Chemical Composition of the Ethyl Acetate Extract of **Retama Raetam**, HPLC-UV-MS Analysis.

Elyacout Chebouat[1], Hayat Zerrouki[2], Imane Chebouat[3], Mohamed Lakhdar Belfar*[4], Manel Zaoui[5]

[1,2,3,4,5]VPRS Laboratory, Chemistry Department, Faculty of mathematics and Matter sciences, University Kasdi Merbah, Ouargla 30000, Algeria.

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Abstract

Since phytochemistry is mainly concerned with the separation, purification, and finally the structural determination of the separated compound, our contribution can be made in this field; Our work is part of the perspective of valuing and developing research on medicinal plants in Algeria and promoting traditional medicine; We chose the Retama Raetam plant from southeastern Algeria for the study.

Through the results of the preliminary phytochemical examination, we confirmed the presence of flavonoids, so we extracted them by applying the most famous extraction methods (ethanol/water) (70/30); Where we got the highest yield in the butanol extract.

The extracts obtained from the Retama Raetam plant were qualitatively analyzed by thin-layer chromatography, where 12 compounds were separated from ethyl acetate extract and 7 compounds from butanol extract.

After that, we conducted a qualitative analytical study of the ethyl acetate extract by high-performance liquid chromatography attached to ultraviolet spectroscopy and HPLC-UV-MS mass spectrometry, through HPLC chromatogram analysis, it was found that there are 22 compounds. We proposed a chemical formula from the interpretation of the UV spectrum and the mass spectrum of the compound with Retention time Rt=16.37.

Finally, phytotherapy constitutes an alternative role to the traditional pharmacy, and the need to find new molecules remains a public health priority.

Keyword : phytochemistry, Retama Raetam, flavonoids, TLC, HPLC-UV-MS.

Corresponding author: Email: mbelfar@gmail.com*

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Introduction :

Plants wield an arsenal of structurally diverse chemical compounds called secondary metabolites A great majority of these compounds do not directly participate in the growth, development, and reproduction of plants. Plants generally secrete these in response to stress. Over the last few decades, secondary metabolites have got great attention due to their potential role in human nutrition, cosmetics, and drugs and their indispensable role in plant defense. These secondary metabolites are very useful for humankind as they possess a wide range of therapeutic activities. Secondary metabolites produced by plants include alkaloids, flavonoids, terpenoids, and steroids. Flavonoids are one of the classes of secondary metabolites of plants found mainly in edible plant parts [2][1]

Flavonoids are a group of plant constituents and are biologically active phytochemicals that are ubiquitous in the plant kingdom and are being used in various herbal medicines for many years now. Flavonoids are generally found in vegetables, seeds, fruits, beverages grains, bark, and stems. These compounds are in the edible parts of plants, They constitute an essential part of our daily diet. Flavonoids are responsible for the red and dark blue color of berries, In the human body, they play a similar role as vitamins. There has been increasing interest in the research on flavonoids from plant sources because of their versatile health benefits reported in various studies. Flavonoids have a long history of medicinal use, mainly for support of healthy, Flavonoids protect the human body from damage caused by oxidizing agents such as ultraviolet rays, environmental pollution, food chemicals, etc . confirming in most investigations the existence of anti-inflammatory effects, antiviral or anti-allergic, and their protective role against cardiovascular disease, cancer, and various pathologies. [4][3]

Retama raetam, commonly known as ‘raetam’ or ‘broom bush’, is a desert shrub native to several countries of North Africa (e.g., Tunisia Egypt, Libya, AlgeriaandMorocco), temperate Asia (e.g., Syria, Jordan, Palestine, and Lebanon). Retem is described as a leguminous green plant, a shrub that belongs to the family Fabaceae. The shrub is normally about 3 meters tall but may reach up to 6 meters. The plant has green stems which are responsible for much of the photosynthesis; the small leaves are dropped immediately to conserve water loss through transpiration (Awen, et al., 2011). especially during the dry season; It is a desert shrub adapted to withstand recurrent drought spells and harsh environments.

R. raetam is traditionally well known for its famous folk medicinal values in many countries. The Bedouins in some areas use Retem as a folk remedy for the treatment of backache, arthralgia, and infertility and for inducing abortions using oxytocic effect on the uterus (Bailey and Dannin,1981).traditionally used for the treatment of renal diseases (Gonzalez-Tejero it is applied against several diseases such as jaundice, joint pains, sore throat, inflammation, fever, and microbial infections. [7][6][5]

The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical, and cosmetic products. It aims to extract the largest amount of chemical molecules present in the plant; By using selective organic solvents to speed up and increase the extraction yield. can be extracted from fresh, frozen, or dried plant samples. Usually, before extraction plant samples are treated by grinding and homogenization, which may be preceded by air-drying or freeze-drying. [9][8]

In the last step, the purified and extracted extracts are normally used for further study by chromatography techniques, usually involving the identification, quantification, and recovery of flavonoid compounds. The isolation and identification of each compound, as well as the correlation of their structures to the observed activity, is often difficult, due to the similarities of their structures and polarities. Notable recent advances have been made in both the analysis and identification of flavonoids due to the combination of instrumentation such as powerful liquid chromatographs and mass spectrometers. [10]

The work of a phytochemist essentially concerns the isolation, purification, and finally the structural determination of the isolated product, and it is in this area that our contribution can be made. Our work is part of a perspective of valorization and development of research on medicinal plants in Algeria and the enhancement of traditional medicine. the interest aroused by the study of natural molecules with biological activity and the knowledge of the chemical elements of plants to improve their use.

Materials and methods:

- preparation of plant extract :

Test plants were collected locally during the flowering period in the Ouargla region (southeast Algeria).plant was dried in the shade away from light, at room temperature.

After drying, plant materials were powdered using a grinder to obtain a fine powder, which was used for the preparation of the various extracts.

- preparation of extracts :

100g of the leaver of Retama Raetam, was soaked in(ethanol -Eau) (70-30) for 48 hours four times. at ambient temperature, The hydro-alcoholic solutions were then filtered using filter paper (Whatman No.1) . The filtrates obtained were concentrated under a vacuum on a rotary evaporator at 40 C.

Extractions with solvents of increasing polarity: petroleum ether, chloroform, ethyl acetate, and, butanol three times for each solvent. The 4 types extracted were concentrated under a vacuum in

the Rotavapor in each organic phase and stored for further use. plant extract obtained by different solvents and several extractions are mixtures of numerous compounds.

The residues obtained by CH2Cl2 were dissolved in Ethanol and subjected to

HPLC-UV-MS analysis.

Isolation of flavonoids

Separation and purification:

Plant extracts are generally complex mixtures that may contain many compounds, the development, and commercialization of which requires an initial step of careful analysis (constituent identification and quantification).

Various analysis techniques are available for the laboratory to separate natural products such as chromatography. [11]

Chromatography:

is a physical method of separation based on the differences in affinity of the substances to be analyzed into two phases, one stationary, the other mobile. Depending on the chromatographic technique involved, the separation of the components entrained by the mobile phase results either from their absorption and desorption they are successive on the stationary phase or from their different solubility in each phase in the case where the two phases are liquids. There are several methods of chromatographic separation depending on the objective set beforehand and the feasibility of the method.[12]

Thin layer chromatography (TLC):

Principle:

TLC is a rapid, simple, and inexpensive analytical technique. It is mainly based on the phenomenon of adsorption. It applies to pure molecules, extracts (complex mixtures of metabolites), and biological samples. It allows us to have an overall idea of the metabolites present in an extract or a fraction and allows easy and fast control of the purity of a compound when the operating conditions are well determined. It also helps to follow the progress of a reaction since it indicates the number of components in a reaction mixture.

Indeed, when the solvent system or the eluent is well chosen, this technique used in its preparative form is often very advantageous because the transposition from analytical TLC is easy. The process is fast and allows the isolation of sufficient quantities of substances for structural analysis. This method allows most of the time to obtain pure products.

Analytical (TLC) method:

Thin layer chromatography (TLC): We mainly use Kieselgel (Silicagel) 60 F254, ready-to-use aluminum-supported silica plates (Merck). The solvent systems employed with these plates are:

1- Ethyl acetate extract :

- Toluene, acetate ethanoate, acetic acid(5.8 , 3.3 , 0.9).

-Toluene, formic acetate (1.4 ,10) .

-Chloroform, methanoate (9.6, 0.4).

- Acetate ethanoate, acetic acid, distilled water (100, 11, 26).

-Butanol, acetate-acetone, distilled water (6, 2, 2).

-Butanol, acetic acid, distilled water (6.5, 1.5, 2.5).

2- Butanol extract :

-Acetate ethanoate, acetic acid, distilled water (100, 11, 26).

-Butanol, acetate-acetone, distilled water (6, 2, 2).

- Butanol, acetic acid, distilled water (6.5, 1.5, 2.5).

The plates are analyzed in visible light and under UV (254 and 356 nm). The use of different reagents Such as (H2SO4, Iodine, AlCl3...) vaporized on the thin layer chromatography plates after elution makes it possible to compare the profiles of the separated fractions and to group them according to their similarities, to obtain additional information on the type of a molecule.

High-Performance Liquid Chromatography (HPLC):

Principle: This is one of the advanced techniques used in analytical studies of non-evaporating particles of high polarity. When it comes to flavonoids, this method is one of the most accurate methods, as it is used in qualitative studies of quantity.

Free flavonoids are separated by HPLC in both normal and reversible polarity methods, while sugar flavonoids are preferred to be separated in the reverse polarity method. We use a mixture of (water/methanol/acetic acid) or a mixture of (water/acetonitrile / acetic acid). It requires the use of high pressures to push the solvent through the column. It is detected by ultraviolet spectroscopy.

HPLC and Mass Spectrometric Analysis:

In the field of natural products, interest has been growing in the last twenty years in developing fast and efficient strategies to enable the discrimination between previously isolated known compounds and new compounds at the level of the crude extract.

Hyphenated techniques, which can be defined as the coupling of high-performance liquid chromatography (HPLC) separation technologies with online spectroscopic/spectrometric detection technologies, are the most commonly used in dereplication strategies. liquid chromatography-mass spectroscopy (LC-MS) has a wide range of applications in the development of dereplication tools for the tentative identification of plant and microbial secondary metabolites.[16][15].

Method:

In our analytical study, we applied the polar opposite method to ethyl acetate extract under the following practical conditions:

- Device type: Agilent Prep - C18 scalar PN 440905 – 902
- SN: USAWSO1038, LN: PR045203
- Chromatographic column: Agilent Colonne: (4,6*250mm,5M)
- Stationary phase: substituted silica gel (C-18).
- Mobile phase: water/methanol / acetic acid.
- Injection volume: lµ10

2-Results and Discussions :

Phytochemical tests:

Phytochemical screening is detecting the different families of compounds existing in the plant parts studied by qualitative characterization reactions. These reactions are based on phenomena of precipitation or coloring by reagents specific to each family of compounds.

Phytochemical screening was carried out due to its simplicity, speed, minimum use of equipment, and selectivity (Nohong 2009). allows us to have a good idea of the phytochemistry of the

medicinal plant. The results obtained from Phytochemical tests are in Table 1.

Table 1: the result of the initial phytochemical screening of . the leaver of Retama Raetam .

the	Active compounds in the aerial	**percentage of**

number	part of the plant	**presence**
01	Flavonoids	+++
02	Glycoside Flavonoids	+++
03	Free Flavonoids	-
04	Alkaloids	+++
05	Cardenolides	++
06	Tannins	+++
07	Saponoids	+++
08	Unsaturated sterols and terpenes	-
09	Unsaturated steroids	+++
10	Steroid derivatives	-

Through the results obtained, the presence of almost all the active compounds, especially the basic ones, in the studied plant are as follows:

Flavonoids, glycoside flavonoids, alkaloids, tannins, saponoids, and Unsaturated steroids are present in a large percentage and a moderate amount of cardenolides, and we note the absence of unsaturated sterols and terpenes, steroid derivatives, and free flavonoids in the plant.

The results obtained confirmed the presence of several phytochemicals (bioactive compounds), that have a crucial role in biological activity and justify plant Retama Raetam therapeutic uses.

Extraction results:

The three fractions recovered were dried over (anhydrous Na2SO4) and subjected to concentration at low pressure at 35° C. Then weighed to determine the extraction yields expressed relative to 100 g of dry matter. Based on these data, we can calculate the extraction yield for each phase according to the following relationship:

$$R\% = \frac{m}{m^{\circ}} \times 100$$

R: extraction yield. **m:** mass resulting from the extraction process. m^c: the soaked mass (100g).

Table 2: extraction yield.

mass of matter	Extract	Mass(g)	Yield (%)
100 g	Dichloromethane	0.2234	0.2234%
	Ethyl acetate	0.3324	0.3324%
	Butanol	3.8055	3.8055%

Through the table, we notice that the extraction yields of dichloromethane and ethyl acetate are very few percentage percentages, and the yield extract of the Butanol phase extraction is relatively large compared to them.

Indeed, the solubility of the substances contained in the vegetable matter in a given solvent the nature and the Physicochemical characteristics of the solvents used, and in particular their polarity. Thus, the extraction yields and the composition of the extracts vary according to the studied plant and solvent.

Results (TLC) :

The obtained extracts are analyzed by thin-layer chromatography (TLC). For rinsing, use several solvents in different proportions, to get the best. Observed by UV lamps (254 and 365 nm) using an NH3 detector, we also determined the (Rf) for each compound. The best results are in the following tables:

Table 3: Results of analysis by TLC (Ethyl acetate extract).

mobile phase	the color of the UV	UV + NH_3	R_f
Toluene, acetate ethanoate, acetic acid (5.8 , 3.3 , 0.9).	Blue	Orange	**0,09**
	Blue	Light Brown	**0,24**
	Blue	Light Blue	**0,17**
	Light Brown	Light Blue	**0,26**
	Orange	Violet	**0,39**

	Light Brown	Blue	0,43
	Light Brown	Orange	0,46
	Blue	Brown	0,50
	Light Blue	Violet	0,59
	Orange	Orange	0,66
	Violet	Violet	0,82
	Violet	Violet	0,88
Toluene, formic acetate (1.4 ,10) .	Yellow	Orange	0,06
	Yellow	Light Green	0,12
	Blue	Light Green	0,16
	Orange	Light Brown	0,21
	Light Blue	Orange	0,24
	Light Blue	Violet	0,31
	Brown	Violet	0,37
	Brown	Orange	0,40
	Brown	Orange	0,45
	Brown	Light Brown	0,54
	-	Violet	0,56
-Chloroform, methanoate (9.6, 0.4).	Violet	Orange	0,05
	Violet	Violet	0,11
	Blue	Violet	0,16
Acetate ethanoate, acetic acid, distilled water (100, 11, 26).	Violet	Orange	0,84
Butanol, acetate-acetone, distilled water (6, 2, 2).	Violet	Light Brown	0, 31
	Light Blue	Orange	0,44

	Light Brown	Orange	0,55
	Yellow	Light Blue	0,83
Butanol, acetic acid, distilled water (6.5, 1.5, 2.5).	Violet	Yellow	0,29
	Violet	Green	0,49
	Brown	Light Blue	0,62
	Brown	Light Brown	0,70
	Orange	Orange	0,83

Table4: Results of analysis by TLC (Butanol extract).

mobile phase	the color of the UV	UV + NH_3	Rf
Acetate ethanoate, acetic acid, distilled water (100, 11, 26).	Brown	Light Brown	0,09
	Yellow	Yellow	0,24
	Light Blue	Yellow	0,17
	Brown	Light Brown	0,26
	Blue	Light Blue	0,39
	dark Brown	Orange	0,43
	Light Brown	Orange	0,46
Butanol, acetate-acetone, distilled water (6, 2, 2).	Orange	Light Green	0,63
	Light Blue	Light Brown	0,68
	Orange	Brown	0,73
	Violet	Orange	0,77
	Light Blue	Violet	0,83
	Light Blue	Light Blue	0,90
- Butanol, acetic acid, distilled water (6.5, 1.5, 2.5).	Green	Orange	0 ,047
	Green	Brown	0,078

	Green	Violet	**0,16**
	Orange	Light Blue	**0,29**
	Brown	Violet	**0,44**
	Brown	Violet	**0,57**
	Violet	Violet	**0,68**

- As expected, TLC analysis shows that the Ethyl acetate extract and Butanol extract contain flavonoids.

-Through the results recorded in the two tables, we notice that (12) compounds were separated from the Ethyl acetate extract, and as for the Butanol extract, (7) compounds were separated,

We also notice the appearance of colors, except for the colors, which we interpret as the possible presence of some types of flavonoid compounds, summarized in the following table.

Table5: Relationship between structure and color of flavonoids under UV radiation:

	Fluorescence	**Possible structures**
Violet	Yellow Yellow-green Brown	A flavone with an OH at the C-5 position, and a 4'-OH - Flavanols substituted at position 3-OH, 5-OH 4'-OH Flavanone 5-OH or 4'-OH A chalcone without an OH on the B ring
	Little or no discoloration	Flavones or 5-OH flavonols without the 4'-O A flavanol or a flavanone has an OH at the 3 C-position. - A flavonol substituted with an OH at the C-3 position and without an OH at the C-5 position.
	Orange	Chalcone and/or without 4-OH

Yellow or light yellow	Little or no discoloration	Flavonols with a free OH at the 3C- position and with or without an OH at the 5C- position
Yellow	Orange	Aurones with 4'-OH
Blue	Yellow or green	A flavanone and a flavone without an OH at the C-5 position Flavanols without 5-OH with 3-OH
Blue	Blue	isoflavones without 5-OH

Structural analysis:

Chromatographic behavior To identify the isolated products, a series of methods are used and applied depending on the nature of these products. Thus, for flavonoids, the Rf values in known solvent systems and the fluorescence of the product in question make it possible to have a first structural approach which will greatly facilitate subsequent operations.

These two operations are applied systematically for this kind of natural product even before the actual physical analysis.

The absorption of flavonoid substances UV at the wavelength of 365 nm gives preliminary information on the chemical structure. It allows one to distinguish between the different classes of flavonoids very quickly.

Rf is defined as being the ratio of the distance between the product spot and the origin on the one hand and the distance between the origin and the solvent front d 'somewhere else. The **Rf** value varies with the nature of the solvent used (organic or aqueous), the type of chromatographic support (silica gel, polyamide, cellulose), the form of the product itself (aglycone or glycosyl), as well as the disposition of different substitutions of the flavonoid skeleton. The Rf does not depend on the concentration of the constituent in the mixture.

The Rf values of flavonoids are related to the following factors:

• The position of the OH in the flavonoid compound: the presence of OH at the C-6 and C-8 positions gives a smaller Rf, while its presence at the C-5 position leads to a larger Rf, in addition to the presence of substituents in the position ortho to OH leads to a higher Rf.

• The saturation of the C cycle: leads to a higher Rf, while its unsaturation leads to a lower Rf, and this is what is observed about the two classes of flavones and flavanones.

• Sugar clusters: The presence of sugar clusters in flavonoids leads to a smaller Rf due to increased polarity, which leads to stronger bonding between flavonoid sugar compounds and silica gal.[26].

Analysis by HPLC-MS-UV:

High-performance liquid chromatography (HPLC):

The chromatographic curve of the ethyl acetate extract of Retama Raetam was obtained by HPLC- UV-MS separation.

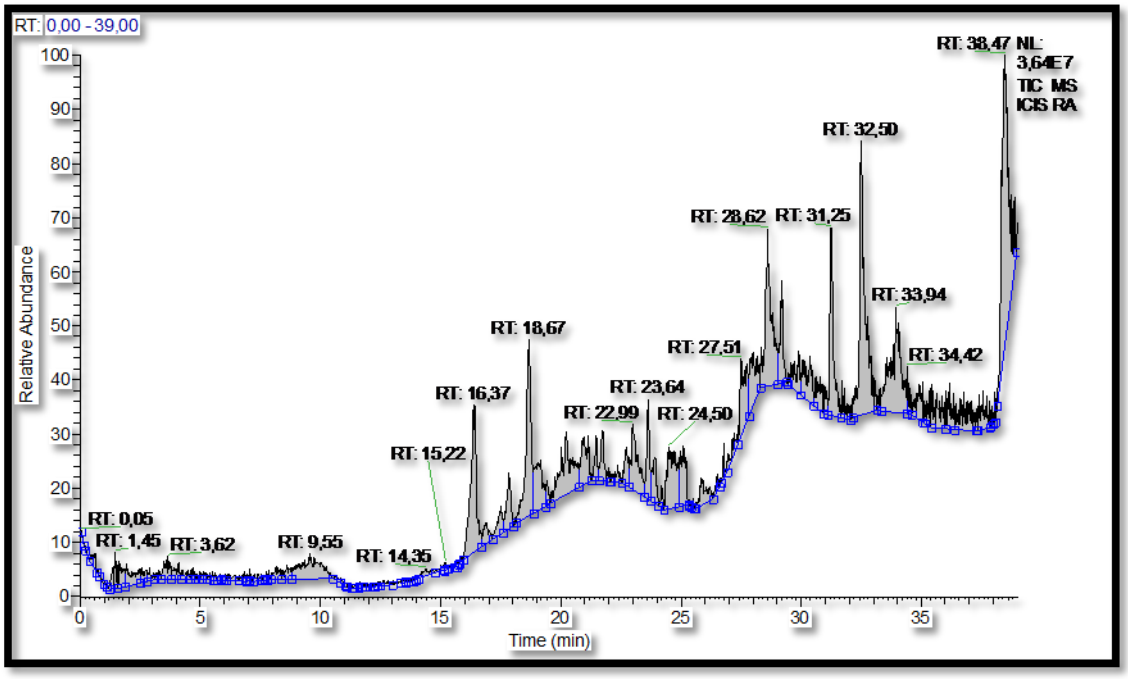


Figure 1:Chromatogram(HPLC-UV-MS).

Through the results obtained from the qualitative analysis of the ethyl acetate extract of the Retama Raetam plant by high-performance liquid chromatography (HPLC) represented by the chromatography curves; We were able to know the number of compounds present in the ethyl acetate extract represented by the peak, as we note through the results the presence of 22 compounds.

- **Structural determination of the compound (Rt=16.37):**

- **Analysis by UV spectroscopy:**

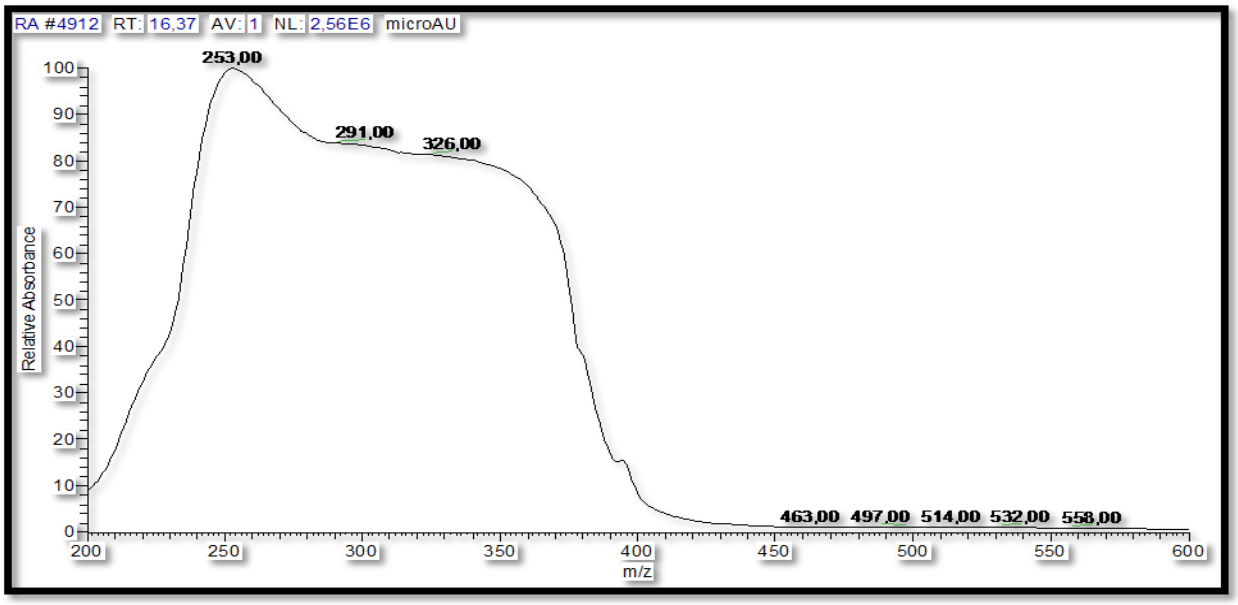


Figure 2: UV spectrum of the compound (A Rt = 16.37).

Flavonoids are a well-defined group of compounds with established physical and chemical characteristics. This especially counts for their absorption of ultraviolet(UV) radiation, which makes their UV spectra very characteristic and UV spectroscopy a method of choice for their characterizations.

Ultra-violet (UV) absorption spectroscopy of flavonoids has two maxima absorptions around 304–285 and 250–280 nm corresponding to bands I and II from A and B rings, respectively.

The position of these bands gives information about the kind of flavonoid and its substitution pattern.

Table 6 : UV spectroscopy:

Flavonoid class	Band II nm	Band I nm
Isoflavone	245-275	310-330
Isoflavone (A Rt = 16.37)	253	326

Analysis by MS mass spectrometry:

The mass spectrometry technique is very helpful in the structure elucidation of flavonoids. It is used in the determination of the molecular weight for establishing the distribution of substituents between the A- and B-rings and in the determination of the nature and site of attachment of the sugar(s) in flavonoid C- and O-glucosides, it allows two types of information to be given:

-Determination of the apex of the molecule: allows estimation of the number and nature of hydroxyl or methoxyl substituents and others. It allows access to the overall formula of the studied molecule with the utmost accuracy.

- designate the underlying fragments; Fractionation peaks that allow knowing the distribution of substituents between nuclei A and B

Peaks obtained during this fragmentation process represent accurately the corresponding ion fragments that are expressed as the mass-to-charge ratio (m/z)

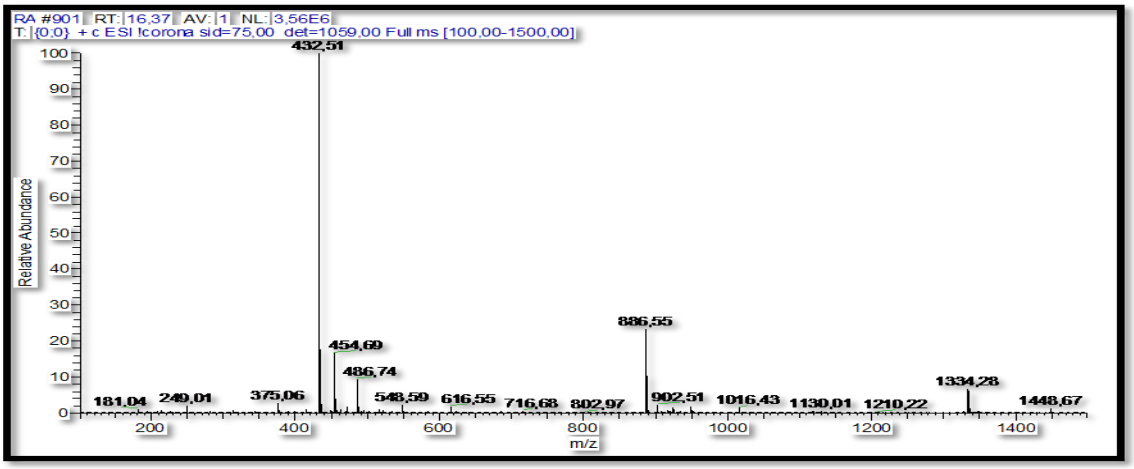


Figure 3: Mass spectrometry of the compound (A Rt = 16.37).

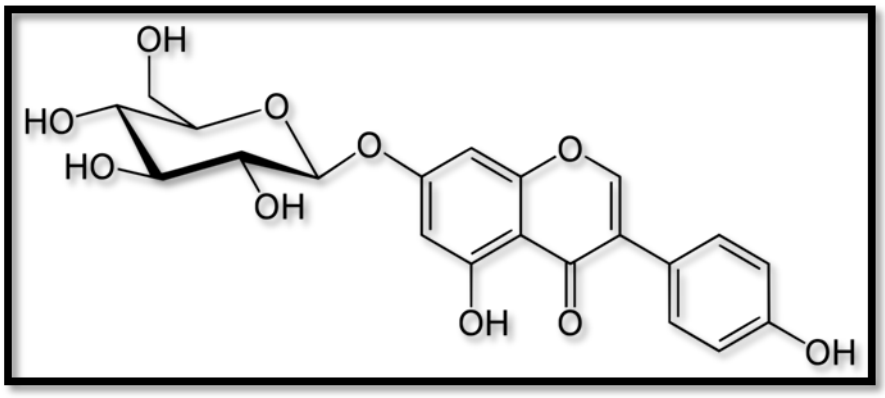
The mass spectrometry results are shown in the following table:

Table 7: Mass spectrometry results:

Fragments agree	m/z
[M]	432
[M+Na]	455
[M+H$^+$]	433
[2M+Na]	887
[M+H-Glu]	180

Structural elucidation of isolated products:

We suggested the chemical formula of the compound by analyzing the HPLC-UV-MS results:



Genistin

Conclusion:

Nowadays, the main concerns about natural medicinal products are effectiveness, safety, and the quality of herbal drugs. Consequently, it is essential to identify and measure all the bioactive constituents of medicinal plants to ensure biological research reliability and repeatability as well as to ensure enhancing the quality control over the pharmacological benefits and/or hazards.

The need for flavonoids in agriculture, food, and drug industries is still one of the worldwide up-to-date research interests. Natural resources and especially medicinal plants are still available to discover novel or efficient antioxidant flavonoids that could be used as drugs to fight against degenerative diseases one of the issues global health is facing today.

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