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Research Article

ISOLATION OF BIOACTIVE COMPOUND OF *MICHELIA CHAMPACA* L. BARK AND ITS ACTIVITY TEST USING MECHANISM-BASED YEAST BIOASSAY

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ABSTRACT

Objectives: This study aimed to isolate the active compound of *Michelia champaca* L. bark and test its activity using mechanism-based yeast bioassay.

Methods: The bark was extracted by methanol; fractionation was done by liquid-liquid extraction (LLE) using n-hexane, ethyl acetate, and water. The activity of LLE fractions was tested by mechanism-based yeast bioassay. The most active fraction was then separated by vacuum liquid chromatography, further separated by classical column chromatography and purified by recrystallization. The isolate was characterized by ultraviolet-visible, infrared spectrophotometric method, nuclear magnetic resonance spectroscopy, and mass spectrometric method.

Results: The isolation process resulted in an isolate named MCET51. Characterization data showed that MCET51 was proved as liriodenine ($C_{17}H_9NO_3$) with molecular weight 275 (m/z), an aporphine alkaloid. The activity assay showed that liriodenine was active against *Saccharomyces cerevisiae* strain 1140, 1353, and 1138 with IC_{12} values were 22.15±1.71, 24.76±0.56, and 7.02±1.85 µg/ml, respectively.

Conclusions: It can be concluded that *M. champaca* L. bark contained liriodenine which was active both as topoisomerase I inhibitor and topoisomerase II inhibitor.

Keywords: *Michelia champaca* L., Topoisomerase inhibitor, Mechanism-based yeast bioassay, Liriodenine.

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INTRODUCTION

Michelia champaca L. or *Cempaka kuning* is a tree with 15-25 m high, grown in Java area in Indonesia, at 1200 m altitude marshy forest. This species is a member of *Magnoliaceae* that usually produces proanthocyanidins and alkaloid, often cyanogenic compounds and volatile oil. Traditional usage of bark is for tonicum, herbal maternity, and curing fever [1,2].

M. champaca bark was active against *Aspergillus versicolor*, *Trichophyton tronsurum*, *Aspergillus niger, and Aspergillus vitis* [3] and against human epidermoid carcinoma of the nasopharynx [4]. The leaves were active as an anti-inflammatory [5], antiarthritic [6], and antifertility [7]. *Michelia* flower was active as burn wound healing [8], antioxidant [9], anti-inflammatory [10], antihyperlipidemic [11], and anticancer on Ehrlich ascites carcinoma cell line [12]. In addition, the flower and leaves of *Michelia* were having antiulcerogenic property [13].

Chemical compounds contained in *C. kuning* were sinapyl 4-O-β-D-glucopyranoside alcohol, sinapyl 4-O-β-D-glucopyranoside aldehyde, syringaresinol and *N*-acetylnonaine [14], michampanolide, 8-acetoxyparthenolide, magnograndiolide, parthenolide, cestunolide [15], quercetin [16], champacaine, anonaine, norushinsunine, ushinsunine, N-acetylanonaine, roemerine, asimilobine, anolobine, isocorydine, liriodenine, atherospermidine, O-methyl moschatoline, syringaresinol, N-trans-feruloyltyramine, 4-hydroxybenzaldehyde, vanillin, vanillic acid, syringic acid, 3,4-dimethoxybenzoic acid, coniferyl aldehyde, syringin, scopoletin, 4-acetonyl-3,5-dimethoxy-p-quinol, sitostenone, stigmasta-4,22-dien-3-one [17], stigmasterol and 3β-16α-dihydroxy-5-cholestene-21-al, *Michelia*-A, and guaianolides [4].

DNA topoisomerases is an important enzyme in cell proliferation phase of developing cancer in all types. Anticancer targeting this enzyme will alter the process of DNA replication and transcription so that inhibit cell division and inactivate cancerous cells from growing and dividing [18]. Mechanism of drug targeting this enzyme and biological roles of DNA topoisomerase can be studied in yeast, as powerful model system [19] because of its genetic and biochemical of yeast resemblances is close to mammalian cells [20]. Hence, microbial yeast bioassay is suitable for evaluating or screening anticancer agents.

In previous research, 23 species of *Apocynaceae*, *Simaroubaceae,* and *Magnoliaceae* have been screened for their anticancer activity, and *M. champaca* L. bark was one of the best active extracts against *Saccharomyces cerevisiae* strain 1140, 1353, and 1138 with IC_{12} values were 3424.54±2806.57, 2124.42±450.40, and 542.6±102.45 µg/ml, respectively. The results mean that *M. champaca* extract active as anticancer, having DNA damaging agent or topoisomerase inhibitor [21]. However, the active ingredients that corresponded with the activity were still unknown. So, this present study aimed to isolate the active compound of *M. champaca* L. bark guided by mechanism-based yeast bioassay.

METHODS

Materials

C. kuning bark, methanol, ethanol, aquadest, n-hexane, chloroform, ethyl acetate, dimethyl sulfoxide, precoated plate silica gel GF_{254}, silica gel H, silica gel 60 (0.063-0.200 mm), agar bacteriological, peptone, dextrose, yeast extract, and sodium chloride.

Instruments and apparatus

Grinder, macerator, rotavapor (Buchi), electric dryer (Philips), separatory funnel, freeze dryer (Telstar), vacuum column chromatography, classical column chromatography, common glassware in laboratory, ultraviolet (UV) lamp (Desaga), UV-visible spectrophotometer (HP 8453), thin-layer chromatography (TLC) densitometer (Camag), infrared spectrophotometer (FT/IR Jasco 4200), mass spectrometry (waters), and nuclear magnetic resonantion spectrometry (Agilent 500 MHz).

Sample preparation

M. champaca L. bark was collected on January 2015 from area of Institut Teknologi Bandung (ITB) West Java Indonesia. Fresh bark was sorted, chopped in small pieces, air-dried in drying cupboard (40°C) for 24 hrs. Plant sample (collection no 12.130) was authentication in Herbarium Bandungense, School of Life Sciences and Technology ITB.

Extraction and fractionation

Extraction of dried bark was done by methanol maceration for 24 hrs in three replication, and the filtrate was concentrated using vacuum rotary vaporator. Condensed extract was fractionated by liquid-liquid extraction (LLE) using n-hexane followed by ethyl acetate and water. All fractions of LLE were tested for their activity using mechanism-based yeast bioassay against *S. cerevisiae* strain 1140, 1353, and 1138.

Activity test using mechanism-based yeast bioassay

The method mechanism-based yeast bioassay was done according to Gunatilaka and Kingston [20], Gunatilaka *et al.* [22], Subong and Primavera [23] as modified by Zuhrotun *et al.* [21] as followed. *Saccharomyces cerevisiae* strains 1140, 1353, and 1138 that cultured in yeast peptone dextrose (YPD) broth was suspended in sterile 0.9% saline solution until the transmitant was 80% at l 600 nm. Yeast inoculums 1 ml and medium YPD agar 20 ml were poured into a petri dish with 9 cm diameter that produced 6 mm layer. Seven wells with 6 mm diameter were made on the plate using perforator. Samples were dissolved in a mixture of dimethyl sulfoxide-methanol (1:1) with variation concentrations. Amount of 50 µl of the mixture was placed in each well. The plates were then incubated at 30°C for 36-48 hrs. The samples were considered active or contain DNA damaging agent if inhibition zone produced as the growth of yeast were inhibited. IC_{12} value was referred to required concentration of sample (in µg/ml) that produced an inhibition zone of 12 mm around a well. The value was determined using linear regression from dose-response curves with log of dose as absis (Y) and zone size as ordinate (X). A topoisomerase I inhibitor defined to sample that active against *S. cerevisiae* strain 1140 and A topoisomerase II inhibitor defined to sample that active against *S. cerevisiae* strain 1353.

TLC bioautography

To guide isolation of active compound, the active fractions were tested by TLC bioautography [24,25] with modification. Chromatography profile was performed by mixture of chloroform-methanol (9:1) as mobile phase and precoated plate silica gel GF_{254} as stationary phase. TLC plate was contacted on agar medium inoculated with the yeast for 15 minutes. After that, the plate removed and agar medium was incubated for 36-48 hrs, and inhibition zones were measured.

Further separation and purification

The active fraction (10.21 g) was separated using silica gel H by vacuum liquid chromatography with isocratic elution using n-hexane-ethyl acetate (1:9) resulted 34 fractions. Fractions 16-31 (0.98 g) were further separated by open-column chromatography using silica gel 60 with isocratic elution of ethyl acetate-ethanol (10:1) resulted 72 subfractions. Subfractions 18-26 (0.27 g) were subjected to open-column chromatography again with same conditions resulted in 53 subfractions. Every fraction was monitored by TLC. The last, subfractions 7-18 purified by recrystallization using chloroform-methanol (9:1) and obtained yellow crystal-needle isolate (30.2 mg) named MCET51.

The purity of MCET51 was tested by TLC using 3 mobile phases and two-dimensional (2D) TLC. The chromatography performed with commercial precoated plate silica gel GF_{254} as solid phase and mixture of ethyl acetate-ethanol (10:1), ethyl acetate-chloroform (6:4), and chloroform-methanol (9:1) as mobile phase.

Characterization of isolate

Characterization of MCET51 was done by color test, UV-visible, infrared spectrophotometric method, mass spectrometric method and nuclear magnetic resonance (NMR) spectroscopy of proton (^1H), carbon (^{13}C), Heteronuclear Single Quantum Coherence and heteronuclear multiple-bond correlation spectroscopy.

RESULTS AND DISCUSSION

The yield of extraction was 5.82% (w/w). Since *M. champaca* bark extract was reported active with the method [21], then it directly fractionated by LLE obtained n-hexane, ethyl acetate, and water fractions. All fractions were tested by mechanism-based yeast bioassay to *S. cerevisiae* strains 1140, 1353, and 1138. Activity results of fractions were shown in Table 1.

Fractions and pure compounds were considered active if IC_{12} values at the lower doses of the extract, where the active extract ranging from 1000 to 8000 µg/ml [20]. Table 1 showed that all fractions were active against *S. cerevisiae* strain 1353 and 1138 with IC_{12} values <8000 µg/ml and n-hexane fraction also active against *S. cerevisiae* strain 1140. These results indicated that the fractions contain active compound as topoisomerase inhibitor.

TLC bioautography is a combination of TLC as simple separation technique and antimicrobial bioassay. In this research, TLC was used to separated quickly the mixture of compounds contained in LLE fractions so that can be readily tested the biological activity of each component (spot). The result of TLC bioautography showed that the Rf of the active compounds were 0.8-0.84 of ethyl acetate fraction and 0.69-0.74 of n-hexane fraction.

According to TLC bioautography results, further separation and purification were focus on the active spot in ethyl acetate fraction. The process was yielded yellow crystal-needle isolate named MCET51. Purity tests showed MCET51 was a single spot with an area under curve 95.25% at 200-700 nm. The isolate had a melting point at 271.5-272.6°C.

The MCET51 gave yellow-red with Dragendorff reagent indicated that was alkaloid. UV-vis spectrum showed peaks absorbance at 205, 248, 268, 309, and 414 nm in methanol that predicted MCET51 was identical to aporphine alkaloid [17,26]. Infrared spectrum showed existence functional group of amines at 3421.1/cm, C-H stretch at 3039.26/cm and 2919.7/cm, carbon aromatic at 1658.48, 1573.63; 1473.35 and 1419.35/cm, and carbonyl at 1307.5/cm [27]. Mass spectrum showed 100% peak ion $[M+1]^+$ 276.14 (m/z), mean that molecular weight of MCET51 was 275 (m/z).

NMR spectrum signals of MCET51 in $CDCl_3$ showed the number of proton and carbon with their 2D correlation in determining structural arrangement [27] as shown in Table 2. These spectrum signals were identical with liriodenine based on previous results [26,28,14]. Based on characterization, data showed that MCET51 was proved as liriodenine, an aporphine alkaloid with molecular formula was $C_{17}H_9NO_3$.

The result of activity assay of isolate showed that liriodenine was active against *S. cerevisiae* strain 1140 (IC_{12} 22.15±1.71 µg/ml), strain

Table 1: The IC_{12} values (µg/ml) of fractions as results of activity test using mechanism-based yeast bioassay

Sample	*S. cerevisiae* strain		
	1140	**1353**	**1138**
n-hexane fraction	1386.55±226.57	111.18±43.09	136.77±30.29
Ethyl acetate fraction	>8000	542.60±102.46	279.43±163.07
Water fraction	>8000	401.62±107.40	242.71±30.27

S. cerevisiae: *Saccharomyces cerevisiae*

Table 2: NMR spectrum signals of MCET51 and liriodenine (δ in ppm, J in Hz)

No atom	MCET51		Liriodenine [24]		Liriodenine [26]	
	C	H	C	H	C	H
	102.44	**6.36 (2H, s)**		**6.39 (2H, s)**	**102.4**	**6.35 (2H, s, O-CH_2-O)**
1	151.71		144.54		151.7	
1a	108.10		107.76			
1b	123.20				123.2	
2	147.90		151.28		148.8	
3	103.23	7.16 (1H, s)	102.15	7.20 (1H, s)	103.2	7.14 (1H, s)
3a	135.69				135.7	
4	124.21	7.73 (1H, d, J=6.16)	122.60	7.79 (1H, d, J=5.19)	124.2	7.73 (1H, d, J=5)
5	144.90	8.86 (1H, d, J=5.15)	135.16	8.90 (1H, d, J=5.4)	144.9	8.86 (1H, d, J=5.5)
6a	145.90		144.15		145.2	
7	182.42		181.8		182.4	
7a	131.28		123.86		131.5	
8	128.78	8.56 (1H, d, J=8)	127.57	8.59 (1H, dd, J=7.95, 0.9)	127.3	8.60 (1H, d, J=8.0) or 8.56 (1H, dd, J=1.5 e8.0)
9	128.55	7.56 (1H, t, J=7.3, J=7.4)	130.5	7.52 (1H, dt, J=7.94, 1.22)	133.9	7.71 (1H, m) or 7.55 (1H, dt, J=1.5 e8.0)
10	133.88	7.72 (1H, m)	133.47	7.58 (1H, dt, J=7.8, 0.9	128.8	7.71 (1H, m) or 7.55 (1H, dt, J=1.5 e8.0)
11	127.32	8.60 (1H, dd, J=3.05, J=10.53)	126.88	8.66 (1H, dt, J=7.95)	128.6	8.60 (1H, d, J=8.0) or 8.56 (1H, dd, J=1.5 e8.0)
11a	132.85		132.34		132.9	

a, b → Position of C in order of liriodenine structure. NMR: Nuclear magnetic resonance

1353 (IC_{12} 24.76±0.56 µg/ml), and strain 1138 (IC_{12} 7.02±1.85 µg/ml). It means that liriodenine is active both as topoisomerase I inhibitor and topoisomerase II inhibitor. These IC_{12} values of isolate were lower than *M. champaca* bark extract [21] and LLE fractions and mean that isolate more potent than the extract and fractions. Hence, it was clearly defined that liriodenine was an active ingredient of *M. champaca* as an anticancer agent or topoisomerase inhibitor.

This research results reported that based on mechanism-based yeast bioassay *M. champaca* L. fractions and liriodenine were proved as Type I and Type II topoisomerase inhibitor, whereas other studies only showed liriodenine as topoisomerase II inhibitor. Activity test of liriodenine by *in vivo* assay using SV40-infected cells and *in vitro* enzymatic assay reported that liriodenine is a potent inhibitor of topoisomerase II [29]. Computational study of liriodenine by molecular modeling and docking technique reported that *liriodenine* is the most effective *aporphine* alkaloids as a novel inhibitor of topoisomerase II [30]. Combination studies by *in silico* absorption, distribution, metabolism, and excretion toxicology analysis, docking and molecular dynamic simulation of 100 anticancerous alkaloids reported that liriodenine as one of six alkaloids inhibited topoisomerase II [18].

CONCLUSIONS

Our research revealed that *M. champaca* L. bark fractions active as topoisomerase inhibitor. Isolation from ethyl acetate fraction guided by mechanism-based yeast bioassay carried out liriodenine that active as topoisomerase I inhibitor and topoisomerase II inhibitor.

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