In Vitro Antioxidant Activity and HPTLC Analysis of *Borago Officinalis* Linn.

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ABSTRACT

Borage (*Borago officinalis* L.) is known drug in Unani system of medicine which is very popular as Gaozaban and used in various khamira (semi solid Unani formulations) for cardioprotection. In the present study, a comparative antioxidant potential of different extracts of borage leaves along with rosmarinic acid (positive marker of the drug) was carried out using DPPH and nitric oxide free radical scavenging method prior to estimated total phenolic content in each extract. The suitable solvent systems were developed for TLC profiling of each extract and results were compared. A HPTLC method was developed for the analysis of rosmarinic acid and it was validated as per the ICH guidelines. The developed method was found simple, selective, economic and accurate for the analysis of rosmarinic acid. The developed method was used for the estimation of rosmarinic acid in different extracts and it was concluded that content of rosmarinic acid is correlated to the antioxidant potential of the drug.

Keywords: *Borago officinalis* L., antioxidant activity, HPTLC, quality control, rosmarinic acid.

INTRODUCTION

The plant *Borago officinalis* Linn. is known as starflower, borage and gaozaban in Unani system of medicine, which is an annual herb found in Syria, Europe, America and Asia. It was popular for its mood elevating properties from ancient time.[1] The drug was cultivated for its culinary and medicinal importance as well as for the valuable seed oil, which contains gamma-linolenic acid and other fatty acids. The drug contains various medicinal properties as antispasmodic, antihypertensive, antipyretic, aphrodisiac, demulcent and diuretic. It has also useful treatment of cramps, diarrhea, palpitations, lungs, throat and kidney disorders.[2,3] The drug is recently studied for anti-inflammatory[4] and calcium antagonist mechanism for its antispasmodic, vasodilator and cardiodepressant activity.[2] Traditionally, the drug is used as nerve and cardiac tonic.[5] It contains different phytoconstituents as ascorbic acid, tannins, resins, beta-carotene, niacin, riboflavin, thiamine, silicic acid and choline arabinose. It also contains unsaturated pyrrolizidines alkaloids including amabiline, lycopsamine and supinidine, which are found to have hepato toxicity.[6,7,8] Phenolic constituents are known for their antioxidant potential of herbs and drug have studied for its antioxidant potential by using *in vitro* 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method and rosmarinic acid (phenolic acid) was demonstrated as one of the main antioxidant constituent of the drug.[9] The cytotoxicity and free radical scavenging activities of the drug were also observed[9,10] which may be due to presence of phenolic acids.[11] In Unani system of medicine the drug is very popular as Gaozaban and used in various khameeras for cardioprotection. The previous studies are indicating that the drug is pharmacologically very useful but further need to explore its protective role for human health. There is no any report

DOI: 10.5530/ijper.47.4.4

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on this drug for marker specific study for its quality control. In a present study, the quality assessment of borage was carried out by doing *in-vitro* antioxidant activity of different extract with their fingerprinting by using thin layer chromatography however few quantitative methods are available for the estimation of rosmarinic acid[12] but we have improved previous method and validated as per the ICH guidelines.[13] The method was found simple, selective precise and accurate for the analysis of rosmarinic acid. The estimation of rosmarinic acid was carried out in different extracts of borage and its relation with antioxidant activity was established.

MATERIAL AND METHODS

Chemicals and reagents

Reference standard ascorbic acid (99%), rosmarinic acid (98%) and catechin were purchased from sigma Aldrich, USA. Borage was purchased from local market of Delhi, India and identified by Dr. H B Singh, Scientist F and Head, Raw Material and Herbarium and Museum, NISCAIR, New Delhi. Chromatographic grade methanol and toluene, ethyl acetate, formic acid and other analytical reagents were purchased from Merck, India. Spectral and absorbance measurements were carried out on a Shimadzu UV-Vis 1601 spectrophotometer by using 1.0 cm quartz cells.

Sample preparation

The air-dried borage leaves (2.0 gm) were powdered and then extracted with 50 mL of methanol by using reflux condenser. The crude extract was filtered and evaporated under reduced pressure and reconstituted using methanol in 10 mL volumetric flask. This was used for the different analysis and *in vitro* antioxidant activity.

Similarly, chloroform and petroleum ether extracts were prepared and used for the analysis.

Total phenolic contents

Estimation of total phenolic contents was carried out according to the Folin-Ciocalteu (F.C.) method using catechin as a standard phenolic constituent.[14] Different dilutions were prepared from 25–300 µg mL^{-1} of standard catechin with methanol. Each standard dilution (0.5 mL) was taken in to a test tube. The 5.0 mL of F.C. reagent (10%) and 4.0 mL of 1 M sodium carbonate (Na_2CO_3) solution were added to the test tubes. The methanolic, chloroform and petroleum ether extracts of borage (0.5 mL) were also taken in to separate test tubes and similarly reagents were added to it and kept for 15 min. The blue color was developed and the absorbance was measured at 765 nm against blank solution. The calibration curve was plotted using standard dilutions and from the curve, linear regression equation was obtained. The concentrations of phenolic contents in different extracts of borage were calculated by using standard calibration curve.

DPPH free radical scavenging method

The free radical scavenging capacity of methanolic, chloroform and petroleum ether extracts of borage and rosmarinic acid was determined using established DPPH method.[15,16] The borage extracts and rosmarinic acid were mixed with 95% methanol to prepare the stock solution (1.0 mg mL^{-1}). The 1.0 mL of DPPH solution (0.10 mM) prepared in 95% methanol was taken in test tubes then 1.0 mL of each sample was added followed by serial dilutions (5.0–200 µg mL^{-1}) to every test tube. Ascorbic acid was used as a reference standard and dissolved in methanol to make the stock solution with the same concentration (1.0 mg mL^{-1}) followed by serial dilutions (5.0–200 µg mL^{-1}). The absorbance was measured after 10 min at 515 nm. The control sample was prepared containing the same volume without any extract and reference ascorbic acid. Methanol (95%) was used as blank. The percentage scavenging activity of the borage against DPPH free radical was measured using the following equation:

$$\% \ Inhibition = [(A_0 - A_1) / A0] \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance of the extract or standard.

Nitric oxide scavenging method

Nitric oxide radical scavenging activity was determined according to the established method.[17,18] Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, it can be determined by the use of the Griess Illosvoy reaction. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. The sodium nitroprusside (2.0 mL of 10 mM in 0.5 mL phosphate buffer pH 7.4 was mixed with 0.5 mL of borage extracts and rosmarinic acid at various concentrations (10–200 µg mL^{-1}) and the mixture was incubated at 25ºC for 150 min. From the incubated mixture, 0.5 mL was taken out and added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for five min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1%, w/v) was mixed and incubated at room temperature for 30 min. The absorbance of the chromaphore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was measured at

540 nm and compared to the absorbance of standard solutions of ascorbic acid treated in the same way with Greiss reagent as a positive control. The nitric oxide radicals scavenging activity of samples was calculated as similar to DPPH method.

Chromatographic estimation

Development of TLC fingerprint

The dry extracts were reconstituted to get 20 mg mL^{-1} concentration and used for TLC analysis. Different solvent systems were tried for separation of constituents by hit and trial method and best ratio of solvent systems were selected for the final analysis.

Samples were applied on precoated silica gel 60 F_{254} TLC plates (5 × 10 cm) by linomat V sample applicator. The samples were applied in triplicate (8.0 µL, each) the width of the track was 5.0 mm and distance between tracks was kept on 14 mm. After sample application, the plates were developed up to 80 mm in development chamber saturated with the selected solvent system. Then plate was scanned 254 and 366 nm followed by spectral analysis. Reprostar Chromatography Documentation Apparatus (RCDA) was used for taking photographs of the HPTLC plates. Plates also scanned at suitable wavelength in visual range after sprayed visualizing reagents.

Development of HPTLC method for analysis of rosmarinic acid

Different solvent systems were tried for separation of rosmarinic acid in sample by hit and trial method and the best solvent system was selected for the final analysis. The samples were spotted in the form of bands of width 3.0 mm using microlitre syringe on pre-coated silica aluminum sheet 60F_{254} (20×10 cm, 0.2 µm thickness) using Camag Linomat V sample applicator (Switzerland). The plates were pre-washed with methanol and activated at 60°C for 20 min prior to chromatography. A constant application rate of 120 nL sec^{-1} was employed and space between two bands was kept at 6.8 mm. The slit dimension was kept at 3.0×0.30 mm and 20 mm sec^{-1} scanning speed was employed. The mobile phase consisted of toluene: ethyl acetate: formic acid (5:4:1, *v/v/v*) and 15 mL of mobile phase was used for per chromatography. Linear ascending development was carried out in 20×10 cm twin trough glass chamber, which was previously saturated with mobile phase for 15 min. The length of the chromatogram run was 80 mm. After the development, TLC plate was dried in a current of air with the help of an air dryer. Densitometric scanning was performed on Camag TLC scanner III operated by winCats software using wavelength 330 nm. The source of radiation utilized was tungsten lamp.

RESULTS AND DISCUSSION

Total phenolic contents

Antioxidant efficacy of the plants is mainly due to presence of phenolic constituents which may contribute directly to the antioxidant properties. Recently, so many reports to suggest that phenolic compounds have inhibitory effect on carcinogenesis and mutagenesis in humans, when ingested high fruits and vegetables rich diet. Different extracts of borage were prepared and total phenolic contents were measured by using F C reagent method. It was found that the maximum amount of phenolics was present in methanolic extract 4.12% w/w whereas chloroform and petroleum ether extract contained 2.35 and 1.36% w/w respectively.

DPPH free radical scavenging method

The borage extracts and rosmarinic acid showed a concentration-dependent antioxidant activity by inhibiting DPPH radicals. The results indicated that as polarity of the solvent for extraction is increasing the antioxidant efficacy is also increasing. The rosmarinic acid and methanolic extract of borage showed significant antioxidant efficacy with an IC_{50} value of 96.4 µg mL^{-1} and 116.2 µg mL^{-1}, respectively whereas IC_{50} value of ascorbic acid was found to be 22.94 µg mL^{-1}. The chloroform and petroleum ether extracts were showing less antioxidant efficacy with IC_{50} values 431.8 and 1059 µg mL^{-1}, respectively (Fig. 1A). The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extracts and rosmarinic acid were having the efficacy to reduce the stable radical DPPH to the yellow-coloured diphenyl picrylhydrazine. It has been found that ascorbic acid, rosmarinic acid and methanol extract considerably reduces and decolorizes 1, 1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability.

Nitric oxide scavenging method

The results of nitric oxide scavenging method were also showed that borage extracts and rosmarinic acid have dose dependent efficacy between 10–50 µg mL^{-1}. The methanolic extract of borage and rosmarinic acid was having maximum efficacy with 60.58 and 41.28 µg mL^{-1} as IC_{50} values, respectively whereas IC_{50} value of ascorbic acid was found 14.37 µg mL^{-1}. The chloroform and petroleum ether extract were showing less antioxidant efficacy similar to DPPH method with IC_{50} values 163.5 µg mL^{-1} and 824.8 µg mL^{-1}, respectively (Fig. 1B). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions. The plant or plant products may have the property to counteract the effect

of NO formation and in turn may be of considerable interest in preventing the disease effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

TLC fingerprinting

TLC profiling of petroleum ether extract showed the presence of 10 constituents at different R_f values at 366 nm. Similarly chloroform extract contained 6.0 spots at 400 nm and methanol extract contained 12 constituents at 530 nm by using anisaldehyde sulphuric acid as visualizing agent. The developed TLC plates and HPTLC chromatograms are given in Fig. 2 and results are summarized in Table 1.

HPTLC method validation

The developed method was validated as per ICH guidelines[13] still, there are several chromatographic HPLC and HPTLC methods have been developed and validated by the laboratory,[19-22] which are in use for the quality assessment of herbal drugs.

Calibration curve for rosmarinic acid

A stock solution of rosmarinic acid having a known concentration 200 µg mL^{-1} was prepared in methanol and different dilutions were prepared and 2.0 µL of each were applied to get the concentration from 20–1000 ng $spot^{-1}$. The calibration curve was plotted using peak area versus drug concentration. For assessing the linearity, the least square regression equation and correlation coefficient were calculated.

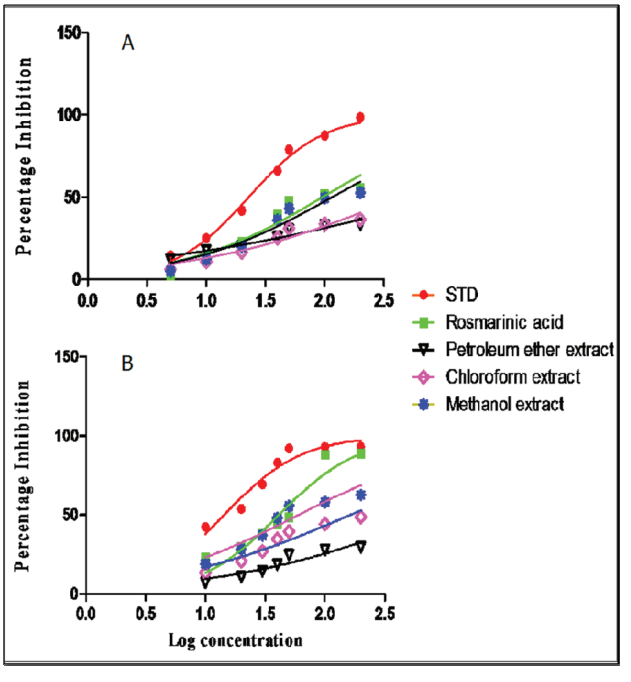

Figure 1: Comparative antioxidant efficacy of borage extracts and rosmarinic acid with ascorbic acid by DPPH (A) and nitric oxide scavenging method (B).

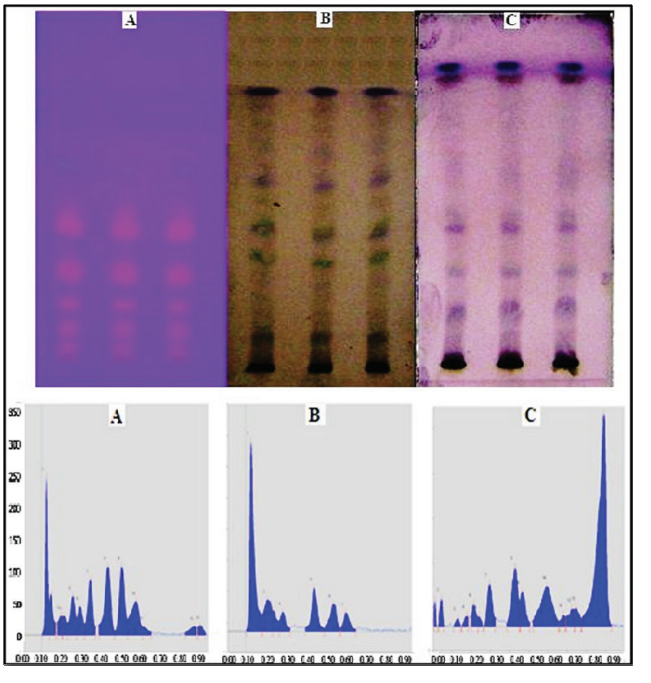

Figure 2: Developed TLC plates and HPTLC chromatograms of petroleum ether (A), chloroform (B) and methanol (C) extracts of borage.

Table 1: HPTLC Profiling Data of Different Extracts of Borage

Sample	Solvent system used	Detection wavelength	No. of peaks and R_f values
A-Petroleum ether extract	Petroleum ether: diethyl ether (1:1, v/v)	366 nm[a)]	(10) 0.1, 0.16, 0.21, 0.27, 0.38, 0.47, 0.55, 0.6, 0.91, 0.95
B- Chloroform extract	Hexane: ethyl acetate (4:1, v/v)	400 nm (sprayed with anisaldehyde sulphuric acid)	(6) 0.11, 0.15, 0.2, 0.39, 0.51, 0.59
C-Methanol extract	Toluene: ethyl acetate (9:1, v/v)	530 nm (sprayed with anisaldehyde sulphuric acid)	(12) 0.13, 0.18, 0.21, 0.24, 0.29, 0.43, 0.47, 0.60, 0.68, 0.72, 0.75, 0.89

[a)]Nanometer

Linearity

The linearity of the calibration plot for analysis of rosmarinic acid by HPTLC method at in the range of 20–200 ng spot^{-1} was good with $r^2 = 0.993$. The calibration curve obtained during analysis could be described by the linear equation y = 377.99 + 35.209x where Y is peak area and X is the concentration. Summary of calibration plot obtained in triplicate were presented in Table 2.

Accuracy

The accuracy of the methods was determined by doing recovery studies. For this, pre analyzed samples were spiked with standard rosmarinic acid in three different levels i.e. 50, 100 and150% and the mixtures were analyzed by the proposed methods. From the data obtained, the developed method was found to be accurate and recovery was found to be within the limit of 97.03–102.76%. The values of recovery % and % RSD were depicted in Table 3.

Precision

Precision of the proposed method was obtained by repeatability and intermediate precision in accordance with ICH guidelines. In the analysis of six different plates of rosmarinic acid in three concentrations were applied and developed. The plates were scanned and the peak area obtained in each chromatogram was comparatively studied by proposed method and % RSD, were calculated. Inter-day and intraday precisions were done by preparing and applying three different concentrations of samples in the same day and in three different days, respectively. The results from the repeatability and intermediate precisions, expressed as % RSD were depicted in Table 4.

Specificity

The specificity of the proposed method was determined by comparing the sample and standard peaks for its R_f and UV spectra. Three point peak purity i.e. peak start, peak apex, and peak end was compared and found superimposed. This indicated that standard rosmarinic acid and in sample peaks were not merging with any other components or impurities. The peak purity of rosmarinic acid was assessed by comparing the spectra at three different levels, i.e. Peak start, peak apex and peak end positions (Fig. 3C).

LOD and LOQ

The limits of quantitation and detection were calculated as per linearity curve method by using the formula LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the response and S is the slope of the calibration plot. For the developed method LOD was found as 5 ng spot^{-1} and LOQ was calculated as 18 ng spot^{-1}.

Robustness

Robustness of the method was carried out by introducing small changes in the composition of mobile phase and detection wavelength, the effect on the results were examined as % RSD. Mobile phase having the compositions of toluene: ethyl acetate 47: 43: 10 and 53: 37: 10, v/v/v were used whereas detection wavelength has changed (±3) and results were observed. The chromatograms were developed and robustness of the method was observed at three different concentration levels 50, 100 and 200 ng spot^{-1} (Table 5).

Table 2: Linearity data of HPTLC Methods

Parameter	Observation
Linearity range	20–200 ng[a] spot - 1
Regression equation	Y = 377.99 + 35.209x
Correlation coefficient ± S D[b]	0.993 ± 0.001
Slope ± S D	35.209 ± 0.93
Intercept ± S D	377.99 ± 0.86

[a]Nanogram, [b]Standard deviation

Table 3: Accuracy of the HPTLC Method (n=6)

% of standard drug added	Theoretical content (ng[a])	Recovered drug (ng)	% of drug recovered	% RSD[b]
0	53.13	53.67	101.01	1.40
50	79.69	77.32	97.03	1.56
100	106.26	109.20	102.76	2.97
150	132.82	131.95	99.34	0.17

[a]Nanogram, [b]Relative standard deviation

Table 4: Precision of the HPTLC Method (n =6)

Concentration (ng[a] spot^{-1})	Inter-day precision		Intra-day precision		Inter-system precision	
	Mean area ± SD[b]	%RSD[c]	Mean area ± SD	%RSD	Mean area ± SD	%RSD
50	2107.41 ± 19.95	0.95	2098.25 ± 44.54	2.12	2105.84 ± 15.45	0.73
100	4134.11 ± 27.83	0.67	4112.20 ± 22.14	0.54	4134.07 ± 42.37	1.02
200	6933.99 ± 42.31	0.61	6985.54 ± 22.80	0.33	6878.06 ± 141.64	2.06

[a]Nanogram, [b]Standard deviation, [c]Relative standard deviation

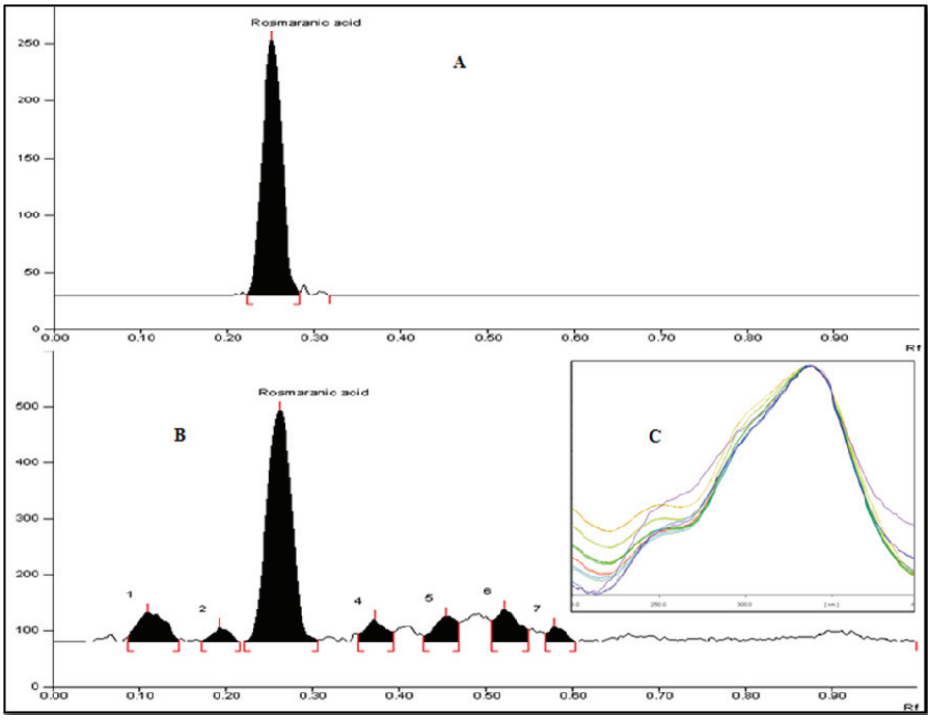

Figure 3: HPTLC chromatogram of standard rosmarinic acid (A), borage extract (B) and superimposed UV spectra (C) of rosmarinic acid standard and in borage extract.

Table 5: Robustness of the HPTLC Method (n=3)

Mobile phase composition (Toluene: ethyl acetate: formic acid, 50: 40: 10, v/v/v)		Mean area ± SD[b]	% RSD[c] of area
Concentration (ng[a] spot^{-1})	Used (v/v/v)		
50	53: 37: 10	2099.73 ± 30.32	1.44
	47: 43: 10	2091.84 ± 41.16	1.97
100	53: 37: 10	4117.68 ± 34.87	0.85
	47: 43: 10	4125.45 ± 30.41	0.74
200	53: 37: 10	6932.54 ± 63.68	0.92
	47: 43: 10	7012.55 ± 75.95	1.05
Detection wavelength (330 nm)		Mean area ± S.D	% RSD of area
Concentration (ng spot^{-1})	Used wavelength (nm)		
50	333	2146.13 ± 41.52	1.93
	327	2117.40 ± 36.11	1.71
100	333	4177.33 ± 86.06	2.02
	327	4184.57 ± 38.55	0.92
200	333	7006.25 ± 79.38	1.13
	327	5052.26 ± 69.42	0.98

[a] Nanogram, [b] Standard deviation, [c] Relative standard deviation

Analysis of sample

The newly developed and validated HPTLC method was applied for the analysis of rosmarinic acid in borage leaves. The peak areas of triplicate samples were analysed by regression equation obtained from calibration plot to get the content of rosmarinic acid by using HPTLC method. The rosmarinic acid content was found to be 0.0378% w/w in methanol extract of borage whereas petroleum ether extract having undetectable amount and chloroform extract was having only 0.0092% w/w of rosmarinic acid.

CONCLUSION

The present study concluded that the methanolic extract of borage have more protective efficacy towards the human body as compared to petroleum

ether and chloroform extract. The methanolic extract showed maximum antioxidant activity with maximum constituents in TLC profiling. The first time we are reporting rosmarinic acid as positive biomarker for the drug and it was estimated in petroleum ether, chloroform and methanol extract by newly developed and validated HPTLC method. It was again evident that the concentration of rosmarinic acid was found maximum in methanol extract. Rosmarinic acid has reported to have antioxidant and various pharmacological properties. The study also showed that rosmarinic acid and methanolic extract having good antioxidant potential, it will be helpful for quality control as well as exploring the usefulness of the drug.

ACKNOWLEDGEMENT

The authors are highly thankful to the Central Council for Research in Unani Medicine (CCRUM), AYUSH and Department of Science and Technology (DST), New Delhi, Govt. of India, for providing the financial assistance for carrying out this research work.

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