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TLC Densitometric Method for the Development and Validation of Gallic Acid, Catechin and Chlorogenic Acid as Markers in Evicapoly-Herbal Formulation.

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ABSTRACT

Rapid and simple high-performance TLC methods were developed for the quantitative estimation of Gallic acid, Catechin and Chlorogenic acid as active principals or marker constituents of poly herbal formulated Evica capsule. Identification and quantification were performed on 20 cm × 10 cm, layer thickness 0.2 mm, aluminum- backed silica gel 60 F254 HPTLC plates previously washed with methanol. Toluene-ethyl acetate-formic acid (5: 4: 1 v/v) used as a mobile phase for Gallic acid, toluene-ethyl acetate-formic acid- methanol (6: 3: 1.6: 0.4 v/v) used as a mobile phase for Catechin, and ethyl acetate-formic acid- acetic acid- water (10: 1.1: 1.1: 2.6 v/v) for Chlorogenic acid. The spots were scanned at λ = 273, 254 and 366 nm for Gallic acid, Catechin and Chlorogenic acid respectively. The suitability of this HPTLC method for simultaneous estimation of the marker constituents were proved by validation in accordance with ICH Guidelines. Determination of methods accuracy by the standard addition method at three concentration levels returned a mean recovery of 98.01 ± 0.16, 98.7 ± 0.24 and 97.5 ± 0.3 for gallic acid, catechin, and chlorogenic acid. The developed method has the advantage of being rapid and easy. Hence it can be applied for routine quality control analysis of these molecules in a poly-herbal formulation.

Keywords: *Gallic acid, Catechin, Chlorogenic acid, HPTLC, Validation, Polyherbal formulations, Calibration curve.*

INTRODUCTION*

Traditional system of medicine is extensively practice in developing countries. There are many plant based medicine that are specific for women related diseases and ailments[1].

The literature search revealed that plants such as *Saracaindica* L. *Vitexagnus-castus* L, *Withaniasomnifera* (L) Dunal, *Valerianahardwickii* Wall, *Matricaria chamomile* L, *Symplocosracemosa* Roxb., Quercusinfectoria Olivier, *Areca catechu* L, *Asparagus racemosus* Willd, *Commiphoramyrrha* Engl, *Bombaxmalbaricum* L, *Buteafrondosa* Taub are use in different gynecological complaints[2]. Evica, therefore, has been developed with afore mentioned plants. Polyherbal dosage form design as Evica manufactured by Herbion Pakistan (Pvt.) Limited is consists of number of herbs and their composition and pharmacological activities listed in Table 1 and the main active makers analyzed include gallic acid, catechin and chlorogenic acid. These selected medicinal plant administered as uterine tonic known for the efficacy disorders. The indications of Evica capsule usage are leucorrhea, menorrhagia,

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metrorrhagia, irregular menstrual cycle, premenstrual syndrome and post menopausal symptoms [3,4]

The medicinal plants elaborate different types of chemical constituents and different chemical compounds bring about synergistic effects for cure and prevent gynecological diseases. In the given formulations the biomarkers play an important role in the quality assurance of herbal dosage form design. Therefore, in this study different physico-chemical parameter shave been applied for the validation.

MATERIAL AND METHODS

Chemicals and Reagents

All reagents used were of analytical grade. Evica capsules were provided from the Herbion Pakistan Pvt. Ltd. The samples were powdered to 100 mesh and stored at 250C under room temperature. All reference standards were purchased from sigma Aldrich, TLC Plates and silica gel G60 F_{254} were purchased from Merck.

Instrumentation

Spotting device: Linomat V Automatic sample applicator linked to Wincats software; Syringe: 100 µL; TLC development chamber: Glass twin trough chamber (20 x 20 cm); Densitometer: TLC Scanner linked to Win Cats software; all were purchased from CAMAG (Muttenz, Switzerland). HPTLC plates: 20 x 10 cm, 0.2 mm thickness pre-coated with silica gel 60 F_{254} was purchased from Merck, Darmstadt, Germany.

Experimental

Standard and Sample Solutions Preparation

Preparation of Standard Solutions

A stock solution of Gallic acid, Catechin and Chlorogenic acid (0.1 mg/ml) was prepared by dissolving 2.5 mg of accurately weighed each reference standard in 25 ml volumetric flask separately and making up the volume with methanol. The aliquots(1 to 6 mL) of each three stock reference standard solution were transferred to 10 mL volumetric flasks separately and the volume of each was adjusted to 10 mL with methanol to obtain standard solutions containing (0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 mg/mL) Gallic acid, Catechin and Chlorogenic acid respectively.

Preparation of Sample Solutions

Accurately weighed 2.5 gm of capsule powder, and extracted with methanol four times (4 × 25 mL) under reflux (30 mins each time) in a water bath and collected in pool. The pooled extract was concentrated under vacuum and transferred to 25 mL volumetric flasks which were used as sample.

Calibration Curve for Gallic acid

10 µL each of the standard solutions of Gallic acid (10, 20, 30, 40, 50 and 60 µg/ml spot) were applied (band width: 6 mm, distance between the bands: 13 mm) in triplicate on a pre coated silica gel 60 F_{254} TLC plate (E. Merck, Cat. no. 1.05554.0001) (0.2 mm thickness) using a CAMAG Linomate IV Automatic Sample Spotter. The plates were developed in a solvent system of toluene-ethyl acetate-formic acid (5 : 4 : 1 v/v) in a CAMAG glass twin trough chamber (20 x 10 cm) up to a distance of 9 cm (temperature 25 ± 2oC, relative humidity 40%). After development, the plate was dried in air and scanned and absorption spectra were recorded at start, middle and end positions of

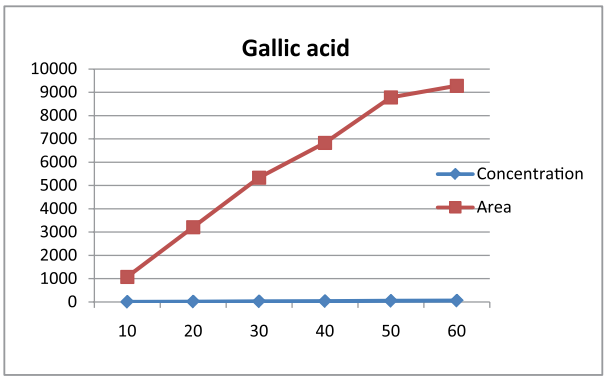

Fig. 1. Calibration curve of Gallic acid by HPTLC method

the band to check the purity. The plates were scanned separately at 273 nm using CAMAG TLC Scanner III and CATS IV software. The peak areas were recorded. Calibration curve of standards were obtained by plotting peak areas vs. concentration of standards applied.

Calibration Curve for Catechin

10 µL each of the standard solution of Catechin (10, 20, 30, 40, 50 and 60 µg/ml spot) were applied (band width: 6 mm, distance between the bands: 13 mm) in triplicate on a pre-coated silica gel 60 F254 TLC plate (E. Merck, Cat. no. 1.05554.0001) (0.2 mm thickness) using a CAMAG Linomat IV Automatic Sample Spotter. The plate was developed in a solvent system of toluene-ethyl acetate-formic acid-methanol (6 : 3 : 1.6 : 0.4 v/v) in a CAMAG glass twin trough chamber (20 x 10 cm) up to a distance of 9 cm (temperature 25 ± 2oC, relative humidity 40%). After development, the plate was treated in same manner but scanned at 254 nm using same instrument as gallic acid. The peak areas were recorded. Calibration curve of Catechin was obtained by plotting peak areas vs. concentration of Catechin applied.

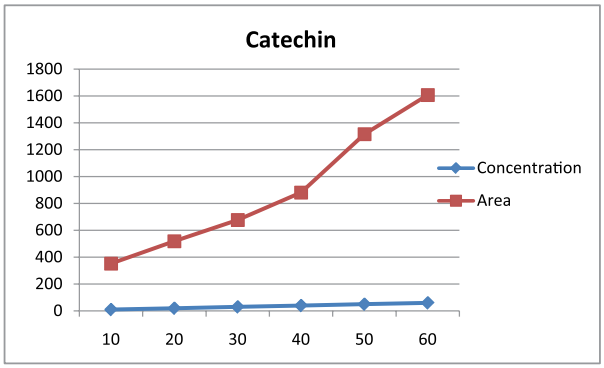

Fig. 2. Calibration curve of Catechin by HPTLC method

Calibration Curve for Chlorogenic acid

10 µL each of the standard solutions (10, 20, 30, 40, 50 and 60 µg/ml spot) were applied (band width: 6 mm, distance between the bands: 13 mm) in triplicate on a precoated silica gel 60 F254 TLC plate (E. Merck, Cat. no. 1.05554.0001) (0.2 mm thickness) using a CAMAG Linomat IV Automatic Sample Spotter. The plate was developed in a solvent system of ethyl acetate-formic acid- acetic acid- water (10 : 1.1 : 1.1 : 2.6 v/v) in a CAMAG glass twin trough chamber (20 x 10 cm) up to a distance of 9 cm (temperature 25 ± 2 C, relative humidity 40%). After development, the plate was dried in air and scanned and absorption spectra were recorded at start, middle and end position of the band to check the purity of the band. The plates were scanned at 366 nm using CAMAG TLC Scanner 3 and CATS 4 software. The peak areas were recorded. Calibration curve of Chlorogenic acid was obtained by plotting peak areas vs. concentration of Chlorogenic acid applied.

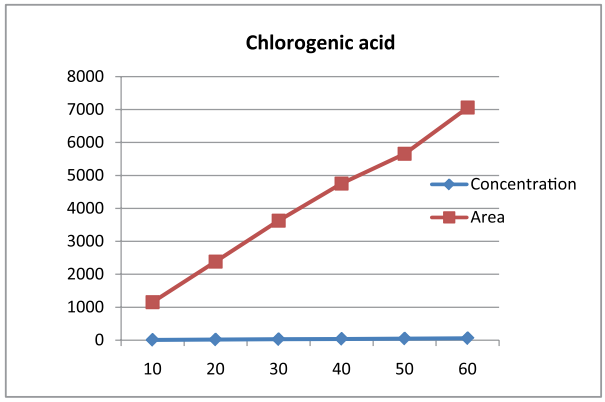

Fig. 3. Calibration curve of Chlorogenic acid by HPTLC method

Quantification of Gallic acid, Catechin and Chlorogenic acid in different samples 10 µL each of sample solutions were applied in triplicate on a pre-coated silica gel 60 F254 TLC plate (E. Merck) (0.2 mm thickness) with CAMAG Linomat IV Automatic Sample Spotter. The plate was developed and scanned as mentioned above. The peak areas were recorded. The amount of standards in different samples was calculated using the calibration curve of standards.

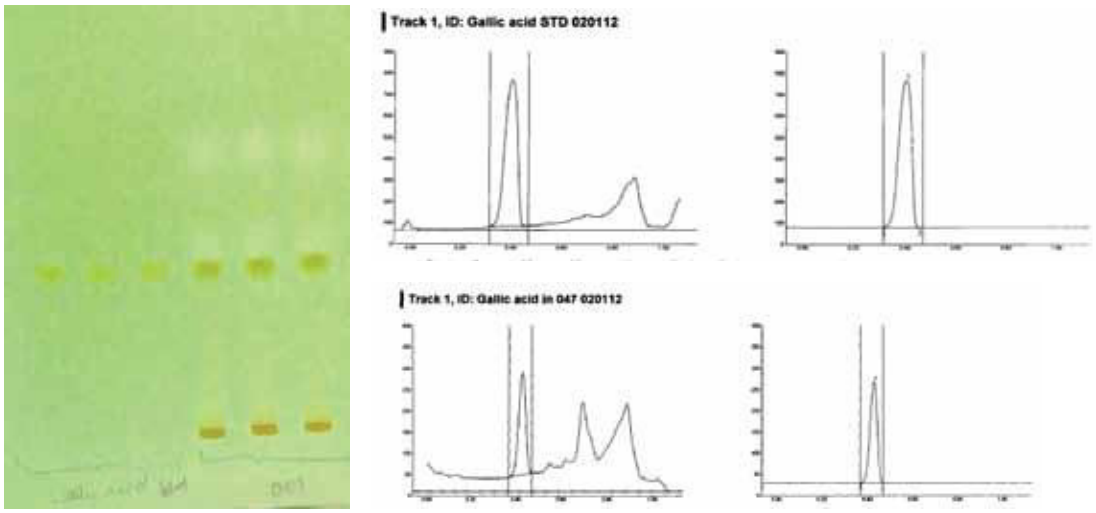

Fig. 4. Typical TLC and HPTLC Chromatogram of Gallic acid in Evica capsule by HPTLC method

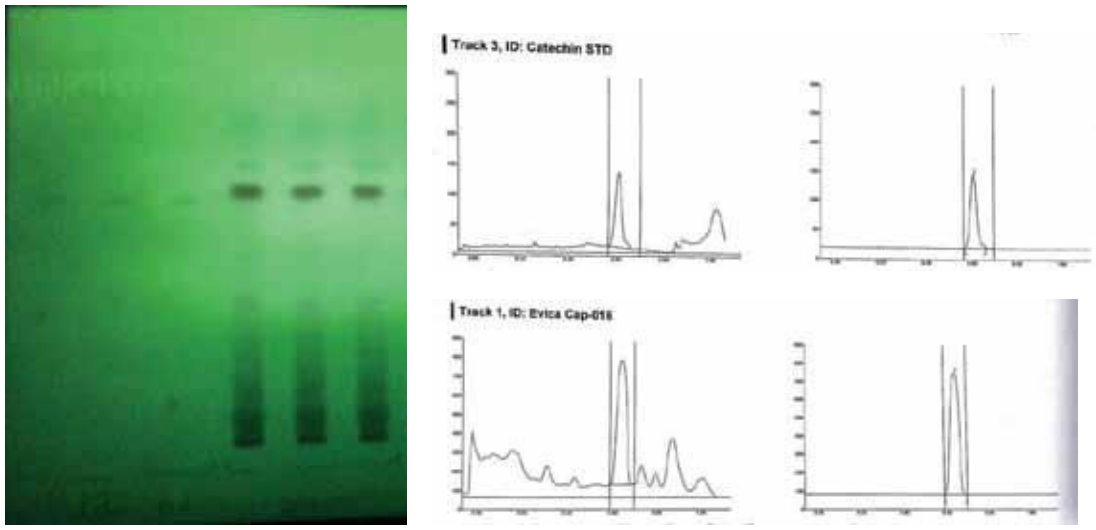

Fig. 5. Typical TLC and HPTLC Chromatogram of Catechin in Evica capsule by HPTLC method.

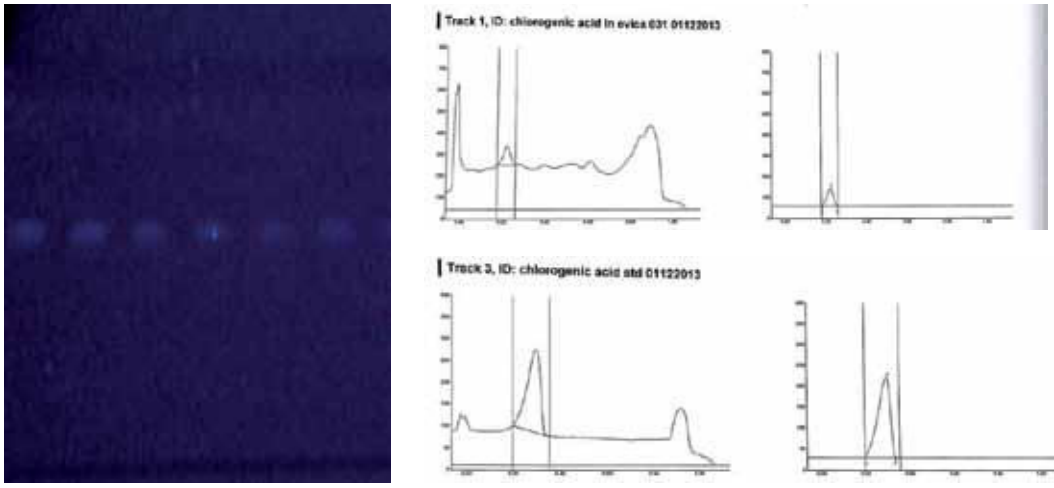

Fig. 6. Typical TLC and HPTLC Chromatogram of Chlorogenic acid in Evica capsule by HPTLC method.

RESULTS

Evica is a poly-herbal formulation, consisting of six ingredients of plant origin (Table 1) and it is widely used for the indication of leucorrhea, menorrhagia, metrorrhagia, irregular menstrual cycle etc. The major active components in this formulation are catechin in Areca catechu L, chlorogenic acid in Valerianawallichi and gallic acid from different herbs that are responsible for its different bioactivities. TLC densitometry methods were developed using HPTLC for the quantitative estimation of three marker compounds from the poly-herbal formulation Evica. Solvent systems were optimized to achieve best separation of the marker components from the other components of the formulation. After several trials of solvent systems, the one containing toluene-ethyl acetate-formic acid (5 : 4 : 1 v/v) gave best resolution of gallic acid (Rf = 0.50), toluene-ethyl acetate-formic acid-methanol (6: 3: 1.6: 0.4 v/v) used as a mobile phase for catechin (Rf = 0.75) and ethyl acetate-formic acid- acetic acid-water (10: 1.1: 1.1: 2.6 v/v) for chlorogenic acid (Rf = 0.55) in the presence of other number of compounds in the sample extract and enabled the quantification of marker compounds.

The presence of compounds in the sample was confirmed by comparing the Rf with Gallic acid standard (Figure 4), catechin standard (Figure 5) and chlorogenic acid standard (Figure 6) and the HPTLC chromatograms by overlaying their spectra with those of their respective standards; gallic acid standard and in sample (Figure 4),catechin standard and in sample (Figure 5), chlorogenic acid standard and in sample (Figure 6) using CAMAG TLC Scanner 3.

Linearity and range

The relationship between the concentration of standard solutions and the peak area was linear. The correlation coefficient of gallic acid, catechin and chlorogenic acid are 0.988, 0.981 and 0.999 respectively(Table 5)

The purity of the bands due to gallic acid, catechin and chlorogenic acid in the sample extract was confirmed by overlaying the chromatogram recorded at start, middle and end position of the band in the sample tracks.

Precision

The methods were validated in terms of precision, repeatability and accuracy shown in Table 5. Method repeatability was obtained of value by repeating the assay three times in same day for intra-day precision (Table 6).The intra- and inter-day precision was carried out at 0.088, 0.156, 0.310 g spot concentration for gallic acid, catechin, and chlorogenic acid respectively. The details are given in Table 6.

Accuracy and percent recovery

The accuracy (average % recovery) at three different levels was found for gallic acid was 102.56 %, for catechin100.21 % and for chlorogenic acid 100.27 % and the results are presented in Table 7.

Limits of detection and limit of quantification

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted three times following the same methods. The LOD was specific for gallic acid, catechin and chlorogenic acid. The LOQ of gallic acid, catechin and chlorogenic acid were 0.088, 0.156 and 0.31, presented in table 5.

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spots for analytes were confirmed by comparing the Rfand spectra of the spot with that of standard. The peak purity of analytes were assessed by comparing the spectra at three different levels, i.e. peak start, peak apex and peak end positions of the spot. The specificity values for gallic acid,

Table 1. Composition of EvicaFormulation

Name of active ingredient	Common names	Qty of Crude herbs	Qty of Thick extracts	Function
Saraca indica L. (Bark)	Ashokachhal	750 mg	180 mg	Endometrium and ovarian tissue stimulant
Symplocos racemosa Roxb. (Bark)	Lodhpathani	250 mg	60 mg	Astringent for abnormal secretions of urogenital organs
Valeriana wallichi DC. (Roots)	Taggar	250 mg	60 mg	Antispasmodic, carminative, relaxant
Matricaria chamomilla L. (Flowers)	Gul-e-baboona	250 mg	60 mg	Anti inflammatory, antiphlogistic,
Vitex agnuscastus L. (Fruits)	Sambhalu	250 mg	60 mg	Aphrodisiac
Areca catechu L. (Nuts)	Chiknisupari	250 mg	60 mg	Emmenagogue, Nervine tonic

Table 2. Values of concentration and peak areas of Gallic acid.

S. No	Concentration (µg/ml)	Area
01	10	1079.9
02	20	3215.65
03	30	5336.75
04	40	6831.6
05	50	8782
06	60	9280.25

Table 3. Values of concentration and peak areas of Catechin.

S. No	Concentration (µg/ml)	Area
01	10	352.2
02	20	518.15
03	30	676.85
04	40	880.9
05	50	1315.45
06	60	1607.25

Table 4. Values of concentration and peak areas of Chlorogenic acid .

S. No	Concentration (µg/ml)	Area
01	10	1155.85
02	20	2386.6
03	30	3632.15
04	40	4756.85
05	50	5659.45
06	60	7063.3

Table 5. Method validation data for estimation of gallic acid, catechin and chlorogenic acid

Parameters	**gallic acid**	**catechin**	**chlorogenic acid**
Instrumental precision	0.25	0.23	0.34
(%CV, n = 7)	99.1	98.6	97.8
Repeatability (%CV, n = 7)	1.2.56	100.21	100.27
Limit of detection (1µg)	Specific	Specific	Specific
Limit of quantification (1µg)	0.088-	10.156-	0.31-2.48
Specificity	0.528	0.78	0.998
Linear range (1µg spot)	0.988±0.0003	0.981±0.00062	0.999±0.00051
Linearity (correlation coefficient)	0.988	0.981	0.99

Table 6. Inter-day and Inter-day precision

Compound	Amount applied (1g spot)1)	Precision inter-day	Inter-day
Gallic acid	0.088	0.23	0.24
Catechin	0.156	0.25	0.21
Chlorogenic acid	0.31	0.32	0.29

Table 7. Recovery of gallic acid, catechin and chlorogenic acid

Compound	**Amount in sample (mg)**	**Amount added to sample (mg)**	**Amount found (mg)**	**Recovery %**	**Average recovery**
Gallic acid	0.583	0.528	1.129	103.41± 1.101	**102.56**
	0.504	0.352	0.871	04.26 ± 0.81	
	0.515	0.088	0.603	100.00 ±0.62	

Compound	Amount in sample (mg)	Amount added to sample (mg)	Amount found (mg)	Recovery %	Average recovery
Catechin	0.213	0.312	0.521	98.72 ± 1.231	**100.21**
	0.184	0.156	0.345	03.20 ± 0.94	
	0.188	0.078	0.265	98.72 ± 0.15	
Chlorogenic acid	1.532	2.48	3.980	98.71 ± 0.21	**100.27**
	1.324	1.24	2.564	100.00 ± 0.07	
	1.352	0.62	1.985	102. 10 ±0.03	

Table 8. Amounts (mg / g) of Gallic acid, Catechin, and Chlorogenic acid in a sample of Poly herbal herbalEvica Capsule

	(Amount mg/gm)
Gallicacid	4.96 ± 0.17
Catechin	1.81 ± 0.05
Chlorogenic acid	13.02 ± 0.32

*Mean ± SD (n = 3)

catechin and chlorogenic acid was 0.528, 0.78 and 0.998 respectively.

Quantification

Gallic acid, Catechin and chlorogenic acid content in a poly-herbal composition evica were quantitatively determined by the proposed methods. The methods developed were found to be suitable for the quantification of these marker compounds. The amount (mg/g) of gallic acid, catechin and chlorogenic acid in a sample of polyherbalevica capsule were quantified as 4.96 ± 0.17, 1.81 ±0.05 and 13.02 ± 0.32 respectively see table 8.

DISCUSSION

The increasing use of herbal medicine to cure diseases has created a necessary thrust that the relevant quality control methods should indicate and identify the chemical markers for the validation. The literature citation to identify chemical markers in polyherbal formulation is being progressively reported. Bonsoussan et al.have communicated in a study on chemical markers for quality assurance of complex seven herbal medicine components for irritable bowl syndrome [5]. Li and coworkers have cassified eight categories of chemical markers and these in turn were correlated with fingerprint spectrum [6]. Thomford et al. described determination of rutin, quercetin and kaempferol contents in the herbal medicinal plants of Ghana by using a simultaneous determination RP-HPLC methods [7]. Therefore in the given study the development of mobile phases for biomarkers were optimized by employing the accustomed solvents. The TLC densiometric method as described in this communication was found to be simple, reliable, and convenient for routine analysis and work confirms such findings. The method can be used conveniently for the estimation of gallic acid, catechin and chlorogenic acid in the Evica herbal formulation.Its main advantages include its simplicity, accuracy and selectivity as well as the present standardization provides

a specific and rapid tool in the herbal research, permitting formulation for the assessment of quality assurance.

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