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NEW GALLIC ACID BASED HYDROGEL FOR PHLORETIN INTESTINAL RELEASE

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ABSTRACT

The present work aims to realizeahydrogelbased ongallic acid, a molecule with antioxidant and antidiabetic activity.The compound has in its structure two different functional groups, carboxyl and hydroxyl, susceptible to derivatization. Due to these characteristics, was developeda hydrogel, potentially useful for oral release of phloretin, a flavonoid of natural origin, found in apples and pears, with glucose transporter (GLUT) inhibitory activity. The obtained gallatehydrogel was characterized by Fourier Transform Infrared spectroscopy (FT-IR). Its equilibrium swelling degree (a%) and the phloretinrelease were evaluated in simulating gastric and intestinal fluids. The antioxidant activity in inhibiting lipid peroxidation, in rat-liver microsomal membranes, induced in vitro by a source of free radicals, was assessed after exposure of gallate hydrogel containing phloretin to gastrointestinal environmental conditions.The results showed that the gallate hydrogel could be successfully applied in pharmaceutical field for phloretinintestinal releaseand maintenance of an appropriate blood glucose level in the diabetic patient.

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1. INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by a congenital(type I insulin-dependent) or an acquired (type IInon-insulin-dependent) inability to transportglucose from the blood to the cells. The chronic hyperglycemia, due to a deficiency of insulin secretion or insulin resistance,[1]leads to glucose toxicity and is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes,

kidneys, nerves, heart, and blood vessels.[2-4]Consequently it is necessary, in particular, in diabetic patient,the maintenance of an appropriate blood glucose level in postprandial state.[5]Carbohydrates in the diet are hydrolyzed by digestive enzymes and thencleaved into monosaccharides, these can be absorbed from the small intestine via influx hexose transporters.[6,7]

There are two type of transporter: Na+-dependent glucose transporter (SGLT1), located at the brush border membrane (BBM), that mediates the uptake of glucose into the cell against its concentration gradient. The other type of transporter GLUT2, transports sugars across the basolateral membrane to the blood.[8]Literature data suggest that GLUT2 can also be found on to the BBM in the presence of glucose in the lumen[9] and can contributeat the glucose absorption process.[10]Thus, these glucose transporters might be an attractive therapeutic target for diabetes[11].
In this work,wasdeveloped a hydrogel for intestinal release of phloretin.

A hydrogel is a three-dimensional, water-swollen structure composed of hydrophilic polymers. This network attains physical integrity and is made insoluble due to the presence of chemical and/or physical crosslinks. [12-14] In addition it is offers excellent potential as oral therapeutic systems due to inherent biocompatibility, diversity of both natural and synthetic material options and tunable properties. In particular, stimuli-responsive hydrogels exploit physiological changes along the intestinal tract to achieve site-specific and controlled release of protein, peptide and chemotherapeutic molecules for local and systemic treatment applications.[15]In this context,was obtained a hydrogel based on gallic acid, a polyphenolic compound commonly distributed in plant-derived foods, such as cereals, legumes, nuts, vegetables, fruits, and in beverages such as green or black tea, wine, fruit juice, beerand etc.[16,17]Literature data proved antidiabetic and antioxidant properties of gallic acid. [18]So the polyphenolic compound was chosen for hydrogel development, both for its properties, both for its chemical structure: in fact,it possessestwo different functional groups, carboxyl and hydroxyl, susceptible to derivatizationand so able to provide polymerizable derivatives.[19]The compound entrapped in ourgallate hydrogel is the phloretin (Figure 1).

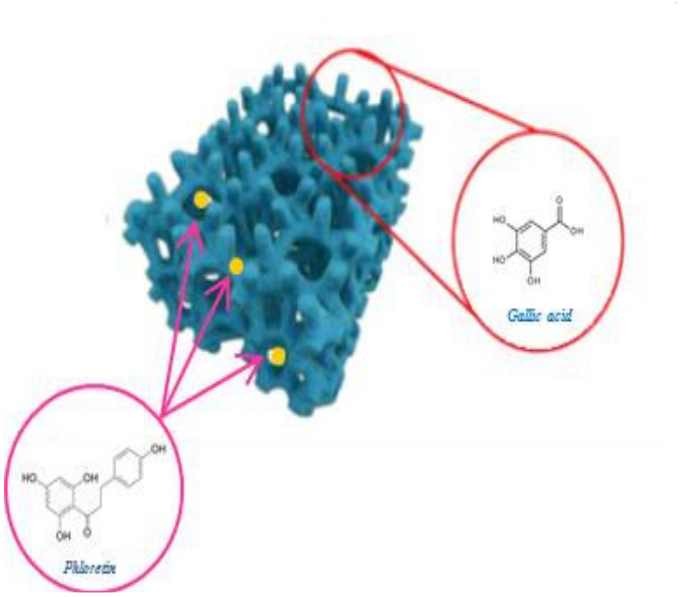


Figure 1

It is a dihydrochalcone flavonoid, found in apples and other fruits,that displays also a potent antioxidant activity in peroxynitrite scavenging and the inhibition of lipid peroxidation.[20]It is known to inhibit the protein kinase C[21-24]and human leukemia cell growth.[25]Literature data suggest that phloretindecreases the glucose absorptionimpeding its intestinal transport mediated by GLUT2.[26,27]On the other hand, the phloretin, as well as other flavonoids,can be easily modified by environmental factors such as temperature, pH and light.[28] These compounds, in fact, are poorly adsorbed in the intestine because are, probably, degraded by intestinal microorganism and/or enzymes, producing different metabolites.For these reasonsflavonoids, when administered through delivery systems, show an improved stability and absorption profile[28] and, consequently,their activity becomes enhanced, more detectable and prolonged.[28] Therefore, our gallate hydrogel has been formulated specifically

tofacilitate the phloretinintestinal release, protect it from chemical degradation in gastrointestinal tract, enhancing its bioavailability.[19,28]

The obtained hydrogel was characterized by Fourier Transform Infrared spectroscopy (FT-IR). Its equilibrium swelling degree (a%) was also evaluated in simulating gastric and intestinal fluids. The phloretinrelease from hydrogel was carried out in the same condition of swelling studies. The antioxidant activity in inhibiting lipid peroxidation, in rat-liver microsomal membranes, induced *in vitro* by a source of free radicals, such as *tert*-butyl hydroperoxide (*t*-BOOH), was assessed after exposure of gallate hydrogel, containing phloretin, to gastrointestinal environmental conditions. The results demonstrate that this hydrogel, could be potentially useful for the maintenance of an appropriate blood glucose level, in particular, in the diabetic patient.

2. Materials and Methods

2.1 Reagents

Acetone, hydrochloric acid, chloroform, diethyl ether, ethanol, isopropanol, methanol, n-hexane, tetrahydrofuran (THF), allyl alcohol and sodium sulfate were purchased from Carlo Erba Reagents (Milan, Italy). Gallic acid (MW = 170.12), phloretin (MW = 274.27), acrylic acid (MW = 72.06, d = 1051 g / ml), dicyclohexylcarbodiimide (DCC), N,N-dimethylaminopyridine (DMAP), potassium tert-butoxide, N,N-dimethylacrylamide (DMAA), ammonium persulfate (NH_4) $2S_2O_8$, N,N'-methylene-bis-acrylamide, sorbitantrioleate (Span85), polyoxymethylenesorbitantrioleate (Tween 85), N,N,N',N'-tetramethyl-ethylenediamine(TMEDA), tert-butylhydroperoxide (*t*-BOOH), trichloroacetic acid (TCA) acid, 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (Sigma Chemical Co, St Louis, MO, USA).

2.2 Instrument

The FT-IR spectra were performed using a spectrometer Perkin Elmer 1720. ^{1}H-NMR spectra were obtained by the use of a spectrophotometer Burker VM30; the chemical shifts were expressed as δ and referring to the solvent. The structures of the compounds obtained were confirmed by mass spectrophotometry using a Hewlett Packard instrument GM-MS 5972. The UV-VIS spectra have been realized by means of UV-530 spectrophotometer JASCO. The samples were lyophilized using "Freezing-drying" Micro Modulyo, Edwards. Dimensional analysis of the microspheres prepared were carried out by means of light scattering using a Brookhaven 90 Plus Particle Size Analyzer.

2.3 Acrylation of 3,4,5-trihydroxybenzoic acid with 2-propenoic acid

Acrylic acid (0.14 ml, $2 \cdot 10^{-3}$ mole) was dissolved in dry THF. Then DCC (0.42 g, 2×10^{-3}mol) and DMAP (0.05 g, 4×10^{-4}mol) were added and the reaction mixture was kept under stirringat 50 °C for 1h. After that, gallic acid (1g, 5.8×10^{-3} moles) was added to the solution. The reaction waskept under magnetic stirring at 50 °C for other 72h and monitored by thin layer chromatography (TLC/silica gel, eluent mixture: chloroform-methanol 7:3). The dicyclohexylurea (DCU), formed during the reaction, was eliminated by filtration. The solvent reaction was removed by evaporation at reduced pressure. The obtained product, witha gelatinous consistency and yellowcolor, was purified throughsilica gel column chromatography (eluent:chloroform). The purified product**(1)** has been characterized by FT-IR, GC-MS and ^{1}H-NMR.

2.4 Preparation of the hydrogel based on diacrylategallic acid

The acrylated derivative of 3,4,5-trihydroxybenzoic acid was solubilized anaqueous solution of NH_3/urea. Subsequently, DMAA (0.035 g, 0.037 ml, 3.5×10^{-4}mol) and (NH_4) $2S_2O_8$ (0.8 g, 3.5×10^{-3} moles)were added. The reaction mixture was heated to 60 °C and left under magnetic stirring until the formation of the hydrogel (2.5 g) which was subsequently washed with distilled water, frozen, lyophilized and characterized by FT-IR and SEM.

2.5 Evaluation of the antioxidant activity

The ability of prepared hydrogel, containing and not containingphloretin, to protect against lipid peroxidationinduced by *tert*-BOOH, was examined in rat liver microsomal membranes during 120 min of incubation andafter hydrogel exposure to gastrointestinal environmental conditions. Aliquots of hydrogel were added to the microsomal suspension. The suspension was then incubated at 37°C in a shaking bath under air in the dark. After incubation, the thiobarbituric acid–malondialdehyde complex (TBA–MDA) formation was monitored by the use of UV–Vis spectrophotometry at 535 nm.[29-30] The experiment was repeated in triplicate (n= 3).

2.9 Swelling studies

The swelling behavior of hydrogel, evaluated in accordance with a procedure reported in literature, [31] was evaluated at two different pHs, which simulated the conditions typical of the gastro-intestinal tract. In particular aliquots of the hydrogel of 0.05 g were placed in glass filters, previously weighed, and immersed in beakers containing buffer solutions of different pHs (1.2 to mimic the acid environment of the stomach and phosphate buffer at 7.4 to mimic the small intestine ones). At predetermined time intervals (1h, 2h, 3h, 6h, 12h, 24h), the excess water has been removed from the filters through percolation. Subsequently, the filters were centrifuged at 8000 rpm for 5 minutes and weighed. The weights, measured at all intervals time,have been used to calculate the hydrogel swelling degree. The swelling was evaluated for the first two hours at pH 1.2and then the pH was adjusted to 7.4 and at this pH the swelling degree was estimated from the third hour onwards.

2.10Release studies

Dried and loaded hydrogel (0.031 g) was placed in flasks containing two solutions at analogous pH conditions used for the swelling tests. The flasks were put in a waterbath at 37 °C under stirring.At predetermined time intervals (1h, 2h, 3h, 6h, 12h, 24h), the samples were centrifuged, 5 ml of supernatant were removed and the medium was replaced with fresh solution to maintain the same total volume throughout the study. The phloretin concentration in the different solutions wasmonitored by the use of UV-Vis spectrophotometry at 288nm. As well as for swelling studies, the releasewas evaluated at pH 1.2 only for the first two hours, while the release at 7.4 was performed by the third hour onwards. The data were calculated in terms of drug release percentage.

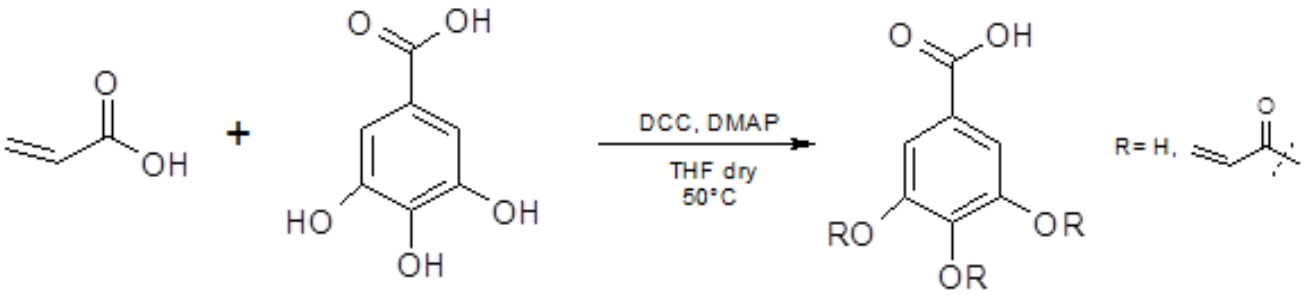
3. Results and discussions

Many literature data, concerning the polymers as drug delivery systems, show an increasing interest in respect ofbiocompatible and site-specific materials exploiting the possibility of combine the controlled release of the drug with it protection from possible degradation causes like light, pH temperature etc. This work fits in this context and had as its purpose the design and synthesis of a gallate hydrogel useful for phloretin intestinal release and maintenance of an appropriate blood glucose level in the diabetic patient. The starting material is gallic acid, organic acid widely present in the plant kingdom, which presents in its structure a carboxyl group and three hydroxyl groups, all susceptible to derivatization and therefore able to provide various derivatives polymerizable. Therefore, through anacrylation reaction has been possible to obtain a diacrylatederivative of gallic acid which was used for the preparation of a hydrogel.

3.1Acrilation of 3,4,5-trihydroxybenzoic acid with 2-propenoic acid

Objective of this reaction has been to synthesize the diacrylatederivative of gallic acid. This reaction provides for the use of DCC (dicyclohexylcarbodiimide) condensing agent, that reacting with the carbonyl group of acrylic acid,allowed the formation of an adduct which subsequently underwent nucleophilic attack by the hydroxyl groups of gallic acid. The DMAP (N,N-dimethylaminopyridine), has also been used as

nucleophilic activatorbecause,deprotonating the hydroxyl group of gallic acid, permitted the alcoholateformation that reacted with the electrophile carbonyl of acrylic acid.In addition,the DMAP, actingas a base, preventing the pH lowering (Scheme 1). The dicyclohexylurea (DCU) that was formed during the reaction was eliminated by treating the product with hot methanol and filtering it. The product was dried under reduced pressure, purified by column chromatography and characterized by FT-IR, ^{1}H-NMR and GC-MS. FT-IR (KBr) ν (cm^{-1}): 3324 (-OH), 3261 (-OH), 3034 (-CH), 1780 (-C = O), 1739 (-C = O), 1710 (- C = O), 1626 (-C = C), 1261 (-CO), 985 (-CH), 905 (-CH). M / Z: 205 (100%), 277 (4%). ^{1}H-NMR (CD_3OD) δ (ppm): 5.916 (2H, dd), 6.378 (2H, dd), 6.675 (2H, dd), 7.651 (2H, d). Yield 70%.



Scheme 1

3.2 Preparation of the hydrogel based on gallic acid diacrylate

The gallic acid diacrylatedwas solubilized in 2.5 ml of an aqueous solution of NH_3/urea and the comonomerN,N-dimethylacrylamide and the radical initiator ammonium persulfate were added. The obtained solution was kept under stirring and warm (60 ° C) up to formation of the hydrogel. This latter, washed with distilled water, frozen and freeze-dried and has been thoroughly characterized by FT-IR that showed the complete disappearance of the bands typical of the acrylic group at 985 cm^{-1} and 905 cm^{-1} and the appearance of a new band of stretching carbonyl at 1640 cm^{-1}.

3.3 Evaluation of the antioxidant

The ability of the gallatehydrogel,loaded and not loaded with phloretin, to inhibit lipid peroxidationinduced by the *t*-BOOH, a source of free radicals, was examined in microsomal membranes of rat liver over a period of 120 minutes of incubation. Both hydrogels were previously exposed for two hours to intestinal environmental conditions (pH 7.4) to verify the preservation of the antioxidant activity ofgallic acid-based hydrogel. The hydrogels antioxidant activity was preserved in the time as shown in the Figure 2. In addition the higher efficiency of loaded hydrogel respect to not loaded one, is probably due to the synergic action of gallic acid and phloretin.

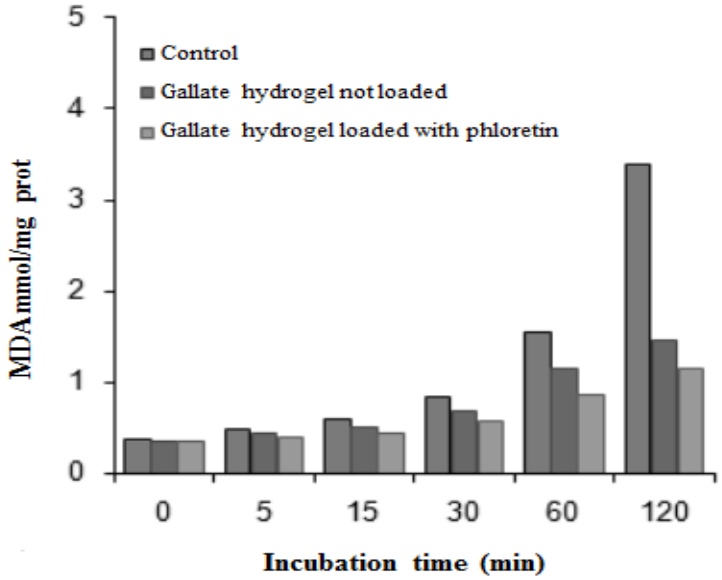


Figure 2

3.4Swelling studies

The swelling degree (% α) of thegallate hydrogel has been evaluated at to two different pHs (1.2 and 7.4) and at predetermined time intervals (1h, 2h, 3h, 6h, 12h, 24h). The degree of swelling was calculated by the equation 1:

$$\alpha(\%) = \frac{(Ws-Wd)}{Ws} \times 100 \text{(Eq. 1)}$$

where Ws is the weight of the polymer swellatedand Wd is the weight of the initial polymer (dry). The Table 1 shows the values of α% at pHs 1.2 and 7.4 and different time intervals.

Time (h)	α% pH 1.2	α% pH 7.4
1	*67%*	-
2	*118%*	-
3	-	*540%*
6	-	*779%*
12	-	*938%*
24	-	*1060%*

Table 1

The obtained data showed the applicability of this hydrogel forphloretinintestinal specific release.

3.5 Impregnation of the hydrogel with phloretin

The hydrogel wasloaded with phloretinusing a drug solution water/ethanol 8/2. The whole was left under constant stirring at 37 ° C in a waterbath for 72 h. The solution was analyzed, after filtration, by UV-VIS (λ = 288nm, ε = 3201.4 mol • dm^{-1} • cm^{-1}). This allowed us to calculate the drug loading efficiency (LE%) of hydrogel through the following equation (Eq. 2):

$$LE\% = \frac{Mi-M0}{Mi} \times 100 \text{(Eq.2)}$$

were Mi and M0 are respectively the mass of the drug in solution before and after impregnation. The hydrogel efficiency of loading was found to be equal to 80% .

3.6 Release studies from hydrogel

The release studies were conducted on fixed aliquots ofhydrogel (0.031 g) at two different pH (1.2, and 7.4), which mimic the conditions of the gastro-intestinal tract, and at different time intervals (1h, 2h, 3h, 6h, 12h, 24h) through the use of a waterbath at 37 ° C and under stirring. The results of release tests revealed that the hydrogel effectively released the drug at higher percentages at pH 7.4 (Figure 3).

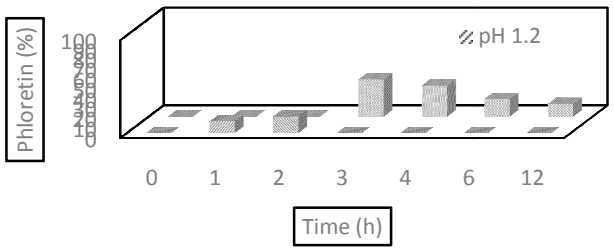


Figure 3

Thus, these data allow to assume an oral administration and the use of this material as a vehicle of phloretinin the intestine.

4. CONCLUSIONS

The aim of this work was the inclusion of phloretin, a natural dihydrochalcone flavonoid with multiple properties including antidiabetic and antioxidant, in a hydrogel based on gallic acidformulated specifically to facilitate the phloretin intestinal release and protect it from chemical degradation in gastrointestinal tract, enhancing its bioavailability. The swelling degree of the hydrogel not containing the phloretin, was carried out attwo different pH and predetermined time intervals. In particular, acidic solutions of HCl at pH 1.2 to mimic the pH of the stomach and 7.4 to mimic the pH of the small intestine, have been used. The obtained results revealed that the gallate hydrogel swells more at pH 7.4. Furthermore,the hydrogel was subjected to release studies in the same conditions of swelling tests.Theresultshave shown the ability of hydrogel to release the drug at pH 7.4. Finally, the hydrogel was also submitted to evaluation of the antioxidant activity. These studies revealed that the antioxidant activity of the prepared materials is time-dependent and is preserved over the time. The obtained data show that the hydrogel based on gallic acid could be used for the controlled release of phloretin through the gastro-intestinal tract, particularly in the treatment of diabetes.

Declaration of interest

None declared

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