

## Original Research Article

# Development of HPTLC method for determination of $\alpha$ -terpinyl acetate, and evaluation of antioxidant properties of essential oils in *Elettaria cardamomum*

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### Abstract

**Purpose:** To develop a simple and reliable protocol for high performance thin layer chromatography (HPTLC) quantification of  $\alpha$ -terpinyl acetate in oils extracted from *Elettaria cardamomum*, and to study relative antioxidant potential of oils obtained from three varieties of fruits of *E. cardamomum*.

**Methods:** Essential oil was extracted separately from the fruits of three varieties of *E. cardamomum*, viz, Valley-green, Palakuzhi, and ICRI-2, using hydro-distillation method. In the development of an HPTLC method, standard  $\alpha$ -terpinyl acetate was subjected to chromatography on aluminium-backed silica gel 60 F<sub>254</sub> plates using a mobile phase of *n*-hexane: ethyl acetate (8:2, v/v), and quantified at 665 nm through densitometric analysis. The antioxidant property of essential oil of each cultivar was determined with respect to 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging assays, as well as ferric reducing assay.

**Results:** The developed HPTLC method showed a good resolution for  $\alpha$ -terpinyl acetate, with  $R_f$  of  $0.64 \pm 0.01$ , and also showed good linearity of the calibration plots ( $r^2 = 0.9982$ ). The HPTLC method was validated as per ICH guidelines, and used for the determination of  $\alpha$ -terpinyl acetate in the essential oils. The valley green variety of *E. cardamomum* had the highest content of  $\alpha$ -terpinyl acetate ( $55.36 \pm 1.33$  %w/w). The inhibitory capacity (IC<sub>50</sub> value) for the oil of valley green variety as determined using DPPH and ABTS methods was 378.2 and 19.87  $\mu$ g/mL, respectively.

**Conclusion:** The proposed HPTLC method is suitable for routine analysis of  $\alpha$ -terpinyl acetate in medicinal herbs, and it reveals the role of  $\alpha$ -terpinyl acetate in the antioxidant efficacy of cardamom oil.

**Keywords:** HPTLC,  $\alpha$ -Terpinyl acetate, *Elettaria cardamomum*, Cardamom oil, Antioxidant

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## INTRODUCTION

*Elettaria cardamomum* (Maton), popularly known as Indian green cardamom, is native to South East Asia. The fruits and seeds of this plant are

sources of very famous and expensive spices all over the world, and they have multiple health benefits. They are used as flavouring agents, food additives and constituents of several aromatic flavours [1]. The anti-inflammatory,

antimicrobial, and antioxidant properties of essential oil of the fruits of cardamom have been previously reported [2,3]. The essential oil and extract of cardamom fruits are also used for treatment of several ailments in the conventional and alternative system of medicine [3]. The natural cultivars of the plant (Mysore, Malabar, and Vazhukka) are cultivated in the southern parts of India [4]. Several varieties of green cardamom have been developed by ICRI-India through breeding technology. These include high-yielding varieties such as Vazhukka (valley green), Malabar (Palakuzhi) and Mysore (ICRI-2) cultivars [4-6].

Alpha-terpinyl acetate, an essential oil related the terpenoid family, contributes to the flavour and fragrance of *E. cardamomum* fruits [7]. Alcohol extract of the seeds or fruits has been previously reported to have several medicinal values such as gastrointestinal protection, as well as anti-inflammatory, analgesic, antimicrobial, antiviral, hypotensive, antispasmodic, mucolytic and antioxidant effects [8,9]. High performance thin layer chromatography (HPTLC) is one of the cost-effective and rapid chromatographic techniques used for quantification of secondary metabolites in the plants and plant parts, and for detection of complex mixtures of drugs, natural products, essential oils and foodstuffs [10].

Worldwide, there is growing demand for natural antioxidants. The essential oil of fruits of *E. cardamomum* might be an alternative source of antioxidant medicine [11]. There are several varieties of *E. cardamomum* which have  $\alpha$ -terpinyl acetate as a major bioactive constituent, but due to the low concentration of  $\alpha$ -terpinyl acetate, their antioxidant potential are not satisfactory.

The aim of the current study was to develop a reliable HPTLC protocol for the quantification of  $\alpha$ -terpinyl acetate and screening of *E. cardamomum* cultivars for contents of  $\alpha$ -terpinyl acetate. Furthermore, the antioxidant potential of  $\alpha$ -terpinyl acetate was evaluated using comparative study of oils from selected cultivars of the plant to confirm the contribution of this compound to the antioxidant effect.

## EXPERIMENTAL

### Materials

Dried fruits of valley green, Palakuzhi and ICRI-2 cultivars of the plant were procured from ICRI, Myladumpara, Idukki, and Kerala, India. Authentication reference (ICRI RES-500), voucher number and invoice reference number-

27414) were provided by ICRI purchase department. The reagents ABTS and DPPH, and standard BHT,  $\alpha$ -terpinyl acetate (purity  $\geq 95\%$ ) were procured from Sigma Aldrich, USA). The solvents used in the chromatography and antioxidant activity were of AR grade.

### Instrumentation

Aluminium-baked pre-coated TLC plates (silica gel 60F<sub>254</sub>, 20 × 20 cm, 0.2 mm thick) were obtained from E. Merck. Camag-Automatic TLC Sampler-4, automatic development chamber (ADC2), Camag UV cabinet-4, Camag-derivatization chamber, Camag-TLC plate heater and Camag-TLC Reprostar 3 were the main instruments used in this study.

### Isolation of cardamom essential oil

The dried fruits (70 g) of each variety were ground to a coarse powder, and essential oils were extracted through hydro-distillation for 4 h using Clevenger-type apparatus. The water moiety of the extracts was slowly drawn off using the anhydrous Na<sub>2</sub>SO<sub>4</sub>, and the resultant oil extract was collected in an amber-coloured bottle and preserved at 4°C for use in subsequent studies.

### Instrumentation conditions used for development of the HPTLC method

Chromatography was executed on TLC plates (20 cm × 10 cm). Samples were applied using TLC Sampler-4 applicator. Several mobile phases containing hexane, toluene, benzene, ethyl acetate, chloroform and methanol in different proportions, or ethanol alone were tried. Finally, a mixture of hexane: ethyl acetate (8: 2; v/v) was found to be the most appropriate for  $\alpha$ -terpinyl acetate studies. The mean retention factor (R<sub>f</sub>) value of  $\alpha$ -terpinyl acetate was 0.64 ± 0.01. The mobile phase (hexane: ethyl acetate (8: 2, v: v; 20 mL) was placed in the twin-trough glass chamber and saturated for 5 min at room temperature (25 ± 5 °C) and relative humidity of 60 ± 2 %. Linear ascending development was performed, and thereafter, the plates were air-dried and screened in UV chamber under low-UV (254 nm), high-UV (366 nm) and white light. The plates were derivatized using anisaldehyde-sulfuric acid reagent, and heated up to 100 °C using plate heater. The plates were then cooled and scanned at 665 nm using -TLC scanner in absorbance mode. The results were calculated using WinCats software.

### Preparation of standard solution of $\alpha$ -terpinyl acetate and calibration curve

$\alpha$ -Terpinyl acetate (10 mg) was transferred into a volumetric flask (10 mL), mixed with methanol (5 mL) and sonicated for 10 min. Then, the volume was made up to the mark with methanol. Different dilutions of standard  $\alpha$ -terpinyl acetate were prepared and used for preparation of standard calibration curve. The regression equation, correlation coefficient, slope and intercept of the curves were calculated from the standard plot.

### Preparation of sample solution

Exactly 20.0 mg of essential oil of each variety of *E. cardamomum* (Valley green, Palakuzhi, and ICRI-2) was transferred to 10 mL volumetric flask; 5 mL methanol was added, and the solution was sonicated for 10 min. Then, the volume was made up to the mark with methanol.

### Method validation

The developed method was validated for accuracy, specificity, sensitivity, precision, and robustness, as per ICH guidelines [12].

### Specificity and sensitivity

The specificity and sensitivity of the HPTLC method were assessed by analysing sample solutions with  $\alpha$ -terpinyl acetate and *E. cardamomum* essential oil subjected to interferences from oils and other compositions. The sample spot was matched with  $R_f$  values of the spot of the standard  $\alpha$ -terpinyl acetate. The limit of quantification (LOQ) and the limit of detection (LOD) were used to measure the sensitivity of the developed method.

The LOQ and LOD were calculated using Equations 1 and 2 below:

$$LOD = 3a/S \dots\dots\dots (1)$$

$$LOQ = 10 3a/S \dots\dots\dots (2)$$

where  $a$  is standard deviation of  $y$ -intercepts, and  $S$  is the slope of the developed calibration curve.

### Accuracy

Accuracy was expressed as % recovery. The measurements were performed by addition of  $\alpha$ -terpinyl acetate solution at four different levels (0, 50, 100 and 150 %) to a pre-analysed sample solution (300 ng/spot). All the experiments were performed in triplicate ( $n=3$ ).

### Precision and robustness

Precision studies were performed to determine the repeatability and reproducibility of the new method. Intra-day precision was determined by analysing standard solutions ((300, 400 and 500 ng/spot) on the same day, while inter-day precision was determined by analysing on three consecutive days. The robustness of the method was aimed at determining the effect of small changes in the ratio of the solvent system, distance development, and chamber saturation time on  $R_f$  value and peak area of  $\alpha$ -terpinyl acetate.

### Determination of antioxidant potential

#### DPPH free radical scavenging assay

In the present study, the DPPH free radical scavenging effect was determined using an earlier method, with slight modifications [13]. Stock solutions of the essential oil of each variety and the standard solutions were prepared in methanol. Stock solutions of different dilutions (1000 to 10  $\mu$ g/mL) were made to assess the antioxidant property of the oils. One millilitre of a diluted solution of each sample or standard was separately mixed with DPPH (90  $\mu$ M, in methanol) and the volume was made up to 4 mL using methanol. The absorbance of the diluted samples and standard were read in a spectrophotometer at 517 nm, and the  $IC_{50}$  of each essential oil was calculated.

#### ABTS free radical scavenging assay

The radical scavenging of ABTS was based on an earlier method with slight modification [14]. Briefly, dilutions (15 - 45  $\mu$ g/mL) of the stock solution of standard and samples were prepared. The working solution of ABTS was prepared using ABTS (7 mM) and potassium persulfate (2.45 mM; 1:1 v: v), and the solution was kept in the dark for 4 h at 25 °C. The working solution was mixed with phosphate buffer to get an absorbance of  $0.70 \pm 0.02$  at 734 nm before use. Then, 3 mL of standard or sample was added to 1 mL of working solution. The absorbance of the diluted samples and standard were read in a spectrophotometer, and  $IC_{50}$  value of each essential oil was calculated.

#### Ferric chloride reducing (FR) power assay

The assessment of FR assay was based on an earlier method of Han et al, with slight modifications [15]. A stock solution was diluted serially (0.25 to 10 mg/ml). Then, 1 ml of sample solution, phosphate buffer (2.5 mL; 0.2 mol/L, pH

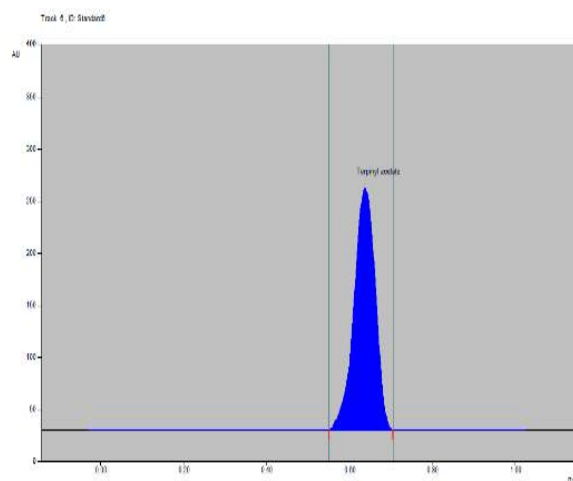
6.6), and potassium ferricyanide (2.5 mL, 1 %) were mixed and incubated for 30 min at 50°C. Trichloroacetic acid solution (10 %, 2.5 ml) was added, followed by centrifugation at 10,000 rpm for 10 min. Thereafter, 2.5 ml of the supernatant was added to 2.5 mL of distilled water and 0.5 mL of 0.1 % FeCl<sub>3</sub> solution. The absorbance of the diluted samples and standard were read at 700 nm in a spectrophotometer.

**Statistical analysis**

Data are expressed as mean ± (SD). The IC<sub>50</sub> values were calculated using non-linear regression, followed by log inhibitor vs. response - variable slope, using Graph-Pad Prism version 7.03.

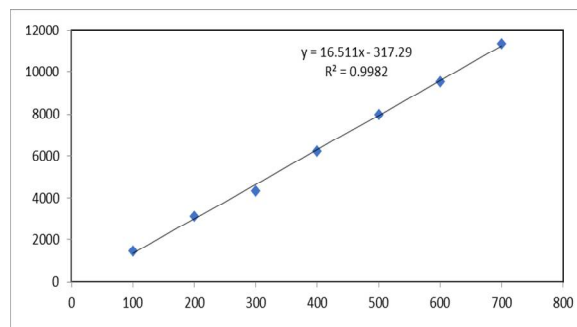
**RESULTS**

The mixture of mobile phase, hexane: ethyl acetate (8: 2; v/v) gave good resolution with R<sub>f</sub> value of 0.64. The thin layer chromatography chamber was saturated for 10 min. The band of TLC plates was visualized after derivatization with spray reagents at 665nm. The identification of α-terpinyl acetate in the essential oil was confirmed by matching with the chromatogram of standard α-terpinyl acetate. Figure 1 shows the densitogram of the developed HPTLC method for standard α-terpinyl acetate.



**Figure 1:** Chromatogram of standard α-terpinyl acetate: peak of 300 ng (R<sub>f</sub> = 0.64)

The peak area of each concentration (100, 200, 300, 400, 500, 600 and 700 ng/band) of α-terpinyl acetate was calculated, and a graph of standard solution versus peak area was plotted. The calibration curve for α-terpinyl acetate has good linearity with a correlation coefficient of is shown in Figure 2.



**Figure 2:** Standard calibration curve of α-terpinyl acetate at different concentrations of α-terpinyl acetate (100 - 700 ng/band)

The regression equation, correlation coefficient, slope, intercept LOD and LOQ of the α-terpinyl acetate were y = 16.5x - 317.29, 0.9982, 16.5 ± 0.3178, 317 ± 142.1, 6.20, and 17.12, respectively. Linearity was found in the range of 100 – 700 ng/band as shown in Table 1.

**Table 1:** Calibration parameters of standard α-terpinyl acetate

Parameter	α-Terpinyl acetate (n=6)
Linearity (ng/spot) range	100-700
Equation of regression	Y = 16.5x - 317.29
Correlation coefficient	0.9982
Slope ± SE	16.5 ± 0.3178
Intercept ± SE	317 ± 142.1
CI (95%) of slope	15.69 to 17.32
CI (95%) of intercept	679.3 to 51.43
F value	2696
DF (nominator per denominator) (DFn/DFd)	1.49
P value	< 0.0001
LOD (ng/band)	6.20
LOQ (ng/band)	17.12

CI, confidence interval, DF, degree of freedom

The accuracy of the method was analysed in the triplicate mode. The effects of recovery were calculated in terms of percentage recovery (%) and the results are shown in Table 2. The recovery of α-terpinyl acetate was in the range of 97.75 – 99.38 %.

The range of RSD for intraday (repeatability) precision was 0.28 - 0.61 %. Inter-day precision study was performed using measurement of peak area for three consecutive days. The range of RSD for interlay (intermediate) precision was 0.39 - 0.71 % (Table 3).

The range of RSD obtained for robustness of method was 0.51 - 0.59 %, after introducing small changes in the developed method. The results are shown in Table 4.

**Table 2:** Accuracy of the HPTLC method standard for determination of  $\alpha$ -terpinyl acetate

Excess $\alpha$ -terpinyl acetate added to analyte (%)	Content (ng)	$\alpha$ -Terpinyl acetate		
		Conc $\pm$ SD (ng)	Recovery (%)	RSD (%)
0	200	195.50 $\pm$ 2.52	97.75	0.79
50	300	291.50 $\pm$ 1.76	97.20	0.94
100	400	398.50 $\pm$ 0.84	99.38	0.21
150	500	493.50 $\pm$ 1.64	98.63	0.85

**Table 3:** Inter-day and intra-day precision

(Conc. (g/spot))	Precision	$\alpha$ -Terpinyl acetate		
		Mean conc. $\pm$ SD (n = 6)	SE (Standard Error)	RSD (%)
300	Repeatability precision (intra-day)	4335.00 $\pm$ 26.36	10.36	0.61
400		6219.00 $\pm$ 31.68	12.93	0.51
500		7998.80 $\pm$ 22.11	9.03	0.28
300	Intermediate precision (inter-day)	4333.00 $\pm$ 30.80	12.57	0.71
400		6221.00 $\pm$ 36.72	15.00	0.59
500		8006.80 $\pm$ 31.38	12.81	0.39

**Table 4:** Robustness of the new HPTLC method

Conc (ng/spot)	Original*	Solvent system	Change	$\alpha$ -Terpinyl acetate		
				Area $\pm$ SD, (n = 3)	R <sub>f</sub>	% RSD
400	8:2	7.9:2.1	-0.1, +0.1	6241 $\pm$ 37	0.66	0.59
		8:2	0.0	6219 $\pm$ 32	0.64	0.51
		8.1:1.9	+0.1, -0.1	6217 $\pm$ 35	0.62	0.56

\*Mobile phase (Hexane: ethyl acetate)

**Table 5:** Percentage of  $\alpha$ -terpinyl acetate in the three varieties of essential oils (n=3).

Essential oil	Content (mean $\pm$ SD, % w/w of oil) $\alpha$ -terpinyl acetate
Valley green	55.36 $\pm$ 1.33
Palakuzhi	41.82 $\pm$ 4.11
ICRI-2	41.42 $\pm$ 2.17

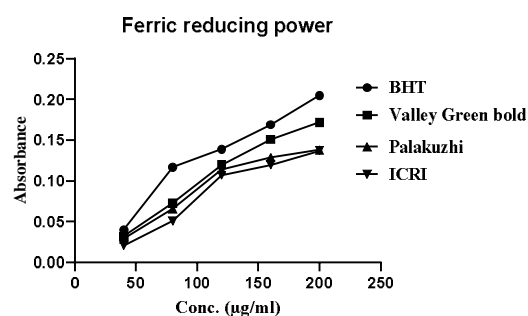
**Table 6:** IC<sub>50</sub> ( $\mu$ g/mL) values of the essential oils of three varieties of *E. cardamomum* (n = 3)

Essential oil	DPPH	ABTS
Valley green	378.2	19.87
Palakuzhi	485.4	20.2
ICRI-2	547.7	23.09
BHT	81.47	19.8

### Antioxidant potential of essential oils

The scavenging power in terms of IC<sub>50</sub> (using DPPH and ABTS methods) for Valley green, Palakuzhi and ICRI-2 varieties of *E. cardamomum* and BHT (standard) are shown in Table 6. In the DPPH method, the essential oil obtained from Valley green had the highest antioxidant potential (IC<sub>50</sub> = 378.2  $\mu$ g/mL), followed by Palakuzhi (IC<sub>50</sub> = 485.4  $\mu$ g/mL) and ICRI-2 (IC<sub>50</sub> = 547.7  $\mu$ g/mL). In the ABTS free radical scavenging assay, the essential oil of Valley green also had higher antioxidant potential (IC<sub>50</sub> = 19.87  $\mu$ g/mL) than any of the other two varieties.

The results of ferric reducing power for the three essential oils are shown Figure 3. The reducing power was in the increasing order of BHT > Valley green > Palakuzhi > ICRI-2.

**Figure 3:** Ferric reducing power of the oils of varieties of *E. cardamomum*, and BHT standard. Values are presented as mean  $\pm$  SD (n = 3).

### DISCUSSION

The proposed HPTLC procedure was optimized with a view to quantifying the presence of  $\alpha$ -terpinyl acetate in the essential oils. The method was validated using ICH guidelines [12]. The solvent mixture of hexane: ethyl acetate (8:2,

v/v) was optimized, and it provided good resolution for  $\alpha$ -terpinyl acetate. The chromatographic conditions of the developed method such as saturation time,  $R_f$  value, spraying reagent, and scanning wavelength were 10 min, 0.64, anisaldehyde-sulfuric acid and 665nm, respectively. These conditions were reliable, precise and good for quantification of  $\alpha$ -terpinyl acetate.

LOD and LOQ values revealed the suitable sensitivity of the developed method. Recovery of  $\alpha$ -terpinyl acetate was nearly 100, indicating that there was no interference in the analysis by the other compounds present in the essential oil, which is evidence of the accuracy of the developed method [16]. The peak area RSD (%) did not exceed two percent (< 2 %), both in the intra-day and inter-day observations, showing the high precision of developed method [17].

The range of relative standard deviation (% RSD) obtained for robustness of the method observed after small changes in the TLC process indicates the robustness of the method [18]. The HPTLC method was used to calculate the amount of  $\alpha$ -terpinyl acetate present in the essential oils of the selected varieties of *E. cardamomum*, and the results indicated that the essential oil of Valley green had the highest content of  $\alpha$ -terpinyl acetate.

In the present study, the essential oil of Valley green fruits had the highest antioxidant potential, when compared to the other oils, with respect to DPPH and ABTS radical scavenging potential. The ABTS free radical has been broadly used to assess the antioxidant ability of essential oils, and it is more reactive than DPPH radical [19]. The antioxidant effect of the essential oils was highly associated with the contents of the bioactive constituent  $\alpha$ -terpinyl acetate.

The reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable, reliable and popular radical used for testing the antioxidant effects of plant extracts or essential oils [20]. The violet colour of the DPPH radical changes to yellow after receiving a free electron. Compounds which undergo this colour transformation are considered as antioxidants. The findings in the present study are in agreement with those obtained in a previous study, where it was reported that different cultivars of *E. cardamomum* exerted different antioxidant properties [21]. The reducing assay method also revealed the high antioxidant potential of the Valley green variety of *E. cardamomum*. The antioxidant property of  $\alpha$ -terpinyl acetate has also been reported by other investigators [5,22].

## CONCLUSION

A simple, rapid, specific, accurate, and precise HPTLC method for the determination of  $\alpha$ -terpinyl acetate in the essential oils of cardamom has been successfully developed. The developed method is sensitive, and is suitable for use in quality control. Based on the method, the content of  $\alpha$ -terpinyl acetate in the essential is high for fruits of Valley green variety. Since  $\alpha$ -terpinyl acetate is a principal component of the *E. cardamomum* essential oil, it may be responsible for the high antioxidant effect of Valley green oil. The findings of the current study may provide a basis for accurate determination of  $\alpha$ -terpinyl acetate in other plant species.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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