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Antibacterial and phytochemical properties of *Aphanamixis polystachya* essential oil

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Abstract: Now a day's rise of new antibiotic resistant bacterial strains is a global threat. Ethnic people of India have been employing *Aphanamixis polystachya* (Wall.) R. Parker wood extract in healing cancerous wounds. The aim of this study was to evaluate the antimicrobial activity and to identify the medicinally potent chemicals in the essential oil extract of *A. polystachya*. The antibacterial properties of various organic extracts were evaluated against a range of bacteria (gram-positive and gram-negative bacteria) based on the disc diffusion method and GC-MS based analysis for finding active oil extract components. All extracts of *A. polystachya* leaves showed potential antibacterial activity, notably ethyl acetate, while petroleum ether extracts revealed highly sensitive activity against all tested bacteria (zones of inhibition ranging from 8.83 to 11.23 mm). In addition, the petroleum ether extract had the lowest MIC value (32 to 256 μg/mL) against *E. coli*, *S. lutea*, *X. campestris*, and *B. subtilis* bacteria. The major compounds detected in oil [*β*-elemene (16.04 %), *β*-eudesmol (12.78 %), *β*-caryophyllene (19.37 %), *β*-selinene (11.32 %), elemol (5.76 %), and *α*-humulene (5.68 %)] are expected to be responsible for the potent antimicrobial activity. The results of this study offer valuable insights into the potent role of *A. polystachya* essential oil extract in pharmaceutical and antibiotic research.

Key words: *Aphanamixis polystachya*, antibacterial activity, disc diffusion assay, minimum inhibitory concentration, GC-MS

1. Introduction

Infectious diseases have always been considered as a major threat to human health and are important causes of morbidity and mortality.[1-3] Among infectious diseases, bacterial infection was the second-leading cause of death worldwide in 2002, according to world health organization's estimates (WHO).[3] As such, antibiotic resistance has drawn a great deal of attention as one of the most important emerging risks

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for human beings worldwide.[4,5] Normally, synthetic antibiotics are the most common practice for the treatment of bacterial infections. Nonetheless, many antibiotics are known to cause several side effects, including hypersensitivity, immune-suppression, and allergic reactions.[6] To help resolve these problems, it is desirable to find new and more potent antimicrobial compounds that can suppress or potentially prevent pathogenic infections. In light of these demands, the use of natural (rather than synthetic) materials from plants could be a safe and reliable choice to treat infectious diseases.[7,8]

Aphanamixis polystachya (Wall.) R. Parker is a large tree belonging to the *Meliaceae* family that is distributed throughout the sub-Himalayan region, Bangladesh, and Indo-Malaysian regions. It is also called locally as Roina, Pitraj (Bengali), Rohitak, Pithraj (English), etc.[9,10] As its roots, leaves, and bark have effective medicinal value, it has been used in various traditional medicines for tumors, cancer, spleen diseases, and rheumatism treatment.[11] In Ayurveda, *A. polystachya* has been used to treat various diseases associated with liver and spleen disorders, tumors, ulcer, dyspepsia, intestinal worms, skin diseases, leprosy, diabetes, eye diseases, jaundice, hemorrhoids, burning sensation, arthritis and leucorrhoea.[12]

The Santanol ethnic people of India were reported to employ the wood extract of *A. polystachya* in healing cancerous wound.[9] The fruits of *A. polystachya* are used as anthelmintic, laxative, and refrigerant and good for ulcers and rheumatism; the seed oils are also used as a liniment in muscular pains.[9] It was also found that the stem bark extracts of *A. polystachya* has significant effects on *in vitro* antibacterial, mild antifungal, cytotoxic and antioxidant treatments.[9,10,12] Likewise, extracts of *A. polystachya* bark were seen to have radioprotective efficacy,[13] while those of seed showed antifeedant, repellant, and contact toxicity to beatles.[14] *A. polystachya* has medicinal value because it contains essential secondary metabolites including aphanamixis and novel triterpene and tetranortriterpenoid (i.e. aphanamixinin, sterol, saponins, flavanone, and anthraquinone glycosides) molecules.[15,16] These metabolites were expected to play a crucial role in antibacterial activity. Several studies have been carried out to assess the ethnomedicinal value of *A. polystachya.*[16-19] Nevertheless, the composition of *A. polystachya* essential oil extracts and their function remain to be elucidated. In this study, we evaluated the effect of essential oil extract of *A. polystachya* on antibacterial activity through the identification of its novel compounds. The results of this study will ultimately help us find the best solution to replace the use of commercial synthetic antibiotics.

2. Experimental

2.1. Plant material

The leaves of *A. polystachya* were collected from Satkhira, Bangladesh in February 2014. This plant was deposited as a voucher (DACB 38572) after the identification by National Herbarium, Mirpur, Dhaka, Bangladesh.

2.2. Preparation of plant extracts

The collected leaves were washed under running tap water and then finally washed with distilled water four times. They were then dried under an air shade for two weeks at room temperature. The dried leaves were pulverized into powder form. Afterwards, to prepare plant extract, 20 g of dried leave powder were added into 100 mL of each organic solvent including ethyl acetate, ethanol, methanol, petroleum ether, dichloromethane, and *n*-hexane. The solvents were then evaporated in an incubator at 37 °C for 48 h. Finally, the extracts were concentrated to 4 mg/mL and were stored at 4 °C in sterile containers for future use.

2.3. Preparation of essential oils

For the preparation of essential oils, the fresh leaf samples were subjected to a solvent extraction process using a Clevenger type apparatus. Petroleum ether was used as the solvent to extract essential oil. Anhydrous Na_2SO_4 was also used for drying the oil, which was then preserved at 4 °C in a sealed vial for further analysis.[20]

2.4. Test organisms for antibacterial activity

A total of seven (i.e., two gram-positive and five gram-negative) bacteria were used in this study, including *Bacillus subtilis* IFO 3026, *Sarcina lutea* IFO 3232, *Escherichia coli* IFO 3007, *Proteus vulgaris* MTCC 321, *Pseudomonas denitrificans*, *Xanthomonas campestris* IAM 1671, and *Klebsiella pneumoniae* ATTC 10031. These pathogens were obtained from the Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh.

2.5. Determination of antimicrobial activity

To evaluate *in vitro* antimicrobial activity, we performed the disc diffusion method.[21] Briefly, Whatman No. 1 filter paper discs (5.5 mm diameter) were impregnated with several extracts such as ethyl acetate, ethanol, methanol, petroleum ether, dichloromethane, and *n*-hexane, respectively. Each disc contained 300 μg of each respective extract. For calculating minimum inhibitory concentration (MIC), blank filter paper discs were impregnated with each extract prepared at six different concentration levels (512, 256, 128, 64, 32, and 16 μg/mL). The test bacteria were cultured on nutrient agar culture media, and the dried discs were placed on the bacteria/agar surface and incubated at 37 °C for 24 h. The culture plates were then examined to assess the zone of inhibition (ZOI) on a millimeter scale. Nalidixic acid (15 μg/mL), a commercial antibiotic, was used as the positive control and a blank disc impregnated with solvents followed by evaporation was used as the negative controls.

2.6. Gas chromatography-mass spectrometry (GC-MS) analysis

For the quantification of oil extracts, GC-MS analysis was carried out by the GC (Agilent Technologies 7890A)-MS (5975C) equipped with an inert triple axis detector (XL EI/CI MSD) at the Bangladesh Council of Scientific and Industrial Research (BCSIR). The basic conditions of GS-MS analysis are described in *Table* 1. Briefly, the essential oil extracts were analyzed using the electron impact ionization (EI) mode. For each analysis, 1 μL of essential oil was injected in the splitless mode and analyzed for 40 min. The spectra were then analyzed and the percent composition (w/w) of the oil was identified by referring to retention indices based on the co-injection of homologous *n*-alkanes (C_6-C_{24}). Then, obtained spectra were interpreted based on the National Institute Standard and Technology (NIST) library with more than 62,000 compounds.[22,23] Library spectra with the major number of peaks in common were seen to match with the unknown spectra. In this procedure, 95 % of the matching compounds were taken, while 5 % of nonmatching compounds were mislaid. To find the best matching quality, we scaled the best matching peaks from 95 % matching compounds by their m/z values. Accordingly, 98 % of compounds were successfully achieved, while 2 % were left as mislaid.

Table 1. Experimental conditions of the GC-MS system used for the detection of compounds in *A. polystachya* essential oil extracts.

Parameter	Value	Units
GC (Agilent Technologies 7890A)		
(a) Oven condition		
Initial temp.	60	°C
Hold time	0	min
Ramping rate	5	°C/min
Final temp	240	°C
Hold time	4	min
Carrier gas	Helium (He)	99.99%
Column flow rate	1	ml/min
Total run time	40	min
(b) Detector (MS) (Agilent Technologies 5975C)		
Ionization mode	EI (70 eV)	
Ion source temp.	230	°C
TIC scan range	50-550	m/z
Threshold	100	
(c) Column (Agilent J&W HP-5ms)		
Length	30	m
Diameter	0.25	mm
Film thickness	0.25	μm
Temperature Limits	-60 – 325/350	°C

2.7. Statistical analysis

The results derived for the ZOI were expressed as the mean ± SD (standard deviation) of three parallel measurements using Microsoft Excel® for windows (version 2010).

3. Results

3.1. Antibacterial activity of *A. polystachya* extracts

The antibacterial properties of various chemicals extracted from the leaves of *A. polystachya* were evaluated against the tested bacteria by examining the presence or absence of inhibition zones *in vitro*. The extracts showed antibacterial activity against two gram-positive (*Bacillus subtilis* and *Sarcina*

Table 2. Antibacterial activity of various *A. polystachya* extracts against different gram-positive and gram-negative bacteria. Values are given as the mean ± S.D. (n= 3).

Bacterium	zone of inhibition (ZOI) in millimeter (mm) scale						
	Nalidixic acid (15 µg/mL)	Ethyl acetate	Ethanol	Methanol	Petroleum ether	Dichloro-methane	n-hexane
B. subtilis	11.50 ± 0.45[a]	9.73 ± 0.31	9.43 ± 0.45	11.57 ± 1.25	8.83 ± 0.57	10.70 ± 0.20	
S. lutea	12.50 ± 0.51	10.63 ± 0.47	8.57 ± 0.51	-	11.23 ± 0.49	8.93 ± 0.12	9.07 ± 0.21
E. coli	10.50 ± 0.17	8.93 ± 0.12	10.90 ± 0.17	-	10.80 ± 0.20	-	7.93 ± 0.12
P. vulgaris	10.00 ± 0.25	9.43 ± 0.51	7.73 ± 0.25	8.27 ± 0.68	9.37 ± 0.32	9.33 ± 0.58	10.17 ± 0.29
P. nitrificans	10.00 ± 0.29	8.93 ± 0.51	8.33 ± 0.29	9.07 ± 0.12	9.30 ± 0.46	8.50 ± 0.20	6.67 ± 0.15
X. campestris	10.00 ± 0.17	-	-	-	10.20 ± 0.20	-	-
K. pneumoniae	8.50 ± 0.12	7.53 ± 0.25	-	-	-	-	-

[a]Mean ± standard deviation (SD)

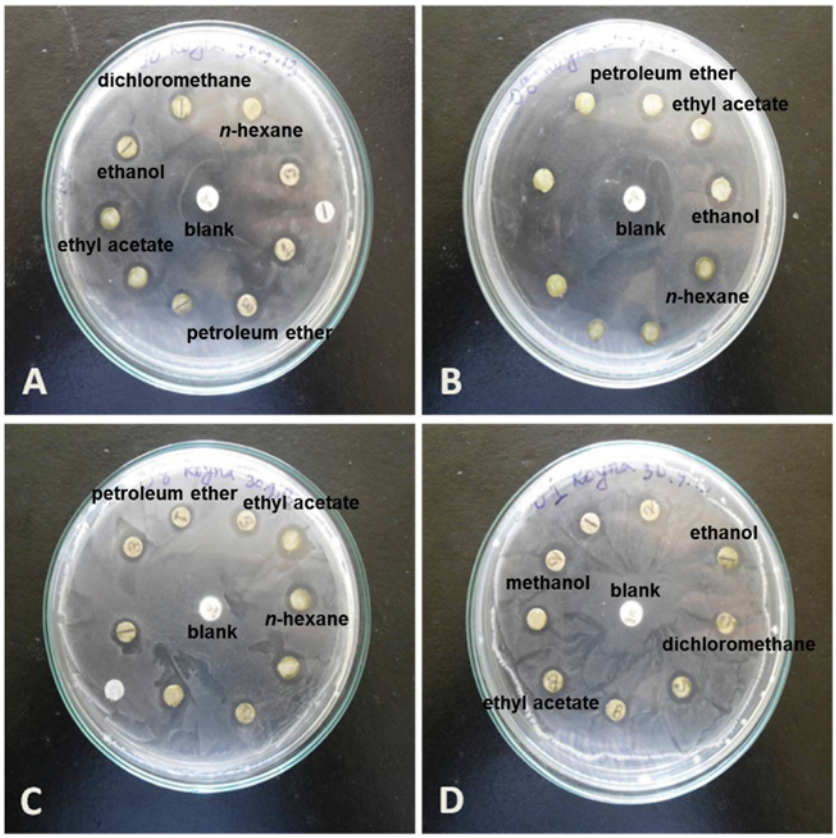

Fig. 1. Zones of inhibition formed by various extracts of *A. polystachya* with lowest minimum inhibitory concentration from *Table* 3 as tested against (A) *S. lutea*, (B) *E. coli*, (C) *P. vulgaris*, and (D) *B. subtilis.*

lutea) and five gram-negative bacteria (*Escherichia coli, Proteus vulgaris, Pseudomonas denitrificans, Xanthomonas campestris,* and *Klebsiella pneumoniae*). Although all *A. polystachya* extracts showed potential antibacterial activity, the petroleum ether extracts revealed considerably higher sensitivity against *E. coli*, *S. lutea*, and *X. campestris* bacteria (ZOI ranging from 10.20 to 11.23 mm, *Table* 2 and *Fig.* 1). The largest ZOI was measured by the methanol extract against *B. subtilis* (11.57 mm) (*Table* 2). Moreover, ethanol and dichloromethane showed moderate antibacterial activity against *B. subtilis*, *E. coli*, *S. lutea*, *and p. vulgaris*, respectively (7.73 to 10.9 mm). Interestingly, only the *A. polystachya* petroleum ether extract inhibited *X. campestris* (10.2 mm) that was similar effects with the commercially available antibiotic (nalidixic acid; 10 mm). Thus, the petroleum ether extract of *A. polystachya* could be replaced nalidixic acid for the inhibition of *X. campestris.* On the other hand, the ethyl acetate extract showed moderate activity against *K. pneumoniae* (7.53 mm, *Table* 2).

In diagnostic laboratories, minimum inhibitory concentrations (MICs) are used to evaluate the potency of new antimicrobial agents and also to confirm the minimum dose of the agent in that microorganisms could not survive.[24] A MIC is generally considered as the primary measurement tool against an organism

Table 3. MIC of different *A. polystachya* solvent extracts against different bacteria.

Name of Bacteria	Ethyl acetate	Ethanol	Methanol	Petroleum ether	Dichloromethane	*n*-hexane
B. subtilis	128*	64	64	256	128	-
S. lutea	128	256	-	128	128	128
E. coli	256	128	-	32	-	128
P. vulgaris	256	256	64	128	256	256
P. denitrificans	256	128	64	128	128	256
X. campestris	-	-	-	128	-	-
K. pneumoniae	256	-	-	-	-	-

*Units-µg/mL

Table 4. Chemical composition of *A. polystachya* essential oil identified by GC-MS.

Peak no.	Retention Index (RI)	Retention time (RT)	Name of the compound	M. W.	Formula	% composition (w/w)
1.	1375	38.523	*β*-Elemene	204.34	$C_{15}H_{24}$	16.04
2.	1418	41.13	*β*-Caryophyllene	204.36	$C_{15}H_{24}$	19.37
3.	1439	42.264	*α*-Guaiene	204.35	$C_{15}H_{24}$	1.65
4.	1456	43.391	*α*-Humulene	204.35	$C_{15}H_{24}$	5.68
5.	1485	45.998	*β*-Selinene	204.35	$C_{15}H_{24}$	11.32
6.	1503	47.81	Germacrene A	204.35	$C_{15}H_{24}$	3.80
7.	1518	48.489	(−)-*α*-Panasinsen	204.35	$C_{15}H_{24}$	2.19
8.	1547	51.205	Elemol	222.37	$C_{15}H_{26}O$	5.76
9.	1564	52.447	(±)-*trans*-Nerolidol	222.37	$C_{15}H_{26}O$	1.68
10.	1581	53.126	Caryophyllene oxide	220.35	$C_{15}H_{24}O$	0.56
11.	1595	54.375	Guaiol	222.37	$C_{15}H_{26}O$	0.77
12.	1647	55.366	Himachalol	222.37	$C_{15}H_{26}O$	0.92
13.	1651	55.882	*γ*-Eudesmol	222.37	$C_{15}H_{26}O$	1.01
14.	1652	57.07	*α*-Eudesmol	222.37	$C_{15}H_{26}O$	3.04
15.	1654	59.792	*β*-Eudesmol	222.37	$C_{15}H_{26}O$	12.78
16.	1666	60.485	Bulnesol	222.37	$C_{15}H_{26}O$	0.96
17.	1949	89.9	*trans*-Phytol	296.53	$C_{20}H_{40}O$	0.61
18.	2026	94.652	Geranylgeraniol	290.48	$C_{20}H_{34}O$	1.03

for a new antimicrobial agent.[25] In this research, the MIC values of the various extracts (against the tested bacteria) are summarized in *Table* 3. The petroleum ether extract exhibited the highest sensitivity against *E. coli* (MIC, 32 μg/mL). In addition, the petroleum ether extract recorded the lowest MIC value (128 μg/mL) against *X. campestris* bacteria. On the other hand, methanol extract showed a significant effect on *B. subtilis*, *P. vulgaris*, *and P. denitrificans* with the lowest MIC (64 μg/mL). Interestingly, *K. pneumoniae* was inhibited by only ethyl acetate extract (256 μg/mL), suggesting that the different *A. polystachya* solvent extracts might exert a potential inhibitory effect against different bacteria.

3.2. Chemical composition of the essential oils

We analyzed the essential oil extracts of *A. polystachya* by GC-MS to determine the active compounds. In *Table* 4, the results of GC-MS analysis are summarized in terms of retention index (RI), retention time (RT), molecular weight (MW), molecular formula, and percent composition (w/w) of the compounds in *A. polystachya* oil extract. Based on the data, it was possible to identify about 90 % of the *A. polystachya* oil extract components, while the remaining portion (~10 %) was unidentified. The major identified compounds in oil were *β*-elemene (16.04 %), *β*-eudesmol (12.78 %), *β*-caryophyllene (19.37 %), *β*-selinene (11.32 %), elemol (5.76 %), *α*-humulene (5.68 %), germacrene A (3.80 %), *α*-eudesmol (3.04 %), and (−)-*α*-panasinsen (2.19 %). Furthermore, *α*-guaiene, nerolidol, *γ*-eudesmol, *trans*-phytol, bulnesol, and geranylgeraniol were found in low concentrations.

4. Discussion

To date, a variety of medicinal plants have been identified and investigated from various parts of the world. The recent literature shows that the majority of the world population is still using medicinal plants for numerous medical purposes.[26] Further, medicinal plants and their phyto-constituents have been screened in the late 19th century for antimicrobial activity.[27] Likewise, pharmaceutical companies are trying to improve the effective drugs extracted from natural resources, especially from plants, to treat pain, fever and other maladies.[28] In this study, various *A. polystachya* extracts were evaluated for antibacterial activity against gram-positive and gram-negative bacterial pathogens based on GC-MS analysis of phyto-constituents of petroleum ether extracted *A. polystachya* essential oil.

In this study, *A. polystachya* organic extract showed significant antibacterial activity against tested bacterium, especially the petroleum ether extract. The antibacterial activity of *A. polystachya* organic extract was in agreement with previous findings,[29-31] which showed that organic extracts of *A. polystachya* fruits and leaves (especially methanolic) have strong antibacterial activity against *S. aureus, S. dysenteriae,* and *C. albicans*. The petroleum ether extract in this study exhibited the highest antibacterial activity against *B. subtilis, S. lutea*, *E. coli, P. vulgaris, P. denitrificans, X. campestris,* and *K. pneumoniae*. In another study, similar antibacterial activity of various *A. polystachya* stem bark extracts was also reported.[32,33] Further, aqueous and methanolic extracts of *A. polystachya* stem bark showed antiulcer and antianxiety activity in Wistar rat models.[34] In contrast, the antibacterial activity of other extracts (e.g., methanol, ethanol, dichloromethane, ethyl acetate, and *n*-hexane) maintained a minimal level.

Generally, plant materials consist of highly complicated phyto-constituents such as monoterpene hydrocarbons, oxygenated monoterpenes, limonene, phytol, and geraniol.[22,23] Hence, to analyze their components, GC-MS is a great tool due to its high sensitivity and selectivity and it is thought that GC-MS gives reliable standards in scientific analysis.[36,37] Recently, many screening studies for phyto-constituents in plant essential oil extracts have been conducted in the pharmaceutical research using GC-MS.[22,23,35,38] As a means to extend such research efforts, this study was conducted to evaluate the phyto-constituents of petroleum ether extracted *A. polystachya* essential oil by GC-MS analysis. This accurate and high throughput analysis revealed several important phyto-constituents

present in the extracts.

According to our GC-MS analysis, *β*-caryophyllene was identified as the most concentrated compound in *A. polystachya* oil extract, which to our knowledge has not been reported previously. Recently, *β*-caryophyllene was reported as a cannabinoid receptor type-2 (CB2) agonist;[39] it was also identified as a neuroprotective, anxiolytic, antidepressant, and anti-alcoholism reagent.[39-41] Moreover, the presence of other highly concentrated compounds (*β*- elemene, *β*-eudesmol, and *β*-selinene) in the *A. polystachya* oil extracts have not been reported in previous studies. Previously, *β*-elemene has been reported as an anti-proliferative agent in various cancer studies.[42-44] Additionally, *β*-eudesmol and *β*-selinene, well-known for antioxidant, antimicrobial, and anti-wood-decay fungal activities, were reported to be extracted from other plants;[45-47] however, no such reports made the case for *A. polystachya*. The antibacterial activity of *A. polystachya* extracts may at least partially reflect the effect of *β*-caryophyllene, *β*-elemene, *β*-eudesmol, and *β*-selinene.

Recently, several studies demonstrated that major compounds (i.e., caryophyllene (12.55 %), *β*-selinene (3.86 %), *α*-humulene (3.98 %), and phytol (2.58 %)) extracted from diverse tree species (*Acanthospermum australe*, *Calea fruticosa*, *Salvia urmiensis* Bunge, *Mikania glauca*, and *Premna integrifolia* Linn.) showed a potential role in preventing bacterial growth.[20,48,49] In the current study, the GC-MS analysis of *A. polystachya* essential oil extract also revealed the presence of oxygenated mono and sesquiterpene hydrocarbons like caryophyllene, *α*-selinene, *β*-selinene, *α*-humulene, and phytol, which may have strong potential for the inhibitory activity on tested bacteria.

5. Conclusion

The current study was carried out with the aim to uncover the ethnobotanical usage of essential oil extract from *A. polystachya* leaves that contain several phyto-constituents with specific bioactive functional potential. From a medicinal viewpoint, *A. polystachya* essential oils are rich in oxygenated mono and sesquiterpene hydrocarbons, especially caryophyllene, α-selinene, β-selinene, α-humulene, and phytol, which were identified by GS-MS analysis. Moreover, this biological study demonstrated that the essential oil extract of *A. polystachya* has great antimicrobial potential, as proven by a large ZOI with MIC against gram-positive and gram-negative pathogens. Hence, it is being concluded that *A. polystachya* has appreciable bioactive anti-pathogen compounds. This study can be extended further for evaluation *in vitro* and *in vivo* to improve novel drug components that may be used for the treatment of microbial infectious diseases.

6. Competing Interests

The authors declare that they have no competing interests.

7. Author Contributions

AA and MSR conducted laboratory analysis and drafted the manuscript. SKS and JH conducted statistical analysis and prepared the Figures and Tables presented in the manuscript and drafted the manuscript. MSR and KHK designed the study and supervised the work. All authors read and approved the final manuscript.

8. Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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