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ANTIOXIDANT ACTIVITIES AND ANTIMICROBIAL PROPERTIES OF VARIETY *Iranian plantago minor* WITH POTATO STARCH BIOFILMS AT THE FLOWERING AND FRUIT SET STAGES

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ABSTRACT

Chemical and herbal materials for pain management have recently attracted attention. Medicinal plants and traditional medicine are being increasingly used in various countries, including Iran, because of the numerous complications and high costs of chemical medicines. Considerable research has revealed that medicinal plants are important sources of antioxidants. This research investigated the antioxidant activities and antibacterial properties of broadleaf *plantago minor* (plantain) leaves (at the flowering and fruit set stages) along or in combination with potato starch films. Results showed no significant differences in the DPPH radical-scavenging activities and FRAP test results between the leaf samples collected at the flowering and fruit set stages ($P > 0.05$). However, significant differences in iron ion trapping and thiobarbituric acid-reactive species test results were observed at all studied concentrations in the flowering and fruit set stages ($P < 0.05$). At the fruit set stage, the extracts exhibited no significant differences in antibacterial properties against different microorganisms ($P > 0.05$). At the flowering stage, the extracts also demonstrated no significant differences in antibacterial properties against different bacteria, except for *Escherichia coli* ($P < 0.05$). Furthermore, the extracts combined with potato extract films revealed significant differences in functional properties at both seasons ($P < 0.05$). Given their desirable antioxidant properties, the extracts can be used in the food industry for delaying oxidation instead of employing synthetic antioxidants. These extracts, especially when combined with potato starch films, can be used in the pharmaceutical and food industries as natural preservatives because of their antibacterial properties.

Keywords: *Plantago minor* leaves, extract, antioxidant properties, antibacterial properties, potato starch film.

INTRODUCTION

Chemical and herbal materials for pain management have recently attracted attention. Medicinal plants and traditional medicine are being increasingly used in various countries, including Iran, because of the numerous complications and high costs

of chemical medicines. Considerable research has revealed that medicinal plants are important sources of antioxidants. Plants rich in antioxidants can protect cells against oxidative damage (Kumaran, 2006). Natural antioxidants increase the power of plasma antioxidants and reduce the incidence of some diseases, such as cancer, heart diseases, and stroke (Prior and Cao, 2000). Plant-derived secondary metabolites, including phenolic compounds and total flavonoids, are found in all plant parts, such as leaves, fruits, seeds, roots and bark, and have a strong potential for removing free radicals (Mathew and Abraham, 2006).

Medicinal plants have always been research hotspots because of their extensive therapeutic properties. The use of chemical materials in producing antibacterial medicines has attracted the interest of researchers with the expansion of various scientific branches, such as phytochemistry and pharmacology, but scientists have been forced to consider utilizing herbal compounds for treating infectious diseases because of the uncontrolled and incorrect use of chemical medicines (Kinghorn, 2001). Some medicinal plants used in traditional medicine exert effective antibacterial properties (Abdolshahi *et al*., 2016; Cowan, 1999; Mohammadhosseini *et al*., 2016). Medicinal plants, mainly their secondary metabolites, are rich sources of antioxidants (Savithramma *et al*., 2011). Most secondary metabolites display pharmaceutical and medical applications (Bourgaud *et al*., 2001; Zakerin *et al*., 2015). Among the identified materials present in active compounds of plant organs, phenolic compounds or non-nitrogenous secondary metabolites are the most important and most frequently found to have various biological effects, including antibacterial properties (Nouri *et al*., 2014; Yadegarinia *et al*., 2006).

Plantago minor is an important medicinal plant because it contains phenolic compounds (derivatives of caffeic acid), flavonoids, alkaloids, terpenoids, and vitamin C. Broadleaf *plantago minor* seed mucilage is used to reduce blood cholesterol levels. The soluble fibers present in this plant considerably reduce bad cholesterol (low-density lipoprotein) in blood, thereby decreasing cardiovascular complications and stroke occurrences. Given its antioxidant activities, broadleaf *plantago minor* can be used as a source of natural antioxidants (Mirzaei *et al*., 2011). Throughout the world, broadleaf plantain is used for pharmaceutical purposes. Broadleaf *plantago minor* seeds and leaves display various biological activities. In specific, they can improve wound healing; exert anti-inflammatory, analgesic, antioxidant, weak antibiotic, anti-gastric ulcer, anti-leukemia, anticancer, antivirus, and antitumor effects; regulate the immune system; and reduce the side effects of anticancer medications and blood pressure (Atta and El-Sooud, 2004; Nezhinskaya *et al*., 2008; Nyunt and Plowe, 2007).

Furthermore, continuous use of chemical medicines causes the development of highly resistant microbes. Hence, patients resort to strong antibiotics and new chemical medicines (Abdollahi *et al*., 2011; Behzadian Nejad *et al*., 2009). Meanwhile, many herbal medicines exhibit numerous positive effects with fewer side effects and complications compared with synthetic drugs (Meshkibaf *et al*., 2010). Thus, many researchers are studying the antibacterial and antioxidant effects of plant extracts (Abdollahi *et al*., 2012).

The main purpose of this research was to study the antibacterial properties and antioxidant activities of broadleaf *plantago minor* leaf extracts (alone or in combination

with potato starch films) obtained at the flowering and fruit set stages.

MATERIALS AND METHODS

Chemicals and equipment

DPPH powder, potassium ferricyanide, Folin–Ciocalteu, methanol (all purchased from Merck, Germany), gallic acid (purchased from Sigma-Aldrich, Germany), a spectrophotometer (Jenway 6305, England), a rotary evaporator and a magnetic stirrer (Heidolph-MR Hei-Standard, Germany), and a desiccator (Gerhard, Germany) were some of the materials and equipment used in this research.

Preparation of extract

Broadleaf (*plantago minor)* plantain leaves were washed with water, air dried at room temperature, and then powdered using an electric grinder. The solid–liquid method introduced by Nawaz *et al*. (2006) was employed as follows to extract the phenolic compounds. The mentioned powder (30 g) was extracted using 100 mL of the water–ethanol (30:70 v/v) solvent. The obtained solutions were placed on a magnetic stirrer in the dark for 1 h at 25 °C and then filtered. The extraction process was repeated twice under identical conditions, the filtered solutions were mixed, and their solvents were evaporated under vacuum using a rotary evaporator at 40°C.

Evaluation of DPPH radical scavenging

The extent of DPPH free radical suppression was evaluated in accordance with the method introduced by Shimada *et al*. (1992) and modified by Chang *et al*. (Chang *et al*., 2016a; Chang *et al*., 2016b). The DPPH methanolic solution (1 mL, 0.1 mM) was added to 3 mL of the extract, and the mixture was vigorously stirred. The test tubes were placed in the dark for 30 min, and the absorbance was read at 517 nm. In the control sample, the extract was replaced with 3 mL of methanol. Finally, the percentage of DPPH free radical suppression by the extract was calculated as follows:

Percentage suppression of DPPH free radical suppression $=\frac{(Ac-As)}{Ac} \times 100$,

where *Ac* and *As* represent absorbance by the control and by the sample, respectively.

Evaluation of ferric-reducing antioxidant power (FRAP)

FRAP was studied in accordance with the methods introduced by Strain and Benzie (1996) and Galletti *et al*. (2005). The FRAP reagent was prepared by mixing acetate buffer with TPTZ in hydrochloric acid and $FeCl_3.6H_2O$. Absorbance values in the solution containing the reagent and various concentrations of the extract were measured after 15 min at 593 nm. The FRAP reagent was used as the control. Moreover, aqueous solutions of $FeSO_4$ $7H_20$ at various concentrations were used to draw the calibration curve. Finally, FRAP was expressed in mmol iron sulfate per gram of the dry extract powder. Ascorbic acid at various concentrations was employed as the standard material.

Measurement of ferrous ion-chelating capacity (FIC)

For this test, orange leaf extracts at concentrations of 100–1000 µg/mL were prepared using the methanol solvent. To begin with, 2 mM solutions of $FeSO_4$ and 5 mM ferroin indicator were prepared. Each solution was diluted 20-fold. Exactly 1 mL of

each diluted iron sulfate solution was mixed with 1 mL of the sample solution, and then 1 mL of the diluted ferroin indicator was added. The obtained mixture was rested for 10 min, and then light absorbance was read at 562 nm. The control sample (i.e., without the extract) was also tested. Results of the test were reported in percentage-chelating capacity using the following relation:

$$\text{Percentage chelating capacity} = (A_{\text{control}} - A_{\text{sample}}) \times 100/A_{\text{control}},$$

where A_{control} represents the light absorbance by the control sample and A_{sample} represents the light absorbance by the sample containing the extract.

Thiobarbituric acid-reactive species (TBARS) test

The amount of TBARS as a secondary product of fat pre-oxidation was determined in accordance with the method introduced by Docker *et al*. Exactly 0.1 mL of the various dilutions of the extract (25, 50, and 100 mg/mL) was added to the mixture of the egg yolk emulsion with 0.1 mol of the phosphate buffer (pH 7.4). The obtained mixture was incubated for 1 h at 37°C and then mixed with 0.5 mL of 15% trichloroacetic acid and with 1 mL of freshly prepared 1% TBA. Furthermore, 100 µL of the supernatant was removed, and light absorbance by the sample was read at 532 nm. BHT and ascorbic acid were used as the standards, and the percentage inhibition was calculated using the following formula:

$$\%\ \text{inhibition} = \left[\frac{A\ \text{control} - A\ \text{sample}}{A}\ \text{control}\right] \times 100,$$

where A is the absorbance, A_{control} is the light absorbance by the control sample, and A_{sample} is the light absorbance by the sample containing the extract.

Disc diffusion method for antimicrobial properties

Using bacteria from Mueller–Hinton agar and Mueller–Hinton Broth culture media, antibacterial tests were performed using the disk diffusion method to study the antimicrobial activities of the extracts (Nassiri and Abdorreza, 2013). In the disk diffusion method, the bacteria were cultured in Mueller–Hinton Broth for 24 h, and microbial suspensions equivalent to a 0.5 McFarland standard were prepared and transferred to the surface of Mueller–Hinton agar medium using a loop to have a uniform culture of the bacteria. Blank disks impregnated with 25 µL of the extracts of interest were placed at the center of the cultured bacteria and incubated for 24 h at 37°C. Zones of inhibition were then measured to determine the sensitivity or resistance of the bacteria to various concentrations of the extract. Then, 96-well microplates were employed to determine the minimum inhibitory and minimum lethal concentrations of the extracts.

Statistical analysis

SPSS 21 was used for statistical analysis of the data, and ANOVA and t test were employed for data evaluation. Diagrams were drawn using Excel 2010.

RESULTS AND DISCUSSION

Evaluation of DPPH free radical scavenging

Results of the study on hydroalcoholic extracts of Iranian plantago minor at their flowering and fruit set stages indicated no significant differences between the samples with respect to their growth stages ($P > 0.05$) (Fig. 1).

Fadavi and Koohsari (2015) studied the antioxidant and antimicrobial effects of orange leaf extracts prepared from trees planted in Iran on soybean oil stability. Results showed that the green leaf extract exerted significant antioxidant effects relative to extracts of black orange leaves and exhibited good antimicrobial effects on *Staphylococcus aureus*. In addition, the green and black leaf extracts showed significant effects on chelating capacity, and these effects improved in a concentration-dependent manner.

Ferric antioxidant and reducing power (FRAP)

Results showed no significant differences between I. plantago minor extracts prepared at the flowering and fruit set stages (P > 0.05), i.e., 0.7 ± 0.13 and 0.5 ± 0.07 power at the flowering and fruit set stages, respectively. Absorbance was greater in all samples of the flowering stage compared with those of the fruit set stage (Fig. 2).

Khalighi-Sigaroodi *et al*. (2013) studied the antioxidant effects and measured the total phenolic and flavonoid contents of extracts prepared from *Nepeta pogonosperma* using the colorimetric method. The antioxidant capacity of the extracts was evaluated using the scavenging method of the free radicals DPPH and ABTS and FRAP methods. Results indicated that the extracts contained greater total phenolic compounds than the total flavonoid compounds. The methanolic extract exhibited average scavenging effects against DPPH free radicals.

Measurement of ferrous ion-chelating capacity (FIC)

Various concentrations (25, 50, and 100 mg/mL) of the extracts were used. The 25 and 100 mg/mL concentrations of extracts prepared at the flowering and fruit set stages showed no significant differences (P > 0.05). However, at the concentration of 50 mg/mL, significant differences were found between the extracts prepared at the flowering and fruit set stages (P < 0.01). Furthermore, results revealed that the level of trapping of iron ions in the samples at the flowering stage was higher than that of in the samples at the fruit set stage, and the highest level of ion trapping (3035.7 ± 187.2) corresponded to 25 mg/mL extract concentration (Fig. 3).

Fig. 1. Means ± standard deviations of antioxidant activities exhibited by the studied extracts against DDPH. Similar letters indicate no significant differences between the values (P > 0.05)

Fig. 2. Means ± standard deviations of FRAP in the studied extracts. Similar letters indicate no significant differences between the values (P < 0.05)

Fadavi and Koohsari (2015) showed that the antioxidant power of extracts prepared from green leaves of orange trees is much greater than that of black leaves and that significant differences exist between the extracts at all concentrations. Their results differ from the present findings, which indicated that oxidative power declined with increasing concentration.

Thiobarbituric acid-reactive species (TBARS) test

In this study, different concentrations (25, 50 and 100 mg/mL) showed no significant differences in both flowering and fructification seasons (P > 0.05). The results also showed that the absorption amount in the flowering season samples was higher than that in the fructification season, with the highest concentration of 25 mg/mL (6941.38 ± 0.88) (Fig. 4).

Microbial test using the disk diffusion method

Results indicated no significant differences between the antibacterial properties of the broadleaf plantain leaf extracts at the flowering stage against *Bacillus*, *Salmonella*, and *Staphylococcus* (P > 0.01). Moreover, significant differences existed between *Escherichia* species and those of the other studied genera (P < 0.01). The largest inhibitory effect was observed at the flowering stage against *Salmonella* and the smallest against *Escherichia coli*. No significant differences between the samples at the fruit set stage were found (P > 0.05). Furthermore, the highest and lowest inhibitory effects were observed using the extract prepared at the fruit set stage against *Bacillus* and *E. coli*, respectively (Fig. 5).

Various studies have shown that the cell walls of Gram-positive bacteria are highly sensitive to many antibiotics, antimicrobial compounds, and even herbal drugs (McDonnell and Russell, 1999; Norajit *et al*., 2007; Schlievert *et al*., 1992). On the contrary, the presence of a lipopolysaccharide layer in the cell walls of Gram-negative bacteria and the plasma-rich environment has increased their relative resistance (Mazzola *et al*., 2006).

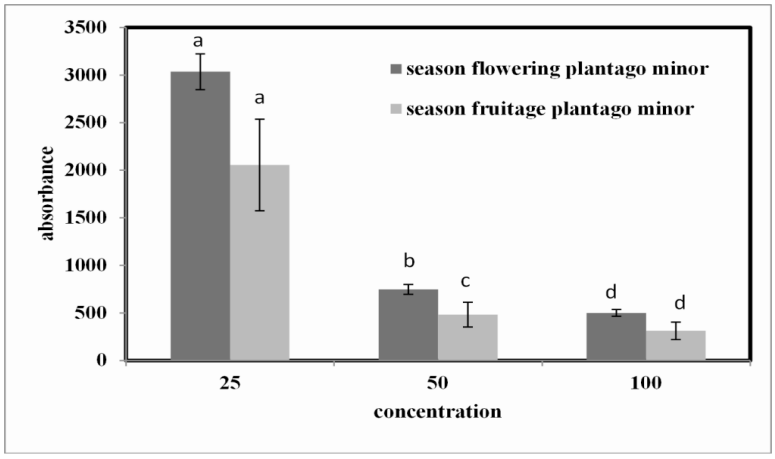

Fig. 3. Means ± standard deviations of the ferrous ion-chelating capacity of broadleaf plantain leaf extract. Similar letters show no significant differences between the values (P > 0.05), but different letters indicate significant differences between the values (P < 0.01)

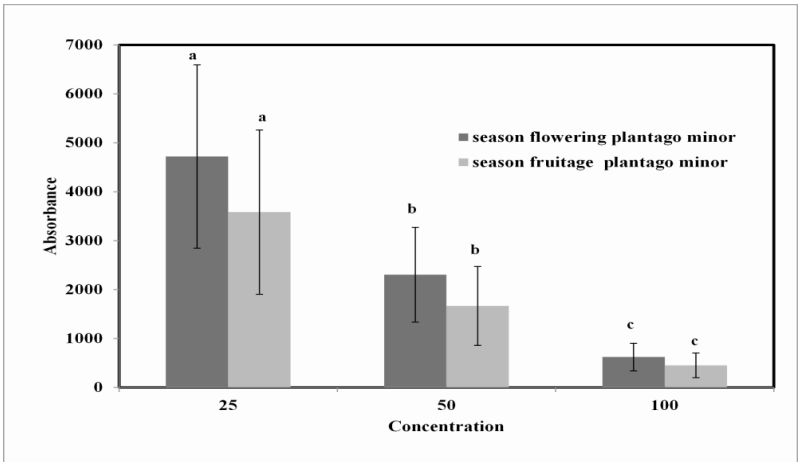

Fig. 4. Means ± standard deviations of assessing thiobarbituric acid in broadleaf plantain leaf extract. Similar letters indicate no significant differences between the values (P < 0.01)

Mirzaei *et al*. (2011) studied the antimicrobial effect of hydroalcoholic oak jaft extract (jaft is a part of the oak fruit), thyme sprouts, and skin of wild pistachio fruit against Listeria monocytogenes. Results showed that jaft and green skin of wild pistachio have the greatest antibacterial properties. The inhibition zone for jaft at 32 mg/mL was almost identical to ampicillin disks; the minimum inhibitory concentration for jaft extract was 125 µg/mL, and its minimum lethal concentration was observed at 250 µg/mL.

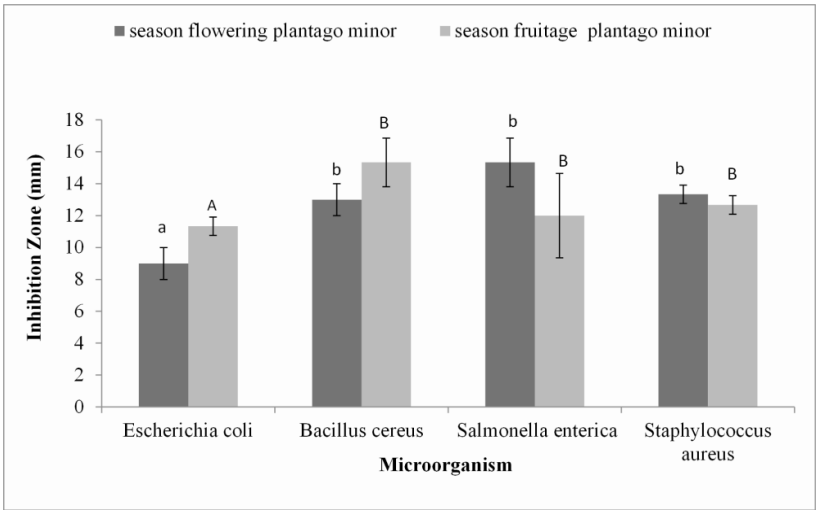

Fig. 5. Means ± standard deviations of antibacterial properties of broadleaf plantain leaf extract. Different letters indicate significant differences between the values (P < 0.05)

Ebrahimi et al. (2010) investigated the antibacterial effect of oak fruit extract against *S. aureus, Staphylococcus epidermis* and *E. coli* and attributed the observed antibacterial effect of the extract against the mentioned bacteria to the presence of phenolic compounds, especially tannins, in the extract.

Mehni and Shahdadi (2014) studied the phenolic compounds and antiradical activities of methanolic extracts of broadleaf plantain and colocynth in Iran. Results showed that the broadleaf plantain extract has the largest while the colocynth extract has the smallest contents of phenolic compounds. Moreover, the extracts of broadleaf plantain and colocynth have the lowest and highest IC50, respectively. These results indicate that broadleaf plantain and colocynth exert antiradical activities and could be used as natural antioxidants in the food and pharmaceutical industries.

Antibacterial properties of potato starch films combined with antibiotics

Films containing amoxicillin, furazolidone, and cloxacillin were used in this research. Results showed significant differences between the effects of the combination of potato starch films with antibiotics and potato starch films with control sample on *Staphylococcus* ($P < 0.01$). The greatest inhibitory effect was exhibited by the combination of potato starch film and amoxicillin. Results related to *Salmonella* indicated that the highest inhibitory effect is exhibited by amoxicillin among the aforementioned antibiotics. Moreover, significant differences were found between all samples containing antibiotics and the control samples ($P < 0.01$). Results of the study on *E. coli* revealed significant differences between samples with antibiotics and the control samples ($P < 0.01$). Furthermore, the largest inhibitory effect was

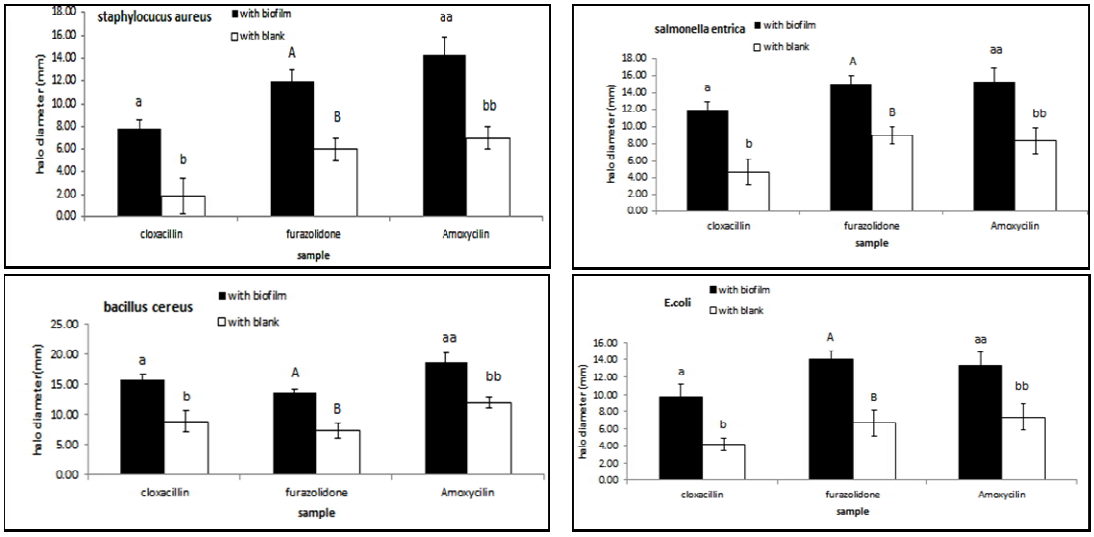

Fig. 6. Means ± standard deviations of antibacterial properties of potato starch films together with antibiotics. Different letters indicate significant differences between the values (P < 0.01)

observed in the film containing furazolidone. In the study on *Bacillus*, the highest inhibitory effect was exerted by the sample containing amoxicillin, and significant differences were found between the samples with antibiotics and the control samples (P < 0.01) (Fig. 6).

CONCLUSION

The antioxidant and antibacterial properties of broadleaf plantain leaf extracts at the flowering and fruit set stages were studied. Results showed the potential antibacterial and antioxidant activities of the extracts. Natural antioxidants are mainly present in plants containing phenolic compounds. The phenolic contents in plants depend on genetic and environmental factors. Many antioxidant compounds exist in plants, and their complete identification is difficult. Therefore, the antioxidant capacities of extracts are evaluated using many assessments (Yazici *et al*., 2012). Moreover, results showed that the samples at the flowering stage had higher activities than those at the fruit set stage and could be used as suitable antioxidants in industries. Pouya *et al*. (2016) studied the effects of hydroalcoholic extracts of thyme, garlic, and licorice and reported that the extracts had suitable antibacterial effects compared with the control samples. Moreover, the combination of potato starch films and antibiotics exhibited stronger inhibitory effects compared with the control. This result indicated a strong synergistic effect between potato starch films and antibacterial materials.

Leaf extracts of the studied plant exerted desirable antioxidant activities. After further research, these extracts could be used in the food and pharmaceutical industries for delaying oxidation instead of employing synthetic antioxidants. In consideration of the global problem of microbial resistance in recent years, these

extracts can be used in the pharmaceutical and food industries as natural preservatives because of their antibacterial properties, especially when they are combined with potato starch films.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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