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Evaluation of *in vitro* efficacy of homeopathic medicine *Secale Cornutum* (*Claviceps purpurea* sclerotium liquid) against molds causing keratitis

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Abstract
Mycotic keratitis/ keratomycosis continues to be a major concern to ophthalmologists. Homeopathic preparations have been employed for a variety of human mycoses. Secale Cornutum drug is used to treat various ailments but has never been explored for treating ophthalmic mycoses. Secale Cornutum is a nosode prepared from *Claviceps purpurea*, an ergot fungus that infects cereal crops. The present study is about isolating and characterising fungi from keratomycoses and exploring the in vitro efficacy of homeopathic preparation of Secale Cornutum on the ocular fungal isolates. *Fusarium* spp. (n=5), *Aspergillus flavus* (n=6), *Bipolaris* spp. (n=3), *Exserohilum* spp. (n=3) and *Curvularia* spp. (n=3) were obtained after processing the corneal scrapings employed for the study. The efficacy of Secale Cornutum against the fungal isolates was evaluated by plate assay, and minimum inhibitory concentrations were determined using broth microdilution. Secale Cornutum 10 M had a good inhibitory effect on the test fungal isolates. Except, for Secale Cornutum Q, all the other potencies tested showed significant ($P<0.05$) growth inhibition. The MICs of Secale Cornutum 30C ranged between $0.5/10^{60}$ and $0.25/10^{60}$ for *Fusarium* isolates. The MFC of Secale Cornutum 10 M was $0.5/10^{20000}$ for all the isolates of *Exserohilum* spp. The dilutions of Secale Cornutum have a significant effect on the inhibition of the growth of molds causing keratitis.

Keywords: Homeopathy, mycotic keratitis, *Secale Cornutum*, *Claviceps purpurea*, human mycoses

Introduction

Keratomycosis is a severe, sight-threatening eye infection caused by fungi. [1, 2, 3] Keratitis can be characterized by defects in the corneal epithelium with inflammation of underlying corneal stroma. [4] Mycotic keratitis/ keratomycosis continues to be a major concern to ophthalmologists. Filamentous fungi especially species of *Fusarium* and *Aspergillus* have been implicated as the predominant agents of ocular infection in most of the reports worldwide. [5,6,7,8] Filamentous fungi involved in keratitis include hyaline fungi, such as species of *Fusarium*, *Aspergillus* etc., and dematiaceous fungi such as species of *Curvularia*, *Bipolaris* and *Exerohilum*. [9]

The clinical management of mycotic keratitis remains a therapeutic challenge for ophthalmologists due to various predisposing factors like injury, ophthalmic ailments, poor lens hygiene, surgical procedures, misuse of ocular anaesthetics, weak immunity, long-term antibiotics, steroid therapy, emergence of resistance in fungal species against available antifungals, relative unavailability of effective antifungals and unaffordability of the public to current surgical therapies (keratoplasty, enucleation, evisceration *etc*). [9] Therefore, it is highly imperative to discover new broad-spectrum antifungals (not only fungistatic but also necessitate the host defence mechanisms to eradicate the mycotic infections) against the fungal species gaining drug resistance.

Homeopathic preparations have been employed for a variety of human mycoses. Also, homeopathic medicines are employed in the management of various ocular complications including cataracts and various infections without any side effects. [10] Homeopathic preparations of nosodes have been utilised for the therapy of various infectious and non-infectious diseases. [11, 12]. Chand [13] and Singh *et al.* [14]

have elaborated on various homeopathic preparations that can be used for ocular disorders. Uttamchandani and Patil [15] have reported the efficacy of homeopathic preparations in the treatment of fungal infections.

Secale Corutum is a nosode prepared from *Claviceps purpurea* commonly referred to as ergot fungus, an ascomycete that infects cereal crops resulting in the replacement of grains with fungal sclerotia. [16] This drug is used in the treatment of various ailments but has never been explored for the treatment of ophthalmic mycoses.

There are only very few reports on the *in vitro* activities of homeopathic drugs against human fungi. Against this background, the current research was undertaken to isolate and characterise molds involved in keratomycoses and to evaluate the *in vitro* effectiveness of the homeopathic drug *Secale Cornutum* against the molds isolated from human keratomycoses.

Materials and Methods

Fungal isolates from Keratitis patients

Corneal scrapings were obtained from keratomycoses patients vising a specialised eye care hospital at Coimbatore, Tamilnadu, India. The specimens were aseptically processed on Sabouraud's dextrose agar (SDA) and incubated at 28 °C for 5 to 7 days until the colonies covered one-third of the plate. The causative agents were identified based on growth morphology and through microscopic observation after subjecting the fungal isolates to standard fungal staining techniques [17].

Secale Cornutum potencies

Homeopathic drug preparations of *Secale Cornutum* were procured directly from the producer and the details are furnished in Table 1.

Table 1: Source, parts used, and potency preparation method of *Secale Cornutum* used in Homeopathic Pharmacopoeia of India (HPI) [18]

Source and part used	Potency Preparation Method in HPI	Potency
Claviceps purpurea	Q Drug strength=1/10 (1X)	Q
Hypocreaceae	[1000 ml of Q] 100g (freshly ground coarse powder): 530 mL purified water: 500 mL strong alcohol	30 C
Ergot of rye	Potencies: 2×= 1 parts of Q: 4 parts of purified water: 5 parts of strong alcohol	200 C
	3× and Higher = with dispensing alcohol	1 M
Whole fungus freshly dried in coarse powder	Old method - Class III	10 M

Evaluation of *in vitro* efficacy of homeopathic drug *Secale Cornutum* against the molds isolated from keratomycoses: Inoculum was prepared by growing the fungal isolates for 8 days on potato dextrose agar (PDA) plates. Sterile PDA was prepared with media and drug concentration in the ratio of 10:0.5, dispensed into the Petri dishes, solidified, aseptically inoculated with 1.5 mm diameter mycelial discs, and incubated at 28°C. The growth (radial diameter in mm) of the isolates was evaluated on 3rd, 4th and 5th day after incubation. Appropriate PDA plates were maintained as growth control (0.5 mL sterile water), spirit control (0.5 mL rectified spirit) and negative control (5 mg ketoconazole). The experiments were performed in triplicates.

The growth inhibition of the test isolates by *Secale Cornutum* was estimated using the following formula:

Percent (%) growth inhibition =

$$\frac{dg-dm}{dg} \times 100$$

Where dg is the radial growth diameter (mm) of the isolate in the control plate and dm is the mean radial growth diameter (mm) of the isolates in the plates amended with drugs.

Determination of minimum inhibitory Concentrations (MICs) of *Secale Cornutum by* broth microdilution method

Spore suspension of the test isolates grown on PDA slants was prepared in the range of 0.4×10^4 - 5×10^4 spores in sterile distilled water. The evaluation of MICs of the dilutions of various potencies of *Secale Cornutum* was done on RPMI - 1640 media as per the guidelines outlined by the Clinical and Laboratory Standard Institute (CLSI) [19] with minor variations (Table 2). Exactly, 100 µL of each of the appropriate drug dilutions was aseptically pipetted into a sterile U-bottomed 96-well microtiter plate. Subsequently, 100 µL of the spore suspension was added to the wells. For growth control and sterility control, 100 µL RPMI-1640 medium & 100 µL inoculum and 100 µL RPMI-1640 medium & 100 µL water, respectively were used. The plates were incubated at room temperature for 48 h and growth in each well was compared with that of the respective growth control with the aid of a reading mirror. The lowest drug concentration that exhibited the complete growth inhibition of the fungal isolate was considered the MIC endpoint for the respective potency of *Secale Cornutum.*

Table 2: Dilution scheme of *Secale Cornutum* 10 M for the determination of minimum inhibitory concentrations against ocular fungal isolates

Dilution Tubes	RPMI 1640 medium (mL)	Drug (mL)	Drug concentration (*Secale Cornutum* 10 M)
Tube 1 (T1)	0	2	$1/10^{20,000}$
Tube 2 (T2)	1	1 from T_1	$0.5/10^{20,000}$
Tube 3 (T3)	1	1 from T_2	$0.25/10^{20,000}$
Tube 4 (T4)	1	1 from T_3	$0.125/10^{20,000}$
Tube 5 (T5)	1	1 from T_4	$0.0625/10^{20,000}$
Tube 6 (T6)	1	1 from T_5	$0.0312/10^{20,000}$
Tube 7 (T7)	1	1 from T_6	$0.0156/10^{20,000}$
Tube 8 (T8)	1	1 from T_7	$0.0078/10^{20,000}$
Tube 9 (T9)	1	1 from T_8	$0.0039/10^{20,000}$
Tube 10 (T10)	1	1 from T_9	$0.0019/10^{20,000}$

Minimum Fungicidal Concentrations (MFC) of *Secale Cornutum*

A loopful of contents from the wells after the MIC well of the previous experiment was inoculated onto the SDA plates and incubated at 25˚C for 72 h. The concentration of the drug that showed no growth was recorded as the MFC value of the drug.

Results

The study involved 35 samples of corneal scrapings taken from keratomycoses patients between October 2012 and January 2013. The microbiological processing of these samples yielded a total of 20 isolates of molds. The fungal genera were identified based on the growth pattern on PDA, potassium hydroxide wet mount and lactophenol cotton blue wet mount. Of the 20 isolates identified, 6 and 5 were *Aspergillus flavus* and *Fusarium* spp., respectively and three isolates each of *Bipolaris* spp., *Exserohilum* spp. and *Curvularia* spp.

For plate assay, the radial diameter (mm) of colonies was measured and was used for the calculation of growth inhibition (%), statistical analysis and comparisons (Tables 2 and 3). From the results of the plate assay, it is evident that all the potencies of *Secale Cornutum* analysed had an inhibitory effect on the test isolates except *Secale Cornutum* Q which could not inhibit the growth of *Exserohilum* sp. ES2. *Secale Cornutum* 30 CH was found to inhibit *Fusarium* isolates (93.04 % for *Fusarium* sp. FS2) effectively when compared to other potencies. *Secale Cornutum* 200 CH, 1M and 10 M were highly effective against *Bipolaris* spp. (≥ 88.53% growth inhibition).

Similarly, *Secale Cornutum* 10 M inhibited *Exserohilum* sp. (82.87 % to 97.50 %) and *Curvularia* sp. (61.96 % to 85.29 %) to a larger extent. *Secale Cornutum* 30 CH, 200 CH and 10 M had inhibitory activity on *Fusarium* spp. *Aspergillus flavus* isolates were found to be least inhibited by all the potencies. Notably, *Secale Cornutum* 1M could inhibit 72.77 % growth of *Aspergillus flavus* AS 5. Of all the potencies tested, *Secale Cornutum* 10 M had a good inhibitory effect on the test fungal isolates (Table 4). Except, for *Secale Cornutum Q*, all the other potencies tested showed significant ($P<.05$) growth inhibition.

Based on the results of the plate assay, *Secale Cornutum* 30C for *Fusarium* spp. (FS) and *Secale Cornutum* 10M for *Exserohilum* spp. were used for the MIC and MFC determinations.

The MICs of *Secale Cornutum* 30C were $0.25/10^{60}$, $0.125/10^{60}$ and $0.0625/10^{60}$ for FS2, FS3 & FS4, FS1 and FS5, respectively. The MFC of *Secale Cornutum* 30C was $0.5/10^{60}$ and $0.25/10^{60}$ for FS2, FS3 & FS4 and FS1 & FS5, respectively. The MIC and MFC of *Secale Cornutum* 10 M were $0.25/10^{20000}$ and $0.5/10^{20000}$, respectively for all the isolates of *Exserohilum* spp. (Table 5).

Discussion

The efficacy of *Secale Cornutum* against 20 fungal isolates from mycotic keratitis was evaluated using plate assay and broth microdilution assay. The results suggested that *Secale Cornutum* 10 M had a commendable inhibitory effect on the test fungal isolates *in vitro*. *Secale Cornutum* Q exhibited poor inhibitory activity against the isolates tested, whereas all the other potencies tested showed significant ($p<0.05$) growth inhibition. The MICs of *Secale Cornutum* 30 C ranged between $0.5/10^{60}$ and $0.25/10^{60}$ for *Fusarium* isolates. The MFC of *Secale Cornutum* 10 M was $0.5/10^{20000}$ for all the isolates of *Exserohilum* spp

Table 3: Effect of *Secale Cornutum* on the radial growth of ocular fungal isolates as evinced by plate assay

Test fungus	*Secale* Cor Q	*Secale* Cor 30 CH	*Secale* Cor 200CH	*Secale* Cor 1M	*Secale* Cor 10M
Bipolaris sp. BS1	26.61%	73.24%	88.53%	96.94%	92.35%
Bipolaris sp. BS2	24.31%	67.13%	83.18%	94.65%	90.06%
Bipolaris sp. BS3	78.80%	97.81%	97.81%	97.81%	97.81%
Exserohilum sp.ES1	26.67%	55.83%	62.50%	38.33%	97.50%
Exserohilum sp.ES2	0.00%	34.64%	49.67%	32.68%	83.01%
Exserohilum sp.ES3	20.95%	47.30%	60.47%	45.32%	82.87%
Curvularia sp.CS1	21.92%	31.93%	57.96%	50.95%	61.96%
Curvularia sp. CS2	35.56%	55.56%	71.11%	54.44%	70.00%
Curvularia sp. CS3	53.92%	88.24%	88.24%	55.88%	85.29%
Fusarium sp. FS1	27.44%	60.04%	63.20%	36.91%	61.09%
Fusarium sp. FS2	91.30%	93.04%	44.30%	42.56%	44.30%
Fusarium sp. FS3	33.93%	44.94%	49.95%	24.92%	51.95%
Fusarium sp. FS4	37.06%	67.20%	45.04%	34.40%	41.49%
Fusarium sp. FS5	5.26%	3.51%	85.96%	10.53%	84.21%
Aspergillus flavus AS1	38.41%	52.90%	34.78%	45.65%	63.77%
Aspergillus flavus AS2	46.43%	44.05%	46.43%	44.05%	20.24%
Aspergillus flavus AS3	39.62%	59.47%	38.79%	22.25%	42.10%
Aspergillus flavus AS4	10.36%	47.71%	15.03%	50.51%	34.64%
Aspergillus flavus AS5	12.91%	49.95%	14.91%	72.97%	27.93%
Aspergillus flavus AS6	24.85%	51.37%	30.15%	55.79%	37.22%

Table 4: Evaluation of *in vitro* efficacy of homeopathic preparation of *Secale Cornutum* against molds isolated from keratomycoses by paired samples t-test

Homeopathic Medicine	Genera	*df*	Mean		mean diff	*t stat*	Sig
			Growth control	Treated Plates			
Sec Cor Q	*Bipolaris* spp.	2	4.43	2.49	-1.94	2.34	ns
Sec Cor Q	*Exserohilum* spp.	2	4.72	4.13	-0.59	1.23	ns
Sec Cor Q	*Curvularia* spp.	2	3.24	2.03	-1.21	3.71	**
Sec Cor Q	*Fusarium* spp.	4	3.2	1.8	-1.4	2.46	**
Sec Cor Q	*Aspergillus flavus*	5	3.38	2.43	-0.95	4.83	**
Sec Cor 30 CH	*Bipolaris* spp.	2	4.43	0.9	-3.53	7.46	**
Sec Cor 30 CH	*Exserohilum* spp.	2	4.72	2.59	-2.13	11.34	**
Sec Cor 30 CH	*Curvularia* spp.	2	3.24	1.33	-1.91	3.34	**
Sec Cor 30 CH	*Fusarium* spp.	4	3.2	1.29	-1.91	3.31	**
Sec Cor 30 CH	*Aspergillus flavus*	5	3.38	1.64	-1.73	10.53	**
Sec Cor 200 CH	*Bipolaris* spp.	2	4.43	0.9	-3.53	7.46	**
Sec Cor 200 CH	*Exserohilum* spp.	2	4.72	2.02	-2.7	14.87	**
Sec Cor 200 CH	*Curvularia* spp.	2	3.24	0.89	-2.35	7.18	**
Sec Cor 200 CH	*Fusarium* spp.	4	3.2	1.46	-1.74	25.81	**
Sec Cor 200 CH	*Aspergillus flavus*	5	3.38	2.38	-1	5.78	**
Sec Cor 1M	*Bipolaris* spp.	2	4.43	0.16	-4.27	43.25	**
Sec Cor 1M	*Exserohilum* spp.	2	4.72	2.89	-1.83	7.82	**
Sec Cor 1M	*Curvularia* spp.	2	3.24	1.5	-1.74	21.67	**
Sec Cor 1M	*Fusarium* spp.	4	3.2	2.17	-1.02	4.23	**
Sec Cor 1M	*Aspergillus flavus*	5	3.38	1.76	-1.62	6.76	**
Sec Cor 10M	*Bipolaris* spp.	2	4.43	0.29	-4.14	25.28	**
Sec Cor 10M	*Exserohilum* spp.	2	4.72	0.61	-4.11	39.1	**
Sec Cor 10M	*Curvularia* spp.	2	3.24	0.89	-2.35	8.62	**
Sec Cor 10M	*Fusarium* spp.	4	3.2	1.49	-1.7	25.93	**
Sec Cor 10M	*Aspergillus flavus*		3.38	2.11	-1.27	6.76	**

The measurements furnished here are the means radial growth diameter (mm) on triplicate plates. (Significance indications - - $p<.05$, - $p<.01$, ns - not significant).

Table 5: Evaluation of the effectiveness of homeopathic preparation of *Secale Cornutum* based on 75%-100% growth inhibition of fungal isolates from keratomycoses

***Secale Cornutum* potencies**	Fungal isolates inhibited	No. isolates inhibited (% of 20)
Secale Cor Q	*Bipolaris* sp. - BS3, *Fusarium* sp. -FS2	2 (10%)
Secale Cor 30 C	*Curvularia* sp. - CS3, *Bipolaris* sp. BS3, *Fusarium* sp. -FS2	3 (15%)
Secale Cor 200 C	*Curvularia* sp. - CS3, *Bipolaris* sp. - BS1, BS2, BS3, *Fusarium* sp. -FS5	5 (25%)
Secale Cor 1M	*Bipolaris* sp. - BS1, BS2, BS3	3 (15%)
Secale Cor 10M	*Curvularia* sp. - CS3, *Exserohilum* sp. - ES1, ES2, ES3 *Bipolaris* sp. -BS1, BS2, BS3, *Fusarium* sp.- FS5	8 (40%)

Table 6: Susceptibility of ocular fungal isolates against various potencies of *Secale Cornutum*

Medicines	MIC	Isolates	MFC	Isolates
Secale Cor 10M	$0.25/10^{20000}$ (3rd dilution)	ES1, ES2, ES3	$0.5/10^{20,000}$ (2nd dilution)	ES1, ES2, ES3
Secale Cor 30	$0.25/10^{60}$ (3rd dilution)	FS3, FS4, FS2	$0.5/10^{60}$ (2nd dilution)	FS3, FS4, FS2
Secale Cor 30	$0.125/10^{60}$ (4th dilution)	FS1	$0.25/10^{60}$ (3rd dilution)	FS1, FS5
Secale Cor 30	$0.0625/10^{60}$ (4th dilution)	FS5	-	-

Fungal keratitis/keratomycosis / mycotic keratitis has been identified as an important cause of blindness in Asian countries and molds have been recorded as the causative agents of 30% to 40% of keratitis. [3] In India, nearly 30-35% of culture-positive corneal ulcers are due to molds. [19] Fungal infections of the eye are usually treated with antifungal agents which are polyenes, 5-fluorocytosines, azoles, echinocandins, and miscellaneous tropical & oral antifungal agents. The clinical management of mycotic keratitis remains a therapeutic challenge for ophthalmologists due to various factors like the emergence of resistance in fungal species against available antifungals, relative unavailability of effective antifungals and unaffordability of the public to current surgical therapy (keratoplasty, enucleation, evisceration *etc*.). Evidence of *in vitro* antifungal resistance due to the commonly used antifungals has been widely reported. [20-23] the present scenario is on the high-time need for new broad-spectrum antifungals which are not only fungistatic but also furthering the host defence mechanisms to eradicate mycotic infections. Therefore, it is inevitable to search for safe and suitable alternative antifungal agents. The main advantage of homeopathic drugs over conventional antifungal agents is that they are prepared from natural resources and hence when used for therapy will not cause any complicacy. Homeopathic medicines are being used to treat ocular infections/disorders without any side effects.

C. purpurea produces a variety of ergot alkaloids belonging to ergoline and clavinet types with extensive biological activities. [24] According to Majrashi *et al.* [25] the ergot derivatives display intriguing pharmacokinetic and pharmacodynamic properties. The synthesis of pharmaceutically interesting ergot alkaloids, tri-or tetra-cyclic derivatives of prenylated tryptophan by the sclerotia has contributed to the significance of this fungus [26]. Reports on antimicrobial activities of ergot alkaloids are scarce. However, Eich [27] reported that clavines inhibited the growth of bacterial isolates *viz*., *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and the fungal isolate, *Candida albicans*.

According to Arora and Arora [28] in homeopathic clinical practice, nosodes have an important and indispensable part. *Secale Cornutum* is one such nosode. Reports on *Secale Cornutum* for the management of fungal infections are rare. The *in vitro* analysis has proved that the dilutions of *Secale Cornutum* have a significant effect on the inhibition of the growth of molds causing keratitis. Jurj [29] has demonstrated the efficacy of *Secale Cornutum*, a rarely used homeopathic remedy for non-infectious dermatological disease, psoriasis, in a case resistant to conventional dermatological topical applications.

Antifungal susceptibility testing is developed to predict antifungal drug resistance of the fungal species to the available drugs. Also, antifungal susceptibility testing might aid in timely & successful intervention on the control, and clinical management of a wide variety of ocular fungal pathogens (*Fusarium* spp., *Aspergillus* spp., *Acremonium*, *Candida* spp., *Curvularia* spp. *etc*.). *In vitro*, experiments are of extreme importance in comparative analysis to understand the homeopathic drug efficacy and its therapeutic potential. The experiments demonstrate mainly the chemical changes and possible advanced pathological changes concerning the drugs; therefore, this would be a proof of quality of a drug, or a method of analysis of the drug.

The *in vitro* susceptibilities of the tested isolates to various potencies and dilutions of *Secale Cornutum* as determined by the MICs of drugs may depend on the inoculum size, composition of the medium used for the susceptibility testing, the nature of the biological materials used as inoculum, and the temperature and duration of growth, Nevertheless, this type of *in vitro* pharmacological evaluations are required to comprehend the potency and efficiency of the homoeopathic preparations against the molds involved in ocular infections. Furthermore, the *in vitro* studies give indications of the homeopathic drug's mode of action, potential targets on the pathogen as well as the inhibitory effects.

The current study proves that various potencies and dilutions of homeopathic preparation of *Secale Cornutum* have significant activity on the molds responsible for human keratomycoses and can be administered for the treatment of the same. The study also establishes that there is no relation between the potencies and inhibition of fungal growth by *Secale Cornutm*. The present study did not evaluate the mode of action of *Secale Cornutum* against the tested isolates. However, the results may help to establish a standard protocol for the analysis of the minimum inhibitory concentrations of homeopathic preparations against fungal isolates, which in turn would help in the fine-tuning of the drug preparation process as per the requirement for a particular infection.

Conclusion

In the present investigation, the inhibitory effect of different potencies of the homeopathic preparations of *Secale Cornutum* was evaluated against 20 filamentous fungi [*Fusarium* spp. (n=5), *Aspergillus flavus (n=6), Bipolaris* spp. (n=3), *Exserohilum* spp. (n=3) and *Curvularia* spp.(n=3)] isolated from keratomycoses using plate assay and minimum inhibitory concentration analysis. The results indicated that all the potencies of *Secale Cornutum*, except *Secale Cornutum Q* had a significant inhibitory effect on the growth of the test isolates. The MICs of *Secale Cornutum* 30C ranged between $0.5/10^{60}$ and $0.25/10^{60}$ for *Fusarium* isolates. The MFC of *Secale Cornutum* 10 M was $0.5/10^{20000}$ for all the isolates of *Exserohilum* spp.

The limitation of the present study is that the efficacy of *Secale Cornutum* was determined under standardised *in vitro* conditions. The mode of action of *Secale Cornutum* was not evaluated in this study and the variation in the efficacies of the potencies of *Seclae Cornutum*, and its effect *in vivo*, can be researched be analysed further. It could be concluded that homeopathic preparations of *Secale Cornutum* at different potencies and dilutions have significant antifungal activity against the molds involved in keratomycoses.

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