Indian Journal of Natural Products and Resources
Vol. 6(3), September 2015, pp. 189-193

Isolation, characterization and quantification of bioactive dibenzylbutyrolactone lignan (-)-cubebin from fruits of *Piper cubeba* L.f.

Mulik M B* and Laddha K S

Medicinal Natural Products Research Laboratory, Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga (East), Mumbai-400 019, India

Received 11 November 2014; Accepted 8 June 2015

The fruits of *Piper cubeba* L.f. have significance in traditional medicine (Ayurveda) and reports say that its activity is due to major lignan (-)-cubebin, which is a bioactive dibenzylbutyrolactone lignan and possess analgesic, anti-histaminic, antimicrobial and cytotoxic activities. Column chromatography is mostly used for the isolation of lignans from plant sources which is laborous and time consuming process. Therefore, the purpose of this research work is to develop simple and convenient method for isolation of (-)-cubebin from fruits of *P cubeba* L.f. The isolated compound was characterised by thin-layer chromatography (TLC), UV/visible spectroscopy (UV), infrared spectroscopy (IR), mass spectroscopy (MS) and nuclear magnetic resonance spectral analysis (NMR). Further high performance liquid chromatography (HPLC) was used to quantify (-)-cubebin in chloroform extract of the fruits.

Keywords: Cubebin, HPLC, Isolation, *Piper cubeba* L.f.

IPC code; Int. cl. (2014.01)–A61K 36/00

Introduction

(-)-Cubebin is an important dibenzylbutyrolactone lignin (Fig. 1) chemically known as 2,3-bis(3,4 methylenedioxybenzyl)**-**butyrolactol. It is found in the plants of family Piperaceae and Aristolochiaceae[1,2]. The primary source of (-)-cubebin is *Piper cubeba* L.f. commonly known as *Kababchini* or Cubeb pepper (family Piperaceae). Presence of other lignans such as (-)-hinokinin, (-)-clusin, (-)-dihydroclusin, (-)-cubebinin and (-)-dihydrocubebin[3] is also reported. (-)-Cubebin is also found in aerial parts of *P. nigrum* L., *P. clusii* (Miq.) C. DC. syn *P. guineense* Schum. & Thonn, *Aristolochia constricta* Griseb. (Family-Aristolochiaceae) and *Zanthoxylum naranjillo* Griseb. (Family- Rutaceae). It has been reported to show various pharmacological activities such as radical scavenging, anti-inflammatory, antitumor, antihistaminic, antispasmodic, antibacterial, antifungal, genotoxic, antileishmanial and trypanocidal[4-15].

Earlier method of isolation using silica gel column chromatography with benzene:ethyl acetate as a solvent system yielded 4 lignan molecules along with (-)-cubebin from leaves of *P. clusii* (Miq.) C. DC.[16]. Also there are reports of isolation of (-)-cubebin being done by column chromatographic techniques using non polar organic solvents. This leads to the well resolved elution pattern of (-)-cubebin and like compounds depending on their polarity[17].

The dimeric structure and oxygenated ring makes (-)-cubebin non-polar in nature which has specific solubility in organic solvents and this property can be used as a tool in isolation. Hence, the rationale of

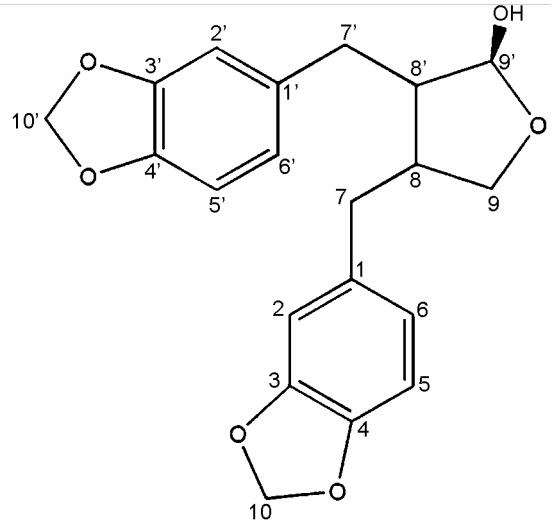

Fig. 1—Structure of (-)-cubebin

*Correspondent author
Phone: +91-9819551756
E-mail: mandarmulik@yahoo.com

this study was to isolate a bioactive lignan using simple solvent extraction technique and its characterization by spectroscopic methods. In addition, the study involved quantification of the same by HPLC method.

Materials and Methods

Plant material

Dried fruits of *P. cubeba* L.f. were procured from the local market of crude herbal drugs, Mumbai and the microscopy of the fruits was studied to authenticate the sample. Voucher specimen (ICT/MNPRL/2014/PC-1) has been deposited at Medicinal Natural Products Research Laboratory, Institute of Chemical Technology, Mumbai.

Chemicals

All solvents used for extraction and isolation were of Laboratory reagent grade and obtained from S.D. Fine Chemicals Limited, India. Isolated compound was identified by Thin-layer Chromatography (TLC) on pre-coated silica gel G60 F_{254} (E. Merck, Mumbai, India) with 50 % sulfuric acid reagent. HPLC grade acetonitrile, methanol and *o*-phosphoric acid were purchased from Merck India. Distilled water and solvents for HPLC were filtered through 0.45 µm filter (Millipore, Bedford, MA, USA) and degassed in an ultrasonic bath (Remi Instruments, Mumbai, India) before use.

Instrumentation

UV/Visible spectrum was recorded on Jasco V-530 spectrophotometer. IR spectra were recorded on Perkin Elmer instrument. Mass spectrum was recorded on Micromass Q-TOF MS Mass Spectrometer (Varian Inc, Peabody, MA, USA). ^{1}H and ^{13}C NMR spectra were recorded on a JOEL 400-MHz instrument (Varian Inc, Peabody, MA, USA) with an internal standard of tetramethylsilane.

HPLC analysis was performed with a Jasco (Hachioji, Tokyo, Japan) system consisting of an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i) equipped with a 20 µL loop and a UV/Vis detector (UV-1575). HPLC column of 250 mm x 4.6 mm internal diameter, 5 mm particle, Hibar LiChrocart Purospher star RP-18 (Merck, Darmstadt, Germany) was used. HPLC was performed at ambient temperature and data were analysed using Borwin software.

Method for isolation

100 g of powdered dried material was subjected to extraction with chloroform (500 mL) for 12 h using Soxhlet *a*pparatus. The chloroform extract was then reduced to 1/4th of its original volume. The oily residue obtained was partitioned with 250 mL water:methanol mixture (60:40) containing 5 % sodium hydroxide. Hydro-alcoholic layer was separated and kept aside overnight to saponify fatty material. The above mixture was concentrated to dryness; viscous liquid obtained was further extracted with petroleum ether (100 mL) to remove unsaponifiable fatty material. Precipitate obtained was dissolved in acetone (60 mL) and solution was refrigerated for 1 h. Cooled acetone solution was filtered to remove precipitate, which was discarded. On drying of remaining acetone solution, about 250 mg crude (-)-cubebin was obtained as yellowish precipitate. Precipitate was further recrystalized using petroleum ether:ethyl acetate (80:20) as solvent. White needles (118 mg) of pure (-)-cubebin were obtained. TLC and HPLC studies were carried out to determine the purity of the sample while the structure was elucidated and confirmed by UV, IR, MS and ^{1}H and ^{13}C NMR spectral analysis.

Preparation of standard solutions

Stock solution (1 mg/mL) of (-)-cubebin was freshly prepared in HPLC methanol. Standard solutions at five concentrations [(-)-cubebin concentrations 5, 10, 15, 20 and 25 µg/mL] were prepared by appropriate dilution.

Preparation of sample

About 5 g dried fruit powder of *P. cubeba* L.f. was weighed and extracted with 60 mL of chloroform in a Soxhlet apparatus for 3 h. The extract was filtered and transferred to 100 mL volumetric flask and the volume was made up with chloroform. 500 µl of this stock solution was transferred to 10 mL volumetric flask and the volume was made up with methanol.

TLC analysis

TLC was performed using toluene:ethyl acetate (70:30) as mobile phase and derivatized using 50 % sulfuric acid reagent[18].

HPLC analysis

The percentage content of (-)-cubebin in fruits of *P. cubeba* L.f. was determined by HPLC method using 70:30 mixtures of acetonitrile and water

containing 0.1 % v/v *o*-phosphoric acid as isocratic mobile phase at a flow rate of 1 mL/min. The injection volume was 20 µL and the detection wavelength was 285 nm. Good resolution was obtained using this method and hence it can be used for quantification of (-)-cubebin in fruits of *P. cubeba* L.f.

Results

Objective of the study was to isolate (-)-cubebin from fruits of *P. cubeba* L.f. on large scale with minimum amount of time and solvent. Different parameters such as polarity, solubility and purity were considered for method development. For isolation of (-)-cubebin from these, various parameters were optimised using different methods. Firstly powdered fruits were extracted using methanol and then partitioned repeatedly with petroleum ether. For 500 mL of methanolic extracts about 500 mL x 6 times of petroleum ether fractions were needed for complete extraction of (-)-cubebin. Petroleum ether layer was separated and concentrated to give crude (-)-cubebin. This method was lengthy and yielded impure (-)-cubebin. Secondly extraction of drug material using petroleum ether alone provided sticky and very less amount of crude (-)-cubebin; hence, it was rejected. Finally drug material was extracted in chloroform to achieve complete extraction of (-)-cubebin. Oleoresin is the major component of *P. cubeba* L.f. fruits, hence fatty acids content in it gets saponified with sodium hydroxide and (-)-cubebin gets entrapped in it. Limited solubility of (-)-cubebin in petroleum ether helps in removing other interfering constituents from saponified material. Extraction with acetone is significant step as it precipitates saponified material on refrigeration and crude (-)-cubebin remains in the solution. The advantage of this method is that it is less time-consuming and the yield was comparatively better as compared to other methods. 99 % pure (-)-cubebin was obtained after recrystalization with petroleum ether:ethyl acetate (80:20).

The UV/Vis maximum in methanol was found to be at 285 nm. Melting point was observed in the range of 130-133 ^{o}C and TLC analysis resulted single band of (-)-cubebin at R_f 0.56 which is in accordance with reported values in literature[18,19]. Derivatization was carried out using 50 % sulfuric acid reagent which showed characteristic pink-violet color on heating. The IR spectrum of (-)-cubebin exhibited absorption bands at 3437/cm due to OH groups and a strong band at 2923/cm (aliphatic C-H stretching) indicating the presence of $–CH_2$- and –C-H. Band of 1599, and 1485-1500/cm indicated the presence of an aromatic ring system, while the bands at 926 and 1038/cm were from C-O absorption bands. In addition the peak at 1242/cm corresponded to a signal of a C-O-C bond. The ^{1}H-NMR spectrum identified the presence of methylenedioxy at δ 5.94 (2 H, s, H-10 H-10´), and signal at δ 5.18 (1 H, d, H-9´a), a signals between δ 2.01 – 2.75 (4 H, m, H-7, H-8, H-7´ and H-8´). The signals of the aromatic region at δ 6.50 - δ 6.80 (6 H, m, H-2´, H-5´, H-2, H-5, H-6, H-6´)[(Ref. 20)]. The ^{13}C-NMR spectrum exhibited signal at δ 72.13 is assigned to C-9 of cubebin. The signal at δ 100.75 and 100.80 corresponded to the pair of $–O-CH_2-O$ for C-10 and C-10´. While resonance of C-9, and C-9´ appeared at δ 98.75 and δ 103.29, the C-6, C-6´, C-5, C-5´, C- 2, C-2´, aromatic rings carbons resonated at δ 121.68 until δ 108.03. Other quaternary carbons appear at δ 42.82 and 51.94 (C-8 and C-8´). Signals due to methylen carbons appeared at δ 33.55 for C-7´at 38.82 for C-7, δ 38.35[(Ref. 20)]. The structure of (-)-cubebin (Fig.1) was confirmed by its mass spectrum which is showed the molecular ion peak $[M]^-$ at m/z 354.9.

Isocratic elution program of 15 min on the HPLC system with the mobile phase acetonitrile:water containing 0.1 % v/v of *o*-phosphoric acid (70:30), showed a peak for isolated (-)-cubebin at R_t of 4.9 min. The presence of (-)-cubebin in the extracts was ascertained by comparing the R_t of the peak with that of the isolated (-)-cubebin. The detection wavelength for (-)-cubebin was set up at 285 nm after recording the UV spectra for the same and choosing the wavelength showing maximum absorption. The linear relationship between detector response and different concentrations of (-)-cubebin (five levels) was confirmed in range of 5-25 µg/mL with correlation coefficient of 0.9886 and equation of y = 23144x-27493. HPLC chromatogram for isolated (-)-cubebin and that for the sample are as shown in Fig. 2.

Discussion

The proposed method is a new approach for isolation of lignan, as earlier reported methods were worked only on principle of column chromatography. HPLC analysis of *P. cubeba* L.f. showed presence of 0.0024 % cubebin[17]. Current study quantified about 0.42 % of cubebin by HPLC and 0.118 % of crystalline compound was isolated using solvent-solvent extraction method. Column chromatography

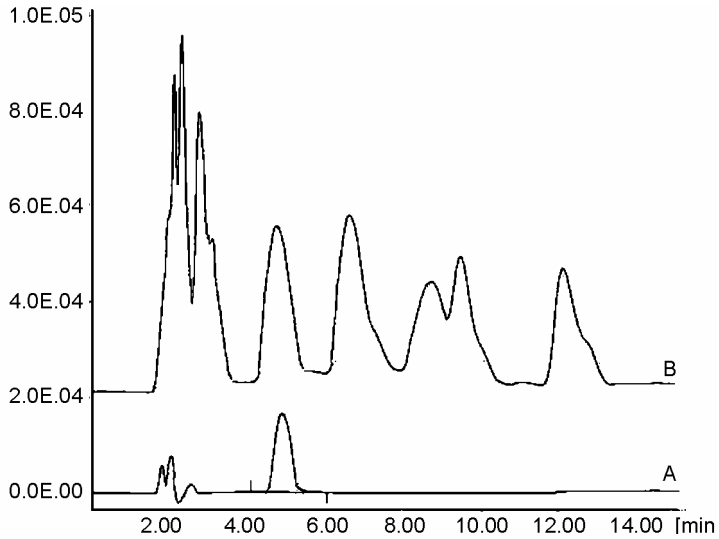

Fig. 2—HPLC chromatograms of isolated (-)-cubebin (A) and extract of *Piper cubeba* L.f. (B)

is the preferred method for separation of lignans but newly established method is advantageous and has a potential to isolate cubebin for better yields. Hence this method can be used for large scale procedure to isolate cubebin from the dried fruits of *P. cubeba* L.f. The structure obtained from spectroscopic data is in consonance with previous reports[20,21]. (-)-Cubebin possesses various bioactivities, viz. analgesic, anti-histaminic, anti-microbial to cytotoxic, anti-tummor and genotoxic. It has been reported that these fruits show similar activities as of (-)-cubebin. Ayurveda describes these fruits as an important ingredient in many preparations. Quantification of (-)-cubebin can be applied for standardization of such preparation and plant materials, hence development of sensitive and selective method for quantification of (-)-cubebin is a need for the future.

Conclusion

The isolation method developed involved simple techniques such as saponification, solvent fractionation and further purification by recrystalization. The method was found to be efficient on laboratory scale for the isolation of (-)-cubebin. Since this method is simple and rapid it can be further optimized to make it suitable for commercial isolation of (-)-cubebin. A HPLC method was established for its quantification. The concentration of (-)-cubebin in dried powder was found to be 0.42 % w/w.

Acknowledgement

The authors would like to thank the University Grant Commission-Rajiv Gandhi National Fellowship for Disability, New Delhi for the financial support for this research work.

References

1 Monograph of *Piper cubeba* Linn. f., *In*: Quality standards of Indian medicinal plants, Vol 11, Tandon N and Sharma P, Eds, Medicinal Plant Unit, Indian Council of Medical Research, New Delhi, 2012, 282-292.

2 Haworth R D, Natural resins, *Ann Rep Prog Chem*, 1936, **33**, 266.

3 Prabhu B R and Mulchandani N B, Lignans from *Piper cubeba*, *Phytochemistry*, 1985, **24**(2), 329-331.

4 de Rezende A A, e Silva M L A, Tavares D C, Cunha W R, Rezende K C, Bastos J K, Lehmann M, de Andrade H H, Guterres Z R, Silva L P and Spanó M A, The effect of the dibenzylbutyrolactolic lignan (-)-cubebin on doxorubicin mutagenicity and recombinogenicity in wing somatic cells of *Drosophila melanogaster*, *Food Chem Toxicol*, 2011, **49**(6), 1235-1241.

5 Souza G H, da Silva Filho A A, de Souza V A, Pereira A C, Royo Vde A and e Silva M L, Analgesic and anti-inflammatory activities evaluation of (-)-*O*-acetyl, (-)-*O*-methyl, (-)-*O*-dimethylethylamine cubebin and their preparation from (-)-cubebin, *Il Farmaco*, 2004, **59**(1), 55–61.

6 Bastos J K, Carvalho J C T, de Souza G H B, Pedrazzi A H P and Sarti S J, Anti-inflammatory activity of cubebin, a lignan from the leaves of *Zanthoxylum naranjillo* Griseb, *J Ethnopharmacology*, 2001, **75**(2-3), 279-282.

7 Hirata N, Naruto S, Ohguchi K, Akao Y, Nozawa Y, Iinuma M and Matsuda H, Mechanism of the melanogenesis stimulation activity of (-)-cubebin in murine B16 melanoma cells, *Bioorg Med Chem*, 2007, **15**(14), 4897-4902.

8 Hirata N, Naruto S, Inaba K, Itoh K, Tokunaga M, Iinuma M and Matsuda H, Histamine release inhibitory activity of *Piper nigrum* leaf, *Biol Pharm Bull*, 2008, **31**(10), 1973-1976.

9 Zhang G, Shimokawa S, Mochizuki M, Kumamoto T, Nakanishi W, Watanabe T, Ishikawa T, Matsumoto K, Tashima K, Horie S, Higuchi Y and Dominguez O P,

Chemical constituents of *Aristolochia constricta*: antispasmodic effects of its constituents in guinea-pig ileum and isolation of a diterpeno-lignan hybrid, *J Nat Prod*, 2008, **71**(7), 1167–1172.

10 Silva M L A, Martins C H G, Lucarini R, Sato D N, Pavan F R, Freitas N H A, Andrade L N, Pereira A C, Bianco T N C, Vinholis A H C, Cunha W R, Bastos J K, Silva R and Da Silva Filho A A, Antimycobacterial activity of natural and semi-synthetic lignans, *Z Naturforsch* C, 2009, **64**(11-12), 779-784.

11 Silva M L A, Coimbra H S, Pereira A C, Almeida V A, Lima T C, Costa E S, Vinholis A H C, Royo V A, Silva R, Filho A A S, Cunha W R, Furtado N A J C, Martins C H G, Carvalho T C and Bastos J K, Evaluation of *Piper cubeba* extract, (-)-cubebin and its semi-synthetic derivatives against oral pathogens, *Phytother Res*, 2007, **21**(5), 420-422.

12 Siddiqui Z N, Khuwaja G, Ahmad J, Antifungal activity of cubebin from *Piper cubeba*, *J Indian Chem Soc*, 2007, **84**(8), 823-824.

13 Maistro E L, Natel A V M, de Souza G H B and Ferreira F F, Genotoxic effects of (-)-cubebin in somatic cells of mice, *J Appl Toxicol*, 2011, **31**(2), 185-189.

14 Bodiwala H S, Singh G, Singh R, Dey C S, Sharma S S, Bhutani K K and Singh I P, Antileishmanial amides and lignans from *Piper cubeba* and *Piper retrofractum*, *J Nat Med*, 2007, **61**(4), 418-421.

15 Bastos J K, Albuquerque S and Silva M L A, Evaluation of the trypanocidal activity of lignans isolated from the leaves of *Zanthoxylum naranjillo*, *Planta Med*, 1999, **65**(6), 541-544.

16 Koul S K, Taneja S C, Dhar K L and Atal C K, Lignans of *Piper clusii*, *Phytochemistry*, 1983, **22**(4), 999-1000.

17 Elfahmi, Ruslan K, Batterman S, Bos R, Kayser O, Woerdenbag H J and Quax W J, Lignan profile of *Piper cubeba*, an Indonesian medicinal plant, *Biochem Syst Eco*, 2007, **35**(7), 397-402.

18 Wagner H and Bladt S, Plant drug analysis, Springer, Germany, 2nd Edn, 2004, 263-273.

19 Cubebin, *In*: The Merck Index: An encyclopedia of chemicals, drugs and biological, 10th Edn, Windholz M and Budavari S, Eds, Merck & Co Inc, New Jersey, 1983, 374.

20 Wahyuono, Wahyuono S, Mursyidi A, Hakim L, Timmerman H and Verpoorte D R, Identification of cubebin and epicubebin isolated from *Piper cubeba* L. fruits with two D-NMR spectroscopy, *Majalah Farmasi Indonesia*, 2005, **16**(4), 232-238.

21 Chatterjee A, Basa S C and Ray A B, Spectral properties of the lignin cubebin, *J Indian Chem Soc*, 1968, **45**(8), 723-725.