ALTERED ANTIOXIDANT ENZYME PROFILE IN WOUND HEALING

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ABSTRACT

The effects of the alcoholic extract of the flowers of *Ixora coccinea* were studied on some of the antioxidant enzymes in dead space wounds created in rats. Increases in the tensile strength of the wound and in the level of lysyl oxidase, the crucial enzyme for collagen maturation, were observed indicating a definite prohealing action. In addition, a highly significant increase in the levels of catalase, glutathione peroxidase and glutathione reductase was observed in the drug-treated group. Thus, the gain in tensile strength may be attributed not only to the better cross-linking but also to the antioxidant properties of the drug.

KEY WORDS

Antioxidant; catalase; glutathione peroxidase; glutathione reductase; Lysyl oxidase

INTRODUCTION

The flowers of *Ixora coccinea* L. (Rubiaceae) have been used in traditional Indian systems of medicine for dysentery, anti-inflammatory action (1), treatment of ulcers and more recently, for anti-tumor activity (2). In an earlier study on the effects of this plant on healing, we had reported that the observed increase in tensile strength of dead space wounds was due to increase in glycosaminoglycan and hydroxyproline contents (3). Very few reports are available (4) on the role of antioxidant enzymes on healing. Since this plant was rich in triterpenoids (attributed to have antioxidant action) we took up this study to investigate whether the prohealing action was due to its antioxidant properties.

MATERIALS AND METHODS

Preparation of herbal extract

Ixora coccinea flowers cultivated for ornamental purposes were collected and identified in the department of Botany, Poornaprajna College, Udupi.

The shade dried, powdered flowers (220 mg) were Soxhlet extracted with 90% ethanol. The solvent was

removed under pressure to yield a solid residue (yield 12%).

Animals

Wistar rats of either sex, bred in the department of Pharmacology, KMC, weighing around 150-200 g were used. They were housed individually in standardised environmental conditions, fed with pellet rodent diet and water *ad libitum*. They were made into two groups (control, test) of 6 animals each.

Wound model

Dead space wound model was used (5,6). Dead space wounds of 500 mm² were created by implantation of a polypropylene tube (0.5 cm x 2.5 cm) beneath the dorsal paravertebral lumbar skin. Controls received 2 ml of 1% carboxymethyl cellulose orally, while the test group received 2 ml of the drug (200 mg/kg body weight) dissolved in 1% carboxymethyl cellulose through the same route, once daily, for a period of 10 days. On the 11th post-operative day, the granuloma tissue formed on the dead space wound was excised. Wet weight of the granuloma tissue was noted. In our earlier study (3) we tried with different doses of the drug, 200 mg of drug per kg body weight was shown to be most effective in the process of wound healing.

Chromatographic techniques and detection of triterpenoids

Crude ethanol extract from flowers of *Ixora coccinea*

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was used for phytochemical screening. Crude ethanol extract, 100 gm (32.5%) was treated with ether, and then the ether extract was washed with acid and then with alkalis. Only 0.7% by weight of the crude extract of the acid dissolved and alkali-dissolved substances were obtained. Ether was completely evaporated from the neutral fraction. The residue of 2.5% by weight of the crude extract. Then analysed by column using alumina as packing material. Pure crystal of 0.20gm by weight (0.1% by weight of the crude) with m.p. 130-140°C was obtained from the petroleum ether-chloroform fraction (3:1). The presence of triterpenoid compounds in the extract was demonstrated by Liebermann-Burchard tests.

Preparation of tissue homogenate

A granuloma tissue was homogenized using 0.02 M potassium-phosphate buffer, pH 7.6 (1:10 w/v). The tissue homogenate was centrifuged at 6000 rpm in a refrigerated centrifuge. The clear supernatant was used for all the assays.

Parameters studied

Tensile strength- This was determined as per the method of Lee (7). The granuloma tissue was held with a tissue holding forceps attached to a pulley. The amount of water pressure, in grams, required to break the tissue gave the tensile strength.

Protein content- Protein content of the tissue homogenate was estimated by the method of Lowry *et al* (8) using bovine albumin as standard.

Estimation of catalase activity (9) -Exactly 0.2 ml of the diluted homogenate (1:500) was added to the reaction mixture, which consisted of 0.2 ml of 0.05 M sodium phosphate buffer and 1.2 mM H₂O₂. After 25 minutes of incubation at room temperature, the reaction was arrested by the addition of 2.5 ml of peroxide reagent containing peroxide and chromogen system. The absorption was measured at 500 nm. A blank without H₂O₂ and control with 1mM sodium azide and catalase enzyme were run simultaneously.

Estimation of glutathione peroxidase (10) - The assay system consisted of 50 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, 1 mM sodium azide, and 0.2 mM NADPH and 1.0 mM GSH. After 2 minutes of incubation at 37°C, 0.1 ml of cumene hydroperoxide was added. The absorbance was measured at 340nm. Readings

were recorded for 5 minutes at 30 seconds interval. A blank with reaction mixture and without test sample and control with test sample but without reaction mixture were run simultaneously.

Estimation of glutathione reductase activity (11). In a tube, 0.12 ml of 0.06% NADPH and 0.12ml of 1.1% oxidized glutathione were taken to which 0.1 ml of tissue homogenate and 1.6 ml of sodium phosphate buffer, pH 6.6, were added and the volume made up to 2 ml with deionized water. Blank and control (without oxidised glutathione) were run simultaneously. The absorbance of the reaction mixture was recorded at 340nm for 3 to 5 minutes at 30-second intervals.

Extraction and estimation of lysyl oxidase from granuloma tissue:

In this case the granuloma tissue homogenate was prepared by homogenising the tissue in 0.02 M phosphate buffer, pH 8.2 containing 1.5 M urea (1:10w/v). This was stirred for 20 min and cold centrifuged. The supernatant served as a source of lysyl oxidase.

Lysyl oxidase activity was assayed spectrofluorometrically, using a monoamine substrate (12) and adaptation of the peroxidase coupled assay (13,14).

Statistical analysis of results:

Results, expressed as mean $\pm$ S.E. were evaluated by student's 't' test. Values of $p < 0.005$ were considered statistically significant.

RESULTS

The effects of the indigenous drug *Ixora coccinea* on wound healing are depicted in Table 1.

Significant increase in the tensile strength ($p < 0.001$) was noticed with the animal treated with extract of *Ixora coccinea* flowers. The lysyl oxidase activity was increased in the test group ($p < 0.001$) when compared with the control group. The antioxidant enzymes catalase, glutathione peroxidase and glutathione reductase were also increased. The increase in catalase and glutathione reductase levels was significantly higher ($p < 0.001$) than the glutathione peroxidase enzyme. Protein content in the granuloma tissue was increased too. The ethanol extract of *Ixora coccinea* flowers responded to Liebermann-Burchard tests for sterol and triterpenoid compounds.

DISCUSSION

Wound healing can be described in three phases (a) fibroblast proliferation and secretion of mucopolysaccharides (b) replacement of mucopolysaccharides by collagen molecules and maturation of these molecules by cross-linking and (c) remodeling and scar formation (15). These processes were demonstrated by workers who studied the effects of vitamins like pantothenic acid and ascorbic acid upon the growth of fibroblasts, obtained from human fetal skin or foreskin. Cell proliferation, protein synthesis and protein release were also evaluated in wound healing process (16).

In our earlier study, we had reported that *Ixora coccinea* had a profound stimulatory effect on the first phase of healing, as evidenced by an increased level of mucopolysaccharides (3). The increased tensile strength was attributed to the interaction between these molecules with the collagen fibres, resulting in better alignment of the collagen bundles in the healing wound. The present study shows that the drug acts at more than one point. It stimulated

the enzyme lysyl oxidase, which is crucial for cross-linking and maturation of collagen (17). It also increased the antioxidant enzymes, thereby affording protection to the developing fibroblasts against free radicals. Wounding initiates inflammation, which in turn stimulates the production of free radicals by phagocytes. Some of the free radicals can leak into the extracellular space and inhibit myofibroblasts thereby retarding the healing process. Whether the drug stimulates the enzymes at gene level is difficult to say at this stage; the increased protein synthesis in the granuloma tissue does hint the possibility of increased enzyme synthesis. Alternately, crude plant preparations have several different constituents and some of these (like triterpenoids) may possess free radical scavenging properties.

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Table 1

Wound healing effects of the ethanol extract of *Ixora coccinea* flowers

Parameter	Control	Drug-treated
Tensile strength (g)	270.0 ± 14.7	564.3 ± 4.1***
Protein content (mg/g)	36.9 ± 3.7	42.3 ± 2.8*
Lysyl oxidase (SFU)	1645 ± 124	3848 ± 240***
Catalase (μMoles H ₂ O ₂ degraded/min/g tissue)	61.77x 10 ³ ± 56	168.45x 10 ³ ± 48***
Glutathione peroxidase (μMoles NADPH oxidised/min/g tissue)	2.0782 ± 0.10	2.9604 ± 0.01*
Glutathione reductase (μMoles NADPH oxidised/min/g tissue)	0.1578 ± 0.07	0.7986 ± 0.01***

Values are mean ± SE; n=6

*p< 0.05

** p< 0.01

*** p< 0.001 vs. control student's t-test

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