Determination of Protocatechuic Acid by HPTLC Method in *Amomum subulatum* Roxb. Fruit Extracts

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ABSTRACT

The aim of present study is development of simple, rapid and accurate HPTLC method for estimation of Protocatechuic acid in various extracts of *Amumum subulatum* Roxb. fruit constituents(Family Zingiberacea), commonly known as 'Badi Elaichi'. The powdered drug was subjected to extraction by soxhlet apparatus using methanol and acetone separately as well as Petroleum ether (40–60), chloroform, methanol and water successively. The extracts were screened for presence of phytoconstituents using preliminary chemical tests. Protocatechuic acid was estimated in methanol and acetone extract by HPTLC method. Detection and quantification was performed at wavelength 254nm. The Acetone and methanol extracts were found to contain 1.04846 and 0.8634 %w/w Protocatechuic acid respectively by using validated method. Since this method resolves and quantifies protocatechuic acid accurately and precisely, it can be useful for quantification of the compound in herbal formulation.

Keywords: Protocatechuic Acid, HPTLC, *Amomum subulatum* Roxb.
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INTRODUCTION

The quality of herbal medicine is implication of safety and efficacy, which is profile of constituents present in it. It is difficult to establish quality control parameters of plant based drug due to complex nature and inheritant variability of chemical constituents. So, modern analytical techniques should be implicated to over come this problem (1).

Since ancient time, seeds of *Amomum subulatum* Roxb. have been valued for its aroma, as spice, flavor and condiment. The seeds are reported in Ayurvedic system of medicine and are an official drug in Ayurvedic Pharmacopoeia and are marketed under the name of '*Elcho*' or '*Badi Elaichi*' (2). Traditionally it has been used for digestive problems treating flatulence, loss of appetite, gastric complaints, congestion of liver and also recommended in cases of inflammatory condition of eyes (3). Orally administered *A. subulatum* could be useful in prevention of hyperlipidaemia and provide antioxidant protection (4). An anti-wrinkle cream containing *A. subulatum* was evaluated in the treatment of facial skin wrinkles by prospective, open, phase III clinical trial and showed that the active constituents of *A. subulatum* (protocatechualdehyde and protocatechuic acid) have potent antioxidant activity (5). Greater cardamom (*A. subulatum* have significant ability to inhibit lipid peroxidation in rat liver homogenate due to their polyphenol content, strong reducing power and superoxide radical scavenging activity (6). Protocatechualdehyde, Protocatechuic acid, 1,7-bis (3,4-dihydroxyphenyl) hepta-4E,6E-dien-3-one and 2,3,7-trihydroxy-5-(3,4-dihydroxy-E-styryl)-6,7,8,9-tetrahydro-5H-benzocycloheptene was isolated from greater cardamom and evaluated for its antioxidant acivity (7).

It was found to possess antioxidant activity, attributed to presence of protocatechuic acid. Since protocatechuic acid is marker compound responsible for its antioxidant activity. So, *A. subulatum* fruit constituent extracts are subjected to HPTLC analysis by developing a method for estimation of protocatechuic acid in methanol and acetone extract. The proposed method has been validated as per ICH guidelines (8–9)

MATERIAL AND METHODS

Present study was conducted at BMCPER, Modasa, Gujarat and RBPMPC, Atkot, Dist Rajkot, Gujarat, India during January 2007 to November 2008.

Collection and authentication of the fruits and seeds

The fruits of *Amomum subulatum* Roxb were collected from local market of Modasa and authenticated by Dr. H. B. Singh Scientist and Head of Raw Materials Herbarium & Museum Dept of National Institute of Science and Communication and Information Resources, New Delhi (NISCAIR) and preserved at the herbarium in Dept. of Pharmacognosy, B. M. Shah College of Pharmaceutical Education and Research, Modasa.

Extraction and Phytochemical Investigations

100g powder of fruit constituents of *A. subulatum* were extracted with Methanol and Acetone separately. Successive extraction was performed by using Petroleum ether (40–60), chloroform and Methanol successively by soxhlet apparatus and lastly remaining marc was refluxed with water. The extracts were concentrated and air dried, weighed and percentage yield was determined. Qualitative chemical tests for identifying various phytoconstituents present were carried out on all extracts of *A. subulatum* Roxb fruit constituents (10).

Estimation of Protocatechuic acid by HPTLC in Methanol and Acetone extract of **A. subulatum Roxb** *fruit constiuents*

Materials: Standard Protocatechuic acid was a purchased from LGC Promochem Pvt. Ltd. Bangalore All the chemicals used in the experiments are of analytical grade.

Experimental condition

Sample applicator: Camag Linomat V Automatic Sample Spotter
Stationary phase: precoated silica gel plates 60 F254 (10 cm × 10 cm, with 0.2 mm thickness, E. Merck, Darmstadt, Germany) The plates were prewashed by methanol and activated at 60 °C for 5 min prior to chromatography.
Solvent system: Chloroform: Acetic acid (9:1) (11)
Development chamber: CAMAG glass twin-through chamber (10 × 10 cm) previously saturated with the solvent for 60 min (temperature 25.2 °C, relative humidity 40%). The development distance was 8 cm. Subsequent to the scanning.
Scanner: Camag TLC scanner III in absorbance mode at 254 nm and operated by Win- Cats software 4.03 version.

Evaluation was *via* peak areas with linear regression

Calibration Curve of Standard Protocatechuic acid

A stock solution of Protocatechuic acid was prepared by dissolving 10mg of compound in ethanol and volume was made up to 10 ml in volumetric flask. From this solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8µl spots were applied on plate as shown in figure 1.

Estimation of Protocatechuic acid in Alcoholic and Acetone Extract

To determine content of Protocatechuic acid in Methanolic and acetone extract, an accurately weighed 50 mg of extracts were transferred to 10ml volumetric

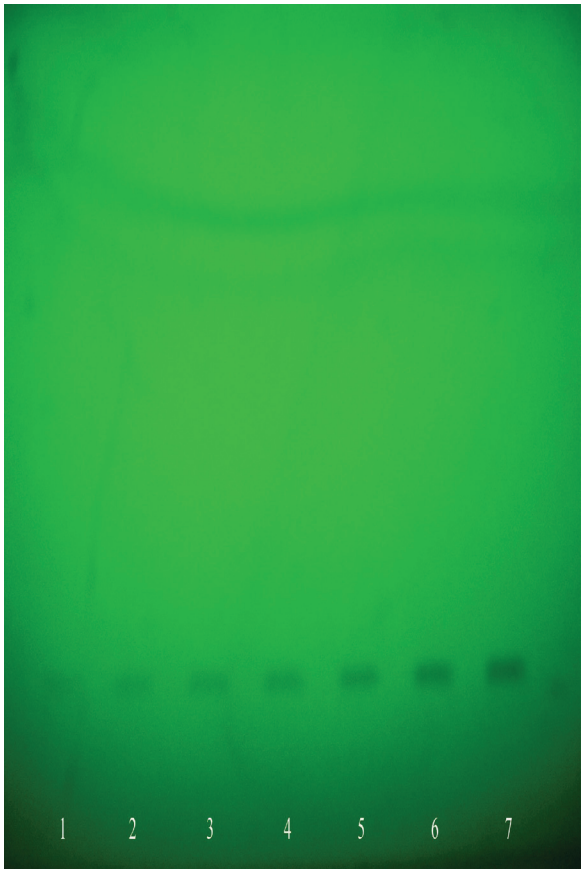

Figure 1. Image of HPTLC plate (254nm) for calibration curve
1 - 100 ng of Protocatechuic acid Standard, 2 - 200 ng of Protocatechuic acid Standard, 3 - 300 ng of Protocatechuic acid Standard, 4 - 400 ng of Protocatechuic acid Standard, 5 - 500 ng of Protocatechuic acid Standard, 6 - 600 ng of Protocatechuic acid Standard, 7 - 800 ng of Protocatechuic acid Standard

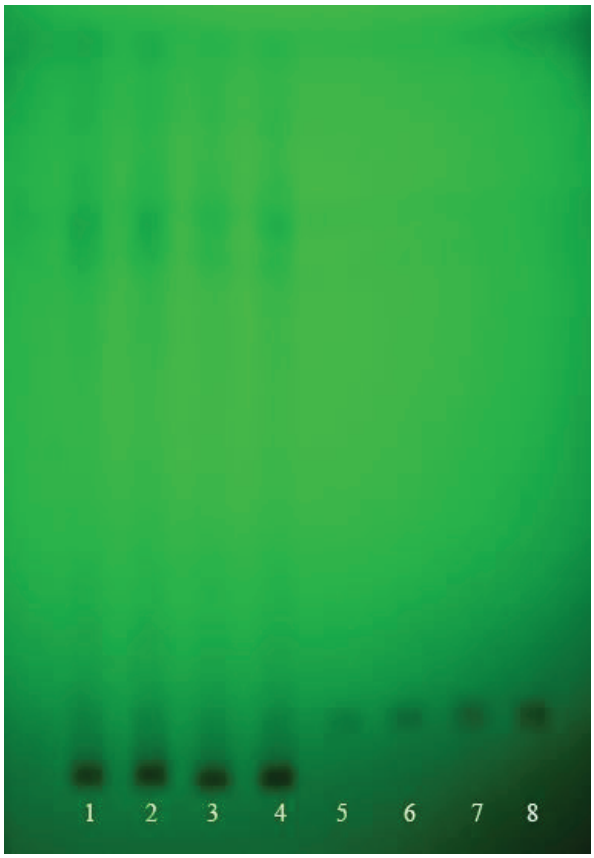

Figure 2. Image of HPTLC plate (254nm)
1. 5µl Acetone extract (5mg/ml), **2.** 10µl Acetone extract (5mg/ml), **3.** 5µl Methanol extract (5mg/ml), **4.** 10µl Methanol extract (5mg/ml), **5.** 0.2µl Protocatechuic acid Standard (1mg/ml), **6.** 0.4µl Protocatechuic acid Standard (1mg/ml), **7.** 0.6µl Protocatechuic acid Standard (1mg/ml), **8.** 0.8µl Protocatechuic acid Standard (1mg/ml), Solvent system Chloroform: Acetic acid (9:1), Detection at 254nm.

flask separately. Then dissolved in ethanol and diluted up to 10ml with ethanol. The solutions were filtered with what man no. 1 filter paper. Spots of 5 and 10 µl of both the solutions were applied to TLC plate along with 0.2, 0.4, 0.6 and 0.8 µl of Protocatechuic acid Standard (1mg/ml) spots on same plate as shown in figure 2. Peak of Protocatechuic acid in extract solution was identified by matching the Rf with peak obtained in Protocatechuic acid Standard solution.

The method was validated in terms of linearity, precision, repeatability, specificity, Limit of detection (LOD), Limit of quantification (LOQ) (8–9).

RESULTS

Result in Table 1 shows that 15.06% w/w methanol extract having dark brownish black color with characteristic odour and semisolid consistency, 14.56% w/w Acetone extract having dark brownish black color with characteristic odour and semisolid consistency were obtained. While successive extraction was performed with Petroleum ether, chloroform, methanol and water successively and it % yield, colour, odour and consistency are shown in Table 1

Qualitative chemical examinations of various extracts revealed the presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides, and tannins. Methanol and Acetone extracts showed presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides, and tannins and phenolics. Petroleum ether showed presence of steroids and terpenoids, while successive chloroform extract showed presence of steroidal compounds. (Table 2)

Estimation of Protocatechuic acid by HPTLC Methanol and Acetone extract of A. subulatum Roxb.

HPTLC Finger Printing of both extract

Figure 3 shows that in Acetone extract 8 peaks were observed its Rf and area is shown in table 3, out of which Peak no 2 at Rf 0.16 was assigned as Protocatechuic acid by matching Rf with standard Protocatechuic acid which is shown in image of HPTLC plate. (Figure 2)

Table 1. Physical characters of various extracts of *A. subulatum* Roxb. Fruit constituents

Extract	% Dry wt in gms.	Colour	Odour	Consistency
Methanol	15.06	Dark Brownish black	Characteristic	Semisolid
Acetone	14.56	Dark Brownish black	Characteristic	Semisolid
Successive extraction				
Petroleum Ether (40–60°C)	08.11	Brownish	Characteristic	Waxy
Chloroform	03.05	Brownish	Characteristic	Semisolid
Methanol	14.16	Dark Brownish black	Characteristic	Semisolid
Aqueous	02.09	Brown	Characteristic	Semisolid

Table 2. Phytochemical screening of various extracts of *A. subulatum* Roxb fruit constituents

Nature	Methanol	Acetone	SPE	SCH	SMET	SAQ
Alkaloids	–	–	–	–	–	–
Carbohydrates	+	+	–	–	+	+
Flavonoids	+	+	–	–	+	+
Amino acids	+	+	–	–	+	+
Steroids	+	+	+	+	+	–
Triterpenoids	+	+	+	–	+	–
Saponins	–	–	–	–	–	–
Glycosides	+	+	–	–	+	+
Tanins & Phenolics	+	+	–	–	+	+

]S.P.E=Petroleum Ether, SCH. = Chloroform, SMET=Methanol, SAQ= Aqueous

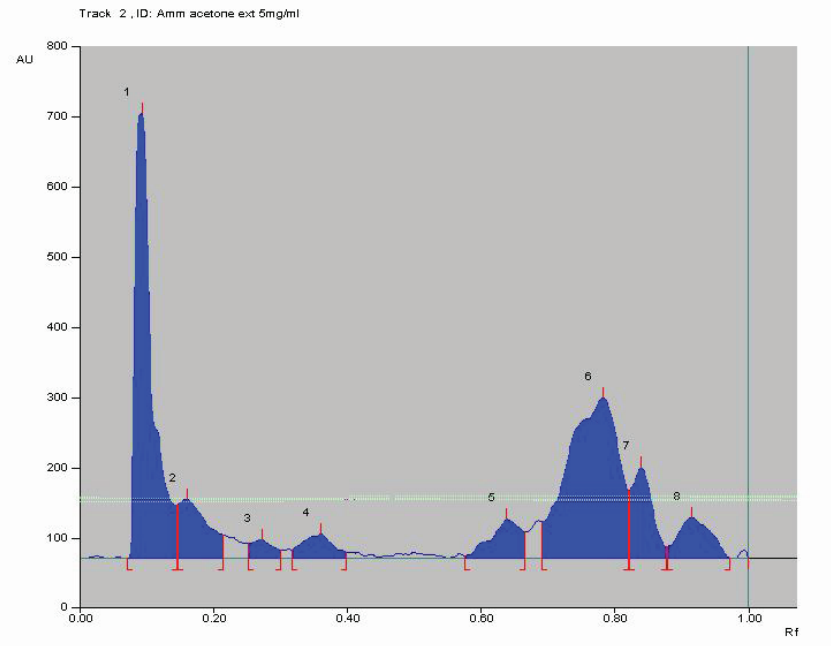

Figure 3. HPTLC finger print chromatogram of 10µl Acetone extract (5mg/ml) solution of *Amomum subulatum* Roxb.

Table 3. Rf and area of peaks observed in HPTLC chromatogram of 10µl Acetone extract (5mg/ml) solution of *Amomum subulatum* Roxb.

Peak No.	Rf	Area
1	0.09	12149.8
2	0.16	2734.5
3	0.27	657.5
4	0.35	1190.8
5	0.64	1891.7
6	0.78	13094.5
7	0.84	2981.1
8	0.91	2119.3

Figure 4 shows that in Methanol extract 11 peaks were observed its Rf and area is shown in table 4. Here, Peak no 2 at Rf 0.16 was assigned as Protocatechuic acid by matching Rf with standard Protocatechuic acid which is shown in image of HPTLC plate. (Figure 2)

Estimation of Protocatechuic acid in Acetone and Methanol extracts

Standard Protocatechuic acid showed single peak in HPTLC Chromatogram and single spots were observed on HPTLC plate as shown in figure 1, 5 and 6. Concentration of Protocatechuic acid in acetone extract and methanol extract were found to be 1.048 and 0.863 %w/w

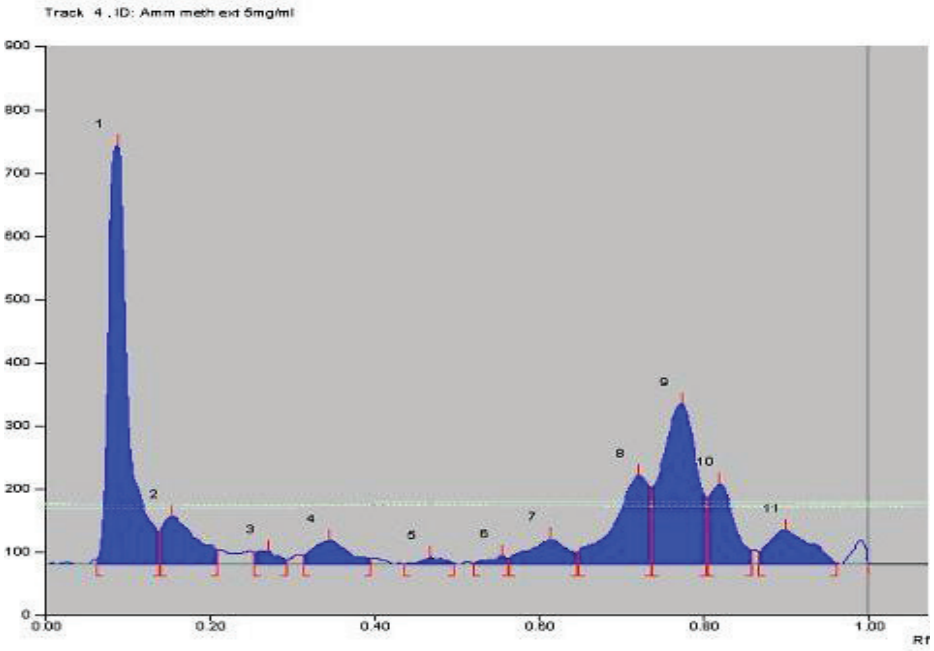

Figure 4. HPTLC finger print of chromatogram 10µl Methanolic extract (5mg/ml) Solution of *Amomum subulatum* Roxb.

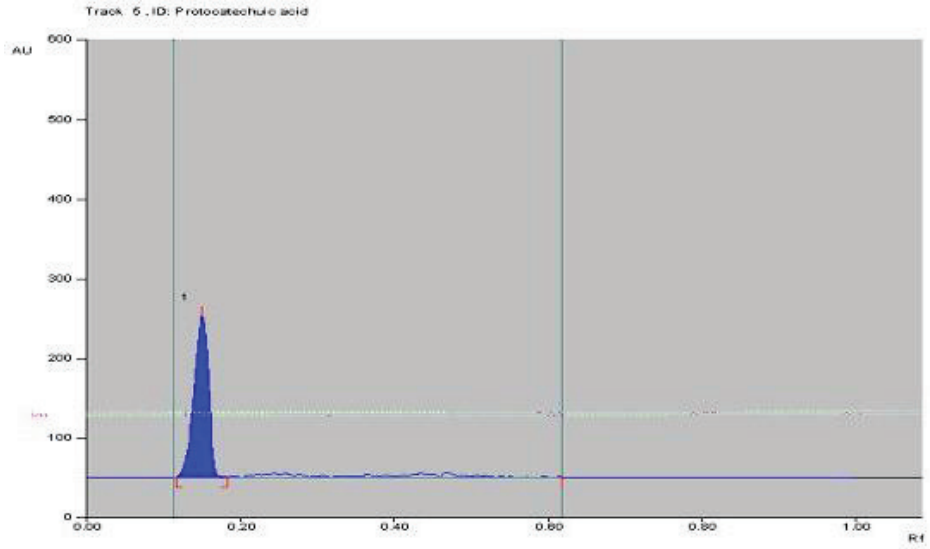

Figure 5. Chromatogram of standard Protocatechuic acid (Rf 0.16); Mobile phase: Chloroform: Acetic acid (9:1)

respectively calculated by regression equation y=4.6142x + 315.61, obtained from calibration curve of standard Protocatechuic acid. (Figure 7)

Validation of HPTLC method

Linearity

As shown in table 5, the correlation coefficient of calibration curve of Protocatechuic acid was found to be 0.9994, thus exhibits good linearity between concentration and area.

Accuracy (% Recovery)

The percentage recovery of Protocatechuic acid in methanol and acetone extract was found to be 99.83% and 98.84%, respectively (Table 6 and 7). Thus accuracy of the method has shown satisfactory results.

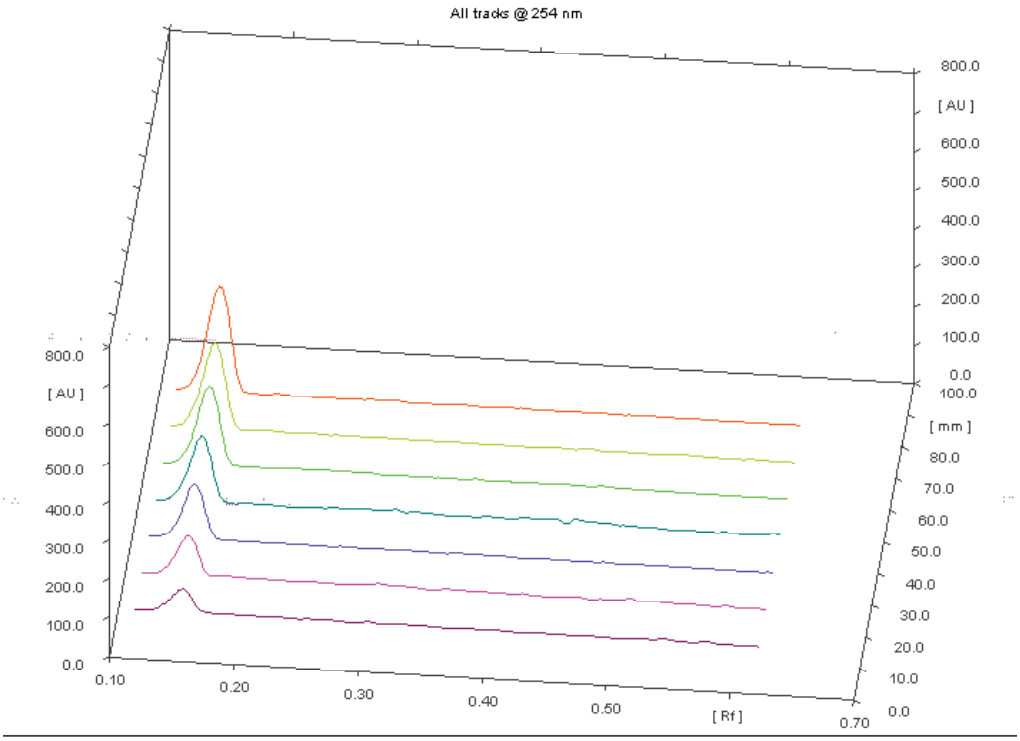

Figure 6. Three dimensional image of calibration spots of Protocatechuic acid (all tracks at 254nm)

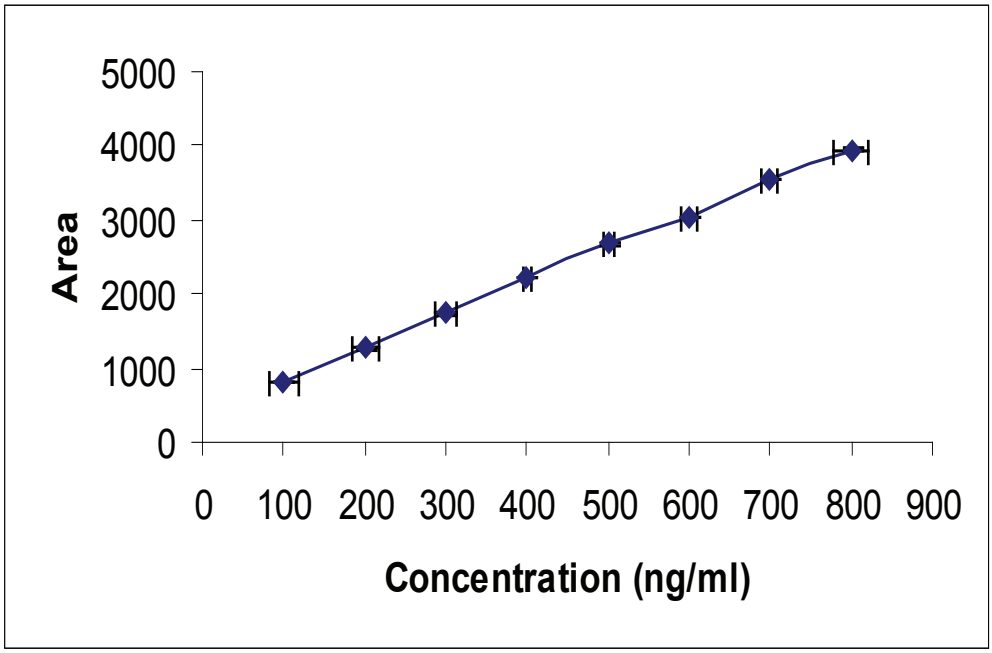

Figure 7. Calibration curve of Protocatechuic acid Standard. (r^2 0.9994), Data expressed as mean ± SEM, n=5

Table 5. Validation parameters for estimation of Protocatechuic acid by HPTLC Method

Parameters	Results
Linearity range	100 - 800 ng/spot
Precision (%CV)	
Repeatability of measurements	0.24 - 0.36 %
Repeatability of application	0.29 - 0.68 %
Interday	1.95 - 2.06 %
Intraday	1.12 - 1.56 %
Correlation coefficient (r2)	0.9994
limit of Detection	33 ng/spot
Limit of Quantification	100 ng/spot
Accuracy (% recovery)	
Methanol extract	97.57–102.01%
Acetone extract	96.65 - 100.79%
Specificity	Specific

Specificity

As Peak of standard Protocatechuic acid as well as Protocatechuic acid peak in sample was matching, therefore the method was found to be specific.

Limit of Detection and Limit of Quantitation

The minimum detectable limit and quantitation limit of Protocatechuic acid was found to be 33 ng/spot and 100 ng/spot, respectively.

DISCUSSION

Methanol and acetone extracts showed presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides, tannins and phenolics which confirms the previous findings (3). HPTLC finger printing of acetone and methanol extract separated 8 components and 11 components, respectively. It confirms presence of protocatechuic acid in both extract (11). Here, acetone extract showed higher concentration of protocatechuic acid as compared to methanol extract. In the past studies only Acetone was used for the extraction of protocatechuic acid (7), but present study revealed that methanol can also extract protocatechuic acid.

Validation of HPTLC method of estimation of protocatechuic acid exhibits good linearity between concentration and area. Precision, accuracy and specificity of the method has shown satisfactory results.

Table 6. Results of recovery study of the method for Protocatechuic acid in Methanolic extract of *Amomum subulatum* Roxb. Fruit constituents

Amount of sample taken	Amount of Protocatechuic acid found (mg)	Amount of Protocatechuic acid added (mg)	Amount of Protocatechuic acid taken (mg)	Amount of Protocatechuic acid found (Mean±SD, n=5) (mg)	% Recovery
25 mg	0.226	2.000	2.226	2.172 ± 0.1181	97.57
50 mg	0.432	2.000	2.432	2.481 ± 0.2477	102.01
75 mg	0.657	2.000	2.657	2.655 ± 0.4182	99.92

Average recovery : 99.83%

Table 7. Results of recovery study of the method for Protocatechuic acid in Acetone extract of *Amomum subulatum* Roxb. Fruit constituents

Amount of sample taken	Amount of Protocatechuic acid found (mg)	Amount of Protocatechuic acid added (mg)	Amount of Protocatechuic acid taken (mg)	Amount of Protocatechuic acid found (Mean±SD, n=5) (mg)	% Recovery
25 mg	0.267	2.000	2.267	2.191 ± 0.8170	96.65
50 mg	0.524	2.000	2.524	2.501 ± 0.4345	99.09
75 mg	0.790	2.000	2.790	2.812 ± 0.5324	100.79

Average recovery : 98.84%

CONCLUSION

The HPTLC method was found to be rapid, simple and accurate for quantitative estimation of Protocatechuic acid in different extracts. The Protocatechuic acid is main bio-marker compound of *Amomum subulatum* Roxb. fruit constituents. Hence the assay results of this compound can be useful for evaluation of marketed product.

REFERENCES

1. Rajani M. and Kanaki N. S. Phytochemical standardization of herbal drugs and polyherbal formulations. In: *Bioactive Molecules and Medicinal Plants*. Editors: Ramawat K.G., Merillon J.M. Springer-Verlag, Berlin Heidelberg; 349–369, (2008). 978-3-540-74603-4.
2. Anonymous. *The Ayurvedic Pharmacopoeia of India, Govt. of India*, First edition, Part I, Vol II, 158–159 (1999)
3. Wealth of India. *A dictionary of Indian Raw Materials and Industrial research*, Revised Edition Reprinted, Vol I, 226–229, (2006)
4. Joshi S.C. and Joshi V. Effect of Amomum subulatum on oxidative stress and atherosclerosis in cholesterol fed rabbits. *Pharmacologyonline*, **1**; 451–463, (2007) http://www.unisa.it/download/1966_10305_1269820156_47_Joshi.pdf.
5. Ravichandran G., Bharadwaj V.S. and Kolhapure S.A. Evaluation of the efficacy and safety of "Anti-Wrinkle cream" in the treatment of facial skin wrinkles: A prospective, open, phase III clinical trial, *The Antiseptic*, **102**(2); 65–70, (2005)
6. Yadav A. S. and Bhatnagar D. Modulatory effect of spice extracts on iron-induced lipid peroxidation in rat liver, *Biofactors*., **29**(2–3);147–57,(2007)
7. Kikuzaki H., Kawai Y. and Nakatani N. 1, 1-Diphenyl-2-picrylhydrazyl radical-scavenging active compounds from greated cardamom (*Amomum subulatum* Roxb.). *J Nutr Sci Vitaminol* (Tokyo), **47**(2): 167–71, (2001)
8. ICH, Q2A, 1994. *Validation of Analytical Procedure: Methodology*. In: Proc. Int. Con. Harmonization, Geneva.
9. ICH, Q2B, 1996. *Validation of Analyatical Procedure: Methodology*. In: Proc. Int. Con. Harmonization, Geneva.
10. Evans W.C. *Trease and Evans Pharmacognosy*, (W B Saunders, London, 15th edn, 202), 513–525. 978-81-312-0087-2.
11. Harborne J.B. *Phytochemical Method - A guide to modern techniques of plant analysis*, (Springer, 3rd edn, 2005), 43–45. 978-81-8128-310-8.