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RESEARCH ARTICLE

Simultaneous Determination of Tannic Acid and Eugenol in Newly Formulated Churna of Tulsi and Parijat

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Abstract

Herbal formulations are widely used to treat a variety of diseases with the use of natural ingredients and have the additional advantage of negligible side effects. Looking towards the wide applicability of herbal formulations, a novel herbal formulation containing Tulsi and Parijat has been formulated and evaluated for different standardisation parameters. Tulsi and Parijat both possess multiple natural effects like antibacterial, antiviral and antipyretic properties along with wide applicability to treat different respiratory problems. Therefore, the combination of these two naturally available herbs can provide good immunomodulatory action for humans. Apart from the various phytoconstituents present in these two herbs, eugenol and tannic acid are found to have versatile effects. They are widely used to treat various common health issues like tonsillitis and various respiratory complaints like cough, cold and sour throat. A successful attempt has been made to develop the HPTLC method for the simultaneous determination of Tannic acid and Eugenol in pure and newly formulated churna containing Tulsi and Parijat as chief herbs. Tannic acid and eugenol mixtures get resolved by toluene:ethyl acetate:formic acid (5:3:2) with R_f of 0.849 ± 0.003 and 0.534 ± 0.009 respectively for tannic acid and eugenol. Linearity is observed within the range of 250–1500 ng/band for both eugenol and tannic acid. According to ICH Q2 R1 advice for analytical method validation, this designed technique has been effectively validated.

Keywords: Churna, Herbal Formulation, High-Performance Thin Layer Chromatography, Immunomodulators, Phytoconstituents, Standardization of Herbs

1. Introduction

1.1 Introduction to Herbal Formulations

Herbal medicines are in high demand in today's global healthcare system due to their low cost of treatment as well as their improved compatibility with the human body and fewer adverse effects[1]. A herb is a fresh or dried, powdered plant material that can be used either raw or after additional processing and preparation to create a completed herbal product, according to the World Health Organization (WHO). Steamed, roasted, decocted, squeezed, infused in water, extracted with alcohol or sweetened, and baked with honey are some of the methods used to create herbal products[2].

Various formulations containing herbs are available for the treatment of a variety of diseases. From a variety of formulations, churna is the most commonly available herbal or Ayurvedic formulation. Churna can be considered a conventional herbal formulation. Churna can be defined as dried raw material which is powdered very finely to produce a small size and again filtered through a cloth's grid to obtain fine powder[2]. A churna containing Tulsi and Parijat is formulated and evaluated successfully. Tulsi and Parijat, both have a wide range of therapeutic benefits, which makes them powerful herbs. Parijat can help with various respiratory problems such as tonsillitis, and asthma and has antiviral, antibacterial and immunostimulant properties[3]. Tulsi is an apoptogenic

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herb that helps in different diseases including respiratory problems and has antiviral, antibacterial and immunomodulatory properties. It is also useful in tonsillitis and fever[4]. These herbs provide such effects due to the presence of various chemical constituents. Tulsi and Parijat have different chemical constituents in their leaves that provide various therapeutic effects. Along with all the phytoconstituents of Tulsi and Parijat, the most commonly found and most widely used active phytoconstituents are eugenol and tannic acid. 2-methoxy-4-(prop-2-en-1-yl) phenol, which is liquid in nature, is the chemical compound that represents eugenol[5]. Similar to tannic acid, 3,4,5-trihydroxybenzoate is a solid that is chemically represented by [2,3-dihydroxy-5-[[(2R,3R,4S,5R,6S)-3,4,5,6-tetracids [[3,4-dihydroxy-5-(3,4,5 trihydroxy benzoyl) ox benzoyl] oxy] oxan-2-yl] methoxycarbonyl] phenyl[6]. Figures 1 and 2 show the structural formulas for eugenol and tannic acid, respectively.

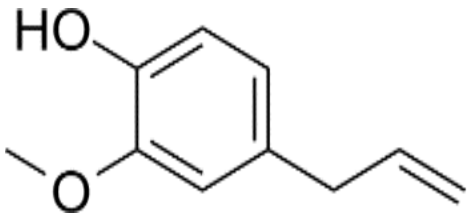

Figure 1. The structural formula for eugenol.

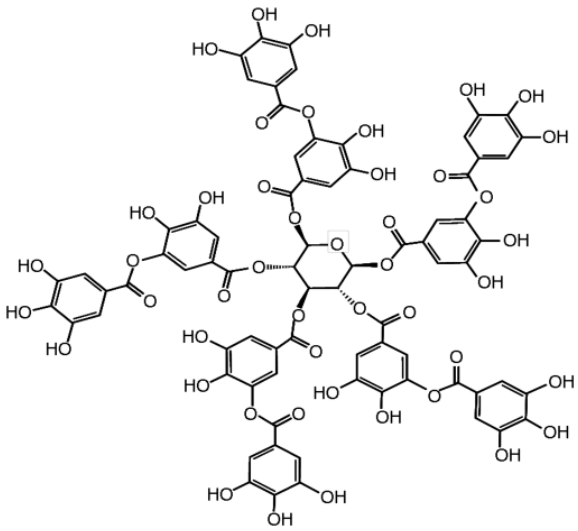

Figure 2. The structural formula for tannic acid.

For the simultaneous detection of eugenol and tannic acid from freshly produced Churna extracts containing the same, a high-performance liquid chromatographic technique has been chosen.

1.2 High-Performance Thin Layer Chromatography (HPTLC)

High-Performance Thin Layer Chromatography (HPTLC) is a more complex version of Thin Layer Chromatography (TLC) that delivers more effective chromatographic layers and makes use of high-end equipment throughout the entire process. The preferred method for identifying different phytoconstituents in herbal products is HPTLC[7-9]. The development and validation of an HPTLC method for the simultaneous determination of tannic acid and eugenol, the principal phytoconstituents of Tulsi and Parijat, respectively, were successful.

2. Materials and Method

2.1 Herbs and Phytoconstituents are combined with Chemicals and Reagents

Tulsi leaf powder was obtained from Harmony Life Science Pvt. Ltd., New Delhi, India. Parijat leaves were obtained from Kamdhenu Laboratories, Jaipur, India. Benched Enterprise Pvt. Ltd., Vadodara, Gujarat, India, supplied the phytoconstituents, eugenol and tannic acid. All of the chemicals and reagents used in developing and validating the method are of analytical grade.

2.2 Apparatus and Equipment

A Camag HPTLC system loaded with Vision CAT software of version 3.0.20196.1 along with a Linomate V sample applicator were used for HPTLC method development. The TLC scanner 4 S/N is attached to the equipment. Along with the HPTLC system, all the required glassware and apparatus were checked and calibrated before use.

2.3 Preparation of Herbal Formulation[10]

The Tulsi and Parijat churna is prepared in accordance with the Ayurvedic Pharmacopoeia. For both plants, a ratio of 6.5:3.5 is chosen for the proportions of Tulsi and Parijat. The standard powders of Tulsi and Parijat were weighed separately and sieved properly by passing them through sieve #85. The powder was dried completely and mixed as per the proportion described

above. The formulated Churna is represented in the following Figure 3.

Figure 3. Churna containing Tulsi and Parijat.

2.4 Evaluation (Standardisation) Methodology of Newly Formulated Herbal Churna[10]

The newly formulated churna is evaluated for various parameters as per the Ayurvedic Pharmacopoeia. The procedure for the same is described below:

2.4.1 Organoleptic Properties

To evaluate the Churna for fundamental qualities including colour, flavour, and aroma of freshly made churna.

2.4.2 The pH of the Newly Formulated Churna

By precisely weighing 1 g of churna powder and combining it with 100 ml of distilled water, the pH can be determined. After filtration, the pH of the final solution is measured with a pH meter.

2.4.3 Moisture Content (Loss on Drying)

For moisture content, the necessary amount of sample is obtained and dried to a consistent weight in a hot air oven at 105°C. The difference between the weights reveals the drug›s moisture content.

2.4.4 Ash Value

2.4.4.1 Total Ash

To determine the entire amount of ash, burn 2/3 gramme of precisely weighed medication in a silica dish at a temperature of no more than 450°C until it is carbon-free, then cool it and weigh it. Make a total ash calculation using the following equation:

%Total ash = Wt. of ash/Wt. of sample X 100

2.4.4.2 Acid Insoluble Ash

To determine how much ash is insoluble in acid, add 25ml of HCl to the total ash and boil for 5 minutes. Gather the insoluble material in the crucible, filter it using ashless filter paper, and then set it ablaze, chilly and heavy. Use the following formula to calculate acid-insoluble ash:

% Acid insoluble ash = Wt. of acid insoluble ash/Wt. of Total ash X 100

2.4.5 Extractive Value

2.4.5.1 Alcohol Soluble Extractive Value

For determination of alcohol soluble extractive value, macerate 5gm of the air-dried drug with alcohol for 24 hr in a closed flask. The flask should then be shaken frequently for 6 hours and left to stand for 18 hours. Filter the filtrate, evaporate it to dryness, and weigh it. Calculate the difference between the initial weight and the final weight of the extract.

2.4.5.2 Water Soluble Extractive Value

To quantify the extractive value that is water soluble, macerate 5 g of the air-dried medication with chloroform water for 24 hours in a closed flask. After that, shake the flask often for 6 hours, and then let it stand for 18 hours. Filter the filtrate, let it dry out, and then weigh it. Determine the difference between the extract's starting weight and ending weight.

2.4.6 Flow Properties

2.4.6.1 Bulk Density

The mass-to-volume ratio of the powder sample that was left untapped determines a substance's bulk density. The equation below can be used to determine bulk density.

Bulk density = Actual weight of powder (g)/Volume (mL) of powder in the cylinder

2.4.6.2 Tapped Density

Mechanically tampering with a graduated cylinder containing the sample until no longer detectable volume changes are observed. The represented value corresponds to the density that was tapped.

Tapped density = Actual powder weight (g)/Volume of powder in the cylinder (mL)

2.4.6.3 Carr's Index

The compressibility of a powder is indicated by Carr's index. Pharmaceuticals typically use Carr's index to determine a powder's flowability.

Carr's index = Bulk density – Tapped density/Tapped density

2.4.7 Hausner's Ratio

Hausner's ratio is a measurement of the flow strength of a powder or granular substance. It is the tapped bulk density of the powder. The value of Hausner's ratio fluctuates depending on how it was determined; hence, it is not thought of as an absolute attribute of a material. The following equation is used to determine the value of Hausner's ratio:

Hausner's ratio = Tapped density/Bulk density

2.4.8 Angle of Repose

The steepest angle of fall or dip that relates to the horizontal plane through which a material can be piled without collapsing is known as a material's angle of repose. The material at the slope face is almost ready to slide at this angle. The following equation can be used to determine the angle of repose:

$\theta = \tan^{-1}(h/r)$

Where, h = Height and r = Radius

2.5 HPTLC Method Development for Simultaneous Determination of Eugenol and Tannic Acid

2.5.1 The Standard Solutions are Made to Create the Calibration Curve

100mg of tannic acid and 0.1 ml of eugenol were accurately measured, respectively, for further dilution. Methanol was added to both samples separately to get the final concentration of both markers at 100µg/ml. A standard laboratory mixture was also prepared to contain eugenol and tannic acid as pure photomarkers.

2.5.2 TLC Method Development

For the optimization of the mobile phase, various TLC trials have been carried out, based on which the mobile phase and other chromatographic conditions are optimized for HPTLC method development and validation.

2.5.3 HPTLC Instrumentation and Experimental Conditions for Simultaneous Determination of Eugenol and Tannic Acid in Pure Form

Aluminium plates 60 F254 (20 × 10) that have been pre-coated with a 0.2mm-thick coating of silica gel (Germany's Merck, Darmstadt, Cat. No. 1.05548) were used. After being prewashed with methanol, the plates were activated at 60°C for 5 minutes before chromatographic operation. Spotter was an automated TLC sampler called Linomate V from Camag in Mittens, Switzerland, which was run with Win CATS software 1.3.3. The 7 mm bandwidth was maintained. 20 × 10 cm twin trough glass chambers (Camag, Muttenz, Switzerland) were utilised to generate the chromatograms. For the optimum mobile phase, the chamber saturation time was 20 minutes at room temperature (30°C 4) with a relative humidity of 22% 5. Toluene:ethyl acetate:formic acid (5:3:2) v/v/v was the ideal mobile phase for both eugenol and tannic acid. The mobile phase was 20 ml. The radiation came from a D2 lamp that continuously emitted ultraviolet radiation between 180 and 400 nm. The medicines were scanned on their respective wavelengths using the densitometric scanner 4 S/N (Camag, Muttenz, Switzerland). Scan speeds of 10 mm/s and slit widths of 6.0 mm × 0.45 mm were used. Detection was done at 281nm and 272nm for eugenol and tannic acid, respectively. Peak regions with linear regression were measured as part of the evaluation process for each plate.

2.6 HPTLC Method Validation[11,12]

2.6.1 Linearity

By spotting the five various concentrations of eugenol and tannic acid between 250 and 1500 ng/spot and 250 to 1500 ng/spot, respectively, the linearity was assessed. The calibration curve for area vs. concentration was drawn.

2.6.2 Precision

The developed method's intraday precision and interday precision were calculated as a percentage of RSD. For intraday precision, the experiments were run six times daily, and for interday precision, on six separate days. Six independent calculations were made to determine the concentration values for both intraday precision

and interday precision. The mean of percent relative standard deviation (% RSD) was then determined (% RSD = [S/X] 100, where S is the standard deviation and X is the mean of the sample under study).

2.6.3 Robustness of the Procedure

The impact on the outcomes was investigated by making slight adjustments to the solvent ratio. Toluene, ethyl acetate, and formic acid in the ratios of (5:3:2) v/v/v, (5:3.5:1.5) v/v/v, and (5.5:3.5:1) v/v/v were tried for both medicines, and their chromatograms were run separately. Before using the chromatographic technique, the plates were prewashed with methanol and activated at 60°C for 25, 27, and 29 minutes, respectively. Variable amounts of mobile phase were present overall. In mobile phases of 10, 15, and 20 ml, plates were produced. For robustness testing and RSD calculations, the times for spotting samples and scanning samples were also adjusted.

2.6.4 Limits of Quantification and Detection

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) of a blank solution (methanol) were estimated using the same procedure six times. Then, the signal-to-noise ratio was calculated. LOD was deemed to be 3:1 and LOQ to be 10:1.

2.6.5 Specificity

By examining the method's capacity to identify the relevant substance in Churna extract even when other components are present, the method's specificity was established. The methanolic extracts of churna, eugenol and tannic acid could be analysed using this technique. By contrasting the Rf values and spectrum of the bands with the standards, the band was identified. By comparing the spectra at three different levels, namely the peak start (S), peak apex (M), and peak end (E) positions of the spot, the peak purity was determined.

2.6.6 Recovery Research (Accuracy)

Recovery studies were used to evaluate the accuracy of the procedure and the interference from excipients. Studies on recovery were carried out using the conventional addition approach. This study was conducted by adding a known concentration of the pre-analyzed churna to known amounts of standard eugenol and tannic acid mixture. Tannic acid and standard eugenol recovery percentages were computed.

2.6.7 System Suitability

The repeatability of the suggested method was used to evaluate the system's appropriateness six times. After inspecting the area, %RSD was calculated.

3. Results

3.1 Evaluation Parameters of Newly Formulated Herbal Churna

The newly created churna that contained Tulsi and Parijat was satisfactorily examined using the Ayurvedic Pharmacopoeia's standard approach. Table 1 lists the findings of various organoleptic examinations of newly designed Churna, and Table 2 lists the findings of various evaluation parameters of newly formulated Churna together with their standard limitations.

Table 1. Organoleptic parameters of newly formulated churna of Tulsi and Parijat

Organoleptic Parameters	Result
Appearance	Powder
Colour	Green
Odour	Characteristics
Taste	Sour

3.2 Development of an HPTLC Method for the Simultaneous Measurement of Eugenol and Tannic Acid in Fresh and Purified Churna

To create the HPTLC method, the TLC method was first optimized. Toluene, ethyl acetate, and formic acid in the ratio of 5:3:2 v/v/v were determined to give adequate migration of the compounds on the created TLC plates out of a number of permutations. As a result, when both eugenol and tannic acid were evaluated simultaneously on distinct plates, this solvent solution was determined to be appropriate. Tannic acid's measured Rf values were 0.849 0.003 and eugenols were 0.534 0.009 when the aforementioned mobile phase was used. Another attempt at developing an HPTLC method was made with the same optimised

Table 2. Results of various evaluation parameters of newly formulated churna

Sr. No.	Parameters	Observed Value	Standard Value
1	pH	6.75 ± 1.114	5.8-8
2	Moisture Content	75. 8 ± 0.942%	-
3	**Extractive Value**		
	Water soluble extractive value	29.33 ± 0.874%	NLT 20%
	Alcohol soluble extractive value	36 ± 0.741%	NLT 10%
4	**Ash Value**		
	Total Ash	1.54 ± 0.277%	Not more than 10%
	Acid insoluble ash	0.65 ± 0.749%	Not more than 1.5%
5	**Flow properties**		
	Bulk Density	0.25 ± 1.314	0.18 – 1.25
	Tapped Density	0.31 ± 0.479	0.6 – 0.9
	Hausner's Ratio	1.14 ± 0.847	1-1.11- Excellent
			1.12- 1.18- Good
			1.19- 1.25- Fair
			21-25- Passable
			26-31-Poor
	Carr's Index	14.16 ± 0.368	<10 – Excellent
			11-15- Good
			16-20- Fair
			21-25- Passable
			26-31- Poor
	Angle of repose	34.86 ± 0.914	25-30- Excellent
			31-35- Good
			36-40- Fair

[a] - Represents the mean values of three determinations.

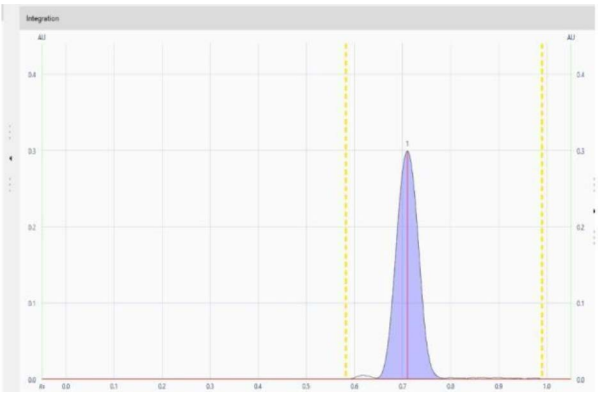

Figure 4. HPTLC chromatogram of eugenol.

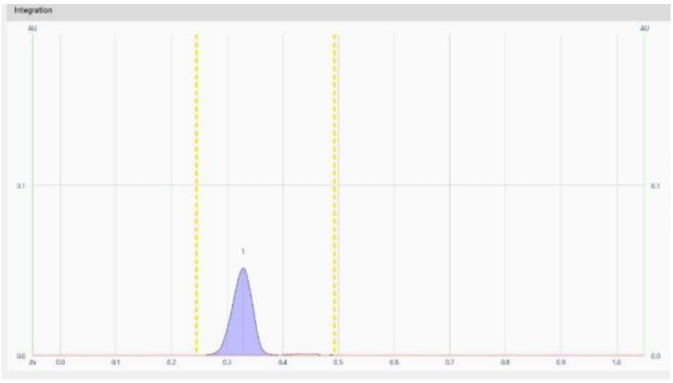

Figure 5. HPTLC chromatogram of tannic acid.

Table 3. Validation factors for a newly created HPTLC method for determining eugenol and tannic acid simultaneously in pure form and from an extract of a newly created churna

Sr. No.	Parameters		Tannic acid	Eugenol
1	Range (ng/spot)		250 – 1500	250 – 1500
2	Slope		0.000002	0.000002
3	Intercept		0.00538	0.00554
4	Correlation coefficient $(R)^2$		0.9983	0.9991
5	Accuracy (% Recovery ± SD)		99.52 ± 0.30	99.1 ± 0.78
6	LOD (ng/band)		16.5	33
7	LOQ (ng/band)		50	100
8	Precision (%RSD)	Intraday	1.26	1.26
		Interday	1.09	1.23

Similarly, the results of robustness were provided in Table 4 below:

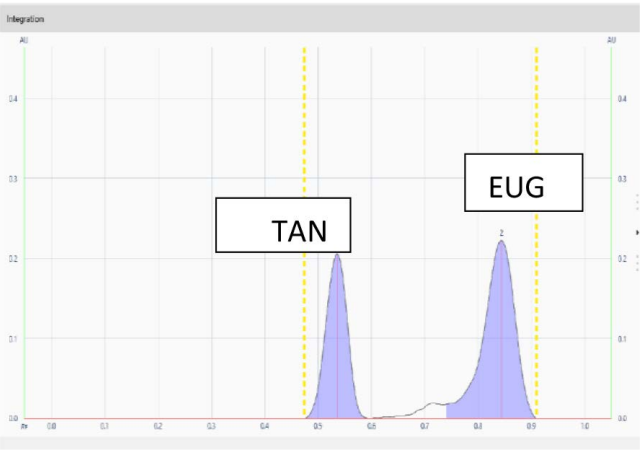

Figure 6. HPTLC chromatogram of eugenol and tannic acid using the optimized mobile phase.

mobile phase, as well as validation of the produced technique in accordance with ICH guidelines, and validation of the developed method in accordance with ICH guidelines for analytical method validation.

Figure 4 depicts the HPTLC chromatogram of eugenol. Similarly, the HPTLC chromatogram for tannic acid was represented in Figure 5.

The HPTLC chromatogram for the simultaneous determination of eugenol and tannic acid was represented in Figure 6.

According to the ICH guidelines for analytical technique validation, the developed HPTLC method was validated. Table 3 provided a representation of the various validation factors.

To determine eugenol and tannic acid in the extracts of recently formulated churna, the established method

Table 4. Results of robustness testing

Sr. No.	Parameters	%RSD [a]	
		Tannic acid	Eugenol
1	Mobile phase composition 1. 5:3:2 2. 5:3:1.5 3. 5.5:3.5:1	1.17 1.80 1.41	0.96 1.10 1.09
2	Mobile phase Volume 1. 10 ml 2. 15 ml 3. 20 ml	1.19 1.01 1.53	1.10 1.15 1.36
3	Saturation time 1. 25 min 2. 27 min 3. 29 min	1.18 1.03 1.20	1.10 0.82 0.98

[a] – Mean value of three determinations.

was successfully used. Figure 7 shows the HPTLC chromatogram of eugenol and tannic acid in the extract of recently produced churna.

In Table 5, the application of the developed HPTLC method for the simultaneous determination of eugenol and tannic acid in the newly formulated churna is shown below.

4. Discussion

Tulsi and Parijat were used as major key ingredients in a novel herbal formulation that was optimised with the specific ratios for the selected herbs. A newly

Table 5. Application of the developed HPTLC method for simultaneous determination of eugenol and tannic acid in the newly formulated churna

Sr. No.	Name of drug	Rf		Area	The amount present in the formulation (mg)
		Test	Reference	Test	
1	Tannic acid	0.534	0.495	0.00633	209.6 mg
2	Eugenol	0.849	0.825	0.00790	625.4 mg

formulated churna is evaluated for various evaluation parameters, and all the parameters are found to be within the acceptance criteria as per the limit prescribed in the Ayurvedic Pharmacopoeia. Eugenol and tannic acid were selected as major phytomarkers for simultaneous HPTLC method development. Various mobile phases were tried for eugenol and tannic acid. The testing showed that a mixture of toluene, ethyl acetate, and formic acid at a ratio of 5:3:2 v/v/v produced the best results. Therefore, this mobile phase was selected for further HPTLC method development. HPTLC chromatograms of pure eugenol and tannic acid along with the mixture of standards and extracts were successfully obtained with the optimised mobile phase. According to the ICH guidelines for analytical method validation, the developed HPTLC method was validated. The acceptance criteria were found to include all of the validation elements. The approach was successfully used to determine eugenol and tannic acid simultaneously in a newly designed churna.

5. Conclusion

A novel herbal formulation in the form of churna is formulated and evaluated successfully using Tulsi and Parijat. The most widely useful constituents of Tulsi and Parijat, i.e., the recently developed HPTLC method, are successfully used to assess eugenol and tannic acid. The established HPTLC method is proven to be reliable, sensitive, and accurate for the simultaneous determination of eugenol and tannic acid in pure form and extracts of recently created churna, including Tulsi and Parijat.

6. Acknowledgement

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