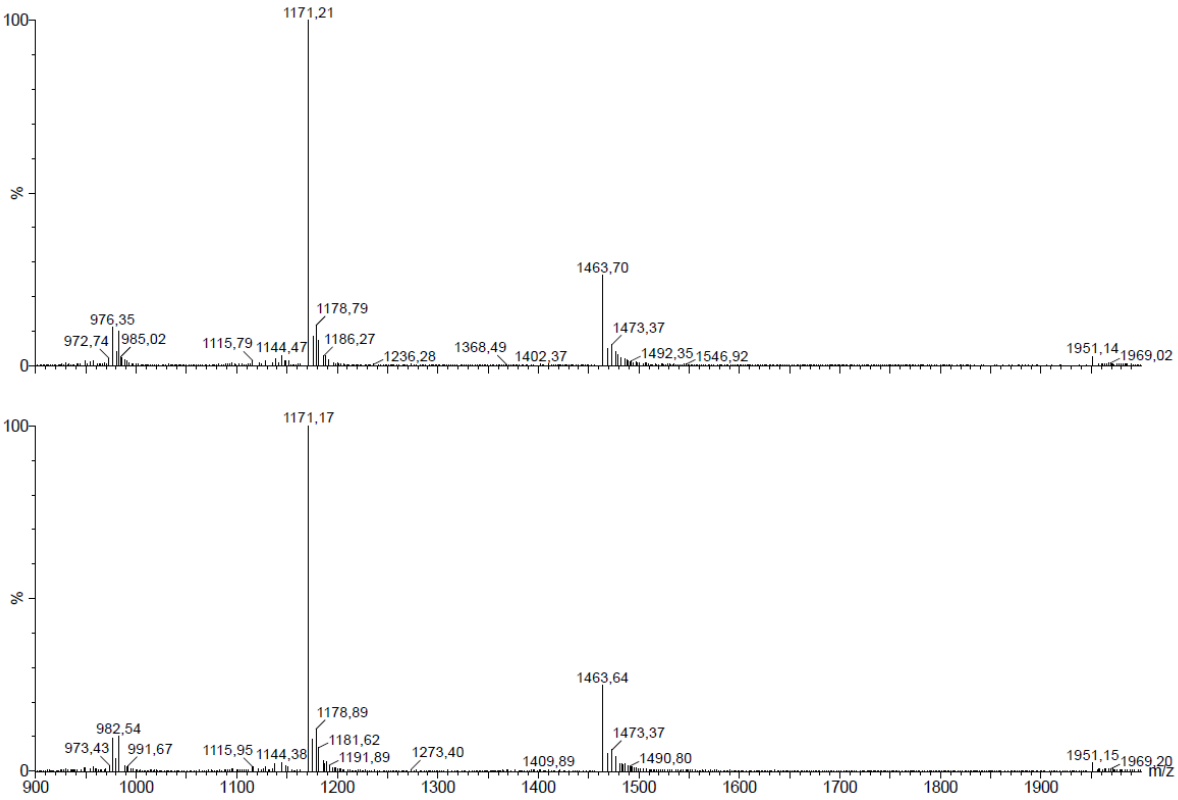


Fig.(2.22) ESI-MS(+) of insulin in the presence of rutin (bottom). No significant change in deuterium-uptake can be observed, when compared with MS spectrum of insulin in the absence of rutin (top).

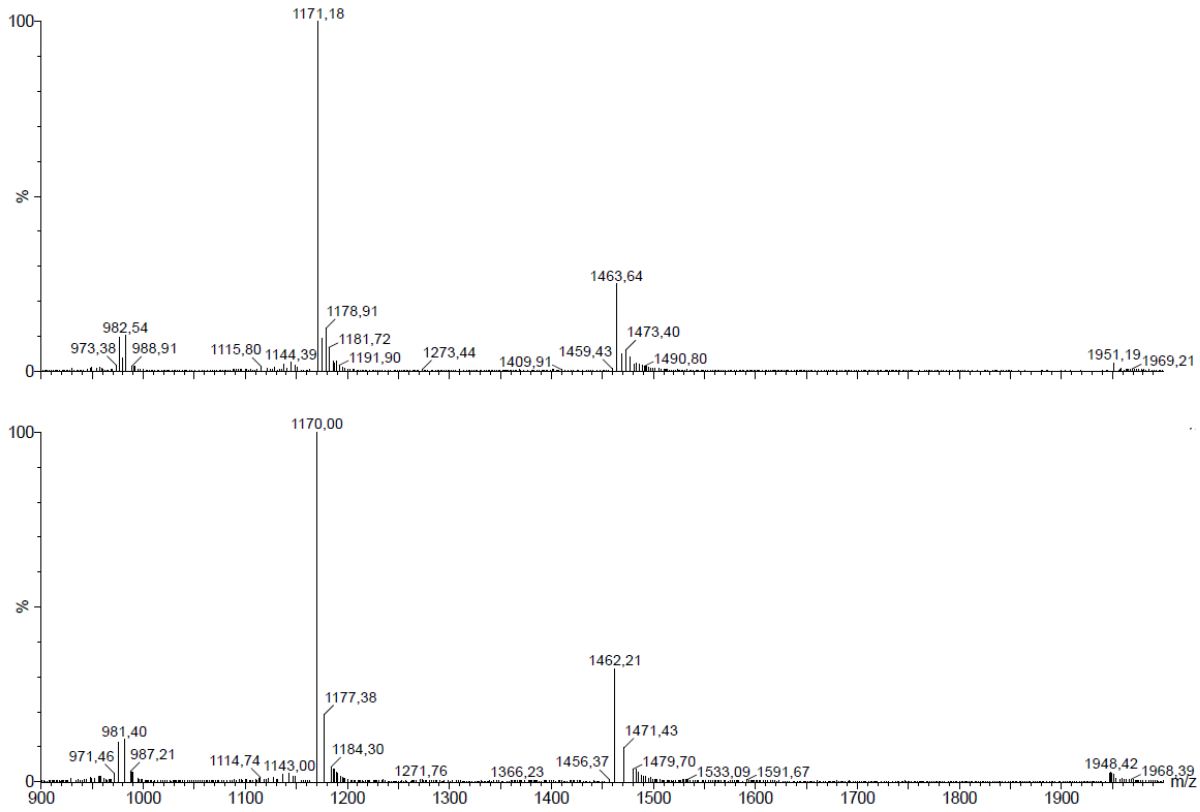


Fig.(2.23) ESI-MS(+) of insulin after been incubated in D_2O water for three hours (top). The deuterium uptake is decreased when insulin incubated in the presence of spiraeoside (bottom).

Because of the flexibly in the chemical structure and the tendency to interact at the polar surfaces through ionic bonds, flavonoid glycosides will not reach the backbone amides. Meanwhile, flavonoid agylcones are less flexible (coplanar) and have lower PSA values in comparison to the glycoside partners, therefore, flavonoid agylcones are capable to reach the non-polar core, and in consequence, are able to build hydrogen bonds with the backbone amides. This explains the decrease in insulin H/D rate of exchange. On the other hand, no significant changes observed when applying flavonoid glycosides. Only spiraeoside, 4'-O-glucoside of quercetin, showed a distinctive shift in the H/D exchanging rate. A Cleavage of the sugar rest at 4' position needs quiet low energy (Petsalo *et al.*, 2006); consequently the aglycone; in this case quercetin, will be set free interacting with the backbone amides causing the shift.

2.4. Conclusion:

Under ecological demands plants evolved to produce complex mixtures of natural products of different structural classes. These substances are sometimes termed as secondary metabolites (SM). Phytochemists have nearly described more than 100000 SM compounds belong to different groups; many of these demonstrate curative properties and had been employed actively throughout centuries in traditional medicines. Nowadays, the modern medicine still recognizes the plants secondary metabolites as a crucial source of new therapeutic agents.

In general, secondary metabolites interact with targets; such as, structural proteins, enzymes, biomembranes, and nucleic acids, inducing changes in the conformation of the targeted molecules preventing them to achieve their assigned functionalities. These interactions could be divided into two major categories from the physicochemical point of view; covalent and non-covalent interactions. In the last few decades, many techniques have been invented and developed to explore the nature of these interactions to understand the mechanism of the bioactivity of natural products. At the early 90s mass spectrometry arose to be a promising technique in the field of drug discovery, due to many attractive features such as; sensitivity, specificity, speed, and amenability to high throughput screening.

In the early stages mass spectrometry was only used to identify the molecular mass of a compound and to elucidate its chemical structure. After the invention of API sources a major breakthroughs took place to enable MS techniques to prevail the field of bio- and macro-molecules. Mass spectrometry techniques have been employed to conduct many useful information concerning ligand-target interactions, such as; exploring of the interaction between antibiotics and bacterial cell wall peptide analogues (Lim et al., 1995), monitoring the interaction with enzymes (Cheng et al., 1995), studying the binding of metal ions to oligonucleotides (Wu et al., 1996), and probing the interaction between flavonoids and DNA triplexes (Liu et al., 2009).

One of the most abundant SM in the nature are polyphenols. Polyphenols have been reported to possess many beneficial aspects to the health of human beings. They possess anti-inflammatory and antimicrobial properties. They also proved to be useful in

cardiovascular disorders, and in the prevention against carcinogens. Moreover, they are recommended as a supportive therapy in neurodegenerative and ageing-related diseases. Polyphenols bioactivity has been mainly related to their anti-oxidative properties and to their ability to scavenge free radicals and suppress oxidative stress, but it has become more clear that the mechanism of action of polyphenols goes beyond these features (Scalbert *et al.*, 2005). Polyphenols can interact with different biomolecules through non-covalent forces modifying and altering their biological structure (Wink, 2008), and leading to achieve a variety of desirable bioactivities.

This work focused on the application of ESI-MS in monitoring the non-covalent interactions of some polyphenols with different peptides and their relevance to drug discovery. Two approaches have been applied for this purpose; the first approach, namely non-covalent MS, was applied to study the ionic interactions with angiotensin (I) and other synthesized peptides. While the second one H/D exchange MS was used to monitor the formation of hydrogen bonds with the backbone amides in insulin.

Polyphenols; and under physiological conditions build spontaneous negatively charged phenolate ions, will target the positively charged amino acid residues in the peptides, thus, forming ionic bonds. Since polyphenols possess many phenolate functional groups, therefore, the targeted peptide would be even immobilized, or its 3D structure would impaired, these all lead to changes in the bioactivity of this peptide.

The obtained ESI-MS spectra proved that polyphenols can interaction with different peptides through non-covalent bonds. MS spectra of mixtures of falvonoids glycosides and angiotensin (I) showed signals related to 1:1 ligand-target complexes. Rutin, as well as both hyperoside and spiraeoside were able to build ionic bonds with angiotensin (I). Nevertheless, the MS spectra of rutin revealed that rutin-angiotensin (I) complexes are more stable than complexes build with hyperoside or spiraeoside. Rutin shares both hyperoside and spiraeoside with the same aglycone, namely quercetin, but rutin has two sugar residues, whereas hyperoside and spiraeoside both has one sugar residue. Therefore, rutin can build more hydrogen bonds with angiotensin (I) making its complex more stable. On the other hand, MS spectra of flavonoid aglycones; such as quercetin, rhamnetin and kaempferol,

exhibited no signals related to complexes with angiotensin (I). This is due to the fact that the complexes build by flavonoids aglycones are not stable enough to be detected using the ESI-MS technique. The reason behind the weakness of these complexes is the co-planarity of flavonoid aglycones making them relatively rigid; therefore, they are more susceptible to the small fluctuations of the targeted biomolecule. On exception was taxifolin, whereas obtained MS spectra proved, that taxifolin is capability to form ionic bonds with angiotensin. The reason is that taxifolin belongs to flavonolol, where **π** system at $\mathbf{C_2}$-$\mathbf{C_3}$ of ring **C** is missing granting taxifolin more flexibility than other studied flavonoid aglycones, and in consequence more compliance to fit on the surface of the biomolecule.

Since that polyphenols build ionic bonds with peptides and proteins through interacting with the positively charged amino acid residues, e.g. lysine, arginine, and histidine. Hence, the increase of the number of such amino acid residues would cause that several polyphenol molecules will interact with one peptide showing binding stoichiometries other than 1:1 polyphenol-peptide. The tested polyphenols formed with angiotensin (I) revealed only 1:1 complexes. When polyphenols were mixed with peptides comprising increasing number of positively charged amino acid residues; e.g. 2, 4 and 8 lysine residues, a complexes such as 2:1, 3:1 and even 8:1 polyphenol-peptide have been monitored in the MS spectra of some tested polyphenols.

When testing rutin with peptides containing the increasing number of lysine residues, complexes 1:1, 3:1 and 6:1 have been observed in the MS spectra. Quercitirn, spiraeoside and hyperoside share with rutin the same flavonoid aglycone core, i.e. quercetin, therefore, they interact with in a similar manner such that rutin does. The only difference that the MS spectra obtained form rutin demonstrates more intensive signals related to rutin-peptide complexes. On the other hand scutellarin comprises fewer phenolic groups than rutin. Thus, scutellarin showed lower binding stoichiometries comparing to rutin, i.e. 1:1 and 2:1 scutellarin-peptide. From the tested polyphenols, EGCG possesses a huge phenolic system showing the highest binding stoichiometries toward lysine rich peptides. EGCG interacted with peptide containing 8 lysine residues forming 8:1 EGCG-peptide complexes. These results concerning EGCG turn in favor of the bioactivity of catechnis toward biomolecules and comply with the findings reported in many publications (Abbas and Wink, 2009; Abbas and Wink, 2010; Henning *et al.*, 2010). Moreover, procyanidin B2 reacted with the peptides

nearly in a similar manner to EGCG. Meanwhile, procyanidin C1 possesses the most phenolic groups; nevertheless, MS data of procyanidin C1 reflect a lower affinity toward the tested peptides as expected. The complexity of the chemical structure of procyanidin C1 is behind this controversy.

Another important, yet weaker, sort of non-covalent interactions is the hydrogen bonds. H/D mass spectrometry is considered as a method of choice to monitor the formation of hydrogen bonds between the polyphenols. When a polyphenol interacts with the a peptide building hydrogen bonds, the hydrogen atoms at the level of the backbone amides therefore this will be mask, and the hydrogen/deuterium exchange reaction is impeded from taking place at levels where the polyphenol interacted. MS spectrum of insulin in the presence of flavonoid aglycones, e.g. quercetin and myricetin, demonstrate a decrease in the deuterium uptake confirming the formation of hydrogen bonds.

Results obtained from quercetin confirm that quercetin is capable to interact with insulin backbone amides masking nearly 7 of the exchangeable hydrogen atoms. Since that myricetin has more OH groups than quercetin; therefore, myricetin could build around 9 hydrogen bonds with insulin. On the other hand, rhamnetin has the least OH groups building less than 4 hydrogen bridges. Moreover, the studied flavonoid glycosides show no significant shifts in deuterium uptakes. Rutin, as well as hyperoside and scutellarin all were not able to build hydrogen bonds with the backbone amides of insulin. Nevertheless, the MS spectra of spiraeoside; a 4'-O-glucoside of quercetin, confirmed an interaction with backbone amides and the masking of nearly 6 of the exchangeable hydrogen atoms.

Insulin in aqueous solutions tends to bury the non-polar amino acid residues in the core reducing the contact surface with the aqueous surrounding. These amino acid residues, also known the backbone amides, form the non-polar core. The backbone amides hydrogen atoms in insulin can undergo H/D exchange reaction in the medium rich with D_2O water, but in the presence of an active ligand, some hydrogen bonds are formed with some backbone amides excluding the last from the H/D exchange reaction. MS spectra of falvonoid glycosides proved their disability to build hydrogen bonds. This due to the fact, that falvonoid glycosides prone to build ionic bonds at the polar surfaces and have less tendency

to form hydrogen bonds with the backbone amides at the non-polar core. One exception was in the case of spiraeoside; 4'-O-glucoside of quercetin. A Cleavage of the sugar bridge at 4' position can occur spontaneously, and the related aglycone; i.e. quercetin, will achieve the interaction with the backbone amides. In the case of flavonoids aglycones, MS data confirm the ability of flavonoids aglycones to build hydrogen bonds with the backbone amides. Flavonoids aglycones are less flexible and have lower PSA values in comparison to their glycoside counter partners; therefore, they are able to reach the non-polar core of insulin, and in consequence, building the hydrogen bonds with the backbone amides.

Abbas S, Wink M (2009) Epigallocatechin gallate from green tea (Camellia sinensis) increases lifespan and stress resistance in Caenorhabditis elegans. *Planta Medica* **75**: 216-221.

Abbas S, Wink M (2010) Epigallocatechin gallate inhibits beta amyloid oligomerization in Caenorhabditis elegans and affects the daf-2/insulin-like signaling pathway. *Phytomedicine* **17**: 902-909.

Baranauskiene L, Petrikaite V, Matuliene J, Matulis D (2009) Titration Calorimetry Standards and the Precision of Isothermal Titration Calorimetry Data. *International Journal of Molecular Sciences* **10**: 2752-2762.

Baytekin B, Baytekin HT, Schalley CA (2006) Mass spectrometric studies of non-covalent compounds: why supramolecular chemistry in the gas phase? *Organic & Biomolecular Chemistry* **4**: 2825-2841.

Bazoti FN, Tsarbopoulos A, Markides KE, Bergquist J (2005) Study of the non-covalent interaction between amyloid-beta-peptide and melatonin using electrospray ionization mass spectrometry. *J Mass Spectrom* **40**: 182-192.

Busch KL (2010) Mass Spectrometry-Mass Spectrometry Retrospective. *Spectroscopy* **25**: 14-+.

Chalmers MJ, Busby SA, Pascal BD, He Y, Hendrickson CL, Marshall AG, Griffin PR (2006) Probing protein ligand interactions by automated hydrogen/deuterium exchange mass spectrometry. *Anal Chem* **78**: 1005-1014.

Cheng XH, Chen RD, Bruce JE, Schwartz BL, Anderson GA, Hofstadler SA, Gale DC, Smith RD, Gao JM, Sigal GB, Mammen M, Whitesides GM (1995) Using Electrospray-Ionization Fticr Mass-Spectrometry to Study Competitive-Binding of Inhibitors to Carbonic-Anhydrase. *Journal of the American Chemical Society* **117**: 8859-8860.

Cheng XH, Harms AC, Goudreau PN, Terwilliger TC, Smith RD (1996) Direct measurement of oligonucleotide binding stoichiometry of gene V protein by mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 7022-7027.

Clegg GA, Dole M (1971) Molecular beams of macroions. 3. Zein and polyvinylpyrrolidone. *Biopolymers* **10**: 821-826.

Cole RB (2003) Focus on noncovalent interactions. *J Am Soc Mass Spectrom* **14**: 417-418.

Curin Y, Andriantsitohaina R (2005) Polyphenols as potential therapeutical agents against cardiovascular diseases. *Pharmacological Reports* **57**: 97-107.

Daniel JM, Friess SD, Rajagopalan S, Wendt S, Zenobi R (2002) Quantitative determination of noncovalent binding interactions using soft ionization mass spectrometry. *International Journal of Mass Spectrometry* **216**: 1-27.

Deng QL, Xu J, Yu B, He J, Zhang KY, Ding XM, Chen DW (2010) Effect of dietary tea polyphenols on growth performance and cell-mediated immune response of post-weaning piglets under oxidative stress. *Archives of Animal Nutrition* **64**: 12-21.

Drummond JT, Loo RRO, Matthews RG (1993) Electrospray Mass-Spectrometric Analysis of the Domains of a Large Enzyme - Observation of the Occupied Cobalamin-Binding Domain and Redefinition of the Carboxyl-Terminus of Methionine Synthase. *Biochemistry* **32**: 9282-9289.

Dunayevskiy YM, Lai JJ, Quinn C, Talley F, Vouros P (1997) Mass spectrometric identification of ligands selected from combinatorial libraries using gel filtration. *Rapid Communications in Mass Spectrometry* **11**: 1178-1184.

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989a) Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246**: 64-71.

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989b) Electrospray Ionization for Mass-Spectrometry of Large Biomolecules. *Science* **246**: 64-71.

Figueirinha A, Cruz MT, Francisco V, Lopes MC, Batista MT (2010) Anti-Inflammatory Activity of Cymbopogon citratus Leaf Infusion in Lipopolysaccharide-Stimulated Dendritic Cells: Contribution of the Polyphenols. *Journal of Medicinal Food* **13**: 681-690.

Frieden E (1975) Non-covalent interactions. Keyto biological flexibility and specificity. *J Chem Educ* **52**: 754-756.

Ganem B, Li YT, Henion JD (1991) Detection of Noncovalent Receptor Ligand Complexes by Mass-Spectrometry. *Journal of the American Chemical Society* **113**: 6294-6296.

Garcia RA, Pantazatos D, Villarreal FJ (2004) Hydrogen/deuterium exchange mass spectrometry for investigating protein-ligand interactions. *Assay Drug Dev Technol* **2**: 81-91.

Glish GL, Vachet RW (2003) The basics of mass spectrometry in the twenty-first century. *Nature Reviews Drug Discovery* **2**: 140-150.

Greig MJ, Gaus H, Cummins LL, Sasmor H, Griffey RH (1995) Measurement of Macromolecular Binding Using Electrospray Mass-Spectrometry - Determination of Dissociation-Constants for Oligonucleotide-Serum Albumin Complexes. *Journal of the American Chemical Society* **117**: 10765-10766.

Griffiths J (2008) A brief history of mass spectrometry. *Anal Chem* **80**: 5678-5683.

He X, Li S, Xu M, Lin S, Zhou J, Yuan G (2011) Investigation of non-covalent interaction of ANF promoter DNA and polyamides containing N-methylimidazole and N-methylpyrrole amino acids by electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* **25**: 993-996.

Henning SM, Wang PW, Aronson WJ, Huang M, Zhang YJ, Lee RP, Heber D (2010) Green Tea Polyphenols and Metabolites in Prostatectomy Tissue: Implications for Cancer Prevention. *Cancer Prevention Research* **3**: 985-993.

Hensley P (1996) Defining the structure and stability of macromolecular assemblies in solution: The re-emergence of analytical ultracentrifugation as a practical tool. *Structure* **4**: 367-373.

Hofstadler SA, Sannes-Lowery KA (2006) Applications of ESI-MS in drug discovery: interrogation of noncovalent complexes. *Nature Reviews Drug Discovery* **5**: 585-595.

Hoofnagle AN, Resing KA, Ahn NG (2003) Protein analysis by hydrogen exchange mass spectrometry. *Annual Review of Biophysics and Biomolecular Structure* **32**: 1-25.

Ito H, Miyake M, Nishitani E, Mori K, Hatano T, Okuda T, Konoshima T, Takasaki M, Kozuka M, Mukainaka T, Tokuda H, Nishino H, Yoshida T (1999) Anti-tumor promoting activity of polyphenols from Cowania mexicana and Coleogyne ramosissima. *Cancer Lett* **143**: 5-13.

Katta V, Chait BT (1991) Observation of the Heme Globin Complex in Native Myoglobin by Electrospray-Ionization Mass-Spectrometry. *Journal of the American Chemical Society* **113**: 8534-8535.

Klebe G, Ritschel T, Atmanene C, Reuter K, Van Dorsselaer A, Sanglier-Cianferani S (2009) An Integrative Approach Combining Noncovalent Mass Spectrometry, Enzyme Kinetics and X-ray Crystallography to Decipher Tgt Protein-Protein and Protein-RNA Interaction. *Journal of Molecular Biology* **393**: 833-847.

Lim HK, Hsieh YL, Ganem B, Henion J (1995) Recognition of Cell-Wall Peptide Ligands by Vancomycin Group Antibiotics - Studies Using Ion-Spray Mass-Spectrometry. *Journal of Mass Spectrometry* **30**: 708-714.

Liu SY, Wan CH, Cui M, Song FR, Liu ZQ (2009) A study of the non-covalent interaction between flavonoids and DNA triplexes by electrospray ionization mass spectrometry. *International Journal of Mass Spectrometry* **283**: 48-55.

Loo JA (1997) Studying noncovalent protein complexes by electrospray ionization mass spectrometry. *Mass Spectrometry Reviews* **16**: 1-23.

Loo JA (2000) Electrospray ionization mass spectrometry: a technology for studying noncovalent macromolecular complexes. *International Journal of Mass Spectrometry* **200**: 175-186.

Mathur S, Badertscher M, Scott M, Zenobi R (2007) Critical evaluation of mass spectrometric measurement of dissociation constants: accuracy and cross-validation against surface plasmon resonance and circular dichroism for the calmodulin-melittin system. *Phys Chem Chem Phys* **9**: 6187-6198.

Pakalapati G, Li L, Gretz N, Koch E, Wink M (2009) Influence of red clover (Trifolium pratense) isoflavones on gene and protein expression profiles in liver of ovariectomized rats. *Phytomedicine* **16**: 845-855.

Palivec L, Urbanova M, Volka K (2005) Circular dichroism spectroscopic study of non-covalent interactions of poly-L-glutamic acid with a porphyrin derivative in aqueous solutions. *J Pept Sci* **11**: 536-545.

Palmer RA, Niwa H (2003a) X-ray crystallographic studies of protein-ligand interactions. *Biochemical Society Transactions* **31**: 973-979.

Palmer RA, Niwa H (2003b) X-ray crystallographic studies of protein-ligand interactions. *Biochem Soc Trans* **31**: 973-979.

Pelaez MV, Costa-Fernandez JM, Sanz-Medel A (2002) Critical comparison between quadrupole and time-of-flight inductively coupled plasma mass spectrometers for isotope ratio measurements in elemental speciation. *Journal of Analytical Atomic Spectrometry* **17**: 950-957.

Petsalo A, Jalonen J, Tolonen A (2006) Identification of flavonoids of Rhodiola rosea by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1112**: 224-231.

Pierce MM, Raman CS, Nall BT (1999) Isothermal titration calorimetry of protein-protein interactions. *Methods-a Companion to Methods in Enzymology* **19**: 213-221.

Poetsch A, Schlusener D, Florizone C, Eltis L, Menzel C, Rogner M, Steinert K, Roth U (2008) Improved identification of membrane proteins by MALDI-TOF MS/MS using vacuum sublimated matrix spots on an ultraphobic chip surface. *J Biomol Tech* **19**: 129-138.

Rodger A, Marrington R, Roper D, Windsor S (2005) Circular dichroism spectroscopy for the study of protein-ligand interactions. *Methods Mol Biol* **305**: 343-364.

Roselin LS, Lin MS, Lin PH, Chang Y, Chen WY (2010) Recent trends and some applications of isothermal titration calorimetry in biotechnology. *Biotechnology Journal* **5**: 85-98.

Scalbert A, Johnson IT, Saltmarsh M (2005) Polyphenols: antioxidants and beyond. *Am J Clin Nutr* **81**: 215S-217S.

Shindo M, Irie K, Fukuda H, Ohigashi H (2003) Analysis of the non-covalent interaction between metal ions and the cysteine-rich domain of protein kinase C eta by electrospray ionization mass spectrometry. *Bioorg Med Chem* **11**: 5075-5082.

Siegel MM (2009) GPC spin column HPLC-ESI-MS methods for screening drugs noncovalently bound to proteins. *Methods Mol Biol* **572**: 231-245.

Smith DL, Deng Y, Zhang Z (1997) Probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry. *J Mass Spectrom* **32**: 135-146.

Smith RD, Loo JA, Edmonds CG, Barinaga CJ, Udseth HR (1990) Sensitivity considerations for large molecule detection by capillary electrophoresis-electrospray ionization mass spectrometry. *J Chromatogr* **516**: 157-165.

Szabo A, Stolz L, Granzow R (1995) Surface-Plasmon Resonance and Its Use in Biomolecular Interaction Analysis (Bia). *Current Opinion in Structural Biology* **5**: 699-705.

Tang XJ, Brewer CF, Saha S, Chernushevich I, Ens W, Standing KG (1994) Investigation of Protein-Protein Noncovalent Interactions in Soybean Agglutinin by Electrospray-Ionization Time-of-Flight Mass-Spectrometry. *Rapid Communications in Mass Spectrometry* **8**: 750-754.

Veenstra TD (1999a) Electrospray ionization mass spectrometry: A promising new technique in the study of protein/DNA noncovalent complexes. *Biochemical and Biophysical Research Communications* **257**: 1-5.

Veenstra TD (1999b) Electrospray ionization mass spectrometry in the study of biomolecular non-covalent interactions. *Biophys Chem* **79**: 63-79.

Wadso I (1997) Trends in isothermal microcalorimetry. *Chemical Society Reviews* **26**: 79-86.

Wallace BA, Janes RW (2003) Circular dichroism and synchrotron radiation circular dichroism spectroscopy: tools for drug discovery. *Biochemical Society Transactions* **31**: 631-633.

Weisse ME, Eberly B, Person DA (1995) Wine as a digestive aid: comparative antimicrobial effects of bismuth salicylate and red and white wine. *BMJ* **311**: 1657-1660.

Whitehouse CM, Dreyer RN, Yamashita M, Fenn JB (1985) Electrospray interface for liquid chromatographs and mass spectrometers. *Anal Chem* **57**: 675-679.

Wilm MS, Mann M (1994) Electrospray and Taylor-Cone Theory, Doles Beam of Macromolecules at Last. *International Journal of Mass Spectrometry* **136**: 167-180.

Wink M (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**: 3-19.

Wink M (2005) Wie funktionieren Phytopharmaka? Wirkmechanismen der Vielstoffgemische. *Phytotherapie* 271-274.

Wink M (2008a) Plant secondary metabolism: Diversity, function and its evolution. *Natural Product Communications* **3**: 1205-1216.

Wink M (2008b) Evolutionary Advantage and Molecular Modes of Action of Multi- Component Mixtures Used in Phytomedicine. *Current Drug Metabolism* **9**: 996-1009.

Woodward C, Simon I, Tuchsen E (1982) Hydrogen exchange and the dynamic structure of proteins. *Mol Cell Biochem* **48**: 135-160.

Woody RW (1995) Circular dichroism. *Methods Enzymol* **246**: 34-71.

Wortmann A, Pimenova T, Alves S, Zenobi R (2007) Investigation of the first shot phenomenon in MALDI mass spectrometry of protein complexes. *Analyst* **132**: 199-207.

Wu QY, Cheng XH, Hofstadler SA, Smith RD (1996) Specific metal-oligonucleotide binding studied by high resolution tandem mass spectrometry. *Journal of Mass Spectrometry* **31**: 669-675.

Zhu MM, Rempel DL, Gross ML (2004) Modeling data from titration, amide H/D exchange, and mass spectrometry to obtain protein-ligand binding constants. *J Am Soc Mass Spectrom* **15**: 388-397.

Zuiderweg ER (2002) Mapping protein-protein interactions in solution by NMR spectroscopy. *Biochemistry* **41**: 1-7.