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PURIFICATION OF ACTIVE COMPOUNDS FROM KECAPI LEAVES THAT HAVE POTENTIAL AS ANTICANCER FOR *IN VITRO* ON MURINE CELLS LEUKEMIA P-388

FEBRIA ELVY SUSANTI[a], PURWANTININGSIH SUGITA[a*] and LAKSMI AMBARSARI[b]

[a]Department of Chemistry, Faculty of Mathematics and Natural Sciences,
Bogor Agricultural University, BOGOR – 16680 (Dramaga) INDONESIA
[b]Department of Biochemistry, Faculty of Mathematics and Natural Sciences,
Bogor Agricultural University, BOGOR – 16680 (Dramaga) INDONESIA

ABSTRACT

Kecapi is many contains secondary metabolites that can prevent a variety of diseases. The aims of this study were to obtain active compounds from the kecapi leaves through a purification, which includes vacuum liquid chromatography, gravity column chromatography, radial chromatography and preparative thin layer chromatography as well as the determination of the molecular structure and bioactivity as anticancer on murine cells leukemia P-388. Characterization of the isolated secondary metabolites is done by measuring the ^{1}H-NMR and MS. The analysis showed that the isolated compounds suspected to contain (-)-Loliolide. Toxicity test using *Brine Shrimp Lethality Test* (BSLT) showed that E fraction was the most active fraction with IC_{50} of 2.03 x 10^{-17} µg mL^{-1}. Anticancer test for *in vitro* on murine cells leukemia P-388 of methanol extract and E fraction result of vacuum liquid chromatography were performed the IC_{50} values decrease of 69.90 µg mL^{-1} to 13.86 µg mL^{-1} while bioactivity increased of 5 times.

Key words: Kecapi, (-)-Loliolide, Anticancer, Leukemia.

INTRODUCTION

Kecapi (*Sandoricum koetjape*) is one of the plants of the family *Meliaceae*. Plant is found in the lowlands to mountainous area with an altitude of 30 meters or more. It is planted in the garden or yard, native to Indonesia, Malaysia, Cambodia, Vietnam and the southern Andaman Islands and is little found in North and South America[1]. Kecapi is many

*Author for correspondence; E-mail: atiek_ps@yahoo.com

benefits in everyday life. Root of kecapi has been used in herbal medicine as antiseptic, pain and reinforcing a woman's body after childbirth. Bark was used for ringworm medicine, antiangiogenic[2], antibacterial[3] and anticancer[4]. Leaves of kecapi have been using as cure skin infections, lower the heat, diarrhea, headache, antibacterial and anticancer[2].

Nagakura et al.[5] has isolates sanjecumin-A and -B and sandrapin-A and –B from kecapi leaves. Sandrapin-A and -B shows inhibitory activity against the production of nitrogen monoxide (NO). Sentulic acid and 3-oxoolean-12-en-27-oic acid isolated from the bark of kecapi[4]. The isolates were cytotoxic activity against leukemia cancer in humans[4]. Triterpenoid compounds isolated from the bark of kecapi[6-7]. The ethyl acetate and hexane extracts were cytotoxic activity[6] while according Famaidi et al.[7] extracts that provided active response to the cytotoxic test only the ethyl acetate extract. Coumarin compounds isolated from the bark of kecapi and the extract was potential as an antibacterial[3].

Secondary metabolites (phytochemicals) test that have been carried out by Susanti et al.[3] showed that the extract of the bark kecapi contains triterpenoids, saponins, alkaloids, phenolics and flavonoids compounds. Based on preliminary testing that has been done, kecapi leaves had higher contents of secondary metabolites than bark. Various compounds have been isolated from kecapi and purification of the active compounds that has potential as anticancer reportedly still little, so in this study conducted purification other active compounds from the leaves of the kecapi as potentially anticancer for *in vitro* on murine cells leukemia P-388.

EXPERIMENTAL

Material, reagents and instrumentation

Kecapi leaves of West Sumatra (Indonesia) were used as material for this research. All reagents were commercially available and were purified before use. Characterization of isolated compounds was carried out using ^{1}H-NMR and MS spectroscopy. The ^{1}H-NMR Agilent (500 MHz) spectrometer using aceton-d*6* as solvent. The MS Agilent 6890 spectrometer using aceton as solvent.

Preparation and isolation

Kecapi (Mesh size 60) was calculated moisture content and phytochemical test. Maceration of kecapi (4 Kg) using methanol with four repetitions for 24 hrs at room

temperature. All filtrates were concentrated with a rotary evaporator to obtain methanol extract. It was removed chlorophyll and tannins extracts then was purified using vacuum liquid chromatography, gravity column chromatography, radial chromatography and preparative thin layer chromatography. Based on the analysis of TLC was set purity of isolates for initial verification. Pure of isolate have been providing a single spot with different three eluents system[8].

Toxicity tests using *Brine Shrimp Lethality* Test (BSLT)

Extracts were prepared in various concentrations. *Artemia salina* larvae (10 tails) were put into a micro plate 96-well and added extracts were diluted with DMSO[9]. LC_{50} values were obtained from the graph relationship between percent mortality and concentration of extract. Percent mortality larvae was obtained from the equation:

$$\text{Larvae mortality (\%)} = \frac{\left(A.\,salina \text{ dead (Sample} - \text{blank)}\right)}{A.\,salina \text{ number of test}} \times 100\%$$

Anticancer test for *in vitro* on murine cells leukemia P-388

MTT assay method was used. Absorbance have been measured at wavelength 540 nm[10]. IC_{50} values were obtained from the graph relationship between concentration of compound test and absorbent materials test after treatment. Statistical analysis was used Origin 8 program.

RESULTS AND DISCUSSION

Determination of moisture and phytochemical contents

Determination of moisture content simplisia[11] of kecapi leaves have been using oven at 105ºC and was yield of 3.32%. Phytochemical test of methanol extract contained phenolic, flavonoids, saponins, tannins, and triterpenoids.

Isolation and purification of the active fraction of methanol extract

Extraction result of dried leaves was yield of 7.50%. Free Chlorophyll-tannins extract was yield of 1.84% and extract (20 g) have fractionated using vacuum liquid chromatography and each fraction were tested toxicity with *Brine Shrimp Lethality Test* (BSLT) (Table 1).

Table 1: Mass of the fraction result of vacuum liquid chromatography

Fraction	Mass (mg)	LC_{50} (µg mL^{-1})
A	0.1	Not tested
B	118.1	1.16
C	521.7	258061.17
D	2946.8	1102.88
E	**2107.5**	**$2.03x10^{-17}$**
F	2294.5	147.27
G	21123.6	459920.09

Based on the order of toxicity from the active to inactive, E > B > F > D > C > G fractions. Total weight from the lot to a little, G > D > F > E > C > B > A fractions. The pattern of separation from profile TLC of the potentially to less potential to be separated, E > F > D > C > A > B > G fractions. Further separation process was performed to the E fraction because was the most active fraction, weight a lot and potentially as blue fluorescent stain (Fig. 1).

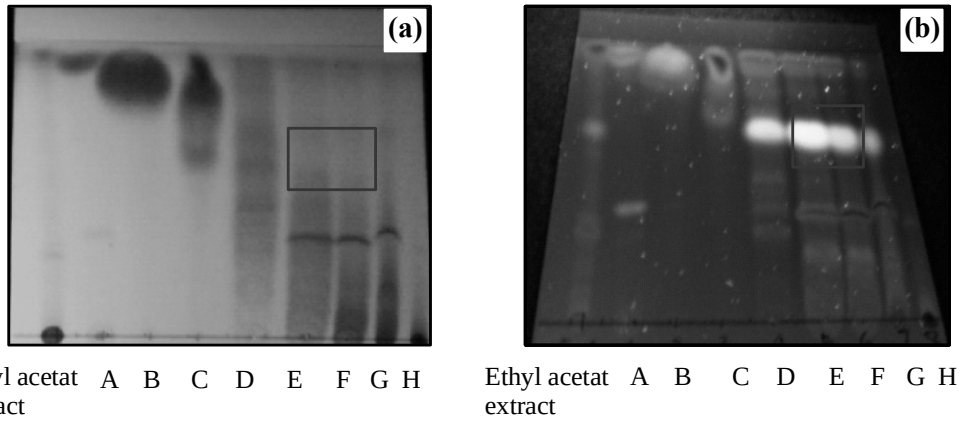


Fig. 1: Profile of TLC free chlorophyll-tannins extract from kecapi leaves result of vacuum liquid chromatography was used. Dichloromethane - methanol (19: 1) eluent under UV lamp (a) λ (254 nm), (b) λ (366 nm)

Separation of E fraction (2000 mg) was done using gravity column chromatography. Further separation process was performed to the E6 fraction caused weight a lot and potentially as blue fluorescent stain (Fig. 2).

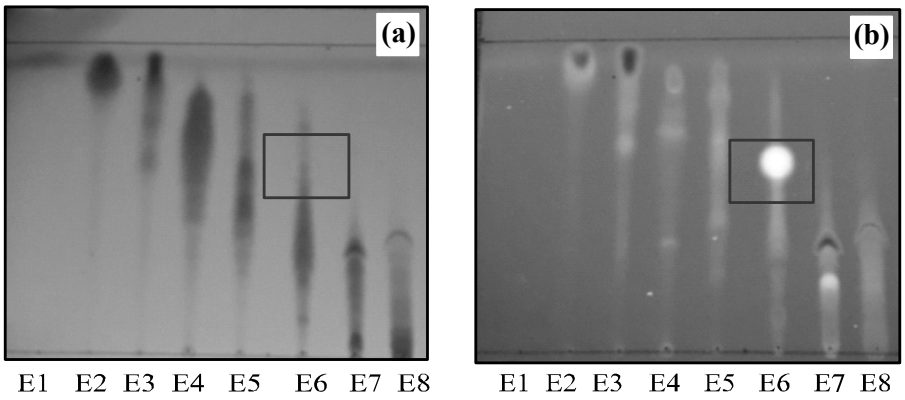


Fig. 2: Profile of TLC E fraction result of gravity column chromatography was used. Dichloromethane-methanol (19: 1) eluent under UV lamp (a) λ (254 nm), (b) λ (366 nm)

Separation further of E6 fraction (438.0 mg) by radial chromatography was produced E61 (296.8 mg) and E62 (21.9 mg) fractions (Fig. 3).

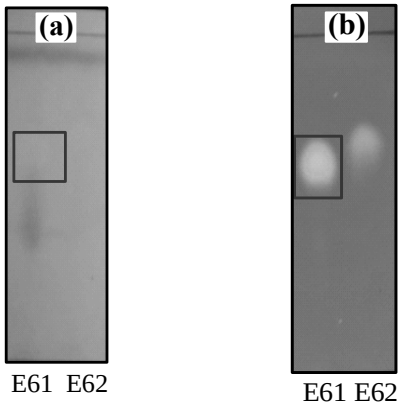


Fig. 3: Profile of TLC E6 fraction result of radial chromatography with dichloromethane-methanol (19:1) eluent under UV lamp (a) λ (254 nm), (b) λ (366 nm)

The separation was performed to the E61 fraction with preparative thin layer chromatography, was separates three stains adjacent with R_f values of 0.78, 0.51, and 0.41, respectively. One stain with R_f values of 0.78 as blue fluorescent stains was thought to be a target of pure compound (E611 compound). Purity test of compound was analyzed by TLC with three eluent systems. Profile TLC was provided a single stain (Fig. 4) then its

characterization was done by ^{1}H-NMR and MS spectroscopy. The R_f values of E611 compound (104.80 mg) have produced of 0.58, having the form gummy yellowish green.

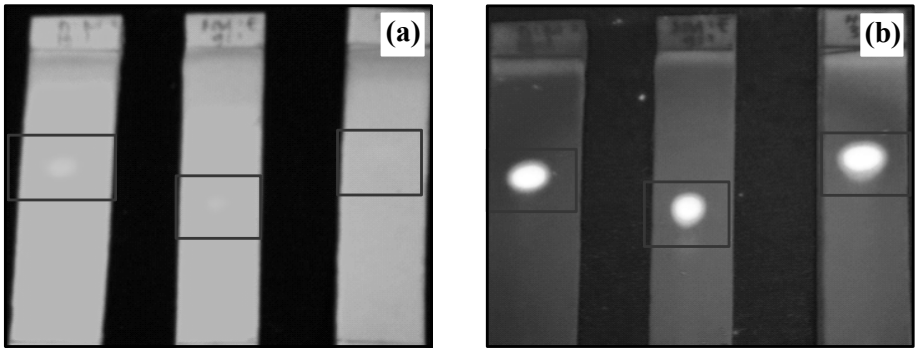
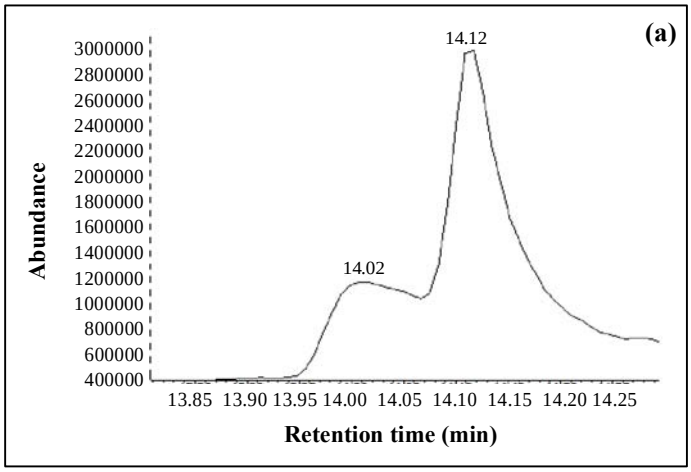


Fig. 4: Profile of TLC E611 compound with eluent system (1) dichloromethane-methanol (19: 1), (2) dichloromethane-ethyl acetate (9: 1), and (3) n-hexane-ethyl acetate (3: 7) under UV lamp (a) λ (254 nm), (b) λ (366 nm)

Characterization of the E611 compound

MS chromatogram showed 5 peaks with a retention time of 14.02, 14.12, 14.58, 14.81 and 15.78 min. However, there were only 2 peaks that has the highest abundance of 14.02 and 14.12 min (Fig. 5).



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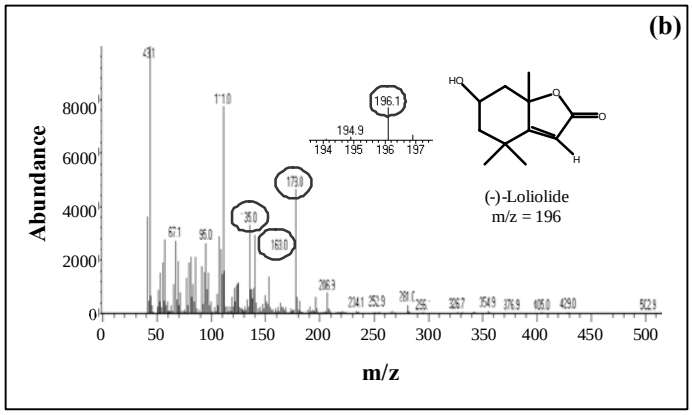

Fig. 5: (a) MS chromatogram of the E611 compound (b) Mass spectra of the E611 compound with a retention time of 14.02 minutes

The similarity of E611 compound was that a retention time of 14.12 minutes was lower than the other peak with WILLEY09TH database because was contained many impurity such as fatty acids. While the compound a retention time of 14.02 minutes have done similarity of 96% with the database so E611 compound was suspected a (-)-Loliolide compound with a molecular weight of 196 g mol^{-1}. E611 compound was supported by ^{1}H-NMR spectrum, was known of 5 signals while the structure of compounds should have 7 signals (Fig. 6). ^{1}H NMR (500 MHz): 4.22 (1H, t), 3.30 (1H, t), 1.92 (1H, s), 1.29 (3H, m), dan 1.26 (3H, m). Based on data, the ^{1}H-NMR spectrum showed that 2 proton signals of 2 methyl groups shows the chemical shift of 1.26 and 1.29 ppm, singlet multiplicity. This signal should have appeared 1 signal as their the same chemical environment.

The proton of carbon methylene have suspected the chemical shift of 1.29 ppm, multiplet multiplicity. The signal of 1 proton of the hydroxyl group the chemical shift of 1.92 ppm, singlet multiplicity. The signal should have appeared the chemical shift at 2.00 ppm. The proton signal of carbon metin the chemical shift of 3.30 ppm, triplet multiplicity. The proton compound should have appeares as a multiplet multiplicity. The proton of ring furanon was appeared chemical shift of 4.22 ppm, triplet multiplicity while should have appeared as a singlet multiplicity. The larger of proton chemical shift have been causing unprotected (deshielding) due to its position near elekton[12] withdrawing group. The existence of two signals that was not appeared methyl carbon (singlet multiplicity) and methylene (multiplet multiplicity) protons. Proton should have appeared the chemical shift of 1.60 and 1.68 ppm. Incompatibility advent of proton signal due to E611 compound was not pure and was contained impurity such as fatty acids.

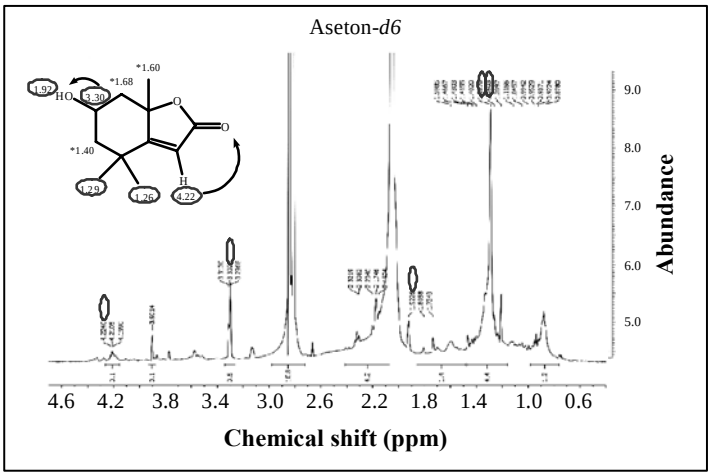

Fig. 6: ^{1}H-NMR spectrum of the E611 compound

Anticancer test for *in vitro* on murine cells leukemia P-388 showed that the IC_{50} values of the methanol extract and free chlorophyll-tannins E fractions result of vacuum liquid chromatography of 69.90 and 13.86 µg mL^{-1}, respectively. Artonin E was used as standard (0.76 µg mL^{-1}).

CONCLUSION

The leaves of kecapi contained active compounds that showed the potential as anticancer leukemia. E611 compound showed that a (-)-Loliolide compound, was isolated from the active fraction of methanol extract from leaves kecapi. Toxicity test *Brine Shrimp Lethality Test* (BSLT) showed that the E fraction was the most active fraction with the LC_{50} values of 2.03 x 10^{-17} µg mL^{-1}. Anticancer test on murine cells leukemia P-388 of the methanol extract free chlorophyll-tannins and the E fraction result of vacuum liquid chromatography showed that the IC_{50} values decreased while its bioactivity were increased of 5 times. Further separation and purification of the E fraction may be expected to enhance the activity of the molecule anticancer on murine cells leukemia P-388.

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