Antioxidant Properties of Selected *Etlingera* and *Zingiber* Species (Zingiberaceae) from Borneo Island

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ABSTRACT

In this study, total phenolic and flavonoid contents as well as antioxidant properties of methanolic extracts of rhizomes and stems of four Zingiberaceae (*Etlingera belalongensis*, *Etlingera velutina*, *Zingiber vinosum* and *Zingiber pseodopungens*) were investigated. Evaluation of antioxidant activity was conducted using 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging (DPPH) assay, 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonate radical scavenging (ABTS) assay and Ferric-Reducing Antioxidant Power (FRAP). The results showed the total phenolic and total flavanoid contents were in the range of 5.3-41.7 mg gallic acid equivalent/g and 1.09-5.86 mg catechin equivalent/g of dry sample, respectively. The antioxidant activities of the extracts as assessed by using DPPH and FRAP assays were strongly correlated with all phytochemical tested (p<0.05). As a conclusion, selected tropical gingers found in Sabah investigated in this study can be developed as natural antioxidant agents.

Key words: Zingiberaceae, antioxidant activity, total phenolic contents, total flavanoid

INTRODUCTION

Zingiberaceae is a valuable herb that has been used in traditional medicine since many years. About 40 species of the genus of *Etlingera* and 29 species of the genus of *Zingiber* can be found in Borneo Island (Poulsen, 2006). Seventy percent (70%) of the total number of *Etlingera* species can be found in Sabah (Poulsen, 2006).

Antioxidant is a substance that can fight and destroy excess free radicals and repair oxidative damage. Nowadays, the synthetic antioxidants are widely used in the food industry and also included in the human diet. The use of natural antioxidant is safer than synthetic antioxidants (Zheng and Wang, 2001). Phytochemicals are compound that benefit to health can be found in herbs, fruit and plants. Polyphenols are the main bioactive phytochemicals that have been shown to prevent many types of chronic diseases such as cancer and cardiovascular diseases.

The natural antioxidant from medicinal plants is widely studied nowadays. Traditionally, Zingiberaceae family are often used as ingredient in 'Jamu' (Indonesian traditional herbal
medicine). In Sabah, at least four medicinal herbs have been used traditionally by ethnic of Kadazan/Dusun. Stem of Costus speciosus or Sibhu-sibhu (in Kadazan language) has been used traditionally to cure asthma and flu. Rhizome of Curcuma longa and Zingiber officinale have been used to treat inflammation. Meanwhile, Kaempferia galangal or 'kusul' in kadazan has been used traditionally to treat women after giving birth. Rhizome of Etlingera belalongensis has been used traditionally to treat fever and urinary ailments (Poulsen, 2006).

Previous studies showed that the highest antioxidant activity of different parts of ginger (Zingiber officinale) was found in the rhizome (Habsah et al., 2000; Zaeeoung et al., 2005). Isolated compound from rhizome of Zingiber cassumuninis showed considerably high antioxidant activity (Masuda and Jtoe, 1994). Zingiber officinale, Zingiber zerumbet and Zingiber octoniisi have been intensively studied in term of their phytochemicals and bioactivity properties (Murakami et al., 2002, Stoilova et al., 2007). Chen and Dou (2008) investigated the antioxidant activity of 26 Zingiberaceae species in Peninsular Malaysia and the results showed that Etlingera species displayed significantly higher antioxidant properties compared to other genus.

Many Zingiberaceae which are endemic to Borneo (especially in Sabah) are not fully investigated. In Sabah, the diversity of Zingiberaceae remains unknown (Theilade and Mood, 1997), although 156 species have already been described (Gobilik and Yusoff, 2005). The purposes of this study were to evaluate the antioxidant activity of two genera of Zingiberaceae using DPPH free radical-scavenging assays, ABTS+ free radical-scavenging assays and Ferric-Reducing Antioxidant Power (FRAP). The total phenolic and total flavonoid contents of the samples were also been measured.

MATERIALS AND METHODS

Plant material: Fresh samples were collected on September 2007 from Tawau Hills Park and November 2007 from Crocker Range Park in Sabah, Malaysia. The herbarium voucher specimen were identified by Mr. Janarius Gobilik from Forest Research Centre, Sandakan, Sabah. The herbarium voucher specimen was deposited in BORNEENSIS, Universiti Malaysia Sabah, Malaysia.

Preparation of the samples: All samples were collected together with the root/rhizome. Two types of Zingiber species were collected, namely Z. pseudopungens and Z. vinosum. Meanwhile, two types of Etlingera species were collected, namely E. velutina and E. belalongensis. All samples were cleaned with water and cut into small pieces. All collected plant materials were dried at 40°C for five to six days. After drying, the samples were ground and stored in air tied plastic bag and kept at -20°C until further used.

Extraction: Fifty gram of sample was extracted with 100 mL methanol for three days. The resulting slurry was vacuum-filtered through a Whatman No. 3 filter paper and filtrate was subjected to vacuum rotary evaporation (Rotavapor model R110, Buchi, Flawil, Switzerland) at 40°C to remove methanol. The concentrated methanolic extracts were stored in amber glass vials at -20°C until used.

Determination of total phenolic contents: Total phenolic content was determined using Folin-Ciocalteu method as describes by Velioglu et al. (1998) with modification. Folin-ciocalteu reagent was diluted 10-fold with distilled water. Three hundred microliters of extract was mixed with 2.25 mL of Folin-Ciocalteu reagent solution. The solution was mixed well using vortex and then
allowed to stand for 5 min under room temperature; 2.25 mL the sodium bicarbonate (60 g L\(^{-1}\)) solution was added to the mixture. After 90 min at the room temperature, absorbance was measured at 725 nm using spectrophotometer. Gallic acid was used as standard. A standard concentration curve from 1 to 5 mg mL\(^{-1}\) at 1 mg mL\(^{-1}\) interval was plotted. Total phenolic content of the extracts were determined from the standard graph. Results were expressed as gallic acid equivalent.

**Determination of total flavonoid contents:** Total flavonoid content was determined according to the colometric assay by Kim et al. (2005), with slight modification. Distilled water (4 mL) was added to 1 mL of extracts. Then, 5% sodium nitrite solution (0.3 mL) was added, followed by 10% aluminium chloride solution (0.3 mL). Test tubes were incubated at ambient temperature for 5 min and then 2 mL of 1 M sodium hydroxide were added to the mixture after 6 min and 2.1 mL of distilled water was added. The mixture was thoroughly vortexed and the absorbance of the pink colour mixture was determined at 510 nm. The calibration curve was prepared using catechin as the standard and the results were expressed as mg catechin equivalents (CEQ)/100 g sample.

**DPPH free radical scavenging assay:** The free radical scavenging ability of the extracts were determined as described by Mensor et al. (2001). Briefly, one milliliter of 0.3 mM DPPH solution was added to 2.5 mL of samples in different concentrations (5, 25, 50, 100, 200, 400, 600, 800 and 1000 µg mL\(^{-1}\)) of sample extracts. The samples were kept at room temperature in the dark for 30 min. The mixture was then measured spectrophotometrically at 518 nm. The free radical scavenging activity was calculated as below:

\[
\text{Scavenging effect} \% = \left[1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right] \times 100
\]

Butylated Hydroxy Toluene (BHT) was used as positive control. Extracts were diluted to nine concentrations to determine the IC\(_{50}\). Triplicate measurements were carried out and their scavenging effect was calculated based on the percentage of DPPH scavenged. Finally, the unit of IC\(_{50}\) was converted to ascorbic acid equivalent capacity (AEAC).

**Abts' radical scavenging activity:** This assay was conducted by using ABTS free radical decolorization assay developed by Re et al. (1999) with some modification. Briefly, the ABTS solution (7 mM) was mixed with 2.45 mM potassium persulfate (K\(_2\)S\(_2\)O\(_8\)). The mixture was allowed to stand for 15 h in the dark at room temperature. The solution was diluted with ethanol to obtain the absorbance of 0.7±0.2 units at 750 nm. The plants extract were separately dissolved in ethanol to yield a concentration of 1 mg mL\(^{-1}\). An aliquat of 20 µL of ethanolic test solution of each sample was added to 180 µL of ABTS free radical cation solution. The absorbance, monitored for 5 min, was measured spectrophotometrically at 750 nm using microtitre plate reader. All measurements were performing in triplicate. The free radical-scavenging activity of each sample was expressed as trolox equivalent antioxidant capacity (TEAC) which was obtained by comparing the absorbance change at 750 nm in the reaction mixture containing a sample of plant extract or test material with that containing trolox. This index is defined as the milimolar concentration of a trolox solution which antioxidant capacity is equivalent to 1.0 mg of the extract. BHT was used as a positive controls.

**FRAP (Ferric reducing/antioxidant power) assay:** This method was adapted and modified from Benzie and Strain (1999). The working FRAP reagent was produced by mixing 300 mM
acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 20 mM FeCl₃·6H₂O in a 10:1:1 ratio prior to use and heated to 37°C in water bath. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate (C₃H₅NaO₃·3H₂O) with 16 mL of glacial acetic acid (C₂H₄O₂) and brought to 1 L with deionized water. The TPTZ solution was prepared by making solution of 10 mM TPTZ in 40 mM HCl. A total of 3.0 mL FRAP reagent was added to cuvette and blank reading was then taken at 593 nm using spectrophotometer. A total of 100 µL samples extract and 300 µL distilled water was then added to the cuvette. After addition of the sample to the FRAP reagent, a second reading at 593 nm was performed after 4 min. The change in absorbance after 4 min from the initial blank reading was then compared with standard curve. Standard of known Fe (II) concentrations from 100 to 1000 µM. A standard curve was then prepared by plotting the FRAP value of each standard versus it concentration. The FRAP values for the samples were then determined using this standard curve.

**Statistical analysis:** All experiments were carried out in triplicate and presented as Mean± Standard Deviation of mean (SD) using SPSS version 15.0. The data were statistically analysed by one-way ANOVA and Duncan’s test. Linear regression test was used to assess correlations and regressions between means. A significance difference was considered at the level of p<0.05.

**RESULTS AND DISCUSSION**

**Total phenolic content:** The Folin-Ciocalteu method was used to determine the total phenolic content. Folin-ciocalteu method is based on the reduction of metal oxides by phenolic acid resulting in a blue solution that has an absorption maximum at 765nm. Phenolic compound is characterized based on a group of chemicals that possess antioxidant activities as well as known powerful chain breaking antioxidants (Shahidi et al., 1992) and have physiological functions including antimutagenic and anticancer activities (Kono et al., 1995). This is believed to be mainly due to their redox properties (Zheng and Wang, 2001) which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The total phenolics in the methanolic extract were in range of 41.7 mg GAE g to 5.3 mg GAE g Dry Weight (DW). Rhizome of *Z. vinosum* and *E. velutina* displayed highest total phenolic content (p<0.05) while the lowest belongs to the stem of *E. velutina*. The results showed that all species contained more phenolic contents in rhizome than stem (Table 1). Previous study reported the rhizome of *Z. officinale*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Part</th>
<th>Total phenolic (mg GAE g⁻¹)</th>
<th>Total flavonoid (mg CE g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. belalongensis</em></td>
<td>Rhizome</td>
<td>17.07±0.38⁴⁺</td>
<td>3.77±0.15⁴⁺</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>10.97±0.25⁴⁺</td>
<td>2.57±0.15⁴⁺</td>
</tr>
<tr>
<td><em>E. velutina</em></td>
<td>Rhizome</td>
<td>25.05±0.48⁴⁺</td>
<td>7.63±0.06⁴⁺</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>5.30±0.14</td>
<td></td>
</tr>
<tr>
<td><em>Z. pseudopungens</em></td>
<td>Rhizome</td>
<td>14.77±0.01⁴⁺</td>
<td>2.87±0.31⁴⁺</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>8.10±0.43⁴⁺</td>
<td>1.07±0.29⁴⁺</td>
</tr>
<tr>
<td><em>Z. vinosum</em></td>
<td>Rhizome</td>
<td>41.70±1.11⁴⁺</td>
<td>8.59±0.2⁴⁺</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>27.97±0.93⁴⁺</td>
<td>3.37±0.06⁴⁺</td>
</tr>
</tbody>
</table>

*Total phenolic was expressed as Gallic Acid Equivalent (GAE) in 1 g of dry sample. Total flavonoid was expressed as Catechin Equivalent (CE) in 1 g of dry sample. Values are presented in Mean±SD (n = 3) these with different letters are significantly different at p<0.05 as measured by Tukey HSD test. ANOVA compares values between rhizomes and stems of each species.*
displayed high antioxidant activity and 6-gingerol is the polyphenol compound that presented in the extracts (Stoilova et al., 2007). Surh (2002) showed that phenolic phytochemicals presented in methanolic extracts of ginger and generally possess strong anti-inflammatory and anti-oxidative properties and exert substantial anti-carcinogenic and anti-mutagenic activities.

**Total flavonoid content:** The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Chang et al., 2002). In addition, aluminium chloride forms acid labile complexes with the orthohydroxyl groups in the A- or B-ring of flavonoids (Chang et al., 2002). From the results, the concentration of flavonoid in the extracts was expressed as mg of catechin equivalents per g of the extract, as shown in Table 1. For total flavonoid content determination, the result showed the same trend with total phenolic content. The total flavonoid in the methanolic extracts was in range of 8.5 mg GAE g to 1.97 mg GAE g Dry Weight (DW). Rhizome of Z. vinosum displayed highest total flavonoid content (p<0.05) while stem of E. velutina is the lowest. Flavonoid compounds in plant have been reported to possess effective antioxidants properties (Cai et al., 2006) and are important phytochemical. Flavonoids are naturally occurring phenolic antioxidants that are present in the human diet. Some flavonoids have been reported to possess a variety of biological functions including antiallergic, antioxidants, anti-inflammatory, antiviral, antiproliferative and anticarcinogenic activities, in addition to having effects on mammalian metabolism (Ren et al., 2003).

**Scavenging activity on 2,2-diphenyl-2-prierylhydrazyl radical:** The proton radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity. DPPH is one of the compounds that possess a proton free radical and shows a maximum absorption at 517 nm. The purple colour fades rapidly when DPPH encounters proton radical scavengers. This assay determines the scavenging of stable radical species of DPPH by antioxidants. The dose-response curves of DPPH radical scavenging activity of the extracts compared with ascorbic acid are shown in Fig. 1. IC$_{50}$ values are defined as the amount of antioxidant needed to decrease the initial DPPH radical concentration by 50%. This means lowest IC$_{50}$ indicates the strongest ability of extracts to act as DPPH scavengers. Ascorbic acid was tested as positive control and showed 50% inhibition at 7.9±0.01 µg mL$^{-1}$ (Fig. 1). From the result, IC$_{50}$ values ranged from 160±0.01 µg mL$^{-1}$ for E. belalongensis (rhizome) to 950±0.01 µg mL$^{-1}$ for Z. vinosum (rhizome).

Meanwhile, no IC$_{50}$ values were obtained for both rhizome and stem of Z. pseudopungens. The final results were expressed as mg ascorbic acid equivalent antioxidant capacity in 1 g of sample (mg AEAC/g). The difference between the extracts and control was statistically significant (p<0.05). Out of the four ginger species studies by DPPH assay, rhizome of E. belalongensis and Z. vinosum (stem) had the highest AEAC with 49.5±3.1 mg AA/g and 47.44±1.87 mg AA/g, respectively. The result was arranged in the order or highest to lowest: E. belalongensis (rhizome)>Z. vinosum (stem)>E. velutina (stem)>E. velutina (rhizome)>Z. vinosum (rhizome) (Table 2).

Shobana and Naidu (2000) reported that alcoholic extracts of Z. officinale have been shown to possess potent antioxidant activity and prevent lipid peroxidation. This study was supported by Stoiilova et al. (2007) which showed that extracts of Z. officinale rhizomes displayed higher free radical-scavenging ability than butylated hydroxytoluene and quercetin. Analysis of correlation revealed that the DPPH free radical scavenging activity was strongly correlated with the total
Fig. 1: DPPH scavenging activity of the extracts. Ascorbic acid was tested as positive control and showed 50% inhibition at 7.9±0.01 µg mL⁻¹. Values are expressed as mean (n = 3)

Table 2: Comparison the antioxidant activity with three different assays

<table>
<thead>
<tr>
<th>Samples</th>
<th>Plant part</th>
<th>DPPH±</th>
<th>FRAP±</th>
<th>ABTS±</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. belalongensis</td>
<td>Rhizome</td>
<td>49.50±3.1^c</td>
<td>2.472±0.07^c</td>
<td>0.33±0.01^a</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>35.40±2.38^c</td>
<td>1.56±0.04^c</td>
<td>3.10±0.09^a</td>
</tr>
<tr>
<td>E. velutina</td>
<td>Rhizome</td>
<td>24.75±1.54^c</td>
<td>2.123±0.06^c</td>
<td>3.62±0.20^a</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>33.07±2.76^c</td>
<td>2.54±0.16^c</td>
<td>0.8±0.08^a</td>
</tr>
<tr>
<td>Z. pseudopungens</td>
<td>Rhizome</td>
<td>N.D</td>
<td>1.235±0.00^b</td>
<td>1.47±0.06^b</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>N.D</td>
<td>1.09±0.05^a</td>
<td>1.13±0.06^a</td>
</tr>
<tr>
<td>Z. vinosum</td>
<td>Rhizome</td>
<td>8.32±0.1^a</td>
<td>1.04±0.02^a</td>
<td>1.78±0.49^b</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>48.47±0.06^d</td>
<td>2.13±0.06^c</td>
<td>2.03±0.10^c</td>
</tr>
<tr>
<td>BHT</td>
<td></td>
<td>138±0.05^b</td>
<td>2.09±0.02^a</td>
<td>3.13±0.23^b</td>
</tr>
</tbody>
</table>

Values are presented in mean±S.D (n=3) which with different letters are significantly different at p<0.05. *DPPH free radical scavenging activity was expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) in 1 g of dry sample. **TEAC (mM trolox equivalents/mg extract) values of samples in sample concentration was 0.8 g mL⁻¹. †FRAP was expressed as mM ferric reduction to ferrous in 1 mg mL⁻¹.

phenolic content and total flavonoid content (R² = 0.826; p<0.05) and (R² = 0.852; p<0.05), respectively. In agreement to this, there are several reported the role of phenolic compounds as scavengers of free radicals (Rice-Evans et al., 1996). The good correlation between the results from total phenolic content and the antioxidant assay has been reported proved by various researchers (Zheng and Wang, 2001; Lamien-Meda et al., 2008; Abu Bakar et al., 2009).

**Scavenging effects of ABTS by extract:** The ABTS test measures the relative antioxidant ability of samples to scavenge the radical-cation ABTS⁺ produced by the oxidation of 2,2-azoniobis-3-ethylbenzothioazoline-6-sulphonate (Re et al., 1999). The calibration curve of trolox standard (R² = 0.655) was used to evaluate antioxidant activity. The calibration of equation of trolox are
The antioxidant capacities ranged from 0.33 to 3.62 mM trolox equivalents mg⁻¹ samples for methanol extracts (Table 2). The results were in the order as follows: *E. velutina* (rhizome) > *E. belalongensis* (stem) > *Z. vinosum* (stem) > *Z. vinosum* (rhizome) > *Z. pseudopungens* (rhizome) > *Z. pseudopungens* (stem) > *E. velutina* (stem) > *E. belalongensis* (rhizome).

Previous researchers reported that the high phenolic contents in extracts have more ability to quench free radicals (ABTS) and the effectiveness of the extracts were depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group substitution (Siddharaju and Becker, 2007; Cai et al., 2006) reported the positive relationships of phenolic compounds from traditional Chinese herbs with antioxidant activity using DPPH and ABTS assay. Analysis of correlation revealed there moderate correlation between total phenolic content and total flavonoid contents.

The results for radical scavenging in ABTS assay showed different patterns compared to DPPH assay. Since both assays have same principle, the results suppose to have same pattern with each other. The results of TEAC value by ABTS assay mostly higher than AEAC value by DPPH assay. Study by Cai et al. (2006) showed that the difference results in radical scavenging activity could be attributed by structural differences in hydroxylation, glycosylation and methoxylation.

**Ferric reduction based on FRAP assay:** The Ferric Reducing Antioxidant Power (FRAP) method is a simple, rapid and reproducible method which can provide a very useful total antioxidant concentration without measurement and summation of the concentration of all antioxidants involved. FRAP method is based on the reduction of Fe³⁺ to Fe²⁺ by antioxidants in acidic medium (Benzie and Strain, 1996). Results are obtained as absorbance increases at 593 nm and expressed as micromolar Fe²⁺ equivalents or related to an antioxidant standard. The calibration curve of FeSO₄·H₂O standard (R² = 0.998) was used to evaluated the FRAP value. The final result was expressed as the concentration of antioxidant having a ferric reducing ability.

FRAP value was highest in *E. velutina* (stem) extract with the value of 2.540 mmol L⁻¹ g⁻¹±0.16 at concentration of 1 g mL⁻¹ (p<0.05). This was followed by *E. belalongensis* (rhizome) with the value of 2.472 mmol L⁻¹ g⁻¹±0.07. Meanwhile, the reducing capacity of *Z. pseudopungens* (stem) and *Z. vinosum* (rhizome) values were 1.093 mmol L⁻¹ g⁻¹±0.05 and 1.048 mmol L⁻¹ g⁻¹±0.02, respectively. The results was arranged from highest to lowest: *E. velutina* (stem) > *E. belalongensis* (rhizome) > *Z. vinosum* (stem) > *E. velutina* (rhizome) > *E. belalongensis* (stem) > *Z. pseudopungens* (rhizome) > *Z. pseudopungens* (stem) > *Z. vinosum* (rhizome) (Table 2).

The reducing ability of the extracts were strongly correlated with the phenolic (R²=0.759; p<0.05) and the flavonoid (R²=0.841; p<0.05). This result was support by Hinneburg et al. (2006) who reported the strong correlation between total phenolic content and FRAP assay. The important mechanism of phenolic antioxidant action is Fe³⁺ reduction which is often used as an indicator of electron-donating activity (Yildirim et al., 2001). Recent study showed that the reducing power was increased concurrent with the increasing concentration of mango ginger (*curcuma amada*) rhizome (Poliegoudra et al., 2007).

By comparing the *Elingera* and *Zingiber* species, *Elingera* species displayed higher antioxidant capacity compared to *Zingiber* species. These results were supported by Chan et al. (2009) who showed that AEAC of *Elingera* is higher compared to *Zingiber*. Since *Elingera* species are relatively larger as compared to *Zingiber* species, they were be able to expose directly to the sunlight (Khaw, 2001) in the rainforest and might have higher antioxidant properties.
CONCLUSION

_Etlingera_ species which possessed the highest polyphenol content exhibited strong antioxidant activity compared to _Zingiber_ species. The correlations indicated that total polyphenols are the major contributors to the antioxidant activity of these Zingiberaceae. With promising antioxidant properties, methanolic extracts of selected _Etlingera_ and _Zingiber_ species endemic to Borneo have great potential to be developed into natural preservatives and herbal products, applicable to the food and nutraceutical industries.

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