Anti-cancer Effects of *Azadirachta indica* in Diethylnitrosamine-Induced Hepatocellular Carcinoma in Wistar Rats

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Abstract: ***Hepatocellular carcinoma (HCC) is a primary liver cancer and has a high rate of mortality. Exploration of local herbal as a chemo-preventive drug has been conducted. This present study aimed to observe the anti-cancer effect of hydroethanolic extracts of Azadirachta indica both Indonesia (HEAII) and the Philippines (HEAIP) on diethylnitrosamine (DEN)-induced HCC on Wistar rat model. VEGF/FGF2/CD166/YAP were evaluated using immunohistochemistry. VEGF/FGF2/CD166/YAP were down-regulated in HCC treated with HEAII and HEAIP compare to that non-treatment HCC. Liver function (aminotransferase (AST) and aminotransferase (ALT) activities) was evaluated from serum. Value of AST and ALT were abnormal in the non-treatment HCC and normal in the HCC treated. These results indicated that both extracts were found to possess anti-cancer activity in HCC.***

Keywords**: *hepatocelullar carcinoma; Azadirachta indica; cluster differential 166 (CD166); fibroblast growth factor 2(FGF-2); yes associated protein (YAP); vascular endothelial growth factor (VEGF)***

I. INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary liver tumor and a major health issue in developing country. The prevalence of HCC is common cause of cancer-related death in Asia Pasific (Zhu *et al.*, 2016). Treatment of HCC remains challenge, due to late diagnosis and short of therapeutic option. Systemic therapeutic as possible chemotherapy is Sorafenib but the efficiency of this drug is limited (Grazie *et al.*, 2017). Therefore, discovering novel therapeutic targets and therapeutic strategies for HCC is still necessary.

Currently, available drugs are plant-based, turning out to be a crucial source of biological compound performing bio activities that can be exploited to produce medicine. Plant contains diverse active ingredients which can be used as medicine for diseases such as cancer. Among the medicinal plants, one of the commonly found plants in tropical countries traditionally named Mimba (*Azadirachta indica).* In the Philippines, it is known as neem. *A. indica* is native plant to South Asia. *A. indica was* widely planted and naturalized in semiarid areas throughout Asia and Africa (Kumar and Navaratnam, 2013). *A*s a medicinal plant, it has been reported as anti-parasitic, anti-inflammatory, and anti-diabetic. Moreover, *A. indica* extract has also anti-tumor activities (Paul *et al.*, 2011).

One of threatening phenomenon in neoplasm is angiogenesis and metastasis. Angiogenesis is establishment process of making new blood vessel. It is a regular part of growth and healing; however it has a role as key player in the growth and metastasis of cancer. Restriction of angiogenesis is a substantial approach for cancer treatment and prevention. Numerous factors expressed when cancer-promoted angiogenesis are vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2). Angiogenesis is also essential for the propagatic and development of tumor metastasis (Folkman, 2002).

Metastatic tumor occurs because of the activation of cluster of differentiation 166 (CD166) and Yes-associated protein (YAP). Their activation promotes proliferation and metastasis. CD166 and YAP can be a potential prognostic marker in cancer malignancy. Previous study by Tang *et al.*,(2015) reports that CD166 and YAP are involved in hepatocarcinogenesis.

This research aimed to evaluate the anti-cancer effects of hydroethalonic extract *A. indica* in diethylnitrosamine inducing hepatocellular carcinoma in wistar rats through the expression of CD166/VEGF/FGF2/YAP using immunohistochemistry and liver function aminotransferase (AST)/aminotransferase (ALT) activities.

II. MATERIALS AND METHODS

A. Chemicals

Diethylnitrosamine (DEN) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Sorafenib was brought from Dr. Soetomo Hospital, Surabaya. All other chemicals used for experiments were analytical grade.

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B. Identification of Plant Material and Preparation of Azadirachta indica Extract

A.indica were collected from the Madura Island, Indonesia and Camiling, Philippines on November 2018. Its leaves were dried under shadow, grinded and extracted by maceration with 80% ethanol at room temperature for 3 days. The extract was concentrated under controlled temperature and pressure (50°C) in a rotary evaporator. The extract was a yellowish brown and was preserved in 4°C.

C. Experimental Animal

Healthy male Wistar rats (*Rattus norvegicus*) were purchased from Institut Biosains, Malang. The animals were caged individually in controlled temperature (25 ± 2˚C) with light cycle (12h dark/light). Rats were fed with pellet and water ad libitum. A total of 27 rats were divided into 3 groups, each group containing nine animals. This research procedure was approved by Institut Biosains, Universitas Brawijaya, Indonesia. No. 1138-KEP-38.

D. Immunohistochemistry

Liver samples were processed in standard protocol of fixation, embedding, deparaffinization, labeling primary antibody (VEGF, FGF-2, YAP, and CD166) and secondary antibody, counterstaining.

E. AST and ALT

AST and ALT were collected from centrifuged blood. Centrifuge was performed by 3000 rpm at room temperature for 10-15 minutes. Then, data was analyzed by One Way Anova statistical test and pass the Tukey test with a confidence of 95%.

III. RESULT AND DISCUSSION

The results were shown in (Figure 1. and Tabel 1). All values are mean ± standard deviation. Data are analyzed by One-way ANOVA followed by Tukey's post hoc method of analysis, where *p< 0

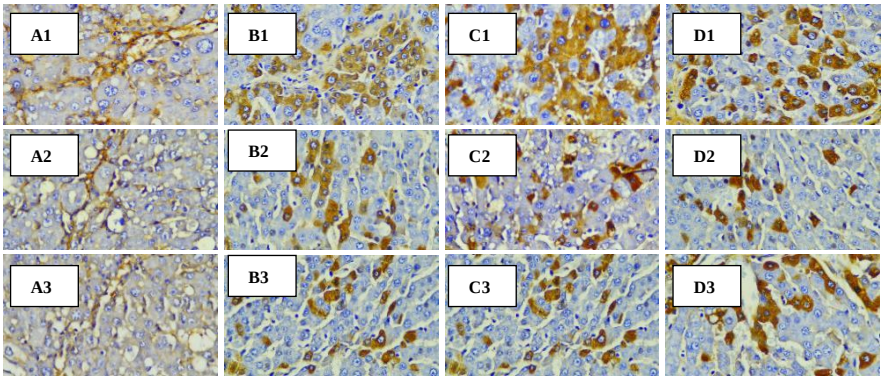

Figure 1. Expression of A (VEGF), B (FGF2), C (CD166), D (YAP) in liver tissues of rats by immunohistochemistry. Visualized by an optical microscope (x400). Group 1 (non-treated HCC), Group 2 (non-treated HCC with HEAII), Group 3 (non-treated HCC with HEAII)

Table 1. Activity of AST and ALT

Groups	AST (average ± SD)	ALT (average ± SD)
Non-Treatment (IU/L)	250.5555^{a} ± 15.28162	83.4444^{a} ± 5.7288
HEAII Treatment (IU/L)	138.6667^{b} ± 37.62978	43.0000^{b} ± 10.53565
HEAIP Treatment (IU/L)	142.7778^{b} ± 33.7074	45.3333^{b} ± 10.35616

IV. DISCUSSION

Previous studies suggest that impaired liver function reflects tumor induced liver damage (Huang *et al.*, 2006). Therefore, we explored the relationships between expression of VEGF, FGF2, CD166, and YAP and liver function test parameters after treatment with HEAII and HEAIF damage induced by HCC after treatment with HEAII and HEAIF.

The present study was aimed to observe the anti-cancer effect of hydroethanolic extracts of *Azadirachta indica* both Indonesia (HEAII) and the Philippines (HEAIP) on diethylnitrosamine (DEN)-induced HCC on Wistar rat model. They were tested for their anticancer effect by imunoexpression of VEGF/FGF2/CD166/YAP and level of AST/ALT level. VEGF/FGF2/CD166/YAP were evaluated using immunohistochemistry. VEGF (A), FGF2 (B), CD166 (C), YAP (D) were down-regulated in HCC treated both HEAII (A2, B2, C2, D2) and HEAIP (A3, B3, C3, D3) compare to those non-treatment HCC (A1, B1, C1, D1).

VEGF and FGF 2 in treatment group is decrease than non treatment group. As we know, VEGF plays as a key pivotal roles in regulating normal and abnormal angiogenesis (Niu and Chen, 2010). VEGF expression increase by FGF-2. FGF-2 is a powerful inducer of angiogenesis and differentiation in numerous organs (Seghezzi *et al.*, 1998). Menchanism of VEGF-FGF2 induce angiogenesis is through intracelullar menchanism (Seghezzi *et al.*, 1998). HCC is a typical hyper vascular tumor; many angiogenic factors have been studied in this cancer (Niu and Chen, 2010). This study showed that HEAII and HEAIP treatment decreased VEGF and FGF2, indicating that the formation tumor angiogenesis decreased because FGF-2 production upregulates VEGF expression. Therefore, it may be said that the anti-angiogenic activity of *A. indica* might correspond to phytochemicals such as terpenoids and flavonoids present in large amount in the HEAII and HEAIP.

CD166 has been indicated to participate in the metastatic cascade of cancer cells. CD166 plays an important role in many biological activities, including T-cell activation and proliferation, angiogenesis, hematopoiesis (Ni *et al.*, 2013). CD166 was linked to YAP in liver cancer, and YAP is over expressed in 62% of HCC patients. Membrane protein CD166 increase YAP to take carcinogenic in HCC (Ma *et al.*, 2014). YAP are nuclear localized in aggressive cancers. Thus, co expression of CD166 and YAP could be prognostic in cancer (Zhang *et al.*, 2018). This study showed CD166/YAP down regulated HCC treated with HEAII and HEAIP compare to that HCC non-treatment. In Figure I, expression of CD166 and YAP decrease in liver tissue of both HCC treated groups. This indicating that both *A. indica* extract has anti metastatic in HCC.

Liver function (aminotransferase (AST) and aminotransferase (ALT) activities) was evaluated from serum. Hepatocyte damage due to hepatocellular carcinoma arising from the emergence of necrotic hepatocyte products in the circulatory system to form aspartate aminotransferase (ALT). Increased activity of the ALT enzyme when liver tissue is damaged allows karmaous plasma membrane damage resulting in protein leakage. This observation looked at the effect of HEAII and HEAIP on the activity of AST and ALT enzymes in the liver of hepatocellular carcinoma rats obtained compared with non treated HCC.

Analysis of AST and ALT enzyme activity was carried out quantitatively by measuring the absorbance of AST and ALT enzyme activity products. Based on the results obtained continued with the One Way Anova statistical test and pass the Tukey test with a confidence of 95% showing the therapeutic choice of AST and ALT enzyme activity in the liver. Tukey test results showed no difference between therapies.

In this study, the high activity of AST and ALT before the administration of HEAII and HEAIP therapy showed that there was damage to the liver tissue. ALT and AST are produced by malignant and non-malignant cells. Compared to normal cells, AST and ALT activity increases after hepatic neoplasia. AST and ALT are found in serum proportional to the amount of cell damage. Most cancer cells produce ATP through glycolysis in aerobic conditions rather than through the tricarboxylic acid cycle. Glycolysis is needed in cancer cells to produce ATP and anabolic precursors needed for survival, growth, and invasion. The aspartate aminotransferase enzyme catalyzes the conversion of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate. (Washington and Hoosier, 2012), in which glutamate functions as a precursor to provide carbon and nitrogen for the biosynthesis of metabolites involved in cancer survival and proliferation. Glutamate has been shown to maintain the TCA cycle and increase gluconeogenesis to increase cancer cell growth. AST functions with malate dehydrogenase to transfer electrons from nicotinamide adenine dinucleotide (NADH) across the inner mitochondrial membrane, which is closely related to glycolysis (Shen *et al.*, 2014; Stocken *et al.*, 2008). Thus, AST can be associated with the prognosis of hepatocellular carcinoma cancer

V. CONCLUSION

In conclusion, these findings suggest that HEAII and HEAIP inhibits migration, invasion, angiogenesis response of HCC and increase liver function toward normal. Our study indicates the importance for further validation of the anti-angiogenic potential in pre-clinical models and in clinical trials, for successful neem treatment into the clinic to prevent tumor progression.

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