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Research Article
Effect of *Musa acuminata* Stem By Immunohistochemistry Test in Ulcer

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Abstract

Background and Objective: Gel of *Musa acuminata* stem from South Borneo had shown to accelerate wound healing by increasing the number of neovasculars on the 5th day. The study aimed to determine the effect of gel of *Musa acuminata* stem extract (EBPM) towards increased expression of HIF-1α, Hsp90α and VEGF in healing traumatic ulcer. **Materials and Methods:** This research was a true experimental with 40 rats of *Rattus norvegicus* of male Wistar strain as the traumatic ulcer models. The biopsy was taken from the ulcer in the left buccal oral mucous. The study was divided into the negative control group (K) given gel, treatment group 1 (P1) given gel of 25% EBPM, treatment group 2 (P2) given gel of 37.5% EBPM and treatment group 3 (P3) given gel of 50% EBPM. On the 3rd and the 5th days, the models were decapitated. They were next biopsied in order to make preparations and to conduct immunohistochemistry test. **Results:** The results proved that the EBPM gel was effective when administered at a concentration of 37.5% in the 5th day because it significantly increased the expressions of HIF-1α, Hsp90α and VEGF. **Conclusion:** It can be concluded that gel of *Musa acuminata* stem extract of 37.5% could increase neovascular number through increasing the expression of HIF1α, HSp90α and VEGF to accelerate oral ulcer healing, including traumatic ulcer. It has indicated that gel of *Musa acuminata* stem extract of 37.5% concentration can be recommended as a natural ingredient of topical drug to accelerate ulcer healing in oral mucous.

Key words: Condensed tannin, *Musa acuminata* stem, HIF-1a, Hsp90a, immunohistochemistry, VEGF, healing, traumatic ulcer

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Traumatic ulcer is one of the most often occurred disorders in the oral mucous. Traumatic ulcer prevalence is quite high, as evidenced by several studies showing varied numbers between 3-24% of the population[1]. Ulceration in the oral cavity will disrupt the process of mastication, resulting in nutrition intake disorders. Therapy administration of the oral mucous ulceration is symptomatically done aiming to reduce inflammation, pain and accelerate wound healing[1,2].

The latest research has proven that wound infection is often triggered by bad circulation of the blood vessels surrounding the wound. Topical medications obtained from plant materials can solve the problem. One of traditional Indonesian plants to accelerate healing time is mauli banana stem (*Musa acuminata*) originating from South Borneo, Indonesia. *Musa acuminata* stem extract is an antioxidant with a heavy metal-binding activity of iron, hydrogen peroxide and hydroxyl. In addition, it is able to reduce levels of malondildehyde (MDA) and increase the activity of Super Oxide Dismutase (SOD) and *catalase* in oral mucosal wound healing process of rats[3,4].

Previous research had shown that the banana stems contain bioactive substance of condensed tanins[5,6]. *Musa acuminata* stem extract contains the most bioactive substance namely tannins reaching approximately 67.59%. This substance causes the *Musa acuminta* stem extract given to wound can increase the number of macrophages on the 3rd day and can also increase the number of neovascular on the 5th day[7,8]. Previous research suggests that condensed tannins may induce tyrosine phosphorylation of insulin receptor on the cell surface of 3T3-L1 adipocytes *in vitro*, so it can activate phosphatidylinositol 3-kinase pathway (PI3K), which plays a role in cell proliferation[9,10].

In the angiogenesis process of wound healing, hypoxia conditions exist physiologically. Furthermore, there are changes in systemic hemodynamics and blood vessels that lead to hypoxia inducable factor-1α (HIF-1α) inducing the expression of VEGF[11]. This process involves Hsp (Heat shock protein) 90α that will govern the process of angiogenesis along with increased expression of VEGF[12]. When extract of *Musa acuminata* stem is applied on the wound, there would be a proliferation of macrophages which results in increased expression of several growth factors in the wound healing process. One of them is Vascular Endothelial Growth Factor[13] (VEGF). The VEGF will trigger the proliferation and differentiation of cells in the angiogenesis area and increase the proliferation, differentiation and migration of endothelial cells[14,15]. The VEGF is an angiogenesis regulator. The HIF-1α affected by Hsp90α affects the VEGF, so it can activate intracellular signaling cascade resulting in an increase in vascular permeability, proliferation, survival, migration and mobilization of endothelial progenitor cells[16,17]. This research aimed to determine the effect of gel of *Musa acuminata* stem extract on the expressions of HIF-1α, Hsp90α and VEGF in healing traumatic ulcer.

MATERIAL AND METHODS

Research design: The type of this study was a true experimental research. The experimental units were 40 rats of Rattus norvegicus of male Wistar strain as the traumatic ulcer models. The biopsy was taken from the ulcer in the left buccal oral mucous. The criteria were healthy, weighed 250-300 g and aged 2-3 months. Ethical approval was was conducted at the Faculty of Dentistry, Airlangga University.

Plant material: *Musa acuminata* plant was taken after producing banana fruit. The used methods were maceration then the ethanol-free test. The gel composition of ethanol extract of *Musa acuminata* stems with a concentration of 25, 37.5 and 50% with 15% HPMC, 1% tween 80, 8% propylene glycol, 5 drops of peppermint oil and distilled water until reaching 100%.

Materials for immunohistochemistry test: Chemicals for immunohistochemistry test were xylol, ethanol, PBS, *trypsin*, alcohol, distilled water, *streptavidin-biotin,* 0.5% H_2O_2, substrate, *phosphotase buffer*, anti-mouse antibody monoclonal to HIF-1α (R and D system), Hsp90α (Santa Cruz Biotechnology, Inc.) and VEGF (BIOSs antibodies). Materials to make the preparation and staining of histopathology were by 10% BNF, alcohol, xylol and paraffin.

Research animals: This study used 40 rats, divided into the negative control group (K) given gel, treatment group 1 (P1) given gel of 25% EBPM, treatment group 2 (P2) given gel of 37.5% EBPM and treatment group 3 (P3) given gel of 50% EBPM applied every 6-8 h day^{-1}.

Research procedure: The treatment to Wistar rats was started by administering inhaled ether anesthesia, then the left buccal oral mucous was injured with biopsy punch with a diameter of 6 mm and a depth of 1 mm and then the wound was taken with a scalpel. The left buccal mucous of the rats was biopsied on the 3rd and 5th day after decapitated. The tissue was soaked in 10% buffered formalin (pH 7.4) and then it was made into preparations and was done immunohistochemistry test to show the expressions of HIF-1α, Hsp90α and VEGF.

RESULTS

HIF-1α expression of macrophage cells of the traumatic ulcer: The following are the results of statistical analysis using Kruskal Wallis. In the time variable of EBPM gel administration on the 3rd day, there was a significant diference in the EBPM gel of 0% concentration (Negative control) compared to all EBPM gels. On the 3rd day, there was a significant difference in the EBPM gel of 25% concentration compared to EBPM gels of 0% and of 50%. On the 3rd day, there was a significant difference in the group of EBPM gel of 37.5% concentration compared to EBPM gel of 0% concentration and 50% concentration. On the 3rd day there was a significant difference in the treatment group of EBPM gel of 50% concentration compared to EBPM gel of 0% and all groups. Overall, in the 5th day there was a significant difference in all treatment groups (EBPM gel of 25, 37.5 and 50% concentrations) compared to EBPM gel of 0% concentration. Mann Whitney statistical analysis indicated a significant difference ($p<0.05$). Based on the timing variable, in all groups of the 3rd day compared to the ones in the 5th day which was gel group had $p = 0.005$, the treatment group of 25% concentration had $p = 0.006$, the group of 37.5% concentration had $p = 0.005$ and the treatment group of 50% concentration had $p = 0.005$. There was an increased HIF-1α expression on the 3rd day than the on the 5th day in all groups. The highest increased expression of HIF-1α occurred in the 37.5% concentration, from 0-6% (Fig. 1).

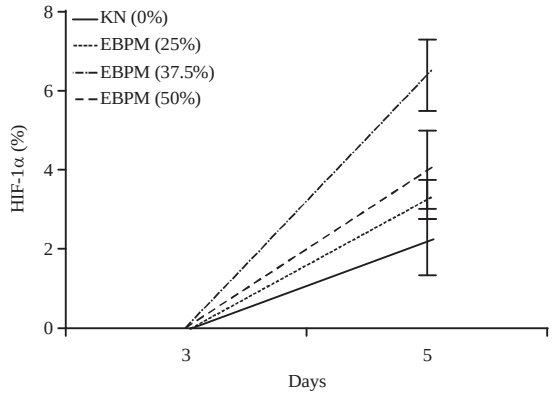

Fig. 1: HIF-1α expression of endothelial cells in the traumatic ulcer on the 3rd until 5th day

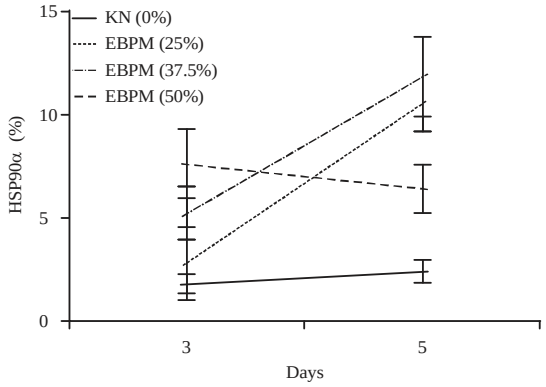

Fig. 2: Hsp90α expression of endothelial cells in traumatic ulcer on the 3rd until 5th day

Hsp90α expression of endothelial cells of the traumatic ulcer: Below are the results of statistical analysis using Kruskal Wallis. In the time variable of EBPM gel administration on the 3rd day there was a significant difference between the group of 0% EBPM gel concentration (negative control) compared to all concentrations of EBPM gel. On the 3rd day there was a significant difference between the treatment group of 25% EBPM gel concentration compared to EBPM gel of 0% and the 50% concentration. On the 3rd day there was a significant difference between the treatment group of EBPM gel of 37.5% concentration compared to EBPM gel of 0% concentration and of 50% concentration. On the 3rd day there was a significant difference between the treatment group of EBPM gel of 50% concentration compared to EBPM gel of 0% concentration and all groups. Overall, on the 5th day there seemed a significant difference between all treatment groups (EBPM gel concentration of 25, 37.5 and 50%) with the group of 0% EBPM gel concentration.

Below are the results of statistical analysis of this study using the Mann Whitney. Based on the administration time variable, there seemed a significant difference ($p<0.05$) in the administration of EBPM gel of 25% concentration, $p = 0.000$ and 37.5% with $p = 0.000$ on the 3rd day compared to the 5th day. In this study there was an increase of Hsp90α expression on the 3rd day compared to the 5th day in all groups, except in the EBPM gel group of 50% concentration with no increase of Hsp90α expression (fixed 7%). The highest increase of Hsp90α expression occurred at the concentrations of 25 and 37.5%. In the group of mauli banana stem extract gel of 25% concentration were these data (3-11%) and of 37.5% concentration (5-12%) (Fig. 2).

VEGF expression of endothelial cells of the traumatic ulcer: Below are the results of statistical analysis using Kruskal Wallis in the administration time variable of EBPM gel. In the administration time variable of EBPM gel on the 3rd day there was a significant difference between the EBPM gel of 0% concentration group compared to groups of EBPM gel of 37.5 and 50% concentrations. On the 3rd day there was a significant difference between the EBPM gel groups of 25% concentration compared to the EBPM gel groups of 37.5 and

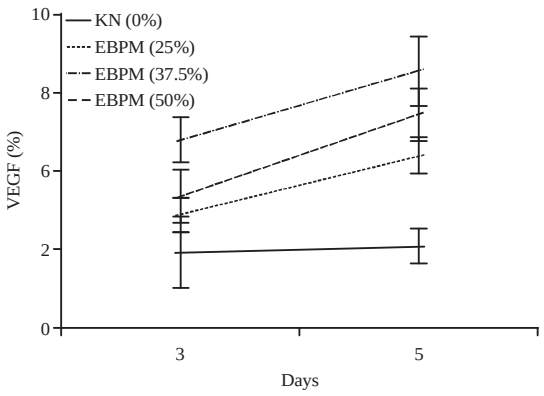

Fig. 3: VEGF expression of endothelial cells in the traumatic ulcer on the 3rd until 5th day

50% concentrations. On the 3rd day there was a significant difference in the EBPM gel group of 37.5% concentration compared to all groups. On the 3rd day there was a significant difference between the EBPM gel group of 50% concentration compared to the EBPM gel groups of 0% concentration and of 37.5% concentration. On the 5th day, it seemed that all treatment groups had a significant difference with the EBPM gel group of 0% concentration.

Below are the results of statistical analysis of this study using the Mann Whitney. There seemed a significant difference, based on the administration time variable, ($p<0.05$) in the administration of EBPM gel of all groups on the 3rd day compared to the 5th day, except in the the EBPM gel groups of 0% concentration which was not significantly different. In the treatment group EBPM gel of 25% concentration was $p = 0.012$, in the treatment group EBPM gel of 37.5% concentration was $p = 0.005$ and in the treatment group EBPM gel of 50% concentration was $p = 0.001$. In this study, there was a significantly increased expression of VEGF on the 3rd day than on the 5th day in all groups of EBPM gel. The highest increased expression of VEGF occurred in EBPM gel of 37.5% concentration (6-9%) and 50% concentration (4-7%) (Fig. 3).

DISCUSSION

Condensed tannins in mauli banana stem extract will stimulate tyrosine phosphorylation in the insulin receptor. Condensed tannins may bind in the α-subunit of the insulin receptor in cell membrane *in vitro* and make kinase tyrosine residue in the β-subunit undergo autophosphorylation.

Autophosphorylation of tyrosine kinase receptors can bind to PI3K and activate it. Active PI3K can catalyze the phosphorylation of PIP3, next PIP3 will recruit two kinase proteins namely AKT and PDK1 heading to the plasma membrane and activating AKT. Active AKT causes Rheb to become active, then triggers mTOR[8,9]. THE mTOR will activate p 70 S6K and phosphorylation of 70 and 85 kDa isoforms of S6K[18]. The S6K later phospolytrates the transcription factors of FoxO3a in the nucleus, which next inhibits CDK inhibitor. As a result, there is CDK activation and it stimulates cell proliferation of macrophages[19].

Condensed tannins has proven to induce tyrosine phosphorylation of the insulin receptor, then as an antioxidant extract of mauli banana stems, it has verified to increase the activity of SOD and catalase, therefore preventing excessive ROS[4,10,20]. This will prevent the expressions of excessive HIF-1α and Hsp90α. Hence it prolongs the healing time. Previous research has shown that administration of antioxidants materials (ROS scavenger) at high doses will cause macrophages to prevent the production of angiogenic factors but when they are given in moderate doses, they will trigger macrophages running angiogenesis processes physiologically[21].

In this study, on the 3rd day it did not seem any HIF-1α expression in the administration of all EBPM gel groups of 25, 37.5 and 50% concentrations and EBPM gel of 0% concentration (negative control). Other studies have also proven that HIF-1α expression was only visible on the 5th day of oral mucous healing. This is because adequate hypoxia in the mucous wound does not happen to trigger the HIF-1α expression. This situation is the one causing wound in the mucous to heal faster when compared to the one on the skin so that in the mucous, scar is never formed, because the faster hypoxia process than on the skin so that the inflammatory process ends quickly. Next decreased expression of HIF-1α would also reduce VEGF expression in mucous wound[21].

Intracellular ROS will form a physiological hypoxic conditions to help the signaling cells through the interaction of HIF-1α and Hsp90 in normal conditions. This will induce HIF-1α and Hsp90. The Hsp90 will bind to HIF-1α causing RNA protein of HIF-1α folding. The process is through HSF induction (Heat shock factor)-1 which causes the inactive monomeric HSF-1 phosphorylated and changed into a trimeric HSF, joining the HSE, then they translocate to the nucleus. The Hsp90 will activate the expression of HIF-1α[22,23].

Heat shock response is regulated by heat shock transcription factors (HSF). One of the HSF genes which is HSF1 is accumulated in the core and joins trimerly in heat shock element (HSES). The HSF1 is modified by phosphorylation, where HSF1 phosphorylation substitutes

(Ser^{303,} Ser^{307} and Ser$^{419)}$ are important in the negative regulation of lowering HSF1 activity, while the ones including (Ser^{230}, Ser^{326} and Ser^{419}) activating phosphorylation activate HSF1 activity. The existence of a balance of kinase and phosphatase activity in activating HSF1 is an important regulation of the heat shock response. That is why there is a negative regulation of HSF1 with the presence of control namely feedback mechanisms in Hsp90α. In cells, the Hsp90α expression will seem weak[24,25]. This is evident in the results of this study on the 3rd day in which there was an increase in the Hsp90α expression of EBPM gel administration of 37.5 and 50% concentrations, whereas on the 5th day there was an increase in the Hsp90α expression of EBPM gel administration of 25, 37.5 and 50% concentrations.

About 50% concentration only increased on the 3rd day. The increased of Hsp90α expression from the 3rd day to the 5th day occured in the EBPM gel of 25 and 37.5% concentrations, while within the 50% concentration there was no such increase. The reason is because in EBPM gel administration of 50% concentration was a high increase of SOD and catalase activity, so finally the intracellular ROS decreased but it was still enough to cause an angiogenesis process. The Hsp90α still plays a role in the activation of HIF-1α without going through the protein pathway of C terminal domain but through hypoxic conditions[25].

The VEGF is one of important angiogenic factors. The beginning of angiogenic effects is the binding of VEGF-A and VEGFR-2. Angiogenesis stimulation through the pathway of PI3K-Akt-eNOS resulting in endothelial cells will lead to migration, proliferation and differentiation. Phosphatidylinositol 3 kinase starts molecule signaling from the serine/threonine kinase Akt/protein kinase B. The Akt/PKB is through a phosphorylation of endothelial nitric oxide synthesis in Ser 1177 which stimulates the production of NO, vasodilatation and migration of endothelial cells[26,27].

Some studies also have shown that plants containing condensed tannins will increase the expression of VEGF such as Red wine and *Terminalia chebula* on the 5th day, in which later on the expression fall on the 7th day 27. In this study, the increased expression of VEGF occured in the EBPM gel of 37, 5 and 50% concentrations on the 3rd day, while on the 5th day of the increased expression of VEGF occurred in the 25%, 37.5 and 50% concentrations. Increased expression of VEGF from the 3rd day to the 5th day occurred in the EBPM gel of 25, 37.5 and 50% concentrations but the highest VEGF expression occurred in the EBPM gel administration of 37.5 and 50% concentrations.

Condensed tannins are bioactive groups of polyphenol Bioflavanoid produced by plants to accelerate wound healing. Previous research has shown that the grape extract has the ability to activate the redox reaction, thereby producing oxidants that could trigger the mRNA expression which later is able to increase VEGF expression[28,29]. The results of this study proved that the EBPM gel was correct when given at a concentration of 37.5% on the 5th day. It can significantly increase the expressions of HIF-1α, Hsp90α and VEGF (Fig. 4a-c and Fig. 5a-c).

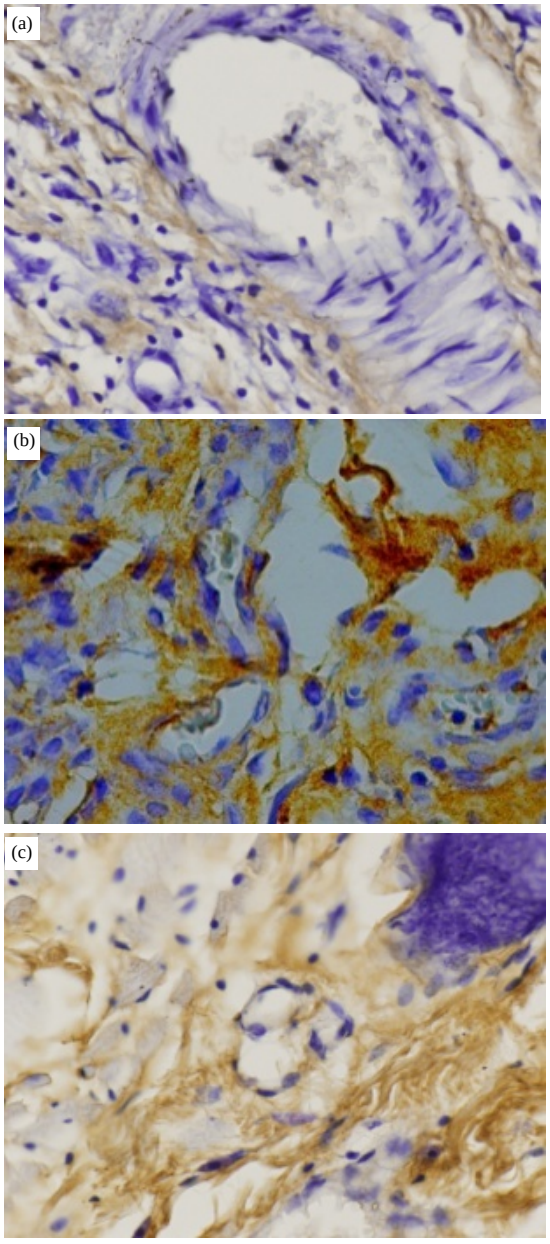

Fig. 4(a-c): Results of immunohistochemistry test (400x) of 37.5% EBPM showed expressions of (a) HIF-1α (b) Hsp90α and (c) VEGF in the endothelial cells of the buccal oral mucous (brown in the cytoplasm) on the 3rd day

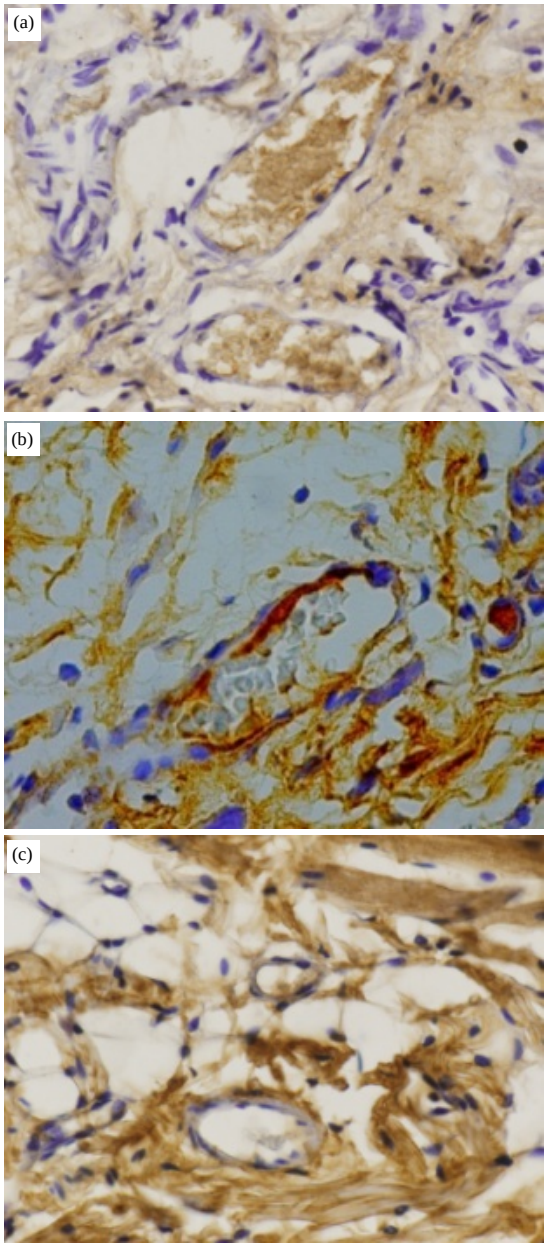

Fig. 5(a-c): Results of immunohistochemistry test (400x) of 37.5% EBPM showed expressions of (a) HIF-1α (b) Hsp90α and (c) VEGF in the endothelial cells of the buccal oral mucous (brown in the cytoplasm) on the 5th day

CONCLUSION

The present study have indicated that gel of *Musa acuminata* stem extract of 37,5% could increase neovascular number through increasing the expression of HIF1α, HSp90α and VEGF to accelerate oral ulcer healing, including traumatic ulcer. This findings that gel of *Musa acuminata* stem extract of 37.5% concentration can be recommended as a natural ingredient of topical drug to accelerate ulcer healing in oral mucous.

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