Topoisomerase Inhibitor Compound Isolated From n-Hexane Fraction of Yellow Champaca Stem Bark (*Michelia champaca* L.)

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ABSTRACT

Aim: Isolation and preliminary identification of active compounds from the n-hexane fraction of *Michelia champaca* L.
Methods: Methanol extract was fractionated by liquid-liquid extraction with three different solvents. The n-hexane fraction separated by vacuum liquid chromatography and column chromatography obtained subfraction VI. This subfraction then purified by preparative thin layer chromatography (TLC) resulted in MCNH-1 and MCNH-II isolates. Activity test of the fraction, sub fractions and isolates were done by mechanism-based yeast bioassay. Identification proceded by TLC with various specific spray agents.
Results: The MCNH-1 isolate was active as topoisomerase 1 inhibitor with IC_{12} 83.004 µg/mL. This isolate identified as a flavonoid.
Conclusion: There is a flavonoid, MCNH-1 isolate, in the *n*-hexane fraction of yellow champaca stem bark (*Michelia champaca L.*) acts as topoisomerase I inhibitor

Keywords: *Michelia champaca* L., yellow champaca bark, mechanism yeast bioassay, topoisomerase I inhibitor.

INTRODUCTION

Along with the increasing incidence and number of deaths due to cancer, effective and efficient research of anticancer agents is needed, as one of them can be derived from plants [1]. Up to these days, research on potential anticancer plants still going on massively. Not less than 3 groups of active compound were found effective as an anticancer agent, such as: flavonoid-alpiniumisoflavone (AIF), steroids-28-homocastasterone (28-homoCS), and taxol-paclitaxel [2]. It shows that plants not only act as an alternative medication, but also useful in the other hands.

Zuhrotun (2015) screened Apocynaceae, Simaroubaceae, and Magnoliaceae plant families for their anticancer activity using *mechanism-based yeast bioassay* on *Saccharomyces cerevisiae* strains 1140 (permeable to topoisomerase I inhibitor), 1353 (rad52.top1), and 1138 (rad52). The result showed that yellow champaca (*Michelia champaca* L.) represented of Magnoliaceae has the best potency as Topoisomerase I and II Inhibitor [3]. Topoisomerase inhibitor includes as one among other anticancer mechanism pathways. Further research shows that n-hexane and ethyl acetate fraction were active as a topoisomerase inhibitor. An isolate of ethyl acetate fraction, liriodenine, was found active as topoisomerase I and II

inhibitors [4]. This research as a continuation of those previous research, that aim to Isolate and identify of active compounds from the n-hexane fraction of *Michelia champaca* L.

MATERIALS AND METHODS

Materials

Methanol extract of yellow champaca stem bark, aquadest, chloroform, dimethyl sulfoxide, ethanol, ethyl acetate, methanol, n-hexane, silica gel 60 size 0.063-0.2mm (Merck), precoated plate silica gel 60 F_{254} (Merck), silica gel H (Merck), Bacteriological Peptone (Oxoid), Dextrose (Glucose), Euro Pharma (Conda), Potato Dextrose Agar (PDA, Oxoid), Potato Dextrose Broth (PDB, Oxoid), Yeast Extract (Oxoid), and *Saccharomyces cerevisiae* normal strain and 1138, 1140, and 1353 mutant strains.

Tools

-5^{o}C refrigerator (Midea), 6 mm perforator, autoclave, classical chromatography column, digital balance scales (Mettler Toledo), magnetic stirrer (Yellow Mag HS7), micropipette 10-100µL, micropipette 100-1000µL, oven (Memmert), rotavapor (Ika RV10), semi micro 1,5mL cuvette (Brand), thermometer, TLC chamber, UV-Vis spectrophotometer (Rayleigh UV-9200), vacuum (Rocker 600), vacuum chromatography column, water bath (Memmert), and standard laboratory equipments.

Isolation Process

The methanol extract of yellow champaca stem bark was fractionated by liquid-liquid extraction (LLE) with n-hexane, ethyl acetate, and aquadest. TLC determined the profile of the fraction. Separation began with vacuum liquid chromatography using gradient solvents of n-hexane and ethyl acetate. Subfractions were grouped by similarity on form and color of TLC profile. Each subfraction was then tested using mechanism-based yeast bioassay. Subfraction with the best activity and considering its adequate quantity, was then further separated by classical column chromatography. The column filled with silica gel 60 and eluted isocratically using optimized solvents. The subfraction was accommodated for each 10mL. TLC then determined profile of the subfraction grouped. Purification was then accomplished by preparative TLC and recristallizations method. Two dimensional TLC was done to know the purity of isolate.

Compound Identification

Identification of groups of active compound was accomplished by TLC using various staining agents. Begin with broad staining agents compatible with various groups of a compound, such as sulfuric acid. Then followed by specific staining agents.

RESULTS AND DISCUSSION

Fractionation and Activity test n-Hexane Fraction

80 g of methanol extract of yellow champaca stem bark was fractionated by LLE method obtained 8.84 g of n-hexane fraction (11.05% w/w). Activity test by mechanism-based yeast bioassay showed that this fraction was active against *Saccharomyces cerevisiae* 1138, 1140, 1353 mutant strains with IC_{12} value were 705, 1393, and 883 µg/ml respectively. Camptothecin used as a positive control due to its well-known anticancer activity as a topoisomerase inhibitor [5]. The IC_{12} value is a minimum concentration that requires to produce diameter 12 mm of zone inhibition. These results are in line with Zuhrotun (2016) which showed n-hexane fraction active as Topoisomerase I and II inhibitors [4]. The chemical compound of n-hexane fraction reflected by TLC profile (Table 1) that showed

minimum eight spots detected under UV 366 nm. Bathochromic shift was detected visually after the addition of H_2SO_4 10% on each plate. Red and blue fluorescent compounds detected on both TLC plate under UV 366 nm.

Table 1: TLC Spot of N-Hexane Fraction

Eluen	Spot Code	Rf	Spot Detection		
			UV 254	UV 366	H_2SO_4 10%
n-Hexane:Ethyl Acetate (8:2)	1	0,94	-	-	Red
	2	0,85	-	-	Yellow
	3	0,66	-	Red	-
	4	0,55	-	Blue	Grey
	5	0,41	-	-	Yellow
	6	0,38	-	Red	Red
	7	0,18	-	-	Violet
	8	0,12	-	Red	Violet
Chloroform:Ethyl Acetate (7:3)	1	0,94	-	Red	Red
	2	0,87	-	Blue	Brown
	3	0,78	-	-	Violet
	4	0,69	-	-	Brown
	5	0,61	-	-	Yellow
	6	0,54	-	Blue	Red
	7	0,45	-	Blue	Brown
	8	0,36	-	Yellow	Brown
	9	0,12	-	Blue	Red

Separation and Activity test of Subfractions

7 g of n-hexane fraction was separated by vacuum liquid chromatography (VLC) using n-hexane:ethyl acetate (10:0) to (0:10) gradually obtained 12 subfractions and based on TLC profiles were grouped to 7 subfractions (I-VII). All seven subfractions tested against *S. cerevisiae* strain 1138. This Strain chosen as screening-type strain due to its representative of topoisomerase I and II inhibitor activity. IC_{12} value was gradually decreased from I to VII subfraction as available in Figure 1. The smaller value of IC_{12} indicates the more potent activity. So, the most potent topoisomerase inhibitor compound in n-hexane fraction belongs to the more polar compound group (subfraction VII).

By considering the quantity and activity of these subfractions, subfraction VI was selected to be separated further using classical column chromatography. Separation was conducted using n-hexane:ethyl acetate (7:3) eluent and obtained 84 subfractions. Considering its TLC profile, subfractions 59-65 were suspected to contain compounds that are active as topoisomerase inhibitors.

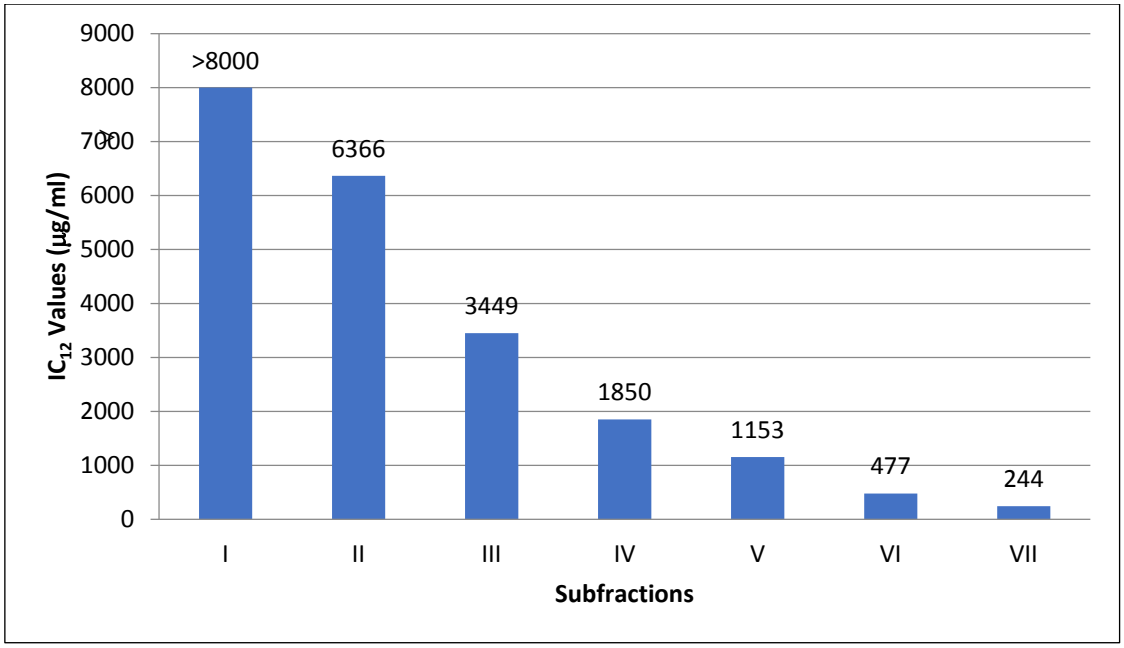

Figure 1: The Activity results of Subfractions against *S.cerevisiae* 1138 mutant strain

Purification and Activity Test of Isolate

The 59-65 subfractions were mixed and purified by preparative TLC using chloroform:ethyl acetate (7:3) obtained two isolates. The MCNH-I isolate (Rf 0,68) 1.1 mg appear as a yellow spot under visible light and UV 254 nm and red under UV 366. The MCNH-II isolate (Rf 0,50) 0.9 mg appears blue fluorescent under UV 366 and colorless under visible light. The purity test of isolates were carried out by two-way TLC. In MCNH-I isolates, the first eluent used was chloroform:ethyl acetate (7:3) followed by chloroform:methanol (9:1) as the second eluent. Whereas in the MCNH-II isolate, the first eluent used was n-hexane:ethyl acetate (9:1) followed by chloroform:ethyl acetate (8:2) as the second eluent. Both isolates were declared pure.

Activity test results can be seen in Figure 2. If inhibitory zone formed was too small or IC_{12} values exceed 1000 µg/mL, so the isolate declared inactive as topoisomerase I and II inhibitors. The MCNH-I isolate only active to S.cervisiae 1140 mutant strain with IC_{12} values of 83,004 µg/mL mean active as topoisomerase I inhibitors. This value is much smaller than camptothecin as positive control mean the MCNH-I isolate more potent than camptothecin [4,5]. However, the MCNH-II has minimal inhibitory zones around the well so that IC_{12} values exceed 1000 µg/mL and are declared inactive as topoisomerase I and II inhibitors.

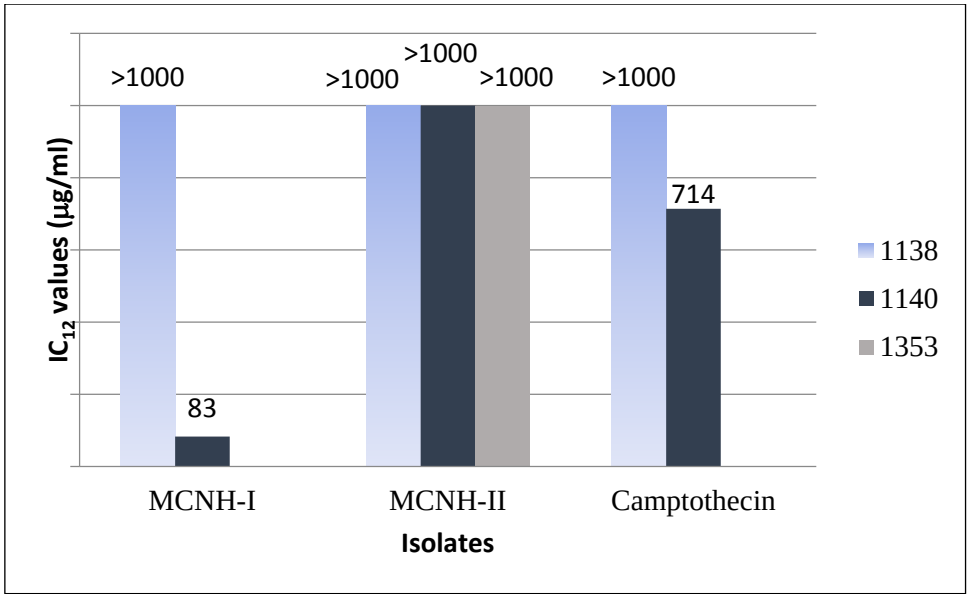

Figure 2: The Activity results of Isolates against *S.cerevisiae* 1138, 1140 and 1353 mutant strain

Identification of Isolate

Identification of MCNH-I isolates was carried out by TLC using specific spray agents. The staining reagent i.e FeCl3 that specific for phenol, Anisaldehyde and Liebermann that specific for terpenoids, Dragendorffs and Mayers that specific for alkaloids, AlCl3, Sitroborates, and ammonia vapors that specific for flavonoids. The results of the TLC with a variety of staining reagents can be seen in Table 2.

Table 2. The Result of TLC Profile With Various Staining Reagents

Staining reagents	UV 254	UV 366	Visible	Interpretation
No staining reagents	-	Red	-	
$FeCl_3$	-	-	Yellow	(-) Phenol
Anisaldehyde	-	-	-	(-) Terpenes
Liebermann	-	-	Brown	(-) Steroids
Dragendorffs	-	-	-	(-) Alkaloids
Mayer	-	-	-	
$AlCl_3$	-	Red	Red	(+) Flavonoid
Sitroborates	-	Red	Yellow	
Ammonia vapors	-	Red	Red	

Based on TLC profiles with various specific spray reagents, MCNH-I isolate was predicted as flavonoids. By the appearance of visually colored spots and fluoridated red patches on UV 366 nm after being sprayed with visible patches of $AlCl_3$, citroboric, and ammonia vapor. The isolated compound is suspected to be a flavonoid compound subclass of chalcone or auron which is red in UV 366 nm after being passed to ammonia vapor [6].

Flavonoid that has reported contain in yellow cempaka is quercetin [7]. This compound, along with fisetin, luteolin, apigenin, and genistein inhibited topoisomerase II activity. While ellagic acid, catechins of green tea, anthocyanidin delphinidin, isoflavonoid genistein demonstrated as topoisomerase II poisons. So there is a correlation between flavonoid with topoisomerases and natural anticancer [8].

This research still needs to be continued to identify the flavonoid compounds that have isolated. So it can be used as a marker compound for topoisomerase inhibitors or natural anticancer from extracts or fractions of yellow cempaka bark. Time and fund needed for more thorough research related to the development of yellow cempaka as a natural anticancer. However, it is still exciting and promising because there are many anticancer derived from plants proven as cancer chemopreventive agents, that are capable of preventing or inhibiting the process of carcinogenesis [9].

CONCLUSION

Isolation process from n-hexane fraction of yellow champaca bark resulted in MCNH-I isolate that active as Topoisomerase I inhibitor with IC_{12} values of 83,004 μg/mL. Preliminary identification showed that this active compound was flavonoid.

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