ANTICANCER ACTIVITY OF ETHANOL EXTRACT OF YELLOW ROOT
(Arcangelisia flava) ON HEPG2 HEPATOCELLULAR CANCER CELLS

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ABSTRACT

This study aimed to evaluate anticancer activity and apoptosis induction of ethanolic extract of Arcangelisia flava (AF) roots on HepG2 cancer cell lines. The AF roots were extracted by maceration using ethanol 80%. MTT assay method was used to evaluate the anticancer activity and the proliferation of HepG2 cells. Flow cytometry method was used to assess the induction of HepG2 cells apoptosis. This study showed that the IC50 of AF ethanol extract against HepG2 cells was 109.14 µg/mL. With IC50 treatment, the apoptosis assay showed a significant decrease in intact cells (80.10±1.7%) and a significant increase in early apoptosis (7.9±0.7%) and late apoptosis cells of HepG2 cancer cells (4.9±0.35%) compared to control cells. Moreover, the proliferation of HepG2 cells was declined significantly in 48 and 72 hours after treatment with IC50 (77.5±5.76% and 64.3±5.37%, respectively) and 2xIC50 of the extract (75.9±1.79% and 70.5±4.27%, respectively). This research suggests that the ethanolic extract of AF roots can potentially be used for hepatocarcinoma treatment.

Key words: anticancer activity, Arcangelisia flava, cancer cell lines, hepatocarcinoma, HepG2

INTRODUCTION

Liver and intrahepatic bile duct cancer is the fifth leading cause of death related to cancer among men and seventh among women in the US (Siegel et al. 2021). In 2015, untreated Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection led to 720,000 deaths due to cirrhosis and 470,000 deaths due to hepatocellular carcinoma (World Health Organization 2017). Hepatocellular carcinoma (HCC) is accounted for more than 90% of liver cancer, with HBV infection being the most common risk factor. Other risk factors included obesity, excess alcohol consumption, cigarette smoking, and hepatitis B virus, and hepatitis C virus infection. Recently, non-alcoholic steatohepatitis (NASH) associated with metabolic syndrome or diabetes mellitus was assigned as one of the etiologies of hepatocellular carcinoma (HCC) (Llovet et al. 2021).

Several drugs such as atezolizumab plus bevacizumab, sorafenib, lenvatinib, regorafenib, cabozantinib, and ramucirumab have been approved by FDA based on phase III trials for liver cancer (Llovet et al. 2021). However, sorafenib could be difficult to tolerate due to side effects such as anorexia, nausea, vomiting, and weight loss, hoarseness of voice, esthesia, and hypertension. Adjustment of dose and treatment interruption is often needed which raises concern about its effectiveness (Balogh et al. 2016).

Sorafenib efficacy in HCC patients with impaired liver function (Child Pugh B) was questioned, significantly when treated with this agent shortened the survival rate compared to a patient with Child-Pugh A and increased the incidence of severe adverse effects (Le Grazie et al. 2017). Combined regimens such as GEMOX (gemcitabine + oxaliplatin) and PIAF (cisplatin + Adriamycin + 5-FU + INF) have shown promising results. However, chemoresistance is still a major issue that can result in a relapse of the diseases. Various mechanisms are involved in chemoresistance, such as apoptosis evasion, autophagy activation, drug expulsion to epigenetic transformation (Loihiest et al. 2018). Therefore, a study on developing novel therapeutic agents in response to the chemoresistance mechanism is necessary.

Arcangelisia flava (AF), known as the yellow root, is widely found in Kalimantan Island, Indonesia. This plant was used by the local community as an ailment for malaria, fever and dysentery (Heryani and Nugroho 2016). Several bioactive compounds have been isolated from this plant, such as berberine, palmitine, and jatrorrhizine, which have shown anticancer activity against MCF 7 breast cancer cells by suppressing the transforming growth factor-beta 1 (TGF-β1) expression (Niwat et al. 2005). Inhibition of TGF-β1 expression (Kim et al. 2018), targeting ephrin-B (Ma et al. 2017), AMPK signaling pathway (Pan et al. 2017), inhibition of specific activator protein-1 (AP-1) activity (Jeong et
al. 2018), triggering to caspase9-dependent apoptosis (Zhao et al. 2017), affecting mRNA levels of chemokine receptors genes such as C-X-C motif chemokine receptor 1 (CXCR1) and C-X-C motif chemokine receptor 4 (CXCR4) (Ahmadiania et al. 2016) and by inducing nucleolar stress and upregulation of p53, a tumor suppressor gene (Sakaguchi et al. 2020) were several mechanisms berberine acts as anticancer against breast cancer cell line. Berberine also showed activity in inhibiting the proliferation and reproduction of several tumorigenic microorganisms such as Helicobacter pylori and hepatitis B virus (Sun et al. 2009). However, the study about the antiproliferative properties of AF against hepatocellular carcinoma is limited. Therefore, this study aimed to determine the anticancer activity of AF extract against HepG2 cell lines.

MATERIALS AND METHODS

Extraction of Arcalengisia flava Roots

As much as 500 g of AF dry root powder was macerated with 1.5 L of 80% ethanol for 24 hours and stirred. After 24 hours the filtrate was filtered, and the maceration was repeated for three times. The obtained filtrate was collected, and vacuum evaporated with a rotary vacuum evaporator at a temperature of 50° C until a thick extract was obtained. Afterwards, the extract was heated at a temperature of 50° C until a constant weight was obtained. The goal was to remove residual ethanol from the extract. The final ethanol extract was weighted and resulted in 22.5 g.

Preparation of HepG2 Cells

HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and cultured at 37° C in a humidified atmosphere containing 5% CO₂ until confluent (70-80% confluent).

Cytotoxic Activity of Ethanol Extract of Arcalengisia flava Roots

The cytotoxic activity was investigated by using MTT colorimetry assay following the previously described method (Yusuf et al. 2020). The ethanol extract of AF was divided into seven different concentrations as follows: 500, 250, 125, 62.5, 31.25, 15.63, and 7.8 µg/mL. HepG2, Vero cells and growth media were seeded into microplates with 24 hours incubation then discarded. Then, 100 µL of each AF concentration was added to the microplate and incubated for 24 hours. Afterwards, MTT solution was added to the microplate and incubated for 4 hours until formazan crystals were formed. Subsequently, 10% SDS solution was added to stop the reaction and incubated overnight. The experiment was repeated three times. The absorbance was read by enzymelinked immunosorbent assay (ELISA) reader at λ 595 nm, which were then converted to the percentage of viable cells by using formula as follows:

% Viable cells = treated cell absorbance - medium absorbance
control cell absorbance - medium absorbance × 100%

The percentage of viable cells was used to calculate the IC₅₀ value using Graph Pad Prism v.8.0.2 (GraphPad Software, CA, USA), with nonlinear regression analysis.

Apoptosis Assay by Flow Cytometry

Apoptosis assay was carried out by seeding the HepG2 cell and growth media into 6-wells plates and then treated with IC₅₀ of AF ethanol extract with 24 hours incubation at 37° C. The cells were then washed and stained with propidium iodide and RNase in PBS (Roche, Mannheim, Germany). The apoptosis phases were visualized by using BD FACS Calibur Flow cytometer (Becton Dickinson, California, US) and analyzed by Modfit Lt. 3.0 (CCRC 2014). This assay was carried out in duplicate.

The Proliferation of HepG2 Cells

The proliferation of HepG2 cells was determined by using the doubling time test. HepG2 cells and growth media were seeded into 96 well plates and then treated with ½IC₅₀, IC₅₀, and 2×IC₅₀ of AF ethanol extracts with 72 hours incubation and observed every 24 hours. Then, MTT was added to the microplate and incubated for four hours until formazan crystals were formed. The stop solution (10% SDS in 0.1N hydrochloric acid) was then added and incubated overnight in a dark place. The absorbance of the plates was read by ELISA reader at λ 550-600 nm. The result was converted to the percentage of viable cells using the aforementioned formula. This doubling time test was conducted in triplicate.

Data Analysis

All data was presented as mean ± SD. All statistical analysis was conducted by using Graph Pad Prism v.8.0.2 (GraphPad Software, CA, USA). Multiple t-test was conducted to determine the significance of apoptosis assay in each phase between control cell and experimental cells with IC₅₀ treatment of AF ethanol extract. Analysis of variance (ANOVA) test followed by a Tukey posttest was used to determine the significance of proliferation test between ½IC₅₀, IC₅₀, and 2×IC₅₀ of AF ethanol extracts at 24, 48, and 72 hours.

RESULTS AND DISCUSSION

Cytotoxic Activity of Ethanol Extract of Arcalengisia flava Roots

The percentage of HepG2 and Vero viable cells against AF ethanol extract was presented in Figure 1. The IC₅₀ of AF ethanol extract against HepG2 cells was 109.14 µg/mL. The viability of HepG2 cells after treatment with 62.5 µg/mL of AF extracts was 50%, while treatment with 500 µg/mL resulted in only 19% living cells. On the other hand, 62.5 µg/mL of AF extracts treatment yielded 95.9% viable Vero cells, while treatment with 500 µg/mL of extracts only decreased the intact Vero cells by 3.2% (92.7%).
The yellow root was also known for its cytotoxicity effect for WiDr colorectal cancer cell line with IC\(_{50}\) varied between 114.119 μg/mL (from Malinau district, North Kalimantan, Indonesia) and 582.857 μg/mL (from Banjarmasin, South Kalimantan, Indonesia) (Mutiah et al. 2020). The potential mechanism by which this plant could interfere with cancer cell growth was based on the blocking of the G1 phase by berberine, one of the compounds found in AF roots. Berberine could bind with DNA and RNA and induce DNA damage in cancer cells by regulating the activity of DNA topoisomerase, leading to cell death (Bhadra and Kumar 2011).

**Apoptosis Assay by Flow Cytometry**

Figure 2 showed the apoptosis analysis using flow cytometry on HepG2 with IC\(_{50}\) of AF ethanol extract treatment and control cells. The intact cells (P1) between HepG2 (80.10±1.7%) and control cells (90.6±1.28%) showed a significant difference (P<0.05). More early apoptosis cells (P2) were observed on HepG2 compared to control cells (7.9±0.7% and 1.35±0.2%, respectively). Meanwhile, HepG2 cells (4.9±0.35%) which were undergone late apoptosis phase/P3 differ significantly when compared to control cells (1.9±0.28%) (P<0.05). However, no significant difference in P4 was observed between both cells.

Study of berberine in combination with curcumin against breast cancer cell lines showed a synergistic growth-inhibitory effect by triggering apoptosis and autophagic cell death, associated with activation of AMP activated protein kinase (AMPK) and an increased expression of the inactive form of acetyl-CoA carboxylase (ACC) (Yu et al. 2014). The apoptosis involved extracellular signal-regulated kinase (ERK) activation and caspase-dependent pathway, while the autophagic cell death involved c-Jun N-terminal kinase (JNK) activation, Bcl-2 phosphorylation and the dissociation of Beclin1/Bcl-2 complex (Wang et al. 2016).

**The Proliferation of HepG2 Cells**

The proliferation of HepG2 cells at 24, 48, and 72 hours were presented on Figure 3. No significant

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**Figure 1.** The percentage of HepG2 and Vero viable cells against ethanol extract of *Arcalengisia flava* roots

**Figure 2.** The apoptosis analysis on HepG2 and control cells with IC\(_{50}\) treatment of ethanol extract of *Arcalengisia flava* roots
difference was observed between treatment with ½ IC$_{50}$, IC$_{50}$, and 2x IC$_{50}$ of AF ethanol extracts on HepG2 viable cells in the first 24 hours with mean±SD for each treatment was 80.8±9.33%, 85.5±4.61%, and 89.3±5.28%, respectively. At 48 hours, the intact cells of HepG2 after IC$_{50}$ (77.5±5.76%) and 2x IC$_{50}$ (75.9±1.79%) treatments were significantly declined compared to ½IC$_{50}$ (59.5±8.49%) treatment with P<0.05. Treatment with IC$_{50}$ and 2xIC$_{50}$ after 72 hours showed decreased in the intact cells of HepG2 substantially compared to ½IC$_{50}$ with P<0.05 (IC$_{50}$= 64.3±5.37% and 2xIC$_{50}$= 70.5±4.27% compared to 50.5±3.68%, respectively).

The proliferation of cancer cells was also affected by Epidermal Growth Factor Receptor (EGFR). Disruption in EGFR can inhibit this process. Berberine was a potential EFGR inhibitor that showed a decline of HepG2 cells after treatment with IC$_{50}$ and 2xIC$_{50}$ (Figure 3). Other molecular docking study showed that berberine gave the most negative bond-free energy and constant least inhibition of all EGFRs, with the highest affinity indicated for EGFR-2 with ΔG of -9.34 kcal/mol and the predictive inhibition constant of 141.81 nM. This result means that berberine has the activity as an EGFR inhibitor, especially for EGFR-2 (Pratama 2016).

Berberine may up-regulate p53 expression by suppressing the inner inhibitor MDM2 at the post-transcriptional level, which inhibits Bcl-2 by BAX, which increases the BAX/ Bcl-2 ratio and induces cell apoptosis by Apaf-1 regulation of the underlying signal caspase 3 (Shukla et al. 2016; Ma et al. 2019; Nkpa et al. 2019). Activating TP53 (wild-type tumour protein p53) by berberine also played an important role in the induction of tumor cell apoptosis (Zhang et al. 2020). Another advantage of berberine is its ability to suppress tumor metastasis by inhibiting the release of MMP-2 (matrix metalloproteinases 2) from tumor cells and thus inhibits tumor cell destruction of the tissue matrix (Serafim et al. 2008; Cai et al. 2014).

Another active compound of AF roots is palmatine. Palmatine was proven to inhibit the viability and proliferation of breast cancer cells selectively, while no effect was observed on normal breast cells. Moreover, palmatine combined with doxorubicin increased breast cancer cells’ sensitization to doxorubicin and decreased cell viability more than each compound alone. Synergistic and additive effect on this combination was shown by isobolographic analysis (Grabarska et al. 2021). A synergistic effect was also observed in combination of palmatine and gemcitabine in inhibiting the growth of pancreatic cancer cell lines (Chakravarthy et al. 2018).

CONCLUSION

Arcalengisia flava roots showed a cytotoxic effect on HepG2 cells by inducing apoptosis and inhibiting cancer cell proliferation. Several potential mechanisms involved include the trigger of apoptosis and autophagic cell death, disruption in EGFR, and upregulation of p53.

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