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EVALUATION OF WOUND HEALING ACTIVITY OF *VITIS VINIFERA* STEM BARK EXTRACTS IN RATS

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ABSTRACT

The present study was aimed for wound healing potential of chloroform and ethanolic extract of *Vitis vinifera* (stem bark) using three types of wound models in rats as incision wound, excision wound and dead space wound. The results were obtained in terms of wound contraction, epithelialization time, tensile strength, hydroxyproline content and granuloma weight. Enhanced wound contraction and decreased epithelialization time were observed in extract-treated animals in excision wound model. The tensile strength of the incision wound was significantly increased in comparison to control group. The granulation tissue weight and hydroxyproline content in the dead space wounds were also increased significantly in treated animals compared with control ($p<0.01$).

INTRODUCTION

Since ancient times plants played an important role in the life of human as the major source of food as well as for the maintenance and improvement of health. The fact that about 80 % of the world's population still relies on plant-based medicines for their primary health care is a clear indication for the role of medicinal plants in the maintenance of health and treatment of diseases as therapeutic alternatives throughout the world, still present century[1].

Tissue repair and wound healing are the complex processes that involve a series of biochemical and cellular reactions, beginning with inflammation and followed by the repair and remodeling of the injured tissue. During inflammatory phase, macrophage is referred to as orchestrator for the removal of fibrin and proliferation of fibroblast, is also responsible for the secretion of TNF and TGF that stimulate fibroblast and keratinocytes, respectively. In the last maturational phase, wound undergoes contraction resulting in reduction of size of apparent scar tissue.

Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1–3% of modern drugs[2]. Reports about medicinal plants affecting various phases of the wound healing process, such as coagulation, inflammation, fibroplasia, collagenation, epithelization and wound contraction are abundant in the scientific literature[3,4,5,6]. However, many traditional remedies are based on systematic observations and methodologies and have been time-tested but for many of them, scientific evidence is lacking.

Vitis vinifera L., the European or Wine grape. *Vitis vinifera* is also called "Old World grape" since most production occurs in Europe and the other species are native to the New World. The plant has been heavily promoted for a wide range of uses because of the presence of active secondary metabolites responsible for the broad pharmacological activities and therapeutic potentials[7]. The present study was designed to test the *in vivo* wound healing activity of the chloroform and ethanol extracts of stem bark of *V. vinifera*.

MATERIALS AND METHODS

Plant material and preparation of the extracts:

The stem bark of mature plants was collected from Sorab region of Shivamogga district, Karnataka, India during August to December (2010). The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number V/005/2010). The bark was shade dried and then powdered using a mechanical grinder; stored in airtight container for extraction. It was then

refluxed successively with 1/10 (w/v) petroleum ether, chloroform and ethanol extracts in a soxhlet apparatus for 48 h in batches of 250 g each. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). The crude chloroform and ethanol extracts were taken for further investigation.

Animal models:

Healthy inbred Albino rats of Wistar strain, weighing about 150-200 g of either sex were obtained from Venkateshwara enterprises, Bangalore. All animals were housed, fed and treated in accordance with the inhouse guidelines for animal protection. Animals were kept for 2 weeks to be acclimatized prior to the investigation. During this time they were given standard pellet diet and water *ad libitum*. Also, they were periodically weighed before and after experiments. Animals were closely observed for any infection; those which showed signs of infection were separated and excluded from the study. The rats were anasthetized prior to infliction of the experimental wounds. Acute toxicity study was performed by stair case method[8]. 200mg/kg body weight was taken as the therapeutic dose of the ethanol extract and aqueous extract of stem bark of *V. vinifera*. The study was performed with due permission from Institutional Animal Ethics committee (SETCP/IAEC/07/462).

The animals were kept under starvation for 12 hours prior to wounding. Wounds were made on the animals under light ether anesthesia. Animals were divided into two groups of six animals each. Control group of animals were given 1 ml of normal saline, first test group animals received the suspension of chloroform extract and second test group animals received ethanolic extract at a dose of 200 mg/kg b.w. by gavage from the day of wounding. Excision wound, incision wound and dead space wound models were used to evaluate wound healing activity.

Excision wound model:

Under light ether anesthesia each animal was secured to operation table in its natural position. An impression was made on the depilated dorsal thorasic central region of the rats, 5.0cm away from the ears, by using a round seal of 2.5cm diameter. The extract was given every day upto 16^{th} day[9].

Incision wound model:

Each animal was secured to operation table in its natural position under light ether anesthesia. Two para-vertebral straight incisions of 6.0cm each were made on the depilated back of the animals by cutting through the entire skin with the help of a sterilized sharp blade. After

complete haemostasis, the wounds were closed (sutured) using 2-zero silk threads as interrupted sutures about 1.0cm apart with the help of a straight round bodied needle. The sutures were removed on 8th post wounding day[10].

Dead space wound model (Granuloma studies):

Under light ether anesthesia, dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5cm X 0.3cm), one on either side of the dorsal paravertebral surface of rat. The granulation tissues formed on the grass piths were excised on 10th post wounding day and the breaking strength was measured. Simultaneously, granulation tissue so harvested was subjected to hydroxyproline estimation[11].

Wound healing evaluation parameters

Wound contraction and epithelialization time:

An excision wound margin was traced after wound creation by using transparent paper and area measured by graph paper. Wound contraction was measured in each 4 days interval, until complete wound healing and expressed in percentage of healed wound area. The percentage of wound closure was calculated. The period of epithelialization was calculated as the number of days required for falling of the dead tissue without any residual raw wound.

Collagen content from regenerated tissues of excision wound:

The regenerated tissue collected from the excision wounds were cut into two pieces. They were washed with 0.5 M sodium acetate and then suspended in ten parts (w/v) of 0.5M acetic acid and stirred intermittently for 48 hrs. The solution was centrifuged at 5600 rpm for 2hrs (intermittently in the micro centrifuge, and then sodium chloride (5% w/v) solution was added to precipitate the collagen. The collagen so precipitated was filtered using a preweighed Whatman filter paper No.1. The weight of the collagen precipitate obtained was calculated by taking difference between the initial and final weights of the filter paper. The same procedure was followed for the animals of the control and both the test groups.

Measurement of tensile strength:

Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists to breaking under tension and may indicate in part the quality of repaired tissue. Sutures were removed on the day 8 after wound creation and the tensile strength was measured. The skin breaking strength of the 10-day-old wound was measured by continuous constant water technique of Lee and Tong[12]. The skin breaking strength is expressed as the minimum weight (in grams) of water necessary to bring about the gapping of the wound.

Hydroxyproline estimation:

Tissues were dried in a hot air oven at 60-70 °C to constant weight and were hydrolyzed in 6N HCl at 130 ºC for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4M perchloric acid and color was developed with the help of Ehrlich reagent at 60°C [11] and measured at 557 nm using a spectrophotometer.

Granuloma weight

The granulomas were excised from the surrounding tissue on 10 post wounding day and were dried at 60 ºC to obtain constant dry weight[13].

Statistical analysis

Results, expressed as mean ± SEM were analyzed statistically using student's t-test to identify the differences between the treated and control. The data were considered at $p < 0.01$.

RESULTS

Wound contraction and epithelialization time

Significant wound healing activity was observed in both the group of animals treated with ethanol and chloroform extracts. The percentage of closure of wound was significant in the animals treated with ethanol extract (92.38±1.01) on day 16th and (99.35±0.15) on day 20th, respectively. While in control animals it was (88.51±0.54) and (94.55±0.33), respectively. Also, the group treated with chloroform extract showed significant value (97.15±0.30) on 20th day in comparison with control group. The time required for complete epithelialization of the excision wound is an important parameter to assess the wound healing process. It was found that the mean time taken for complete epithelialization of the excision wound in ethanol extract treated group was less than the animals treated with chloroform extract and the data are shown in Table 1.

Collagen content from regenerated tissues of excision wound (mg/kg)

The collagen content was estimated from regenerated tissue for control as well as treated groups. There was a significant increase in collagen content on 4th, 8th, 12th, 16th and 20th day in ethanol extract treated group compared to the chloroform extract treated and control group. The increase in collagen content in chloroform extract treated group was also significant except for the 20th day (42.71±0.75) compared to the control group. The ethanol extract was found to be highly effective than chloroform extract with high collagen content of 51.58±0.64 on 20th day (Table 2).

Measurement of tensile strength

In incision wound model, significant increase in the tensile strength was observed in ethanol extract treated groups of animals (421.6±17.30) followed by chloroform extract treated group of animals (328.3±15.54), indicating the effect of *V. vinifera* stem bark extract in maturation of collagen fibers (Table 3). The values were highly significant when compared to control group ($p < 0.01$).

Hydroxyproline estimation and Granuloma weight

The extract treated group showed significant increased hydroxyproline level when compared to control group ($P < 0.01$) in Table 4. Granuloma weight of treated animal groups was found to be increased when compared with control group. The hydroxyproline level was highest in ethanol treated group (8.91±0.13) compared to chloroform extract treated animals (6.91±0.23). However, both the extracts were effective in comparison with the control group (6.2±0.10). The granuloma dry weight estimated was high in ethanol extract treated animals (47.48±2.08) while it was moderate in rats treated with chloroform extract (45.28±1.89). The values were significant at $P < 0.01$ compared to control.

DISCUSSION

Wound healing is a complex cellular event by which a damaged tissue restored as closely as possible to its normal stage. The healing process depends upon the reparative abilities of the tissue, the type and extent of damage and general state of health of the tissue. The process consists of different phases such as granulation, collagenation, collagen maturation and scar maturation which are concurrent but independent to each other. The results in this study are in support that the wound healing and repair is accelerated by administering *V. vinifera* stem bark extracts which was high-lighted by the full thickness coverage of the wound area by an organized epidermis in the presence of mature scar tissue in the dermis. In excision wound model significant decrease in the period of epithelialization and increase in wound contraction rate were observed in the extract treated groups of animals. In both extract treated animals, epithelialization was completed on 20^{th} and 21^{st} post wounding day respectively. While in control animals, the rate of wound contraction was slow and the complete epithelialization of the excision wound was extended up to 24^{th} post wound day.

In the present investigation, significant increase in the tensile strength was observed in the animals treated with the plant extracts on the 10^{th} post wounding day. Similar observations have

been reported by Shirwaikar *et al.*[14] and Singh *et al.*[15]. Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema, and new small blood vessels. The increase in dry granulation tissue in the treated groups is an indication of higher protein content. The chloroform as well as ethanol extracts of *V. vinifera* demonstrated a significant increase in the hydroxyproline content in the granulation tissue indicating increased collagen turnover. Collagen, which strengthens and supports the extra cellular tissue is composed of amino acid, hydroxyproline, which has been used as a biochemical marker for tissue collagen[16]. The enhanced capacity of wound healing with the plant extracts could be explained on the basis of the anti-inflammatory effects of the plants that are well documented in the literature[17,18]. However, in order to unravel the possible mechanism involved in the process of wound healing either by regulating body's defensive mechanism or by direct action of drug on wound, more work is required.

Table 1: Effect of stem bark extracts of *V. vinifera* on excision wound model

Treatment	***Percentage of closure of excision wound area***					***Epithelialization in days***
	Day 4	***Day 8***	***Day 12***	***Day 16***	***Day 20***	
Control	*20.67±0.54*	*62.31±0.53*	*79.18±0.61*	*88.51±0.54*	*94.55±0.33*	*24.93±0.61*
Chloroform extract	*25.08±1.8**	*65.98±0.82*	*81.72±0.26***	*90.48±0.36*	*97.15±0.30***	*21.67±0.76*
Ethanol extract	*31.20±1.24***	*68.04±0.79***	*84.23±0.56***	*92.38±1.01***	*99.35±0.15***	*22.61±0.21*

Values are mean±S.E.; n = 6 in each group. **P* < 0.01 is compared to control.

Table 2: Effect of stem bark extracts of *V. vinifera* on collagen content from regenerated tissues of excision wound (mg/kg)

Treatment	***Day 4***	***Day 8***	***Day 12***	***Day 16***	***Day 20***
Control	*10.11±0.82*	*17.79±0.97*	*23.63±1.38*	*32.23±0.87*	*40.97±0.70*
Chloroform extract	*15.93±0.65**	*22.46±0.81**	*30.91±0.66**	*33.68±0.35**	*42.71±0.75*
Ethanol extract	*22.52±1.6***	*31.90±0.96***	*39.76±0.66***	*45.59±0.94***	*51.58±0.64***

Values are mean±S.E.; *n* = 6 in each group. **P* < 0.01 is compared to control.

Table 3: Wound healing effect of *V. vinifera* stem bark extracts on incision wound model

Treatment	Tensile strength (g)
Control	276.3±8.074
Chloroform extract	328.3±15.54
Ethanol extract	421.6±17.30

Values are mean±S.E.; $n = 6$ in each group. $^*P < 0.01$ is compared to control.

Table 4: Effect of stem bark extracts of *V. vinifera* on dead space wound model

Treatment	***Hydroxyproline content (µg)***	***Tensile strength (g)***	***Granuloma dry weight (mg/100g)***
Control	*6.2±0.10*	*287.4±4.23*	*35.17±2.97*
Chloroform extract	*6.917±0.23**	*312.6±5.37**	*45.28±1.89**
Ethanol extract	*8.917±0.13***	*378.4±19.12**	*47.48±2.08**

Values are mean±S.E.; n = 6 in each group. $^*P < 0.01$ is compared to control.

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