Tamarind Seed Extract Enhances Epidermal Wound Healing

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Abstract

Traditional healing power of tamarind fruits and the established antioxidant activity of the seeds drive the present study. Wound healing efficiency of tamarind seed was evaluated. Different solvents: phosphate buffer saline (PBS), water, methanol and ethanol were used to prepare the extract. Circular wound was inflicted on the nape. 10 µl of 5 µg/ml of extract was applied topically twice daily. Wound area was measured using Adobe ®Photoshop C3 Extended version and the percentage of wound reduction was calculated. PBS extract treatment induced complete wound healing in shortest period (10 days) while water extract, methanol extract and Solcoseryl ointment treatment induced complete wound healing in 11 days and control groups without any treatment took 14 days to heal. Phytochemical screening and Bradford method for protein quantification reveals the presence of alkaloid, saponin and tannin in all samples except PBS extract which tested negative to tannin. Flavonoid tested positive in methanol and ethanol extracts.

Keywords: Wound healing, Tamarind seed extract, Solcoseryl, Phytochemicals

1. Introduction

Plant materials have been used traditionally as medicine for treating ailments and maintaining health *Tamarindus indica* L. is one of the reported ancient herbal medicine plants (Soemardji, 2007). The healing power of tamarind is first mentioned in the traditional Sanskrit literatures. In Europe, the medical properties of tamarind were well known after it has been introduced by the Arab traders. *Tamarindus indica* L. fruit is useful as an agent of antihelmintic, antidiarrheal and anti-emetic (Khan *et al*., 2005). Apart from the other components of the fruit, seeds of tamarind are commercially and nutritionally valuable.

Tamarind seed coat exhibit antioxidant activity when extracted with ethyl acetate and ethanol (Tsuda *et al*., 1994; Luengthanaphol *et al*., 2004).Tamarind seeds inhibit activities of snake envenomation enzymes which are responsible for local tissue damage, inflammation and hypotension (Ushanandini *et al*., 2006). Polysaccharide isolated from tamarind seeds has biological applications. It has immunomodulatory effect and lacks carcinogenic and cytotoxic activities (Sreelekha *et al*., 1993; Sano *et al*., 1996; Ieda *et al*., 1978). Water extract of tamarind seed was found to reduce blood sugar level in Streptozotocin-induced diabetic male rats (Maiti *et al*., 2004). In addition, tamarind seeds known to have high inhibitory activity against human neutrophil elastase (Fook *et al*., 2005). Human neutrophil elastase is release by neutrophil during inflammation but excessive production will lead to emphysema. Tamarind seed polysaccharide (TSP) has been shown to improve dry eye syndrome, to assist release of drug in human body and intraocular penetration of Rufloxacin (Rolando & Valente, 2007; Sumathi & Ray, 2002; Ghelardi *et al*., 2004). Wound healing is one of the major concerns among health care practitioners and scientists. Poor wound healing not only cause trauma to the patient but increase the burden of financial resources and requirement for cost effective management within health care system (Bowler *et al*., 2001).

In the present study, influence of tamarind seeds on wound healing was demonstrated. Different types of tamarind seed extracts, Phosphate Buffer Saline (PBS), water, methanol and ethanol extract have been used as the treatment groups in this study. The aim of this study is to evaluate the effect of these extracts on wound healing. The findings of this study may become a starting point for a better formulation of treatment for wound healing.

2. Methods

2.1 Collection of Tamarind seeds

Raw tamarind seeds were collected from Perusahaan Sinarmaju located in Kempas, Johor, Malaysia. The size of the tamarind seeds were approximately $3\times2\times 2cm^3$.

2.2 Preparation of the extracts

Four types of extracts were prepared namely PBS, water, methanol and ethanol. The air dried seeds were grounded using mechanical grinder several times until it became homogenous powder. PBS and water extract were prepared by mixing each 100g of grounded powder with 400 ml of solvents (sterile distilled water, methanol, ethanol or PBS) according to 1:4 w/v ratios in volumetric flask. The mixture was placed in incubator-shaker for 8 hours at 37°C. The supernatant from the flask was centrifuged at 200 g at 27 °C for 30 minutes. The supernatant was then freeze-dried to obtain the final extraction in powder form. Methanol and ethanol extraction were prepared using Soxhlet apparatus for 10 hours and the supernatant was then freeze-dried to obtain the final extract in powder. Weights of the final form of the extract were recorded to determine the yield.

2.3 Inducing wounds in test animals (mice) and application of the extract

Female ICR mice were used as the test sample to observe the wound healing rate. The mice were purchased from Institute of Medical Research, Kuala Lumpur, Malaysia and housed under standard experimental conditions of temperature 25±2°C with a 12 hour light/dark cycle and fed on normal pellet diet and tap water *ad libitum*. The mice were 7-8 weeks old and 25-30 g at the time when wound was induced. A uniform circular epidermal wound was made at the dorsal part of the mice using a 6 mm biopsy punch. Prior to excision, the mice were anesthetized using intraperitoneal administration of 6% Nembutal [Ceva Sante Animale™] 0.1 mL/10g of body weight. The fur was shaved off and the skin was swabbed with alcohol pads and let dry. The extract (~10 µL) was applied twice a day, once in the morning,

2.4 Observation of wound healing rate

Pictures of the wounds from the first day of the wound induction until the day of complete wound closure (CWC), i.e., complete healing were taken on each alternative day to measure the rate of wound healing and comparative wound healing efficiency of the extracts. A Canon Powershot 5.0MP digital camera (Canon, NY, USA) was used for taken the wound pictures. Pictures were taken with insistent features such as distance, aperture. A 15 cm ruler was used as a scale to measure the size of the wound. The pictures were analyzed for wound surface area using Adobe ® CS3 Photoshop (Extended Version), and the data analyzed using SPSS for Windows version 16.0 software.

Percent relative healing efficiency (RHE) of the extracts was calculated to measure how fast the extracts can completely heal the wound when compared with the time required for complete healing without any medicine or extract (natural healing). Percent RHE was calculated using the following formula:

$$\text{\% relative wound healing efficiency} = \frac{T_N - T_E}{T_N} \times 100$$

T_N=Time required for natural wound healing i.e., CWC without any drug/extract. T_E=Time taken for wound healing i.e., CWC with drug/extract. In the current study solcoseryl and series of extracts were used as shown in Table 2.

2.5 Phytochemical analysis of the extract

A collection of tests was used for qualitative analysis of the phytochemicals present in the extract. The test used in this experiment was to determine the presence or absence of alkaloids, terpenoids, steroids, flavanoids, saponins and phenols. Bradford method was also used to quantify the protein content of the extracts.

2.5.1 Protein analysis using the Bradford assay (Bradford, 1976)

Briefly, the extracts were mixed together in a clear plastic 1 mL microcuvette with sodium chloride (NaCl) and Bradford reagent (20 µL of extract and 80 µL of NaCl with 1000 µL Bradford reagent) and the absorbance was

read at 595 nm. The resulting reading was compared to the standard solution of bovine serum albumin (Amresco™).

2.5.2 Test for alkaloid (Dragendorff's Test)

Two ml of the extract was mixed with a few drops of concentrated hydrochloric acid and a few drops of Dragendorff's reagent in a glass test tube. Yellow precipitate indicated the presence of alkaloids (Mojab *et. al.,* 2003).

2.5.3 Test for Terpenoid and Steroid (Libermann-Burchard Test)

Two ml of the extract was mixed with 3-5 drops of acetic anhydride in a test tube and stirred to allow mixing. Then, 1-2 drops of concentrated sulphuric acid were added to the mixture by slowly dripping them down the test tube wall. Blue and purple coloration indicated the presence of terpenoids and steroids, respectively (Mojab *et. al.,* 2003).

2.5.4 Cyanidin Testing

A piece of magnesium ribbon was dropped into a test tube containing 2 ml of extract, followed by a few drops of concentrated hydrochloric acid. The solution is allowed to mix and settle for 10 minutes. Colour change and precipitate formation (if any) were observed. Yellow substance formation indicated presence of flavonoids (Mojab *et. al.,* 2003).

2.5.5 Tannin Testing

Two ml of the extract was mixed with a few drops of iron (III) chloride solution in a test tube. Brownish green to blue black precipitate formation was indicative of tannins in the sample (Mojab *et. al.,* 2003).

3. Result

3.1 Total yield of the extracts

Ethanol extract of tamarind seeds produced the highest yield with 66.2g freeze-dried powder in 840g of seeds (7.88%). This is followed by methanol extraction with 119.5g freeze-dried powder in 1.7kg of seeds (7.03%), PBS extraction with 4.62g freeze-dried powder in 840g seeds (0.55%) and water extraction with 3.05g freeze-dried powder in 1.14 kg of seeds (0.27%).

3.2 Presence and absence of phytochemicals

The presence of alkaloid, flavanoid, saponin, terpenoid, steroid and phenol in different extract were analyzed. Terpenoid and steroid were absent in all samples. Alkaloid and saponin were tested positive in all samples. Only methanol and ethanol extract gave positive result on flavonoid testing. Tannin was detected in all samples except PBS extract (Table1). Total protein content of the extract can only be determined for PBS extract. Average protein content in PBS extract was 40 µg/100µg of extract.

3.3 Tamarind seeds extract (TSE) significantly reduced wound healing time

TSE were found to require significantly shorter time for wound healing. Time (days) required for complete wound healing in different TSE are as follow: PE-10, WE-11, ME-11 and EE-12 (Table 2). PE showed the fastest healing time which was at day 10±0.00. It was followed by positive control at day 11±0.37, WE at day 11±0.40, ME at day 11±0.40, EE at day 12±0.00 and negative control at day 14±0.68 (Table 2). The rate of wound healing (% reduction of wound area per day) varies according to the types of extract. For 50% reduction of the initial wound area, approximately 3, 3.5, 3.5, and 4.5 days were required for PE, WE, ME and EE respectively. For 75% wound reduction, nearly 5.8, 6, 6 and 6.5 days were required for WE, PE, ME and PE respectively (figure 1).

3.4 Ethanol extract exhibited faster wound closure in the early phase

About 47% of the initial wound area was reduced in the first two days when EE was applied. At day 4, all treatment except ME showed noticeable wound reduction, PE was the highest with 58.32% followed by WE (54.19%) and EE (51.27%). At day 6, all treatments were significant and demonstrated high percentage of wound reduction, WE was the highest with 80.56%, PE (77.28%), ME (75.72%) and EE (71.20%) figure 1.

3.5 PBS extract exhibits the fastest wound closure rate at later phase

PE demonstrated better wound reduction compared to all treatment group while EE showed the lowest. At day 8, WE continue to become the highest in wound reduction with 94.49%, PE (93.28%), ME (91.76%) and EE (89.33%). Complete wound healing was observed at day 10 only with PE. At day 10, ME (98.63%) and WE (98.61%) have almost similar rate of reduction and this rate is significantly higher than control group. However,

percent reduction of wound size with EE (94.61%) was not significant when compared with the control. Mostly on day 12, treatment with all TSE resulted in complete wound healing.

4. Discussion

About 80% of world population in Asia and Africa depend on traditional medicine for primary health care (WHO, 2008). Tamarind is considered as one of the useful traditional medicine used in South East Asia. Different parts of tamarind with different types of solvent have been used by researcher for investigating its medicinal properties. In present study, PBS, water, methanol and ethanol were used as the extraction solvent. PBS and water are chosen because of their isotonic and physiological features that are favorable for extracting protein and water soluble bioactive components (Anuar, *et al*., 2008). Non polar compounds that are present in tamarind seeds can be extracted by organic solvent. Thus, methanol and ethanol was used as the extraction solvent. The extraction was carried out at 37ºC equal to normal physiological temperature and samples were incubated for 8 hours to preserve and maximize the protein yield. More than 8 hour incubation is not recommended for possible degradation by microbial contamination. The freeze-dried product was kept at -80 ºC to minimize protein loss due to enzyme degradation. Extracts were applied by mixing in deionized water to provide moisture in the wound bed which is essential for the enzymatic activity (Anuar, *et al*., 2008). Only female mice were chosen for this study because they are less aggressive compare to male mice. The excision was done at dorsal region of the mice to reduce the possible scratching of the wound. Female mice of known age and body weight range were used to maintain the constant physiological activity. Specific concentration of anesthetic agent (Nembutal) base on the animals' body weight was used to allow constant time of unconsciousness. The extract was applied few minutes after wounding and not immediately to avoid flush out by the wound. Wound healing is classified into inflammatory phase, proliferative phase, and maturational phase. Extract of tamarind seeds used in this study have been shown to have better wound healing. This result was expected due to several consistent findings on immunomodulatory, anti-toxicity, anti-venom, and antioxidant activity of tamarind seeds (Sreelekha *et al*., 1993; Sano *et al*., 1996; Ieda *et al*., 1978).

Tamarind seed flavonoids may be involved in stabilizing highly reactive, potential harmful free radicals and protect cells from oxidative damage. The ability of antioxidants to destroy highly reactive free radicals serves to protect the structural integrity of immune cells and prevents the loss of essential functions. This study proposes that tamarind seed flavonoids might contain bioactive components which act as antioxidant responsible for neutralizing the effect of free radicals which are normally generated by Oxygen-dependent mechanisms for intracellular killing by leukocytes as reported earlier (Sudjaroen *et al*., 2005). PE exhibits the fastest rate of healing that contains highest protein content. The fastest rate of healing could be attributed to Trypsin inhibitor, an important protein molecule involved in proliferation of fibroblast and collagen synthesis during wound healing (El-Siddig *et al*., 2006).

Phytochemical result showed that alkaloid and saponin were present in PE and additional tannin was present in WE. Alkaloid is known to assist in epithelization of wound and chemotaxis in fibrosis (Azeez *et al*., 2007). Saponin on the other hand stimulates angiogenesis by modifying the balance of protease/protease inhibitor secretion in human endothelial vascular cells (Morisaki, 1995). Tannin is believed to have haemostatic activity, arresting bleeding from damaged or injured vessels by precipitating proteins to form vascular plugs (Okoli, 2007). Possible combination of these chemicals may explain the fluctuation of wound reduction of PE and WE on day 4 to 8. Infections by a number of microorganisms such as *P. aeruginosa, S. aureus, S. faecalis, E.coli, Clostridium perfringens*, *Clostridium tetani*, *Coliform bacilli* and *enterococcus* are reported to be responsible for delayed wound healing (Bowler *et al*, 2001). The presence of tannin in WE therefore could be associated with faster wound closure due to its antimicrobial property.

Total protein content of PE was measured using Bradford method. The result indicated high presence of protein in the sample, 40% of the composition of the seeds. The finding is different when compared to the available literature. Different methods of extraction especially the incubation time in extraction may explain the difference in the amount of protein. In contrast, protein could not be detected in WE, ME and EE. The most likely reason for this is the occurrence of polyphenols in the samples. Bradford method is an easy and rapid protein quantitative method. The assay relies on the binding of the dye Commasie blue G250 to protein. The dye binds readily to arginiyl and lysyl residues of proteins but does not bind to free amino acids. This specificity leads to variation in response to different proteins. In addition, sodium ascorbate, phenol compounds, and metal ions existed in the samples may cause interference.

In conclusion, the present study has succeeded to achieve the objective of the research. The different effect of the tamarind extract on wound healing was observed. All extracts exhibited significant increase in the rate of healing.

PBS extract was the most effective in increasing the rate of healing. Ethanol extraction on the other hand was the most effective in yielding tamarind seeds product. Phytochemical screening of the extracts reveals the presence of secondary metabolite such as alkaloid, flavonoid, saponin and tannin. PBS extract only contains protein in a very significant amount.

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Table 1. Qualitative phytochemical analysis of tamarind seed extract

Samples	PE	WE	ME	EE
Alkaloid	✓	✓	✓	✓
Terpenoid	×	×	×	×
Steroid	×	×	×	×
Flavonoid	×	×	✓	✓
Saponin	✓	✓	✓	✓
Tannin	×	✓	✓	✓
Note: Present			× Absent	

Table 2. Number (mean) of days required for complete healing of induced wounds

Treatment group (n=7)	Days required for complete healing (mean ± S.E.M)	% RHE
NC	14 ± 0.68	-
PC	11 ± 0.37	0.21
PE	10 ± 0.00[a]	0.29
WE	11 ± 0.40[a]	0.21
ME	11 ± 0.40[a]	0.21
EE	12 ± 0.00[a]	0.14
Note: [a] Total number of days required for complete wound healing is significantly shorter than negative control ($p < 0.001$).		

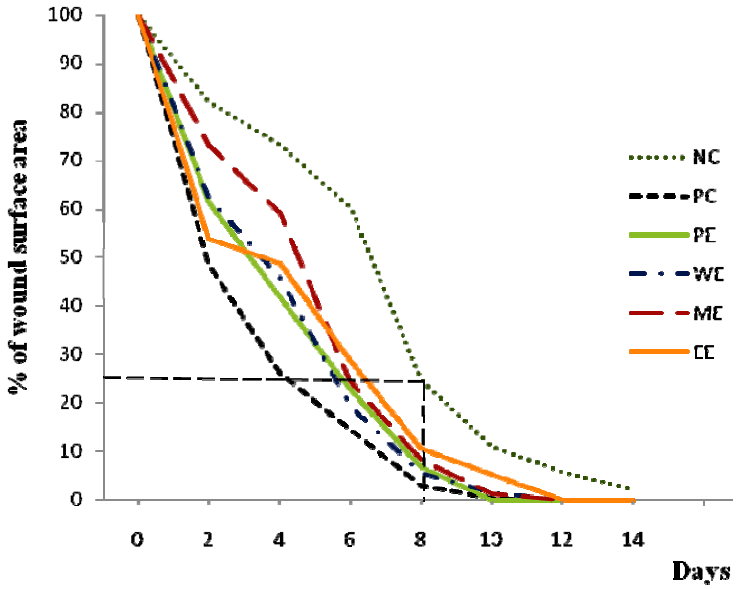

Figure 1. Percentage wound reduction at different time intervals in the treated and the untreated wounds

NC= Control (no treatment), PC= Solcoseryl, PE= PBS seed extract, WE= Water seed extract, ME= Methanol seed extract, EE= Ethanol seed extract.

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