ejpmr, 2018,5(8), 352-355

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

LUPEOL AND EUGENOL ENRICHED FRACTION FROM *PIPER BETEL* LEAF OIL AND THEIR QUANTITATIVE ESTIMATION BY HPTLC FOR QUALITY CONTROL PURPOSES

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Article Received on 30/05/2018 **Article Revised on 20/06/2018** **Article Accepted on 10/07/2018**

ABSTRACT

Eugenol and lupeol are reported from the leaf oil of *Piper betle* Linn.(Piperaceae). Both the compounds have been shown to give interesting biological activities and hence serve as biomarkers. We report extraction of *Piper betle* leaf oil, fractionation and identification by a simple TLC densitometric method for the quantification of eugenol and lupeol in *Piper betle* leaf oil. The contents of eugenol and lupeol in the samples of *Piper betle* leaf oil, as estimated by the proposed method, were found to be 8.321 µg and 634.94 n.g respectively. The proposed HPTLC method for the estimation lupeol and eugenol was found to be simple, precise, specific, sensitive and accurate and can be used for routine quality control of *Piper betle* leaf oil.

KEYWORDS: Thin layer chromatography, Eugenol and lupeol, Medicinal plants, *Piper betle.*

1 INTRODUCTION

The deep green heart shaped leaves of betel vine are popularly known as Paan in India. The scientific name of betel vine is *Piper betle* it belongs to the family Piperaceae, i.e. the Black Pepper family.[1,2] There are about 100 varieties of betel vine in the world, of which about 40 are found in India and in West Bengal. The parts of *Piper betel* utilized, are leaves, roots, stems, stalks and fruits. *Piper betel* has light yellow aromatic essential oil, with sharp burning taste. Leaf posses activity like antidiabetic, antiulcer, antiplatelet aggregation, antifertility, cardiotonic, antitumour, antimutagenic, respiratory depressant and antihelminthic. *Piper betel* is used to treat alcoholism, bronchitis, asthama, leprosy and dyspepsia. Earlier, anti-ulcerogenic activity of *Piper betel* was attributed to its antioxidative property.[3] A preliminary study has reported *Piper betel* leaves extracts contains large numbers of bioactive molecules like polyphenols, alkaloids, steroids, saponins and tannins. The leaves extract of *Piper betel* have also been reported to exhibit biological capabilities of detoxication, antioxidation, and antimutation that suggested the chemopreventive potential of extracts against various ailments including liver fibrosis and carcinoma.[4,5]

After exhaustive literature survey via Scopus, science Direct and Pubmed, it was found that certain marker compounds such as lupeol, β-sitosterol, allylpyrocatechol 3,4 diacetate, eugenol, safrole, methyl chavicol etc. are present in good percentage in *Piper betle* leaf oil but out of them lupeol was not scientifically reported yet and it is a novel bioactive molecule which has potent antidiabetic and anticancer agent.[6,7,8] Lupeol is a lupane type triterpenoid.[9] Some of the methods reported so far, for the estimation of eugenol are based on Gas Liquid Chromatography and High Performance Liquid Chromatography. From our laboratory, we have reported an economical method to develop eugenol and lupeol enriched fraction by normal phase chromatography and HPTLC method for the quantification of lupeol and eugenol from *Piper betel* leaves oil. HPTLC densitometry has emerged as an efficient tool for the phytochemical evaluation of herbal drugs because of its simplicity and minimum sample clean-up requirement. To the best of our knowledge, so far no method has been reported for developing eugenol and lupeol enriched

fraction by normal phase column chromatography and quantification of lupeol by HPTLC method from *Piper betel* leaf oil.

2 Experimental Designs

2.2 Materials and Reagents

In case of extraction of *Piper betle* leaf oil, An authentic leaf sample of *Piper betle* Linn. (Piperaceae) was collected from local cultivator of Haldia (Distt. East Medinipore, West Bengal, Kolkata, India). The plant is commonly known as Bangla Pata in local areas. After authentication of leaf sample, a good amount (around 1K.gms.) of betel leaf was collected from same cultivator and exposed for further processing. After collection of betel leaves, the fresh leaves were washed with de-ionized water and wiped carefully then exposed to size reduction (approximately 5×5 $m.m^2$) and extracted with distilled water by Clevenger's apparatus.[10,11]

Four parallel assemblies of Clevenger's apparatus with 250 ml capacity of round bottom flask were sat up. Around 250 grams. of chopped betel leaf were poured in to each assembly and distilled water was added into it up to 60% of its capacity, glass beads were added into each round bottom flask to avoid bumping. All the heating mantels were switch on and a continuous cold water supply was provided. As the oil collected in side tube was mixed with water and no separate layer of oil was seen so small amount of xylene was added in the side tube then three clear visible layers (oil + water + xylene) were seen and oil layer was separated in container very carefully and small amount of xylene present with oil was evaporated and pure oil sample was stored in dry and cool place for further analysis. Around 1.8 ml of oil was collected.

2.3 Preliminary phytochemical analysis

The extracted oil was subjected to preliminary phytochemical investigations to confirm the presence of various phytochemical groups such as alkaloids, glycosides, terpenoids, tennins etc. in *Piper betle* leaf oil sample.[12,13]

2.4 Fractionation of major groups in *Piper betle* leaf oil by normal phase column chromatography

The *Piper betle* leaf oil was then subjected to normal phase column chromatography, where silica gel (Silica gel 100-200 mesh for column chromatography, MERCK) was used as adsorbent and various solvents from lower polarity such as hexane (MERCK) to higher polarity such as methanol were used as eluent. The collected hexane fractions were again fractionated two to three times with close polarity solvents such as benzene and toluene and stored in dry and cool place for further studies.[14]

2.5 Thin Layer Chromatography (TLC) of collected column fraction for identification of major groups

The standard marker compounds were purchased from SIGMA ALDRICH and lupeol and eugenol were identified by performing thin layer chromatography (TLC) by using readymade pre-coated silica gel plates (TLC Silica gel 60 F_{254}, MERCK, Germany) and collected column fractions with specific solvent system for respective class. The compounds were identified by matching color and Rf value with the parallel spot of marker compound of respective class.

2.6 Further confirmation of lead bioactive molecules by sophisticated analytical technique (HPTLC)

Out of two identified compounds, lupeol was the unexplored one. Again the fractions having lupeol and eugenol were subjected to High Performance Thin Layer Chromatography (HPTLC) for further confirmation and quantification of lead bioactive molecules.[15]

2.6.1 Preparation of Standard Solutions of lupeol and eugenol

A stock solution of lupeol and eugenol (1 mg mL^{-1}) was prepared separately by dissolving 1 mg of accurately weighed compound and making up the volume of the solution to 1 ml with methanol.

2.6.2 Preparation of Sample Solutions

Out of all column fractions hexane-toluene and chloroform fractions have shown the presence of eugenol and lupeol respectively by TLC. Hence, it was found to be necessary to dilute the sample extract, so that the concentration of eugenol and lupeol in the sample extract applied falls within the calibration range. For the estimation of lupeol, 1 µL aliquot (chloroform fractions) was applied and in case of eugenol, 1 µL and 2 µL of aliquot (hexane-toluene fraction) was applied on plate.

2.6.3 Calibration curve of lupeol and eugenol

2.6.3.1 Calibration curve for lupeol

1.5 µL of the standard solution of lupeol was applied (band width- 8 m.m.) distance between the bands was 8 m.m. in single inject on separate pre-coated silica gel 60 F_{254} plate (E.MERCK KGaA, thickness 0.2 m.m.) using a CAMAG Linomate 5 automatic sample spotter. The plate was developed in a solvent system of Toluene: Methanol (9:1), 10 mL in CAMAG twin trough chamber (20x 10 c.m.) up to a distance of 84.5 m.m. after development, the plate was dried with hair drier (at 60° C) and scanned at 550 n.m. using a CAMAG Scanner 3and CATS 4 software. The peak area was recorded. Calibration curve of lupeol was obtained by plotting peak area Vs applied concentration of lupeol.

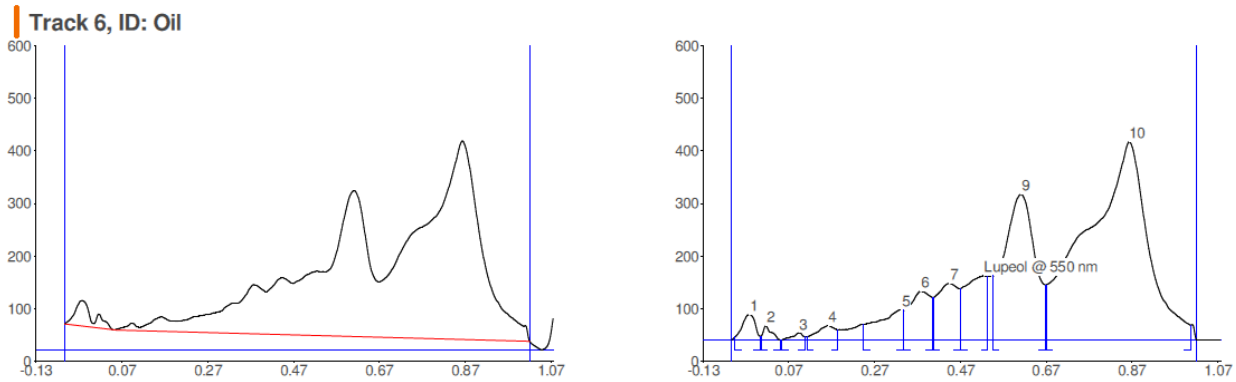

Fig. 01: Overlay absorption spectra of lupeol in sample track with their respective standard Calibration curve for lupeol.

2.6.3.2 Calibration curve for eugenol

2.00 µL and 5.00 µL of the standard solution of eugenol was applied (band width- 8 m.m.) distance between the bands was 8 m .m. each in single inject on separate pre-coated silica gel 60 F_{254} plate (E.MERCK KGaA, thickness 0.2 m.m.) using a CAMAG Linomate 5 automatic sample spotter. The plate was developed in a solvent system of Toluene: Ethyl acetate (9:3), 10 mL in CAMAG twin trough chamber (20x 10 c.m.) up to a distance of 84.00 m.m. after development, the plate was dried with hair drier (at 60°C) and scanned at 281n.m. using a CAMAG Scanner 3and CATS 4 software. The peak area was recorded. Calibration curve of eugenol was obtained by plotting peak area Vs applied concentration of eugenol.

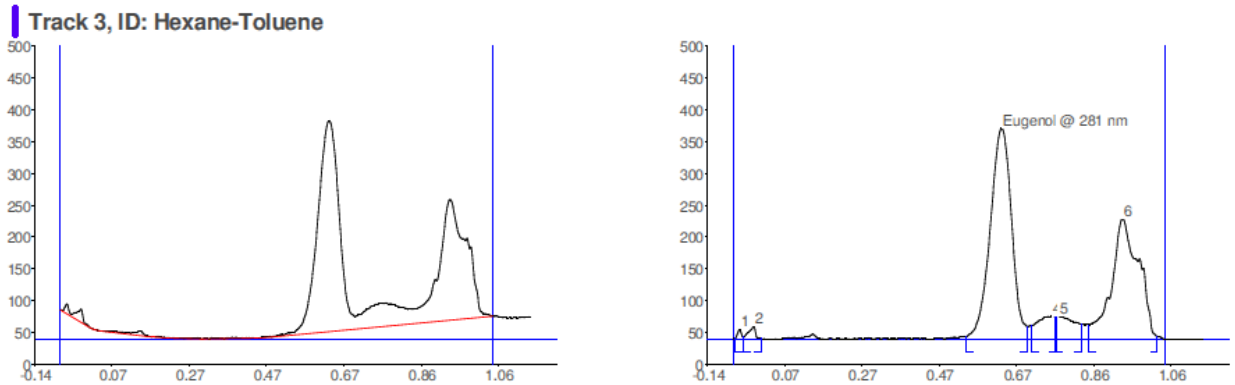

Fig. Overlay absorption spectra of eugenol in sample track with their respective standard Calibration curve for eugenol.

2.6.4 Estimation of lupeol and eugenol in *Piper betle* leaf oil sample

1µL of chloroform fraction (for lupeol) and 1µL and 2µL (for eugenol) was applied on a pre-coated silica gel 60 F_{254} TLC plate (E. Merck, 0.2 m.m. thickness) with CAMAG Linomate 5, automatic sample spotter. The plate was developed and scanned as mentioned above. The peak area and absorption spectra was recorded. The amount of lupeol and eugenol in different samples of *Piper betle* leaf oil sample was calculated using the respective calibration curve of both compounds.

3. RESULT

3.1 Extraction of oil by Clavenger's apparatus

A pale yellowish color oil was collected. The percentage yield was 0.23%.

3.2 Preliminary phytochemical analysis

When oil was subjected to chemical tests for confirmation of major phytochemical groups, tritepene, sterols, glycosides and carbohydrates were positive.

3.3 Fractionation by Column chromatography

Different fractions such as toluene, benzene, chloroform were collected and stored in refrigerator(–18°C) for further analysis.

3.4 HPTLC analysis of lupeol and eugenol

After trying various mobile phase to perform TLC, toluene: methanol (9: 1 v/v) was given the best resolution for lupeol (Rf. 0.52) and toluene: Ethyl acetate (9: 3 v/v) was given the best resolution for eugenol (Rf. 0.63) in the presence of other compounds in the *Piper betle* leaf oil fractions. Preliminary TLC experiments showed that lupeol and eugenol content was significant in *Piper betle* leaf oil sample. The identity of the bands of lupeol and eugenol in *Piper betle* leaf oil fractions was confirmed

by overlying their U.V. absorption spectra with those of the respective reference standard using CAMAG TLC Scanner 3. The purity of each of these band in *Piper betle* leaf oil sample was confirmed by comparing the absorption spectra recorded at start, middle and end positions of band. After the analysis of calibration result the lupeol content was 634.94 n.g and eugenol content was 8.321 µg.

4. CONCLUSION

In conclusion, one can say that, all the above techniques used for fractionation and identification of compounds were economical, conventional, user friendly and reliable. The identified and quantified compounds have great therapeutic potential for the treatment of various ailments, lupeol have anti-cancer and anti-tumor activity where as eugenol is used as dental analgesics and treatment of various dental diseases. The reported research work could further used for quality control purposes. To find simple and economic procedure for isolation of above identified compounds is still need to evaluate.

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