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Antimicrobial and antioxidant potentials of crude extracts of culturally dissimilar endophytic fungi

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Abstract

Endophytes from an unexplored plant such as Spatterdock (SD) *Nuphar lutea variegata* could be a dependable producer of novel secondary metabolites active against drug resistance organisms. This study explored the diversity of fungal endophytes from mid-rib (MR) and leaf blade (LB) of *Nuphar lutea variegata* as well as the antimicrobial and antioxidant potentials of their secondary metabolites. Morphological features observed showed four species were isolated. A broad-spectrum antimicrobial activity observed to be concentration-dependent was observed for all the fungal crude extracts 1 mg/mL. Crude extract of *SD-LB3* showed the best antibacterial activities against *S. aureus* and *P. aeruginosa* with a minimum inhibitory concentration of 0.5 and 0.25 µg/mL respectively. The antioxidant capacities of the fungal crude extracts showed a better neutralizing effect of free radicals compared to the standard Quercitin. Effective inhibition of free radicals was observed for all the fungal extracts from 20 to 100 mg/mL (96.7 – 99.3 %). The highest antioxidant activity was shown by the extracts of fungi isolated from the leaf blade with an average of 98 to 99.2 % at the tested concentrations of 20 to 100 mg/mL. Our study provides evidence of diverse endophytic fungi associated with *Nuphar lutea variegata,* and the antibacterial and antioxidant activities observed confirm their capacities as producers of bioactive secondary metabolites.

This study is the first to isolate endophytic fungi form *Nuphar lutea variegata* and screen the antimicrobial and antioxidant activities of their secondary metabolites.

Keywords: Spatterdock; *Nuphar lutea variegata*; Endophytic fungi; Antimicrobial; Antioxidant; Secondary metabolites

1. Introduction

Over the years, the search for agents needed for the management of a variety of diseases affecting humans, plants, and animals has intensified. This is in response to halting the further deterioration of the fight against diseases. A typical example is the prolonged treatment of infections caused by resistant microbes. This has been attributed mainly as a result of the development of resistance by the infectious particles to medicines currently available [1]. There is a need for the development of new therapeutic interventions for the management of a variety of diseases especially those caused by resistant microorganisms. Endophytic fungal natural products have been identified as a resource for new chemical entities regulated by regulatory proteins [2]. The chemo-diversity of bioactive metabolites of endophytic and marine-associated fungi has redirected the focus of investigators in recent times, given their unique structural and pharmacological effects and potential pharmaceutical and agricultural applications [3-6]. This is hugely a driving force behind the relentless research efforts directed towards discovering novel bioactive molecules from fungi endophytes. Our search for bioactive secondary metabolites was focused on the leaves of spatterdock, a submerged and floating-leaved hydrophyte aquatic plant.

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Spatterdock (*Nuphar lutea*), an aquatic plant of the family *Nymphaeaceae*, is a rhizomatous aquatic herb found in ponds and lakes in Nigeria. They are normally called water lilies rooted in soil in bodies of water with leaves and flowers floating on the surface [7], Spatterdock is more common in open stagnant water systems and slow-moving streams. Life forms involve the plant's morphology [7,8], and vegetation are similar to those found in China, Taiwan, Japan, Korea, Mongolia, Russia, and Europe [8]. A few chemical and pharmacological analyses of *Nuphar lutea* have shown the promising pharmaceutical application of this plant.

The rhizome can be cooked or dried and ground into flour for baking. It has been used for the treatment of inflammation and to restore normal circulation [9]. Other pharmacological properties of *Nuphar lutea* include blood clotting, relief of joint pains in elderly people [8]; also, acute septic shock, arthritis, fevers, and aches [10]. The pharmacological effects have been attributed to several compounds: tannin [9]; "the quinolizidine sesquiterpene alkaloids nupharopumiline, deoxynupharidine, 7-*epi*-deoxynupharidine, nupharidine, 7-*epi*-nupharidine, nupharopumiline and nupharolutine; the dimeric quinolizidin esesquiterpene thioalkaloids 6-hydroxythiobinupharidine, 6,6′-dihydroxythiobinupharidine, 6-hydroxythionuphlutine B, 6′-hydroxythionuphlutine B, neothiobinupharidine, thionuphlutine B, 6,6′-hydroxythionuphlutine B, thiobinupharidine, syn-thiobinupharidinesulfoxide, thionuphlutine B β-sulfoxide and neothiobinupharidine β-sulfoxide" [8].

Moreover, the anti-inflammatory property has been linked to the phytoconstituent deoxynupharidine, which also showed inhibitory activities against the secretion of interleukin 1 (IL-1) and tumor necrosis factor (TNF) by murine peritoneal macrophages in vitro [8]. This work was carried out to investigate the presence of endophytic fungi in healthy leaves of *Nuphar lutea* and also their antimicrobial potentials and antioxidant capacity of the fungal crude extracts.

2. Material and methods

2.1. Plant material

Healthy leaves of the aquatic plant *Nuphar lutea* were collected from Agu Ngwu swamp in Ugwuoba, Enugu State South-Eastern Nigeria (Figure 1). The plant samples were collected from the bottom of the swamp with the help of a local diver and transported to the laboratory in plastic bags. The plant material was identified as *Nuphar lutea variegata* and authenticated by a plant taxonomist at the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Awka, Nigeria, where a voucher specimen with the identification code (PCG/474/A/067) was deposited.

Figure 1 *Nuphar lutea variegata* (Nymphaeaceae)

2.2. Isolation and colonial examination

Harvested *Nuphar lutea variegata* samples were properly disinfected step-wisely with 2.5 % Sodium hypochlorite, 70 % ethanol, and sterile double distilled water. This was done after the samples were thoroughly washed with running tap water for about 10 minutes. Surface-disinfected samples were aseptically cut to about 2 cm and inoculated into sterile Malt Extract Agar (MEA) plates (containing 500 mg/L Chloramphenicol). The plates were incubated for 5 days whilst being observed for the development of mycelium. Isolation of pure cultures was achieved through multiple sub-culturing of isolates on fresh MEA. Examination of the colonial/morphological characteristics of the fungal isolates was carried out by observing selected observable phenotypic character keys such as colony texture and color mycelium and pigmentation [11,12].

2.3. Fermentation and metabolites extraction

Each pure fungal isolate was grown in 1 L Erlenmeyer flasks containing sterilized rice medium, previous autoclaved at 121ºC at 15 psi for 1 h was employed [5]. The fermentation flasks were properly sealed and incubated under static conditions at 28°C for 21 days. Extraction of biosynthesized fungal metabolites was achieved using 500 mL of ethyl acetate. The filtrates were concentrated by evaporating the solvent at 40ºC using a rotary evaporator.

2.4. Bioassay

2.4.1. Antimicrobial assay

The antimicrobial effects of the fungal extracts were tested *in vitro* against two pathogenic bacteria strains, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and one fungal strain, *Candida albicans* [5]. The microbiostatic effect of the fungal extracts was evaluated at different concentrations on microbial growth. A stock concentration of 1 mg/mL of each fungal extract reconstituted in dimethyl sulfoxide (DMSO, 100 % v/v) was further diluted (to 0.5, 0.25, 0.125, 0.065 mg/mL) adopting two-fold serial dilution. Sterile Mueller-Hinton (for the bacterial test isolates) and Sabouraud dextrose agar plates (for the fungal test isolate) were inoculated with 0.5 McFarland suspension of each test organism using sterile cotton swabs. After inoculation, a volume of 80 µl of the extract solution was transferred into the wells made in the agar using a 6 mm sterilized cork borer and incubated at 37 and 25 ºC for 24 and 48 h, for the bacterial and fungal plates respectively. Ciprofloxacin (5 µg/mL) and Miconazole (50 µg/mL) served as the positive controls while DMSO served as the negative control.

2.4.2. Antioxidant assay

The free radical scavenging potentials of the endophytic fungal extracts were carried out as described by [13], with some modifications. The free radical scavenging properties of the extracts against 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical were measured at 490 nm. The concentrations of the extracts and ascorbic acid used were 20, 40, 60, 80, and 100 µg/mL The reaction mixture consists of 25 µl of the stock, 25 µl of DPPH (0.1 mol/L), and 150 µl of methanol solution. These were added into their respective wells in the microtiter. The plate was incubated at 27ºC for 30 min. The absorbance of the mixtures was measured at 517 nm using a UV-vis spectrophotometer (06452; USA). The experiment was done in triplicate for each fungal extract. Free radical scavenging activities were expressed as the percentage inhibition of each extract and calculated using the formula:

$$\%\ Inhibition = \frac{A0 - A1}{A0}\ x\ \frac{100}{1}$$

where A0: Absorbance of blank; A1: Absorbance of test sample

3. Results and discussion

The unidentified endophytic fungi *SD-LB3* and *SD-LB5* (from Spatterdock-Leaf Blade) and *SD-MR2 and SD-MR4* (from Spatterdock-Mid-Rib), were isolated from the leaf blade and the mid-rib sections of *Nuphar lutea variegata* leaf on Malt extract agar (Figure 2). Their colonial features indicate different species of endophytic fungi associated with the leaves of *Nuphar lutea variegata* (Table 1). Fungal endophytes from the leaf blade produced more yield than those isolated from the mid-rib (Table 1; Figure 3).

Table 1 Colonial features and Yield of extracts

Isolate code	COLOUR	TEXTURE	PIGMENT	Yield of fungal extract (g)
SD-MR2	White	Velvety	No pigment	0.40
SD-MR4	Milky	Fuzzy	No pigment	0.70
SD-LB3	Gray	Cottony	black	1.10
SD-LB5	light green and white	Soft slippery	Light green	0.40

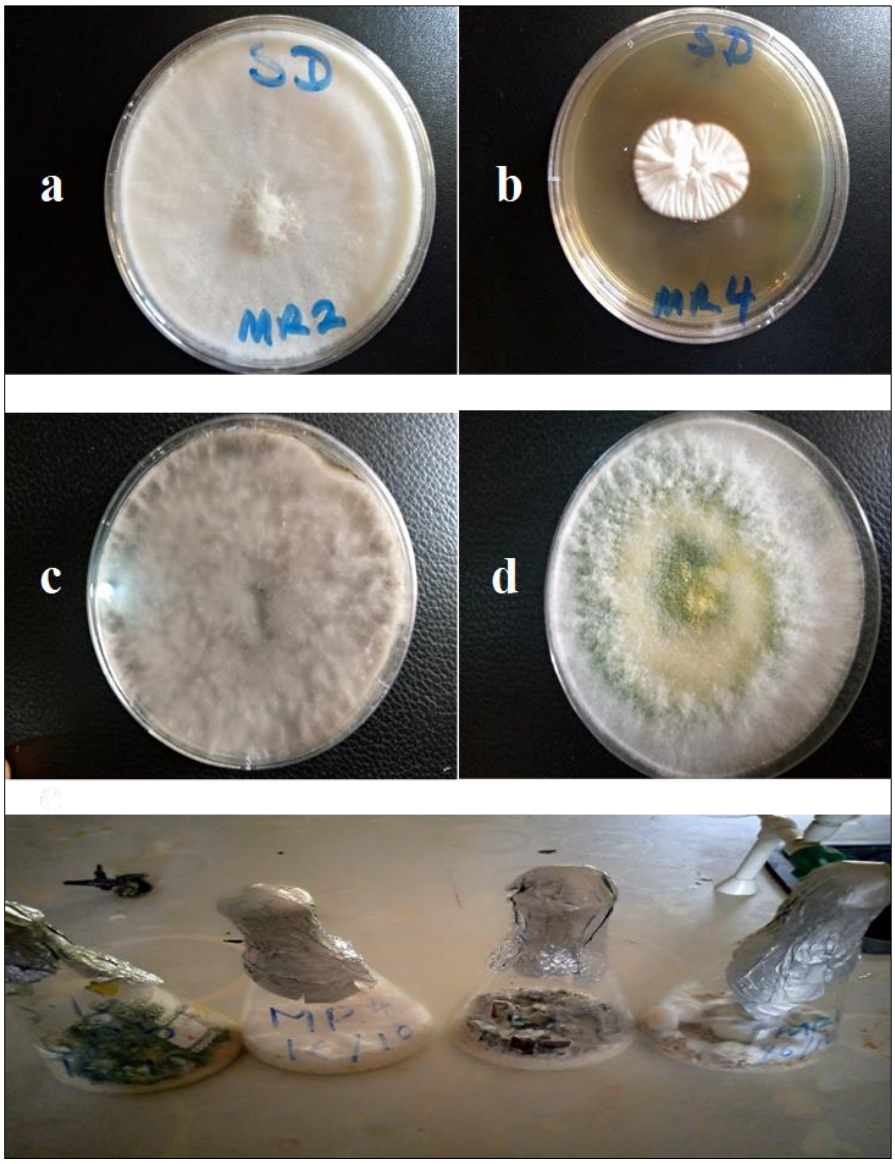

Figure 2 *SD-MR2* (a), *SD-MR4*, (b), *SD-LB3* (c) and SD-*LB5* (d). Fermentation of pure isolates on local rice medium (e)

Figure 3 Secondary metabolites extracted using EtoAc (f), concentrated crude extracts of the isolated fungal endophytes (g)

Data presented in Tables 2 - 5 shows that the ethyl acetate extracts of the fungal endophytes showed broad-spectrum antibacterial activity. The fungal extracts showed concentration-dependent growth inhibition. The highest antibacterial

effect was observed on the pathogenic *Staphylococcus aureus* with the minimum inhibitory concentration of 0.5, 0.25, 0.25, and 0.5 mg/mL of *SD-LB5, SD-MR4, SD-MR2,* and *SD-LB3* respectively. The pathogenic *C. albicans* was resistant to the fungal extracts.

Table 2 Antibacterial potentials of SD-MR2

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)		
	P. aeruginosa	***S. aureus***	***C. albicans***
2	3.5±0.1	3.6±0.1	0±0
1	2.5±0.5	2.5±0.5	0±0
0.5	2.2±0.3	2.3±0.4	0±0
0.3	0±0	0±0	0±0
0.1	0±0	0±0	0±0
Positive control	8.5±0.7	9.5±0.7	16±0
DMSO	0±0	0±0	0±0

The antimicrobial potential was indicated by the inhibition zone produced around each well and measured in millimeters. The positive controls used were ciprofloxacin 10 µg/mL and miconazole 50 µg/mL.

Table 3 Antibacterial potentials of SD-MR4

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)		
	P. aeruginosa	***S. aureus***	***C. albicans***
2	4.4±0.3	3.3±0.2	0±0
1	3.2±0.2	3±0	0±0
0.5	2.6±0.2	2.6±0.1	0±0
0.3	2±0	2.1±0.1	0±0
0.1	0±0	0±0	0±0
Positive control	8.5±0.7	9.5±0.7	16±0
DMSO	0±0	0±0	0±0

Table 4 Antibacterial potentials of SD-LB3

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)		
	P. aeruginosa	***S. aureus***	***C. albicans***
2	3.3±0.4	4.9±0.2	0±0
1	2.8±0.1	3.7±0.1	0±0
0.5	2.3±0	2.7±0.3	0±0
0.3	0±0	2.1±0.1	0±0
0.1	0±0	0±0	0±0
Positive control	8.5±0.7	9.5±0.7	16±0
DMSO	0±0	0±0	0±0

The antimicrobial potential was indicated by the inhibition zone produced around each well and measured in millimeters. The positive controls used were ciprofloxacin 10 µg/mL and miconazole 50 µg/mL.

The antimicrobial potential was indicated by the inhibition zone produced around each well and measured in millimeters. The positive controls used were ciprofloxacin 10 µg/mL and miconazole 50 µg/mL.

Table 5 Antibacterial potentials of SD-LB5

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)		
	P. aeruginosa	***S. aureus***	***C. albicans***
2	3.4±0.5	3.8±0.1	0±0
1	2.7±0.2	2.9±0.4	0±0
0.5	2.1±0.1	2.3±0.1	0±0
0.3	0±0	0±0	0±0
0.1	0±0	0±0	0±0
Positive control	8.5±0.7	9.5±0.7	16±0
DMSO	0±0	0±0	0±0

The antimicrobial potential was indicated by the inhibition zone produced around each well and measured in millimeters. The positive controls used were ciprofloxacin 10 µg/mL and miconazole 50 µg/mL.

The fungal extracts showed a good capacity to neutralize free radicals (Figure 4). Percentage radical inhibition was higher for all the fungal extracts compared with that of Quercitin (positive control).

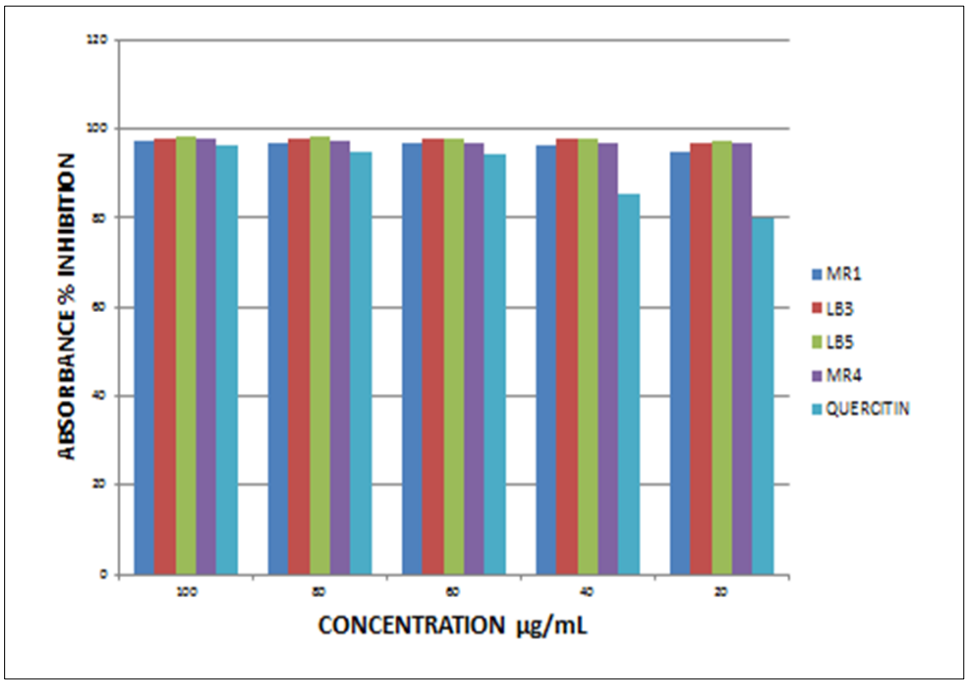

Figure 4 Antioxidant effects of crude extracts of fungal endophytes isolated from *Nuphar lutea variegate* leaf

The majority of the studies on microbial natural products, conducted so far, involve the investigation of different endophytic fungi strains; the chemo-diversity of produced fungal secondary metabolites, the chemical analyses, and pharmacological potentials of the metabolites. This is the first report on the isolation and bioassay of extract of endophytic fungi isolated from *Nuphar lutea variegata* leaf, an aquatic plant.

A Previous study carried out on *Nuphar lutea* L. SM. (Nuphar) species, focused on the inhibition of NFkappaB by the leaf and rhizome extracts [8]. Similarly [14], reported the Anti-leishmanial activity of partially purified alkaloid fraction of *Nuphar lutea* extract. In their work, *Nuphar lutea* extract showed good anti-trypanosomal activity with an IC50 value of 0.42 µg/mL. Flavonoids and phenolic contents have ascribed the antioxidant activities of natural compounds.

In the present study, fungal endophytes isolated from the leaf blade section of the *Nuphar lutea variegata* leaf showed better biosynthetic capacities than strains isolated from the mid-rib as observed by their extract yields. Also, extracts of the fungi isolated from the leaf blade showed good *in vitro* growth inhibitory activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Among the fungal extracts investigated*, SD-LB3* showed the best antibacterial activities against *Staph. aureus* and *P. aeruginosa* with a minimum inhibitory concentration of 0.5 and 0.25 µg/mL respectively. This could be ascribed to the possibility of the presence of more bioactive secondary metabolites produced by this fungus present in the crude extract.

Moreover, all the fungal extracts showed broad-spectrum antimicrobial activities. The antimicrobial effects showed by the fungal extracts against the test organism were observed to be concentration-dependent with the least and maximum inhibition zones observed to be 2 and 4.9 mm respectively. A critical observation of the antibacterial activities of the fungal extracts showed that *S. aureus* was the most susceptible test organism. This may be due to the presence of compounds capable of disrupting the integrity of the cell wall of Gram-positive microorganisms. Similar investigations on the medicinal potentials of *Nuphar* extracts showed anti-bacterial activity [15]. Another study carried out on the extract of *Nuphar lutea* showed inhibitory effects on some bacteria [16,17] and against pathogenic fungi [18].

The antioxidant capacities of the fungal extracts exhibited better neutralizing effects of free radicals compared to the standard Quercitin. The percentage inhibition of free radicals by the fungal extracts was higher than quercetin. Effective inhibition of free radicals was observed for all the fungal extracts from 20 to 100 mg/mL (96.7 – 99.3 %). The highest antioxidant activity was shown by the extracts of fungi isolated from the leaf blade with an average of 98 to 99.2 % at the tested concentrations of 20 to 100 mg/mL.

Study on the anti-inflammatory properties of *Nuphar lutea* (L.) Sm. (Nymphaeaceae family) showed "significant anti-inflammatory potential protecting the mice used through the prevention of acute septic shock" [10]. This is in part due to the antioxidants present in these extracts capable of neutralizing inflammatory diseases through their antioxidant activities.

Plants are abundant sources of natural antioxidants, that are cost-effective and, without side effects. Inhibition of free radicals is a mechanism by which natural compounds exhibit their antioxidant-induced anti-inflammatory potentials. This is a preventive role that helps in the reduction of illnesses that result from oxidative stress [19].

The isolation of endophytic fungi from *Nuphar lutea variegata* leaf, with potential as producers of novel natural compounds, suggests an untapped reservoir. These natural-based antioxidants can be developed into important therapeutic agents capable of halting illnesses triggered by oxidative stress.

Fungal metabolites have shown antimicrobial and antioxidant effects. For instance, the antioxidant potentials of bioactive compounds isolated from fungal extracts have been ascribed to some detected constituents including flavonoids, phenols, ascorbic acid, and tannin [20]. Similarly, saponins have been detected in the crude extract of Penicillium species an endophytic fungus [21]. Cajaninstilbene acid is also an antioxidant produced by *F. solani*, a fungal endophytes isolated from pigeon pea. While antimicrobial activities have been observed by two solanapyrone analogues (solanapyrones N and O) produced by *Nigrospora sp.* YB-141 and endophytic fungi isolated from *Azadirachta indica* A. Juss [22]. Similarly, evaluation of secondary metabolites from *Penicillium* species isolated from the leaf of neem plant produced secondary metabolites with antimicrobial potentials [21].

In addition, similar chemical constituents have been detected in the crude extracts of *Nuphar luteum* which include anthraquinone and cardiac glycosides in petroleum ether; anthraquinone, alkaloids, terpenoids in chloroform extract, and alkaloids, anthraquinone glycosides, terpenoids, saponins, flavonoids, tannins and phenolic compounds, in chloroform and methanol extract [23].

The antimicrobial and antioxidant effects shown by the extracts of fungi isolated from *Nuphar lutea variegata* is an indication that this species harbor endophytic fungi with biosynthetic capacities for a new bioactive compound.

4. Conclusion

Our findings presented in this paper suggest the possibility of the isolation of novel lead molecules produced by uncharacterized endophytic fungi. It is also important to note that the unique environment of the plant under study may be a key player in determining the number and type of secondary metabolites produced by the endophytic fungi associated with this *Nuphar lutea variegata* and other aquatic plants. Therefore, the application of bioassay-guided isolation will increase the chances of isolation of a novel bioactive compound.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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