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High Performance Liquid Chromatography Analysis Using Rutin Marker and Estimation of Phenolic & Flavonoid Compounds in the Extracts of Indian Medicinal Plant *Morus nigra* L.

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Morus nigra L. (black mulberry) belongs to moraceae family of Indian medicinal plants. Black mulberry has significant anticancerous, antioxidant, antidiabetic, antimicrobial and antiobesity activities. Rutin marker was used for high performance liquid chromatography technique. We used gallic acid and quercetin for phenolic compound determination and flavonoid content determination in plant leaf extract. Plant extract of this species was analyzed quantitatively and qualitatively through high pressure liquid chromatography. Rutin compound was the main marker, which is a flavonoidal compound. Standard solution (1 mg/mL) of rutin was prepared by using CH_3OH. Methanol:water (80:20, v/v) ratio was used to dissolve powdered *M. nigra* plant leaves (100 mg), in which 0.01 mg/g rutin content was found. Total phenolic content in *M. nigra* was found 43.15 ± 0.68 mg/g GAE (gallic acid equivalents). Total flavonoid content in *M. nigra* was found 5.8 ± 0.46 mg/g QE. It was found that the *M. nigra* has not significant rutin potency and found to be as 0.01 mg/g.

Keywords: *Morus nigra* L., HPLC, Total phenolic, Flavonoids, Rutin.

INTRODUCTION

Plants contain biologically active compounds to treat severe as well as infectious diseases. Herbal drugs have no side effects and less expensive as compared to synthetic drugs, so it may easily reachable to poor people. Almost all parts of the plant are used as medicine such as leaves, fruits, flowers, seeds, roots, barks, stems and peels [1-4].

Morus nigra (black mulberry) belongs to moraceae family. It is known as 'Shahtoot' (Hindi),Tuta (Sanskrit), Tuti (Marathi) and Toot (Persian). Plant contain tannins, saponins, terpenoids, flavonoids, sitosterols, morusimic acid, anthocyanins glycosides and alkaloids are main active principles [5-8]. Mulberry plants are widely cultivated to feed the silkworm. It is an economically important plant used in sericulture. The pupa (cocoon) which is used to make silk. It is also used as diuretic, antiulcer, antibacterial, laxative, anticancer, antimicrobial, antioxidant, antihypertensive, antihyperglycemic, antihyperlipidemic, brain tonic and antidiabetic [9-11].

Morus nigra is a medium or small sized deciduous tree, 6 to 9 m in height and 1.5-3.0 m in diameter. Leaves are variable, in size and shape, usually 7 to 12 cm long, simple, alternate , broadly ovate-cordate, serrate usually undivided, sometimes 1-2 lobed, thick, 3-nerved. Flowers are monoecious or dioecious, greenish yellow with brown stigma branches. Inflorescence are catkin type, sepals and styles are densely hairy [12-15]. Trunk bark of grown-up trees are brownish gray, consisting of narrow strips that are separated by shallow furrows. It is native of south western Asia and cultivated in many countries for its edible fruits. It has an ovoid to oblong composite fruit. Colour of fruits is dark purple, almost black after ripenning, 2-3 centimeters in length. It is a compound cluster of several tiny drupes. The black colour of fruits is due to the presence of anthocyanins [16-18].

High performance liquid chromatography (HPLC) technique is used for the separation, detection, purification and quantification of the various components of the natural products (bioactive compounds) such as rutin, quercetin, ellagic acid, chloro-

genic acid, *etc*. [19]. Its fingerprinting patterns show the presence of multiple compound in the sample. By comparing the retention times of the samples against the standards, known peaks were identified. This method is also used for the determination of meloxicam in human serum. Meloxicam, a non-steroidal drugs, is used as antipyretic, anti-inflammatory and analgesic drug [20].

EXPERIMENTAL

HPLC analysis: The sample of dried leaves (100 mg) of *Morus nigra* was subjected to solvent (3 × 10 mL, H_2O:CH_3OH 20:80, v/v) in ultrasonic exposure (30.0 cm × 25.0 cm × 12.5 cm at 34 ± 3 kHz, Mumbai, India) at 40 ºC. Centrifugation (3920 g for 10 min) was also applied. Standard solution (1 mg/mL) of rutin was prepared in CH_3OH [21-24].

Fingerprint profile of *Morus nigra* was developed according to previous HPLC reported methods with some changes. Separation was done with optimized solvent composition of acidified H_2O and CH_3OH by applying Phenomenax Luna (250 × 4.6 mm, 5 µm) C_{18} column [25-27]. Applied marker, rutin was confirmed by retention time and UV-spectra matching in the sample and quantified by external standard method [28,29].

Estimation of total phenolic contents: Folin-Ciocalteu reagent and gallic acid were used for the determination of phenolic compounds in alcoholic extract of *M. nigra* [30,31]. 100 mg of gallic acid was dissolved in 100 mL of CH_3OH, for the preparation of stock solution (1.0 mg/mL). Different curves (for calibration) were found by using 1 mL aliquots (gallic acid solutions) of 2.5, 5.0, 10, 20, 30, 40 and 50 µg/mL solutions of gallic acid with 5.0 mL of Folin-Ciocalteu reagent and 4 mL of 7.5% Na_2CO_3 solution [32-34]. Then, 100 mL of ethanol, having purity of 95 % was mixed with 10 mL of extracts (morus leaves) for the preparation of stock solution. The concentration of 100 µg/mL of leaf extracts were also made by using ethanol having purity of 95 %. The measurement of absorbance of reaction samples were done at 760 nm by using UV-visible spectrophotometer (Lasany, Li-2800 series). For the better estimation, every run was analyzed in triplicate [35,36].

Estimation of total flavonoid contents: As a standard compound, quercetin was used for estimation of flavonoids [37]. The concentration of 100 µg/ml of leaf extracts were made by using CH_3OH having purity of 95 %. Every sample of 0.5 mL was introduced into a separated test tubes and added with 1.5 mL of CH_3OH, 0.1mL of 10 % $AlCl_3$, 0.1 mL of 1.0 M CH_3COOK and 2.8 mL of distilled water. The measurement of absorbance of reaction samples were done at 760 nm by using a UV spectrophotometer (Lasany, Li-2800 series). For the better estimation, every run was analyzed in triplicate [38-40].

RESULTS AND DISCUSSION

HPLC chromatogram of standared compound *i.e*. rutin is the process of confirmation of the responsible for antidiabetic activity of *Morus nigra*. Fig. 1 shows the HPLC chromatogram indicating the detection of rutin (39.531 min) in *Morus nigra* L. species. The values were estimated at 254 and 4 nm.

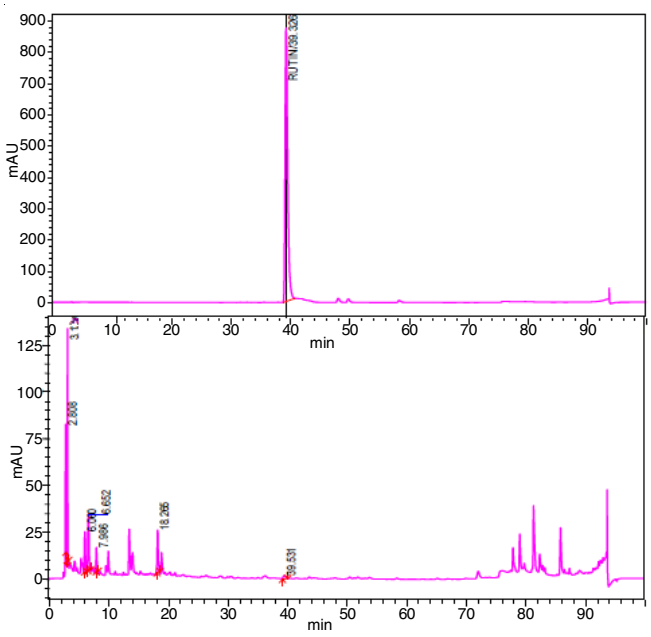

Fig. 1. The comparative HPLC chromatogram for rutin determination in *M. nigra* species

Based on the modified HPLC method by modifying the mobile phase gradient and the sample dilution and also including the markers of quercetin and total flavonoids besides rutin. All chromatographic peaks exhibited typical flavonoid UV absorption profiles, and so peaks 3 (rutin), 4 (isoquercitrin), 5 (unknown) and 7 (quercetin) (Fig. 2) were expressed as rutin to monitor the stability of the *Morus nigra* L. species. Thus total phenolic and flavonoid contents can be determined and the results are shown in Table-1.

Excel 2007 software was used for data analysis. The spectrophotometric determinations represent the average (mean) ± standard deviation in triplicate. The quantity of total phenolic content in extract was estimated by a linear gallic

TABLE-1
TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENTS OF *M. nigra*

Total phenolic: Standard compound (gallic acid) λ_{max} 760 nm				Total flavonoid: Standard compound (quercetin) λ_{max} 760 nm			
Concentration (µg/mL)	Absorbance (nm)			Concentration (µg/mL)	Absorbance (nm)		
	A1	A2	A3		A1	A2	A3
2.5	0.0299	0.0389	0.0412	0.8	0.0416	0.0451	0.0463
5	0.0335	0.0457	0.0435	1.6	0.0465	0.0513	0.0476
10	0.0386	0.0597	0.0489	3.12	0.0532	0.0557	0.0518
20	0.0478	0.084	0.0598	6.25	0.0746	0.0682	0.0624
30	0.0587	0.109	0.0703	12.5	0.1083	0.0905	0.0782
40	0.0676	0.1337	0.0823	25.0	0.1887	0.1431	0.1065

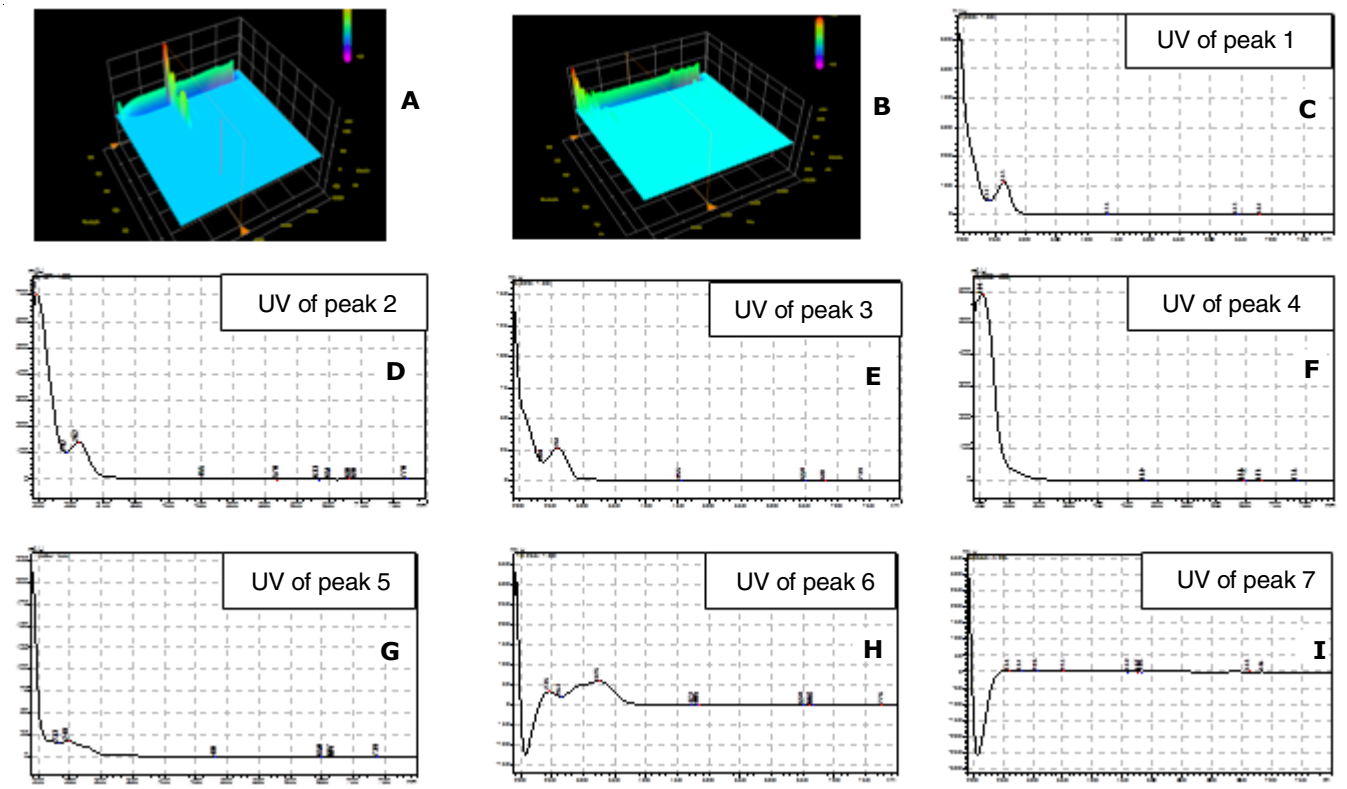

Fig. 2. The rutin marker in *M. nigra* was confirmed by UV-spectra matching in the samples and quantified by external standard method. **A:** fingerprint of rutin marker, **B:** fingerprint of rutin in *M. nigra*, **C, D, E, F, G, H, I:** are peaks at retention times 2.808, 3.129, 6.060, 6.652, 7.986, 18.265, 39.531 respetively

acid standard curve (standard curve equation y = 0.001x + 0.0281, R^2 = 0.9986). Standard compound (gallic acid) and the total phenolic content was expressed as mg/g GAE (gallic acid equivalents). The total phenolic content in *Morus nigra* was found 43.15 ± 0.68 mg/g GAE. Moreover, linear standard quercetin curve was used to estimate flavonoid compounds in the extract (standard curve equation y = 0.0061x + 0.0357, R^2 = 0.9989), while total flavonoid contents in *Morus nigra* was found to be 5.8 ± 0.46 mg/g QE (quercetin equivalents).

Conclusion

Plant leaf extract of *Morus nigra* was analyzed by high pressure liquid chromatography (HPLC) for qualitative and quantitative analyses. The total phenolic contents in *Morus nigra* was estimated as 43.15 ± 0.68 mg/g GAEm while the total flavonoid contents in *Morus nigra* was found to be 5.8 ± 0.46 mg/g QE. It was also found that *Morus nigra* has not significant rutin potency due to its low contents (0.01 mg/g).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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