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Research Article

**SEPARATION AND QUANTIFICATION OF  
PHARMACOLOGICALLY ACTIVE MARKERS p-METHOXY  
BENZOIC ACID, 3, 4-DIHYDROXYBENZOIC ACID AND  
GALLIC ACID FROM CAPPARIS SPINOSA AND FROM  
MARKETED FORMULATION BY HPTLC**Vikas V. Vaidya<sup>1</sup>, Manjiri A.Shinde<sup>1\*</sup>, Pushkar M. Pradhan<sup>1</sup><sup>1</sup>Department of Chemistry, Ramnarain Ruia College, Matunga, Mumbai**Abstract:**

A simple, accurate, precise and reproducible High Performance Thin Layer Chromatographic (HPTLC) method for simultaneous quantification of p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and Gallic acid in the leaf extract of Capparis spinosa was developed. Chromatography was performed on silica gel 60 F<sub>254</sub> pre-coated HPTLC plates with double development using two solvent systems. Using first solvent system, Chloroform: Ethyl acetate: Formic acid: Glacial acetic acid 7.5 : 2 : 0.5 : 0.5 (v/v/v/v) the plate was developed till 80 mm while the second development was carried out till 85 mm using Chloroform: Ethyl acetate: Formic acid: Glacial acetic acid 6 : 2 : 1 : 1 (v/v/v/v) as the mobile phase. After development the HPTLC plate was dried on a hot plate at 40° C for 5 min and scanned at 254 nm. The method was found to give well separated sharp bands of p-methoxybenzoic acid, 3,4-dihydroxybenzoic acid and gallic acid at R<sub>f</sub> of 0.87, 0.66, 0.43 respectively. The quantity of p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and gallic acid was found to be 0.00666 %, 0.02626 % and 0.01095 %; in plant and 0.01291 %, 0.02423 % and 0.06381 % in formulation respectively. The method was validated in terms of linearity, specificity, precision and recovery. Statistical analysis proved that the proposed method is accurate and reproducible. The developed method can be used as a quality control tool for simultaneous quantification of these markers from raw material as well as marketed formulation.

**Keywords:** Capparis spinosa, p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid, Gallic acid, HPTLC, simultaneous quantification

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**INTRODUCTION:**

*Capparis spinosa* Linn. (syn. *C. aculeata* Steud, *C. microphylla* Ledeb) belongs to the family Capparaceae and is known as Himsra in Sanskrit and Caper bush in English. *Capparis spinosa* Linn. is a shrub growing in dry rocky and stony soils of North-Western India, through Punjab, Rajasthan and Deccan peninsular regions. *Capparis spinosa* Linn. is widely used in the traditional medicine of several American, African and Asian countries. *Capparis spinosa* is one such plant established to have highly diverse economic and medicinal value in different system of medicines like in Iranian, Unani, Chinese, Ayurvedic and Greco-Arabi System of medicines [1]. In Ayurveda it is used for the treatment of oedema, dermatopathies, heart disorders, anaemia, renal disorders, hepatic disorders and inflammatory disorders [2].

*Capparis spinosa* is commonly used medicinal plant, contained many biologically active chemical groups including, alkaloids, glycosides, tannins, phenolics, flavonoids, triterpenoids steroids, carbohydrates, saponins and a wide range of minerals and trace elements. It exerts many pharmacological effects including antimicrobial, cytotoxic, antidiabetic, antiinflammatory, antioxidant, cardiovascular, bronchorelaxant and many other effects. [3]

Different flavonoids were identified in caper bush and capers like rutin (quercetin 3-rutinoside), quercetin 7-rutinoside, quercetin 3-glucoside-7-rhamnoside, kaempferol-3-rutinoside, kaempferol-3-glucoside, and kaempferol-3-rhamnourutinoside, p-hydroxy benzoic acid; 5- (hydroxymethyl) furfural; bis (5-formylfurfuryl) ether; daucosterol;  $\alpha$ -D-fructofurano sides methyl; uracil; and stachydrine [3]. From *Capparis spinosa* fruits eight were isolated by chromatographic methods and their structures were established by spectroscopic methods as  $\beta$ -sitosterol, vanillic acid, p-hydroxybenzoic acid, protocatechuric acid, daucosterol, uracil, butanedioic acid and uridine. [3].

The three phytoconstituents selected has various pharmacological activities like p-methoxybenzoic acid shows anti-hepatotoxic activity [4], Gallic acid shows both antioxidant and anti-hepatotoxic activity [5] and 3, 4-dihydroxybenzoic acid shows hepatoprotective, antioxidant, anti diabetic activity [13]. The identification of these three phytoconstituents namely p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid, Gallic acid in various extracts were performed using HPLC [6] was addressed in some reports.

To the best of our knowledge, no efforts have yet been made to examine this drug combination by any HPTLC method. The objective of the present investigation is the development of a simple, precise and reproducible HPTLC method for the simultaneous quantification of three pharmacologically active markers p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid, and gallic acid from the leaf of *Capparis spinosa* Linn. and from marketed formulation Liver detox capsules of Planet Ayurveda. There are no methods reported for simultaneous separation and quantification of these three markers from any plant matrix.

This HPTLC method could serve as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

**MATERIALS AND METHODS:****Chemicals**

HPLC grade chloroform, ethyl acetate and methanol were procured from Merck Specialities Private Ltd, Mumbai, India.

Analytical grade Formic acid and glacial acetic acid were procured from Merck Specialities Private Ltd, Mumbai, India.

Reference Standards of p-methoxy benzoic acid (99 %) and 3, 4-dihydroxy benzoic acid (97 %) were purchased from Sigma-Aldrich.

Reference Standards of gallic acid (99 %) was purchased from Alpha Aesar.

**Plant material**

Plant specimen of *Capparis spinosa* was collected from Tamhini ghat, Pune. Herbarium sample was prepared and authenticated by Blatter Herbarium, St. Xavier's College, Mumbai, India. The whole plant was washed with water to remove dust particles, dried in shade, powdered and then sieved through mesh size 85 and stored in an airtight container.

Polyherbal formulation "Liver Detox capsules" manufactured by Planet Ayurveda, India were procured from Planet Ayurveda manufacturing unit, Punjab.

**Preparation of stock solutions**

Preparation of stock solution of p-methoxybenzoic acid 1000 ppm, 3, 4-dihydroxybenzoic acid 1000 ppm and of Gallic acid 1000 ppm

Standard stock solutions of pure drugs (p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid Gallic acid) were prepared separately by accurately weighing 25 mg of each drug in 25 mL of methanol to get concentration of 1000 µg/mL.

#### Sample Preparation

About 5 gram of dried leaf powder of Capparis spinosa was weighed into a round bottom flask. 100 mL of methanol was added to the flask and the mixture was kept on shaker at 100 rpm speed for 24 hours. The extract was then filtered through Whatmann filter paper no. 41 (E. Merck, Mumbai, India). The filtered solvent was evaporated to dryness. The residue (extracted material) so obtained was reconstituted into methanol with effective concentration of 1000 µg / 1000 µL. This solution was used for assay.

The formulation capsules were opened to get powder material out of them. 5 gram of this powder was put into round bottom flask and 100 mL of Diethyl ether was added to this flask and the mixture was kept on shaker at 100 rpm speed for 24 hours. The extract was then filtered through Whatmann filter paper no. 41 (E. Merck, Mumbai, India). The filtered solvent was evaporated to dryness. The residue (extracted material) so obtained was reconstituted into methanol with effective concentration of 1000 µg / 1000 µL. This solution was used for assay.

#### Chromatographic procedure

The stationary phase was HPTLC pre-coated silica gel aluminium plate 60 F<sub>254</sub>. The sample application was performed as per the chromatographic conditions mentioned in table 1. This was followed by detection at 254 nm for all the three markers (refer Figure 2).

**Table 1: Instrumentation and Chromatographic Conditions**

Parameters	Description
Stationary Phase	Silica gel 60 F <sub>254</sub> pre-coated on aluminium sheet
Mobile Phase	First Development : Till 80 mm Chloroform: Ethyl acetate : Formic acid : Glacial acetic acid 7.5 : 2 : 0.5 : 0.5 v/v/v/v Second development : Till 85 mm Chloroform : Ethyl acetate : Formic acid : Glacial acetic acid 6 : 2 : 1 : 1 v/v/v/v
Development Chamber	CAMAG Twin Trough Chamber
Chamber saturation	20 mins for both mobile phases
Sample Applicator	CAMAG LINOMAT IV
Band	8 mm
Speed	0.5 µL/sec
Development Distance	80 mm for first development 85 mm for second development
Drying of plate	Hot plate 40° C for 5 mins
Densitometric scanner	CAMAG TLC SCANNER III
Lamp	Deuterium
Wavelength	254 nm
Chromatographic evaluation	CAMAG TLC software winCATS 3

#### Validation of the Method [9]

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method. The summary of the validation parameters have been summarized in Table 2.

#### Specificity

Specificity was ascertained by analyzing standard compounds and samples. The bands from sample solutions were confirmed by comparing the R<sub>f</sub> and spectra of the bands to those of the standards. The

peak purity of all the compounds was analyzed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

#### Inter-Day and Intra-Day Precision

Variability of the method was studied by analysing quality control samples of p-methoxybenzoic acid (50, 200, 350 ng/spot), 3, 4-dihydroxybenzoic acid (100, 400, 700 ng/spot) and Gallic acid (50, 200, 350 ng/spot) on the same day (intra-day precision) and on

different days (inter-day precision) and the results were expressed as % RSD.

#### Linearity

Linearity of the components was determined at six different concentrations for Gallic acid, 3, 4-dihydroxybenzoic acid, p-methoxybenzoic acid. Calibration curve was plotted as mean peak area versus concentration.

#### Limit of Detection (LOD) and Limit of Quantification (LOQ)

Sensitivity of the method was evaluated by determining the values of LOD and LOQ. LOD and LOQ were calculated using the formula.

#### Recovery

The accuracy of the method was assessed by performing recovery studies at three different levels i.e 80%, 100% and 120 % , spiking p-

methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and Gallic acid in plant matrix and marketed formulation. The percent recovery and the average percent recovery for each component were calculated.

#### Stability

The stability of the stock solutions of all the three standards was evaluated by storing the stocks in refrigerator at 2-8° C and at room temperature for 72 hours. This was followed by comparing the concentrations of these stocks against freshly prepared stocks for each standard.

#### RESULTS AND DISCUSSION:

A HPTLC method for separation and simultaneous quantification of p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and Gallic acid in the leaf extract of Capparis spinosa and formulation was developed and validated successfully in the present research work.

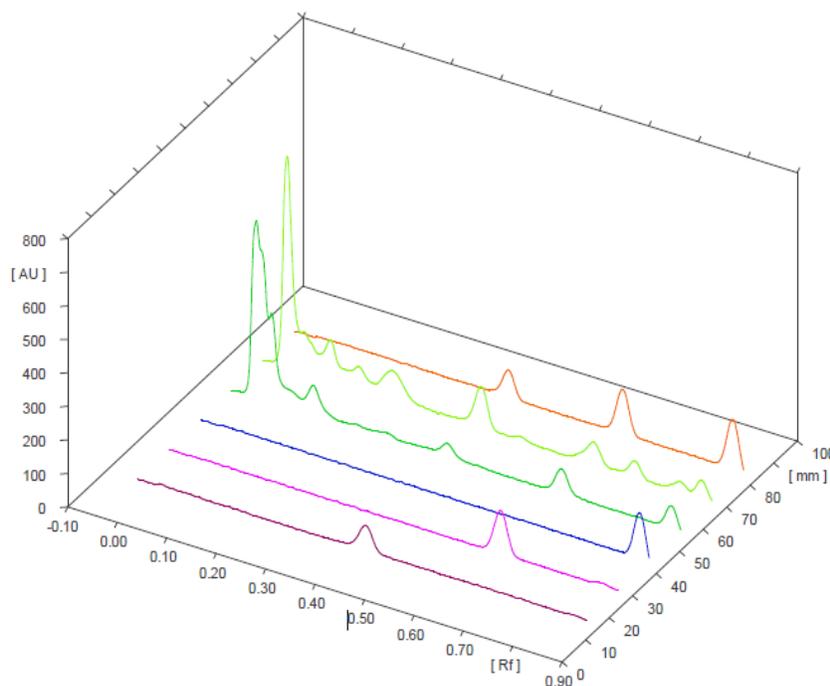
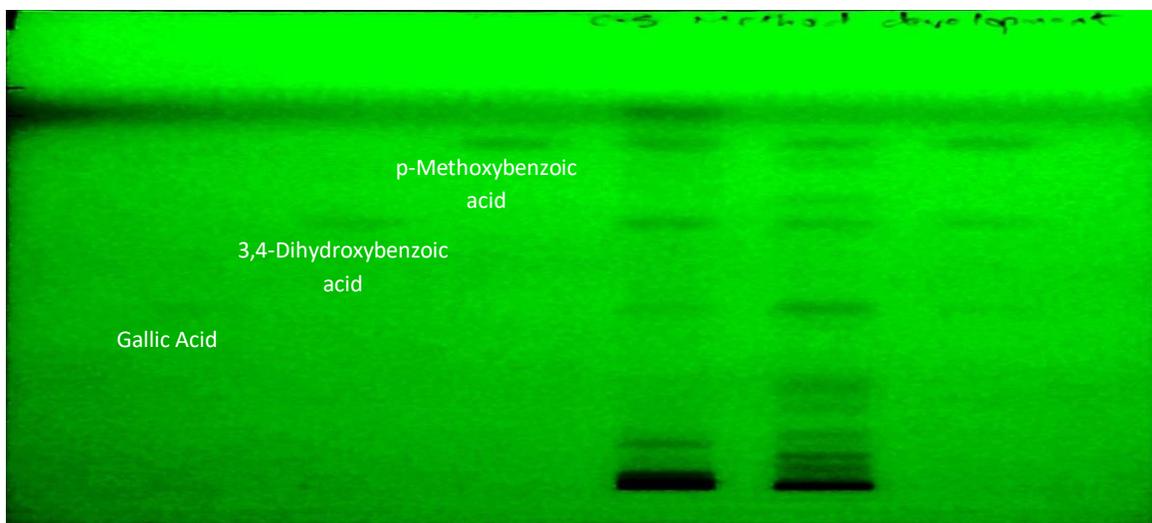


Fig. 1: 3D-Plot of Densitogram of three standards, plant, formulation and mixture of standards along with peak area and retention factor

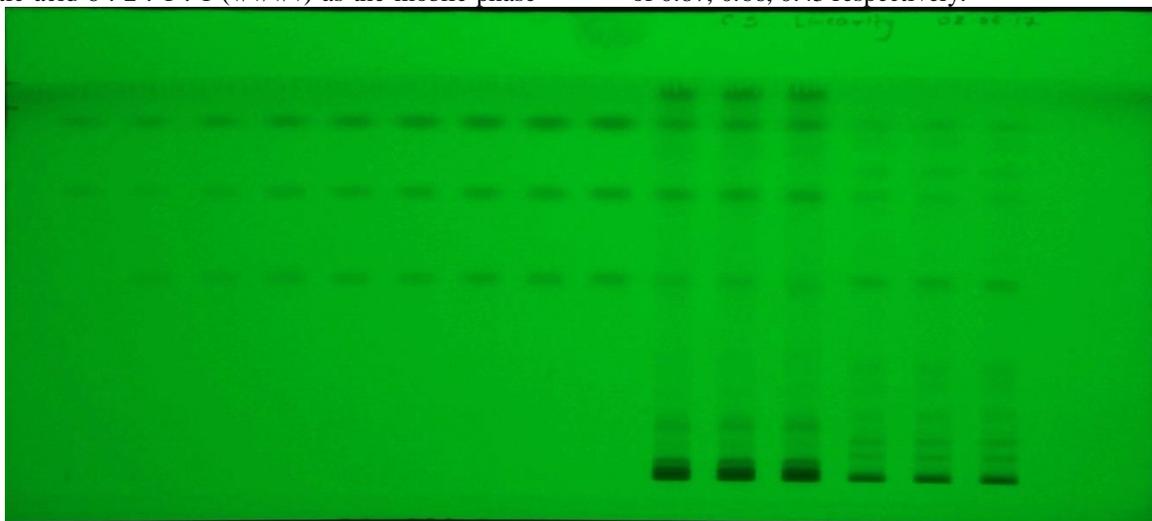


**Fig. 2: HPTLC plate for method development of p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and Gallic acid**

**Track1: Gallic acid, Track 2: 3, 4-dihydroxybenzoic acid, Track 3: p-methoxybenzoic acid, Track 4, 5: Plant Track 6, 7: Formulation**

Different mobile phases containing various ratios of Toluene, methanol, ethyl acetate, n-hexane were tried [6]. Also HPLC grade acetonitrile and 0.01M NaH<sub>2</sub>PO<sub>4</sub> was used as mobile phase containing different ratios [7]. Among the different solvents systems investigated, a double development method with mobile phase Chloroform: Ethyl acetate: Formic acid: Glacial acetic acid 7.5 : 2 : 0.5 : 0.5 (v/v/v/v) the plate was developed till 80 mm and second development was carried out till 85 mm using Chloroform: Ethyl acetate: Formic acid: Glacial acetic acid 6 : 2 : 1 : 1 (v/v/v/v) as the mobile phase

demonstrated compact spots with typical Gaussian shaped peaks for p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and Gallic acid with good resolution between other peaks of the extract. The above mobile phase composition enabled the peak shape and elution of the component. Also in this separation, the UV detector gave good spectra of the separated components. The detection wavelength was confirmed at 254 nm. The method was found to give well separated sharp bands of p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and gallic acid at R<sub>f</sub> of 0.87, 0.66, 0.43 respectively.



**Fig. 3: HPTLC plate for Linearity of standards  
Tracks 1-9: Standard mixture of different concentrations, Track 10-12: Plant extract,  
Track 13-14: Formulation**

The method was validated for linearity, precision, specificity, recovery and stability. The method was found to be linear from 50-350 ng for p- methoxybenzoic acid and gallic acid, 100-700 ng for 3, 4- dihydroxybenzoic acid.



**Fig. 4: HPTLC plate for Reproducibility of standards  
Track 1 to 12: Standard mixture of different concentrations**

The correlation coefficient was found to be  $\geq 0.99$  for all the three components. The limit of detection and limit of quantification for p-methoxybenzoic acid and gallic acid were 16.67 ng and 50 ng respectively while for that for 3, 4-dihydroxybenzoic acid were 33.33 ng and 100 ng respectively. The precision (%RSD) of the method was found to be  $< 2\%$ , indicating that the proposed method is precise. The recovery values for p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and gallic acid were 109.29 %, 108.38 % and 102.61 % in plant whereas in Formulation the recovery values for p-

methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and gallic acid were 94.66%, 98.37% and 101.30% respectively.

Solution stability was evaluated by monitoring the peak area response. Standard solutions were analysed right after its preparation and after 72 hrs. There was no significant change ( $\% RSD \leq 2\%$ ) in the  $R_f$  and area values of standard peak. The quantity of p-methoxybenzoic acid, 3, 4-dihydroxybenzoic acid and gallic acid was found to be 0.00666 %, 0.02626 % and 0.01095 %; in plant and 0.01291 %, 0.02423 % and 0.06381 % in formulation respectively.

**Table 2: Summary of method validation parameters**

Parameter	p-Methoxybenzoic acid	3,4-Dihydroxybenzoic acid	Gallic acid
Specificity	Specific	Specific	Specific
Precision (RSD)	$< 2\%$	$< 2\%$	$< 2\%$
LOD (ng)	16.67 ng	33.33 ng	16.67 ng
LOQ (ng)	50 ng	100 ng	50 ng
Linearity (ng)	50-350	100-700	50-350
Assay(Plant)	0.00666 %	0.02626 %	0.01095 %
Assay (Formulation)	0.01291 %	0.02423 %	0.06381 %
Stock Solution stability	Until 72 hrs at RT	Until 72 hrs at RT	Until 72 hrs at RT
Recovery (Plant)	109.29 %	108.38 %	102.61 %
Recovery (Formulation)	94.66 %	98.37 %	101.30 %

**CONCLUSION:**

A precise, accurate and reproducible HPTLC method is validated for simultaneous quantification of three pharmacologically active markers. This HPTLC method can aid in confirming adulteration in the raw material as well as serve as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

**CONFLICTS OF INTEREST**

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

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