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SR Selvarani
Department of Seed Science and Technology, TNAU, Coimbatore, Tamil Nadu, India

G Sasthri
O/o the Controller of Examination, TNAU, Coimbatore, Tamil Nadu, India

R Jerlin
Department of Seed Science and Technology, TNAU, Coimbatore, Tamil Nadu, India

L Nalina
Department of Medicinal Crops, HC & RI, TNAU, Coimbatore, Tamil Nadu, India

Hemnath Elango
Karpagam Academy of Higher Education, Coimbatore, Tamil Nadu, India

Corresponding Author:
SR Selvarani
Department of Seed Science and Technology, TNAU, Coimbatore, Tamil Nadu, India

A validated RP-HPLC-PDA method for quantification of Eugenol in primed seeds of tomato, Brinjal, and Chilli using plant based Eugenol and commercial Eugenol

SR Selvarani, G Sasthri, R Jerlin, L Nalina and Hemnath Elango

Abstract
A laboratory experiment was conducted to quantify the eugenol in seeds primed with plant based eugenol (tulsi leaf extract, clove bud extract) and commercial eugenol, and also in the roots of resultant seedlings by developing and verifying a rapid and accurate Reverse Phase High-Pressure Liquid Chromatography method. The tomato, brinjal, and chilli seeds were primed (12hrs, 6hrs, and 12hrs respectively) with a standardized concentration of plant based and commercial eugenol. The different priming treatments such as T1-Control, T2-Hydropriming, T3-Purple tulsi extract (3%), T4-Clove bud extract (2%), and T5-Commercial eugenol (0.25%) were adopted in this experiment. The results revealed that the maximum quantity of eugenol was recorded in seeds as well as roots of tomato and brinjal which were primed with clove bud extract (2%), whereas in chilli, the seeds primed with commercial eugenol (0.25%) followed by clove bud extract (2%) recorded the maximum quantity of eugenol in seeds and roots of resultant seedlings, respectively. Eugenol content was absent in control and hydroprimed seeds and seedlings (root). The results also indicate that more than 90 per cent of eugenol was transferred from primed seeds to the roots of all three crops in the case of plant based eugenol.

Keywords: Eugenol, priming, purple tulsi, clove, HPLC

1. Introduction
Eugenol is a phenolic phytochemical present in essential oils such as clove, nutmeg, cinnamon, basil, and bay leaf. It has antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, and anticancer properties. Eugenol got its name since it was initially isolated from the buds and leaves of Eugenia caryophyllata (Clove). Eugenol has been certified safe by the United States Food and Drug Administration (FDA), as well as non-carcinogenic and non-mutagenic [1]. Eugenol has been investigated as a possible biomarker [2].
The chemical formula of eugenol is $C_{10}H_{12}O_2$ and the molecular weight is 164.20gmol^{-1}. The IUPAC name for eugenol is 4-Allyl-2-methoxy phenol. The physical state of eugenol is liquid at 25ºC/1ATM. Its colour ranges from colourless to light yellow and it exhibits a clove odor. Eugenol has a melting point of 9.1-9.2ºC, is insoluble in water, and is extremely soluble in organic solvents [3].
The *in-vitro* and *in-vivo* activities of two eugenol formulations (eugenol-Tween®; eugenol-ethoxylate) inhibited the growth of the pathogens *viz*., *Phlyctema vagabunda*, *Penicillium expansum*, *Monilia fructigena*, and *Botrytis cinerea*, which damage apples. In addition, application of 150 µl/L of volatile eugenol completely inhibited the mycelia growth of the four test pathogens [4]. The effect of eugenol on various pathogens causing postharvest decay of fruits was studied [5]. The result revealed that spore germination of *Alicyclobacillus acidoterrestris* was inhibited by 80 ppm eugenol or through a combination of 40 ppm + 20 ppm cinnamaldehyde, and also that eugenol exhibited good activity against white rot fungus. Wheat seed germination was inhibited by clove oil and it was found that eugenol was responsible for the inhibitory effect [6, 7] investigated the repellant effects and fumigant potentiality of *Ocimum gratissimum* oil (64% of methyl eugenol) and eugenol against the rice weevil. They concluded that the fumigant activity and repellency of the oil and eugenol were significantly influenced by concentration and time after treatment.
Ethanol extract of holy basil (Tulsi) at 2% concentration improved the seed germination, seedling length, and vigour index values in redgram, blackgram, and greengram compared to unprimed seeds [8].

He concluded that the invigorating effect might be due to the eugenol dissolved in higher order with the ethanol extract.
Rapid and uniform field emergence are vital conditions for high-quality, healthy seedling output, particularly for transplanted crops such as tomato, brinjal and chilli. As a result, pre-sowing treatments such as seed priming can be highly beneficial for uniform emergence with enhanced seed quality.
Because of the diverse properties of eugenol, the research was conducted by priming the seeds of tomato, brinjal, and chilli with standardised concentrations of plant based eugenol (tulsi leaf extract and clove bud extract) and commercial eugenol for better seedling establishment and root anchorage.
Seed priming is a controlled hydration process which involves exposing seeds to low water potentials that restrict germination (radicle protrusion), but permits pre-germinative physiological and biochemical changes to occur [9, 10, 11]. Upon rehydration, primed seeds may exhibit a faster rate of germination, more uniform emergence, and greater tolerance to environmental stresses and reduced dormancy in many species [11]. High-pressure liquid chromatography (HPLC) methods for the quantitative estimation of bioactive phytochemicals are widely reported [1], but reversed-phase HPLC equipped with a photodiode array detector (RP-HPLC-PDA) methods for the quantification of eugenol are available with the flaw of a high run time of 20 min [1].
The present study aims to develop and validate a rapid, sensitive, accurate and reliable method for the quantification of eugenol in seeds primed with plantbased eugenol (tulsi leaf extract, clove bud extract), and commercial eugenol, and also to identify the amount of eugenol transmitted from primed seeds to the roots of the seedling.

2. Materials and Methods

2.1 Chemicals, reagents and seed materials

Eugenol (reagent plus 99%) was purchased from Sigma Aldrich Co. (USA). HPLC grade Acetonitrile was acquired from Merck (Mumbai, India). Tomato seed obtained from the Department of Vegetable Science, Horticultural College and Research Institute, Periyakulam; brinjal seed obtained from the Department of Vegetable Science, Horticultural College and Research Institute, Coimbatore; and chilli seed obtained from the Agricultural Research Station, Kovilpatti were used as source seeds in this study.

2.2 Seed Treatment

Seed priming with plantbased (tulsi leaf extract, clove bud extract) and commercial eugenol was conducted on tomato, brinjal, and chilli seeds. Various treatments (standardized) adopted for the study were T1-Control, T2-Hydropriming, T3-Purple tulsi extract (3%), T4-Clove bud extract (2%) and T5-Commercial eugenol (0.25%). Tomato, brinjal, and chilli seeds were soaked in different sources of eugenol for the duration of 12hrs, 6hrs, and 12hrs respectively.

2.2.1 Preparation of 3 % purple tulsi leaf extract

Fresh Ocimum *sanctum* (Tulsi) leaves were gathered from healthy plants and washed for 2 to 3 times in tap water.10 g of leaves were homogenized with a sterile pestle and mortar in 10 ml of 95 percent ethanol (1:1 w/v).The solution was then centrifuged for 10 min at 5000 rpm [12]. The collected supernatant was subjected to overnight evaporation using a magnetic stirrer, and it was considered as 100 per cent leaf extract. From that, a 3 per cent purple tulsi leaf extract was prepared by dissolving 3ml of crude extract in 97 ml of 95% ethanol.

2.2.2 Preparation of 2 per cent clove bud extract

Unprocessed clove buds were dried and powdered. The extraction was done using the SOCS PLUS Four Place Automatic Solvent Extraction System (Model name: SCS 04 R TS). Then 30 g of finely ground clove bud powder was placed in an extraction thimble with petroleum ether at the temperature of 80o C for the first two phases and 170° C in the third phase. The crude extract was collected and from that, a 2 per cent clove bud extract was prepared by dissolving 2ml of crude extract in 98 ml of ethanol [13].

2.3 Preparation of samples and standards

2.3.1 Preparation of seed samples

Seed samples of 0.5 g were taken from each treatment and macerated with 1.5 ml of HPLC grade methanol using a sterile pestle and mortar. After that, the solution was centrifuged at 10000 rpm for 20 min. The supernatant was then filtered using a 0.2µm syringe filter.

2.3.2 Preparation of root samples

Seeds of all the treatments were sown in a pro tray nursery in the recommended media for all three crops [14].Then, 20-day-old seedlings in the pro tray nursery were collected for root sample preparation. Root samples of 0.5 g were taken from each treatment and macerated with 1.5 ml of HPLC grade methanol using a sterile pestle and mortar. The solution was centrifuged at 10000 rpm for 20 min. Then the supernatant was filtered through a 0.2µm syringe filter.

2.3.3 Preparation of standard solution of eugenol

105.63 µg of standard eugenol was dissolved in 10 ml of HPLC grade methanol for stock. From the stock solution, 1ml was taken and the volume made up to 10 ml using HPLC grade methanol (Solution A). From the solution A, 2.5 ml was taken and volume made up to 25 ml using HPLC grade methanol (Solution B). From this solution B, five different concentrations were made for standard (0.11 µg to 0.53 µg).

2.4 HPLC instrumentation and chromatographic condition

Quantification of eugenol was done by using a Waters Alliance 2695 EB separating module (Waters Co., MA, USA) using 21 CFR Part 11 complaint software. The chromatographic conditions are given in table1.

Table 1: Chromatographic condition

Composition	Value
Column	C18 reverse phase column (250x4.6mm, particle size 5µm;Sunfire®)
Flow rate	1 ml min^{-1}
Retention time	6.14 ± 0.036 min
Detector	PDA detector (Waters 2998)
Detection wavelength	280nm
Injection volume	10 µl
Temperature	25°C
Elution Type	Isocratic
Run time	10 min

2.4.1 Mobile phase

As for the mobile phase, HPLC grade Acetonitrile and Millipore water in the ratio of 60:40 (*v/v*).

2.4.2 Preparation of mobile phase
HPLC grade acetonitrile and Millipore water were filtered through a 0.22 µm cellulose nitrate filter and sonicated in an ultrasonic water bath for 15 min. Then the HPLC grade acetonitrile was taken in channel A and Millipore water was taken in channel B in the ratio 60:40.

2.5 Method validation
ICH guidelines were used to validate a proposed technique for linearity, detection and quantification limit, precision and accuracy of the HPLC method.

2.5.1 Linearity and calibration curve
Five standard eugenol solutions were prepared in concentrations ranging from 0.11 µg ml^{-1} to 0.53µg ml-1.Under optimised chromatographic conditions, all five standard concentrations were injected into the HPLC. The calibration curve was constructed by graphing peak area vs concentration.

2.5.2 Limit of detection and limit of quantification
The method signal to noise ratio was used to establish the detection and quantification limits using the equations given below.

QL= 10σ/S ---- equation1

DL= 3:3σ/S---- equation2

where σ is the standard deviation of the intercept of the calibration plot and *S* is the slope of the calibration curve [15].

2.5.3 Precision
The intraday (repeatability) and interday (intermediate precision) fluctuations for eugenol determination were performed at three concentration levels, *viz*., 0.21 µg, 0.32 µg, and 0.42 µg. The determinations were performed in triplicate [1].

2.5.4. Accuracy
The accuracy of the method was evaluated by carrying out a recovery experiment using the standard addition method. The preanalysed sample was spiked with 0.16 µg ml-1, 0.32 µg ml-1, and 0.42 µg ml-1of standard eugenol, then the combination was analysed in triplicate using the standard addition method [1].

2.6 Quantification of eugenol in various treatments
The RP HPLC system was injected with 10 µl of sample solution (seed and root samples of all treatments) under optimum chromatographic conditions. The quantity of eugenol was determined by comparing the peak area in the chromatogram of each sample solution to that of standard eugenol.

3. Results and Discussion
3.1 Method Validation
3.1.1 Linearity and calibration curve
The linearity was tested at five concentration levels, i.e., from 0.11 µg ml-1 to 0.53µg ml-1. The calibration curve was constructed by graphing peak area against concentration (µg ml-1) which is shown in figure 1. The linear equation was y = 1E+07x + 37782. The calibration curve's correlation coefficient was 0.9996, suggesting a good linear relationship between peak area and concentration.

3.1.2 Limit of detection and limit of quantification
The detection and quantification limits were determined to be 8.5 ngml-1 and 25 ng ml-1, respectively, according to ICH guidelines Q2 (R1) 2005.

3.1.3 Precision
The intraday and interday variance indicate the HPLC method's repeatability and intermediate accuracy. The results are presented as a percentage of the RSD (Table 2). The low percent RSD for repeatability and intermediate accuracy demonstrated that the technique devised performed precisely well.

Table 2: Repeatability and intermediate precision of HPLC method

Concentration (µg/ml)	Repeatability (n=3)		Intermediate precision (n=3)	
	Mean peak area ±SD	% RSD	Mean peak area ±SD	% RSD
0.21	2905483±65765.2	2.26	2862656±70813.76	2.47
0.32	4326678±181129.3	1.41	4177816±60497	1.44
0.42	5492580±84106.9	1.53	5536397±42787.77	0.77

3.1.4 Accuracy
The accuracy of the method was tested by evaluating three concentrations of the standard solution that had previously been examined using the standard addition technique. The recovery tests were conducted in order to test the HPLC method's sensitivity to estimate eugenol. The standard addition technique was used, in which 0.16 µg ml^{-1}, 0.32 µg ml^{-1} and 0.42 µg $ml^{-1}$of standard eugenol concentration were added to the sample. The percentage of recoveries for three concentrations ranged from 99.36 to 99.60 per cent (Table 3), indicating good accuracy.

Table 3: Recovery data for accuracy of HPLC method

Excess of eugenol added (µg ml^{-1})	Concentration of sample (µg ml^{-1})	Theoretical concentration of spiked sample (µg ml^{-1})	Concentration of spiked sample ±SD (µg ml^{-1}) (n=3)	Recovery ± SD (%)	%RSD
0.16	0.32	0.48	0.47±0.005	99.36±1.16	1.17
0.32	0.32	0.64	0.63±0.006	99.60±0.97	0.97
0.48	0.32	0.80	0.79±0.004	99.60±0.97	0.58

3.2 Quantification of Eugenol in various treatments
By comparing the chromatograms of each sample solution to those of standard eugenol, the identities of the eugenol peaks were quantified. Under the optimum chromatographic conditions, the RP HPLC system was injected with 10 µl of sample solution (seed and root samples of all treatments, along with untreated and hydroprimed seeds of tomato, brinjal, and chilli). The amount of eugenol found in primed seeds and roots of tomato, brinjal, and chilli seedlings was calculated and reported in tables 4, 5, and 6, respectively.

Table 4: Concentration of eugenol present in primed seeds and roots of resultant seedling of tomato

Treatments	Concentration of eugenol in seed (µg ml^{-1})	Concentration of eugenol in root (µg ml^{-1})
Untreated (control)	0.00	0.00
Hydropriming	0.00	0.00
Purple Tulsi leaf extract 3%	0.27	0.24
Clove bud extract 2 %	0.96	0.98
Commercial eugenol 0.25%	0.89	0.24

Table 5: Concentration of eugenol present in primed seeds and roots of resultant seedling of brinjal

Treatments	Concentration of eugenol in seed (µg ml^{-1})	Concentration of eugenol in root (µg ml^{-1})
Untreated (control)	0.00	0.00
Hydropriming	0.00	0.00
Purple Tulsi leaf extract 3%	0.53	0.49
Clove bud extract 2 %	0.91	0.92
Commercial eugenol 0.25%	0.78	0.34

Table 6: Concentration of eugenol present in primed seeds and roots of resultant seedling of chilli

Treatments	Concentration of eugenol in seed (µg ml^{-1})	Concentration of eugenol in root (µg ml^{-1})
Untreated (control)	0.00	0.00
Hydropriming	0.00	0.00
Purple Tulsi leaf extract 3%	0.34	0.32
Clove bud extract 2 %	0.86	1.00
Commercial eugenol 0.25%	0.93	0.31

Irrespective of priming treatments, eugenol was transferred from all the treatments, i.e. purple tulsi extract, clove bud extract, and commercial eugenol, to seeds and thereby to seedlings (root). However, the concentration of eugenol transferred varied according to the base plant material used for extraction of eugenol. Tables 4, 5 and 6 revealed that the maximum quantity of eugenol was recorded in seeds as well as roots of resultant seedlings of tomato and brinjal which were treated with clove bud extract (2%), whereas in chilli, the seeds primed with commercial eugenol (0.25%) and clove bud extract (2%) showed the maximum quantity of eugenol in seeds and roots of resultant seedlings, respectively. Eugenol content was absent in both the seeds and roots of control and hydro primed seeds and roots of resultant seedlings of tomato, brinjal and chilli.

The results of this investigation revealed that the transfer of eugenol from primed seeds to roots is comparatively lower in the case of commercial eugenol than in clove bud extract and purple tulsi extract in all three crops. In the case of purple tulsi extract, 89, 92, and 94 per cent in tomato, brinjal, and chilli, respectively, whereas in the case of clove bud extract, 100 per cent was transferred from seed to root in all three crops. In the case of commercial eugenol, however, only 27, 44, and 33% of eugenol were transferred from seed to root in tomato, brinjal, and chilli, respectively. The reason for the low amount of eugenol transfer from commercial eugenol compared to purple tulsi and clove bud extract was not known, which needs in-depth study.

Except clove bud extract, in other two sources of eugenol (purple tulsi and commercial eugenol), seeds possessed higher amount of eugenol i.e. in tomato 0.27 µg ml^{-1} and 0.89 µg ml^{-1}; in brinjal 0.53µg ml^{-1} and 0.78 µg ml^{-1} and in chilli 0.34 µg ml^{-1} and 0.93 µg ml^{-1} respectively in purple tulsi and commercial eugenol than resultant seedlings evaluated in terms of root i.e. in tomato 0.24 µg ml^{-1} and 0.24 µg ml^{-1}; in brinjal 0.49 µg ml^{-1} and 0.34 µg ml^{-1} and in chilli 0.32 µg ml^{-1} and 0.31µg ml^{-1} respectively. In case of clove bud extract, the resultant seedlings i.e. root recorded higher amount of eugenol 0.98 µg ml^{-1}, 0.92 µg ml^{-1} and 1.00 µg ml^{-1} respectively in tomato, brinjal and chilli than seeds i.e. 0.96 µg ml^{-1}, 0.91 µg ml^{-1} and 0.86 µg ml^{-1} respectively.

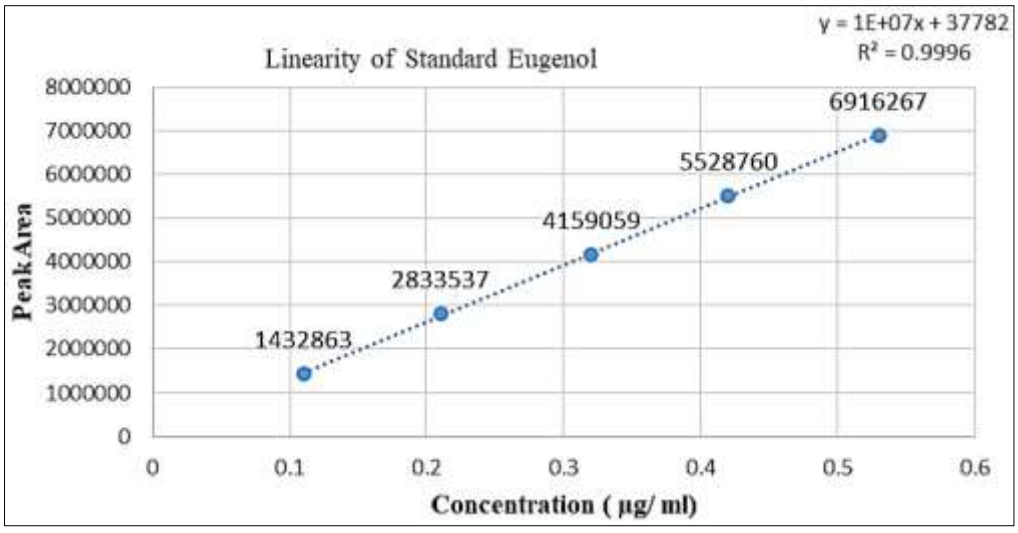

Fig 1: Calibration curve for standard eugenol

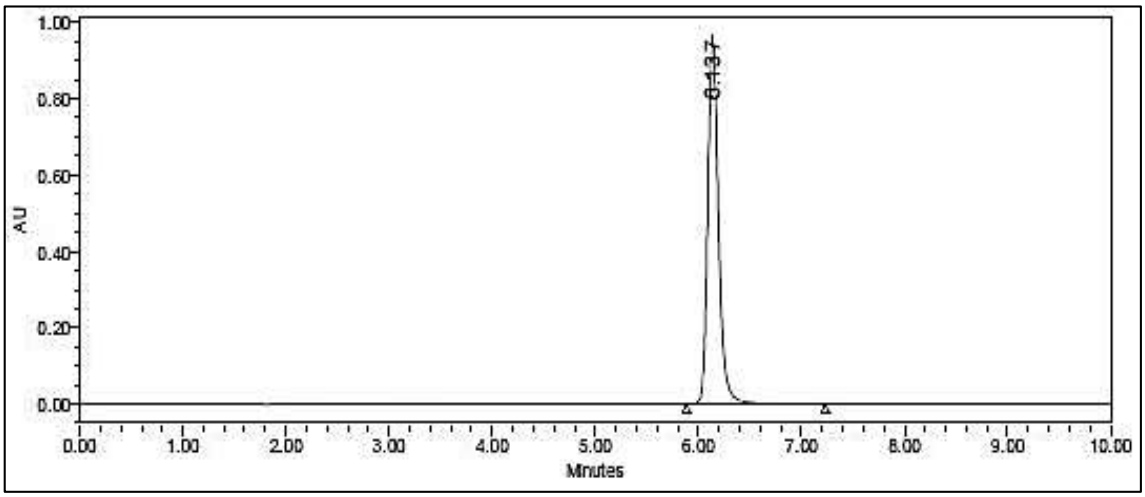

Fig 2: Standard eugenol

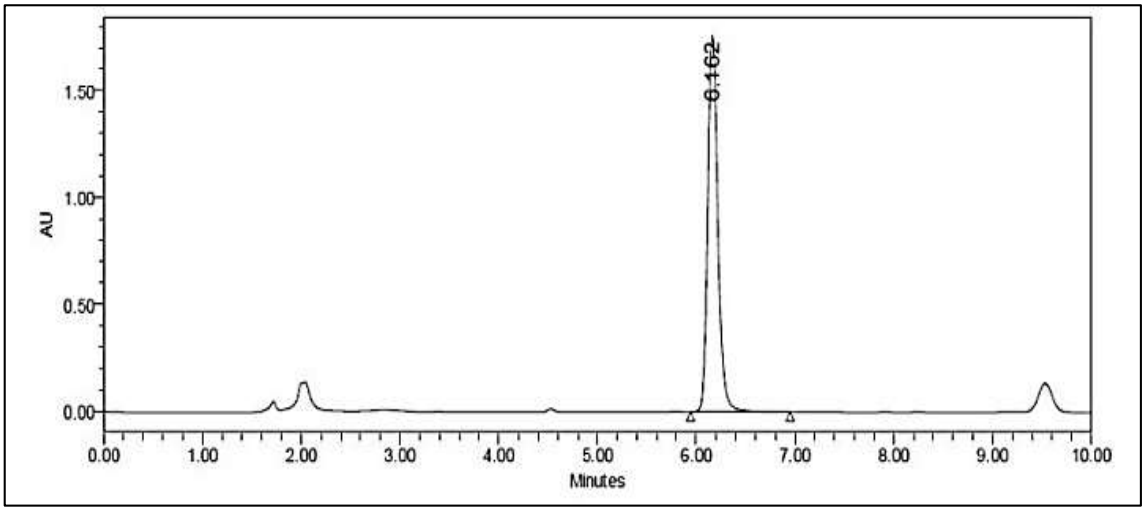

Fig 3: Tomato seeds treated with 2% clove bud extract

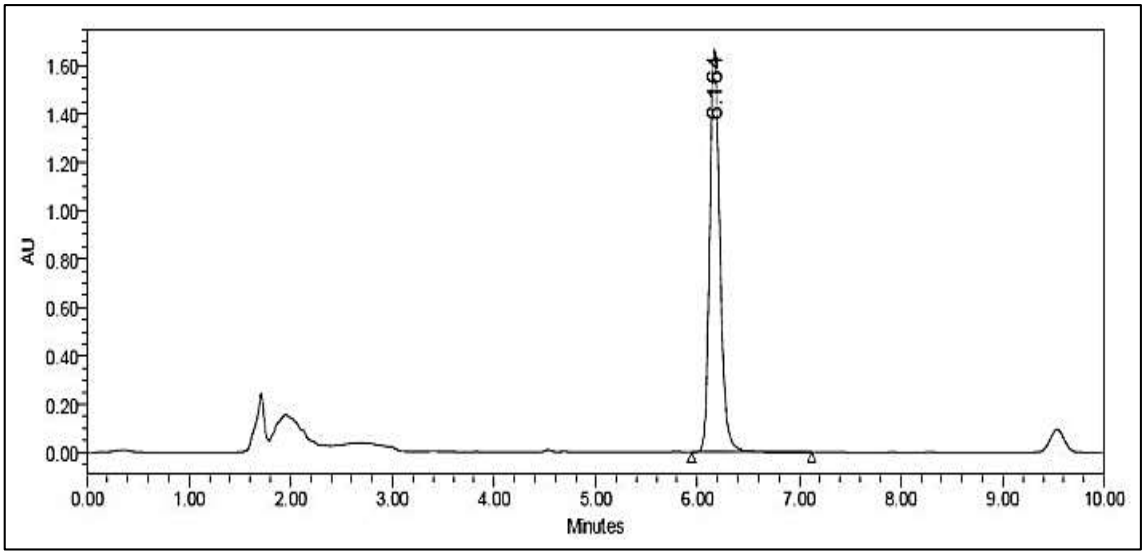

Fig 4: Brinjal seeds treated with 2% clove bud extract.

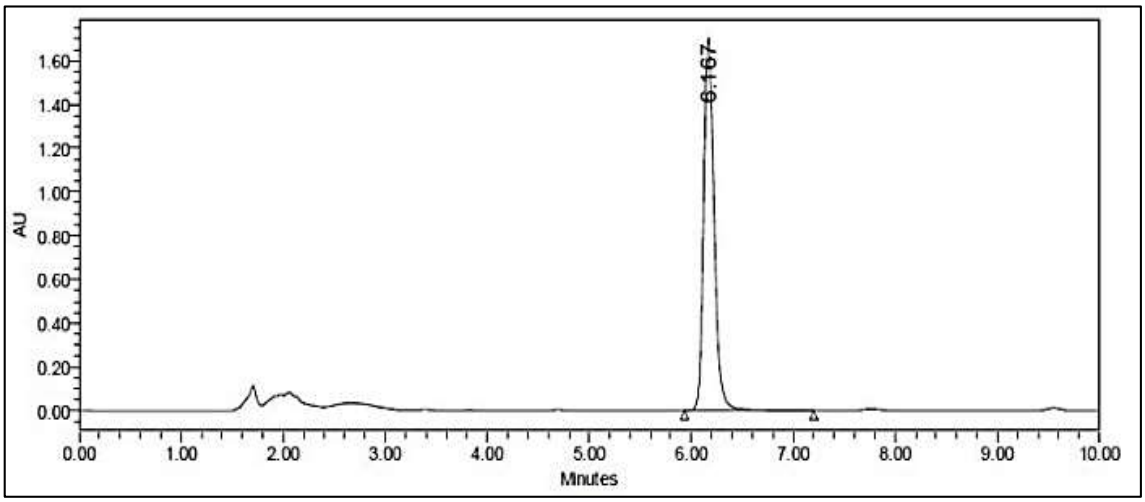

Fig 5: Chilli seeds treated with 0.25% commercial eugenol

4. Conclusion

The increase in the concentration of eugenol in the roots compared to primed seeds of 2 percent clove bud extract in all the three crops might be due to the activation of the metabolic pathway for the synthesis of eugenol in roots, which opens an avenue for further research in the future.

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6. Conflict of interests

The authors declare that there is no conflict of interest.

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