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journal homepage: www.elsevier.com/locate/apjtbOriginal article <http://dx.doi.org/10.1016/j.apjtb.2016.04.014>Cytotoxic activity and phytochemical standardization of *Lunasia amara* Blanco wood extractMuhammad Sulaiman Zubair^{1*}, Syariful Anam¹, Subehan Lallo²¹Department of Pharmacy, Faculty of Science, Tadulako University, Kampus Bumi Tadulako Tondo, 94118, Palu, Indonesia²Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Hasanuddin University, 90245, Makassar, Indonesia

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ABSTRACT

Objective: To evaluate the cytotoxic activity of wood extracts of *Lunasia amara* Blanco (*L. amara*) and to perform further phytochemical standardization.**Methods:** The wood extracts of *L. amara* were assessed for cytotoxic activity by *in vitro* tetrazolium bromide (MTT) method against two human cancer cell lines, cervical cancer cells (HeLa) and breast cancer cells (T47D). Thin layer chromatography, Dragendorff, acetic anhydride-sulfuric acid and ferric chloride were used to detect alkaloids, steroids and polyphenols, respectively. Furthermore, quantitative determination of total alkaloid by ultra fast liquid chromatography-photodiode array detection using lunacrine as a marker compound was performed as well.**Results:** The ethyl acetate extract exhibited higher cell-growth inhibition than methanol and *n*-hexane extracts on HeLa and T47D cancer line cells with the IC₅₀ of 71.15 and 79.04 µg/mL, respectively. Total alkaloid in ethyl acetate extract was counted as (10.46 ± 0.28)% (w/w), while lunacrine determined by ultra fast liquid chromatography-photodiode array detection method was found to be (3.55 ± 0.26)% (w/w).**Conclusions:** The high total alkaloid and lunacrine concentration on the extract confirm the potential cytotoxic property of ethyl acetate wood extract of *L. amara*.

1. Introduction

Cancer is one of the most non-communicable diseases in humans. According to the American Cancer Society, there were annually increasing about 2%–3% of deaths caused by cancer reported worldwide. Thus, around 3.5 million people all over the world were killed by cancer. Several chemically synthetic anti-tumor agents are used to treat cancer, but the wide range of side effects encourages many cancer patients to seek alternative or complementary methods of treatment [1–3]. Natural product-

based medicine becomes one option to treat cancer. Nowadays, natural product or herbal-based medicine represented about 60%–80% of all drugs in use by 1990. Moreover, 70%–95% of world populations applied herbal-based medicine for their primary care, particularly in developing countries [4,5].

Lunasia amara Blanco (*L. amara*), belongs to the Rutaceae family, is a small tree widely distributed in tropical forests of the Philippines, Eastern Java, Borneo, Sulawesi, Moluccas, Papua New Guinea and Australia [6]. It is a popular traditional medicine, used traditionally in Indonesia, particularly in South Sulawesi Province either as a dried small wood or in a mixture with some herbs called as *jamu* for enhancing sexual aggressiveness. Moreover, it is also regularly used by local people for antibacterial purpose such as in treatment of swollen limbs, skin diseases, and inflamed or irritated eyes [7].

Some studies revealed that quinoline alkaloids and sesquiterpenes were the major compounds in this plant. The plant mostly contains quinoline and quinolone type alkaloids such as lunamarine, lunacrine, hidroxy lunacrine, lunacridine, and hydroxyl lunacridine [6]. Two new acridone type alkaloids namely 5-hydroxygraveroline and 8-methoxyifflaiamine were also reported

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[7]. Biological activity investigations related to antimicrobial and cytotoxic properties have been associated with the quinoline alkaloids content [6]. Quinoline alkaloids from *L. amara* can inhibit *Mycobacterium tuberculosis* H37Rv [8]. Moreover, one of quinoline alkaloids, namely, lunacridine was reported as DNA topoisomerase II inhibitor compound [9]. The type of this mechanism will lead to antimicrobial and cytotoxic activities based on the inhibition on cell proliferation.

In our effort to develop a standardized herbal-based medicine from Indonesia medicinal plants, we previously conducted the physicochemical standardization of the wood extract of *L. amara* collected in South Sulawesi [10]. In this recent paper, further phytochemical standardization including the determination of total alkaloid and the concentration of lunacrine as a marker compound were performed to ensure the extract purity and quality which is responsible for affecting biological activity. Cytotoxic activity of wood extract was also performed to prove the potential bioactivity of standardized extract.

2. Materials and methods

2.1. Plant material

The wood of *L. amara* was collected from Siawung Village, Barru, South Sulawesi and identified at Sulawesi Biodiversity Foundation (Yayasan Keanekaragaman Hayati Sulawesi) Indonesia. Voucher specimen (code: LA071301) was deposited at Laboratory of Pharmacognosy and Phytochemistry, Department of Pharmacy, Tadulako University.

2.2. Preparation of extracts

The wood of *L. amara* (2.6 kg) in powdered form was initially extracted by reflux method using methanol solvent. The resulted residue was then subjected to solid–liquid extraction with successive solvent of *n*-hexane and ethyl acetate. The remain solvent of ethyl acetate was then removed *in vacuo* on rotary evaporator until obtained the viscous extract of *n*-hexane extract (2.04 g) and ethyl acetate extract (4.06 g). The last traces of solvent were evaporated in freeze dryer.

2.3. Cell cytotoxicity assay

The cytotoxicity of all those extracts on both HeLa and T47D cell lines was determined by MTT colorimetric assay. The cell lines used in this study were obtained from Laboratory of Parasitology, Faculty of Medicine, Gajah Mada University, Indonesia. Cells (1×10^4 cells/well) in RPMI-1640 containing 10% fetal bovine serum were seeded into 96-well flat bottom culture plates (Nunc A/S, Denmark). After 24 h of incubation, various concentrations of each extract (final concentrations from 31.25 to 1000.00 $\mu\text{g/mL}$) were added to the culture and incubated for 24 h at 37 °C in 5% CO₂ humidified atmosphere. Then cells were added with 10 μL /well of MTT (5 mg/mL) and incubated for 4 h in incubator at 37 °C in 5% CO₂ humidified atmosphere. The reaction was stopped by 100 μL sodium dedosyl sulfate (10% in HCl 0.01 mol/L). The plate was then shaken in shaker for 10 min and incubated overnight in the shaded room. The absorbance of each well was read at 550 nm wavelength in Elisa Reader (Biorad, USA), using wells without cells as blanks. All experiments were performed in triplicate. The effect of all extracts on the

proliferation of human breast and cervical cancer cells was expressed as the % cytoviability, using the following formula:

$$\% \text{ Cytoviability} = \text{Absorbance of treated cells} / \text{Absorbance of control cells} \times 100\%$$

2.4. Phytochemical analysis

The extract material was subjected to further phytochemical analysis using thin layer chromatography (TLC) method, following by spraying with Dragendorff, acetic anhydride-sulfuric acid, and ferric chloride reagents. Meanwhile, the total alkaloid was determined using Harborne gravimetric method [11]. Further quantitative standardization of lunacrine as a marker compound on ethyl acetate extract after confirmation of its presence by TLC was performed by ultra fast liquid chromatography-photodiode array detection (UFLC-PDA). A stock solution of both ethyl acetate extract and standard lunacrine in methanol was prepared in concentration of 0.7 mg/mL and 0.2 mg/mL, respectively. UFLC-PDA analysis was carried out using a system with LC-solution software. The separation was carried out on a Shim-Pack-Vd-Ods column VP-ODS size 150 mm \times 4.6 mm (inside diameter). Optimum efficiency of separation was obtained using methanol: water (90:10, v/v) with a flow rate 1 mL/min. Other parameters adopted were as follows: injection volume 5 μL , column temperature 4 °C, and detection wavelength at 258 nm. Linear regression analysis using SPSS 17.0 (SPSS, Inc, Chicago IL, USA) was applied to correlate the peak area (Y) against the concentration of lunacrine (X). The quantification of lunacrine in the extract was quantified in reference to the calibration curve.

3. Results

3.1. Cytotoxic activity

The result of cytotoxic activity of *L. amara* wood extract against HeLa and T47D cancer cell lines are presented in Figures 1 and 2. It was observed that both cancer cells treated with *L. amara* extract showed a decrease in cell proliferation with the increase of concentration. Ethyl acetate extract showed higher inhibition to both cancer cell lines than the other two extracts. At the concentration of 62.50 $\mu\text{g/mL}$, ethyl acetate extract inhibited cell proliferation on HeLa and T47D cells at 56.16% and 69.97%, respectively. Meanwhile, at the same concentration, that of *n*-hexane was

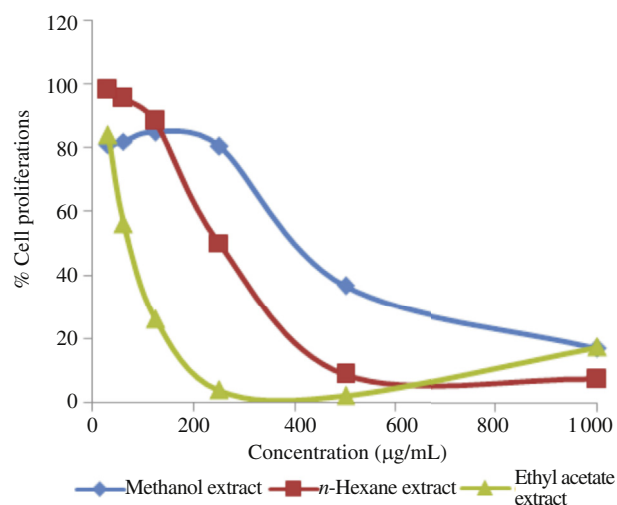


Figure 1. Cytotoxic activity of *L. amara* extract against HeLa cancer cells.

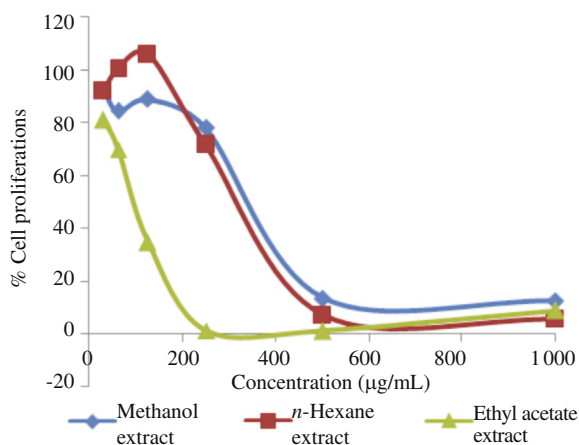


Figure 2. Cytotoxic activity of *L. amara* extract against T47D cancer cells.

95.59% and 100.43%, and methanol extract was 82.25% and 84.43%, respectively. Only ethyl acetate extract was able to inhibit the cell proliferation of both cancer cell lines as revealed by the value of IC_{50} of 71.15 and 79.04 $\mu\text{g/mL}$, respectively (Table 1).

Table 1

Cytotoxic activity (IC_{50}) of *L. amara* Blanco wood extract ($\mu\text{g/mL}$).

Extract/Standard	Cell line type	
	HeLa	T47D
Methanol extract	412.46	337.63
<i>n</i> -Hexane extract	248.33	309.47
Ethyl acetate extract	71.15	79.04
Doxorubicin	8.92	104.96

3.2. Phytochemical analysis

Identification of chemical constituents on the *n*-hexane extract was performed by TLC using stationary phase silica gel 60 F254 and mobile phase *n*-hexane-chloroform-methanol (5:9:0.5) showed that the extract contains alkaloids (two spots with R_f 0.752 and 0.811 after spraying Dragendorff reagent) and steroids (four spots with R_f 0.423, 0.764, 0.917 and 0.929 after acetic anhydride-sulfuric acid spraying). Meanwhile, the TLC analysis of ethyl acetate extract revealed that the extract contains alkaloids (four spot with R_f 0.305, 0.741, 0.858, and 0.941), polyphenols (one spot with R_f 0.705) and steroids (four spots with R_f 0.458, 0.776, 0.929, and 0.941). Quantitative total alkaloid content determined by Harborne gravimetric method showed that methanol extract contains (19.44 ± 1.72)%, *n*-hexane extract contains (8.55 ± 1.08)% and ethyl acetate extract contains (10.46 ± 0.28)%. Further quantitative standardization by UFLC-PDA using lunacrine as a marker compound, chromatogram of the ethyl acetate extract showed a sharp peak for lunacrine which can be comparable with the standard lunacrine (RT 2.48) (Figure 3). Figure 3B represents good separation of lunacrine with adequate peak resolution that indicates that the used method is selective for identification of lunacrine. The calibration curve was based on the analysis of working solutions at five concentration levels for lunacrine standard as shown in Figure 4. The calibration curve has good linearity relationship in the specified concentration range (5–25 $\mu\text{g/mL}$) with a correlation coefficient (R^2) greater than 0.98. The quantity of lunacrine in the extract was found to be (3.55 ± 0.26)% (w/w).

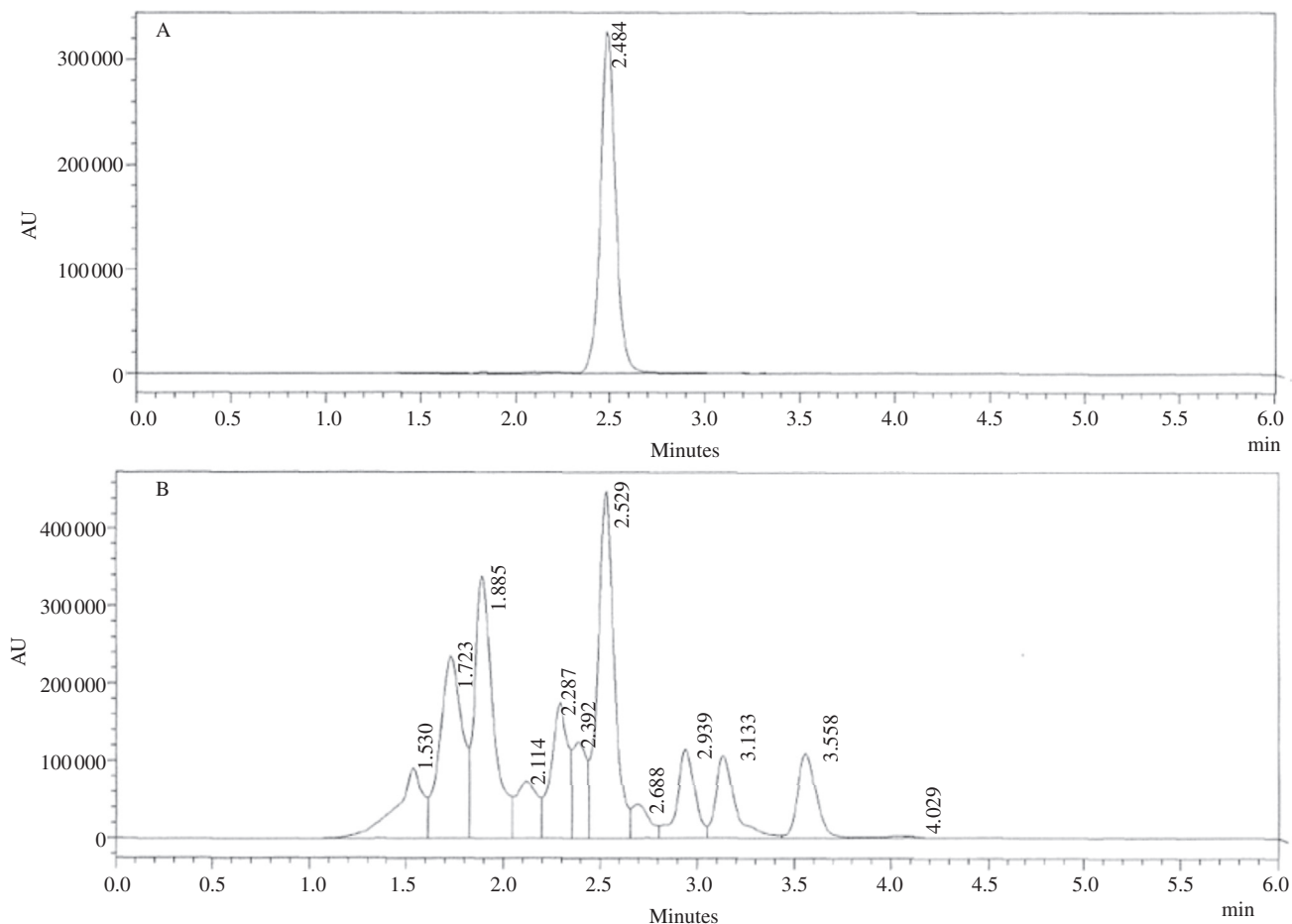


Figure 3. UFLC chromatogram profile of lunacrine in ethyl acetate extract from *L. amara* Blanco wood extract. In figure A: standard peak of lunacrine and B: peak of lunacrine present in ethyl acetate extract.

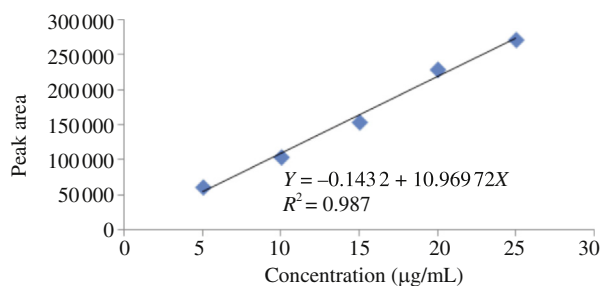


Figure 4. Calibration curve of standard lunacrine.

4. Discussion

It is well established that plants based compounds have been playing an important source of several clinically anticancer agents. The predominant goals of the assessment of cytotoxic activity from the crude plant extracts are either to isolate bioactive agents that can be as a lead molecule for anticancer drugs therapy or to develop the crude plant extract itself to become standardized herbal medicine that can be used as co-chemopreventive agents [2,12]. In the other hand, to ensure the therapeutic importance of herbal medicine, correct identification and quantification of active principal, proper purity assessment and quality measurement of the starting materials are an essential prerequisite to obtain the safety and efficacy of herbal medicine [4,13–16].

In the present study, *L. amara* wood extract was prepared using methanol as the first solvent to dissolve any types of organic compounds in the wood, and then continued by solid–liquid extraction using successive solvent of *n*-hexane and ethyl acetate to differentiate the extract into less polar compounds fraction in *n*-hexane extract and more polar compounds fraction in ethyl acetate extract. We found that there is a significant difference of cytotoxic activity of each extract in inhibiting the cancer cells growth of HeLa and T47D cell lines as we can see in Table 1. Ethyl acetate extract exhibited higher inhibition to both cancer cell lines (IC₅₀ of 71.15 and 79.04 µg/mL, respectively) than *n*-hexane and methanol extracts (both of IC₅₀ > 100 µg/mL). This results indicated that ethyl acetate extract was potential to be a starting material for anticancer herbal-based medicine.

The biological activity of crude extracts are usually attributed by their chemical constituents. TLC identification using several spraying reagents revealed the presence of alkaloids, steroids and polyphenols compounds. We suggested alkaloids as a main source for cytotoxic activity. Alkaloids have been reported to exhibit broad pharmaceutical action ranging from cytotoxic, antimalaria, pain killer, antihypertension, antiparkinson, central nervous system disorders to anti-inflammation activities [15,17]. Moreover, one of reported alkaloids, namely lunacridine, which is converted to 2'-O-trifluoroacetyl lunacridine based on stability reason, posses intercalation mechanism to DNA topoisomerase II enzyme leading to apoptotic cancer cells death via activating caspase 3/7. The cytotoxic activity of lunacridine against P388 murine leukemic cell lines was also reported [9,18]. The potent cytotoxic activity in ethyl acetate extract can be related to the high levels of total alkaloid number. This result was justified by the higher total alkaloid number in more polar extract than less polar extract. This study also further measured the UFLC-PDA quantification of lunacrine on the ethyl acetate extract exhibiting potential cytotoxic activity. UFLC-PDA method was chosen based on its reproducible and accurate analysis in organic acid extraction from plants and edible mushrooms [19,20]. Lunacrine is

a quinoline alkaloid found in *L. amara* reported to posses central nervous system stimulator and low CYP3A4 inhibitory activities [6,21]. Therefore, lunacrine can be used as a chemical marker for standardization of wood from *L. amara*. This investigation is the first report of cytotoxic activity of *L. amara* wood extract that confirm the potential cytotoxic activity from alkaloid based extract. Also the first report dealing with the quantitative estimation of lunacrine from this plant extract.

Ethyl acetate wood extract of *L. amara* showed potential cell-growth inhibition to both HeLa and T47D cancer cell lines that can be correlated to the high concentration of total alkaloid content and lunacrine concentration. This study can be used also as a referential source of valuable information for purity and quality of extract as starting material for herbal based medicine.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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