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Screening of anticancer, hepatoprotective and nephroprotective effects of ethanol extract of *Elephantopus scaber* L.

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Abstract: 7.12-dimethylbenz (α)anthracene (DMBA) is a carcinogenic compound. It is metabolized in the liver by cytochrome P450 enzyme into 7.12-dimethylbenz (α) anthracene-3.4-diol-1.2-epoxide (DMBA-DE) which is more reactive and can damage the hepatic cell. Furthermore, DMBA also can damage the kidneys and cause Reactive Oxygen Species (ROS) accumulation resulting in oxidative stress. This study aimed to determine the activity of *Elephantopus scaber* L as anticancer, hepatoprotector and nephroprotector. Forty female Sprague Dawley (SD) rats were divided into five groups: One control group and four treatment groups. Treatment group II was given 20mg/kg body weight (BW) of DMBA. Groups III, IV, and V were given DMBA and *E. scaber* extract at 50mg/kg BW, 100mg/kg BW and 200mg/kgBW, respectively. Those treatments were orally administered for 5 weeks. Week 6-15 is a time to observe the appearance of nodules and body weight. At the beginning of the 16th week, blood was collected to calculate the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine. Data were analyzed using one way ANOVA method with 95% confidence level. Based on the AUC value, body weight showed no significant difference in each group ($p>0.05$). The highest potential for tumor inhibition was obtained at the dose of 200mg/kg BW. The results of AST, ALT, urea and creatinine levels showed a significant difference between all dose treatment groups with negative controls ($p<0.05$). Ethanol extract of *E. scaber* possibly inhibits the appearance of nodules, protects liver and kidneys cell.

Keywords: *E. scaber*, DMBA, cancer, hepatoprotector, nephroprotector.

INTRODUCTION

Cancer is responsible for the death of most world population. Cancer ranks second place in the cause of death in the world (Khan *et al.*, 2015). Cancer is characterized by unchecked and uncontrolled proliferation of cells caused by the dysfunction of various essential genes which code for vital proteins including anti-apoptotic proteins, transcription factors, tumor suppressors, growth factors and growth factors receptors (Khan *et al.*, 2016). Abnormal cell growth is triggered by various factors, one of which is pollutants such as cigarette and vehicle smoke containing DMBA (Hodgson, 2001). The active metabolite of DMBA is DMBA-3, 4-diol-1,2 epoxide (DMBA-DE) which is a free radical that able to bind DNA, this interaction can lead to cell mutations (Hakkak *et al.*, 2005). Besides causing the formation of cancer cells, DMBA is also toxic for liver and kidneys by interfering mitochondrial performance through Reactive Oxygen Species (ROS). Excessive ROS can lead to oxidative stress (Sharma *et al.*, 2012).

Cell mutations have been linked to oxidative stress initiated by the reaction of free radicals with biological macromolecules such as proteins, lipids, and DNA. Antioxidants, preferably from natural sources, are considered effective treatment for cancer. Previous studies have shown that *E. scaber* contains sesquiterpenes as antitumor (Farha, *et al.*, 2014). Deoxyelephantopin (ES-4), isodeoxyelephantopin (ES-5), scabertopin (ES-2) and isoscabertopin (ES-3) are sesquiterpenes lactone compounds isolated from *E. scaber* leaves which have antiproliferation activity in cancer cells (Xu, *et al.*, 2006). *E. scaber* also contains flavonoid luteolin-7-glucoside as antioxidant compound (Wan *et al.*, 2012). The current study investigated the effect of anticancer in an animal model by measuring the nodule appearance and body weight. The hepatoprotective effect was measured by the AST and ALT levels, while the nephroprotective effect was measured by creatinine and urea levels. DMBA was used to chemically induce cancer.

MATERIALS AND METHODS

E. scaber leaves was collected from Sentolo, KulonProgo, Yogyakarta, Indonesia and the plant was identified from Laboratory of Biology, Universitas Ahmad Dahlan Yogyakarta with No. 011/Lab.Bio/B/II/2018. Ethanol 70%, Tween 80 and distilled water were purchased from Brataco. DMBA was purchased from Sigma. Corn oil was purchased from a local supermarket (Superindo). Nodule Appearance, Creatinine and Urea levels, also AST and ALT levels were analyzed using commercial reagent kits.

Plants extraction

The leaves of *E. scaber* were cleaned, dried in an oven at 50-60°C and powdered. An ethanol extract was obtained by maceration using 70% ethanol. The extract obtained

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was then evaporated using rotary evaporator at 50°C and heated over the waterbath until thick extract was obtained.

Drug and administration

5% Tween 80 solution

2.5grams of Tween 80 was put in a 50ml measuring flask and then added to the mark with distilled water, then stirred until it dissolves completely.

E. scaber extract dosage

This research used 3 dose groups namely 50mg/kgBW, 100mg/kgBW and 200mg/kgBW. Each extract dosage solution was obtained by suspending the thick extract in tween 80% solution.

DMBA solution

The dose of DMBA solution induced to rats was 20mg/kg BW with a concentration of 0.4% dissolved in corn oil.

Animal and experimental groups

The animals were caged at room temperatures of 30°C and humidity 55% with a 12/12 hours light-dark cycle. The rats had free access to water and were fed with a standard pellet diet (BR-2) *ad libitum*. Forty female Sprague Dawley rats divided into five groups. Group I was the control group. Group II was given DMBA solution. Groups III, IV, and V were given DMBA solution and *E. scaber* extract at 50mg/kg BW, 100mg/kg BW, and 200 mg/kg BW, respectively. DMBA solution was administered orally twice a week for 5 weeks while *E. scaber* extract was administered daily by oral gavage for 5 weeks. During the 6th to 15th week, nodules growth was observed. Blood sampling of female SD strains was carried out at the orbital sinus (eye) at the beginning of the 16th week. Ethical approval of this study has been accepted by Ethical Research Committees Universitas Ahmad Dahlan Yogyakarta with No. 011606117.

Observations and analysis

Nodule appearance

Palpation was done to find out the development of nodules from 6th week. The observation was done until the 16th week.

AST and ALT levels

Blood serum was obtained by centrifugation at a speed of 6000 rpm for 15 minutes. AST and ALT blood serum activity was measured by using the principle of a kinetic method that was determined by the International Federation of Chemical Chemistry (IFCC) using UV-Vis spectrophotometer. AST and ALT activity calculated by multiplying the average absorbance of blood serum by a factor.

Urea and creatinine levels

Blood serum was obtained by centrifugation at a speed of 4000 rpm for 10 minutes. Creatinine blood serum activity was measured using the Jaffe Method. While Ureum blood serum activity was measured by using enzymatic Urease-GLDH UV test method.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS 21. One way analysis followed by ANOVA was used to analyze the significance of data. Results were considered significant when $p<0.05$.

RESULT

Body weight

Rats were weighed every week on the same day in all groups of rats, both the healthy control group, negative controls, and the treatment group doses of 50, 100 and 200mg/kgBW which were weighed using a digital scale (Neraca Ohaus). Observations were begun at 6th week after DMBA induction until the 16th week (fig.1, table 1).

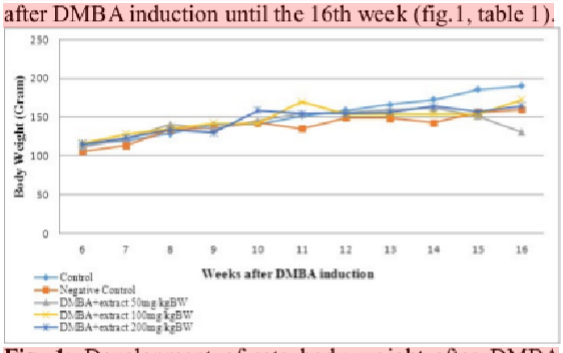

Fig. 1: Development of rats body weight after DMBA induction and administration of *E. scaber* ethanol extract.

Table 1: AUC value of the body weight development curve

Groups	X ± SD
Control	126.47 ± 48.00
Negative Control	62.86 ± 35.20
DMBA+extract 50 mg/kgBW	106.49 ± 55.11
DMBA+extract 100 mg/kgBW	134.18 ± 41.20
DMBA+extract 200 mg/kgBW	94.20 ± 64.25

Nodule appearance

The observation of nodul appearance on 16th week. Surgery was performed by taking nodules on all parts of the body which showed the appearance of nodules in each group of rats (fig. 2).

Tumor incidence by DMBA-induced

The percentage of normal control was 0%. The nodules appeared on the negative control group reached 75%. The other groups on 100mg/kgBW revealed 62.5% of rats produced nodules. Furthermore, the dose 50mg/kgBW and 200mg/kgBW had only 37.5% and 25% rats with nodules respectively (table 2).

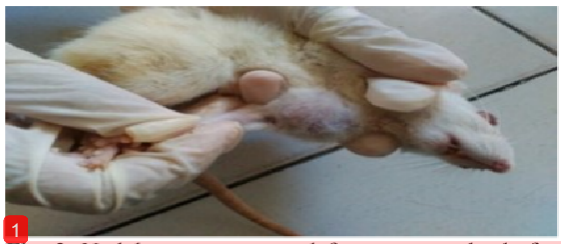

Fig. 2: Nodule appearance on left mammary gland after DMBA induction

Potential Inhibition of Ethanol Extract E. scaber

The subcutaneous administration of active fraction of *E. scaber* significantly inhibited the growth of subcutaneously transplanted DLA and EAC solid tumors, delayed the onset of tumor formation and increased the life span of tumor-bearing rats (Geetha *et al.*, 2010). The highest percentage of tumor inhibitory potential was in the extract dose of 200mg/kg BW, whereas the lowest percentage was in the dose of 100mg/kg BW (table 3).

Multiplicity of tumors

Multiplicity tumors provide synchronous results on the potential of tumor inhibition and tumor incidence proportionally. The dose 200mg/kgBW has the lowest number of the tumor (table 4).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity

AST and ALT activity calculated by multiplying the average absorbance of blood serum by a factor. The negative control group has the AST level 142.8±25.92 U/L and ALT level 74.31±18.51 U/L (figs. 3, 4, table 5).

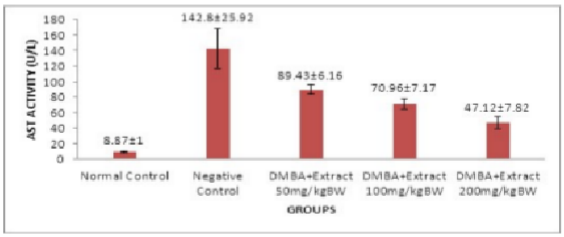

Fig. 3: Average of AST levelin rats blood serumafter DMBA induction and administration of *E. scaber* ethanol extract

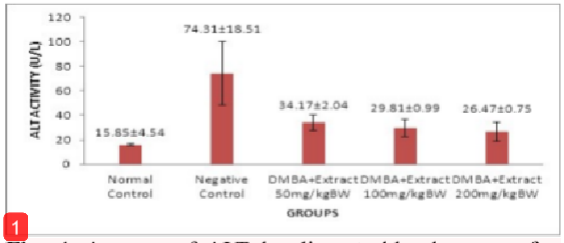

Fig. 4: Average of ALT levelin rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract.

Nephroprotector

Urea Levels

Ureum levels were determined using enzymatic Urease-GLDH UV test method. Urease enzyme catalyzes the hydrolysis of urea into ammonia and carbonate. NADH oxidation causes a decrease in absorbance at 340 nm (table 6).

Creatinine levels

Creatinine levels examined using the Jaffe method. The principle of this method is a complex compound formed by the reaction of creatinine with picric acid in an alkali environment. The amount of complex compound formed has a linear correlation with creatinine levels (table 7).

DISCUSSION

Body weight

Based on fig.1, the body weight of all groups was increased significantly. Meanwhile, the negative control was the lowest weight among treatment period caused by DMBA induction. DMBA caused palpated nodules in all of the group treatments. The highest palpation nodules were found in the negative control group, while others were not as high as negative controls because they were treated with extracts. The nodule has a linear correlated to inflammation. Furthermore, inflammation affects stress management which caused loss of appetite.

Based on Honors and Kinzig (2014), cancer incidence is followed by a loss of appetite and body weight. Moreover, DMBA induction orally leads to loss of body weight caused by tumor soreness level.

The table 1 shows that body weight of group treated by *E. scaber* on 100mg/kgBW was dramatically increased. The condition has a possibility of *E. scaber* as a chemo-preventive effect. Based on statistical analysis using Kolmogorov-Smirnov obtained significant value 0.038 ($p<0.05$) that means data was not normally distributed. Then, data were analyzed by Kruskal-Wallis to determinate the differences between control and treatment groups. The significant value shows 0.079 (>0.05) that means there are no differences between control and treatment groups.

Nodule appearance

Effect of 20mg/kg BW DMBA induction twice a week for 5 weeks seen by surgery on week 6th (fig. 2). Based on necropsy result, the appearance of nodules was found in the lower of the armpit, whereas the nodule appearance on left mammary gland has appeared in the dose of extract 50mg/kg BW and 100mg/kg BW. Based on previous research by Thu *et al* (2018), showed that all groups that were induced by DMBA were not produced the palpable nodules on mammary glands macroscopically, whereas a rat with DMBA-induced has diagnosed with breast cancer

Table 2: Tumor incidence in rats after DMBA induction and administration of *E. scaber* ethanol extract

Groups	Number of Rats	Number of Rats With Nodul	Incidence (%)	Latency Time (weeks, after final induction)
Normal control	8	0	0	0
Negative control	8	6	75	2
DMBA+extract 50 mg/kgBW	8	3	37.5	7
DMBA+extract 100 mg/kgBW	8	5	62.5	4
DMBA+extract 200 mg/kgBW	8	2	25	4

Table 3: The calculation of potential inhibition of tumors in DMBA-induced rats

Groups	Potential Inhibitory (%)
DMBA + extract 50 mg/kgBW	50
DMBA + extract 100 mg/kgBW	16.67
DMBA + extract 200 mg/kgBW	66.67

Table 4: Average of tumors multiplicity in ratsafter DMBA induction and administration of *E. scaber* ethanol extract

Groups	Tumor multiplicity (Means)
Normal control	0
Negative control	1.13
DMBA + extract 50mg/kgBW	0.88
DMBA + extract 100mg/kgBW	1.50
DMBA + extract 200mg/kgBW	0.50

Table 5: Average of AST and ALT activityin rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract

Groups	Mean±SD (U/L)	
	AST	ALT
Normal Control	8.87± 1.00*	15.85±4.54*
Negative Control	142.8±25.92	74.31±18.51
DMBA + extract 50 mg/kgBW	89.43±6.16*	34.17±2.04*
DMBA + extract 100 mg/kgBW	70.96±7.17*	29.81±0.99*
DMBA + extract 200 mg/kgBW	47.12±7.82*	26.47±0.75*

*: showing the significant difference to negative control ($p<0.05$)

Table 6: Urea levels in rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract

Groups	Urea levels (mg/dL) ± SD
Normal Control	45.64 ± 1.53*
Negative Control	57.87 ± 8.84
DMBA + extract 50 mg/kgBW	36.13 ± 2.72*
DMBA + extract 100 mg/kgBW	31.24 ± 3.58*
DMBA + extract 200 mg/kgBW	27.98 ± 3.36*

*: showing the significant difference to negative control ($p<0.05$)

Table 7: Creatinine levels examination in rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract

Groups	Creatinine Level (mg/dL) ± SD
Normal Control	0.66 ± 0.07*
Negative Control	0.9 ± 0.16
DMBA + extract 50 mg/kgBW	0.49 ± 0.09*
DMBA + extract 100 mg/kgBW	0.45 ± 0.09*
DMBA + extract 200 mg/kgBW	0.28 ± 0.07*

*: showing the significant difference to negative control ($p<0.05$)

microscopically. Based on that, DMBA-induced on rats were not given a specific appearance of nodules in mammary glands.

Tumor incidence by DMBA-induced

Based on Choi *et al* (2018), squamous cell carcinoma was successfully maturated after 5 months treatment with DMBA and 12-O-tetradecanoyl phorbol-13-acetate (TPA). Squamous cell hyperplasia with chronic inflammation was detected in the skin dermis of rats (Choi *et al.,* 2018). Moreover, Kwon *et al* (2018) said that DMBA significantly upregulated the expression of specificity protein 1 (Sp1), a transcription factor, and the carcinogenic effects of DMBA were blocked via the suppression or interruption of Sp1 activity. That means DMBA induced carcinogenic effects by upregulating Sp1 activity (Kwon *et al.,* 2018). Table 2 shows the reduction of tumor incidence for all of the groups. The percentage of normal control was 0% that means there was no appearance of nodules founded. The nodules appeared on the negative control group reached 75%. The other groups on 100mg/kgBW revealed 62.5% of rats produced nodules. Furthermore, the dose 50mg/kgBW and 200mg/kgBW had only 37.5% and 25% rats with nodules respectively. That means DMBA is a carcinogenic compound capable of inducing rats into tumor models.

Potential Inhibition of Ethanol Extract E. scaber

Based on similar research by Kabeer *et al* (2017), showed that Deoxyelephantopin, sesquiterpenes from *E. scaber* increased the expression of p53, phospho-Jun amino-terminal kinases (p-JNK), p-p38, increased the expression of phospho-signal transducer and activator of transcription 3 (p-STAT3), and phospho-mammalian target of rapamycin (p-mTOR) in cancer cell. These findings are kindly useful as a chemotherapeutic agent against cancer (Kabeer *et al.,* 2017). Geetha *et al* (2010) said that their present studies significantly inhibited the incidence of sarcomas and reduced the tumor diameter. The table 3 shows that the highest percentage of tumor inhibitory potential was in the extract dose of 200mg/kg BW, whereas the lowest percentage was in the dose of 100mg/kg BW. Based on the previous data of tumor incidence, the data were related to potential inhibitory. The extract dose 200mg/kg BW has the lowest incidence and the highest potential inhibitory than others. That means the dose of 200mg/kg BW can reduce the incidence of tumors and inhibit tumor growth.

Multiplicity of tumors

Ethanol extract of *E. scaber* in addition to inhibiting tumor incidence, it was also able to reduce the average tumor multiplicity as presented on table 4. It can be observed that the 200mg/kg BW has the lowest number of the tumor. Based on Mehmood *et al.* (2017), Deoxyelephantopin from *E. scaber* induces apoptosis through multiple signaling pathways which are deregulated in cancer cells and suggested that by targeting multiple pathways simultaneously, these compounds could selectively kill cancer cells (Mehmood *et al.,* 2017). Moreover, Meiyanto (2012) said that ethanol extract of *Gynura procumbens* L had similar content with ethanol extract *E. scaber*, which is an antioxidant compound. The antioxidant compounds were also able to reduce the average multiplicity tumor in DMBA-induced on rats by the dose of 20mg/kg BW 2 times a week for 5 weeks. That means the antioxidant compounds have the ability to obstruct cancer by inhibiting cancer proliferation.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity

Normally, AST and ALT activity has a range between 40-80 U/L (Lin *et al.,* 2018). Based on other researches, normal control group has the AST level 55.45±20.29 U/L and ALT level 75.67±2.08 U/L (Yadie, 2012). The other research shows AST level 70.36±10.93 U/L and ALT level 33.97±11.43 U/L (Raghavendra *et al.,* 2014). Meanwhile, the data above has the AST level 8.87±1.00 U/L and ALT level 15.85±4.54 U/L, lower than normal level. The differences between AST and ALT level normal control (experimental) and normal level (theory) can be caused by several factors which are stress, treatments, and method (Yadie, 2012).

Table 5 shows that the negative control group has the AST level 142.8±25.92 U/L and ALT level 74.31±18.51 U/L. The ratio between normal control and negative control are significantly increased by negative control. That means DMBA caused hepatocyte cell damage on rats. Loss of structural integrity at the cellular level due to stress-induced oxidative damage was demonstrated by significant increases in the hepatic levels of intracellular marker enzymes (Suhail *et al.,* 2011). Furthermore, the AST and ALT level of each dose were respectively decreased compared with normal and negative control. The higher dose of ethanol extract *E. scaber*, the higher decreasing level of AST and ALT. Decreasing AST and ALT level was completely performed with various doses (Fig. 3, 4).

Fig. 3 and 4 shows that ethanol extract *E. scaber* with various doses 50mg/kg BW, 100mg/kg BW and 200mg/kg BW were decreased AST and ALT level after treatment, respectively. *E. scaber* contains flavonoids and phenolic compounds that have high antioxidant values (Wan *et al.,* 2012). Several studies have also shown that the hepatoprotective activity is highly correlated to the phenolic content of a given plant and the flavonoid content was suggested as the main contributor to this bioactivity (Akanitapichat *et al.,* 2010). Decreasing the level of AST and ALT was indicated as restoration or recovery. The restoration of AST and ALT levels suggested that *E. scaber* could potentially restore hepatic cells or more specifically the integrity of cell membrane

and the normalization of hepatocytes into their normal tissue structure (Wan *et al.*, 2012).

Based on the analysis of statistical data, there were significant differences between AST and ALT levels in group I and group II with a significant value of 0.020 ($p<0.05$). It can be concluded that DMBA lead to a significant increase in AST and ALT levels. Groups III, IV, and V had lower AST and ALT levels compared to group II and showed statistically significant differences with a significance value of 0.000 ($p<0.05$). This means that the ethanol extract of *E. scaber* is able to lower blood AST and ALT levels significantly.

Nephroprotector

Urea Levels

Normal urea level in rats is 15-21mg/dL (Nikolic *et al.*, 2003). Urea levels in group II was higher than group III, IV, and V. Based on table 6, Group II's urea level was the highest among all of the experiment groups. That was similar to a research conducted by Ozdemir *et al.*, (2007) that rats injected by DMBA at a dose of 50mg/kg BW lead to increase of urea levels by 24.0±0.45. DMBA induced ROS accumulation ROS (Sharma *et al.*, 2012). Excessive ROS induces oxidative stress resulting in glomerulus damage. Disability of glomerulus to filter urea causes accumulation of urea in the blood resulting in increasing of urea levels (Dakrory, 2015).

Based on statistical analysis, it was found that there were significant differences between group I and group II with a significant value of 0.029 ($p<0.05$). It means that DMBA injection causes urea levels to increase significantly. Groups III, IV and V had lower urea levels than group II, which were 36.13mg/dL, 31.24mg/dL, and 27.98mg/dL, respectively. The results showed that the ethanol extract of *E. scaber* has the ability to decrease urea levels. Statistical analysis showed that urea levels of group III, IV and V differed significantly from group II with a significant value of 0.029 ($p<0.05$). This result indicated that ethanol extract of *E. scaber* at a dose of 50mg/kg BW, 100mg/kg BW, and 200mg/kgBW can reduce urea levels significantly. That was similar to research conducted by Marcelo *et al* (2014) that 70% ethanol extract of *Mimosa pudica's* roots and stems contain flavonoid have the ability to repair kidney damage by lowering urea and creatinine levels in rats blood.

Creatinine levels

Normal creatinine levels in rats are 0.2-0.8mg/dL (Nikolic *et al.*, 2003). Based on table 7, creatinine level of SD rats injected by DMBA 20mg/kg BW was the highest among all of the experimental group. That was similar to a research conducted by Ozdemir *et al.* (2007) that rats injected by DMBA at a dose of 50mg/kg BW lead to increase of urea levels by 24.0±0.45.

Based on the analysis of statistical data, there were significant differences between creatinine levels in group I and group II with a significant value of 0.010 ($p<0.05$). It can be concluded that DMBA lead to a significant increase in creatinine levels. Groups III, IV, and V had lower creatinine levels compared to group II and showed statistically significant differences with a significance value of 0.000 ($p<0.05$). This means that the ethanol extract of *E. scaber* is able to lower blood creatinine levels significantly.

Both urea and creatinine are kidney performance parameter where that level above normal indicated kidney damage. Kidney damage resulted in excessive ROS from oxidative stress. *E. scaber* extract contains flavonoid glycoside luteolin, flavonoid glycoside luteolin-7-glucuronide 6-methyl ester, and luteolin-4-O-β-D glucoside that act as antioxidant. The secondary metabolites are strong antioxidant to scavenge free radical and repair kidney damage (Daenen *et al.*, 2018).

CONCLUSION

Ethanol extract of *E. scaber* at a dose of 50mg/kg BW, 100mg/kg BW, and 200mg/kg BW orally were not cause a significant increase on body weight in Sprague Dawley rats induced by DMBA. However, it effectively reduced the levels of AST, ALT, Ureum, and Creatinine at the same dose. Whereas, the optimum dose to decrease nodule appearance by decreasing tumor incidence, increasing tumor inhibitory potential and decreasing multiplicity tumors is 200mg/kg BW.

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