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PRACTICAL AUTHENTICATION AND QUALITY CONTROL OF *TILIA* FLOS

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ABSTRACT

Different *Tilia* species are used extensively worldwide for many health complications. The present work was designed to evaluate and characterize *Tilia* flos available in the herbal market compared with different organs (leaves and flos of three different *Tilia* species native to western European countries (e.g. Germany and Belgium). The Hyphenated HPLC-DAD-ESI-MS technique, quantitative estimation of total flavonoid contents (TFC) and total polyphenolic contents (TPC) are used extensively in this study including two methods of extractions. This study revealed that TFC and TPC in the flowers are generally more than those in the leaves. *Tilia tomentosa* flos contain the highest percentage of flavonoids among all samples based on infusion method of extraction while *Tilia* flos available in the Egyptian market showed the lowest percentages of both TFC and TPC among all flower samples. LC/MS analysis revealed the presences of 30 compounds, ten of which were tentatively identified here for the first time in *Tilia* species based on ESI/MS and characteristic UV spectra. The present study are simple, easy applicable and valuable in the practical authentication, quality assurance and prevention of adulteration of the crude *Tilia* drug.

Keywords: Total flavonoid contents (TFC); Total phenolic contents (TPC); Flavonol glycosides; *Tilia* leaves; *Tilia* flos; *T. cordata*; *T. platyphyllos*; *T. tomentosa*; HPLC–DAD–ESI-MS.

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INTRODUCTION

Tilia flos (linden or lime flowers) consists of the inflorescences of *Tilia cordata* Miller, *Tilia platyphyllos* Scop. and *Tilia x vulgaris* Heyne (*Tiliaceae*) are known herbal medicine used worldwide traditionally as sedative, antispasmodic, raised arterial pressure associated with arteriosclerosis and nervous tension **(Barnes et al., 2007)**. It is listed by the Council of Europe as a natural source of food flavouring (category N2) **(Barnes et al., 2007)**. *Tilia* flos have a prominent importance in phytotherapy. It is stated to possess expectorant, diaphoretic and diuretic activities. It has been used for the treatment of flu, cough, migraine, nervous tension, indigestion, various types of spasms, liver and gall bladder disorders **(Toker et al., 2001)**. Medicinal properties claimed for the drug have been attributed to its flavonoid, volatile oil and mucilage components **(Toker et al., 2001)**. Three *Tilia* species are predominant in middle Europe they are T. *cordata, T. platyphyllos and T. tomentosa* **(Senghas and Seybold et al., 2003)**. *T. cordata* flowers are the most preferred medicinal species. *T. platyphyllos* flowers are also used and somewhat interchangeably. The dried flowers are mildly sweet and sticky with a pleasing taste, due to the aromatic volatile oil found in the flowers **(Bradley, 1992)**. Infusions of *Tilia* are widely used in traditional medicine in Europe and Latin America for the treatment of enterocolitis, gastroenteritis, liver disorders and renal colic, **(Aguirre-Hernàndez et al., 2007, Aguirre-Hernàndez, 2010, Pèrez-Ortega et al., 2008)** it was also reported to have tranquilizing activity **(Cárdenas-Rodríguez et al., 2014)**.

Many of *Tilia* constituents were reported to have an interesting pharmacological actions, for example, tiliroside has anti-diabetic effects through inhibitory effects on carbohydrate digestion and glucose uptake in the gastrointestinal tract **(Goto et al., 2012)**, *antihypertensive and vasorelaxant effects* **(Silva et al., 2013)** anti-hyperglycemic, anti-hyperlipidemic and antioxidant effects **(Qiao et al., 2011, Goto et al., 2012)**. Proanthocyanidins, which are a species of polyphenol known to possess a variety of physiological activities, including radical scavenging activity **(Vennat et al., 1994)**, antioxidative properties **(Haslam et al., 1996)**, antifungal effects **(Eberhardt and Young, 1994)**, anti-allergic activity **(Kanda et al., 1998)** and antihypertensive activity **(Cheng et al., 1993)**, Proanthocyanidins have been used as a treatment for capillary stabilization **(Brasseur et al., 1989)**, and also they are reported to possess hair growth-promoting activity **(Takahashi et al., 2005)**. The flavonoid constituents of *Tilia* flos exerted some interesting biological activities such as selective antiproliferative action on tumoral cells, acting through the modulation of H_2O_2 levels, **(Brizi et al., 2012)** total extract of some *Tilia* species

shows antiproliferative action against BW 5147 cells **(Barreiro et al., 2006)**, anexiolytic/sedative activity (Cotrim et al., 1999) and anticonvalsant **(Cárdenas-Rodríguez et al., 2014)**.

In European Pharmacopoeia (EP), tests given for the analysis of official drug are mainly based on the morphological characters. Moreover, there is also a thin-layer chromatographic (TLC) technique in the monograph, which based on the detection of flavonoid components. Using a mixture of EtOAc–HCO_2H–$C_2H_5OCH_3$–H_2O (50:10:30:10) as mobile phase and caffeic acid, rutin and hyperoside as reference compounds, and the spots are then visualized by diphenylboric acid aminoethyl ester/MeOH reagent. The plate is evaluated according to the characteristic color of spots and relative retention times compared with those of reference compounds **(Toker et al., 2001)**. But the resolution of this technique seems, practically, not sufficient specially for the compounds with nearly identical R_f-values **(Wagner et al., 1983)**. Some research articles discussed the utilization of HPLC-DAD and HPLC-DAD-ESI/MS for the analysis of flavonoid contents of *Tilia* (*T. platyphyllos* **(Toker et al., 2001, Wagner et al., 1983, Piettta et al., 1993, Karioti et al., 2014)**, *Tilia rubra* **(Toker et al., 2001)** and *Tilia argentea* **(Toker et al., 2001)**. Results of the previous studies revealed that flavonoid composition of each lime species possesses a specific fingerprint HPLC chromatogram depending upon the parts used.

The present work involves the determination of TFC, TPC of both Flowers and leaves of three European species, T. *cordata, T. platyphyllos and T. tomentosa*, in comparison with the Egyptian sample and qualitative estimation of phenolic and flavonoid constituents using HPLC-DAD-ESI/MS. evaluation of the data might be helpful in the quality assurance as well as determination of adulteration of the crude drug.

The foliage of *T. platyphyllos* **(Fig. 1)** consists of simple, alternately arranged leaves. They are ovate to cordate, mid to dark green above and below, with white downy hair on the underside, particularly along the veins, tapering into a mucronate tip. The leaves of *T. cordata* are alternately arranged, mostly hairless (unlike the related *T. platyphyllos*) except for small tufts of brown hair in the leaf vein axils the leaves are distinctively heart-shaped. The leaves of *T. tomentosa* are alternately arranged, green and mostly hairless above, densely white tomentose with white hairs below **(Maes, 1990, Invanov et al., 2014, Rajendra, 2009)**.

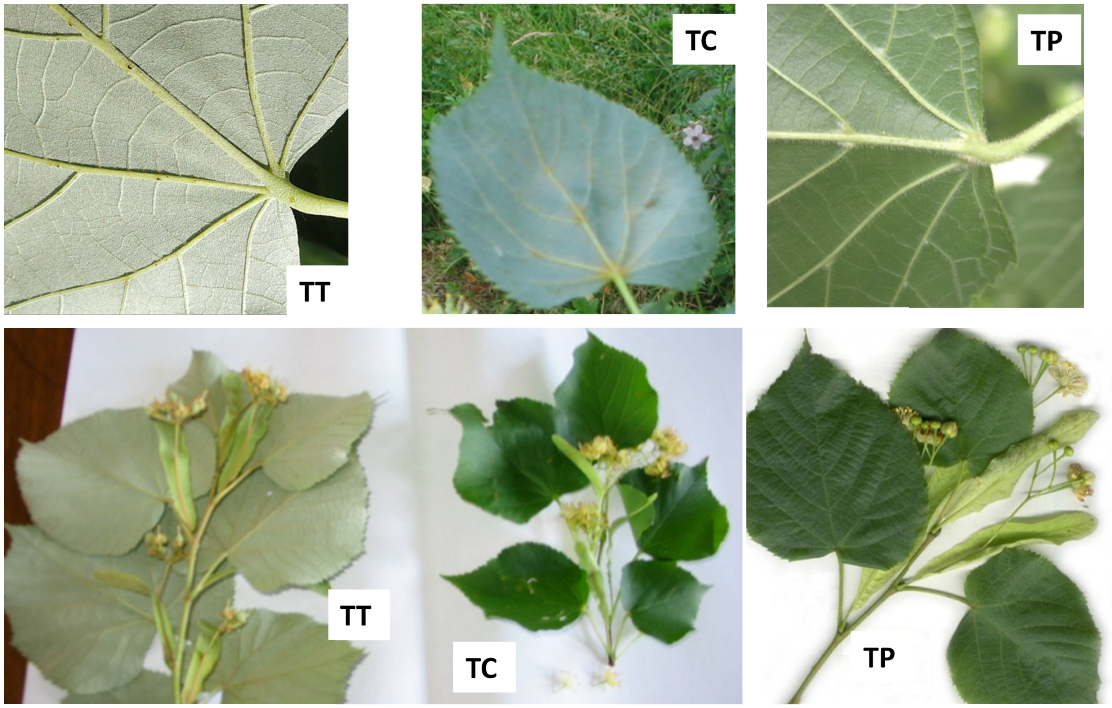

Figure 1: The morphology of *T. cordata* (TC), *T. tomentosa* (TT) *T. platyphyllos* (TP). The leaf dorsal sides (above) show a characteristic hair distribution for each species, while the pictures (below) shows the young twigs with yellowish-white flowers that arranged in drooping, cymose clusters and whitish-green, leaf-like bracts with alternately arranged foliages

The present study was designed to evaluate the chemical profiles of non volatile constituents of the flos, bracts and leaves of Three European *Tilia* species namely *T. cordata* flos (TCF), *T. tomentosa* flos (TTF) *T.platyphyllos* (TPF) flos which are commonly used as herbal medicines and their leaves (TCL, TTL and TPL), which are commonly not used in comparison with the Egyptian sample (TF_{Eg}) of unknown origin. The investigation comprises quantitative estimation of total flavonoid contents (TFC) and total polyphenolic contents (TPC) that were determined using two methods of extractions (infusion method and ultrasonication in 50% methanol) of all samples, in addition to qualitative estimation of the chemical profile of (TF_{Eg} , TTL, TPL, TCL and TTF) using hyphenated HPLC-DAD-ESI-MS technique. Study of leaves of the selected *Tilia* species was included in this work in order to explain the percentage similarity between the flos (the commonly used part) and leaves of the same plant and to determine the differences between the leaves and bracts which are often sold among the official *Tilia* flos.

MATERIAL AND METHODS

Plant materials

Leaves and inflorescences including a whitish green bracts of *T. platyphyllos* Scop., *T. cordata* Miller and *T. tomentosa* Moench., family *Tiliaceae* were collected from the botanical garden of Düsseldorf University (Germany) in addition to the Egyptian *Tilia* flos sample (composed mainly of greenish yellow bracts with few broken inflorescences) that was purchased from a famous herbal market (Harraz

Market) in middle Cairo, Egypt. All voucher specimens are kept in herbarium of the pharmacognosy dept., faculty of pharmacy, (Boys) Al-Azhar University, Cairo, Egypt.

Chemicals

Rutin (quercetin-3-*O*-rutinoside), $AlCl_3$, $NaNO_2$, Folin ciocalteu reagent (2M according to acid contents), Gallic acid, Na_2CO_3 (Sigma-Aldrich, Switzerland). All other reagents and solvents used in the study were of analytical grade. Solvents for HPLC were HPLC grade.

Lab equipments and apparatus

Rotary evaporators: Büchi Rotavap RE 111; Buchi Rotavap R -200 and Buchi V-700, Buchi Labourtechnik AG. 9230 Flawi/ Switzerland

Analytical HPLC (LC /UV/ MS hyphenated system): MS spectrometer : Finnigan LC Q-DECA, HPLC system (Pump, Detector and autosampler) : Agilent 1100 series . Column: Knauer (125mm L, 2 mm ID), prepacked with Eurosphere- 100 C-18 (5 µm) and with integrated pre-column. Mobile Solvents: Acetonitrile or Methanol LiChrosolv HPLC (Merck), *ortho*-phosphoric acid 0.15 %, pH 2.0 (prepared from *ortho*-phosphoric acid 85% p.a., Merck) Nanopure water Barnstead. LC /ESI-MS was carried out using a Finnigan QDECA-7000 mass spectrometry connected to a UV detector. The sample is dissolved in methanol and injected to HPLC/ESI-MS hyphenated system. HPLC was run on Eurospher C-18 reversed phase column. The mass spectra were generated on a dual octopole ion trap mass spectrometer operated in positive and negative ion modes and fitted with an atmospheric pressure electrospray-ionization sample introduction device. Fragmentation experiments were performed by automatic MS technique.

Spectrophotometer for TPC and TFC: UV/Vis spectrophotometer (6105, Jenway LTD, England).

Sample preparations

1) Plant extraction and sample preparation for quantitative estimation of total flavonoid contents (TFC) and total polyphenolic contents (TPC) using 50% MeOH (Method A): Powdered sample (1.0 g, each) was extracted two times with 50% MeOH (100 ml) using ultrasonic bath at 38^{o}C two hours each. Combined extract was filtered and centrifuged and the supernatant was adjusted to 200 ml for TFC experiment. While the extract was adjusted to 1000 ml for TPC (sample was 1ml of the final dilution for all experiments).
2) Plant extraction and sample preparation for quantitative estimation of TFC and TPC using boiling water (infusion method, method B): 100 ml boiling water was added to 1.0 g Powdered samples, after 5 minutes each sample was filtered and the filtrate was adjusted with deionized H_2O to 200 ml for TFC. While it was adjusted to 1000 ml for TPC (sample was 1ml of the final dilution for all experiments).

3) Sample preparation for standard calibration curve for TFC: 1ml of standard rutin concentrations (5µg - 150µg/ml) were prepared in MeOH: H_2O (1:1).
4) Sample preparation for standard calibration curve for TPC: samples of 1ml of standard gallic acid concentrations (5µg - 75µg/ml) were prepared in deionized H_2O.
5) Plant Preparation for HPLC-ESI-MS analysis: fine powdered samples of **TF_{Eg}**, **TTL**, **TPL**, **TCL**, **TTF** (5.0 g, each) was extracted three times with 70% MeOH (50 ml) overnight. Combined extract was filtered and centrifuged and evaporated in vacuo to dryness. (Percentage of solid extracts were 13.5%, 16.0%, 15.2%, 13.7%, 15.5% respectively). The crude extract of each sample was re-dissolved in 10 ml of 70 % methanol and defatted with n. Hexane 10 ml two times, final methanol extracts were adjusted to 10 ml each. 1 ml of defatted extract of each sample was diluted to 5ml methanol (HPLC grade) where only 20 µl is injected into an HPLC-ESI-MS by auto-sampler.

Standard Calibration curves

Standard Calibration curve was established for rutin (as standard flavonoid compound) by preparation of serial dilutions ranging from 5 - 150 µg/ml. The results (absorbance values) were plotted versus corresponding rutin concentrations where the calibration curve and regression equation were obtained.

For TPC, standard Calibration curve was established for gallic acid (as standard phenolic compound) by preparation of serial dilutions ranging from 5 - 75 µg/ml. The results (absorbance values) were plotted versus corresponding gallic acid concentrations where the calibration curve and regression equation were obtained.

Methods

Determination of Total Flavonoid Content by $AlCl_3$ Colorimetric Method

The aluminum chloride colorimetric method was applied according to (Marinova et al., 2005), with slight modifications. Briefly, An aliquote (1 ml) of extracts or standard solution of rutin (5- 150 µg/ml) was mixed with 2 ml of distilled water and 0.3 ml of 5% $NaNO_2$. Five minutes later, 0.3 ml of 5% $AlCl_3$ was added. After another minute, 1 ml of 1 M NaOH was added and the total volume was made up to 5 ml with distilled water. The absorbance at 510 nm was recorded using spectrophotometer (UV/Vis, model 6105, Jenway LTD England). Total flavonoid content was expressed as mg rutin equivalents (RE) per gram dry plant sample using an equation obtained from standard calibration curve of rutin concentrations. The determination of total flavonoid contents in the samples was carried out in triplicate and the results were averaged.

Determination of total polyphenolic content by Folin–Ciocalteu reagent

The level of total polyphenolic contents in each sample was determined by using Folin–Ciocalteu reagent and standard calibration curve obtained by different concentrations of gallic acid according to **(Singelton et al., 1999)**. Briefly; 1 mL of standard or sample solution and 1ml deionized H_2O were mixed, 1.0 ml of Folin–Ciocalteu reagent (10 fold diluted) was added and the contents mixed thoroughly, after 4 min, 1 ml of 10% Na_2CO_3 was added, and then the mixture was allowed to stand for 90 minutes at room temperature. The absorbance was measured at 750 nm using a spectrophotometer (UV/Vis, model 6105, Jenway LTD England). The concentration of the total phenolics was calculated as mg of gallic acid equivalent (GAE) using an equation obtained from gallic acid calibration curve. The determination of total polyphenolic contents in the samples was carried out in triplicate and the results were averaged.

HPLC/ESI-MS analysis.

LC/ESI-MS was carried out using a Finnigan LC QDECA mass spectrometer connected to a UV detector. This hyphenated technique gives a good information (molecular ions, main fragment ions, and characteristic UV absorption maxima) about the individual compounds. The samples were dissolved in methanol and injected into HPLC/ESI-MS set-up. HPLC was run on a Nucleosil C-18 reversed-phase column. The results of LC/ESI-MS analysis were displayed in Table 2.

RESULTS

Total flavonoid contents

The results of TFC Experiments for all *Tilia* samples which obtained from two methods of extraction were shown in (Table 1). TFC for each sample was calculated as mg rutin equivalent (mg RE) using the regression equation Y= 384.19 X-0.4774 obtained from the standard calibration curve, where Y is the calculated flavonoid content for each sample, X is the corresponding absorbance.

Total polyphenolic contents

The results of TPC Experiments for all *Tilia* samples which obtained from two methods of extraction were shown in (Table 1). TPC for each sample was calculated as mg gallic acid equivalent (mg GAE) using the equation Y= 43.292x - 2.3474 obtained from the standard calibration curve, where Y is the calculated polyphenolic content for each sample, X is the corresponding absorbance.

Results of HPLC/ESI-MS analyses

The LC/ESI-MS chromatogram of each sample (Suppl. 1), showing both positive and negative ion fragments, explain a specific LC/MS profile of each sample. It was very obvious that some compounds are identical in all samples but differs in the quantity (based on the area under the peak), while some others are not identical. The results of HPLC/ESI-MS analyses are found in (Table 2).

Table 1: Results of TFC and TPC determination of all samples using two methods of extraction

Tilia sample	Method A	Method B	Method A	Method B
	TFC, mg RE / g		TPC, mg GAE / g	
TF_{Eg}	32.94486	25.56841	35.05689	35.31664
TTL	29.48715	23.95481	30.16489	28.77955
TPL	38.24668	32.71435	50.1225	44.711
TCL	35.48051	31.48494	47.48169	37.87087
TTF	48.54297	41.78123	70.08012	48.39082
TCF	44.16321	37.70882	52.02735	42.5464
TPF	57.22567	40.55182	67.69906	52.41698

Table 2 HPLC-DAD-ESI-MS/MS results of different samples of *Tilia* sp. A, Aglycone; coum, coumaroyl; glc, glucosyl; K, kaempferol; min, minutes; M, molecular ion; Occ., Occurrence; Q, quercetin; Ref conf., reference confirmation; Rt, Retention time; rha, rhamnosyl; TF_{Eg}, Egyptian sample; TTL, *T. tomentosa* leaves; TPL, *T. platyphyllos leaves*; TCL, *T. cordata* leaves; TTF, *T. tomentosa* flos; TCF, *T. cordata* flos; TPL, *T.platyphyllos* flos

No.	Rt (min)	Negative ion mode	Positive ion mode	Identification	Occ.	Ref conf.
1	7.13	577 $(M-288-H)^-$, 695 $(M-H-170)^-$, 713 $(M-H-152)^-$, 739 $(M-H-126)^-$, 865 $(M-H)^-$	291, 579 $(M-288+H)^+$, 697 $(M-170+H)^+$, 715 $(M+H-152)^+$, 867 $(M+H)^+$	Procyanidin-trimer I	TTL, TCL	18
2	7.34	445 $(M-H)^-$, 491 $(M+HCOO)^-$	910$(2M+H_2O)^+$, 464 $(M+H_2O)^+$, 285 $(M-glc+H)^+$, 325 $(M-glycone+H)^+$, 123 $(A+H)^+$	2-phenylethyl-O-β-gentobioside	TTF	18
3	7.43	353 $(M-H)^-$, 294, 191$(M-glc-H)^-$, 179(glucose $-H)^-$	355 $(M+H)^+$, 163$(glc+H)^+$, 145$(M-(glc+OCH_2O)+H)^+$	3,4-(methylenedioxy)cinnamoyl glucoside	TPL	
4	8.26	289$(M-288-H)^-$, 425$(M-H-152)^-$, 577 $(M-H)^-$	291,427$(M+H-152)^+$, 579$(M+H)^+$	Procyanidin-dimer I	TTL,TTF	18
5	8.90	289$(M-288-H)^-$, 425$(M-H-152)^-$, 577 $(M-H)^-$	291,427$(M+H-152)^+$, 579$(M+H)^+$	Procyanidin-dimer II	TF_{Eg}, TTL, TPL, TCL, TTF	18
6	9.6	179, 205, 245, 289 $(M-H)^-$	123, 139, 145, 165, 291 $(M+H)^+$	Catechin	TF_{Eg}, TTL, TCL, TTF	18
7	9.82	593 $(M-288-H)^-$, 881 $(M-H)^-$	595 $(M-288+H)^+$, 883 $(M+H)^+$	Prodelphinidin trimer (gallocatechin-catechin-catechin isomer)	TTL	18
8	9.93		191 $(dihydrosinapoyl-H_2O+H)^+$, 209 $(dihydrosinapoyl)^+$, 227$(M-glc+H)^+$, 327$(M-(OCH_3)_2+H)^+$, 357$(M-OCH_3+H)^+$, 389$(M+H)^+$	Dihydrosinapoyl glucoside	TPL	
9	10.54	463$(M-H-glc)^-$, 478$(M-H-rha)^-$, 625$(M-H)^-$	319(aglycone $+H)^+$, 465$(M+H-glc)^+$, 480$(M+H-rha)^+$, 627$(M+H)^+$	Myricetin-3-O-glucoside7-O-rhamnoside	TTL	
10	10.65	577 $(M-288-H)^-$, 695 $(M-H-170)^-$, 713 $(M-H-152)^-$, 739 $(M-H-126)^-$, 865 $(M-H)^-$	291, 579 $(M-288+H)^+$, 697 $(M-170+H)^+$, 715 $(M+H-152)^+$, 867 $(M+H)^+$	Procyanidin-trimer II	TF_{Eg}, TTL, TPL, TCL, TTF	18
11	11.13	577 $(M-288-H)^-$, 695 $(M-H-170)^-$, 713 $(M-H-152)^-$, 739 $(M-H-126)^-$, 865 $(M-H)^-$	291, 579 $(M-288+H)^+$, 697 $(M-170+H)^+$, 715 $(M+H-152)^+$, 867 $(M+H)^+$	Procyanidin-trimer III	TCL	18

12	**11.7**	**301 $(A-H)^-$, 447 $(M-glc-H)^-$, 463 $(M-rha)^-$, 609 $(M-H)^-$**	**303 $(A+H)^+$, 449 $(M-glc+H)^+$, 465 $(M+H-rha)^+$, 611 $(M+H)^+$**	**Q-3-O-glucoside-7-O-rhamnoside**	**TF_{Eg}, TTL, TPL, TCL, TTF**	**18**
13	**11.95**	**317$(M-H-glc)^-$, 479$(M-H)^-$**	**319(aglycone $+H)^+$, 481$(M+H)^+$**	**Myricetin-3-O-glucoside**	**TTL**	
14	**12.09**	**577 $(M-288-H)^-$,695 $(M-H-170)^-$, 713 $(M-H-152)^-$, 739 $(M-H-126)^-$, 865 $(M-H)^-$**	**291, 579 $(M-288+H)^+$, 697 $(M-170+H)^+$, 715 $(M+H-152)^+$, 867 $(M+H)^+$**	**Procyanidin-trimer IV**	**TTF**	**18**
15	**12.17**	**301 $(A-H)^-$, 433 $(M-rha-H)^-$, 447 $(M-pentosyl)^-$, 579 $(M-H)^-$**	**303 $(A+H)^+$, 449 $(M-pentosyl+H)^+$, 435 $(M+H-rha)^+$, 581 $(M+H)^+$**	**Q-3-O-rhamnoside-7-O-pentoside**	**TPL**	
16	**12.35**	**285 $(A-H)^-$, 431 $(M-glc)^-$, 447 $(M-rha)^-$, 593 $(M-H)^-$**	**287 $(A+H)^+$, 433 $(M-glc+H)^+$, 595 $(M+H)^+$**	**K-3-O-glucoside-7-O-rhamnoside**	**TF_{Eg}, TTL, TPL, TTF**	**18**
17	**12.73**	**301 $(A-H)^-$, 447 $(M-rha)^-$, 593 $(M-H)^-$**	**303 $(A+H)^+$, 449 $(M-rha+H)^+$, 595 $(M+H)^+$**	**Q-3,7- O- di-rhamnoside**	**TPL**	**18, 35**
18	**12.84**	**577 $(M-288-H)^-$, 695 $(M-H-170)^-$, 713 $(M-H-152)^-$, 739 $(M-H-126)^-$, 865 $(M-H)^-$**	**291, 579 $(M-288+H)^+$, 697 $(M-170+H)^+$, 715 $(M+H-152)^+$, 867 $(M+H)^+$**	**Procyanidin-trimer V**	**TCL, TTF**	**18**
19	**13.07**		**287 $(A+H)^+$, 433$(M-pentosyl+H)^+$, 419 $(M+H-rha)^+$, 565 $(M+H)^+$**	**K-3-O-rhamnoside7-O-pentoside**	**TPL**	
20	**13.44**	**301 $(M-glc-H)^-$, 463 $(M-H)^-$**	**303 $(A+H)^+$, 465 $(M+H)^+$**	**Q-3-O-glucoside (isoquercitrin)**	**TF_{Eg}, TTL, TPL, TCL, TTF**	**18, 35**
21	**13.91**	**285 $(A-H)^-$, 431 $(M-rha-H)^-$, 577 $(M-H)^-$**	**287 $(A+H)^+$, 433 $(M-rha+H)^+$, 579 (M+H)+**	**K-3,7-O-di-rhamnoside**	**TF_{Eg}, TTL, TPL**	**18, 35**
22	**14.39**	**301 $(A-H)^-$, 433 $(M-H^-$**	**303 $(A+H)^+$, 435 $(M+H)^+$**	**Q-3-O-pentoside**	**TF_{Eg}, TPL, TCL**	
23	**14.65**	**285 $(A-H)^-$, 447 $(M-H)^-$**	**287 $(A+H)^+$, 449 $(M+H)^+$**	**K-3-O-glucoside (astragalin)**	**TF_{Eg}, TTL, TPL, TTF**	**18, 35**
24	**14.79**	**301 $(A-H)^-$, 447 $(M-H)^-$**	**303 $(A+H)^+$, 449 $(M+H)^+$**	**Q-3-O-rhamnoside (quercitrin)**	**TF_{Eg}, TTL, TPL, TTF**	**18, 35**
25	**14. 97**	**301 $(A-H)^-$, 477 $(M-H)^-$**	**303 $(A+H)^+$, 479 $(M+H)^+$**	**Q-3-O-glucouronide**	**TCL**	
26	**15.48**	**285 $(A-H)^-$, 417 $(M-H)^-$**	**287$(A+H)^+$, 419 $(M+H)^+$**	**K-3-O-pentoside**	**TPL**	
27	**16.55**	**285 $(A-H)^-$, 431 $(M-H)^-$**	**287 $(A+H)^+$, 433 $(M+H)^+$**	**K-3-O-rhamnoside**	**TF_{Eg}, TPL, TTF**	**18**
28	**17.24**	**269 $(A-CH_3)^-$, 283$(A-H)^-$, 591 $(M-H)^-$, 637 (M+ HCOOH$-H)^-$**	**285 $(A+H)^+$, 447 $(M-rha+H)^+$, 593$(M+H)^+$**	**4'-O- methylapigenin- 7-O-rutinoside**	**TCL**	
29	**17.36**	**285 $(A-H)^-$, 447 $(M-glc-H)^-$, 593 $(M-H)^-$**	**147 (p-Coum)$^+$, 287 $(A+H)^+$, 595 $(M+H)^+$, 617 $(M+Na)^+$**	**trans -tiliroside**	**TF_{Eg}, TTL, TPL, TCL, TTF**	**18, 35**
30	**19.25**	**285 $(A-H)^-$, 447 $(M-glc-H)^-$, 593 $(M-H)^-$**	**147 (p-Coum)$^+$, 287 $(A+H)^+$, 595 $(M+H)^+$, 617 $(M+Na)^+$**	**cis -tiliroside**	**TF_{Eg}, TTL, TPL, TCL, TTF**	**18, 35**

4. DISCUSSION

Tilia flos is an important herbal remedy which is used extensively in folk and modern medicine. Due to the great similarity in the anatomical and chemical properties of many *Tilia* species, many of these species are used for the same purposes and may be interchanged. The samples of the same species from different geographical areas are often varied especially in the type and / or the amount of active principles which are responsible for their biological activities. It is well known that, the active constituents of *Tilia* drug are composed of phenolic compounds, mainly flavonoid constituents, and the volatile constituents which impart the agreeable aroma of the drug (Toker et al., 2001, Wagner et al., 198, Piettta et al., 1993, Karioti et al., 2014). The Egyptian country is not native origin for *Tilia* plants and the available *Tilia* drugs in Egypt are mostly imported from abroad, namely from Asia, Europe or America. According to method A of extraction, TPF contain the highest percentage of TFC (5.72 % w/w) and the second highest percentage of TPC (6.77 % w/w), and TTF sample contain the second highest percentage of TFC (4.85 % w/w) and the highest percentage of TPC (7.01 % w/w). In contrast, according to method B of extraction which is the most suitable method of administration for the Egyptian people, TTF contain the highest percentage of TFC (4.18 % w/w) and the second highest percentage of TPC (4.84 % w/w), and TPF sample contain the second highest percentage of TFC (4.06 % w/w) and the highest percentage of TPC (5.24 % w/w). HPLC-DAD-ESI-MS analysis gave additional information about the major flavonoids and phenolic compounds in each sample which act as finger print for each drug. RP-HPLC- ESI-MS analysis is accepted and greatly applied method for on-line analysis of the flavonoid constituents (Toker et al., 2001, Karioti et al., 2014, Abdel-Hameed et al., 2014, Keinänen and Julkunen-Tiitto, 1998, Rodríguez–Rivera et al., 2014). ESI-MS positive ($ESI\text{-}MS^+$) and/or negative mode ($ESI\text{-}MS^-$) shows pseudomolecular ions of the compound, where ($ESI\text{-}MS^+$) shows $(M+H)^+$, $(M+Na)^+$, and /or $(M+H_2O)^+$, while ($ESI\text{-}MS^-$) displays peaks corresponding to $(M-H)^-$ and/or $(M+HCOOH-H)^-$. Furthermore daughter fragments of those precursor ions can be detected through $ESI\text{-}MS/MS^+$ and $ESI\text{-}MS/MS^-$ spectra. Retro Diels-Alder (RDA) fragmentations (Fig. 2) gave an additional information about the substructures or substitutions on Ring A, B, and C of the main skeleton (Tsimogiannis et al., 2007). Application of this LC- MS technique in the present work results in identification of thirty compounds, ten compounds of which are reported here for the first time from *Tilia* species.

The identification of the compounds was based on retention times, ESI/MS analysis and UV spectra where the compounds were classified

into 4 chemical classes according to their UV spectra into:

Group 1: (phenyl propionic acid derivatives): includes different cinnamic acid derivatives which show main absorption maxima at the range of 300 - 326 nm (Fig. 3 A).

Group 2: (catechin derivatives): includes catechin, procyanidin dimers and trimers, showing UV maxima at 280 nm (Fig. 3 B)

Group 3: (flavonol glycosides attached to cinnamic acid derivatives): includes cis tiliroside (kaempferol 3-O-β-D-(6-O-cis-*p*-coumaroyl) glucopyranoside) and trans tiliroside (kaempferol 3-O-β-D-(6-O-trans-*p*-coumaroyl) glucopyranoside) which show three absorption maxima around 234, 268 and characteristic maxima around 314 nm (Fig. 3 C).

Group 4: (flavonol glycosides): includes different kaempferol, Myricetin and quercetin glycosides which show three absorption maxima around 224, 266 and 350 nm (Fig. 3 D).

The compounds were characterized based on their mass spectra, using the precursor ion, fragment ions, and comparison of the fragmentation patterns with molecules described in the literatures. The putative identification of these compounds is discussed below and summarized in (Table 2) where the compounds are numbered according to their retention times in the total ion chromatograms (suppl. 1).

According to on-line HPLC-DAD-ESI-MS analyses thirty compounds were identified, from which, twenty compounds were identified by their comparison with those reported previously by (Toker et al., 2001, Karioti et al., 2014, Negri et al., 2013) from *Tilia* species collected from Turki, Syria, Italy and Brazil. The comparison was based on the relative retention times, UV absorption maxima, +ve and/or –ve ESI/MS and their characteristic fragments (suppl. 2). The rest of these compounds, ten compounds **(3, 8, 9, 13, 15, 19, 22, 25, 26** and **28)** are tentatively identified and reported here for the first time from *Tilia* species:

3',4'- methylenedioxy cinnamoyl glucose, **(Comp 3),** showed a typical UV spectrum (Fig. 3 A) of hydroxycinnamic acids λ_{max} at 326 nm (Karioti et al., 2014), ESI/MS$^+$ spectrum showed pseudomolecular ion peak at m/z 355 $(M+H)^+$, and its characteristic fragment ions at m/z 163 $(glc+H)^+$, 145 $(M-(glc+OCH_2O)+H)^+$, while ESI/MS$^-$ shows pseudomolecular ion peak at m/z 353 $(M-H)^-$, 294, 191 $(M-glc-H)^-$, 179 $(glucose\ -H)^-$. Dihydrosinapoyl glucose **(Comp 8)** showed ESI/MS$^+$ pseudomolecular ion peak at m/z 389 $(M+H)^+$, 2 mass units more than the previously detected sinapoyl glucose in *T. platyphylos* flos by Karioti *et al* 2014 (Karioti et al., 2014) and its characteristic ion fragments at m/z 163 $(glc+H)^+$, 191 $(dihydrosinapoyl-H_2O+H)^+$, 209 $(dihydrosinapoyl)^+$, 227 $(M-glc+H)^+$, 327

$(M-(OCH_3)_2+H)^+$, 357 $(M-OCH_3+H)^+$. Myricetin-3-O-glucoside-7-O-rhamnoside, **(Comp 9)** shows a typical UV spectrum of flavonol derivatives λ_{max} at 256 and 356 nm. (Pinheiro and Justino, 2002, Wollenweber, 1982) ESI/MS^+ spectrum showed pseudomolecular ion peak at m/z 627 $(M+H)^+$, and its characteristic fragment ions at m/z 319 (aglycone $+H)^+$, 465 $(M+H-glc)^+$, 480 $(M+H-rha)^+$, while ESI/MS^- spectrum showed pseudomolecular ion peak at m/z 625 $(M-H)^-$ and its characteristic fragment ions at m/z 463 $(M-H-glc)^-$, 478 $(M-H-rha)^-$, in addition to characteristic RDA fragment $^{0,4}B^+$ at m/z 373, $^{0,3}B^-$ at 343. Myricetin-3-O-glucoside, **(Comp 13)** shows a maximum absorption at 266 and 356 nm. ESI/MS^+ spectrum showed pseudomolecular ion peak at m/z 481 $(M+H)^+$, and its characteristic fragment ion at m/z 319(aglycone $+H)^+$, in addition to characteristic RDA fragment $^{0,4}B^+$ at m/z 209, while ESI/MS^- spectrum showed pseudomolecular ion peak at m/z 479 $(M-H)^-$ and its characteristic fragment ions at m/z 317 $(M-H-glc)^-$, 179 $(glucose-H)^-$ and RDA fragment $^{1,3}A^-$ at 151. Quercetin-3-O-rhamnoside-7-O-pentoside, **(Comp 15)** shows λ_{max} at 264 and 350 nm. ESI/MS^+ spectrum showed pseudomolecular ion peak at m/z 581 $(M+H)^+$, and fragment ions at m/z 303 $(A+H)^+$, 449 $(M-pentosyl+H)^+$, 435 $(M+H-rha)^+$, in addition to characteristic RDA fragment $^{0,3}A^+$ at m/z 269 including the pentosyl moiety , and $^{0,4}B^+$ at m/z 340 including the rhamnosyl moiety, while ESI/MS^- spectrum showed pseudomolecular ion peak at m/z 579 $(M-H)^-$ and its characteristic fragment ions at m/z 301 $(A-H)^-$, 433 $(M-Rha-H)^-$, 447 $(M-pentosyl)^-$. Kaempferol-3-O-rhamnoside-7-O-pentoside, (C**omp 19)** shows λ_{max} at 266 and 348 nm. ESI/MS^+ spectrum showed pseudomolecular ion peak at m/z 565 $(M+H)^+$, and fragment ions at m/z 287 $(A+H)^+$, 433$(M-pentosyl+H)^+$, 419 $(M+H-rha)^+$. Quercetin-3-O-pentoside, **(Comp 22)** shows absorption maxima at 266 and 350 nm. ESI/MS^+ spectrum showed pseudomolecular ion peak at m/z 435 $(M+H)^+$, and fragment ions at m/z 303 $(A+H)^+$. ESI/MS^- spectrum showed pseudomolecular ion peak at m/z 433 $(M-H)^-$, and fragment ions at m/z 301 $(A-H)^-$, in addition to characteristic RDA fragment $^{0,4}B^-$ at 325 including a pentosyl moiety. Quercetin-3-O-glucouronide **(Comp 25)** shows absorption maxima at 256 and 350 nm. ESI/MS^+ spectrum showed pseudomolecular ion peak at m/z 479 $(M+H)^+$, and fragment ion at m/z 303 $(A+H)^+$. ESI/MS^- spectrum showed pseudomolecular ion peak at m/z 477 $(M-H)^-$, and fragment ions at m/z 301 $(A-H)^-$ by loss of 176 mass unit corresponding to glucuronyl moiety (Prasain and Barnes, 2007). ESI/MS^+ spectrum of Kaempferol-3-O-pentoside **(Comp 26)** displayed pseudomolecular ion peak at m/z 419 $(M+H)^+$, and fragment ion at m/z 287 $(A+H)^+$. While ESI/MS^- spectrum showed pseudomolecular ion peak at m/z 417 $(M-H)^-$,

and fragment ions at m/z 285 $(A-H)^-$. UV absorption maxima of 4'-O- methylapigenin- 7-O-rutinoside, **(Comp 28)** were found at 230, 270, and 332 nm, typical for Flavone derivatives, (Wollenweber, 1982, Mabry et al., 1970) ESI/MS^+ spectrum displayed pseudomolecular ion peak at m/z 593 $(M+H)^+$, and fragment ions at m/z 285 $(A+H)^+$, 447 $(M-rha+H)^+$, ESI/MS^- spectrum showed pseudomolecular ion peak at m/z **637** $(M+HCOOH-H)^-$ and fragment ions at m/z 591 $(A-H)^-$, 269 $(A-CH_3)^-$ and 283 $(A-H)^-$.

CONCLUSIONS

Considering the results of TFC and TPC and the number of identified compounds, the present study confirmed the lower quality of the Egyptian sample compared with other samples of *Tilia* flos (Fig. 4). The leaf samples contain many compounds similar to those in flower samples but each sample (leaves or flowers) possesses a unique chemical profile including the percentage and type of flavonoid constituents. In spite of the aroma of volatile constituents of the flowers, the leaves may be used alone or mixed with other aroma modifier as substitute for the *Tilia* flowers but this idea should be firstly estimated through a biological trials. The methods applied in this work were simple and time saving. The Results of the present study may be helpful in the practical authentication, quality assurance and prevention of adulteration of the crude *Tilia* drug specially for countries in which *Tilia* is usually not cultivated like Egypt.

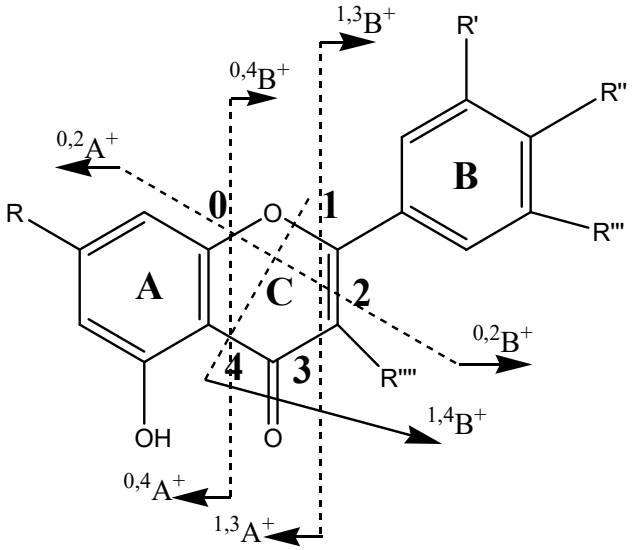

Figure 2: Possible RDA fragmentations of flavonoids, adopted from (Tsimogiannis *et al*; 2007) where A and B letters mean involvement of ring A or ring B respectively in the RDA fragment, the superscript numbers denote the site of fragmentation, and the sign – or + denotes the negative or positive mode of ionization.

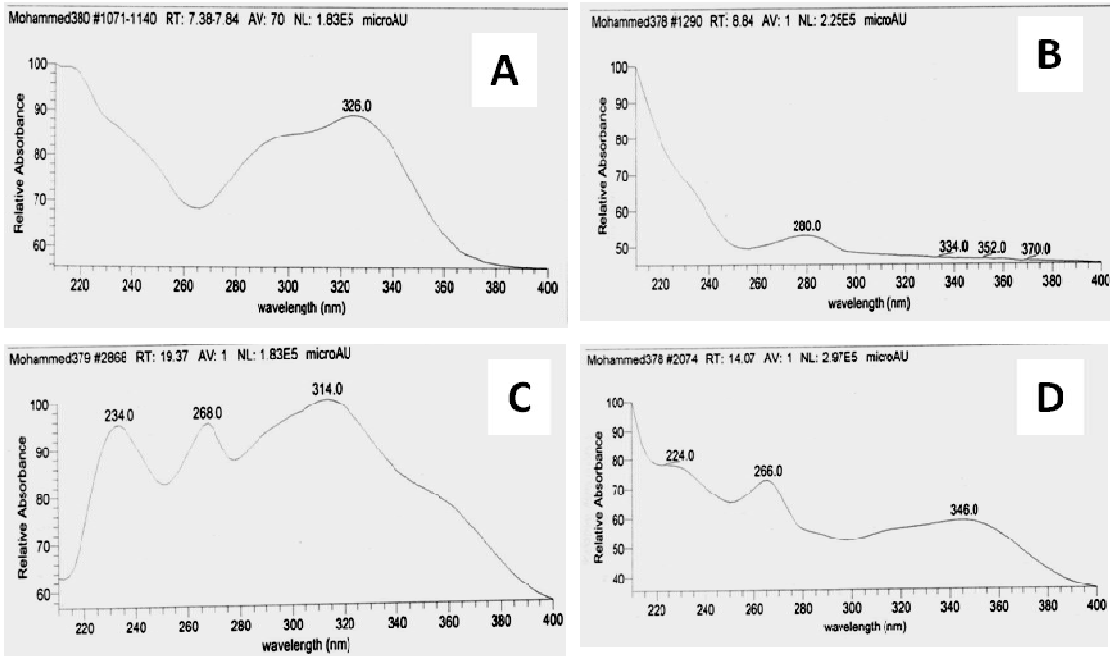

Figure 3: UV spectra of different chemical groups; (A) cinnamic acid derivatives; (B) procyanidin derivatives; (C) flavonol glycosides + cuomaric acids; (D) Flavonol glycosides

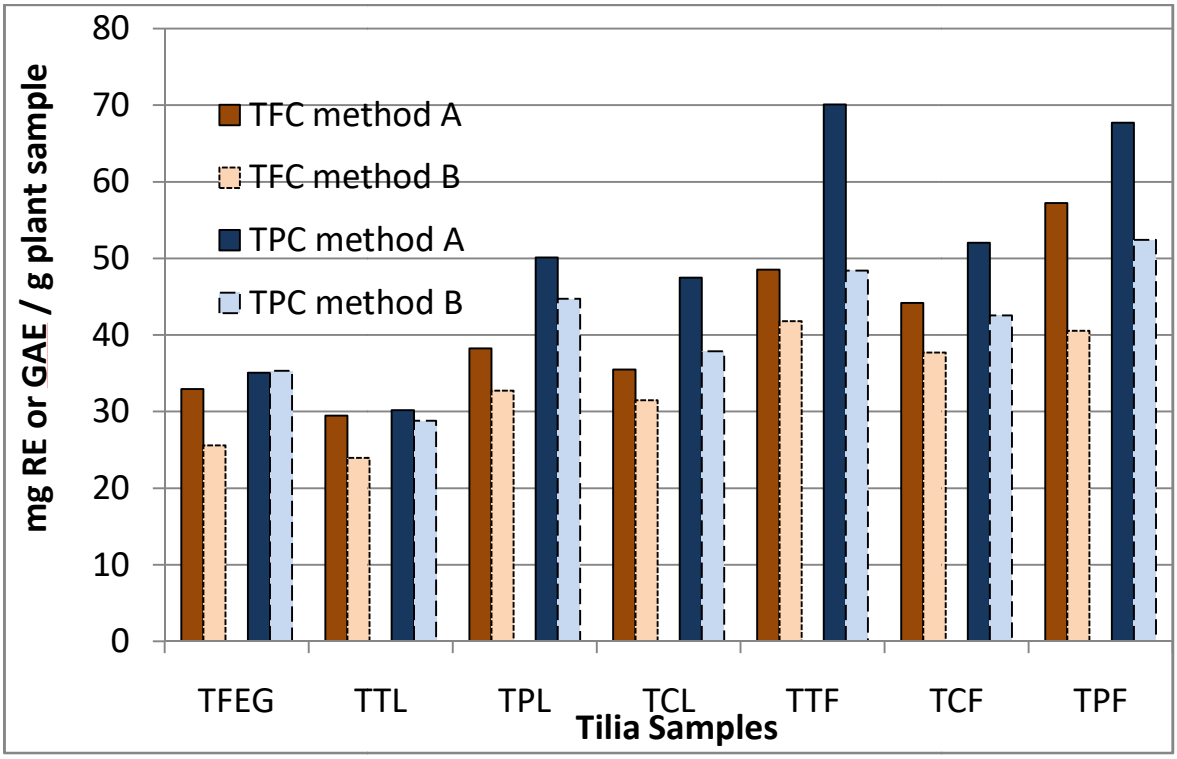

Figure 4: The results of TFC and TPC determination of all *Tilia* samples

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REFERENCES

[1] Abdel-Hameed, E-S.S.; Bazaid, S.A.; Shohayeb, M.M. RP-HPLC–UV–ESI-MS phytochemical analysis of fruits of *conocarpus erectus* L. *Chem. Papers* **2014**, 68, 1358-67.

[2] Aguirre-Hernàndez, E.; Gonzàlez-Trujano, M.E.; Martínez, A.L.; Moreno, J.; Kite, G.; Terrazas, T.; Soto-Hernández, M. HPLC/MS analysis and anxiolytic-like effect of quercetin and kaempferol flavonoids from *Tilia Americana* var. *Mexicana*. *J. Ethnopharmacol.* **2010**, 127, 91–97.

[3] Aguirre-Hernàndez, E.; Martínez, A.L.; Gonzàlez-Trujano, M.E.; Moreno, J.; Vibrans, H.; and Soto-Hernàndez, M. Pharmacological evaluation of the anxiolytic and sedative effects of *Tilia americana* L. var. *mexicana* in mice. *J. Ethnopharmacol.* **2007**,109, 140–145.

[4] Barnes, J.; Anderson, L.A.; Phillipson, J.D. *Herbal medicines*, 2nd ed; Pharmaceutical Press: London, United kingdom, 2007; pp. 409–410.

[5] Barreiro Arcos, M. L.; Cremaschi, G.; Werner, G.S.; Coussio, J.; Ferraro, G.; Anesini, C. *Tilia cordata* Mill extracts and scopoletin (isolated Compound): Differential cell growth effects on lymphocytes. *Phytother. Res*. **2006**, 20, 34-40.

[6] Bradley, P. *The British Herbal Compendium: v. 1: A Handbook of scientific information on widely used plant drugs*, British Herbal Medicine Association: Bournemouth, United kingdom, 1992; pp. 142–144.

[7] Brasseur, T. Flavonoids as medicines (Médicaments renfermant des flavonoïdes). *J. Pharm. Belg.* **1989**, 44, 403–10.

[8] Brizi, M.R.; Marrassini, C.; Zettler, G.; Ferraro, G.; Anesini, C. Comparative antiproliferative action of two extracts from *Tilia x viridis* on normal and tumoral lymphocytes: Relationship with antioxidant activity. *Chinese Medicine* **2012**, 3. 20-29.

[9] Cárdenas-Rodríguez, N.; González-Trujano, M. E.; Aguirre-Hernández, E.; Ruíz-García, M.; Sampieri III, A.; Coballase-Urrutia, E.; Carmona-Aparicio, L. Anticonvulsant and antioxidant effects of *Tilia americana* var. *mexicana* and flavonoids constituents in the pentylenetetrazole-induced seizures. *Oxid. Med. Cell. Longev.* **2014**, 2014, 1-10.

[10] Cheng, J.T.; Hsu, F.L.; Chen, H.F. Antihypertensive principles from the leaves of *Melastoma candidum*. *Planta Med.* **1993**, 59, 405– 7.

[11] Cotrim, M.D.; Figueiredo, I.V.; Cavadas, C.; Proençada Cunha, A.; Caramona,

M.M.; Macedo, T.R.A. Pharmacological properties of *Tilia Europeae* aqueous extract: screening anexiolytic/ sedative activity in mice. *ARQ. PAroL* **1999**, XXXI, 23-29.

[12] Eberhardt, T.L.; Young, R.A. Conifer seed cone proanthocyanidin polymers: characterization by ^{13}C NMR spectroscopy and determination of antifungal activities. *J. Agric. Food. Chem*. **1994**, 42, 1704 – 8.

[13] Goto, T.; Horita, M.; Nagai, H.; Nagatomo, A.; Nishida, N.; Matsuura, Y.; Nagaoka, S. Tiliroside, a glycosidic flavonoid, inhibits carbohydrate digestion and glucose absorption in the gastrointestinal tract, *Mol. Nutr. Food Res.* **2012**, 56, 435-445.

[14] Goto, T.; Teraminami, A.; Lee, J.Y.; Ohyama, K.; Funakoshi, K.; Kim, Y.I.; Hirai, S.; Uemura, T.; Yu, R.; Takahashi, N.; Kawada, T. Tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via activation of adiponectin signaling followed by enhancement of fatty acid oxidation in liver and skeletal muscle in obese-diabetic mice. *J. Nutr. Biochem*. **2012**, 7, 768-76.

[15] Haslam, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* **1996**, 59, 205–15.

[16] Invanov, P.; Loghin, C.; Enescu, C.M. Morphological differentiation between Romanian lime species (Tilia spp.) A case study. *Bulletin of the Transilvania University of Braşov • Series II*. **2014**, 7, 21-28.

[17] Kanda, T.; Akiyama, H.; Yanagida, A.; Tanabe, M.; Goda, Y.; Toyoda, M.; Teshima, R.; Saito, Y. Inhibitory effects of apple polyphenol on induced histamine release from RBL- 2H3 cells and rat mast cells. *Biosci. Biotechnol. Biochem* **1998**, 62, 1284 – 9.

[18] Karioti, A.; Chiarabini, L.; Alachkar, A.; Fawaz Chehna, M.; Vincieri, F.F.; Bilia, A.R. HPLC–DAD and HPLC–ESI-MS analyses of *Tiliae* flos and its preparations. *J. Pharm. Biomed. Anal*. **2014**, 100, 205–214.

[19] Keinänen, M.; Julkunen-Tiitto, R. High-performance liquid chromatographic determination of flavonoids in *Betula pendula* and *Betula pubescens* leaves. *J. Chromatogr. A* **1998**, 793, 370 –377.

[20] Mabry, T.J.; Markham, K.R.; Thomas, M.B. The systematic identification of flavonoids, Springer-Verlag: New York, United states, 1970; pp. 41-61.

[21] Maes, B. Linden trees in the Netherlands. *Gorteria* **1990**, 16, 61-81.

[22] Marinova, D.; Ribarova, F.; Atanassova, M. Total phenolics and total flavonoids in Bulgaria fruits and vegetables. *J.*

Univ. Chem. Technol. Metallurgy **2005**, 40, 255-260.

[23] Negri, G.; Santi, D.; Tabach, R. Flavonol glycosides found in hydroethanolic extracts from *Tilia cordata*, a species utilized as anxiolytics. *Rev. Bras. Pl. Med*. **2013**, 15, 217-224.

[24] Pèrez-Ortega, G.; Guevara-Fefer, P.; Chàvez, M.; Herrera, J.; Martínez, A.; Martínez, A.L.; González-Trujano, M.E. Sedative and anxiolytic efficacy of *Tilia americana* var. *mexicana* inflorescences used traditionally by communities of state of Michoacan, Mexico. *J. Ethnopharmacol*. **2008**, 116, 461– 468.

[25] Piettta, P.; Mauri, P.; Bruno, A.; Zini, L. High-performance liquid chromatography and micellar electrokinetic chromatography of flavonol glycosides from *Tilia*. *J. Chromatogr. A*. **1993**, 638, 357–361.

[26] Pinheiro, P. F.; Justino, G.C. Structural Analysis of Flavonoids and Related Compounds – A Review of Spectroscopic Applications. In *Phytochemicals – A Global Perspective of Their Role in Nutrition and Health*, 1st ed; Rao V. Ed.; InTech, Rijeka, Croatia, 2002; pp. 33-56.

[27] Prasain, J.K.; Barnes, S. Metabolism and bioavailability of flavonoids in chemoprevention: Current analytical strategies and future prospectus. *Mol. Pharm*. **2007**, 4, 846-864.

[28] Qiao, W.; Zhao, C.; Qin, N.; Zhai, H.Y.; Duan, H.Q. Identification of trans-tiliroside as active principle with anti-hyperglycemic, anti-hyperlipidemic and antioxidant effects from Potentilla chinesis. J. Ethnopharmacol. **2011**, 135, 515-21.

[29] Rajendra, K.C. Species differentiation in *Tilia*: A Genetic, Approach. Master of Science Thesis. Georg-August-University, Goettingen, Germany , January 2009.

[30] Rodríguez–Rivera, M.P.; Lugo-Cervantes, E.; Winterhalter, P.; Jerz, G. Metabolite profiling of polyphenols in peels of *Citrus limetta* Risso by combination of preparative high-speed countercurrent chromatography and LC–ESI–MS/MS. *Food Chem.* **2014**, 158, 139–152.

[31] Senghas, k.; Seybold, S. *Schmeil – Fitschen: Flora von Deutschland and angrenzender Laender*, 92nd ed; Quelle & Meyer verlag: wiebelsheim, Germany, 2003; pp. 401.

[32] Silva, G.C.; Pereira, A.C.; Rezende, B.A.; da Silva, J.P.; Cruz, J.S.; de Souza, Mde, F.; Gomes, R.A.; Teles, Y.C.; Cortes, S.F.; Lemos, V.S. *Mechanism of the antihypertensive and vasorelaxant effects of the flavonoid tiliroside in*

resistance arteries. Planta Med. **2013, 79**, 1003-8.

[33] Singelton, V.R.; Orthifer, R.; Lamuela – Raventos, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods Enzymol.* **1999**, 299, 152–178.

[34] Takahashi, T.; Kamimura, A.; Kagoura, M.; Toyoda, M.; Morohashi, M. Investigation of the topical application of procyanidin oligomers from apples to identify their potential use as a hair-growing agent. *J. Cosmet. Dermatol*. **2005**, 4, 245– 249.

[35] Toker, G.; Aslan, M.; Yeşilada, E.; Memişoğlu, M.; Ito, S. Comparative evaluation of the flavonoid content in officinal *Tiliae* flos and Turkish lime species for quality assessment. *J. Pharm. Biomed. Anal.* **2001**, 26, 111–121.

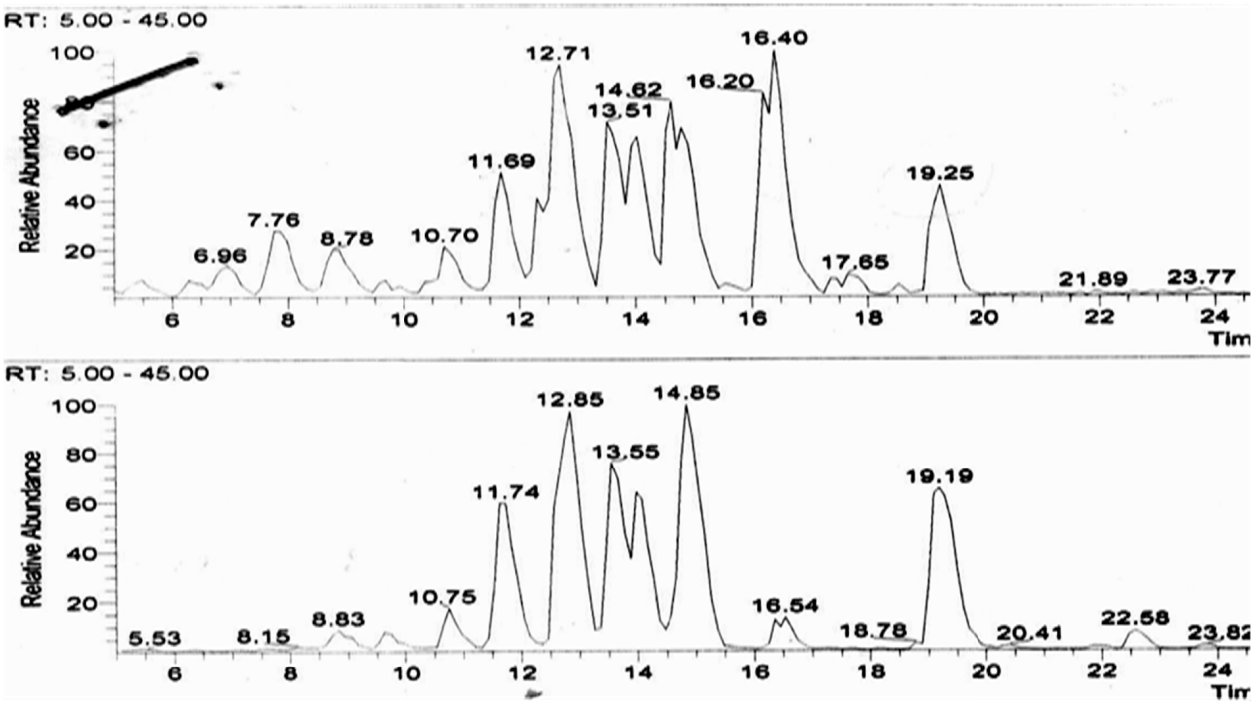
[36] Tsimogiannis, D.; Samiotaki, M.; Panayotou, G.; Oreopoulou, V. Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS. *Molecules* **2007**, 12, 593-606.

[37] Vennat, B.; Bos, M.A.; Pourrat, A.; Bastide, P. Procyanidins from tormentil: fractionation and study of the anti-radical activity towards superoxide anion. *Biol. Pharm. Bull*. **1994**, 17, 1613 – 5.

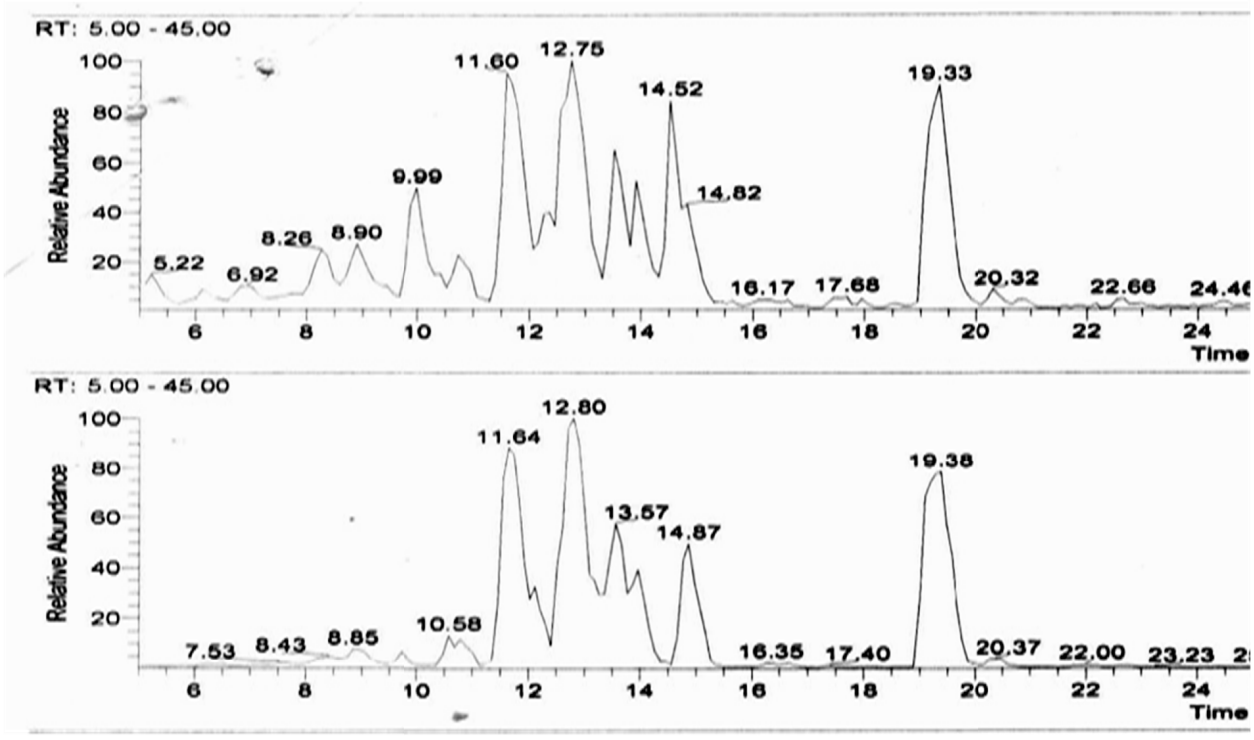
[38] Wagner, H.; Tittel, G.; Bladt, S. Analyse und Standar disierung von Arzneidrogen und Phytopraparaten durch hochleistungesflussige Chromatographie (HPLC) und andere chromatographische Verfahren. *Dtsch. Apoth. Ztg*. **1983**, 12, 515–521.

[39] Wollenweber, E. Flavones and Flavonols, In: Harborne, J.B.; Mabry, T.J. (Eds.), The flavonoids, Advances in research, Chapman &Hall, London, United kingdom, 1982; pp. 189–259.

Supplementary Data 1



Suppl. 1-1 Total ion chromatogram, +ve mode (up) and –ve mode (down) of TF_{Eg}



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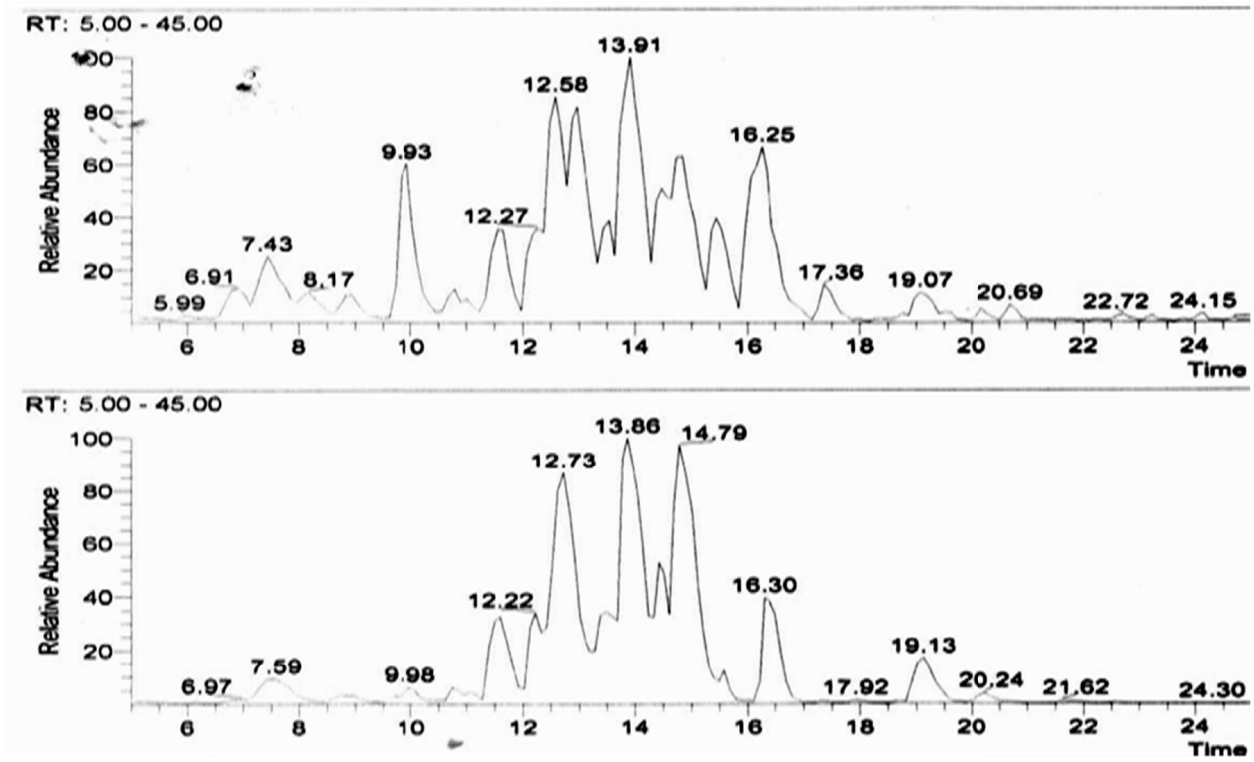

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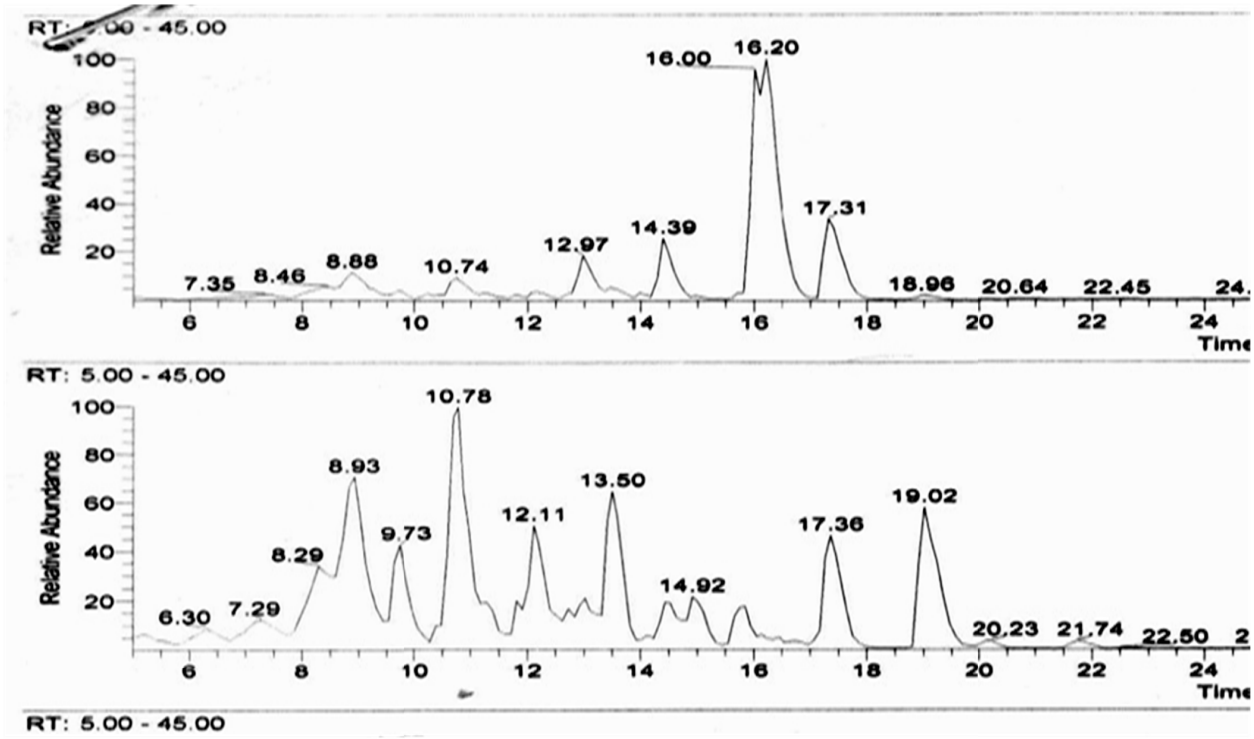
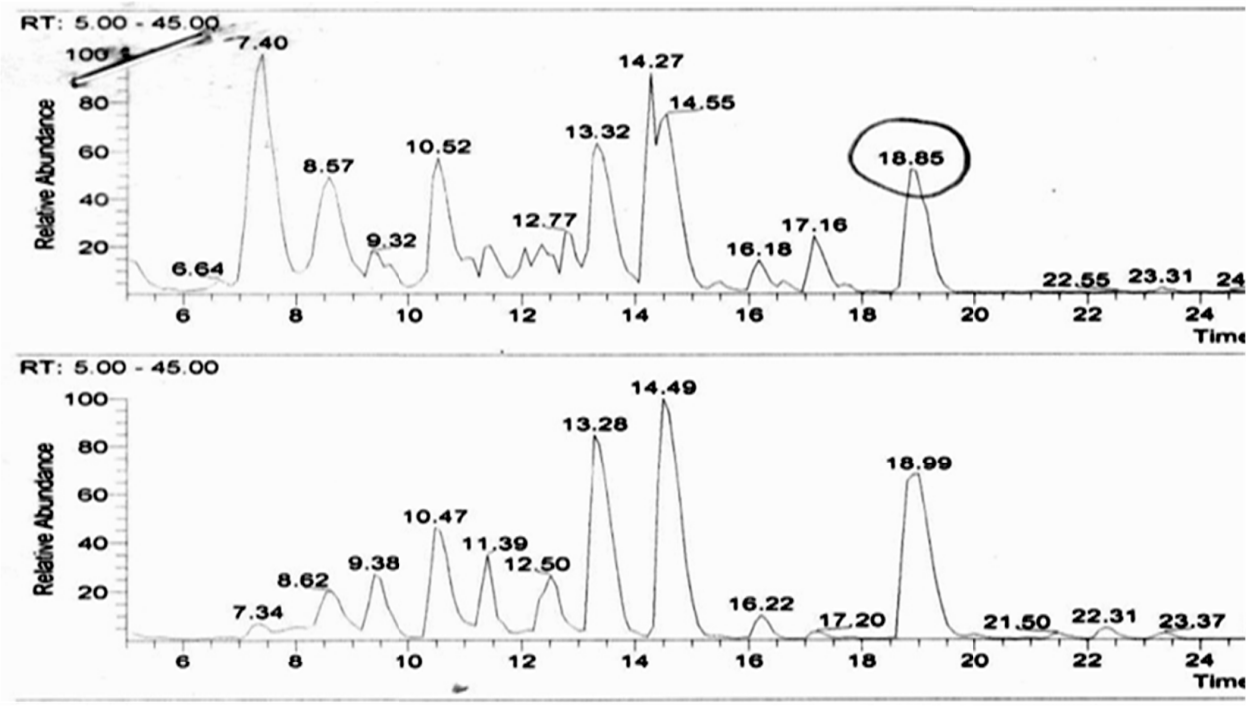
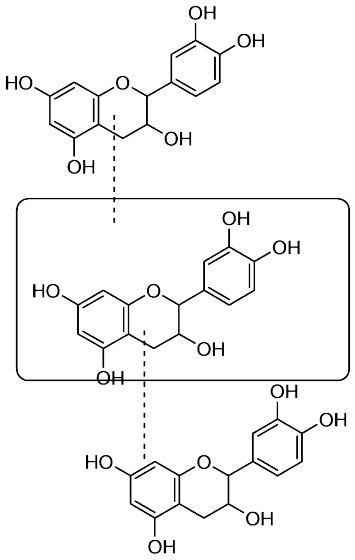


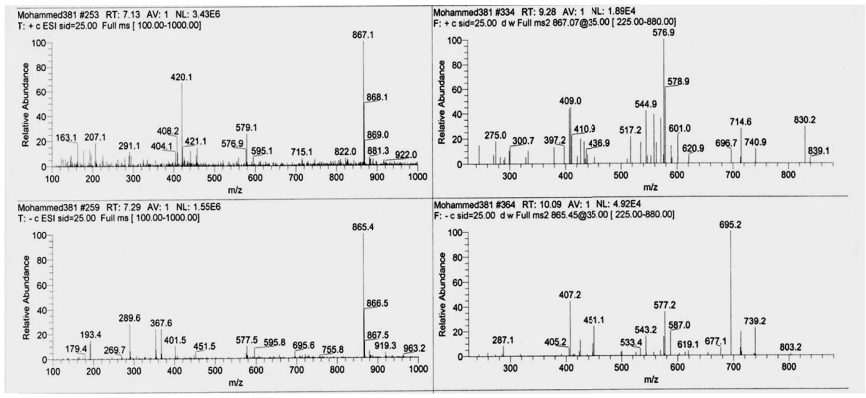
Suppl. 1-4 Total ion chromatogram, +ve mode (up) and –ve mode (down) of TCL



Suppl. 1-5 Total ion chromatogram, +ve mode (up) and –ve mode (down) of TTF

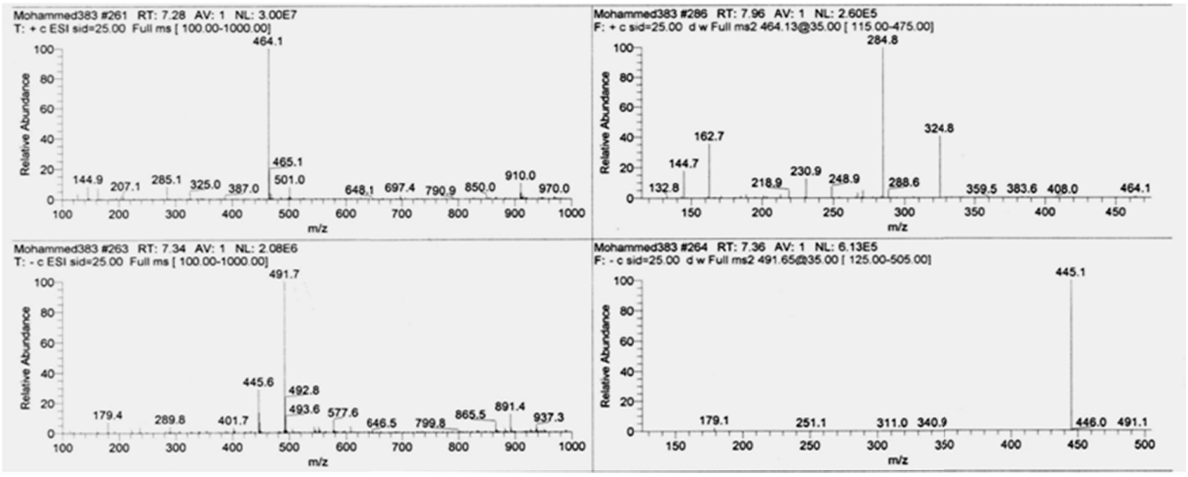
Supplementary Data 2

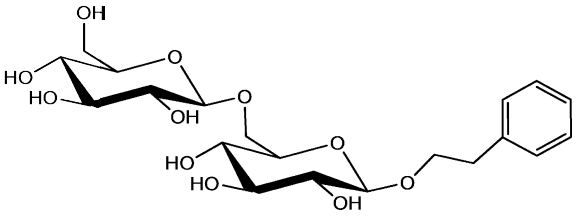




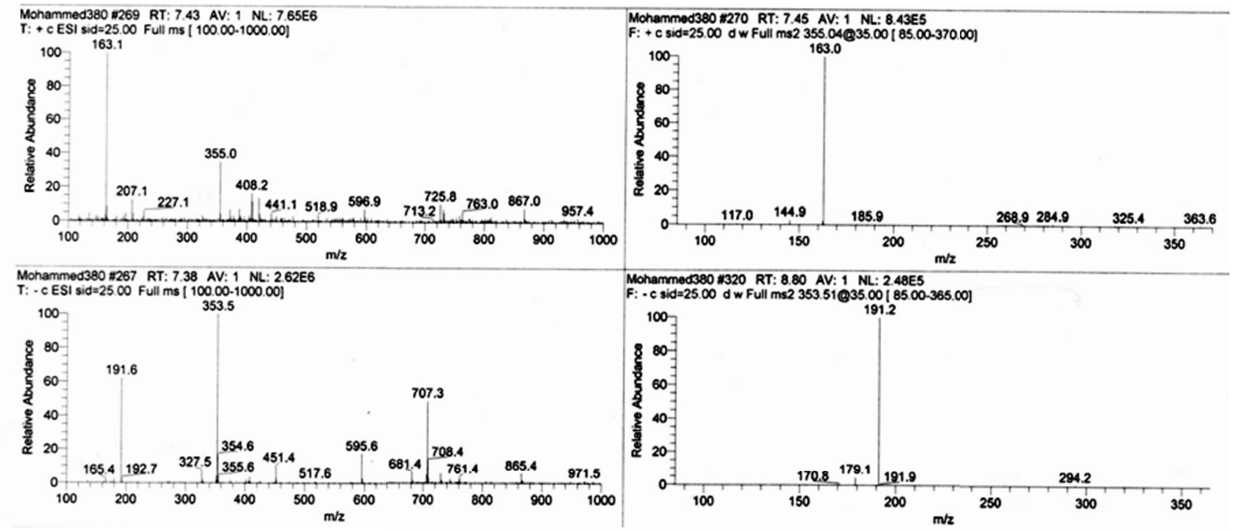
Procyanidin trimer 1 MW. 866 g/mol

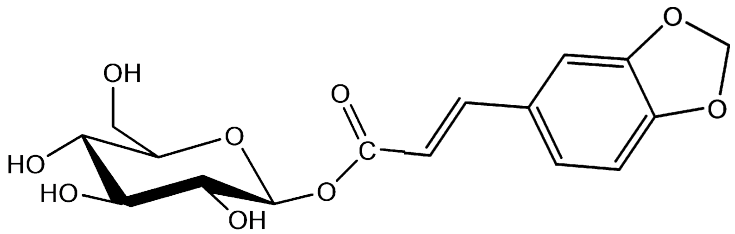
Supplementary Data 2



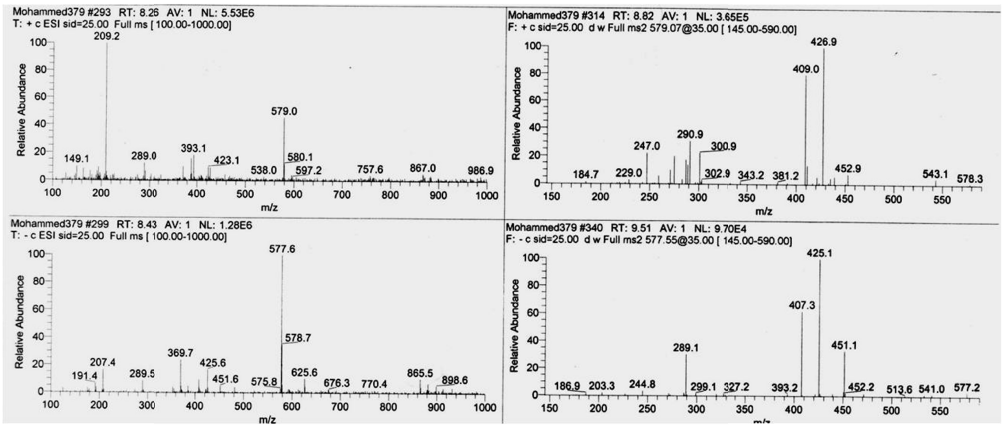


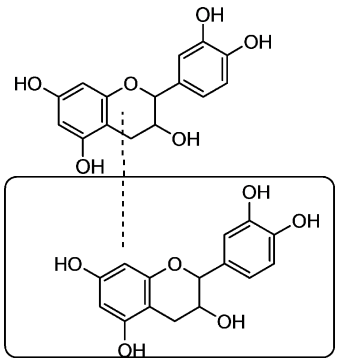
2-phenylethyl-O- β-gentobioside 2 MW. 446 g/mol



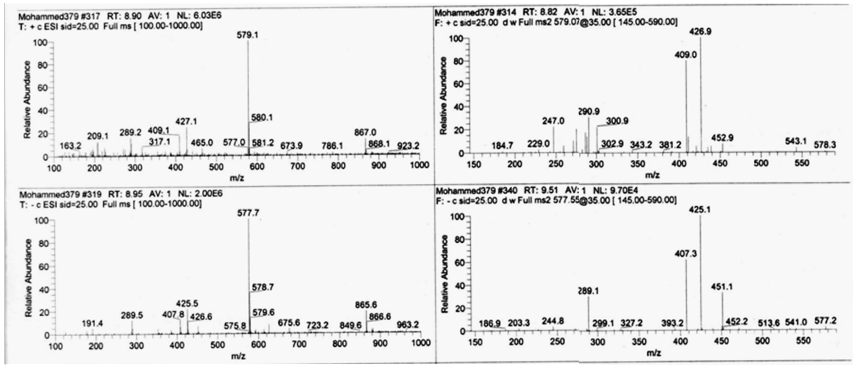


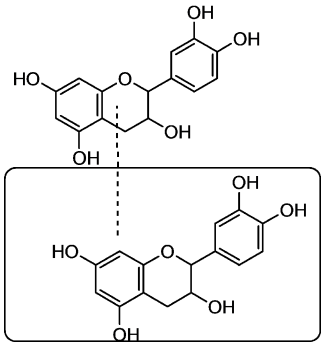
3,4-(methylenedioxy)cinnamoyl glucose 3 MW. 354 g/mol



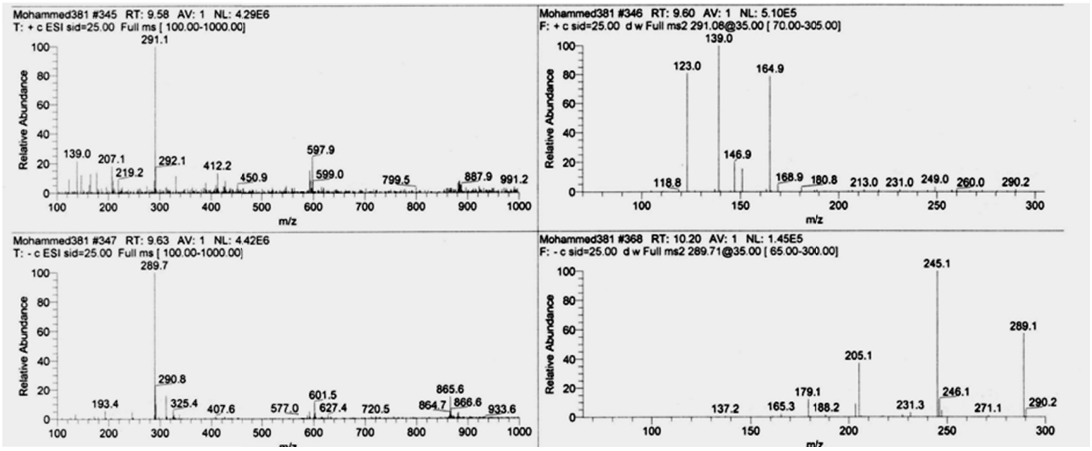


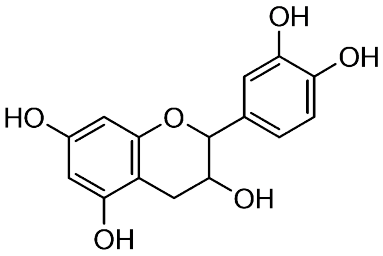
Procyanidin-dimer I 4 MW. 578 g/mol



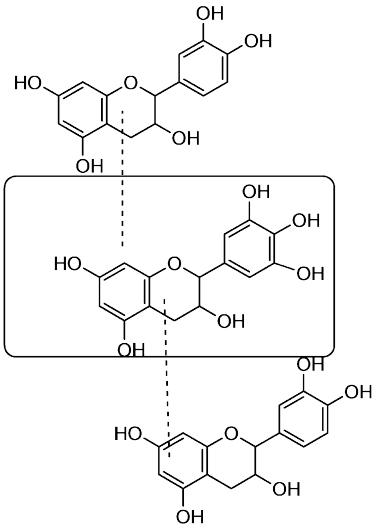


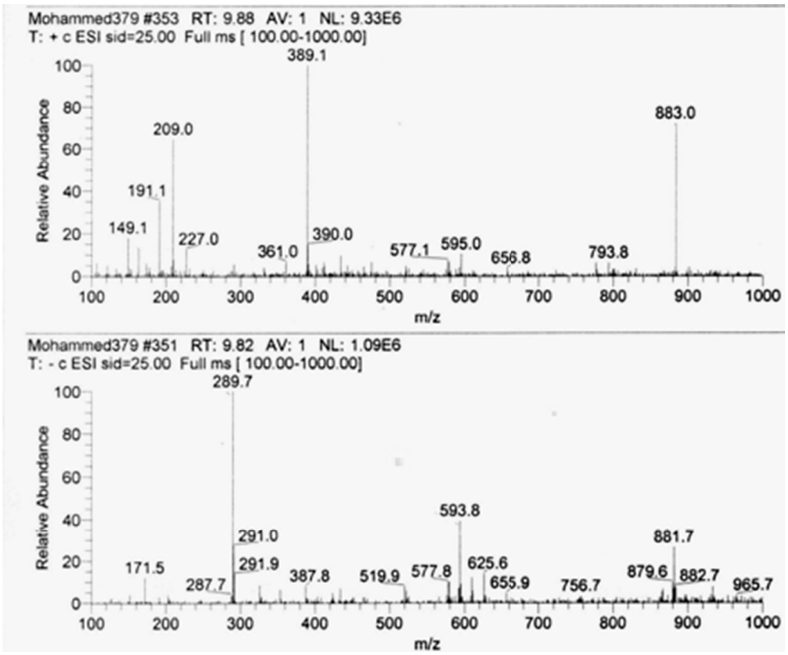
Procyanidin-dimer II 5 MW. 578 g/mol



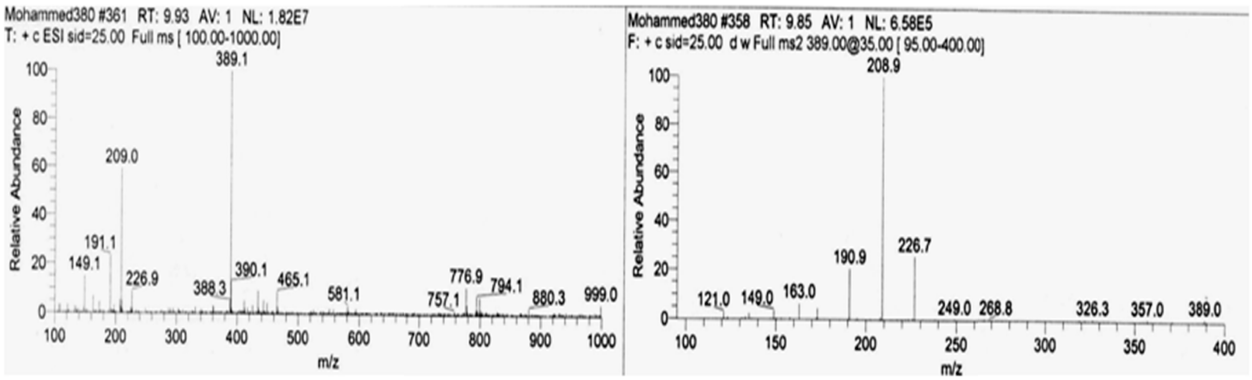


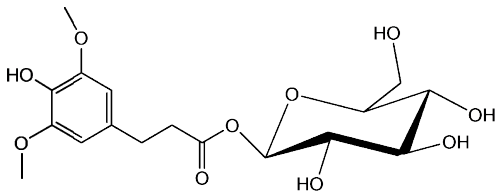
Catechin 6 MW. 290 g/mol



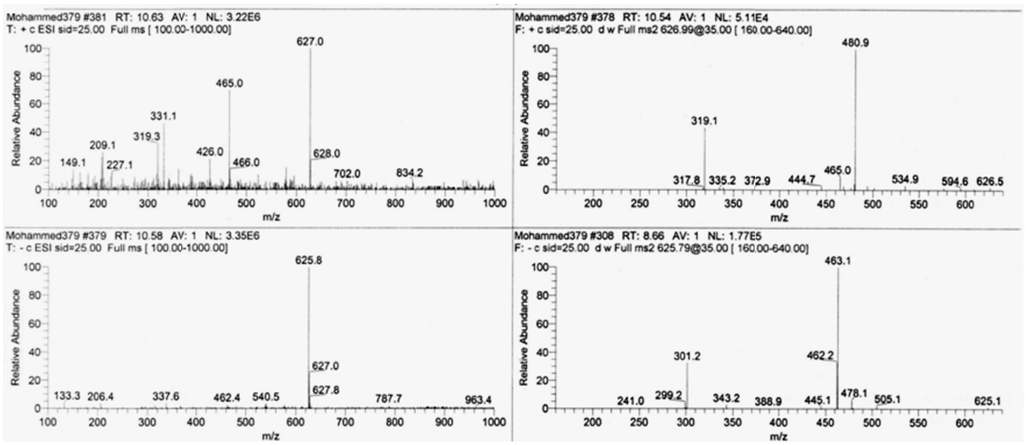


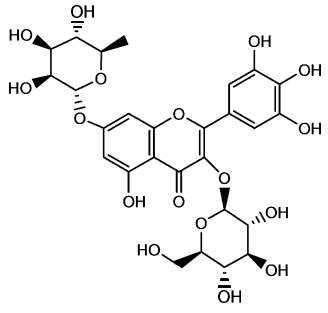
Prodelphinidin trimer 7
(gallocatechin-catechin-catechin isomer) MW. 882 g/mol



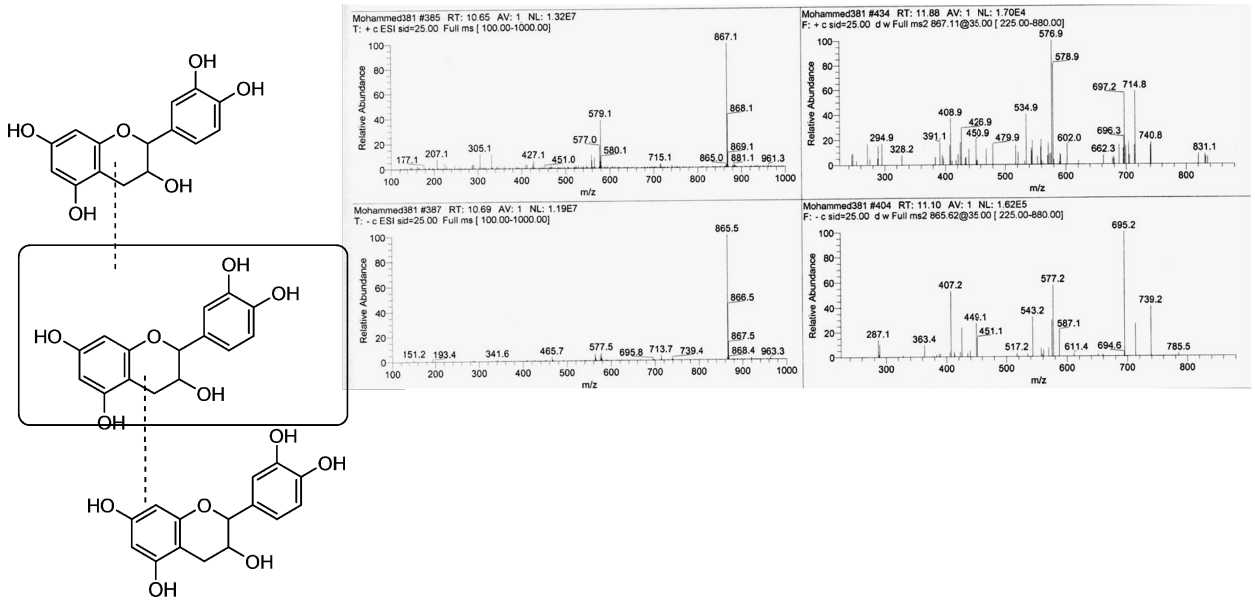


Dihydrosinapoyl glucoside compound 8 MW. 388 g/mol

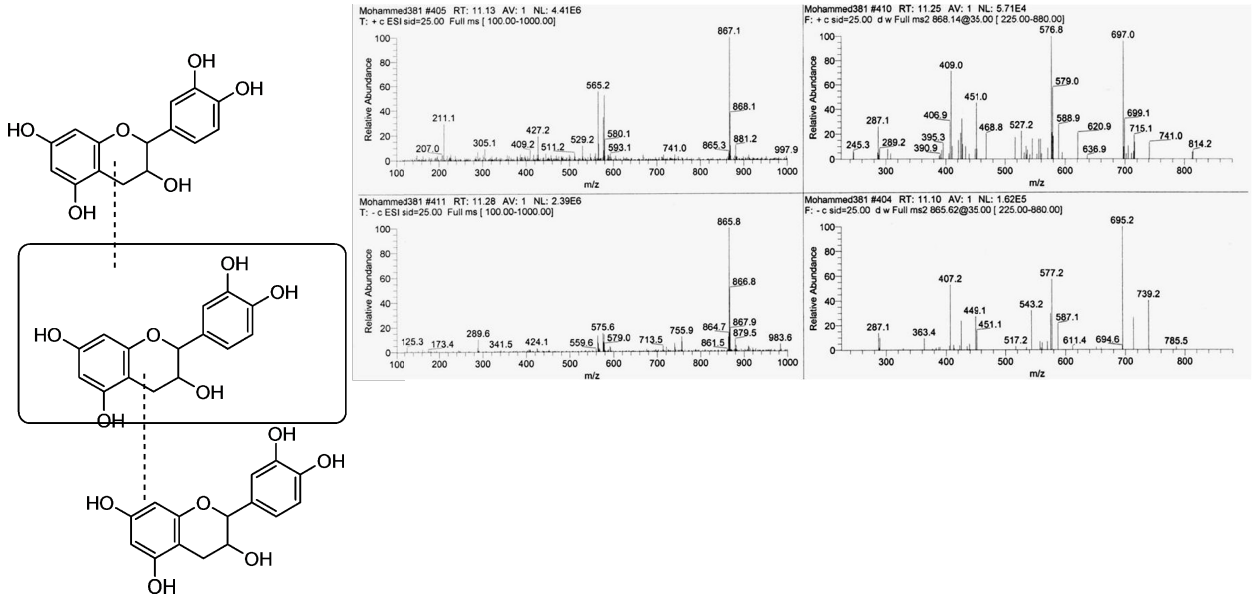




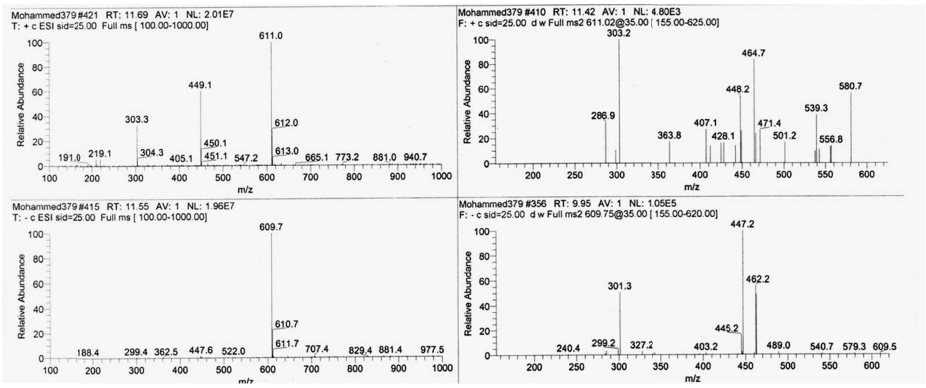
Myricetin-3-O-glucoside-7-O-rhamnoside compound 9 MW. 625 g/mol

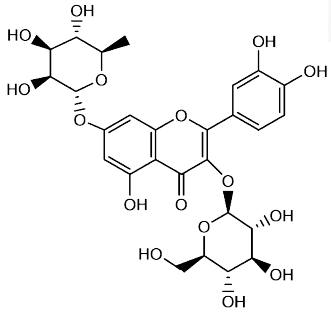


Procyanidin trimer II 10 MW. 866 g/mol

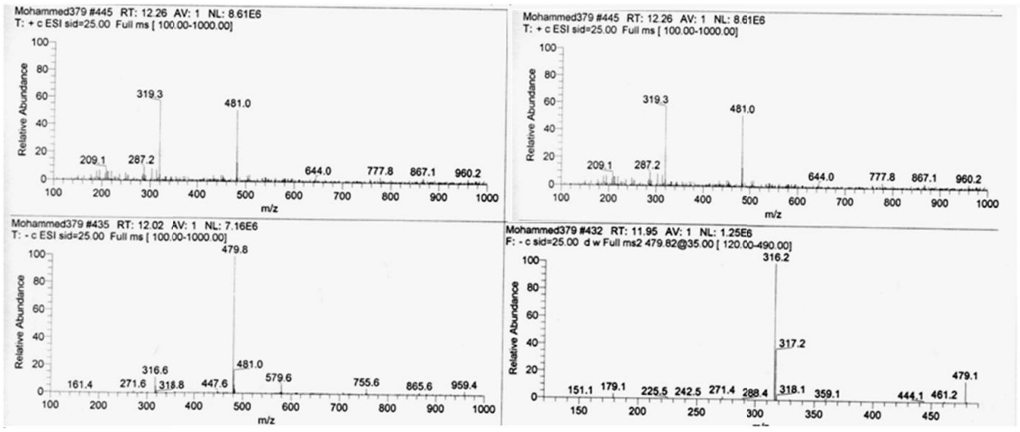


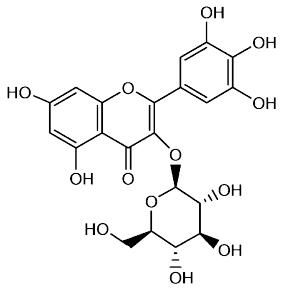
Procyanidin trimer III 11 MW. 866 g/mol



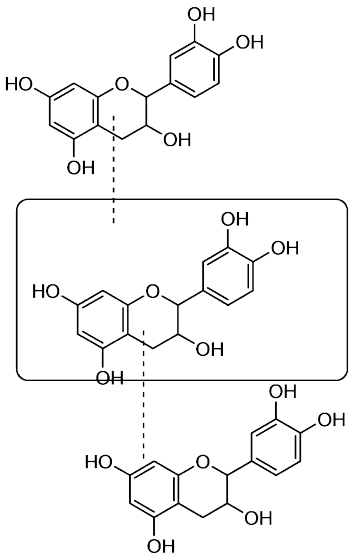


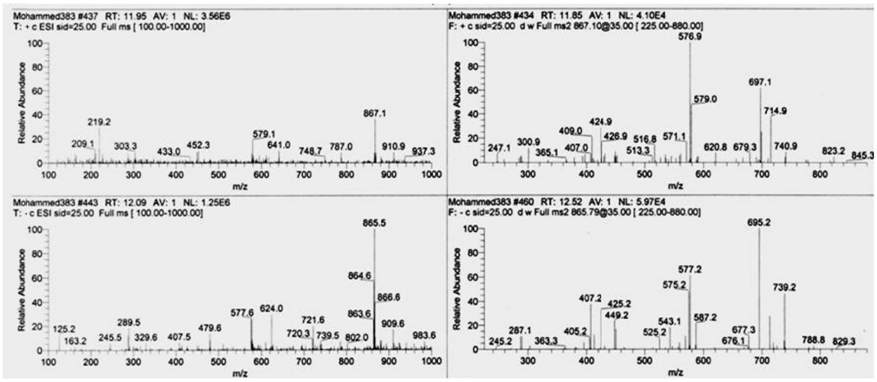
Quercetin-3-O-glucoside-7-O-rhamnoside 12 MW. 610 g/mol



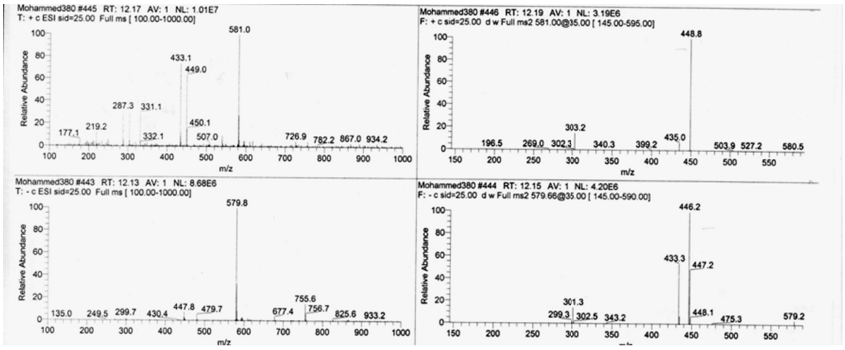


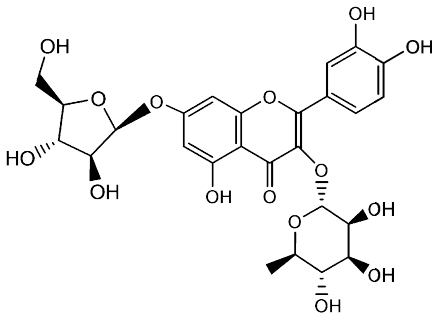
Myricetin-3-O-glucoside 13 MW. 480 g/mol



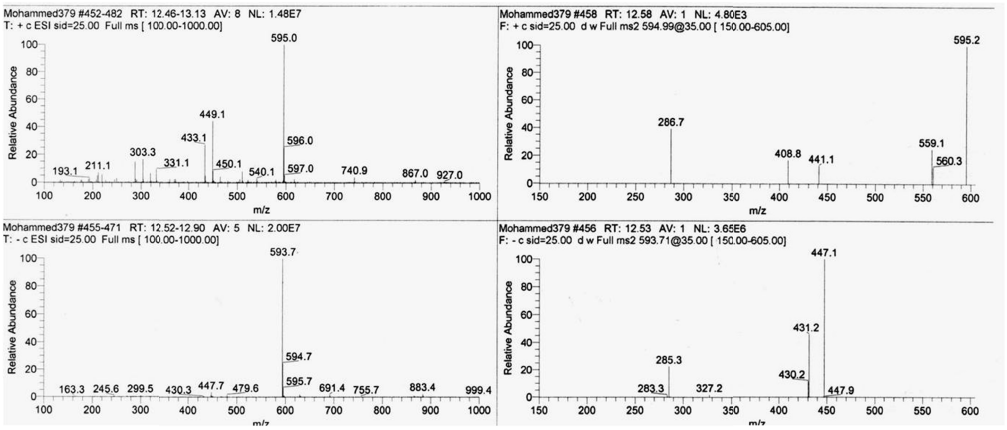


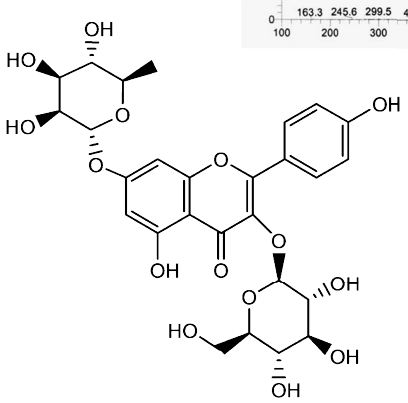
Procyanidin trimer IV 14 MW. 866 g/mol



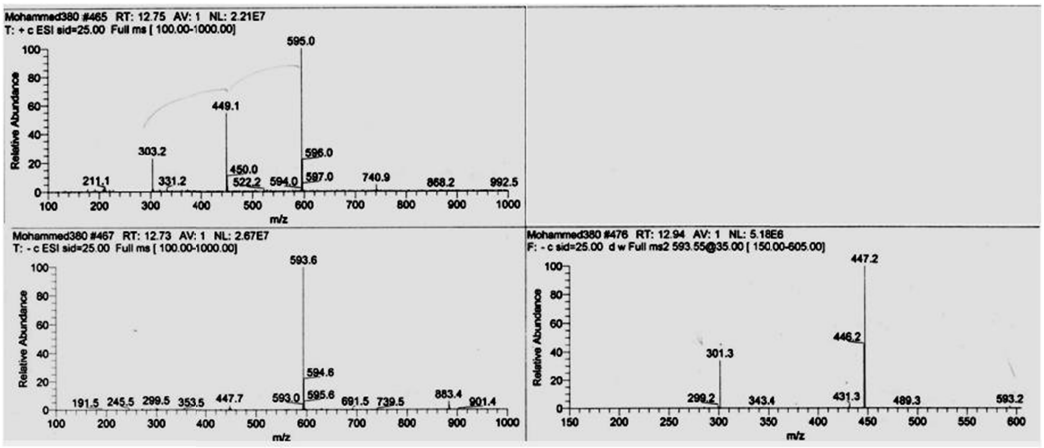


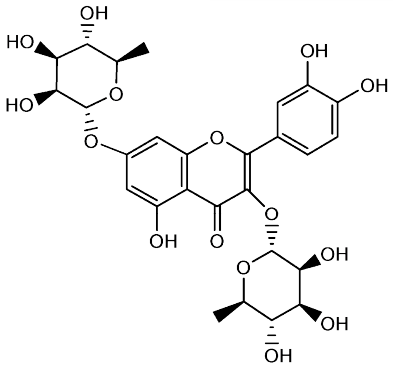
quercetin-3- O-rhamnoside-7-O-pentoside 15 MW. 580 g/mol



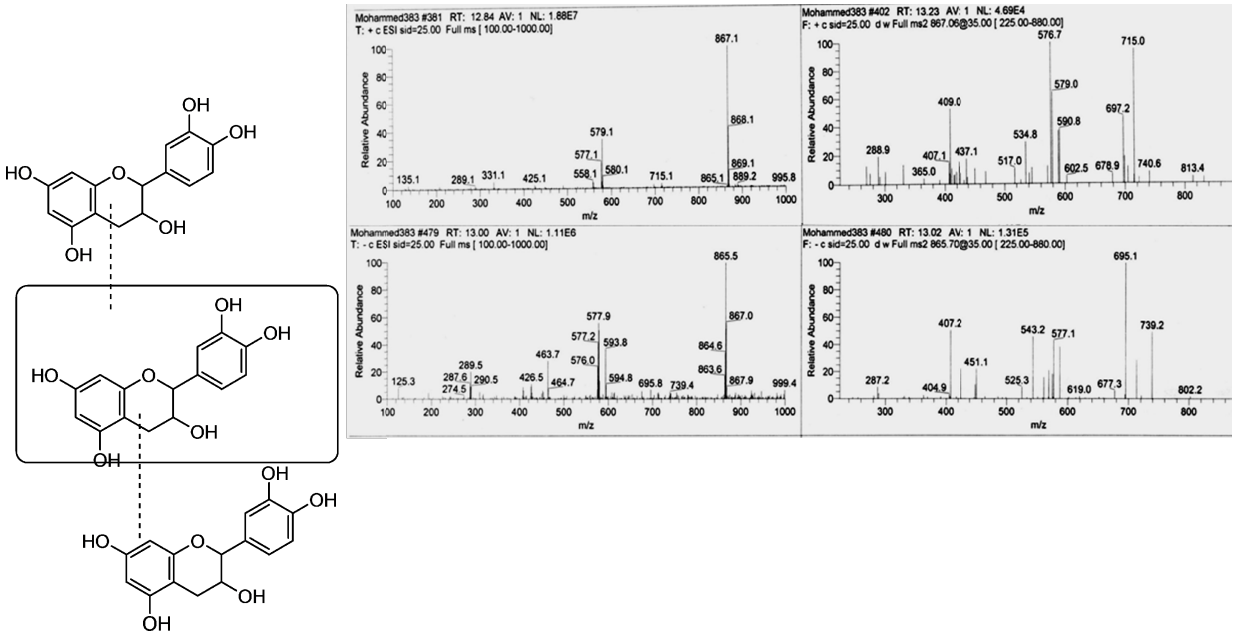


Kaempferol-3- O-glucoside-7-O-rhamnoside 16 MW. 594 g/mol

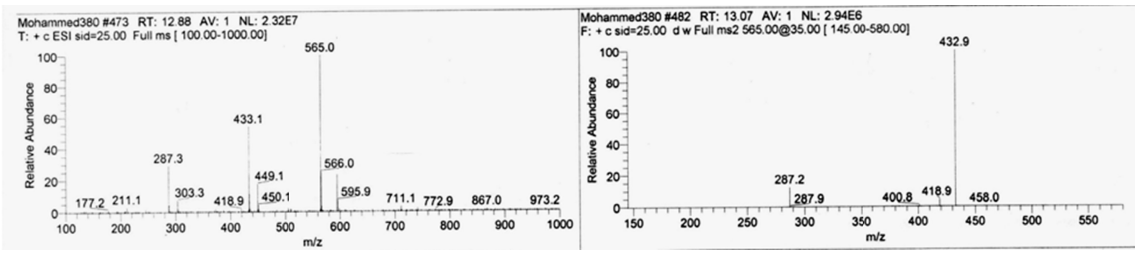


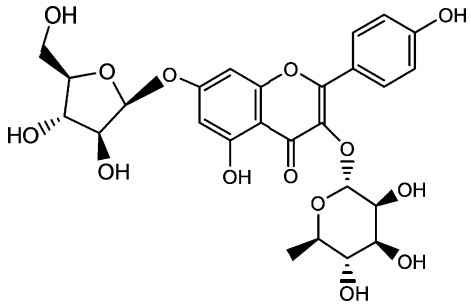


Quercetin-3,7-di-O-rhamnoside 17 MW. 594 g/mol

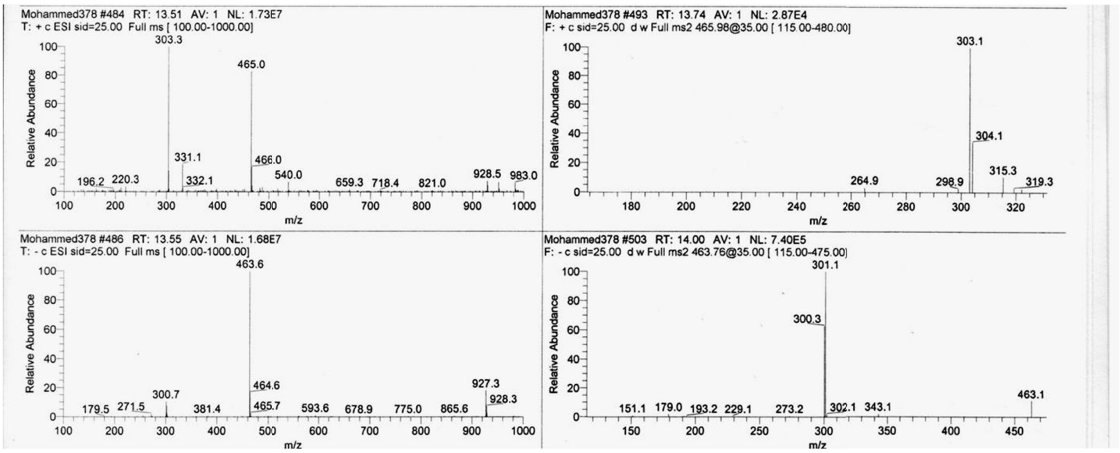


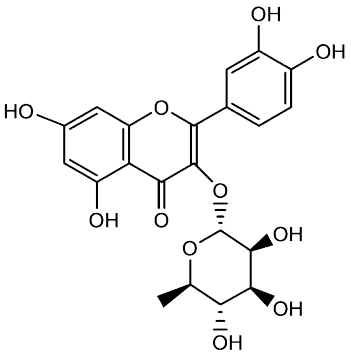
Procyanidin trimer 18 MW. 866 g/mol



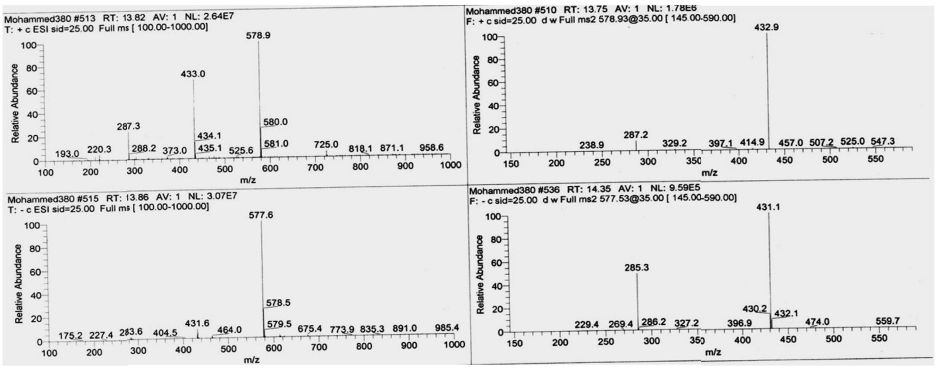


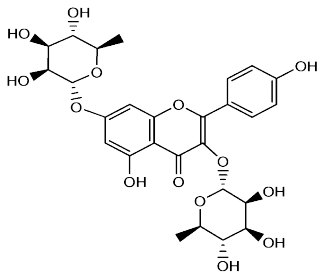
Kaempferol-3- O-rhamnoside -7-O-pentoside 19 MW. 564 g/mol



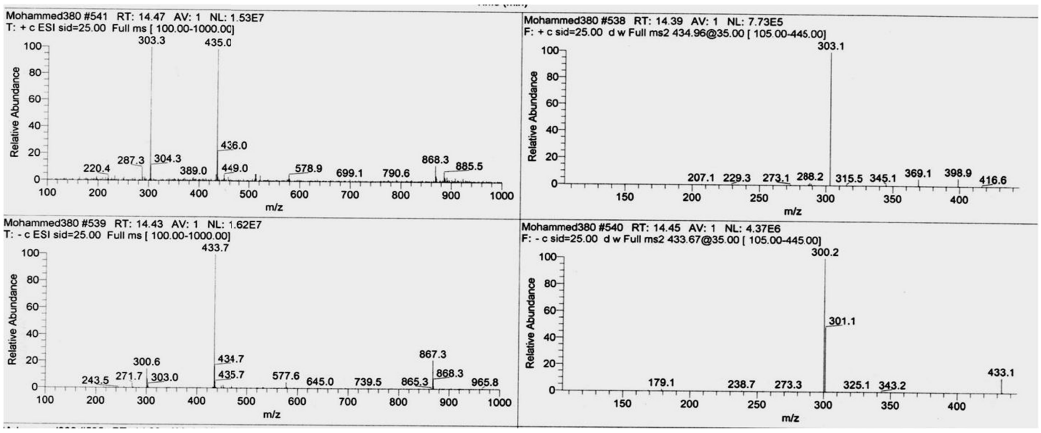


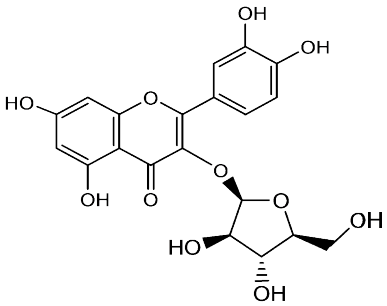
Quercetin-3-O-glucoside 20 MW. 464g/mol



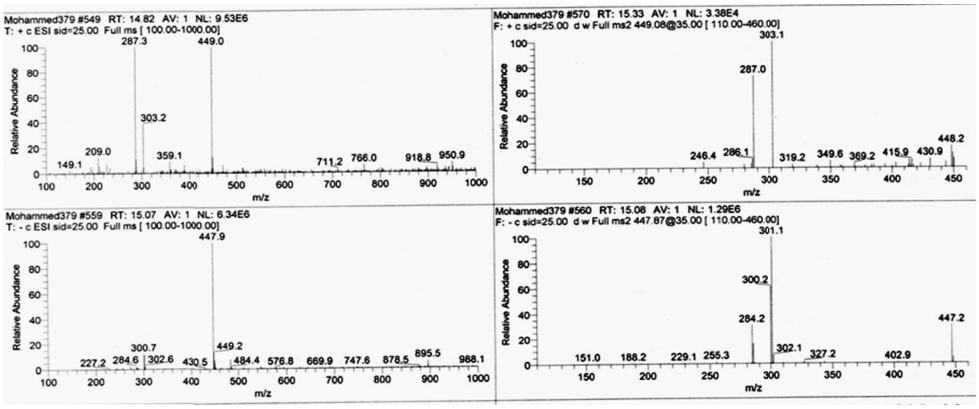


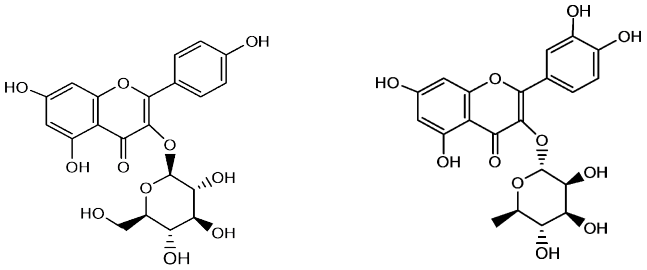
Kaempferol-3,7-di O-rhamnoside 21 MW. 578 g/mol



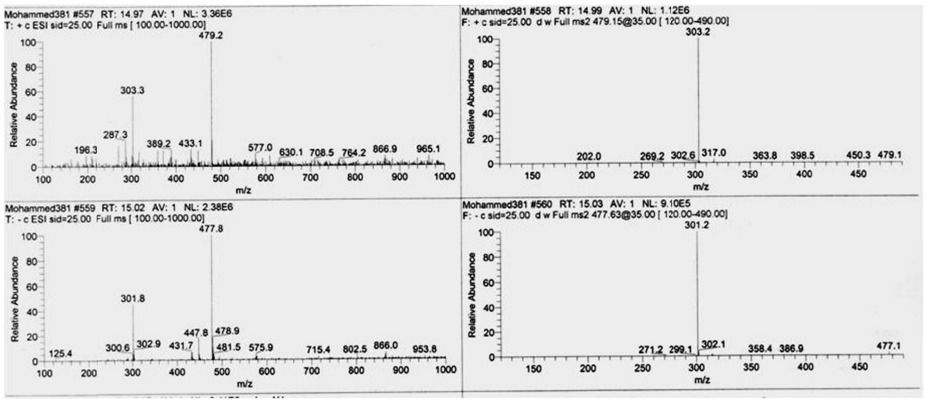


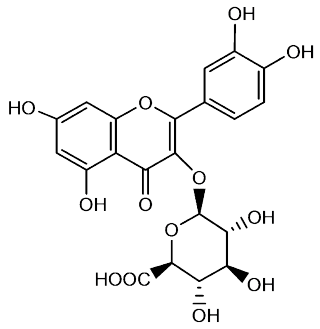
Quercetin-3-O-pentoside 22 MW. 434 g/mol



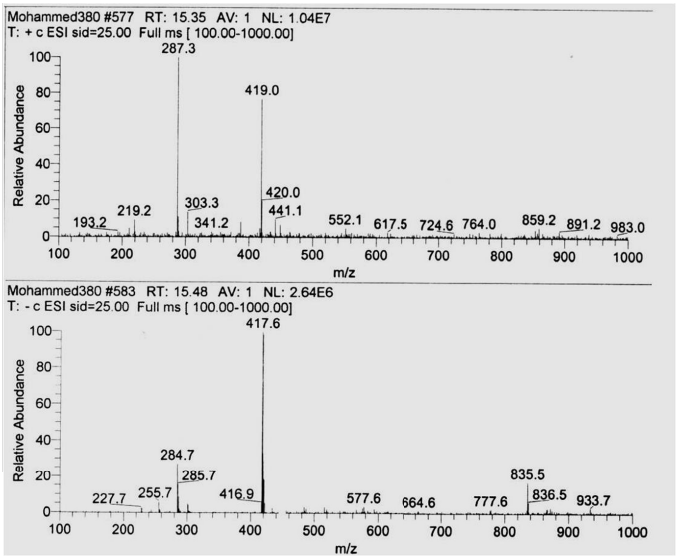


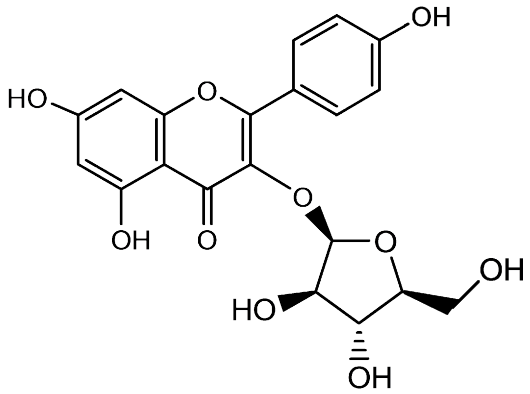
kaempferol-3-O-glucoside 23 MW. 448 g/mol
quercetin-3-O-rhmnoside 24 MW. 448 g/mol



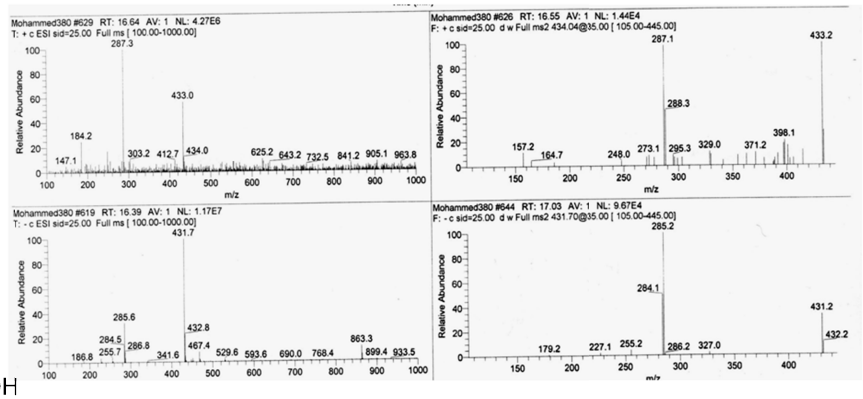


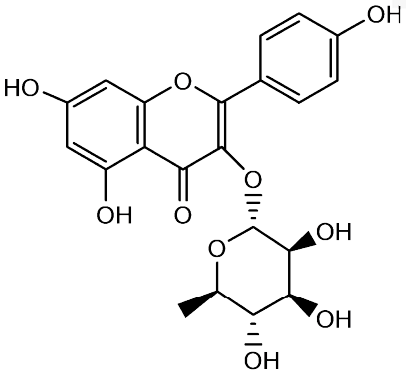
quercetin-3-O-glucouronide 25 MW. 478 g/mol



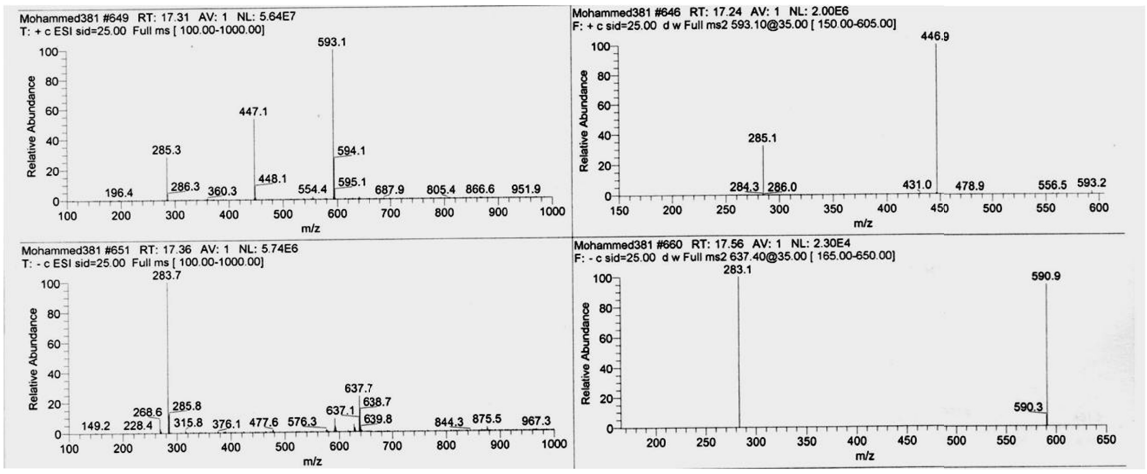


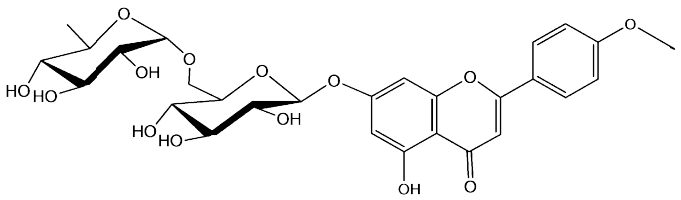
Kaempferol-3-O-pentoside 26 MW. 418 g/mol



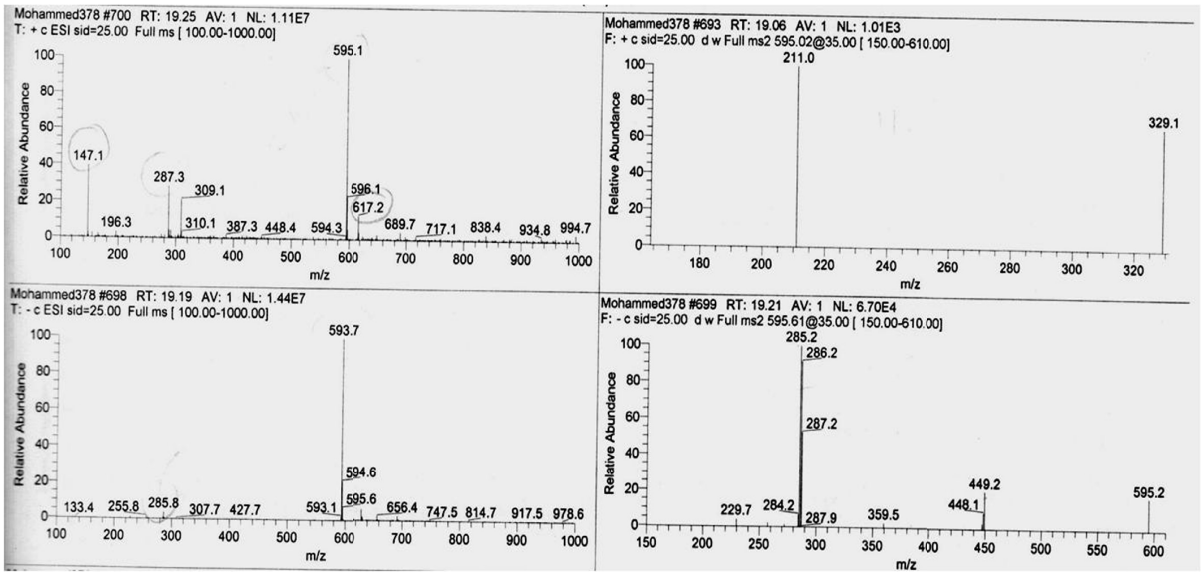


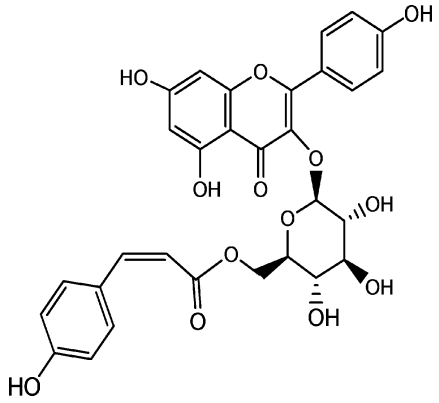
Kaempferol-3-O-rhamnoside 28 MW. 432 g/mol





4'-O- methylapigenin- 7-O-rutinoside 28 MW. 592 g/mol





cis -tiliroside 30 MW. 594 g/mol