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ANALYTICAL METHOD DEVELOPMENT FOR DETERMINATION OF EUGENOL CONCENTRATIONS IN SELECTED SPECIES OF OCIMUM

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ABSTRACT

Eugenol is used as a flavor in the food industry, has a variety of biological activity, and can serve as a biomarker. Because eugenol is present in the leaves of *Ocimum*, which are used as an herbal medicine, a sensitive and reliable quantitative Ultra-violet spectroscopy and high-performance liquid chromatographic method has been established for quantification of the compound in the leaves of the plant. A methanol extract of the powder of dried leaves of *Ocimum*, was spotted on the Merck aluminium plate precoated silica gel F254 with 0.2 mm thickness. Meoh:Chloroform (95:5) and MeOH: H_2O: ACN (50:25:75) used as mobile phase to isolate the eugenol and to prepare the sample for UV and HPLC analysis respectively. The UV and HPLC method proposed for the quantitative monitoring of eugenol in *Ocimum* leaf powder is rapid, simple, and precise. Hence from that UV and HPLC analysis it was concluded that the *Ocimum sanctum linn* contains higher concentration of *Eugenol.*

KEYWORDS: *Ocimum*; Ultra-violet spectroscopy; high-performance liquid chromatography; Merck aluminium plate precoated silica gel.

1. INTRODUCTION

Medicinal properties of *Ocimum* are known for thousand years to various civilizations of the world. This medicinal herb is considered as a sacred plant by the Hindus in the Indian subcontinent. Scientific explorations of traditional belief of medicinal properties of *ocimum* have got momentum mostly after the middle of the 20th century.

There are mainly three types of tulsi mentioned in ayurvedic texts – Rama or green leaf tulsi (*O. gratissimum*), Shyama or krishna or purple leaf tulsi (*O. sanctum*) and Vana or wild leaf tulsi (*O.canum*). Although, all three types of Tulsi have their uses in ayurveda, the Rama and Krishna are the most widely used.

Eugenol is used as a flavor in the food industry, has a variety of biological activity, and can serve as a biomarker. Because eugenol is present in the leaves of *Ocimum*, which are used as a herbal medicines. Sophisticated chromatographic and spectrophotometric techniques are used for isolation, qualitative and quantitative estimation of eugenol from extracts of different species of Tulsi.

2. MATERIALS AND METHODS

There are three varieties of Tulsi namely, Rama Tulsi (*Ocimum gratissimum*), Krishna Tulsi (*Ocimum sanctum*) and Vana Tulsi (*Ocimum canum).*leaves were collected from local area of Bhopal Madhya Pradesh India and authenticated at Department of Pharmacy Barkatullah University, Bhopal.

Extraction of plant material [5-7, 10]

The collected, cleaned leaves of *ocimum* were used for the extraction process. 200g of powder of Leaves were macerated with Methanol, shaking frequently during first 6 hours and allowing stand for 18 hours. The extracts were filtered through what Mann filter paper to remove any impurities if present.

The extracts were concentrated by vacuum distillation to reduce the volume 1/10. The concentrated extracts were transferred to 100 ml beaker and to removing solvent were evaporated on the water bath. The dried extracts were packed and labeled in air tight container for the further studies.

2.1 Estimation of Eugenol contents

2.2.1 TLC profile of eugenol and its sample preparation

Standard preparation[6-8,14-16]**:** Around 2 ml of 99% eugenol was taken in a clean and dry test tube. The standard eugenol was spotted on the Merck aluminium plate precoated silica gel F254 with 0.2 mm thickness.

Preparation of mobile phase: 95% of methanol was mixed with 5% of chloroform by ultra sonication and taken around 100 ml in a chromatographic chamber. The chamber was

saturated for 30 mins to avoid edge effect.

***Method*:** After chamber saturation the spotted plate was placed in the chamber. Then the mobile phase was allow running, the present eugenol in the spot according to its affinity towards mobile phase moved and its retardation factor (Rf) was calculated.

***Preparation of sample for Thin layer chromatography*:** 175mg of each extract was drawn and dissolved in 10 ml of solvent (Meoh:Chloroform 95:5) So 1 ml of solution Contains 17.5 mg of extracts. This 1ml from the above solution was applied to the silica gel percolated plate(For Thin layer Chromatography) in the form of band and all the procedure was maintained as like as standard.

2.3 UV analysis

2.3.1 Determination of λ max: Solvent was prepared by mixing 95 parts of methanol with 5 parts of chloroform with proper sonication. Since standard eugenol contains 99%, hence 99 gm in 100ml and 1000 µg contained in 1.01 ml.

1.01ml-----dissolved up to 10 ml of solvent to give--100 µg / ml of eugenol.
From the above 10ml contains 100 µg of eugenol 1ml was drawn and again dissolved up to 10 ml of solvent to give 10 µg / ml of eugenol. Again From the above 10ml contains 10 µg of eugenol 1ml was drawn and dissolved up to 10 ml of solvent to give 1 µg / ml of eugenol.The prepared three concentrations (1 µg / ml, 10 µg / ml, 100 µg / ml) of eugenol was then carried out for determination of λ max.

2.3.2 ***Preparation of standard curve*:** From 99% eugenol stock solution was prepared. that is 10.1 ml of solution contains 99% was dissolved in 10 ml of solvent to give 1000µg. from that stock solution different Eli quotes of 1,2,3,4,8,10,20 & 25µg/ml was prepared and scanned in UV. The corresponding absorbances were noted. Then a calibration curve was plotted by taking Absorbance vs. Concentration.

2.3.3 UV analysis of eugenol

Ocimum gratissimum linn

By comparing the Rf value of standard eugenol from Thin layer chromatography, the band of eugenol obtained from extract of *Ocimum gratissimum* was identified and scrub out. The scrub silica gel containing eugenol was thoroughly mixed with 5 ml of solvent and filtered. Then the solution was taken for UV Analysis.

Ocimum Sanctum linn

By comparing the Rf value of standard eugenol from Thin layer chromatography, the band of eugenol obtained from extract of *Ocimum Sanctum* was identified and scrub out. The scrub silica gel containing eugenol was thoroughly mixed with 5 ml of solvent and filtered. Then the solution was taken for UV Analysis.

Ocimum canum linn

By comparing the Rf value of standard eugenol from Thin layer chromatography, the band of eugenol obtained from extract of *Ocimum canum* was identified and scrub out. The scrub silica gel containing eugenol was thoroughly mixed with 5 ml of solvent and filtered. Then the solution was taken for UV Analysis.

2.4 HPLC analysis

2.4.1 Development of method: After all trial and error, the following developed method having 50 parts of methanol, 25 parts of water and 75 parts of acetonitrile was fit strong for eugenol in a Isocratic system and this method was developed for both standard eugenol and eugenol present in alcoholic extracts of different species of *ocimum.*

2.4.2 Calibration of eugenol by HPLC: 1.01ml of 99% v/v eugenol when diluted up to 10 ml of solvent (MeOH: H_2O: ACN, 50:25:75) to give100 µg / ml of eugenol. From this stock solution aliquots of 25, 50, 75, 100, 125nano gram per ml of eugenol were prepared. After sufficient 30 min sonication and filtration through 0.45 micron filter paper those solutions were injected to HPLC for Calibration.

3. RESULT

3.1 **Quantitative estimation of eugenol by UV:** The concentrations of eugenol can be determined by UV- VIS spectrophotometer by putting the absorbances of eugenol obtained from various extracts of *ocimum* in the standard calibration curve.

From the spectrum of fig.2 it was found that the λ max of eugenol (Peak-2) is 281 nm.

Ocimum gratissimum linn: In fig.3 peak of eugenol of *Ocimum gratissimum l.* having absorbance 0.456 A at near about the λ max of standard eugenol. It was found from calibration curve 7.26 µg of Eugenol present in 1ml of Solvent. Hence 5ml of solution contains = 7.26×5=36.3 µg =0.036 mg So 1ml of Solvent which Contains 17.5 mg of extract contains 0.036 mg of Eugenol. Hence 5000mg (5gm) of Extract contains = 0.036/17.5×5000

mg of Eugenol=10.38 mg of Eugenol. Hence % of Eugenol contains in *Ocimum gratissimum* = 10.38 /5000 × 100 = 0.20%.

Ocimum sanctum linn: In fig.4 peak of eugenol of *Ocimum sanctum l.* having absorbance 0.678 A at near about the λ max of standard eugenol. It was found from calibration curve 9.74 µg of Eugenol present in 1ml of Solvent. Hence 5ml of solution contains = 9.74×5= 48.7 µg = 0.0487 mg So 1ml of Solvent which Contains 17.5 mg of extract contains 0.0487 mg of Eugenol. Hence 5000mg (5gm) of Extract contains = 0.0487 /17.5×5000 mg of Eugenol=13.91 mg of Eugenol. Hence % of Eugenol contains in *Ocimum sanctum l.*= 13.91 /5000 × 100 = 0.27%.

Ocimum canum linn: In fig.5 peak of eugenol of *Ocimum canum l* having absorbance 0.540 A at near about the λ max of standard eugenol. It was found from calibration curve 8.10 µg of Eugenol present in 1ml of Solvent. Hence 5ml of solution contains = 8.10×5= 40.5 µg = 0.0405 mg so 1ml of Solvent which Contains 17.5 mg of extract contains 0.0405 mg of Eugenol. Hence 5000mg (5gm) of Extract contains = 0.0405 /17.5×5000 mg of Eugenol= 11.57 mg of Eugenol. Hence % of Eugenol contains in *Ocimum canum l* = 11.57 /5000 × 100 = 0.23%.

3.2 Quantitative estimation of eugenol by HPLC method :

The concentrations of eugenol isolated by thin layer chromatography from alcoholic extracts of different species of *ocimum* can be determined by putting the peak areas of eugenol in the standard calibration curve of HPLC.

The fig.7 shows the spectrum of standard eugenol (Peak-1) at Rt 12.79.

Ocimum gratissimum lin: In Fig.8 spectrum peak no 9 is the peak of eugenol of *Ocimum gratissimum l* at near about the Rt of standard eugenol and having peak area 1535.96.

175 mg of Extract was dissolved in 10ml of solvent. So 1 ml of solution Contains 17.5 mg of extracts. This 1ml was taken as a band in silica gel precoated plate (For thin layer chromatography). The developed Eugenol band was scrub out & dissolved in again 2ml of Solvent and taken for sonication and filtration then from this 2 µl was injected to HPLC. It was found from calibration curve, 29.5 ng of Eugenol present in 2 µl of Solution. Hence 2ml of solution contains = 0.029 mg. So 1ml of Solvent which Contains 17.5 mg of extract contains 0.029 mg of Eugenol. Hence 5000mg (5gm) of Extract contains = 0.029/17.5 ×5000

= 8.28 mg of Eugenol Hence % of Eugenol contains in *Ocimum gratissimum* = 8.28/5000×100 = 0.16%.

Ocimum sanctum linn: In Fig.9 spectrum peak no 8 is the peak of eugenol of *Ocimum sanctum l.* at near about the Rt of standard eugenol and having peak area 1849.

175 mg of Extract was dissolved in 10ml of solvent. So 1 ml of solution Contains 17.5 mg of extracts. This 1ml was taken as a band in silica gel precoated plate (For thin layer chromatography). The developed Eugenol band was scrub out & dissolved in again 2ml of Solvent and taken for sonication and filtration then from this 2 µl was injected to HPLC. It was found from calibration curve, 35.3 ng of Eugenol present in 2 µl of Solution. Hence 2ml of solution contains = 0.035mg. So 1ml of Solvent which Contains 17.5 mg of extract contains 0.035mg of Eugenol. Hence 5000mg (5gm) of Extract contains = 0.035mg/17.5×5000 = 10.08 mg of Eugenol Hence % of Eugenol contains in *Ocimum sanctum l.* = 10.08 /5000×100 = 0.20%.

Ocimum canum lin: In Fig.10 spectrum peak no 9 is the peak of eugenol of *Ocimum canum l.* at near about the Rt of standard eugenol and having peak area 1598.79. 175 mg of Extract was dissolved in 10ml of solvent. So 1 ml of solution Contains 17.5 mg of extracts. This 1ml was taken as a band in silica gel precoated plate (For thin layer chromatography). The developed Eugenol band was scrub out & dissolved in again 2ml of Solvent and taken for sonication and filtration then from this 2 µl was injected to HPLC. It was found from calibration curve, 30.6 ng of Eugenol present in 2 µl of Solution. Hence 2ml of solution contains = 0.030mg. So 1ml of Solvent which Contains 17.5 mg of extract contains 0.030mg of Eugenol. Hence 5000mg (5gm) of Extract contains = 0.030/17.5×5000= 8.5 mg of Eugenol Hence % of Eugenol contains in *Ocimum canum l* = 8.5/5000 ×100 = 0.17%.

Table 1. Calibration Table of Eugenol (UV)

Concentration µg/ml	Absorbance
0	0
5	0.322
10	0.623
15	0.922
20	1.272
25	1.552

Table 2: Calibration Table of Eugenol (HPLC)

Concentration ng/ml	Peak area
25	1290
50	2680
75	3976
100	5356
125	6678

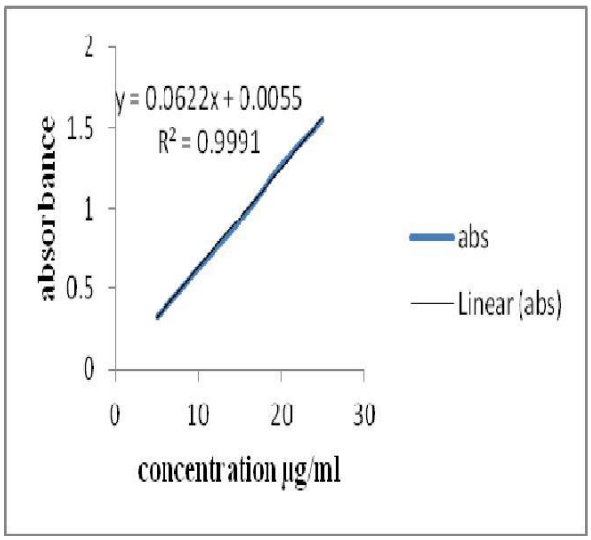

Fig . 1: Standard Curve of Eugenol

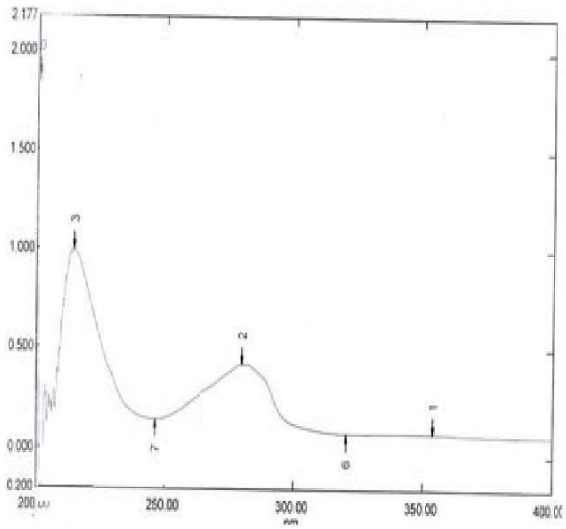

Fig. 2: UV Spectrum of Standard Eugenol

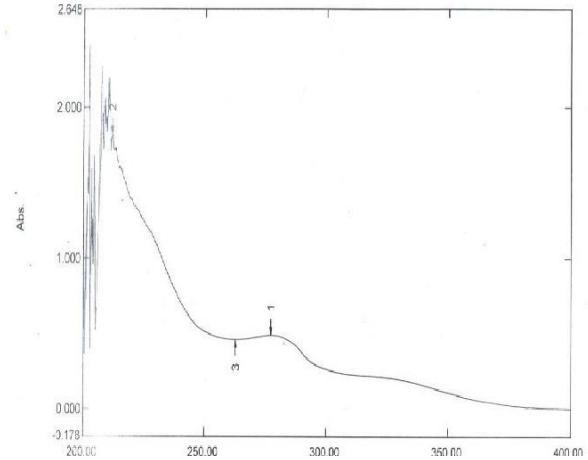

Fig. 3: UV Analysis of *Ocimum Gratissimum*

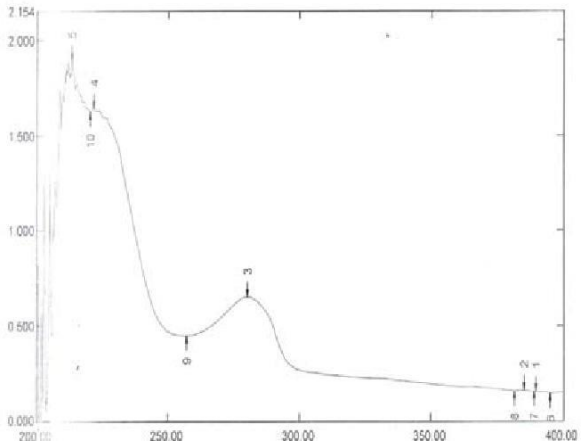

Fig. 4: UV Spectrum of *Ocimum sanctum*

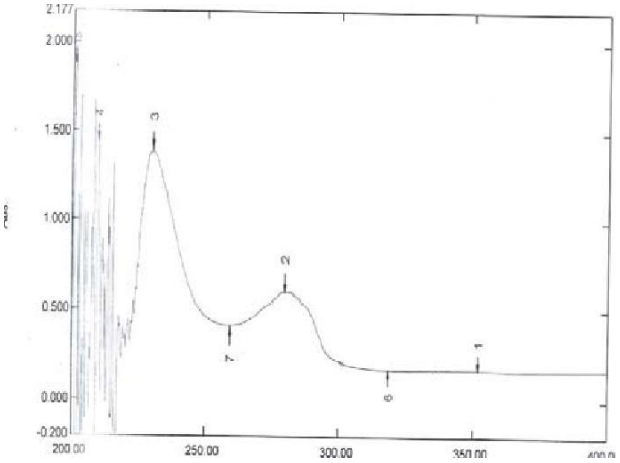

Fig. 5: UV Spectrum of *Ocimum Canum Linn*

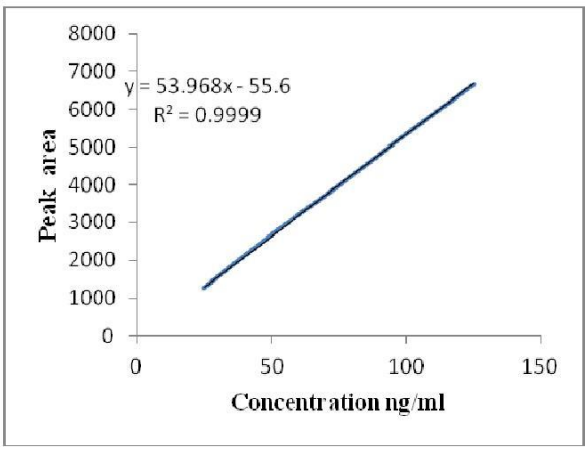

Fig. 6: HPLC Calibration Curve of Eugenol

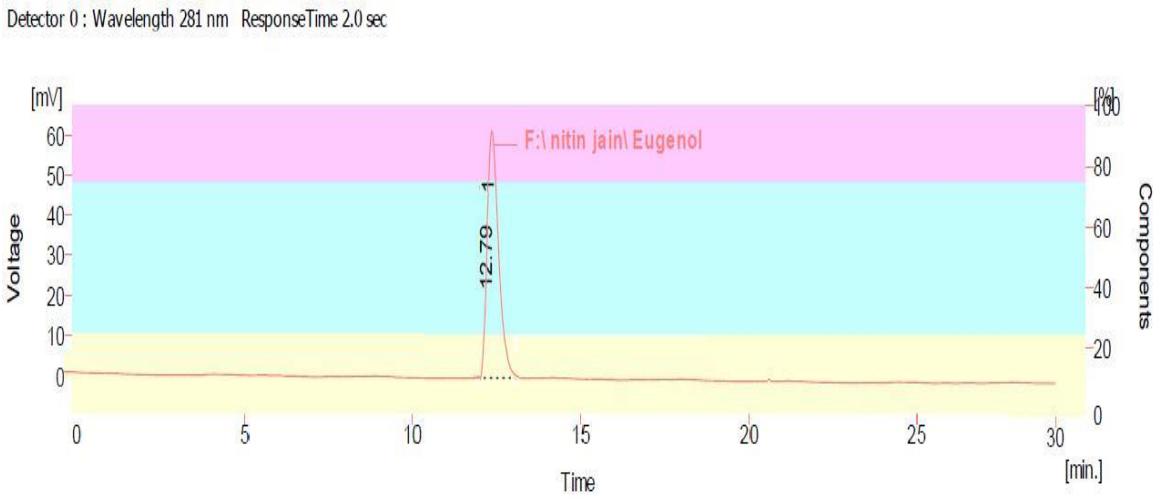

Fig. 7: HPLC Chromatogram of Eugenol

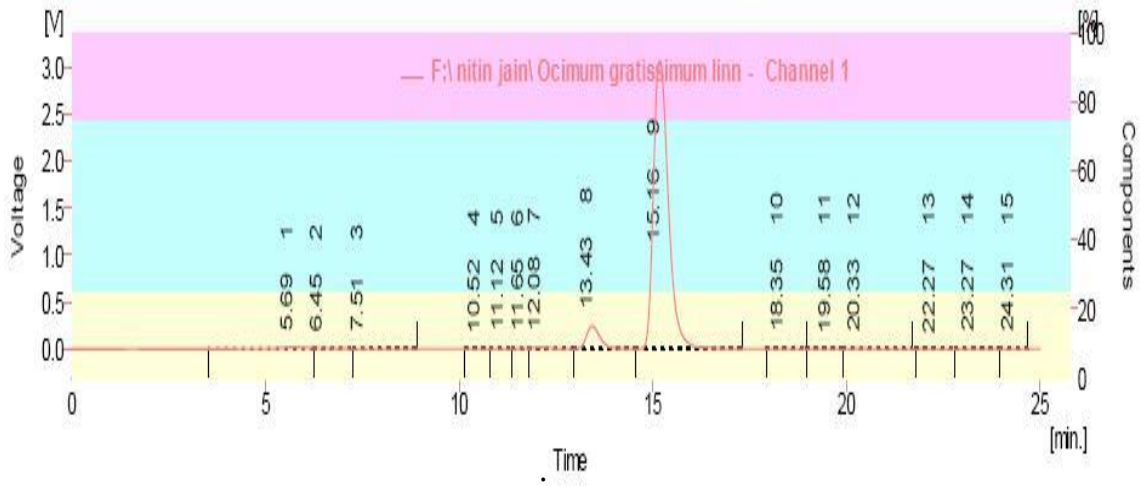

Fig. 8: HPLC Chromatogram of *Ocimum Gratissimum Linn*

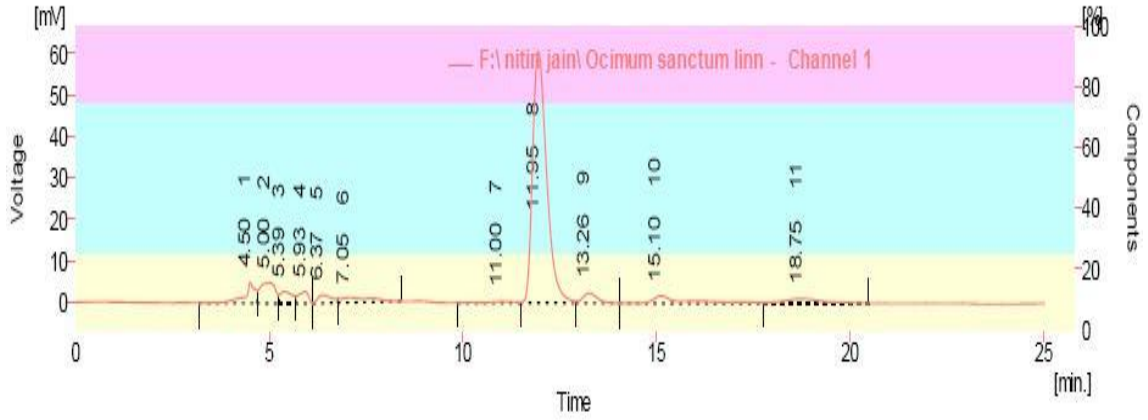

Fig. 9: HPLC Chromatogram of *Ocimum Sanctum Linn*

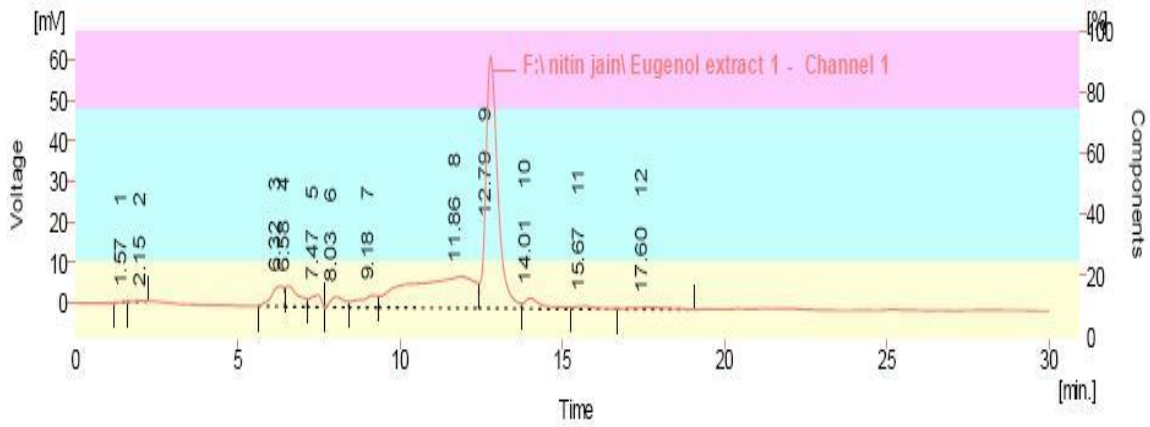

Fig. 10: HPLC Chromatogram of *Canum Linn*

DISCUSSION

The result from the UV analysis it was conclude that the *Ocimum gratissimum linn* contains 10.38 mg (0.20%) of Eugenol. *Ocimum sanctum linn* contains 13.91 mg (0.27%) of Eugenol. *Ocimum americanum linn* contains 11.57 mg (0.23%) of Eugenol From 5gm of Extracts. And from the HPLC analysis it was conclude that the.*Ocimum gratissimum linn* contains 8.28 mg (0.16%) of *Eugenol.Ocimum sanctum linn* contains 10.08 mg (0.20%) of Eugenol. *Ocimum canum linn* contains 8.5 mg (0.17%) of Eugenol From 5gm of Extract. The result obtain from UV and HPLC analysis shows that the, *Ocimum sanctum linn* contains higher amount of eugenol and established Ultra-violet spectroscopy and high-performance liquid chromatographic method for quatification of phenolic compound or eugenol in leaves of the plants has been sensitive and reliable.

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