QUANTIFICATION OF EUGENOL IN VARIOUS SPICES USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT:-*Today there is greater scope of herbal medicines in the whole world due to their less or negligible side effects .The scarcity of data regarding the parameters and methods employed for assessing the quality of medicine prevent lots of medicines to be formed using the herbs and spices present in nature .The spices cloves, dalchini , tejpatta ,nutmeg and tulsi all of them contain an active component Eugenol which can be helpful in preparation of various medicines.*

In this study, an HPTLC – densitometric method was developed for the determination of Eugenol in above mentioned five spices as cloves dalchini ,tejpatta ,nutmeg and tulsi. Chromatographic separation was performed on precoated Si gel F_{254} (0.2mm thickness) plates using toluene :ethyl acetate :Glacial acetic acid(9:1:0.12 v/v/v) as the mobile phase. A TLC scanner set at 278nm was used for the direct evaluation of the chromatogram .The method was found to be linear over the range of 10- 50 µg/spot with a correlation coefficient(r^2) of 0.998, indicating good relationship between peak area and concentration .Recovery value was obtained from 98.32 % to 99.8 % showing excellent accuracy of method .Validation of method was done according to the ICH protocol for repeatability , precision and accuracy. The HPTLC method proposed for quantitative analysis of eugenol in above spices is found be rapid, simple and precise which will have applicability in quality control.

Keywords- HPTLC, Eugenol , validation , correlation coefficient

1.INTRODUCTION:-

The spices form an inseparable part of Indian food. They are the dried parts of plants which have been used as diet component mainly to improve colour , aroma and palatibility of food.[1]

Some of the spices and herbs which contain a bioactive component eugenol in common , are studied in this work to quantify eugenol in them.

Syzigium aromaticum commonly known as Clove is in the bud form and is used as spice in food industry. The essential oil obtained from the buds of this plant (clove oil) finds extensive use in dental formulations, toothpaste, breath freshener, mouthwashes, soaps, cosmetic items and insect repellent. The oil possesses anti bacterial, antifungal, antioxidant and cytotoxic properties .[2]

Dalchini is a tropical evergreen tree native to the Malabar coast of India. Its botanical name is Cinnamon zeylanium belonging to lauraceace family. In South East Asia , America and some European countries , it has been used as an important f lavouring agent in food , pickles , chutneys and beverages.[3]

Medicinally it is used to treat poor apetite , fever, arthritis, hypertension ,nervous disorder , cough, vomiting, skin antiseptic , kidney problems, [4-8].Along with its anti-microbial effect ,it has been found to show anti-diabetic ,analgesic effect also.

Cinnamomum tamala commonly known as tejpatta belongs to family lauraceae and is found in tropical Asia ,Australia .[9]. The C .tamala leaves are used as spice in food. It is used in treatment of mouth dryness, nausea, it has hypoglycemic prop. It is also used as carminative, a duretic and to treat heart disorders.

Ocimum sanctum commonly Known as Tulsi , a sacred plant belongs to labiatae family. It is a hub having many medicinal uses.[10,11] The whole plant is used as a source of remedy. It has aromatic nature . Eugenol is one of the important phenolic component found in it. Essential oil obtained from tulsi contain carvaciol , and Eugenol which are responsible for various antimicrobial activities.

HPLC and GC methods for estimation of eugenol have been reported earlier in the literature . HPTLC has proved to be an efficient tool for an evaluation of the herbal drugs,[12-15]. In the present work, Here in HPTLC , method has been developed for comparative analysis of eugenol and as an alternative method for quantification of the component in above mention 5 spices and for the standardization of the these spices using eugenol as a main component. The proposed method is sensitive , precise and simple.

2. MATERIALS AND METHODS:-

2.1COLLECTION OF PLANT SAMPLES:-

Scientific and common names of the studied spices

The experimental material consists of 5 spices: cloves, cinnamom , nutmeg , tejpat and tulsi.

All the spices except tulsi herb were purchased from the local market in Nagpur, Maharashtra , India. Tulsi plants were grown in nagpur farm itself , then the leaves were collected ,washed and dried in the shade They were classified according to their English name , scientific name and the used part of the plant.(Table 1).All the spice samples were authenticated from the department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University ,Nagpur, where the herbarium specimens were identified. A set of authenticated herbarium is kept in the department for further references.

Common name	Scientific name	Family	Used parts
Cloves	Syzygium aromaticum	Myrataceae	Bud
Dalchini	C.Zeylanicum	Lauraceae	Bark
Tejpat	C.Tamala	Lauraceae	Leaves
Nutmeg	Myristica fragrans	Myristicaceae	Seed
Tulsi	Ocimum sanctum	Labiateaes	leaves

Table 1 - Scientific and common names of the studied spices

2.2 REAGENTS AND STANDARDS:-

Analytical grade methanol, toluene , ethyl acetate and acetic acid were obtained from Merck company Eugenol standard (≥ 99 % purity) was purchased from Sigma-Aldrich(Bangalore).

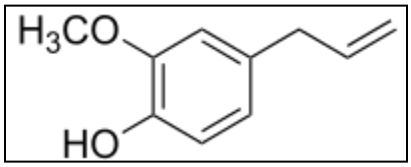


Figure 1 – Structure of eugenol.

Proximate analysis of raw materials:
To assess the quality of raw materials, proximate parameters like ash values (total ash, acid insoluble ash and water soluble ash), loss on drying and foreign matter were determined using standard pharmacopoeial methods as per WHO guidelines[10] .

Preliminary phytochemical evaluation:
Phytoconstituents in above mentioned five spices clove, Dalchini , Nutmeg, Tejpatta and Tulsi were evaluated by performing preliminary phytochemical tests for flavonoids, essential oils, tannins, glycosides, alkaloids and resins as per standard methods[11].

Chromatographic Evaluations –

HPTLC conditions:
HPTLC was performed on 20 cm x 20 cm TLC plates precoated with (0.2 mm) layer of silica gel $^{60}F_{254}$ (Merck). The plates were thoroughly washed with methanol before using them . Working standard solutions of Eugenol (10µl) of different concentration were applied to the plates as 6mm bands using CAMAG Linomat 4 automatic sample applicator . The plates were developed at 25+_ 3 c in a glass twin trough chamber presaturated with mobile phase.The development distance was 8.0 cm .The plates were then dried in air and scanned at 278nm by using a CAMAG TLC scanner and CATS 3 software .Peak areas were recorded for Eugenol and a calibration plot was obtained by plotting peak area against eugenol concentration. Linear plot was obtained in the range 10 to 50 µg/ml, with a correlation coefficient of 0.98 . The limit of quantification was found to be 5µg/ml and the limit of detection was found to be 0.9 µg/ml.

Preparation of Standard solutionsof Eugenol:
A stock solution of Eugenol (1µg/ml) was prepared by dissolving 10 mg accurately weighed Eugenol in methanol and diluting it to 10ml of methanol .From this stock solution , solutions of 10µg/ml - 100µg/ml was prepared for calibration curve of Eugenol. The three quality control samples of 10 , 50 and 80µg/ml were prepared for studying precision, accuracy and ruggedness.

Solvent System-

Various solvent systems were investigated , of which toulene : ethyl acetate : glacial acetic acid (97:3:0.1) v/v/v gave the best Eugenol resolution at (R_f = 0.49-0.53) from various other components of the methanolic extracts of above mentioned spices viz, Clove, dalchini , tejpatta nutmeg and tulsi. The identity of eugenol band in all the samples were confirmed by their UV absorption spectra with that of the standard,

obtained by use (fig. 2) shows an HPTLC separate of Eugenol standard and methanolic extract of clove, dalchini , tejpat , nutmeg and tulsi ,visualized in UV light.)of the CAMAG TLC scanner .

Preparation of sample solutions:-All the spices were dried properly in the shade , powdered and seived through a mesh .They were stored in an air tight container in the refrigerator till further use.

The powdered sample of 50 g clove was placed in a soxhlet extraction setup using methanol solvent for about 10 -12 hours. The extract was then concentrated by distillation process .In the same way extracts of all other samples as dalchini , tejpatta ,nutmeg and tulsi were obtained. 50 mg of each methanolic extract was taken in 10 ml volumetric flask and the solution was made in methanol upto 10 ml .This solution was ready for the assay experiment .Same procedure was done for rest of the sample extracts.

Method Validation-Validation of the developed HPTLC method was carried out as per the International Conference of Harmonization (ICH) guidelines for specificity, sensitivity, linearity, accuracy, precision repeatability, and robustness.

Specificity: Specificity was ascertained by analyzing standard compound with sample. The band of Eugenol from sample solution was confirmed by comparing its Rf and spectra with that from standard. The peak purity of all the compounds was analyzed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

Instrumental Precision and Repeatability: . Instrumental precision was checked by scanning the same eugenol spot (5µg/spot) 10 times and was expressed as a coefficient of variance (%RSD) . The repeatibility of the method was confirmed by analysis of 10 spots (5 µg/spot) of eugenol solution and all the answers were given in the (%RSD).

Inter-Day and Intra-Day Precision: Variability of the method was studied by analyzing Quality control samples of Eugenol (50, 85 and 145 µg mL-1) on the same day (intra-day precision, n = 3) and on different days (interday precision, n = 3). The results were expressed as % RSD.

Limit of Detection and Limit of Quantitation: Limit of detection (LOD) and limit of quantitation (LOQ) of the developed method was affirmed by analyzing progressively low concentrations of Eugenol along with methanol as blank. Limit of detection (LOD) and limit of quantitation (LOQ) were established at a signal to noise ratio of 3:1 and 10:1 respectively.

Recovery: The accuracy of the method was assessed by performing recovery studies at three different levels (0,10,20 µg). Appropriate concentrations of Eugenol were spiked into the sample spices and then each was analyzed as per the developed method. The percent recoveries at each level were calculated to deduce average percent recovery .

Ruggedness: Ruggedness of the method was assessed by deliberately incorporating the small variations in the optimized chromatographic condition. Effect of change in analyst, change in mobile phase composition [Toluene: Ethyl Acetate: Glacial Acetic Acid (8.1: 1.9: 0.1, v/v/v) and Toluene: Ethyl Acetate: Glacial Acetic Acid (9.5: 0.5: 0.1, v/v/v)] and change in spotting volume (9 µl and 11 µl) on the response and Rf of quality control samples was observed.

2.6 Estimation of eugenol in samples of spices :

The sample solution (10µl) was applied in duplicates to the precoated silica gel $60F_{254}$ plates , again with the Camag linomat IV each plate was developed and scanned as described above. The peak areas and absorption spectra were recorded . The amount of eugenol in S.aromaticum , C.zey lanicum, C. tamala , M.fragrans and O.sanctum were calculated for each solution by use of the calibration plot.

RESULTS AND DISCUSSION -

The results for the proximate analysis of raw materials for some parameter like ash values (total soluble ash) , loss on drying moisture content and extraction values were calculated and they were found to be quiet in compliance with the pharmo copeial limits. (data not mentioned)

The phytochemical evaluations of above mentioned spice extracts show the presence of different phyto constituents.

Various solvent systems were investigated , of which toulene : ethyl acetate : glacial acetic acid (9:1:0.1) v/v/v gave the best Eugenol resolution at (R_f = 0.49-0.53) from various other components of the methanolic extracts of above mentioned spices viz, Clove, dalchini , tejpatta nutmeg and tulsi. The identity of eugenol band in all the samples were confirmed by their UV absorption spectra with that of the standard, obtained by use (fig. 2) shows an HPTLC separate of Eugenol standard and methanolic extract of clove, dalchini , tejpat , nutmeg and tulsi ,visualized in UV light.)of the CAMAG TLC scanner .

Table.2 - Method Validation parameters of Eugenol.

Parameters	Results
Linear working range(µg/ml)	10µg/ml - 50µg/ml
Regression equation	101.78x + 118.17
Correlation coefficient (r^2)	0.996
Instrumental precision	0.02-0.07
Repeatability (% RSD,n=5)	0.29%
LOD (µg/ml)	0.9µg/ml
LOQ (µg/ml)	10µg/ml
Specificity	Specific
Ruggedness	Rugged

Pharmacologically , Eugenol was quantified from the spices extract by HPTLC densitometric terms of precision , repeatability and accuracy as per the ICH guidelines. The corelation coefficient (r^2) of 0.998 (fig.4) which resulted in a regression equation y =10.178x+118.17 was obtained..The equation was then used to determine the respective eugenol content of all the mentioned samples. The assay results of the same are presented in table 3, 4, 5.

(I) *Sensitivity*

Sensitivity of the method was tested with respect to LOD and LOQ of eugenol.
Series of concentrations of eugenol in the range of 0.5-5.0 µ/spot was applied on the plate and analyzed to determine LOD and LOQ. Under the experimental conditions employed, LOD and LOQ were determined to be 0.9 µ/spot and 5 µ/spot , respectively . (Table 1). The developed TLC densetometric

method was found to be precise with %R.S.D. in the range of 0.02 % and 0.07 % for intraday precision respectively ,indicating that the proposed method is precise and reproducible.

(II) *Linearity and Calibration Curve*

The linearity was tested at ten concentration levels i.e. 10 – 100 µ/spot A calibration plot was constructed by plotting peak area against the concentration (µg/spot) with the help of the win-CATS software. The linear regression value indicated a linear relationship over a concentration range of 10-50 µ/spot and its linear equation was y = 101.78x+118.17; the slope, intercept, and correlation co-efficient were also determined.

The correlation co-efficient of the calibration plot was 0.99 (Table 1), indicating a good linear relationship between peak area and concentration (Figure 3).

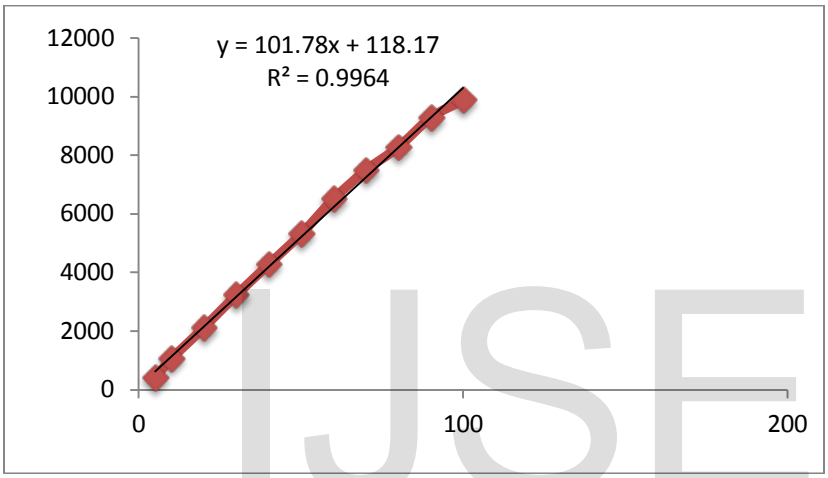


Fig. 1 -. Linear calibration plot for eugenol

(III) *precision*

Obs no.	*Conc (µ/spot)*	*Intraday Precision*			*Inter Day Precision*		
		Mean Area	*S.D.*	*%RSD*	*Mean Area*	*S.D.*	*%RSD*
1	*10*	*1050.7*	*1.44*	*0.14*	*1051.07*	*0.51*	*0.05*
2	*50*	*5309.91*	*1.55*	*0.02*	*5310.23*	*1.02*	*0.02*
3	*80*	*8274.5*	*0.7*	*0.08*	*8274.9*	*0.6*	*0.07*

Table3 – Intra- and inter-day precision study of HPTLC method[a]
[a] n = 6

Instrumental precision was studied by scanning five bands of eugenol at a concentration of 50 µ/spot . The coefficient of variation of measurement of the peak area was taken to evaluate the system precision and was found as (Table 1). Intra-day precision of the method within a laboratory over a short period of time by the same operator using the same equipment was evaluated for eugenol at three different concentrations (10, 50 , 80 µg/spot) over entire calibration range for six time (n = 6) on the same day, whereas inter-day precision was checked at the same concentration level over entire calibration range for six time (n = 6) on

the consecutive days. In both cases, the coefficient of variation of peak area of spots was used to evaluate method precision and the results obtained are shown in Table 2. In all the condition , % RSD values were less than 2% confirming the precision of the method.

(IV) *Accuracy*

FOR CLOVE,

Table 4. Results from the recovery study of eugenol by the proposed HPTLC method

Amt. Of sample(mg/ml) Extract	*Amt. Of eugenol Present (mg)*	*Amt. of eugenol Added (mg)*	*Total amount (mg)*	*Total amount Found (mg)*[a]	*Recovery %*	*Average recovery*
100	*1.468*	*0*	*1.468*	*1.440*	*98*	
101	*1.52*	*10*	*1.62*	*1.58*	*98.2*	*98.73%s*
102	*1.67*	*20*	*1.87*	*1.87*	*100*	

a =Each value is the mean of three analyses

The accuracy of the analytical method was determined from recovery experiments, performed by adding a known amount of eugenol at 3 different levels with 3 replicates for each level and the % RSD was calculated. Recoveries obtained were satisfactory in the range of 98 % to 100 % giving an average of 98.73 %. The results are presented in Table 3 indicating that the method provides sufficient accuracy. Similarly, the recovery of eugenol for Dalchini , Tejpatta , nutmeg and tulsi were also calculated. The result of it is shown in the table given below.

Sr. no.	Samples (methnaolic extracts)	% recovery (average)
1	Dalchini	98.70
2	Tejpatta	98
3	Nutmeg	98.1
4	Tulsi	98.2

Table 5 :-Recovery studies for Eugenol in taken spices

(V) Specificity –

The specificity of the developed method was confirmed by comparing the retention factor (R_f value) of eugenol standard with sample. The identity of eugenol peak was confirmed by UV – visible absorbance spectrum of the peak from the standard with concentration peak of the sample (Fig. 4).

4. Quantification of eugenol

The above developed method was applied to determine the eugenol content in the spices mentioned above

The Eugenol content of the sample Clove was found to be 1.48% by the above proposed method; in dalchini it was 0.30% , nutmeg showed 0.05 % of Eugenol content in it , tejpatta contained 0.05% and tulsi contained 0.2% Eugenol in it.(fig .3) shows the chromatogram of Standard Eugenol and samples of methanolic extract .

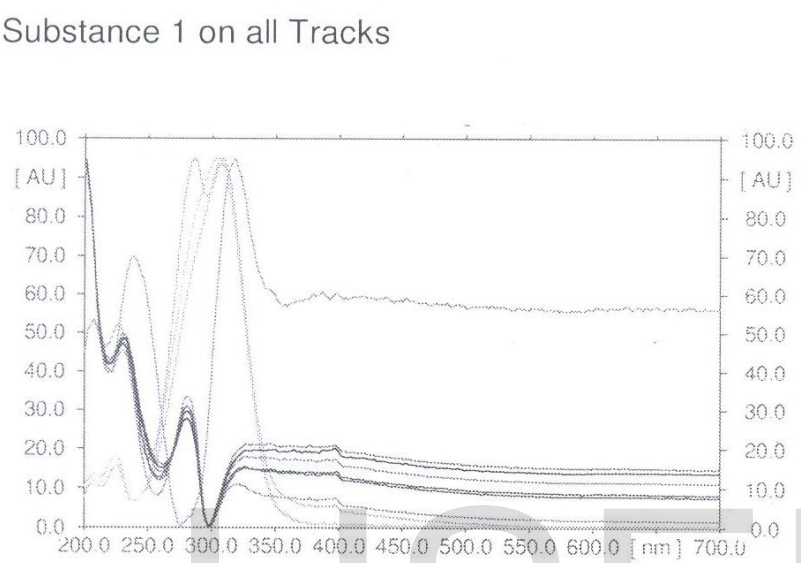
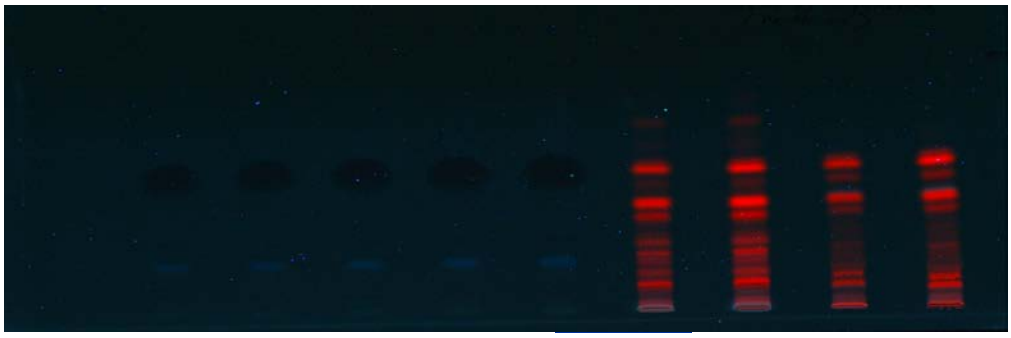


Figure 3 - Chromatogram of Standard Eugenol and sample spices in methanolic extract .

Table 4 – Results showing the percentage of eugenol quantified in spices taken for HPTLC.

Sample(methanolic extract)	Plant part used	% of Eugenol
Clove	Buds	1.46
Dalchini	Bark	0.30
Tejpat	Leaves	0.54
Nutmeg	Seed	0.65
Tulsi	Leaves	0.93



(X) A B C D

Figure 2 – HPTLC separation of methanolic extract of clove (A), dalchini (B) , Tejpatta (C), Nutmeg (D) and standard eugenol (X)

CONCLUSION:-

Eugenol is found to be one of the major contents in the taken spices clove , dalchini , nutmeg , tejpat and tulsi . The method developed for estimation and comparison of eugenol in above samples can be applied to various polyherbal formulation containing Eugenol. The developed HPTLC method provides a simple, precise , and accurate analytical method for the identification and exact quantification of eugenol in different spices mentioned above. A good separation of analyte was achieved using toluene : ethyl acetate : acetic acid (9 : 1 : 0.12, v/v/v)as a mobile phase on precoated silica-gel 60 F254 plates. The method was successfully validated as per the ICH guidelines and statistical data proved that the developed HPTLC method may be used as a tool for routine analysis of this bioactive marker. It is evident from the comparative analysis that all the above spices contain eugenol in given percentage in their specified regions which was not reported earlier . Moreover, these spices may be used as an alternative source for this important bioactive molecule.

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