

Duku (*Lansium domesticum*) Leaves Extract Induces Cell Cycle Arrest and Apoptosis of HepG2 Cells via PI3K/Akt Pathways

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Abstract

Liver cancer is a type of malignant cancer that causes many deaths in the world. Efforts to find natural ingredients that can be used to treat liver cancer are very important. This study investigated the anticancer activity of ethanol extract of Duku (*Lansium domesticum*) leaves (LDE) through the cytotoxic activity, cell cycle inhibition mechanism and apoptosis influence through the PI3K/Akt pathways. Secondary metabolites of LDE were determined by common qualitative method. Cell cycle arrest was carried out by flow cytometer at inhibitory concentration 50 (IC₅₀), while the apoptosis test was obtained using double staining method. The IC₅₀ value was used to identifying the expression of PI3K/Akt genes. The analyzed results were proven LDE can induce HepG2 cell death which IC₅₀ value of 19.93 ± 0.93 µg/mL (ethanol extract), 267.06 ± 3.24 µg/mL (ethyl acetate extract), and 216.47 ± 2.87 µg/mL (n-hexane extract). Cell cycle arrest activity in HepG2 cells which IC₅₀ value of ethanol extract of LDE occurs in S phase. Observation of apoptosis occurrence was obtained getting apoptosis in cell line. PI3K/Akt genes expression of ethanol extract of LDE was obtained using RT-PCR method. The bands density of PI3K/Akt at IC₅₀ value treatment of 0.55 ± 0.02; 0.25 ± 0.01. The LDE was determined to contain secondary metabolites which can inhibit the growth of HepG2 cells through PI3K/Akt pathways.

Keywords: *Lansium domesticum* corr, HepG2 cell, Cell cycle arrest, Apoptosis, PI3K/Akt

Introduction

The PI3K/Akt (Phosphatidylinositol 3-Kinase/ Protein Kinase-B) pathway is a signaling pathway that is widely used as a target for anticancer testing [1]. Intracellular pathways that are responsible for the regulation of cell death pathways, cell growth, and cell metabolism. This pathway is directly involved in oncoproteins and tumor suppressors that influence the regulation of cell metabolic signaling and thus affect the formation of cancer cells [2]. Several cancer drugs have been developed to inhibit the PI3K/Akt pathway because they are considered effective in affecting cancer cells [3]. HepG2 cells are cancer cells that express PI3K/Akt abnormally, causing the cell cycle and apoptosis processes become to abnormal. Suppression of PI3K/Akt expression will have an impact on the death process of HepG2 cells. This is based on the activity of several natural products that are efficacious as anticancer through their activity in the PI3K/Akt pathway [4,5]. Several compounds from natural ingredients have been reported to have activity in inhibiting PI3K/Akt expression. Flavonoid compounds from *Tephrosia kirilowii* were able to inhibit the expression of these genes *in silico* and *in vitro* test, causing cell cycle inhibition and triggering apoptosis in MCF-7 cells [6]. The same thing was confirmed by steroid compounds from *Trillium tschonoskii* which had an impact on RAW 264 cell death [7].

The use of natural products to treat cancer has increased. The number of studies that state the resistance of several types of chemotherapy and the side effects of these drugs are the reasons natural products are developed as cancer drugs [8,9]. The genus *Lansium domesticum* Corr. is a member of the *Meliaceae* family and is locally known as Duku. Duku is a well-known fruit in Southeast Asia, with

Malaysia, Thailand, the Philippines, and Indonesia producing the most of it. Duku tree bark and seeds have traditionally been used to treat diarrhea, cancer cell removal, and as an antipyretic and anthelmintic. Studies have extensively concentrated on the antimalarial, anticancer, and antibacterial properties of Duku [10]. This study focused on activity of Duku leaves extracts as anticancer in HepG2 cell. There has been no research on the secondary metabolite content of Duku leaves until recently. Alkaloids, flavonoids, tannins, triterpenoids, and saponins have been identified in Duku seeds [11]. The presence of these secondary metabolites is most likely found in Duku leaves. The investigation of Duku leaves action as an anticancer agent will begin with the discovery of secondary metabolites in the extract, cytotoxic tests, cell cycle and apoptosis testing, and the expression of the PI3K/Akt gene as the major cancer cell signaling pathway.

Materials and methods

Extract preparation

The maceration technique was used to make the Duku leaves extracts. Duku leaves was collected from Deli Serdang, North Sumatera, Indonesia. Duku leaves was identified as *Lansium domesticum* corr (Research Center for Biology, Indonesian Institute of Science). Duku leaves were washed and dried in a drying cabinet at a temperature of 40 - 50 °C. The sample is weighed 500 g and put into a container. The sample was steeped in 5 L of n-hexane for 6 h, agitated intermittently, and kept out for 18 h in the first maceration. Another sample was extracted using a same method, ethyl acetate and ethanol become a next solvent for extraction. The filtrate was again separated from the residue. Three times each the sample was soaked with solvents. A rotary evaporator has been used to evaporate the macerate from the 3 solvents, creating a crude extract. After evaporation, the extract is put into a tightly closed container, and stored at 2 - 8 °C [12].

Chemicals and equipments

DMEM medium, 0.25 % trypsin EDTA, Fetal bovine serum, Fungizone® (Gibco, USA), 0.4 % trypan blue, Dimethyl sulfoxide (DMSO), Penicillin-streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) (Sigma, USA), 96-well plate, 24-well plate (Corning, USA), Ethanol, Ethyl acetate, n-hexane (Merck, USA), Propidium Iodide kit (Biolegend), PI3K and Akt genes (Biossusa). Elisa Reader (Merck), FACS Flowcytometer (Biorad), Confocal Microscope, Poly Chain Reaction Instrument, Electrophoresis systems (Thermo Scientific), Rotary Evaporator (Heidolph, Germany).

Cell cultures

HepG2 cells were obtained from Parasitology Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia. HepG2 was cultured in DMEM medium and supplements with 10 % (v/v) fetal bovine serum, 1 % penicillin and streptomycin, and 0.5 % fungizone were inserted inside an incubator at 37 °C with 5 % CO₂. After cell growth at 95 % confluence, the cells were trypsinized by trypsin-EDTA to harvest the growing cells [13].

Phytochemical screening

Phytochemicals screening to determine alkaloids, flavonoids, steroid/ triterpenoids and tannins were carried out according to standard procedures. Alkaloids were identified using color reagents such as Mayer, Bouchardat, and Dragendorff. Shinoda and cyanidin test for flavonoids, tannin with FeCl₃ solution, and steroid/triterpenoids with Liebermann Bouchardat reagent [14].

Cytotoxic activity of extract

Various extracts of Duku leaves and sorafenib (as a control positive in this test) have been used to treat the cells. To obtain optimum growth, HepG2 cells were plated in a 96-well microplate at a density of 1×10⁴ cells/mL and incubated for 24 h. The medium was removed after 24 h with a new one. After that, the HepG2 cells after being mixed with the test solution that had been pre-dissolved with DMSO and incubated for 24 h at 37 °C in a 5 % CO₂ incubator. The media and test solution were removed at the end of the incubation, and the cells were cleaned with PBS. Then each well was loaded with 100 µL of culture medium and 10 µL of MTT 5 mg/mL. The cells were cultured in a 5 % CO₂ incubator at 37 °C for another 4 - 6 h to determine viability. The MTT reaction was ended with a reagent stopper (10 % SDS in 0.1 N HCl), and the plate was wrapped in aluminum foil and left out for 1 night at room temperature. MTT would induce living cells to react, producing in a purple color. At a wavelength of 595 nm, the absorbance (abs) were read by an ELISA reader [15]. The percent cell viability was calculated using the equation;

% Cell viability = $(\text{abs.sample} - \text{abs.blank}) / (\text{abs.control} - \text{abs.blank}) \times 100 \%$

Cell cycle arrest induce

In a 6-well plate, HepG2 cells (5×10^3 cells/well) were placed and incubated 24 h. The cells were then incubated 24 h after being treated with IC₅₀ concentration of active extract. The 0.025 % trypsin was used to collect the associated floating cells, which were then placed into the cone tube. Then 1 mL of PBS was added, and PBS was extracted with such a micropipette and centrifuged for 5 min at 2500 rpm. The liquid was extracted, and the RNase/propidium iodide (PI) dye solution was added for 10 min at 37 °C in a dark (light-free) condition. The proportion of cells collected in each cell cycle phase (G1/S and G2/M) could then be determined using Modfit Lt. The 3.0 s utilizing the FACS can flow cytometer [16].

Observation of apoptosis

HepG2 cells were seeded on coverslips and incubated for 24 h (1×10^5 cells/well, 24 well plate). The cells were then treated with active extract at the IC₅₀ concentration and incubated for 24 h. PBS was used to remove the medium and wash it. Coverslips were placed on a glass slide and incubated for 15 min with 10 µL of acridine orange-ethidium bromide (AO/EB). The samples were then examined using a confocal microscope [17].

Analysis of gene expressions *in vitro* with RT-PCR

The total ribonucleic acid (RNA) utilized (3000 ng) was reverse transcribed with a 1 g random primer and ReverTra Ace (Toyobo) for 10 min at 30 °C, 60 min at 42 °C, and 5 min at 99 °C, according to the manufacturer's procedure, to generate complementary deoxyribonucleic acid (cDNA) (20 L). The resultant cDNA mixture was diluted in TE buffer solution before being utilized directly in the PCRs. 1 µL cDNA was added to the 25 µL PCR Master Mix (12.5 µL GoTaqGreen, 9.5 µL DNase/RNase free water, 1 µL primer forward and 1 µL primer reverse, as shown in **Table 1**) to determine semiquantitative RT-PCR for the PI3K and AKT genes [18]. The combined solution was then added in 8 µL (5 RT-buffer 4 µL, random primary 1 µL, deoxynucleoside triphosphate 2 µL, and Revers Tra-Ace 1 L). 30 °C for 10 min, 42 °C for 60 min, and 99 °C for 5 min were used for PCR. The RT-PCR product was obtained as cDNA and kept at 20 °C. 1 µL of cDNA was added to 25 µL of PCR Master Mix, and 35 - 40 cycles were run for 15 - 30 s at 94 °C, 45 s at 94 °C, and 10 s at 55 - 60 °C, with a final extension phase at 72 °C for 5 min; the PCR products were then kept at 4 °C [19,20].

Table 1 Forward and reverse primer sequences used in this study were derived from previous report [21].

Genes		Primer sequence (5'-3')	Product length (bp)
PI3K	F	5'-GGA CAA TCG CCA ATT CAG-3	195
	R	5'-TGG TGG TGC TTT GAT CTG-3'	
Akt	F	5'-ATG AGC GAC GTG GCT ATT GTG ATT-3'	330
	R	5'-GAG GCC GTC AGC CAC AGT CTG GAT G-3'	

The bands were photographed using a Dox XR (Bio-Rad) Gel image scanner. Testing for activity avoidance from the genes PI3K and Akt were done by amplifying cDNA in HepG2 cells using a test solution, then electrophoresis on agarose 2 % with medium 10 % TBE to add the amplified cDNA findings [22].

Statistical analysis

Data were presented as mean \pm SEM. One-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test was used to analyze the data.

Results and discussion

Secondary metabolites content of Duku leaves extracts

The extracts were obtained using maceration multilevel method. A qualitative test was conducted to determine the secondary metabolite concentration of various Duku leaf extracts. Color reagents were used to determine the number of secondary metabolites such as alkaloids, flavonoids, steroid/terpenoids, and tannins in each Duku leaf extract [23]. % Yield of Duku leaves extracts and phytochemicals content was shown in **Table 2**.

Table 2 Phytochemical screening performed on ethanol, ethyl acetate, and n-hexane extract of Duku leaves.

Extract	% Yield	Alkaloids	Flavonoids	Steroid/Terpenoids	Tannins
Ethanol	42.9	+++	+++	+++	+++
Ethyl acetate	23.3	++	++	–	++
n-Hexane	12.4	–	–	+++	–

+++ : very abundant ++ : moderately abundant + : present – : absent

Identification of secondary metabolites was carried out on ethanol, ethyl acetate, and n-hexane extracts from Duku leaves. Duku leaf extract extracted using ethanol as a solvent gave the most optimal results. Alkaloids, flavonoids, terpenoids, and tannins were detected quite strongly in these extracts. Ethyl acetate solvent gave the second dominant yield after ethanol. In this extract, the presence of terpenoids was not detected. Meanwhile, the use of n-hexane solvent can only attract terpenoid compounds. Of course, this extract becomes more specific because it only attracts 1 type of secondary metabolite compound [24].

Cytotoxic activity of Duku leaves extracts

The MTT assay technique was used to test the cytotoxicity of extracts against HepG2 cells. Some extracts doses (31.25, 61.5, 125, 250 and 500 $\mu\text{g/mL}$) were used to treat HepG2 cells. Then, at 595 nm, the IC_{50} was determined using a microplate reader. Absorbance obtained from the test results are used to calculate cell viability. The results of % cell viability can be seen in **Figure 1**.

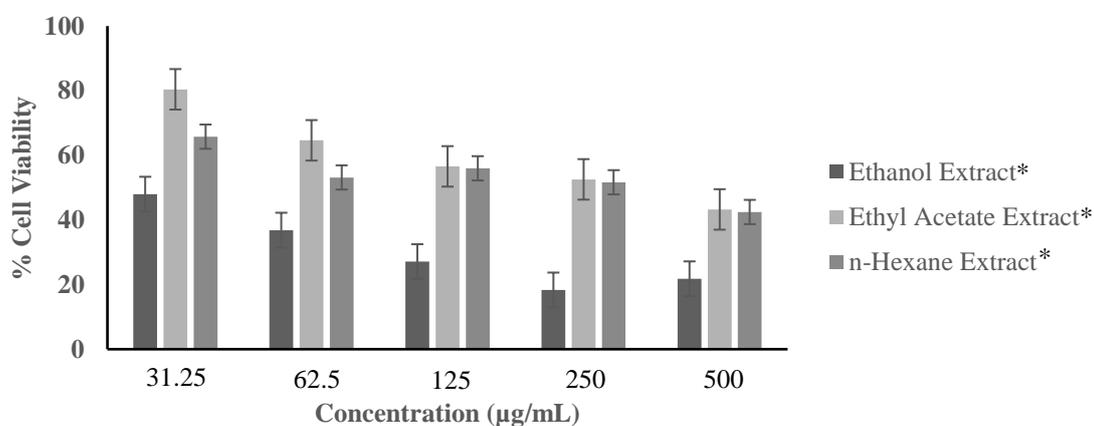


Figure 1 Inhibition of HepG2 cell population growth by Duku leaves extracts (mean \pm SE, $n = 3$), $*p < 0.05$.

According to **Figure 1**, each Duku leaf extract has a different influence on the development of HepG2 cells. Increased concentration leads to an increase in cell death. In comparison to other extracts, ethanol extract had the highest effect on HepG2 cell death ($p < 0.05$). These findings will be used to compute the IC_{50} value, which will be used to identify the optimum extract for future testing. In **Table 3**, the IC_{50} value may be found.

Table 3 IC₅₀ value of ethanol, ethyl acetate, and n-hexane extract of Duku leaves.

Extract	IC ₅₀ (µg/mL)
Ethanol	19.93 ± 0.93
Ethyl acetate	267.06 ± 3.24
n-Hexane	216.47 ± 2.87
Sorafenib	2.02 ± 0.53

Table 3 shows the results of the IC₅₀ test. It was discovered that extracts have cytotoxic properties. The ethanol extract has a better IC₅₀ value than another extract like ethyl acetate and n-hexane is each 19.93 ± 0.93, 267.06 ± 3.24 and 216.47 ± 2.87 µg/mL. Secondary metabolites content of extracts is influencing cytotoxic activity [25]. Alkaloids, flavonoids, terpenoids, and tannins were reported to inhibit the growth of cancer cells [26]. The mechanism action of Duku leaves extract absolutely need to know. LDE was to choose as a better extract than ethyl acetate and n-hexane. The IC₅₀ of LDE was categorized in good range and able to try in next test like the ability to inhibit cell cycle, inducing apoptosis, and affect gene expression of PI3K/Akt [27].

Cell cycle arrest activity

The flow cytometry technique revealed that there was an inhibition in the cell cycle phase. Flow cytometry was a technique for detecting each phase of the cell cycle based on the number of chromosomes present in each phase (G1, S, and G2/M) [28]. The distribution of cells in each phase of the cell cycle after treatment can be determined using this flow cytometry technique. In addition, the effectiveness of the extract in stopping the cell cycle can be calculated [29]. The effect of LDE on the cell cycle of HepG2 can be seen in **Figure 2**.

Flow cytometry was used to evaluate cell cycle distribution based on the cytotoxic activity described earlier. The dispersion of HepG2 cells treated with IC₅₀ doses of LDE (20 µg/mL) and sorafenib (2 µg/mL) during a 24-h incubation period is shown in **Figure 2**. To examine the distribution of HepG2 cells accumulating throughout the cell cycle, we utilized an untreated cell HepG2 as a control cell [30]. HepG2 cells treated with 20 µg/mL accumulated in the S phase, increasing from 19.58 % in untreated cells to 22.51 % in treated cells. In HepG2 cells, sorafenib has been shown to promote cell accumulation in the S phase, which is 24, 33 %.

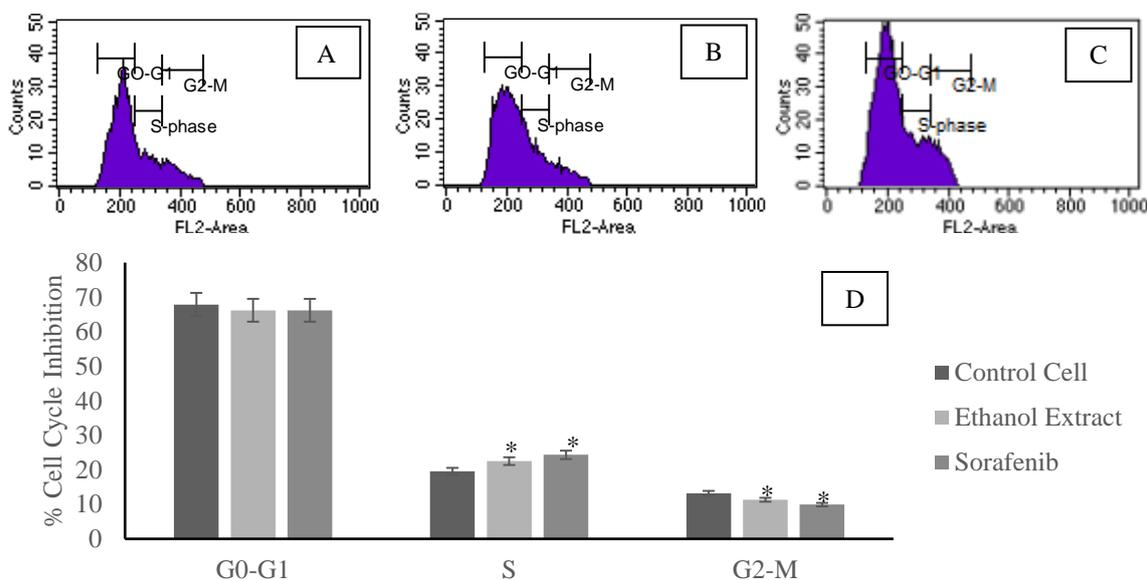


Figure 2 Cell cycle inhibition of ethanol extract against HepG2 cells (mean ± SD, n = 3). A) control cell, B) LDE in 20 µg/mL, C) sorafenib in 2 µg/mL, D) % cell cycle inhibition in G0 - G1, S, and G2-M phase, **p* < 0.05.

HepG2 cells treated with LDE produced cell cycle arrest in the S phase, according to this study. This finding suggests that one of LDE anticancer mechanisms may be the suppression of cell growth. Cyclins and CDKs are required for proper cell cycle control, and changes in the production of cyclin/CDK complexes have been demonstrated to impact cell growth, proliferation, and differentiation by death [31]. The S phase of the cell cycle is governed by Cyclin A, CDK2, and CDC25A. Cyclin A/CDK2 complexes activate CDC25A [32]. These complexes allow the cell cycle to proceed, and higher CDC25A expression promotes cell proliferation. As a result, more research into the extract's action on the production of the protein responsible for the S phase is required [33].

Apoptosis induction activity

After treatment with Duku leaves ethanol extract, AO/EB staining techniques clearly indicated induction of apoptosis in HepG2 cells. LDE treated HepG2 cells had obvious apoptotic characteristics that grew more apparent as the treatment period progressed (**Figure 3**). Observation of cell apoptosis was carried out subjectively using a confocal microscope.

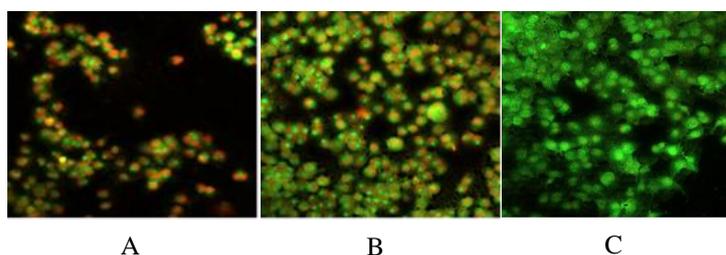
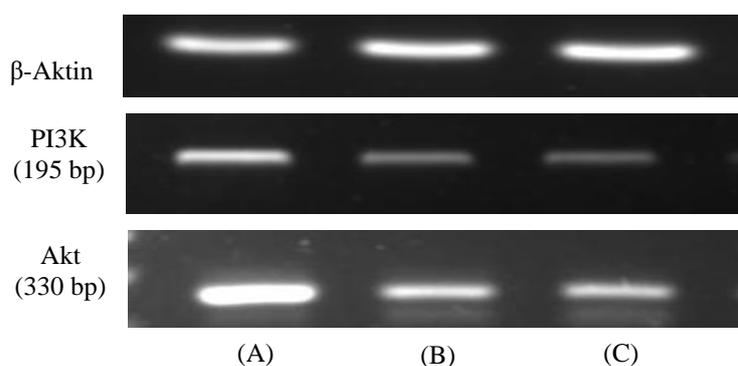


Figure 3 Images of stained HepG2 cells treated with Duku leaves ethanol extract (20 $\mu\text{g/mL}$) and sorafenib (2 $\mu\text{g/mL}$). Cells were cultured in serum-free DMEM medium and maintained at 37 $^{\circ}\text{C}$ and 5 % CO_2 . AO/EB fluorescence images of HepG2 cells with red showing the apoptotic cell. A) Control cell, B) LDE in 20 $\mu\text{g/mL}$, C) Sorafenib in 2 $\mu\text{g/mL}$.

In terms of color and shape, the AO/EB staining pictures revealed apoptotic alterations in LDE treated cells. The first visible difference between the untreated and treated cells was the apparent luminous coloration. The control cells were brilliant green in color and had an intact nuclear structure, indicating that they were healthy and alive [34]. Treatment cells, on the other hand, showed a strong orange stain, indicating the presence of apoptotic cells. The decrease of membrane permeability in the cells was shown by red fluorescence in the cells, indicating that leded ethidium bromide could enter the cells. It served as a warning sign that cells were dying [35].

PI3K/Akt genes expression using RT-PCR

By amplifying the cDNA HepG2 cells given a test solution, the expression of the genes PI3K/AKT was measured. Following that, the amplified cDNA was separated using electrophoresis on agarose 2 % with medium tris-borate ethylenediaminetetraacetic acid (TBE) 10 % and a 70 mV electric current. **Figure 3** shows the electrophoresis findings of HepG2 cells. The PI3K and Akt bands are more prominent than in the control group (**Figure 3**). The electrophoresis data were processed with Quantity One software to get the density of bands in the order in which they occurred, followed by the average and standard deviations.



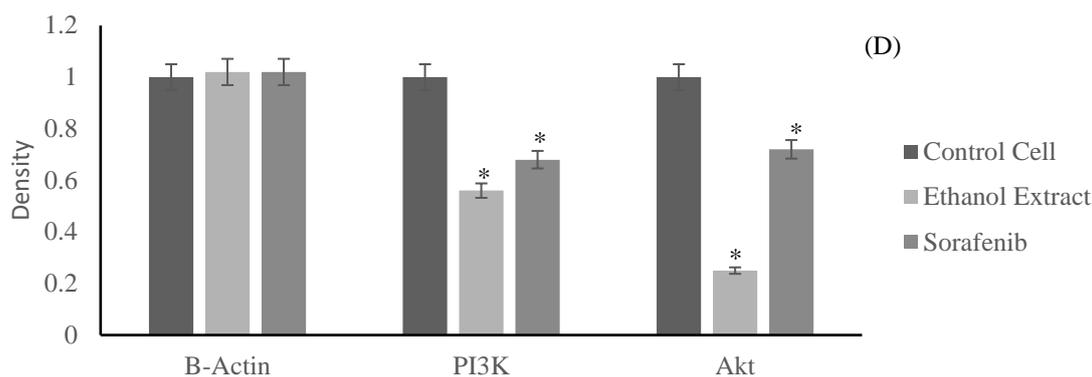


Figure 3 Gene expression of PI3K and Akt on the treatments of HepG2 cell line. (A) Band density of control cell, (B) Band density of LDE treatment, (C) Band density of sorafenib treatment, (D) The density value on the treatments, * $p < 0.05$ (mean \pm SD, $n = 3$).

Figure 3 shows the value of band density on treatments. LDE and sorafenib can decrease the density of PI3K and Akt band compared with the control cell. It means the LDE has an activity to influence the cancer cell growth through PI3K/Akt pathway. The value of band density on control cell, LDE, and sorafenib is 1.00 ± 0.00 ; 0.56 ± 0.02 ; and 0.68 ± 0.03 for PI3K, and 1.00 ± 0.00 ; 0.25 ± 0.01 ; and 0.72 ± 0.04 for Akt. The PI3K/AKT pathway is involved in the development of cancer cells and promotes cell division and death [36]. Because the activity of the PI3K/AKT gene reduces in a direct manner, cell cycle inhibition and apoptosis are impeded, and cancer cell growth is hindered [37]. Can be seen the influence of the effect on the inhibition of the cell cycle. The LDE caused the cells to be inhibited in the S phase with a percentage of 22.5 %, compared to the control only 19.58 %. Likewise, its effect on promoting apoptosis. Based on the test results, the cells given the LDE can trigger apoptosis when compared to control cells. The terpenoids compound of plant reported have activity to inhibit the expression of PI3K/Akt. Polyisoprenoid in *Nypa fruticans* reported effective to inhibit WiDr cell through PI3K/Akt pathways [38]. Then, in breast cancer cells, a combination of sesquiterpene lactones, such as vernodalol, vernodalinol, and vernomygdin, was found to suppress PI3K/AKT1 phosphorylation [39].

Conclusions

The LDE was shown to have anticancer activity in HepG2 cells in a particular way in this investigation. Duku leaves ethanol extract was able to inhibit the cycle in the S phase, induce apoptosis in HepG2 cells, and affect the expression of PI3K and Akt genes. More research into the selectivity of possible chemicals, as well as effective molecular methods for apoptotic cell death induction, is required to gain a better understanding of the mechanism.

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