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Original Article

Bioactivities of Methanol and Ethyl Acetate Mace Extracts of *Myristica fragrans* Houtt

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ABSTRACT

Introduction: *Myristica fragrans* Houtt is used as a spice in Indonesia. This study aimed to scientifically validate the traditional use of mace from *M. fragrans*. **Methods:** The mace was extracted with methanol and ethyl acetate, and the chemical composition was analysed by GC–MS. The extracts were evaluated for their antibacterial activities against seven different bacteria based on a well diffusion method, their antioxidant activities based on DPPH radical scavenging assay, and their α-glucosidase inhibitory activities *in vitro*. **Results:** Four major components were identified from both extracts: sabinene, methoxyeugenol, myristicin, and elemicin. The methanol extract (ME) had higher methoxyeugenol, myristicin and elimisin levels, whereas sabinene was dominant in ethyl acetate extract (EAE). Both extracts showed good antibacterial activity against *S. aureus* only. Both extracts showed good antioxidant activities, in which EAE showed stronger activity than ME (IC_{50} 94 µg GAE/ml and IC_{50} 162 µg GAE/ml, respectively). Furthermore, both extracts inhibited α-glucosidase more strongly than the standard acarbose, in which ME (IC_{50} 7.50 µg GAE/ml) exhibited stronger α-glucosidase inhibitory capacity than EAE (IC_{50} 10.65 µg GAE/ml). **Conclusion:** The results suggest that ME and EAE of *M. fragrans* have biological activities with high potential for pharmacological uses.

Key words: Antibacterial, Antioxidant, GC-MS, α-glucosidase inhibition, *Myristica fragrans*.

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INTRODUCTION

Natural products derived from plant materials are increasingly exploited in a number of fields, such as pharmaceutical, cosmetics, and food. In recent decades, around 25% of the commercial drugs are developed from natural products, many of them have been prescribed as treatments for chronic degenerative diseases and infectious diseases.[1] Some of these illnesses are related to oxidative stress and are the main causes of mortality in the world (59.7% chronic degenerative diseases; diabetes 2.0%; infectious diseases 16.2%).[2] Treatments against such diseases are not always effective. In many cases, bacterial resistance is reported, making prognosis of infectious diseases worsen. From this point of view, new drugs are needed, and plants are still the main source.

The nutmeg tree, *Myristica fragrans* Houtt (family: Myristicaceae) is originally from the Moluccas in Indonesia. However, it has been successfully cultivated in other Asian countries, such as India, Malaysia, Sri Lanka, and in the Caribbean islands, mainly in Grenada and Trinidad. This aromatic tree can grow 9-12 m high with spreading branch and yellowish flesh fruit. Inside the ripe fruit, is the brown seed kernel and the fleshy scarlet mace covering the kernel.

Nutmeg is widely used a spice and flavour for foods and beverages. In addition, it has been traditionally used to treat a number of medical conditions such as diarrhoea and kidney disorders. Studies have reported antioxidant, antimicrobial, antidiarrheal, and anti-inflammatory activities of the species.[3-4] Despite the numerous pharmacological studies, *M. fragrans* Houtt had not been comprehensively studied. Most of the previous studies have been focused on the essential oil or the organic extracts from the seed and the mace parts of *M. fragrans* Houtt.[5-6] However, less work has been done in relation to the crude extracts from the mace using methanol and ethyl acetate as solvent extractor. Thus, the objective of this study was to investigate antibacterial, antioxidant and α-glucosidase inhibition activities of methanol and ethyl acetate extracts (ME and EAE) obtained from *M. fragrans* Houtt's mace. Both extracts were analysed for their total phenolic compounds and GC-MS profiles.

MATERIALS AND METHODS

Plant material, chemicals, and bacteria

The maces of *M. fragrans* Houtt were collected from Halmahera (North Moluccas Province) in March 2016. These specimens were identified by one of the authors (KHT). A voucher specimen (KWF007) was kept in the laboratory.

All solvents and chemicals used in the experiments were analytical grade. Folin and Ciocalteu's phenol reagent, 2, 2-diphenyl-picryl-hydrazyl (DPPH), 3, 5-di-tert-butyl-4- hydroxytoluene (BHT), α-glucosidase from yeast *Saccharomyces cerevisiae* and *p*-nitrophenyl-α-D-glucopyranoside as the synthetic substrate for the enzyme were purchased from Sigma-Aldrich (St. Louis, USA). Gallic acid was purchased from Santa Cruz Biotechnology (Dallas, USA). Sodium carbonate (Na_2CO_3) was purchased from Merck (Darmstadt, Germany). Ascorbic acid was purchased from VWR BDH Prolabo Chemicals (Tingalpa, Australia).

Seven human pathogen bacterial strains were used for the antibacterial activity test: *Staphylococcus epidermidis ATCC 12228, Staphylococcus epidermidis FNCC 0048, Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Pseudomonas aeruginosa PAO I, Staphylococcus aureus COWAN I* and *Streptococcus mutants ATCC 14721*. The bacteria used in this study were obtained from our university culture collection. The original strains were obtained from Prof Hwang (Yonsei Christian Private University, South Korea) and Gadjah Mada University, Jogjakarta.

Preparation of the extracts

The maces were rinsed with distilled water and dried in the shade at room temperature. Then, they were crushed and ground into coarse powder to maximize the extraction yield. The extraction was performed by successive maceration. The powdered mace (50 g) was macerated by ethyl acetate overnight. Following filtration, the residue was further macerated by methanol (1:5 w/v) overnight. Each supernatant was reduced to dryness using rotary evaporator (Buchi, Switzerland) and further dried under N_2 stream to obtain a deep orange liquid. The liquid was stored at 4°C before use.

GC-MS analysis

The GC-MS analysis was performed as for previous experiments.[7] A Shimadzu GCMS-QP2010S system was used for the analysis with an AGILENT HP column, 30m length, ID 0.25 mm, film 0.25 µm. The carrier gas was helium. Its ionisation was EI at 70 eV. The column oven temperature was 70°C. The injection temperature was 300°C, while the injection mode was splitless. Sampling time was 0.20 min. Flow control mode: pressure was 13.7 kPa. Total flow was 40.0 ml/min. Column flow was 0.50 ml/min. The split ratio was 73.0 and the oven temperature program was set with rate 5.00, temperature 70 – 300°C, and hold time was set 5-29 min. The start time for data acquisition was 3 min and the end time was 80 min. The obtained spectra were identified by comparing with the library NIST12LIB.

Total phenolic content

The ME and EAE of *M. fragrans* Houtt were analysed for their phenolic compounds spectrophotometrically based on reported procedure with slight modification.[8] The extract (5 µl) was mixed with DMSO (5 µl) and 490 µl of distilled water. The diluted sample was mixed with 2.5 ml of Folin-Ciocalteu reagent (10%), and the solution was left to stand for 10 min at room temperature. The reaction was then neutralized using saturated Na_2CO_3 (75 g/l). After being incubated for 2 h in darkness at room temperature, the absorbance was measured at 765 nm. The experiment was carried out triplicate. The total phenolic content was estimated from a standard curve of gallic acid (12.5 – 200 µg/ml) in methanol, and the results were expressed as µg gallic acid equivalent per ml (µg GAE/ml).

Antibacterial activity

Antibacterial test activity had been conducted by applying agar well diffusion method. Seven strains used in this test were *Staphylococcus epidermidis ATCC 12228, Staphylococcus epidermidis FNCC 0048, Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Pseudomonas aeruginosa PAO I, Staphylococcus aureus COWAN I* and *Streptococcus mutans ATCC 14721*. Mueller-Hinton Agar (OXOID CM0337) was used as growth medium. 100 µl bacterial suspension (McFarland 0.5) was spread on the agar plate. The well diameter was 0.5cm and filled with 20 µl of the extracts. The incubation was done at 37°C for 24 h. The inhibition zone was observed and recorded.

DPPH radical scavenging activity

The antioxidant potential of ME and EAE from *M. fragrans* Houtt was determined based on the reported procedure[9] with minor modification.[10] In this procedure, antioxidant activity was evaluated in terms of radical scavenging ability of the sample using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The reduction of DPPH radicals to the reduced form of DPPH-H created changes in colour (from deep purple to light yellow) that could be monitored by a spectrophotometer. The degree of decolourization indicated the scavenging activity of the extract. DPPH solution (0.6 mM in ethanol) was prepared, and 1ml of this solution was added to 3 ml of extract and standard solutions (BHT, ascorbic acid, and α-tocopherol) at various concentration (10 – 100 µg/ml). The reaction was incubated for 30 min in darkness at room temperature, and the absorbance was read at 517 nm using spectrophotometer (BioChrom Libra S22). Ethanol (3 ml) in place of the extract was used as a control. The percentage inhibition activity was calculated according to the following equation:

$$\%\ \text{Inhibition on DPPH radicals} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$ is absorbance of control and A_{sample} is absorbance of the sample. The percentage inhibition was then plotted against concentration, and IC_{50} was determined using a linear regression analysis. The values expressed as µg GAE/ml and were compared to the standard solutions. All measurements were carried out three times.

The antioxidant activities were also expressed as the antioxidant activity index (AAI).[11] The constants AAI allows for antioxidant activities to be evaluated independent of the concentrations of DPPH and extracts used. AAI was calculated as follows:

$$AAI = \frac{\text{Final concentration of DPPH } (\mu g\ ml^{-1})}{IC_{50} (\mu g\ ml^{-1})}$$

The antioxidant activities were categorized as poor (AAI < 0.5), moderate (AAI = 0.5-1), high (AAI = 1 – 2), and very high (AAI > 2).

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity of *M. fragrans* Houtt extracts was performed according to the reported method[12] with some modifications. In brief, appropriate dilution of samples (total volume 50 µl) were mixed with 5 µl of DMSO, 45 µl phosphate buffer (50 mM, pH 6.8) and 50 µl of α-glucosidase (0.5 unit/ml). After being pre-incubated for 5 min at 37°C, 100 µl of the substrate (1 mM p-nitrophenyl-α-D-glucopyranoside) was added to the reaction mixture and further incubated for 20 min at 37°C. After the incubation, the reaction was stopped by the addition of 750 µl of Na_2CO_3 (100 mM). The absorbance was recorded at 405 nm using a spectrophotometer (Biochrom Libra S22). For the control solution, all procedures were followed except that the sample was replaced by a buffer. Acarbose at varying concentrations (0.0625 – 4 mg/ml) was used as a positive control. The inhibition percentage was calculated using the following equation:

$$\%\ \text{Inhibition of } \alpha - \text{Glucosidase} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$ is absorbance of control and A_{sample} is absorbance of the sample. The α-glucosidase inhibitory activity was expressed as IC_{50} values (µg GAE/ml) and was determined from the graph plotted against the percentage inhibition.

Statistical analysis

All experiments were performed in triplicates, and the data were reported as mean ± SD. The differences between extracts obtained from the two solvent systems were analysed by the Student t-test ($\alpha = 0.05$) by SPSS v. 23.0 program.

RESULTS AND DISCUSSION

Extracts yields, total phenolic content (TPC), and chemical composition

The yields of ME and EAE obtained from the mace of *M. fragrans* Houtt were 0.048% (w/w) and 0.218 % (w/w). The total phenolic content of the extracts was measured based on Folin-Ciocalteu method and the results were expressed as gallic acid equivalents (GAEs). A gallic acid standard curve was plotted with a linear correlation value of 0.9976 and was used for determining the GAE of the extracts. The TPC was found to be affected by the kinds of extracting solvent used. The TPC of ME was higher than EAE ($t = 17.67$, $p < 0.05$); 13.43 ± 0.83 and 11.56 ± 0.62 µg GAE/mg dried extract for ME and EAE, respectively. This might indicate that methanol was more effective than ethyl acetate for extracting polyphenolics.

The chemical compositions analysed by GC-MS showed that both extracts had similar major components, *i.e.* sabinene, methoxyeugenol, myristicin and elemicin. ME was rich in methoxyeugenol, myristicin, and elemicin, whereas sabinene was the most abundant in EAE (Table 1 and Figure 1). The difference in chemical compositions might be caused by the use of different solvent systems.[13]

Table 1: Main components of ethyl acetate and methanol extracts obtained from mace of M. fragrans Houtt as analysed by GC- MS (area %).

Compound	RT	Area (% v/v)	
		MeOH	EAE
Sabinene	6.773	1.92	14.83
Limonene	8.53	1.28	5.75
Terpinene	9.488	nd	4.85
Methoxyeugenol	25.532	31.19	25.85
Myristicin	29.505	15.64	nd
Benzeneacetic acid	45.854	2.071	nd
Elemicin	47.046	12.75	13.99
Yield (mg/g)		**48**	**218**

*nd: not detected
**RT: retention time

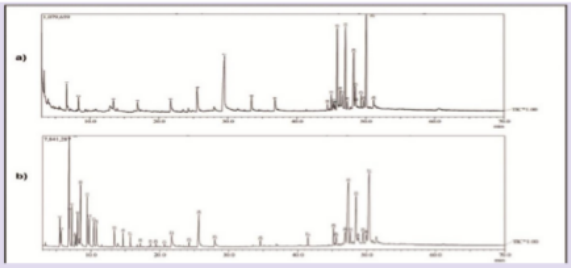

Figure 1: Chromatograms of methanol (a) and ethyl acetate (b) extracts
Peaks for:
Sabinene at a (no.1) and b (no. 3)
Limonene at a (no. 2) and b (no. 10)
Terpinene at b (no. 11)
Methoxyeugenol at a (no. 6) and b (no. 24)
Myristicin at a (no. 7)
Benzeneacetic acid at a (no. 15)
Elemicin at a (no. 31) and b (no. 18)

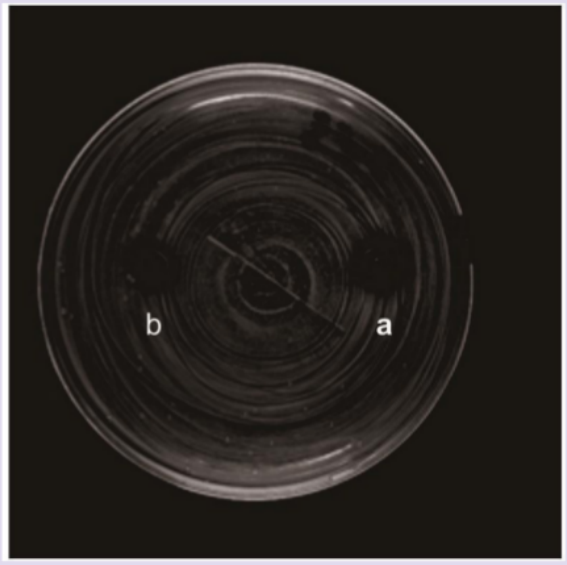

Figure 2: Inhibition clear zone of methanol (a) and ethyl acetate (b) extract

Antibacterial activity

In this study, ethyl acetate and methanol mace extracts only gave positive results against *S. aureus* ATCC 25922 (Figure 2). The result is in accordance with a previous report.[14] In the present study, the methanol extract showed a stronger antibacterial activity than the ethyl acetate extract. In another study, mace extracts showed antimicrobial action against several oral pathogens, in particular against *S mutans, S mitins*, and *S salivarius*, with an ethanol extract showing stronger activity than an ethyl acetate extract [4] Many plants polyphenolics have been reported to have an antimicrobial activity againts *S. aereus*.[15-16] Another important component that had antimicrobial activity could be sabinene, as has been reported previously.[17] The present study showed that ME and EAE can be further exploited as natural antibacterial agents.

DPPH radical scavenging activity

One of the pathways in which antioxidant may protect biological molecules from oxidative damages is through the removal of free radicals.[18] This can be achieved by donating hydrogen or electron from antioxidant compounds to free radicals, which results in the reduction of the radicals into a more stable compound. In this direction, antioxidant potential of extracts from *M. fragrans* Houtt was evaluated based on its ability to scavenge DPPH free radical, which directly measured the hydrogen donating ability of the antioxidant compounds in the extracts. The DPPH radical scavenging assay was widely employed for samples including plant extracts due to its sensitivity and ease of use.

The results of the DPPH radical scavenging activities of the extracts are expressed as gallic acid equivalent (μg GAE/ml) and shown in Table 2. Both extracts were shown to have radical scavenging activities in a concentration-dependent manner, with moderate activities compared to references (ascorbic acid, BHT, and α-tocopherol). This suggests that ME and EAE extracts are able to reduce DPPH into its non-radical form DPPH-H. Both ME and EAE showed high concentration of total phenolics, which could be associated with their radical scavenging activities on DPPH. It has been widely reported that phenolic-type compounds derived from plant sources are strongly associated with most antioxidant activities.[19-20] This could be understood from the acidic property of the phenolic (-OH) moiety and the nucleophilic character of the benzene ring that plays a crucial role in scavenging free radicals. It should be noted however, that EAE extracts exerted stronger activity than that of ME, (IC_{50} of 94.29 ± 0.68 and 162.39 ± 0.49 μg GAE/ml, respectively). The activity found in ME was stronger than that found in the previous report (201.91 μg/ml).[21] The dissimilarity may be due to the differences in extraction methods used. In another study, similar results were obtained when the mace was extracted using 80% methanol (160.9 ± 13.9 μg/mL) as reported previously.[22] This may suggest that increasing the solvent polarity by the addition of water does not alter the antioxidant activity. When the activities were expressed as antioxidant activity index (AAI), ME was considered to have a poor activity, obtaining an AAI of 0.36, whereas EAE showed a moderate activity, AAI of 0.63. As expected, the references showed strong (ascorbic acid) and very strong activities (BHT and α-tocopherol). It is worth noting that EAE has the higher concentration of total phenolics than ME. This indicates that other components may contribute to the radical scavenging activity of the extracts. From these results, it is likely that sabinene, which is present in

Table 2: The DPPH free radical scavenging activity of methanol extract from *M. fragrans* Houtt.

	Concentration (ug GAE/mL)	Inhibition (%)	IC_{50} (ug GAE/mL)	AAI
	14.82	5.86±0.15		
	29.64	12.47±0.29		
	59.28	21.11±0.33		
MeOH	88.93	32.34±0.66	162.39±0.49	0.36
	118.57	39.76±0.74		
	148.21	46.44±0.61		
	177.85	51.23±0.63		
	12.33	5.39±0.18		
	24.65	9.09±0.17		
	36.98	18.85±0.48	94.29±0.68	0.63
EAE	49.31	27.89±0.78		
	61.63	38.22±1.01		
	73.96	41.41±0.92		
	98.61	47.27±1.20		
	Ascorbic acid		53.24±0.82	1.11
	BHT		21.36±0.80	2.76
	Tocopherol		1.710±0.01	34.59

Table 3: The inhibitory α-glucosidase activity of methanol extract from *M. fragrans* Houtt

	Concentration (ug GAE/mL)	Inhibition (%)	IC_{50} (ug GAE/mL)
	2.22	10.2±07.32	
	4.45	16.77±1.66	
MeoH	6.67	26.40±1.44	7.50±0.58
	8.89	69.31±10.89	
	11.12	89.90±8.27	
	1.85	11.83±3.02	
	5.55	33.59±3.42	
EAE	9.25	44.81±2.53	10.65±0.52
	12.95	62.54±0.4	
	14.8	63.49±3.58	
	Acarbose		2,300±0.01

abundance in the EAE, is a good radical scavenger. Similar results have been shown that sabinene has strong activity on DPPH.[23] These results suggest that the mace of *M. fragrans* Houtt can be a good source of natural antioxidant and can be exploited for food and pharmaceutical uses.

α-glucosidase inhibitory activity

Inhibition of key enzymes is considered to be an effective strategy for the treatments of diseases. Digestive enzymes (such as α-glucosidase and α-amylase) are crucial enzymes in the hydrolysis of carbohydrates. Therefore, inhibition of these enzymes is an important strategy in managing blood glucose level. The synthetic α-glucosidase inhibitors (such as acarbose, voglibose, etc) are reported to cause unfavourable side effects such as diarrhoea and flatulence.[24] Thus, there is a considerable need for the development of safe α-glucosidase inhibitors, particularly from plant-based natural products.

Antidiabetic activity of *M. fragrans* Houtt extracts was investigated using an *in-vitro* α-glucosidase inhibitory assay. The activities are presented as IC_{50} values in gallic acid equivalent (μg GAE/ml) as shown in Table 3. The extracts exhibited good inhibition against α-glucosidase, with both extracts having IC_{50} values much lower than the standard acarbose (7.50 ± 0.58 and 10.65 ± 0.52 μg GAE/ml for ME and EA, respectively). Previous study has reported a lower activity of ME (75.7 μg/ml) when using mace commercial dried powder.[25] The ME was observed to have stronger inhibition than EAE ($t = 7.96$, $p < 0.05$). A strong relationship between α-glucosidase inhibitory activity (IC_{50}) and the total phenolic content was previously demonstrated in the literature[26] and gallic acid-type compounds may be responsible for the α-glucosidase inhibitory activity as reported previously.[27] In this sense, the inhibitory activities of ME and EAE may be associated with the high concentration of phenolic compounds in the extracts. The results indicate that extracts obtained from the mace of *M. fragrans* Houtt could be a potential source of inhibitors for the treatment of diabetes mellitus.

CONCLUSION

Results obtained from this study clearly demonstrate the bioactivities (*i.e.* antibacterial, antioxidant, and α-glucosidase inhibitory activities) of methanol and ethyl acetate extracts from the mace of *M. fragrans* Houtt. Our study supports the ethno-pharmacological use of this plant for the prevention and treatment of various oxidative stress-related diseases and infections. Further studies are required to isolate and characterize bioactive compounds in order to investigate structure-activity relationships in the discovery of new drugs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BHT: 3, 5-di-tert-butyl-4- hydroxytoluene; **DPPH:** 2, 2-diphenyl-1-picryl-hydrazyl; **EAE:** Ethyl Acetate Extract; **GAE:** Allic Acid Equivalence; **ME:** Methanol Extract.

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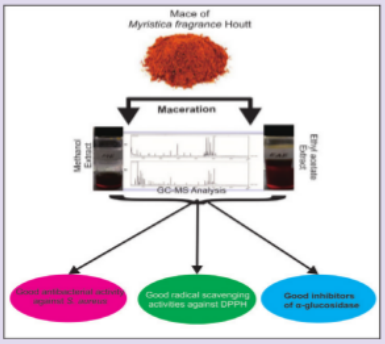
PICTORIAL ABSTRACT



SUMMARY

- Methanol (ME) and ethyl acetate mace extracts (EAE) of *M. fragrans* Houtt had similar major components identified by GC-MS, i.e. sabinene, methoxyeugenol, myristicin and elemicin.
- ME and EAE were strong inhibitors of α-glucosidase (IC_{50} of 7.50 and 10.65 µg GAE/ml, respectively).
- Both extracts showed good radical scavenging activity against DPPH (IC_{50} of 162 and 94 µg GAE/ml for EAE and ME, respectively).
- Both ME and EAE exhibited good antibacterial activity against *S. aureus*

ABOUT AUTHORS

Adelina Simamora, MS, MSc (Pharm): researcher in the centre for herbal medicine studies and lecturer in Biochemistry, faculty of medicine Krida Wacana Christian University. At present, her research focus on the bioactive potential of medicinal plants, in particular various enzyme inhibition properties.

Adit Widodo Santoso, BSc: researcher in the centre for herbal medicine studies. His interest is mainly in the areas of microbiological activities of variety of plant species. Currently, he is focusing on zymography studies and their applications.

Prof Dr Kris Herawan Timotius is lecturer in microbiology and biochemistry at the Faculty of Medicine Krida Wacana Christian University. His research focus on herbal medicine including natural antibiotics, antiquorum sensing, antibiofilm, enzyme therapy, and anti enzyme activities such as alpha glucosidase and xanthine oxidase.

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