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Research Article

# VALIDATED RP-HPLC METHOD TO ESTIMATE EUGENOL FROM JATIPHALADI *CHURNA*

**Baljeet Singh*, Sourabh Jain, Karunakar Shukla**

*Faculty of Pharmacy, Dr. A.P.J. Abdul Kalam University, Indore, Madhya Pradesh, India*

**Corresponding author: jeet.khurana@gmail.com*

## ABSTRACT

Eugenol is a significant phytochemical biomarker compound of ayurvedic and other marketed herbal formulations. It shows anti-inflammatory, anti-bacterial and anti-tubercular activity. Thus, it is a suitable bioactive constituent to establish the quality of commercial drug and its formulation. The aim is to develop and validate an efficient and effective RPHPLC method for quantification of eugenol from commercial marketed formulations. Separation was carried out on a cosmosil $C_{18}$ column, mobile phase composition was methanol: distilled water (60:40, v/v) at flow rate of 1mL/min. Detection was carried out at 215 nm using a photodiode array detector (PDA). Commercial Ayurvedic formulation such as Jatiphaladi *Churna* was further subjected to RP-HPLC for estimation of eugenol. The RP-HPLC method was validated as per ICH guidelines. The LOD and LOQ level were found to be 25.00ng/mL and 50.00 ng/mL, respectively. Detector response was found to be linear from 50.00ng/mL to 50,000.00 ng/mL. The method was found to be simple, accurate, reproducible and rugged. This method was used to estimate the content of eugenol in commercial formulations and the method is found suitable for quality assurance and marker based standardization of ayurvedic formulations containing eugenol.

**Keywords:** Jatiphaladi *Churna*, RP-HPLC, Eugenol, Formulation.

## 1. INTRODUCTION

Medicinal plants have been used since thousands of years from the beginning of human civilization for its therapeutic properties, containing inherent active ingredients that have properties to heal sores, relieve pain; cure diseases [1] and maintenance of overall good health [2]. Medicinal properties of plants provide ample opportunity for development and obtaining a wide variety of drugs. Therefore should be investigated further to better understand their safety and efficacy [3].

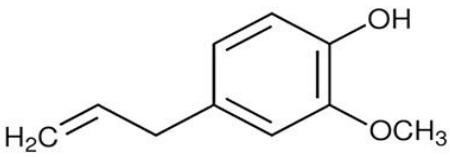


**Fig. 1: Molecular Structure of Eugenol**

The property of herbal medicine is highly dependent upon the composition of chemical phytoconstituents in their extracted final product. There is current need for development of method for establishing quality control parameters for ayurvedic formulations owing to variability and complexity of chemical constituents present in herbal plant based drugs [4]. Identification, isolation, purification and characterization of active ingredients in crude extracts from herbal plants is now possible relatively easily because of development and implementation of high resolution separating analytical techniques like RP-HPLC [5, 6]. Among these bioactive compounds, there has been current explosion of interest in areas of distilled essential oils from fresh leaves, roots, stems and root sources of plant parts. These essential oils of plant contain phytochemicals. Among these phytochemicals, the major essential oil eugenol, a phenolic compound (L-hydroxy-2-methoxy-4- allyl benzene) is widely distributed [7]. Eugenol can be predominantly extracted from various species and families of aromatic plants and comprise about 70-85 % in many essential oils [8]. Several studies have reported pharmacological mode of action of eugenol from medicinal plants such as *Ocimum sanctum* (leaf), *Anethum sowa* Roxb (leaf), *Pimpinella anisum* Linn (leaf), *Alpinia galanga* wild (rhizome), *Salvadora persica* Linn. (leaf) and *Vetiveria zizanioides* (root) in experimental animal systems [9-14] as hepato-protective agent, vasorelaxing-action [15], as an attractant to fruit fly [16], membrane stabilizing properties useful in the treatment of

neurological, allergic disorders, anti-tubercular activity [7], and has anti-nociceptive potential to be used as dental analgesic [10]. Various methods such as HPLC mass spectrophotometry using offline dansyl chloride derivatization has been carried for detection of lower limit of eugenol [17, 18]. Additionally, HPLC-UV method has been successfully used for determination of eugenol in *Syzygium aromaticum* Linn (Clove) and *Cinnamomum zeylanicum* (cinnamon oils) by using NDBD-F as a labeling reagent [19]. However, these systems are relatively costly and are increasingly complicated. The use of costly polymer based columns and absence of organic phase have contributed to difficulties in developing viable and cheaper RP-HPLC analysis. Although there are many chromatographic methods currently utilized for quantification of eugenol from various fruits, vegetables, leaves etc but virtually not much work has been validated and used for estimation and quantification of eugenol from commercial formulations. Hence, an alternative method needs to be developed for determination of such essential oil which is simpler, reliable and offers results in shorter span of time.

Present study aims in development of reliable, cost effective and validated analytical method for separation and quantification of eugenol from commercial formulation of Jatiphaladi *Churna* like commonly eugenol containing formulation by HPLC using photodiode array detector. The estimation of eugenol from ayurvedic formulations can be useful for quality control analysis and for safety purposes, due to possible toxic effects.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals and reagents

Eugenol (99 %, C10H12O2) was obtained from Aldrich, USA. Commercial Ayurvedic formulations (plants) like Jatiphaladi *Churna* (Cannabis sativa jatiphala, lavanga, ela, patra, tvak, nagakesara, candana, tila, tvaksiri, tagara, amla, talisa, pippali, pathya, sthulajiraka) [20], containing eugenol were purchased from local markets. HPLC grade methanol was procured from Merck Specialist Private Limited (Mumbai, India). Distilledwater was prepared in-house using Millipore (Millipore SA Molsheim, France). All other chemicals used were of analytical grade.

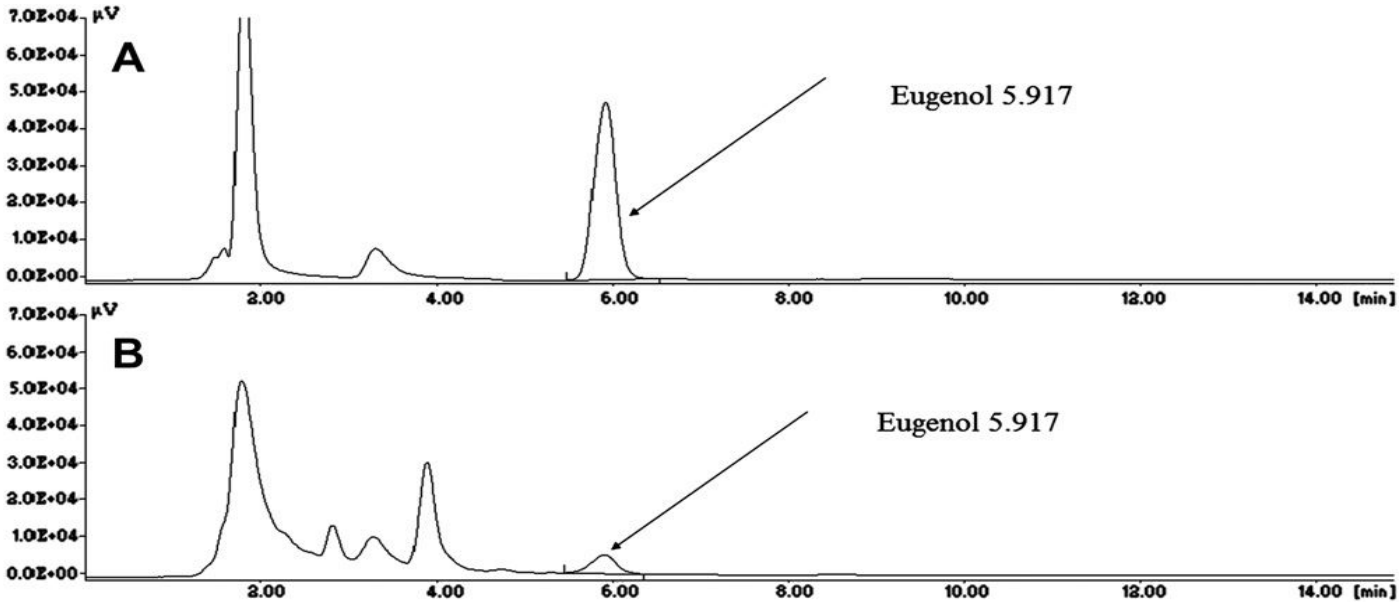


**Fig. 2: Representative HPLC chromatogram of (A) standard sample 10 ppm and (B) Jatiphaladi *Churna***

### 2.2. Preparation of standard solution

A stock solution of 1000 ppm was prepared by accurately weighing 10 mg of eugenol standard in a 10 mL volumetric flask and it was further diluted with HPLC grade methanol up to the mark. The solution was vortexed for 10s.

### 2.3. Preparation of sample solution

One g of ayurvedic formulations were taken in 10 mL of methnol and then solvent extraction was performed using a rotary shaker for 24 h. The tubes were centrifuged at 4000 rpm for 10 min and the solution was filtered with Whatman filter paper no. 41. The

filtrate was collected in polypropylene tubes and stored at 4°C until further analysis. Furthermore, the filtrate was given appropriate dilution in mobile phase prior to injection on to the HPLC system.

### 2.4. Chromatographic conditions

The HPLC system used for quantification of eugenol consisted of a Jasco PU-980 pump, AS-2057 auto sampler and Jasco UV-970detector. The chromatogram peaks were quantified by means of PC based Borwin software (Version 1.5). Chromatographic separation for analyte was achieved on cosmosil C18 analytical column (150 mm X 4.6 mm, 5 m) maintained at ambient temperature. The mobile phase was pumped at a flow rate of 1mL/min. The mobile phase was filtered through a 0.45 m nylon membrane filter and degassed in an ultrasonic bath prior to use. The injection volume was 30 mL, the flow rate was 1.0 mL/min and a chromatographic peak was detected at 215nm.

### 2.5. Method validation

The entire experimental analysis was according to the ICH guidelines and was validated for calibration curve, limit of detection, limit of quantification, system suitability, precision, accuracy, solution stability and ruggedness [21]. Marketed commercial formulation samples of Jatiphaladi *Churna* were accurately weighed in weighing balance. Later they were transferred to Tarson tubes, filled with methanol and kept overnight on rotator shaker. These tubes were subsequently centrifuged, filtered and stored in refrigerator for further HPLC analysis.

### 2.6. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection is defined as the smallest concentration that can be detected but not necessarily quantified as an exact value whereas the limit of quantification is termed as the lowest amount of analyte in the sample that can be quantitatively determined with precision and accuracy that provided a peak area with signal to noise ratio higher than 10, with precision (% CV) and accuracy with (±) 10%.

### 2.7. Calibration curve and linearity

Linearity was determined by means of calibration graph. The graph was further analyzed by using an increasing amount of each analyte and further evaluated by visual inspection of a calibration graph. These calibration curves were plotted over different concentration ranges. The absorbance of the analyte was determined at 215 nm. Regression equation was calculated by constructing calibration curves by plotting absorbance v/s concentration. The results of linearity ranges, plots and curves are shown in fig. 3.

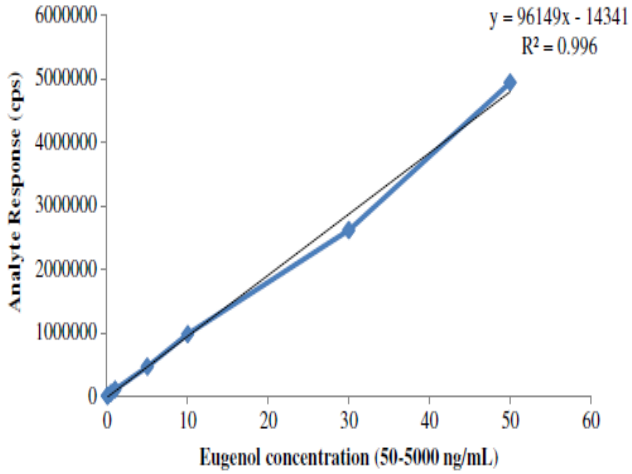


**Fig. 3: Linearity plot of eugenol**

### 2.8. System suitability

The system performance parameters of the developed HPLC method were evaluated by six replicate analysis of the formulation at a concentration of 10ppm. The retention time of their areas were recorded subsequently. Mean area and SD was calculated to determine relative SD and the criteria is >2% respectively.

### 2.9. Precision and accuracy

Accuracy was determined for the assay method at two levels: i.e. repeatability and intermediate precision. The repeatability was evaluated by means of intraday variation and intermediate precision was determined by measuring inter-day variation in the assay method of formulation in six replicate runs. Accuracy and precision of the method assay was performed by injecting three samples spiked at 500ng/mL, 1000 ng/mL and 5000 ng/mL of drug in the *placebo* triplicate sets at three different levels LQC, MQC and HQC respectively for inter day and intraday batch respectively. Mean was determined by, S.D, CV % and % nominal of three different levels was calculated.

### 2.10. Solution stability

The solution stability of working standard solution of eugenol was tested at day 0, 24 h and 20 days

respectively. The important criterion for selecting the solution stability is by comparing percent area and peak purity of the eugenol from chromatograms.

### 2.11. Ruggedness and robustness

The ruggedness of the method is defined as its capacity to remain unaffected by minuscule changes in method conditions. The ruggedness was evaluated by deliberate changes in composition of mobile phase and flow rate.

## 3. RESULTS AND DISCUSSION

### 3.1. Method validation

The principle objective of the proposed research work was to develop method for analytical quantification of eugenol from Jatiphaladi *Churna* and to validate the developed method according to ICH guidelines for its further estimating pharmaceutical formulation. Based on different validation parameters used for detection of eugenol from HPLC analysis, this method offers reliable estimation of eugenol from commercial formulations.

#### *3.1.1. Method optimization*

The project was found to be rapid, simple, accurate and reliable for routine estimation of eugenol in commercial formulations by RP-HPLC. HPLC conditions were optimized to enable separation of eluted compounds. Methanol: water (60:40, v/v) was successfully employed as the mobile phase and it gave symmetry and well resolved peaks for eugenol. The retention time of eugenol were recorded at 5.91 min and detected at UV absorbance at 215nm at flow rate of 1.00 mL/min (Fig. 2A). The use of PDA detector allows optimum utilization of online UV spectra to assess peak purity. The peaks recorded with a retention time in all the chromatograms of eugenol from ayurvedic formulations resulted to be within the peak purity limits. These data excludes the presence of significant interference by other plant constituents.

#### *3.1.2. Calibration curve and linearity*

A good linearity was successfully achieved in the concentration range of 50.00 ng/mL to 50,000.00 ng/ mL. The regression equation and correlation coefficient was found to y ¼ 96149x -14341 and $R^2$ ¼ 0.996.

#### *3.1.3. Method application*

The relative retention time (RRT) and relative peak area (RPA) of each characteristic from samples related to the reference peak was calculated for quantifying eugenol from ayurvedic formulations: Jatiphaladi *Churna* and clove oil. The concentration (mg/ gm) and % CV are shown below in table 1.

**Table 1: Application of method to quantification of eugenol from commercial formulation**

| Sample | Concentration (mg/gm)[a] | % CV |
|---|---|---|
| Jatiphaladi *Churna* | 0.54±0.04 | 6.61 |

*a, Mean ± S.D., n = 3 (batches).*

#### *3.1.4. Limit of determination and limit of quantification (LOD and LOQ)*

The LOD and LOQ were determined from both the values of calibration curve and with signal to noise ratios of 3 and 10 respectively. The LOD and LOQ were found to be 25.00 ng/mL and 50.00 ng/mL. The acceptance criterion for system suitability is >2% for the percent coefficient of the variation of the peak area and retention time of the drug. The values are depicted in table 2 which indicated good performance of the system.

#### *3.1.5. Precision and accuracy*

The precision and accuracy % RSD values for recovery at each level was not more than >0.2% for accuracy and were within the acceptable limits to meet the guidelines for analytical method validation. The accuracy was determined by means of recovery of the added analytes at three different concentrations (low, medium and high level) as well as S.D. of the assays. The results recorded for accuracy studies mean recovery values for all ayurvedic formulations were always higher than 85% as indicated in table 2. The % CV intraday and inter day results were obtained in the values ranging between 0.33-1.21 and 1.08-1.58 individually. The mean assay result for intraday and inter day precision was found to be 103.87 % and 104.30 % respectively. Since there was no impurity of peaks in the chromatograms, the values obtained indicate that solution is stable for at 24 days at ambient temperature. The accuracy of both the methods was good with the deviation between the nominal concentration and calculated concentration well below the limits of 15%. Thus, intraday and inter day precision and accuracy data indicated that the method is validated, highly reproducible reliable and satisfactory.

#### *3.1.6. Stability*

Stability of eugenol from Jatiphaladi *Churna* for 12 h and 24 days was evaluated. The experimental conditions

were deliberately altered for determining the robustness of the assay method and check the reliability of an analysis with respect to deliberate variations in method parameters. The method conditions such as flow rate and mobile phase concentrations were altered and the values are illustrated in Table 2.

**Table 2: Method validation parameters for quantification of eugenol from commercial formulations**

| Method validation parameters | Results |
|---|---|
| LOD (ng/mL) | 25 |
| LOQ (ng/mL) | 50 |
| Linear range (ng/mL) | 50-50000 |
| Mean correlation coefficient (r2) | 1 |
| System suitability (% CV, n ¼ 6) | 0.88 |
| Retention time (min) | 5.91 |
| Accuracy and precision | (% CV, n=3) |
| Intraday | 0.33-1.21 |
| Inter day | 1.08-1.58 |
| Stability | 24 h |
| Jatiphaladi Churna | 92.81% |
| Stability | 20 days |
| Jatiphaladi Churna | 102.31% |
| Ruggedness | Mobile phase |
| (Methanol: d/w, v/v) | Retention time (min) |
| 59:41 (v/v) | 6.3 |
| 60:40 (v/v) | 6.54 |
| 61:39 (v/v) | 5.21 |
| Flow rate change (mL/min) | Retention time (min) |
| 0.9 mL/min | 6.2 |
| 1.0 mL/min | 6.33 |
| 1.1 mL/min | 5 |

### *3.1.7. Ruggedness/robustness*

Ruggedness was studied by using different composition of mobile phase and changing flow rate. The retention time recorded for our parameters was well within the limit 1 min, which indicated that this method is robust as indicated in table 2.

### *3.1.8. System suitability*

System suitability for six replicate analyses (% CV) was found to be 0.88 which is completely within the acceptable analytical range 0.999, which proves the method validated is highly accurate and sensitive and meets with ICH guidelines.

Several variations in factors like temperature, storage, packaging, drying, etc affects both the quality of photothera peutic agents and their therapeutic value in plant constituents. Therefore, not only standardization but also method validation is becoming increasing important for routine quality control analysis of raw materials and for to carry out quality evaluation of marker substances whose active principle is unknown [22]. Despite the number of studies published on standardization of in house and marketed herbal medicinal formulations, our knowledge regarding quantification of phytochemicals from commercial ayurvedic formulation to set quality specification, stability profiles and chemical analysis of analyte of interest is largely unknown mainly due to lack of simple, reliable and sensitive validated analytical methods. In this contribution, we developed completely simple and new experimental chromatographic set up method for separation and quantification of phyto-chemical eugenol from Jatiphaladi *Churna* based on classical RP-HPLC using photodiode array detector (PDA) and methanol: distilled water (60:40,v/v) as mobile phase [23-26].

Additionally, alternative synthetic drugs produced are very expensive, produce adverse side effects and therefore, an alternative approach is needed for formulating ayurvedic drugs having potent antibacterial properties. Recent finding confirms Jatiphaladi *Churna* as having strong anti-bacterial activity in inhibiting and preventing chronic enteric bacterial infections using disc diffusion method [27]. Currently no reported analytical validation data is available which can be further carried for routine quality control analysis in formulation for this *Churna*. The analytical separation technique validated in this paper demonstrates for the very first time quantification and separation of eugenol (anti-bacterial and antioxidant) phytochemical from Jatiphaladi *Churna* with very short retention time. This finding can be further used for critical simultaneous quantification of other marker compounds such as active markers (possess therapeutic activity) from Jatiphaladi *Churna* and other marketed herbal medicines, thus facilitating easy separation and detection of phyto-chemicals for development of herbal medicines against multidrug resistant microbial pathogens. Eugenol present in clove oil has been proved to possess anti-microbial activity against bacteria species such as *S. aureus* ATCC25923, *K. pneumoniae* species, etc [28]. Gas chromatography mass spectrophotometer (GC-MS) technique has been used for detection of eugenol. In principle, the main shortcomings of this technique for

quantification of phytochemical are that the results are not of very high resolution, difficult to record and not automated. GC-MS operates at high temperature and this may affect the stability of thermally labile phytochemical constituents in herbal formulations. On the other hand, validated RP-HPLC method developed in this paper for detection of detection of eugenol was found to be highly sensitive and flexible technique. This was evident from Ruggedness validation parameter data, in which chromatographic conditions such as Mobile phase concentration and Flow rate change were deliberately changed without use of any heating protocols and need of high temperature. The retention time recorded completely satisfies the acceptance criteria >1% (Table 2). Thus, the validated analytical chromatographic method reported is highly rugged, sensitive, requires less retention time and is not affected by minuscule changes in the chromatographic conditions. Fishes get easily get spoilt at room temperature and therefore, increasing the lifespan of fishes is now big issue in food technology industry [29]. Eugenol has scientifically been proven to have anti-microbial activity because of it significant anti-oxidant capacity and has currently acquired large interest among food scientists to incorporate this phytochemical as natural anti-microbial agent in the form of natural preservative in extending shelf life of fishes. This report is further supported by research carried out for studying the effect of 0.5% of eugenol oil on fresh carp, *Cyprinus carpio* L. fillets during storage in fish industry. This breakthrough research suggests very high demand for isolation and quantification of eugenol from herbal formulations.

With increasing human population food requirements and growing interest in need of animal protein sources from fishes, there is high demand for development of analytical method which can easily separate and quantify eugenol from other plant interfering constituents, to be safely used in food preservation industry worldwide. Thus, validated RP-HPLC method demonstrated in this paper quantifies micrograms of eugenol in short span of time and is thus highly sensitive. This method will definitely aid in quantifying, separating potential anti-microbial commercial phytochemical like eugenol and provide highly reproducible data for quality control analysis infood technology related industries. In conclusion, solvent extraction methods by RP-HPLC PDA detection method was developed and validated for quantitative estimation of eugenol from Ayurvedic formulations of Jatiphaladi *Churna* successfully. The developed analytical chromatographic method offered adequate calibration curve/linearity, LOD, LOQ, system suitability, precision, accuracy, solution stability, robustness method application and has been fully validated as per ICH guidelines.

## 4. CONCLUSION

This method can be successfully applied for quality control of herbal medicines containing eugenol to screen toxic botanicals, microbial toxins, pesticides, fumigation, foreign organic matter, fingerprinting/ marker compound for identification and standardization of botanical drugs containing eugenol. This will aid in identification of chemical constituents marker compounds such as chemical and active marker compounds that possess therapeutic activity of the herbal drug which are major constituents of plant materials, identifying herbal materials and standardize botanic preparations during all aspects of manufacturing process.

### *Conflicts of interest*

All authors have none to declare.

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