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## The inhibitory effect of *Andrographis paniculata* extract on proliferation of breast cancer cell line

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**Abstract.** Aerobic glycolysis is commonly used as the main energy source for cancer cell growth. Adenylate kinase 2 (AK2) plays an important role in maintenance of ATP production in the mitochondria. *Neoandrographolide* in *Andrographis paniculata* potentially inhibit AK2 activity *in silico*. This study aimed to investigate the inhibitory effect of *A. paniculata* extract on proliferation of breast cancer cell line. This experimental laboratory used MCF-7 cell line and *A. paniculata* plants, which were extracted using the maceration method with 70% ethanol. *Neoandrographolide* concentration in *A. paniculata* extract was determined using HPLC. A total  $1 \times 10^4$  MCF-7 cells were incubated for 24 hours with or without 0.4% (v/v) dimethyl sulfoxide as solvent (SC) or negative control (NC) group. The same amount of MCF-7 cells was treated with *A. paniculata* extract with 3 different dosages: 222 ppm (AE1), 111 ppm (AE2) and 55.5 ppm (AE3). The inhibition of cell proliferation used the 3-(4,5-dimethylthiazol-2-yl)-2,5-difeniltetrazolium bromide (MTT) assay. Data were analyzed using ANOVA and LSD tests with p value <0.05. Ethanol extract of *A. paniculata* contained 4.43  $\mu$ M *neoandrographolide*. The inhibition of MCF-7 cell proliferation was found in AE1 (47.98%) and AE2 (30.50%) groups and was significantly different from the NC group. While the percentage of MCF-7 cell inhibition in the AE1 group differed from NC (p = 0.002) and SC (p = 0.013) groups. Ethanol extract of *A. paniculata* can inhibit MCF-7 cell proliferation. Further Study is needed to investigate the role of *neoandrographolide* on inhibition of AK2 activity in MCF-7 cells.

**Keyword:** Adenylate kinase 2, Neoandrographolide, *Andrographis paniculata*, MCF-7 cell line.

### 1. Introduction

Cancer has become the second main cause of death around the world after cardiovascular diseases. World Health Organization (WHO) reported that there are 18 million new cases of cancer and it causes 9.6 million deaths worldwide in 2018 [1]. Meanwhile, the cancer prevalence in Indonesia has increased from 1.4% in 2013 to 1.8% in 2018 [2]. Breast cancer contributes to 43.3% cancer cases in female and affects 40 females per 100.000 in Indonesia. In recent years, breast cancer has become the highest cancer prevalence that increases mortality among females [3].



Surgery, radiotherapy and chemotherapy are the standard therapy for cancer [4]. Based on cancer type, grading and staging, such therapy can be given alone or combination [5]. Chemotherapy is usually used to inhibit cancer cell growth and metastasis in advanced stages [6] to improve survival rate of cancer patients [7]. So far, existing chemotherapeutic drugs have different action mechanisms. For example, cyclophosphamide and ifosfamide cross-link DNA and 5-fluorouracil inhibit activity of thymidylate synthetase. Meanwhile, methotrexate inhibit activity of dehydrofolatereductaese and doxorubicin and epirubicin directly bind to DNA by intercalation [4,8]. However, cancer therapy using these drugs not only kill cancer cells but also high proliferative normal cells, which lead to serious adverse effects like neuropathy and cardiomyopathy [9,10]. Furthermore, cancer cells have abilities to induce chemotherapy resistance [11].

Breast cancer cells proliferate through heterogenous pathways as it has overexpression of different receptors, corresponding for uncontrolled proliferation [12,13]. Approximately 60% breast cancer cases overexpress estrogen and/or progesterone receptors (ER+PR+) and 20% cases overexpress human epidermal growth factor receptor 2 (HER2+) and other cases do not express those receptors, well known as triple negative [14]. These molecular profiles of breast cancer cell are used not only for prediction of clinical prognosis but also for determination of appropriate chemotherapies [13,14], resulting in improvement of chemotherapy outcome and reduction of morbidity and mortality [12]. For example, breast cancer patients with ER+PR+ should be treated with endocrine therapy such as selective estrogen-receptor response modulators or SERM (e.g. tamoxifen) and aromatase inhibitors or AI (e.g. anastrozole, letrozole) [15]. Meanwhile, patients with triple negative receptors have only cytotoxic chemotherapy as the primary option [14,15]. Although tamoxifen and anastrozole have high efficacy and benefits, some adverse effects has been reported like pulmonary embolism, deep vein thrombosis, thrombophlebitis, osteoporosis and stroke [15,16]. Therefore, alternative drugs are needed to improve survival rate of patients with breast cancer.

In nature, many plants have secondary metabolites that are effective to kill cancer cells [17]. Indonesia has 30.000 plant species and approximately 9.600 species have pharmacological activities [18]. From biocomputational study, some phytochemical have been identified as new cancer drugs [19]. For example, neoandrographolide is able to interact with AK2 enzyme, better than a AK global inhibitor (ap5a). This phytochemical is found in *A. paniculata* plants [20], which are well known as “King of Bitters” and belong to acanthaceae family [21]. The bitter taste of this plant comes from its major secondary metabolite of diterpenoid groups such as andrographolide and neoandrographolide [17]. *A. paniculata* extracts have been used to treat fever, common cold, diabetes, hypertension and inflammation [22]. In vitro study showed that 111 ppm *A. paniculata* extract could inhibit 50% proliferation of MCF-7 breast cancer cell line. In addition, a mixture of 70:30% (v/v) ethanol and water can dissolve diterpenoid group compounds [23]. This study therefore aimed to investigate the effect of neoandrographolide in ethanol extract of *A. paniculata* plants on cell proliferation of MCF-7 breast cancer cells.

## 2. Methods

### 2.1. Extraction of *A. paniculata* plants and measurement of neoandrographolide concentration

Dried *A. paniculata* plants were obtained from plantations in Pakem, Sleman Regency, Yogyakarta Province, Indonesia and manually extracted using the maceration method with 70:30% (v/v) ethanol and water solvents in Phytochemical Laboratory, Faculty of Pharmacy, Gadjah Mada University. Pure neoandrographolide powder (Sigma-Aldrich®, USA) was used as a standard for measurement of neoandrographolide in *A. paniculata* Extract (AE). Before determination of neoandrographolide concentration, standard neoandrographolide was diluted using 55% (v/v) methanol (Merck®, Germany) and 45% (v/v) of aqua pro injection (Ikapharmindo®, Indonesia) to make 1,10,20,30,40 and 50 ppm final concentrations whereas AE was diluted with the same solvent to make 50 ppm concentration. A total of 20 µL diluted standard neoandrographolide and samples were injected into a column C18 (ACE-5, 250×4.6 mm) and automatically run into the HPLC device (waters e2695®

separation module, France) with (55:45) methanol and water as the mobile phase at 0.9 mL/min flow rate. HPLC was conducted in Central Laboratory, Faculty of Life Sciences, Universitas Sebelas Maret. Retention time and peak area on HPLC chromatograms were used to determine neoandrographolide compound and its concentration in EA samples. Linear regression equation was generated by plotting diluted concentrations of standard neoandrographolide and peak areas. This equation was then used to calculate the concentration of neoandrographolide in AE samples.

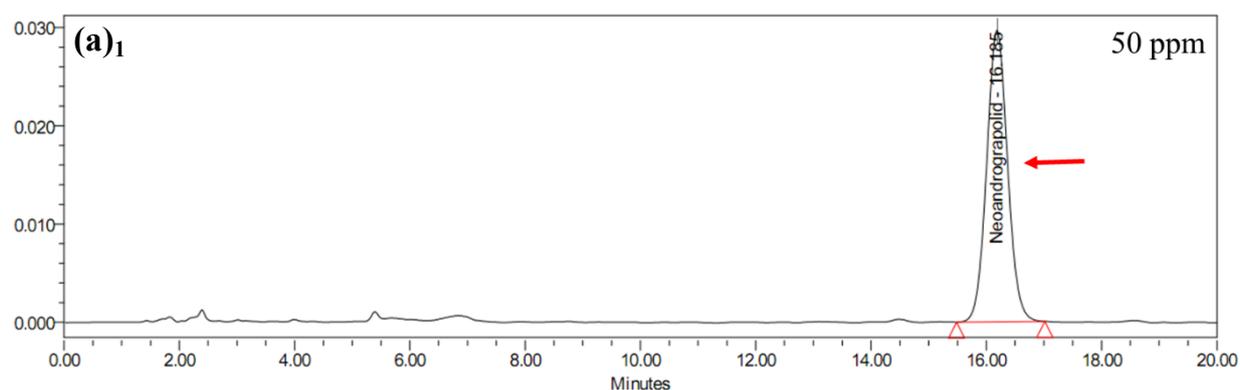
### 2.2. Inhibitory Assay of AE in MCF-7 Cells

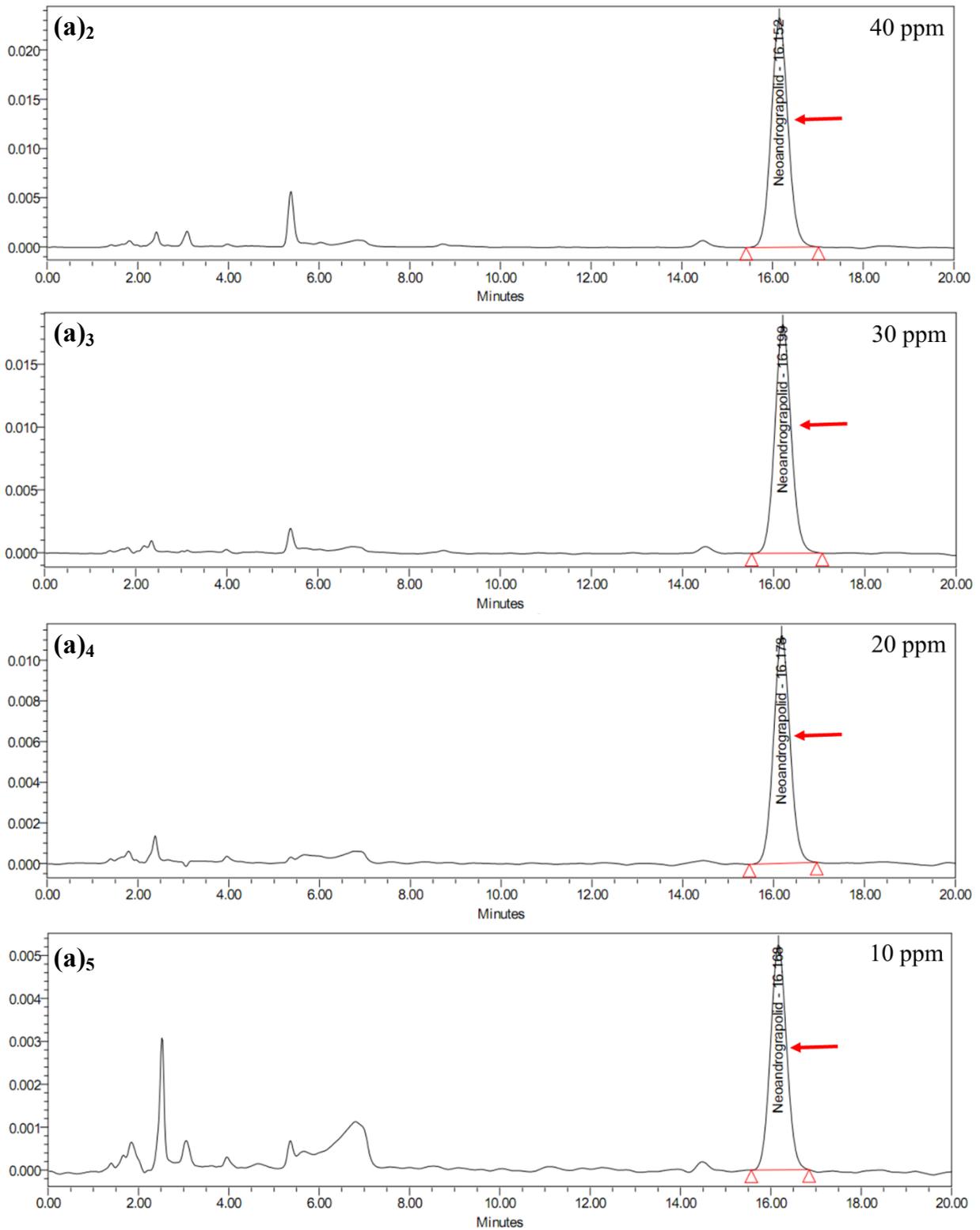
The protocol of cellular inhibition of MCF-7 cells using the MTT assay was adopted from the Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Gadjah Mada University, Yogyakarta. MCF-7 cell line stock was provided by the Biological Pharmacy Laboratory, Faculty of Pharmacy, Muhammadiyah Surakarta University. A total of  $1 \times 10^4$  cells of MCF-7 breast cancer cell line were seeded into 96-well microplate that contained Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco®, USA) and 1% (v/v) penicillin-streptomycin (Penstrep; Gibco®, USA) and incubated in a CO<sub>2</sub> incubator at 37 °C to reach confluence. Before treatment, AE was dissolved in dimethyl sulfoxide (DMSO) to make 3 different dosages. 222 ppm (AE1), 111 ppm (AE2) and 55.5 ppm (AE3). The following day, MCF-7 cells only were used as the negative control (NC) group while other MCF-7 cells were treated with 0.4% (v/v) DMSO as the solvent control (SC), and treated with 3 different doses of AE as treatment groups. At the end of incubation, all MCF-7 cell were added 100 µL (v/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Merck®, Germany) into each well and then incubated for 4 hours at 37°C. After adding 100 µL (v/v) SDS (Sigma-Aldrich®, USA) stop solution, absorbance values of MCF-7 cell were spectrophotometrically measured at 595 nm wavelength to detect formazan crystals. The difference of inhibitory effect of AE samples in MCF-7 cells was analyzed using the analysis of variance test and followed by the post hoc LSD test with p value <0.05.

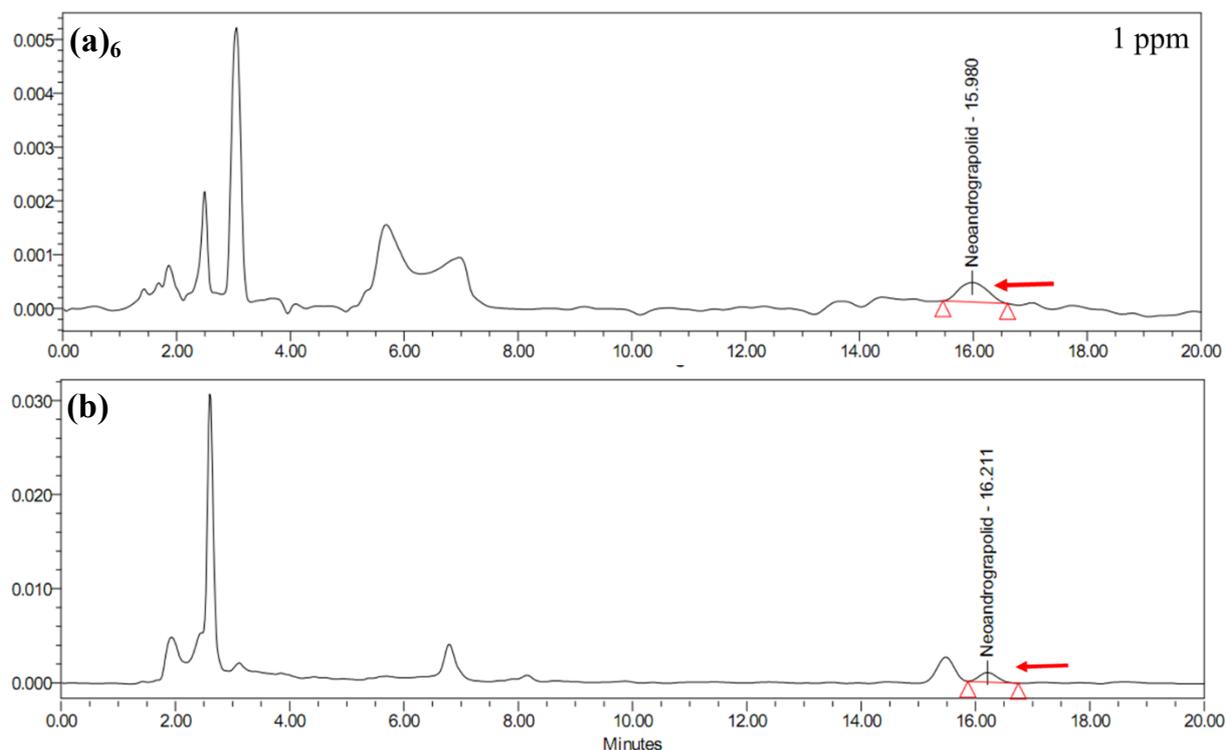
## 3. Result and discussion

From HPLC analysis, chromatograms and peak areas of 50 ppm standard neoandrographolide and AE were shown in Figure 1. Both samples had single peak and 16.2 min retention time. After generating a regression linear equation from six different concentration of neoandrographolide standard, neoandrographolide concentration in AE was 2.13 ppm or 4.43 µM. Our result is different from a research study conducted by Patarapanich et al. They have reported that methanol extract of *A. paniculata* derived from 4 different areas in Thailand contained different concentration of neoandrographolide. So, different results of these studies are perhaps related to different plantation conditions (soil fertility, rainfall, climate, season, etc.) and age plant on harvesting time [24].

Neoandrographolide belongs to the diterpenoid group that consists of andrographolide, isoandrographolide, dehydroandrographolide, 14-deoxyandrographolide and 14-deoxyandrographiside [25]. This compound is the second highest diterpenoid that is widely distributed in leaves, stem, twig and root of *A. paniculata* plants [26].



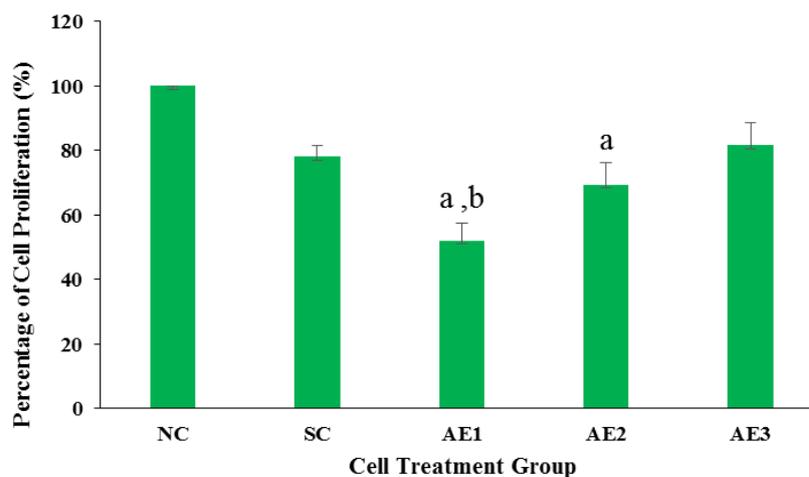




**Figure 1.** Results of HPLC chromatograms of **(a<sub>1</sub>-a<sub>6</sub>)** 6 diluted standards neoandrographolide and **(b)** 50 ppm AE. A total of 20  $\mu$ L diluted standard neoandrographolide and AE was injected into the C18 column that linked to a HPLC device. Each sample was performed in triplicate.

The next experiment was to evaluate inhibitory effect of AE using the MTT assay. Figure 2 showed that inhibitory effect of AE on MCF-7 breast cancer cell line was dose-dependent manner. Administration of 222 ppm AE (AE1) could significantly inhibit 47.98% MCF-7 cell proliferation, compared to SC ( $p=0.013$ ) and NC ( $p=0.002$ ) groups. In addition, a significant inhibition was observed in MCF-7 treated with 111 pm AE (AE2), compared to NC group ( $p=0.039$ ). According to American National Cancer Institute, crude extract is considered potentially as anticancer if it can inhibit 50% cell proliferation under 30 ppm [27]. So, ethanol extract of AE is potential to inhibit MCF-7 breast cancer cell but with a higher dose.

Our results of cell proliferation were different from Tarwadi et al. study. They reported that 111 ppm ethanol extract of *A. paniculata* can inhibit 50% cell proliferation [23], higher effect than our finding. The discrepancy of these results may be caused by cell passage number, which results in differences of phenotype, genotype, morphology, response to stimuli, growth rate and protein expression [28].



**Figure 2.** Inhibitory effect of AE on MCF-7 breast cancer cell line. A total of  $1 \times 10^4$  MCF-7 cells were grown into a 96 well plate for 24 hours at 37 °C. The following day cells were no treatment (NC), treated with 0.04% (v/v) DMSO (SC) and treated with 3 different doses of AE (AE1-3) for 24 hours at 37 °C. Cell proliferation rate was assessed using a MTT assay. a and b letters indicated statistical differences between treatment groups and NC/SC groups respectively.

We also found that inhibitory effect of AE can be derived from other secondary metabolites. Study has reported that *A. paniculata* plants also contain alkaloid, flavonoid, glycoside, aglycon flavonoid, tannin and steroid [29]. Some polyphenols, flavonoids and steroids were known having anti cancer effects [30]. Furthermore, other studies have documented that AE is able to inhibit cancer cells growth such as HT-29 colon cancer, IMR-32 neuroblastoma [31], human oral squamous cell carcinoma [32], HeLa cervical cancer, Widr colorectal cancer, T47D breast cancer [33] and A549 nonsmall cell lung cancer through several mechanisms [34]. At first, diterpenoid groups inhibit G0/G1 phase in cell cycle through induction of inhibitory protein p27 and decreased expression of cyclin dependent kinase 4 (CDK4) [35].

Diterpenoid groups also induce apoptosis through activation of caspase 8 and caspase cascade, change of Bax conformation and expression of Bcl-2 [36]. Inhibition of tyrosine kinase activity has an important role in lowering cancer cell proliferation, survival, invasion and metastasis [29]. Neoandrographolide is also recognized as a chemosensitizer agent, which inhibits DNA topoisomerase II in DNA synthesis and promotes cell apoptosis in S and G2 phase of cell cycle [37]. However, further study is needed to investigate the inhibitory effect of neoandrographolide in AE toward AK2 activity.

#### 4. Conclusion

Ethanol extract of *A. paniculata* plants contains 2.13 ppm (4.43  $\mu$ M) neoandrographolide and shows an inhibitory effect in MCF-7 cells with higher doses. Further investigation is required to find out the molecular mechanism of neoandrographolide that inhibits cancer cell growth.

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