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Research Article

REACTIVE OXYGEN SPECIES CONTROL BY PLANT BIOPOLYMERS INTENDED TO BE USED IN WOUND DRESSINGS

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ABSTRACT

Reactive oxygen species (ROS) production in wounds is a key factor in the healing process. Excess or uncontrolled ROS is a characteristic feature of chronic wounds they can slow healing by interfering with metabolic enzymes causing oxidative degradation of lipids in the cell membrane with increase in wound inflammation. Efficient therapy thus entails controlling ROS at the wound site. In the present study biopolymers from *Moringa oleifera* pods and gum of *Acacia* plant have been suggested as good antioxidants for controlling ROS. Gum of *Acacia* showed better superoxide scavenging ability while *Moringa* seed associated biopolymer had more potential to scavenge hydrogen peroxide, hydroxyl and nitric oxide radicals. Both the biopolymers had sufficient reducing power and thus have great potential as wound management aids.

Keywords: Antioxidants, Biopolymer, Gum acacia, *Moringa oleifera*, Wound healing

INTRODUCTION

Response to trauma begins at the moment of injury with activation of first line of defence, contributed by polymorphonuclear neutrophils (PMNs), macrophages and monocytes. This is followed by an oxidative burst which releases a large amount of ROS intra and extracellularly. Intracellularly combined action of ROS and proteolytic enzymes kill infecting bacteria [1, 2] while extracellularly excessive generation of ROS induce severe tissue damage and even leads to neoplastic transformation decreasing the healing process by damaging cellular membranes, DNA, protein and lipids, especially in acute and chronic wounds [3]. Typically, burn injuries show excessive activity of free radicals [4, 5]. Enhanced ROS concentrations in chronic wounds are thought to drive a deleterious sequence of events finally resulting in the non healing state. Thus, elimination of ROS could be an important strategy in healing of wounds [6]. Antioxidants are expected to decrease the oxidative challenges at the wound site by removing products of inflammation. Excess ROS kill fibroblasts and make skin lipids less flexible. They counter the excess proteases and ROS often formed by neutrophil accumulation in the injured site and protect protease inhibitors from oxidative damage [7]. Because of these, the overall role of antioxidants appears to be significant in the successful treatment and management of wounds.

There are two basic categories of antioxidants namely synthetic and natural ones. Although synthetic antioxidant like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and t- butyl-hydroquinone (TBHQ) are known to ameliorate oxidative damages but they may be carcinogenic [8]. Therefore, research for the determination, development and utilization of more effective antioxidants of natural origin that have significant scavenging properties, are less toxic and inherently safer than synthetic antioxidants is desired [9].

Plants are a potential source of natural antioxidants and produce various antioxidative compounds to counteract ROS in order to survive [10]. A large number of plants, their extracts, decoctions and pastes have been used by tribals and folklore traditions for treatment of cuts, wounds and burns. Natural gums and mucilage have been widely explored as pharmaceutical excipients. Gum of *Moringa oleifera* has been used as disintegrant tablet for fast release of drug [11] on the other hand extracts of *Moringa oleifera* and *Acacia* have been traditionally used in skin care [12-14]. Gum acacia extracted from *Acacia* plant and polymers from aqueous extracts of *Moringa oleifera* were found to be non haemolytic, non cytotoxic and thrombogenic in our lab and thus have potential for being used in wound management aids.

In the present study we shall determine whether these polymers have ROS scavenging activity that would further enhance their wound healing ability. Different antioxidant compounds may act through different mechanisms, consequently one method alone can't be utilised to fully evaluate the antioxidant capacity of the material. As reported earlier the results from different antioxidant assays are even difficult to compare because of the difference in substrates, probes, reaction conditions and quantification methods[15]. We therefore assessed the antioxidant properties of the above mentioned plant biopolymers using different tests with different approaches and mechanisms.

MATERIAL AND METHODS

Chemicals

All the chemicals used are of high analytical grade. All solutions were prepared in freshly prepared doubled distilled water. Deoxyribose, trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), ascorbic acid were purchased from Hi-Media, Mumbai. Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), N-(1-naphthyl) ethylene-diamine-dihydrochloride were purchased from SRL, Mumbai.

Polymer Extraction

Moringa pods were collected from local plantations and dried. Precisely the seeds and the mucilaginous part around it was ground to a fine powder. Aqueous extracts were prepared using 10g powder in 100mL of distilled water and stirred for 30 min [16]. After filtration, the filtrate was treated with three volumes of chilled isopropyl alcohol and refrigerated overnight for precipitation of biopolymer. In the same way water soluble polymeric part of gum acacia was separated from a 20% solution. The gum was dissolved in distilled water at 80^{o}C with stirring, filtered and subsequently precipitated with isopropyl alcohol.

Carbohydrate and protein estimation of plant biopolymers

Carbohydrate content of the plant polymers was measured by anthrone method using glucose as standard [17], and protein was estimated using BSA as a standard [18].

Superoxide scavenging assay

The activity was evaluated using nitro blue tetrazolium (NBT) reduction method [19]. The reaction mixture consisted of 1mL of NBT solution (156µM) and 4mL biopolymer solution of different concentrations (100-1000µg mL^{-1}). The reaction was started by adding 100µL of phenazine methosulfate solution (60µM, PMS) in phosphate buffer (pH 7.4) to the reaction mixture followed by incubation at 25^{o}C for 5 min and then absorbance at 560nm was measured against blank (phosphate buffer). Ascorbic acid (100-1000µg mL^{-1}) was used as the standard.

$$\text{Superoxide scavenging activity (\%)} = \frac{\text{Ac (control)} - \text{As (sample)}}{\text{Ac (control)}} \times 100 \quad \text{Eq. 1}$$

Where, Ac (control): Absorbance of the control and As (sample): Absorbance of the biopolymers/standard.

H_2O_2 radical scavenging assay

The ability of the plant biopolymers to scavenge hydrogen peroxide was determined after the method of Ruch *et al.* (1989) [20]. A solution of hydrogen peroxide (2mmol L^{-1}) was prepared in phosphate buffer (pH 7.4). 0.6mL of this solution was added in 4mL of biopolymer solution of variable concentration (100 - 1000µg mL^{-1}). Absorbance of the reaction mixture at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with ascorbic acid, the reference compound. Percentage inhibition was calculated according to Eq. 1.

Hydroxyl radical scavenging assay

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al. (1989) [21]. Stock solutions of EDTA (1mM), $FeCl_3$ (10mM), ascorbic acid (1mM), H_2O_2 (10mM) and deoxyribose (10mM) were prepared in distilled deionized water. The assay was performed by adding 0.1mL of EDTA, 0.01mL of $FeCl_3$, 0.1mL of H_2O_2, 0.36mL of deoxyribose,1.0mL of biopolymer solution (100 - 1000µg mL^{-1}), 0.33mL of phosphate buffer (50 mM, pH 7.4) and 0.1mL of ascorbic acid in this sequence. The mixture was incubated at 37°C for 1hr. Then 1.0mL of 10% TCA and 1.0mL of 0.5% TBA was added in the reaction mixture and kept at 100° C for 20 min. to develop the pink chromogen. Absorbance was measured at 532 nm. Ascorbic acid with same concentrations was used as a reference compound. Percentage hydroxyl scavenging activity was calculated according to Eq. 1.

Assay of Nitric oxide scavenging activity

The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, 1mL of sodium nitroprusside (5mM) in phosphate buffered saline (PBS) was mixed with 4mL of biopolymer solutions (100 - 1000µg mL^{-1}) and incubated at 28°C for 150 min. The same reaction mixture without the biopolymer but with an equivalent amount of PBS served as control. After the incubation period, 1mL aliquot of reaction mixture was diluted with 4mL distilled water and then 0.1mL Griess reagent (1% sulfanilamide, 5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylene-diamine-dihydrochloride) was added in it. The absorbance of the chromophore formed was read at 545 nm [22]. Ascorbic acid and BHT with same concentrations were used as the standards and PBS was used as the blank in the experiment. Percentage inhibition was calculated as per Eq. 1.

Reducing power

The reducing power of plant biopolymers was determined after the method of Oyaizu [23]. One mL of different concentrations of the biopolymer (100– 1000 µg mL^{-1}) were mixed with 2.5mL of phosphate buffer (0.2 M, pH 6.6) and 2.5mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 min. To this 2.5mL of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (5mL) and 0.02mL $FeCl_3$ (0.1%). After proper mixing, absorbance was measured at 700 nm against phosphate buffer (pH 6.6) as a blank. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid (100– 1000 µg mL^{-1}) was used as the standard.

Determination of IC_{50} value

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. IC_{50} value was calculated by linear regression method that represents the level where 50% of the radicals were scavenged by test samples.

Statistical analysis

All the experiments were conducted in triplicate. Data presented in means, standard deviation and variance. One-way ANOVA was calculated by Smiths statistical package (SSP). Differences were considered statistically significant at $p < 0.001$.

RESULTS AND DISCUSSION

Wound healing is a complex programmed sequence of processes including inflammation, cell migration, granulation, collagenation, maturation, re-epithelisation and scar formation that run concomitantly but independent of each other. A characteristic feature of inflammatory phase is the oxidative burst that leads to production of high level of radicals and non-radical ROS that play an important protective role [24]. However the oxidative stress created at the wound site if not controlled by the host's antioxidative capacity leads to inhibition of cell migration and proliferation. This results in tissue damage and perpetuation of inflammation [25, 26]. Delayed wound healing in type 2 diabetic patients and in chronic wounds has been correlated to ROS generation [27, 28]. Thus control of ROS produced *in vivo* is an important strategy in healing of wounds.

Gum acacia (GA) and *Moringa* seed polymer (MSP) were primarily polysacchridic in nature containing 762 and 564µg of anthrone reactive carbohydrate and 47 and 325µg protein. mg^{-1} dry weight respectively.

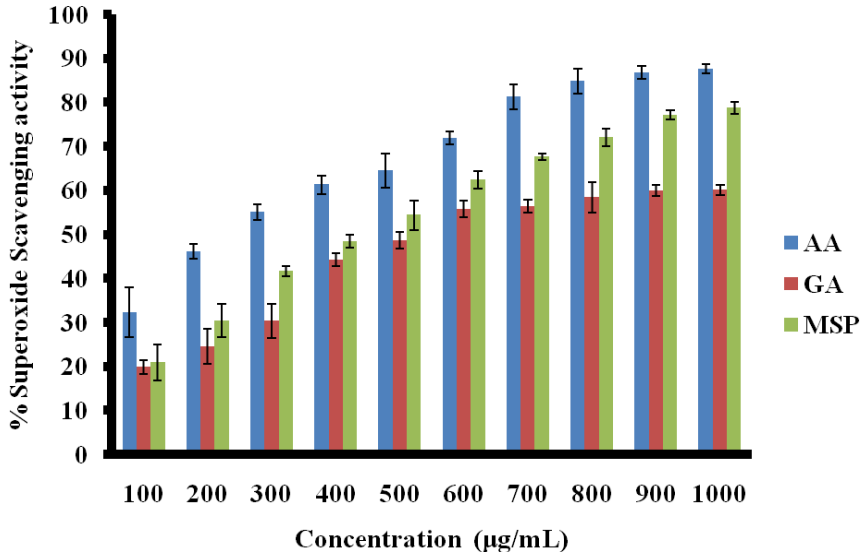

Fig. 1: Superoxide radical scavenging activity of gum acacia (GA), *Moringa* seed polymer (MSP) compared with ascorbic acid (AA). (Values are mean±SD, n=3)

Large amount of superoxide radicals are released by the NADPH-oxidase enzymatic complex of polymorphonuclear cells and macrophages migrating into the wound sites. Non phagocytic cells like fibroblasts also release ROS. This anion does not cause any tissue damage but its conversion to hydroxyl radical, hypochlorite anion and/or peroxynitrate may eventually have detrimental effects [29, 30]. In the present study superoxide anion radicals are derived in PMS-NADH-NBT system, where the decrease in absorbance at 560nm with both biopolymers indicates the consumption of superoxide anion in the reaction mixture, thereby exhibiting a dose dependent increase in superoxide scavenging activity as shown in Figure 1. GA with an IC_{50} of 502 µg mL^{-1} showed a higher scavenging activity than MSP that had an IC_{50} of 637 µg mL^{-1}. On the basis of present investigation the plant biopolymers showed high significance ($p< 0.001$) when compared to standards.

H_2O_2 a non radical ROS is present in wound fluids in micro molar concentrations. It plays an important role in fast recruitment of inflammatory cells and latent neutrophil collagenase to the wound site that may degrade collagen in the wound [31]. A concentration of 0.05-0.5 mM H_2O_2 leads to apoptosis of fibroblasts [32]. H_2O_2 is a weak oxidising agent that inactivates enzymes directly by oxidation of essential thiol groups. It can cross the cell membrane barrier and react with Cu^{+2} or Fe^{+2} to form the more potent $OH^{\cdot}$ radical [33]. As demonstrated in Figure 2 that both the plant biopolymers were capable of controlling the oxidative stress created by H_2O_2 in a dose dependent manner. MSP proved to be a better scavenger of H_2O_2 with an IC_{50} value of 652.52 µg mL^{-1} against GA (738.09 µg mL^{-1}), however both showed lower scavenging activity than ascorbic acid (423.58 µg mL^{-1}). They exhibited significant H_2O_2 scavenging activity ($p< 0.001$) when compared to standards.

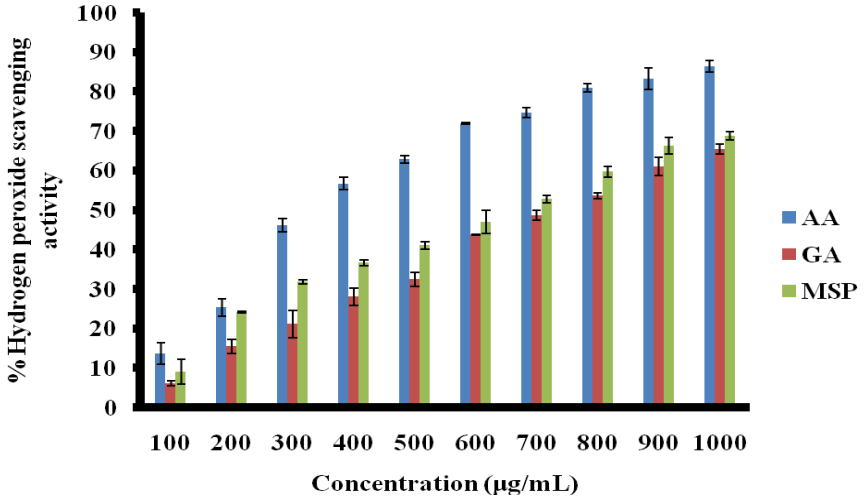

Fig. 2: Hydrogen peroxide radical scavenging activity of gum acacia (GA), *Moringa* seed polymer (MSP) in comparison to ascorbic acid (AA) used as standard. (Values are mean±SD, n=3)

Another key activity of an antioxidant material is its ability to inhibit the hydroxyl radical formation. Hydroxyl radicals, indeed, exhibit very high reactivity and tend to react with a wide range of molecules found in living cells [34]. Due to the high reactivity, the radicals have a very short biological half-life and thus an effective scavenger must be present at a high concentration or possess high reactivity toward these radicals to control their reaction [35]. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism *in vivo* is the Fenton reaction [36]. Hydroxyl radical can be generated *in situ* by decomposition of hydrogen peroxide by high redox potential EDTA–Fe^{2+} complex, and in the presence of deoxyribose substrate, it forms thiobarbituric acid-reactive substances (TBARS) which can be measured. Antioxidant activity is detected by decreased TBARS formation, which can come about by donation of hydrogen or electron from the antioxidant to the radical or by direct reaction with it [37]. Hydroxyl radical scavenging activity of the two biopolymers was assessed by observing the inhibition of free radical mediated deoxyribose damage. As can be observed by Figure 3, MSP could scavenge 76% hydroxyl radicals while GA scavenged 65% as against AA that removed 83% hydroxyl radicals. IC_{50} values of GA, MSP and AA in this assay were 473 µg mL^{-1}, 300 µg mL^{-1} and 72 µg mL^{-1}, respectively, with the highest value representing least hydroxyl radical scavenging ability. The values were statistically significant at $p < 0.001$ level.

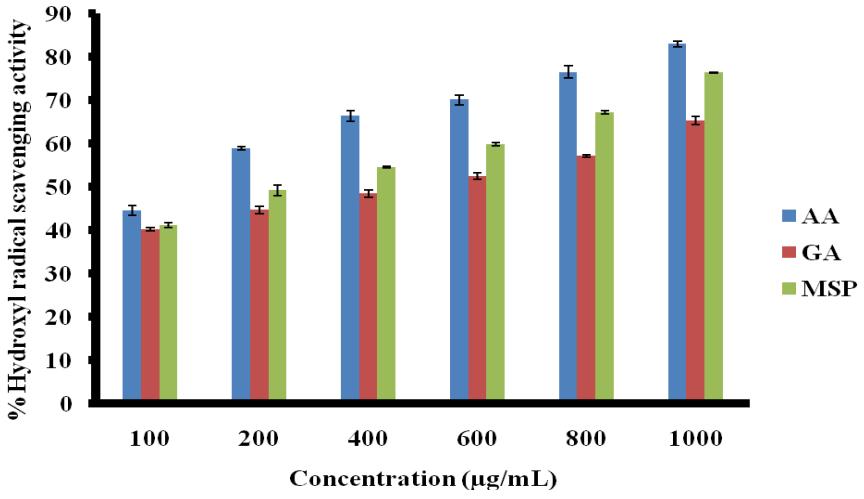

Fig. 3: Percentage hydroxyl radical scavenging activity of gum acacia (GA), *Moringa* seed polymer (MSP) and ascorbic acid (AA). (Values are mean±SD, n=3)

At the site of inflammation in the wounds, mitogen-activated cells, such as macrophages, neutrophils, eosinophils, and epithelial cells, synthesize and release various pro-inflammatory mediators. Prostaglandin E2 (PGE2) and nitric oxide (NO•) are two pivotal pro-inflammatory mediators [38]. Despite the possible beneficial effects of NO•, its contribution to oxidative damage is increasingly becoming evident. This is due to the fact that NO• can react with superoxide to form the peroxynitrite anion, which is a potential strong oxidant that can decompose to produce •OH and NO_2 [39]. Its protonated form, peroxynitrous acid (ONOOH), is a very strong oxidant [40]. The main route of damage is the nitration or hydroxylation of aromatic compounds, particularly tyrosine. Under physiological conditions, peroxynitrite forms an adduct with carbon dioxide dissolved in body fluid and is responsible for oxidative damage of proteins [41, 42]. As can be observed from Figure 4 different concentration of GA, MSP, BHT and AA showed dose dependent inhibition of nitric oxide radical. GA was found to be a weak nitric oxide scavenger with 56% NO• scavenged and an IC_{50} value of 791 µg mL^{-1}, while MSP, BHT and AA had IC_{50} value of 474, 353 and 236 µg mL^{-1} with 63 %, 74% and 79% removal of NO•. There was a statistical significant relationship between the nitric oxide scavenging activity of GA and MSP ($p< 0.001$).

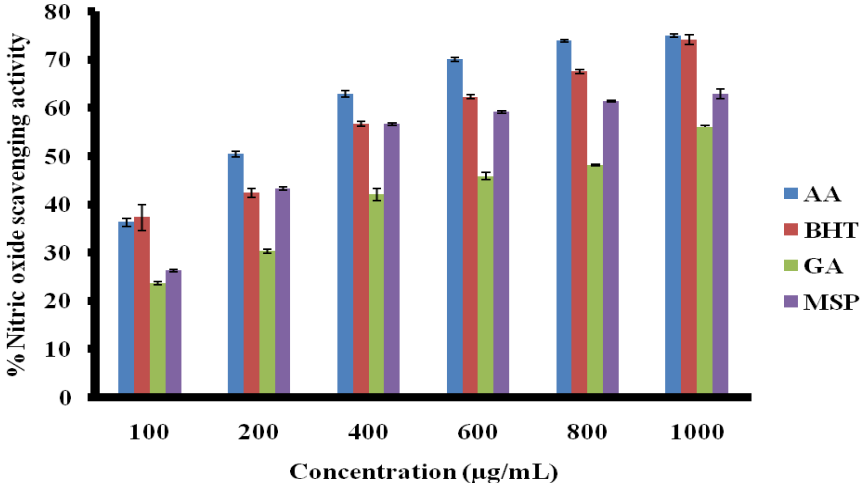

Fig. 4: Nitric oxide radical scavenging activity of gum acacia (GA) and *Moringa* seed polymer (MSP) compared with butylated hydroxy toluene(BHT)and ascorbic acid (AA) used as standards. (values are mean±SD, n=3)

The reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity [43]. Unless an antioxidant prevents the generation of ROS by metal chelation or enzyme catalysed removal [44, 45] a redox reaction occurs. Non enzymatic oxidants can thus be described as reductants. In this context antioxidant power may be analogously referred to as reducing ability. Reducing power reflects the electron donating ability of bioactive compounds [46]. The reducing ability of both plant polymers is measured in this study by the demonstration of reduction of $Fe[(CN_6)]_3$ to $Fe[(CN)_6]_2$. Based on the principle, the reductive ability of plant biopolymers was measured with AA as a reference compound. Addition of free Fe^{+3} to reduced product leads to the formation of the complex that is measured at 700 nm. The observed increase in reducing power of the biopolymers in a concentration dependent manner suggested that they are good electron donors. The total reduction ability of both GA and MSP were more or less similar as showed in Figure 5 however AA showed higher reducing ability than the biopolymers. From the results it was evident that that the studied biopolymers possess significant ($p<0.001$) reducing power as compared to standards.

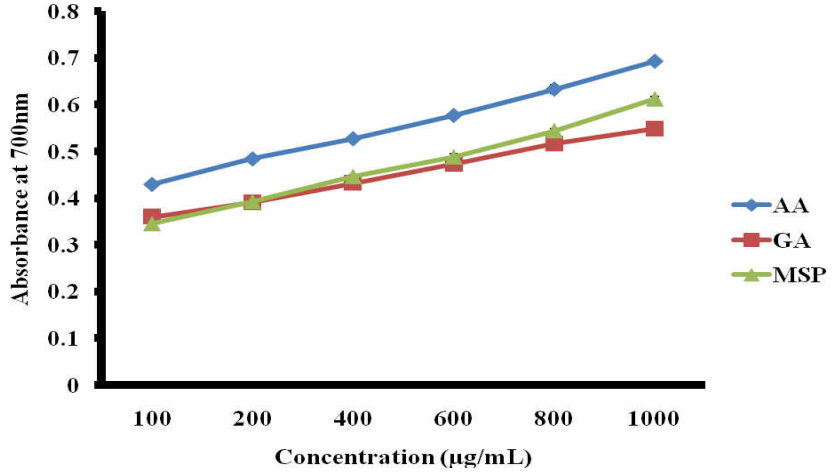

Fig. 5: Total reducing ability of gum acacia (GA), *Moringa* seed polymer (MSP) and ascorbic acid (AA) used as standard. (Values are mean±SD, n=3)

CONCLUSION

The study generates sufficient evidence from the *in vitro* antioxidant assays that the biopolymers from MSP and GA are important sources of natural antioxidants, which might be useful as preventive agents against oxidative stress however, MSP with a higher antioxidant potential when incorporated in wound healing preparation would efficiently accelerate the wound healing capacity by diminishing the oxidative stress at wound site.

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