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Chromatography Profile from the Methanol Extract of *Abelmoschus esculentus* L Flowers

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ABSTRACT

Herbal medicine is as primordial as mankind and has appreciably contributed to the health care of the human society. The study is to develop the high performance thin layer chromatography (HPTLC) fingerprint profile of *Abelmoschus esculentus* (L). HPTLC method for the separation of the active constituents in extracts has been developed using solvent system Toluene: Ethyl acetate: Formic acid (5:4:1). In HPTLC analysis, it showed the presence of quercetin in standard as well as the sample with Rf value 0.56. The fingerprinting images will be helpful in the identification and quality control of the drug and ensure therapeutic efficacy. The methanol extracts of *Abelmoschus esculentus* (L) flowers showed the presence of molecules such as Protocatechuic Acid-4-o- Glucoside, Kaempferol-3-o-Hexoside, Isorhamnetin-3-0- Glucoside, Quercetin Dimer and Quercetin derivative by LCMS. Liquid chromatography-mass spectrometry (LCMS) is proved to be a very useful technique for plant metabolite and allows the identification of a large variety of common plant metabolites in a single chromatogram.

INTRODUCTION

Almost 25 centuries ago, Hippocrates, the Father of Medicine, stated, "*Let food be the medicine and let medicine be the food".* Amidst ancient civilisations, India has been known to be rich repository of medicinal plants [1]. Today a number of chemicals obtained from plants are used as vital drugs in more countries in the world [2]. This knowledge is accessible from thousands of medical texts and manuscripts. The substances having medical value have been extensively used for treating various disease conditions. Herbs being easily available to human beings have been explored to the maximum for their medicinal properties. Products of primary metabolism such as amino acids, carbohydrates and proteins are vital for the maintenance of life processes, while others like alkaloids, phenolics, steroids, terpenoids are products of secondary metabolism and have toxicological, pharmacological and ecological importance [3]. Many medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system (Ayurveda) and proposed for their interesting multilevel activities. Amongst the medicinal plants used in Ayurvedic preparations for their therapeutic action, some have been thoroughly investigated and some need to be explored [4]. Ayurveda, Unani, Siddha and Folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda and Unani Medicine are most developed and widely practiced in India [5].

Abelmoschus esculentus. (L) is an important medicinal plant of tropical and subtropical India. Its medicinal usage has been reported in the traditional systems of medicine such as Ayurveda, Siddha and Unani. *Abelmoschus esculentus*. (L) is a flowering plant in the mallow family. *Abelmoschus esculentus. L* plays an important role in the human diet by supplying carbohydrates, minerals, and vitamins and its flower have been consumed as health tea and herbal medicine for hundreds of years. It is reported to have many curative effects, such as antioxidant, anti-inflammatory and antitumor activities [6].

High performance liquid chromatography (HPLC) and Liquid chromatography mass spectrography (LCMS) profiles were also recorded for which the plant extracts act as a fingerprint for future investigations. HPTLC allows for the separation of the chemical compounds that possess varying polarities thereby allowing for the profiling of the compounds found in the plant species and thus serving as a fingerprint for the chemical constituents of the plant species. HPTLC and LCMS analysis of plant species provide a

means for the correct identification of the plant species and also to profile the chemical composition of the plants [7].

MATERIALS AND METHODS

Flower sample collection

The fresh flower samples were collected in the month of October, from in and around Nagapattinam, Nagapattinam district, Tamil Nadu, India. The flower samples were thoroughly washed under running tap water to remove adhering dust particles and blotted dry under shade for about two weeks, ground into milled powder and stored in an airtight container used for further investigations.

Preparation of extracts

The powdered *Abelmoschus esculentus* (L) flower samples (1000g) were weighed and mixed with 2000 ml of methanol. Then it is kept in an orbital shaker at 190-220 rpm for 48 hours. The supernatant was collected, filtered through Whatman No.1 filter paper and then concentrated by evaporating to dryness which gave a solid amorphous residue and it was dried thoroughly to remove the solvent used. The obtained dried extract was then accurately weighed, stored in small vials and used for the subsequent studies.

High performance thin layer chromatography (HPTLC) Analysis

Instrument : CAMAG Automatic TLC Sampler 4(ATS4) with win CATS software.

Stationary phase : Plates silica gel 60 F_{254} pre coated layer

Mobile phase : Toluene: Ethyl acetate: Formic acid (5:4:1)

Standard : Quercetin

Sample : Brown powder

Solubility : Methanol Standard concentration 1µl/ml

Standard preparation : Weigh 5mg of standard Quercetin in 5ml of methanol

Standard injection volume : 1, 2, 4, 6, 8, 10,12, 14, 16, 18µl

Sample concentration : 5, 10µl

Weigh about 100mg of

Hydro-alcoholic extract : Dissolved in 5ml of 7:3 Alcohol: Water

and Sample preparation

Sample application volumes : 1, 2,4,8,12,16

(µl)

Development mode : Ascending mode

Scanning wavelength : 254 nm and 366nm

Measurement mode : Absorbance

Evaluation: A band Rf value of 0.61 corresponding to Quercetin is visible in test solution tracks

Preparation of the plates

The plates used for HPTLC was silica gel 60 F 254 (E.MERCK KGaA). 0.5µl to 3.5µl of standard solution was applied in the form of bands using LINOMAT IV applicator. The sample concentration was 10mg/ml and the different volumes were 10 to 50µl. The mobile phase used was toluene: ethyl acetate: formic acid: methanol (5.5:3:1:0.5). The chromatograph was developed for 15 minutes, dried at room temperature and scanned at 254nm and 366nm.

Procedure

Applied 1µl of standard solution and 5, 10µl of test solution on a percolated silica gel 60F254TLC Plate of uniform thickness 0.2mm using sample applicator. The plate was developed in the solvent system to a distance of 8cm. The plate was scanned densitometrically at 254 nm using TLC Scanner. The plate was observed under UV light at 254nm and 366nm using CAMAG REPROSTAR 3.

Liquid chromatography is a fundamental separation technique used in life sciences and related fields of chemistry. Liquid chromatography (LC) combined with mass spectrometry (MS) is a powerful tool for qualitative and quantitative analytics of organic molecules from various matrices, and the use of this hyphenated technique is very common in bioanalytical laboratories. In the present study, LC/MS methods and the required sample preparation applications were developed for detection of compounds such as flavones, terpene and sesquiterpenes lactones.

Methanolic extract was used for the analysis. For determination of secondary metabolites, micro TOF-Q II (Bruker, Germany), UV detector at 330nm and Quadrupole II for mass analysis and TOF for mass detection was utilized. The column used was UHPLC Dionex C18 RP Acclaim 120Å, 2.1 × 150mm, 3.0µm column (Dionex, USA). Solution A: ACN (1% Acetic acid) and Solution B: Water (1% Acetic acid) was used as mobile phase [8].

RESULTS

The study revealed that *Abelmoschus esculentus.* (L) flower showed best results in Toluene: Ethyl Acetate: Formic acid 5:4:1 solvent system for methanolic extracts. After scanning and visualizing the plates in absorbance mode at both 254nm, 366 nm and visible light range (400-600nm after spraying with anisaldehyde sulphuric acid reagent) best results were shown at 375nm. The HPTLC images shown in Figure 4 indicate that all sample constituents were clearly separated without any tailing and diffuseness.

The results from HPTLC fingerprint scanned at wavelength 375nm for methanol extract of *Abelmoschus esculentus.* L flower revealed the presence of seven polyvalent phytoconstituents in standard Table 1 and five polyvalent phytoconstituents in sample (Table 2). The Rf values ranged from standard 0.62 to 0.60 and Rf values ranged from sample 0.64 to 0.63. It is also clear from Table 1 and Table 2 and the chromatogram as shown in Figure 2 and 3 that out of 12 components, the component with sample Rf values 0.63, 0.63 were found to be more predominant as the percentage area is more with 100.00% and 100.00% respectively. TLC plate showed different colour phytoconstituents of *Abelmoschus esculentus.* (L) flower methanol extract (Track 1-2). The bands revealed presence of one blue, light blue, three greenish, purple, one red and two light yellowish orange bands showing the presence of steroids, terpenoids and saponins (Figure 4) after spraying with anisaldehyde sulphuric acid reagent.

The identity of the quercetin bands in sample chromatograms was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution. HPTLC analysis of methanol extract of *Abelmoschus esculentus.* (L) flower was carried out along with the standard quercetin and toluene: ethyl acetate: formic acid: methanol (5.5:3:1:0.5) as the mobile phase. The identity of the bands of quercetin in the methanol extract was confirmed by comparing the UV-Vis absorption spectra with those of standards using a CAMAG TLC scanner3 (Figure 1). The standard quercetin has Rf value of 0.56 for quercetin (Table 1 and Figure 2). The 3-D spectrum of all tracks scanned at 254nm is shown (Figure 4).

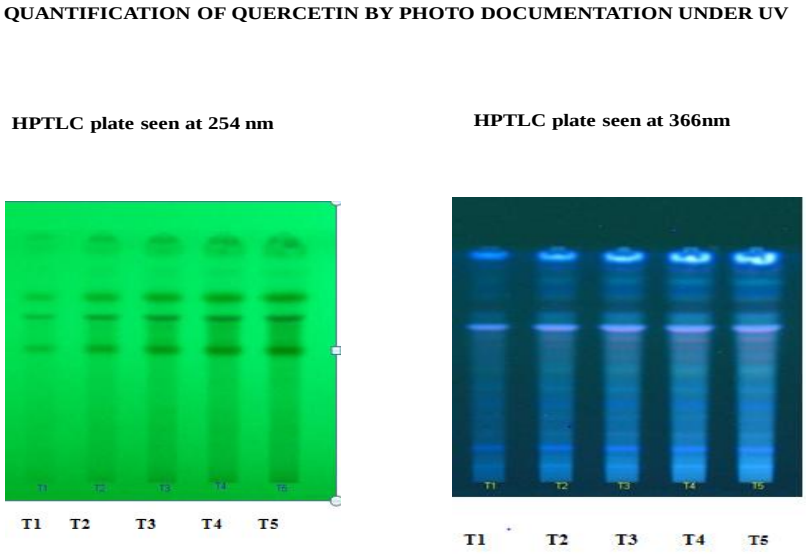
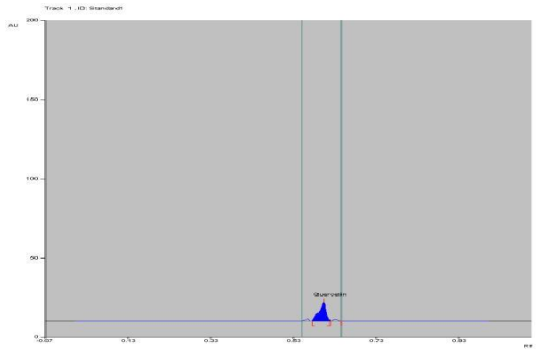
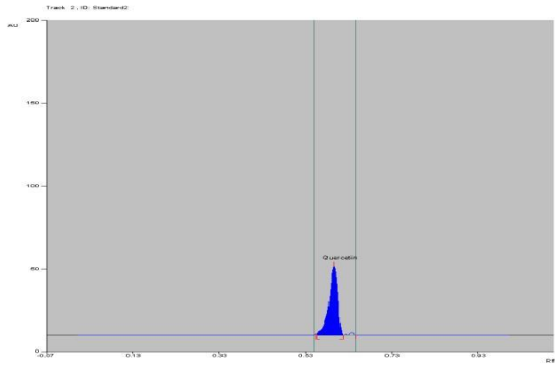


Figure 1: Chromatogram after derivatization, A) Under UV 254nm B) Under UV 366nm.

PEAK DISPLAY (0.5µl of Standard)

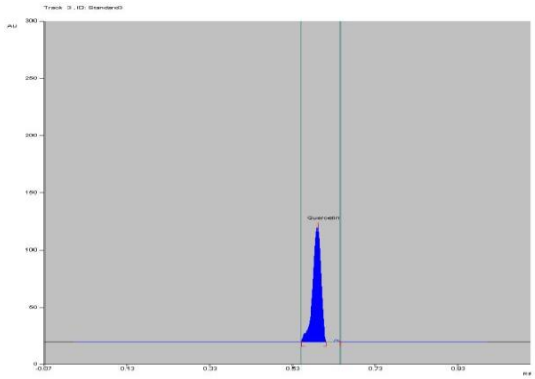


PEAK DISPLAY (1µl of Standard)

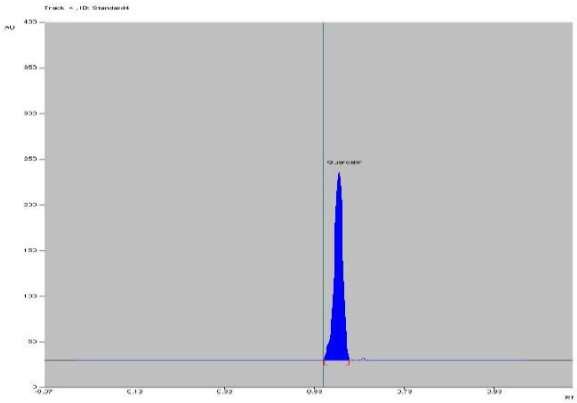


PEAK DISPLAY (1.5µl of Standard)

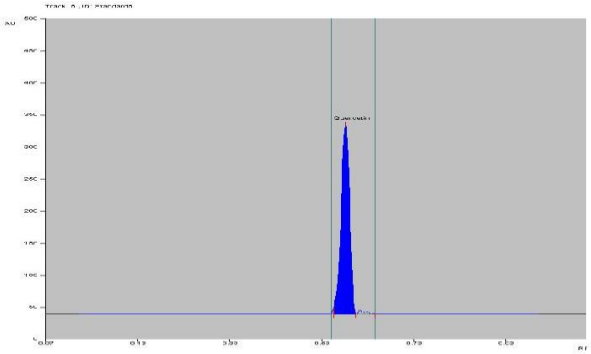
PEAK DISPLAY (2µl of Standard)



PEAK DISPLAY (2.5µl of Standard)



PEAK DISPLAY (3µl of Standard)



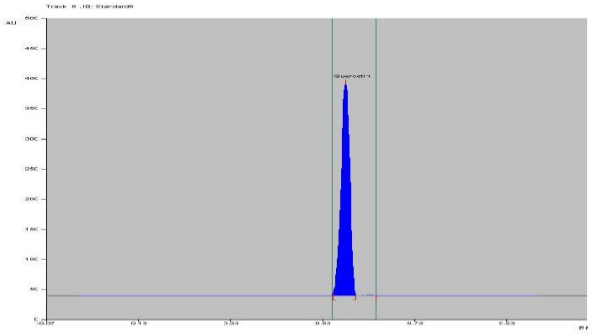
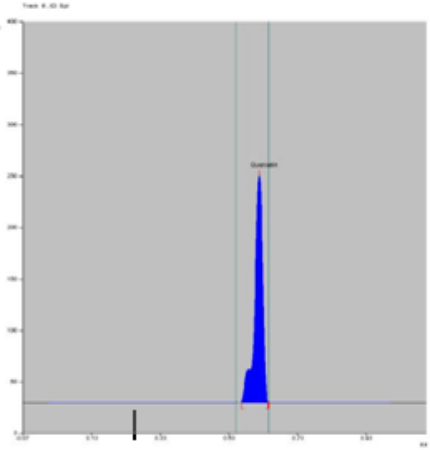


Figure 2: Chromatogram of Standard Quercetin (0.5µl to 3µl of standard)

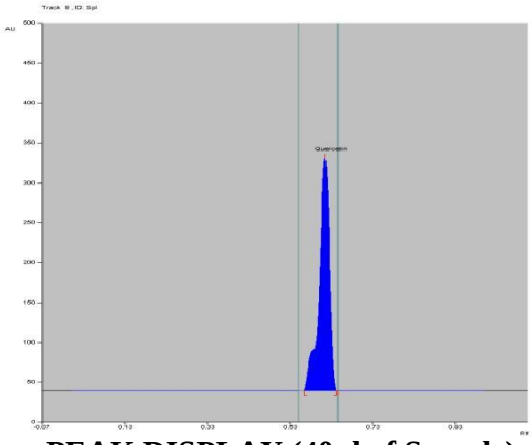
Table 1: Shows peak table with Rf values, height and area of Quercetin sample in methanol extract of *Abelmoschus esculentus.* (L) flower

Peak	Standard (µl)	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1.	0.5	0.57	0.0	0.60	119	100.00	0.62	0.7	166.8	100.0
2.	1.0	0.55	1.2	0.59	418	100.00	0.61	0.1	668.0	100.0
3.	1.5	0.55	0.9	0.59	100.4	100.00	0.61	0.2	1510.7	100.0
4.	2.0	0.55	1.6	0.58	206.8	100.00	0.61	0.5	3041.3	100.0
5.	2.5	0.55	9.6	0.58	292.4	100.00	0.60	1.4	4289.2	100.0
6.	3.0	0.55	4.3	0.58	352.0	100.00	0.60	1.3	5229.0	100.0
7.	3.5	0.55	4.6	0.58	385.7	100.00	0.60	0.8	5990.0	100.0

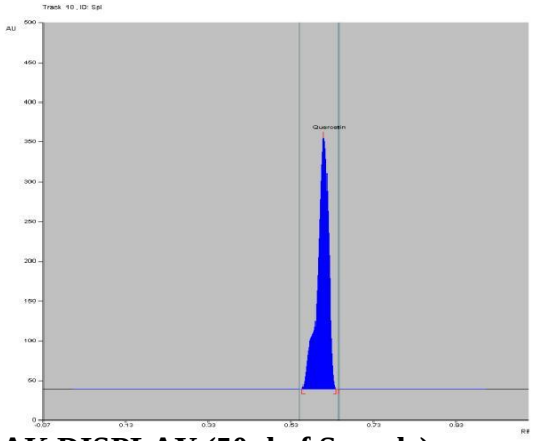
PEAK DISPLAY (10µl of Sample)



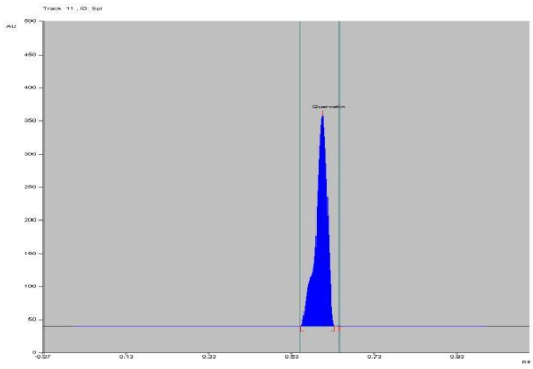
PEAK DISPLAY (20µl of Sample)



PEAK DISPLAY (30µl of Sample)



PEAK DISPLAY (40µl of Sample)



PEAK DISPLAY (50µl of Sample)

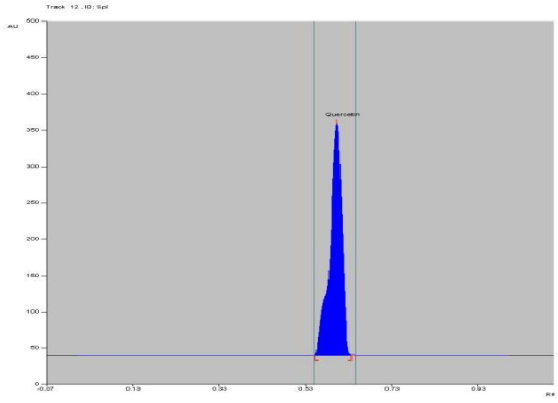


Figure 3: Chromatogram of *Abelmoschus esculentus.* (L) flower extract (10µl to 50 µl of sample)

Table 2: Shows peak table with Rf values, height and area of sample in methanol extract of *Abelmoschus esculentus.* (L) flower

Peak	Sample (µl)	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1.	10	0.56	0.6	0.62	221.2	100.00	0.64	0.6	4091.4	100.0
2.	20	0.56	0.5	0.61	290.1	100.00	0.64	0.2	5689.3	100.0
3.	30	0.56	0.3	0.16	316.3	100.00	0.64	0.2	6683.7	100.0
4.	40	0.55	0.1	0.60	318.8	100.00	0.63	0.6	6839.2	100.0
5.	50	0.55	1.0	0.60	319.1	100.00	0.63	1.6	7084.8	100.0

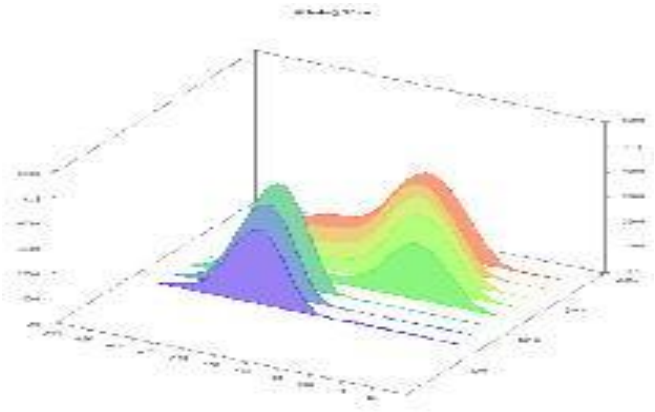


Figure 4: 3D display of HPTLC chromatogram of flower extracts of *Abelmoschus esculentus.* (L)

TABLE 3: COMPOUNDS IDENTIFIED BY LC-MS

Sr. No.	R.T (min)	Compound	[M _ H]	[M + H]
1	11.5-11.7	PROTOCATECHUIC ACID-4-*O*-GLUCOSIDE	315.1	-
2	19.8-19.9	KAEMPFEROL-3-O-HEXOSIDE	447.1	-
3	19.9-20.3	I SORHAMNETIN-3-O-GLUCOSIDE	477.1	-
4	24.0-24.1	QUERCETIN DIMER	603.1	-
5	26.6-26.9	QUERCETIN	301.0	-

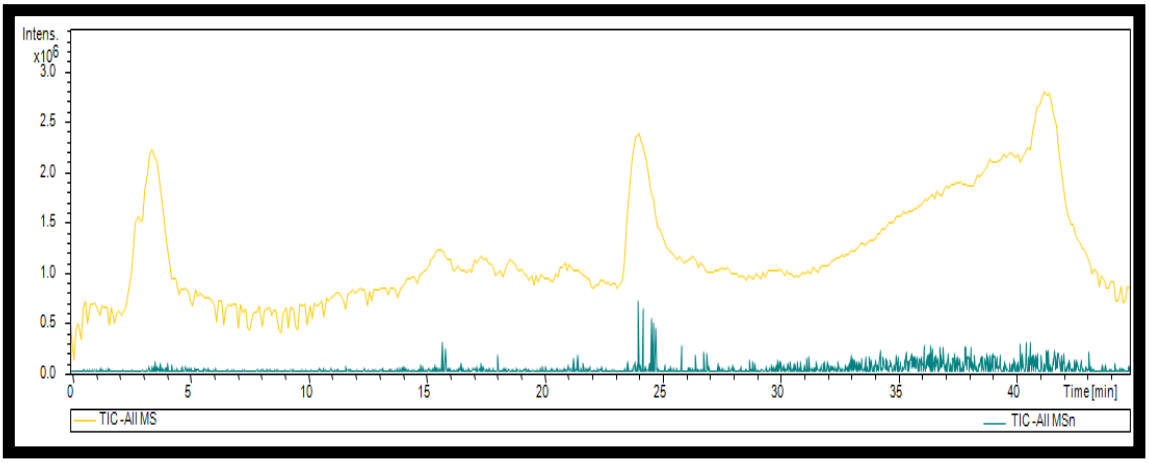


FIGURE 5: Total Ion Chromatogram obtained from LCMS (Negative mode)

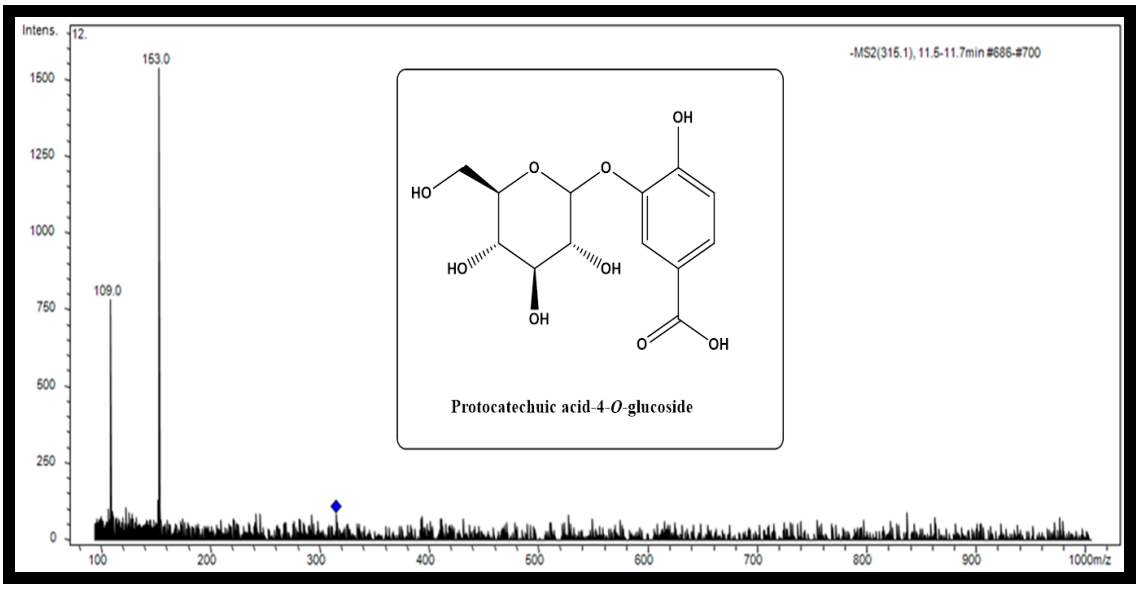


FIGURE 6: MSMS obtained from LCMS

PROTOCATECHUIC ACID-4-*O*- GLUCOSIDE

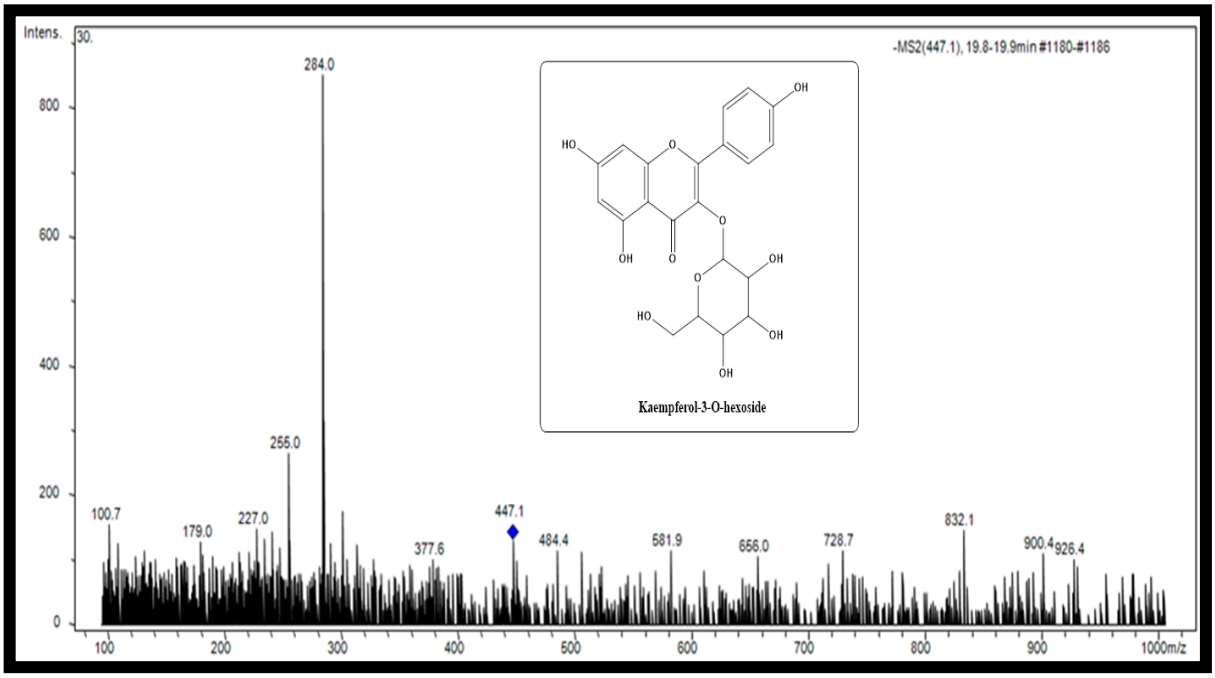


FIGURE 7: KAEMPFEROL-3-O-HEXOSIDE

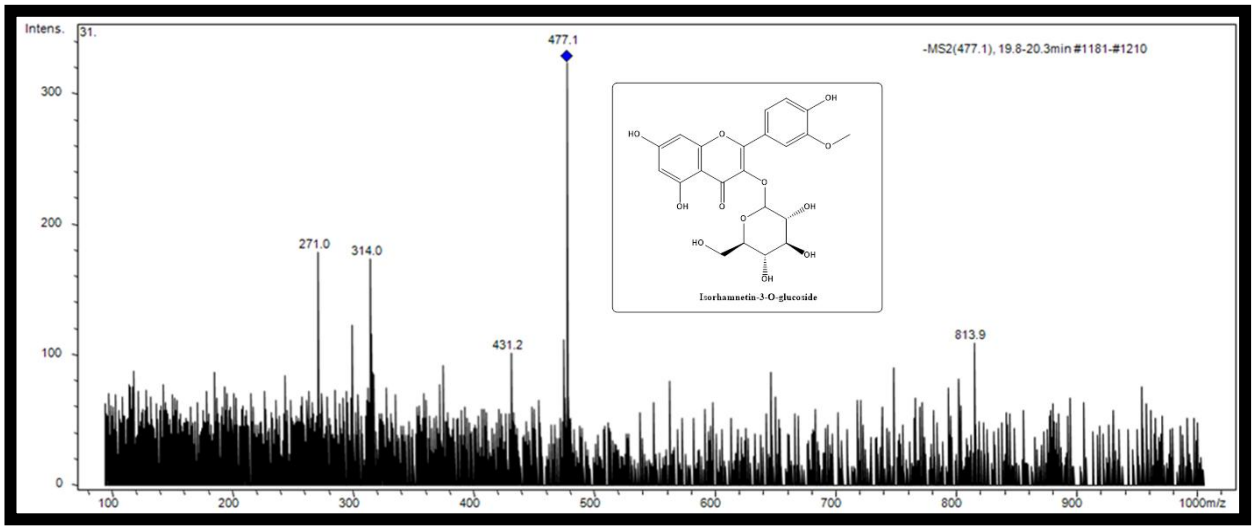


FIGURE 8: ISORHAMNETIN-3-O-GLUCOSIDE

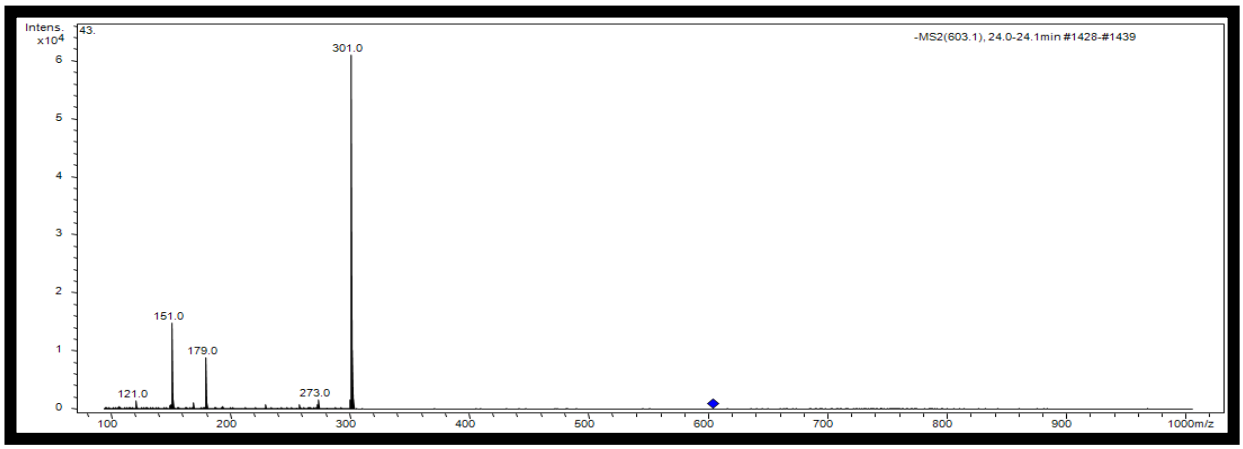


FIGURE 9: QUERCETIN DIMER

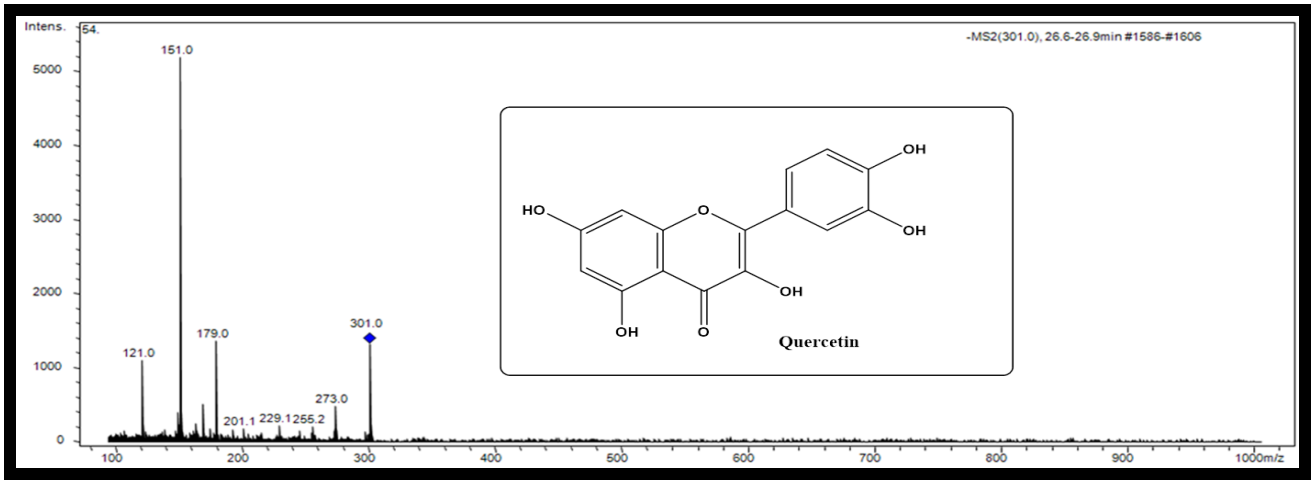


FIGURE 10: QUERCETIN

The methanolic extract was subjected to LCMS analysis. Secondary metabolites present in methanol extracts of *Abelmoschus esculentus (L)* flower such as terpenoids, saponins, flavonoids, tannins, steroids and alkaloids were identified with the help of this technique. The active principles with their molecular weight, retention time and structure are presented in Table 3. The chromatogram and the double mass spectrum of the methanolic extract of the test drug are shown in Figure 5 to 10.

The methanolic extract was subjected to LCMS analysis to understand the major molecules present in the selected plant. In the methanol extracts of *Abelmoschus esculentus (L)* flower molecules such as Protocatechuic Acid-4-o-Glucoside, Kaempferol-3-o-Hexoside, Isorhamnetin-3-0- Glucoside, Quercetin Dimer and Quercetin derivative were identified.

DISCUSSION

In HPTLC profile, each and every metabolite has played specific role and function in harmony with other metabolites within the organization framework of the cells in the defense mechanism of the plants. Chromatographic fingerprinting of phytoconstituents can be used for the assessment of quality consistency and stability of herbal extracts or products by visible observation and comparison of the standardized fingerprint pattern [9]. WHO has emphasized the need to ensure the quality of medicinal plant products by using modern controlled techniques and applying suitable standards [10].

HPTLC profile was carried out as it could be used as a chemical standard to detect the genuineness of the selected plant drug. The pharmaceutical and nutraceutical industries are nowadays confronted with adulteration and cheating[11]. Determination of salient standards for herbal drug is inevitable in this field to check adulteration and substitution. HPTLC fingerprinting observed in the present study could serve as a chemical standard to check the quality and genuineness of the selected plant drug.

As mint showed highest concentration of quercetin it can be used as good sources of quercetin. Herbs are enormously important in both traditional and western medicine[12]. Quercetin is useful in some of the allergies such as hay fever, hives. It inhibits the production and release of histamine and other allergic/inflammatory substances possibly by stabilizing cell membranes of mast cells [13,14]. Quercetin is xanthine oxidase inhibitory by nature and hence prevents the production of uric acid, thereby easing the gout symptoms [15,16].

Liquid Chromatography -Mass Spectrometry (LC-MS) is proved to be a very useful technique for plant metabolite profiling and allows the identification of a large variety of common plant metabolites in a single chromatogram. The research reveals the potential of *Abelmoschus esculentus (L)* flower as a good source of bioactive compounds such as Protocatechuic Acid-4-*O*- Glucoside, Kaempferol-3-O-Hexoside, Isorhamnetin-3-O-Glucoside, Quercetin Dimer and Quercetin derivatives that justify the use of this plant for its various ailments by traditional practitioners [17,18].

CONCLUSION

The HPTLC fingerprinting analysis showed that quercetin is present within the methanol extract of *Abelmoschus esculentus (L)* flower. HPTLC fingerprinting observed in the present

study could serve as a chemical standard to check the quality and genuineness of the selected plant drug. The methanolic extracts of *Abelmoschus esculentus (L)* flower confirmed the presence of molecules such as Protocatechuic Acid-4-o-Glucoside, Kaempferol-3-o-Hexoside, Isorhamnetin-3-0- Glucoside, Quercetin Dimer and Quercetin derivatives by LCMS analysis.

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