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In vitro wound healing and cytotoxic effects of sinigrin–phytosome complex

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ABSTRACT

Sinigrin is a class of glucosinolates found naturally in plants of the Brassicaceae family. Lately, studies have shown that sinigrin possesses anticancer, antibacterial and anti-inflammatory activities. Since its efficacy has not been explored on wound healing, we examined the effect of sinigrin on HaCaT cells. We also aimed at formulating sinigrin into phytosome to form a sinigrin–phytosome complex and study the wound healing and cytotoxic activities on A-375 and HaCaT cells. Sinigrin was efficiently formulated into the phytosome with an average particle size of 153 ± 39 nm, zeta potential of 10.09 ± 0.98 mV and complex efficiency of $69.5 \pm 5\%$. The formation of the sinigrin–phytosome complex was confirmed by DSC and FTIR analysis. The sinigrin–phytosome complex significantly exhibited wound healing effects when compared to sinigrin alone. After 42 h, the phytosome complex completely healed the wound, whereas sinigrin alone showed only 71% wound closure. The sinigrin–phytosome complex displayed minimal toxicity towards HaCaT cells and at higher concentrations, it showed potent activity towards A-375. The results indicated that sinigrin–phytosome complex augmented the therapeutic potential of sinigrin towards the wound healing activity and this approach should be explored further for cancerous wound treatment.

1. Introduction

A wound is any type of injury, scrape, bruise, burn, defect or a break in the skin arising from physical, chemical or thermal damage (Lazarus et al., 1994). Wounds are generally classified as acute or chronic. Acute wounds are generally tissue injuries which heal entirely, with little scarring and within a relatively short time period (generally 8 to 12 weeks) (Percival, 2002). The main causes of acute wounds involve mechanical injuries such as abrasions and torn skin, penetrating wounds caused by knives or as a result of surgery. Wound from burns and chemical injuries, which result from sources such as radiation, electricity, chemicals and thermal sources, are also acute wounds (Munday and Munday, 2002). Chronic wounds develop from tissue injuries and heal very slowly (beyond 12 weeks) and often relapse (Harding et al., 2002), this kind of wound does not heal due to physiological conditions such as prolonged infections, diabetes, malignancies and tumours (Moore et al., 2006). Healing of wound is a complex, dynamic and specific biological process associated with the phenomena of growth and tissue regeneration. The wound healing process consists of four overlapping stages: haemostasis, inflammation, proliferation and maturation. A healed wound is one that has returned to its normal anatomic structure, function and appearance (Boateng et al., 2008).

Sinigrin is one of the major glucosinolates found in plants of the Brassicaceae family, such as Brussels sprouts, broccoli and the seeds of black mustard (*Brassica nigra*) (Jie et al., 2014). Glucosinolates are a class of secondary metabolites that are characteristic of plants of the mustard family (Brassicaceae). Glucosinolates are broken down enzymatically by myrosinase, mainly to isothiocyanates, cyanides and thiocyanates which are the main bioactives known for biological activity (Chew, 1988). The metabolic activation of sinigrin results in the formation of isothiocyanates which are attributed to the anti-tumour effects (Krul et al., 2002; Munday and Munday, 2002). Sinigrin has been studied for its different biological activities such as anticancer (Lazarus et al., 1994; Rungapamestry et al., 2008a) antimicrobial (Shofran et al., 1998) and anti-inflammatory (Lee et al., 2014), but the wound healing effects of sinigrin are unknown. In the present study, we investigated the wound healing action of sinigrin on normal human keratinocytes cells.

It is possible to make phytoactives or plant extracts more effective by formulation it to increasing the bioavailability and resolve solubility issues, thus enhancing the biological profile or

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the therapeutic potential. In this study, we have decided to formulate sinigrin into phytosomes, also known as herbosomes, to increase the bioavailability and enhance the therapeutic efficacy. Phytosomes are formed by a process in which the plant extract or bioactives are bound to phospholipids, mainly phosphatidylcholine, making a lipid compatible molecular complex. The water-soluble phytoconstituents are converted into lipid-compatible molecular complexes (Raju et al., 2011). The lipid phase substances employed to make phytoconstituents lipid compatible are phospholipids, generally phosphatidylcholine. In the last few years phytosome technology has achieved prominence in the area of pharmaceuticals, cosmeceuticals and nutraceuticals for its preparations and formulations in the form of powder, granules, tablets, gels, emulsions, lotions, solutions and capsules. Since phytosomes exhibit a chemical bonding between phospholipid molecules and phytoconstituents, they are known for their greater stability. Apart from behaving as a carrier, phosphatidylcholine also have several therapeutic properties giving it a synergistic effect when used for the formation of phytosomes. Phospholipid molecules represent an important vehicle made up of a water soluble head and two fat soluble tails and due to this; they acquire dual solubility and thus behave as effective emulsifiers (Singh et al., 2014). Phospholipid molecules have emerged as a potential vehicle system for improving the bioavailability of poorly absorbed extracts or phytoconstitutents due to their distinctive structural components, which are equivalent to the lipid content of the mammalian cell membrane making them compatible with the physiological system (Virtanen et al., 1998).

Phosphatidylcholine acts as a dual functional compound, the phosphatidyl moiety being lipophillic and the choline moiety being hydrophilic in nature. The choline head of the phosphatidylcholine molecule attaches to the compound and the lipid soluble phosphatidyl portion, comprising the body and tail, which then sheaths the choline-bound material. Thus the phytoactive constituents produce a lipid compatible molecular complex with phospholipids, which is also known as phytophospholipid complex (More et al., 2012). Substantial efforts have been made by researchers to explore phytosome technology and studies have indicated improved bioavailability and therapeutic activity of the phytosome complexes such as anticancer, antioxidant, anti-inflammatory, anti-wrinkles, anti-aging and wound healing, skin disorders, etc. Hence, the compositions of phytosomes are relatively safe and are recommended for pharmaceutical and cosmeceutical use. Phytosome complexes also increase the permeability of the bioactive across the biological membranes.

To date, the wound healing effects of sinigrin have not been explored yet. It was also of interest to determine whether sinigrin formulated in phytosome, could enhance the wound healing activity of sinigrin. Hence we endeavoured to prepare sinigrin–phytosome complex (phytolipid vesicle) and examined its wound healing activity, along with sinigrin alone, on HaCaT cells. The cytotoxic effect was also studied on melanoma cells (A-375) and normal human keratinocytes (HaCaT).

2. Materials and methods

Sinigrin (purity ≥ 98) was purchased from Santa Cruz Biotechnology, Germany. L-α-phosphatidylcholine hydrogenated (soya bean) (≥99%) was obtained from Sigma–Aldrich, USA. Methylthiazoletetrazolium [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Life Technologies (USA). Dimethyl sulfoxide (DMSO), phosphate buffer solution (PBS) tablets and 0.4% trypan blue solution were obtained from Sigma–Aldrich (USA). Solvents such as dichloromethane (DCM) and *n*-hexane were purchased from Sigma–Aldrich, USA. Cytoselect™ wound healing and cell migration kits were procured from Cell Biolabs, Inc., USA.

2.1. Preparation of sinigrin–phytosome complex

Phosphatidylcholine was mixed with sinigrin, in a ratio of 1:1 (w/w), with 5 ml of DCM and stirred well while evaporating the DCM. Once the DCM was completely removed, 5 ml of *n*-hexane was added to the thin film, stirred well and then left open in the fume hood for the complete removal of the solvent. After the *n*-hexane was completely removed, the thin film was hydrated and probe sonicated (Hielscher UP 200ST ultrasonic device, South Africa) in order to obtain desired size phytosome complex.

2.2. High performance liquid chromatographic analysis of sinigrin

The method for the analysis of sinigrin was developed and validated according to ICH guidelines (ICH, 2005). The HPLC (Agilent 1100 series, Agilent, Palo Alto, USA) system comprised of an auto-sampler, isocratic pump and a variable wavelength detector (VWD). For analytical separation, a chromatographic column (Venusil XBP C_{18}, 5 μm, 150 × 4.6 mm, Agela Technologies, Newark, USA) was used. The mobile phase consisted of a mixture of tetrabutyl ammonium bisulfate ($TBASO_4$) and acetonitrile (80:20, v/v). $TBASO_4$ was prepared by dissolving it in deionised water (1.7 g/l) and adjusting the pH to 7.3 ± 02. The flow rate was set at 1.0 ml/min, along with an injection volume of 10 μl and UV detection at 228 nm.

2.3. Particle size and zeta potential

The particle size and zeta potential of the phytosome vesicles were determined by the dynamic light scattering (DLS) technique (Malvern Zeta Sizer, Malvern Instruments, Malvern, UK), at a scattering angle of 90° at 25 °C. Samples of the prepared phytolipid vesicles were diluted with the deionised water and measured in disposable polystyrene cuvettes at 25 °C. To examine the stability of the sinigrin–phytosome complex, it was lyophilised and stored at room temperature. The phytosome complex was tested for its particle sizes and zeta potential and an HPLC analysis was performed at regular intervals (0, 1, 2 and 3 months).

2.4. Transmission electron microscopy

Morphological examination of phytosome vesicles was done with transmission electron microscopy (TEM) (model FEI Tecnai™ G2, USA), at an accelerating voltage of 120 kV, with images captured with a GATAN bottom mount camera using digital micrographics. Samples were diluted with distilled water, and then a drop of the phospholipid vesicles was placed onto a copper grid, leaving a thin liquid film. The films on the grid were stained with 1% osmium tetroxide, excess staining solution was removed with the help of filter paper, and it was left to dry for about 15 min. The stained films were observed and photographed with the TEM.

2.5. Complex formation efficiency

Free sinigrin and its phospholipid complex dissolve well in methanol, therefore the original content of the sinigrin can be determined by dissolving amounts of sinigrin–phytosome complex in methanol and measuring the content of the sinigrin dissolved in methanol by HPLC. The same amount of sinigrin–phytosome complex was dissolved in chloroform and the free sinigrin, precipitated as it was insoluble in chloroform. The precipitates were removed by filtration and the content of the sinigrin dissolved in chloroform *i.e.* combined sinigrin was