Journal of Sustainability Science and Management Special Issue No. 3 2017
Improving the Health of Setiu Wetlands Ecosystems and Productivity of Crustacean Resources for Livelihood Enhancement:14-22
ISSN: 1823-8556

ANTIOXIDATIVE ACTIVITIES AND FLAVONOIDS CONTENTS IN LEAVES OF SELECTED MANGROVE SPECIES IN SETIU WETLANDS EXTRACTED USING DIFFERENT SOLVENTS

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Abstract: Mangrove plant possess special properties and are an important source of nutritional and medicinal uses. Several mangrove species from Setiu wetland were analysed and compared for their antioxidant activity value. Different solvents namely, methanol, water and hexane were used for the antioxidant activity extraction. DPPH was used to measure the antioxidant activity as well as the Total Phenolic and Total Flavonoid content. Total flavonoid content varied from 25.67 ± 0.06 mg QE/g to 37.90 ± 4.76 mg QE/g for each of the species. Total phenolic content ranged from 2.39 ± 4.97 mg GAE/g to 64.28 ± 3.05 mg GAE/g.The scavenging activity DPPH indicated that *B. sexangula* holds the highest antioxidant activity for both methanol and water extraction samples with 99.33% and 99.28%, respectively. Mangrove leaves extract exhibit high total flavonoids and phenolics contents along with strong scavenging activity. It is hoped that the results of this study will be beneficial in contributing towards food industry and perhaps might invoke further research on understanding the different antioxidant mechanisms.

Keywords: Antioxidant activity, DPPH, flavonoids, mangroves, phenolics, Setiu Wetlands.

Introduction

Setiu Wetland are located in the north of Kuala Terengganu, Malaysia and encompasses many ecosystems such as estuary, mangrove and wetland (Suratman *et al*., 2014). Mangroves are known as salt tolerant trees and shrubs that grow in muddy and wet soil in intertidal regions of the tropical and subtropical coastlines (Satyavani *et al*., 2015). These wetland ecosystems are among the most productive and diverse in the world and also can be considered as a reservoir of contaminates (Song *et al*., 2012). Mangroves forests have been well recognized recently based on their significance towards all organisms and play an important role in sustaining and maintaining the ecosystem conditions (Azmi *et al*., 2012). Mangroves were not fully explored previously are now believed to have nutritional and medicinal uses with high antioxidant activity and antiradical scavenging activity (Shamsuddin *et al*., 2013). Mangrove plants may contain phenolic compounds such as phenolic acid, flavonoids, quinones, coumarins, lignans, stilbenes and tannins as free radical scavenging molecules (Cai *et al*., 2004; Roby *et al*., 2013).

Antioxidants are compounds that can delay or protect the oxidation of lipids or free radicals by inhibiting the initiation or propagation of oxidizing chain reactions (Zheng & Wang, 2001). The antioxidant activity is the most studied property attributed to flavonoids (Asha *et al*., 2012). The other properties of flavonoids including anti-allergic, anti-inflammatory, antimicrobial, antiviral, antioxidant, oestrogenic, enzyme inhibition, vascular and cytotoxic anti-tumour activity (Asha *et al*., 2012) antioxidant activity is the

most studied property attributed to flavonoids instead of many others useful properties including anti-allergic, anti-inflammatory, antimicrobial, antiviral, antioxidant, oestrogenic, enzyme inhibition, vascular and cytotoxic anti-tumour activity (Asha *et al*., 2012). The antioxidant activities were determined by DPPH free radical scavenging ability. In order to determine the antioxidant activity, the extraction process is needed by using three types of solvents (methanol, water and hexane). This is because, these solvents contain different of polarity. Using solvents with different polarities consequence the production of polyphenol-enriched *Humulus lupulus* (plant) extracts (Kowalczyk *et al.,* 2013). In addition, the extracts are safe to be consumed because the extraction procedure includes evaporation process.

There is scarce information on antioxidant activity and flavonoids contents in leaves of mangrove species (*Bruguiera sexangula, Bruguiera cylindrica*, *Rhizophora apiculata*, *Aviccenia alba* & *Lumnitzera racemosa*) in Setiu Wetlands. The species selected according to their popularity, benefits, most studied and uses on antioxidant activity and flavonoids contents. Yet, therefore, the main objectives of this study are to identify the morphology characteristics of mangrove species in Setiu Wetlands; to determine the antioxidant activity and the flavonoids contents in each of the mangrove species and to compare the extracted using different solvents.

Materials and Methods

Samples collection and preparation

Five dominant species of mangrove such as *Bruguiera sexangula*, *Bruguiera cylindrica*, *Rhizophora apiculata*, *Aviccenia alba* and *Lumnitzera racemosa* were collected from Setiu Wetlands, Terengganu. The morphology characteristics of the species were determined and identified. The specimens were preserved in herbarium of Universiti Malaysia Terengganu (UMT). The plant parts were shed-dried, finely ground and stored in airtight containers for further extractions (Banerjee *et al.,* 2008).

Extraction of plants

The extraction process was determined as previously described by (Cai *et al.,* 2004). The dried samples undergo three types of extraction process. First, water extraction: 5 g of the powdered sample with 100 mL ultra-filtered water were extracted at 80°C for 20 min in a water bath shaker (Shaking Bath 5B-16) (Techne, Ltd., UK). The cooled extracts were centrifuged at 5,000 rpm for 10 min and filtered by a Millipore filter with a 0.45-AM nylon membrane under vacuum at 23°C. The filtrate was stored at 4°C until use within 24 hr. The methanol extraction procedures were the same as in the water. By the other hand, the temperature used in water bath shaker was lowered in hexane extraction (40°C) due to easily evaporated property of hexane.

Total Phenolic Contents

Total phenolic results quantified in triplicates and expressed as gallic acid equivalent. Total phenolic contents were determined according to Roby *et al*., (2013) following the Folin-Ciocalteau method with a few modifications. Samples 0.5 ml were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagents. After 5 min later, the solution was then added with 2.0 ml of 75 g/l sodium carbonate. Then, the solution was incubated at room temperature for 2 h before the absorbance reaction were taken at 760 nm with UV/Visible spectrophotometer. The standard curve was prepared using 25 to 200 mg/ml solutions of gallic acid and results were expressed as gallic acid equivalent (mg/g of dry mass).

Total Flavonoid Contents

Total flavonoid contents were estimated using the method of Ebrahimzadeh *et al.* (2008) with a few modifications. Briefly, 5mg/10ml methanol of sample was mixed with 1.5 mL of

methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 490 nm.Total flavonoid contents were quantified in triplicates and expressed as mg of quercetin equivalent

DPPH Free Radical Scavenging Activity

The DPPH free radical scavenging activity was quantified in triplicates. The capability of free radical scavenging was determined as described previously (Rahim *et al.,* 2008). An appropriate amount of DPPH in methanol is needed to prepare 1, 1-Diphenyl-2-picrylhydrazil (DPPH) radical solution to a concentration of 1 mM. The concentration of L(+) –ascorbic acid used was 100ug ml^1. To 0.5 ml were prepared and 4ml of DPPH solution was added to it. The absorbance of the mixture was measure after 30 min at 517 nm. The procedure was repeated with standard solutions of butylated hydroxytoluene (BHT) and mangrove samples.

The following Equation 1 is the capability to scavenge the DPPH radical:

$$DPPH\ scavenge\ activity\ (\%) = \frac{Ao-A}{Ao} \times 100\% \qquad \text{Equation 1}$$

Where Ao is the absorbance of the control reaction and A is the absorbance in the presence of samples.

Results and Discussion

Sample Description

Five mangrove species selected were *B. sexangula, B. cylindrical, R. apiculata, A. alba and L. racemosa* according to their uses, popularity, beneficial to the community and favourable from previous studies as shown in Table 1:

Table 1: Previous studies on mangrove plants and their references

Mangrove Genus/ Species	References
Rhizophora species	Rahim *et al*. (2008)
Bruguiera cylindrical and *Rhizophora apiculata*	Ravikumar *et al*. (2011)
Rhizophora apiculata	Asha *et al*. (2012)
Bruguiera cylindrica, Lumnitzera racemosa and *Rhizophora apiculata*	Poompozhil and Kumarasamy (2014)
Rhizophora apiculata	Satyavani *et al*. (2015)

The identification based upon leaves of these mangrove species indicated that several differences were observed between them in terms of colour, size, thickness and texture. These mangrove species can grow up the in range of 15m to 33m tall except for *L. racemosa* which only 5m tall (Ibrahim & Husain, 2012). According to the observations, *B. sexangula* leaf measurement was (11cm x 4.5cm). Leaf of *B. sexangula* (Figure 1a) is yellow-green with shorter leafstalk compared to *B. cylindrica*.

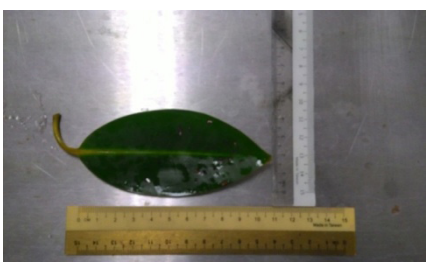

Figure 1a: *Bruguiera sexangula* leaf (11cm x4.5cm)

Bruguiera cylindrica leaf is simple with measurement of (7.5cm x 3.5cm) and smooth green leaf with pointed end (Figure 1b).

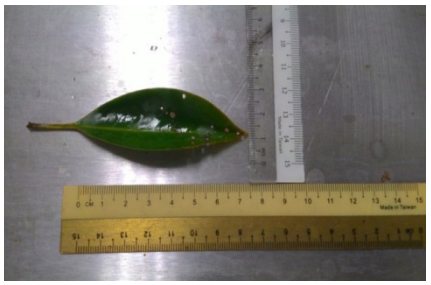

Figure 1b: *Bruguiera cyilindrica* leaf (7.5cm x 3.5cm)

Rhizophora apiculata (12cm x 4.5cm) has tiny black spot underside of the leaf and has reddish leafstalk (Figure 1c).

Figure 1c: *Rhizophora apiculata* leaf (12cm x 4.5cm)

Avicennia alba was found to be dark green colour with greyish underside (Figure 1d).

Figure 1d: *Avicennia alba* leaf (7cm x 3.5cm)

Lumnitzera racemosa has the smaller size than other species.The leaf texture is distinct than others because *L. racemosa* leaf is thicker with leathery and fleshy texture (Figure 1e).

Figure 1e: *Lumnitzera racemosa* leaf (6cm x 2.3cm)

Yield

Figure 2 shows the yield produced from the different mangrove species extracted using with different solvents namely methanol, water and hexane. It was necessary to undergo extractions process to affect total phenolic content and antioxidant activity of each plant (Skotti *et al*., 2014). The extraction processes were needed prior to antioxidant activity analyses.

The different solvents used due to the different reactions in terms of their polarity and solubility towards samples used. Four species exhibited higher yield in water extraction except for *B. sexangula* where methanol extraction illustrated the highest yield. On the other hand, hexane extraction was found to be the lowest yield (0.21% to 0.60%). The extractions were quantified in duplicates.

Total Phenolic Contents

Total phenolic content (TPC) of mangrove samples for three different solvents used varied from 2.39 ± 4.97 mg GAE/g to 64.28 ± 3.05 mg GAE/g (Figure 3). Gallic acid as the most important polyphenol in natural product being used as standard for comparison and to determine the phenolic of tested plants (Zahin *et al*., 2009). The highest phenolic content was observed in polar extract of *B. sexangula* which exceed than 60 mg GAE/g. The observations showed that Folin-Ciocalteau reagent gave distinct reactions towards mangrove samples and gallic acid.

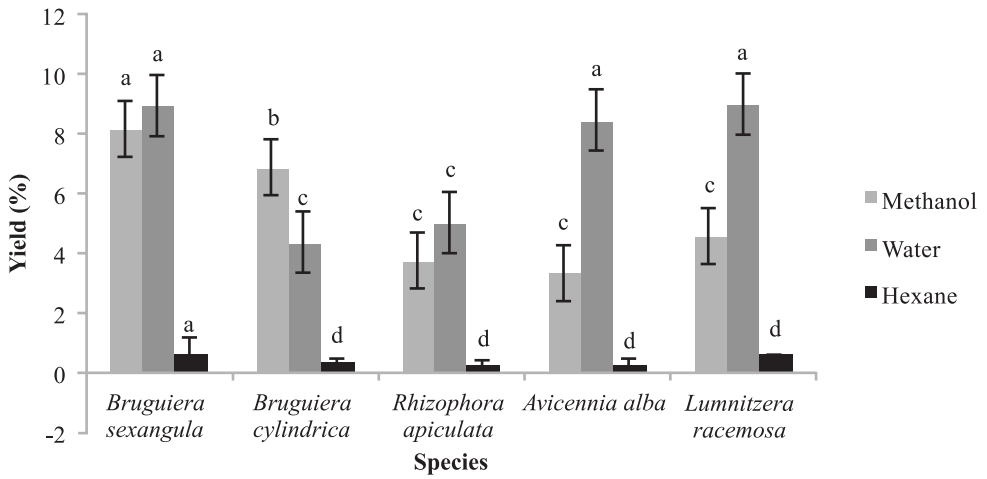

Figure 2: Yield of mangrove species with different types of solvents.
*Values with different letter (a,b,c,d) are significantly different ($p<0.05$) between samples.

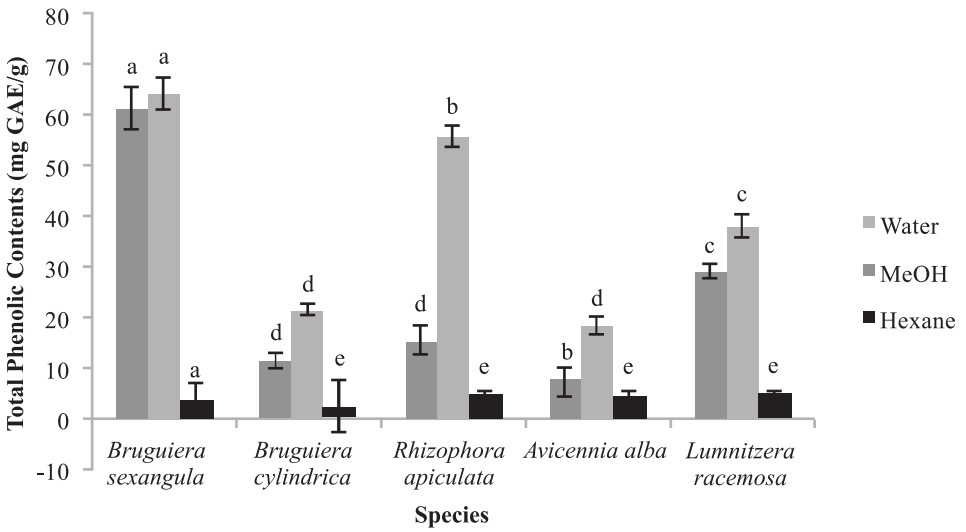

Figure 3: Total phenolic contents of mangrove species expressed as gallic acid equivalent/gram of sample (mg GAE/g).
*Values with different letter (a,b,c,d,e) are significantly different ($p<0.05$) between samples.

The reactions from this experiment were mainly due to redox properties which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Javanmardi *et al*., 2003). The highest TPC was found in methanol extract, followed by water extract and finally in hexane extract. This is because, methanol extract contained high polarity compared to water and hexane. Low yield of phenolics probably contributed to low polarity of the solvent (Kowalczyk *et al.,* 2013).

The plant extracts with higher level of polyphenolic compounds such as *B. sexangula* (64.28 ± 3.05 mg GAE/g) showed excellent antioxidant contents. There were no significant differences ($p<0.05$) between five mangrove species extracts in methanol, water and hexane

solvents. It has been considered that, the higher phenolic contents in the plant (leaves) contributed to the greater protection of the plants towards herbivores and photo damage (Banerjee *et al*., 2008).

Total Phenolic Contents

The flavonoid content (TFC) was expressed as mg quercetin equivalent (QE) per gram of dried sample. Figure 4 shows the crucial results of total flavonoid contents where methanolic extract gave the highest TFC compared to water and hexane. The result is in accordance to the study carried out by Kowalczyk *et al.* (2013) methanol is the better hydrogen bond donor and acceptor than water or hexane.

There was a positive and highly significant linear correlation ($R^2 = 0.92$) between antioxidant activity and total flavonoid content for all tested leaves of mangrove species. Generally, the structure and the substitution pattern of hydroxyl groups determined the antioxidant activity of flavonoids (Wojdylo *et al*., 2007). According to Banerjee *et al.* (2008) flavonoids are of paramount important in plants where they act as shield protection from ultraviolet radiation. Using the standard curve generated by quercetin ($R^2 = 0.92$), the total flavonoid content varied from 25.67 ± 0.06 mg QE/g to 37.90 ± 4.76 mg QE/g for each of the species.

No significant differences ($p<0.05$) were observed between the mangroves extracts. Yet samples with methanolic extract exhibit most TFC compared to the other two solvents. On the other hand *B. sexangula* in methanol and water extract revealed that they have the highest TFC than other species 37.90 ± 4.76 mg QE/g and 30.53 ± 0.01 mg QE/g, respectively. The terminology in hexane extract gave not far different for each of the species which not exceed than 30mg QE/g. The lowest value 25.67 ± 0.06 mg QE/g was observed in *B. sexangula* hexane extract.

DPPH Free Radical Scavenging Activity

DPPH free radical scavenging activity is widely used in various types of sample especially in plant type (Lee *et al*., 2003). This method used to assess the scavenging activity of sample used against free radical (Dehpour *et al*., 2009). All samples and standards had substantial DPPH scavenging activity. Figure 5 shows the results of DPPH scavenging activity in mangrove species and selected standards, ascorbic acid and BHT. Ascorbic acid was the highest inhibition with the value of 99.57%.

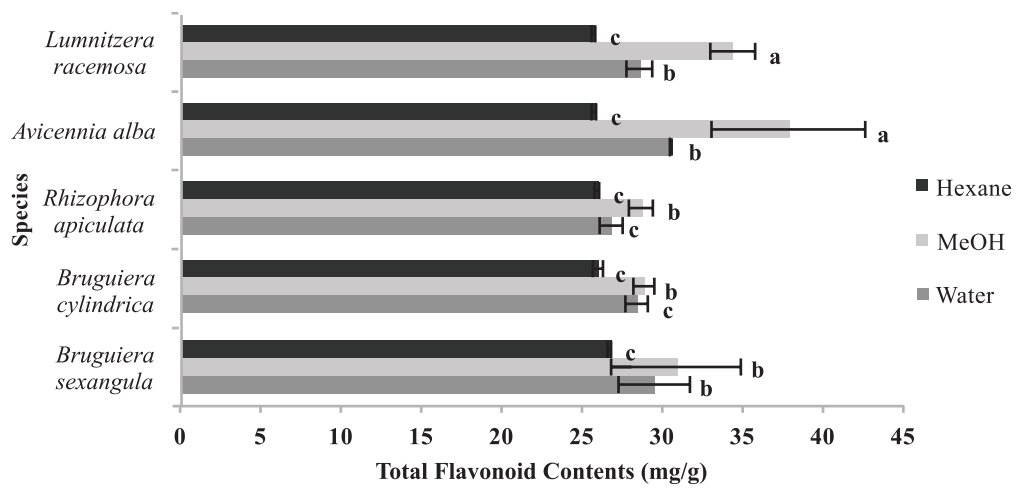

Figure 4: Total flavonoids contents of mangrove species that expressed as mg quercetin equivalent/g of samples. *Values with different letter (a,b,c) are significantly different ($p<0.05$) between samples.

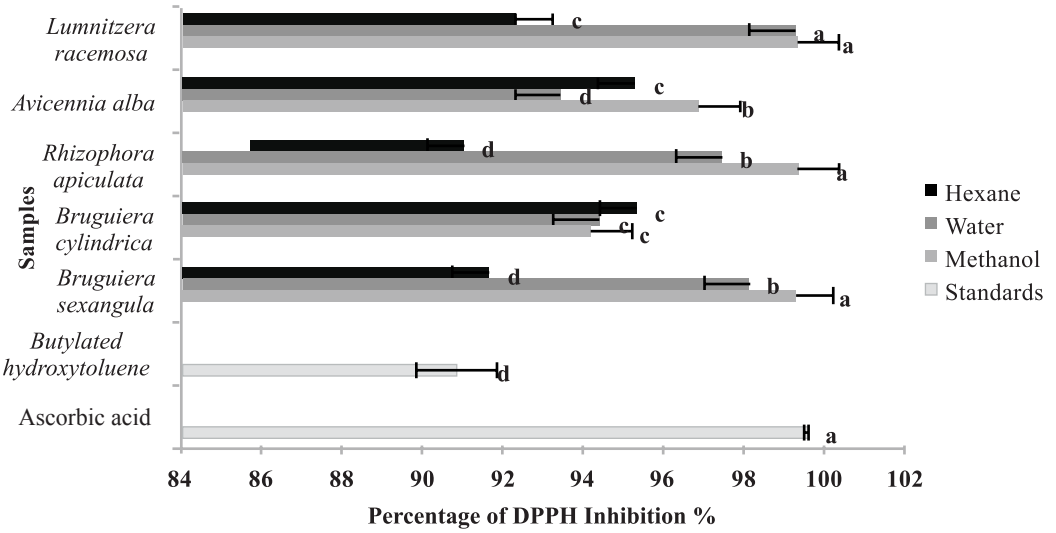

Figure 5: DPPH inhibition percentage of mangrove species differentiated with polar and non-polar solvent extraction. *Values with different letter (a,b,c) are significantly different ($p<0.05$) between samples.

The mangrove species samples show quite extending results of scavenging activity where they exceeded 90%. *L. racemosa* holds the highest DPPH inhibition for both in methanol and water extracts which 99.33% and 99.28%, respectively. *B. sexangula* hexane extract was among the lowest inhibition which is 91.67% instead of BHT which only 90.88%. The concentration of samples and standards used were 100ug ml^1. At 10ug ml^1, the scavenging activity was between 40% and 60% and scavenging activity increased as the tannin concentration increased with a maximum scavenging activity of more than 90% (Rahim *et al*., 2008).

The analysed data depicted that mangrove extract of five different species from neither polarnor non-polar solvents had no significant different ($p < 0.05$). From the observation during this experiment, the reduction process happened when mangrove extract were mixed up with DPPH solution where the violet colour from DPPH solution bleaching. It showed that mangrove extract had strong antioxidant scavenging activity. DPPH is a stable nitrogen centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation (Dehpour *et al*., 2009). The compound in the extract samples can react with DPPH solution by donated an electron or hydrogen to DPPH (Poorna *et al.*, 2013).

Organisms or samples can be considered as both antioxidants and radical scavengers if they are capable to accomplish this reaction (Dehpour *et al*., 2009). In correlation with other methods, high total phenolic and total flavonoid contents can contribute to its good DPPH scavenging activity (Dehpour *et al.,* 2009).

Conclusion

The leaves of five mangrove species illustrated outstanding antioxidant contents and antioxidant activity. All analysis showed no significant different ($p > 0.05$) on five mangrove species with different extractions. Total flavonoid contents (TFC) method showed that *B. sexangula* extract with polar solvents hold high flavonoid content than other species which are 30.53 ± 0.01 mg QE/g and 37.90 ± 4.76 mg QE/g.

Using Total Phenolic Content (TPC) method, the highest phenolic content was methanolic *B. sexangula* extract which is (64.28 ± 3.05 mg GAE/g). In DPPH method, the most active radical scavenger activity was showed by *L. racemosa* with methanol and water as solvents (99.33 ± 0.03 and 99.28 ± 0.03) instead of ascorbic acid (99.57 ± 0.04) as the standard. According to the results obtained, the leaves of mangroves species displayed high antioxidant activity with polar solvents. This might be because of the low soluble properties in hexane. The flavonoids contents in leaves of selected mangroves are good enough to proof that mangroves have their own values to be introduced and commercialised. Also, the scavenging antioxidant activity showed that mangroves have strong inhibition against free radical. Therefore, the crucial results from this research might invoke further research on understanding the differences of antioxidant mechanisms.

Acknowledgements

This research was supported by a grant (NRGS/53131) from the research council of Universiti Malaysia Terengganu. We are thankful to the department of Food Science, Biology and Central Lab for providing basic facilities and instruments for this research.

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