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Isolation and Screening of Antibacterial Activity of Endophytic Fungi Isolates from *Ocimum citriodorum* Vis. Leaves

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ABSTRACT

Endophytic fungi is believed to resided in the leaves of medicinal herb plant and live symbiotically with their host plant. The aim of the study was to isolate endophytic fungi from Lemon Thai Basil leaves using different agar media and leaf age stages. A total of 167 endophytic fungi were isolated from different stages of Ocimum citriodorum leaves; young leaves (19), mature leaves (41), old leaves (65) and senescent leaves (42). The isolation were carried out on six different growth media, in which 4 of them were supplemented with either host plant powder (HP) or host plant extract (PE). The number of isolates varies based on the growth medium; 25 endophytes were isolated from PDA, 47 from PHP, 30 from PPE, 16 from MEA, 29 from MHP and 20 from MPE. Most of the isolated endophytes were obtained from PDA supplemented with HP. Ten endophyte isolates showed antibacterial activities in the primary screening. From these, two isolates namely TLBML-M1 and TLBML-PHP2 showed the most significant antibacterial activities against Methicillin-resistant Staphylococcus aureus (MRSA), Klebsiella pneumoniae and Shigella boydii. Four organic solvents; hexane, dichloromethane, ethylacetate and isobutanol were used to extract the active compound from fungal biomass and fermentative broth of the endophytic isolates. Results showed high concentration of antibacterial compound were extracted from fungal biomass compared to fermentative broth. The fungal biomass inhibited all bacteria except gram-positive Staphylococcus aureus. The compounds extracted using ethyl acetate and dichloromethane showed high antibacterial activity presence in the endophytes broth, with almost all test bacteria were inhibited. This indicates that antibacterial compound from fermentative broth can be effectively extracted by ethyl acetate and dichloromethane.

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INTRODUCTION

Medicinal herbs are widely use in healing, treating and health maintenance purposes. There are more than 20 000 species of angiosperms and 600 species of ferns can abundantly be found in Malaysia tropical rain forest. Among these, 1082 angiospermand 76 fern species were reported to have medicinal properties (Mansor, 2005). *O. citriodorum* was selected for this study as it has a great potentia lin immunostimulant, antioxidant, antidiabetic, cardioprotection and antifungal agent. *O. citriodorum* Vis is belong to Family Lamiacea and derive from natural hybrid between *Ocimum basillicum* and *Ocimum americanum.*Essential oils from *O. citriodorum* extract are used in traditional remedies for colds, inflammation, stomach disorders, heart disease, malaria and headaches (Murkherjee *et al.*, 2005; Samson *et al.*, 2007).

Nowadays, medicinal herbs have become important sources for new drug discovery as it contains various chemical compounds contribute multifaceted therapeutic powers. The uses of medicinal herbs as an alternative medicine possessed less negative impacts compared to the synthetic drug. The selection of endophytic fungi in the current research is due to its biomass regeneration and conservation of the plant resources. Hence, the current study was conducted to isolate the endophytic fungi at different stages of maturity of *O. citriodorum* Vis. leaves. The results from this study will reveal the best leaf age stages and growth media for fungal isolation in order to study the antimicrobial activities of endophytic fungi isolated from *O. citriodorum.*

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MATERIALS AND METHODS

Preparation of plant material:

O. citriodorum leaves samples at different maturity stages with no visibilityof disease symptoms were collected and kept in a ziplock bag prior to processing within 4 hours after collection. The leaves were stripped off from its stalk using scissors or plucked by hand, washed under the tap water and were scattered in a basket to dry in a room temperature. The dried leaves were ground to a fine powder using dry blender and kept in a ziplock bags stored in a desiccator.

Extraction of O. citriodorum leaves:

The *O. citriodorum* leaves were extracted using distilled water. 1.25 g of finely ground *O.citriodorum* leaves was soaked in 125.0 ml distilled water and kept in the room temperature for 24 hours. The extracts were filtered through Whatman No. 1 filter paper and kept at 4 °C.

Estimation of chlorophyll content:

Fully expanded leaf samples from *the O. citriodorum plant* were obtained at the 5 months in cultivation. The leaves were selected at different growth stages; young, mature, senescent and old. The chlorophyll content was measured in triplicate using SPAD-502 meter (Konica-Minolta, Japan) around the midpoint near the midrib of each leaf sample. The average SPAD meter values were calculated to estimate the amount of chlorophyll present in the leaf.

Isolation of endophytic fungus:

The isolation of fungi was conducted using method described by Okuda *et al*., (2005) with some modifications.

Leaf-print analyses:

Vitality test:

The top and bottom of each leaf samples was printed onto PDA agar. The plates were incubated at 30 °C for 7 days and the viability of the epiphytic microorganisms was observed. The samples that showed no visible of epiphytic microorganisms on PDA plates were further examined for surface imprint test.

Surface imprint test:

The leaf samples were washed under running tap water and soaked in 1% of sodium hypochlorite with immersion time of 1, 5, 10 and 15 minutes respectively. This method was conducted to determine the optimal immersion time to remove epiphytes and other contaminants. The top and bottom part of the leaves were then printed onto fresh growth medium (Potato Dextrose agar + plant powder). The plates were incubated at 30 °C for 7 days.

Isolation of endophytic fungus using different growth media:

Preparation of growth agar media:

Six growth media were used to grow endophytic fungi; Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), PDA plus host plant powder (PHP), PDA plus host plant extract (PPE), MEA plus host plant powder (MHP) and MEA plus host plant extract (MPE). PDA and MEA were prepared according to the manufacturer's protocol. The growth media supplemented with host plantwere prepared in 500 ml distilled water by mixing 5 g plant powder/extract with 20 g and 24 g PDA and MEA powder respectively. All media were autoclaved at 121°C for 15 minutes and supplemented with 0.1 g chloramphenicol.

Isolation of endophytic fungi:

The leaf samples were sterilized by immersing in 1% of sodium hypochlorite. The samples were then aseptically cut into small pieces, placed onto different growth media and incubated at 30°C for 7 days. The endophytic fungi can be observed by the growth of hyphal tips on the media. The hyphal were aseptically cut into small fragments and transferred onto fresh agar media. The endophytic fungi were subcultured several times to ensure the genetic purity. The pure culture was grown on slant agar and kept at 8°C with proper labelling.

Bacterial strain:

The test microorganismused in this study were gram-positive bacteria; *S. aureus, Enterecoccus faecalis, Bacillus subtilis,* MRSA and gram-negative bacteria*; Escherichia coli, Pseudomonas aeruginosa, K. pneumoniae* and *S.boydii*. The bacteria were maintained on Nutrient agar every 2 weeks. All cultures were kept at 4°C for further experiment. Bacterial inoculum was prepared by adding 4 ml of sterile physiological saline tothe bacterial slant culture, followed by vigorous shake to get the cell suspension.

Preliminary screening of endophytic fungi:

Preliminary screening of the isolated endophytic fungi was carried out by agar plug method. The endophytic fungi were first grown as lawn for 7 days. Cork borer was used to cut the agar plug from the prepared lawn. A starter culture was prepared by growing a loopful of test bacteria in 8 ml Luria Broth medium at 37°C, 150 rpm for 24 hours. The bacteria with the inoculum size of 10^5 (CFU)/mlwere prepared by diluting 10 µl of the starter culture into 100 mlsterile distilled water. The bacterial inoculum was then spread onto Mueller Hilton (MH) medium. The prepared agar plug was then transferred onto the MH medium containing bacterial inoculum. The plate was incubated at 4°C for 7 days, followed by 24 hours incubation at 37°C. During the incubation period, presence or absence of inhibition zone was

observed and recorded. Two endophytic fungi that showed good inhibitory activities were selected for secondary screening.

Metabolites extraction from endophytic fungi:

The selected endophytic fungi from primary screening were first grown as lawn for 7 days. Cork borer (1 cm diameter) was used to cut the agar plug from the prepared lawn. Fungal inoculum were prepared by transferring the agar plug into250 ml Erlenmeyer flasks containing 100 ml Yeast Sucrose broth (20 g/L yeast extract, 40 g/L sucrose, 0.5 g/L $MgSO_4$) and Potato Dextrose Broth (24 g/L potato dextrose powder, 0.2 g/L chloramphenicol) respectively. The cultures were incubated at 30 °C, 120 rpm for 20 days. Fermented broth and fungal biomass were separated by centrifugation at 5311 x g (Sigma, Model 4K15). The fungal biomass were freeze-dried followed by metabolite extraction. The freeze-dried fungal biomass were soaked in methanol overnight at ratio of 1:50 (w/v). Liquid phase was collected and further extracted with equal volume of hexane, dichloromethane, ethyl acetate and butanol for 3 times.

Disc and agar diffusion assay:

Preparation of bacteria plates and disc diffusion assay:

A total of 1.0 L Mueller Hilton agar (MH agar) was prepared for disc diffusion assay and agar well assay. Fresh bacteria were prepared by inoculating one loop of bacteria taken from the bacteria culture plate onto 8 ml sterile Luria Broth (LB). The bacteria were incubated in the incubator shaker at 37°C for 24 hours with 150 rpm of rotational speed. The bacteria with the inoculum size of 10^5 (CFU)/ml) then were prepared by dilute 10 µl of one day old bacteria onto 100ml sterile distilled water. The 10^5 (CFU)/ ml) bacteria were swabbed onto MH agar. The screening of antimicrobial activity was conducted according procedures defined by Jorgensen and Turnidge (2007). The crude extracts were dissolved in 50 % dimethy sulfoxide (DMSO). The test organisms with the inoculum size of 4 x 10^5 colony-forming units (CFU)/ml were transferred by streaking the inoculum on the MH agar (Hi-media) surface using sterile cotton swab. The Sterile Whatman antibiotic disc impregnated with 20.0 µl of each extracts with 20.0 mg/ml concentration was placed on the inoculated medium surface. 20 % of DMSO was applied as a control to detect the solvent effects. A total of 30.0 µg/ml chloramphenicol (Sigma) was used as the positive control for the bacteria. The bacteria were incubated at 37°C for 24 hours. The inhibition zone diameter that appeared around the disc was measured.

RESULT AND DISCUSSION

Estimation of chlorophyll content:

Chlorophyll content and photosynthetic pigment assessments in every leaf with different growth stages is very important indicator to measure the leaf senescence since the chlorophyll will loss due to the environmental stress (Yamamoto *et al*., 2002). The estimation of chlorophyll content using SPAD 502 meter is performed to select the best accurate stages of leaf maturity(Netto *et al*., 2005).

Table 1 shows the colour and relative chlorophyll content of *O. citriodorum* leaves at different growth stages. The green pigmentation become low when the leaf getting mature. The chlorophyll content of leaf peaked at 60 days old and continued decreasing at 30, 90 and 120 days old (Zhang *et al*., 2008). The finding from the current study shows the chlorophyll content low when young stages while high at mature stage because green pigmentation was found higher at mature stage. During old stage and senescent stage, the green pigmentation (chlorophyll) degraded.

Table 1: Colour and relative chlorophyll content of *O.citriodorum* leaves at different growthstages

Criteria	Leaf growthstages			
	Young	Mature	Old	Senescent
Colour	Light green	Dark green	Yellowish	Dark yellowish
Relative chlorophyll content (SPAD) unit	20 ± 0.4	38 ± 0.5	32 ± 0.1	12 ± 0.8

Surface sterilization of the leaf sample:

The surface sterilization of leaf samples can be performed using sodium hypochlorite, ethanol and distilled water. The most frequent choice for surface sterilization in laboratories is using sodium hypochlorite to remove epiphyte fungi, dirt and other contaminants on leaf samples (Oyebanji *et al*., 2009). In the current study, the leaves were immersed in 1% of sterile sodium hypochlorite for 1, 5, 10 and 15 minutes to remove the epiphyte fungi followed by 60 second of immersion in ethanol.

Table 2 shows the old and senescent leaves took a longer immersion time compared toyoung and matured leaves. These show that epiphytes are more colonized on senescent leaves than young leaves. The same result was reported in the previous study which showed the old leaves tend to have more epiphytes than the young leaves (Angsupanich, 1996).

Table 2: The immersion time of *O. citriodorum* leaves followed by their leaf age stages in 1% Sodium hypochlorite

Leaf age stages	Immersion time (minutes)
Young	1
Matured	3
Old	7
Senescent	10

Isolation of Endophytic Fungi from Ocimum citriodorum Vis. Leaves:

A total of 169 fungal were successfully isolated from different growth stages of *O. citrodorum* leaves. Surface imprint method and viability test conducted were used to confirm that only endophytes can be isolated instead of epiphytes fungal as epiphytes will be killed during the immersion of leaf in 1% Sodium hypochlorite and ethanol (Sanchez- Marquez *et al*., 2007, Yenn *et al*., 2014). Out of 169 isolates, only two isolates failed the vitality and surface imprint test which are TLBSL-PDA8 and TLBSL-PDA5.

Table 3 shows the 167 endophytic fungi that successfully isolated from different growth stages of *O.citriodorum* leaves. Six different growth media; PDA, MEA, PHP, PPE, MHP and MPE were used in order to isolate the endophytic fungi. The tendency of the endophytic fungi to grow with the supplement of their host plant were determined. Results showed that endophytic fungi from *O. citriodorum* leaves tend to grow on PDA (Difco and BBL Manual, 2009; Griffith *et al*., 2007) and media for endophytic fungal isolation supplemented with their host can promote and enhance their growth (Tong, 2014; Yenn *et al*., 2014).

Table 3: Endophytic fungiisolated from *O. citriodorum*leaves using 6 different growthmedia

Leaf growthstages	Growth media						Total
	[1]PDA	[2]PHP	[3]PPE	[4]MEA	[5]MHP	[6]MPE	
Young	1 ± 0.2	8 ± 0.4	4 ± 0.2	1 ± 0.1	3 ± 0.5	2 ± 0.4	19
Mature	6 ± 0.4	12 ± 0.1	7 ± 0.1	3 ± 0.2	8 ± 0.3	5 ± 0.8	41
Old	11 ± 0.4	16 ± 0.3	11 ± 0.3	7 ± 0.5	11 ± 0.1	9 ± 0.2	65
Senescent	7 ± 0.7	11 ± 0.4	8 ± 0.5	5 ± 0.8	7 ± 0.4	4 ± 0.3	42
Total	25	47	30	16	29	20	167

[1]Potato dextrose agar [2]Potato dextrose agar + Host plant powder Potato dextrose agar+Host Plant extract [4]Malt host extract agar [5]Malt extract agar + Host plant powder [6]Malt extract agar+ host Plant extract

Primary screening:

The primary screening of endophytic fungi was carried out to determine the antibacterial activity of ten endophytic fungi isolated from *O. citriodorum* leaves against *S.aureus, E.faecalis, B.subtilis,* MRSA, *E.coli, P.aeruginosa, K.pneumoniae and S.boydii* (Table 4). Most of endophytic fungi isolates showed a good inhibition on gram-negative bacteria compared to gram-positive bacteria. Cell wall layer of peptidoglycan in gram negative bacteria are much thinner than in gram positive bacteria. Hence, endophytic fungi compound can easily destroy the synthesis of cross linked peptidoglycan in gram-negative bacteria. *K. pheumoniae and S. boydii* were found to be the most susceptible test bacteria to the endophytes isolates.

Table 4: The primary screeningof antimicrobial activity of 10 Endophytic fungi isolated from *O. citriodorum* leaves.

No	Endophytic fungi	Bacteria								Total
		[1]S. a	[2]E. f	[3]B. s	[4]MRSA	[5]E.c	[6]P. a	[7]K. p	[8]S. b	
1	TLBML-M1	–	–	+	+	-	+	+	+	5
2	TLBML-M2	–	–	–	–	+	–	+	+	3
3	TLBML-P1	–	–	+	–	–	–	+	+	3
4	TLBML-PHP1	–	–	–	–	+	+	–	+	3
5	TLBML-PHP2	+	–	+	–	+	+	+	+	6
6	TLBOL-P1	–	+	+	–	–	–	–	–	2
7	TBLOL-P2	–	–	–	–	–	–	+	+	2
8	TLBSL-MHP1	+	–	+	–	–	–	–	–	2
9	TLBOL-PHP1	+	+	–	–	+	–	–	–	3
10	TLBOL-PHP3	+	–	–	–	+	–	+	–	3

[1]*Staphylococcus aureus* [2]*Enterococcus faecalis* [3]*Bacillus subtilus* [4]Methicillin-resistant *S.aureus* [5]*Escherichia coli* [6]*Pseudomonas aeruginosa* [7]*Klebsiella pheumoniae* [8]*Shigella boydi*

Metabolite extraction from endophytic fungi:

Based on preliminary screening, only two endophytic fungi were selected for metabolite extraction. Isolate TLBML-M1 and TLBML-PHP2 showed a positive result which is these endophytic fungi inhibited the higher number of test bacteria included the most pathogenic bacteria. TLBML- M1 shows inhibitory activities against 5 test bacteria while TLBML-PHP2 against six test bacteria. These two endophytic fungi, isolate TLBML-M2 and TLBML-PHP2 inhibited most gram-negative bacteria rather than gram-positive bacteria. These two endophytic fungi were fermented for 20 days prior to metabolite extraction. The extraction of endophytic fungi is very important as it affects the yield and activity of bioactive compound in the

extract. In this method, the dry process is very crucial because the water content in the extract can interfere the extraction process that will bring the wrong separation during partitioning process (Sticher, 2008). The metabolites were extracted from endophytic fungi using 4organic solvents which are hexane, dicloromethane, ethyl-acetate and iso-butanol (Khadhasamy and Arunachalam, 2008). Solvent partitioning method is a common method used for separation and extraction of compound from natural products before the chromatographic technique was introduced (Otsuka, 2005). This method also knownas liquid-liquid extraction which separate the compounds based on their relative solubility in two immiscible liquids (Mensor *et al*., 2001).

Antimicrobial assay:

The antimicrobial activities of TLBML-M1 and TLBML- PHP2 were observed using disc diffusion assay (Table 5). Based on the results, most of antimicrobial compounds were mainly extracted in ethyl acetate (Yenn*et al*., 2014)compared to hexane (non-polar).

Table 5: Antimicrobial activity of TLBML-M1 and TLBML-PHP2 cultured in YES medium on disc diffusion assay.

Test Bacteriabacteria	Diameter of inhibition zone (mm)					
	Isolate TLBML-M1			Isolate TLBML-PHP2		
	Hexane	[1]DCM	[2]EtAc	Hexane	[1]DCM	[2]EtAc
Gram-positive						
S. aureus	-					
E. faecalis	-	-	25.3 ± 0.8	-	31.3 ± 1.2	27.3 ± 1.2
B. subtilus	-	12.3 ± 2.3	22.0 ± 0.3	-	27.7 ± 0.9	23.3 ± 1.9
MRSA	15.3 ± 2.1	22.7 ± 0.9	20.0 ± 1.1	12.7 ± 1.2	37.7 ± 0.9	12.7 ± 0.6
Gram-negative						
E. coli	11.3 ± 1.2	-	24.0 ± 0.9	-	32.7 ± 0.4	22.3 ± 2.1
P. aeruginosa	-	-	-	19.7 ± 1.1		-
K. pheumoniae	-	-	20.7 ± 1.4	-	29.7 ± 0.9	22.0 ± 0.4
S. boydii	-	11.3 ± 1.1	26.3 ± 2.1	12.3 ± 1.2	30.7 ± 0.3	28.0 ± 0.4

[1]Dichloromethane [2]Ethyl-acetate

Fig. 1: The ethyl acetate extract of the isolate TLBML-M1,TLBML-PHP2 and positive control.

Fig. 2: The ethyl acetate extract of the isolate TLBML-M1,TLBML-PHP2 and positive control.

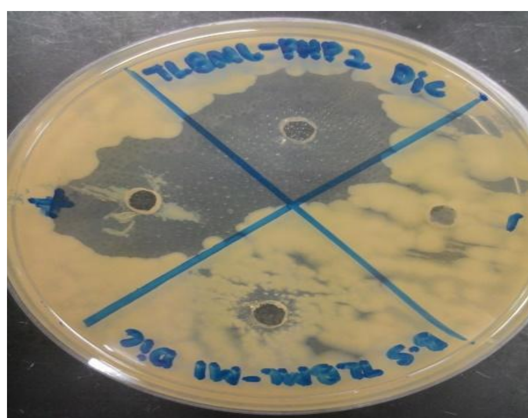

Fig. 3: The dichloromethane extract of the isolate TLBML-M1,TLBML-PHP2 and positive control.

Figure 1 shows the inhibition zones observed from isolates TLBML-M1, TLBML-PHP2 extracted using ethyl acetate against *K. pneumoniae* with positive control, P-30µg/ml chloramphenicol. The inhibition zones indicated by the clear zone surrounding the paper disc. No inhibition zone was detected for negative control, N-20% DMSO.

Figure 2 showed that ethyl acetate extract of the isolate TLBML-M1, TLBML-PHP2 and positive control, P-30µg/ml chloramphenicol showed inhibitory activity against *B. subtilus* on disc diffusion assay, which indicates by the clear zone surrounding the paper disc. No inhibition zone was detected for negative control, N-20% DMSO.

Figure 3 showed that dichloromethane extract of the isolate TLBML-M1, TLBML-PHP2 and positive control, P-30µg/ml chloramphenicol showed inhibitory activity against *B. subtilus* on agar well diffusion assay, which indicates by the clear zone surrounding the paper disc. No inhibition zone was detected for negative control. N-20% DMSO. The diameter of zone of inhibition of the ethyl extract of both isolates showed a same diameter as positive result. This indicates that, the antimicrobial compounds of these endophytic fungi extract were having a same antimicrobial activity as antibiotic use for positive result against the bacteria.

Agar well diffusion assay:

The antimicrobial activities of TLBML-M1 and TLBML- PHP2 in a disc diffusion assay are shown in Table 6. The crude extract of both endophytic fungi showed high positive results on Gram – negative bacteria. The finding was similar as the antimicrobial activity of both endophytic fungi using disc diffusion method where almost all bacteria inhibited by ethyl acetate crude extract. These showed that the antimicrobial compounds were most present in polar fraction which is in ethyl acetate and also dichloromethane. Less antimicrobial compound present in hexane (non-polar) fraction as the results shows only two bacteria were inhibited by the hexane extract of both endophytes.

Table 6: Antimicrobial activity of TLBML-M1 and TLBML-PHP2 cultured in YES medium on agar well diffusion assay.

Test Microorganism (Bacteria)	Diameter of inhibition zone (mm)					
	Isolate TLBML-M1			Isolate TLBML-PHP2		
	Hexane	[1]DCM	[2]EtAc	Hexane	[1]DCM	[2]EtAc
Gram-positive						
S. aureus	-	-	-	-	-	-
E. faecalis	-	-	-	-	-	-
B. subtilus	-	6.30 ± 1.1	25.0 ± 0.6	-	31.0 ± 2.5	29.3 ± 0.6
MRSA	-	-	13.7 ± 0.4	11.7 ± 2.2	14.7 ± 0.9	20.7 ± 2.1
Gram-negative						
E. coli	-	11.3 ± 0.2	23.3 ± 0.2	-	-	19.3 ± 1.1
P. aeruginosa	-	-	-	-	-	14.0 ± 1.2
K. pheumoniae	-	-	24.7 ± 0.9	-	-	26.0 ± 0.9
S. boydii	-	13.7 ± 0.9	29.0 ± 2.2	14.7 ± 0.9	31.7 ± 1.2	26.7 ± 2.4

[1]Dichloromethane [2]Ethyl-acetate

Conclusion:

Based on the resuts of the study, atotal of 167 endophytic fungi were isolated from different growth stages of *O. citriodorum* leaves. 39%of the isolates that most densely colonized by endophytic fungi were an old leaves stages. From the total of 167 endophytic fungi isolated from different agar media, PHP is the most suitable growth media for endophytic fungi as it gave the highest endophytic fungi isolates. The bioactive compound was highly produced by the fungus when the host plant was supplemented into the culture medium. Two isolated namely TLBML-M1 and TLBML-PHP2 exhibited significant antibacterial activities on the test

microorganisms. Fungal biomass of both endophytic fungi, TLBML-M1 and TLBML-PHP2 showed highest antibacterial activities on the test microorganisms compared to fermented broth extract. For fermentative broth, ethyl acetate and dichloromethane extracts gave the highest yield of antimicrobial activities for both endophytic fungi isolates.

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