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Development and characterization of fish myofibrillar protein/chitosan/rosemary extract composite edible films and the improvement of lipid oxidation stability during the grass carp fillets storage

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23 **Abstract:** Biofilm composition from fish myofibrillar protein (FMP) and chitosan solution (CS)  
24 incorporated with rosemary extract (RE) was developed and applied to monitor the freshness of fish  
25 fillets. The effects of different concentrations of RE (0.05%, 0.1%, 0.15%, 0.2%, v/v) as well as  
26 physical, mechanical, structural and functional properties of FMP/CS films were investigated. Films  
27 containing RE showed reduced water solubility and water vapor permeability (WVP) and enhanced  
28 tensile strength and elongation at break. Results of X-ray diffraction (XRD), scanning electron  
29 microscopy (SEM) and atomic force microscopy (AFM) suggest that there was good compatibility  
30 of the components and good dispersion of RE in the matrix. However, the content of RE (0.2%, v/v)  
31 added in the composite films produced aggregations and had negative effects on their film-forming  
32 properties. The antioxidant capacity of composite films was related to the level of RE and  
33 demonstrated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. Chilled  
34 grass carp fillets wrapped with different films to evaluate the preservative effect were stored at 4 °C.  
35 Results of thiobarbituric acid reactive substances (TBARS), pH value, Free amino acid (FAA) and  
36 total volatile basic nitrogen (TVB-N) indicated that FMP/CS/RE composite film could protect the  
37 fish fillet well and inhibit the lipid oxidation. In summary, the developed FMP/CS/RE composite  
38 films possess the potential to be applied as edible films in the food packaging industry and food  
39 cold chain transportation.

40 **Keywords:** Fish myofibrillar protein; Rosemary extract; Composite edible films; Biofilm  
41 composition;

42

## 43 1. Introduction

44 During the past decade, composite edible films and packaging have drawn more attention due  
45 to their excellent barrier, edibility, renewable and biodegradable properties compared with their  
46 synthetic counterparts [1]. Various natural resources such as polysaccharides, proteins, lipids and  
47 carbohydrates are often used to develop environmentally friendly biopolymer films [2]. Proteins are  
48 one of the biopolymer materials empirically used as edible food packaging materials on account of  
49 their good film-forming ability [3]. To enhance the mechanical and physicochemical properties of  
50 edible films, proteins are usually mixed with polysaccharides, carbohydrates, polyphenols *etc.* [4-  
51 6].

52 Myofibrillar protein (MP) is a popular protein-based hydrophilic polymer extract from aquatic  
53 products. Fish myofibrillar protein (FMP) can form a continuous matrix during the drying process,  
54 which can be used to prepare transparent films with certain mechanical properties. However, due to  
55 the existence of disulfide bonds, hydrogen bonds, electrostatic forces and hydrophobic interactions  
56 in intermolecular MP, the individual MP-based biofilm is more fragile. Therefore, some researchers  
57 have focused on the modification of FMP-based edible films by adding different kinds of additives.  
58 FMP-based films incorporated with procyanidins and tea polyphenol could improve the thermal  
59 stability and rigidity of films [7]. FMP based film modified with phenolic compounds suggest  
60 stronger and stiffer film structure by allowing crosslinking of MP and polyphenols [8]. Bacterial  
61 cellulose nanofibers were employed in order to reinforce the various properties of edible FMP  
62 nanocomposite films [9]. Furthermore, technological properties were improved by adding chitosan  
63 solution (CS) to MP based films [10].

64 Presently, rosemary extract (RE) is widely used in the inhibition of lipid oxidation, meat and

65 aquatic products preservation. For example, the application of RE in glazing treatment on  
66 preservation of mud shrimp significantly reduced loss of quality protein and lipid changes [11]. RE  
67 is also useful in decreasing quality loss and inhibiting lipid oxidation of minced sea salmon muscles  
68 during frozen storage [12], inhibiting lipid oxidation and maintaining color stability during storage  
69 of beef burgers [13]. Furthermore, there are many more papers in which RE was added to the edible  
70 film. For example, when RE was added to the cellulose/gelatin hydrolysate composite film it  
71 improved the mechanical and ultraviolet light barrier properties of the film [14]. The oxidative and  
72 microbial stability of smoked eel fillets were also enhanced by the edible coating enriched with RE  
73 [15].

74         However, there is few reports with regards to the application of RE to FMP/CS composite  
75 film. In this study, the modification of RE to FMP/CS composite film and the corresponding  
76 properties including mechanical properties, water solubility, light transmittance, water vapor  
77 transmission rate and morphology features were measured. Meanwhile, the effects of RE on the  
78 microstructure of FMP/CS composite film were further explored using X-ray diffraction (XRD),  
79 scanning electron microscopy (SEM) and atomic force microscopy (AFM). Moreover, the quality  
80 changes and lipid oxidation during inhibition of fresh fish fillets wrapped with FMP/CS/RE  
81 composite film, polyvinyl chloride (PVC) film and the untreated group were investigated and  
82 compared. The developed composite films possess the potential to be applied as edible films in the  
83 food packaging industry and food cold chain transportation.

84

## 85 **2. Materials and methods**

### 86 **2.1 Materials**

87         The fresh grass carp (~ 2.5 kg) was purchased from the local market in Huazhong Agricultural

88 University (Hubei, China). The fish were gutted, cleaned, filleted and mechanically deboned. The  
89 dorsal white muscle samples were frozen in liquid nitrogen and kept at  $-80\text{ }^{\circ}\text{C}$  until used for MP  
90 extraction. Chitosan with 90% degree of deacetylation was purchased from Sinopharm Chemical  
91 Reagent Co., Ltd. (Shanghai, China). RE was obtained from Hangzhou Linran Biotechnology Co.,  
92 Ltd. (Purity 80%, Hangzhou, China). All other reagents used in the present study were of analytical  
93 grade and acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

## 94 **2.2 Myofibrillar Protein extraction**

95 MP was extracted from grass carp as described in our previous work [16]. Briefly, dorsal white  
96 fish muscle was minced and rinsed in a low-salt phosphate buffer (0.05 mmol/L NaCl, 3.38 mmol/L  
97  $\text{NaH}_2\text{PO}_4$ , 15.5 mmol/L  $\text{Na}_2\text{HPO}_4$ , pH 7.5) in order to remove the water-soluble protein and other  
98 substances using a homogenizer (T18 digital ULTRA TURRAX, IKA, Germany) at a speed setting  
99 of 9000 g for 1 min. The homogeneous liquid was further centrifuged at 8000 g for 5 min by a  
100 refrigerated centrifuge ( $4\text{ }^{\circ}\text{C}$ , Avanti J-26 XP, Beckman Coulter, CA, USA). Then the obtained  
101 pellets were extracted at  $4\text{ }^{\circ}\text{C}$  in a high salt phosphate buffer (0.45 mmol/L, pH 7.5). After  
102 centrifugation, the supernatant was poured into deionized water at  $4\text{ }^{\circ}\text{C}$  to precipitate MP. Finally,  
103 the precipitate (MP) was collected by centrifugation, then diluted with 0.6 M NaCl Tris-HCl (pH  
104 7.5) buffer and kept in a fridge at  $4\text{ }^{\circ}\text{C}$  for further analysis. The MP concentration was determined  
105 by the Lowry method [17], with serum albumin used as the standard. The purity of MP was about  
106 8% (w/v).

## 107 **2.3 Edible film preparation**

108 The edible film-forming solution was prepared according to a previous method [18] with little  
109 modification. Distilled water was added to MP to obtain the protein concentration at 2% (w/v), then

110 homogenized at 9000 g for 1 min and the pH was adjusted to 3.0 with 1 M HCl. It was centrifuged  
111 at room temperature with a centrifugal force of 3000 g for 10 min and then the supernatant was  
112 collected. Glycerol was added as a plasticizer to the solutions at a concentration of 1.63% (w/v) and  
113 stirred at room temperature for 30 min. Chitosan was dissolved in 1% acetic acid to get the chitosan  
114 fully dissolved, and stirred overnight to make the final 2% CS. Finally, the solution was mixed with  
115 CS using a homogenizer to obtain the MP/CS (4.33:3.67) composite film forming solution,  
116 according to previous results of response surface optimization (data not shown).

117 To ensure full hydration of the biopolymer dispersions (MP, CS, and MP/CS), the dispersions  
118 were mixed together and homogenized for 30s [18] Then, different amounts of RE were added to  
119 achieve the final volume ratios of 0.05%, 0.1%, 0.15%, 0.2% similar to the former mixture of  
120 composite film (MP/CS). After mixing evenly, a volume of 40 mL of each film-forming solution  
121 was cast in polystyrene petri dishes (13 cm diameter), dried at 50 °C for 16 h. The edible composite  
122 films were stored in a desiccator at 25 °C and 50% relative humidity (RH) for 24 h. Dried films  
123 were manually peeled off from the surface for further analysis.

#### 124 **2.4 Film thickness and mechanical properties**

125 Film thickness was determined using digital micrometer (PosiTector 6000, DeFelsko  
126 Corporation, USA) with a precision of 0.001 mm. Five measurements at different positions were  
127 taken from each film sample to obtain the mean film thickness[19]. These measurements were  
128 obtained after a conditioning period at 25 °C with a relative humidity of 50%. Tensile strength (TS,  
129 MPa) and elongation at break (EB, %) of film samples were determined as formerly described [7],  
130 using a Texture Analyzer (TA.XT.plus, Stable Micro Systems, UK). The films were cut into 50  
131 mm×20 mm strips, then the initial grip separation and cross-head speed were set at 30 mm and 2

132 mm/s, respectively. TS was calculated by dividing the maximum force with the initial cross-  
133 sectional area of the film; EB (%) was calculated by dividing the film elongation at break with the  
134 initial gauge length of the specimen.

## 135 **2.5 Determination of moisture content and water solubility**

136 Film samples (4×4 cm<sup>2</sup>) were prepared and weighed ( $W_s$ ), then dried in an oven at 105 °C until  
137 constant weight was reached ( $W_i$ ). The moisture content (MS) was calculated as follows [20]:

$$138 \quad MS = \frac{W_s - W_i}{W_s} \times 100\% \quad (1)$$

139 Where  $W_s$  is the initial film weight (g) and  $W_i$  is the final film dry weight (g).

140 Water solubility (WS) of film samples in water was determined based on a former method [9].  
141 The conditioned film samples (4×4 cm<sup>2</sup>) were weighed and dipped into 50 mL of distilled water  
142 with 0.1% (w/v) sodium azide as an antimicrobial agent. The samples were then shaken at a speed  
143 of 250 rpm for 24 h at 25 °C. After centrifugation at 8000 g for 10 min, samples were dried at 105 °C  
144 for 24 h to determine the weight of the remaining pieces of films ( $W_f$ ). WS was calculated according  
145 to the following equation:

$$146 \quad WS = \frac{W_i - W_f}{W_i} \times 100\% \quad (2)$$

147 Where  $W_i$  is the final film dry weight (g) and  $W_f$  is the final dry weight of the remaining film  
148 (undissolved in water) weight (g). All tests were carried out in triplicate.

## 149 **2.6 Water vapor permeability (WVP) and water contact angle**

150 The WVP values of films were determined in triplicate (n=3) and measured gravimetrically  
151 according to the ASTM E96-05 Standard Method E96-00 [21]. A test cup (3 cm (depth) × 5 cm  
152 (diameter)) was filled with distilled water (15 mL) and the film (6 cm diameter) was placed over  
153 the opening. Then, the cups were placed in the desiccator at 25 °C and 50% relative humidity (RH).

154 Due to the difference in the water vapor pressure inside and outside the test cup, water was  
155 transferred through the film. The weights of the cups were recorded at 2 h intervals over a 12 h  
156 period. The measured WVP ( $\text{g}\cdot\text{mm}/\text{m}^2\cdot\text{h}\cdot\text{kPa}$ ) of the films was calculated as follows:

$$157 \quad WVP = \frac{\Delta m \cdot d}{A \cdot t \cdot \Delta p} \quad (3)$$

158 where  $\Delta m$  is the weight change of the cup (g),  $D$  is the mean film thickness (mm),  $A$  is the area  
159 of exposed film ( $\text{m}^2$ ),  $t$  is the time interval (2 h), and  $\Delta P$  is the water vapor pressure difference (kPa)  
160 on both sides of the film. All tests were performed at least in triplicate.

161 Contact angles of the composite films were tested by means of an OCA20 contact angle  
162 analyzer (Dataphysics, Germany). Briefly, 5  $\mu\text{L}$  per drop of distilled water was carefully deposited  
163 onto the surface of the film and angles were measured in five different regions of each surface and  
164 averaged. In addition, the image of the distilled water droplet was captured with a camera [9].

## 165 **2.7 Opacity (OP)**

166 To determine the opacity of the film, the ultraviolet spectrophotometer (Genesys 10S UV-VIS,  
167 Thermo Scientific, Thermo Fisher Scientific, USA) was used to measure the transmittance of the  
168 composite film at 350 nm to 800 nm. Meanwhile, absorbance of the film sample at 600 nm was used  
169 to calculate the opacity of the thin film. Each sample was repeated three times and the calculation  
170 formula was as follows:

$$171 \quad OP = \frac{A_{600}}{d} \quad (4)$$

172 where  $OP$  is opacity,  $A_{600}$  is the absorbance value at 600 nm;  $D$  is the film thickness (mm). The  
173 higher value of OP indicates higher opacity and low degree of transparency [22].

## 174 **2.8 Film morphology and color**

175 After the films were dried, the prepared film was cut into  $4 \times 4 \text{ cm}^2$  size and conditioned at  $50 \pm$   
176  $5\%$  RH (25 °C, 48 h), the visual aspect of film samples was examined using a digital camera

177 (Fujifilm Finepix S4900; acquired from Fujifilm Thailand Co. Ltd., Bangkok, Thailand).

178 A precision Colorimeter (Shang Guang WSC-S, Shanghai, China) was used to determine color  
179 attributes of the film and expressed as  $L^*$ ,  $a^*$ , and  $b^*$ .  $L^*$  (brightness),  $a^*$  (redness -greenness),  $b^*$   
180 (yellowness-blueness) values were recorded. The whiteness ( $W$ ) value was calculated using the  
181 following equation [23] :

$$182 \quad W = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (5)$$

### 183 **2.9 Thermogravimetric analysis (TGA)**

184 TGA of the samples was performed by a thermogravimetric analyzer (PYRIS 1, Perkin-Elmer,  
185 Waltham, Mass, USA) [24]. Each film sample (3-5 mg) was sealed in an aluminum plate and heated  
186 from 30 °C to 600 °C at a heating rate of 10 °C/min in a nitrogen atmosphere (20 ml/min).

### 187 **2.10 X-ray diffraction (XRD)**

188 XRD was used to examine the crystallinity of MP-based composite films according to a former  
189 method [25] with few modifications. All specimens were examined with a D8 Advance XRD  
190 (Bruker, Germany) at room temperature and a scan angle from 5-80° with a step size of 0.02° 2 $\theta$   
191 intervals using Ni-filtered  $K_{\alpha}$  Cu X-ray radiation (wavelength of X-ray was 1.54 Å).

### 192 **2.11 Scanning electron microscopy (SEM)**

193 SEM was used to capture the image of the surface and cross-section of films (JSM-6390LV,  
194 JEOL, Tokyo, Japan). The films (0.5 cm × 0.5 cm) were fixed on the bronze stub using double-sided  
195 tape and were sputtered with gold using Sputter Coater SCD 005 (BAL-TEC AG, Balzers,  
196 Liechtenstein). The voltage and the magnification used were 15 kV and 5000×magnification,  
197 respectively [26].

### 198 **2.12 Atomic force microscopy (AFM)**

199 The surface topography of the prepared films was determined using AFM (MFP-3D, Asylum  
200 Research, USA) operated in intermittent contact/tapping mode [27]. The images were collected at a  
201 fixed scan rate of 0.5 Hz. The sampling rate was 512 lines. The morphology and the roughness data  
202 were processed using MF3D software (version 111111 + 1219).

### 203 2.13 Antioxidant activity

204 The antioxidant activity of the different composite films was assessed using DPPH (2,2-  
205 diphenyl-1-picrylhydrazyl) free radical scavenging assay according to the method described by  
206 Siripatrawan et al. with slight modification [28]. Film samples (10 mg) were added to 10 mL  
207 methanol. After they had completely dissolved, the film samples were oscillated at 25°C for 2h. Then,  
208 500 µL extraction of the composite film was mixed uniformly with 4.0 mL 150 µmol/L DPPH, and  
209 incubated in the dark for 30 min. The absorbance of the solution was measured at 517 nm, the  
210 percentage of DPPH free radical scavenging activity was calculated according to the following  
211 equation:

$$212 \text{ DPPH scavenging Activity(\%)} = \frac{A_b - A_f}{A_b} \times 100 \quad (6)$$

213 Where  $A_b$  is the absorbance of the control and  $A_f$  is the absorbance of the film sample.

## 214 2.14. Effect of different composite films on fish meat

### 215 2.14.1. Preparation of fish meat wrapped with films

216 Samples of fish meat (approximately 4×4 cm) were randomly divided into five groups. Five  
217 different kinds of films (Control, PVC, FMP/CS, FMP/CS/RE (0.1%) and FMP/CS/RE (0.2%))  
218 prepared as described above were used to wrap fish meat samples. Samples without packaging were  
219 regarded as the control group. All samples were kept at 4 °C for 10 days and taken out for quality  
220 measurement on day 0, 2, 4, 6, 8 and 10.

221 **2.14.2 Thiobarbituric acid reactive substances (TBARS) and pH analysis**

222 TBARS were measured according to a former method [29]. Minced fish muscle (~5.00 g) was  
223 homogenized (IKA Ultra-Turrax T18 Basic, Staufen, Germany) with 25 mL 7.5% (w/v)  
224 trichloroacetic acid (containing 0.1% EDTA) for 1 min at 3000 rpm, then centrifuged (Fitchell SF-  
225 TDL-5A, Shanghai, China) at a speed of 4000 g for 5 min. The supernatant (~5.0 mL) was mixed  
226 with TBA (20 mM, 5 mL) solution; the mixture was incubated in a water bath at 100 °C for 30 min  
227 and cooled with flowing water. The absorbance was measured with ultraviolet-visible  
228 spectrophotometer (UV-VIS) at 532 nm (UNIC UV-2600 UV-Vis, Shanghai, China). The standard  
229 curve was prepared using 1, 1, 3, 3-tetraethoxypropane. The results were expressed as milligrams  
230 of malondialdehyde (MDA)/kg of fish sample. For measuring pH, ~3.0 g of the samples were  
231 homogenized in 27.0 ml distilled water for about 30.0 min and centrifuged at a speed of 8000 g for  
232 10.0 min. Then, pH was evaluated by placing the pH electrode in the samples.

233 **2.14.3 Free amino acid (FAA) and total volatile basic nitrogen (TVB-N)**

234 The content of FAA was determined by titration [30]. The minced fish sample (~5.00 g) was  
235 homogenized with 15.0 ml ether-ethanol solution (2:1, v/v) at 3000 g for 1-2 min, then centrifuged  
236 at a speed of 4000 g for 5.0 min and the supernatant collected. The same procedure was repeated  
237 once as described above. The supernatant (~10.0 mL) was drawn off into an erlenmeyer flask, using  
238 1% phenolphthalein as an indicator; the mixture was titrated with potassium hydroxide solution  
239 (0.05 M). The FAA content was calculated and expressed as FAA in 100 g fish muscle sample.

240 The homogenized fish sample (~5.00 g) was extracted with 50 mL of deionized water for 30  
241 min. After filtration, 5.0 mL filtrate and 10.0 mL flushing deionized water were mixed with 5.0 mL  
242 of 0.1% (w/v) MgO. Then it was transferred to a 750 mL digestion tube. The tube was then installed

243 into an auto Kjeldahl nitrogen analysis system (FOSS Kjeltex 8400, Hilleroed, Denmark) for TVB-  
244 N measurement [31]. The TVB-N content was calculated and expressed as mg/100 g sample. The  
245 experiments were repeated in triplicate.

## 246 **2.15 Statistical analysis**

247 Results were analyzed using the statistical software SPSS 23.0 (SPSS Inc., Chicago, Illinois,  
248 USA). Data are presented as mean  $\pm$  SD (standard deviation). Turkey test was used to determine the  
249 significant differences of the results ( $p \leq 0.05$ ). All the tests in this study were carried out in  
250 triplicates, unless otherwise specified.

251

## 252 **3. Results and discussion**

### 253 **3.1 Mechanical behavior analysis of the composite film**

254 TS and EB of the composite films were used to assess the capability of the film's mechanical  
255 resistance to break and the stretching degree, respectively [32]. Generally, the mechanical behavior  
256 of a composite film changes with the corresponding composition variation. The TS and EB values  
257 of the composite film affected by different concentrations of RE are illustrated in Fig. 1. Results  
258 indicate that the addition of RE to FMP/CS films could significantly improve the TS of films,  
259 especially when the RE content was 0.05%. However, the TS of the FMP/CS/RE film showed a  
260 decreasing trend by increasing RE% to ratios higher than 0.05%. This may be due to the  
261 hydrophobic interaction formation between the benzene ring and carbonyl group which exist in RE  
262 and the hydrophobic regions of the myofibrillary protein. Nevertheless, EB of FMP/CS/RE films  
263 were reduced compared to the control. There are two possible explanations for this result. Firstly,  
264 the hydroxyl and carboxyl groups in RE bound with amino groups in the FMP which formed the

265 compact protein-phenolic network structure. The tight network structure leads to reduced film  
266 flexibility of the FMP/CS/RE films. Secondly, the enhanced intermolecular forces between RE and  
267 MP led to the destruction of the mobility of the film's molecular chains and decrease in flexibility  
268 of the film which contributed to the lowering of EB values of the composite film.

### 269 **3.2 Physical characteristics of different composite films**

270 The thickness, water content, solubility and WVP of the composite films are presented in Fig.  
271 2. With the increase in RE contents, the thickness of the composite film gradually increased from  
272 69.67 to 78.22  $\mu\text{m}$ . The reason could be that the addition of RE increased the dry matter/film-  
273 forming substance content of the FMP/CS composite film. Furthermore, the increase in thickness  
274 of the composite films, might be related to the structure changes of the FMP/CS film with the  
275 addition of RE. This phenomenon is consistent with the results of Li et al. [33], namely the  
276 interaction between hydroxyl in polyphenol and glycerol and the film-forming matrix may have  
277 changed the structure of the film to some extent, thereby causing the thickness of the film to increase.

278 Compared with the control group, with the increase in content of RE, the water content of the  
279 composite film was significantly increased ( $p < 0.05$ ). The reason could be that the presence of  
280 phenolic compounds led to the hydrophilic functional groups ( $-\text{OH}$ ,  $\text{NH}_2$  and  $-\text{COOH}$ ) of the film  
281 to increase and strengthen the interaction (hydrogen bond) of water molecules [34]. In this study,  
282 the water solubility of the FMP/CS/RE composite film was higher than that in the control group,  
283 mainly due to the hydrophilicity of RE which was stronger than both the FMP and CS. The water  
284 solubility of the film in the groups containing RE was higher than that in the control group, arguably  
285 because the hydrophilicity of the RE was stronger than that of chitosan molecules and proteins,  
286 which is consistent with the results of Ashrafi et al. [35].

287 Generally, the WVP depends on the ratio of hydrophilic to hydrophobic components in the film  
288 [36]. When RE was added, the values of WVP of composite films were higher than that of the  
289 control. Basically, the trend of WVP of film kept rising as the RE concentration increased. This may  
290 be caused by the existence of RE which reduced the crystallinity of the film and increased the  
291 content of hydrophilic components within the film network structure which led the value of WVP  
292 to increase. Binding of hydrophilic rosemary with macromolecular chains in film forming  
293 components has been found to promote WVP in film substrates [37].

### 294 **3.2 Water contact angle (WCA)**

295 The hydrophilic and hydrophobic degree of the composite film surface were evaluated by  
296 measuring the water contact angle between the film surface and water droplets [38]. The contact  
297 angle of composite films is presented in Fig. 3. Essentially, the water contact angles of all groups  
298 are less than 90°, indicating that the surfaces of all composite films are hydrophilic. The water  
299 contact angle of the FMP/CS composite film was the largest and the WCA of the composite  
300 membrane decreased following the increased content of RE. This means that the addition of RE  
301 made the surface of the composite film more hydrophilic due to the hydrophilic nature of RE. This  
302 outcome is also in agreement with the result of increased WVP and consistent to those obtained  
303 from the bilayer edible films made by hydrophobic ethyl cellulose and hydrophilic carboxymethyl  
304 chitosan hydrogel [39]. Nonetheless, these results are distinct from findings on films based on  
305 cassava starch films modified with polyphenols-rich RE [40]. This different behavior can be  
306 explained on the basis that hydrophobicity of the main bioactive compounds is isolated from the  
307 aqueous RE (such as rosmarinic acid and carnosic acid). A comparison of these two compounds  
308 shows that, carnosic acid is more hydrophobic with two OH groups and a COOH group, whereas

309 rosmarinic acid has four OH groups and a COOH group. In this sense, the increase in the film  
310 surface hydrophobicity could be attributed to the amounts of carnosic acid, which could migrate to  
311 the film surface during the drying process. The purity of rosmarinic acid used in our study was very  
312 high, it is about 80%, but the RE used in that reference were extracted from dried and milled  
313 rosemary leaves only. Thus, the purity of rosmarinic acid is far below that used in this study, as it is  
314 only about 2.6% after estimation.

### 315 **3.3 Appearance and color analysis**

316 The color of the film affects the appearance of the food. Thus, it is an important parameter for  
317 the preparation process of the edible film. Plant extracts can usually change the color of the film.  
318 Numerous studies have reported that the degree of color change by the addition of plant extracts  
319 depends on the species and the concentration of the plant extract [41]. Usually, the color difference  
320 is determined by three parameters, such as  $L$ ,  $a$  and  $b$ . The values of these three parameters are  
321 collected in Table 1. When RE was added, all color parameters of the film showed significant  
322 differences. As the rosemary concentration increased, a decrease in the  $L$  value indicated that the  
323 film became darker and an increase in the  $a$  and  $b$  values suggests that the color of the film tended  
324 to be redder and yellower. In addition, an increase in  $\Delta E$  was observed. The above data shows that  
325 as the concentration of rosemary increased, the film became darker, yellower and slightly reddish.  
326 The oxidation of phenolic compounds in rosemary can change the color and as the temperature  
327 increases, the color of phenolic compounds becomes darker. The reason may be due to two aspects.  
328 On the one hand, Maillard reaction may have occurred between the fish protein and carbohydrates,  
329 while on the other hand, yellow pigments could have formed in acidic conditions causing the degree  
330 of color change to be affected by processing conditions and moisture content [42].

### 331 **3.4 Light transmittance and opacity**

332 In general, plant extracts can cause an increase in the opacity of composite films due to their  
333 color [36]. The effect of adding RE onto the transmittance and opacity of the FMP/CS film is shown  
334 in Table 2. It can be seen that the composite film with RE has lower light transmittance and higher  
335 opacity when compared with the control. Moreover, the opacity of the composite film was positively  
336 correlated with the RE concentration. With the increase of RE concentration, the opacity of  
337 FMP/CS/RE films displayed excellent visible light blocking performance. Wen et al. also found that  
338 light transmittance of polyvinyl alcohol composite film decreased with the increase in green tea  
339 extract [43]. The low light transmittance and high opacity can effectively prevent the decomposition  
340 of photosensitive ingredients in foods, which is extremely important for food packaging and this  
341 result is also consistent with the color measurement of composite films. The decrease in  
342 transparency and increase in opacity may be due to the presence of coloring RE which contributes  
343 significantly to reducing of the transmission of light [44]. In addition, the light selective absorption  
344 of polyphenol compounds exist in the extracts at low wavelengths which expresses a reddish color  
345 in the films, thus decreasing the light transmittance and opacity [45].

### 346 **3.5 TGA analysis**

347 The decomposition pattern and the thermal stability of different films were evaluated utilizing  
348 TGA analysis, which are presented in Fig. 4. Following the water desorption, a three-step  
349 decomposition curve was observed. The first stage (30-150°C, weight loss: 20-25 %) was possibly  
350 due to loss of free water, bound water and acetic acid which remained in the film [46]. The second  
351 stage (150-300 °C, weight loss: 25-60%) could mainly be attributed to thermal degradation of the  
352 glycerol rich phase, FMP and skeleton protein that exist in film substrates. Compared with the

353 FMP/CS composite film, the weight loss of the composite film was reduced by 5-15 % when 0.05-  
354 0.2 % RE was added. The reason may be that the addition of RE enhanced the thermal stability of  
355 the composite film, thus, the degradation temperature was increased. The trend of increasing  
356 decomposition temperature indicates that there are stronger interactions between RE and FMP/CS  
357 film matrix [47]. The existence of polyphenol- cross-linked FMP networks led to the higher thermal  
358 degradation temperature [8]. Furthermore, the higher concentration of RE (0.1-0.2%) increased the  
359 hydrophilicity of the MP/CS/RE composite film, which could lead to the increase of water content  
360 in the film, hence, the thermal stability was lower than that of RE content (0.05%). The thermal  
361 decomposition of the third stage which occurred at 300-600 °C could be attributed to the further  
362 degradation and carbonization of the remaining products after thermal degradation during the first  
363 two stages. This could explain the low content of flame retardant material in the prepared composite  
364 film [48].

### 365 **3.6 XRD analysis**

366 An amorphous structure predominates in composite films as demonstrated by the X-ray  
367 diffraction pattern in Fig. 5. The composite films of all samples show two specific peaks at  $2\theta$  of  $8^\circ$   
368 and  $20^\circ$ . The same two peaks appeared in XRD spectrums of a series of chitosan/zein blend films  
369 with different proportions [47]. Owing to the amorphous crystal structure that exist in the composite  
370 film, there are no high-intensity diffraction peak which appeared in the spectrum. The presence of  
371 RE with different percentages in the FMP/CS polymer substrate led to the intensity of diffraction  
372 peaks at  $2\theta$  to increase. When the concentration of RE was 0.2%, the intensity of the diffraction  
373 peaks at  $2\theta$  significantly increased, which indicates that the compatibility of FMP, CS and RE was  
374 slightly decreased [49]. The result is confirmed by SEM and AFM analysis.

### 375 3.7 Composite film morphology observed by SEM

376 SEM morphology was used to explore the uniformity and network structure of the film forming  
377 matrix. The surface and cross-sectional SEM micrographs of FMP/CS films incorporated with  
378 different concentrations of RE are illustrated in Fig. 6. It can be observed that there are some  
379 differences in the surface and cross-section structure morphology between the FMP/CS film and  
380 FMP/CS/RE films. The surface of the FMP/CS composite film (Fig. 6A) is uniform and smooth,  
381 without holes, pores or cracks in the cross-sectional view, which indicates that the FMP and CS have  
382 good compatibility and film-forming properties. The addition of RE had an obvious effect on the  
383 surface of the FMP/CS film. When the concentration of RE was 0.05%, the surface of the composite  
384 film remained smooth, and there were no obvious holes in the cross section, indicating that the low  
385 concentration of RE was well-distributed into the film matrix with strong interactions and all the  
386 film-forming matrix demonstrated better compatibility (Fig. 6B). With the increase in concentration  
387 of RE, the surface of the composite film exhibited "protrusions", which could be related to the  
388 interaction between RE and the film-forming matrix. The excessive amount of RE (0.2%) destroyed  
389 the internal structure and weakened the intermolecular forces between polymer chains, making the  
390 film relatively loose (Fig. 6C and 5D). Furthermore, more pores or cavities appeared in the cross-  
391 section structure morphology of FMP/CS/RE films with excessive RE%, which resulted in the  
392 aggregation and non-uniform dispersion of the RE in the film forming matrix, and led to  
393 irregularities [50]. Similar results are reported were rougher film surface was obtained in alginate  
394 composite films with the addition of green tea extract and a grape seed extract [45]. The holes  
395 observed in the cross-section structure of the film are enlarged, which somewhat just explains the  
396 increase in the WVP of the film. In addition, when comparing the scale of the cross-sectional view

397 (multiplier: 1000), it can be seen that the higher the concentration of RE is, the thicker of composite  
398 film, which is consistent with the results of thickness.

### 399 **3.8 AFM image**

400 The morphology and roughness of FMP/CS composite film affected by different concentration  
401 of RE treated composite films is shown in Fig. 7. It can be seen very clearly that the surface of the  
402 FMP/CS composite film is overall flat with slight concaves. There are a few light spots on the 3D  
403 image, which can be explained as the product generation of the interaction between FMP and CS.  
404 When 0.05% concentration of RE was added, the composite film displayed a smooth surface  
405 compared with the control film, indicating that a good distribution of RE in the blend of FMP/CS  
406 matrix was attained due to the cross-linking of FMP and CS, which is in agreement with the results  
407 obtained by SEM. However, as soon as the concentration of RE reached 0.10–0.20%, the surface  
408 roughness of the composite film increased and some of "gulls" emerged. This result may be due to  
409 the molecular interaction between the composite FMP/CS film and RE, which is in agreement with  
410 the previous report that reported that the surface roughness of films was increased because of the  
411 interaction between film-forming substances[51]. An adverse result was found in study on edible  
412 film based on potato starch, olive oil and zein nanoparticles[27].

### 413 **3.9 Antioxidant activity**

414 The addition of antioxidants can change the color of the film and inhibit the oxidation of lipid  
415 components, thus extending the shelf life of packaged products. The major polyphenolic compound  
416 in RE is rosmarinic acid, which is known for its oxidation resistance, anti-inflammatory,  
417 antibacterial and other properties[52]. Therefore, adding RE to the composite film can increase the  
418 antioxidant capacity of the film to some extent. DPPH radical scavenging assay has been widely

419 used to test the antioxidant activity of polyphenolic compounds. Antioxidant activity expressed in  
420 terms of DPPH radical scavenging activity of FMP/CS films adding different concentrations of RE  
421 is shown in Fig.8. The DPPH radical scavenging ability of the control FMP/CS film was low, while  
422 the antioxidant activity capacity of the FMP/CS/RE composite film increased with the level of RE.  
423 The maximum antioxidant activity was observed in film containing RE concentration of 0.20%.  
424 This could be due to the antioxidant activity of the composition of film-forming materials, such as  
425 MP, CS and RE. This phenomenon is consistent with former results [53] - the antioxidant activity  
426 of the MP film without the addition of catechin-Kradon extract had the lowest value. Furthermore,  
427 it fundamentally shows a positive correlation between the amount of polyphenol and antioxidant  
428 capacity, which is identical with the conclusion of Gomez-Estaca et al [54]. Rosemary and oregano  
429 extracts were added into tuna-skin and bovine-hide gelatin based films, the strength of antioxidant  
430 activity of the gelatine films attributed to the content of phenolic compounds of additives.

### 431 **3.10 Quality changes in fish fillets during storage**

#### 432 **3.10.1 Thiobarbituric acid reactive substances (TBARS)**

433 TBARS is an indicator of the degree of lipid oxidation, which is induced by the accumulation  
434 of lipid peroxides and the corresponding secondary metabolites [55]. As shown in Fig. 9A, the initial  
435 TBARS was 0.29 mg MDA/kg per sample and the TBARS of each group did not indicate any  
436 significant difference ( $p>0.05$ ). From the six days of storage, the TBARS value of RE groups were  
437 significantly lower than that of the control. On account of the extension of the storage periods,  
438 peroxides like malondialdehyde (MDA) are produced and increased with the oxidation of  
439 unsaturated fatty acids [56]. During the late storage period (10 days), the TBARS (1.24 mg MDA/kg  
440 sample) of the control was significantly higher than that of the other groups. This result is consistent

441 with RE or other phenolic compounds that have strong free radical scavenging capability, which can  
442 decrease the degree of lipid oxidation [57]. Furthermore, the content of phenolic compound of the  
443 film-forming materials restricts the lipid oxidation and decreases the TBARS values.

#### 444 **3.10.2 pH**

445 The pH of fish is an indicator reflecting the freshness of the fish quality after death. The change  
446 in pH of grass carp flesh during cold storage is illustrated in Fig. 9B. It can be seen that the pH of  
447 all groups showed a similar trend -a decreasing and then an increasing trend. During the early stage  
448 of refrigeration (0-4 days), the pH values were decreased which could be due to the glycolysis of  
449 grass carp flesh that led to the accumulation of lactic acid and the degradation of adenosine  
450 triphosphate (ATP) to release inorganic phosphate. With the extension of refrigeration time (4-10  
451 days), the autolysis of fish protein and the formation of basic compounds (ammonia and trim-  
452 ethylamine) caused by the action of microorganisms has been shown to lead to a gradual increase  
453 in the pH value [58]. During the storage period, compared with the control group, the pH value of  
454 both the PVC film and FMP/CS composite film groups increased slowly, but the pH of the  
455 FMP/CS/RE composite film exhibited the slowest increase among all sample groups. The pH  
456 increase rate of the group was opposite to the concentration in RE, which could be due to certain  
457 inhibitory effects of RE on the microorganisms of fish meat. This is consistent with the study of the  
458 pH value of mud shrimp (*Solenocera melantho*) treated with RE which was significantly lower than  
459 that of the control during the frozen storage [11].

#### 460 **3.10.3 Changes in FAA in fish fillets**

461 The content of FAA in fish fillets is a common indicator for evaluating the spoilage of aquatic  
462 products. The changes in FAA detected in grass carp flesh wrapped with different composite

463 film/coating are illustrated (Fig. 9C). Firstly, it can be seen that the content of FAA of grass carp  
464 flakes increased with the extension of storage time, indicating that the lipids in fish flesh are  
465 continuously hydrolyzed during cold storage. In this study, the sample wrapped with the films  
466 inhibited lipid hydrolysis to some extent. The speed of FAA production rate for fish flesh under  
467 different treatments were as following: the control group > the PVC film group > the FMP/CS group >  
468 the FMP/CS/RE (0.1%) > the FMP/CS/RE (0.2%). The major factor affecting the lipid hydrolysis  
469 during the fish late storage period is microbial activities [59]. Therefore, the results indicate that RE  
470 could affect microbial activities in the fish muscle during the cold storage of grass carp flakes.

#### 471 **3.10.4 Volatile base nitrogen (TVB-N)**

472 The value of TVB-N is an intuitive reflective indicator of food spoilage. TVB-N is positively  
473 correlated with the activity of endogenous enzymes in fish and the growth of microorganisms [60].  
474 The changes in TVB-N detected in grass carp flakes during cold storage are shown in Fig. 9D.  
475 Compared with the TVB-N value of grass carp meat on 0 day (9 mg/100g), the TVB-N values of all  
476 groups were increased. After the 4<sup>th</sup> day, the TVB-N values of FMP/CS/RE groups were  
477 significantly lower than those of the control and the PVC film groups ( $p>0.05$ ). The result illustrates  
478 that the RE composite film could extend the shelf life of grass carp flakes and is more effective  
479 compared to the plastic fresh-keeping film. This is consistent with the finding of edible coating  
480 enriched with RE that enhanced the oxidative and microbial stability of smoked eel fillets [15].

#### 481 **4. Conclusion**

482 In the present study, composite films based on FMP/CS incorporated with RE were developed.  
483 The composite films showed considerable mechanical properties, acceptable moisture barrier  
484 capability as well as good compatibility which are supported by results of SEM and AFM images.  
485 Moreover, the composite films were applied in order to preserve grass carp fillets. Results indicate

486 that FMP/CS/RE (0.20%) composite films possessed the best protective effect on fish muscle,  
487 maintaining a low pH value and FAA values. The changes in TVB-N and TBARS show that the  
488 degree of lipid oxidation of fish muscle decreased during the seven days refrigerated storage. It was  
489 therefore suggested that the prepared composite film FMP/CS/RE could have potential for  
490 improving the quality and shelf life of fish muscle.

491

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657 [60] M.Y. Arancibia, M.E. López-Caballero, M.C. Gómez-Guillén, P. Montero, Chitosan coatings enriched  
658 with active shrimp waste for shrimp preservation, *Food Control*, 54 (2015) 259-266.  
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661 **Table 1: Appearance and color parameters of different composite films.**

RE content (%)	Appearance	L*	a*	b*	ΔE
FMP/CS		93.31±0.64 <sup>a</sup>	1.33±0.17 <sup>c</sup>	9.00±1.32 <sup>e</sup>	8.68±1.34 <sup>e</sup>
FMP/CS/ RE (0.05%)		89.67±0.64 <sup>b</sup>	0.58±0.05 <sup>b</sup>	12.59±1.26 <sup>d</sup>	12.86±1.37 <sup>d</sup>
FMP/CS/RE (0.10%)		88.06±0.82 <sup>c</sup>	0.63±0.09 <sup>b</sup>	15.20±1.33 <sup>c</sup>	15.85±1.53 <sup>c</sup>
FMP/CS/RE (0.15%)		86.74±0.21 <sup>d</sup>	0.20±0.17 <sup>a</sup>	17.44±0.54 <sup>b</sup>	18.43±0.56 <sup>b</sup>
FMP/CS/RE (0.20%)		85.56±0.93 <sup>e</sup>	0.04±0.22 <sup>a</sup>	19.68±2.03 <sup>a</sup>	20.94±2.21 <sup>a</sup>

662 Note: Values are presented as mean ± standard error. Means in the same line with different

663 subscripts (a, b, c, d, e) are significantly different (p<0.05).

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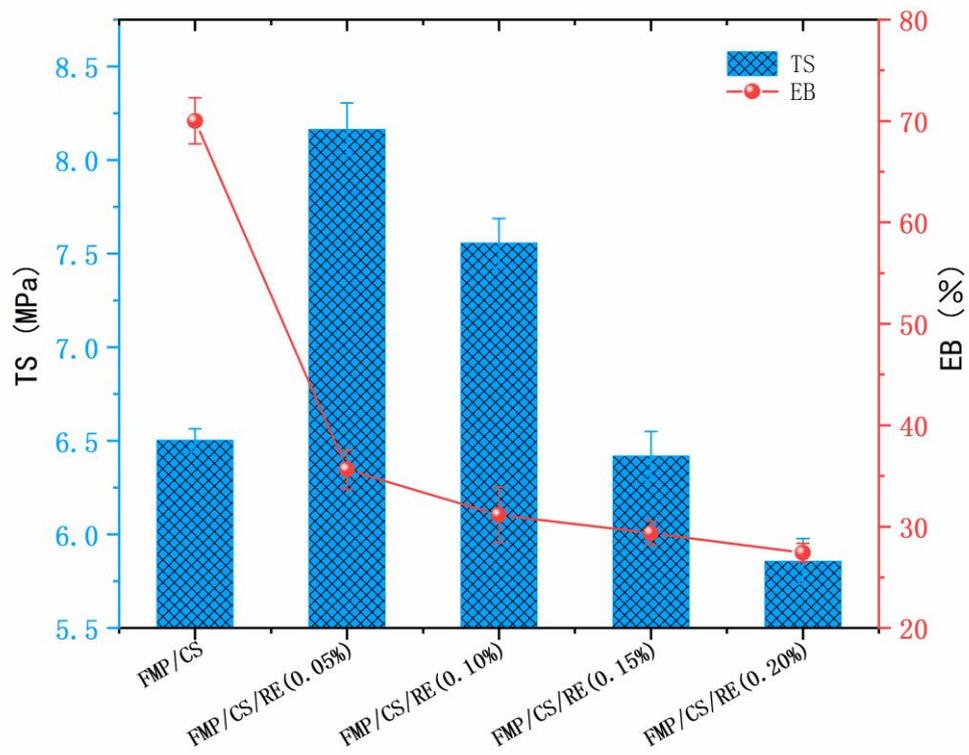
665 **Table 2: Light transmittance and transparency of composite films treated with different**  
 666 **concentrations of RE.**

RE (%, W/W)	Transmittance (%T) at wavelength (nm)						Opacity
	350	400	500	600	700	800	
0	47.1	56.77	64.34	72.34	73.21	73.78	2.1±0.05 <sup>e</sup>
0.05%	0.063	46.28	50.31	63.85	64.34	64.75	3.16±0.07 <sup>d</sup>
0.10%	0.046	42.43	48.32	58.73	59.25	60.02	3.50±0.14 <sup>c</sup>
0.15%	0.022	39.35	44.76	49.37	50.23	51.05	5.01±0.12 <sup>b</sup>
0.20%	0.002	34.25	37.46	40.14	41.05	41.94	5.75±0.11 <sup>a</sup>

667 Note: Different superscripts a, b, c, d, e in each column indicate significant differences (p<0.05).

668 Values are given as mean ± SD from triplicate determinations.

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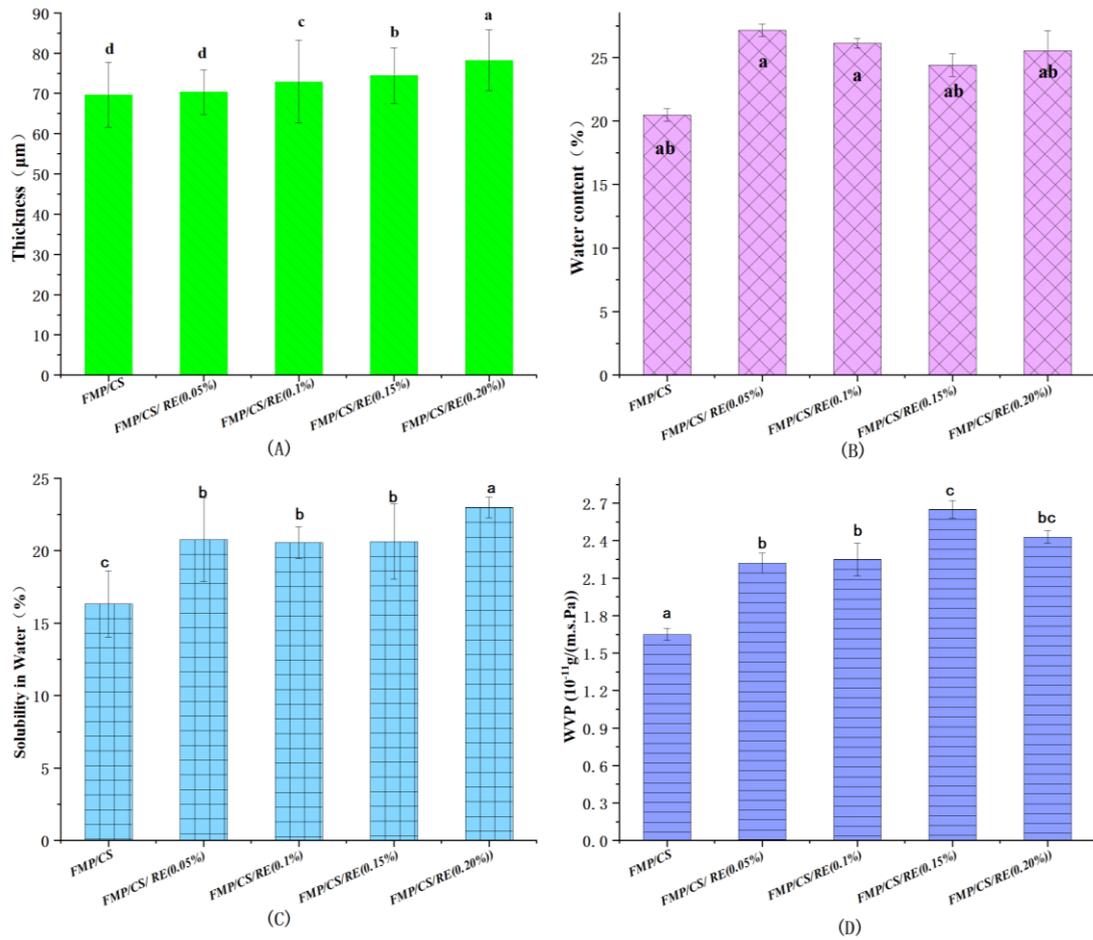
**Fig. 1: The mechanical properties of different composite films**

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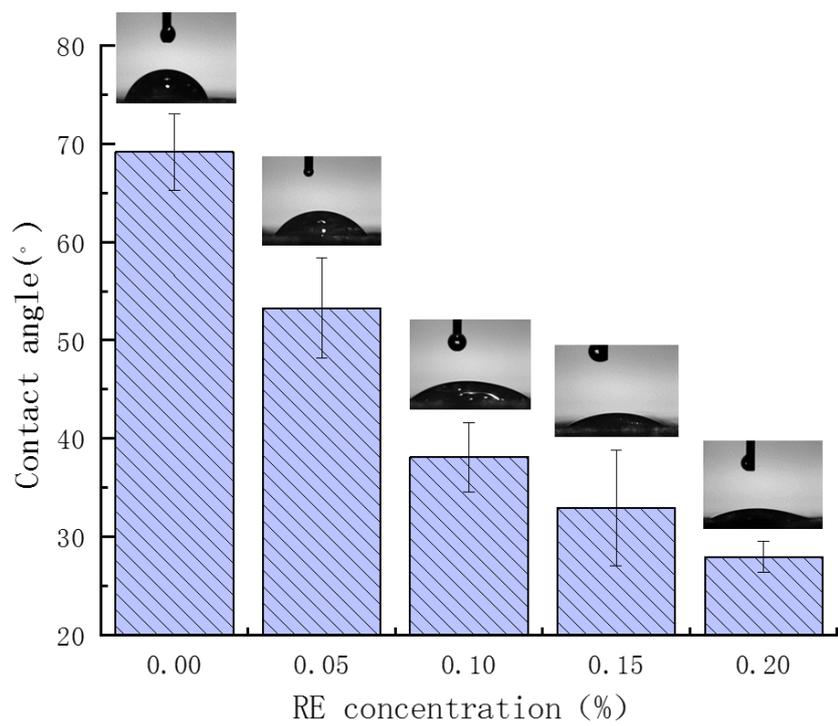
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675 **Fig. 2: Thickness, water content, solubility and water vapor permeability of different**

676 **composite films.** Note: The letters a, b, c, d (different letters in the same column) indicate significant

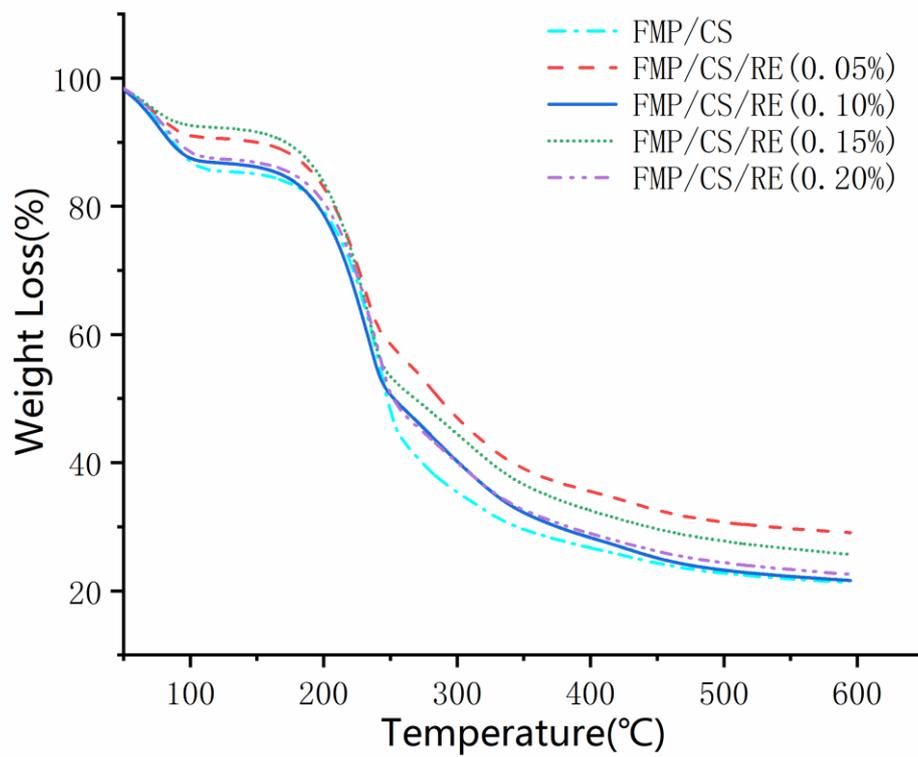
677 differences ( $p < 0.05$ ).

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**Fig. 3: Water contact angle of the composite films.**



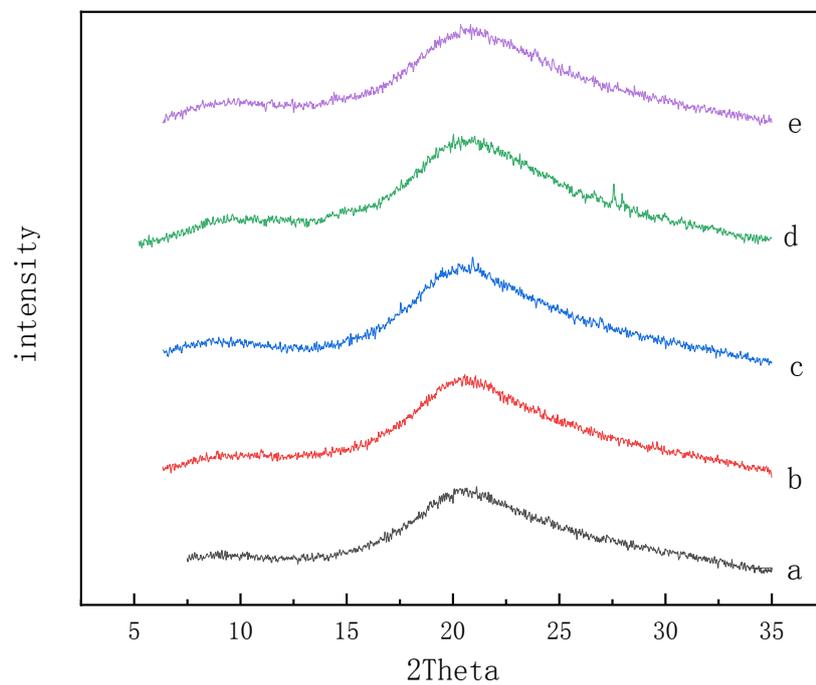
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**Fig. 4: TGA curves of different composite films**

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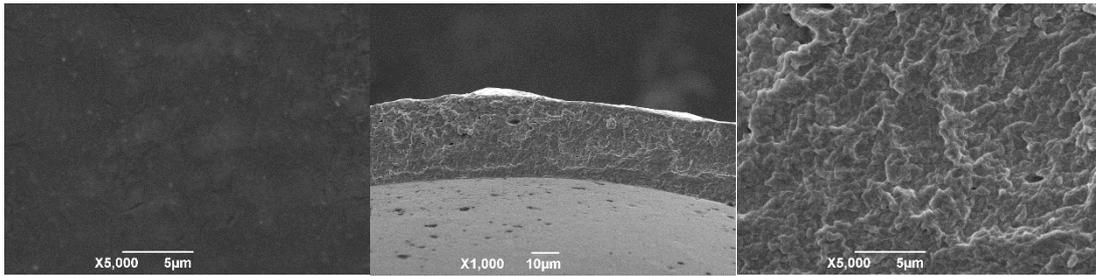


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690 **Fig. 5: X-ray diffraction of fish myofibrillar protein based composite films with different**  
691 **rosemary concentrations (a: FMP/CS, b: FMP/CS/ RE (0.05%), c: FMP/CS/RE (0.10%), d:**  
692 **FMP/CS/RE (0.15%), e: FMP/CS/RE (0.20%))**

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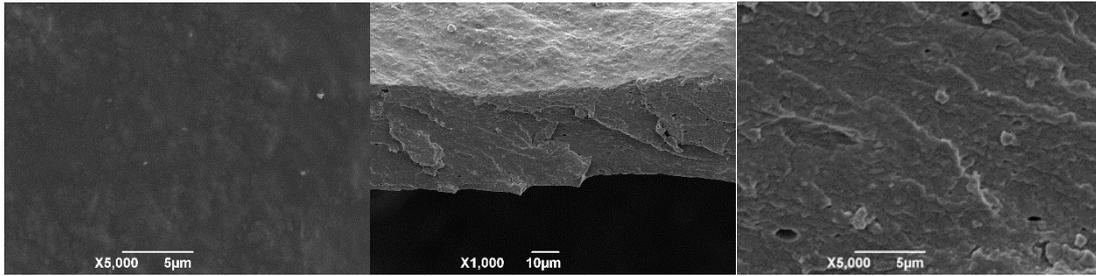
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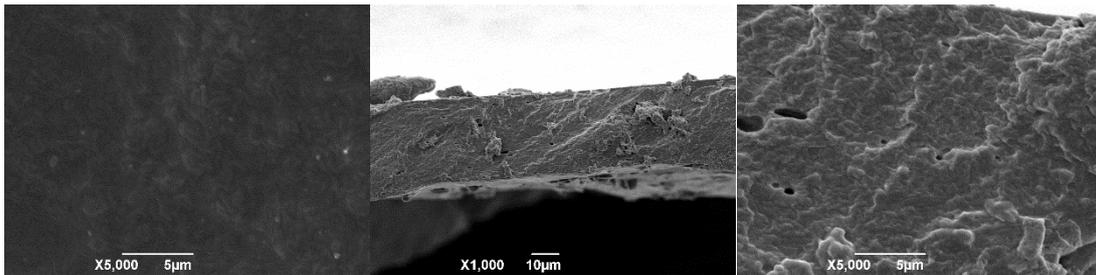
**a. FMP/CS composite films**



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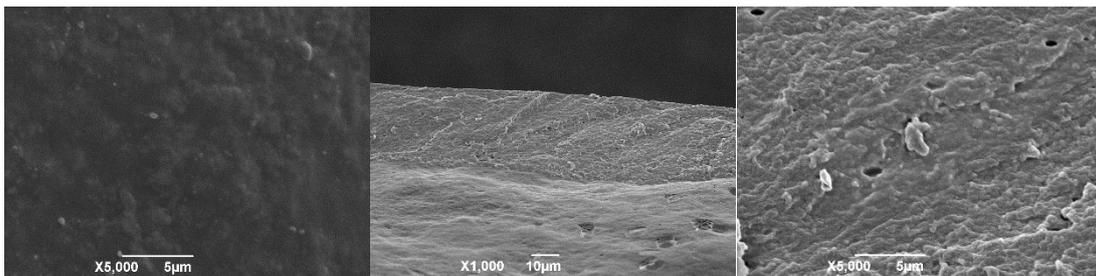
**b. FMP/CS/RE (0.05%) composite films**



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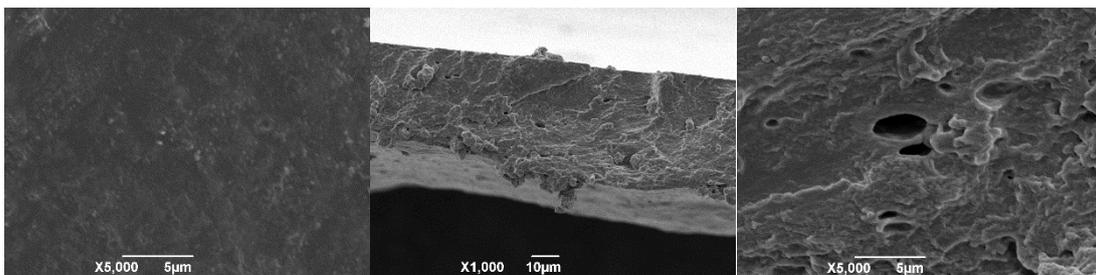
**c. FMP/CS/RE (0.10%) composite films**



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**d. FMP/CS/RE (0.15%) composite films**



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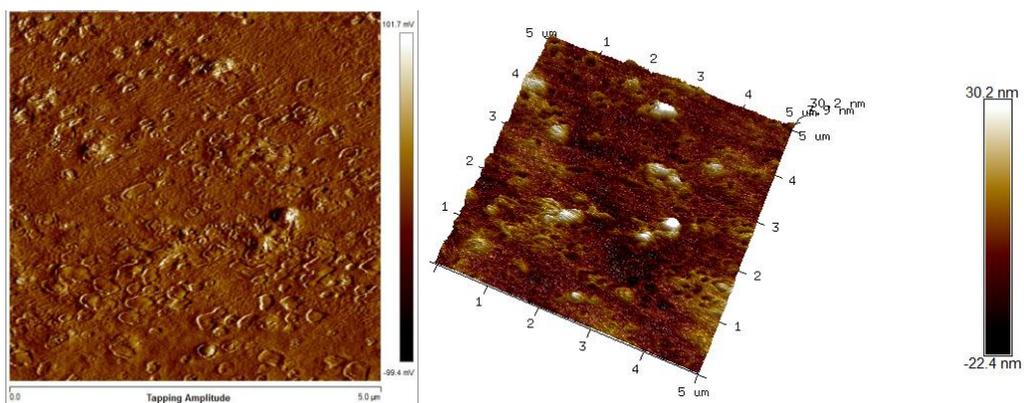
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**e. FMP/CS/RE (0.20%) composite films**

705 **Fig. 6: Scanning electron microscopy (SEM) images of the surface and cross-section of fish**

706 **myofibrillar protein based composite films with different concentrations of RE. Surface**  
707 **(5000× magnification), cross section (1000× magnification), cross section (5000×**  
708 **magnification).**  
709

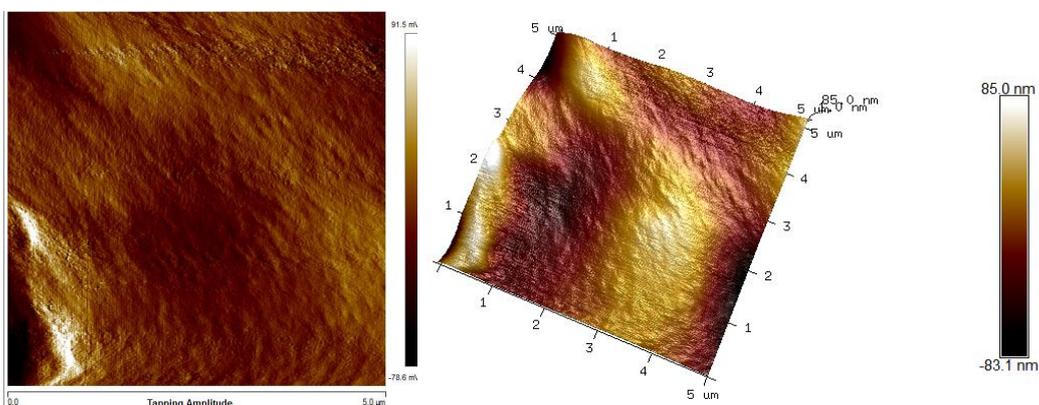
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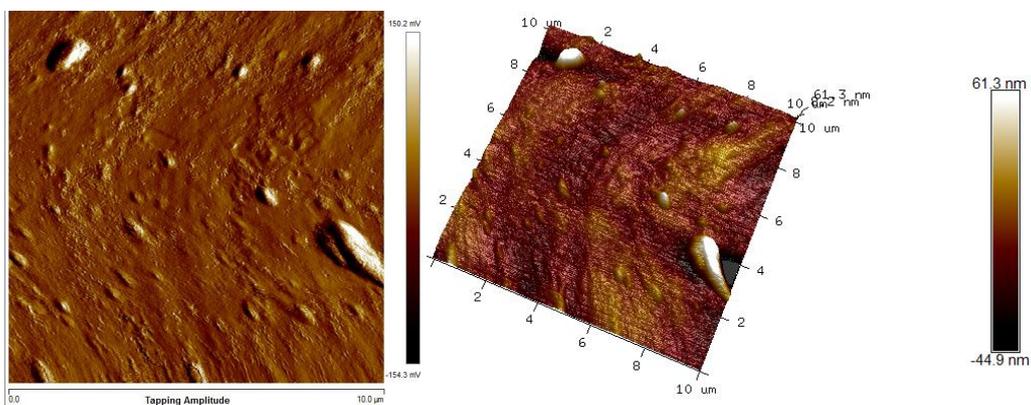
**a. FMP/CS**



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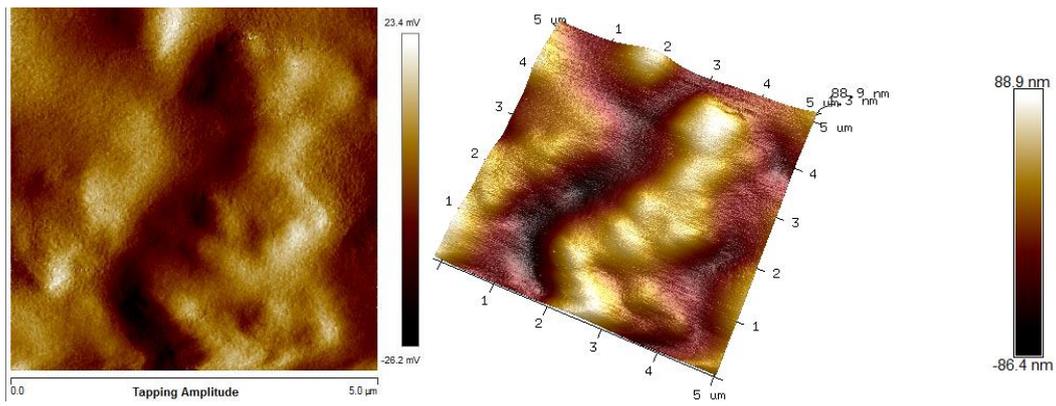
**b. FMP/CS/RE (0.05%)**



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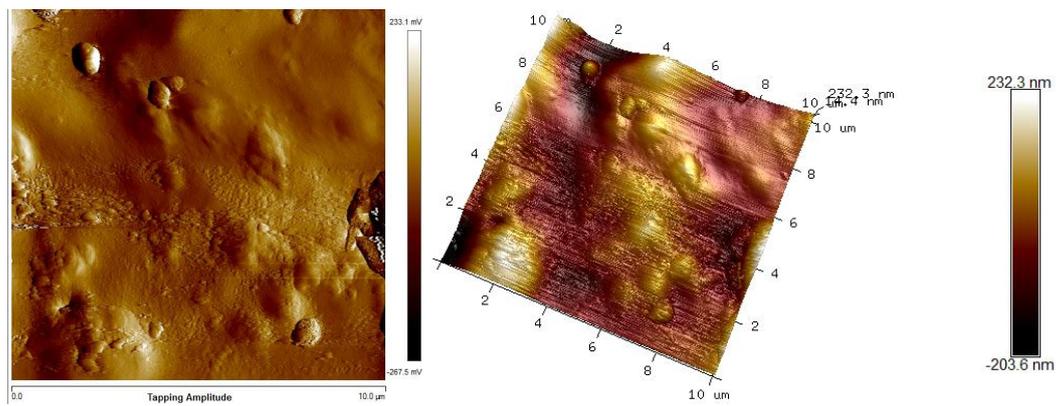
**c. FMP/CS/RE (0.10%)**



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**d. FMP/CS/RE (0.15%)**



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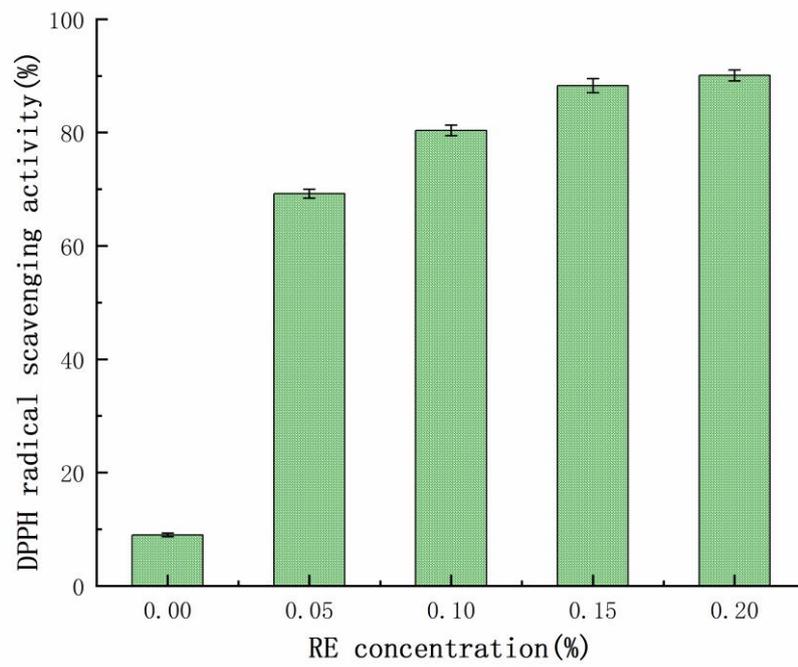
**e. FMP/CS/RE (0.20%)**

**721 Fig. 7: Surfaces morphology of composite membrane containing different concentrations of**

**722 rosemary extract (left: 2D, right: 3D)**

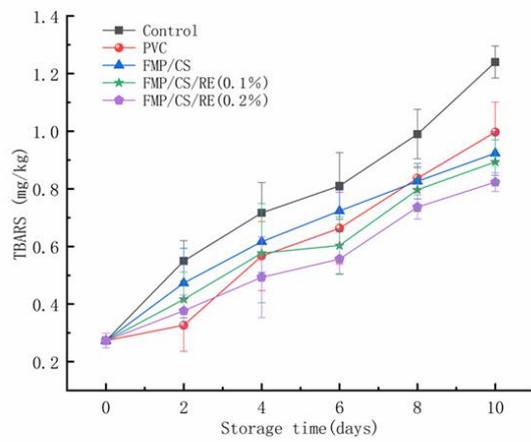
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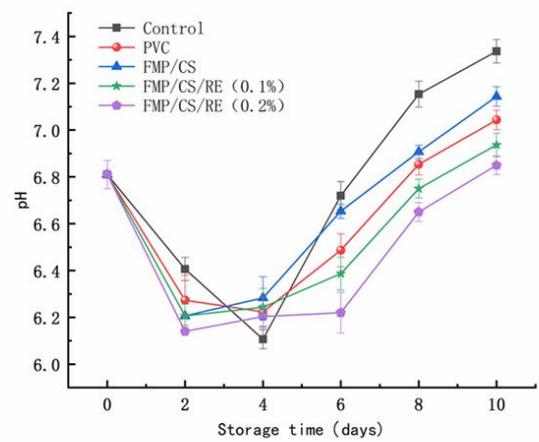


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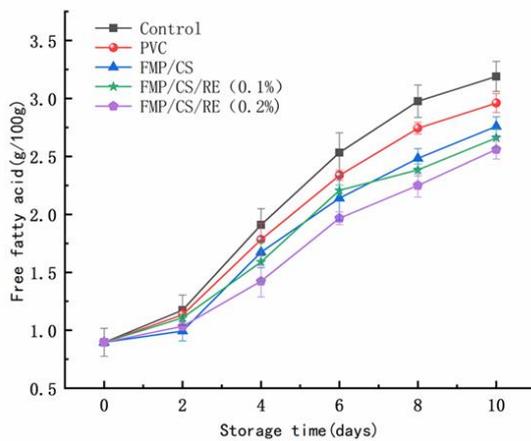
Fig. 8: The DPPH free radical scavenging activity of the composite films containing different concentrations of RE.



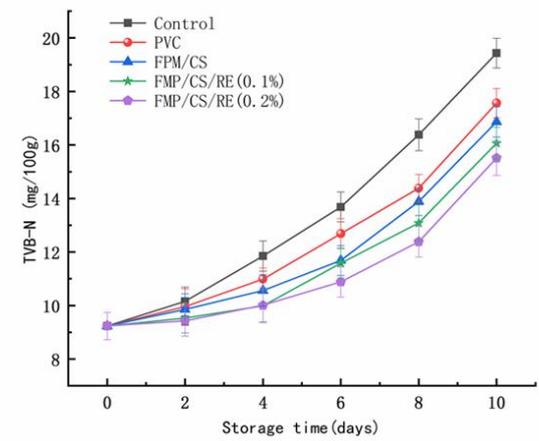
(A)



(B)



(C)



(D)

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730 **Fig. 9: Changes in TBARS, pH, FAA, TVB-N values of grass carp fillets during cold storage.**

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(A) TBARS; (B) pH; (C) FAA; (D) TVB-N.

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