DETERMINATION OF MARKER COMPOUND OF ARTOCARPUS CHAMPEDEN SPRENG. STEMBARK EXTRACT AND VALIDATION OF THE ANALYSIS METHOD USING HPLC

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DETERMINATION OF MARKER COMPOUND OF *ARTOCARPUS CHAMPEDEN* SPRENG. STEMBARK EXTRACT AND VALIDATION OF THE ANALYSIS METHOD USING HPLC

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Abstract: *Artocarpus champeden* SPRENG. (Moraceae), commonly known as "cempedak", has been traditionally used for malarial remedies. Several studies also reported in vitro and in vivo antimalarial activity of this plant. In order to developed *A. champeden* stembark as antimalarial phytopharmaceutical product, marker approach was chosen for quality control purpose of the extract as raw material. The aims of this study are to determine marker compound of *A. champeden* stembark extract and develop an analysis method for quantification of marker compound in ethanol extract of *A. champeden* SPRENG. stembark using HPLC. Isolation of marker isolate was carried out by column chromatography and preparative TLC techniques. Based on UV, IR, 1H NMR, 13C NMR, 2D NMR (COSY, HMBC and HMQC) and MS spectra and comparison with other references, confirmed that marker isolate was identical with known prenylated chalcone, Morachalcone A. The HPLC profile showed that marker compound has time retention shown as Mean ± SD (RSD) of 12.547 minutes ± 0.39 (3.08%). Validation of analysis method for quantification of marker in ethanol extract was conducted. The analyte was chromatographed on C8 250x4.6 mm column, methanol-water as mobile phase (65:35 v/v) by isocratic elution at flow rate of 1 ml/minute, column temperature of 30ºC, stop time of analysis at 20 minutes and detection wavelength at 385 nm. The method was validated in terms of selectivity, specificity, linearity, accuracy and precision. The calibration curve was linear within a high range concentration of 25 - 50 μg/ml and low range concentration of 3.5-25 μg/ml. The accuracy and intraday precision were found to be well within acceptable limits. The marker

content in ethanol extract of *A. champeden* SPRENG. stembark was shown as Mean ± SD (RSD) of 0.3361%±0.03 (8.45%).

Keywords: *A. champeden* SPRENG., antimalarial activity, ethanol extract, marker compound, morachalcone A, HPLC analysis

INTRODUCTION

Artocarpus champeden SPRENG. belongs to Moraceae, commonly known as "cempedak", is widely distributed in Indonesia and has been traditionally used for malarial remedies [Hakim et aI.,2006]. Previous studies reported that several prenylated flavonoids isolated from *A. champeden* SPRENG. has potent antimalarial activity in vitro against *P. falciparum* strains 3D7 and in vivo against *P. berghei* strains ANKA (unpublished). This indicates that *A. champeden* is prospective to be developed as phytomedicine product for antimalarial. The pharmaceutical requirements for a herbal product destined for a multi-target therapy are very complex [Kroll and Cordes, 2006]. The constituents of medicinal herbs can vary greatly as a result of genetic factors, climate, soil quality and other external factors ISchulz et al.,2004]. Therefore, despite the use of authenticated botanical voucher specimens to help assure proper identity, modern concepts and methods relating to the quality (i.e., chemical consistency) of herbal materials and products pertain to phytochemical markers and fingerprint analyses. These markers are the threads that tie together production and quality control [Reif et al., 2004]. Ideally, chemical marker should be unique components that contribute to the therapeutic effects of herbal medicine [Li et al., 2008]. The standardized extract should have consistent constituent in order to ensure the consistency of quality, safety and efficacy of the product. The marker approach to ensure consistency is based upon the assumption that the content of other constituents will vary in proportion to the marker compound, that if each batch contains the same standardize amount of marker, the content of other constituents will also be relatively consistent [Lazarowych and Pekos, 19981 McCutcheon, 2002]. In order to develop *A. champeden* as antimalarial phytomedicine product with multi-component approach, it is needed to standardize *A. champeden* extract as raw material. Therefore, the study to obtain standardized extract of *A. champeden* SPRENG. stembark as raw material of antimalarial phytomedicine product using active marker compound was conducted. The preliminary study showed that 80% ethanol extract of *A. champeden* stembark was active extract that inhibit parasite growth both in vitro against

Plasmodium falciparum strains 3D7 and in vivo against *Plasmodium berghei* strains ANKA. Then, from this ethanol extract has been identified an isolate belongs to chalcone group, based on UV spectra, as characteristic orange spot, detected in extract, fraction and subfraction using TlC-Densitometer and HPLC. This isolate also showed, in vitro antimalarial activity against *P. falciparum* strains 3D7 with an IC_{50} value of 0.69 µg/ml, therefore this isolate can be used as marker candidate from 80% ethanol extract of *A. champeden* stembark. The present study aims to determine and isolate marker compound from 80% ethanol extract of *A. champeden* stembark, then develop an analysis method for quantification of marker content in ethanol extract of *A. champeden* stembark. Therefore, in the beginning we isolate and identify the structure of an isolate that we used as marker candidate for ethanol extract of *A. champeden* stembark. This study consist of several steps, are begun with isolation of marker compound using conventional column chromatography and preparative TLC techniques, followed by identification of isolate based on UV-Vis, IR, 1H NMR, 13C NMR, 2D NMR (COSY, HMBC and HMQC) and MS spectra. Further step is development of analysis method for quantification of marker content in ethanol extract of *A. champeden* stembark using HPLC that conducted by validation of an analysis method.

MATERIALS AND METHODS

Plant material

The stembark of *Artocarpus champeden* SPRENG. was collected from Bogor, West Java, Indonesia, in June 2007 and 2008. A voucher specimen was identified and deposited at the Herbarium Bogoriense, Bogor Botanical Garden, Bogor, Indonesia.

Chemicals and Reagents

All reagents were analytical grade, otherwise reagents for HPLC analysis. Methanol for HPLC analysis was purchased from Mallinckodt, inc. (USA). Samples for HPLC were filtered through a 0.45 pm membrane filter (Millipore, Bedford, MA, USA). Reference standard, marker compound Morachalcone A, was isolated from the stembark of *Artocarpus champeden* SPRENG. In our laboratory; and mass spectrometry, NMR spectrometry and infrared spectrometry were applied to determine compound characteristics.

General Experimental Techniques and Instruments

Silica gel was used for column chromatography (Merck, 0.063-0.200 mm) and silica gel for preparative thin layer chromatography (Merck). Spots on plates were detected under UV light (254 and 366 nm) and by spraying with l0% H2SO4 in water followed by gentle heating. IR spectrum obtained on a Shimadzu spectrometer IR Prestige-2l type. The NMR experiments (both lD and 2D) were obtained on a JEOL spectrometer ECA 500 type operating at 500 MHz. HPLC analysis was performed with Hewlett Packard Agilent 1100 series, an Agilent 1100 series Degasser Gl322A, a Rheo-dyne 7725 injection valve with a 20-pL loop, Agilent I 100 series Quarternary Pump Gl3llA, Agilent I100 series Column Compartment Gl3l6A, Agilent 1100 series diode array detector (DAD) Gl3l5A. Compounds were separated on a 250x4.6 mm Varian Microsorb MV 100-5 C8 column.

Extraction and Isolation

One kilogram of *Artocarpus champeden* SPRENG. stembark was extracted with 80% ethanol at 60°C in rotavapor, yielded 74.6368 g of crude extract. This extract was applied to ODS column chromatography, using methanol-water (4:lv/v)-methanol-methanol-acetonitril (l:l v/v) and acetonitril as eluent, resulting in l0 major fractions (fraction l-10). Fraction 4 (686.9 mg) was applied to silica gel column and eluated with $CHCl_3$ followed by increasing polarity of $CHCl_3$-MeOH 1 –10% by gradient elution, yielded 9 major subfraction. Further separation of subfraction 4.8 (47.1 mg) was conducted with several steps of Preparative TLC techniques using silica RP-18 as stationary phase with MeOH-H2O (7:3 vlv) mixtures as mobile phase yielded active subfraction 4.8.8 (18.7 mg). This was then continued using silica as stationary phase with CHCI:-MeOH (9.5:0.5 v/vi mixtures as mobile phase yielded active subfraction 4.8.8.7 (12.2 mg). Purification of this subfraction was conducted by reverse-phase PTLC with MeOH-H_2O (4:1 v/v) mixtures as mobile phase resulting in active marker isolate, Morachalcone A (7.2 mg).

Morachalcone A: orange powder; UV (MeOH) λ nm 250, 316 and 385; IR (KBr) cm^{-1}: 3,456, 1,697. 1H and '3C NMR data are given in Table l.

Antimalarial activity Testing

The antimalarial activity of fractions and the isolated compound was determined by the procedure described by Budimulya et al. (1997). In brief, each fractions and compound

was separately dissolved in DMSO (10^{-2} mol L^{-1}; and kept at -20°C until used. The malarial parasite *P. falciparum* 3D7 clone was propagated in a 24-well culture plate in the presence of a wide range of concentration of each fractions or compound. The growth of the parasite was monitored by making a blood smear fixed with MeOH and stained with Giemsa stain. The antimalarial activity of each fractions or compound was expressed as an IC_{50} value, defined as the concentration of the compound causing 50% inhibition of parasite growth relative to an untreated control.

HPLC Conditions

HPLC analysis was conducted with methanol-water (65:35 v/v) as mobile phase by isocratic elution, C8 250x4.6 mm Varian Microsorb MV 100-5 column at flow rate of 1 ml/minute, column temperature of 30°C, stop time of analysis at 20 minutes and detection wavelength set at 385 nm.

Preparation of standard solution

The standard stock solution (1 mg/ml) was prepared by dissolving 5 mg of standard to 5 mL of methanol. The stock solution was stored at 4°C.

Sample preparation

Ten mg of ethanol extract of *A. champeden* SPRENG stembark was accurately weighed and dissolved in methanol using vortex for 5 minutes. Sample solution then filtered through a 0.45 pm syringe membrane filter.

Calibration

The standard stock solutions of marker substance was diluted by methanol to give serial concentrations are: at 25;30;35;40;45 dan 50 μg/ml (high range concentration) and at 25;20;10;7;6;5 and 3.5 μg/ml (low range concentration). After filtering through a 0.45 pm membrane filter, 20 μl of each concentration was injected into the HPLC column for analysis. Linear regression model was used to describe the relationship between peak area and concentration of standard solution.

Method validation

Method validation for quantification of marker content in ethanol extract of *A.champeden* stembark using HPLC was carried out in terms of selectivity, specificity, linearity, limit of detection, limit of quantitation, accuracy and precision.

Selectivity and Specificity

Selectivity and specificity was checked by using an extract of *Artocarpus champeden* SPRENG. stembark and marker compound Morachalcone A and optimizing separation and detection. The purity of the peaks was checked by acquisition of spectra (λ 200-400 nm) by use of the DAD detector. Spectra were acquired at the upslope, apex, and downslope of each peak, computer normalized, and superimposed. Peaks were considered pure when there was coincidence between the three spectra (match factor >950.000). Selectivity study resulting in methanol-water (65:35 v/v) as mobile phase by isocratic elution, C8 250x4.6 mm Varian Microsorb MV l0O-5 column at flow rate of 1 ml/minute, column temperature of 30°C, stop time of analysis at 20 minutes and detection wavelength set at 385 nm.

Limits of Detection and Limit of Quantification

Limits of detection and quantification were determined as the amounts for which the calculated signal-to-noise ratios were approximately 3: 1 and 10:1, respectively.

Precission

Standard stock solution was diluted in methanol for intra-day test (injecting certain concentration of standard six times within 24 hours). The standard deviation (SD) and relative standard deviation (RSD) were calculated.

Accuracy

Accuracy study was carried out by spiking certain concentration of marker to sample Recovery rate (%) was calculated.

RESULT AND DISCUSSION

The dried stem bark of *A. champeden* was extracted with 80% ethanol. In a preliminary test of in vitro antimalarial activity against *P. falciparum* 3D7 clone of this ethanol extract showed significant inhibition (unpublished). Marker isolation from ethanol extract of *A. champeden* stembark was done by bioactivity guided fractionation.

The ethanol extract was fractionated with open column chromatography using ODS as stationary phase and methanol-water as mobile phase, resulting in l0 major fractions and fraction 4 was proved to be active against *P. falciparum* strains 3D7 in vitro. Fraction 4 was applied to silica gel column and eluated with $CHCl_3$ followed by increasing polarity of $CHCl_3$-MeOH l-l0% by gradient elution, yielded active subfraction 4.8 with an IC_{50}

value of 0.03 μg/ml. Further separation oi subfraction 4.8 with several steps of PTLC techniques using silica RP-Cl8 as stationary phase with MeOH/H_2O mixtures as mobile phase yielded active subfraction 4.8.8 (IC_{50} value of 0.10 μg/ml), then silica as stationary phase with $CHCl_3$-MeOH mixtures as mobile phase yielded active subfraction 4.8.8.7 (IC_{50} value of 0.39 μg/ml). Purification of this subfraction by reverse-phase PTLC with MeOH-H_2O mixtures as mobile phase resulting in active marker isolate.

Identification of isolate based on UV-Vis, IR, ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, HMBC and HMQC) spectra and comparison with other references. Marker compound was isolated as orange powder. The UV-Vis absorptions at 250,316 and 385 nm were suggestive of a chalcone skeleton [Mabry, et al., 1970]. The IR spectrum of morachalcone A contained- absorption bands at 3,456 cm^{-1} and 1,697 cm^{-1} corresponding to hydroxyl and carbonyl groups, respectively [Silverstein et al., l99l]. The ^{1}H NMR spectrum contained characteristic signals ascribable to an isoprenyl group (6H 1.65, 1.17, 5.22, 3.34). Two proton signals at δ 7.73 (lH, dd, J=15.25 Hz) and 8.1 (lH, dd, J=15.9 Hz) form an AB system, the large coupling constant indicating the trans geometry of a double bond. The 1H-NMR spectrum (table l) also indicated signals for ortho coupled aromatic protons in ring A (δ 6.36 (ZH, m) and δ 7.52 (lH, dd, J= 8.55,3.7 Hz) and two aromatic protons in ring B δ 6.43 (lH, q, J=8.55, 17.75 Hz) and δ 7.76 (lH, d, J=".55 Hz). The presence of proton signals δ13.93 and δ 14.48 indicated that the hydroxyl groups are located at C-2 and C-4 in ring A and C-2' and C-4' in ring B. The ^{13}C-NMR spectrum contained signals from 20 carbon atoms including that of a ketone carbonyl carbon at δc 194.25 (table l).

On the basis of HMQC and HMBC spectral analysis, all protons and carbon signals were fully assigned and the positions of the substituents on the aromatic rings were determine. The HMBC correlations for H-1"/C-2', C-3' and C-3" confirmed that the 3,3dimethylallyl group is located at C-3'. Structure determination for marker compound also conducted based on comparison with their spectroscopic data from literature values [Abegaz et al., 1998; Monache et al., 1995]. Thus, the structure of marker compound was deduced as known prenylated chalcone, Morachalcone A. Morachalcone A was previously isolated from callus culture of *Maclura pomifera* (Moraceae) [Monache et al., 1995] and also from methanol extract of *A. champeden* stembark (unpublished).

The marker compound revealed inhibitory activity against *P. falciparum* strain 3D7 with an IC_{50} value of 0.18 μg/ml. The inhibitory activity of this compound showed

that the isolated compound was an active marker. The result of this study showed that active marker compound, Morachalcone A, can be used to standardize ethanol extract of *A. champeden* stembark as antimalarial phytomedicine product. Morachalcone A, obtained from this study was then used as reference standard for analysis of marker content in ethanol extract of *A.champeden* stembark using HPLC. According to Reif [2004], marker compound can be isolated in own laboratory if it is not commercially available and used as reference standard with defined identification, purity and content. The development of analysis method for quantification of marker content in ethanol extract of *A. champeden* stembark using HPLC was conducted by validation of an analysis method, Validation of the method was done in terms of selectivity, specificity, linearity, accuracy, precision, limit of detection and limit of quantitation. The chromatographic condition was optimized through comparison of different solvents and solvent ratio. Compared with other solvents, the mixture of methanol and water (65:35 v/v) as mobile phase by isocratic elution, C8 250x4.6 mm Varian Microsorb MV 100-5 column at flow rate of 1 ml/minute, column temperature of 30°C, stop time of analysis at 20 minutes and detection wavelength set at 385 nm showed the best separation and minimize peak tailing. Under this condition, the chromatogram profile of ethanol extract (Figure l) showed that peak at time retention shown as Mean ± SD (RSD) of 13.001 minutes a 0.37 (2.87%), has resolution of 1.42, plate number of 3524, peak width at half height of 0.5200, symmetry factor of 0.82 and selectivity factor of l. The purity factor of this peak was 997.034, indicated that targeted peak fall within acceptable purity. The identity of this peak against marker spectrum was shown as match factor value of 981.261 (>950.000), indicated that targeted peak analyzed in ethanol extract was marker compound that has been detected in ethanol extract of *A. champeden* stembark. Linearity study showed that analysis within the high range concentration of 50 - 25 μg/ml and low range concentration of 3.5 - 25 μg/ml resulting in response proportional against concentration with correlation coefficient ® of 0.9989 and 0.9997, Vo value of 1.26 % and 2.ll% respectively, indicated linear correlation. Limit of detection was 1.57 μg/ml and limit of quantitation was 4.71 μg/ml, The compound analysis were obtained with the recovery rate shown as Mean ± SD (RSD) of 102.5991% ± 5.62 (5.48%), with intra-day for instrument precision shown as Mean ± SD (RSD) of 31.0668 ± 1.88 (6.06 %) and

method precision shown as RSD of 8.45%. Based on parameter values obtained from validation study, the analysis method can be assumed to be valid for quantification of marker content in ethanol extract of *A. champeden* stembark using HPLC. Amount of Morachalcone A as marker in ethanol extract of *A. champeden* stembark shown as Mean ± SD (RSD) was 0.3361% ± 0.03 (8.45%).

CONCLUSION

Results of this study showed that prenylated chalcone, Morachalcone A, can be used as marker for ethanol extract of *Artocarpus champeden* SPRENG. stembark. HPLC method also has been developed for quantitation of Morachalcone A in ethanol extract of *A. champeden* SPRENG. stembark. Results indicate that the present method was valid in controlling the quality of ethanol extract of *A. champeden* SPRENG. stembark. The quantitative analysis showed that the content of marker Morachalcone A in ethanol extract of *A. champeden* was shown as Mean±SD (RSD) of 0.3361% ± 10.03 (8.45%).

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GENERAL COMMENTS

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