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A Facile Conjugation of 6-Hydroxylflavone Biomolecule with Polyethylene Glycol for Enhancing Conjugate Stability

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Abstract. 6-Hydroxyflavone (6HF) contains attractive biological properties of significance pharmacologically and has been discovered as an effective diabetic medication. However, due to its high degradation in aqueous solution, its efficacy in biological treatment remains a considerable obstacle. Thus, conjugating a polymer, polyethylene-glycol, to 6HF by direct esterification between the carboxyl group of PEG and the hydroxyl group at the sixth carbon of the 6HF biomolecule is one of the approaches applied in this research to increase its stability while maintaining the inherent biological characteristics**.** This study examined the optimum esterification reaction conditions for conjugate PEG-6HF utilizing EDC and DMAP as conjugation reagents in various solvents, such as DMSO, PBS, and PBS 10 mM HEPES pH7.4 with the assistance of HOBt, including its stability in the biomimicking environment. For this purpose, PEG-6HF connected through the ester bond was validated using various analytical techniques such as FTIR, UV-Vis spectroscopy, and HPLC. , Notably, esterification at 25 °C for 24 hours in a 10 mM HEPES pH 7.4 buffer solution using EDC with HOBt resulted in the most significant conjugation percentage, 42 percent. Furthermore, PEG-6HF revealed 1.3 times lower degradation percentages of 6HF biomolecules than unconjugated-6HF following 6 hours of incubation in 10 mM HEPES pH 7.4 at 37 °C. Hence, the optimal conditions and the resulting conjugation percentage with high stability are expected to be a fundamental approach to conjugated polymer with a biomolecule.

Keywords: Conjugation; Esterification; 6-Hydroxyflavone; PEG-6HF; Stability

1. Introduction

Recently, a breakthrough has emerged in polymer chemistry development by applying a fundamental theory of polymer phase behavior at site-specific modification of peptides, synthetic biology, and single-chain polymer behavior (Shu et al., 2013; Dey et al., 2015; Krisanti et al., 2020). However, there have been slight changes in obtaining successful selective molecular transport, hierarchical structure control, modulated responsiveness to small perturbation, and long-term enzymatic activity (Shu et al., 2013). Therefore, researchers have agreed that polymer-biomolecule conjugate would improve this limitation. Currently, few conjugation methods have been discovered, for example, click chemistry, amidation, thiol-maleimide, and esterification (Shimokawa et al., 2009; Liechty et al., 2010; Shu et al., 2013; Che-Harun et al., 2016). Nevertheless, esterification is seen as a better alternative due to its simplicity, eliminating the need for further biomolecule

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modification. Therefore, polymer-biomolecule conjugates formed by direct covalent conjugation through the esterification process are a new class of soft materials since each component is complementary (Shu et al., 2013; Dey et al., 2015).

6-Hydroxyflavone (6HF) is a group of naturally derived bioactive polyphenolic compounds that possess tremendous medicinal assets that have potential roles in preventing chronic diseases, including effectiveness against some neurological disorders paraplegia or sciatica (Wang et al., 2021). 6HF has pharmacologically significant biological characteristics such as neuroprotection, antimicrobial, anti-inflammatory, anticancer, and antioxidants and has been discovered to be an effective treatment for diabetic patients against glomerulonephritis and glomerulosclerosis (Iwakiri et al., 2013; Wang et al., 2015; Das et al., 2018; Das et al., 2019; Stompor et al., 2019; Wang et al., 2021). Stompor et al. (2019) and Mikell et al. (2015) revealed that the hydroxyl group or the propionyl group located in the A ring of the flavones at the C-6 positions has an inhibitory effect on hormone production in the process of steroidogenesis and has cytotoxic solid and apoptotic activities against cancer cell (Iwakiri et al., 2013; Mikell et al., 2015; Wang et al., 2015; Stompor et al., 2019). A hydroxyl group at the sixth carbon in the 6HF compound chemical structure makes 6HF easily esterifiable. Even though much research focuses on intramolecular chemical changes of 6HF for certain specific applications, the stability of 6HF and its derivatives in an aqueous solution remains a big challenge.

Previously, Bayard et al. (2013) reported the successful conjugation between PEG with low molecular weight hydrophobic biomolecules, including hormones and antioxidants, through esterification leads to excellent pharmacokinetic properties of the drug. The objective of developing PEG conjugates was to enhance water solubility and stability while also lessening clearance through the kidney, which prolonged circulation in the bloodstream and increased the drug molecule's biocompatibility (Bayard et al., 2013; Hamley, 2014; Cui et al., 2021). Additionally, PEG has good solubility and stability, increasing membrane permeability and enhancing oil recovery (Febriasari et al., 2021; Irawan et al., 2017). Furthermore, PEG is appropriate for biological applications due to its biological inertness and low toxicity (Rashmi et al., 2020; Turecek & Siekmann, 2019). Moreover, there has been no fact-finding discussion on the conjugation of a 6HF biomolecule with a polymer unit to the authors' knowledge. Thus, researchers were inspired to direct conjugate a molecule of 6HF with a hydrophilic biodegradable polymer, polyethylene glycol (PEG), to improve 6HF stability. Researchers investigated the optimum esterification conditions of a PEG having a carboxyl functional end-group with a 6HF molecule with an active hydroxyl functional group using various solvents such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), and phosphate buffer saline (PBS) to expand the compatibility of the conjugation process and evaluated PEG—6HF conjugates stability in the biomimicking environment. Figure 1 depicts the general route for the conjugation of PEG-6HF. The produced PEG-6HF conjugates were characterized by their physicochemical properties by RP-HPLC analysis, FTIR, and UV-Vis analysis. The results revealed that the conjugation of PEG-6HF in 10 mM HEPES with pH 7.4 buffer solvent with the assistance of additive peptide coupling, HoBt, demonstrated the highest percentage of conjugation. This is the first research to develop a conjugation between a 6HF biomolecule with a unit of polymer and the conjugate enhanced 6HF stability in a biological environment. Moreover, this research analyzes the optimum conjugation conditions of the PEG and 6HF through esterification, which may aid in understanding the new molecular construction, which is expected to be a new methodology for material chemistry research to provide crucial information for future bioorganic and medicinal chemistry studies.

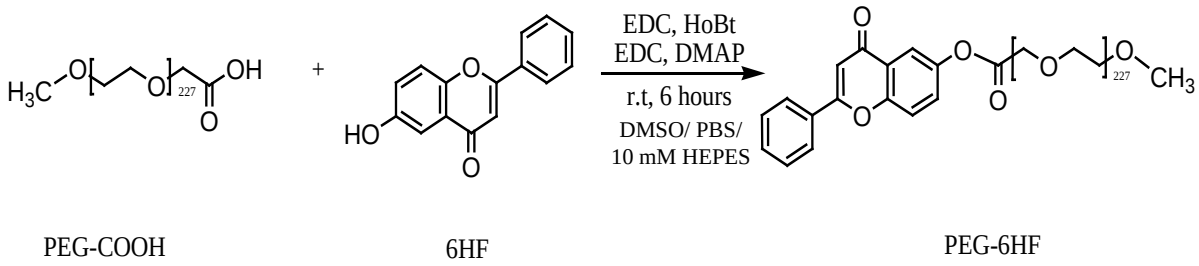

Figure 1 Synthetic scheme of PEG-6HF

2. Methods

2.1. Materials

6-Hydroxyflavone (6-Hydroxyflavone; 98% purity of 6-Hydroxyflavone) and 1M N- (2-Hydroxyethyl) piperazine-N'-(2-ethane sulfonic acid) (HEPES) were acquired from Sigma-Aldrich (USA). Advanced BioChemicals (USA) supplied methoxy polyethylene glycol carboxyl (mPEG-COOH, Mw 20 KDa) (USA). For the conjugation, Sigma-Aldrich (USA) provided dimethyl sulfoxide (DMSO), 4-dimethyl aminopyridine (DMAP), and 1-hydroxy benzotriazole hydrate (HOBt), N-ethyl-N'-(3-dimethyl aminopropyl) carbodiimides (EDC), and ethanol. Phosphate buffer saline (PBS) was prepared according to the standard formula. Ultra-pure water produced from the Mili-Q water purification system was utilized throughout the reaction and the purification process. All analytical-grade chemical agents were obtained from commercial sources in Malaysia. The reaction and purification process used ultra-pure water obtained from the Mili-Q water purification system.

2.2. Synthesis of PEG-6HF in DMSO or PBS

The synthesis of PEG-6HF conjugate is based on methodology from Dey et al. (2015) and Stompor et al. (2019) with few modifications. PEG solution was synthesized with a ratio of 1:1 v/v in 4 ml of a mixture of DMSO and water by dissolving 30 mg of mPEG-COOH. The mixture was vigorously stirred using a magnetic bar stirrer for three hours at 25 °C. After a fine suspended PEG was visible, 1 mg and 3 mg of DMAP and EDC were added as condensation reagents. Afterward, the carboxylate group was activated by continuously stirring the mixture at 25 °C for an hour. The stock solution of the 6-Hydroxyflavone was prepared in 1 mL of DMSO by dissolving 1 mg of 6-Hydroxyflavone. While stirring, the solution (294.12 M of 6-Hydroxyflavone in DMSO) was progressively added to the activated PEG under nitrogen conditions. . The product was purified against DMSO after 7 hours of stirring, then deionized water using an Amicon Ultra-15 Centrifugal Filter Unit (Membrane NMWL= 3KDa) to eliminate unreacted molecules. Then, produced PEG-6HF was stored in the refrigerator in an amber-colored glass bottle for further experiments. Furthermore, PEG-6HF prepared using PBS solvent was implemented and analyzed similarly to PEG-6HF produced with DMSO solvent.

2.3. Synthesis of PEG-6HF in 10 mM HEPES pH 7.4 with the assistance of HOBt

PEG solution was synthesized in 4 ml of 10 mM HEPES with pH 7.4 buffer solvent by dissolving 30 mg of mPEG-COOH. For 3 hours at 25 °C, the mixture was vigorously stirred with a magnetic bar stirrer. Following the formation of a fine suspended PEG, 1 mg and 3 mg of HoBt and EDC as condensation reagents were added. Then, the following procedure similar to that for synthesis PEG-6HF in DMSO or PBS.

2.4. UV-Vis Analysis

UV-vis analysis of the emission and absorption spectra of the unconjugated 6HF and produced PEG-6HF conjugates was carried out on a double beam PerkinElmer Lambda

EZ210 spectrophotometer used 1 cm length of quartz cuvette in a range of 200 to 400 nm to identify the wavelength and the absorbance range of the conjugates. The produced PEG-6HF was analyzed at room temperature, and all the spectra were corrected to the blank data.

2.5. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis

Reverse Phase High-performance liquid chromatography (HPLC) using an Ultimate 3000 DIONEX chromatograph equipped with a WPS-3000 autosampler, a TCC-3100, a thermostated column compartment, Diode Array Detector DAD-3000, and LGP-3400 RS dual-pump fluid control module, was used to identify the purity of the unconjugated-6HF and produced PEG-6HF conjugate (Shelton, USA). The Chromeleon 6.80 software (Dionex Corporation) was used for the data acquisition. RP-HPLC analysis was performed using a reversed-phase C18 column as the stationary phase (Brownlee Analytical C18 5µm, 150 mm × 4.6 mm) connected to a guard column (Brownlee Analytical C18) (PerkinElmer Life and Analytical Sciences, Shelton, USA). The analysis of the produced 6HF-PEG using HPLC was carried out following the method from (Iwakiri et al., 2013; Dey et al., 2015; Stompor et al., 2019) with some modifications. The mobile phase consisted of two components, A: 0.1 % HCOOH in H2O and B: 0.1 % HCOOH in MeCN. The components A and B ratio was set at 40:60 (v/v). The flow rate was set at 1 mL/min, and the total analysis time was 15 minutes. The injection volume was 10 µL, and the temperature of the samples and column was set at 25 °C. The detector of UV-visible was set at 280 nm to detect the content of the unconjugated-6HF and PEG-6HF conjugate.

2.6. Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

Fourier Transform Infrared (FTIR) spectroscopy was used to identify the chemical structure characterization between PEG and 6HF using PerkinElmer Spectrum RX1. Chemical structure characterization aims to analyze different functional groups which vibrate at a particular frequency. In this experiment, the spectra were obtained by analyzing PEG-6HF conjugate using FT-IR with a range of 250 – 4000 cm^{-1} with a spectral resolution of 1 cm^{-1} at 25 °C equipped with a single beam purgeable and single compartment.

2.7. Stability Analysis

1 mL of PEG-6HF conjugate and unconjugated-6HF, respectively, was added into 4 mL of 10 mM HEPES pH 7.4 buffer solution, and subsequently, the solutions were incubated at 37 °C for 6 hours. PEG-6HF conjugate and unconjugated-6HF samples were taken at one-hour intervals and subjected to UV-Vis spectrometer analysis to determine the change in solution absorbance.

3. Results and Discussion

In this study, the conjugation was conducted using the commercialized PEG-COOH with 6HF in the presence of the EDC as a coupling agent and DMAP as a catalyst. The reaction was conducted using three types of solvents, i.e., DMSO, PBS, and 10 mM of HEPES with pH 7.4 with the assistance of HOBt at 25°C for 7 hours. The RP-HPLC was used to collect and purify the products. The conjugation of produced PEG-6HF was achieved by direct esterification and was confirmed by the FTIR spectrum. The chemical structure characterization of produced PEG-6HF conjugates was verified by the FTIR spectra, as shown in Figure 2. As a result, a broad brand at a range of 3200 cm^{-1} to 3500 cm^{-1} was assigned to O-H stretching. It demonstrates that the conjugate contains phenolic OH- group moieties from the 6HF compound. Due to aliphatic C-H stretching, the peak appeared at about 2900 cm^{-1} in produced PEG-6HF conjugates. The existence of C-O and C=O carboxylate stretching frequencies at 1100 cm^{-1} and 1650 cm^{-1} proved the ester bond in

PEG-6HF conjugates (Esmaeili et al., 2020), thus, indicating the successfulness of PEG-6HF conjugation.

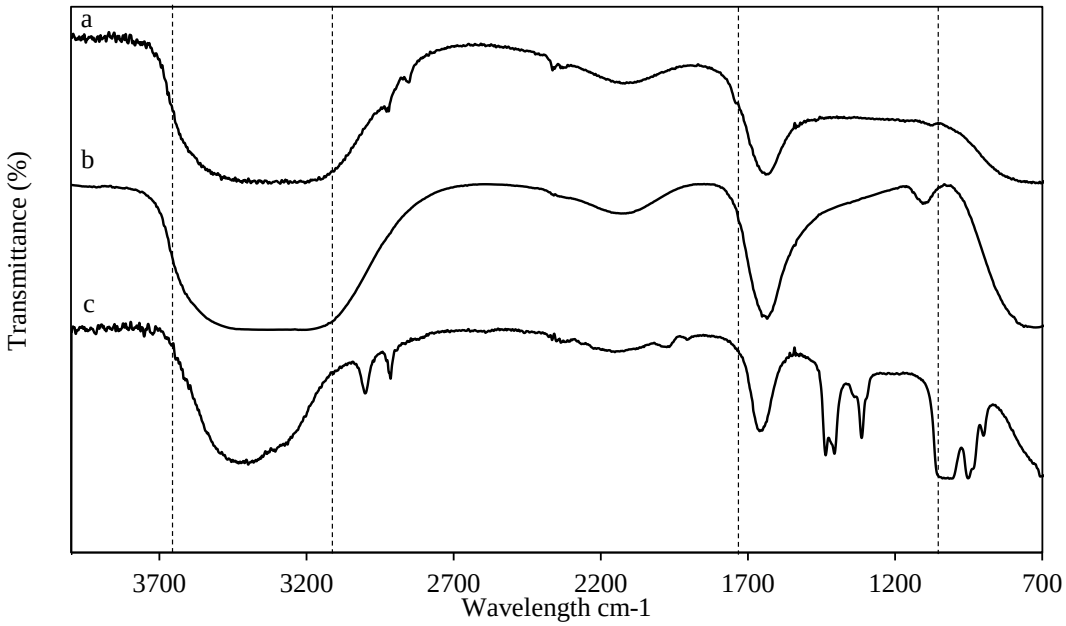

Figure 2 The FTIR spectra of the produced PEG-6HF conjugates were prepared (a) in PBS, (b) in 10 mM HEPES with pH 7.4, and (c) in DMSO

To further confirm the formation of produced PEG-6HF, the UV-Vis study of the emission and absorption spectra was performed from 200 nm until 340 nm. Figure 3 depicts the characteristics of the UV-Vis absorption spectra of PEG-6HF produced in different solvents. The peak of UV-Vis absorption of unconjugated-6HF was observed at the wavelength 210 nm; however, once conjugation occurred, the peak shifted to 280 nm. The emergence of a new absorption peak at 280 nm for each PEG-6HF conjugate indicated the covalent conjugation and confirmed that the 6HF was successfully conjugated onto the PEG. Following conjugation with PEG, the position of these bands shifts, implying a conformational change in the 6HF biomolecule (Ta-Aithuak et al., 2020). This result revealed that the size of the PEG-6HF conjugate increased after the 6HF biomolecule adsorbed onto the PEG compound, as demonstrated by a wavelength shift in the UV-Vis spectrum.

The produced PEG-6HF was purified by RP-HPLC to identify the conjugation percentages. The percentage conjugation of the produced PEG-6HF was calculated based on the 6HF calibration curve (R^2 = 0.999). According to Figure 4, the percentage conjugation yields of PEG-6HF conjugate performed in 10 mM HEPES pH 7.4 buffer solvent were 42% and PEG-6HF conjugate performed in PBS was 34%. Meanwhile, the percentage conjugation yield of PEG-6HF conjugate performed in DMSO was 11percent. The highest percentages of conjugation yield of PEG-6HF conjugate in 10mM HEPES pH 7.4 buffer solvent suggested that water-based solvent is the most suitable medium for the esterification process of polymer-biomolecule conjugation.

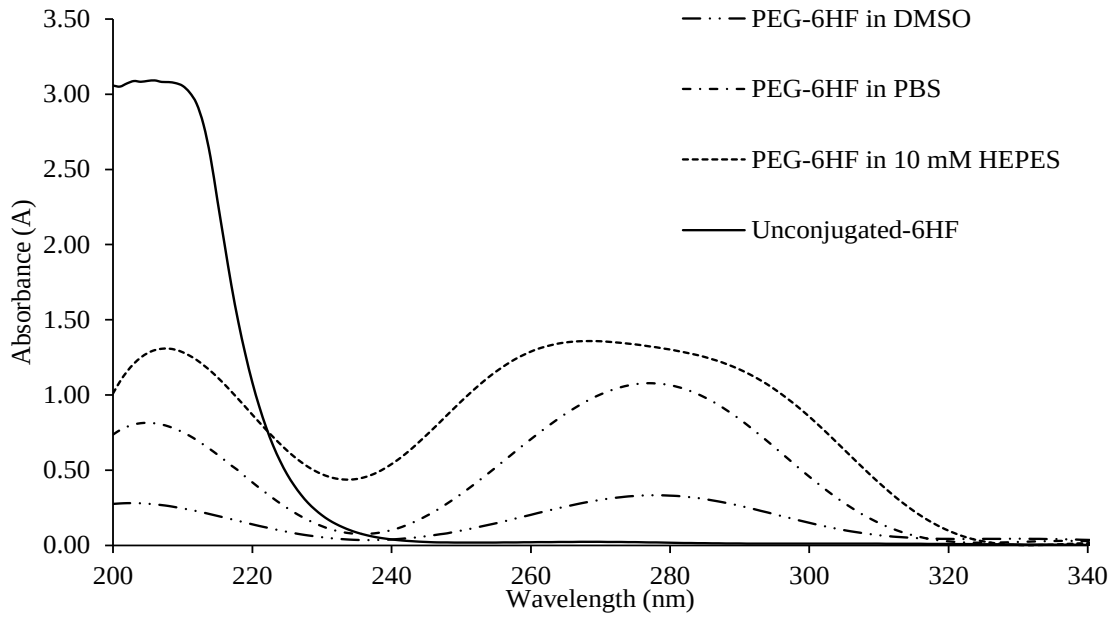

Figure 3 UV-Vis absorption spectrum of produced PEG-6HF in DMSO, PEG-6HF in PBS, PEG-6HF n 10 mM HEPES with pH 7.4 and unconjugated-6HF

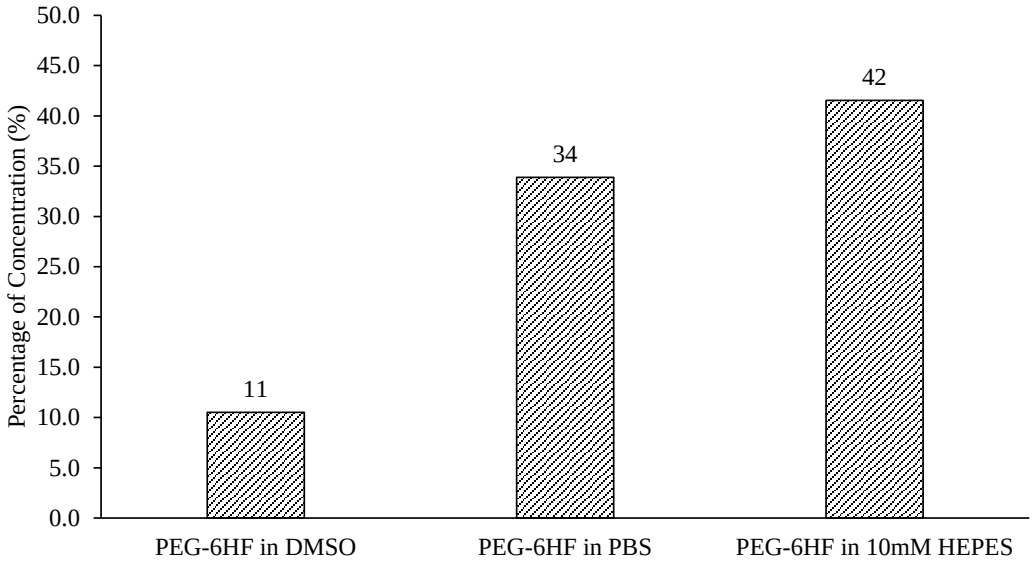

Figure 4 Percentage concentration of produced PEG-6HF in DMSO, PEG-6HF in PBS, and PEG-6HF in 10 mM HEPES with pH 7.4 at 25 °C for 6 hours

Furthermore, to further investigate the ability of produced PEG-6HF, the stability test of PEG-6HF was performed by incubating the unconjugated-6HF. It produced PEG-6HF in 10 mM HEPES pH 7.4 solution at 37 °C for 6 hours, mimicking the biological environment. A sufficient number of samples were collected every hour, and the changes were constantly monitored using a UV-Vis spectrophotometer (Charoensit et al., 2019; Liu et al., 2020). The percentages of the degradation 6HF were calculated based on the initial absorbance of both samples. Figure 5 shows the percentage of remaining 6HF in PEG-6HF conjugate after 6 hours was 80%. The remaining 6HF in PEG-6HF conjugate was 1.3 times higher than unconjugated-6HF (60 percent). The significantly higher stability of 6HF in PEG-6HF conjugate compared to unconjugated-6HF might indicate that the conjugated PEG to the 6HF protects the 6HF molecule in the biological environment.

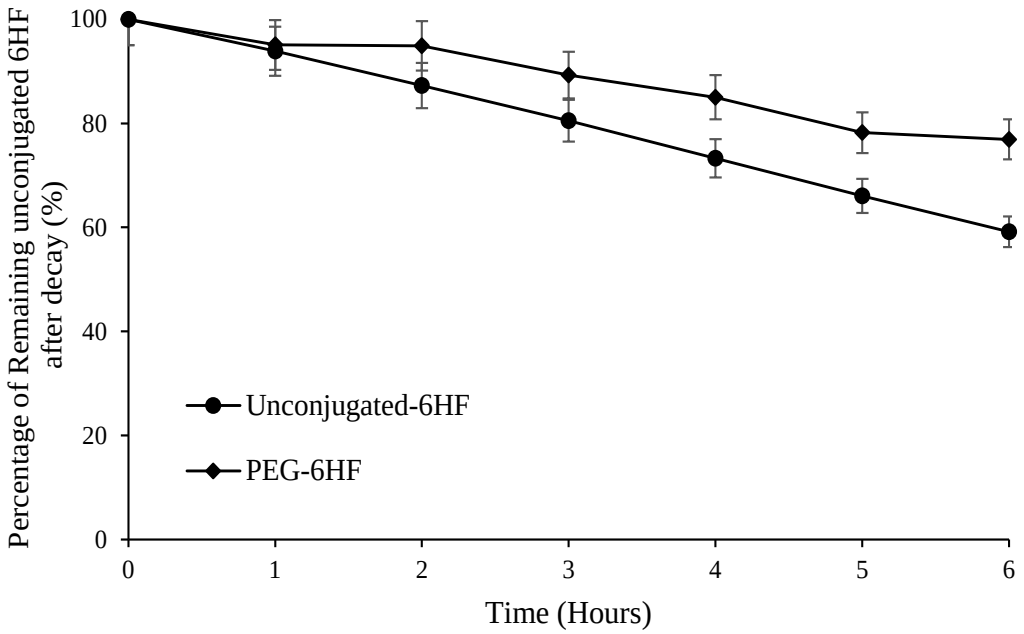

Figure 5 The degradation of unconjugated-6HF and PEG-6HF conjugate in 10 mM HEPES pH 7.4 buffer solution at 37 °C for 6 hours

4. Conclusions

In conclusion, a successful conjugation with a high percentage yield (42 percent) between polyethylene glycol (PEG) and 6-Hydroxyflavone (6HF) synthesized by direct esterification in 10 mM HEPES with pH 7.4 at 25°C was observed in comparison to the PEG-6HF conjugate that prepared in DMSO (11 percent). Moreover, the PEG-6HF conjugate significantly improved its stability, which was 20 percent more stable than unconjugated-6HF due to 1.3 times lower degradation in physiological conditions for 6 hours. The antimicrobial activity of PEG-6HF conjugate is currently being studied. Hence, this new conjugation can be a fundamental methodology for material chemistry field study and provide critical information for future bioorganic and medicinal chemistry studies.

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