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Preparation and characterization of materials based on fish scale collagen and polyphenols extracted from *Camellia chrysantha*

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Abstract. This paper presents the preparation and some characteristics of collagen/polyphenols materials containing different polyphenols contents. Collagen was extracted from fresh-water fish scales and polyphenols were extracted from *Camellia chrysantha* (Tam Dao, Vinh Phuc, Viet Nam). A comparison between these polyphenols and green tea polyphenols (commercial product) was carried out in this study. Functional groups and morphology of collagen/polyphenols materials were determined by Infrared spectroscopy (IR) and scanning electron microscope (SEM). Ultraviolet-visible spectroscopy (UV-Vis) was used to evaluate the release of polyphenols from collagen/polyphenols materials in simulated body fluids. In addition, the anti-oxidation ability of collagen/polyphenols materials was also investigated by 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging method. The obtained results showed that polyphenols with the size of *ca.* 100 nm were dispersed and adhered well to the collagen matrix. The release of polyphenols from the materials follows two stages: fast release during the first 2 hours of testing and controlled release in the following hours. The content of polyphenols released from the collagen/polyphenols materials in pH 2.0 solution was higher than that in pH 7.4 solution. After 8 hours of testing, the content of polyphenols released from the collagen/polyphenols materials reached 15-65 % depending on the polyphenols content, type of polyphenols and pH of solution. The anti-oxidation ability of polyphenols extracted from *Camellia chrysantha* is better than that of green tea polyphenols and collagen/polyphenols materials.

Keywords: Tea polyphenols, Camellia chrysantha, fish scale collagen.

Classification numbers: 2.5.2, 2.7.1, 2.9.4.

1. INTRODUCTION

Tea (*Camellia sinensis*, Theaceace) is one of the healthiest beverages in Viet Nam as well as other countries. Polyphenols are known as the most potential substances in tea which have many valuable bioactivities such as anti-oxidation, anti-bacteria, anti-cancer, anti-inflammatory, anti-thrombotic, anti-angiogenesis and so on. The tea polyphenols mainly include epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC). EGCG is the most abundant catechin and may account for 50 - 75 % of total catechins [1].

Camellia chrysantha (yellow flower tea), a tea rich in polyphenols, is commonly cultivated and exploited in Ba Che District, Quang Ninh Province (Viet Nam) and Tam Dao District, Vinh Phuc Province (Viet Nam). The polyphenols in *Camellia chrysantha* are characterized by di- or tri-hydroxyl groups on ring B and meta-5,7-dihydroxyl groups on ring A. These structures have strong anti-oxidation ability. Moreover, the anti-oxidant activity of tea polyphenols is enhanced by the presence of a tri-hydroxyl structure in the D (gallate) ring in EGCG and ECG [2-4].

Andzi Barhé *et al.* showed that over 95 % of the anti-oxidation effectiveness of tea extracts is due to polyphenols [5]. The IC50 value of tea extracts determined by the DPPH method was 2.24 µM and was significantly lower than that of ascorbic acid (4.62 µM). The strong anti-oxidation activity of polyphenols in yellow flower tea was also confirmed by Song *et al*. [6]. In addition, the compounds in yellow tea showed positive effects on cardiovascular system and anti-cancer, especially breast cancer [7-8]. In an *in vivo* study, Yokozawa Kitani *et al*. reported the effectiveness of green tea polyphenols against obesity in cholesterol-enhanced mice (obese mice) [9]. Tea polyphenols may induce thrombotic anti-ulcer responses due to their anti-oxidation properties and increase levels of high-density lipoprotein cholesterol.

Ngo Thi Thao extracted polyphenols from yellow flower tea in Ba Che District, Quang Ninh Province (Viet Nam) and found that the total polyphenol content in *Camellia sp1*. leaves (CA2), *Camellia chrysanthoides* (CA3), *Camellia chrysantha* (CA4.1, CA4.2), and *Camellia sp2*. (CA5) was 20.104, 6.213, 7.787, 6.522, and 8.449 %, respectively. The EGCG content in *Camellia sp1*. leaves and *Camellia chrysanthoides* was 3.64 and 0.40 %, respectively. All *Camellia* samples exhibited over 80 % anti-oxidation activity at a concentration of 100 µg/mL. These polyphenols exhibited a moderate inhibitory effect on liver cancer cells (Hep-G2), breast cancer cells (MDA-BA-231) and rectal cancer cells (SW480), and a low effect on skin cancer cells (SK-Mel2) and lung cancer cells (LU-1) [10]. Nguyen Thi Ha Ly also obtained polyphenols from yellow *Camellia* leaf extract in Thai Nguyen Province (Viet Nam). The leaf extract had anti-oxidation effect with SC50 value of 42.62 µg/mL according to DPPH method [11].

The applications of tea polyphenols in the food and pharmaceutical industries are limited due to their low chemical stability, low solubility, and slow absorption into the blood. Therefore, to overcome these limitations, polyphenols are loaded with natural polymers. Polymeric drug delivery systems are known as excellent materials for encapsulating drugs [12 - 17]. The combination of polyphenols with natural polymers can improve solubility and bioavailability of polyphenols. Debnath *et al*. prepared polymer nanoparticles carrying polyphenols in green tea to inhibit protein agglutination, and reduction of cytotoxicity [18]. The composites based on biocompatible polymers such as chitosan, poly (caprolactone) (PCL), poly (lactide-co-glycolide) and tea polyphenols can enhance the solubility of tea polyphenols, leading to a positive effect of polymer/polyphenols systems on anti-cancer and necrosis of injury, etc. [19 - 20]. Cao *et al*. fabricated a PCL/tea polyphenol complex and applied it in the treatment of DNA reduction caused by benzo[α]pyrene. The authors reported that the PCL/tea polyphenol complex is highly stable and can significantly reduce the DNA caused by benzo[α]pyrene [21].

Collagen is the most common protein in vertebrates. It plays an important role in linking cells together into tissues and organs and ensuring the mechanical stability and durability of the tissues [16]. Besides, collagen is non-toxic, highly biocompatible and rapidly absorbed. In addition, collagen contains hydroxyl, carboxyl and amine groups which can interact with hydroxyl groups in tea polyphenols through hydrogen bonds [22 - 24]. Therefore, in this study, collagen was chosen as a carrier for *Camellia chrysantha* (Tam Dao District, Vinh Phuc Province, Viet Nam). Collagen from fish exhibits many advantages such as high absorption rate, causing no infectious diseases, and availability of fish scales or fish skins from seafood processing. In our previous study, collagen was successfully extracted from fresh-water fish scales [23]. Thus, the highlight of this paper is to evaluate the anti-oxidation ability of the materials based on collagen from fresh-water fish scales and polyphenols from *Camellia chrysantha*. Thereby, some characteristics, properties of the materials are determined and discussed.

2. MATERIALS AND METHODS

2.1. Materials

Green tea polyphenols (≥ 98 % (HPLC)) (PPc) is a commercial product of China. *Camellia* polyphenols (PP) were extracted from *Camellia chrysantha* leaves (Tam Dao District, Vinh Phuc Province, Viet Nam) at the National Institute of Medicinal Materials. The polyphenol content in PP sample was 30.25 mg/kg (determined according to FIRLM277 method (TCVN 9745-1:2013) by the National Center for Food Analysis and Assessment). Collagen was extracted from fresh-water fish scales at the Institute for Tropical Technology, Vietnam Academy of Science and Technology (VAST) according to the procedure reported in our previous literature [23]. Some other analytical chemicals such as glutaraldehyde (50 % in water), acetic acid 99.5 %, ethanol 99.9 %, HCl 37 %, $Na_2HPO_4.2H_2O$, $NaH_2PO_4.7H_2O$, and NaOH are commercial products of China. The chemicals are used as received without further refining.

2.2. Preparation of collagen/polyphenols

Preparation of collagen mixture (A) using glutaraldehyde as a crosslinker

1 g of collagen was added to 50 mL of 1 % CH_3COOH solution. The mixture was then stirred on a magnetic stirrer with a rotor speed of 400 rpm at room temperature until collagen was completely dissolved. Then, glutaraldehyde solution was slowly dropped into the collagen solution and the mixture was continuously stirred for 30 minutes to obtain mixture A.

Preparation of polyphenol solution

Polyphenols (PPc or PP) was added to 10 mL of ethanol. The mixture was stirred until polyphenols were completely dissolved to obtain solution B.

Preparation of collagen/polyphenol materials

Solution B was dropped slowly into solution A while solution A was stirred continuously. Next, the solution was ultrasonicated at a speed of 18000 rpm for 30 minutes to obtain a homogenous solution. The solution was then stirred on a magnetic stirrer for 60 minutes to reach a stable status.

After that, the solution was poured into a Petri dish and allowed to dry at room temperature to obtain the collagen/polyphenols materials in powder form as shown in Table 1.

Table 1. Component ratio and abbreviation of collagen/polyphenols materials.

Collagen (g)	PP (g)	PPc (g)	Glutaraldehyde (g)	Abbreviation
1	0.05	0	0.005	COLPP5
1	0.10	0	0.005	COLPP10
1	0	0.05	0.005	COLPPc5
1	0	0.10	0.005	COLPPc10

2.3. Characterization

Infrared spectroscopy (IR): IR spectra of collagen/polyphenols materials were recorded on a Nicolet iS10 spectrometer (Thermo Scientific, USA) using attenuated total reflectance (ATR) technique at the Institute for Tropical Technology, VAST in the wavenumber range 4000 - 400 cm^{-1} with a resolution of 8 cm^{-1} and an average of 32 scans at room temperature.

Field Emission Scanning electron microscopy (FE-SEM): FESEM images of collagen/polyphenols materials were analyzed on a FESEM S4500 (Hitachi, Japan) at the Institute of Material Science, VAST under a voltage of 80 kV and a magnification of 50,000. Samples were coated with silver or platinum to improve the quality of the FESEM images at high magnification.

Ultraviolet-visible (UV-Vis) spectroscopy was used to determine the concentration of polyphenols released from the materials in simulated intestinal fluid (pH 7.4) and simulated gastric fluid (pH 2). The tests were carried out on a S80 Libra (Biochrom, United of Kingdom) at the Institute for Tropical Technology, VAST.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method was used to evaluate the antioxidant activity of tea polyphenols and collagen/polyphenols materials. The antioxidant activity shown by the color reduction of DPPH was determined by UV-Vis measurement at a maximum wavelength of 517 nm. The tests were carried out at the Institute of Natural Products Chemistry, VAST. DPPH free radical scavenging percentage of samples was calculated according to the following formula:

$$SC\% = (OD\ control - OD\ sample)/OD\ control\ (\%) \quad (1)$$

SC_{50} (concentration at which 50 % of free radicals was neutralized) was calculated as an SC value relative to different concentrations of the samples [25].

2.4. Polyphenols release study

Study on the release of polyphenols from collagen/polyphenols materials and free polyphenols was carried out in pH 2 and pH 7.4 buffer solutions. 0.1 g of samples was added to a 250 mL Erlenmeyer flask. Next, 200 mL of fresh buffer solution was poured into the flask. The mixture was then stirred continuously on a magnetic stirrer at 37 ^oC for 8 hours at a speed of 400 rpm.

After every 1 hour of testing, 5 mL of solution was withdrawn and 5 mL of fresh buffer solution was added into the flask. The withdrawn solution was subjected to UV-Vis spectroscopy at 200-400 nm. The amount of polyphenols release was calculated based on the calibration equations to determine the polyphenols concentration in solutions with different pH using Excel software. The amount of polyphenols release (X) was calculated according to the following formula:

$$X(\%) = \frac{Polyphenols\ at\ t\ time}{Polyphenols\ in\ samples} \times 100 \quad (2)$$

3. RESULTS AND DISCUSSION

3.1. Infrared spectra of collagen/polyphenol materials

IR spectra of collagen, polyphenols and collagen/polyphenols materials containing different polyphenols contents are shown in Figure 1. The vibrations of O-H, C-H, C = O, C = C and C-O groups in PP and PPc were similar. The polyphenols extracted from *Camellia chrysantha* have less purity than commercial tea polyphenols, therefore, an additional peak appeared at 1515 cm^{-1} characteristic of the bending vibration of the N-H group in the impurity.

The IR spectrum of collagen shows peaks representing the vibrations of amide A at 3277 cm^{-1}, amide B at 3068 cm^{-1}, C-H at 2926 cm^{-1}, amide I at 1630 cm^{-1}, amide II at 1538 cm^{-1}, and amide III at 1238 cm^{-1} [24].

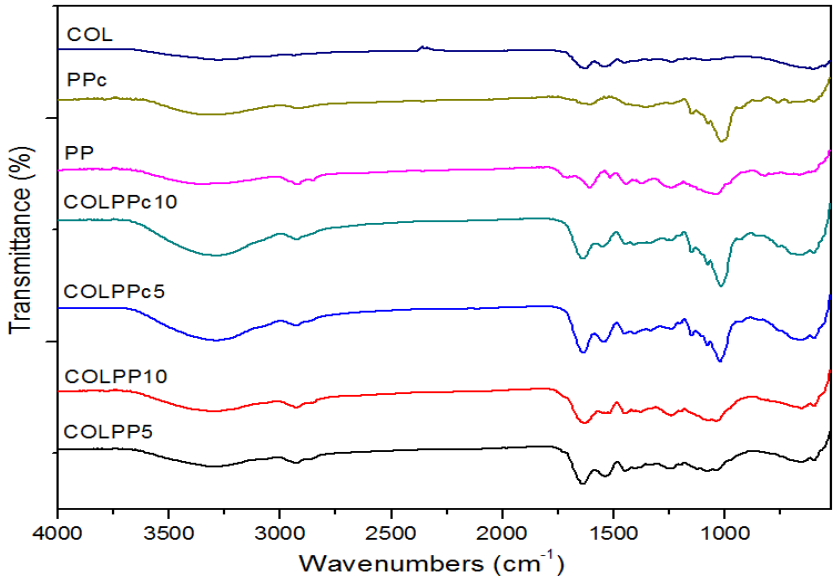

Figure 1. IR spectra of collagen/polyphenols materials.

The vibrations of functional groups in collagen and polyphenols were also indicated in the IR spectra of collagen/polyphenols materials. Owing to the slight shift of wavenumbers characteristic of the vibrations of the O-H and N-H groups in the IR spectra of the samples as listed in Table 2, it can be recognized that collagen can weakly interact with polyphenols through hydrogen bonding between the O-H groups in polyphenols and the amide groups in collagen [22]. The peaks of the C=O group of glutaraldehyde at about 1700 cm^{-1} cannot be seen on the IR spectra of collagen/polyphenol materials suggesting that glutaraldehyde was crosslinked with collagen during processing.

Table 2. Wavenumbers of characteristic vibrations in IR spectra of collagen, PP, PPc and collagen/polyphenols materials.

Sample	Wavenumbers (cm^{-1})				
	Amide A, Amide B, N-H, O-H	C-H	Amide I, C=O, C=C, O-H	Amide II, N-H,	Amide III, C-O, C-C
Collagen	3277 3068	2926 1452 1336	1630	1538	1238 1081
PP	3347	2924 1444 1377	1709 1609	1515	1241 1041
PPc	3317	2921 1408 1355	1709 1608	-	1242 1015
COLPP5	3293	2927 2844 1447	1637	1536	1242 1077
COLPP10	3294	2926 1447 1381	1631	1517	1241 1038
COLPPc5	3288	2927 1449 1335	1634	1547	1240 1021
COLPPc10	3288	2928 1407	1639	1550	1244 1018

3.2. Morphology of collagen/polyphenols materials

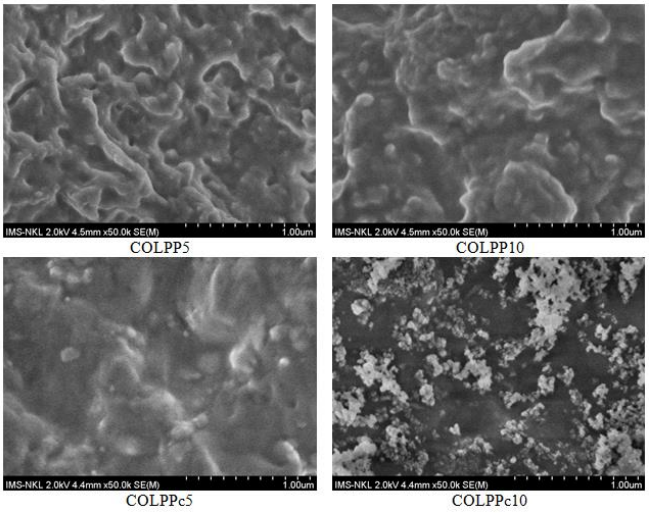

Figure 2. SEM images of collagen/polyphenols materials.

SEM images of collagen/polyphenols materials containing different polyphenols contents in Figure 2 indicate that the polyphenols were uniformly dispersed in the collagen matrix with a

size of *ca.* 100 nm. The adhesion of the polyphenols extracted from *Camellia chrysantha* to the collagen matrix was better than that of the commercial tea polyphenols (less portion of surface light particles). When using a large amount of PPc, the light particle portion on the surface of COLPPc10 was dense, indicating that PPc was poorly adhered to the collagen matrix.

3.3. Amount of polyphenols released from collagen/polyphenols materials

Calibration equations are necessary for calculating the amount of polyphenols released in solution. Table 4 displays the calibration equations for determining polyphenol concentrations in pH 2 and pH 7.4 solutions (simulated gastric and intestinal fluids) at the maximum absorption wavelength of PP and PPc, in which x is concentration of polyphenols and y is optical density. The linear regression coefficients (R^2) of these equations are nearly 1, thus, these equations can be used for calculating the polyphenols amount released from collagen/polyphenols materials in pH 2 and pH 7.4 solutions.

Table 4. Calibration equations for calculating the polyphenols amount in solution and linear regression coefficients (R^2).

pH of solution	λ_{max} (nm)	Calibration equation	R^2
PP			
2	222	y = 5823.3x + 0.0606	0.9991
7.4	226	y = 13716x + 0.048	0.9990
PPc			
2	215	y = 9465.7x + 0.1213	0.9990
7.4	217	y = 8366.4x + 0.1255	0.9998

Figures 4 and 5 demonstrate the amount of polyphenols released from free polyphenols and collagen/polyphenols materials in pH 2 and pH 7.4 solutions versus time of testing. It can be seen that polyphenols were released quickly from samples during the first 2 hours of testing and then, gradually and in a controlled manner over the subsequent testing hours.

In the pH 2 solution, the PP amount released from free polyphenols was lower than PPc at the same testing time. Polyphenols were released from COLPP5, COLPP10 and COLPPc5 faster than others and tended to increase continuously during the testing process. An increase in the amount of polyphenols released from samples in acid environment could be caused by the protonation of amine groups in collagen, leading to the swelling and dissolution of collagen. As a result, polyphenols adhered to collagen was released into the solution together with collagen. With the same initial amount of polyphenols, the COLPP5 and COLPP10 samples had a higher amount of released polyphenols than the COLPPc5 and COLPPc10 samples. This could be explained by good interaction and adhesion of PP with the collagen matrix as compared with PPc as seen on SEM images (Figure 2). That made the amount of PP released into the solution higher than that of PPc when the structure of collagen was changed by the protonation. The amount of released polyphenols of the COLPPc5 sample was higher than that of COLLPPc10 while for the COLPP samples, a reversal change to the above trend was observed. This could be due to the difference in purity of the polyphenols samples. As commercial polyphenols had a purity of 98 %, it could be compatible with collagen when used in low amounts but it was poorly dispersed and adhered to collagen in large amounts (as can be seen in the light particle part in Figure 2).

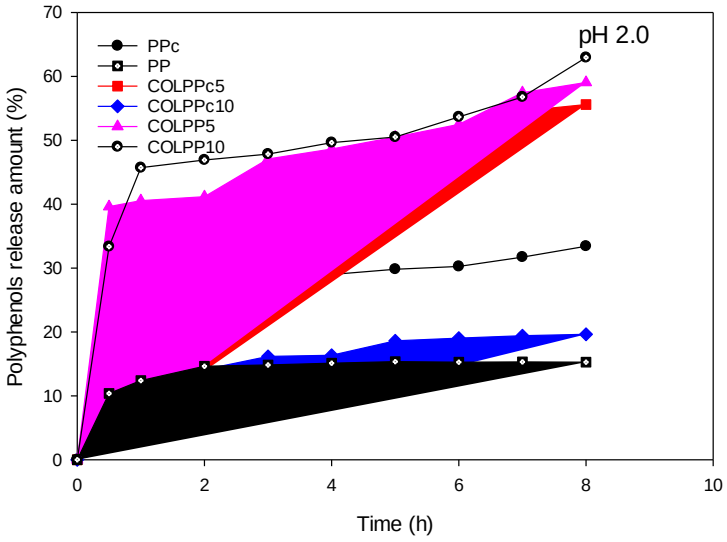

Figure 4. Polyphenols release amount from free polyphenols and collagen/polyphenols materials in pH 2 solution.

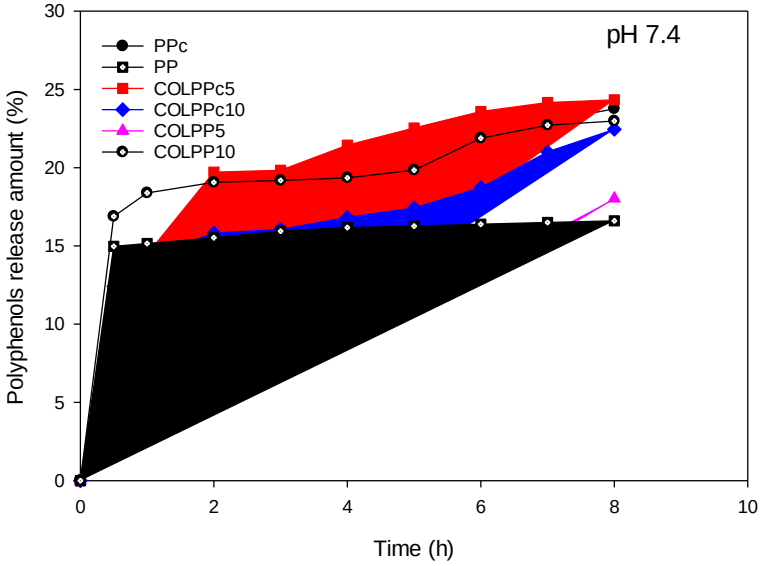

Figure 5. Polyphenols release amount from free polyphenols and collagen/polyphenols materials in pH 7.4 solution.

Comparing the amount of polyphenols released from samples in pH 2 and pH 7.4 solutions, the amount of polyphenols released from samples in the pH 2 solution was higher than that in the pH 7.4 solution at the same time because collagen was protonated in the pH 2 solution as mentioned above. In the pH 7.4 solution, the polyphenol content released from both free polyphenols and collagen/polyphenols materials tended to increase over time of testing. The amount of polyphenols released from the PP and COLPP samples was arranged as COLPP10 > PP > COLPP5, while the amount of polyphenols released from the PPc and COLPPc samples was in the order of COLPPc5 > COLPPc10 > PPc (for the first 3 hours of testing) and COLPPc5

> PPc > COLPPc10 (in the next 5 hours). The release of polyphenols from samples was not systematical due to the difference in composition between the polyphenols extracted from yellow flower tea (Tam Dao District, Vinh Phuc Province, Viet Nam) and the commercial polyphenols. In general, the COLPPc5 and COLPP10 samples had lower polyphenols release content as compared to free polyphenols in the pH 7.4 solution, demonstrating an advantage of collagen/polyphenols materials.

3.4. Anti-oxidation activity of polyphenols and collagen/polyphenols materials

The results of anti - oxidation activity assessment of polyphenols and collagen/polyphenols materials are presented in Table 5, showing that the anti-oxidation activity of polyphenols samples is different. The SC_{50} values of PP and PPc are 79.13 and 159.35 µg/mL, respectively, demonstrating that PPc has less anti-oxidation activity than PP. However, compared to polyphenols extracted from *Camellia chrysantha* in (Ba Che District, Quang Ninh Province, Viet Nam), polyphenols extracted from *Camellia chrysantha* in this study had lower anti - oxidation activity (SC_{50} of the polyphenols extracted from *Camellia sp.1* (Ba Che District, Quang Ninh Province, Viet Nam) is 25.88 µg/mL [10]). The ability to neutralize free radicals (SC, %) of collagen/PPc materials is lower than that of collagen/PP materials when using the same polyphenol amount. COLPP5 exhibits much better anti - oxidation activity than COLPP10 (SC_{50} of 239 and 970 µg/mL for COLPP5 and COLPP10, respectively). When combining collagen with polyphenols, because the polyphenol content in the sample only accounts for 5 %, the anti - oxidation activity of the composite is about 3 times lower than that of the polyphenols.

Table 5. Results of assessment of anti - oxidation activity of polyphenols and collagen/polyphenols materials.

Sample	The ability to neutralize free radicals (SC, %)	SC_{50} (µg/mL)
Control (+) [Ascorbic acid]	87.53 ± 0.3	11.50
Control (-) [DPPH/EtOH+DMSO]	0.0 ± 0.0	-
PP	71.36 ± 1.2	79.13
PPc	57.80 ± 1.3	159.35
COLPP5	68.62 ± 0.5	239
COLPP10	55.56 ± 1.1	970
COLPPc5	15.40± 2.0	≥ 250
COLPPc10	14.01± 0.1	≥ 250

4. CONCLUSION

In this paper, collagen/polyphenols materials were succesfully prepared in powder form by solvent evaporation method combined witth ultrasonication using glutaraldehyde as a crosslinker and collagen extracted from fresh water fish scale as a carrier. A comparison between the characteristics of collagen materials loaded with green tea polyphenols and collagen materials loaded with polyphenols extracted from yellow flower tea (*Camellia chrysantha*, District, Vinh

Phuc Province, Viet Nam) was made. The IR and SEM results indicate that yellow *Camellia* polyphenols can interact with and adherse to the collagen matrix better than commercial polyphenols. The collagen matrix contains particles with a size of *ca.* 100 nm. Polyphenols can release from free polyphenols and collagen/polyphenols materials in pH 2 and pH 7.4 solutions with two stages: fast release for the first 2 hours of testing and stable release for the next testing hours. The collagen/polyphenols materials can improve the release of polyphenols in both pH 2 and pH 7.4 solutions. The anti-oxidation activity of yellow *Camellia* polyphenols is better than that of commercial polyphenols. The collagen/polyphenols materials have less anti-oxidation activity than polyphenols.

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CRediT authorship contribution statement. Nguyen Thuy Chinh: Writing paper, Methodology, Investigation, Funding acquisition. Mai Duc Huynh: Formal analysis. Ly Thi Ngoc Lien: Formal analysis. Tran Thi Mai: Formal analysis. Thai Hoang: Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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