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Evaluation of Antibacterial Activity of Selected Edible Herbs Against Genital Mycoplasmas Among University Students in Enugu State, Nigeria

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Abstract: *The prevalence rates of Mycoplasma hominis and Ureaplasma Spp* and the evaluation of edible herbs against genital mycoplasmas were determined among university students in Enugu state, Nigeria. Specimens from 2400 subjects comprising of 1200 male and female subjects were tested for *Mycoplasma hominis* and *Ureaplasma Spp*. Cultures were done on Mycoplasma agar, A7agar and urea-arginine LYO2 broth. Antimicrobial susceptibility pattern was determined by Mycoplasma IST2 kit. Preliminary qualitative phytochemical analysis was done using standard methods to reveal the presence of basic phytochemicals. Rizomes of *Curcuma longa*, garlic and ginger, cloves and seeds of *Garcina kola* were extracted with water, methanol and ethanol sequentially and reconstituted with dimethyl -sulfoxide to concentrations (mg/ml) of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56. Mycoplasma isolates were screened for sensitivity to the extracts using agar well diffusion and broth diffusion methods. *Mycoplasma hominis* occurred more in women at (81.6%) while *Ureaplasma Spp* occurred more in men at (88%). *Mycoplasma hominis* (98%) and *Ureaplasma Spp* (91.6%) showed high sensitivityto ciprofloxacin. The test plants all showed presence of alkaloids, tannis, flavonoids, terpenoids, saponins, phenols and cardiac glycosides. At 200mg/ml *Garcina kola* and *Curcurma longa* showed higher zones of inhibition at 35mm on *Mycoplasma hominis* and *ureaplasma Spp*. Syngestic activities of ethanolic extracts of Curcuma longa and garlic showed high zones of inhibition at 45mm on *Mycoplasma hominis* while at 200mg/ml *Garcina kola* and cloves showed zones of inhibition at 45mm on *ureaplasma spp*. The minimum inhibitory concentration (MIC) results of the syngestic plants were 1.56mg/ml on all the isolates. The Results from the study showed that there is high prevalence of *Mycoplasma hominis* in women while *Ureaplasma Spp* while *Ureaplasma Spp* occurred more in men in the study area. The synergistic activities of selected edible plants showed higher efficacy on resistant isolates and suggest its use in the treatment of genital Mycoplasmas.

Keywords: Genital Mycoplasmas, Syngestic Activities, Phytochemical, Edible Plant, Antibiotics

1. Introduction

Annually, there are an estimated 500 million new cases of sexually transmitted infections (STIs) which are acquired, worldwide according to (W.H.O) [8]. If diagnosed in time, these infections can be treated easily with minimal morbidity as well as decreased economic burden [14]. *Mycoplasma genitalium, Mycoplasma hominis* and *Ureaplasma Spp* (collectively, genital mycoplasma) are most often implicated in genital or reproductive health conditions [15]. Transmission is often by sexual contact and from mother to child during vaginal delivery. Mycoplasmatales are associated with the infection of the genitourinary tract, neonatal morbidity, mortality and reproductive failure. Infection with genital mycoplasmas has been linked with infertility [11]. *Ureaplasma Spp*. is the main cause of nonchlamydial, nongonococcal urethritis and acute prostatitis. *U. urealyticum* in a large proportion of healthy women complicates the assessment of the pathogenic roles of this organism, but several studies have indicated that genital colonization of the *U. urealyticum* can be associated with an

increased risk of developing certain pathogenic conditions. Both *U. urealyticum and M. hominis* have been linked to bacterial vaginosis, causing 62-92% and 58-76% of cases, respectively. A large study of women with clinically suspected PID has shown that *Mycoplasma genitalium* was as prevalent as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in this disease [16]. *Mycoplasma genitalium* is highly specific for men with non-gonococcal urethritis, and also linked to a high-grade urethritis and also causes balanoposthitis (inflammation of the glans and the foreskin). Several studies have implicated *Ureaplasmaurealyticum, Mycoplasma hominis* and *M. genitalium* in prostatitis syndromes. During the past decade, evidence has accumulated the causative role of *U. urealyticum* in human infertility [17].

This study is aimed at assessing the occurrence and aetiology of significant *Ureaplasmaurealyticum* and *Mycoplasma hominis* among male and female students of higher institutions in Enugu State and to screen for edible herbs as potential antimicrobial agents on genital mycoplasmas.

Problem Statement/Justification

There are an estimated 500 million new cases of sexually transmitted infections which are acquired, worldwide according to [8]. Most colonized people remain asymptomatic and are prone to an increased risk of developing certain pathogenic conditions and pregnancy complications. Treatment of genital mycoplasmas varies with geographic region and there are increasing reports of antimicrobial resistance on genital mycoplasmas. If the burden of the infection of mycoplasmal infections are clearly evaluated and physicians and health authorities alerted on the pathogenic potential of these organisms, the spread of mycoplasmal infections would be greatly reduced in Enugu State. Also the use of available edible herbs as treatment options for *M. hominis* and *U. urealyticum* would reduce drug resistance, toxicity and drug interactions in existing antibiotics.

2. Materials and Methods

2.1. Study Design

A randomized cross sectional study involving 1200 female students and 1200 male students in the 6 higher institutions in Enugu State were used (giving a total of 200 male and female students each from the six higher institutions). Asymptomatic and symptomatic male and female students within the age bracket of 18 to 30 years were selected. This is because the study considered mostly sexually active males and females of child bearing age as mycoplasmal infections are more rampant among these set of individuals.

2.2. Ethnical Consideration

Ethnical clearance was obtained from the Ethnical Committee of the Enugu State University College of Medicine prior to the commencement of the study. Consent for the participation in the study was obtained from the students in the study area. The study had no adverse effect on the environment.

2.3. Sample Collection

It was stipulated that all persons participating in the study had not taken any antimicrobial agent prior to sampling so that the growth of mycoplasma would not be affected. Sterile cottons swab sticks were given to each of the female students for sample collection (HVS) after which it was placed directly into RI tubes (transport medium) while universal bottles were given to each of the male students for sample collection (urine) and subsequently taken to the clinical laboratory for analysis.

2.4. Isolation of Organism

2.4.1. Isolation of Mycoplasma Hominison Mycoplasma Agar

Methods of Whit hear, 1989 was employed to screen for Mycoplasma. A primary inoculation was made directly on Mycoplasma agar and spread over the reminder of the agar surface with an inoculating loop. Plates were incubated at 37°C under humidified microaerophilic atmosphere with a candle jar to provide additional carbondioxide. Mycoplasma colonies were purified by transferring micro-colonies to mycoplasma broth and incubated at 37°C until turbidity was observed. Mycoplasma colonies were stored in mycoplasma agar slants at 40°C for further use.

2.4.2. Isolation of Organisms on Urea-arginine LYO2 Broth

Vaginal swabs were inoculated into the Mycoplasma RI vial solution and 3ml transferred to Mycoplasma R2 vial, vortexed to dissolve completely and incubated aerobically at 36°C for 48h. 2 drops of the urine samples were transferred to Mycoplasma R2 vial, vortexed to dissolve completely and incubated aerobically at 36°C for 48h.

2.4.3. Isolation of Ureaplasma Spp on A7 Agar

From the R2 positive tube, 0.1ml were inoculated ontoA7 agar plates and incubated at 37°C humidified microaerophilic atmosphere with a candle jar to provide additional CO_2 checking characteristic colony morphology. Results were interpreted after 24 and 48hours of incubation. Colonies presenting with a fried egg appearance suggest the presence of *M. hominis* whereas colonies of *U. urealyticum* appear tiny granular and dark due to accumulation of manganese oxide brown. Resultant Ureaplasma colonies were purified by removing a block of agar containing micro-colonies and transferred to R2 vial and incubated aerobically at 37°C for 48h, Ureaplasma colonies were stored on A7 slants at 4°C for further use.

2.5. Identification Test

Resultant colonies on each medium were aspectically isolated and characterized using established microbiological methods.

2.6. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility pattern of the *M. hominis* and *U. urealyticum* isolates were determined using the

Mycoplasma IST2 kit (Biomeriux, France). The kit contained strips that gave information on the presence or absence of *M. hominis*and *U. urealyticum* and also provided additional information on antibiotic susceptibility to doxycycline, josamycin, ofloxacin, erythromycin, tetracycline, ciprofloxacin, azithromycin, clarithromycin and pristinamycin. The Mycoplasma IST2 strip consisting of 22 wells were inoculated with the rehydrated R2 growth medium, 55 ul per well was overlaid with drops of mineral oil and incubated aerobically at 36°C for 48h. Positive reading showedcolour change from orange to red in each cupule. Negative reading indicated yellow colour in the test cupules. *M. hominis* ATCC 15488 and *U. urealyticum* ATCC 27813 strains were used as controls.

2.7. Collection and Preparation of Aqueous, Methanol and Ethanol Extract of Plant Material

The seeds of Garcinia kola, cloves and the roots of turmeric, garlic and ginger were purchased from Ogbete market, Enugu State. The plants were identified and authenticated at the herbarium of the Department of Applied Biology, Enugu State University of Science and Technology Enugu. Fifty (50) grams each of the pulverized powder of plants were dissolved in 200ml of sterile distilled water, ethanol and methanol respectively for 24h. The mixtures were filtrated using what man's filter paper no. 1 to obtain solutions free of solids. The filtrate was concentrated by drying at 37°C and stored at 4°C. The stored extracts will be reconstituted using dimethyl-sulfoxide to obtain extracts of several concentrations: 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56mg/ml and stored at 4°C to determine the minimum inhibitory concentration.

2.8. Antimicrobial Assay

Antimicrobial activity of the extracts of the various plants was determined by agar well diffusion method as described by Lino and Deogracious (2006). The plates containing A7 and mycoplasma agar was spread with 0.1ml of the bacterial inoculums. Wells of 6mm in diameter were cut from the agar plates using sterilized stainless steel borer and the wells filled with 0.1ml of each extract. The plates were incubated at 37°C for 24h and the diameter zones of inhibition measured. The MIC is the least concentration that inhibits growth.

2.9. Broth Diffusion Assay

MIC was determined by broth diffusion method according to methods by [17]. 0.1ml of the bacterial inoculums already matched with MacFarland's turbidity standard were transferred to the R2 vial and 0.1ml of each extract was also transferred to each tube and incubated aerobically at 37°C for 48h.

2.10. Synergy Test

To test the combined extracts, selected extracts were used. Equal volumes (0.1:0.1) of each extracts were tested on the organisms. 0.1ml of each mixture was added into the wells which were inoculated with the test organisms and incubated at 37°C for 24h. The zones of inhibition were noted.

3. Results

Ethno botanical Result of the plant Species

Ethnobotanical information of the plants used included their botanical, local names and parts used (Table 1).

Table 1. *Ethnobotanical result of plant species.*

Plant Species	Local name	Part used	Ethnomedical uses
Garcina kola	Bitter kola	Seed	Purgative, antiparasitic, antimicrobial agent, throat infection, diarrhea, bronchitis
Cloves	-	Flower buds	Antioxidant, (eugenol) antimicrobial, Antidiabetic
Curcuma longa	Turmeric	Rhizome	Antiinflamatory, antioxidant, antimicrobial, treatment of Alzheimer's disease, delays ageing
Garlic	-	Underground stem	Treatment of cold, reduction of blood pressure, improve cholesterol levels
Ginger	-	Rhizome/root	Used in treating colic, stomach upset, gas, diarrhea, irritable bowel syndrome

Table 2. *Characteristics and Percentage yield of crude extract.*

Plant Sp	Extracting solvent	Percentage yield (%)	Extract code
	Water	2.48	E1
Garcina kola	Ethanol	3.24	E2
	Methanol	3.20	E3
	Water	1.28	E4
Cloves	Ethanol	2.08	E5
	Methanol	2.06	E6
	Water	3.20	E7
Curcuma longa	Ethanol	5.48	E8
	Methanol	6.10	E9
	Water	2.10	E10
Garlic	Ethanol	3.28	E11
	Methanol	2.78	E12
	Water	3.10	E13
Ginger	Ethanol	5.28	E14
	Methanol	5.10	E15

Key: E1-15 indicates Extracts.

Table 2 shows the percentage yield and grouping of each extract.

Table 3. *Qualitative Phytochemical Screening of Extracts.*

ExtractAlkaloidCode	Saponins	Reducing sugar	Flavonoids	Tannis	Terpenoids	Cardiacglycosides	Phenols
E1+	-	-	+	+	-	+	-
E2+	+	+	+	+	+	+	+
E3+	+	-	+	+	-	+	+
E4-	+	-	-	-	+	+	+
E5+	+	-	+	+	+	+	+
E6+	+	-	+	-	+	+	+
E7+	+	-	-	-	+	+	+
E8+	+	-	+	+	+	-	-
E9+	+	-	+	+	+	+	+
E10-	-	-	+	-	-	-	-
E11+	+	+	+	+	+	-	+
E12+	+	+	+	+	+	-	+
E13+	+	-	+	+	+	+	-
E14+	+	-	+	+	+	+	+
E15+	+	-	+	+	+	+	+

Key +=positive
-=negative
- E=Extract.

Table 4. *Occurrence of Isolates from Samples.*

Organism Identified	Specimen analysed	Sex	No of samplesPositive	No of sampleNegative
MycoplasmaHominis	HVS	F	980 (81.6%)	220 (18.3)
	Urine	M	260 (22%)	940 (78.3)
Total			1240 (51.6%)	1160 (48%)
Urea plasma	Urine	M	1058 (88%)	142 (12%)
Spp	HVS	F	570 (48%)	622 (57%)
Total			1628 (68%)	764 (31%)

Key: HVS: High Vaginal Swab
F: Female
M: Male.

Table 4 shows that both organisms had a high occurrence but *Ureaplasma Spp* occurred highest in males at 1058 (88%).

Table 5. *Pattern of Antibiotic Susceptibility in M. hominis Isolatesn=(1, 240).*

Antimicrobials Agents	S	I	R
Doxycline	1008 (81%)	21 (1.6%)	211 (17%)
Ofloxacin	630 (51%)	48 (3.9%)	562 (45.3%)
Erythromycin	1089 (88%)	-	151 (12.2%)
Josamycin	-	240 (19.3%)	1000 (81%)
Tetracycline	985 (79%)	48 (38.7%)	207 (17%)
Ciprofloxacin	1220 (98%)	-	20 (1.6%)
Azithromycin	388 (31.2%)	28 (2.2%)	824 (66.4%)
Clarythromycin	450 (36%)	320 (25%)	470 (38%)
Pristinamycin	-	300 (24%)	940 (76%)

Key: S- Sensitive,I – intermediate, R- Resistant.

Table 6. *Pattern of Antibiotic Susceptibility in theUreaplasma Spp Isolates (n=1636).*

S/N	AntimicrobialsAgents	S	I	R
1.	Doxycline	1800 (75%)	204 (8.5%)	396 (16.5%)
2.	Ofloxacin	700 (29.2%)	350 (14.5%)	1350 (56.2%)
3.	Erythromycin	1100 (46%)	480 (20%)	820 (34.2%)
4.	Josamycin	-	41 (1.7%)	2,359 (98%)
5.	Tetracycline	1840 (76%)	-	560 (23.3%)
6.	Ciprofloxacin	2200 (91.6%)	200 (8.3%)	-
7.	Azithromycin	680 (28.4%)	441 (18.3%)	1279 (53.2%)
8.	Clarythromycin	741 (31%)	567 (23.3%)	1092 (46%)
9.	Pristinamycin	-	530 (22%)	1870 (78%)

Key: S- Sensitive,I – intermediate, R- Resistant.

Table 7. *Zones of inhibition (mm) of Ethanol, Aqueous and methanol Extract of Garlic kola, Cloves, Curcuma longa and Garlic against M. hominis.*

Organism	Extracts (mg/ml)	200	100	50	25	12.5	6.25	3.125	1.56
M. hominis	E1	10	8	6	-	-	-	-	-
	E2	35	20	17	8	4	-	-	-
	E3	28	17	11	5	2	-	-	-
E4		8	-	-	-	-	-	-	-
E5		28	17	10	6	-	-	-	-
E6		26	21	18	7	-	-	-	-
E7		15	10	6	4	-	-	-	-
E8		35	23	16	9	4	-	-	-
E9		30	26	20	10	5	-	-	-
E10		12	8	6	5	-	-	-	-
E11		28	20	13	7	2	-	-	-
E12		25	20	18	8	-	-	-	-
E13		15	10	6	4	-	-	-	-
E 14		30	25	20	18	10	8	15	-
E 15		35	30	18	14	10	10	8	4

Table 8. *Zones of inhibitions (mm) of Ethanol, Aqueous and Methanol Extracts of Garlic kola, cloves, Curcuma longa and Garlic against Ureaplasma Spp.*

Extract Code (mg/ml)	200	100	50	25	12.5	6.25	3.125	1.56
E1	32	24	16	7	3	-	-	-
E2	15	10	8	4	3	-	-	-
E3	33	25	16	8	4	2	-	-
E4	28	18	10	6	4	3	-	-
E5	10	8	6	3	2	-	-	-
E6	33	25	17	8	4	-	-	-
E7	35	30	24	18	10	8	4	2
E8	18	15	10	8	4	-	-	-
E9	35	28	26	20	10	8	6	4
E10	28	18	10	8	4	-	-	-
E11	10	8	6	4	3	-	-	-
E12	25	20	11	9	5	-	-	-
E13	33	25	10	18	10	8	4	2
E14	16	10	8	4	3	-	-	-
E15	30	26	20	16	10	8	8	4

Table 9. *Zones of Inhibition (mm) of Combined Extracts (mg/ml) on Isolates.*

Test Organism	Extracts (mg/ml)	200	100	50	25	12.5	6.25	3.125	1.56
M. hominis	E7+E11	45	40	38	35	34	30	25	18
U. spp	E7+E11	43	40	37	36	33	30	23	15
M. hominis	E9+E12	42	38	36	35	35	31	25	15
U. spp	E9+E12	44	40	38	34	34	30	26	15
M. hominis	E2 +E5	40	44	35	35	33	28	25	14
U. spp	E2 +E5	45	40	35	36	32	30	24	18
M. hominis	E3 +E6	44	42	32	35	33	30	21	17
U. spp	E3 +E6	43	40	35	34	32	31	21	16

Table 10. *Minimum Inhibitory Concentration of Extracts (mg/ml) on M. hominis.*

Test Organisms *M. hominis*	Extracts (mg/ml)	MIC
	E1	25
	E2	12.5
	E3	6.25
	E4	25
	E5	12.5
	E6	12.5
	E7	25
	E8	12.5
	E10	6.25
	E11	50
	E12	12.5
	E13	6.25
	E14	6.25
	E15	6.25

Table 11. *Minimum Inhibitory Concentration of Extracts (mg/ml) on Ureaplasma Spp.*

Test OrganismsU. spp	Extracts (mg/ml)	MIC
	E1	50
	E2	6.25
	E3	12.5
	E4	50
	E5	6.25
	E6	6.25
	E7	50
	E8	12.5
	E9	6.25
	E10	50
	E11	12.5
	E12	6.25
	E13	50
	E14	6.25
	E15	6.25

Table12. *Minimum Inhibitory Concentration of Combined Extracts (mg/ml) on Isolates.*

Test organisms	Extracts (mg/ml)	MIC
M. hominis	E7 + E11	1.56
U. spp	E7 + E11	1.56
M. hominis	E9 + E12	1.56
U. spp	E9 + E12	1.56
M. hominis	E2 + E5	1.56
U. spp	E2 + E5	1.56
M. hominis	E3 + E6	1.56
U. spp	E3 + E6	1.56

4. Discussion

The ethnobotanical survey showed that all the test plants are noted in their use for the treatment of several disease [10]. According to the World Health Organization, over 85% of the population in sub-sahara Africa, including Nigeria still depend on herbal traditional medicine for their health care needs [8]. Results from qualitative phytochemical screening of *Garcina kola,* cloves*, Curcuma longa*, ginger and garlic are illustrated in Tables 2 and 3. Table 3 revealed varied reactions in the qualitative phytochemical screening of plants. Only E2 revealed the presence of alkaloids, saponins, reducing sugars, flavonoids, tannis, terpenoids, cardiac glycosides and phenols. Ethanol and methanol gave higher extract yields from all the test plants compared to water extracts. This observation is in accordance with extraction of solvents on plant in the works of [3], who observed that methanol extracted more of the active ingredients in plants than water and hexane. The highest extract yield was obtained from methanol extraction of *Curcuma longa*. The presence of phytochemicals in plants is responsible for their antioxidant and antibacterial activity [5]. Table 3 shows a high occurrence of *M. hominis* in females at (81.61%) and a higher occurrence of *Ureaplasma Spp* in men at (88%). *Ureaplasma Spp* and *M. hominis* are undonotedly the most significant in terms of disease inducing potential, their presence might be high among healthy people and are frequently encountered in the inner genitourinary tract of sexually active men and women [16]. According to [11] 40 – 80% and 21 – 53% of asymptomatic and sexually active women respectively harbor *Ureaplasma Spp* and *Mycoplasma hominis*. Genital mycoplasma lack cell wall and are resistant to antimicrobial agents that are active against this structure. Therefore, penicillins, cephalosporins and vacomycin are inactive in the treatment of conditions caused by the organisms. Antimicrobial agents that halt protein synthesis are active against Mycoplasmas but there is an increase in the occurrence of resistance to the group of drugs among clinical isolates [19]. Results in Tables 5 and 6 showed that *M. hominis* and *Ureaplasma Spp*. was highly sensitive to ciprofloxacin at 98% and 91.6% respectively. Both spp were highly resistant to Josamycin at 81% and 98%. There was variation in the pattern of antibiotic susceptiblity of isolates to other agents. Variation in pattern of antibiotic susceptibility has been observed in other studies [5, 19]. The in-discriminate use of antibiotics [9], the adverse effects of some antibiotics on host such as hypersensitivity, immune – suppression, allergic reactions and even loss of hearing [10] and emergence of multiple drug resistance among pathogens are challenges in the health care system which necessitate the need for new antimicrobial substances. Studies by [6, 7] advocate the use of medicinal plant as an alternative to the present antimicrobial therapy. The *in-vitro* antimicrobial activities of the plant extracts inhibited *M. hominis* and *Ureaplasma Spp* (Tables 7 and 8) and compared well with the control (ciprofloxacin). At 200mg/ml *Garcina kola* and showed the highest inhibition zones at 35mm. Complete inhibition by *Curcuma longa* on other organisms has been observed by [10, 20]. At 200mg/ml *Garcina kola,*

Curcuma longa and ginger extracts showed zones of inhibition at 33mm, 35mm and 33mm respectively on *Ureaplasma Spp*. It was observed that methanol and ethanol extracts inhibited the test organisms more than the aqueous extracts. These results are in accordance with [5] who found antimicrobial activity of cloves active against Mycoplasma isolates. The result presented in tables 8 and 9 indicate that the MIC of ethanol and methanol extract of the plants was higher than the MIC of the aqueous extracts of the test plants, The MIC results ranged from 6.23mg/ml to 12.5mg/ml. lower MIC were observed in the studies of [5]. Table 7 revealed that synergestic actions of extract on isolates showed a higher inhibition zones than when applied singly. At 200mg/ml inhibition zones ranged from 42mm to 45mm and were higher than those observed in the control. The MIC results of the synergestic plants were 1.56mg/ml in all the isolates and showed complete inhibition. Ethanolic extracts of *Curcuma longa* and Garlic (E7 and E11) on *M. hominis* showed the highest inhibition rates at 45mm while ethanol extracts of *Garcina kola* and cloves exhibited high inhibition zones at 45mm on *Ureaplasma Spp* (Table 12). This can be expected as the selected extracts showed high inhibition zoneson the test organisms when applied simply.

5. Conclusion

Genital mycoplasmal infections by *Ureaplasma Spp* and *Mycoplasma Hominis* was considerably high in the study. Both isolates were predominately resistant to josamycin and showed multiple drug resistance to other agents. Edible plants are consumed often without detrimental effects and are a source of medicine used to combat various aliments. From the study, all the selected plants possessed phytochemical constituents and may suggest antimicrobial activities. They differed in their antimicrobial activities and showed higher inhibition rates when extracted with ethanol and methanol. ynergestic activities of the plants showed higher inhibition activities. This investigation suggests further studies on the combination of edible herbs in the treatment of resistant pathogens.

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