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Thangavel Ponrasu
Department of Biochemistry, Central Leather Research Institute, Council of Scientific and Industrial Research, Adyar, Chennai 600 020, India.
Email: slonchin@yahoo.co.uk
Tel: +91-44-24911386

Muthaiya Kannappan Subamekala
Department of Biopharmaceutics, Anna University, Chennai, India.

Moorthy Ganeshkumar
Department of Biochemistry, Central Leather Research Institute, Council of Scientific and Industrial Research, Adyar, Chennai 600 020, India.
Email: slonchin@yahoo.co.uk
Tel: +91-44-24911386

Lonchin Suguna
Department of Biochemistry, Central Leather Research Institute, Council of Scientific and Industrial Research, Adyar, Chennai 600 020, India.
Email: slonchin@yahoo.co.uk
Tel: +91-44-24911386

Correspondence:
Lonchin Suguna
Department of Biochemistry, Central Leather Research Institute, Council of Scientific and Industrial Research, Adyar, Chennai 600 020, India.
Email: slonchin@yahoo.co.uk
Tel: +91-44-24911386

Role of *Annona squamosa* on antioxidants during wound healing in streptozotocin-nicotinamide induced diabetic rats

Thangavel Ponrasu, Muthaiya Kannappan Subamekala, Moorthy Ganeshkumar, Lonchin Suguna

ABSTRACT

Annona squamosa is known for its antioxidant, antidiabetic and wound healing activity. We examined the efficacy of *A. squamosa* on the levels of enzymatic and non-enzymatic antioxidants during wound repair in normal and streptozotocin induced diabetic rats. An excision wound was made on the back of rat and 200 µL (100 mg/kg b.wt) of *A. squamosa* extract was applied topically once daily for the treated wounds. The control wounds were left untreated. The wound tissues formed were removed on day 4, 8, 12 and 16 of post wounding. Levels of enzymatic and non-enzymatic antioxidants were estimated in control and treated wound tissues. Enzymatic antioxidants like Catalase, Superoxide dismutase, Glutathione peroxidase and Glutathione-S-transferase were significantly ($p<0.05$) increased in *A. squamosa* treated animals especially on day 8. Diabetes is characterized by deficiency of nitric oxide (NO) at the wound site, which is an essential key factor in diabetic wound management. NO was also high in *A. squamosa* treated wound tissues. Non-enzymatic antioxidants like ascorbic acid, vitamin-E and reduced glutathione were also significantly ($p<0.05$) increased on day 4 and 8 in treated animals. *A. squamosa* promotes increased levels of enzymatic and non-enzymatic antioxidants in wound tissues for better wound repair mechanism in normal and diabetic rats.

Keywords: *Annona squamosa*, Antioxidant, GSH, SOD, Streptozotocin, Wound healing

1. Introduction

Diabetic wounds are defined as chronic wounds or lesions that take long time to heal, fail to heal or recur [1]. The problems of defective wound repair mechanism in diabetes are not yet completely understood. However, all phases of the healing process like inflammation, proliferation, maturation and reepithelialization are disrupted [2].

Reactive oxygen species (ROS) play an important role in variety of pathological conditions such as respiratory disorders, diabetes, cancer, inflammatory disorders, rheumatoid arthritis, atherosclerosis and neurodegenerative diseases [3, 4]. Various inflammatory cells like macrophages, neutrophils, fibroblasts and endothelial cells generate ROS[5]. Oxidative damage is characterized by ROS, produced through cell death, oxidative stress or formation of excess free radical metals [6]. Diabetic wounds have low levels of free radical scavengers, i.e. both enzymatic and non-enzymatic antioxidants. The recovery of antioxidant activities might avoid the delay in healing in diabetic patients [7].

Nitric oxide (NO) was found to be an essential signal molecule and mediator during wound healing. Subsequently, elevated levels of NO production in the early phase of healing process accelerate wound healing significantly [8, 9]. Earlier experimental reports suggest that NO played a crucial role in the anti-microbial activity of macrophages against various intracellular pathogens like *L. major, Trypanosoma cruzi* and *Toxoplasma gondii* [10].

Antioxidant protective system arises from both enzymatic and non-enzymatic antioxidant compounds. Antioxidants have been shown to accelerate wound healing and antioxidant levels defence the wound against ROS, either partially or completely in impaired wound healing [7]. Without proper antioxidant activities, wound healing might be delayed, or severe tissue damage can occur.

Therefore, normal wound repair mechanism accelerates the expression of many antioxidant genes such as glutathione peroxidase (GPx), catalase (CAT) and SOD [11].

Plant products seem to play a key role in accelerating wound healing due to the presence of antioxidant principles such as alkaloids, flavonoids and phenolic acids [12]. We have reported the efficacy of some plant extracts like *Terminalia chebula, Centella asiatica, and Butea monosperma* on wound healing in rats [13-15]. Recently, we have reported the role of *Annona squamosa* on diabetic wound healing in rats [16]. In this manuscript, we describe the potential role of *A. squamosa* in accelerating both normal and diabetic wound healing by increasing the antioxidant levels through its active principles such as alkaloids, flavonoids and phenolic acids.

2. Materials and methods

2.1 Chemicals

Ascorbic acid, Catalase, Vitamin-E, Glutathione, 1-Chloro-2, 4-dinitrobenzene (CDNB), Epinephrine, N-(1-naphthyl)-ethylenediamine dihydrochloride (NEED) and all chemicals were purchased from Sigma Chemical Company, St. Louis, USA. All the reagents are used of high analytical grade.

2.2 Plant material and solvent extraction

The leaves of *Annona squamosa* was collected, confirmed and authenticated with herbarium of Botanical Survey of India (southern circle, Coimbatore). The voucher specimen was deposited in Department of Botany, Bharathiar University, Coimbatore, India (Voucher specimen VSN-MS 89). Briefly, 100 g of fine powder of shade dried leaves was macerated with 70% ethanol in dark and filtered to harvest a viscous supernatant. The supernatant was then dried under vacuum below 40 °C. The viscous residue was collected, weighed and kept at 4 °C until use.

2.3 Determination of preliminary phytochemicals

The chemical tests were performed to find out different chemical groups present in *A. squamosa* extract, which are responsible for antioxidant property. The analysis was carried out as described by Chulet Rahul *et al* [17].

2.4 Animals, diabetes induction and experimental protocol

Healthy male Wistar rats, weighing between 150-200 g were used for the experiment. The rats were housed in wire topped cages with sterilized rice husk bedding under controlled conditions, of light/dark cycle (12:12 hrs.), temperature at 29-31 °C and rats were fed with commercial rat feed and water *ad libitum*. All procedures were carried out according to the stipulations of the Institutional Animal Care and Use Committee (IACUC). A formal approval from the Institutional Animal Ethical Committee has also been obtained. (Registration Number: 466/01a/CPCSEA dated 24th August 2001: proposal approval number: IAEC No.07/01/2011)

Diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg kg^{-1}) dissolved in 0.1 M of cold citrate buffer (pH 4.5); 15 min after the intraperitoneal administration of nicotinamide (110 mg kg^{-1}) in overnight fasted rats [18]. Diabetic condition was confirmed by tail vein blood glucose determination using glucometer (One Touch Horizon, Johnson & Johnson, and Mumbai, India) after 72 hrs. Two weeks after diabetic induction, rats with blood glucose level > 250 mg dl^{-1} were used for the study.

The rats were divided into four groups comprising six rats in each group as follows:

Group I: Control rats, left untreated.

Group II: Rats treated with *A. squamosa* (200 µl) 100 mg/kg. b.wt)

Group III: Diabetic control rats also left untreated.

Group IV: Diabetic rats treated with *A. squamosa* (200 µl) 100 mg/kg b.wt)

2.5 Creation of full thickness skin wound and drug administration

After confirming the induction of diabetes, six animals per group were used to create wounds. Rats were anaesthetized by mild dosage of diethyl ether. A 2 cm^2 (2 x 2) full thickness open excision wound (Fig 1) was made on the back of the rat as reported in our earlier studies [16]. The control rats were left untreated and the treated rats were topically administered daily with 200 µl (100 mg/Kg. b.wt) of the extract, until the wounds healed completely. The wound tissues were removed on day 4, 8, 12 and 16 post-wounding and used for different antioxidant analyses.

Fig 1: Open excision wound

2.6 Nitric oxide

Nitric oxide (NO) biosynthesis was measured by determining nitrite levels in the wound tissues. Wound tissues were homogenized in hypotonic saline and centrifuged. Nitrite concentrations were determined with Griess reagent [19]. Briefly, the supernatant was mixed with freshly prepared Griess reagent

(0.1% NEED, 1% sulphanilamide and 5% phosphoric acid in a 1:1:1 ratio), incubated at 37 ºC for 30 mins and the absorbance at 543 nm was measured using UV-visible spectrophotometer. Sodium nitrite was used as standard. Nitrite levels are expressed in terms of µM/100 mg dry wound tissue.

2.7 Assay of antioxidants

2.7.1 Catalase (CAT)

The levels of CAT in the granulation tissue were analyzed by the method of Aebi *et al* [20]. In short, to 1.2 ml of phosphate buffer pH 7.0, 0.5 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of 30 mM hydrogen peroxide (H_2O_2), the substrate for catalase. The decrease in absorbance was measured at 240 nm for 3 min in UV-Visible spectrophotometer. An enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

2.7.2 Superoxide dismutase (SOD)

SOD activity was measured by the method of Misra and Fridovich [21]. To 0.5 ml of tissue homogenate, 0.75 ml of ethanol and 0.15 ml of chloroform (chilled in ice) were added and centrifuged at 2000 rpm for 20 min. To 0.5 ml of supernatant, added 0.5 ml of 0.6 mM EDTA solution and 1 ml of carbonate bicarbonate buffer (0.1M, pH 10.2). The reaction was initiated by the addition of 0.5 ml of 1.3 mM epinephrine and the increase in absorbance at 480 nm was measured in spectrophotometer.

2.7.3 Glutathione peroxidase (GPx)

GPx levels of the granulation tissues were evaluated by the method of Rotruck *et al* [22]. To 0.2 ml tissue homogenate, 0.2 ml of 0.8 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of 4 mM reduced glutathione, 0.4 ml of phosphate buffer (0.4 M, pH 7.0) were added and incubated at 37 ºC for 10 min. The reaction was arrested by adding 0.5 ml of 10% TCA and the tubes were centrifuged at 2,000 rpm for 20 min. To the supernatant, 3 ml of disodium hydrogen phosphate (0.3 M**)** and 1.0 ml of 40% 5, 5'-Dithiobis-2-nitrobenzoic acid (DTNB) were added and the color developed was read immediately at 420 nm in spectrophotometer.

2.7.4 Glutathione-S-transferase (GST)

GST was estimated as described by Habig *et al* [23]. Briefly, To 0.5 ml of tissue homogenate, 1.0 ml of phosphate buffer, 1.7 ml of water and 0 .1 ml of CDNB were added. After incubation at 37 ºC for 15 minutes, 0.1 ml of GSH was added and change in absorbance was read at 340 nm for 3 minutes at an interval of 30 seconds.

2.7.5 Assay of ascorbic acid

The amount of ascorbic acid in the wound tissues was measured by the method of Omaye and Turnbull [24]. Wound tissue was homogenized in 9 ml of 5% ice cold TCA per g of tissue and centrifuged for 30 mins at 5000 rpm. 0.5 ml of supernatant was mixed with 0.1 ml of DTC reagent (3g of 2, 4-dinitro phenyl hydrazine DNPH, 0.4 g of thiourea, 0.05 g of copper sulphate dissolved in 100 ml of 9 N H_2SO_4) was added and incubated for 3 hrs. at 37 ºC. Then 0.75 ml of ice cold 65% sulphuric acid was added and mixed well and the solutions were allowed to stand at room temperature for 30 min. The colour developed was read at 520 nm using spectrophotometer.

2.7.6 Assay of vitamin-E (α-Tocopherol)

Vitamin E content in wound tissues was estimated by the method of Quaife and Dju [25]. Briefly, 0.5 ml of tissue homogenate, 0.5 ml of standard and 0.5 ml of water were pipetted out separately. To all the tubes, 0.5 ml of ethanol and 0.5 ml of xylene were added, mixed well and centrifuged. Then xylene layer was transferred into another tube. To each tube, 0.5 ml of dipyridyl reagent was added and mixed well. 0.2 ml of ferric chloride reagent was added and mixed well. The red colour developed was read exactly after 15 minutes at 520 nm in spectrophotometer.

2.7.7 Assay of reduced glutathione (GSH)

The amount GSH in wound tissues was assayed by the method of Moran *et al* [26]. Tissue homogenate was precipitated with 10% TCA and centrifuged at 2000 rpm for 20 min. To 1.0 ml of aliquot, 1.0 ml of 0.6 mM 5, 5'-Dithiobis-2-nitrobenzoic acid (DTNB) was added and the final volume was made up to 5.0 ml with 0.33 M phosphate solution. The color developed was read at 420 nm in spectrophotometer.

2.7.8 Statistical analysis

Data were expressed as mean ± S.D of six animals in each group and the results were statistically evaluated using one-way ANOVA and Students paired t-test. All statistical analyses were performed using graph pad prism (version 5.0; Graph Pad software Inc. San Diego CA, California, USA). Values are corresponding to $p<0.05$ were considered as significant.

Results

3.1 Phytochemicals

Preliminary phytochemical screening revealed that ethanolic extract of *A. squamosa* contains considerable amount of alkaloids, flavonoids, saponins and triterpenes (Table 1).

Table 1: Preliminary phytochemical screening of leaves of *Annona squamosa*

S. No	Types of constituent	Ethanolic extract of *A. squamosa*
1	Flavanoids	++
2	Saponins	++
3	Carbohydrates	+++
4	Steroids	-
5	Tannins	-
6	Alkaloids	+++
7	Proteins	-
8	Amino acids	-
9	Glycosides	-
10	*Triterpenes*	+++

†Values are expressed as mean of three values; +++ High amount; ++ Moderate amount; - Completely absent

3.2 Nitric oxide (NO)

Level of nitric oxide in wound tissues of the control and *A. squamosa* treated wounds was shown in figure 2. The production of NO was found to be high ($p<0.05$) in group II rats till day 16 of post wounding. But in diabetic treated rats (Group IV), it was very less on day 4 and then increased significantly to higher amount on day 8 and 12 (46 to 51%) then decreased.

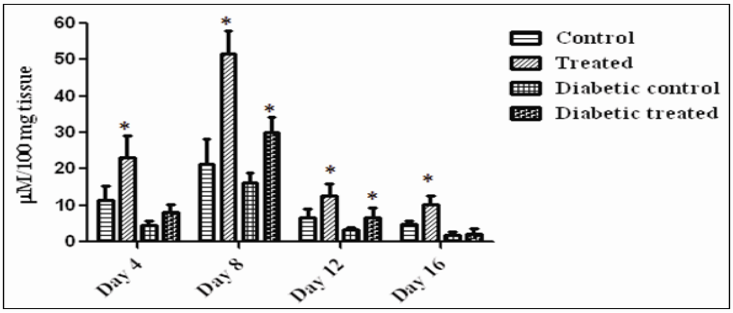

Fig 2: Level of nitric oxide in control and *A. squamosa* treated wound tissues on various days. Values are expressed as mean ± S.D for six animals and level of significance is expressed as *$p<0.05$ respectively, compared with the corresponding control.

3.3 Effect of *A. squamosa* on enzymatic antioxidants

Table 2 shows the levels of *A. squamosa* on enzymatic antioxidants like CAT, SOD, GPx and GST of control and treated wounds. In group II, *A. squamosa* treatment significantly increased the catalase level on day 4 (17%), day 8 (22%), day 12 to day 16 by 32% respectively when compared to respective control (Group I). A similar trend has been observed in the group IV treated (diabetic) rats, with significant increase on day 4 (30%), day 8 (56%) and from day 12 to 16 by 55 to 23%. A linear increase in levels of SOD (67 to 78%) was found on day 4 to day 8 and then it was slightly decreased on day 12 (55%) in Group III rats. Significantly increased levels (25% to 74%) of SOD were observed in day 4 to day 12 ($p<0.05$) in treated (Group IV) rats and decreased thereafter. About 32 to 64% ($p<0.05$) of increased GPx content was noted in treated rats (Group II) until day 12. But it was increased 39 to 48% in diabetic treated rats on day 8 and then slightly decreased. A significant increase in GST level from day 4 to 12 by 58 to 65% was observed in group II rats. In diabetic treated rats, about 60 to 65% of increased GST content was found on day 4 to 8. Subsequently, GST content was decreased from 8 to 4% on day 12 to 16.

Table 2: Changes in the levels of enzymatic antioxidants in wound tissues of control and *A. squamosa* treated rats†

Group	Day 4	Day 8	Day 12	Day 16
	Catalase (µmoles of hydrogen peroxide decomposed per minute per milligram of protein)			
Control	11.34 ± 2.95	12.52 ± 1.75	2.57 ± 0.97	1.54 ± 0.50
Treated	13.58 ± 5.34*	15.96 ± 2.25*	3.81 ± 1.25*	2.29 ± 0.78*
Diabetic control	4.61 ± 0.35	8.02 ± 2.20	5.60 ± 2.10	3.37 ± 0.45
Diabetic treated	6.55 ± 0.35*	18.32 ± 3.80*	12.52 ± 3.86*	4.41 ± 0.60*
	Superoxide dismutase (mg of protein required to give 50% inhibition of epinephrine auto oxidation)			
Control	0.27 ± 0.16	0.34 ± 0.09	0.068 ± 0.03	0.061 ± 0.03
Treated	0.84 ± 0.42*	1.57 ± 0.05*	0.15 ± 0.03*	0.064 ± 0.04
Diabetic control	0.21 ± 0.16	0.14 ± 0.07	0.07 ± 0.04	0.15 ± 0.03
Diabetic treated	0.28 ± 0.16	0.34 ± 0.10*	0.27 ± 0.10*	0.11 ± 0.06
	Glutathione peroxidase (nmol of CDNB conjugated per minute per milligram of protein)			
Control	5.41 ± 0.29	8.31 ± 0.91	1.25 ± 0.09	0.85 ± 0.09
Treated	8.03 ± 1.40*	9.77 ± 1.83*	3.51 ± 0.63*	0.89 ±0.27
Diabetic control	2.81 ± 0.89	4.41 ± 0.83	2.57 ± 0.72	1.06 ± 0.40
Diabetic treated	4.64 ± 0.65*	8.60 ± 0.95*	3.63 ± 0.45*	1.17 ± 0.49
	Glutathione–S-transferase (µmol of glutathione utilized per minute per milligram of protein)			
Control	0.38 ± 0.18	1.49 ± 1.10	0.17 ± 0.05	0.19 ± 0.09
Treated	0.92 ± 0.33	3.74 ± 1.15*	0.49 ± 0.19*	0.21 ± 0.14
Diabetic control	0.30 ± 0.03	0.66 ± 0.41	0.22 ± 0.11	0.22 ± 0.08
Diabetic treated	0.76 ± 0.21*	1.86 ± 0.04*	0.24 ± 0.03	0.23 ± 0.11

†Values are expressed as mean ± SD of six animals in each group; *$p<0.05$ as significant compared to corresponding control

3.4 Effect of *A. squamosa* on non-enzymatic antioxidants

Non- enzymatic antioxidant levels were observed periodically in control and treated rats from day 4 to day 16. Figure 3 compares the ascorbic acid content in control and treated wound tissues. A significant increase (25 to 29%) in ascorbic acid was observed on day 4 to 8 in group II and it was slightly higher on day 12 (35%) then decreased. In group IV rats, a significantly increased level was noticed till day 8 (26 to 31%). A similar kind of results was obtained for vitamin-E levels in *A. squamosa* treated wounds from day 4 to day 12. Figure 4 explores increased ($p<0.05$) vitamin-E content in Group II and Group IV animals.

Reduced glutathione plays an important antioxidant role in treated wounds. Glutathione content of group II rats on day 4, day 8 and day 12 was 46%, 51% and 73% respectively ($p<0.05$). In group IV animals, it was 39%, 42% and 44% in respective intervals of day 4 to 12 when compared to control ($p<0.05$). Finally it was decreased

to 13% on day 16 (Fig 5).

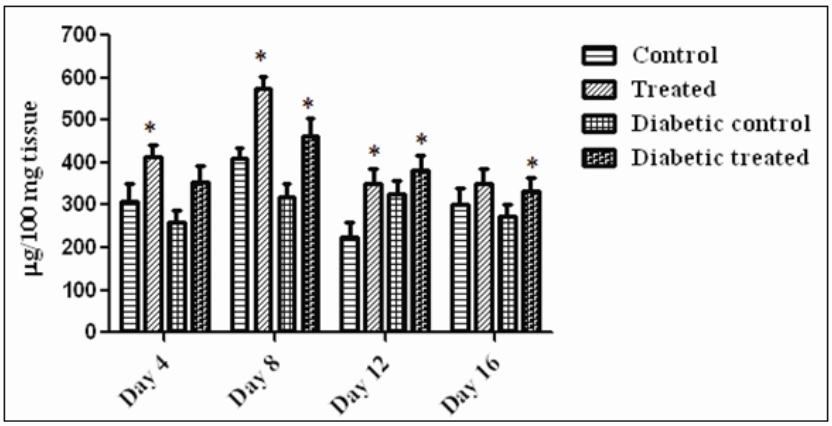

Fig 3: Ascorbic acid content in control and treated rats on various days. Values are expressed as mean ± S.D for six animals *p<0.05, as significant when compared with the control.

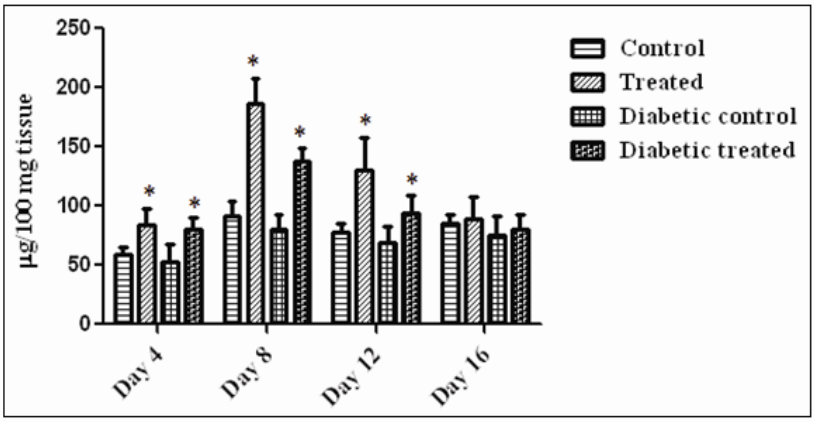

Fig 4: Graphical representation of Vitamin E on various days of control and *A. squamosa* treated wounds. Values are expressed as mean ± S.D for six animals *p<0.05, as significant compared with the control.

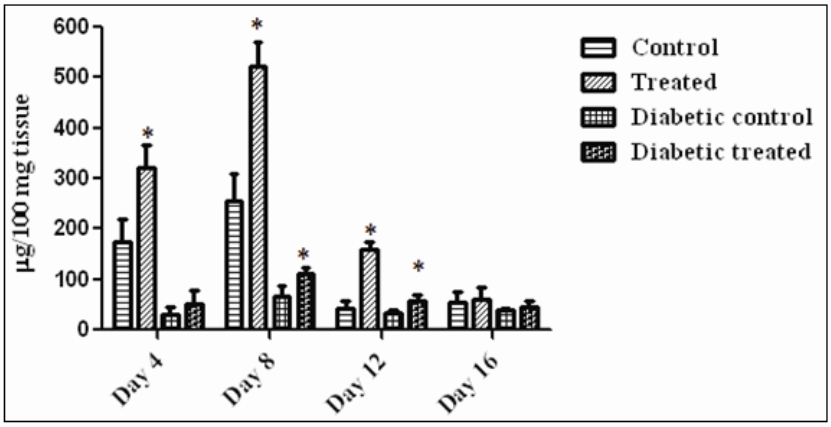

Fig 5: Glutathione content in control and treated wound tissues on various days. Values are expressed as mean ± S.D for six animals *p<0.05, as significant when compared with the control.

4. Discussion

Diabetes mellitus is a condition which is known to be associated with a variety of connective tissue abnormalities. Collagen, being an important connective tissue protein, provides strength and integrity to the dermis [27]. The collagen content of the skin is decreased as a result of reduced biosynthesis and/or accelerated degradation of newly synthesized collagen. These abnormalities contribute to the impaired wound healing observed in diabetes [28].

Our earlier experimental results supported that plant products are promising agents for wound healing because of their widespread availability, non-toxicity, effectiveness and absence of unwanted side effects as crude preparations [16]. Plant extracts and medicinal herbs have been shown to possess variety of potent antioxidant principles.

Presence of large amount of bioactive principles like alkaloids, flavonoids in *A. squamosa* and the well-known antioxidant

property helped the wounds to heal significantly faster in treated animals than the untreated [29]. *A. squamosa* leaves possess variety of antioxidants like total phenolic compounds, considered as a major contributor for their antioxidant and antibacterial properties [30]. Recently, we have reported the role of alcoholic extract of *A. squamosa* on the amelioration of diabetic wound healing in rats [16]. Here, we report the role of *A. squamosa* on its free radical quenching capacity which would substantiate its potential as a wound healer.

Antioxidants have been clearly known to accelerate wound healing [31]. Decreased levels of antioxidants have been observed initially in cutaneous wound healing, which is slowly recovered after complete healing [7]. The mechanism of free radical generation and their disposal procedures are changed in delayed wound healing and shown that oxygen free radicals consists major role in impaired wound healing[32]. Also, altered antioxidant levels during wound healing in diabetic and aged animals have also been reported [33].

Promotion of free radical generation occurred by direct and frequent exposure of the skin to various environmental pollutants and pro-oxidants. Antioxidants play a vital role as primary defense against tissue damage of the skin. ROS considered as specific secondary messengers in signaling cascades in angiogenesis [34-36]. Neutrophils and macrophages donate variety of antioxidants like H_2O_2, which itself acts as primary antioxidant at the wound site [37].

Increased nitric oxide level directly stimulates the production of cellular inflammatory cytokines, subsequently promotes the development of the various phases of wound healing in diabetic rats [38]. NO is involved in various phases of physiological and pathophysiological reactions in skin (e.g. differentiation and proliferation of epidermal cells, hair growth, wound- healing processes, antimicrobial activities, antigen presentation, allergic skin reactions, regulation of innate immune reactions and inflammatory responses)[39]. Previous findings also report that decreased nitric oxide levels at the wound site contribute to the delayed healing in diabetes [40]. Diabetic wounds are more susceptible to nitric oxide donor because wound is deficient in NO [8]. Topical administration of nitric oxide donors increased the collagen synthesis in fibroblasts *in vitro* and promotes wound repair in excision wound model [41].

Ascorbic acid (AA) is found in variety of biological species including plants, animals and single cell organisms [42]. AA generally known as an antioxidant, which can eradicate chained radical reactions by being as a strong (electron + proton) donor in interactions with free radicals [43]. Vitamin E (α tocopherol), a major lipid soluble compound in skin, is an essential antioxidant for speedy wound healing [44]. Musalmah *et al* showed that vitamin E enhances wound healing by reducing free radicals and increasing GPx content [45].

Glutathione (GSH) is a major non protein thiol antioxidant compound present in living organisms, which serves a significant role in antioxidant defense mechanism [46]. GSH, a tripeptide, possess an unusual peptide linkage between the amine group of cysteine and the carboxyl group of glutamate side chain. GSH acts as an effective antioxidant, protecting the cellular components from oxidative damage caused by ROS [47].

A significantly increased catalase activity observed in *A. squamosa* treated rats revealed that accumulation of excessive H_2O_2 in wound tissues due to increased activity of SOD might be properly neutralized by catalase [48]. SOD is considered to be an inducible key enzyme and its activity depends on O_2^- concentration in the biological system [49]. Elevated levels of SOD might be the adaptive nature of enzyme against increased oxidative stress and tissue damage in diabetic rats. Similar trend has been observed in the previous report of Govan *et al* [50]. SOD catalyzes the dismutation of O_2^- into oxygen and H_2O_2, which has been known that SOD decreases ROS generation and oxidative stress [51].

Increased levels of SOD and GPx in treated animals are known to quench the superoxide radical and thus protect the tissue damage by free radicals [52]. GPx is considered to be an essential factor in defense against oxidative tissue damage and the cellular function. It was reported that nutritional GPx reduces oxidative stress *in vivo*, protecting diabetes-related complications [53].

Severe tissue damage can occur during the prolonged overproduction of reactive oxygen species which leads to impaired wound healing in diabetes [54]. Glutathione-S-transferase (GST) and heme oxygenase-1 (HO-1) are the two ROS-inducible enzymes, extremely important in the cytoprotection during cutaneous wound healing [55].

In conclusion, our experimental study clearly indicates that the topical administration of ethanolic extract of *A. squamosa* promotes levels of enzymatic and non-enzymatic antioxidants in wound tissues, thus detoxifying free radicals to promote better wound healing in normal and diabetic rats. Also the increased levels of antioxidants in *A. squamosa* treated rats could have supported the improved wound healing observed in our earlier report [16].

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