

Original Article

## ***A Simple Liquid-Liquid Fractionation (LLF) Method for Isolating Deoxyandrographolide dan Andrographolide from Herbs of *Andrographis paniculata* (Burm., F) Ness and Its Cytotoxic Activity on 3T3-L1 Preadipocyte Cells***

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Received: 26 October 2020; Revised: 22 November 2020; Accepted: 23 November 2020; Published: 31 December 2020

**Abstract:** The main bitter constituents of sambiloto (*Andrographis paniculata* (Burm., F) Ness) are diterpene lactones, namely andrographolide and deoxyandrographolide which have been reported to have antidiabetic, cytotoxic, antiatherosclerosis, anti-inflammatory, and antimicrobial activity. There are many studies that performed the isolation of deoxyandrographolide and andrographolide from *A. paniculata* herbs, but most of them included several steps that make them not efficient. This research was conducted to do an isolation of deoxyandrographolide and andrographolide through liquid-liquid fractionation (LLF) due to its simplicity, low cost, and time efficient. The extraction of deoxyandrographolide and andrographolide from the herbs was carried out using chloroform as the solvent by using Soxhlet apparatus, and LLF was performed to isolate the compounds. The identities of the compounds were confirmed by TLC scanner compared to its standard references. Hence, these present methods were successfully isolated and determined deoxyandrographolide and andrographolide of *A. paniculata*. The compounds were also showed relatively moderate cytotoxicity on 3T3-L1 cell lines using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, with LC<sub>50</sub> of deoxyandrographolide and andrographolide; 29.32 µg/mL and 37.70 µg/mL, respectively.

**Keywords :** *Andrographis paniculata*, isolasi, Fraksinasi Cair-Cair, 3T3-L1, MTS

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### 1. INTRODUCTION

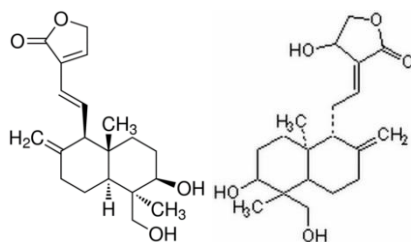
In recent years, treatment of diseases with traditional herbs has become an alternative. The main reasons regarded as relatively safe in long term use and it has minimal side effects. *Andrographis paniculata* (Burm.f.) Nees is known as a medicinal plant which can be used as an alternative treatment for various diseases. It is known as king of bitter due to the very bitter taste of all part of the plant and in Indonesia its name is sambiloto. In pharmacological research, it shows antidiabetic [1],

cytotoxic effect [2], antimicrobial activity [3], anti-inflammatory [4], anti-hyperglycemia [5], antiatherosclerosis [6], antimalaria [7], hepatoprotective [8], and anticancer activity [9]; in vitro and in vivo studies.

The phytochemical contents of *A. paniculata* include diterpenoids, polyphenols and flavonoids [10]. Andrographolide ( $C_{20}H_{30}O_5$ ) is the first major diterpene lactone. Andrographolide exhibits several pharmacological properties and a potential chemotherapy agent [11]. Andrographolide contains  $\alpha$ -alkylidene  $\gamma$ -butyrolactone and three hydroxyl groups in C-3, C-19 and C-14 (Fig.1) which are responsible for cytotoxic activity against many cancer cells [11].

Several methods of isolation deoxyandrographolide and andrographolide compounds from sambiloto have been carried out. Isolation of deoxyandrographolide has been evaluated by several methods, including not only the methods by Rashid et al., 2018 and Hermawan et al., 1997 using Preparative Thin Layer Chromatography (PTLC), but also the method by Imanta, 2017 [12-14]. Isolation of deoxyandrographolide has been performed by Rashid et al., 2018 from ethanolic crude extract which then was subjected to silica gel column chromatography eluted with chloroform-methanol (99:1) [12]. More than 20 years ago Hermawan et al., 1997 isolated deoxyandrographolide using Preparative Thin Layer Chromatography (PTLC) [13]. Chromatographic methods were needed not only for isolation of deoxyandrographolide but also for separation of andrographolide and deoxyandrographolide. Isolation of andrographolide has been reported from cold maceration of leaf powder in a 1:1 mixture of dichloromethane and methanol followed by recrystallization [15]. The second major diterpene lactone of *A. paniculata* is 14-deoxyandrographolide (Fig. 1). It has immunomodulatory and antiatherosclerotic effects [16,17]. Therefore, the chosen isolation method must be selective to provide the expected isolation results precisely and quickly. This research focused on the isolation of deoxyandrographolide and andrographolide from the herbs of *A. paniculata* without chromatographic method. So, this research became a new thing to separate andrographolide and deoxyandrographolide.

On the other hand, the use of *A. paniculata* as remedy for curing diabetes is usually in relatively long period of time, so a question of safety arises. Beside the chronic toxicity test, the approach of safety concern can be performed by observation of cytotoxicity. Based on this approach, this research focused also on the effect of deoxyandrographolide and andrographolide on viability of pre-adipocyte cells.



**Figure 1.** Chemical structure of deoxyandrographolide and andrographolide

## 2. MATERIALS AND METHODS

### 2.1. Materials

The herbs of *A. paniculata* were collected during April 2019 from Kulonprogo Region of Yogyakarta Province, Indonesia. Authentication of herb was conducted by a botanist at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia and the

voucher specimen were kept in herbarium of that department. The high glucose of Dulbecco's Modified Eagle Medium (DMEM), bovine serum (BS), and Penicillin/Streptomycin were purchased from Gibco (Gaithersburg, USA), whereas Dimethyl Sulfoxide (DMSO), andrographolide and deoxyandrographolide standard references were obtained from Sigma Aldrich. Pro analysis degree of silica gel plate, chloroform, *n*-hexane, ethyl acetate and methanol were obtained from Merck. 3T3-L1 fibroblast cells were procured from American Culture Collection (ATCC) (Manassas, VA).

#### 2.2. Liquid-Liquid Fractionation (LLF) Method for Isolation of 14-deoxyandrographolide and andrographolide

Briefly, the chloromic extract, the non-hexanic fraction, the isolated andrographolide and deoxyandrographolide were applied on silica gel F<sub>254</sub> HPTLC plate with *n*-hexane: ethyl acetate (1:4) v/v as mobile phase and detected under ultraviolet lamp 254 nm. The authentication of isolated andrographolide and deoxyandrographolide was checked by comparison with their standard references on HPTLC system. Therefore, the pattern spectrums of each isolates were compared to each standard reference.

#### 2.3. Cell Culture

3T3-L1 fibroblast cells were cultured in high glucose DMEM supplemented with 10% bovine serum (10% B/S), 1% penicillin/streptomycin (1% P/S)] and incubated at 37°C in a condition of humidified atmosphere of 5% CO<sub>2</sub>. Cells were sub-cultured every 2-3 days at confluence at approximately 90%.

#### 2.4. Cytotoxicity Test of Deoxyandrographolide and Andrographolide on 3T3-L1 Pre-adipocyte Cells

Deoxyandrographolide and andrographolide were tested on 3T3-L1 pre-adipocyte cells based on the previous studies by Tandrasasmita et al., 2011 with any modifications [18]. The aim of this method was to examine the safety concentration of the deoxyandrographolide and andrographolide on 3T3-L1 pre-adipocyte cells. The first step, we should make a standard curve of the cells to convert the cells number in a toxicity cells curve. 3T3-L1 pre-adipocyte cells were plating in 96-well plate. Series cells curve that was made has the concentration of 1x10<sup>4</sup>, 3x10<sup>4</sup>, 5x10<sup>4</sup>, 7x10<sup>4</sup>, 9x10<sup>4</sup>, and 1x10<sup>5</sup> cell/mL in DMEM+1% P/S 1%+10% B/S. There were incubated in 12 hours (to avoid doubling time of cells) in 37°C, 5% CO<sub>2</sub>. Thereafter, cells were observed with microscope. The medium was changed to 100 µL PBS 1x, then it was added by 20 µL MTS reagent. The mixture was then incubated in 37°C, 1 hour 30 minutes. From these steps, the equation of the lines would be concluded.

The cytotoxicity of deoxyandrographolide and andrographolide were examined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Briefly, 3T3-L1 pre-adipocyte cells were cultured in 96-well plate until the confluence of 5x10<sup>4</sup> cells/mL in high glucose DMEM+1% P/S 1%+10% B/S. Cells were incubated in 24 hours, 37°C, 5% CO<sub>2</sub>. Cells were then observed microscopically. Thereafter, cell became 80% confluence, the medium was changed to high glucose DMEM free serum, and it was incubated until 4 hours, 37°C, 5% CO<sub>2</sub>. After incubated for 4 hours, the high glucose DMEM free serum was discarded and then it was treated by deoxyandrographolide with the doses 2.5, 5, 10, 12.5, 25, 50, 75 and 100 µg/mL in 0,1% DMSO. Whereas the dose of andrographolide for the treatment were 3.125, 6.25, 12.5, 25, 50, 75 and 100 µg/mL in 0,1% DMSO. The mixtures were incubated in 37°C, 5% CO<sub>2</sub> until 24 hours. The media

was changed to 100  $\mu$ L PBS 1x, then it was added by 20  $\mu$ L MTS. Cells were incubated in 37°C, 1 hour 30 minutes. The LC<sub>50</sub> in standard cells curve was analyzed by probit analyze in *Biostat v5 software*.

### 2.5. Statistical Analysis

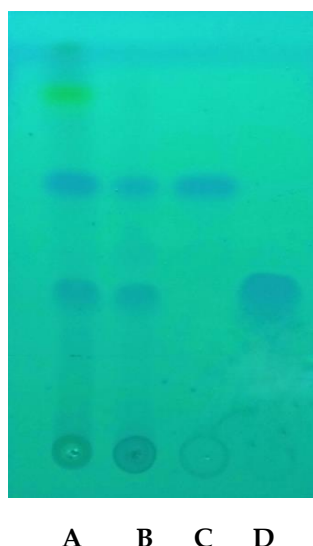
Data were statistically analyzed using IBM PASW SPSS Statistic version 18.0 (SPSS IBM, NY USA). Normality test were conducted using Saphiro Wilk. Statistical significance ( $p < 0.05$ ) was assessed by one-way analysis of variance (ANOVA) coupled with Dunnett's T-tests.

## 3. RESULT AND DISCUSSION

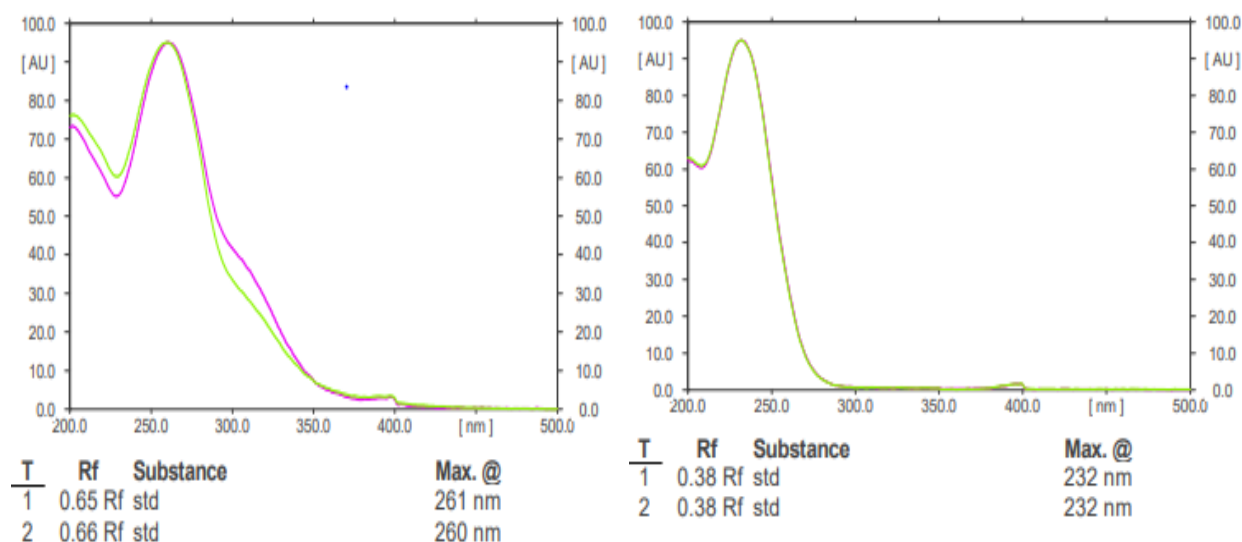
### 3.1. Isolation of Deoxyandrographolide and Andrographolide

Extraction of *A. paniculata* herbs using Soxhlet apparatus gave a result of 8.26 g crude extract with dark green color, whereas the Liquid-Liquid Fractionation (LLF) method was successfully isolated the deoxyandrographolide and andrographolide from the extracts. Firstly the fractionation with *n*-hexane eliminated perfectly chlorophyll and other lypophilic substances without interfere deoxyandrographolide and andrographolide such as shown by HPTLC data (Fig. 2). Further, as we know that in comparison to andrographolide, deoxyandrographolide lack of oxygene moiety at 14 position in the structure (Fig. 1). That will increase lypophilicity of the structure and we found that the mixture of *n*-hexane: chloroform (3:2) dissolved deoxyandrographolide but not andrographolide. The last mentioned compound was dissolved in chloroform. Both deoxyandrographolide and andrographolide were then recrystallized and the crystals had white color. Thus, LLF was chosen for this experiment due to its simplicity, low cost, and time efficient. A simple LLF method has been developed for the isolation of deoxyandrographolide and andrographolide from chloroformic extract of *A. paniculata*. As the isolate was formed, the weight of deoxyandrographolide and andrographolide were measured and showed yields of 0.248 g and 0.502 g respectively, or 3.0% and 6.07% from chloroformic extract. In comparison to other publication that the percentage of deoxyandrographolide in ethanol extract of dried whole plant, stems and herbs were 4%, 0.8-1.2% and 0.5-6%, respectively [19]. Indonesian Herbal Pharmacopoea mentions the percentage of andrographolide in ethanolic extract of *A. paniculata* herbs are not more than 3.80% [20]. The percentage of isolated deoxyandrographolide and andrographolide from chlorofomic extract in this research are relatively higher than ethanolic extract mentioned in the litterature due to lower percentage of polar impurities. As we know that ethanol is an universal or non selective solvent. It dissolves lypophylic and hydrophylic substances whereas chloroform dissolves only lypophylic ones.

In this study, deoxyandrographolide and andrographolide appeared on silica gel F<sub>254</sub> as quenching spots under UV<sub>254</sub> at R<sub>f</sub> value 0.66 and 0.38, respectively (Fig.2) These spots are not only similar to those of standard references, the pattern of spectrums are also similar to those of standard references according to the data evaluated by TLC scanner (Fig.3).



**Figure 2.** HPTLC silica gel 254 profile of *A. paniculata* herbs. A: Chloroformic extract, B: n-Hexanic insoluble fraction, C: Isolated deoxyandrographolide, D: Isolated andrographolide. Mobile phase: n-Hexane-Ethyl acetate (1: 4)

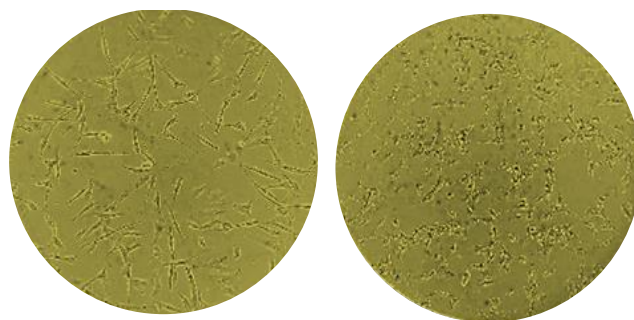


**Figure 3.** UV Spectrum profiles of isolated deoxyandrographolide with standard reference and andrographolide with standard reference

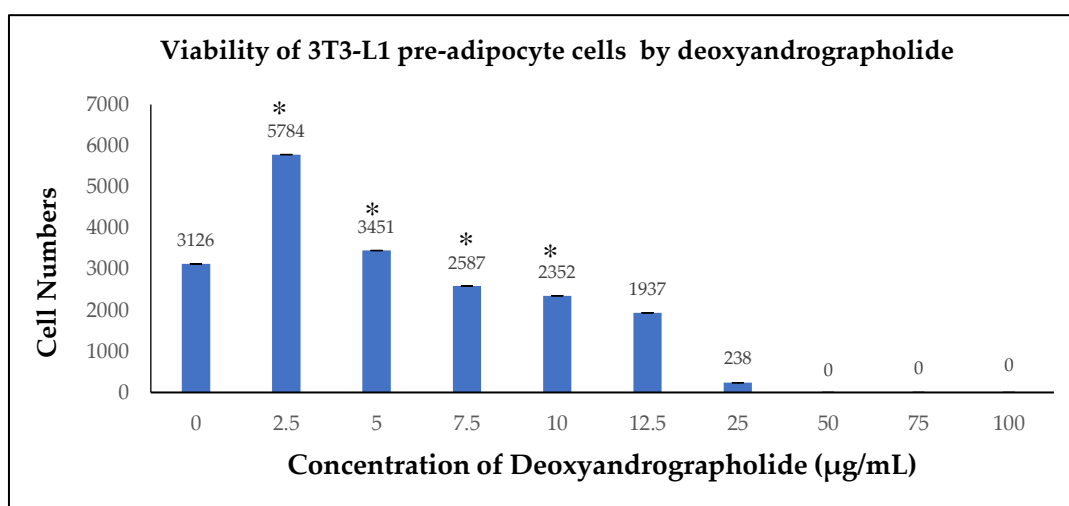
### 3.2. Cytotoxicity Test Result of Deoxyandrographolide and Andrographolide on 3T3-L1 Pre-adipocyte Cells

Further study was designed to examine the cytotoxic effect of deoxyandrographolide and andrographolide on 3T3-L1 pre-adipocyte cells. The equation of standard curve cells was  $y=2063x-545.69$ . This equation was important because it was to convert the number of cells. Fig. 4 showed examples of microscopic view of cells of the control group treated with DMSO and the group treated with deoxyandrographolide 50 µg/mL. Almost all cells of the control group without any treatment except the media were 3126 cells, whereas after treatment with 5.0, 10.0 and 12.5 µg/mL of deoxyandrographolide the viability of cells were lower than the control, and then the viability decrease significantly at the concentration of 25 µg/mL and at concentration more than 50 µg/mL all cells were death. It means that 12.5 µg/mL were limit of concentration which did not influence significant viability of cells (Fig. 5). Thus, the cell viability was dramatically increased (almost 100%)

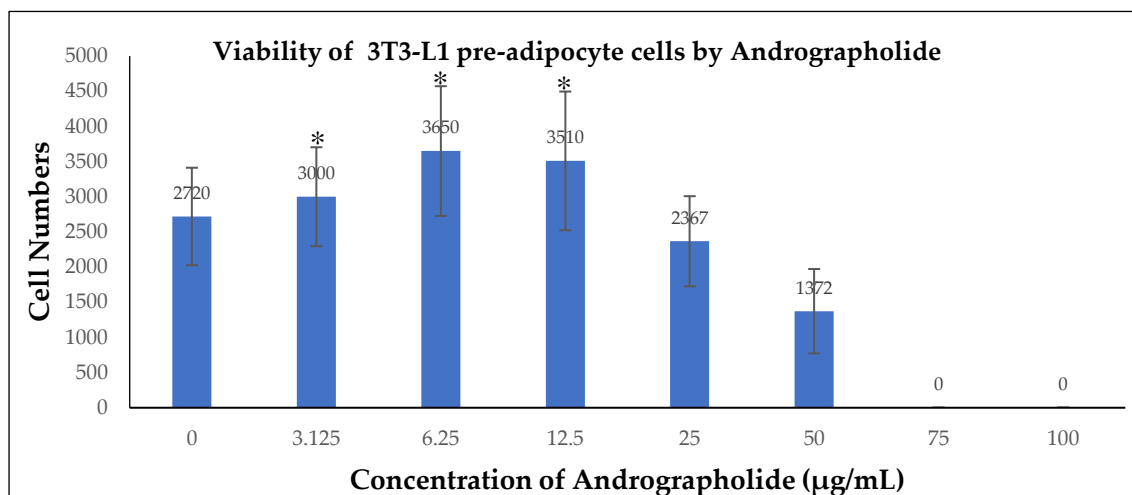
upon 2,5 ug/ml deoxyandrographolide treatment (Fig. 5), it was because in that dose could be happen proliferation cells. The viability of cells without any treatment except the media were 2720 cells, whereas after treatment with 3.125, 6.25, and 12.5  $\mu\text{g}/\text{mL}$  of andrographolide the viability of cells were still upper than the control. At the concentration of 25  $\mu\text{g}/\text{mL}$  the viability of cells were lower than the control, and than the viability decrease significantly at the concentration of 50  $\mu\text{g}/\text{mL}$  and at concentration more than 75  $\mu\text{g}/\text{mL}$  all cells were death, so the concentration of 25  $\mu\text{g}/\text{mL}$  was limit of concentration which did not influence significant viability of cells (Fig. 6). It meant that for further experiment the use of 12.5  $\mu\text{g}/\text{mL}$  deoxyandrographolide would be safe, whereas the safe concentration of andrographolide were 25  $\mu\text{g}/\text{mL}$ . The  $\text{LC}_{50}$  value was calculated from the correlation between percentage of death cells with the concentration using probit analysis according to Biostat Pro 5.9.8 software. The  $\text{LC}_{50}$  value of deoxyandrographolide and andrographolide was counted to see the effect of each isolates which was represent the 50% the death of cells. These were 29.32  $\mu\text{g}/\text{mL}$  and 37.70  $\mu\text{g}/\text{mL}$ , respectively. The statistical data of ANOVA showed that both isolates were significant compared to DMSO as control group ( $p < 0.05$ ). That was important to give not only the information about cytotoxic activity but also the cell viability for the next in vitro experiment.



**Figure 4.** Microscopic view of 3T3-L1 pre-adipocyte cells after treatment with DMSO (control group) and deoxyandrographolide 50  $\mu\text{g}/\text{mL}$



**Figure 5.** Viability of 3T3-L1 pre-adipocyte cells after treatment different concentration of deoxyandrographolide (average  $\pm$  SD,  $n=3$ ). Information: (\*) Significant difference result ( $p < 0.05$ ) compared to control



**Figure 6.** Viability of 3T3-L1 pre-adipocyte cells after treatment with different concentration of andrographolide (average  $\pm$  SD, n=3). Information: (\*) Significant difference result ( $p < 0,05$ ) compared to control

#### 4. CONCLUSION

By this study, we have evaluated the liquid-liquid fractionation method for isolating deoxyandrographolide and andrographolide from *A. paniculata* herbs. The method was found to be suitable for analysis because its simplicity, low cost, and time efficient. The cytotoxicity of deoxyandrographolide and andrographolide on 3T3-L1 pre-adipocyte cells was also evaluated and the LC<sub>50</sub> were 29.32 and 37.70 µg/mL, respectively.

**Acknowledgements:** We thank to Dexa Laboratories of Biomolecular Sciences (DLBS) for provision of research facilities. Great appreciation is also addressed to Direktorat Peneliti UGM through “Hibah Program Asistensi Riset 2019” for the financial support of the research.

**Conflict of interest:** There is no conflict of interest.

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