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Antibacterial and DPPH Free Radical-scavenging Activities of the Ethanol Extract of Propolis Collected in Taiwan

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ABSTRACT

In the present study, ethanolic extracts of propolis (EEP) collected from various regions (Taipei, Mingchen and Fangliao) in Taiwan during different time periods (June, August and October-November, 2000) were tested for their antibacterial and antioxidative activities. In addition, the thermal stabilities of these activities exerted by EEP were also investigated.

It was found that the EEP samples, depending on collecting location and time period, exerted various antioxidative activities in terms of scavenging α,α-diphenyl-2-piorylhydrasyl (DPPH) free radicals and showed various extents of antibacterial activity against *Staphylococcus aureus* and *Listeria monocytogenes*, but not against *Escherichia coli* and *Salmonella typhimurium*. In general, the Taiwanese propolis extract collected in June showed the most profound antibacterial and free radical-scavenging activities than those collected during other time periods. Among all of the samples tested, EEP collected from the Mingchien area in June exhibited the highest antibacterial activity, while that collected from the Taipei area during the same time period showed the highest free radical-scavenging activity. Further tests of EEP collected from Taipei in June revealed that its DPPH free radical-scavenging effects reduced significantly after heating at 50, 80 or 100°C for 1 hr, while its antibacterial activity remained unchanged.

Key words: ethanolic extract of propolis, antibacterial and DPPH free radical-scavenging activity, thermal stability

INTRODUCTION

Propolis is a natural product derived from plant resins collected by honey bees. It is used by bees as glue, a general-purpose sealer, and as draught-extruder for beehives. Propolis has been used in folk medicine for centuries[(1)]. It is known that propolis possesses antimicrobial, antioxidative, anti-ulcer and anti-tumor activities[(2,3)]. Therefore, propolis has attracted much attention in recent years as a useful or potential substance used in medicine and cosmetics products[(4)]. Furthermore, it is now extensively used in foods and beverages with the claim that it can maintain or improve human health[(5,6)].

The chemical composition of propolis is quite complicated. More than 150 compounds such as polyphenols, phenolic aldehydes, sequiterpene quinines, coumarins, amino acids, steroids and inorganic compounds have been identified in propolis samples[(2,7)]. The contents depend on the collecting location, time and plant source[(7-9)]. As a consequence, biological activities of propolis gathered from different phytogeographical areas and time periods vary greatly. In Taiwan, Chang et al.[(10)] reported that the three Taiwanese propolis samples examined contained various amounts of flavonoids, which are generally considered to be the key active biological compounds in propolis.

Although numerous reports concerning the biological activities of propolis collected in Europe and South America have been documented, information concerning the characteristics of Taiwanese propolis is still quite limited. Therefore, antibacterial and antioxidative activities of the ethanolic extract of propolis (EEP) collected from different regions of Taiwan at different time periods in 2000 were determined.

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MATERIALS AND METHODS

I. *Propolis Origins*

Taiwanese propolis samples tested in the present study were all obtained from Professor K. K. Ho, Dept. of Entomology, National Taiwan University, Taipei, Taiwan. These propolis samples were originally collected from beehives located at different regions in Taiwan: Taipei (northern part), Mingchien (middle part) and Fangliao (southern part) in June, August and October-November, 2000.

Brazilian propolis and Chinese propolis samples, originally obtained from Research & Development Division, Institute of Agricultural Research, Chinese Academy of Agricultural Science, Beijing, China, were also provided by Prof. Ho. These propolis samples were stored at -20°C.

II. *Preparation of Ethanolic Extracts of Propolis (EEP) Solution*

Propolis samples were cut into small pieces, ground and extracted with 80% ethanol (1:10, w/v) by shaking (150

rpm) at 25˚C for 48 hr. The ethanolic extract solution was then filtered through a Whatman # 1 filter paper and restored to the original volume with 80% ethanol. Based on the individual dry weight determined in the solution, the EEP solution was further adjusted with appropriate amounts of 80% ethanol to obtain solutions containing various amounts of EEP.

III. *Heat Treatment of EEP Solution*

Ethanol solution containing EEP (Taipei-6) was heated at 50, 80 or 100˚C for 1 hr by a refluxing system. The heating sample was restored to its original volume with the addition of 80% ethanol and served as the heating sample.

IV. *Microorganisms and Preparation of Inoculum*

To determine the antibacterial activity of EEP, *Staphylococcus aureus* CCRC 12657 and *Listeria monocytogenes* CCRC 19730 were obtained from the Culture Collection and Research Center, Food Research and Development Institute, HsinChu, Taiwan.

After two successive transfers of the test organism in tryptic soy broth (TSB, Difco, Detroit, MI, USA) at 37˚C for 12 hr, the activated culture was inoculated into TSB and incubated at the above temperature 37˚C for 12 hr. When the population was about 3~7 $\times 10^8$ CFU/mL, it was appropriate to serve as the inoculum.

V. *Determination of Balsam Content*

The EEP solution was evaporated under 105˚C until dry. Weight was determined and expressed as weight percentage of balsam in the ethanolic extract solution.

VI. *Measurement of Antibacterial Activity*

Saline solution (0.85% NaCl) in a quantity of 8.9 mL was first added with 0.1 mL of the prepared EEP solution (750 μg/mL) or 0.1 mL 80% ethanol, which served as the control. The mixture was then inoculated with 1.0 mL of the test organism at an initial concentration of 10^7 CFU/mL. Viable population of the test organism was determined after 6 hr of incubation at 37˚C.

To enumerate the viable population of the test organism, cultures were first serially diluted with saline solution. One mL of the serially diluted sample was pour-plated onto tryptic soy agar (TSA, Difco, Detroit, MI, USA). Colonies appearing on the plates after 48 hr of incubation at 37˚C were counted. In addition, the population reduction (log CFU/mL) was obtained by subtracting the final population (log CFU/mL) in test sample from that in the control.

VII. *Measurement of α,α-Diphenyl-2-piorylhydrasyl (DPPH) Free Radical Scavenging Activity*

The DPPH radical scavenging ability of EEP was determined basically according to the method of Shimada et al.[11]. The EEP solution (20-640 μg/mL) was mixed with 400 μM DPPH (Sigma Chemical Co., St Louis MI.) methanol solution at a ratio of 1:3. The mixture was left in the dark at room temperature for 90 min. The absorbance of the resulting solution was measured by a spectrophotometer at 517 nm. The capability of scavenging DPPH radicals was then calculated by the following equation:

$$\text{Scavenging effect \%} = [1-(A_{517} \text{ of sample}/A_{517} \text{ of control})]$$

VIII. *Statistical Analysis*

The mean values and the standard deviations were calculated from data obtaining triplicate trials. These data were then compared with the least significant difference test[12].

RESULTS AND DISCUSSION

I. *Color and Balsam Content of Propolis*

Differences in the color of Taiwanese propolis harvested at different time periods were noted. In general, the Taiwanese propolis collected in June, regardless of collecting location, appeared yellowish-green, while those collected in August and October-November appeared yellowish-brown and brown, respectively. On the other hand, the color of the Brazilian propolis, similar to that of Taiwanese propolis collected in August, appeared yellowish-brown in color; while the color of the Chinese propolis was dark brown.

In the present study, propolis was extracted with 80% ethanol. The fraction of the propolis soluble in alcohol was usually called “propolis balsam” and it leaves the alcohol-insoluble or wax fraction separate[1]. Balsam contents have been reported to vary with the source of propolis[13,14]. As shown in Table 1, the balsam contents in Taiwanese propolis, ranging between 24.3% and 70.9%, varied not only with collecting regions but also with the collecting time of propolis. In general, a relatively higher content of

Table 1. Balsam contents of various propolis extracted with 80% (v/v) ethanol

Source of propolis	Balsam contents (%, w/w)
Taipei-6	53.7
Taipei-8	31.4
Taipei-11	24.3
Mingchien-6	70.9
Mingchien-8	56.7
Mingchien-10	49.6
Fangliao-6	56.8
Fangliao-8	42.7
Fangliao-10	37.3
Brazil	45.2
China	49.5

Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003

Table 2. Antibacterial activities of the ethanolic extracts of Taiwanese propolis obtained from different regions during different time periods

	Staphylococcus aureus		*Listeria monocytogenes*		*Salmonella typhimurium*		*Escherichia coli* O157:H7	
EEP[a]	Final population[b] (log CFU/mL)	Population reduction[c] (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)
Control[d]	6.95 ± 0.05A[e]		7.02 ± 0.05A		7.41 ± 0.05AB		7.10 ± 0.10A	
Taipei-6	0.84 ± 0.33FG	6.11	0.78 ± 0.28I	6.24	7.38 ± 0.01AB	0.03	7.08 ± 0.10A	0.02
Taipei-8	2.74 ± 0.12C	4.21	2.55 ± 0.25EF	4.47	7.39 ± 0.04AB	0.02	7.11 ± 0.03A	-0.01
Taipei-11	4.54 ± 0.22B	2.41	3.20 ± 0.12D	3.82	7.45 ± 0.03A	-0.04	7.07 ± 0.01AB	0.03
Mingchien 6	0.48 ± 0.41G	6.47	0.10 ± 0.17J	6.92	7.40 ± 0.03AB	0.01	6.95 ± 0.15B	0.15
Mingchien 8	2.06 ± 0.09E	4.89	2.02 ± 0.21G	5.00	7.37 ± 0.01B	0.04	7.07 ± 0.06AB	0.03
Mingchien 10	2.24 ± 0.22DE	4.71	2.25 ± 0.44FG	4.77	7.41 ± 0.04AB	0.00	7.08 ± 0.03A	0.02
Fangliao 6	0.98 ± 0.60F	5.97	0.44 ± 0.47IJ	6.58	7.35 ± 0.03B	0.06	7.06 ± 0.04AB	0.04
Fangliao 8	2.61 ± 0.42CD	4.34	1.54 ± 0.19H	5.48	7.41 ± 0.08AB	0.00	7.09 ± 0.10A	0.01
Fangliao 10	2.76 ± 0.31C	4.19	2.72 ± 0.42E	4.30	7.40 ± 0.05AB	0.01	7.08 ± 0.05A	0.02
Brazil	6.89 ± 0.09A	0.06	4.88 ± 0.02C	2.14	7.39 ± 0.04AB	0.02	7.12 ± 0.08A	-0.02
China	6.95 ± 0.06A	0.00	6.51 ± 0.10B	0.51	7.41 ± 0.04AB	0.00	7.16 ± 0.06A	-0.06

[a]Saline solution (0.85% NaCl) containing 7.5 μg/mL EEP was inoculated with test organism at initial concentration of 10^7 CFU/mL.
[b]Determined after 6 hr of incubation at 37°C.
[c]Population reduction = log (final population in control)- log (final population in test sample).
[d]EEP was substituted with ethanol solution.
[e]Values with the same organism with different capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

balsam was noted in propolis collected in June, regardless of collecting area, than those collected in other months. Among the propolis samples tested, that collected from Mingchien in June showed the highest balsam content. Besides, the Brazilian and Chinese propolis tested showed a balsam content of 45.2% and 49.5%, respectively.

II. *Antibacterial Activity of the Ethanol Extract of Propolis*

It is reported that the antimicrobial activity of propolis reflected its constituent, which may differ from area to area, and season to season depending on its chemical composition[15-18]. Flavonoids and esters of phenolic acids are regarded to be responsible for the anti-microbial activity of propolis[18,19]. Kujumgiev et al.[13] found that tropical propolis did not contain such substances but still showed similar antibacterial activity and indicated that different substance combinations in the propolis are essential for its biological activity. On the other hand, Kedzia et al.[20] reported that the mechanism of anti-microbial activity is complicated and could be attributed to the synergy between flavonoid hydroxyacids and sesquiterpenes. Krol et al.[21] also observed this effect.

Antibacterial activities of the ethanolic extract of propolis gathered from different regions at different time intervals against test organisms are summarized in Table 2. In agreement with the reports of Kujumgiev et al.[13], Nieva Moreno et al.[14] and Dobrowalski et al.[22], the ethanolic extracts of propolis tested did not show antibacterial activity against *Sal. typhimurium* and *E. coli* O157:H7 which were gram (–) bacteria. On the other hand, the Taiwanese propolis extracts exhibited various extents of antibacterial activity against *Sta. aureus*, which showed a marked population reduction, ranging from 2.41 to 6.11 log CFU/mL, under the present test conditions. A population reduction ranging between 6.92 and 3.82 log CFU/mL was also observed with *L. monocytogenes* after 6 hr of exposure to the Taiwanese propolis extracts tested. In general, *L. monocytogenes* was more susceptible to the EEP than *Sta. aureus*. The ethanolic extracts of Chinese and Brazilian propolis tested also showed antibacterial activity against *Sta. aureus* and *L. monocytogenes*, while not against *Sal. typhimurium* and *E. coli* O157:H7.

Sforcin et al.[18] reported that there are no significant difference between the antibacterial activity of Brazilian propolis collected during different seasons. However, we do find a significant difference in the antibacterial activity of Taiwanese propolis due to differences in the collecting time as shown in Table 2. This is in agreement with the report of Santos et al.[23] who found that the propolis extract collected in summer exhibited higher antibacterial activity against *Actinobacillus actinomycetemcomitans* than those collected during other seasons. Among the Taiwanese propolis extracts tested, in general, propolis collected in June exhibited the most profound antibacterial activity than those collected during other time periods against *Sta. aureus* or *L. monocytogenes*. For example, Taipei-6 EEP reduced the viable *Sta. aureus* population by 6.11 log CFU/mL, while Taipei-11 EEP caused a smaller population reduction of only 2.41 log CFU/mL under similar test conditions. Variations in the antibacterial activity of propolis collected in the same time period, while from different locations were also observed. Among the samples collected at the same period, in general, EEP from the Mingchien area exhibited a higher antibacterial activity than those from other areas.

III. *Antioxidative Activity of Propolis Extracts*

Various investigators have reported that propolis

possesses antioxidative activities[24-28]. Among them, Nagai et al.[28] demonstrated the antioxidative activity in commercially available propolis. They postulated that flavonoids, such as quercetin, flavones, isoflavones, flavonones, anthocyanins, catechin and isocatechin may contribute to the antioxidative activity they observed. Nieva Moreno et al.[14] also found that the ethanolic extracts of the Argentina propolis showed free radical-scavenging activity. However, they indicated that different flavonoid compositions and other factors might be involved in the free radical-scavenging activity.

In the present study, the antioxidative activity, in terms of the scavenging of the radical DPPH, of the ethanolic extracts of various propolis was determined and compared. The proton-radical scavenging action has been known as an important mechanism of antioxidation. DPPH was used to determine the proton-radical scavenging action of the propolis extract, since it possesses a proton free radical and shows a characteristic absorption at 517 nm. The purple color of the DPPH solution would fade rapidly when it encounters proton-radical scavengers[29].

Figure 1 shows the dose-response curve for the radical-scavenging activity of the EEP. While Table 3 summarizes the calculated half-inhibition concentration (IC_{50}), the efficient concentration required for decreasing initial DPPH concentration by 50%. IC_{50} was obtained by interpolation from linear regression analysis of data shown in Figure 1. It was found that the propolis extracts tested showed various potencies for free radical-scavenging activity with an EEP where IC_{50} ranged between 17.90 and 108.05 μg/mL (Table 3). The antioxidative activity of all the EEP tested, except Taipei-6 propolis extract, increased with the concentration of propolis extract to 80 μg/mL (Figure 1). From the same region, the Taiwanese propolis extract collected in June exhibited a higher activity than those collected at other time periods. Variations in the free radical-scavenging activity were also noted on the extracts of propolis collected from different regions. Among the propolis extracts tested in the present study, propolis from Taipei collected in June (Taipei-6) exhibited the highest free radical-scavenging activity, while extract of Taipei-11 propolis showed the lowest activity. On the other hand, the IC_{50} for the propolis extract from Brazil and China was found to be 41.41 and 24.53 μg/mL, respectively.

Table 3. Half-inhibition (IC_{50})[a] of EEP in scavenging DPPH radicals

EEP	IC_{50} (μg/mL)
Taipei-6	17.90 ± 0.22I[b]
Taipei-8	42.33 ± 0.12C
Taipei-11	108.05 ± 1.75A
Mingchien-6	28.17 ± 0.32G
Mingchien-8	37.40 ± 1.09E
Mingchien-10	40.12 ± 2.00D
Fangliao-6	31.16 ± 1.48F
Fangliao-8	37.14 ± 0.42E
Fangliao-10	51.48 ± 2.26B
Brazil	41.41 ± 0.46CD
China	24.53 ± 0.57H

[a]IC_{50}, the efficient concentration decreasing initial DPPH concentration by 50%, was obtained by the interpolation from linear regression analysis.

[b]Each value is given as means ± standard deviation (n = 3). Values with capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

IV. *Thermal Stability of the Antibacterial and Antioxidative Activities of Taiwanese Propolis Extract*

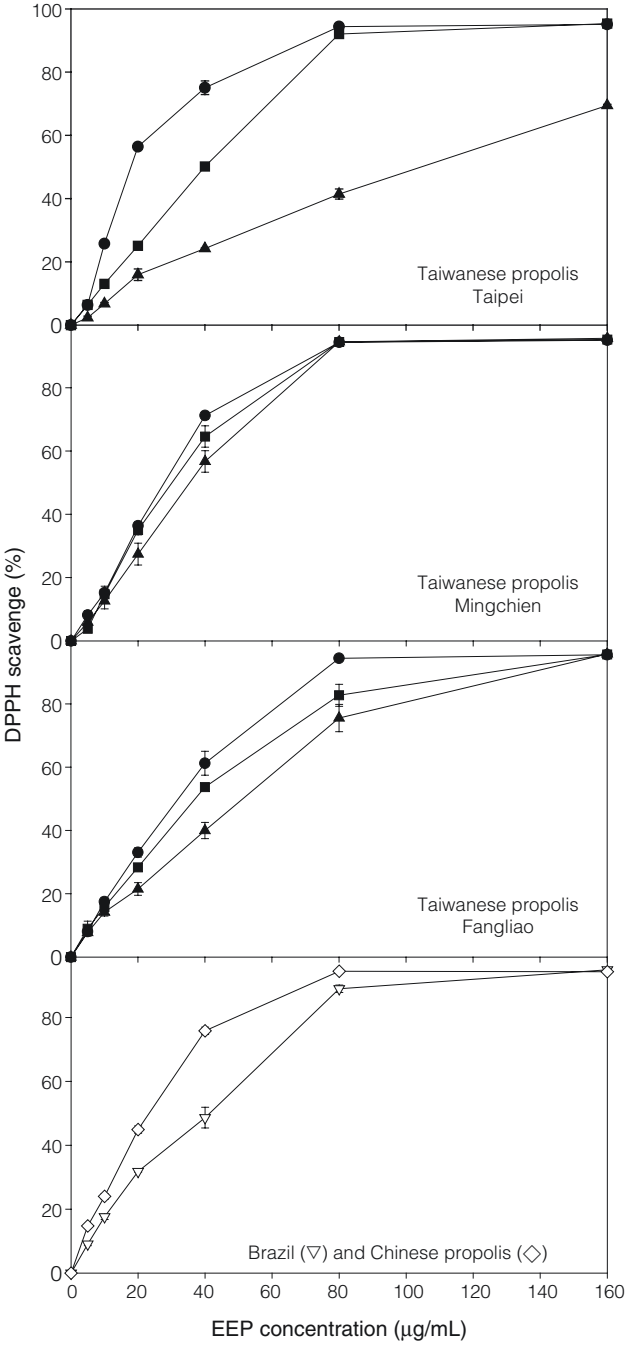

Figure 1. DPPH free radical scavenging effects of EEP obtained from different regions during different time periods. Correcting time: June, ●; August, ■; October (Mingchien and Fangliao) and November (Taipei), ▲.

Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003

Table 4. Effects of heating on antibacterial activities of EEP[a]

Heating temperature (˚C)	*Staphylococcus aureus*		*Listeria monocytogenes*	
	Final population[b] (log CFU/mL)	Population reduction[c] (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)
Control[d] without heating	6.95 ± 0.05A[e]		7.02 ± 0.05A	
50	0.84 ± 0.33B	6.11 ± 0.33A	0.78 ± 0.28B	6.24 ± 0.28A
80	0.60 ± 0.39B	6.35 ± 0.39A	0.69 ± 0.09B	6.33 ± 0.09A
100	0.97 ± 0.23B	5.98 ± 0.23A	0.33 ± 0.56B	6.69 ± 0.56A
	0.72 ± 0.47B	6.23 ± 0.47A	0.29 ± 0.11B	6.73 ± 0.11A

[a]Saline solution (0.85% NaCl) containing 7.5 μg/mL Taipei-6 EEP which has been heated at the specified temperature for 1 hr, was inoculated with test organism at an initial concentration of 10^7 CFU/mL.
[b]Determined after 6 hr of incubation at 37˚C.
[c]Population reduction = log (final population in control)- log (final population in test sample).
[d]EEP was substituted with ethanol solution.
[e]Values with the same organism with different capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

The effect of heat treatment on the antibacterial and antioxidative activities was investigated on Taipei-6 propolis extract. After each sample was heated at 50, 80 and 100˚C for 1 hr, the antibacterial and antioxidative activities were measured.

As shown in Table 4, the finial populations of either *Sta. aureus* or *L. monocytogenes* in saline solution containing EEP were all less than that found in saline solution containing no EEP (control). However, the final population or population reduction of each test organism noted in the saline solution containing EEP with or without heat treatments did not show any significant difference ($p > 0.05$). This indicated that antibacterial activity of the propolis extract was quite stable under the heat treatments tested.

Table 5 shows the IC_{50} of the Taipei-6 propolis extract after subjecting to various heat treatments. IC_{50} of propolis extracts subjected to 50, 80 or 100˚C heat treatment showed no significant difference ($p > 0.05$) among them. While they are all higher than that of propolis extract without heat treatment, this demonstrated that heat treatments, contrary to that observed on antibacterial activity, reduced the antioxidative activity of propolis extract. The antioxidative activity of propolis was not thermally stable. Porpolis components associated with the antibacterial and antioxidative activities may be different or not entirely the same and thus led to the observed difference in the thermal stability. However, the exact reason remained to be further examined.

Table 5. Half-inhibition (IC_{50}) of heated EEP[a] in scavenging DPPH radicals

Treatment temperature (˚C)	IC_{50} (μg/mL)
50	28.48 ± 0.28A[b]
80	28.25 ± 0.46A
100	28.42 ± 0.98A
Without heating	17.90 ± 0.22B

[a]Taipei-6 EEP was subjected to heating for 1 hr.
[b]Each value is given as means ± standard deviation (n = 3). Values with capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

CONCLUSION

The results of this study demonstrated that ethanolic extracts of Taiwanese propolis possess antibacterial activities and DPPH radicals-scavenging effects which varied with the source and collecting time. Regardless of collecting locations, the extracts of propolis harvested in June, in general, exhibited higher antibacterial and DPPH radical-scavenging activity than those harvested during other time periods. Among the samples tested, extract of propolis collected from Taipei in June (Taipei-6) exhibited the highest DPPH radicals scavenging activity, while Mingchien-6 propolis extract showed the highest antibacterial activity. In addition, although the DPPH radicals scavenging effects of Taipei-6 propolis extract significantly reduced after heating at 50, 80 or 100˚C for 1 hr, its antibacterial activity remained unchanged.

Difference in the plant source available to honey bees at different locations and time periods might lead to differences in biologically active components present in the propolis[7-9]. This may in turn result in the variations of the antibacterial and antioxidative activities of propolis observed in the present study. Therefore, both the identity of the plant source available in Taiwan and the biological active components in Taiwanese propolis merit further investigation. However, results obtained from the present study, along with reports of other investigators[7-9], further stress the importance of quality control when commercial propolis products for these biological activities are being prepared from raw materials.

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