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ISOLATION AND CHARACTERIZATION OF QUERCETIN AND KAEMPFEROL FROM *Ginkgo biloba* LEAVES GROWN IN EGYPT

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ABSTRACT: In the present study, *Ginkgo biloba* leaves were extracted with methanol 80% and fractionated by sequential extraction with petroleum ether (Fr-l), ethyl ether (Fr-II) and ethyl acetate (Fr-III) separately. Fraction III was co-chromatographed after hydrolyzed with 7% sulphuric acid with 3 solvent systems. Paper chromatography on Whatman cellulose chromatography papers 3 CHR with benzene, acetic acid and water (125: 72: 3) as mobile phase enabled good separation of quercetin at 0.6 R_F and kaempferol at 0.78 R_F. In the present study, quercetin and kaempferol were isolated from *Ginkgo biloba* leaves grown in Egypt and characterized by various spectral studies. The ethanolic extract of *Ginkgo biloba* leaves was subjected to high-performance liquid chromatography analysis for the identification of major phenolics and flavonoids. Eleven components were detected. Most flavonoids in *Ginkgo biloba* leaves are the derivatives of quercetin, and kaempferol. The results were obtained from UV and FTIR indicated for the molecular structure of quercetin and kaempferol. The mass spectrum of kaempferol and quercetin was observed at m/z 286.8 and 302.9, respectively. The results of the present study established the presence of biologically active phytochemicals in the *Ginkgo biloba* leaves. The results also suggested that the *Ginkgo biloba* leaves extract contain significant amounts of quercetin and kaempferol. Thus, it may be concluded that the *Ginkgo biloba* leaves have great potential for producing healthy and highly nutritive products.

Key words: *Ginkgo biloba* leaves, quercetin, kaempferol, FTIR, mass analysis

INTRODUCTION

Ginkgo biloba L. (common name-maiden hair tree; family Ginkgoaceace) is traditionally as well as an economically important plant. The medicinal parts of Ginkgo (fresh or dried leaves, and seeds separated from their fleshy outer layer) are known for antioxidant, anti-asthmatic, wound healing, neuroprotective and antimicrobial properties and to improve the mental capacity in Alzheimer's patients **(Mazzanti *et al.,* 2000; Sati *et al.,* 2012; Xu *et al.,* 2012).** The medicinal and the antimicrobial properties of Ginkgo can be attributed to two important chemical constituents, *viz.* terpenes trilactone (ginkgolides and bilobalide) and flavonoid glycosides **(Craig, 1999; Van Beek and Montoro, 2009)**. Flavonoids, abundant in vegetables, fruits, medicinal plants, teas have attracted the greatest attention and have been studied extensively because they are a kind of highly effective antioxidants with lower toxicity than synthetic antioxidants such as butylated hydroxyl touline (BHT) and butylated hydroxy anisole (BHA) **(Pekkarinen *et al.,* 1999)**. *Ginkgo biloba* leaf has a long history of medicinal use for the treatment of numerous conditions, going back thousands of years. Extracts of *Ginkgo biloba* leaves contain a wide variety of active compounds and are a particularly rich source of flavonoids, primarily quercetin, kaempferol, and isorhamnetin. Ginkgo has become an increasingly well-known medicinal plant worldwide and is now among the best-selling phytomedicines in Europe, where it is prescribed as a treatment for peripheral vascular disorders,

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particularly cerebral insufficiency, including general dementia and Alzheimer's disease **(Itil and Martorano, 1995)**. The most important substances are flavonoids (ginkgo flavone glycosides) and terpenoids (ginkgolides and bilobalide) **(Craig, 1999)**. The various compounds found in ginkgo may play a protective role in different stages of the decline of intellectual function *via* several mechanisms of action: vasoregulating activity of arteries, capillaries, and veins (increased blood flow); platelet activating factor (PAF) antagonism; homeostasis of inflammation and oxidative stress; prevention of cell membrane damage caused by free radicals and neurotransmission modulation **(Itil and Martorano, 1995)**. Therefore, in the present study quercetin and kaempferol were isolated from *Ginkgo biloba* leaves and characterized by various spectral studies.

MATERIALS AND METHODS

Collection of Plant Materials

Leaves of *Ginkgo biloba* L were collected from Orman Park, Giza, Egypt. *Ginkgo biloba* L leaves were manually cleaned and ground for 3 min using a Moulinex mixer (Type 716, France) at the maximum speed setting. Ground leaves were passed through a 1 mm^2 sieve.

Extraction Procedure

Sample of twenty grams was extracted individually with 200 ml ethanol 99.7 using soxhlet apparatus for 6 hr., at 60°C followed by filtration through Whatman No. 42 filter paper. The soxhlet was hydrolyzed by the reflux with 3 ml of conc. HCl and 5 ml of H_2O for 2.5 hr., **(Kaur *et al.,* 2012)** to detect the flavonoid glycosides in the HPLC. The final hydrolyzed filtrate was thereof filtered using Whatman filter paper (No. 42). The filtrate was concentrated using a rotary evaporator to obtain the constant mass of respective extract. Concentrated extracts were dissolved in Methanol and sonicated for 15 min at 40°C. The prepared samples were filtered through a 0.45 mm filter prior to HPLC analysis and kept in airtight at 4°C until further analysis.

HPLC Analysis

HPLC analysis was carried out according to **Sati *et al.* (2019)** with slight modifications using an Agilent Technologies 1100 series liquid chromatograph equipped with an auto-sampler and a diode-array detector. The analytical column was Agilent Eclipse XDB C18 (150 x 4.6 µm; 5 µm) with a C18 guard column. The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (*V/V*) (solvent B). The flow rate was kept at 0.8 mL min^{-1} for a total run time of 70 min and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. There was 10 min of post-run for reconditioning. The injection volume was 10 µl and peaks were monitored simultaneously at 280, 320 and 360 nm for the benzoic acid, cinnamic acid derivatives and flavonoids compound, respectively. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectrum and compared with those of the standards.

Quercetin and Kaempferol Isolation and Purification

Chromatography

Paper chromatography (PC): Whatman cellulose chromatography papers (3 CHR, W × L 460 mm × 570 mm, pkg of 100 each, WHA3003917 Aldrich). Solvent systems: system 1 (S-1): n-butanol, acetic acid, water (4:1:5 (*V/V*), upper layer), system 2 (S-2): n-butanol, acetic acid, water (3:1:1) (*V/V*) and system 3 (S-3): benzene, acetic acid and water (125: 72: 3) (*V/V*).

Column chromatography: Polyamide column (S-7). Fractions were eluted with 40% aqueous methanol.

Isolation and purification

Ginkgo biloba leaves powder (50 g) were Soxhlet's extracted with 80% methanol for 24 hr. The methanol soluble fractions were filtered, concentrated in vacuum and the aqueous

fractions were fractioned by sequential extraction with petroleum ether (Fr- l), ethyl ether (Fr-II) and ethyl acetate (Fr-III) separately. Each step was repeated thrice for complete extraction, fraction I was discarded because it contained fatty substances, whereas fraction II and III were concentrated and used for flavonoids separation.

Fraction III was further hydrolyzed by refluxing with 7% sulphuric acid (10 ml/g plant material for 2 hr.), filtered and the filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled together separately, neutralized by distilled water with repeated washings, and concentrated in vacuum. Both fraction II and fraction III were taken up in small volume of ethanol (2-5 ml) before chromatographic examination.

Each of the extract was co-chromatographed with authentic samples of flavonoids (Kaempferol and quercetin) as markers. Papers were developed in an air- tight chromatographic chamber saturated with solvent mixture .The developed sheets were air dried and visualized under UV light and by exposure to ammonia fumes. The mouth of a 100 ml bottle containing concentrated NH_4OH was held in contact with each spot for about 5-10 seconds and fluorescent spots corresponding to that of standard markers were marked. The colored spots thus developed were noted and the R_F. value of each spot was calculated. Several other solvent systems such as n-butanol, acetic acid, water (4:1:5(*V/V*), upper layer), n-butanol, acetic acid, water (3:1:1) (*V/V*) were also tested, but the solvent system containing benzene, acetic acid and water (125: 72: 3) (*V/V*) gave better results.

Kaempferol and quercetin have been obtained from Fr-III (ethyl acetate fraction) according to the observation on paper chromatography as compared to authentic samples. Then, Kaempferol and quercetin was eluted from paper chromatograms by dispersion in 40% aqueous methanol (40% H_2O-MeOH) and was filtered using Whatman filter paper (No. 42). The filtrate was purified on a polyamide column (S-7).

Kaempferol and quercetin identification

R_f value

The R_f value is defined as the ratio of the distance moved by the solute and the distance moved by the solvent along with the paper.

$$Rf=\frac{\text{Distance moved by the solute}}{\text{Distance moved by the solvent}}$$

Fourier transform infrared (FTIR) spectroscopy

Kaempferol and quercetin were prepared with potassium bromide (KBr) pellet method **(Souillac *et al.*, 2002)**. Infrared spectra were measured with a FT-IR spectrometer (NICOLET NEXUS 470, DTGS, Thermo Scientific, Waltham, MS, USA) at 25°C. For each spectrum 256 interferograms were collected with a resolution of 4 cm^{-1} with 64 scans and a 2 cm^{-1} interval from the 4000 to 400 cm^{-1} region. The system was continuously purged with dry air. Second derivation spectra were obtained with Savitsky–Golay derivative function soft **(Surewicz and Mantsch, 1988)**.

UV spectroscopy (selection of λ_{max})

Kaempferol and quercetin were diluted with methanol to get (10 µg/ml) concentration. This solution was scanned between the wavelength regions of 200-400 nm against methanol as blank. The maximal absorption was recorded as λ_{max} **(Telange *et al.*, 2014)**.

Mass analysis

An advion compact mass spectrometer (CMS) NY/USA instrument equipped with an electro spray ionization source (ESI) was employed to analyze the composition of isolated compounds (diluted with methanol to get 10 µg/ml concentration) with the following specification:

Ion source: ESI; Polarity: Positive and negative ion switching in a single analysis; Flow rate range: 10 µl/min to 2 ml/min; m/z range: expression S m/z 10 to 1.200 and expression L m/z 10 to 2.000; Acquisition speed: 10.000 m/z units/sec., Sensitivity: 10 pg reserpine; Accuracy: ± 0.1 m/z units of the entire acquisition range; Stability: 0.1 m/z units at m/z 1.200 over 12 hr.

period operating temperature of 20°C ±1°C; Polarity switching speed: 50 ms; dynamic range: 4.5 orders of magnitude.

RESULTS AND DISCUSSION

HPLC Analysis

The bioactive phenolic compounds identified in the *Ginkgo biloba* leaves are listed in Table 1 and Fig. 1. 17 components were represented. Gentisic acid, chlorogenic acid, caffeic acid, vanillic acid, sinapic acid, and rutin were dose not detected. 11 components were detected (gallic acid, protochatchuic acid, catachine, syrngic acid, ferulic acid, coumarin, rosmarinic acid, cinnamic acid, quercetin, kaempferol and chyrsin). The main components were catachine, chyrsin, rosmarinic acid, protochatchuic acid and coumarin (64.632, 43.546, 41.301, 20.191 and 12.512 µg/ml, respectively). Most flavonoids in *Ginkgo biloba* leaves are the derivatives of isorhamnetin, quercetin, and kaempferol **(Ding *et al.*, 2009)**. In the present study quercetin, and kaempferol was recorded 9.964 and 6.157 µg/ml, respectively at retention time 43.4 and 46.4 min, respectively. These results are in agreement with those obtained by **Ding *et al.* (2009) and Sati *et al.* (2019)**.

Quercetin and Kaempferol Identification

R_F value

Paper chromatography on Whatman cellulose chromatography papers 3 CHR with benzene, acetic acid and water (125: 72: 3) (*V/V*) as mobile phase enabled good separation of quercetin at 0.6 R_F and kaempferol at 0.78 R_F.

FT-IR spectroscopy

The IR spectrum of the quercetin is shown in Fig. 2A. The absorption around 3417/cm is due to the presence of phenolic hydroxyl groups in the compound. The intense absorption band at 1644/cm is due to the presence of ν(C=O). The band around 1458/cm is due to the occurrence of the aromatic group in an isolated compound. The IR spectrum of the kaempferol is shown in Figure 2B. The FT-IR spectrum revealed broad absorption bands at 3391/cm represents to OH group stretching. The absorption band occurs at 1664/cm for the carbonyl group (C=O) and the absorption band at 1567, 1449/cm denotes the presence of aromatic ring. Moreover, the band around 2900/cm corresponds to the presence of C-H stretching. This result is in good agreement with the previous literature for molecular structure of quercetin and kaempferol **(Chourasiya *et al.*, 2012; Sathyadevi and Subramanian, 2015; Sambandam *et al.*, 2016)**.

UV-spectrum

The absorption maximum (λ_{max}) was found to be 258 nm and 375 nm for quercetin and 265 nm and 365 nm for kaempferol. This result is closely to those obtained by **Chaudhari *et al.* (2014) and Telange *et al.* (2014)**.

Mass analysis

An advion compact mass spectrometer (CMS) NY/USA instrument equipped with an electro spray ionization source (ESI) was employed to analyze the composition of isolated quercetin and kaempferol. The mass spectrum of quercetin is illustrated in Fig. 3. 100% base peak for compound, was observed at m/z 302.9 in the mass spectrum indicating the compound as quercetin. The mass spectrum of kaempferol is illustrated in Fig. 4. 100% base peak for compound, was observed at m/z 286.8 in the mass spectrum indicating the compound as kaempferol. This result is closely to those obtained by **Chaudhari *et al.* (2014) and Telange *et al.* (2014)**.

Conclusions

The results of the present study established the presence of biologically active phytochemicals in the *Ginkgo biloba* leaves. The data also suggested that the *Ginkgo biloba* leaves extract to contain significant amounts of quercetin and kaempferol. Thus, it may be concluded that the *Ginkgo biloba* leaves have great potential for producing healthy and highly nutritive products.

Table 1. Bioactive phenolic compounds (µg/ml) in *Ginkgo biloba* leaves

Compound	Retention time (min)	Conc. (ug/ml)
Gallic acid	5.6	2.314
Protochatchuic acid	9.7	20.191
Gentisic acid	16.7	ND
Catachine	18.4	64.632
Chlorogenic acid	20.3	ND
Caffeic acid	21	ND
Syrngic acid	22.5	4.587
Vanillic acid	24.1	ND
Ferulic acid	32	4.610
Sinapic acid	33.5	ND
Rutin	36.1	ND
Coumarin	36.7	12.512
Rosmarinic acid	40.1	41.301
Cinnamic acid	42.7	0.909
Quercetin	43.4	9.694
Kaempferol	46.4	6.157
Chyrsin	51.7	43.546

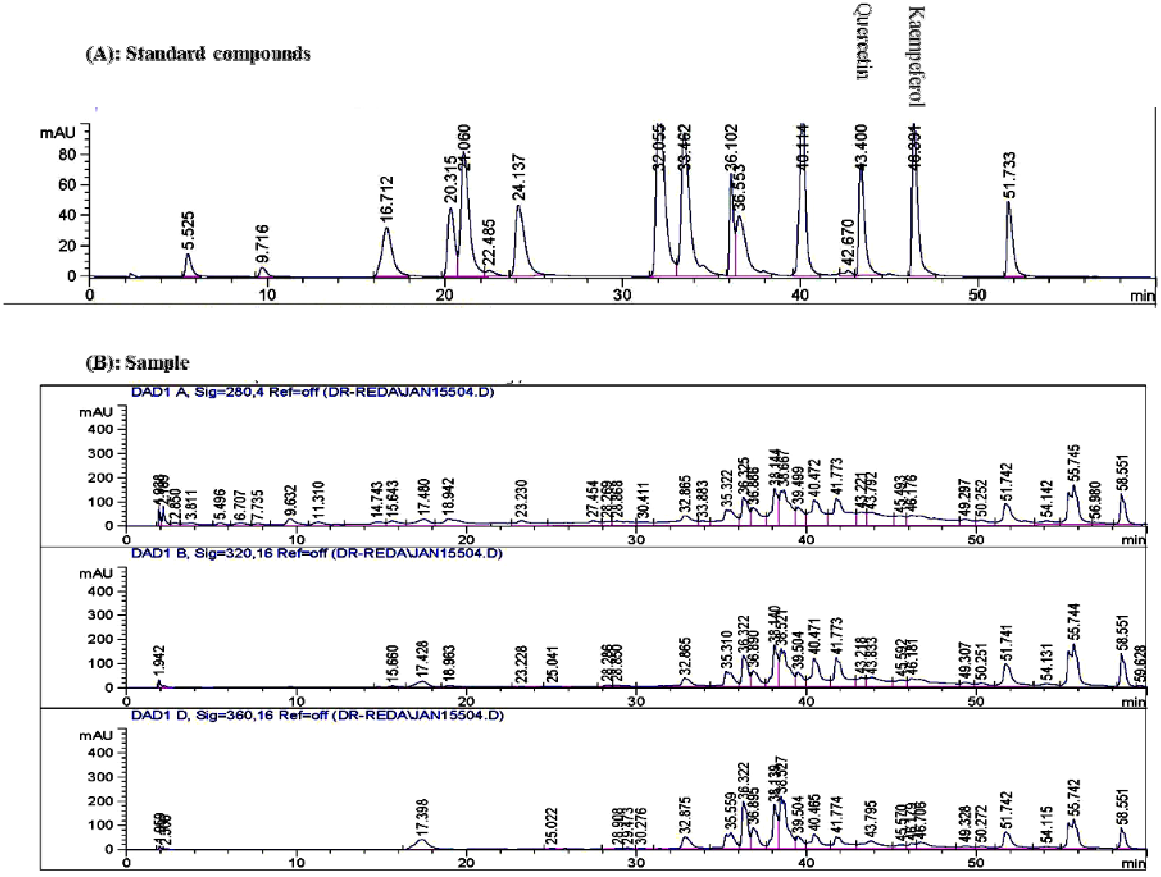

Fig. 1. Representative chromatogram of *Ginkgo biloba* leaves flavonoid glycosides. A: standard compounds and B: sample measured at 280, 320 and 360 nm for the benzoic acid, cinnamic acid derivatives and flavonoid compounds, respectively

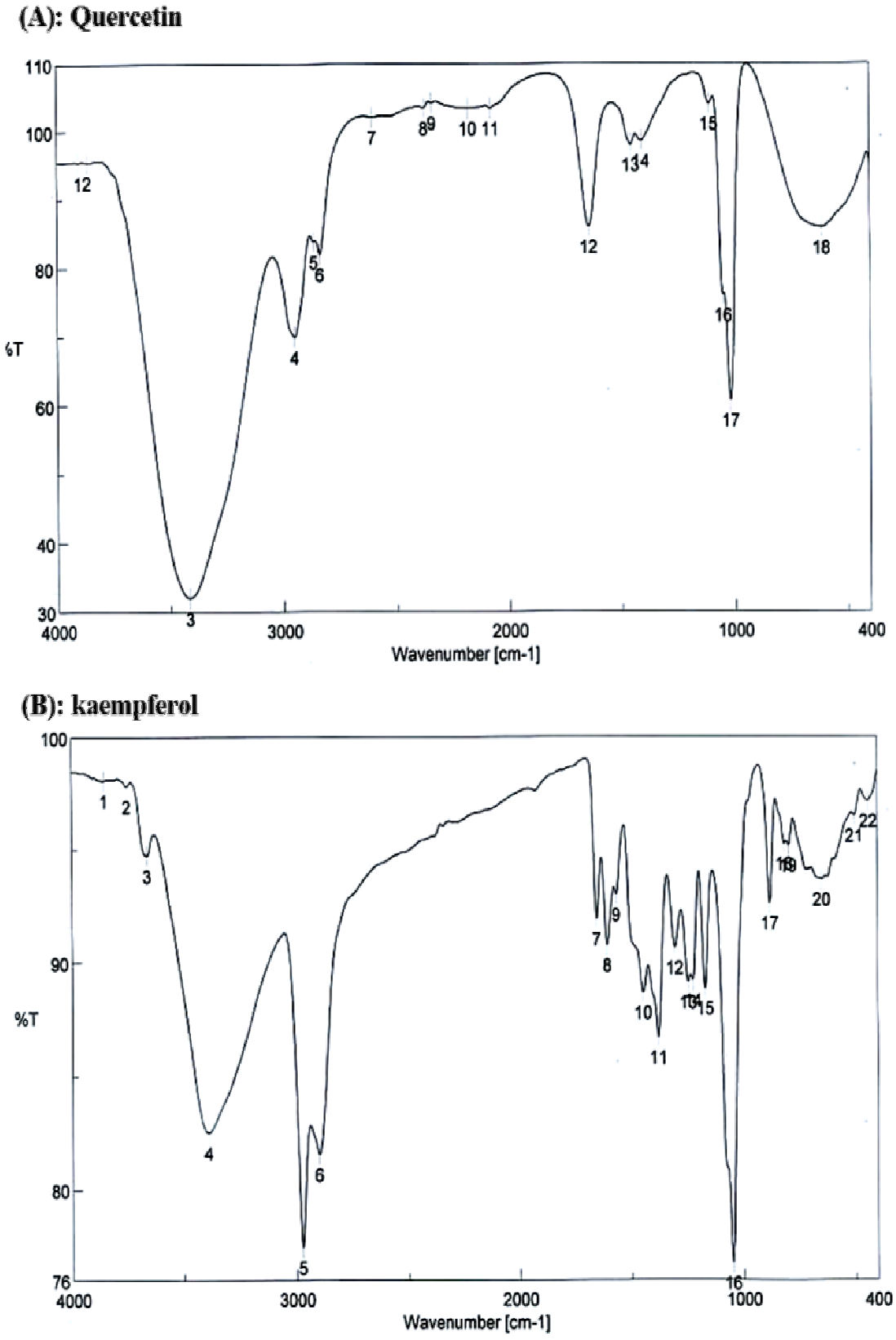

Fig. 2. FT-IR spectrum of isolated compounds (quercetin A and kaempferol B)

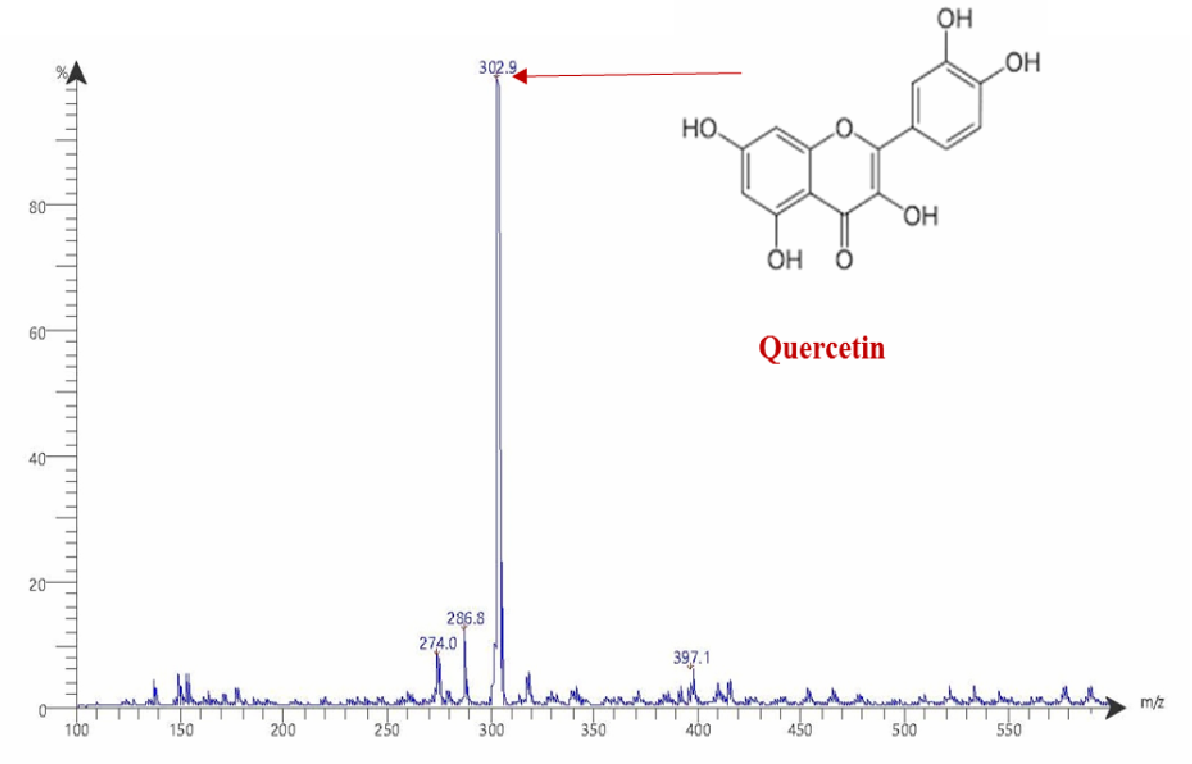

Fig. 3. Mass spectrum of quercetin stated by Chaudhari *et al.* (2014) and Telange *et al.* (2014)

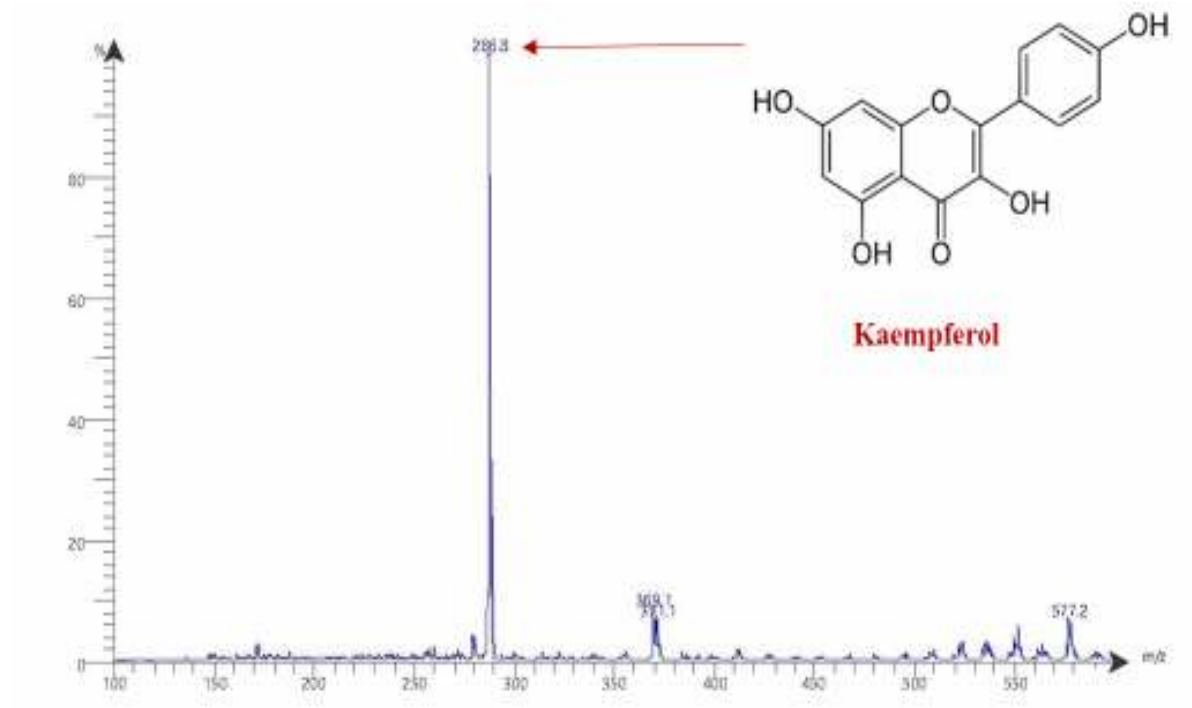

Fig. 4. Mass spectrum of kaempferol stated by Chaudhari *et al.* (2014) and Telange *et al.* (2014)

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عزل وتوصيف الكيرسيتين والكمبيفيرول من أوراق الجنكه بيلوبا المزروعة في مصر

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تم الكشف عن ١١ مركب فينولى، معظم مركبات الفلافونويد الموجودة في أوراق الجنكة بيلوبا هي مشتقات الكيرسيتين والكمبيفيرول، من ناحية أخرى، تم الاستخلاص من أوراق الجنكه بيلوبا بواسطة الميثانول ٨٠% وتقسيمها عن طريق استخلاص متتالي مع الإيثر البترولى، الايثيل ايثر وخلات الإيثيل بشكل منفصل، تمت عملية التحليل اللوني للمستخلص الثالث (خلات الايثايل) بعد تحلله بحمض الكبريتيك تركيزه ٧% مع ٣ أنظمة للمذيبات لتحديد أفضل نظام من المذيبات لفصل الكيرستين والكمبيفيرول على ورق الكروماتوجرافى Watman3, CHR، أوضحت الدراسات أن النظام الثالث المكون من البنزين وحمض الأسيتيك والماء (١٢٥: ٧٢: ٣) هو أفضل مذيب حيث تمكن من الفصل الجيد بين الكيرسيتين عند R_F ٠,٦ والكمبيفيرول عند R_F.٠,٧٨، في الدراسة الحالية، تم فصل وتنقية الكيرسيتين والكمبيفيرول من أوراق الجنكة بيلوبا المزروعة في مصر والتي تم تعريف المركبات المفصولة بواسطة الدراسات الطيفية المختلفة، تعرض المستخلص الإيثانولي لأوراق الجنكه بيلوبا لتحليل كروماتوجرافي سائل عالي الأداء لتحديد أهم الفينولات والفلافونويدات، أظهرت النتائج المتحصل عليها من الدراسات التطبيقية بواسطة الأشعة فوق البنفسجية والتحليل الطيفى بالأشعة تحت الحمراء أن المركبات المفصولة هى الكيرستين والكمبيفيرول، لوحظ التحليل الطيف الكتلي للكيرستين والكمبيفيرول عند ٣٠٢,٩ و٢٨٦,٨ على التوالي، كما أوضحت هذه الدراسة وجود المواد الكيميائية النباتية النشطة بيولوجيا في أوراق الجنكه بيلوبا، تشير النتائج أيضًا إلى أن مستخلص أوراق الجنكه بيلوبا يحتوي على كميات من الكيرسيتين والكمبيفيرول، وبالتالي، فإن أوراق الجنكه بيلوبا لديها إمكانات كبيرة للحصول على منتجات كيميائية حيوية ذات قيمة صحية وغذائية.

المحكمـــــون:

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