IJPSR (2020), Volume 11, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 27 March 2019; received in revised form, 22 July 2019; accepted, 02 September 2019; published 01 January 2020

VALIDATED STABILITY INDICATING HPTLC METHOD FOR PROTOCATECHUIC ACID

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Keywords:

Protocatechuic acid, Stress degradation, HPTLC

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Abstract: Protocatechuic acid (PCA) is a type of widely distributed naturally occurring phenolic acid, commonly found in bran, grain, brown rice, fruits such as plums, gooseberries, grapes and also in onion peels. A new, simple, precise, accurate and sensitive stability-indicating HPTLC method for Protocatechuic acid was successfully developed. This method is based on HPTLC separation followed by UV detection at 258 nm. The HPTLC method is used to determine the presence and quantify the protocatechuic acid in onion peel extract. The separation was carried out on Merck TLC aluminum sheets precoated with silica gel 60F254 using Toluene: Ethyl Acetate: Formic acid (6:6:1.2 v/v/v) as a mobile phase and scanning was done by using TLC Scanner III. Protocatechuic acid gave well defined and sharp peak at R_f 0.52 ± 0.03 at 258 nm. The calibration curve was linear in range 100-500 ng/band. Protocatechuic acid was subjected to stress conditions like hydrolysis under acidic, basic and neutral conditions, oxidation, heat, and photolysis.

INTRODUCTION: Protocatechuic acid (PCA) is a type of naturally occurring phenolic acid. PCA is chemically 3, 4-dihydroxybenzoic acid. PCA has structural similarity with gallic acid, caffeic acid and vanillic acid which are well-known antioxidant compounds. The chemical formula is C7H6O4, and molar mass is 154.12 g/mol. It is freely soluble in methanol and sparingly soluble in water, insoluble in benzene [1, 2]. Protocatechuic acid has many pharmacological activities such as anti-bacterial, anti-inflammatory, hepatoprotective, anti-cancer, anti-diabetic, anti-oxidant, anti-ulcer, anti-mutagenic, analgesic, *etc* [3-10].

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DOI: 10.13040/IJPSR.0975-8232.11(1).130-36

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(1).130-36

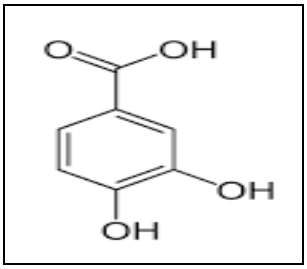

FIG. 1: STRUCTURE OF PROTOCATECHUIC ACID

As per literature search there is no stability-indicating method reported for determination of protocatechuic acid in onion peel by HPLC and HPTLC. Development of SIM is based on systematic exposure of API to various stress conditions. Systematic optimization trials are required to arrive at combination of "concentration of stress reagent and duration of exposure," to obtain degradation preferably in the 10-20% range. Typical degradation conditions involve hydrolysis under different pH conditions, photolysis, oxidation and thermal studies [12 - 13].

MATERIALS AND METHODS:

Reagents and Chemicals: Protocatechuic acid was procured from SRL. Pvt. Ltd. Methanol (AR grade), Toluene (AR grade), Ethyl Acetate (AR), Formic acid (AR grade) Hydrochloric acid (HCl), 6% w/v Hydrogen peroxide (H_2O_2) and Sodium hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. Ltd. Mumbai. Instruments: Linomat-5 sample applicator (Camag, Switzerland), twin trough chamber (10 × 10 cm; Camag, Switzerland), TLC scanner 3 (Camag, Switzerland), WinCATS version 1.4.3 software (Camag, Switzerland), Photostability chamber (Newtronics NEC103RSPI), Shimadzu balance (Model AY-120), Camag 100 µl sample syringe (Hamilton, Switzerland) were used in the study.

Preparation of Standard Stock Solution: Standard stock solution of protocatechuic acid was prepared by dissolving 10 mg of drug in 10 ml of methanol (concentration of 1000 µg/ml). The standard stock solution was suitably diluted to obtain 25 µg/ml solutions as working standard using methanol.

Selection of Detection Wavelength: The λ_{max} of Protocatechuic acid was determined using UV spectrophotometer (V-730 model) Make JASCO. From the standard stock solution, further dilutions were prepared using methanol and scanned over the range of 200 - 400 nm and the spectrum was obtained to finalize wavelength for detection.

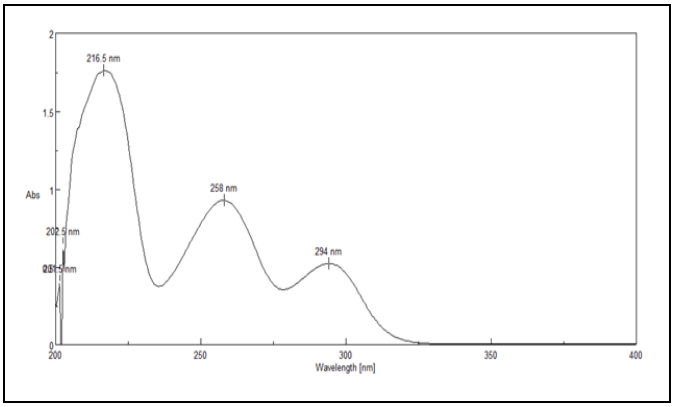

FIG. 2: UV SPECTRUM OF PROTOCATECHUIC ACID

Chromatographic Conditions and Instrumentation Chromatographic separation of the drug was performed on Aluminium plates precoated with silica gel 60 F_{254}, (10 cm × 10 cm with 250 µm layer thickness). Protocatechuic acid was applied on the plate which was prewashed, dried and activated, as a band with 6 mm width using CAMAG 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (CAMAG, Switzerland). Development of densitogram was done in twin trough glass chamber previously saturated with the mobile phase Toluene: Ethyl Acetate: Formic acid (6:6:1.2 v/v/v) for 15 min. Densitometric scanning was performed at 258 nm on CAMAG TLC scanner 3. by using WINCATS software (Version 1.4.3, CAMAG), slit dimensions were 4.00 × 0.45 mm and Deuterium lamp was used as a radiation source.

Chromatographic separation of the drug was performed on Aluminium plates precoated with silica gel 60 F254, (10 cm × 10 cm with 250 µm layer thickness). Protocatechuic acid was applied on the plate which was prewashed, dried and activated, as a band with 6 mm width using CAMAG 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (CAMAG, Switzerland).

Development of densitogram was done in twin trough glass chamber previously saturated with the mobile phase Toluene: Ethyl Acetate: Formic acid (6:6:1.2 v/v/v) for 15 min. Densitometric scanning was performed at 258 nm on CAMAG TLC scanner 3. by using WINCATS software (Version 1.4.3, CAMAG), slit dimensions were 4.00 × 0.45 mm and Deuterium lamp was used as a radiation source.

Mobile Phase Optimization: The mobile phase was optimized for protocatechuic acid such that the R_f should be in the range of 0.2 - 0.8 and the band should be compact.

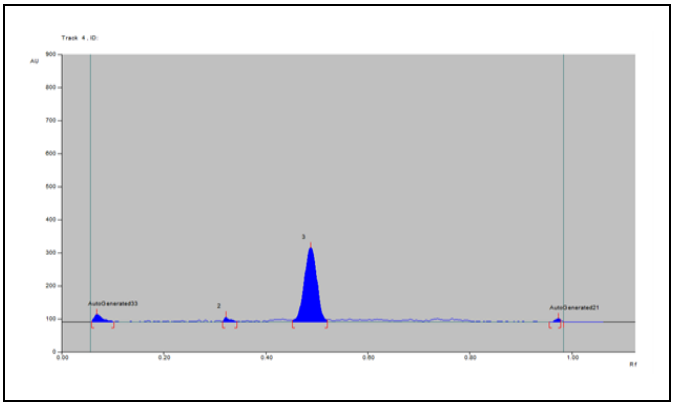

FIG. 3: DENSITOGRAM WITH OPTIMIZED MOBILE PHASE

Forced Degradation Studies: [14, 15] Stress studies were performed on the drug to provide evidence on how the quality of drug varies under the influence of variety of stress conditions like hydrolysis under different pH conditions, oxidation. Dry heat and photolytic were carried out in solid state. All studies were carried out at concentration level of 300 ng/band.

Optimization of stress conditions was done by changing the strength of reagent and duration of exposure to obtain degradation preferably in the 10-20% range.

Optimization Trials: Initially trials were conducted using various normalities of HCl and NaOH by keeping the sample solution for different hours. For the thermal study sample was heated at 80 of or 3 h and for oxidation, trials were conducted using 1%v/v H_2O_2. It was observed that the drug gets degraded partially.

Confirmation of Degradation Product: By increasing the conc. of the stock solution and stress reagent by 10 times we check for the presence of the degradation product peak.

Optimized Stress Conditions:

Photolytic Degradation: The standard Protocatechuic acid was exposed to UV light (200-watt h / square meter) and cool white fluorescent lamp (1.2 million lux h). 10 mg was accurately weighed and dissolved in few ml of methanol into volumetric flasks (10 ml) and made up the volume with methanol. Further diluted with methanol to attain the working standard of 25 µg/ml concentration. 12 µl of the resultant solution was applied at the TLC plate, and densitogram was developed. Average 88.25% and 85.87% was recovered in UV and Fluorescence respectively with no peak of degradant.

Alkali Hydrolytic Condition: For alkali degradation study, 1 ml of 250 ppm solution of Protocatechuic acid was taken into 10 ml volumetric flask and 1 ml of 0.01 N sodium hydroxide was added and diluted with methanol to make up the volume 10 ml (25 µg/ml), kept for 2 h. 12 µl of this solution was then applied at TLC plate and densitogram was developed, 85.91% of Protocatechuic acid was recovered with two peaks of degradant.

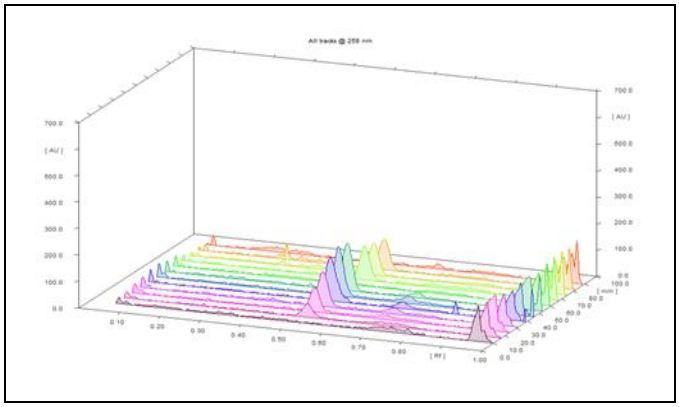

FIG. 4: DENSITOGRAM OF PROTOCATECHUIC ACID AFTER ADDITION OF 0.01 N NaOH FOR 2 HRS AT-RT (TRACK 1 METHANOL, TRACK 2-6 LINEARITY, TRACK 7 NAOH BLANK, TACK 8-10 NAOH STRESS)

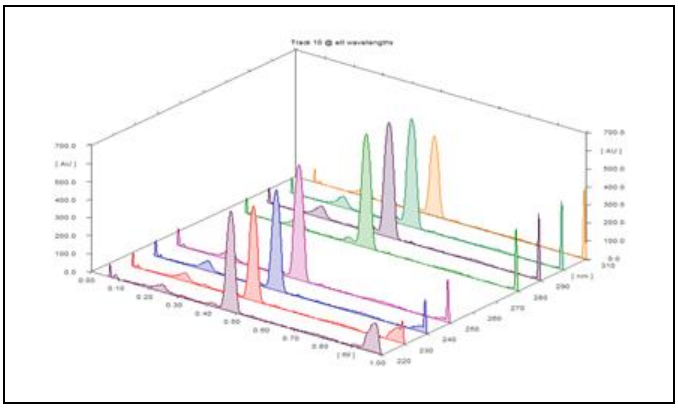

FIG. 5: MULTIWAVELENGTH SCANNING OF TRACK 9 (NAOH STRESS)

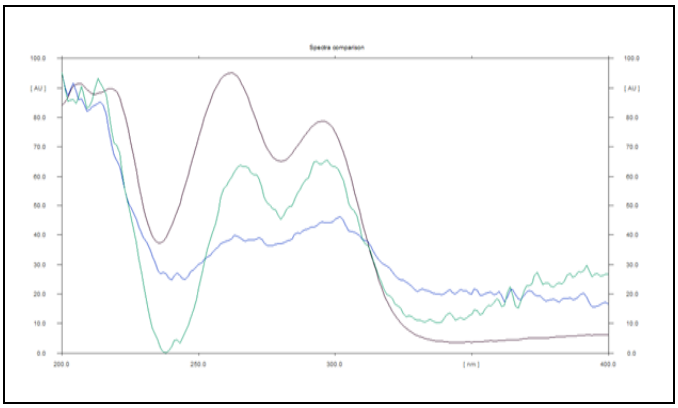

FIG. 6: SPECTRAL SCANNING OF TRACK 4 AT R_F 0.52 AND TRACK 9 AT RF 0.27 AND 0.43

The degradation product may be in **Fig. 7** and **8**.

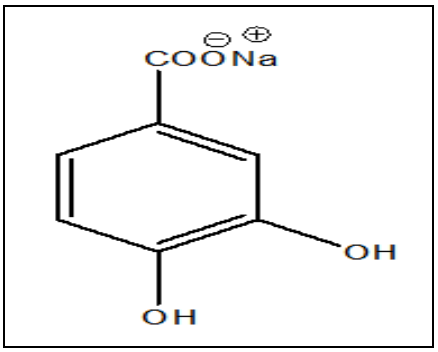

FIG. 7: DEGRADATION PRODUCT AT R_f 0.27

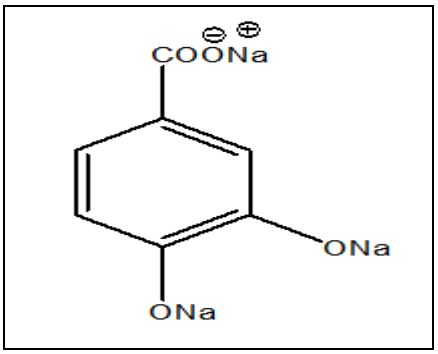

FIG. 8: DEGRADATION PRODUCT AT R_f 0.43

Acid Hydrolytic Condition: For acid degradation study, 1 ml of 0.01 N hydrochloric acids was added, and rest procedure was same as alkali hydrolytic condition. Average 84.10% of protocatechuic acid was recovered with no peak of degradant.

Neutral Hydrolytic Condition: For neutral degradation study, 1 ml of distilled water was added and further procedure same as alkali hydrolytic condition. 89.67% of protocatechuic acid was recovered with no peak of degradant.

Oxidative Stress Degradation: For oxidative stress degradation, 1 ml of stock solution of protocatechuic acid was taken into 10 ml volumetric flasks and 1 ml of 1 % v/v hydrogen peroxide (H_2O_2) were added and volume was made up with methanol. The above solution was kept for 15min at room temperature. 12 µl of this solution was then applied at TLC plate and densitogram was developed. 87.58% of protocatechuic acid was recovered with no peak of degradants.

Dry Heat Degradation: Dry heat studies were performed by keeping drug sample in oven (80 °C) for a period of 3 h. The sample was withdrawn, dissolved in methanol and diluted to get 25 µg/ml. 12 µl of the resultant solution was applied on TLC plate and densitogram was developed. Average 85.57% protocatechuic acid was recovered with no peak of degradants.

RESULTS AND DISCUSSION:

Optimization of Densitometric Conditions: The first step in developing this stability-indicating HPTLC method is to achieve the resolution of Protocatechuic acid with R_f in the range of 0.2 to 0.8. Various binary combinations of solvents were tried. The densitometric was achieved by linear ascending development in 10 cm × 10 cm twin trough glass chamber. The optimized mobile phase was Toluene: Ethyl Acetate: Formic acid 6:6:1.2 v/v/v, and detection were carried out at 258 nm. The retention factor for protocatechuic acid was found to be 0.52 ± 0.03. Representative densitogram of standard solution of protocatechuic acid is shown in **Fig. 9**.

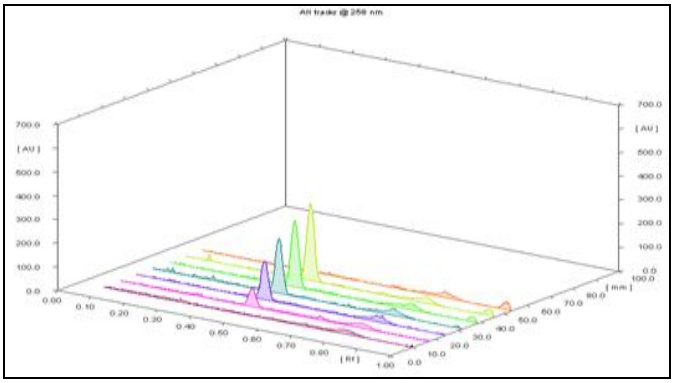

FIG. 9: DENSITOGRAM OF STANDARD SOLUTION OF PROTOCATECHUIC ACID 100ng/band (R_f 0.52 ± 0.03)

Result of Forced Degradation Studies: After the optimization of the different stress conditions, there was a well-resolved degradation product peak observed. Only for alkali hydrolysis it was confirmed by applying 10 times higher concentration spotting (3000 ng /band) and further confirmed by multi wavelength scanning to observe if any degradation products are present. Peak purity is comparison of absorbance spectra from the start to middle (s, m) and from middle to end (m, e), ensured non-interference by-product of degradation of Protocatechuic acid in all condition.

No peak for degradation product was observed for protocatechuic acid. During either of the stress conditions like oxidation, dry heat, and photolysis. Results of the stress degradation studies are presented in **Table 1**.

TABLE 1: SUMMARY OF STRESS DEGRADATION

S. no.	Parameters	Condition	% Recovery	Peak purity	R_f of degraded product
1	Alkali hydrolysis	0.01 N NaOH 2 h at RT	87.68	0.999	0.990
2	Acid hydrolysis	0.01N HCl 2 h at RT	84.10	0.999	0.998
3	Neutral hydrolytic	H_2O at RT	89.67	0.999	0.998
4	Oxidative stress Degradation	1% v/v H_2O_2 15 min at RT	87.58	0.999	0.997
5	Dry heat degradation	80˚C for 3 h	85.57	0.999	0.996
6	Photolytic Degradation	UV light (200 watt hours/square meter)	88.20	0.999	0.998

Validation of the Method: [16] The method was validated for various parameters in accordance with ICH guidelines [17].

Specificity: The specificity of the method was determined by peak purity profiling studies. The peak purity values were found to be more than 0.99, indicating the non-interference of any other peak of degradation product or impurity.

Linearity: The calibration curve was obtained in the range of 100-500 ng/band for PCA by applying different volumes of stock solution (25 µg/ml) and peak areas were recorded. The standard calibration graph was plotted of peak area Vs amount applied. The equation of the calibration curve found Y = 12.43x + 176.8. The coefficient of correlation (r^2) was found to be 0.994 for PCA shown in **Fig. 10**.

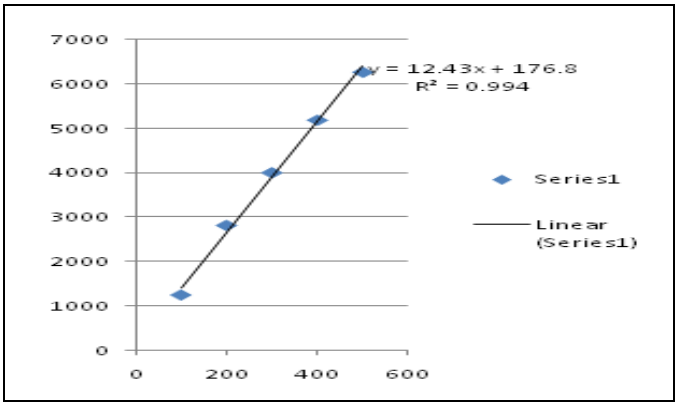

FIG. 10: CALIBRATION CURVE OF PCA (100-500ng/BAND)

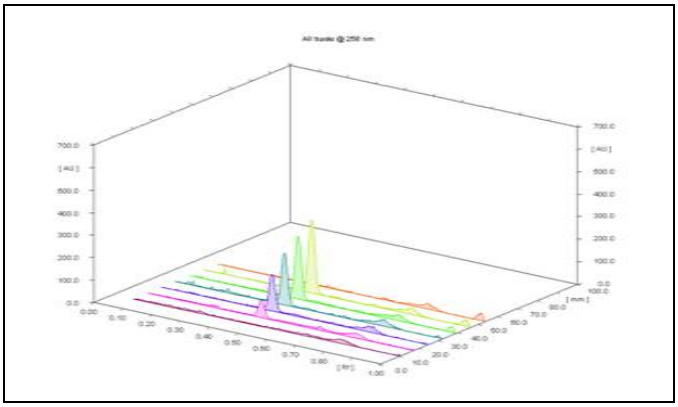

FIG.11: DENSITOGRAM OF PROTOCATECHUIC ACID LINEARITY 100ng/BAND-500ng/BAND (R_F 0.52 ± 0.03)

Precision: The precision of the system was demonstrated by intra-day and inter-day studies. In both the studies 6 replicates of 1 standard concentration (100 ng/band) were analyzed in a day and percentage RSD was calculated. For intraday and intraday system precision RSD was found to be 0.85, 1.13, 1.44 and 1.11, 0.85, 1.13 respectively for PCA

TABLE 2: INTERDAY AND INTRADAY PRECISION

Concentration (ng/band)	Intraday			Intraday		
	Mean Area	SD	% RSD	Mean Area	SD	% RSD
100	1251.483	10.69662	0.854715	1252.9467	13.977999	1.1156101
100	1245.1667	14.118168	1.1338376	1251.4833	10.696619	0.8547153
100	1263.65	18.22358	1.442139	1259.7883	14.236948	1.1301064

Limit of Detection (LOD) and Limit of Quantitation (LOQ): LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively; where σ is the standard deviation of the lowest concentration-response and S is the slope of the calibration plot. The LOD and LOQ were found to be.

LOD of PCA = 3.74 ng/band

LOQ of PCA = 11.35 ng/band

Robustness: Robustness of the method was determined by carrying out the analysis under different conditions during which chamber saturation time was altered, Time was also changed from spotting to development and development to scanning, and the effects on the peak area was noted.

Assay: Assay was performed for onion peel extract. Assay was determined by extrapolation of

peak area from linearity equation, which was found to be 10.20% for PCA.

Accuracy: To check the accuracy of the method, recovery studies were carried out by adding standard drug to assay at three different levels 80, 100 and 120%. The drug concentrations were calculated from respective linearity equation. The results of the recovery studies indicated that the method is accurate for estimation of drug in the onion peel extract.

TABLE 3: ACCURACY STUDY OF PCA WITH EXTRACT

Level (%)	From extract	From std. addition	Total Conc. With extract (ng/band)	Conc. Recovered (ng/band)	% Recovery
80	120	75	195	196.72	100.88
100	120	100	220	220.54	100.24
120	120	125	245	247.39	100.97

Solution Stability: Standard stock solution of PCA was found to be stable for 96 h if stored at 2-8 °C.

TABLE 4: SUMMARY OF VALIDATION

Sr. no.	Validation Parameters	Protocatechuic acid
1	Linearity Equation (r^2)	Y = y = 12.43x + 176.8
	Range	R^2 = 0.994
		100-500 ng/ band
2	Precision	(% RSD)
		0.85
	Intraday	1.13
		1.44
	Intraday	1.11
		0.85
		1.13
3	Accuracy	% Recovery
	80%	100.88
	100	100.24
	120%	100.97
4	Limit of Detection	3.74 ng/band
5	Limit of Quantitation	11.35ng/band
6	Specificity	Specific
7	Robustness	Robust
8	Solution stability	Stable

Discussion: During the literature survey, two articles based on HPTLC work were found. Manek Ravi et al and Pandey Shivanand *et al*., have reported use of chloroform and acetic acid as mobile phase, wherein the R_f observed was not in the ideal range of 0.2-0.8. In our work the R_f of Protocatechuic acid is 0.52 ± 0.03, which is in the ideal range. The values of LOD and LOQ as per our method are relatively low thus indicating that the developed method is more sensitive.

CONCLUSION: As per literature survey there is no stability-indicating assay method on protocatechuic acid. The present study was aimed to develop stability-indicating method for protocatechuic acid that may be used to monitor stability of protocatechuic acid. Validation of stability indicating method for Protocatechuic acid using HPTLC confirms that the developed method is precise, specific, and accurate. The present study of stability indicating method for protocatechuic acid may be used to monitor stability of protocatechuic acid.

ACKNOWLEDGEMENT: Authors are thankful to the Principal and the management of AISSMS College of Pharmacy, Pune for providing the necessary facilities for research work.

CONFLICTS OF INTEREST: NIL

REFERENCE:

1. https://en.wikipedia.org/wiki/Protocatechuic_acid (Accessed on 12-07-2018).
2. https://www.drugbank.ca/drugs/DB03946 (Accessed on 13-07-2018).
3. kakkar S: A review on Protocatechuic Acid and Its Pharmacological Potential by ISRN Pharmacology 2014; 1- 9.
4. Khan AK: pharmacological activities of protocatechuic acid. Acta Poloniae Pharmaceutica and Drug Research 2015; 72(4): 643-50.
5. Arslan R: The possible mechanisms of protocatechuic acid induced central analgesia. Saudi Pharmaceutical Journal 2018; 541-45.
6. Misana AC, Mimica-Dukicb NM, Mandica AI, Sakaca MB, Milovanovića IL, Sedeja IJ: Central European Journal of Chemistry 2011; 9(1): 133-42.
7. Vaidy VV: Separation and quantification of pharmacologically active markers p-methoxybenzoic acid, 3, 4dihydroxybenzoic acid and gallic acid from capparis spinos and from marketed formulation by high performance liquid chromatography. International Journal of Research In Pharmacy and Chemistry 2017; 7(4): 437-42.
8. Osman M: Indian Journal of Science and Technology 2014; 7(11): 1847-51.
9. Ming yan, chen: Study on HPLC fingerprint and determination of six components including vanillic acid of

the stems and leaves of *Veronicastrum axillare* Yamazaki, Chinese Journal of Pharma Analysis 2017; 37: 663-70.

10. xie L and Guo J: Efficient determination of protocatechuic acid in fruit juices by selective and rapid magnetic molecular inprited solid-phase extraction coupled with HPLC. Journal of Agri and Food Chem 2014; 8221-28
11. Damle MC, Tembhurne SV and Todkar S: Evaluation of hepatoprotective activity of hydroalcoholic onion peels extract and fingerprinting of protocatechuic acid by HPTLC method. Journal of Drug Delivery and Therapeutics 2019; 9(4): 82-8.
12. Damle MC and S Todkar: Stability indicating Rp-HPLC method for protocatechuic acid, an active phytoconstituent of onion peels. Journal of Emerging Trend and Innovative Research 2019; 6(4): 91-8.
13. ICH Harmonised Tripartite Guideline, Stability Testing: Photostability Testing of New Drug Substances and Products, Q1B. International Conference on Harmonisation, Current Step 4 version, dated 6 November 1996.
14. ICH guidelines. Q2 (R1): Validation of Analytical Procedures: Text and Methodology. International Conference on Harmonization Geneva. ⟨http://www.ich.org/products/ guidelines/quality/article/quality guidelines/html), 2005.
15. ICH guidelines. Q1A (R^2): Stability Testing of New Drug Substances and Products. International Council for Harmonisation Geneva. ⟨http://www.ich.org/products/ guidelines/quality/article/quality-guidelines/html), 2003.

How to cite this article:
Damle MC and Todkar SR: Validated stability indicating hptlc method for protocatechuic acid. Int J Pharm Sci & Res 2020; 11(1): 130-36. doi: 10.13040/IJPSR.0975-8232.11(1).130-36.