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Enhancing stability and antioxidant efficacy of fisetin by encapsulating as β-cyclodextrin inclusion complex with porous polylactic acid film from breath figure

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Abstract

This research aims to investigate the scope of fisetin (FIT)/β-cyclodextrin (β-CD) inclusion complexes with the ratios of 1:2 and 2:1 mole by means of freeze-drying, kneading and physical mixing methods (controlled process). Moreover, the complex compounds are analyzed by Fourier-Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC). The results show that the most suitable preparation method of fisetin (FIT)/β-CD complex is the freeze-drying method with the fisetin (FIT) to β-CD ratio at 1:2. The emission of FIT/β-CD was investigated and it is found that the emission rate of freeze-dried FIT/β-CD in 1:2 ratio by freeze-drying method is lower than that by kneading and physical mixing methods. These results lead to an initiation of the innovative active packaging materials with synthetic polylactic acid (PLA) porous film by breath figure (BF) method in order to entrap FIT/β-CD inclusion complex. The honeycomb structure with and without the FIT/β-CD complex were analyzed by Scanning Electron Microscopy (SEM). Thereafter, the effectiveness of Antioxidant Activities (%AA) of the porous PLA films is measured by the anti-oxidation caused by 2,2-diphenyl-1-picrylhydrazyl (DPPH method). The result shows that PLA entrapping complex fisetin is more effective than PLA entrapping pure fisetin, by result of 53.0% and 48.6%, respectively.

1. Introduction

At present, fisetin (FIT) (3,3',4',7-tetrahydroxyflavone, 2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-chromen-4-one, Figure 1 [1]) is widely used for several beneficial human actions in terms of health due to its therapeutic activities and harmacological properties, such as antibacteria [2], anti cancer [3,4], anti-inflammatory [5,6] and anti-angiogenic [7]. In addtition, fisetin's property as an anti-oxidant agent is able to prevent and delay the oxidation reaction of free radicals and to eliminate the molecules. Antioxidant agent can be applied to develop healthy food products by decreasing the undesired substances in a food product without risk [8,9]. In recent years, there is a concern about the safety and potential toxicity problems associated with synthetic antioxidants. As such, the importance of natural antioxidants have significantly increased, and various researchers have studied on the application of natural antioxidants.

For this reason, fisetin is being proposed as a new approach to develop healthy food and active packaging. However, fisetin is practically sensitive to poor water solubility and easily degradable in light and heat. To diminish the limitations, fisetin is used with other water soluble components, e.g. β-Cyclodextrin (β-CD) [10-12], in order to form the inclusion complexes. β-CD is a cyclodextrin comprising seven glucopyranose units which is linked by α-(1,4) glycosidic bonds. Due to its cone-shape cavity which has hydroxyl

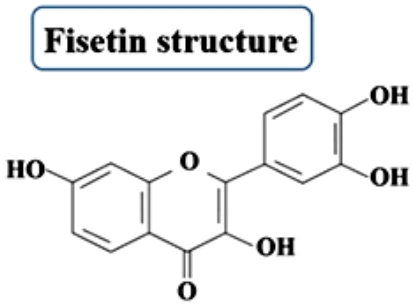

Figure 1. Chemical structure of fiestin.

groups at its rims, β-CD is used as a host molecule to encapsulate guest apsules, such as fisetin, and the guest molecules will arrange inside the cavity of β-CD. The inclusion complexes are obtained with van der Waals force, as opposed to covalent or ionic bond. Their structure may slightly be deformed, but the host molecules hardly change in its size and shape whilst the guest molecules' structure is fairly stable [13]. β-CD has thus a pivotal role in controlling inclusion complexion between the host and guest molecules, and improves the solubility and stability of the guest molecules. In addition, as β-CD is a nontoxic substance, it has been approved by the FDA and the WHO/FAO Joint Committee with the GRAS status (list of food additives that are 'generally recognized as safe') [14] and hence

used as an encapsulating agent for pharmaceutical application (e.g. drug carrier), food additives and vitamins. This method classified as encapsulation is very attractive and effective because it can obviously increase stability, dissolution rate and bioavailability [15]. Furthermore, using biodegradable polymer such as PLA [16], Poly-(ε-caprolactone: PCL) [17] encapsulating antioxidant has increasingly gained interest to maintain the effective antioxidant cavity and other properties related to heat-seal package.

Polylactic acid (PLA) porous film is an attractive substrate because of its properties such as ability to entrap the active compound, noncomplex process, biodegradation and non-toxicity [18], so it can be used as functional food packaging [19]. The porous PLA films can be easily fabricated by using a breath figure (BF) method [20-22]. Briefly, the BF technique is the method to form a porous surface. The method commences when water droplets from high humidity environment contact with cold surface of PLA in organic solvent. The endothermic evaporation of the organic solvent decreases the temperature of solution leading to water condensation on solution surface. The porous PLA is formed when the solvent is completely evaporated. Porous PLA film allows the active molecule to be entrapped onto the surface in functional film packaging.

In this research, fabrication of cyclodextrin inclusion complexes of fisetin (FIT/β-CD) methods consist of freeze-drying, kneading and physical mixing methods with the ratios in mole of 1:2 and 2:1 between β-CD and fisetin. Afterward, they are analyzed by FTIR to investigate heat capacity. FIT/β-CD inclusion complexes then are packed in porous PLA film by BF method and analyzed for antioxidant activity by means of DPPH in order to be an original design of innovative and economical active packaging materials containing appropriate environmental-friendly active agents for food package in the future.

2. Material and experimental

2.1 Materials

PLA (Polylactic acid) was obtained from Nature work PLA Polymers 2002D, USA. Fisetin (C15H10O6, FW=286.23, purity > 99%), β-Cyclodextrin: β-CD (FW=1135, purity > 99%) and DCM (Dichloromethane, purity > 99%) purchased from Merck Co., Ltd. to be used without further purification. Apart from substances mentioned, the others were analytical reagent grade, and ultrapure water was used in all experiments.

2.2 Preparation of the FIT/β-CD inclusion complexes

FIT/β-CD inclusion complexes with the ratios in mole of 1:2 and 2:1 were successfully prepared by 3 main methods, including freeze drying, kneading and physical mixing, which is altogether a controllable method. Details are as follows:

2.2.1 Freeze-drying method

Fisetin was dissolved in ethyl alcohol as solvent whilst β-CD was dissolved in water. Both solutions were then mixed together with 200 rpm at 32°C for 2 h. The mixed solution were centrifuged until the solid inclusion complex was completely precipitated out of the solution. The complex particles were frozen at -40°C for 48 h and subsequently operated by Freeze Dryer (CHRIST, Alpha 1-2 LD plus) at -40°C for another 48 h to dry. The FIT/β-CD inclusion complexes were obtained.

2.2.2 Kneading method

Fisetin powder and β-CD powder were mixed in a medicine mortar and milled for 15 min. Then, ethanal and water were poured into the mixture with the ratio 1:3 (v/v) 0.5 mL and mixed for another 15 min until the mixture became well combined. The mixture was brought to bake at 60°C for 24 h or until the substance was dried.

2.2.3 Physical mixing method

Fisetin powder and β-CD powder were mixed in a medicine mortar with the ratios in mole of 1:2 and 2:1 (or in weight (g), 0.063:0.5 and 0.5:0.99, respectively) for 5 min.

2.3 Characterization of the obtained FIT/β-CD inclusion complexes

2.3.1 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra were analyzed on the Fourier transform infrared spectrometer (Nicolet 6700, Thermo Fisher Scientific, Waltham, MA). The FTIR measurements were performed in the scanning range of 4000-400 cm^{-1} at ambient temperature.

2.3.2 Studies of release profile of FIT/β-CD inclusion complex

2.3.2.1 Maximum absorption spectra of fisetin (λmax)

Fisetin was dissolved in 100% ethanol and the solution was poured into Quartz Cuvette to measure λmax by spectrophometer which wavelength range is 300-500 nm and ethanol was used and originally set zero for light absorbance.

2.3.2.2 Release profile of FIT-β-CD inclusion complex studies

100 mg of fully prepared FIT/β-CD inclusion complexes from all methods was melted in 20 mL water in beaker. The beaker was then brought to thermostatic water bath in controlled 37°C at 0, 10, 30 and 60 min and 1 mL of each solution was transferred through 0.45 μm nylon membrane filter. To further analyze, spectrophometer was used with a wavelength range that Fisetin can absorb the light in absorbance maximum in order to measure the emission rate of FIT/β-CD inclusion complexes.

2.3.3 Differential scanning calorimetry (DSC)

Measurements were conducted on a Mettler Toledo instrument and Linses Sta PT1600, respectively, and 3.0-3.5 mg of each sample was heated at a rate of 10°C·min^{-1} from room temperature to 350°C under dynamic nitrogen atmosphere and at a flow rate of 10 mL·min^{-1}.

2.4 Fabrication of porous PLA film

The solution was prepared by dissolving PLA in Dichloromethane (DCM) as solvent at a concentration of 3 wt%. The mixture was then poured onto 16.5 × 13.0 × 0.5 cm glass mold, left to dry at room temperature (32°C) in a closed-chamber at 80% RH controlled by aqueous solution of sodium chloride (NaCl). The result was measured by means of hygrothermograph. The evaporation of DCM solvent reduced temperature of the surface of the PLA solution until it was lower than the dew point temperature. Then, the moisture vapor condensed into water droplets. The water droplets then orderly assembled on the surface of the PLA solution. After the solvent and water droplets completely evaporated, the porous PLA films were obtained. The honeycomb-structure film on the surface was analyzed by Scanning Electron Microscopy (JEOL, Model JSM-6400, Japan) and the pore size was measured by ImageJ version 1.34e software. The specific surface area of the obtained PLA film was measured by Brunauer–Emmett–Teller (BET) analyzer (Micromeritics ASAP® 2420 Accelerated surface area and porosimetry system).

2.5 FIT-β-CD inclusion complex incorporate in porous PLA film

FIT/β-CD inclusion complex was entrapped on the porous PLA substrate by using adsorption method. The procedure was carried out by soaking the porous PLA film in 0.3% (w/v) FIT/β-CD inclusion complex dissolved in absolute ethanol for 24 h and then the film was left to dry at room temperature overnight. The porous PLA film then was soaked in fisetin complex solution and porous PLA finally was dried at 50°C in a desiccator cabinet.

2.6 Antioxidant activity

The antioxidant activities of porous PLA film were evaluated using the slightly modified DPPH assay which is the popular Colorimetric method as it is accurate, convenient and time-saving. Each sample (100 mg of films) was dissolved in 10 mL of a 7:3 v/v DCM/ DMF solution. Then, the solution was diluted with 10 mL of methanol. 1 mL of each sample was transferred to separate vials, and 3 mL of methanol solution of DPPH (100 µmol·L^{-1}) was added to each of the vials and the mixture was incubated in darkness at room temperature for 30 min. After incubation, the absorbance (A sample) of the solution was spectrophotometrically measured at 517 nm with a Perkin-Elmer Lambda 2 UV-vis spectrometer (PerkinElmer Inc., Waltham, MA). The absorbance of the solution contains fisetin complex solution and vitamin C (ascorbic acid) as a control factor (was also measured as a reference) [23]. The antioxidant activity (%AA) was shown as a percentage of the absorbance value for DPPH radical species which was decreased in comparison with that of the reference sample, according to the following equation:

$$\%AA = \frac{A_{control} - A_{sample}}{A_{sample}} \times 100\% \quad (1)$$

3. Results and discussion

3.1 Fourier-transform infrared spectroscopy (FTIR)

Figure 2 shows FTIR spectra of β-CD, fisetin, and FIT/β-CD inclusion complexes prepared by freeze-drying method, kneading method and physical mixing method with the mole ratio of fisetin: β-CD at 1:2 and 2:1 which were analyzed at room temperature (32°C) in a spectral region between 4000 and 450 cm^{-1}.

β-CD, fisetin and FIT/β-CD inclusion complexes were characterized using FTIR spectroscopy as tool for confirming the formation of the inclusion complexes. As shown in Figure 2 [24,25], the characteristic peaks of β-CD due to glucose macromolecule appear at 3299 cm^{-1} (OH stretching), 2923 cm^{-1} (C-H stretching), 1640 cm^{-1} (C-O stretching) and 1154 cm^{-1} (C-O stretching). For fisetin, the bands of strong intensity appear at 3517 cm^{-1} (OH stretching), 1600 cm^{-1} (aromatic C=C group), 1440 cm^{-1} (in-plane C-H bending vibration), 1270 cm^{-1} (C–O–H bending) and 1165 cm^{-1} (C-O-C group). IR spectra of the mixture of fisetin and β-CD from kneading and physical mixing methods show the peaks similar to the former found in fisetin. Its peaks of FTIR spectrum are clearly observed at 1600 cm^{-1}, 1440 cm^{-1} and 1165 cm^{-1} from physical mixing method while the ones from kneading method are also noticeable but less sharp at 1600 cm^{-1}, 1440 cm^{-1} and 1165 cm^{-1}. These results which are corresponding to those of fisetin could thus be concluded that FIT/β-CD inclusion complex is not constituted or, possibly, is constituted in a small amount. Conversely, the FTIR spectra of the mixture of fisetin and β-CD from freeze-drying method show some significant changes, compared to the pure fisetin spectrum, which evidences the formation of the FIT/β-CD inclusion complex.

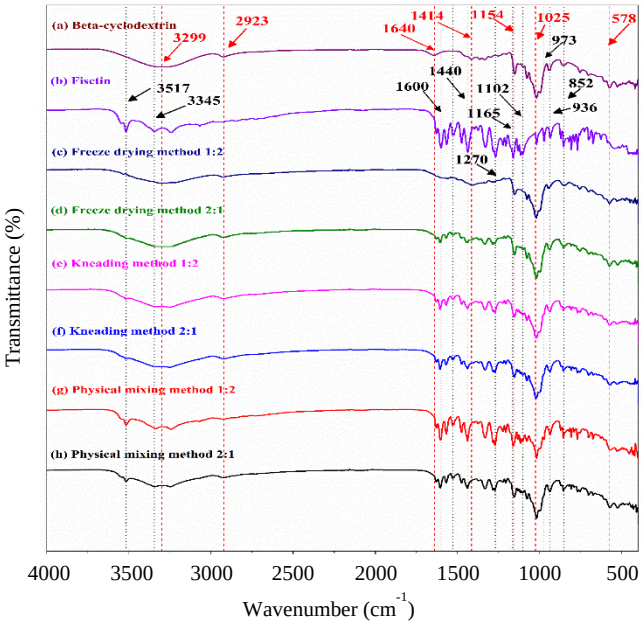

Figure 2. FTIR spectra (a) β-CD, (b) fisetin (FIT), (c)-(h) FIT/β-CD inclusion complexes prepared by freeze drying method, kneading method and physical mixturing method at the mole ratio of fisetin:β-CD.

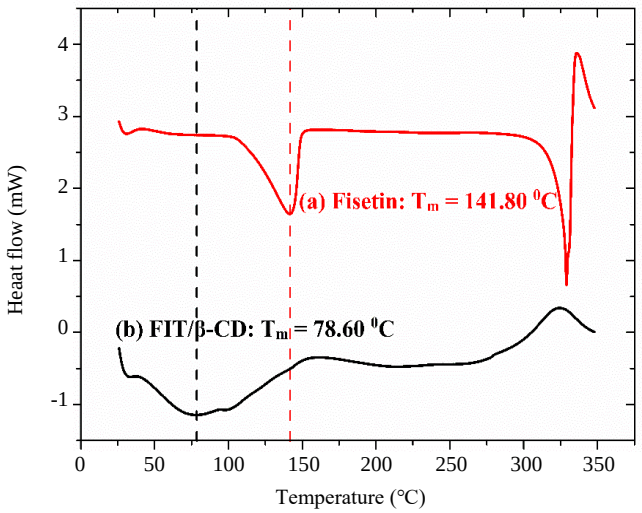

Figure 3. The analysis of heat capacity by DSC showing thermogram and melting point of fisetin and FIT/β-CD inclusion complex from freeze drying method.

In particular, some distinctive bands are observed from the mixture of fisetin and β-CD by freeze-drying method with reducing the peak intensities. The C-C stretching vibration in aromatic ring appeared at 1440 cm^{-1} significantly decreases in the inclusion complexes as it is more preferable for phenyl ring of fisetin to stay in the β-CD cavity [26]. No peak is found at 1600 cm^{-1} and 1270 cm^{-1}. In addition, the peak width of O-H of pure β-CD at 3299 cm^{-1} was found to be narrowed in the FTIR spectrum of the inclusion complex. All of the above results are a good indication of an interaction between fisetin and β-CD, which is van der Waals force, for β-CD to encapsulate fisetin into its cone cavity and form an inclusion complex [27].

Furthermore, Figure 3 shows the DSC analysis between fisetin and the FIT/β-CD inclusion complexes through freeze-drying method with the mole ratio of fisetin: β-CD at 1:2. Melting point of the inclusion complexes is at 78.60°C which differs from melting point of fisetin at 141.80°C. Additionally, as pure fisetin is encapsulated in the β-CD cavity, no sharp endothermic peak is observed from such mixture in the volatilization range of pure fisetin [28]. These can be an obvious evidence that the mixture of fisetin and β-CD originated from freeze-drying method at the mole ratio of 1:2 is an inclusion complex between fisetin and β-CD.

3.2 Release profile of FIT/β-CD inclusion complex

According to λmax of fisetin and inclusion complex prepared by freeze-drying, kneading and physical mixing methods in 300-500 nm wavelength measured by spectrophotometer, the maximum light absorption rate is at 367 nm as shown in Figure 4. Thus, the wavelength in this research will be set as 367 nm for analyzing the release profile of FIT/β-CD inclusion complex.

Figure 5 illustrates the amount of fisetin which was emitted from FIT/β-CD inclusion complex prepared with the mole ratio of 1:2 and 2:1 by freeze drying, kneading and physical mixing methods as shown in Table 1. It was clear that the fastest emission rate of FIT/β-CD inclusion complex was the one prepared by physical mixing, kneading and freeze-drying methods, respectively, according to the graph of % cumulative release versus time. The result was caused by mostly fisetin of the mixture from physical mixing method unsealed by leading to be attacked by solvents and disintegration, considering from the outcome of FTIR characteristic obviously shown in Figure 2(g)-2(h), whereas FIT/β-CD inclusion complex prepared by freeze-drying method has the highest ability to maintain fisetin.

In addition, the synthetic FIT/β-CD inclusion complexes with the mole ratio of fisetin:β-CD at 2:1 can emanate fisetin faster than its ratio at 1:2. The reason behind this property is that the sufficient amount of molecule as a host is able to maintain much more and longer fisetin. Hence, it can be concluded that FIT/β-CD inclusion complex by freeze-drying method with the mole ratio of 1:2 of fisetin to β-CD constantly emit fisetin at the slowest rate. This matches with food packaging production requiring active packaging materials which antioxidant effectively works throughout the lifespan of particular substance. In other word, this FIT/β-CD inclusion complex is the most effective maintenance of fisetin and can be the most suitable process for food packaging development.

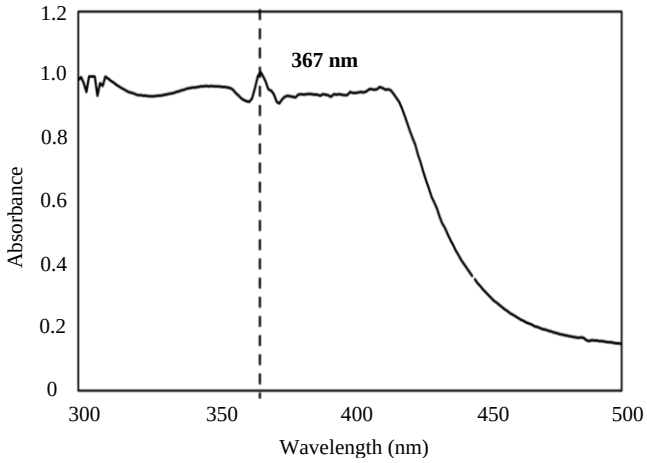

Figure 4. Absorption spectrum of light of fisetin.

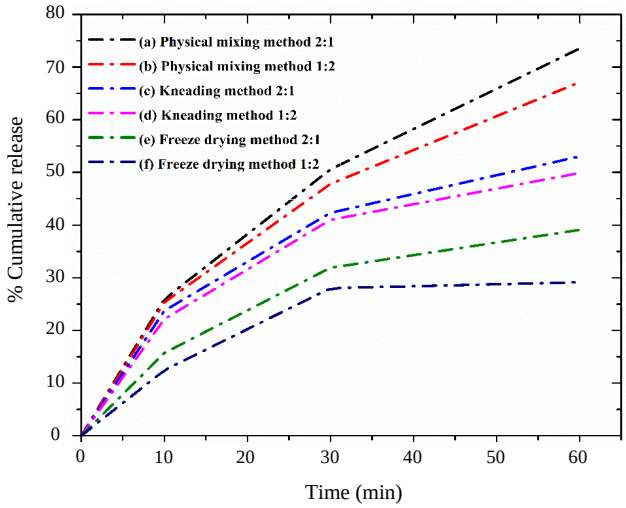

Figure 5. The amount of fisetin which was emitted from FIT/β-CD inclusion complex prepared with the mole ratios of 1:2 and 2:1 (fisetin:β-CD by freeze drying, kneading and physical mixing methods.

Table 1. The percentage of fisetin which were emitted fisetin from FIT/β-CD inclusion complex prepared with the mole ratio 1:2 and 2:1(fisetin: β-CD) by freeze drying, kneading and physical mixing methods.

Time (min)	The percentage of fisetin which were emitted fisetin from FIT/β-CD inclusion complex (%)					
	Physical mixing method		Kneading method		Freeze drying method	
	FIT:β-CD at 2:1	FIT:β-CD at 1:2	FIT:β-CD at 2:1	FIT:β-CD at 1:2	FIT:β-CD at 2:1	FIT:β-CD at 1:2
0	0	0	0	0	0	0
10	25.96	25.35	23.75	22.15	15.74	12.46
30	50.57	47.81	42.34	40.99	31.90	28.02
60	73.56	67.11	53.04	49.89	39.09	29.14

3.3 PLA porous film and PLA-FIT/β-CD inclusion complexes incorporate in porous films

The surface morphology of the PLA porous film was characterized by scanning electron microscope (SEM). Figure 6(a) shows the microporous structure of PLA film in honeycomb structure which approximated diameter of pore size is 23.22±4.10 μm and the smaller average pore size of 8.53±3.61 μm occurred in the main pore and the specific surface area is 9 $m^2 \cdot g^{-1}$. The PLA porous film with high surface area provides high entrapment capacity for the FIT/β-CD inclusion complexes which these particles will be trapped in the pore and results in attaching with PLA porous film by van der Waals force. In addition, these particles of FIT/β-CD inclusion complex can generate gravitation that creates loosening attachment or aggromolation illustrated in Figure 6(b) and it is considered a suitable substrate for food packaging application.

3.4 Antioxidant activity by DPPH assay

The antioxidant activities of PLA porous film and PLA-FIT/β-CD inclusion complexes porous film samples were evaluated by DPPH scavenging assay. This method allows to determine the percentage of free radicals scavenged by the sample. DPPH is a stable free radical and its color is purple. Due to its presence, an odd electron DPPH exhibits maximum absorbance at 517 nm. In presence of antioxidants such as a hydrogen donor, the odd electron of DPPH is paired off, resulting in a decrease in absorbance. This results in decolorization of the solution which the color changes from purple to pale yellow. The extent of loss in color is thus dependent on the reducing ability of the antioxidant species. Fisetin is a well known antioxidant that acts as hydrogen atom donor. In this study, the color of DPPH solution in presence of pure fisetin, FIT/β-CD inclusion complex and PLA-FIT/β-CD inclusion complex changes from purple to pale yellow and the absorbance decreases while PLA porous film does not change in color of DPPH solution. Decolorization of the solution affirms the antioxidant activity of fisetin and FIT/β-CD inclusion complex. It have been calculated to the percentage of DPPH radical scavenging activity using equation (1). This is found to be 48.6%, 53.0%, 69.3% and 81.8% for fisetin incorporating in PLA porous film (PLA-FIT), PLA-FIT/β-CD inclusion complex (PLA-FIT/β-CD), pure fisetin (FIT) and ascorbic acid standard (vitamine C), Figure 7.

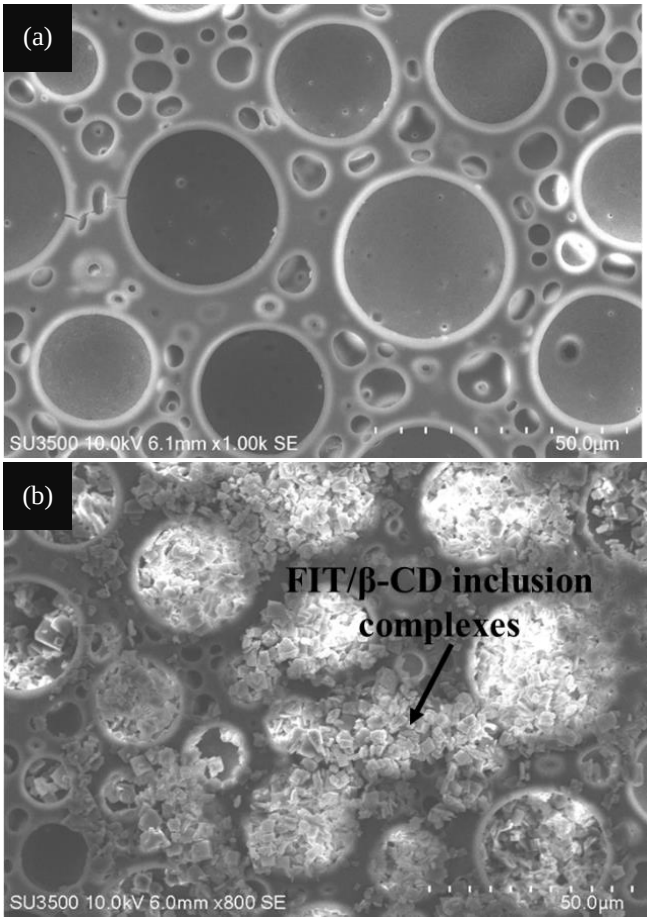

Figure 6. SEM microstucture of (a) PLA porous film fabricated by breath figure method and (b) FIT/β-CD inclusion complexes incorporated in porous films.

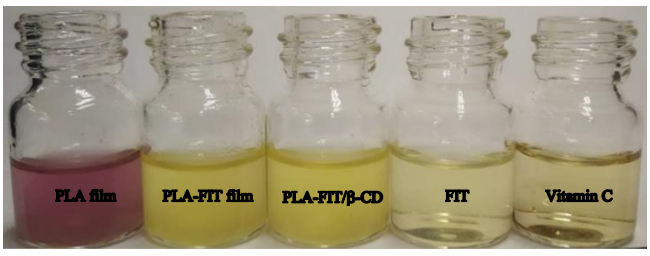

Figure 7. Color change of DPPH radical scavenging activity of pure PLA film, PLA-FIT, PLA-FIT/β-CD, pure fisetin (FIT) and vitamin C (from left to right).

4. Conclusion

Maintaining effectiveness of fisetin can be executed through an encapsulation of fisetin by freeze-drying method with the mole ratio of 1:2 and 2:1 of fisetin: β-CD. Not only does it preserve but also control fisetin emission better than kneading and physical mixing method. Freeze-drying method has an ability of synthetic of FIT/β-CD inclusion complex which can be analyzed from FTIR and the result of DSC of melting point which decreases the point from 141.80°C to 78.60°C effecting the most effectiveness in maintenance. It can also control consistent and stable fisetin emission which turns into the most suitable active agents for food package. Last but not least, smart active packaging is synthesized by porous PLA film from BF method entrapping fisetin and FIT/β-CD inclusion complex. The results show that it is beneficial to be an antioxidant resource by DPPH method containing %AA (antioxidant activity) equal to 48.6% and 53.0%, respectively. Thus, it becomes the most appropriate material for smart active packaging which can retain food quality and nutrition.

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