Majalah Farmasi Indonesia, 14(3), 119 - 123 , 2003

Isolasi Senyawa Tracheopasmolytic Dari Buah *Piper cubeba*

Isolation of Tracheopasmolytic Compounds From *Piper cubeba F*ruits

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Abstrak

Buah dari tanaman *Piper cubeba* (kemukus) sudah lama digunakan secara tradisional untuk pengobatan asma. Pembuatan ekstrak untuk penelitian dengan jalan maserasi bertingkat dari buah yang sudah dikeringkan dengan n-heksana kemudian dengan ethanol. Ekstrak ini dilakukan uji trakeospasmolitik pada trakea marmut terisoler yang sudah dikontraksi lebih dahulu dengan metakolin dengan dosis masing-masing 0.25 mg/ml Kedua ekstrak menunjukkan penghambatan namun ekstrak etanolik lebih rendah, dan kromatogram KLT keduanya sama, sehingga dilakukan partisi dengan heksana terhadap ekstrak etanolik. Kombinasi ekstrak tersebut kemudian dilakukan fraksinasi dengan kromatografi cair hampa.

Fraksi-fraksi yang diperoleh dilakukan uji trakeospasmolitik. Dua fraksi menunjukkan penghambatan pada uji trakeospasmolitik. Dengan pemisahan menggunakan kromatografi kolom yang dilanjutkan dengan kromatografi preparatif didapatkan satu senyawa yang berkhasiat sebagai trakeospasmolitik yang diidentifikasi sebagai senyawa lignan dihidrokubebin
Kata Kunci : *Piper cubeba*, lignan, dihidrokubebin, trakeospasmolitik

Abstract

The fruits of Piper cubeba (kemukus;Javanese)have been used in traditional medicine to treat illnesses such as asthma. Extracts were prepared by gradual maceration of the dried fruits with n-hexane, and by extraction of the residue with ethanol, and were tested for their tracheospasmolytic effects on isolated guinea-pig trachea contracted with metacholine at a dose of 0.25 mg/ml. Both extracts demonstrated spasm inhibition but the effect of the ethanol extract was lower than that of the n-hexane extract. However, TLC analysis did not distinguish between the extracts. The ethanol extract was then partitioned with n-hexane, and the first and second n-hexane extracts were combined and dried before further fractionation using vacuum liquid chromatography. The fractions were tested for tracheospasmolytic effects. Two active fractions were obtained and separated by column chromatography and preparative TLC. Four pure compounds were obtained. One of these is a lignan compound and identified as dihydrocubebin, had tracheospasmolytic activities.
***Key words**: Piper cubeba; lignan, dihydrocubebin; tracheospasmolytic effect.*

Introduction

Piper cubeba (*kemukus;Javanese*) is a tropical plant which is common in Indonesia. Local people use the fruits of the plant as an anti-asthma traditional medicine called *jamu*. Some people prefer to use anti-asthma *jamu* instead of a modern medicine, as it is much cheaper. The research on the effect of *P.longum* in bronchial asthma (Upadhay *et al*., 1982) and its efficacy of asthma treatments for children has been done by Danahukar *et al*., (1984). Chang *et al.,* (1985) isolated three neolignans from *P.futokadsura* and reported thad kadsurenon was the most effective in inhibiting the binding of platelet activity to receptor site on isolated rabbit platelet plasma membranes. Three neolignan compounds e.g. puberelin A, B and C were isolated from *P.puberulum* which proved active to inhibit the binding of PAF (Zhang *et al*., 1995). Prabhu and Mulchandani., (1985) have isolated some lignans from *P.cubeba*, and neolignans have been isolated by Badheka et al., (1986; 1987). Preliminary experiments have shown that n-hexane and ethanol extracts of *P. cubeba* have the capacity to reduce methacholine-induced contraction of the trachea (Wahyuono, *et al*., 1999), suggesting that they contain spasmolytic compounds which reduce the agonist effect of metacholine. The aim of this research was the isolation and structure elucidation of the compounds from fruits of *P. cubeba* which have a tracheospasmolytic effect. The compounds could be new leads from natural sources which have the potential to provide a more effective drug for the treatment of bronchial asthma.

Methodology

Plant and extracts

Fresh fruits of *P. cubeba* were collected from the garden plantation at the Center of Plant Drug Research, Department of Health, Tawangmangu, Indonesia in August, 1999. Voucher specimens were stored at Department of Pharmaucetical Biology, Faculty of Pharmacy, Gadjah Mada University, Jogjakarta, Indonesia.

The plant extracts were prepared as follows:

Hexane extract: 2 kg fruit of *P. cubeba* was macerated with 10 L hexane for 24 hours, 3 times. After filtration the resulting extracts were combined and evaporated to dryness.

Ethanol extract: The residue from hexane extract was macerated with 10 L ethanol for 24 h, 3 times. The ethanol extracts were combined and evaporated to dryness.

Chemicals

Anisaldehyde was purchased from Acros Organic (Geel, Belgium). All organic solvents (analytical grades) were purchased from J.T.Baker (Devente, the Netherlands). Metacholine, indomethacine were from Sigma (Deisenhofen, Germany).

VLC fractionation

The dried ethanolic extract was partitioned with n-hexane and was compared on TLC with the ethanol extract before and after partitioning. Five gram of the n-hexane extract were fractionated by VLC using gradient elution with dichloromethane - ethylacetate (9 : 1 v / v) ; dichloromethane - ethylacetate (8 : 2 v / v) and dichloromethane/ethylacetate (6 : 4 v / v). The subfractions, were similar on TLC analysis, were combined to give seven fractions. Each fraction was tested for tracheospasmoytic effect.

Tracheospasmolytic assay

Guinea-pigs (weighing 400 – 500 g), were killed by decapitation, the thoracic part of the trachea was rapidly removed and immersed in Kreb's solution [composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.15, NaH_2PO_3 1.17, $NaHCO_3$ 25, glucose 14.4] at 37^0 C, and aerated in carbogen (95% O_2 , 5% CO_2) to maintain a pH-value of 7.4. The trachea was dissected free of adhering adipose and connective tissue under a stereomicroscope and cut into eight rings. A cotton thread was tied to the cartilage of each tubular segment on both sides adjacent to the *Paries membranaceus* before the rings were opened ventrally by cutting the cartilage opposite to the *Musculus transversus tracheae*. The preparation allows the measurement of contraction of the tracheal smooth muscle uninfluenced by the elasticity of the cartilage. The contraction was measured using a Grass force-displacement transducer, model 388 connected to a Kipp & Zone polygraph BD 41.

Each preparation was mounted isometrically in a 20 ml organ bath filled with Kreb's solution (containing 3 x 10^{-6} *u*l indomethacine). The organ bath was gassed continuously at 37^0 C with carbogen. The organ preparation was given an initial resting tension of 1 gram and was allowed to equilibrate for 60 min

(the bathing medium was changed after 15 min). When a stable baseline was obtained, the organ was contracted by addition of the spasmogen under investigation at a concentration provoking 90% maximal contractions (as induced by metacholine 10^{-3} M). After washing the muscle was allowed to relax for about 60 min a stable baseline was reached (Williamson *et al.*, 1996). The spasmogen was then added a second time and contraction again determined. A dose response curve was obtained by using spasmogen concentrations ranging from that which produced a response equivalent to 2.10^{-9} M metacholine up to that inducing a maximal contraction (as induced by 10^{-4} M metacholine). After washing, when the stable basal tension of the tracheal strip had again been reached, the test compound or extract was added to the bath for 30 min. In order to distinguish between the effects of the solvent (DMSO) and the active compound(s) of *P. cubeba*, the control solvent was also tested.

Isolation of active compound

The active VLC fractions were separated with preparative column chromatography (Li Chroprep ® Si 60, Merck Darmstadt, Germany). The column was subsequently eluted with 8:2 (v/v) dichloromethane/ethylacetate . The fractions that showed similarity on TLC were combined and evaporated under reduced pressure. Analysis of the purity of the compounds was carried out by TLC. The final purification of the active compound was carried out by HPLC. The HPLC system consisted of 2150 HPLC pump from LKB, a Rheodyne 7010 injector with a 100 *u*l loop and a UV detector operating at 280 nm. All analyses were performed at room temperature on a 4.0 mm (i.d.) x 250 mm LiChrospher® 60 RP selected column with a particle size of 5 *u*m at flow rate 1ml/m. A guard column was always used in combination with the analytical column. The eluent consisted of MeOH and 50 mM aqueous H_3PO_4 (30:70, v/v). The pH was adjusted to 2.5 with 8 M NaOH. The eluent was filtered through a 0.45 um pore size nylon filter and degassed under vacuum. A fluorescence detector (RF-10A xL, Shimadzu, Japan) was used with an emission and an excitation wavelength of 407 nm and 305 respectively. The pure compounds were tested for bronchospasmolytic effect and the structure of compounds were determined with UV, IR, MS and NMR spectrometry. The MS was measured by a Finnigan MAT TSQ-700 triple quadrupole equipped with custom made Electro Spray Interface (ESI). The UV spectrum (in methanol) was measured on a Cary 1Bio UV-Visible spectrophotometer. The IR was measured by IR-Spect, Sp_3-200, PYE-UNICAM and NMR spectra were measured on a Bruker DPX-600 spectrometer.

Result And Discussion

The tracheospasmolytik activity

At a level of 0.25 mg/ml the crude ethanol and hexane extracts relaxed the contraction of the guinea-pig trachea. However, they had different levels of contraction. The hexane extract caused a 100% relaxation of the contraction of trachea relative to metacholine, but the ethanol extract caused a lower level of contraction (Fig.1). TLC analysis of both of extracts showed no differences. Therefore, the ethanol extract was partitioned with n-hexane to separate the non-polar compounds in the ethanol extract. The same compounds also disappeared from the ethanol extract. All of the hexane extracts were collected and used for fractionation using VLC. Two fractions had spasmolytic effect (Fig.2) and the other fraction not active.

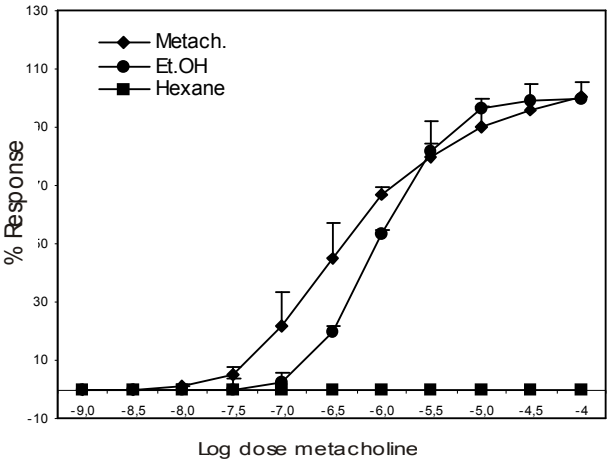

Figure.1 Inhibition of metacholine-stimulated smooth muscle contraction on the isolated guinea-pig trachea preparation in the presence of ethanol or n-hexane extract

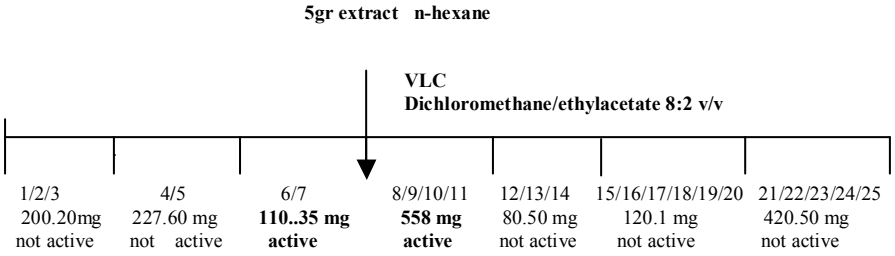

Figure.2 Results of separation by VLC

The VLC fractions, which had a tracheospasmolytic effect, were further separated by column chromatography followed by preparative TLC. The TLC spots were scraped and diluted in a mixture of chloroform/methanol 1:1 (v/v). After filtration, the pure compounds were collected the purity of the compounds were tested by HPLC. From the 5 gram hexane extract subjected to VLC, 5 mg compound was collected .

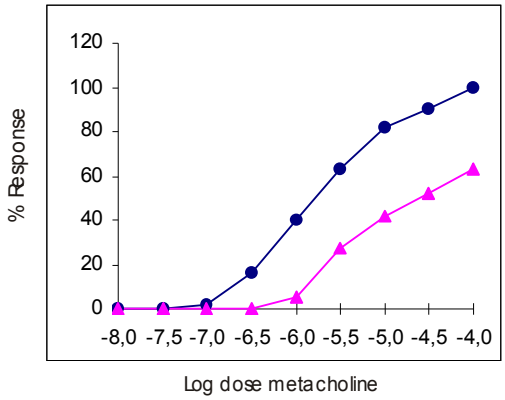

Figure 3.Inhibition of metacholine-stimulated smooth muscle contraction on the isolated guinea-pig trachea (•) and in the presence of compound (♦, 3.14x10^{-5}M)

Structural determination

Crystals were colourless needles, m.p 101-102^{o} soluble in ethanol, chloroform, dichloromethane and not soluble in water; Rf 0.34 (DCM:EtOAc, 8:2). This compound furnished a pink coloured spot when the TLC plate was heated 100^{o}C after spraying with anisaldehyde-H_2SO_4. UVλmax nm, 233, 286. M^+ 358, IR Vmax (KBR cm^{-1}) 3250, 2880, 1605, 1480, 1435, 1355, 1240, 1100,1030. ^{1}H NMR signals δ 1,85 (2H,m,-CH-), 2.66 (4H,d, J=7Hz, Ar-CH_2-), 3.6(6H,m,-CH_2OH), 5.9(4H,s,-O-CH_2-O), 6.62(6H,s,Ar-H). 13C NMR (600 MHz), δ 35.7 (C-7,C-7'), 44.1 (C-8,C-8'), 59.8 (C-9,C-9'), 100.6(C-10,C-10'), 107.9(C-2,C-2') 121.7(C-6,C-6'), 134.3(C-1,C-1'), 145.5(C-4,C-4'), 147.3(C-3,C-3').

The mass spectrum of this compound indicated a MW of 358, corresponding to $C_{20}H_{22}O_6$, The UV spectrum maximal at 233 and 286 nm showed a typical lignanoid absorption . The IR spectrum showed the presence of hydroxyl (3250 cm^{-1}) and methylenedioxy (1030) groups in the IR spectrum. In the NMR spectrum this gave the four methylene proton of two –CH_2OH groups appearance as a multiplet at δ3.60. The two methylenedioxy protons were observed as a singlet at δ5.9, and an aromatic proton as a singlet at δ 6.62. It is clearly that the compound is dihydrocubebin.

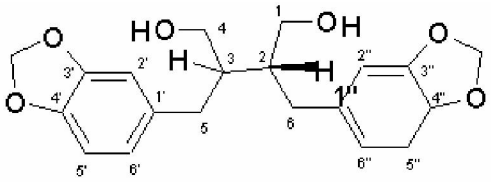

Figure 4 : *(3.4).(3'.4')-Bis-methylendioxy-9'-9 dihydroxy-lignan*
(dihydrocubebin)

P*iper cubeba* contains (3.4),(3'.4')–Bis - methylendioxy-9'-9 dihydroxy - lignan (dihydrocubebin). which have tracheospasmolytic effect (Fig.3) at a dose of

3.14x 10^{-5} M, reduced the effect of contraction from metacholine. The possible action of the compounds is the $ß_2$-adrenergic receptor located on bronchial smooth muscle. Smooth muscle relaxation induced by a $ß_2$-adrenergic agonist is reduced if the bronchial epithelium is removed from the preparation. This could indicate that the release of smooth muscle relaxing factor from the bronchial epithelium is other a response to the $ß_2$-adrenergic stimulation or due to enhancement of the effect of $ß_2$ adrenergic agonist on the smooth bronchial muscle.

A tracheospasmolytic effect is reported for these compounds for the first time. Dihydrocubebin is usually as an antimicrobial against *Mycobacterium smegmatis* (Harborne and Baxter, 1993). Therefore, this work could be a lead for also finding potential tracheospasmolytic compounds.

Conclusion

From four compounds isolated from piper cubeba fruits, one has tracheospasmolytic effect and is identified as dihydrocubebin

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