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Carvacrol/clay hybrids loaded into in situ gelling films

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ABSTRACT

The aim of the present work was the development of polymer films loaded with a carvacrol (CVR)/clay hybrid (HYBD) for the delivery of CRV in infected skin ulcer treatment. Different clays were considered: montmorrilonite, halloysite and palygorskite (PHC). CRV incorporation in PHC reduced its volatility. HYBD showed 20% w/w CRV loading capacity and was able to preserve CRV antioxidant properties. HYBD was characterized by improved antimicrobial properties against *S. aureus* and *E. coli* and cytocompatibility towards human fibroblasts with respect to pure CRV.

Films were prepared by casting an aqueous dispersion containing poly(vinylalcohol) (PVA), poly (vinylpyrrolidone) (PVP), chitosan glutamate (CS), sericin and HYBD. Optimization of film composition was supported by a Design of Experiments (DoE) approach. In a screening phase, a full factorial design (FFD) was used and the following factors were investigated at two levels: PVA (12–14% w/w), PVP (2–4% w/w) and CS (0.134–0.5% w/w) concentrations. For the optimization phase, FFD was expanded to a "central composite design". The response variables considered were: elongation, tensile strength and buffer absorption of films, durability of the gels formed after film hydration. Upon hydration, the optimized film formed a viscoelastic gel able to protect the lesion area and to modulate CRV release.

1. Introduction

Chronic wounds, such as diabetic foot ulcers, pressure ulcers and venous leg ulcers represent a worldwide health problem (Boateng et al., 2008; Boateng and Catanzano, 2015). Advanced therapeutic dressings, designed to take an active role in the wound healing process, represent an interesting approach in the treatment of chronic wounds. Their biological properties are related not only to the bioactive agents loaded into dressing, but also to the presence of biomaterials able to improve tissue repair (Boateng and Catanzano, 2015). Among these biomaterials, chitosan, a linear polysaccharide obtained by chitin deacetylation, has been recognized as a biopolymer able to promote tissue repair and to avoid the onset of infections (Muzzarelli, 2009; Rossi et al., 2010, 2013; Mori et al., 2016; Tenci et al., 2016).

The association of synthetic and natural polymers in composite dressings has been recognized useful to control drug delivery in the wound site (Boateng et al., 2008). Among synthetic polymers, poly(vinylalcohol) (PVA) and poly(vinylpyrrolidone) (PVP) have been largely employed for biomedical applications, such as controlled release systems and tissue engineering (Li et al., 2010; Vicentini et al., 2010).

Carvacrol (CRV), a monoterpene phenolic compound, is the major component (up to 80%) of oregano essential oil (EO) (*Origanum vulgare)* (Burt, 2004). It possesses antioxidant, antifungal and antimicrobial properties (Ben Arfa et al., 2006; Safaei-Ghomi et al., 2009; Tunç et al., 2011).

Different strategies were proposed to reduce evaporation of EOs. One of these provides the inclusion of EOs in montmorrilonite (MMT) and halloysite (HAL). Recently, some authors proposed the use of such clays as packaging materials and demonstrated their capability to enhance the thermal stability and to preserve the antimicrobial properties of EOs (Efrati et al., 2014; Shemesh et al., 2015). Gorrasi (2015) proved that packaging hybrid/polymer films were able to control EOs release.

So far, to the best of our knowledge, no papers have been published on the use of EO/clay hybrids loaded into films for cutaneous application. Clays generally employed in the

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pharmaceutical and cosmetic fields belong to smectites, kaolin and fibrous clay groups (López-Galindo et al., 2007; Viseras et al., 2008, 2010; Sanchez-Espejo et al., 2014).

Given these premises, the aim of the present work was the preparation of CVR/clay hybrids to be loaded into gelling viscoelastic films for the treatment of infected skin lesions. Hybrids should prevent CVR evaporation and maintain its antioxidant and antimicrobial properties. Three different commercial clays have been considered: MMT, HAL and palygorskite (PHC).

In a first phase of the research, CVR and CVR/clay hybrids were prepared by using two different approaches: adsorption in saturated atmosphere and shear mixing. Hybrids were subjected to thermal analysis in order to study the effect of clay type and of preparation method on CRV volatility. On the basis of the results obtained, clay and preparation method which allowed the highest CRV loading were chosen for the continuation of the research. The chosen CRV/clay hybrid (HYBD) was investigated *in vitro* for cell viability and antioxidant activity on human fibroblasts and for antimicrobial properties on *S. aureus* and *E. coli*, in comparison with pure CRV.

A second phase of the research was devoted to the preparation and optimization of films to be used as vehicle for HYBD. Films should be characterized by suitable mechanical properties (high flexibility and resistance to rupture) and by the capability to absorb wound exudate forming a viscoelastic persistent gel, able to protect the lesion area without impairing CRV release.

Films were composed by poly(vinylalcohol) (PVA), poly(vinylpirrolidone) (PVP), chitosan glutamate (CS), sericin (SER) and glycerol. PVA was used for its excellent film-forming capacity (Guo et al., 2011; Comolli et al., 2012). PVP was combined with PVA as a controller of mechanical properties because this polymer had good aqueous solubility and extremely low cytotoxicity (Contardi et al., 2017; Sreedharan and Sujith, 2015; Seabra and De Oliveira, 2004; Singh and Pal, 2011). CS was chosen for its capability to enhance wound healing (Rossi et al., 2015). Sericin (SER) was added to improve HYBD antioxidant properties (Mori et al., 2016), while glycerol was used as plasticizing agent. To obtain films of optimized composition, a DoE approach was employed. The experimental design provided a screening and an optimization phase. In the screening phase a full factorial design (FFD) was used not only to investigate the effect of each factor (PVA, PVP and CS concentrations) on the response variables considered (flexibility, mechanical strength, hydration capability of films and gel durability), but also to individuate the main influencing factors. To find the optimal formulation, the screening design was expanded to a central composite design (CDD) (Dejaegher and Vander Heyden, 2011). The experimental results characterizing the optimized formulation were compared to those predicted by the model in order to confirm its predictive power. Moreover, film of optimized composition was subjected to rheological (viscoelastic) characterization upon hydration in a medium mimicking wound exudate and to *in vitro* CRV release measures.

2. Materials and methods

2.1. Materials

The following materials were used: antibiotic/antimycotic solution (100×), containing 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B (Sigma Aldrich, Milan, I); carvacrol (natural, 99%, FG; CRV) (Sigma Aldrich, Milan, I); chitosan low MW (CS) (DD: 80%) (Sigma Aldrich, Milan, I); dimethyl sulfoxide (DMSO) (Sigma Aldrich, Milan, I); Dulbecco's Modified Eagles Medium (DMEM) (Lonza, BioWhittaker, Walkersville, MD, USA); Dulbecco's Phosphate Buffer Solution (Sigma Aldrich, Milan, I); glutamic acid (Sigma Aldrich, Milan, I); glycerol (Carlo Erba, Milan I); Hank's balanced salt solution (HBSS) (Sigma Aldrich, Milan, I); inactivated foetal calf serum (Biowest, Nuaillé, F); hydrogen peroxide (Carlo Erba, Milan, I); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, Milan, I); $NaH_2PO_4 \cdot H_2O$ (Carlo Erba, Milan I); $Na_2HPO_4 \cdot H_2O$ (Carlo Erba, Milan, I); NaCl (Carlo Erba, Milan, I); *n*-hexane (Carlo Erba, Milan I); Pharmasorb© colloidal (Palygorskite, PHC) (BASF Aktiengesellschaft, Ludwigshafen, G); poly(vinylalcohol) (PVA) (Sigma Aldrich, Milan, I); poly(vinylpyrrolidone) K90 (PVP) (BASF Aktiengesellschaft, Ludwigshafen, G); trypan blue solution (Biological Industries, Beit-Haemek, IL); trypsin–EDTA solution (Sigma Aldrich, Milan, I); Veegum© HS (montmorillonite, MMT) (Vanderbilt Minerals, LLC, Norwalk, USA); Halloysite (HAL) (Sigma Aldrich, Madrid, S).

2.2. Preparation of CVR/clay hybrids

Three different clays, halloysite (HAL), montmorrilonite (MMT) and a commercial palygorskite (PHC) were considered. CVR/clay hybrids were prepared according to two different methods: adsorption in saturation conditions and shear mixing.

The first method provided to create an ambient saturated with CVR. To this aim, a beaker containing 10 ml of CVR was put in the middle of an hermetically sealed glass chamber (489 cm^3 volume). 50 mg of each clay was layered on a watch glass inside of the chamber. The chamber was thermostatated at a constant temperature (20, 40, 60, 80 and 120 °C) for a fixed time period (2 or 5 days).

Shear mixing technique provided to disperse the clay in CVR (2:1 w/w ratio) by ultra-sonication at room temperature for 1 h (Shemesh et al., 2015). CVR not adsorbed onto clay was removed by centrifugation (4218 centrifuge, ALC International s.r.l., Milan, I) at 1000*g* for 15 min and by evaporation in oven (Vismara Laseleletronics s.r.l., Lodi, I) for 24 h at 80 °C.

To assess CRV loading, CVR/clay hybrids were subjected to thermogravimetric analysis (TGA). Pure CVR and clays were used as references. TGA analysis was carried out by a Shimadzu mod. TGA-50H apparatus, in the temperature range 36–950 °C at a heating rate of 10 °C/min. The results were expressed as% mass loss as a function of temperature. From the comparison of TGA profiles of hybrids with those of pure CVR and clays, % CRV loading into hybrids was calculated (% LC) (see Eq. (1)).

$$\%LC = \frac{\text{CVR loaded (mg)}}{\text{hybrid (mg)}} \times 100 \quad (1)$$

TGA analysis was also used to assess HYBD stability upon storage for 1 month at 20 °C in a desiccator.

To confirm the data obtained from TGA analysis, the amount of CRV loaded into HYBD was assessed by an extraction method. In particular, 2 mg of HYBD was weighted and added to 10 ml *n*-hexane (Tunç and Duman, 2011). The dispersion was maintained under stirring overnight, centrifuged at 3000 rpm for 20 min and then filtered (cellulose acetate, 0.45 μm; Sartorius, Muggiò, I). The supernatant was recovered and analyzed for CRV content by means of a UV–vis spectrophotometer (Perkin Elmer, Lambda 25) at wavelengths ranging from 200 to 500 nm. A calibration curve was obtained using CRV hexane solutions having the following concentrations: 50, 25, 20, 10 and 5 μg/ml.

2.3In vitro *cytocompatibility assessment of CRV and HYBD*

NHDF (normal human dermal fibroblasts from foreskin) (Promocell GmbH, Heidelberg, G) 6th to 16th passage were used.