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Improvement of Chemical and Physical Properties and Antioxidant Evaluation of Eugenol – PEG adduct

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Eugenol (EU) – PEG adduct was synthesized to improve the chemical and physical properties of eugenol. The phenolic group was covalently bound to the carboxyl group of PEG and the release kinetics were studied *in vitro* in buffer solution at pH 7.4, in simulated gastric fluid and in mouse plasma. Studies *in vitro* on the release of the parent drug from the prodrug in various media indicate that the adduct may be sufficiently stable to pass intact into the gastrointestinal tract and release EU into the circulation. The antioxidant activity of PEG-EU adduct was also evaluated. Scavenging activity was absent in the original PEG-EU adduct but gradually increased on the basis of drug delivery.

Keywords: Eugenol, PEG, Drug delivery, Antioxidant activity.

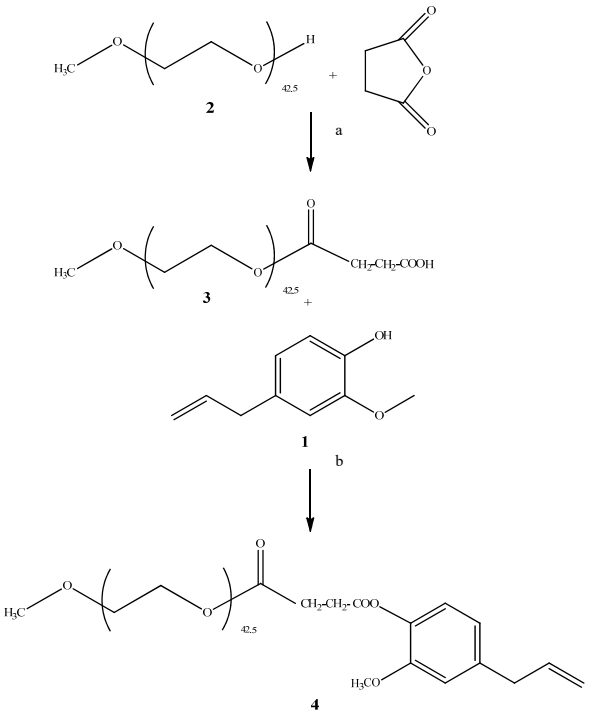
Eugenol ($C_{10}H_{12}O_2$) is an allyl chain-substituted guaiacol (**1**), weakly acidic, slightly soluble in water and soluble in organic solvents. It is the major volatile constituent of clove essential oil obtained through hydrodistillation of mainly *Eugenia caryophyllata* (*Syzygium aromaticum*) buds and leaves [1]. In biological applications, eugenol was widely used in dentistry, and as a flavoring agent in cosmetic and food products since eugenol was recognized as a safe material by the US Food and Drug Administration (FDA) [2]. Recently, eugenol has attracted attention because of its pharmacological properties including anti-inflammatory and anticancer effects, as well as anti-oxidant activity due to the presence of its phenolic group [3]. However, different limitations are associated with eugenol such as low solubility, degradation, adverse effects and a pungent taste which make it unsuitable to use as such.

It is well known that the creation of poly(ethylene) glycol (PEG) prodrugs that are minimally or partially active could potentially make it possible to administer higher doses and so achieve a longer duration of action of compounds that have toxicities related to C_{max} in their fully active form [4]. Besides, PEG appears to be particularly convenient as a biocompatible support, since it is available in a wide range of molecular weights and possesses a well-defined macromolecular structure. PEG is also known to be non-toxic, non-antigenic, soluble in both water and most organic solvents, and has also been approved by the FDA [5].

Since eugenol is a very promising candidate for versatile applications, the design of a new prodrug based on the pharmacological effects of eugenol could be beneficial. We have previously reported the synthesis of a bi-functionalized eugenol-PEG-Ibuprofen derivative with the aim of reducing the common side effects caused by traditional NSAIDs [6]. The objective of the present study was to improve the chemical-physical properties of eugenol after preparing a simple PEG-eugenol derivative which can provide valuable information regarding its pharmacological activity. An inclusion complex of eugenol with hydroxyl-propyl-β-cyclodextrins and hydrogels composed of chitosan and eugenol have been reported to enhance and sustain antioxidant activities [7, 8]. In the present study the phenolic group of eugenol was covalently bound to the carboxyl group of PEG and the release kinetics were studied *in vitro* in buffer solution at pH 7.4, in simulated gastric fluid and in plasma. The antioxidant activity of PEG-EU adduct was also evaluated with the aim to establish the role of the phenolic group in the eugenol moiety on the basis of drug delivery.

Preparation and characterization of mPEG-EU: The water solubility of eugenol is limited, as far as the use of the compound both *in vitro* and *in vivo* biological experiments. Efforts have been made to improve the water solubility of EU by non-covalent complexes with hydrophilic cyclodextrins and chitosans [7,8], but only moderate solubilizing capacity for EU has been obtained with the methods reported. PEG is the most common polymer used for conjugation. We have previously conjugated EU with PEG together with ibuprofen in order to obtain a synergistic anti-inflammatory effect without the side effects due to NSAIDs [6], but no pharmacological study has been conducted. In this work, PEG was used to covalently modify eugenol in order to improve its chemical-physical properties and to provide valuable informations regarding its radical scavenging activity. Eugenol has an excellent reducing power and exerts antioxidant activity against DPPH and lipid peroxidation [19]. For synthesis of the EU prodrug, $mPEG_{1900}$carboxylate (**3**) was used. The succinyl derivative of $mPEG_{1900}OH$ (**2**) was prepared by reaction with succinic anhydride in toluene at 150°C. The carboxylate polymer (**3**) was obtained with a yield of 95% after precipitation in Et_2O, filtration and recrystallization from EtOH hot/cold. TLC showed only one spot and no succinic anhydride. The product titrated with 0.01 N NaOH yielded 100% of free carboxylic groups. The subsequent esterification reaction between the carboxyl group of the activated polymer (**3**) and the phenolic hydroxyl group of eugenol (**1**) allowed us to obtain the desired adduct (**4**) [Scheme 1].

An ester bond links mPEG-COOH to EU. Esters with PEG as an electron-withdrawing substituent (alkoxy) in the α-position proved to be especially effective linking groups in the design of prodrugs since they aid in the enzymatic hydrolysis of the ester carbonyl bond, and thus are able to release alcohols in a continuous and effective manner [10]. The conjugation was carried out in a single



Scheme 1: Synthesis of $mPEG_{1900}$-EU. Reagents and conditions: a) Toluene, 150°C, 5 h; b) DCC, DMAP, CH_2Cl_2, 5 h

step by adding EU (**1**), under stirring, to a solution of $mPEG_{1900}$-COOH (**3**) in CH_2Cl_2 in the presence of DCC and DMAP, respectively [Scheme 1]. The adduct (**4**) was obtained with a yield of 94%, after precipitation in Et_2O, filtration and recrystallization from EtOH hot/cold. The absence of free EU was verified by HPLC analysis. The amount of EU linked to the functionalized polymer was evaluated by measuring the coefficient molar extinction of EU solution in methanol to 281 nm, taking advantage of the transparency of the polymer at longer wavelengths at 205 nm [11]. The conjugation does not change this property and, therefore, the spectrum of absorption of the adduct and EU standard is perfectly superimposed. The degree of modification was 98.8%. The ^1H NMR spectrum shows the set of expected signals and confirmed the presence of the methylene protons, ascribed to the PEG backbone (***δ*** = 3.36-3.75 ppm), together with the two signals at δ 2.74 and 2.84 (*J* = 6.0 Hz) due to the methylene groups adjacent to the carboxyl group of the polymer. The singlet at δ 3.77 is due to the *ortho* methoxy group of the eugenol moiety.

Unlike free EU, the drug–polymer conjugate was freely water-soluble at room temperature.

Hydrolytic stability: The hydrolysis rate of the conjugate $mPEG_{1900}$-EU was studied *in vitro* in a phosphate buffer solution, pH 7.4, and in artificial gastric juice, pH 1.2.The degradation of the adduct was followed by RP-HPLC analysis of the released free drug. Figure 1 shows the hydrolysis rate of the mPEG-EU in phosphate buffer solution, pH 7.4, at 37°C; about 7% of EU was released from the polymer over 24 h. Figure 1 depicts also the hydrolytic behavior for the same conjugate in artificial gastric fluid (0.32% of gastric protease) at pH 1.2, 37°C. Less than 2% of EU was released over 6 h. On the basis of the above data, the hydrolytic stability is also good in gastric juice.

The $mPEG_{1900}$-EU was almost hydrolytically stable in phosphate buffer pH 7.4, and in simulated gastric medium showing good resistance in the stomach acidic medium.

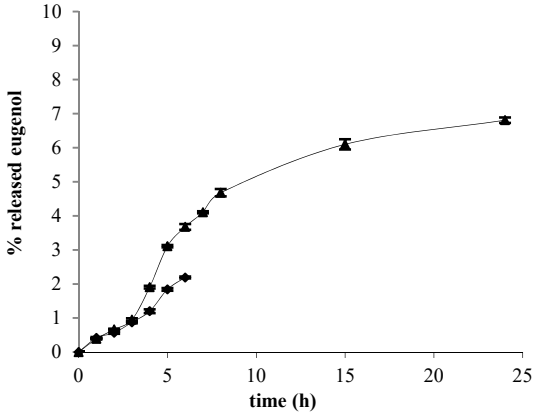


Figure 1: Release of EU from $mPEG_{1900}$- EU in buffer solutions, ■ pH = 1.2 and **Δ** pH = 7.4 at 37 ± 0.1°C

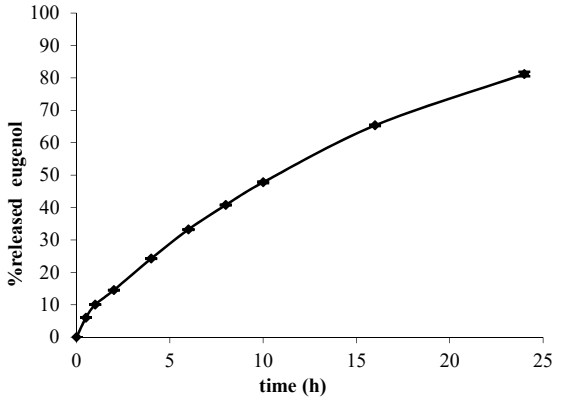


Figure 2: Release of EU from $mPEG_{1900}$- EU in mouse plasma at 37 ± 0.1°C.

Stability in plasma: The macromolecular prodrug's ability to release the free drug was evaluated in mouse plasma. More than 85% of linked EU was released from the polymeric conjugate within 24 h (Figure 2). This indicates the possibility for this macromolecular conjugate to be a good substrate for the plasma enzymatic complex and its capacity to deliver free and active drug in a prolonged manner. The structure of released EU was confirmed by mass spectrometric analysis. The molecular ion at *m/z* = 164 confirmed the presence of EU and the fragments at *m/z* = 149 and 91 were due to the loss of a methyl and of C_2H_3 group; respectively.

Free radical scavenging activity: The free radical scavenging activity was assayed spectrophotometrically using the DPPH method [12-14]. The activity of EU, EU from mouse plasma, $mPEG_{1900}$ and $mPEG_{1900}$-EU was measured at concentrations of 0.0008–0.05 mg/mL of EU, and the results are shown in Figure 3.

The scavenging activity was the same for EU and EU released from the adduct by enzymatic reactions in plasma. Otherwise, the mPEG-EU adduct did not present activity, because the phenolic group in eugenol is engaged in the polymer bond. Thus, the scavenging activity of mPEG-EU, by enzymatic hydrolysis of the ester bond, gradually increases on the basis of drug delivery.

Experimental

General: Eugenol (EU, purity > 98%), trifluoroacetic acid (TFA), ascorbic acid and mouse plasma were purchased from Sigma Chemicals Co. (St. Louis, USA), and methoxypoly(ethylene glycol)

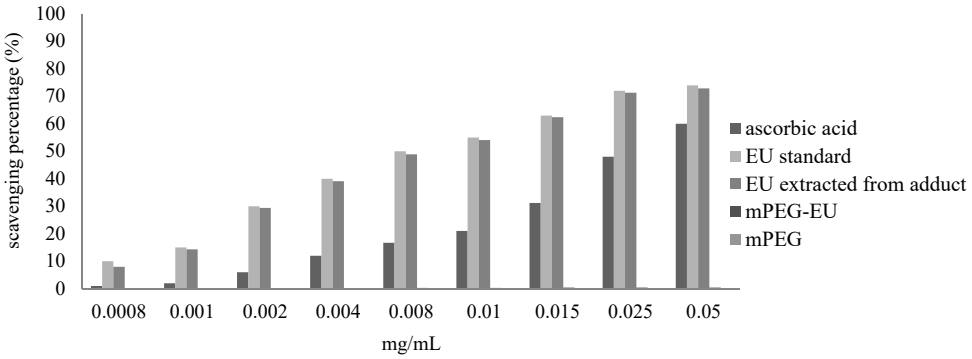


Figure 3: DPPH scavenging ability of eugenol when the assay was carried out in different samples

{MW=1900 (mPEG)}, succinic anhydride, *N*,*N*'dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Fluka (Buchs, Switzerland). All the other chemicals and solvents used in this work were of the highest quality commercially available. Organic solvents were dried over molecular sieves (3 Å).

^{1}H NMR spectra were recorded on a Jeol EX400 spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as internal standard (δ = 0.0 ppm). Electro spray ionization (ESI) mass spectra were obtained on a PE-API 1 spectrometer by infusion of a solution of the polymeric sample in a 1:1 solution of CH_3OH/CH_3COONa 5% (ionizing potential: 5600 V; infusion 0.1 mL/h; curtain gas N_2, 1.2 L/min; nebulizing gas, air 0.6 L/min). Spectrophotometric UV/VIS analysis was performed on a Helios β UNICAM spectrophotometer.

Potentiometric titrations were carried out using a TTT80 pH stat equipped with an ABU80 autoburette, a titrigraph module REA 160 and a REA 070 pH stat unit, radiometer.

HPLC analysis: HPLC analyses were carried out with a Perkin-Elmer HPLC connected to a variable-wavelength UV detector. A LiChrosorb RP18 column (5 µm, 250 mm x 4.6 mm i.d., Perkin-Elmer) was used. A mixture of water–acetonitrile–methanol–trifluoroacetic acid (20:10:70:0.1, v/v) was employed as mobile phase; the detection wavelength was 281 nm and the flow rate 1 mL/min. Quantification was performed on the basis of a linear calibration plot of peak area against concentration. Each curve was based on 7 concentrations of the standard. Identification of the drug was made by comparison of the retention times with those of pure standard.

Preparation of mPEG-COOH: $mPEG_{1900}$-COOH was synthesized according to a previously published procedure [15]. Briefly: $mPEG_{1900}$ (**2**) (0.005 mol) was dissolved in toluene (100 mL) and dried by distilling off most of the toluene. Succinic anhydride (0.02 mol) was added and the mixture stirred for 5 h on an oil bath at 150°C. The mixture was cooled, taken up in CH_2Cl_2 and the polymer precipitated by diethyl ether. The product was recrystallized twice from CH_2Cl_2/diethyl ether. Yield 90%.
TLC: BuOH/AcOH/H_2O, 4:1:1, v/v.
^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.20 (4H, t, CH_2OCO), 3.60 (br s, CH_2-O-PEG), 3.34 (3H, s, CH_3-OPEG), 2.60 (4H, br s, CH_2COO-).

Preparation of mPEG-EU: Eugenol (**1**) (2 mmol; 308 µL) and DCC (0.66 mol; 134 mg) were added in small portions to a solution of $mPEG_{1900}$-COOH (**3**) (0.5 mmol; 1g) and DMAP (0.12 mol; 15.27 g) in CH_2Cl_2 (50 mL); the mixture was stirred for 5 h at room temperature. The precipitate was filtered and the filtrate evaporated to dryness. The residue was extracted with acetone, filtered and the product precipitated by diethyl ether. The $mPEG_{1900}$-eugenol (mPEG-EU, **4**) was recrystallized twice from ethanol. Yield 94 %. The absence of the free drug in the adduct was confirmed by HPLC analysis. The degree of functionalization was confirmed measuring the absorbance of the adduct at 281nm in methanol.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.69-6.93 (3H, m, H-arom.), 5.93 (2H, m, CH_2CH=CH), 5.02-5.09 (2H, m, CH_2CH=CH_2), 4.21 (2H, t, CH_2CH_2OCO), 3.90 (2H, t, CH_2CH_2OCO), 3.77 (3H, s, CH_3O-EU), 3.60 (br s, CH_2-PEG), 3.34 (3H, s, CH_3O-PEG), 3.25 (2H, d, CH_2CH=CH_2), 2.84 (2H, t, J = 6.07 Hz, CH_2COO), 2.74 (2H, t, J = 6.0 Hz, CH_2COO).

Conjugate stability in simulated extra-cellular fluid and in simulated gastric medium: The mPEG-EU (5mg/mL) was incubated in phosphate buffer solution (10 mM NaH_2PO_4, 0.15 M NaCl), pH 7.4, and artificial gastric juice (2.0 g NaCl, 3.2 g pepsin powder, 80 mL HCl 1 M in 1 L of water), pH 1.2. The solutions were incubated at 37 ± 0.1°C and sampled by RP-HPLC analysis at scheduled times on the basis of formation of free EU. The quantity of released EU by hydrolysis was quantified with the HPLC method previously described. Every experiment was repeated in triplicate.

Stability in plasma: Stability in plasma of the mPEG-EU was assessed by incubating the conjugate at 37°C ± 0.1°C in mouse plasma for 24 h. The conjugate was dissolved in PBS and sonicated for 2 min. Then, 100 µL of solution was added to the plasma to obtain a final concentration of 1.0 mg/mL of EU. At scheduled times the samples were analyzed after solid-phase extraction (SPE) by HPLC and EI-MS analysis. Every experiment was repeated in triplicate.

SPE analysis extraction: EU in plasma was extracted employing a solid-phase extraction technique (SPE). Each tube containing 1 mL of plasma was vortexed for 30 sec. The sample was applied into RP Supelclean® LC18 solid-phase extraction columns pre-treated with 1.0 mL of methanol, followed by 1.0 mL of water. The LC18 cartridges were set in a Visiprep® sample processing station and subsequently washed twice with 1.0 mL of acidic water (at pH 5) under vacuum. The EU was eluted from LC18 SPE column with 1 mL of methanol. The eluent was evaporated under a stream of nitrogen at 40°C and the residue was redissolved in 50 µL of methanol. Subsequently, the samples were centrifuged for 10 min at 10000 *g*, 20 µL aliquots were injected into the HPLC (EU recovery: 98%) and other 20 µL aliquots were used for the free radical scavenging activity test.

Free radical scavenging activity test: Sample solutions of SPE extraction, solutions of mPEG, mPEG-EU and EU standard were diluted to final concentrations of eugenol 0.0008-0.05 mg/mL in 70% methanol, and used for the test. Five mL of 0.2 mM DPPH ethanol solution was added to 1 mL of sample solution of different concentrations, shaken well by vortex, and allowed to react at room temperature. The absorbance values were measured after 10 min at 525 nm by a UV/Vis spectrophotometer. The free radical scavenging activity of samples was calculated according to the formula:

DPPH radical scavenging activity (%) = [1 - (*Abs* sample – *Abs* blank)/*Abs* control] × 100

where *Abs* sample is the absorbance of the experimental sample, *Abs* blank is the absorbance of the blank, *Abs* control is the absorbance of the control.

As a blank, 70% methanol (0.5 mL) and sample solution (1.0 mL) were used. DPPH solution (0.5 mL, 0.2 mM) and 70% methanol (1.0 mL) was used as a negative control. Vitamin C at 0.1% was used as a positive control. Each treatment was replicated thrice.

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