Bukti Korespondensi publikasi artikel
Pakistan Journal of Pharmaceutical Sciences (PJPS)

Artikel disubmit pada 14 November 2018 dengan judul :

Effect of Ethanolic Extract from Elephantopus scaber L as Anticancer, Hepatoprotector and Nefroprotector

Namun, berdasarkan email 6 Februari 2019, ada saran dari reviewer untuk merubah judul menjadi : **Screening of anti cancer, hepatoprotective and nephroprotective effects of ethanol extract of Elephantopus scaber L**."

Berdasarkan saran tersebut, selanjutnya naskah kami kirimkan dengan judul baru.

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Tanggal	Korespondensi		Hal
	Author	Editor Jurnal	
14 November 2018	Artikel disubmit melalui email dengan judul awal : **Effect of Ethanolic Extract from Elephantopus scaber L as Anticancer, Hepatoprotector and Nefroprotector.** (lampiran 1. Bukti email dan lampiran manuskrip)		4
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27 Nopember 2018	Email pertanyaan author tentang progres artikel (Lampiran 3. Bukti email)	Email jawaban dari Editor tentang progres artikel masih preliminary approval. (Lampiran 4. Bukti Email)	21 & 22
22 Januari 2019	Email pertanyaan author lagi tentang progres artikel (Lampiran 5. Bukti email)	Email jawaban dari Editor bahwa progres artikel masih menunggu comments dari reviewer (Lampiran 6. Bukti Email)	23 & 24
6 Februari 2019		Email tentang penyampaian saran/rekomendasi dari reviewer beserta lampiran artikel hasil review dan form response sheet. Salah satu	25

		rekomendasi yantertulis di email adalah pergantian judul menjadi "**Screening of anti cancer, hepatoprotective and nephroprotective effects of ethanol extract of Elephantopus scaber L**" yang kemudian digunakan oleh author sebagai judul pada artikel menggantikan judul yang lama. (Lampiran 7. Bukti Email dan naskah hasil review)	
20 Februari 2019	Email tentang pengiriman perbaikan artikel beserta lampiran artikel dan response sheet. Artikel sudah menggunakan judul baru : "**Screening of anti cancer, hepatoprotective and nephroprotective effects of ethanol extract of Elephantopus scaber L**" (Lampiran 8. BUkti email, artikel hasil revisi dan response sheet))		37
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Lampiran 1. Email pengiriman artikel

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Submission Journal Manuscript

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Kepada: pjps@uok.edu.pk
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Catatan : Judul saat submission adalah yang tertera dalam email ini, namun ada saran perbaikan judul oleh reviewer berdasarkan email 6 Feb 2019 dari PJPS, sehingga judul berubah sesuai masukan.

Dear:

Editor in Chief

Pakistan Journal of Pharmaceutical Sciences **(PJPS)**

Submission of manuscript to Pakistan Journal of Pharmaceutical Sciences

It is my great pleasure to submit our paper entitled **"Effect of Ethanolic Extract from Elephantopus scaber L as Anticancer, Hepatoprotector and Nefroprotector"** to be published in your esteem journal. Please contact me if you need further information regarding the paper.

As a corresponding author, we stated that:

1. That the work described has not been published before
2. That it is not under consideration for publication elsewhere;
3. That all authors have contributed significantly;
4. That all authors are in agreement with the content of manuscript is included;
5. That its publication has been approved (tacitly or explicitly) by the responsible authorities at the institution where the work is carried out.

Thank you in advance for your cooperation.
Sincerely,

The corresponding author

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2 lampiran

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Manuskrip :

Effect of Ethanolic Extract from Elephantopus scaber L as Anticancer, Hepatoprotector and Nefroprotector

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ABSTRACT

7.12-dimethylbenz(a)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 nefroprotector.

Fourty 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. Group III, IV, and V were given DMBA and Elephantopus scaber L extract at 50mg/kgBW, 100mg/kgBW 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 the 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 tumour inhibition were obtained at the dose 200mg/kgBW. The results of AST, ALT, urea and creatinine levels showed a significant difference between all dose treatment groups with negative controls ($p < 0.05$).

Ethanolic extract of Elephantopus scaber L possibly inhibits appearance of nodules, protects liver and kidneys cell.

Keywords: Elephantopus scaber L, DMBA, cancer, hepatoprotector, nefroprotector.

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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, tumour suppressers, 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 smokes 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 into 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 into 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 Elephantopus scaber L.contains sesquiterpen as antitumour (Farha, et al., 2014). Deoxyelephantopin (ES-4), isodeoxyelephantopin (ES-5), scabertopin (ES-2) and isoscabertopin (ES-3) are sesquiterpen lactone compounds isolated from Elephantopus scaber L leaves which have antiproliferation activity in cancer cells (Xu, et al., 2006). Elephantopus scaber L also contains flavonoid luteolin-7-glucoside as antioxidant compound (Wan et al, 2012).

The current study investigated the effect of anticancer in animal model by measuring the nodule appearance and body weight. The hepatoprotective effect was measured by the AST and ALT levels, while the nefroprotective effect was measured by creatinine and urea levels. DMBA was used to chemically induce cancer.

MATERIALS AND METHODS

Materials

Elephantopus scaber L. leaves were collected from Sentolo, Kulon Progo, Yogyakarta, Indonesia in March 2017. Ethanol 70%, Tween 80, and distilled water were purchased from Brataco. DMBA was purchased from Sigma. Corn oil was purchased from local supermarket (Superindo). Nodule Appearance, Creatinine and Urea levels, also AST and ALT levels were analysed using commercial reagent kits.

Plants Extraction

The leaves of Elephantopus scaber L. 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

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

Making Test Solution

- 5% Tween 80 solution

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

- Elephantopus scaber L. extract dosage

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

- DMBA solution

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

Animal and Experimental Groups

The animals were kept under standard conditions of temperature and humidity and fed with a standard pellet diet and allowed *ad libitum* water. Forty female Sprague Dawley rats divided into five groups. Group I was the control group. Group II was given DMBA solution. Group III, IV, and V were given DMBA solution and Elephantopus scaber extract at 50 mg/kgBW, 100 mg/kgBW, and 200 mg/kgBW, respectively. DMBA solution was administered orally twice a week for 5 weeks while Elephantopus scaber L extract was administered daily by oral gavage for 5 weeks. During the 6^{th} to 15^{th} week nodules growth was observed. Blood sampling of female SD strains was carried out at the orbital sinus (eye) in the beginning of the 16^{th} week.

Observations and Analysis

- Body Weight

Mice 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 200 mg/kg BW which were weighed using a digital scale (Neraca Ohaus). Observations were begun at 6^{th} week after 5 weeks of DMBA induction until the 16^{th} week.

- Nodule Appearance

Palpation was done every week to find out the development of nodules from 6^{th} week. Observation was done until the 16^{th} week. On the 16^{th} week, surgery was performed by taking nodules on all parts of the body which showed the appearance of nodules in each group of rats.

- 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 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 Jaffe Method. While, Ureum blood serum activity was measured by using enzymatic Urease-GLDH UV test method.

STATISTICAL ANALYSIS

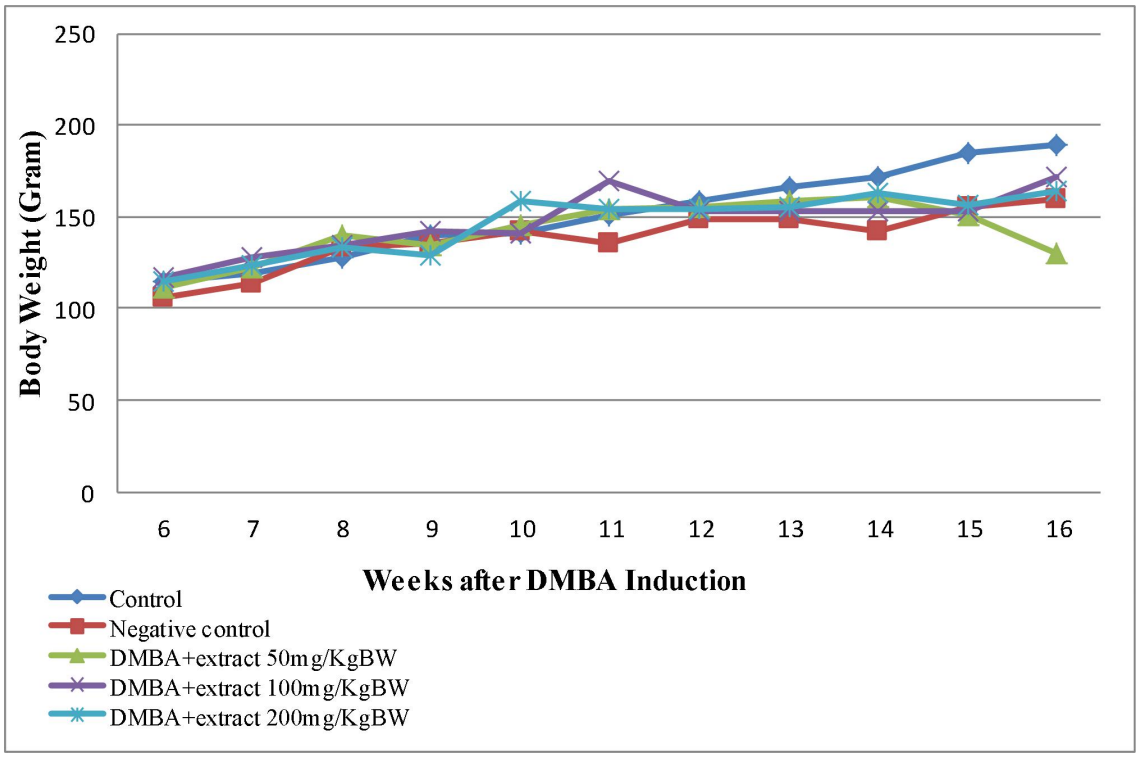
Statistical analysis was carried out using SPSS 21. One way analysis followed by Kruskal-Wallis and Mann-Whitney was used to analyse significance of data. Results were considered significant when $p<0.05$.

RESULT AND DISCUSSION

Body Weight

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

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



Graphic 1. Development of rats body weight after DMBA induction and administration of Elephantopus scaber L ethanolic extract.

Table I. Results of AUC Calculation

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

The table shows that body weight of group treated by Elephantopus scaber L on 100mg/kgBW was dramatically increased. The condition has a possibility of Elephantopus scaber L as 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 analysed by Kruskal-Wallis to determinate the differences between control and treatment groups. The significant value shows 0.079 (>0.05) that means there is no differences between control and treatment groups.

Nodul Appearance

Effect of 20 mg/kgBW DMBA induction twice a week for 5 weeks seen by surgery on weeks 16th (Figure 1). Based on necropsy result, the appearance of nodules was found in the lower of armpit, whereas the appearance of nodules on left mammary gland has appeared in the dose of extract 50 mg/kgBW and 100 mg/kgBW. Based on previous research by Thu et al (2018), showed that all groups that was induced by DMBA were not produce the palpable nodules on mammary glands macroscopically, whereas a rat with DMBA-induced has diagnosed with breast cancer microscopically. Based on that, DMBA-induced on rats were not given specific appearance of nodules in mammary glands.

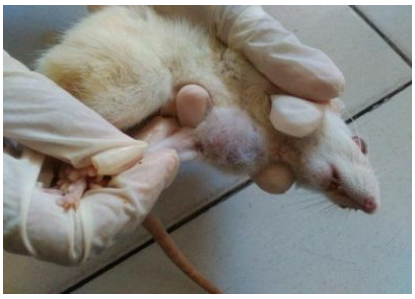


Figure 1. Nodule appearance on left mammary gland (Self Documentation)

Tumor Incidence by DMBA-Induced

Table II. Tumor Incidence in Rats

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/kg BW	8	3	37.5	7
DMBA+extract 100 mg/kg BW	8	5	62.5	4

DMBA+extract 200 mg/kg BW	8	2	25	4

Based on Choi et al (2018), squamous cell carcinoma was successfully maturated after 5 months treatment with DMBA and 12-O-tetradecanoylphorbol-13-acetate (TPA). Squamous cell hyperplasia with chronic inflammation was detected in the skin dermis of mice (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 II shows the reduction of tumor incident for all of 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 100 mg/kgBW revealed 60% of rats produced noduls.. Furthermore, the dose 50 mg/kgBW and 200 mg/kgBW had only 40% and 25 rats with nodules respectively. That means DMBA is a carcinogenic compound which capable of inducing mice or rats into tumor models.

Potential Inhibition of Ethanolic Extract Elephantopus scaber L

Table III. Results of Percentage of Potential Inhibition of Tumors in DMBA-Induced Mice

Groups	**Potential Inhibitory (%)**
DMBA + extract 50 mg/kg BW	50
DMBA + extract 100 mg/kg BW	16,67
DMBA + extract 200 mg/kg BW	66,67

Based on similar research by Kabeer et al (2017), showed that Deoxyelephantopin (DEO), sesquiterpenes from Elephantopus scaber L increased the expression of p53, phospho-Jun amino-terminal kinases (p-JNK), p-p38, increased the expression of phospho-signal tranducer and activator of transcription 3 (p-STAT3), and phospho-mammalian target of rapamycin (p-mTOR) in cancer cell. These findings of DOE 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 subcutaneous administration of active fraction of Elephantopus scaber L 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 mice (Geetha et al, 2010).

The data above shows that the highest percentage of tumor inhibitory potential was in the extract dose of 200mg/kgBW, whereas the lowest percentage was in the dose of 100mg/kgBW. Based on the previous data of tumor incidence, the data were related to potential inhibitory. The extract dose 200mg/kgBW has the lowest incidence and the highest potential inhibitory than others. That means the dose of 200mg/kgBW can reduce the incidence of tumors and inhibit tumor growth.

Multiplicity of Tumors

Table IV. Results of Tumors Multiplicity in DMBA-induced rats

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

Ethanolic extract of Elephantopus scaber L in addition to inhibit tumor incidence, it was also able to reduce the average tumor multiplicity (number of tumor). It can be observed that the 200mg/kgBW has the lowest number of tumor. Multiplicity tumors provide synchronous results on the potential of tumor inhibition and tumor incidence proportionally.

Based on Mehmood et al (2017), DEO from Elephantopus scaber L induce 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 ethanolic extract of Gynura procumbens L had similar content with ethanolic extract Elephantopus scaber L, 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 20 mg/kgBW 2 times a week for 5 weeks. That means the antioxidant compounds has the ability to obstruct cancer by inhibiting cancer proliferation.

Aspartate Transaminase (AST) and Alanine Aminotransferase (ALT) Activity

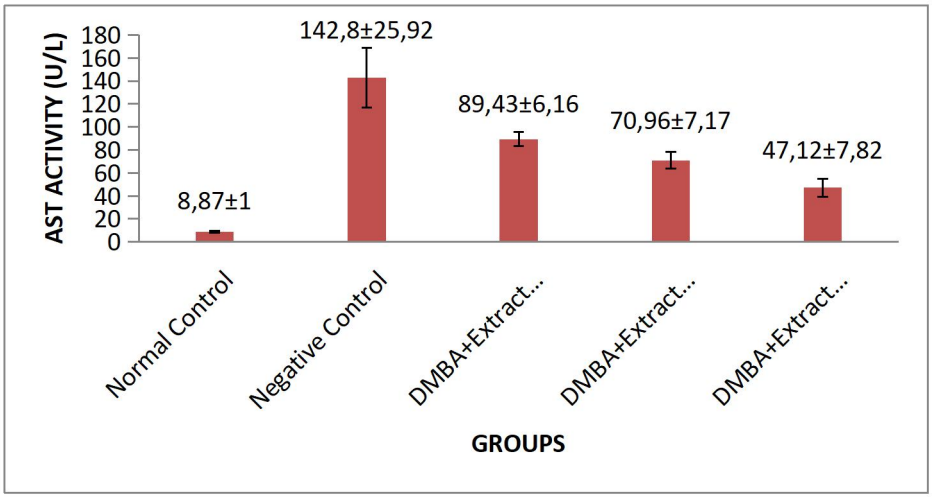
Table V. Average of AST and ALT Activity

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

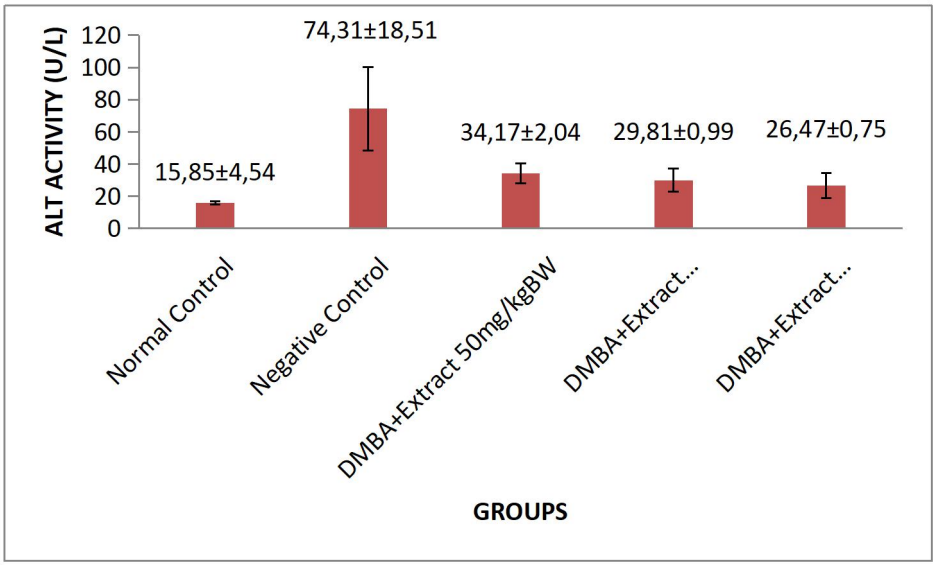
Normally, AST and ALT activity has the range between 40-80 U/L (Lin et al, 2018). Based on other researches, normal control group has the AST level 55.45±20.29 U/L and ALT level 75.67±2.08 U/L (Yadie, 2012). The other research shows AST level 70.36±10.93 U/L and ALT level 33.97±11.43 U/L (Raghavendra 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).

Tabel V shows that 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 on 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 compare with normal and negative control. The more higher dose of ethanolic extract Elephantopus scaber L, the more higher decreasing level of AST and ALT. Decreasing AST and ALT level was completely performed with various of doses (Graphic 2,3).



Graphic 2. Average of AST Level



Graphic 3. Average of ALT Level

Graphic 2 and 3 shows that ethanolic extract Elephantopus scaber L with various doses 50mg/kgBW, 100mg/kgBW and 200mg/kgBW were decreased AST and ALT level after treatment, respectively. Elephantopus scaber L was presence a high flavonoid and phenolic compounds as 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 level of AST and ALT were indicate as restoration or recovery. The restoration of AST and ALT levels suggested that Elephantopus scaber L 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).

Nefroprotector

Urea Levels

Ureum levels determined using enzymatic Urease-GLDH UV test method. Urease enzyme catalyzes hydrolysis of urea into ammonia and carbonate. NADH oxidation causes a decrease in absorbance at 340 nm. The result of urea levels determination shown in the table below.

Tabel VI. Urea levels examination in each group of SD rats given Ethanolic Extract of Elephantophus scaber L.

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 ($p<0.05$)

Normal urea level in mice is 15-21 mg/dL (Nikolic et al, 2003). Urea levels in group I was higher than group III, IV, and V. Based on the table I, Group II's urea level was the highest among all of experiment group. That was similar to a research conducted by Ozdemir *et al*., (2007) that mice injected by DMBA at 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 causing 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 analysis obtained that there was a significant difference between group I and group II with significant value of 0.029 ($p<0.05$) that means injection of DMBA causing urea level increase significantly. Group III, IV, and V have average urea levels of 36.13 mg/dL, 31.24 mg/dL, and 27.98 mg/dL, respectively lower than group II. The results showed that Ethanolic extract of Elephantophus scaber L has ability to decrease urea levels. Based on the statistical data processing showed that Group III, IV, and V result a significant difference compare to group I with significant value of 0.029 ($p<0.05$). This result indicated that Ethanolic extract of Elephantophus scaber L at dose of 50 mg/KgBW, 100 mg/KgBW, dan 200 mg/KgBW can reduce urea levels significantly.That was similar to research concucted by Marcelo et al (2014) that 70% ethanol extract of Mimosa pudica's roots and steems contain flavonoid have ability to repair kidney damage by loweing urea and creatinine levels in mice blood.

Creatinine Levels

Creatinine levels examined using Jaffe method. Principle of this method is complex compound formed by reaction of creatinine with picric acid in alkali environment. The amount of complex compound formed has linier correlation with creatinine levels. The result of Creatinine levels examination can be seen in the table below.

Table VII. Creatinine levels examination in each group of SD rats given Ethanolic Extract of Elephantophus scaber L.

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 ($p<0.05$)

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

Based on statistical data analysis there is a significant difference between group I and group II with significant value of 0.010 ($p<0.05$) that means DMBA causing an increase in creatinine levels significantly. Group III, IV, and V have the lower creatinine levels compare to group II. The result shows that Ethanolic extract of Elephantophus scaber L has activity to lowering blood creatinine levels. Statistical data shows that Etanolic extract of Elephantopus scaber L has a significant difference with group II with significance value of 0.000 ($p<0.05$). That means Ethanolic extract of Elephanthopus scaber L. have ability to lower creatinine levels in the blood significantly.

Both urea and creatinine are kidney performance parameter where that level above normal indicated kidney damage. Kidney damage resulted by excessive ROS from oxidative stress. Elephantophus scaber extract contains flavonoid aglicoside luteolin, flavonoid glycoside luteolin-7-oglucuronide 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.

CONCLUSION

Ethanol extract of Elephantopus scaber L at a dose of 50 mg / KgBW, 100 mg / KgBB and 200 mg / KgBB orally were not cause a significant increase on body weight in Sprague Dawley rats induced by DMBA. However, it is effectively decreased AST, ALT, Ureum, and Creatinine level 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 200 mg/kgBW.

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Lampiran 2. Email tentang pemberian informasi bahwa artikel sudah diterima, informasi **Serial No.6863** dan akan diproses selanjutnya.

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Submission Journal Manuscript

Pakistan Journal of Pharmaceutical Sciences Faculty of Pharmacy <pjps@uok.edu.pk> 15 November 2018 16.21
Kepada: naniksulistyani@gmail.com

Dr. Nanik Sulistyani
Faculty of Pharmacy,
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Yogyakarta, Indonesia
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Dear Dr. Nanik Sulistyani,
Your article entitled: "Effect of Ethanolic Extract from *Elephantopus scaber* L as Anticancer, Hepatoprotector and Nefroprotector" (authors: Nanik Sulistyani and Nurkhasanah) has been received for publication in Pakistan Journal of Pharmaceutical Sciences, listed for publication vide Serial No.6863 and will be communicated with this serial number in future.
Publication fee @ US $ 50.00 per page (single side of the leaf) will be charged at the time of publication.

Prof. Dr. Iqbal Azhar
Editor-in-Chief, PJPS & Dean
Faculty of Pharmacy & Pharmaceutical Sciences
University of Karachi
[Kutipan teks disembunyikan]

Lampiran 3. Email pertanyaan kami tentang progres artikel

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Submission Journal Manuscript

nanik sulistyani <naniksulistyani@gmail.com> 27 November 2018 11.35
Kepada: pjps@uok.edu.pk

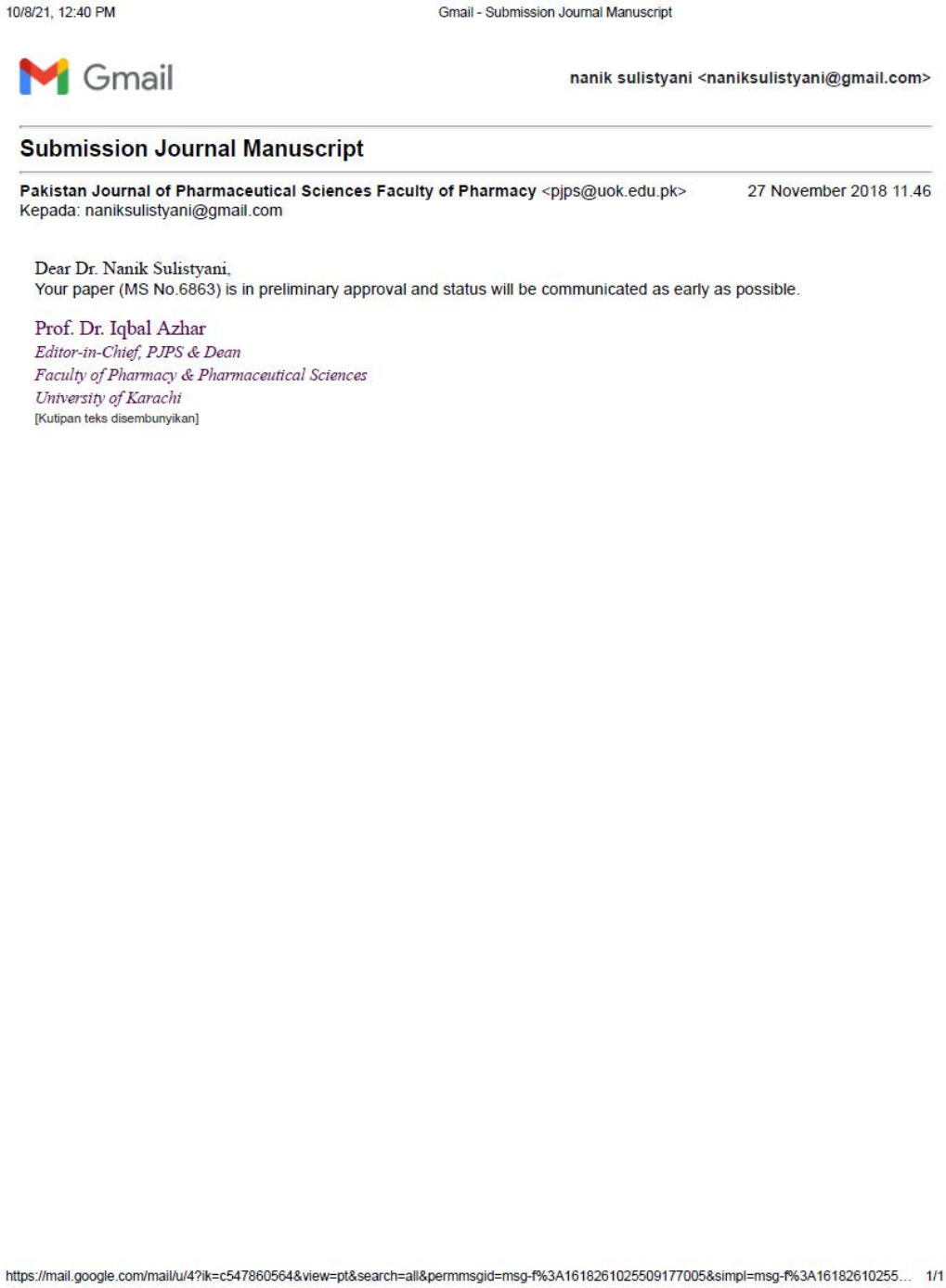
Dear: **Prof. Dr. Iqbal Azhar**
Editor in Chief, PJPS & Dean
Faculty of Pharmacy & Pharmaceutical Sciences
University of Karachi

Regarding with submission manuscript entitled "Effect of Ethanolic Extract from *Elephantopus scaber* L as Anticancer, Hepatoprotector and Nefroprotector" (authors: Nanik Sulistyani and Nurkhasanah) with serial number of **manuscript references 6863**, we would like to know about review progress of the manuscript.
Thank you in advance. Thank you for your information.

Best regards,

Dr. Nanik Sulityani, M.Si., Apt
[Kutipan teks disembunyikan]

Lampiran 4. Email jawaban dari Editor tentang progres artikel masih preliminary approval.



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Submission Journal Manuscript

Pakistan Journal of Pharmaceutical Sciences Faculty of Pharmacy <pjps@uok.edu.pk> 27 November 2018 11.46
Kepada: naniksulistyani@gmail.com

Dear Dr. Nanik Sulistyani,
Your paper (MS No.6863) is in preliminary approval and status will be communicated as early as possible.

Prof. Dr. Iqbal Azhar
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Lampiran 5. Email pertanyaan author lagi tentang progres artikel

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Manuscript Progress

nanik sulistyani <naniksulistyani@gmail.com> 22 Januari 2019 11.25
Kepada: pjps@uok.edu.pk

Dear: **Prof. Dr. Iqbal Azhar**
Editor in Chief, PJPS & Dean
Faculty of Pharmacy & Pharmaceutical Sciences
University of Karachi

Regarding with submission manuscript entitled "Effect of Ethanolic Extract from *Elephantopus scaber* L as Anticancer, Hepatoprotector and Nefroprotector" (authors: Nanik Sulistyani and Nurkhasanah) with serial number of **manuscript references 6863**, we would like to know about review progress of the manuscript.
Thank you in advance. Thank you for your information.

Best regards,

Dr. Nanik Sulityani, M.Si., Apt

Lampiran 6. Email jawaban dari Editor bahwa progres artikel masih menunggu comments dari reviewer

11/10/21, 3:43 PM Gmail - Manuscript Progress

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Manuscript Progress

PJPS <pjps@uok.edu.pk> 22 Januari 2019 12.07
Kepada: nanik sulistyani <naniksulistyani@gmail.com>

Dear Dr. Nanik Sulityani
Your paper (MS No.6863) is in evaluation queue and comments of the referee will be communicated immediately when this office received from referee's end.

Prof. Dr. Rahila Ikram
Editor-in-Chief: PJPS & Dean
Faculty of Pharmacy & Pharmaceutical Sciences
University of Karachi
[Kutipan teks disembunyikan]

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Lampiran 7. Email tentang penyampaian saran/rekomendasi dari reviewer beserta lampiran artikel hasil review dan form response sheet

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MS No.6863

Pakistan Journal of Pharmaceutical Sciences <pakjps@hotmail.com> 6 Februari 2019 13.38
Kepada: "naniksulistyani@gmail.com" <naniksulistyani@gmail.com>

Dr. Nanik Sulistyani
Faculty of Pharmacy,
Universitas Ahmad Dahlan,
Yogyakarta, Indonesia

Dear Dr. Nanik Sulistyani,

Reference to your e-mail regarding publication of your article entitled: "Effect of Ethanolic Extract from *Elephantopus scaber* L as Anticancer, Hepatoprotector and Nefroprotector" (authors: Nanik Sulistyani and Nurkhasanah). This is to inform you that your paper has been examined by the referee whose comments are as under:

catatan : Comment no 2 adalah judul yang disarankan oleh reviewer sehingga judul berubah sesuai saran tersebut

Comments

This paper is very interesting and good work however it is poorly written and presented, I offer some major / minor comments to improve it

1. The study claims hepatoprotecton and nephroprotection of the plant extract then what about histo-pathological examination of hepatic and renal tissues??? To confirm the claim there must be histopathological study of vital tissues.
2. Modify the title you cannot directly report as anticancer or hepatoprotective or nephroprotective. You can write in this manner" screening of anticancer, hepatoprotective and nephroprotective effects of ethanol extract of Elephantopus scaber L."
3. Abstract need to be restructured, poor grammatical sentences are observed. You mentioned one way ANOVA was used (line 15) however in statistical analysis (line 108) there is no information about ANOVA, please make corrections.
4. Please mention list of abbreviation.
5. In the whole manuscript the plant name must be written in italic
6. Why the authors used word "BW" with weight every where in the text, when they are writing mg/kg it is understood that they are talking about weight of the animals.
7. Correct the spell of "Nefroprotective" as Nephroprotective throughout the text.
8. The voucher number for plant identification must be mentioned in line no.54
9. The sub heading in line no. 65 must be replaced with drug and administration
10. Under the sub heading mentioned in line no.76, please mention detail of animals used?? What were housing conditions like temperature, humidity, light/dark cycle and diet.
11. Please clear the weeks where ever you mentioned, line 92, 93 and 94.
12. Line 186 clear the abbreviation DOE
13. Remove the word self documentation in line no. 161
14. Clarify the captions of tables (see table V)
15. The placement of stearic (*) on values of tables VI and VII. Also mention caption of table VI
16. Grammatical errors are observed throughout the manuscript careful proof reading required.
17. Below the graphs do not write graphics rather write it figure.

Lampiran 7. Email tentang penyampaian saran/rekomendasi dari reviewer beserta lampiran artikel hasil review dan form response sheet

Gmail nanik sulistyani <naniksulistyani@gmail.com>

MS No.6863

Pakistan Journal of Pharmaceutical Sciences <pakjps@hotmail.com> 6 Februari 2019 13.38
Kepada: "naniksulistyani@gmail.com" <naniksulistyani@gmail.com>

Dr. Nanik Sulistyani
Faculty of Pharmacy,
Universitas Ahmad Dahlan,
Yogyakarta, Indonesia

Dear Dr. Nanik Sulistyani,

Reference to your e-mail regarding publication of your article entitled: "Effect of Ethanolic Extract from *Elephantopus scaber* L as Anticancer, Hepatoprotector and Nefroprotector" (authors: Nanik Sulistyani and Nurkhasanah). This is to inform you that your paper has been examined by the referee whose comments are as under:

catatan : Comment no 2 adalah judul yang disarankan oleh reviewer sehingga judul berubah sesuai saran tersebut

Comments

This paper is very interesting and good work however it is poorly written and presented, I offer some major / minor comments to improve it

1. The study claims hepatoprotecton and nephroprotection of the plant extract then what about histo-pathological examination of hepatic and renal tissues??? To confirm the claim there must be histopathological study of vital tissues.
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14. Clarify the captions of tables (see table V)
15. The placement of stearic (*) on values of tables VI and VII. Also mention caption of table VI
16. Grammatical errors are observed throughout the manuscript careful proof reading required.
17. Below the graphs do not write graphics rather write it figure.

18. Ethical approval of study is not mentioned in the paper.
19. References are not as per PJPS style, go through Instructions for Authors.

Editor's note:

Recent references of five years must be included.

You are required to revise above paper (attached) as per above comments of the referee and send us alongwith RESPONSE SHEET (attached) showing queries and their responses, also revise version of manuscript with highlighted revised portions as soon as possible for further process.

Prof. Dr. Rahela Ikram
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Artikel hasil review :

Effect of Ethanolic Extract from Elephantopus scaber L as Anticancer, Hepatoprotector and Nefroprotector

ABSTRACT

7.12-dimethylbenz(a)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 nefroprotector. Fourty 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. Group III, IV, and V were given DMBA and Elephantopus scaber L extract at 50mg/kgBW, 100mg/kgBW 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 the 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 tumour inhibition were obtained at the dose 200mg/kgBW. The results of AST, ALT, urea and creatinine levels showed a significant difference between all dose treatment groups with negative controls ($p < 0.05$).

Ethanolic extract of Elephantopus scaber L possibly inhibits appearance of nodules, protects liver and kidneys cell.

Keywords: Elephantopus scaber L, DMBA, cancer, hepatoprotector, nefroprotector.

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, tumour suppressers, 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 smokes 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 into 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 into 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 Elephantopus scaber L.contains sesquiterpen as antitumour (Farha, et al., 2014). Deoxyelephantopin (ES-4), isodeoxyelephantopin (ES-5), scabertopin (ES-2) and isoscabertopin (ES-3) are sesquiterpen lactone compounds isolated from Elephantopus scaber L leaves which have antiproliferation activity in cancer cells (Xu, et al., 2006). Elephantopus scaber L also contains flavonoid luteolin-7-glucoside as antioxidant compound (Wan et al, 2012).

The current study investigated the effect of anticancer in animal model by measuring the nodule appearance and body weight. The hepatoprotective effect was measured by the AST and ALT levels, while the nefroprotective effect was measured by creatinine and urea levels. DMBA was used to chemically induce cancer.

MATERIALS AND METHODS

Materials

Elephantopus scaber L. leaves were collected from Sentolo, Kulon Progo, Yogyakarta, Indonesia in March 2017. Ethanol 70%, Tween 80, and distilled water were purchased from Brataco. DMBA was purchased from Sigma. Corn oil was purchased from local supermarket (Superindo).

Nodule Appearance, Creatinine and Urea levels, also AST and ALT levels were analysed using commercial reagent kits.

Plants Extraction

The leaves of Elephantopus scaber L. 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 was then evaporated using rotary evaporator at 50 °C and heated over waterbath until thick extract was obtained.

Making Test Solution

- 5% Tween 80 solution

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

- Elephantopus scaber L. extract dosage

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

- DMBA solution

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

Animal and Experimental Groups

The animals were kept under standard conditions of temperature and humidity and fed with a standard pellet diet and allowed *ad libitum* water. Forty female Sprague Dawley rats divided into five groups. Group I was the control group. Group II was given DMBA solution. Group III, IV, and V were given DMBA solution and Elephantopus scaber extract at 50 mg/kgBW, 100 mg/kgBW, and 200 mg/kgBW, respectively. DMBA solution was administered orally twice a week for 5 weeks while Elephantopus scaber L extract was administered daily by oral gavage for 5 weeks. During the 6^{th} to 15^{th} week nodules growth was observed. Blood sampling of female SD strains was carried out at the orbital sinus (eye) in the beginning of the 16^{th} week.

Observations and Analysis

- Body Weight

Mice 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 200 mg/kg BW which were weighed using a digital scale (Neraca Ohaus). Observations were begun at 6^{th} week after 5 weeks of DMBA induction until the 16^{th} week.

- Nodule Appearance

Palpation was done every week to find out the development of nodules from 6^{th} week. Observation was done until the 16^{th} week. On the 16^{th} week, surgery was performed by taking nodules on all parts of the body which showed the appearance of nodules in each group of rats.

- 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 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 Jaffe Method. While, Ureum blood serum activity was measured by using enzymatic Urease-GLDH UV test method.

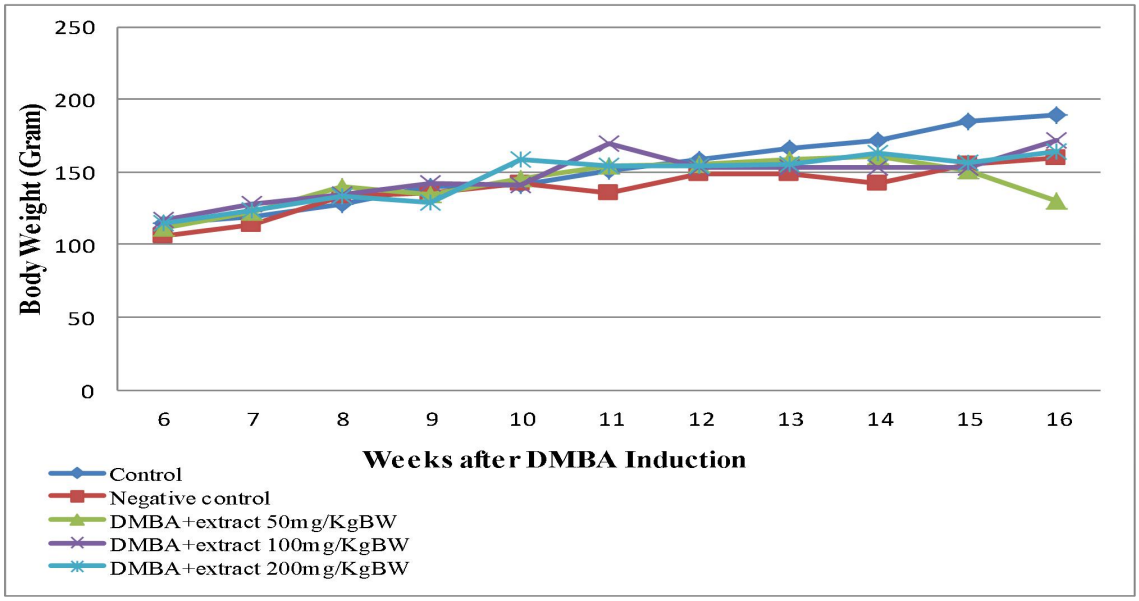
STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS 21. One way analysis followed by Kruskal-Wallis and Mann-Whitney was used to analyse significance of data. Results were considered significant when $p<0.05$.

RESULT AND DISCUSSION

Body Weight

Based on the Graphic 1, body weight of all groups was increased significantly. Meanwhile, negative control was the lowest weight among treatment period caused by DMBA induction. DMBA caused palpated nodules in all of group treatments. The highest palpated nodule was found in negative control group, while others not as high as negative control cause treated by extracts. Nodule



has a linear correlated to inflammation. Furthermore, inflammation affects stress management which caused loss of appetite.

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

Graphic 1. Development of rats body weight after DMBA induction and administration of Elephantopus scaber L ethanolic extract.

Table I. Results of AUC Calculation

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

The table shows that body weight of group treated by Elephantopus scaber L on 100mg/kgBW was dramatically increased. The condition has a possibility of Elephantopus scaber L as 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 analysed by Kruskal-Wallis to determinate the differences between control and treatment groups. The significant value shows 0.079 (>0.05) that means there is no differences between control and treatment groups.

Nodul Appearance

Effect of 20 mg/kgBW DMBA induction twice a week for 5 weeks seen by surgery on weeks 16th (Figure 1). Based on necropsy result, the appearance of nodules was found in the lower of armpit, whereas the appearance of nodules on left mammary gland has appeared in the dose of extract 50 mg/kgBW and 100 mg/kgBW. Based on previous research by Thu et al (2018), showed that all groups

that was induced by DMBA were not produce the palpable nodules on mammary glands macroscopically, whereas a rat with DMBA-induced has diagnosed with breast cancer microscopically. Based on that, DMBA-induced on rats were not given specific appearance of nodules in mammary glands.

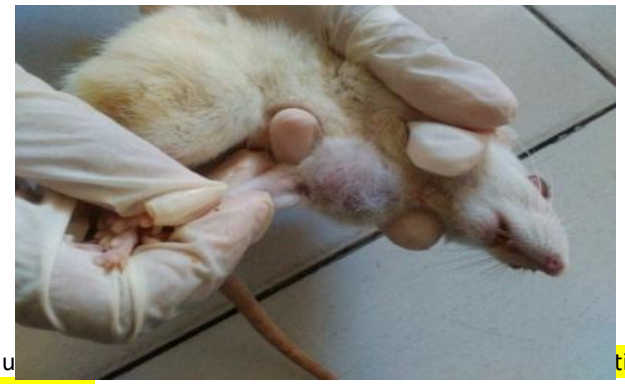


Figure 1. Nodu tion)

Tumor Incidence by DMBA-Induced

Table II. Tumor Incidence in Rats

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/kg BW	8	3	37.5	7
DMBA+extract 100 mg/kg BW	8	5	62.5	4
DMBA+extract 200 mg/kg BW	8	2	25	4

Based on Choi et al (2018), squamous cell carcinoma was successfully maturated after 5 months treatment with DMBA and 12-O-tetradecanoylphorbol-13-acetate (TPA). Squamous cell hyperplasia with chronic inflammation was detected in the skin dermis of mice (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 II shows the reduction of tumor incident for all of 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 100 mg/kgBW revealed 60% of rats produced noduls.. Furthermore, the dose 50 mg/kgBW and 200 mg/kgBW had only 40% and 25 rats with nodules respectively. That means DMBA is a carcinogenic compound which capable of inducing mice or rats into tumor models.

Potential Inhibition of Ethanolic Extract Elephantopus scaber L

Table III. Results of Percentage of Potential Inhibition of Tumors in DMBA-Induced Mice

Groups	**Potential Inhibitory (%)**
DMBA + extract 50 mg/kg BW	50
DMBA + extract 100 mg/kg BW	16,67
DMBA + extract 200 mg/kg BW	66,67

Based on similar research by Kabeer et al (2017), showed that Deoxyelephantopin (DEO), sesquiterpenes from Elephantopus scaber L increased the expression of p53, phospho-Jun amino-terminal kinases (p-JNK), p-p38, increased the expression of phospho-signal tranducer and activator of transcription 3 (p-STAT3), and phospho-mammalian target of rapamycin (p-mTOR) in cancer cell. These findings of DOE 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 subcutaneous administration of active fraction of Elephantopus scaber L 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 mice (Geetha et al, 2010).

The data above shows that the highest percentage of tumor inhibitory potential was in the extract dose of 200mg/kgBW, whereas the lowest percentage was in the dose of 100mg/kgBW. Based on the previous data of tumor incidence, the data were related to potential inhibitory. The extract dose 200mg/kgBW has the lowest incidence and the highest potential inhibitory than others. That means the dose of 200mg/kgBW can reduce the incidence of tumors and inhibit tumor growth.

Multiplicity of Tumors

Table IV. Results of Tumors Multiplicity in DMBA-induced rats

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

Ethanolic extract of Elephantopus scaber L in addition to inhibit tumor incidence, it was also able to reduce the average tumor multiplicity (number of tumor). It can be observed that the 200mg/kgBW has the lowest number of tumor. Multiplicity tumors provide synchronous results on the potential of tumor inhibition and tumor incidence proportionally.

Based on Mehmood et al (2017), DEO from Elephantopus scaber L induce 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 ethanolic extract of Gynura procumbens L had similar content with ethanolic extract Elephantopus scaber L, 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 20 mg/kgBW 2 times a week for 5 weeks. That means the antioxidant compounds has the ability to obstruct cancer by inhibiting cancer proliferation.

Aspartate Transaminase (AST) and Alanine Aminotransferase (ALT) Activity

Table V. Average of AST and ALT Activity

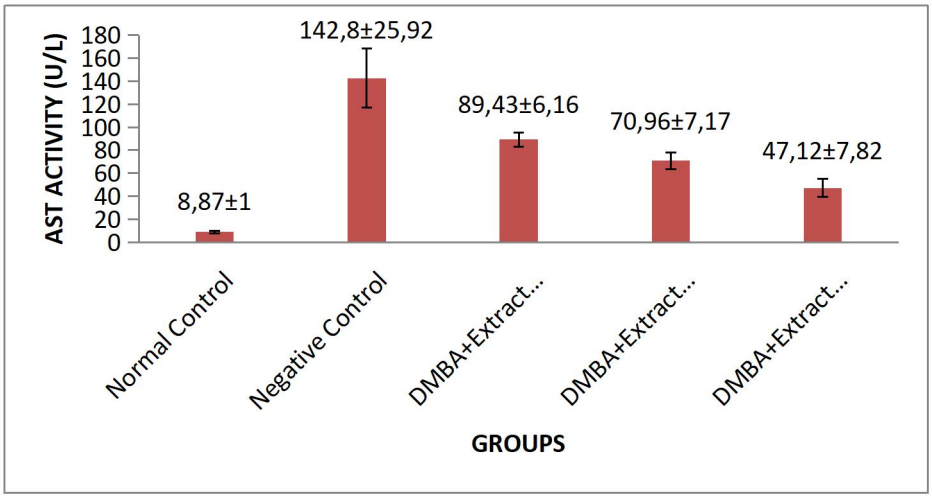
Groups	Mean±SD (U/L)	
	AST	ALT
Normal Control	8.87± 1.00	15.85±4.54
Negative Control	142.8±25.92	74.31±18.51
DMBA + extract 50 mg/KgBW	89.43±6.16	34.17±2.04
DMBA + extract 100 mg/KgBW	70.96±7.17	29.81±0.99
DMBA + extract 200 mg/KgBW	47.12±7.82	26.47±0.75

Normally, AST and ALT activity has the 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).

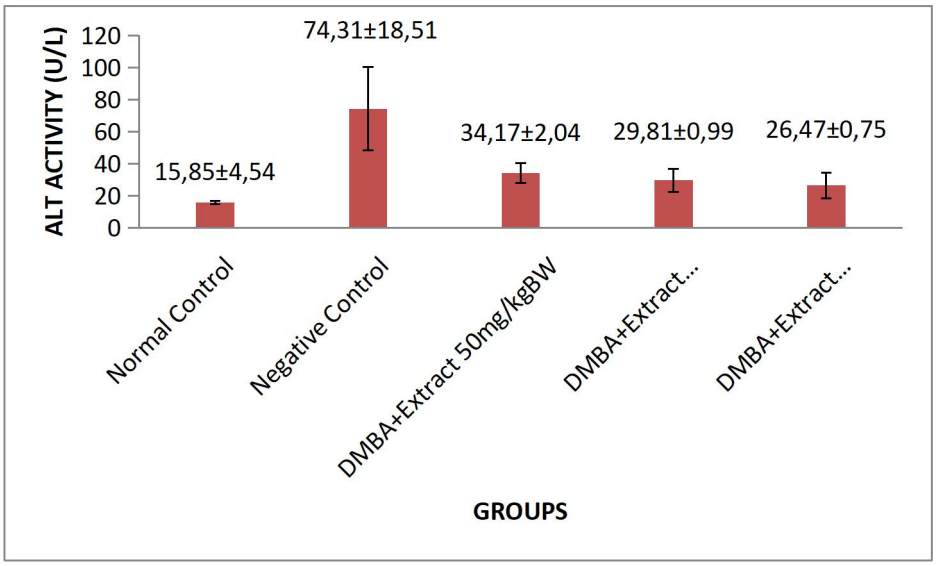
Tabel V shows that negative control group has the AST level 142.8±25.92 U/L and ALT level 74.31±18.51 U/L. The ratio between normal control and negative control are significantly increased on negative control. That means DMBA caused hepatocyte cell damage on rats. Loss of structural

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 compare with normal and negative control. The more higher dose of ethanolic extract Elephantopus scaber L, the more higher decreasing level of AST and ALT. Decreasing AST and ALT level was completely performed with various of doses (Graphic 2,3).



Graphic 2. Average of AST Level



Graphic 3. Average of ALT Level

Graphic 2 and 3 shows that ethanolic extract Elephantopus scaber L with various doses 50mg/kgBW, 100mg/kgBW and 200mg/kgBW were decreased AST and ALT level after treatment, respectively. Elephantopus scaber L was presence a high flavonoid and phenolic compounds as 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 level of AST and ALT were indicate as restoration or recovery. The restoration of AST and ALT levels suggested that Elephantopus scaber L 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).

Nefroprotector

Urea Levels

Ureum levels determined using enzymatic Urease-GLDH UV test method. Urease enzyme catalyzes hydrolysis of urea into ammonia and carbonate. NADH oxidation causes a decrease in absorbance at 340 nm. The result of urea levels determination shown in the table below.

Tabel VI. Urea levels examination in each group of SD rats given Ethanolic Extract of Elephantophus scaber L.

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 ($p<0.05$)

Normal urea level in mice is 15-21 mg/dL (Nikolic et al, 2003). Urea levels in group I was higher than group III, IV, and V. Based on the table I, Group II's urea level was the highest among all of experiment group. That was similar to a research conducted by Ozdemir *et al*., (2007) that mice injected by DMBA at 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 causing 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 analysis obtained that there was a significant difference between group I and group II with significant value of 0.029 ($p<0.05$) that means injection of DMBA causing urea level increase significantly. Group III, IV, and V have average urea levels of 36.13 mg/dL, 31.24 mg/dL, and 27.98 mg/dL, respectively lower than group II. The results showed that Ethanolic extract of Elephantophus scaber L has ability to decrease urea levels. Based on the statistical data processing showed that Group III, IV, and V result a significant difference compare to group I with significant value of 0.029 ($p<0.05$). This result indicated that Ethanolic extract of Elephantophus scaber L at dose of 50 mg/KgBW, 100 mg/KgBW, dan 200 mg/KgBW can reduce urea levels significantly.That was similar to research concucted by Marcelo et al (2014) that 70% ethanol extract of Mimosa pudica's roots and steems contain flavonoid have ability to repair kidney damage by loweing urea and creatinine levels in mice blood.

Creatinine Levels

Creatinine levels examined using Jaffe method. Principle of this method is complex compound formed by reaction of creatinine with picric acid in alkali environment. The amount of complex compound formed has linier correlation with creatinine levels. The result of Creatinine levels examination can be seen in the table below.

Table VII. Creatinine levels examination in each group of SD rats given Ethanolic Extract of Elephantophus scaber L.

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 ($p<0.05$)

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

Based on statistical data analysis there is a significant difference between group I and group II with significant value of 0.010 ($p<0.05$) that means DMBA causing an increase in creatinine levels significantly. Group III, IV, and V have the lower creatinine levels compare to group II. The result shows that Ethanolic extract of Elephantophus scaber L has activity to lowering blood creatinine levels. Statistical data shows that Etanolic extract of Elephantopus scaber L has a significant difference with

group II with significance value of 0.000 ($p<0.05$). That means Ethanolic extract of Elephanthopus scaber L. have ability to lower creatinine levels in the blood significantly.

Both urea and creatinine are kidney performance parameter where that level above normal indicated kidney damage. Kidney damage resulted by excessive ROS from oxidative stress. Elephantophus scaber extract contains flavonoid aglicoside luteolin, flavonoid glycoside luteolin-7-oglucuronide 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.

CONCLUSION

Ethanol extract of Elephantopus scaber L at a dose of 50 mg / KgBW, 100 mg / KgBB and 200 mg / KgBB orally were not cause a significant increase on body weight in Sprague Dawley rats induced by DMBA. However, it is effectively decreased AST, ALT, Ureum, and Creatinine level 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 200 mg/kgBW.

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Lampiran 8. Email tentang pengiriman perbaikan artikel beserta lampiran artikel dan response sheet. Artikel sudah menggunakan judul baru :
"**Screening of anti cancer, hepatoprotective and nephroprotective effects of ethanol extract of Elephantopus scaber L**"

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Screening of Anticancer, Hepatoprotective and Nephroprotective Effects of Ethanol Extract of *Elephantopus scaber* L

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/kgBW, 100mg/kgBW 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/kgBW. 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

Materials

E. scaber leaves was collected from Sentolo, KulonProgo, Yogyakarta, Indonesia and the plants identification was 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 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

2.5 grams of Tween 80 was put in a 50 ml 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/kgBW 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 50 mg/kgBW, 100 mg/kgBW, and 200 mg/kgBW, 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

- 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 200 mg/kgBW which were weighed using a digital scale (Neraca Ohaus). Observations were begun at 6th week after DMBA induction until the 16th week.

- Nodule Appearance

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

- 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 AND DISCUSSION

Body Weight

Based on Figure 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.

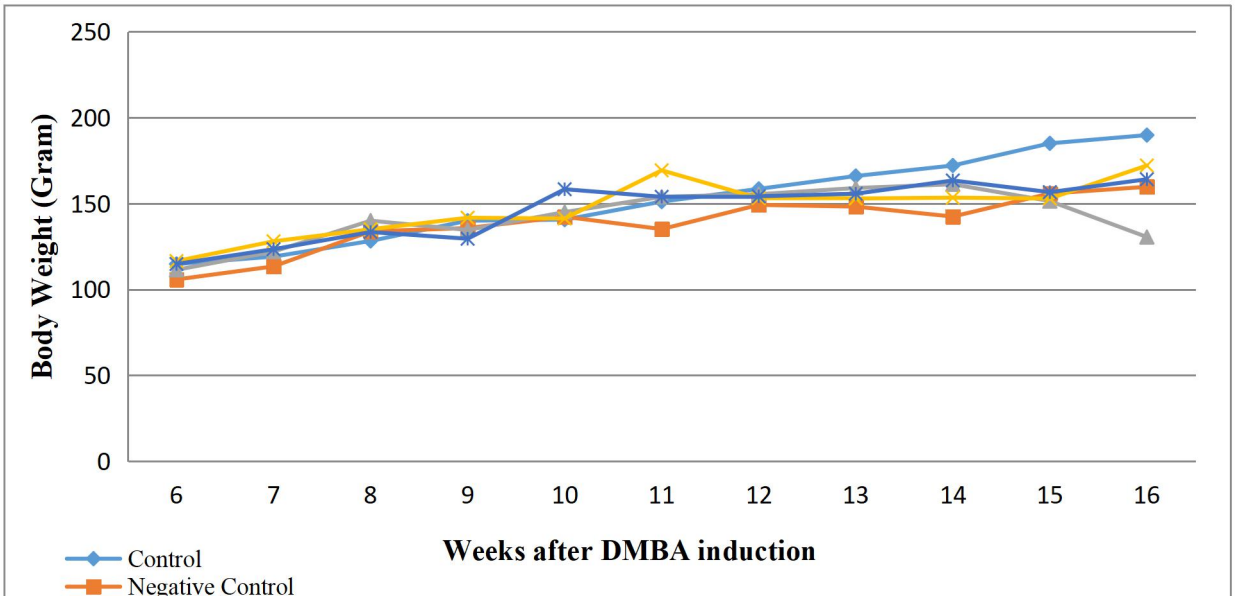


Figure 1. Development of rats body weight after DMBA induction and administration of *E. scaber* ethanol extract.

The AUC value of the body weight curve (Figure 1) is presented on Table I.

Table I. 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

The table 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/kgBW DMBA induction twice a week for 5 weeks seen by surgery on week 6th (Figure 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/kgBW and 100mg/kgBW. 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 microscopically. Based on that, DMBA-induced on rats were not given a specific appearance of nodules in mammary glands.

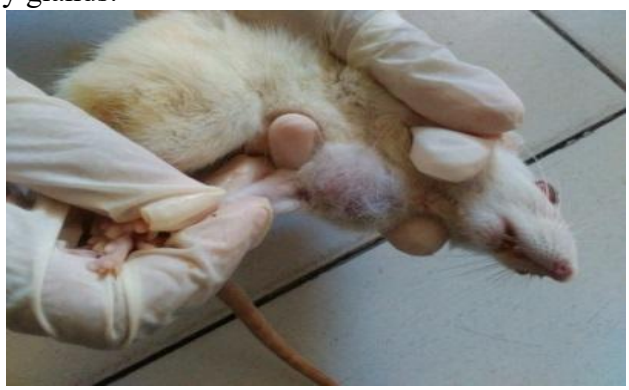


Figure 2. Nodule appearance on left mammary gland after DMBA induction

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 II 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.

Table II. Tumor incidence in ratsafter 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

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 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 Table III shows that the highest percentage of tumor inhibitory potential was in the extract dose of 200mg/kgBW, whereas the lowest percentage was in the dose of 100mg/kgBW. Based on the previous data of tumor incidence, the data were related to potential inhibitory. The extract dose 200mg/kgBW has the lowest incidence and the highest potential inhibitory than others. That means the dose of 200mg/kgBW can reduce the incidence of tumors and inhibit tumor growth.

Table III. 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

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 IV. It can be observed that the 200mg/kgBW has the lowest number of the tumor. Multiplicity tumors provide synchronous results on the potential of tumor inhibition and tumor incidence proportionally.

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/kgBW 2 times a week for 5 weeks. That means the antioxidant compounds have the ability to obstruct cancer by inhibiting cancer proliferation.

Table IV. 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

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 V 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).

Table V. 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

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 (Figure 3,4).

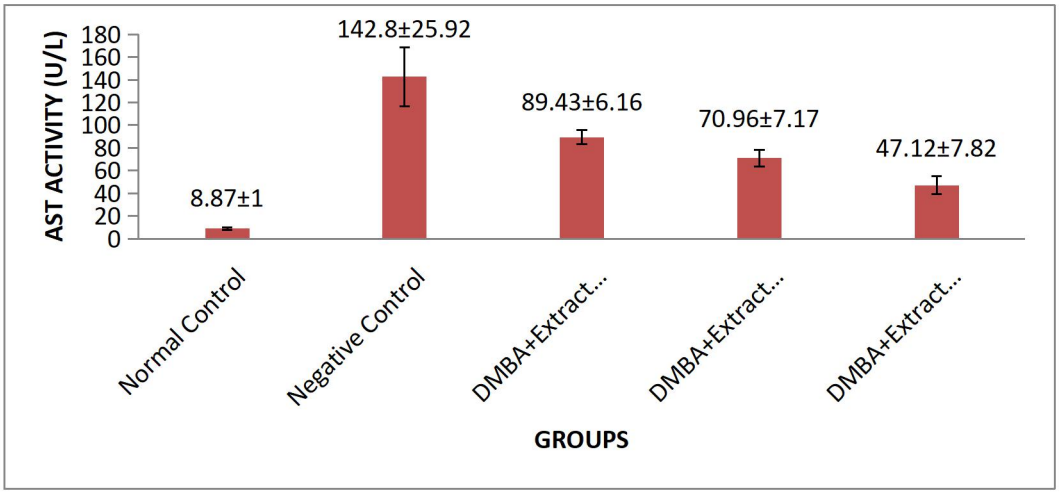
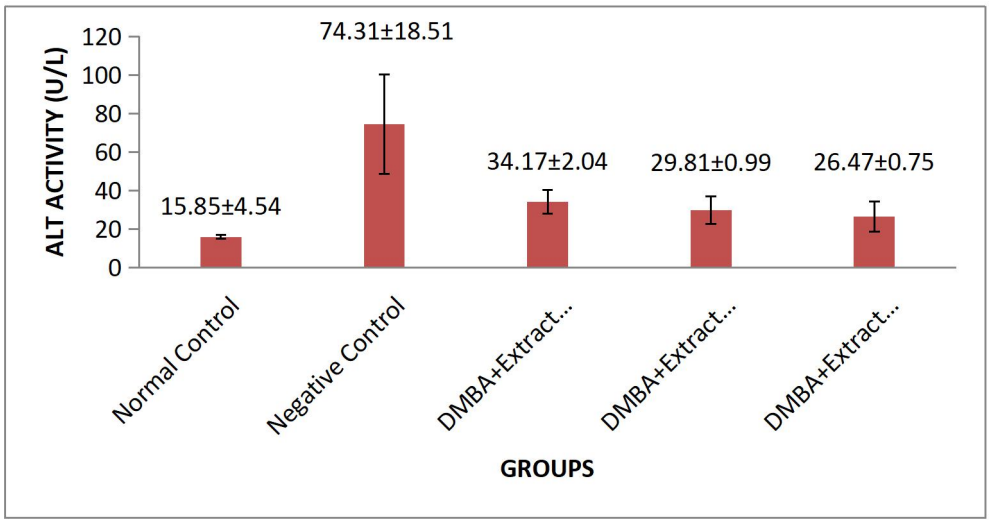


Figure 3. Average of AST levelin rats blood serumafter DMBA induction and



administration of *E. scaber* ethanol extract

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

Figure 3 and 4 shows that ethanol extract *E. scaber* with various doses 50mg/kgBW, 100mg/kgBW and 200mg/kgBW 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).

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. The result of urea levels determination shown in the table VI.

Table VI. 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 ($p<0.05$)

Normal urea level in rats is 15-21 mg/dL (Nikolic et al, 2003). Urea levels in group I was higher than group III, IV, and V. Based on table I, 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/kgBW 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 levelsto increase significantly. Groups III, IV, and V had lower urea levels than group II, which were 36.13 mg/dL, 31.24 mg/dL, and 27.98 mg/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 I with a significant value of 0.029 ($p<0.05$). This result indicated that ethanol extract of *E. scaber* at a dose of 50 mg/kgBW, 100 mg/kgBW, and 200 mg/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

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. The result of creatinine levels examination can be seen in the table VII.

Table VII. 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 ($p<0.05$)

Normal creatinine levels in rats are 0.2-0.8 mg/dL (Nikolic et al, 2003). Based on table VII, creatinine level of SD rats injected by DMBA 20 mg/kgBW 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 50 mg/kgBW 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/kgBW, 100mg/kgBW, and 200mg/kgBW 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/kgBW.

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RESPONSE SHEET

On
MS No. 6863

S. No.	Queries	Replies
1.	The study claims hepatoprotection and nephroprotection of the plant extract then what about histo-pathological examination of hepatic and renal tissues??? To confirm the claim there must be histopathological study of vital tissues.	Histopathological study have not been conducted
2.	Modify the title you cannot directly report as anticancer or hepatoprotective or nephroprotective. You can write in this manner, "screening of anticancer, hepatoprotective and nephroprotective effects of ethanol extract of elephantopus scaber L"	Effect of Ethanolic Extract from *Elephantopus scaber* L as Anticancer, Hepatoprotector and Nephroprotector → Screening of Anticancer, Hepatoprotective and Nephroprotective Effects of Ethanol Extract of *Elephantopus scaber* L
3.	Abstract need to be restructured, poor grammatical sentences are observed. You mentioned one way ANOVA was used (line 15) however in statistical analysis (line 108) there is no information about ANOVA, please make	Data were analyzed using one way ANOVA method with 95% confidence level. STATISTICAL ANALYSIS Statistical analysis was carried out using SPSS 21. One way analysis followed by ANOVA was used to analyse significance of data. Results were considered significant when $p<0.05$.

	corrections.	
4.	Please mention list of abbreviation	**LIST OF ABBREVIATION** **1.** ***DMBA*** *:7,12-dimethylbenz(α)anthracene* **2.** ***DMBA-DE***: *7,12dimethylbenz(α)anthracene-3,4-diol-1,2-epoxide* **3.** ***ROS***: *Reactive Oxygen Species* **4.** ***SD***: *Sprague Dawley* **5.** ***BW*** : *Body weight* **6.** ***AST***: *Aspartate Aminotransferase* **7.** ***ALT***: *Alanine Aminotransferase* ***8.*** ***ES-4***: *Deoxyelephantopin* ***9.*** ***ES-***5: *Isodeoxyelephantopin* ***10.*** ***ES-***2: *Scabertopin* ***11.*** ***ES-***3: *Isoscabertopin* ***12.*** ***IFCC***: *International Federation of Chemical Chemistry* ***13.*** ***TPA***: *12-O-tetradecanoylphorbol-13-acetate* ***14.*** ***DEO***: *Deoxyelephantopin* ***15.*** ***p-JNK***: *phospo-Junamino-terminal kinase* ***16.*** ***p-STAT3*** : *phospo-signal transducer and activator of transcription 3* ***17.*** ***p-mTOR***: *phospo-mammalian target of rapamycin*
5.	In the whole manuscript the plant name must be written in italic	*Elephantopus scaber* L and *E. scaber*
6.	Why the authors used word “BW” with weight everywhere in the text, when they are writing mg/kg it is understood that they are talking about weight of the animals.	Because of mg/kgBW shows a dosage based on body weight. For instance : 20 mg/kgBW If body weight = 60 kg, the dosage must be 60 x 20 mg = 1200 mg or 1,2 g for one dosage
7.	Correct the spell of “Nefroprotective” as Nephroprotective throughout the text	Nefroprotector (line 8, 23, 281) → Nephroprotector Nefroprotective (line 48) → Nephroprotective
8.	The voucher number for plant identification must be mentioned in line No. 54	*E. scaber* leaves was collected from Sentolo, KulonProgo, Yogyakarta, Indonesia and the plants identification was from Laboratory of Biology, Universitas Ahmad Dahlan Yogyakarta with No. 011/Lab.Bio/B/II/2018.
9.	The sub heading in line No. 65 must be replaced with drug and	Making test solution → Drug and Administration

<table>
<tr><td></td><td>administration</td><td></td></tr>
<tr><td>10.</td><td>Under the sub heading mentioned in line No. 76, please mention detail of animal used?? What where housing conditions like temperature, humidity, light/dark cycle and diet.</td><td>The animals were caged at room temperatures of 30°C and humidity 55% with a 12/12 hours light-dark cycle. The mice had free access to water and were fed with a standard pellet diet (BR-2) ad libitum.</td></tr>
<tr><td>11.</td><td>Please clear the weeks where ever you mentioned, line 92, 93 and 94.</td><td>Observations were begun at 6th week after 5 weeks of DMBA induction until the 16th week → Observations were begun at 6th week afterDMBA induction until the 16th week
Palpation was done every week to find out the development of nodules from 6th week → Palpation was done to find out the development of nodules from 6thweek</td></tr>
<tr><td>12.</td><td>Line 186 clear the abbreviation DOE</td><td>These findings of DOE kindly useful as a chemotherapeutic agent against cancer (Kabeer et al, 2017)→ These findings are kindly useful as a chemotherapeutic agent against cancer (Kabeer et al, 2017).</td></tr>
<tr><td>13.</td><td>Remove the word self documentation in line No. 161</td><td>Figure 2. Nodule appearance on left mammary gland (Self Documentation)→ Figure 2. Nodule appearance on left mammary gland after DMBA induction</td></tr>
<tr><td>14.</td><td>Clarify the captions of the tables (see table V)</td><td>Aspartate Transaminase (AST) and Alanine Aminotransferase (ALT) Activity → Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Activity</td></tr>
<tr><td>15.</td><td>The placement of stearic (*) on values of tables VI and VII. Also mention caption of table VI</td><td>Table VI. Urea levels in rats blood serum after DMBA induction and administration of E. scaber ethanol extract
<table><tr><th>Groups</th><th>Urea levels (mg/dL) ± SD</th></tr><tr><td>Normal Control</td><td>45.64 ± 1.53*</td></tr><tr><td>Negative Control</td><td>57.87 ± 8.84</td></tr><tr><td>DMBA + extract 50 mg/KgBW</td><td>36.13 ± 2.72*</td></tr><tr><td>DMBA + extract 100 mg/KgBW</td><td>31.24 ± 3.58*</td></tr><tr><td>DMBA + extract 200 mg/KgBW</td><td>27.98 ± 3.36*</td></tr></table>*: showing the significant difference to negative control (p<0.05)

Table VII. Creatinine levels examination in rats blood serum after DMBA induction and administration of E. scaber ethanol extract
<table><tr><th>Groups</th><th>Creatinine Level (mg/dL) ± SD</th></tr></table></td></tr>
</table>

		<table><tr><td>Normal Control</td><td>0.66 ± 0.07*</td></tr><tr><td>Negative Control</td><td>0.9 ± 0.16</td></tr><tr><td>DMBA + extract 50 mg/KgBW</td><td>0.49 ± 0.09*</td></tr><tr><td>DMBA + extract 100 mg/KgBW</td><td>0.45 ± 0.09*</td></tr><tr><td>DMBA + extract 200 mg/KgBW</td><td>0.28 ± 0.07*</td></tr></table> *: showing the significant difference to negative control ($p<0.05$)
16.	Grammatical errors are observed throughout the manuscript careful proof reading required	Grammatical errors have been checked
17.	Below the graphs do not write graphics rather write it figure	Graphic 3. Average of AST Level → Figure 3. Average of AST level in rats blood serumafter DMBA induction and administration of *E. scaber* ethanol extract Graphic 4. Average of ALT Level → Figure 4. Average of ALT levelin rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract
18.	Ethical approval of study is not mentioned in the paper	Ethical approval of this study has been accepted by Ethical Research Committees Universitas Ahmad Dahlan Yogyakarta with No. 011606117
19.	References are not as per PJPS style, go through Instruction for Authors	References have been changed as PJPS Style

Lampiran 9. Email balasan bahwa artikel sudah diterima Editor

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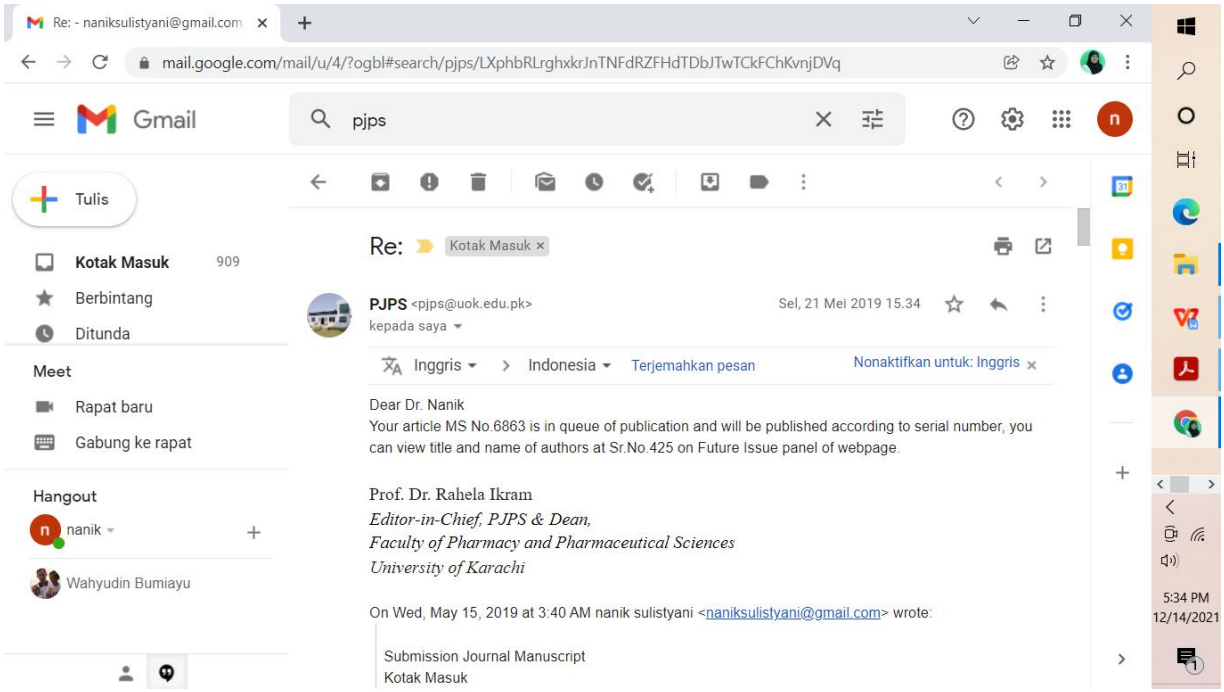
Lampiran 11. Email jawaban dari Editor yang menyatakan bahwa artikel dalam antrian publikasi.



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Dr. Nanik Sulistyani
Faculty of Pharmacy, Universitas Ahmad Dahlan,
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Kindly refer to our e-mail regarding publication of your article entitled: "Effect of Ethanolic Extract from Elephantopus scaber L as Anticancer, Hepatoprotector and Nefroprotector" (authors: Nanik Sulistyani, Nurkhasanah) As per galley proof (attached), your manuscript is consisting of 7 pages, you are required to send us an amount of US $ 350.00 as Publication charges through on-line banking details of our account are as under:

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Screening of anticancer, hepatoprotective and nephroprotective effects of ethanol extract of *elephantopus scaber* l

Nanik Sulistyani and Nurkhasanah
Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia

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 2 was given 20mg/kg body weight (BW) of DMBA. Groups 3, 4, and 5 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

Materials

E. scaber leaves was collected from Sentolo, KulonProgo, Yogyakarta, Indonesia and the plants identification was 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

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by maceration using 70% ethanol. The extract obtained was then evaporated using rotary evaporator at 50°C and heated over the waterbath until thick extract was obtained.

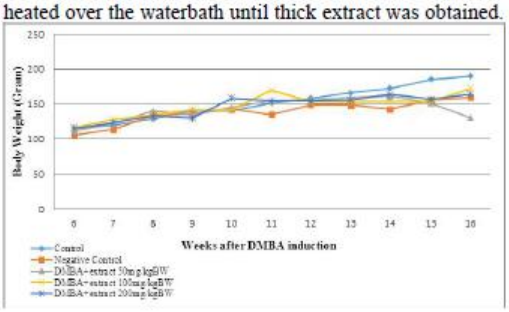

Fig. 1: Development of rats body weight after DMBA induction and administration of *E. scaber* ethanol extract.

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

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

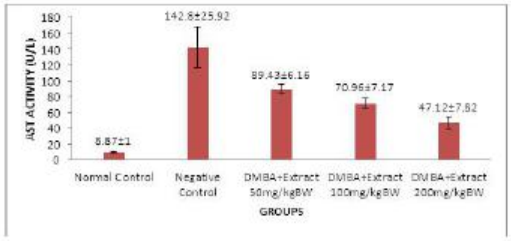

Fig. 3: Average of AST levelin rats blood serumafter DMBA induction and administration of *E. scaber* ethanol extract

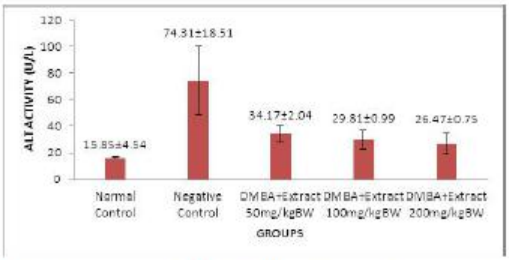

Fig. 4: Average of ALT levelin rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract

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

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.

Table 2: Tumor incidence in ratsafter 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

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 ($p<0.05$)

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

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 AND DISCUSSION

Body Weight
Based on Figure 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 AUC value of the body weight curve (fig. 1) is presented on table 1.

The table 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 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 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 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. Multiplicity tumors provide synchronous results on the potential of tumor inhibition and tumor incidence proportionally. 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).

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. The result of urea levels determination shown in the table 6.

Normal urea level in rats is 15-21mg/dL (Nikolic *et al*, 2003). Urea levels in group 1 was higher than group 3, 4, and 5. Based on table 1, Group 2'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 1 and group 2 with a significant value of 0.029 ($p<0.05$). It means that DMBA injection causes urea levels to increase significantly. Groups 3, 4 and 5 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 3, 4 and 5 differed significantly from group I 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/kg BW 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

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. The result of creatinine levels examination can be seen in the table 7.

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 1 and group 2 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 3, 4, and 5 had lower creatinine levels compared to group 2 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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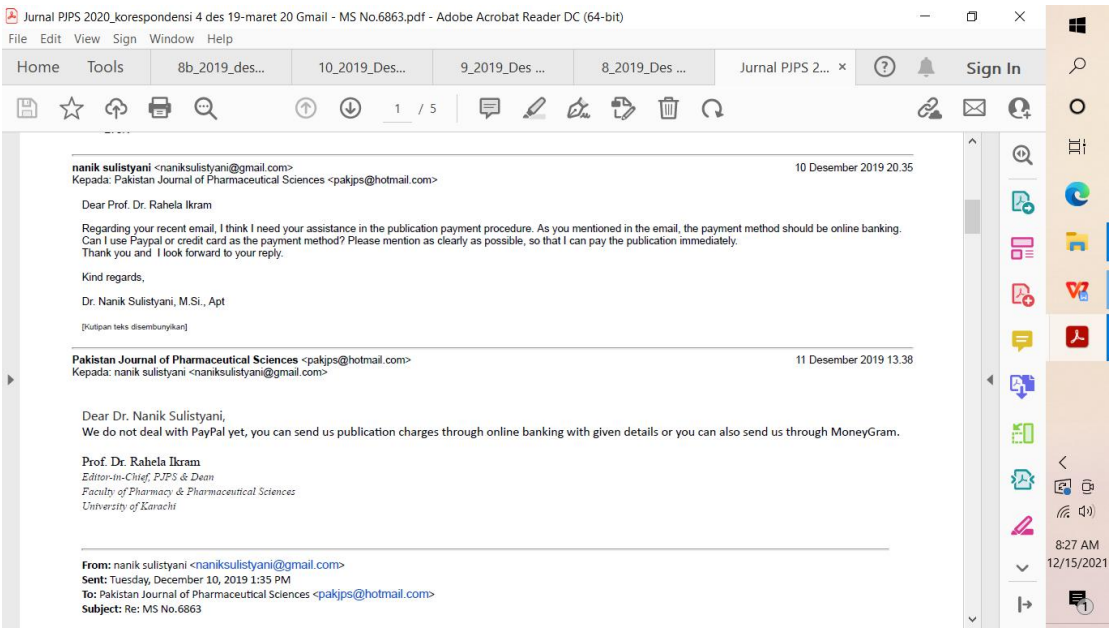

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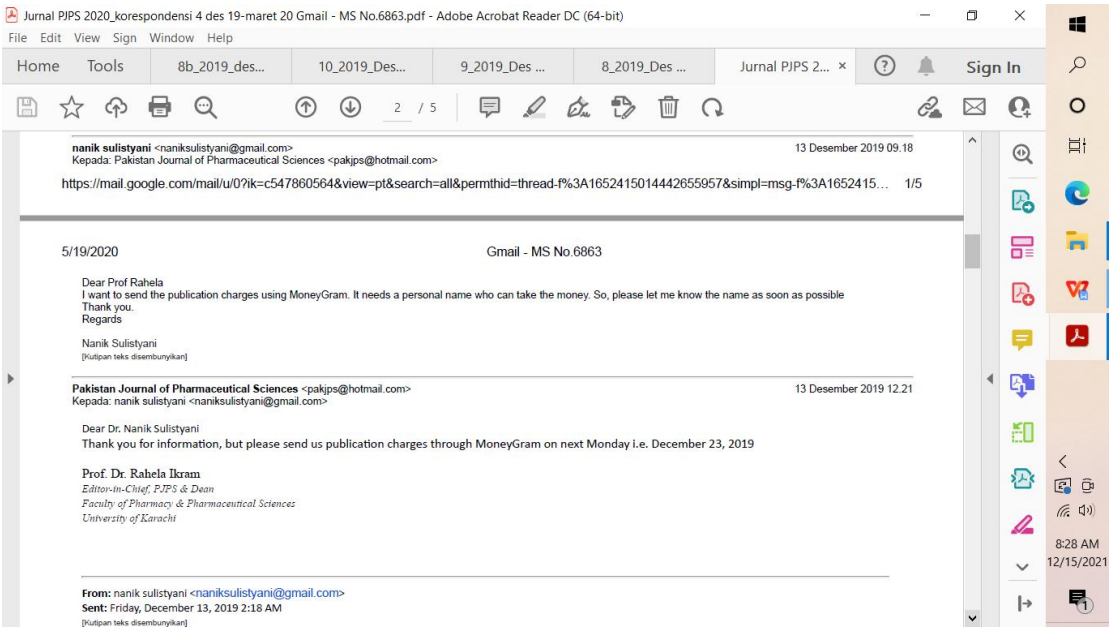

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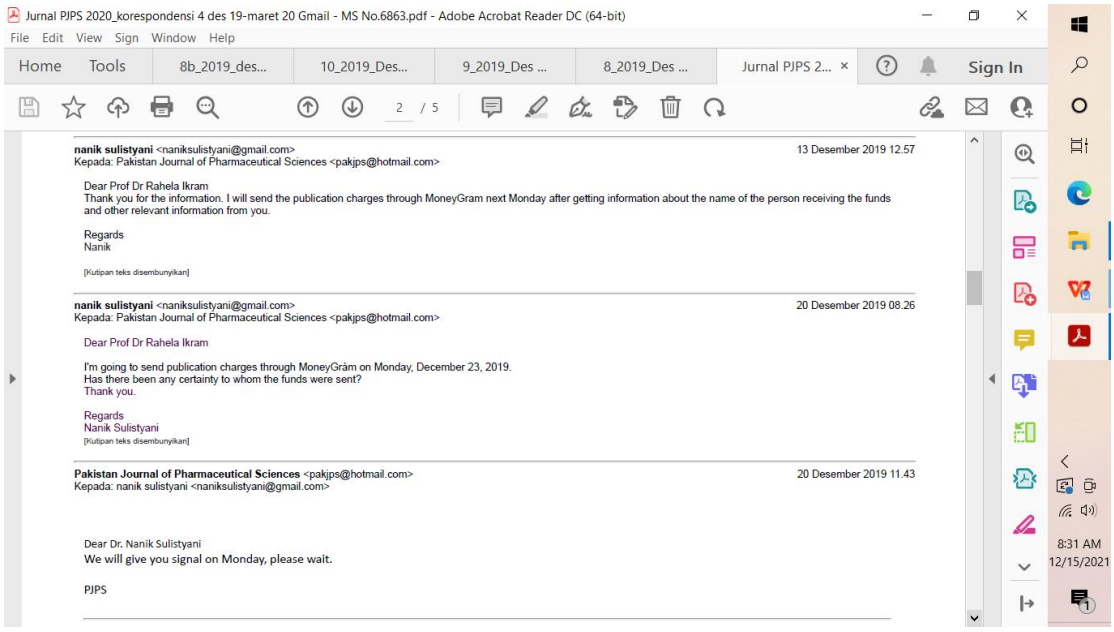
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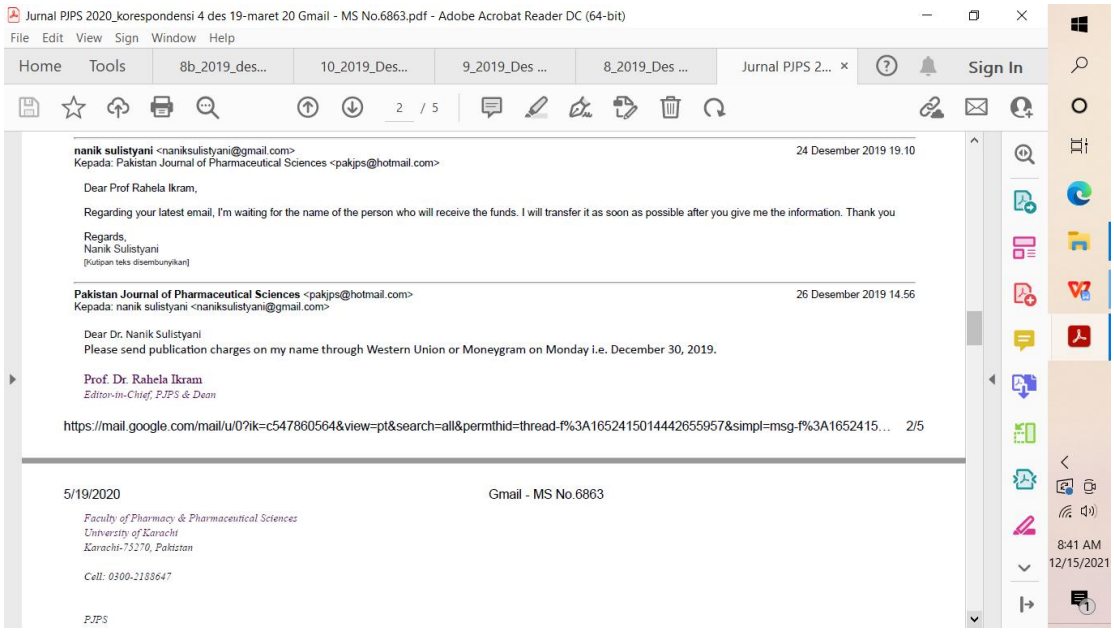
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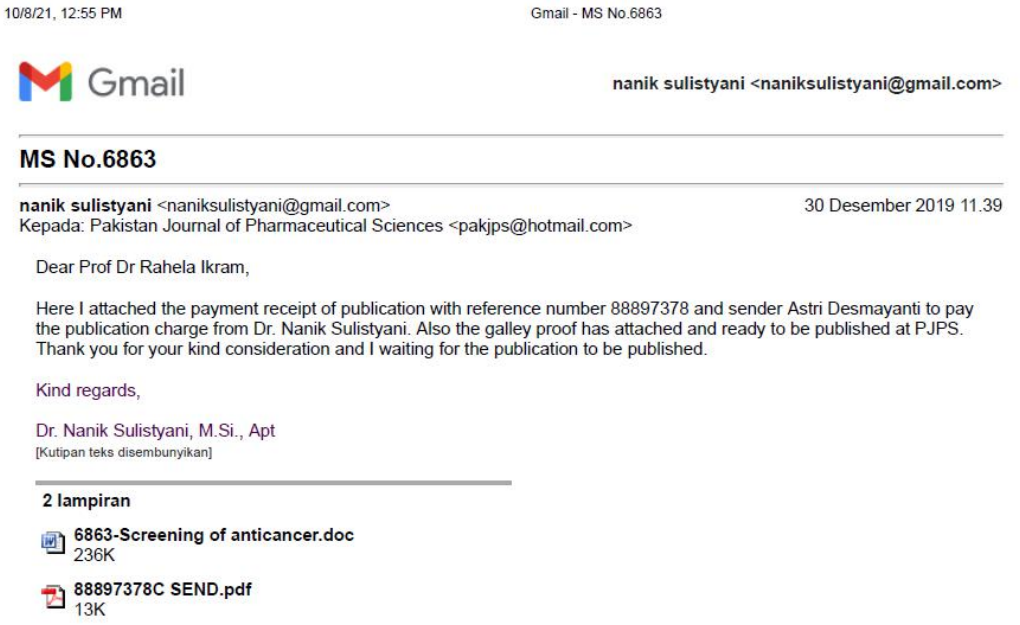

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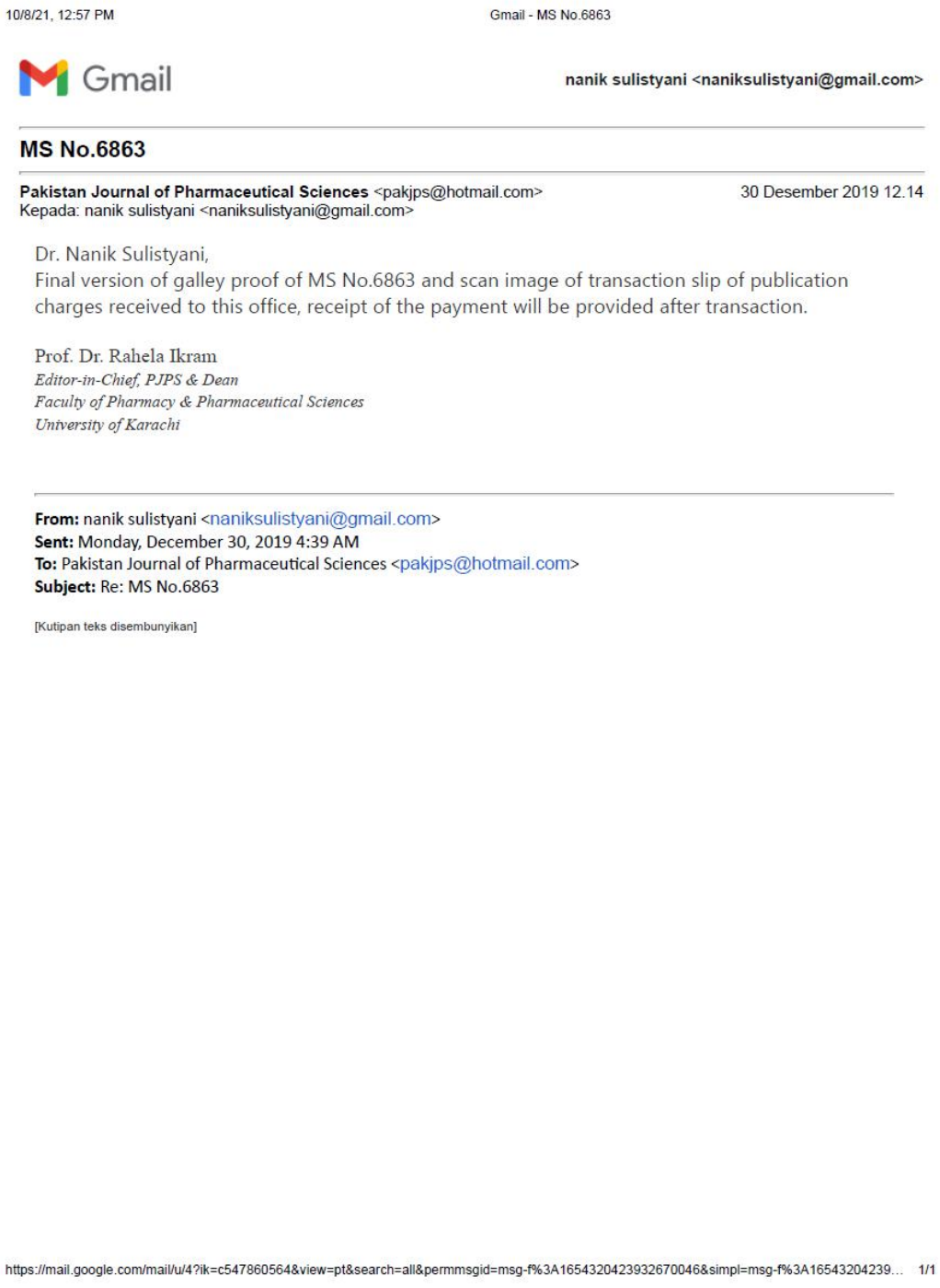
Lampiran 15. Email Jawaban dari Editor tentang penerimaan Naskah Galleyproof dan bukti pembayaran



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Corrections:
Comparison of significance has not been done for table 5.
In area levels correct table no to 6.
Also in labeling of table 6 it is shown that comparison to group II but in result text group I is written recheck?
Results and Discussion should be separated as per format of PJPS

Please note also:

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Artikel :

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

Nanik Sulistyani and Nurkhasanah
Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia

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 2 was given 20mg/kg body weight (BW) of DMBA. Groups 3, 4, and 5 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

Materials

E. scaber leaves was collected from Sentolo, KulonProgo, Yogyakarta, Indonesia and the plants identification was 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

**Corresponding author:* e-mail: naniksulistyani@gmail.com

by maceration using 70% ethanol. The extract obtained was then evaporated using rotary evaporator at 50°C and heated over the waterbath until thick extract was obtained.

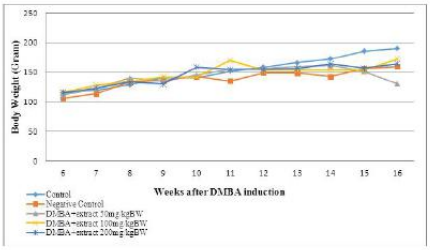

Fig. 1: Development of rats body weight after DMBA induction and administration of *E. scaber* ethanol extract.

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

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

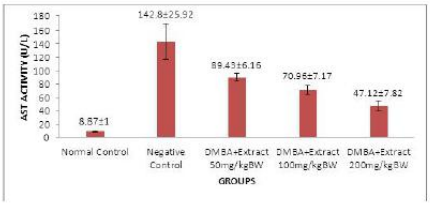

Fig. 3: Average of AST levelin rats blood serumafter DMBA induction and administration of *E. scaber* ethanol extract

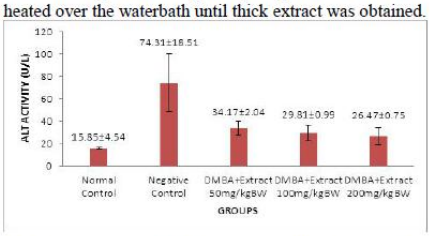

Fig. 4: Average of ALT levelin rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract

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

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.

Table 2: Tumor incidence in ratsafter 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

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*

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Negative Control	142.8±25.92	74.31±18.51
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DMBA + extract 200 mg/kgBW	0.28 ± 0.07*

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

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 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 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. Multiplicity tumors provide synchronous results on the potential of tumor inhibition and tumor incidence proportionally. 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).

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. The result of urea levels determination shown in the table 6.

Normal urea level in rats is 15-21mg/dL (Nikolic *et al*, 2003). Urea levels in group 1 was higher than group 3, 4, and 5. Based on table 1, Group 2'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 1 and group 2 with a significant value of 0.029 ($p<0.05$). It means that DMBA injection causes urea levels to increase significantly. Groups 3, 4 and 5 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 3, 4 and 5 differed significantly from group I 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/kg BW 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

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. The result of creatinine levels examination can be seen in the table 7.

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 1 and group 2 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 3, 4, and 5 had lower creatinine levels compared to group 2 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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Khan M, Maryam A, Qazi JI and Ma T (2015).Targeting apoptosis and multiple signaling pathways with

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 3, 4 and 5 differed significantly from group I 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/kg BW 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

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. The result of creatinine levels examination can be seen in the table 7.

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 1 and group 2 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 3, 4, and 5 had lower creatinine levels compared to group 2 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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Lampiran 17. Bukti Email Pengiriman naskah yang sudah direvisi beserta naskah dan response sheet

10/8/21, 1:01 PM Gmail - MS No.6863

Gmail nanik sulistyani <naniksulistyani@gmail.com>

MS No.6863

nanik sulistyani <naniksulistyani@gmail.com> 30 Maret 2020 00.35
Kepada: Pakistan Journal of Pharmaceutical Sciences <pakjps@hotmail.com>

Dear Prof Rahela Ikram,

Regarding to your corrections, here I attached the correction of article.

Regards,
Nanik Sulistyani
[Kutipan teks disembunyikan]

2 lampiran

6863-Screening of anticancer-30-12-2019.doc
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Response Sheet.docx
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Artikel :

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

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Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia

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

Materials

E. scaber leaves was collected from Sentolo, KulonProgo, Yogyakarta, Indonesia and the plants identification was 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

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by maceration using 70% ethanol. The extract obtained 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).

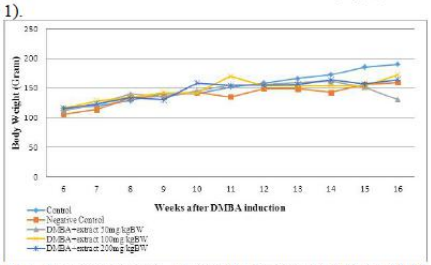

Fig. 1: Development of rats body weight after DMBA induction and administration of *E. scaber* ethanol extract.

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 (Fig.3, Fig.4, Table 5).

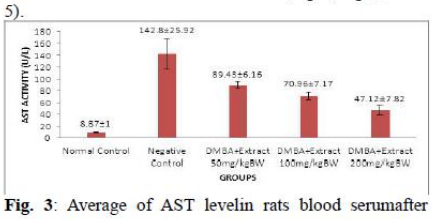

Fig. 3: Average of AST levelin rats blood serumafter DMBA induction and administration of *E. scaber* ethanol extract

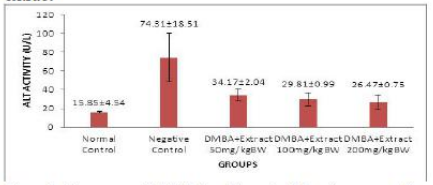

Fig. 4: Average of ALT levelin rats blood serum after DMBA induction and administration of *E. scaber* ethanol extract

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 ratsafter 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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Response sheet

RESPONSE SHEET

On

MS No. 6863

<table>
<tr><th>S. No.</th><th>Queries</th><th>Replies</th></tr>
<tr><td>1.</td><td>Comparison of significance has not been done for table 5</td><td>
<table>
<tr><th rowspan="2">Groups</th><th colspan="2">Mean±SD (U/L)</th></tr>
<tr><th>AST</th><th>ALT</th></tr>
<tr><td>Normal Control</td><td>8.87± 1.00*</td><td>15.85±4.54*</td></tr>
<tr><td>Negative Control</td><td>142.8±25.92</td><td>74.31±18.51</td></tr>
<tr><td>DMBA + extract 50 mg/kgBW</td><td>89.43±6.16*</td><td>34.17±2.04*</td></tr>
<tr><td>DMBA + extract 100 mg/kgBW</td><td>70.96±7.17*</td><td>29.81±0.99*</td></tr>
<tr><td>DMBA + extract 200 mg/kgBW</td><td>47.12±7.82*</td><td>26.47±0.75*</td></tr>
</table>
*: showing the significant difference to negative control ($p<0.05$)
</td></tr>
<tr><td>2.</td><td>In area levels correct table no to 6.</td><td>Done</td></tr>
<tr><td>3.</td><td>Also in labeling of table 6 it is shown that comparison to group II but in result text group I is written recheck?</td><td>Normal urea level in rats is 15-21mg/dL (Nikolic et al, 2003). Urea levels in group 2 was higher than group 3, 4, and 5. Based on table 6, Group 2'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 1 and group 2 with a significant value of 0.029 ($p<0.05$). It means that DMBA injection causes urea levels to increase significantly. Groups 3, 4 and 5 had lower urea levels than group 2, 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 3, 4 and 5 differed significantly from group 2 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</td></tr>
</table>

		BW, 100mg/kg BW, and 200mg/kg BW 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.
4.	Results and Discussion should be separated as per format of PJPS	Done

Lampiran 18. Bukti Email dari Editor tentang penerimaan naskah yang sudah direvisi

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