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## CHEMICAL CHARACTERISATION, EVALUATION OF ANTIOXIDANT ACTIVITY AND ACUTE TOXICITY OF POLAR EXTRACTS OF *TABERNAEMONTANA CRASSA* BENTH (FAMILY APOCYNACEAE)

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### Keywords:

*Tabernaemontana crassa* Benth, Chemical characterisation, Phenolic compounds, Antioxidant activity, acute toxicity

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**ABSTRACT:** *Tabernaemontana crassa* Benth, synonym *Tabernaemontana jollyana* Pietre ex Stapf, is a Central African pharmacopoeia plant whose stem bark is widely used in traditional medicine. The aim of this study was to evaluate the acute toxicity of aqueous extracts of this species. Preliminary phytochemical tests carried out on the different crude extracts of *T. crassa* revealed the following chemical groups: alkaloids, quinones, triterpenes and sterols. Quantitative analysis showed that the hydro-methanolic and methanolic extracts of *T. crassa* had a high content of total polyphenols and flavonoids ( $247,33 \pm 2,05 \mu\text{g Eq GA/g Ms}$  and  $1543,93 \pm 10,15 \mu\text{g Eq Rut/g Ms}$ , respectively). Evaluation of the free radical scavenging capacity of *T. crassa* extracts (methanolic, hydro-methanolic and aqueous) using the DPPH free radical scavenging method on 96-well microplates showed moderate  $\text{IC}_{50}$  values ( $0,2714 \pm 0,0020$ ,  $0,2480 \pm 0,0006$  and  $0,3129 \pm 0,0005 \text{ mg/mL}$ ), but still low compared to the reference molecule, ascorbic acid ( $0,064 \pm 0,000 \text{ mg/mL}$ ). The acute toxicity study of the aqueous extract showed no clinical signs of toxicity at single doses of 2000 and 5000 mg/mL body weight in mice.

**INTRODUCTION:** The use of medicinal plants has experienced a revival in recent decades. Today, medicinal plants occupy a very important place in the African pharmacopoeia, because the population often has to rely on the empirical use of « drugs », essentially consisting of plant matter that grows in their environment, to maintain and restore their

health, due to lack of access to medicines prescribed by modern medicine, but also because these plants are often truly effective and much cheaper than synthetic medicines <sup>1,2</sup>. Today, plants play a crucial role in the treatment of diseases such as diabetes, hypertension, malaria, epilepsy and bacterial infections.

*Tabernaemontana crassa* Benth, is a plant of the family Apocynaceae, synonym *Conopharyngia crassa* (Benth.) Stapf, or *Tabernaemontana jollyana* Pietre ex Stapf. The genus *Tabernaemontana* comprises 100 to 110 species, of which about 18 are found on the African continent and 15 on Madagascar <sup>3</sup>. *T. crassa* is widely used in

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traditional African medicine for its many therapeutic properties. It is a shrub or medium-sized tree that can reach a height of 15 to 23 meters, with a hairless body and a trunk up to 30 cm in diameter. The bark of the trunk is grey-brown or light to dark brown, with large lenticels. The leaves are evergreen, opposite, simple and entire, 3 to 25 cm long, with a milky sap. The flowers are fragrant, white and 1 to 5 cm in diameter. The fruits consist of 2 separate, obliquely spherical follicles, 5-12 cm in diameter, pale green to glaucous, with 2 valves containing numerous seeds surrounded by a fleshy pulp. *T. crassa* is found in humid tropical Africa, from Angola, Cameroon, Ivory Coast, Sierra Leone, Ghana, Benin, Equatorial Guinea, Gabon, Liberia, Central African Republic, Congo and DR. Congo.

Ethnobotanical studies have reported that *T. crassa* Benth is widely used in the treatment of many ailments such as: wound infections, leprosy wounds, headaches, stomach disorders<sup>4</sup>, skin infections and fungal disorders<sup>3</sup>.

Previous studies have also shown antibacterial<sup>3, 5</sup> and anti-gonorrhoea activity. Previous chemical studies have reported the presence of several indole alkaloids in the roots, seeds and bark of this plant<sup>3, 6, 7</sup>: vobasine, tabersonine, conopharyngine, ibogaine, ibogamine, isovoacangine and others.

Despite an available bibliography and interesting therapeutic knowledge, phytochemical screening, assessment of antioxidant activity and study of acute toxicity of the Central African species have never been carried out to our knowledge.

The present work, which is a contribution to the development of this Central African medicinal species, aims to identify secondary metabolites in the stem bark of *T. crassa* Benth using tube reactions, to assess for the first time the antioxidant activity on TLC plates and 96-well microplates, and to experimentally assess the acute toxicity of aqueous extracts of *T. crassa* Benth in mice.

## MATERIALS AND METHODS:

**Plant Material:** Bark from the trunk and root of *T. crassa* was collected in the village of Yamboro, located at 18°23'6" eastern longitude and 4°19'23" east-south latitude in the Ombella M'poko region, 30 km south of Bangui (Central

African Republic). The plant was botanically identified at the Plant Biodiversity Laboratory of the Faculty of Science at the University of Bangui. The bark of the trunk of this species was dried in a dark, dry place and then ground. The powder obtained was stored in glass bottles for later analysis.

**Animal Material:** Adult male and female albino mice weighing between 20 and 26 g were used. These mice, provided by the Animal House of the Faculty of Science and Technology of the University Marien N'gouabi (Congo), were acclimatised in the Biochemistry and Pharmacology.

Laboratory of the Faculty of Science and Health of the University Marien N'gouabi (Congo) for 5 days prior to the experiments. Mice were maintained under standard conditions (12 hours light, 12 hours dark) at a temperature of  $27 \pm 2^\circ\text{C}$  with free access to standard chow and tap water.

**Preparation of Crude Extracts for Quantitative Determination:** Two grams of *T. crassa* stem bark powder were cold macerated in 40 mL of methanol, hydro-methanol (v/v) and aqueous under magnetic stirring for 24 hours, then filtered. The different filtrates obtained were stored in a cool place ( $4^\circ\text{C}$ ) for the different analyses.

**Preparation of Aqueous Extracts for Acute Toxicity Test:** 50 grams of *T. crassa* stem bark powder was decocted in 500 ml distilled water for 30 min. After cooling and filtration, the filtrate obtained was evaporated to dryness under reduced pressure in a Büchi R-200 rotary evaporator at  $60^\circ\text{C}$ . The dry concentrate obtained was weighed and stored in a glass bottle for acute toxicity testing.

**Preliminary Phytochemical Screening:** The qualitative composition of the extracts of *T. crassa* Benth by tube reactions was carried out according to the classical method<sup>8, 9, 10</sup> based on colouring and/or precipitation reactions to identify the presence of chemical substances. Tannins<sup>11</sup> and polyphenols were identified by the ferric chloride test and the Stiasny reagent, flavonoids by the cyanidine reaction, triterpenes and sterols by the Libermann-Bürchard test and alkaloids by the Dragendorff and Mayer tests.

**Determination of Total Polyphenols:** The content of total phenolics in the crude extracts of *T. crassa* was determined spectrophotometrically, using the colorimetric method according to the Folin-Ciocalteu reagent<sup>11, 12</sup> with a slight modification.

A quantity of 100 µL of each crude extract (methanol, methanol-water and aqueous) was mixed with 900 µL of distilled water, then with 900 µL of Folin-Ciocalteu (1 N) and 200 µL of sodium bicarbonate solution (20%). The mixture was shaken and incubated for 40 minutes in the dark. Absorbance was measured at 725 nm using an AL 800/Spectro Direct UV/Visible spectrophotometer against a methanol solution used as a blank. The results obtained were expressed as micrograms of gallic acid equivalent per gram of dry matter (µg Eq GA/g Ms).

**Determination of Total Flavonoids:** The flavonoid content of crude extracts of *T. crassa* was determined by the aluminium trichloride colourimetric method. The protocol used is that described by<sup>12, 13, 14</sup> with some modifications.

A quantity of 250 µL of each extract (methanol, methanol-water and aqueous) was mixed with 1000 µL of distilled water. At time zero (t = 0), 75 µL of a 5% sodium bicarbonate solution was added to the mixture. After 5 minutes (t = 5 min), 75 µL of a 10% aluminium trichloride (AlCl<sub>3</sub>) solution was added. After incubation for 6 minutes at room temperature, 500 µL of 1N sodium hydroxide (NaOH) solution and 2,5 mL of distilled water were added to the reaction mixture. The mixture was immediately vortexed vigorously. Readings were taken at 510 nm using an AL 800/Spectro Direct UV/Visible spectrophotometer against a methanol blank.

#### Evaluation of Antioxidant Activity:

**Thin Layer Chromatography Method:** Protocol 15 was used for the evaluation of antioxidant activity. Five microlites (5 µL) of each extract (methanol, ethanol, ethanol-water and aqueous) were chromatographed on a Silicagel 60 F254 Merck plate with an aluminium support. The plates were placed in a chromatographic cell containing the following mobile phase Ethyl acetate/formic acid/water (8:1:1; V/V/V). After migration, the chromatograms were dried and then developed with

0.1% DPPH in methanol. Components of each extract with antioxidant activity appeared as light yellow spots on a purple background.

**2,2-diphenyl-1-picrylhydrazyl (DPPH) Method in a 96-well Microplate:** This method is based on the use of a free radical: DPPH described by 16, 17 with a slight modification. The reduction of DPPH is accompanied by a change in the colour of the solution from purple to yellow, which can be measured spectrophotometrically at 525 nm. This indicates antioxidant activity. The intensity of the colour change measured by the spectrophotometer is inversely proportional to the antioxidant activity of the extracts whose activity we wish to determine.

For our extracts, we prepared a solution of DPPH at a concentration of 7 mg in 20 ml of methanol. This solution was diluted 10 times and stored in the refrigerator for a maximum of two days. Four dilutions of different concentrations (60, 150, 240 and 300 mg/L) were prepared from the stock solution of each extract (MeOH, MeOH-water and aqueous). A pasteurised pipette was used to dispense 20 µL of each extract and 180 µL of DPPH into each microplate well. The blank was 20 µL DMSO and 180 µL MeOH. Readings were taken at 524 nm after 25 minutes incubation in the dark. Ascorbic acid was used as a standard, prepared under the same conditions as the extracts. All extracts were reproduced at least 4 times to minimise error.

The results were expressed as percentage inhibition (%I):  $\text{Abs blanc} - \text{Abs extracts} / \text{Abs blanc} \times 100$

IC<sub>50</sub> values were calculated graphically using the linear regression method of the graphs tested percentage inhibition as a function of different concentrations of crude extracts.

**Acute Toxicity Assessment:** The acute toxicity of the aqueous extract of the stem bark of *T. crassa* was assessed in mice in accordance with the OECD (Organisation for Economic Co-operation and Development) guideline No. 423 of 17 December 2001 on the testing of chemical substances and the protocol described by<sup>18, 19</sup> with a slight modification. Nine (9) mice, fasted for 18 h prior to the experiment, were divided into three (3) groups of three (3) mice each and treated orally as follows:

the first group received distilled water (0,5 ml/100 g body weight). The second and third groups received single doses of 2000 and 5000 mg/kg body weight, respectively, of the aqueous extract of the stem bark of the plant. After administration of the products (water and extract), the mice were observed for thirty 30 minutes and then hourly for four 4 hours. Observations included ptosis, aggressiveness, mobility, vigilance, vomiting, vocalisation, faecal condition, convulsions and spontaneous locomotor activity. The number of dead mice in each batch was recorded for 48 hours after dosing to determine the LD50.

**Analysis of Results:** Results are expressed as the mean with standard error of the mean. Comparison

of means between treated and control batches were performed using Student's test followed by analysis of variance (ANOVA). The significance level was set at  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Qualitative Composition by Tube Reactions:

The qualitative analysis by tube reactions carried out on the crude extracts of *T. crassa* trunk bark is presented in **Table 1**. It can be seen that alkaloids, quinones, saponosides, triterpenes and sterols are very abundant in the extracts of the trunk bark of *T. crassa*. Anthocyanins, flavonoids, tannins and reducing sugars were also completely absent.

**TABLE 1: RESULTS OF PHYTOCHEMICAL SCREENING OF EXTRACTS FROM THE STEM BARK AND ROOT OF *T. CRASSA***

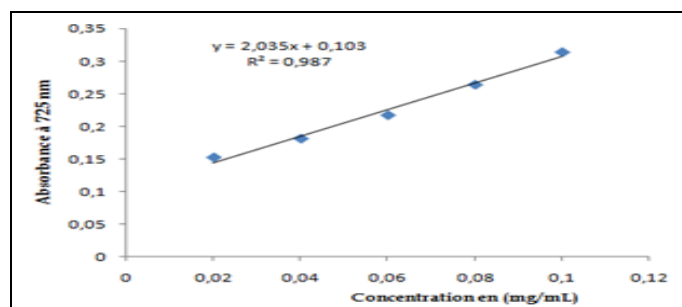
Chemical groups	<i>Tabernaemontana crassa</i>	
	Barks	Root
Alkaloids	++	++
Flavonoids	-	-
Quinones	++	++
Catechic tannins	-	-
Gallic tannins	-	++
Saponins	++	++
Triterpenes et Steroids	++	++
Anthocyanins	-	-
Reducing sugar	-	-

++: Abundant - : Absent.

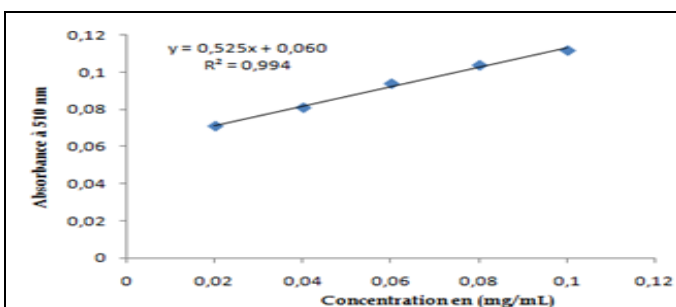
The results of qualitative analyses by tube reactions show that the stem and root barks of *T. crassa* are rich in alkaloids, quinones, saponosides, triterpenes and sterols. Flavonoids, catechic tannins, anthocyanins and reducing sugars are also absent. The presence of these chemical families in the stem bark of varieties of the Cameroon species has also been demonstrated. This study also showed the absence of flavonoids<sup>20</sup>. Previous work on ethereal, methanolic and aqueous extracts of the trunk bark of *T. crassa* showed the presence of

alkaloids, sterols, reducing sugars and coumarins in the form of glycosides. In addition, saponins and alkaloids were found in the root bark of the Ghanaian species, while terpenoids, tannins and flavonoids were absent<sup>21</sup>.

**Total Phenolic and Flavonoid Content:** The determination of the total phenolic and flavonoid content in the different extracts of *T. crassa* was carried out separately by colourimetric methods (Folin-Ciocalteu and aluminium trichloride).



**FIG. 1: GALLIC ACID CALIBRATION CURVE OF FOR DETERMINATION OF TOTAL PHENOLICS**



**FIG. 2: RUTIN CALIBRATION CURVE FOR THE DETERMINATION OF TOTAL FLAVONOIDS**



The contents of total phenolics and flavonoids in the different crude extracts of *T. crassa* Benth were determined from the linear regression equations for each calibration curve, expressed successively in  $\mu\text{g}$  gallic acid equivalent and  $\mu\text{g}$  rutin equivalent per gram dry matter **Fig. 1** and **2**.

The results obtained **Table 2** show that the total phenolic content varied from  $299,91 \pm 1,61$  to  $227,19 \pm 2,27 \mu\text{g Eq AG/g Ms}$  and from  $471,58 \pm 2,01$  to  $372,31 \pm 2,27 \mu\text{g Eq AG/g Ms}$ , respectively, for the bark of the stem and the root of *T. crassa*. On the other hand, the total flavonoid content determined by the aluminium trichloride method showed that the extracts from both organs had high flavonoid contents in relation to the total polyphenols in both the bark and the roots of *T. crassa*.

Furthermore, the results in **Table 2** show that the methanolic and hydro-methanolic extracts of *T.*

*crassa* bark have high total polyphenol content. The lowest content is found in the aqueous extract. Similarly, the methanolic and hydro-methanolic extracts of the two organs (bark and root) of *T. crassa* have higher levels of total flavonoids. Furthermore, the results in **Table 2** show that the methanolic and hydro-methanolic extracts of *T. crassa* bark have high total polyphenol content. The lowest content is found in the aqueous extract. Similarly, the methanolic and hydro-methanolic extracts of the two organs (bark and root) of *T. crassa* have higher levels of total flavonoids.

We can confirm that the polar extracts of *T. crassa* have high flavonoid contents compared to total polyphenols in both the stem bark and roots. The work 21 showed the presence of a low content of phenolic compounds in the bark of the root of *T. crassa* Benth.

**TABLE 2: TOTAL POLYPHENOL ( $\mu\text{G GA EQ/G MS}$ ) AND FLAVONOID ( $\mu\text{G RUT EQ/G MS}$ ) CONTENTS OF CRUDE EXTRACTS OF CRUDE EXTRACTS OF *T. CRASSA***

Organ	Extracts	Total polyphenols	Total flavonoids
Trunk bark	MeOH	$299,91 \pm 1,61$	$1543,93 \pm 10,15$
	MeOH-H <sub>2</sub> O	$247,33 \pm 2,05$	$666,69 \pm 11,65$
	Aqueous	$227,19 \pm 2,27$	$667,95 \pm 6,04$
Overall average		$258,14 \pm 1,97$	$959,52 \pm 9,28$
Root bark	MeOH	$372,31 \pm 2,27$	$1285,84 \pm 12,69$
	MeOH-H <sub>2</sub> O	$471,58 \pm 2,01$	$667,14 \pm 6,25$
	Aqueous	$386,89 \pm 0,43$	$837,52 \pm 3,23$
Overall average		$410,26 \pm 1,57$	$930,16 \pm 7,39$

Values are the mean of three replicates  $\pm$  sem

### Evaluation of Antioxidant Activity:

#### DPPH Radical Reduction Test on TLC Plate:

Chromatogram no1 of the different polar extracts of the bark of *Tabernaemontana crassa* Benth, obtained with a 0,1% DPPH solution in methanol,

shows faint yellow-white spots on a violet background in the methanolic and ethanolic extracts, demonstrating the low antioxidant activity of these compounds in these polar extracts.



**CHROMATOGRAM N°1: CHROMATOGRAPHIC PROFILES OF THE ANTIOXIDANT ACTIVITY OF VARIOUS POLAR EXTRACTS OF *TABERNAEMONTANA CRASSA* BENTH**

**DPPH Radical Reduction Test in a 96-well Microplate:** The antioxidant activity of polar extracts of *T. crassa* stem bark and the reference compound (ascorbic acid) against the free radical DPPH was evaluated using a MULTISKAN FC version 100-79 plate reader. The reduction of DPPH is accompanied by its change from violet (DPPH<sup>•</sup>) to yellow (DPPH-H), measured at 524 nm. This reduction capacity is determined by a decrease in absorbance induced by the antiradical substances. The results of the evaluation of the antiradical capacity carried out on the polar extracts (methanolic, hydro-methanolic and aqueous) of *T. crassa* are shown in **Table 3**. It can be seen that the reducing power is proportional to the increase in concentration. The SC50 values **Table 3** show that all the polar extracts have high SC50 values of

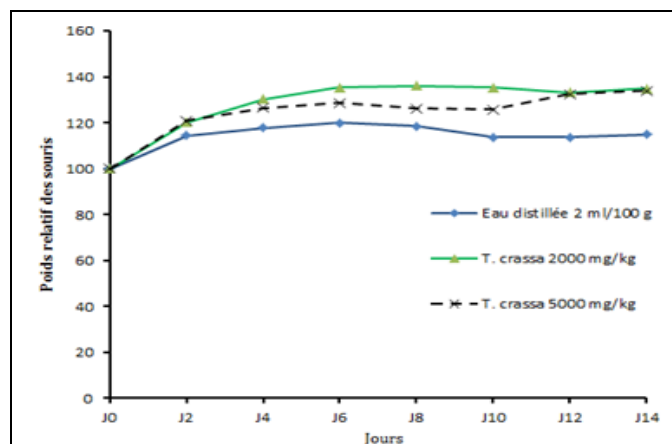
0,2714 ± 0,0020 mg/ml; 0,2480 ± 0,0006 mg/ml and 0,3129 ± 0,0005 mg/ml for the methanolic, hydro-methanolic and aqueous extracts, respectively, compared to vitamin C (0,064 ± 0,000 mg/ml). We note that it is the fraction of the hydro-methanolic extract (0,2480 ± 0,0006 mg/mL) that has a low antioxidant activity, comparable to that of the reference compound, vitamin C (0,064 ± 0,000 mg/mL).

It should be noted that the higher the 50% trapping concentration, the lower the anti-free radical activity **Table 3**. This low activity indicates that the different polar extracts of *T. crassa* contain few substances that react with the DPPH radical, as confirmed by the phytochemical tests carried out on extracts of this species.

**TABLE 3: SC50 CONCENTRATION VALUES FOR POLAR EXTRACTS OF T. CRASSA STEM BARK**

Extracts	MeOH	MeOH-H <sub>2</sub> O	Aqueous	Vitamin C
Mean IC <sub>50</sub> (mg/mL)	0,2714 ± 0,0020	0,2480 ± 0,0006	0,3129 ± 0,0005	0,0064 ± 0,000

**Acute Toxicity of Aqueous Extract:** Acute *per os* toxicity tests of the aqueous extract of *T. crassa* (2000 and 5000 mg/kg) did not cause any change in the behaviour or general condition of the mice compared with the control. In addition, no mortality was observed up to 48 h as in the control mice. However, mice given a dose of 2000 mg/kg body weight of the aqueous extract of *T. crassa* showed some signs of transient diarrhoea. For the acute toxicity test, we monitored the changes in body weight of the mice on days (D0 to D14). The results are shown in **Fig. 1**, which represents the relative weight of the mice (%) as a function of day.



**FIG. 3: EFFECT DESEXTRAITS AQUEUX DE T. CRASSA BENTH SUR L, EVOLUTION PONDERALE DES SOURIS**

We note **Fig. 3** that the mice receiving the aqueous extract of *T. crassa* showed an increase in body weight from D8 to D10 and then stabilised from D12. We also noted that mice receiving aqueous extracts, particularly at a dose of 2000 mg/kg bw, gained more weight than the control; this would be due to the fact that the aqueous extract of *T. crassa* Benth stimulates appetite in mice.

The lack of observed signs of toxicity suggests that the aqueous extract of the stem bark of *T. crassa* is well tolerated by mice at doses of 2000 and 5000 mg/kg. No mortality was observed up to a dose of 5000 mg/kg. This result suggests that the lethal dose 50 (LD<sub>50</sub>) of the aqueous extract of this plant is greater than 5000 mg/kg body weight.

It should be noted that it has been shown that LD<sub>50</sub> values below 5000 mg/kg correspond to highly toxic substances and those above 5000 mg/kg to slightly toxic substances. The aqueous extract of the stem bark of *T. crassa* would therefore be slightly toxic. Work by <sup>3, 22</sup> on ethanolic and hydroethanolic extracts of *T. crassa* trunk bark and roots showed similar results. However, mice given a dose of 15,000 mg/kg bw of ethanolic extracts of *T. crassa* Benth stem bark showed some external signs of toxicity: a rather severe rash on the tail <sup>23</sup>.

**CONCLUSIONS:** This work identified the following main families: alkaloids, quinones, saponins, triterpenes and sterols in *T. crassa* extracts. The lowest levels of total polyphenols were found in the aqueous extracts. Similarly, the highest levels of total flavonoids were observed in the methanolic and hydro-methanolic extracts of both *T. crassa* organs.

The antioxidant activity of polar extracts of *T. crassa* using both TLC and 96-well microplate methods showed moderate antiradical activity. Assessment of acute toxicity in the aqueous extract of *T. crassa* showed no clinical signs of toxicity up to a dose of 5000 mg/kg body weight in mice, proving that the aqueous extract is considered to be a non-toxic substance by the oral route.

However, if its pharmacological properties are to be exploited, further research is required to identify, isolate and purify its constituents and to carry out other tests such as antibacterial, antimicrobial, antifungal and spontaneous motor activity tests.

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**CONFLICTS OF INTEREST:** Nil

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