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Antioxidant activity of essential oil of three cultivars of *Amomum subulatum* and standardization of high performance thin layer chromatography (HPTLC) method for the estimation of 1,8-cineole

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Essential oils of the fruits of three cultivars of *Amomum subulatum* (Family-Zingiberaceae), such as varlangy, seremna and sawney were isolated. Antioxidant potential was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging and $FeCl_3$-reducing models and the presence of 1,8-Cineole was quantified using the developed high performance thin layer chromatography (HPTLC) method. In the DPPH scavenging model, the IC_{50} value of the essential oil of the seremna, varlangy and sawney were found to be 172.3, 216.9 and 274.3 µg/ml, respectively, while in the ABTS scavenging model, the IC_{50} value of the seremna, varlangy and sawney were found to be 27.96, 31.34 and 32.49 µg/ml respectively. The antioxidant power of the $FeCl_3$ reducing model, the absorbance value of the essential oil of the seremna was found comparatively higher than the essential oils of varlangy and sawney. The content of 1,8-cineole was determined by the developed HPTLC densitometric method, and the value of the percentage (mean ± SD, % w/w) content of the essential oils of the seremna, varlangy and sawney were found to be 69.59 ± 1.45%, 48.78 ± 3.21 and 47.84 ± 1.76 respectively. The highest antioxidant value of seremna cultivar may be due to the presence of high content of 1, 8-cineole. The HPTLC method, therefore confirms that monoterpene 1,8-cineole is the main antioxidant compound present in the fruits of *A. subulatum*.

Key words: *Amomum subulatum*, cultivars, essential oil, 1,8-cineole, high performance thin layer chromatography (HPTLC), antioxidants.

INTRODUCTION

The fruits of *Amomum subulatum* or greater cardamom is one of the most expensive fruits of the large family, Zingiberaceae of the Kingdom Plantae (Kumar et al., 2013). It is a perennial herb consisting of subterranean rhizomes and leafy aerial shoots which stands erect at 1.7 to 2.6 m depending on the cultivars. Inflorescence of this plant has a condensed spike, commonly found, and has 10-40 fruits in each spike depending on the cultivars. Flowering season starts during March–April at lower altitudes, and in May, at higher altitudes. Harvesting starts during August–September at lower altitudes and October–December at higher altitudes (Gupta and John,

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1987). It is also known by other common names such as, English name: black cardamom, hil cardamom, Bengal cardamom, Nepal cardamom; Sanskrit: Aindri, Sthula ela, Brihatupakunchika, Hindi, Punjabi and Gujarati kali ilaichi; Malayalam: karutta elakka; Marathi: Moto-eldori, Moteveldode and Thorveldoda. There are a number of cultivars of *A. subulatum* species grown worldwide, with some of the cultivars such as ramsey, sawney, golsey, varlangy, seremna, etc. popularly cultivated in the Sikkim State and North Bengal, Darjeeling (West Bengal State) of India. Sikkim is one of the leading states in India, which produce huge quantity of the fruits of *A. subulatum* (Dubey and Yadav, 2001). In the area, Sikkim ranked 28th; situated in the north eastern side of India and contributes approximately 53% of the world production of the greater cardamom (Sharma et al., 2000; Agnihotri and Wakode, 2010; Joshi et al., 2013). In India, this perennial herb is commonly planted at an elevation of 700 to 1650 m above sea level and is widely used for the condiments, homemade remedies, and Indian and Chinese systems of medicine (Mukherjee, 1972; Madhusoodhanan and Rao, 2001). In addition to India, the other cultivars of *A. subulatum* is also cultivated in the several other countries such as Nepal, Bhutan, China and Indonesia.

Dried fruits of spices and its essential oils are a good source of an antioxidants. Commercial synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are used to avert lipid peroxidation of commercial food products but these chemicals also which have carcinogenic properties. The essential oil of *A. subulatum* has already been used as a preservative in the juice industry (Kapoor et al., 2009). The essential oil of the fruits of Greater cardamom has already been evaluated for the antioxidant and other activities (Sharma et al., 2017). The other activities such as antiviral, antitussive, antiallergic, anti-inflammatory, bronchodilator, mucolytic, gastroprotective, antitumor, antimicrobial and insecticidal activities of the 1,8-cineole has also been reported (Juergens et al., 2003; Lima et al., 2013). Essential oil obtained from the fruits of *A. subulatum* using hydro-distilled methods, mainly contain monoterpenes 1,8-cineole (Kaskoos et al., 2013). Along with 1,8-cineole, the other monoterpenes such as α-terpineol, DL-limonene, nerolidol, 4-terpineol, δ-terpineol, δ-3-carene, β-myrcene, germacrene D, α-terpinene and longifolenaldehyde have been reported in the hydrodistilled essential oil of the fruit (Vilela et al., 2009; Hussain et al., 2011).

Nowadays, research is concerned with finding the safe and natural antioxidant for the commercial food products to avoid the carcinogenicity of the food products linked with synthetic antioxidants, but in the most of the investigations, the natural antioxidants showed comparatively lower potency than synthetic antioxidants (Ramalho and Jorge, 2006). There is need to investigate the antioxidant potential of the natural sources in the whole extract, because in addition to the principal compounds several other agonist and antagonist compounds present in it, which may together show synergistic potential or other compounds may revert the foremost side effects (Jimenez-Andrade et al., 2003). The oxidative stress is an imbalance between free radical production and antioxidant defences, due to overproduction of free radical through metabolic process and caused several diseases (Uttara et al., 2009; Lobo et al., 2010). Numerous research suggests that consumption of oils, fruits and vegetables or other natural products are important for decreasing the ROS oxidative stress by a variety of mechanisms (Rahal et al., 2014). HPTLC is one of the most advanced form of chromatography, highly applicable for the quantification of active compounds in the extracts, oils and other natural products and it is widely accepted in all over the world (Attimarad et al., 2011; Kathirvel et al., 2012). The main purpose of this study was to select a highly antioxidant varieties of fruits of A. *subulatum* species following *a* new approach, that is, comparing the essential oil of three popular varieties of *A. subulatum fruits,* using DPPH, ABTS and ferric reducing methods along with quantifying the main active constituents 1-8 Cineole using newly developed, reproducible and accurate HPTLC method.

MATERIALS AND METHODS

Three different cultivars of *A. subulatum* fruits (sawney, seremna and varlangy) were obtained from ICRI Tadong, Sikkim, India, in 2016. The reagents such as DPPH, ABTS and BHT (3,5-ditert-4-butylhydroxy toluene were obtained from Sigma, USA. The markers 1,8 cineole (purity > 99%) was procured from the supplier (Sigma Aldrich), Polaris Bioscience, Delhi, India. The pre-coated aluminium-backed TLC plates (silica gel, 0.2 mm thick, 60$F_{254,}$ 20 × 20 cm) were purchased from E. Merck, Germany.

Separation of essential oil

The fruits of sawney, seremna and varlangy cultivars of *A. subulatum* were coarsely powdered in the mixer grinder and passed through sieve No. 60. About 100 g of each powder was hydro-distilled for 4 h using Clevenger apparatus (lighter than water). Further, distilled essential oil was collected and dried over anhydrous sodium sulphate; the obtained essential oils were transferred using a glass vial, and stored in the deep freezer for further study.

Antioxidants activity

Free radical scavenging activity (DPPH model)

The DPPH scavenging activity of the essential oil was assessed by using reported method (Asnaashari et al., 2016), with slight modification. In short, essential oils and standard (BHT) were separately diluted in methanol to obtain 1000 µg/ml stock solution. 80 µg/ml of DPPH solution was prepared. Six dilutions (500 to15. 6 µg/ml) of samples and standard were prepared separately. Accurately, 5 ml of DPPH was mixed with 5 ml of samples and

standard solution and the mixtures were kept in the dark at 25°C for 30 min. The absorbance was recorded immediately at 517 nm by UV spectrophotometer. Percentage inhibition of free radical was calculated by the following formula:

$$\% \text{ inhibition} = \frac{B - A}{B} \times 100$$

Where, A was the absorbance of sample and standard solution and B was the means absorbance of blank (without essential oil or standard, BHT). Further, a plot between percentage inhibition and concentration of different dilutions of samples and standards was plotted for the estimation of IC_{50} (50% inhibition).

Free radical scavenging activity (ABTS model)

The ABTS antiradical activity of essential oils and the standard was assessed using reported methods (Yang et al., 2009) that was slightly modified. In short, 1000 µg/ml stock solution was used to prepare the serial dilutions (200 - 12.5 µg/ml) of samples and standard. The ABTS solution was prepared by reacting equal amount of ABTS (7 mM) with potassium persulfate (2.45 mM). The reaction mixture was kept in the dark for at least 4 h at 25°C. Further, it was diluted with 0.1 M sodium phosphate buffer (pH 7.4) to acquire an absorbance of 0.70 ± 0.02 at 734 nm. Accurately, 2.99 ml of ABTS solution was mixed with 10 µl of serial diluted solution of samples and standard and kept in the dark at 25°C for 30 min. An absorbance was recorded immediately at 734 nm by UV spectrophotometer. Percent inhibition and IC_{50} were calculated by equation mentioned in DPPH assay.

Reducing power assay (Ferric chloride model)

The reducing power assay of the essential oils was assessed using reported methods (Olugbami et al., 2015) with slight modification. In short, a stock solution of samples and standard were used for the serial dilutions (200 - 25 µg /ml). About 1 ml of solution from each dilution was mixed with 2.5 ml of phosphate buffer of 0.2 mol/l (pH 6.6) and 2.5 ml of 1% $C_6N_6FeK_3$ solution and were incubated at 50°C for 30 min. About 2.5 ml of 10% trichloroacetic acid (TCA) solution was added into it and was then centrifuged for 10 min at 3000 *g*. Further, about 2.5 ml of the supernatant was pooled with 2.5 ml of distilled water, shaken with 0.5 ml of 0.1% $FeCl_3$ solution (freshly prepared) and incubated at room temperature for 10 min. The absorbance was recorded at 700 nm against blank (n=3). A graph was plotted between the average of absorbance and concentration for estimation of antioxidant activity.

Chromatographic conditions

The high performance thin layer chromatography (HPTLC) method was developed on a system consisting; TLC applicator Linomat V, automatic development chamber 2 (ADC2), derivatization chamber, TLC plate heater, TLC Scanner equipped with WinCATS software (version 1.4.6) and TLC Reprostar 3 (all from Camag, Muttenz, Switzerland). Separation, derivatization and identification were done with *TLC Silica Gel 60 F254 Glass* plates, 20 × 20 cm and derivatization of plates was developed by automatic TLC sprayer using Vanillin-H_2SO_4 reagents.

Preparation of standard and sample solutions

1000 µg/ml w/v of the standard solution of 1,8-cineole, and the sample solution of the essential oils were prepared separately in the solvent methanol.

Chromatographic conditions

Estimation of 1,8 cineole was performed on a TLC plate without pre-washing. Standard and samples of essential oils were applied to the silica gel plates as 8 mm bands with Linomat V. The plates were developed in automatic development chamber 2 to a distance of 80 mm, relative humidity 50-60%, at 25 ± 5°C, using mobile phase composed of hexane: ethyl acetate (8:2), saturation for 25 min. After development, the plates were air-dried and observed in a visualizer at white light, 254 nm and 366 nm. The reagent Vanillin-H_2SO_4 was used for the derivatization and then the plates were heated at 100°C using a TLC plate heater. The plate was then cooled and scanned in the scanner at UV 665 nm for the quantification.

Calibration curve for standard

The calibration curve for the standard was prepared after application of 1 to 7 µg on a TLC plate. It was then developed and scanned as per the conditions of chromatography. The peak areas were recorded and the calibration curve of standard 1,8-cineole was made by plotting peak area verses concentration.

Method validation

The intended method was validated, referring to the International Conference on Harmonization guidelines (ICH, 2005). The linearity of the current method for the 1,8-cineole was checked (100 to 700 ng/spot) and concentration was plotted against peak area. LOD (Limit of detection) and LOQ (limit of quantification) were determined from the slope of the calibration curve. The equation $3.3\times SD\times S^{-1}$ and $10\times SD\times S^{-1}$ were used for the determination LOD and LOQ respectively. The accuracy of the present method was tested by the pre examined samples spiked with 1,8-cineole (0, 50, 100, and 150%); the mixtures were re-examined; and the percentage of recovery and RSD (% Relative standard deviation) were calculated. The precision of standard was determined by study of repeatability and intermediate precision. Repeatability precision was measured in 6 replicates of a standard solution at three different concentration levels 300, 400 and 500 ng/spot on the same day (intra-day precision). Intermediate precision was also measured in 6 replicates of a standard solution at three different concentration levels on different days (inter-day precision). Robustness of the method was determined by estimating the effect on small changes in the polarity of the mobile phase.

Statistical data

The mean ± standard deviation (SD), relative to percentage of scavenging activity by means of non-linear regression, followed by log (inhibitor) vs. response - Variable slope (four parameters) was used to estimate the IC_{50} using software GraphPad Prism, version 7.03 (San Diego, CA).

RESULTS AND DISCUSSION

DPPH free radical scavenging activity

Figure 1 shows the percentage DPPH radical scavenging

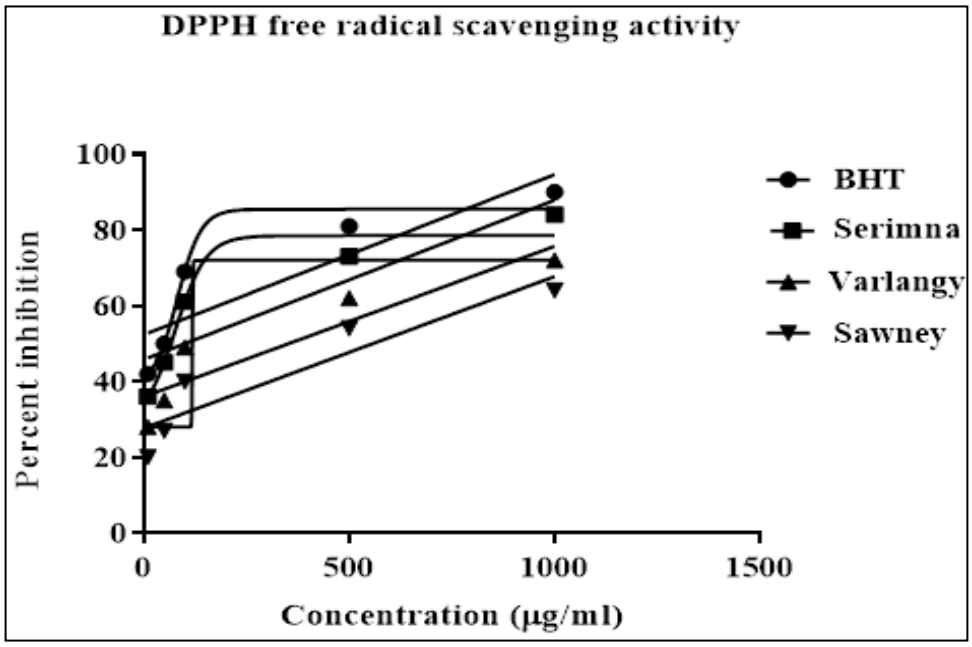

Figure 1. DPPH scavenging activity of essential oils obtained from the fruits of seremna, varlangy and sawney. Values are expressed as mean ± SEM (n=3).

Table 1. Antioxidant activity of essential oils obtained from the fruits of seremna, varlangy and sawney determined using three different methods such as DPPH, ABTS and $FeCl_3$-reducing models.

Essential oils	DPPH method	ABTS method	$FeCl_3$-reducing method
	IC_{50} (µg/ml)		Absorbance
Seremna	172.3	27.96	1.453
Varlangy	216.9	31.34	1.011
Sawney	274.3	32.49	1.001
BHT	84.54	22.77	1.858

activity of the essential oil of three different cultivars of *A. subulatum* fruits and result revealed the different levels of scavenging activities. The percentage scavenging of free radical by Seremna was 84.74% comparatively higher than for varlangy (72.38%) and sawney (64.42%). Table 1 shows the IC_{50} value of essential oils and standard; 84.54, 172.3, 216.9 and 274.3 µg/ml of BHT, seremna, varlangy and sawney respectively. The previous study confirmed that the *in vitro* DPPH free radical scavenging assay is a reliable and stable method for the investigation of antioxidant activity (Amiri, 2012). Pathogenesis of various diseases were caused by free radicals, which are produced during metabolic reaction. Free radicals can oxidize all types of biomolecules, spread in all the body parts and damage cells. The antioxidant compounds that are present in extracts or essential oil of spices and herbs scavenge the free radical and prevent the various diseases. The DPPH antioxidant study of essential oil of fruits of *A. subulatum* has been previously reported by some of the investigators (Bisht et al., 2011; Dhuley, 1999; Kapoor et al., 2008), but as per our knowledge, this is the first report pertaining to the comparative antioxidant activity of essential of fruits of three cultivars. DPPH and ABTS methods have been widely used for the study of the essential oils (Ballester-Costa et al., 2017; Afoulous et al., 2013). The most powerful scavenging constituent by DPPH was found in the essential oils of seremna cultivar.

ABTS free radical scavenging activity

Figure 2 shows the different levels of ABTS radical scavenging activity in the essential oil of each selected cultivar. The percentage scavenging of essential oil of fruits of seremna was 84.1% comparatively higher than for varlangy (52.6%) and sawney (44.7%). Table 1 shows the IC_{50} value of essential oils and standard such as

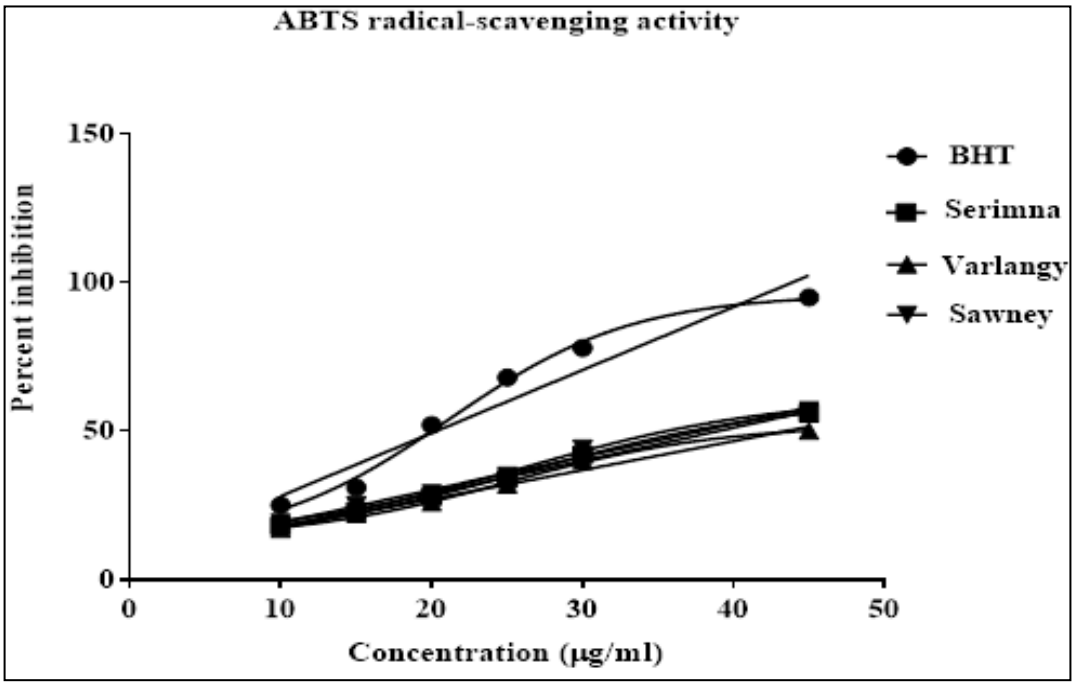

Figure 2. ABTS scavenging activity of essential oils obtained from the fruits of seremna, varlangy and sawney. Values are expressed as mean ± SEM (n=3).

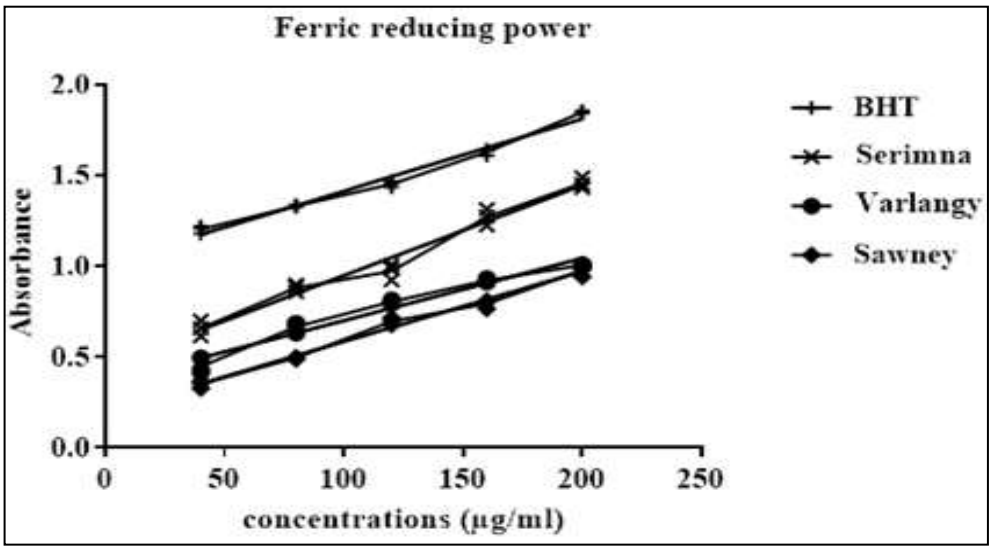

Figure 3. Ferric chloride reducing assay of essential oils obtained from the fruits of seremna, varlangy and sawney. Values are expressed as mean ± SEM (n=3).

27.96, 31.34 and 32.49 µg/ml of seremna, varlangy and sawney respectively. Like DPPH, the ABTS radical scavenging activity also reproduces hydrogen-donating ability. ABTS method is commonly used for the antioxidant profiling of the essential oils; it is a method that is generally superior to DPPH especially for the essential oil activity, due to the complexity and polarity of compounds (Okoh et al., 2016; Kaviarasan et al., 2007). The most powerful scavenging using the ABTS method was found in the essential oils of seremna cultivar, showing IC_{50}, 27.96 µg/ml.

Ferric chloride reducing assay

Figure 3 shows the reductive capabilities of the essential

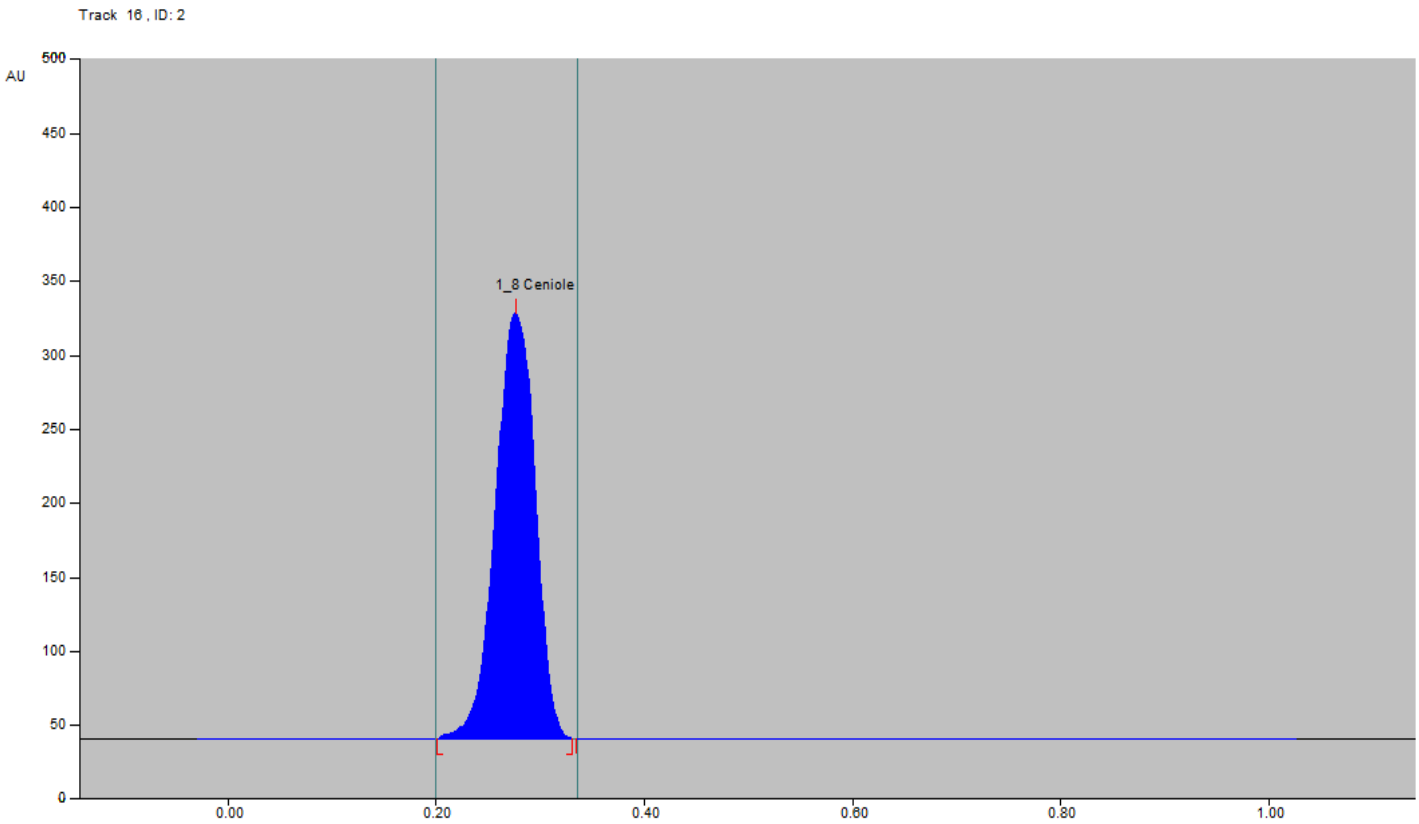

Figure 4. HPTLC densitogram of 1-8 cineole at UV 665 nm.

oils of fruits of cultivars of *A. subulatum* cultivars compared with BHT and the results showed different reducing levels. The reducing power of the essential oil of seremna fruit, 1.453 was found to be higher than the varlangy, 1.011 and sawney, 1.001 at 200 μg/ml, which increased gradually with a rise in the concentration. The method of reducing power assay based on the reaction between antioxidant compounds and potassium ferric cyanide to form potassium ferrous cyanide, which then reacts with ferric chloride to form complex mixture of ferric–ferrous that has an absorption maximum at 700 nm. The reducing power of the essential oils and BHT increases with the increase in the concentration of antioxidant compounds (Bhalodia et al., 2013).

Method validation

Selection of mobile phase

Figure 4 shows the HPTLC densitogram of the developed method for the standard 1,8-cineole. For the optimization of mobile phase, different trials were made using several solvents alone and in different proportions. Solvent system consisting of hexane and ethyl acetate was used in the ratio of 8:2, v/v, where a spot was observed at the R_f value 0.28±0.12 for the standard.

Specificity

The specificity of the method was determined by analysing samples and standard spectra at the peak apex, peak start and end positions of the peak. When excess standards were added to the essential oil of fruits of cultivars to check the specificity of the method, the spectro-densitogram of standard 1,8-cineole and essential oil at λ 665 nm was observed. A satisfactory peak purity was obtained and it was observed that the other essential oil present in the essential oil did not interfere with the standard peak and it was found that chromatogram was stabled in solution and on the TLC plate at room temperature.

Linearity

Table 2 shows the linearity of the developed method, for which a calibration curve of five dilution was prepared by plotting peak area against concentrations. It was evaluated by applying different concentrations of 100 to 700 ng/spot for standard solution 1,8-cineole. A good

Table 2. Linear regression data for the calibration curve (n=6).

Parameter	Value
Linearity range (ng/spot)	100-700
Regression equation	Y = 12.34x + 214.9
Correlation coefficient	0.9977
Slope ± SE	12.34 ± 0.2664
Intercept ± SE	214.9 ± 119.1
95% Confidence Interval of slope	11.66 to 13.03
95% Confidence Interval of intercept	521.1 to 91.36
F	2147
DFn/DFd	1, 5
P value	< 0.0001
LOD	5.14 $ngband^{-1}$
LOQ	14.66 $ngband^{-1}$

Table 3. Accuracy of the proposed method (n=6).

Excess drug added to analyte (%)	Theoretical content (ng)	Concentration found (ng) ± SD	% Recovery	% RSD
0	200	196.17 ± 1.94	98.08	0.99
50	300	294.00 ± 2.45	98.00	0.83
100	400	397.33 ± 1.03	99.33	0.26
150	500	494.00 ± 4.00	98.80	0.81

Table 4. Precision of the proposed method (n=6).

Concentration (ng/spot)	Precision	Average concentration ± SD (n = 6)	Standard error (SE)	% RSD
300		3567.00 ± 30.77	12.56	0.86
400	Repeatability precision (Intraday)	4545.40 ± 19.21	7.84	0.42
500		6055.80 ± 27.58	11.26	0.46
300		3573.00 ± 42.15	17.21	1.18
400	Intermediate precision (Interday)	4537.40 ± 28.81	11.77	1.64
500		6037.80 ± 41.41	16.91	0.69

linear relationship was found with r^2 value of 0.9977, intercepts 214.9 ± 119 and slopes 12.34 ± 0.2664, that endorse the accuracy of the present method.

LOD and LOQ

Table 2 also shows the limit of detection (LOD) and limit of quantification (LOQ) of the developed method for the identification of 1,8-cineole. The LOD 5.14 ng/b and LOQ, 14.66 ng/b respectively, were calculated for standard solution and validate the sensitivity of the present method.

Accuracy

Table 3 shows the accuracy of the present method; for this the pre-analysed samples were spiked with 50, 100 and 150% of the standard 1,8-cineole and the mixtures were re-analysed using the proposed method. The range of percentage recovery (98 to 99.33%) and percentage relative standard deviation, (0.26 to 0.99 % RSD) for standard 1,8-cineole endorse the accuracy of the present method.

Precision

Table 4 shows the precision data on the intraday and inter-day variation for three different concentration levels. The results of repeatability were stated in terms of the relative standard deviation (% RSD). The low % RSD showed the method is precise for the analysis of 1,8-cineole.

Table 5. Robustness of the proposed HPTLC method.

Concentration (ng/spot)	Original*	Used	Changed	Area ± SD (n = 3)	R_f	% RSD
		7.9:2.1	-0.1, +0.1	4537 ± 28	0.30	0.61
400	8:2	8:2	0.0	4539 ± 26	0.28	0.57
		8.1:1.9	+0.1, -0.1	4545 ± 23	0.27	0.52

Table 6. The contents of 1-8 Cineole in the essential oils obtained from the fruits of Seremna, Varlangy and Sawney determined using developed HPTLC method.

Essential oils of cultivars	Contents (mean ± SD, % w/w)	% RSD
Seremna	69.59 ± 1.45	3.47
Varlangy	48.78 ± 3.21	4.18
Sawney	47.84 ± 1.76	2.53

Robustness

Table 5 shows the robustness of the present method of the introduction of small changes in the composition of the mobile phase and the effects on the results were observed. Low % RSD indicated that the developed HPTLC method is robust.

Quantification of 1,8-cineole in the essential of *A. subulatum* cultivars

Table 6 shows the percentage contents of 1,8-cineole in the essential oils of fruits of seremna, varlangy and sawney and these were analysed using developed HPTLC methods. The contents of 1,8-cineole were 69.59 ± 1.45%, 48.78 ± 3.21% and 47.84 ± 1.76% of the essential oils of fruits of seremna, varlangy and sawney, respectively.

Conclusion

The developed HPTLC method was found to be accurate, specific, precise and easy to use for the quantitative analysis of 1,8-cineole. Quantification of the 1,8-cineole using developed method confirmed that the monoterpenes, 1,8-cineole is a principal ingredient found in the essential oil of fruits of *A. subulatum* cultivars. 1,8-Cineole was found to be higher in seremna cultivar fruit in comparing to varlangy and sawney fruits. So, the seremna fruits obtained essential oil is a good choice of antioxidant cultivar in comparison to the other two cultivars fruit. The comparative antioxidant activity of fruit oil further confirmed the antioxidant nature of monoterpene, 1,8-cineole and the study concluded that the higher the concentration of 1,8-cineole, the greater the value of antioxidant. This method can be adopted for the quality control of essential oils and formulations that contains 1,8-ineole as active compounds as well as for the selection of good antioxidant cultivars that contain 1,8-cineole as an active marker.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Afoulous S, Ferhout H, Raoelison EG, Valentin A, Moukarzel B, Couderc F, Bouajila J (2013). Chemical composition and anticancer, antiinflammatory, antioxidant and antimalarial activities of leaves essential oil of *Cedrelopsis grevei*. Food and Chemical Toxicology 56:352-362.

Agnihotri S, Wakode S (2010). Antimicrobial activity of essential oil and various extract og greater cardamom. Indian Journal of Pharmaceutical Sciences 72:657-659.

Amiri H (2012). Essential Oils Composition and Antioxidant Properties of Three Thymus Species. Evidence-Based Complementary and Alternative Medicine 2012.

Asnaashari S, Afshar FH, Ebrahimi A, Moghadam SB, Delazar A (2016). Chemical composition and radical scavenging activity of essential oil and methanolic extract of *Eremostachys azerbaijanica* Rech.f. from Iran. Research in Pharmaceutical Sciences 11:113-119.

Attimarad M, Ahmed KKM, Aldhubaib BE, Harsha S (2011). High-performance thin layer chromatography: A powerful analytical

technique in pharmaceutical drug discovery. Pharmaceutical methods 2:71-75.
Ballester-Costa C, Sendra E, Fernández-López J, Pérez-Álvarez JA, Viuda-Martos M (2017). Assessment of Antioxidant and Antibacterial Properties on Meat Homogenates of Essential Oils Obtained from Four Thymus Species Achieved from Organic Growth. Foods 6:59.
Bisht VK, Negi JS, Bhandari AK, Sundriyal RC (2011). *Amomum subulatum* Roxb. traditional, phytochemical and biological activities-A review. African Journal of Agricultural Research 6:5386-5390.
Dhuley JN (1999). Anti-oxidant effects of cinnamon (*Cinnamomum verum*) bark and greater cardamom (*Amomum subulatum*) seeds in rats fed high fat diet. Indian Journal of Experimental Biology 37:238-242.
Dubey AK, Yadav DS (2001). Comparative performance of different varieties of large cardamom (*Amomom subulatum* Roxb.) under mid altitude of Amnachal Pradesh. Journal of Spices and Aromatic Crops 10:119-122.
Gupta U, John TD (1987). 'Floral biology of large cardamom'. Cardamom 20:8-15.
Hussain AI, Anwar F, Nigam PS, Sarker SD, Moore JE, Rao JR, Majumdar A (2011). Antibacterial activity of some Lamiaceae essential oils using resazurin as an indicator of cell growth. LWT-Food Science and Technology 44:1199-1206.
ICH (2005). Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2 (R1)Geneva, Switzerland: International Conference on Harmonization.
Jimenez-Andrade JM, Ortiz MI, Perez-Urizar J, Aguirre-Banuelos P, Granados-Soto V, Castaneda-Hernandez G (2003). Synergistic effects between codeine and diclofenac after local, spinal and systemic administration. Pharmacology Biochemistry and Behavior 76:463-471.
Joshi R, Sharma P, Sharma V, Prasad R, Sud RK, Gulati A (2013). Analysis of the essential oil of large cardamom (*Amomum subulatum* Roxb.) growing in different agro-climatic zones of Himachal Pradesh, India. Journal of the Science of Food and Agriculture 93:1303-1309.
Juergens UR, Dethlefsen U, Steinkamp G, Gillissen A, Repges R, Vetter H (2003). Anti-inflammatory activity of 1,8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial. Respiratory medicine 97:250-256.
Kapoor IPS, Singh B, Singh G (2009). Essential oil and oleoresins of cardamom (*Amomum subulatum* ROXB.) as natural food preservatives for sweet orange (Citrus sinensis) juice. Food Process Engineering 34:1101-1113
Kapoor IPS, Singh B, Singh G, Isidorov V, Szczepaniak L (2008). Chemistry, antifungal and antioxidant activities of cardamom (*Amomum subulatum*) essential oil and oleoresins. International Journal of Essential Oil Therapeutics 2:29-40.
Kaskoos RA, Mir SR, Kapoor R, Ali M (2013). Essential Oil Composition of the Fruits of *Amomum subulatum* Roxb. Journal of essential oil-bearing plants 11(2):184-187.
Kathirvel S, Prasad KR, Babu KM (2012). Development and validation of HPTLC method for the determination of mycophenolate mofetil in bulk and pharmaceutical formulation. Pharmaceutical Methods 3:90-93.
Kaviarasan S, Naik GH, Gangabhagirathi R, Anuradha CV, Priyadarshini KI (2007). In vitro studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum* graecum) seeds. Food Chemistry 103:31-37.
Kumar KMP, Asish GR, Sabu M, Balachandran I (2013). Significance of gingers (Zingiberaceae) in Indian System of Medicine - Ayurveda: An overview. Ancient science of life 32:253-261.
Lima PR, Melo TSd , Carvalho KMMB , Oliveira IBd, Arruda BR , Castro Brito GAd, Rao VS, Santos FA (2013). 1,8-cineole (eucalyptol) ameliorates cerulein-induced acute pancreatitis via modulation of cytokines, oxidative stress and NF-κB activity in mice. Life Sciences 92:1195-1201.
Lobo V, Patil A, Phatak A, Chandra N (2010). Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Reviews 4:118-126.
Madhusoodhanan KJ, Rao YS (2001). Cardamom (large), in *Handbook of herbs and spices*, vol I, 1st edn, edited by K V Peter (Woodhead Publishing Limited, England) pp. 134-141.
Mukherjee DK (1972). Large cardamom. World Crops 25:31-33
Okoh SO, Iweriebor BC, Okoh OO, Nwodo UU, Okoh AI (2016). Antibacterial and Antioxidant Properties of the Leaves and Stem Essential Oils of *Jatropha gossypifolia* L. BioMed Research International 2016.
Olugbami JO, Gbadegesin MA, Odunola OA (2015). *In vitro* free radical scavenging and antioxidant properties of ethanol extract of *Terminalia glaucescens*. Pharmacognosy Research 7:49-56.
Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, Dhama K (2014). Oxidative Stress, Prooxidants, and Antioxidants: The Interplay. BioMed Research International 2014:1-19.
Ramalho VC, Jorge N (2006). Antioxidantes utilizados em óleos, gorduras e alimentos gordurosos. Quimica Nova. 29:755-760.
Sharma E, Sharma R, Singh KK, Sharma G (2000). Large Cardamom Farming in the Sikkim Himalaya. Mountain Research and Development 20:108-111.
Sharma V, Lohia N, Handa V, Baranwal M (2017). *Amomum subulatum* seed extract exhibit antioxidant, cytotoxic and immune suppressive effects. Indian Journal of Biochemistry and Biophysics 54:135-139.
Uttara B, Singh AV, Zamboni P, Mahajan R (2009). Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Current Neuropharmacology 7:65-74.
Vilela GR, Almeida GS, D'Arce MABR, Moraes MHD, Brito JO, da Silva MFGF, CruzSilva S, Piedade SMS, Calori-Domingues MA, Gloria EM (2009). Activity of essential oil and its major compound, 1,8-cineole, from *Eucalyptus globulus* Labill., against the storage fungi *Aspergillus flavus* Link and Aspergillus parasiticus Speare. Journal of Stored Products Research 45:108-111.
Yang S-A, Jeon S-K, Lee E-J, Im N-K, Jhee K-H, Lee S-P, Lee I-S (2009). Radical Scavenging Activity of the Essential Oil of Silver Fir (*Abies alba*). Journal of Clinical Biochemistry and Nutrition 44:253-259.