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Morpho-anatomical, Physicochemical and Anti-oxidant studies of *Saussurea hypoleuca* spreng root

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Natural medicines obtained from different sources have been consumed throughout the world for their valuable therapeutic effects. *Saussurea hypoleuca*, a medicinal plant root has been used as a liver tonic in native population of Baluchistan. The current study was premeditated to document morpho-anatomical, physicochemical and antioxidants assays which will be beneficial in future to ensure the safety, purity and biological efficacy of the plant root. Morpho-anatomical, physicochemical parameters were applied as a diagnostic tool for identification and standardization of the plant root. Antioxidants assays were done with 2, 2-diphenyl -1-picryl-hydrazyl (DPPH) and Ferric Reducing Power Assay (FRAP). Morpho-anatomical evaluation has depicted Xylem vessels, fibers, tracheids, starch granules, cork cells and trichomes. Fluorescence analysis under short (254nm) and long (365nm) ultraviolet wavelength exhibited fluorescent compounds. Phytochemical analysis revealed the presence of saponins, tannins, alkaloids, glycosides, polyphenols, flavonoids, carbohydrates, lipids and proteins. Estimation of total ash values (15.8%), water soluble ash (20.9%), acid insoluble ash (9.93%), sulphated ash (19.1%) and total moisture content (2.5%) were determined by physicochemical analysis. DPPH and FRAP has shown the antioxidant potential in all fractions of plant root. This article entitled the standardization, purity and biological efficacy of *Saussurea hypoleuca* root.

Keywords: Morpho-anatomical, Physicochemical, DPPH, FRAP, *Saussurea hypoleuca*.

INTRODUCTION

Medicinal plants have been playing a key role in improving the human health since ancient decades. The use of plant extracts in ethno medicines is still significant and almost more than half of the clinically administered drugs have been isolated either from natural products or their derivatives (Dar et al. 2017). These herbal medicines were often used in different countries for racial, ancient, spiritual and environmental reasons (Shakya, 2016). These medicines have also been formulated in the form of capsules,

tablets, syrups or given as fresh extract juices and teas. There are many motives to consume these herbs, as people consider them safe, secondly they have low cost and minimum side effects (Umair et al. 2017). Cognizance of these herbal medicines have increased the hassles of phytomedicines. Worth of these phytomedicines contingent upon their proper authentication and standard procedures, as the plants have been used to cure various infirmities. So there is a need to standardize these herbs due to the gambles of adulteration. Morpho-anatomical, physicochemical

are vital tool for illustrating of these herbs for imperiling them into clinical trials (Akbar et al. 2014).

Saussurea hypoleuca (Asteraceae) is locally known as Qust. The root has been consumed in herbal formulation for liver tonic. Extensive review on literature has shown that no phytoconstituents have been isolated from this plant root. Proximate analysis and in vitro biological assays have shown that root contains a lot of phytochemicals (Arshad and Ishtiaq, 2019). GC-MS analysis, anticancer and anti-inflammatory activities of crude extract have been investigated which directs that root holds strong pharmacological potential (Arshad et al. 2021). This study documents the standardization, purity and biological efficacy of the plant root for its future use.

MATERIALS AND METHODS

Plant collection

Plant roots have been collected from the mountains of Quetta, Baluchistan, Pakistan and verified by a taxonomist, Prof Dr. Zaheer Khan, Department of Botany, GC University Lahore, Pakistan under the voucher number GC.Herb.Bot.3453.

Solvent extraction and fractionation

The plant root was pulverized into powder, followed by triple extraction by using cold maceration at room temperature. Methanolic extract was made under reduced pressure by using rotary evaporator at 45-50°C. Fractionation of methanolic extract was conceded through different solvents of increasing polarity order including n-hexane, chloroform, ethyl acetate and n-butanol (El-Rafie and Sleem, 2016). These fractions were then dried and preserved in tightly closed container at refrigerator for pharmacological activities.

Microscopy and Micrometry

Microscopy was performed by passing through sieve No. 40 to obtain content uniformity. Plant root powder was subjected to slides with 2-3 drops of each different mounting medias such as chloral hydrate (50 g in 20 mL DW), chloral hydrate glycerin solution, phloroglucinol HCl and slides were covered with cover slip to examine under microscope and photographed (Mehlhorn 2011). Powder micrometry was accomplished by employing the sample on microscope stage and focus on each entity to be measured. Superimposed the ocular micrometer scale and

read off the dimensions of each entity. Multiply the number of scale divisions by the micrometer value to give the actual dimension in micrometers. Method was developed according to the WHO guidelines for quality control methods of medicinal plant material (Ishtiaq et al. 2018).

Scanning Electron Microscopy (SEM)

It produces three-dimensional image by passing a beam of intensive electrons over the sample in cathode ray tube and reading both the electrons scattered and produced by the test sample. SEM gives comprehensive and three dimensional representation of observed macrostructures by using electromagnetic lenses and focusing is carried out by moving the current to produce the object on photographic plates. All these diagnostic features provide well understanding of ultra-structures of the plant cells. It also reveals the spatial relations, unpredicted details and previously non- recognized features. In other words, the micrographs obtained by SEM, show the best possible structural details of the specimens (Robards 1970 and Heywood 1972). In SEM samples were fixed on the specimen stub using adhesive tape. Coated sample was analyzed in a JEOL scanning electron microscope operated at 15 KV and photographed.

Fluorescence Screening

Fluorescence screening was executed with standard procedures (Gayathri and Kiruba, 2015). It is a phenomenon in which many phytoconstituents fluorescence in visible range in day light and it is significant parameter for standardization of herbs. Root powder was treated with different acidic and basic solvents to observe simultaneously under UV-chamber having short and long wave length. The change in colour and appearance was documented.

Phytochemical Analysis

Extract and fractions were tested for various phytoconstituents as per documented procedures. Saponins were detected with foam and lead acetate test (El-Lemy *et al.*, 1994). Presence of flavonoids were determined with ammonia sulfuric acid, dilute ammonia and aluminum chloride (Harborne 1973). Alkaloids were screened with Mayer's and Dragendorff's reagents to observe the precipitations as mentioned by (Brain and Turner 1975). Tannins were detected with ferric chloride test and bromine water test as reported by (Ali 1998). Triterpenoids and anthraquinone

glycosides were screened as reported by (Sofowora 2008; Evans 2002) respectively.

Physicochemical analysis

Physicochemical analysis was performed by adopting the standard protocol and procedures as written in WHO guidelines quality control methods for herbal medicines (Abere and Okpalaonyagu, 2015). Ash contents determination is important to identify the quality and purity of the powder. It also helpful to determine the presence of sodium, potassium, chloride and other minerals. Total ash was determined by igniting the powder in crucible furnace at 675°C until it turned into white, the resultant powder was desiccated and weighed. % age of total ash was determined in accordance to dried sample weight. Acid insoluble ash was calculated by heating a part of total ash with 25 mL hydrochloric acid followed by collecting, washing on ashless filter paper, cooling in desiccator and weighing. Similarly water soluble ash was determined from total ash by solubilizing in water. Sulphated ash was also determined by igniting powder with sulphuric acid under fume hood to observe the white fumes followed by cooling and weighing the powder. Extractive values of root powder in different solvents were estimated by weighing 2 gm of sample macerated with solvents of increasing polarity and stirred on mechanical shaker for six hours. Extracts were filtered, concentrated to dryness and weighed. Total ash and extractive values were recorded according to reported protocols (WHO guidelines 2002). Moisture contents in root powder were determined by subjecting the sample in hot oven at 105°C. Dryness was sustained until two successive weights did not diverge by 5 mg (WHO guidelines 2002).

Antioxidant Activity

DPPH Assay

Antioxidant activity of root methanolic extract (RME) and fractions was executed by using DPPH as explained by (Verru et al. 2009). Stock solutions (1 mg/mL) of RME and fractions were prepared in methanol served as control. Different concentrations (50, 100, 150, 200 & 250 µg/mL) of RME, fractions and ascorbic acid (standard compound) were made. All samples and standard were incubated at RT for 30 minutes. UV spectrophotometer was operated at 517 nm for absorbance. Assay was repeated in triplicates and decrease in absorbance was recorded. Following

equation was used to determine the percentage of inhibition.

$$\%inhibition = \frac{Abs(control) - Abs(sample)}{Abs(control)} * 100$$

1C50 of RME and fractions was calculated having antioxidant potential.

FRAP Assay

Antioxidant potential of RME and fractions was assessed by FRAP as reported in (Yen *et al.*, 2000). Different concentrations (25, 50, 100, 200, 400, 800 & 1000 µg/mL) of standard, RME and fractions were dissolved in 1 mL of distilled water up to 1000 µg/mL final concentrations. 2.5 mL of sodium phosphate buffer (7.4 pH) and 2.5 mL of 1% potassium ferricyanide were added in test tubes. Incubate the samples at 50°C for 20 minutes. 2.5 mL of 10% trichloroacetic acid was added in test tube after incubation and centrifuge them at 2700 rpm for 10 minutes. After centrifuge, a supernatant (2.5 mL) from all test samples was taken, followed by addition of 0.5 mL 1% ferric chloride and 2.5 mL of distilled water into this system. Absorbance was recorded at 760 nm by UV spectrophotometer. Increased in reducing power was determined by higher absorbance of the sample (Chen et al. 2007).

Statistical analysis

All results are expressed as mean ± SD.

RESULTS

Powder Microscopy and Micrometry

Powder microscopy and micrometry has revealed various structures under light microscope. Details of microscopic analysis and micrometry are depicted in Fig.1 (1-20). This study elaborated the characteristic features of powder and dimensions of various microscopic structures.

SEM

A large number of information about the structures were obtained by employing SEM. It plays vibrant role to find the structures which were not defined previously. Further determination of these microstructures generate data which would be helpful in relation to plant family and phytochemicals. Photomicrographs of root were taken at 20 µm, 10 µm and 5 µm while powder were at 50 µm, 20 µm and 10 µm as given in Fig.2 respectively.

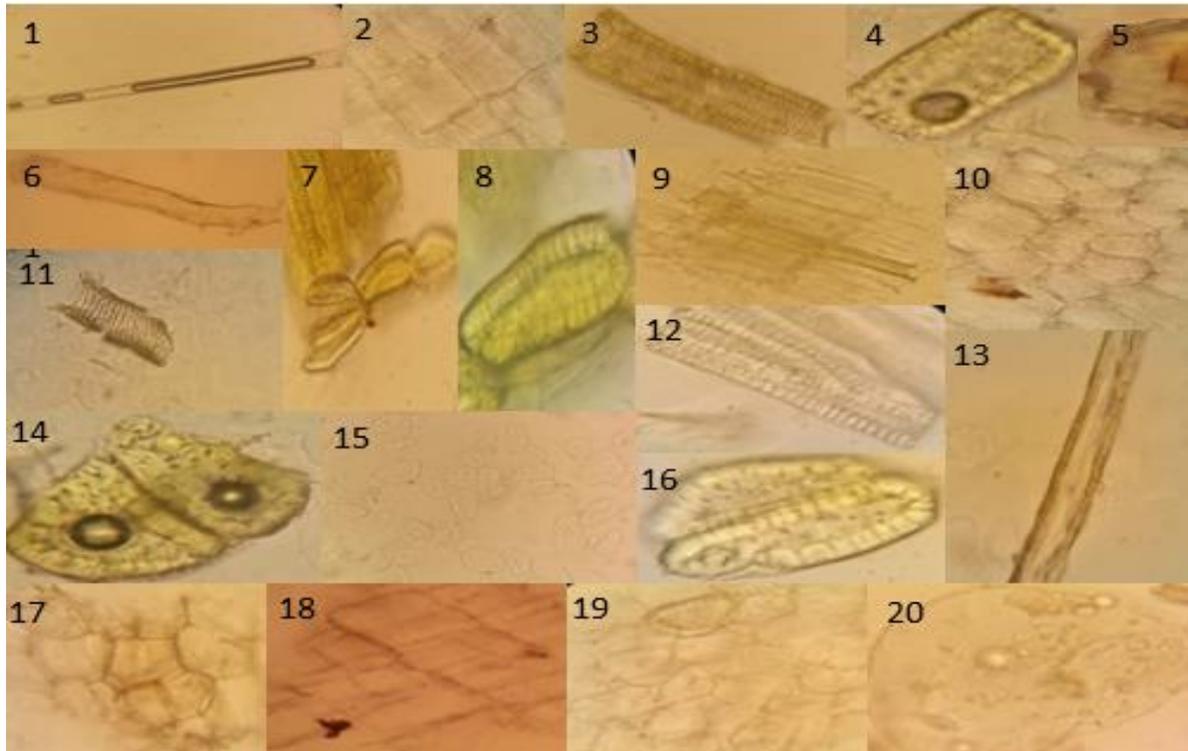


Figure 1: Powder Microscopy and Micrometry of *S. hypoleuca* Root

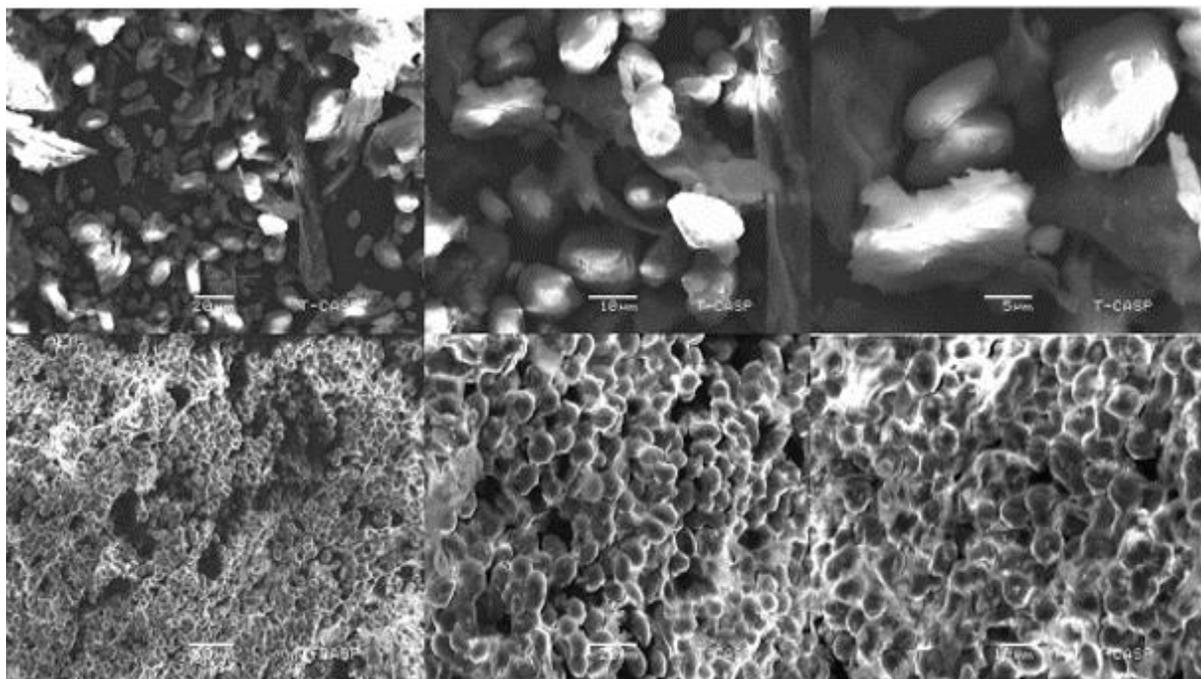


Figure 2: SEM of *S. hypoleuca* Root

Fluorescence Screening

Root powder was macerated with various chemicals and reagents to determine the presence of fluorescent compounds under day light and UV light. The results of fluorescence analysis are compiled in Table 1.

Phytochemical Screening

Qualitative phytochemical screening was performed according to the USP standards to determine the existence of numerous phytochemicals in RME and fractions. The verdicts are presented in Table 2.

Table 1: Fluorescence Analysis of *S. hypoleuca* Root Powder

Protocol	Ordinary light	Short wavelength (254nm)	Long wavelength (365nm)
PRP+50%HNO ₃	Reddish brown	Lemon green	Pale yellow
PRP + Dil HCl	Light brown	Light green	Sandy colour
PRP + 66%H ₂ SO ₄	Black soil	Blackish green	Dark purple
PRP +50% H ₂ SO ₄	Brownish black	Green	Dark black
PRP + 5%NaOH	Chocolate brown	Milky green	Bluish green
PRP +5% FeCl ₃	Pale yellow	Yellowish green	Dark brown
PRP + 50% KOH	Yellowish brown	Light green	Dark green
PRP + Methanol	Pale yellow	Light green	Milky white
PRP + Iodine solution	Reddish brown	Dark green	Blackish green
PRP + Aniline	Dark red	Dark brown	Dark purple
PRP + Chloroform	Turbid	Light green	Milky white
PRP +Water	Turbid brown	Green	Blue
PRP	Brown	Light green	Dark green

Table 2: Phytochemical Screening of extract and Fractions of *S. hypoleuca*

Tests	RME	CF	n-BF	E.AF	n-HF	AF
Protein test						
Ninhydrin test	-	-	+	-	-	+
Million's test	-	+	+	-	+	+
Carbohydrates						
Molish test	+++	+++	+++	+++	+++	++
Benedict's test	+++	++	-	-	+	++
Glycosides						
Keller Killani test	++	++	+	-	+++	++
Legal's test	++	++	++	++	+++	+
Tannins						
Ferric chloride	-	-	-	-	-	-
Bromine water	+	+++	++	+++	++	++
Fat and fixed oil	+	+	-	+	+	-
Saponins						
Foam test	-	-	-	-	-	-
Flavonoids						
Lead acetate	+++	+++	+	+	-	-
Alkaline reagent	+++	-	+	+	-	-
Alkaloids						
Hager's reagent	+	+	++	+++	++	+
Wagner's reagent	+	+	++	++	++	+++
Dragendroff's t	+	++	++	++	+++	++
Mayer's test	-	+	++	++	+	-
Terpenoids						
Salkovaski	+	++	+	+	+++	+++
Lieberman Burchard test	+	++	+	+	+++	+++
+ = present, - = absent						

Physicochemical properties

Physicochemical properties including total ash, acid insoluble, water soluble, sulphated ash and moisture contents of the test plant root powder are outlined in Table 3.

Table 3: Physicochemical Properties of *S. hypoleuca* Powder

Parameters	Contents (% W/W)
Total ash	15.8 ± 0.35
Acid insoluble ash	9.93 ± 0.15
Water soluble ash	20.9 ± 0.15
Sulphated ash	19.1 ± 0.25
Moisture contents	2.5 ± 0.2

Extractives Values

Extractives values of PRP in various solvents are summarized in Table 4.

Table 4: Determination of Extractive Values of *S. hypoleuca* Root

Parameters	Values (%W/W) Mean ± SD
RME	8.13 ± 0.31
CF	0.96 ± 0.05
n-BF	4.00 ± 0.1
n-HF	0.96 ± 0.05
E.AF	2.01 ± 0.02
AF	23.9 ± 0.15

Antioxidant Activity

DPPH

DPPH is a rapid method for in vitro estimation of

antioxidant potential of the plant extract and fractions. Discoloration of DPPH solution is correlated to the antioxidant potential in the examined sample. Percentage inhibition was calculated for samples as well as for standard. n-HF has highest percentage inhibition and very minor IC 50 values which was evaluated of each fractions of the test plant given in Table 5. All these verdicts revealed that all fractions of plant root possess strong and comparable antioxidant activity.

FRAP

FRAP was also used to study the antioxidant potential of extracts and fractions when compared with standard (A.A). RME has significant antioxidant potential ($p < 0.005$) at all concentrations as compared to others fractions as presented in Fig.3.

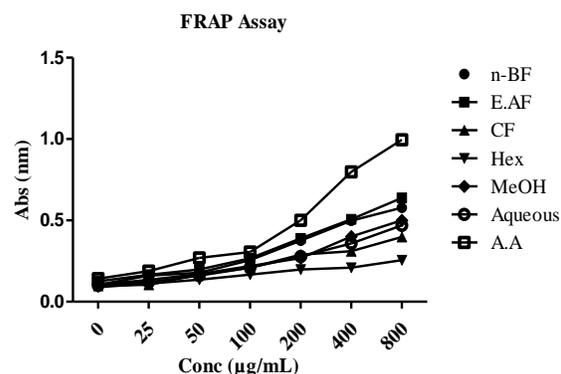


Figure 3: FRAP Assay of RME and Fractions of *S. hypoleuca*

Table 5: IC 50 Values of Extracts and Fractions of *Saussurea hypoleuca* root

Samples Tested	Concentrations (mg/mL)	% Inhibition Mean ± SD	IC ₅₀ (µg/mL)
E.AF	1	44.84 ± 17.7	173.1
n-BF	1	47.44 ± 18.21	161.16
CF	1	48.91 ± 17.97	154.83
RME	1	55.31 ± 17.62	126.03
n-HF	1	68.29 ± 14.35	47.18
AF	1	55.85 ± 15.95	120.94
A.A	1	57.56 ± 13.50	105.26

Results are presented as Mean ± SD. A.A (Ascorbic acid) served as standard.

DISCUSSION

Natural products have gained a huge importance due to their therapeutic values. Pakistan is rich with this floral biodiversity and a large number of people use these medicinal herbs because of low cost and minimum side effects. However, rational use of these herbs relies on the phytochemical screening and Pharmacognostic evaluations because these are important tools for the quality assurance and identification of these herbs. The root of *Saussurea* have been used in Ayurvedic medicines for the treatment of different diseases (Madhuri et al. 2012). There is no scientific evidence available to develop their real therapeutic values. The present study on plant root was conducted to strengthen its therapeutic importance in modern medicine and to standardize it.

Pharmacognostic investigations are simplest and easiest process for the authentication and identification of material from natural origin. Macroscopic together with microscopic study gives a complete and comprehensive detail that is helpful to distinguish the plant from other closely related species within the same genus (Thirumalai et al. 2013). Powder microscopy of root was performed which showed characteristic structures; cork cells, parenchymatous tissues, fibers, sclerides and vessels. Starch granules were observed which depicts the nutritional value of this herb (Ishtiaq et al. 2018). Parenchymatous cells store food and photosynthesis, fibers provide mechanical strength, cork cell give protection, vessels and tracheids are important in the transport system of the plant (Singh et al. 2018).

Extractive potential of alcohol is more than water for plant root powder, as it is revealing from the presence of alcohol soluble phytoconstituents such as glycosides, phenols and flavonoids, etc. Methanol and ethanol are best solvents for the extraction of different constituents from plant material (Rizwan et al. 2012) because they penetrate the plant cell wall and infiltrate the internal phytoconstituents. Mainly, methanol is an important solvent to isolate the flavonoid and phenolic components and the present results also strength this suggestion. These data would be supportive for determination of purity and identification of the genuine sample of the plants.

UV fluorescence analysis is also a good stricture for evaluations of herb. Different colors were observed indicating the presence of florescent compounds (Joshi, 2012).

Physicochemical factor further provides superfluous support for the authentication and

standardization of test plant which showed that the care has been done during collection, drying and storage of the plant. Total ash values, water soluble, acid insoluble and extractive values indicate the existence of inorganic compounds like carbohydrates, phosphorous or other added adulterants. These all outcomes obtained should be within specified official limits. Moisture contents also assumes the greater stability and quality control of the herb material. The standard moisture content value in the crude powder has been suggested to be less than 14% (United States Pharmacopoeia, 2009). It should be within official limits to avoids the decomposition of the phytoconstituents as well as to prevent the microbial attack.

CONCLUSION

Plant derived medicines have recently gained great interest owing to their versatile benefits and minimum side effects. Medicinal plants are great resources of traditional medicines, modern medicines, pharmaceutical, nutraceutical, food, folklore medicines and chemical entities of synthetic drugs. Furthermore, the presence of active ingredients offer confidence to the use of plant for the treatment of diseases. However, plant always used as catalyst for healing. Using plant as stimulus of innovative drugs provides infusion for novel substances. In current research work Pharmacognostic, phytochemical and physicochemical properties of the *Sasussurea hypoleuca* root extracts have been screened which have a potential for the source of novel drug against ailments. The results are very auspicious but scientific authentication is necessary before putting into modern practice.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NA wrote the manuscript and performed the experimental work, SI rechecked the manuscript and designed the experimental work. SA and SR helped in anatomical study AM and SR assisted in micrometry and SEM, SR, UR and AR assisted throughout the experimental as well as theoretical work.

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