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Potential of Malaysian White Type Edible Jellyfish, *Lobonema smithii* as Antioxidant and Collagen Promoter in Dermal Wound of Sprague Dawley Rats

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Abstract: In this study, for the first time we reporting the potential of white type edible jellyfish *Lobonema smithii* as antioxidant and collagen stimulator in wound healing process. Antioxidant activity of methanolic extract and crude protein of the species were determined using 1, 1-dipheyl-2-picrylhrazyl (DPPH) free radical scavenging. In vivo, full thickness wounds were created on the dorsal area of control and treatment rats. The treatment rats were orally administrated with freeze-dried jellyfish powder (1000 mg/kg) while control rats were fed with normal pellet daily. Physical observation and wound contraction were recorded daily for 21 days. Granulation tissues, kidney and liver were removed and blood was drawn from each rat on days 7, 14 and 21 post-wounding and used for histopathological, hematology and toxicity study. Antioxidant assay showed the methanolic crude extract has higher antioxidant activity (46.78%) when compared to crude protein (37.60%). Epithelialization, wound contraction rate, and collagen deposition were significantly higher ($p<0.05$) in treated animals compared to control. No toxicity and allergic reaction observed in all animals. These findings clearly substantiate the beneficial effects of Malaysian white type edible jellyfish *L.smiithi* consumption as antioxidant and stimulator of collagen deposition in wound healing process.

Key words: Collagen · Wound Healing · Jellyfish · *Lobonema smithii* · Antioxidant

INTRODUCTION

Nowadays, non-healing chronic wounds have become new challenge to the patient and wound care clinician worldwide. According to statistics for injury in Malaysia, open wound contributed 29.2% of incidence leading to injuries [1]. Wound is a rupture in the epithelial integrity of the skin and may be accompanied by disruption of the anatomic structure and function of underlying normal tissue caused by violence or other means [2, 3]. It is important to treat wound immediately in order to restore the integrity of the skin as it plays a very important role in maintaining homeostasis [3, 4]. Untreated wound leads to challenging clinical problem and complications that can cause morbidity and mortality [5]. Healing of wound, whether from accidental injury or surgical intervention, involves an orderly progression of events which comprise an intricate network of blood cells, tissue types, cytokines and growth factors [6]. Generally, wound healing process can be broadly categorized into three main stages; inflammatory phase (consisting the establishment of homeostasis and inflammation), proliferate phase (comprises of granulation, contraction and re-epithelization) and finally the remodeling phase which ultimately determines the strength and appearance of healed tissue [2, 5]. Wound usually came along with a short period of pain, swelling and reddening an indicator of inflammatory reaction that triggered by the release of eicosanoids, prostaglandins, leukotrienes and reactive oxygen species (ROS) [7]. ROS is produced in high

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amounts at the sites of wound as a defense mechanism against infection of bacteria, however the healing process may be impeded and become chronic by the presence of excessive free radicals or microbial infection [8]. Natural product or substances that are having antioxidant capability has been widely reported to help and accelerates wound healing process [9-12]. Therefore, it is important to develop and utilize effective natural antioxidants so that they can protect the human body from free radicals and stimulate the occurrence of others chronic diseases.

In Malaysia particularly in Sarawak state, some large edible jellyfish including dried product are considered to be delicacies in Chinese cooking and become an important fishery commodity in Southeast Asia [13]. The white type jellyfish *Lobonema smithii* is a type of edible jellyfish which has flat bell, besprinkled exumbrella, 8 separated mouth-arms, 16 radial-canals, 8 rhopalar and 8inter-rhopalar [14]. The medicinal values of jellyfish has long been recognized [15] as its body mainly consist of collagen protein [16-19] which are believed to be effective in treating ailments such as arthritis, high blood pressure, back pain, ulcer and indigestion, to treat fatigue, stimulate blood flow during menstruation, anti-aging, cartilage repair and reduce swelling [13, 19-21]. Furthermore, protein isolated from other edible red jellyfish *Rhopilema esculentum* has been shown to inhibit free radical scavenging activities studied using various *in vitro* antioxidant assays [18, 22].

To the best of our knowledge, until now there are lacks of researchers looking at the potential of Malaysian white type edible jellyfish *L.smithii* as antioxidant and wound healing promoter. Realizing the potential of other types of jellyfish protein and collagen as described earlier, hence the present study was conducted to investigate the potential of antioxidant activity of Malaysian white type edible jellyfish *L.smithii* methanolic extract and its crude protein and also to study the efficacy of processed *L.smithii* consumption as a collagen promoter in wound healing process.

MATERIALS AND METHODS

Sampling and Materials: Fresh salted jellyfish was obtained from Sea Horse Corporation Sdn. Bhd, Kuching, Sarawak, Malaysia. The jellyfish type is locally known as white type jellyfish and determined as *Lobonema smithii.* The organs consist of whitish exumbrella with numerous 1-3cm long, pointer papillae and umbrella up to 500mm in diameter [14][23]. Only the exumbrella (bell) part of the jellyfish was used in this study.

Sample Preparation: Salted samples were cut into small pieces of 3-5cm length and desalted by soaking into distilled water for 36 hours followed by washing three times per day respectively. The desalted samples were then frozen at -80°C and freeze dried under vacuum pressure for 72 hours. All dried samples were ground into fine powder for further extraction process and used for *in vivo* study.

Methanolic Extract: Powdered dried jellyfish (1000 g) sample was soaked into 1000ml (w/v) of 95% (v/v) methanol for 5 consecutive days. The sample was filtered and underwent rotary evaporation process to remove the organic solvent and to obtain the crude yield for antioxidant activity screening.

Crude Protein Isolation: Method was modified from Yu *et al*., [22]. Frozen desalted *L.smithii* jellyfish exumbrella was immediately homogenized in cold (4°C) phosphate buffer solution (0.01M, pH 7.4) four times for 60 s each time. The homogenized fluid was centrifuged at 5000 rpm for 5 minutes to separate the jellyfish residues. Remaining supernatant was then slowly added with cold acetone (-20°C) in ratio of 1:3 (v/v) for protein precipitation. Crude protein was obtained by centrifugation (15,000 rpm) for 45 minutes at 4°C. Protein pellets then were rinsed with cold acetone and allowed for air dry. The crude protein was kept in -20°C for antioxidant activity screening.

DPPH Free Radical Scavenging Assay: The DPPH (1,2-Diphenyl-2-picrylhydrazyl radical) free radical scavenging abilities of the test compounds were determined by measuring the change in absorbance of DPPH at 517 nm by the spectrophotometric method described by Thitilertdeca *et al*., [24]. All measurements were conducted in replicate. The ability to scavenge the DPPH radical was calculated as percentage of DPPH using the following equation:

$$\% \text{ DPPH scavenging} = [(A_0-A_1)/A_0)] \times 100$$

A_0= absorbance of the control, A_1= absorbance of the extract mixed with DPPH.

Quercetine (Sigma Aldrich Gmbdh, Germany), was used as standard for comparison to determine IC_{50} value of the extracts concentration.

Wound Healing Study

Animal: Thirty six clinically healthy Sprague Dawley's female rats weighing between 200 -250 g were randomly

selected for wound healing study. All animals were housed in standard environmental conditions with temperature of 25 ± 1°C with 12 hours light and 12 hours dark cycle. All experimental animals were acclimatized to hygienic laboratory condition for 7 days before the start of experiment. Animals were fed with standard commercial pellet of 10g/kg body weight and tap water *ad libitum*.

Experimental Design: A total of 36 rats were divided equally into two groups (A and B) with 18 animals each. Group A (the control group) where no treatment was given while Group B was subjected to oral treatment of processed *L.smithii* jellyfish powder (1000 mg/kg). All animals in each group were anaesthetized with light ether prior to the wound incision and 70% of alcohol was applied as topical disinfection on shaved selected areas, preferably on dorsal thoracic region part of animal skin. An 8 mm diameter of wound was created by using sterile wound biopsy punch (FRAY, USA). Each animal was wounded with uniform two circular excisions on the dorsal part individually to represent duplicate. Wound contraction and measurements were taken daily and animals were euthanized for skin tissues collection at day 7, 14 and 21 days.

Histopathological Studies: Granulation tissue specimens, liver and kidneys from control and treated rats were collected and fixed in 10% buffered formalin. All granulation tissues were subjected to standard histology procedures and stained with modified Masson's Trichrome stain [25]. Slide specimens were assessed under light microscope to evaluate infiltration of leukocytes, fibroblast proliferation, collagen deposition and re-epithelization [7]. Standard hematoxylin and eosin stain was used to stain the liver and kidney section to study the side effect of *L.smithii* jellyfish consumption.

Collagen Density Evaluation: Method was followed as described by Suvik & Effendy [25]. All slides stained with Masson's Trichrome stain were examined using polarized light microscope (Leica, Germany) and with the aid of a software image analyzer (Video Test-Master 4.0 software), measurements were made at the intensity of blue colour which represent the collagen density. Collagen density was measured under the wounded area and compared to normal dermis at 20X magnification.

Differential Leukocytes Count: Approximately 5 µl of blood was drawn from each rat's tail vein at different time intervals of 1, 7, 14 and 21 days post-wounding. A thin film of blood smear was prepared on glass slide using cover slip. Samples were stained with 2 ml Wright's solution for 5 minutes and washed with distilled water. Stained samples were air dried at room temperature and covered with cover slip by using DPX. The total number of white blood cells was counted under light microscope following standard technique.

Statistical Analysis: All data were analysed statistically using Prism (Version 6.0 GraphPad Software Inc., San Diego, CA, USA). The differences in wound contraction and collagen density between control and treatment group were analyzed using Student's *t-test*. The data were considered significantly different at $p<0.05$.

RESULTS

Antioxidant Activity: Screening of antioxidant activity of *L.smithii* jellyfish has showed that the *L.smithii* methanolic extract has a higher antioxidant activity compared to crude protein at any given concentrations ranging from 0-10 mg/ml (Fig. 1). At high concentration (10 mg/ml) tested; the methanolic crude extract has an antioxidant activity of 46.78 ± 0.03% when compared to crude protein which has 37.60 ± 0.03% of activity. The IC_{50} value from both extracts could not be determined as the antioxidant activity was more than 10 mg/ml compared to standard Quercetine which its IC_{50} value was 0.19 ±0.02 mg/ml.

Wound Contraction and Gross Observation: Daily physical gross examination revealed wounds in animal fed with jellyfish powder has better and faster ephiteliliazation period when compared to normal wound.

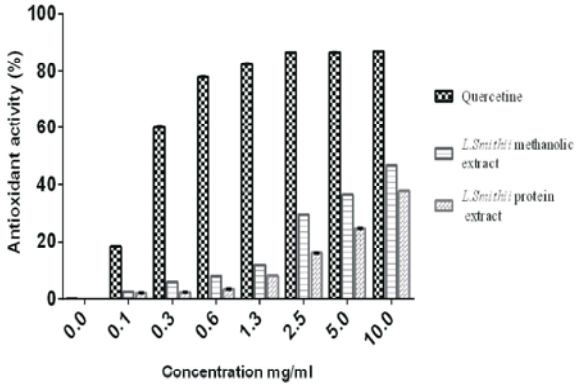

Fig. 1: Antioxidant activities of white type edible jellyfish *L.smithii* methanolic extract and crude protein at different concentrations ranging from 0-10 mg/ml. Quercetine was used as a standard.

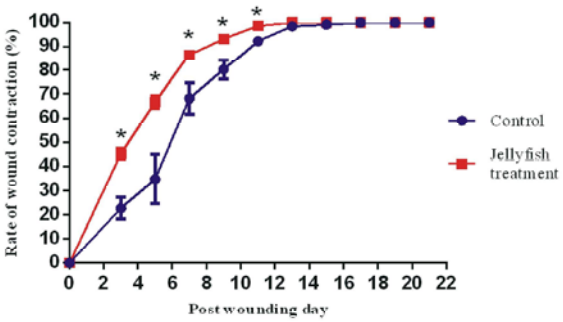

Fig. 2: Rate of wound contraction and period of re-ephiteliliazation of control and treatment group.

Fig. 3: Gross development of wound healing process of control (A) and treated (B) groups at different day. Rat fed with jellyfish powder (1000 g/kg) daily showed faster wound healing process indicated by early scab formation, increased wound contraction, promotion of hair growth and less scar formation when compared to normal wound.

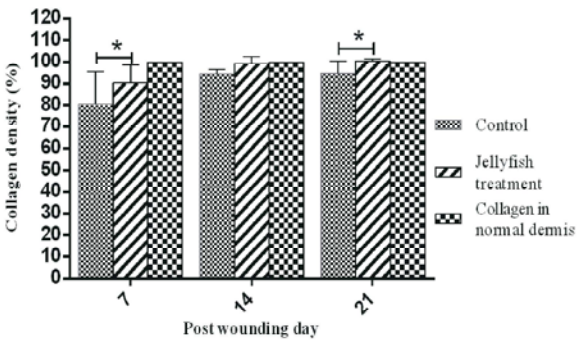

Fig. 4: Rate of collagen deposition and density under wounded area of normal and treatment group. *p<0.05 in comparison between normal and treatment of collagen density mean ± S.D.

There were significant (p<0.05) higher rates of wound contraction in treatment wound when compared to normal wound at the first two weeks post wounding (Fig 2). No significant different observed in the rate of contraction after day 15 to day 21. In addition, physical examination has also showed that treatment group was having faster wound healing effects indicated by the early formation of scab, higher wound closure rate, re-establishment of hair growth and reduced scar formation on the wound area from day 3 to day 15 when compared to control group (Fig. 3).

Collagen Density Evaluation: To evaluate the collagen deposition rate, computerized quantification revealed that the collagen density measured at the center of treated wound area was significantly higher when compared to control group (Fig. 4) on every post wounding day. On day 21 of post wounding the collagen density in the treatment group has completely been synthesized (100.54%) compared to control (97.82%) which quantified not completely deposited. Histopathological analysis confirmed the physical gross observations where wound treated orally with jellyfish powder has better view of tissues reconstruction and faster healing process, indicated with clear reduction of leucocytes infiltration (reduced inflammation), faster scab formation, complete ephiteliliazation, higher fibroblast aggregation and collagen deposition in the wounded area when compared to control group at day 7, 14 and 21 (Fig. 5).

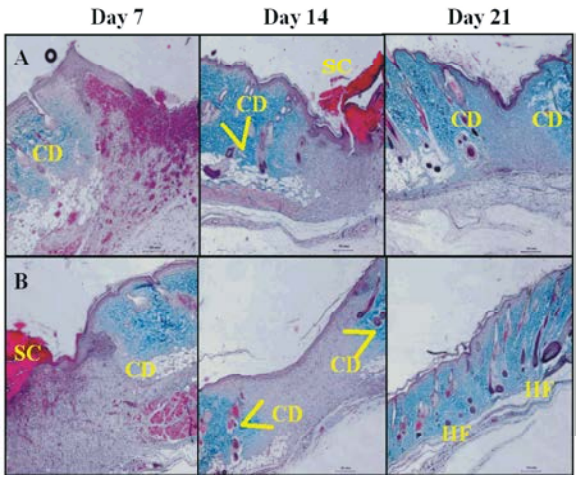

Fig. 5: Photomicrograph of histopathological analysis of wound healing process at wounded area of normal (A) and jellyfish treated animal (B). Skin samples stained with Masson's Trichrome staining. Treatment group has faster wound healing indicated by reduced inflammation on day 7, complete epitheliliazation and higher collagen deposition on day 14 and faster hair promotion with no scar formation on day 21 when compared to control wound. **SC**: Scab, **CD**: Collagen deposition, **HF**: Hair follicle. Images captured using light microscope with 20X objective lens.

Leucocytes Counts and Toxicity Study: There was no significant difference in leucocytes count (neutrophil, monocytes, lymphocytes, basophil, eosinophil) between control and treatment ($P>0.05$) indicating that the treated rats was in normal condition and did not experience any allergic reaction with consumption of the jellyfish powder at dosage of 1000 mg/kg daily for 21 days. Histopathological analysis on liver and kidney also revealed there were no lesion and abnormalities encountered in both organs. Indicating that ingestion of the jellyfish powder is not toxic and safe.

DISCUSSION

Wound healing is a complicated and dynamic process which involves a series of events that restore the continuity and function of the damaged tissue [2, 5, [26]. It is a combination process of coagulation, inflammation, formation of granulation tissues, epithelialization, wound contraction, collagen deposition and remodeling [27, 28]. The whole process can be divided into 3 major phases which are inflammatory phase, proliferative phase and remodeling phase. Wound must be treated immediately to restore the integrity of the skin because it plays a very important role in maintaining homeostasis [3, 28]. The process of wound healing may be impeded by presence of oxygen free radicals or bacteria invasion. Therefore, substances with antioxidant and anti-bacterial activity can speed up the wound healing process [7, 9, 29]. Recently, there has been considerable amount of interest in the finding of wound healing promoter from natural product especially from marine sources. Hence in this study, for the first time we investigated the potential of white type edible jellyfish *Lobonema smithii* as antioxidant and collagen promoter in wound healing process on Sprague dawley rats.

So far, there was no other report looking at the antioxidant activity of *L.smithii*. Here in this study, we have showed that the methanolic extract and crude protein of white type edible jellyfish *L.smithii* were having antioxidant activities. The antioxidant activity was assessed by DPPH scavenging method where the methanolic crude extract was found to be higher with 46.78 ±0.03% of activity compared to its crude protein which gave 37.60 ± 0.03% of activity as shown in Fig. 1. However the IC_{50} value from both jellyfish extracts could not been determined as the activity was more than 10 mg/ml when compared to standard, Quercetine.

Meanwhile *in vivo*, wound healing study has discovered that consumption on dried edible jellyfish *L.smithii* at dosage 1000 mg/kg daily for 21 days promoted faster wound healing process when compared to normal wounds with significant increased ($p<0.05$) on the rate of wound closure time and contraction (Fig. 2) along with the promotion of hair growth and less scar formation (Fig 3). These findings were confirmed by the histopathological analysis where treatment wounds were showed to have better tissue reconstruction, high fibroblast aggregation and faster collagen formation when compared to control wounds on day 7, 14 and 21.

Hematological and toxicity study were done to investigate the inflammatory and allergic response evoked by the oral consumption on *L.smithii* jellyfish powder. Results obtained indicates there was no significant difference ($p>0.05$) in leucocytes count between treatment and control group. It clearly shows that the oral consumption on jellyfish powder is safe and did not have any harmful substance that can trigger allergic or inflammatory reaction even at high dosage of 1000 mg/kg. In addition, no significant changes in the kidney and liver observed.

The abilities of the consumption on edible jellyfish to promote wound healing process in this study might in some extent related to its antioxidant abilities and also the

high collagen protein content in the *L.smithii* jellyfish [22, 30]. In the inflammation phase during the wound healing process, one of the first line defense mechanism are infiltration of leucocytes which function to identify, phagocytize, kill and digest invading microorganism and eliminate wound debris [29]. These leucocytes together with damaged cells through their 'respiratory burst' activity produces Reactive Oxygen Species (ROS) or oxidants acting as antimicrobial and cellular messengers which activate various beneficial wound healing pathways [7, 29]. However at high concentration, ROS can promotes severe tissue damage and delays the normal wound healing process. In order to overcome the problem, cells have developed mechanisms to detoxify ROS. Hence, natural product with antioxidant or antimicrobial activity can be a good therapeutic agent for accelerating the wound healing process [9, 29].

Besides having antioxidant, collagen from the *L.smithii* jellyfish itself may play a role in stimulating collagen synthesis which later promotes high rate of ephiteliliazation period. We also noticed that rats fed with jellyfish powder were having less scar formation and enhanced hair growth. Collagen identified from jellyfish was comprised of 3 different alpha chains as á1, á2 and á3 which homologous to collagen type I, II and type V in human skin [16]. The balance between the synthesis of collagen type I and degradation of type III influences the scar formation [31]. Moreover, collagen also known as source for antioxidant and hair grow stimulant [20, 32, 33]. The amount of collagen cross linked into a more organized structure determines greater tensile strength in healed skin [34]. If insufficient collagen is deposited, weak wound that break easily will be resulted and can caused wound healing process delayed [27].

Apart of the data presented in this paper, we also have studied the specific immune cells that infiltrated in the wounded area. In immunohistochemistry analysis (data not presented) we have found that the number of T-lymphocytes $CD4^+$ and $CD8^+$ were significantly higher in wound treated with jellyfish powder when compared to normal wound. The capability of the *L.smithii* consumption to enhance the production of T cells also play a vital role as a stimulator to collagen synthesizes in the wound area which was proven in our findings. The $CD4^+$ and $CD8^+$ T-lymphocytes play major roles in the early phase of wound repair which essential for regulation of fibroblast migration, replication and collagen synthesis via secreted lymphokines and growth factors [35, 36]. In addition, T lymphocytes modulate fibroblast activity during normal wound healing and its depletion significantly impairs wound breaking strength and wound collagen deposition.

CONCLUSION

It could be concluded that consumption on processed white type edible Malaysian jellyfish *Lobonema smithii* has many beneficial effects and this species could be used and developed for future pharmaceutical and nutraceutical product either as natural antioxidant, immunomodulatory and wound healing agent or as collagen stimulator in skin.

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