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Induced oxidative stress management in wounds through phenolic acids engineered fibrous protein: An *in vitro* assessment using polymorphonuclear (PMN) cells

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ABSTRACT

The present study explores the preparation, characterization and the role of phenolic acid tethered fibrous protein in the management of induced oxidative stress studied under *in vitro* conditions. In brief, the biomaterial is prepared by engineering the fibrous protein with dihydroxy and trihydroxy phenolic acid moieties and subjected to characterization to ensure the tethering. The resultant biomaterial studied for its efficacy as a free radical scavenger using polymorphonuclear (PMN) cells with induced oxidative stress and also as an agent for cell migration using fibroblasts cells. Results revealed that induced oxidative stress in PMN cells after exposure to UVB radiation managed well with the prepared biomaterial by reducing the levels of superoxide anion, oxygen and hydroxyl radicals. Further, the protein and the phenolic acid interaction supports the cell migration as evidenced from the scratch assay. In conclusion, though phenolic acids are well known for their antimicrobial and antioxidant potential, indenting these acids directly to the wounds is not sensible, but tethering to protein explored the scavenging activity as expected. The present study infers that phenolic acid engineered protein has a significant role in managing the imbalance in the redox state prevailing in wounds and supports the healing at appreciable level.

1. Introduction

By definition, oxidative stress is the excess production of reactive oxygen and nitrogen species despite the presence of antioxidative defense mechanism resulting with imbalance in the redox state. Oxidative stress has high implications on cells, repair or damage cellular or sub cellular membranes, DNA and protein. To alleviate the oxidative stress, antioxidant enzymes or molecules having scavenging property has been supplied to restore the redox state. Nevertheless, for each radical, the quantity of antioxidant molecule required needs intensive research study, since; most of the antioxidant molecules at high concentration may also behave as a pro-oxidant [1].

With respect to chronic wounds, the high titer values of inflammatory signals implied the status of the oxidative stress [2]. The application of simple protein or polymer based biomaterial fails to perform the healing process under these conditions [3] and demand antioxidant based therapy [4]. However, incorporating or encapsulating antioxidant molecules during the preparation of biomaterial though satisfy the current need, the variation in release pattern alter the scavenging of free radicals and subsequent healing process. Alternatively, antioxidant biomaterial (biomaterial itself act as an antioxidant system) has been suggested [5], but care is required on the nature of the basic material, which should not induce or increase the stress upon application [6]. All these descriptions suggest the need for potential free radical scavenging biomaterial.

With regard to biomaterial, natural, synthetic and composite materials are being studied in detail, but each material has its own drawbacks [7]. Most of the materials try to satisfy the mechanical stability, biocompatibility, biodegradability properties, but slighted for not having the most important antioxidant property. Other than synthetic and semi synthetic based biomaterials, protein based biomaterial especially collagen based materials are well studied as the supporting material in wound healing. Thus, incorporating antioxidant property to the already recognized material will add value to the material at the greater extent.

In general, most of the natural and traditional medicinal plants and fruit extracts display antioxidant property, but exploiting the extracts as such for the preparation of biomaterial is troublesome. Instead, the active constituents of the extracts or the polyphenolics available in the market that has been already recognized as a food grade product may find application in the preparation of

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the product. In this context, the three phenolic acids recognized for antioxidant property have been selected to impart antioxidant property to the chosen protein based biomaterial.

Thus, the present study explores tethering of phenolic acids with fibrous protein and assessed the antioxidant profile of the product under *in vitro* condition using PMN cells as a model. Further, the study investigates the cell migration and cell proliferation efficacy of the products developed using 3T3 fibroblasts cells.

2. Materials and methods

Protein form porcine skin type A, DPPH (2,2-diphenyl-1-picrylhydrazyl), caffeic acid, protocathechuic acid, gallic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), phorbal 12-myristate, (PMA) thiobarbituric acid (TBARS), ctyochrome C Horse heart type III, superoxide dismutase, catalase and ascorbic acid were purchased from Sigma Aldrich, USA. The salts of ferrozine, ferricchloride, potassiumferricyanide were obtained from SRL India. Trichloroacetic acid, density gradient HiSep LSM1084, RPMI culture media were obtained from HiMedia, India.

2.1. Engineering fibrous protein

The preparation of engineered fibrous protein performed according to the published protocol [8]. Briefly, the first step involves dissolution of fibrous protein. About 20 wt% fibrous protein was dissolved in HEPES buffer pH (6.5–8.0) at 50 °C under constant stirring. In the second step, the chosen phenolic acids, Caffeic (Ca), Gallic (Ga) and Protocathechuic (Pa) were dissolved separately in PBS buffer (pH 4.5–6.0) and mixed with EDC and NHS at 1:2:2 mol ratios under stirring for 45 min at 25 °C. The solutions obtained from step I and II were mixed and subjected to extensive dialysis against water containing1% NaCl. The final dialysis made against 0.1 M phosphate buffer (pH 6.5–7.5). The samples were then lyophilized and stored at 4 °C for further analyses.

2.2. Characterization

The samples obtained from the above said paragraph was subjected to UV–visible and FTIR instrumental analyses to confirm the tethering of phenolic acids to the protein backbone. UV–visible spectra for Protein, Caffeic (Ca), Gallic (Ga) and Protocathechuic (Pa), and the respective engineered products Ceg, Peg and Geg samples were recorded using UV–vis- 2450 (Shimadzu, Japan). In brief, 1 mg of each sample was dissolved separately in 1 ml of phosphate buffer (pH 6.5) and the spectrum was recorded in the wavelength region of 200–700 nm. With respect to FTIR spectral analysis, the samples were mixed with KBr and the pellet obtained upon hydraulic press subjected to FTIR analysis. All spectra were recorded at the resolution of 4 cm^{-1} and in the range of 400–4000 cm^{-1}.

2.3. Antioxidant profile of the phenolic acids tethered protein product

2.3.1. DPPH radical scavenging effect – spectrophotometric method

Free radical scavenging activity of the fibrous protein, phenolic acids and the engineered products obtained was measured using DPPH radical. In brief, solution of DPPH radical (1 mM/L) in ethanol was prepared. The experimental samples were mixed with DPPH radical and incubated for 30 min at room temperature [9]. The reduction in colour intensity measured at 570 nm using UV–visible spectrophotometer supplied by Shimadzu, Japan with respect to varied concentrations (75, 125, 225, 300 μg/ml) of the samples. The concentration of sample required to inhibit 50% of the DPPH radical was measured as IC_{50} using Log dose inhibition curve. The percent DPPH radical scavenging activity of the sample was calculated using the following equation:

$$\text{DPPH scavenging effect (\%) or Percent inhibition} = (A0 - A1)/A0 \times 100$$

Where, A0 was the Absorbance of control reaction and A1 was the Absorbance in presence of test or standard sample.

2.3.2. DPPH radical scavenging assay – EPR method

In addition to the spectrophotometric assay, DPPH radical scavenging activity was also measured by EPR (Electron paramagnetic resonance) (Bruker EMX EPR controlled spectrometer operating at X-band frequencies (9.5GH_Z)) as per the procedure described elsewhere, with slight modifications. DPPH radical at 3 mM concentration dissolved in ethanol was mixed with the samples chosen and measured the EPR spectrum using flat cell with the defined instrumental parameters: center field 3466G, sweep width 100G, microwave frequency 9.775 GH_Z, microwave power 3.196 mW, receiver gain 7.10×10^3, modulation frequency of 100 KH_Z, modulation amplitude 3G, temperature set to 298 K.

2.3.3. Examination of reducing power of the engineered protein samples

The reducing power of the samples assessed according to the method suggested by Oyaizu [10] using potassium ferricyanide. In brief, phenolic acids before and after tethering with protein were mixed with 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide, incubated at 50 °C for 20 min. The reaction was arrested using 2.5 ml of 10% trichloroacetic acid and centrifuged at 6500 RPM for 10 min. The supernatant was separated and diluted with deionized water and then mixed with 1 ml of 0.1% ferric chloride and measured the absorbance at 700 nm.

2.4. Induced oxidative stress management using engineered protein

2.4.1. Isolation of PMN cells

Polymorphonuclear cells (PMN) isolated according to the procedure summarized by Mosely et al. [11]. In brief, 10 ml of random blood samples were collected from each apparently healthy and nonsmoker human volunteers of age group 20–40 years from the Government Hospital, Taramani, Tamil Nadu, Chennai -20 after the informed consent. Followed by collection, blood samples were mixed with HiSep LSM (density gradient) solution in the ratio of 1:1 and allowed to stand for few minutes. The mixture was then centrifuged at 1000 rpm for 45 min at room temperature and carefully withdrawn the PMN cells and immediately transferred to clean conical bottom tubes. Equal volume of phosphate buffered saline (PBS) added to the collected PMN layers and centrifuged at 2300 RPM for 5 min at room temperature. The PMN pellet thus obtained was initially washed with PBS and then with RPMI-1640 medium.

2.4.2. UVB – irradiation

For UVB-irradiation, cells ((1×10^6) in 2 ml of PBS) in 35 mm petri dishes covered with a UV permeable membrane (transpire surgical tape # 1527-3) to prevent contamination and irradiated [UVB using a battery of TL 20 W/20 fluorescent tubes (Heber Scientific, India) in the wavelength range of 280–320 nm (maximum peak at 312 nm) with an intensity of 2.2 mW/cm^2 for 9 min] for 30 min. For the experimental groups, the cells were pre-exposed to the phenolic acids (0, 10, 20 μg) and then engineered product (100 and 400 μg) for the period of 60 min and then preceded for irradiation. Followed by irradiation for scheduled time period, cells