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Quantitative analysis of biomarkers in hydro-ethanolic extract of *Aegle marmelos* by High Performance Thin Layer Chromatography (HPTLC)

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Abstract

In the present study the quantitative phytochemical analysis of extract of unripe fruits of *Aegle marmelos* was carried out with specific biomarkers like gallic acid, quercetin and rutin by high-performance liquid chromatography (HPLC). The detection and quantification of these markers were performed at 200 - 450 nm wavelength with reference to standards in mobile phase Toulene: Ethyl Acetate: Formic Acid: Methanol (3:3:0.3:0.2). The hydro-ethanolic extract of unripe fruits of *Aegle marmelos* showed peak *R_f* values which were coinciding with standard *R_f* value of gallic acid and quercetin. The *R_f* values for gallic acid and quercetin in sample and reference standard were found comparable. These observations indicated the presence of gallic acid and quercetin in hydro-ethanolic extract of unripe fruits of *Aegle marmelos*. Results of this study concludes that the presence of gallic acid and quercetin that might be responsible for its antidiarrhoeal, antibacterial, astringent and anti-inflammatory activity in extract.

Keywords: Gallic acid, *Aegle marmelos*, HPTLC, biomarkers, quercetin

Introduction

Aegle marmelos is an important medicinal plant with wide ethno-medicinal applications in traditional and folk medicinal systems. Antidiarrheal activity is one of the major medicinal properties of *A. marmelos* which have been extensively used to control chronic diarrhea and dysentery^[1, 2]. Recently, several *in vitro* and *in vivo* studies have been conducted to confirm the antidiarrheal property of *A. marmelos*. It has also been traditionally used for the treatment of various infectious diseases. Many workers reported antibacterial activity of the leaves, fruits and barks of *Aegle marmelos*^[3, 4, 5].

Decoction of unripe fruit is astringent, useful in diarrhea and chronic dysentery. *Aegle marmelos* fruit is astringent, laxative, digestive, stomachic, brain and heart tonic as reported by several researchers^[6]. The ripe fruit promotes digestion and is helpful in treating inflammation of rectum. Methanol and aqueous extract of Beal fruit have shown strong activity against multi drug resistant *S. typhimurium*. The effectiveness of *Aegle marmelos* fruit in diarrhea and dysentery has resulted in its entry into the British pharmacopoeia^[7].

A. marmelos plant has been blessed with seven major phytochemicals such as alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids and steroids which are biologically active and have been the major source in curing different diseases. Extensive investigations have been carried out on different parts of *A. marmelos*^[8]. Anti-diarrhoeal and anti-dysenteric of medicinal plants were found to be due to the presence of tannins, alkaloids, saponins, flavonoids, steroids and terpenoids^[9]. In the past, many research has been done on this phytochemical aspects and the studies have shown that the fruit pulp of *A. marmelos* contains bio active substance like carotenoids, tannins, flavonoids, terpenoids, alkaloids, pectins, reducing sugar, saponins, carbonyls, phlobatanins and steroids^[10, 11, 12].

However, very scanty information is available on quantification of phytochemical evaluation of extract of Beal fruit. Hence, unripe fruits extract of *Aegle marmelos* was selected to undertake quantitative phytochemical studies. In the present study, quantitative phytochemical analysis (HPTLC) of unripe fruits extract of *Aegle marmelos* was carried out to know the active bioactive compounds responsible for anti-diarrhoeal and antibacterial activity present in

this plant. Gallic acid, rutin and quercetin which are important active constituents of medicinal plants selected in the present study were estimated by High performance thin layer chromatography (HPTLC) method.

Materials and Methods

Preparation of Cold Extract

The unripe fruits of *Aegle marmelos* were collected and shade dried and processed to get fine powder with the help of pulverizing machine. Freshly prepared powder (25 g) was immersed in hydro-ethanolic solution (40% distilled water + 60% ethanol) in a flask stoppered tightly with cotton plug and kept on orbital shaker at room temperature for 48 hours at 150 rpm. The contents of the flask were filter through Whatman No. 1 filter paper. Final filtrate was evaporated and per cent extractability was determined. The extract was store in airtight screw cap vials and kept in the desiccator until further use in this study.

High Performance Thin Layer Chromatography [HPLC] Methodology

The present study was directed towards the quantitative estimation of biomarkers gallic acid, Rutin and Quercetin in hydro-ethanolic extracts of unripe fruits of *Aegle marmelos*.

Instrumentation

Analysis was performed on a CAMAG HPTLC system equipped with a sample applicator Linomat V, 100 ml syringe (Hamilton, Bonaduz, Switzerland), twin trough development chamber (20 cm x10 cm) size, TLC Scanner III linked to WinCats software (CAMAG), 0.2 mm thickness pre-coated with silica gel 60 F254 (Merck) were used in this study.

Reagents and Chemicals

Analytical grade Toluene, Ethyl acetate, Methanol, Formic acid, was obtained. Gallic acid, Quercetin and Rutin were obtained from the Natural Remedies Ltd, Bangalore. Precoated TLC aluminium sheets silica gel 60F254 (10 x10 cm, 0.2 mm thick) were obtained from E. MERCK KGaA.

Procedure

Analysis was performed on 20 cm x 10 cm HPTLC silica gel G60 F254 plates with fluorescent indicator. Before starting the analysis, HPTLC plates were cleaned by predevelopment with methanol by ascending method. HPTLC plate was immersed in a CAMAG glass chamber (20 cm x 10 cm), containing 30 ml methanol (HPLC grade) as solvent system. The chamber was covered with glass lid and left till development of the plate to the top with methanol. After complete development, the plate was removed from TLC glass chamber and dried in an oven. Three spots of 10 µl were applied (in the form of band) of standard preparation along with three spots of 10 µl of sample preparation as the bands on the same plate by means of a CAMAG Linomat 5 (automated spray-on applicator equipped with a 100 µl syringe and operated with the settings band length 6 mm, distance between band 15 mm, distance from the plate side edge 15 mm and distance from the bottom of the plate 15 mm).

TLC Development and Scanning (Chromatographic Conditions)

Samples of extracts and standard Gallic acid and Rutin were spotted on a Precoated TLC aluminium sheets silica gel 60 F254 (10x10cm, 0.2 mm thickness) as 8 mm wide band width

by using automatic TLC applicator Linomat V, 10 mm from the bottom. The Mobile phase used was Toluene: Ethyl Acetate: Formic Acid: Methanol (3:3:0.3:0.2) for Gallic acid, Quercetin and Rutin. The plates were kept for saturation in twin trough chamber for 15 min. After development the plates were dried in oven and scanned at 200, 250, 300, 350, 400, 450 nm for Gallic acid, Quercetin and Rutin by using CAMAG Scanner III.

Calibration Curve for Standards

The standard solutions were applied on TLC plate and further it was developed and scanned as per the chromatographic conditions mentioned above. The peak areas were recorded. Calibration curve of Gallic acid, Quercetin and Rutin was prepared by plotting peak area against concentration of standard applied.

Results and Discussion

In the present analysis, the given samples were measured at wavelength 200 to 450nm with reference to Gallic acid, Rutin as Rutin trihydrate and Quercetin as Quercetin dihydrate as standards in mobile phase Toluene: Ethyl Acetate: Formic Acid: Methanol (3:3:0.3:0.2).

The HPTLC profile for hydro-ethanolic unripe fruits extract of *Aegle marmelos* is presented in Table 1. The R_f value of standard gallic acid scanned at 200, 250, 300, 350, 400 and 450 nm were found to be 0.19, 0.18, 0.16, 0.19, 0.20 and 0.20, respectively. The average R_f value of gallic acid was 0.18, showed peak of gallic acid. In gallic acid standard peak number 3 at wavelength 250nm, 350nm, 400nm and 450nm found in average 83.05% area i.e. more pure form of gallic acid at R_f 0.18 and at wavelength 200 and 300nm found R_f 0.18 shows 67.21% and 88.23% area in peak number 4 and 2, respectively. It confirmed that gallic acid was present in the sample.

The standard rutin was scanned at 200, 250, 300, 350, 400 and 450 nm, however, no peak was seen at wavelength 200 to 450nm. It confirmed that rutin was absent in the sample.

The R_f value of standard quercetin scanned at 200, 250, 300, 350, 400 and 450 nm were found to be 0.32, 0.30, 0.28, 0.29, 0.29 and 0.30, respectively. The average R_f value of quercetin was 0.29, showed peak of quercetin. In Quercetin dihydrate standard peak no. 2 at 250 to 450 nm and peak no. 3 at 200nm found highest purity i.e. in average 85.23% area and R_f 0.29. It found that sample contain Quercetin dihydrate.

In track 5 of *Aegle marmelos* at wavelength 200 to 350 nm compound gallic acid was found at R_f 0.21 i.e. very nearer to standard of gallic acid peak, showed presence of gallic acid. No peak for gallic acid was evident at wavelength 450nm. At wavelength 200 to 350nm compound quercetin was found with R_f 0.33 i.e. R_f value was coinciding with standard R_f value of quercetin, showed presence of quercetin. No peak was seen in track 5 and 6 of standard Rutin. It confirmed the absence of rutin in the sample.

The R_f values for gallic acid and quercetin in sample and reference standard were found comparable. These observations indicated the presence of gallic acid and quercetin in hydro-ethanolic unripe fruits extract of *Aegle marmelos* (Table 1). Some workers also characterized the various phenolics in the fruit extract of *Aegle marmelos* as chlorogenic acid, gallic acid, ellagic acid and quercetin through LC-MS and HPLC studies^[13].

The HPTLC chromatograms are useful in identification of biomarkers in various herbal formulations by comparing the fingerprints with standards. Gallic acid; rutin and quercetin

are important active constituents of many herbal plants. Rutin and quercetin are polyphenol flavonoids found in many herbal plants. They mainly include flavonoids (e.g. quercetin), glycosides (e.g. rutin) and simple phenols (e.g. gallic acid). Flavonoids is a group of polyphenol compounds, which are widely distributed through the plant tissues. Flavonoids occur in plant tissues in a free state, (Quercetin: 5, 7, 3', 4'-tetrahydroxy flavonol), or in glycosides (Rutin: 5, 7, 3, 4, tetrahydroxy flavonol-3-rhamnoglucoside). Quercetin and rutin are the most common flavonoids found in plant tissues. [14] Gallic acid is a type of simple phenol compounds (3, 4, 5-trihydroxybenzoates) found commonly in plant tissues. It possesses an astringent activity. Tannins and flavonoids in general have been reported to have antidiarrhoeal activity through inhibition of intestinal motility, antimicrobial action and antisecretory effects [11]. Therefore, in the present quantitative estimation, the gallic acid is well represented in chromatogram.

Many workers reported presence of carbohydrates, glycosides, amino acids, proteins, tannins, flavanoids, phytosterols in extract of dried unripe fruit pulp of *A. marmelos*. [10, 11]. Some workers reported the presence of chemicals like Alkaloids, Cardiac glycosides, Terpenoids,

Saponins, Tannis, Flavonoids and Steroids in crude ethanolic extracts of Bael fruit which was effective against the tested organisms *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* due to the presence of these bioactive constituents in the extract [9]. Many researchers reported alkaloids, *N*-2-[4-(3',3'-dimethylallyloxy) phenyl] ethyl cinnamide, *N*-2-hydroxy-2-[4-(3',3'- dimethylallyloxy) phenyl] ethyl cinnamide or marmeline, *N*-4- methoxystyryl cinnamide and *N*-2-hydroxy-2-(4-hydroxyphenyl) ethyl cinnamide and aegeline from fruits of *A. marmelos* [15, 16].

The present quantitative analysis of three biomarkers by the HPTLC methods indicated that the gallic acid was the most abundant biomarker present in the extract, as it is well represented in chromatogram. HPTLC study of extract showed presence of gallic acid and quercetin that might be responsible for its anti-diarrhoeal, antibacterial, astringent and anti-inflammatory activity in extract. Thus, the results of the present study support the anti-diarrhoeal, antibacterial, astringent and anti-inflammatory activity in extract of unripe fruits of *Aegle marmelos*. In the present investigation, HPLC method was found effective for the separation of rutin, quercetin and gallic acid.

Table 1: HPTLC profile for standard biomarkers and hydroethanolic extract of unripe fruits of *Aegle marmelos*

S. No.	Name of sample	Wave length (in nm)						Observation	Inference
		200	250	300	350	400	450		
1	Gallic acid std	Rf - 0.19 % Area -67.21 Peak no.- 4	Rf - 0.18 % Area -78.09 Peak no.-3	Rf -0.16 % Area-88.23 Peak no.-2	Rf - 0.19 % Area -82.63 Peak no.- 3	Rf - 0.20 % Area - 86.11 Peak no.-3	Rf -0.20 % Area -85.37 Peak no.-3	At Rf 0.18 shows peak of Gallic acid	Gallic acid is present
2	Rutin trihydrate std							No peak	No peak
3	Quercetin dihydrate std	Rf - 0.32 % Area-72.35 Peak no.- 3	Rf - 0.30 % Area -78.35 Peak no.-2	Rf -0.28 % Area -83.24 Peak no.-2	Rf -0.29 % Area-90.02 Peak no.-2	Rf -0.29 % Area - 18.7 Peak no.-2	Rf -0.30 % Area 94.54 Peak no.-2	At Rf 0.29 shows peak of Quercetin dihydrate	Quercetin dehydrate present
4	Sample - <i>Aegle marmelos</i> Track 5	Rf- 0.20 Rf- 0.38	Rf- 0.21 Rf- 0.33	Rf- 0.21 Rf- 0.29	Rf- 0.21 Rf- 0.30		No peak	No peak at 450nm	Gallic acid and Quercetin may be present
	Track 6	Rf- 0.19 Rf- 0.31	Rf- 0.21 Rf- 0.34	Rf- 0.22 Rf- 0.29	Rf- 0.21 Rf- 0.32	Rf- 0.23	No peak	No peak at 450 nm	Gallic acid and Quercetin may present

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