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ORIGINAL PAPER

# Phytochemical Profile, Antioxidant and Cytotoxic Activities of the Carob Tree (*Ceratonia siliqua* L.) Germ Flour Extracts

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**Abstract** This work aimed to evaluate the phytochemical content and to determine the antioxidant and cytotoxic activities of methanol extracts of the carob tree (*Ceratonia siliqua* L.) germ flour. The extracts were rich in phenolic compounds, had considerable antioxidant activity, and reduced the viability of cervical (HeLa) cancer cells. The chemical content and the biological activities of the extracts were significantly affected by gender and cultivar. Female cultivar Galhosa had the highest levels of phenolic compounds, and the highest antioxidant activity. Extracts from the hermaphrodite trees and from the female cultivars Galhosa and Costela/Canela exhibited the highest cytotoxic activity. The most abundant compound was theophylline. The phenolic content was correlated to both antioxidant and cytotoxic activities. Our findings provide new knowledge about the health implications of consuming food supplemented with carob germ flour.

**Keywords** Alkaloids · Antioxidant · Antiproliferative · Oxidative stress · Phenolic compounds · ROS · Theophylline

**Abbreviations**
RSA Radical scavenging activity

## Introduction

The carob tree (*Ceratonia siliqua* L.) is one of the most useful trees of the Mediterranean basin with application in

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the food industry as a source of gum extracted from the seeds (LBG-E410). The germs are used to produce germ flour with a high content of proteins and highly unsaturated oil, and employed as a dietetic human food [1, 2]. The carob bean embryo contains caroubin, a protein with properties similar to gluten, which confers carob germ flour the potential as a gluten replacement in cereal-derived foods for celiac people [3].

Germ flour contains polyphenols, proanthocyanidins, and ellagi- and gallotannins [4]. These phytochemicals protect the organism from excessive production of free radicals and reactive oxygen species (ROS), which are involved in the development of the pathology of numerous diseases such as Alzheimer and cancer [5]. Since the prevention of chronic diseases is a more effective strategy than their treatment, many functional foods are nowadays aimed at boosting intakes of antioxidants in order to reduce the risk of diseases linked to oxidative stress. Moreover, there is increasing evidence that some synthetic antioxidants such as butylated hydroxytoluene (BHT) have severe side effects such as carcinogenicity [6], and thus, studies on natural antioxidants as nutritional supplements and health food have gained increasingly greater importance.

To the best of our knowledge, there is no information on the bioactivity of carob germ flour, and no comparative studies of the phytochemical contents between genders and/or cultivars. This study aimed to characterize the phytochemical profile and to determine the antioxidant and the *in vitro* cytotoxic properties of germ flour extracts from different female cultivars and hermaphrodite carob trees. In addition, we assessed the contribution of the major compound theophylline to the antiproliferative activity of the crude extract against HeLa cells.

## Materials and Methods

### Plant Material

Mature fruits were sampled during August and September of 2005 in the Algarve region (Portugal) from six female Portuguese cultivars of carob tree, namely Mulata, Galhosa, Aida, Costela/Canela, Gasparinha, and Preta de Lagos, and from two hermaphrodite trees. Fruits were manually deseeded, and the seeds were used to produce germ flour by DANISCO Portugal Industrias de Alfarroba, LDA.

### Preparation of Germ Flour Extracts

The methanol extracts were prepared by Soxhlet extraction as described previously [7]. For the cell experiments, the solvent was evaporated and the extracts were re-dissolved in the appropriate culture medium. All the other assays were performed with the methanol extracts.

### Preparation of Theophylline Solution

The stock solution of theophylline was prepared on phosphate-buffered saline (PBS, pH 7.4). Immediately before use, working solutions were prepared in culture medium in concentrations corresponding to the amounts quantified in the crude extracts.

### Phytochemical Evaluation of the Extracts

*Total Phenolic Content* Total phenolic content was determined by the Folin–Ciocalteu method [8], and expressed as gallic acid equivalents in milligrams per gram of extract (dry weight, DW). Total condensed tannins content was analyzed by the vanillin assay [9] and expressed as catechin equivalents in milligrams per gram DW. Total flavonoid content was estimated by the aluminium chloride method [10] and expressed as rutin equivalents in milligrams per gram DW.

*High-performance Liquid Chromatography* The extracts (10 mg $ml^{-1}$) were analyzed on an Agilent 1100 Series liquid chromatograph with a UV–Vis DAD system (Agilent Technologies, Germany). Analyses were performed on a Tracer excel 120 ODS-A column (150 mm× 4.0 mm, 5 μm particle size, Teknokroma, Spain). The mobile phase was a mixture of 2.5% acetic acid in water (A) and methanol (B), the applied gradient was 0–50 min: 30–80% B, 50–55 min: 80–30% B, hold for 5 min and the flow rate was 0.5 ml $min^{-1}$. The analyses were performed at 25 °C, and the injection volume was 40 μl with a draw speed of 200 μl $min^{-1}$. For the identification of the phenolic compounds, the retention parameters of each assay were compared with the standard controls and the peak purity with the UV–visible spectral reference data. The levels of the different compounds were extrapolated from calibration standard curves.

### Determination of Antioxidant Activity

The antioxidant activity was evaluated on the extracts at the concentration of 10 mg $ml^{-1}$. The absorbances were measured on a Shimadzu UV-160A spectrophotometer. BHT (E321) was used as the positive control at the concentration of 1 mg $ml^{-1}$.

*Radical Scavenging Activity* Radical scavenging activity (RSA) was evaluated against 1-diphenyl-2-picrylhydrazyl (DPPH) [11] and 2,2′-azino-bis(3-ethylbenzthiazoline-6-

sulphonic acid (ABTS) radicals [12]. Results were expressed as percent inhibition relative to a blank containing methanol.

*$Fe^{3+}$-Reducing Power Assay* The $Fe^{3+}$-reducing power was determined by the method described by Choi et al. [13]. The intensity of blue green colour was measured at 700 nm, with a high absorbance of the reaction mixture indicating an elevated reducing power.

## Evaluation of the *in vitro* Cytotoxic Activity and Reactive Oxygen Species Production

*Cell Culture and Viability Assays* The 4-(3-4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio)-1,3-benzenedisulfonate (WST-1) colorimetric assay was used to assess the effect of the extracts on (HeLa) cells viability [7]. Cells were cultured in Dulbecco's modified eagle medium with 1,000 mg $ml^{-1}$ of glucose, 10% (*v*/*v*) heat-inactivated fetal bovine serum, L-glutamine (2 mM), sodium pyruvate (111 mg $l^{-1}$), penicillin (50 U $ml^{-1}$) and streptomycin (50 μg $l^{-1}$), and were grown in a incubator at 37 °C, 5.1% $CO_2$ in humidified atmosphere. Cells were seeded on 96-well plates ($7\times10^3$ cell/well), incubated for 24 h and treated for 6–72 h with the extracts (2.5–40 mg $ml^{-1}$). Then, 20 μl of WST-1 were added to each well, and the absorbance measured 2 h later at 450 nm on a Power Wave XS spectrophotometer. Results were expressed in terms of cell viability (%), half maximal inhibitory concentration ($IC_{50}$, mg $ml^{-1}$), and maximal degree of inhibition Max.,%. To determine the effect of theophylline on HeLa cells viability, cells were seeded in 96-well plates and treated with culture medium containing theophylline in concentrations corresponding to the amounts contained in the complete extract at the concentration of 10 mg $ml^{-1}$ (60–220 μg $ml^{-1}$). After 24–72 h of incubation cell viability was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [14]. Results were expressed in terms of cell viability (%).

*ROS Production* The Mulata extract effect on ROS production was measured in HeLa cells using 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA) [15]. Cultivar Mulata was chosen because it is the most common female cultivar present in commercial orchards in the southern of Portugal and the most important in terms of carob production. Cells were exposed to the extract (10 mg $ml^{-1}$, 24 h), trypsinized and washed with PBS. Treated and control cells were resuspended in PBS containing 10 μM DCFH-DA at 37 °C for 30 min and then incubated with 4 mM $H_2O_2$ for 30 min at 37 °C. Relative amount of intracellular ROS (%) was subjected to evaluation by fluorescence-activated cell sorting in a flow cytometer (Coulter XL) and calculated according to the following equation:

Relative amount of intracellular ROS (%)=$(FI_1/FI_0)\times$ 100%, where $FI_0$ was the fluorescence intensity of the

**Table 1** Total phenolic content (TPC), total tannin content (TTC), total flavonoid content (TFC), and antioxidant activity measured by the DPPH and ABTS assays (% inhibition), and $Fe^{3+}$–$Fe^{2}$ reducing power assay (absorbance at 700 nm) of germ flour extracts from different origins

| Cultivar/tree | Yield (%) | Phenolics content | | | Antioxidant activity | | |
|---|---|---|---|---|---|---|---|
| | | TPC[a] | TTC[b] | TFC[c] | DPPH | ABTS | $Fe^{3+}/Fe^{2}$ |
| Female | | | | | | | |
| Mulata | 11.6±1.8 | 21.9±0.5 cd | 4.2±0.4b | 11.08±0.0e | 57.5±1.2d | 78.8±3.0ab | 0.9±0.0c |
| Galhosa | 4.3±1.2 | 32.9±0.9a | 6.8±0.4a | 22.3±0.1a | 89.4±1.1a | 85.4±3.6a | 1.5±0.1a |
| Aida | 3.2±0.7 | 19.8±0.6 cd | 2.2±0.2c | 10.8±0.1f | 45.2±1.1e | 84.7±3.0a | 0.5±0.0d |
| Gasparinha | 5.6±0.7 | 24.8±0.6bc | 1.9±0.2 cd | 17.4±0.4c | 68.2±0.9b | 68.4±6.8b | 1.4±0.1ab |
| Costela/Canela | 6.3±1.1 | 28.1±7.8ab | 3.9±0.4b | 14.7±0.3d | 61.2±0.3c | 82.8±3.1a | 0.8±0.0c |
| Mean[d] | 6.2 | 25.5 | 3.8 | 15.4 | 64.3 | 80.0 | 1.0 |
| Hermaphrodites | 7.4±1.8 | 28.7±0.3ab | 4.5±0.2b | 18.8±0.2b | 86.7±0.8a | 65.6±4.1b | 1.3±0.0b |
| BHT[e] | – | – | – | – | 77.6±1.6 | 56.2±0.4 | 3.0±0.0 |

For each column, statistical analysis was made between cultivars/trees. Values followed by different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test)

[a] mg GAE $g^{-1}$ extract (DW; *GAE* gallic acid equivalents)

[b] mg RE $g^{-1}$ extract (DW; *RE* rutin equivalents)

[c] mg CE $g^{-1}$ extract (DW; *CE* catechin equivalents)

[d] Means of all the values of the cultivars

[e] Reference compound, 1 mg $ml^{-1}$

**Table 2** Phytochemical analysis of germ flour extracts of carob tree from different origins by HPLC-DAD (mg/g extract, DW)

| | Compound | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cultivar/tree | C | GEac | CLac | Ct | Fac | GA | M | MG | Q | R | Sac | V | Total | T |
| Female | | | | | | | | | | | | | | |
| Mulata | – | – | 1.5 | – | 0.2 | 0.3 | – | – | – | – | – | 0.1 | 2.1 | 1.6 |
| Galhosa | 3.7 | 2.0 | – | – | – | 0.6 | – | 0.3 | 0.2 | – | – | – | 6.8 | 0.5 |
| Aida | – | – | – | – | – | 0.6 | 0.5 | 0.6 | 0.2 | – | 0.4 | – | 2.3 | 1.0 |
| Gasparinha | – | – | – | – | – | 0.4 | 1.1 | 0.5 | – | – | 0.4 | – | 2.4 | 1.3 |
| Costela/Canela | – | – | – | – | – | 0.4 | 0.9 | 0.5 | – | – | 0.3 | – | 2.1 | 1.3 |
| | | | | | | | | | | | | Mean | 3.1 | 1.1 |
| Hermaphrodites | – | – | – | – | – | 0.5 | 1.5 | 0.6 | – | – | 0.4 | – | 3.0 | 1.4 |

*C* (+)-catechin; *GEac* gentisic acid, *CLac* chlorogenic acid; *Ct* catechol; *Fac* ferulic acid; *GA* gallic acid; *M* myricetin; *MG* methyl gallate; *Q* quercetin; *R* rutin; *Sac* syringic acid; *T* theophylline; *V* vanillin

[a] As identified by comparison of the retention parameters with the standard controls and the peak purity with the UV–visible spectral reference data

negative control and $FI_1$ the fluorescence intensity in the presence of the extract at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

### Statistical Analysis

Values were expressed as mean±standard error of mean (SEM) for at least three experiments and were subjected to analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 16.0, SPSS Inc). Significance between means was tested by Duncan's New Multiple Range Test ($P$=0.05). Correlations between parameters were investigated using the correlation and regression program of Microsoft Excel. The $IC_{50}$ values were calculated with the GraphPad Prism 4.

## Results and Discussion

Cross-varietal screening tests show that certain genotypes within a plant species can have widely divergent levels of antioxidants [16]. Our results support those findings, since a significant variation in the phenolic profile was observed between samples from different female cultivars (Table 1). The extract yield ranged from 3.2% in Aida to 11.6% in Mulata (Table 1). Hermaphrodites exhibited higher amounts of phenolic compounds than females (Table 1), except than Galhosa cultivar. Germ flour extract from Galhosa exhibited the highest content of the three classes of phenolic compounds ($P$<0.001, Table 1).

Gallic acid was present in all samples, while other compounds were only detected in some cultivars (Table 2). (+)-Catechin and gentisic acid were only identified in Galhosa, and chlorogenic, ferulic and vanillin, only in Mulata (Table 2). These differences can be due to the high genetic diversity occurring between cultivars of this species [17]. On the other hand, some compounds could be present in the extracts, but at concentrations not detectable by the high-performance liquid chromatography (HPLC) conditions used in this study.

The most abundant identified compound was the alkaloid theophylline. Theophylline was already detected in aqueous extracts of carob pods [18] and in this work it was identified for the first time as the major compound in

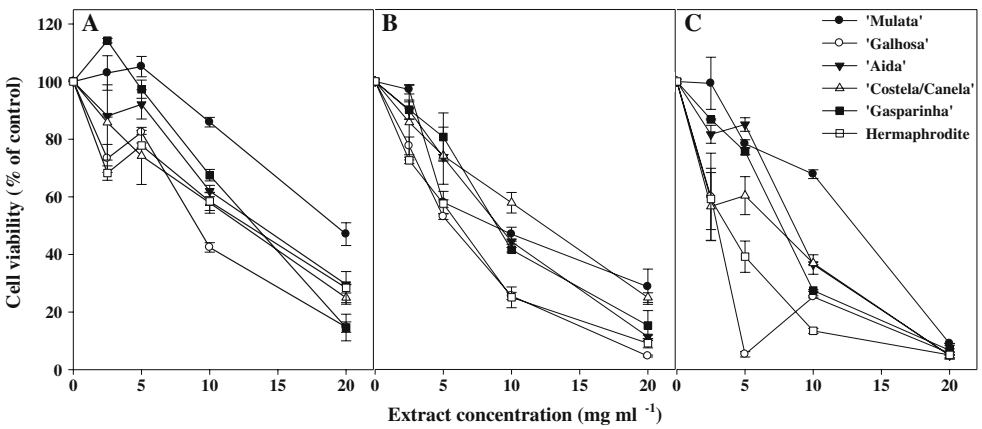


**Fig. 1** Effect of treatment with extracts from carob tree germ flour on HeLa cells viability after 24 h (**a**), 48 h (**b**) or 72 h (**c**) of incubation. Values represent means±SEM ($n$=3)

**Table 3** Half maximal inhibitory concentration ($IC_{50}$, mg $ml^{-1}$) and maximal degree of inhibition (Max., %) of carob germ flour extracts on HeLa cell line

| Cells/incubation | Parameter | Cultivar | | | | | |
|---|---|---|---|---|---|---|---|
| HeLa | | Mulata | Galhosa | Aida | Gasparinha | Costela/Canela | Hermaphrodites |
| 24 | $IC_{50}$ | 10.3±0.0b | 6.9±0.3b | 8.9±0.5a | 10.3±0.1b | 6.9±1.9b | 5.5±0.3b |
| | Max. | 52.9±3.9b | 85.2 ±1.7b | 70.4±0.1b | 85.37±4.6a | 75.0±1.6b | 71.6±5.6b |
| 48 | $IC_{50}$ | 5.1±0.9a | 4.8±0.1b | 7.2±0.0a | 7.3±1.1a | 5.9±0.3ab | 4.5±0.0ab |
| | Max. | 71.2±6.1b | 95.3±0.3a | 88.6±0.5ab | 84.7±5.1a | 89.8±0.6a | 90.5±1.2a |
| 72 | $IC_{50}$ | 9.9±0.6b | 2.7±0.1a | 7.8±0.1a | 6.6±0.0a | 4.4±0.1a | 3.7±0.1a |
| | Max. | 91.0±0.0a | 94.3±1.4a | 95.0±1.2a | 93.3±1.6a | 95.1±1.0a | 94.9±0.0a |

For each analysed parameter ($IC_{50}$ or Max.), statistical analysis was made between periods of incubation for the same cultivar/tree. For the same cultivar and parameter, values followed by different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test

methanol extracts of carob germ flour. Theophylline is widely used as a smooth muscle relaxant, and has application in the treatment of asthma as a bronchodilator. At low dose, theophylline is an effective add-on therapy to corticosteroids in controlling asthma, due to its anti-inflammatory action [19]. Due to the stimulatory influence of theophylline and other methylxanthines in the central nervous system (CNS) [20], future work is needed to evaluate the CNS effects of carob germ flour.

The extracts showed a significant RSA, more pronounced on the ABTS radical (Table 1). Extracts from Galhosa exhibited the highest RSA and also the highest reductive ability (Table 1). Compounds with reducing power indicate that they are electron donors, and can reduce the oxidized intermediates of lipid peroxidation processes, therefore they can act as primary and secondary antioxidants [21]. The antioxidant activity was correlated with the content of phenolic compounds, but not with theophylline (data not shown), and varied according to the extract, probably due to the different phytochemical profile (Tables 1 and 2). A positive correlation between phenolics and antioxidant activity is common in natural extracts [22, 23]. Both the phenolic compounds present in the extract and the synergistic mechanisms between them may be responsible for the antioxidant activity displayed by carob germ flour.

Extracts from leaves and fruit pulps from carob tree reduce cell viability of different cancer cell lines [7, 18, 24, 25]. This work provides the first evidence of *in vitro* cytotoxic activity of carob germ flour extracts on HeLa cells. The reduction on cell viability was dose-dependent and already evident after a 24-h treatment (Fig. 1), indicating that the phytochemicals in the extracts promptly triggered a series of cellular events leading to the reduction of cell viability and/or the induction of cell death. The $IC_{50}$ values where generally lower for the 48 and 72 h of incubation and ranged from 2.7 mg $ml^{-1}$ in Galhosa (72 h) to 10.3 mg $ml^{-1}$ in Gasparinha and Mulata (24 h; Table 3). It is note worthy that the obtained $IC_{50}$ values, while somewhat high, still indicate some interesting activity. These values are likely due to the presence of low

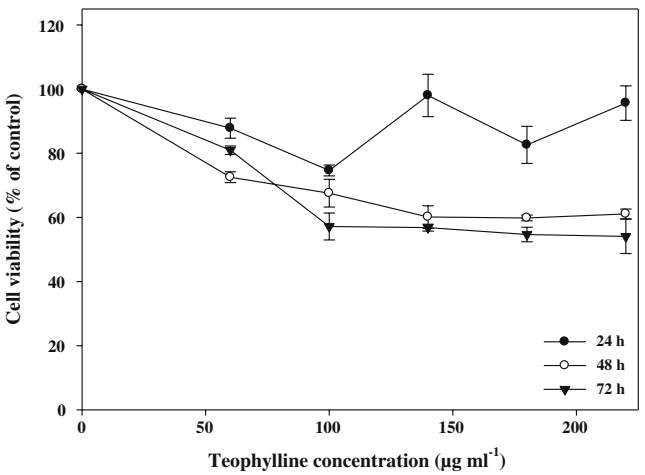


**Fig. 2** Effect of treatment with theophylline on HeLa cells viability after 24, 48 and 72 h of incubation. Values represent means±SEM ($n=3$)

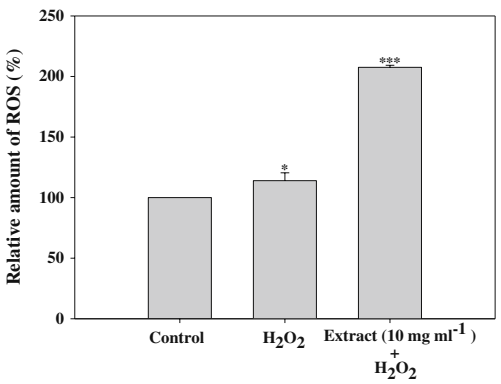


**Fig. 3** Effect of the application of 'Mulata' extract on intracellular ROS production by HeLa cells. Significant differences with control: $*P<0.05$, $**P<0.01$, $***P<0.001$

concentrations of compounds of interest in the crude extract, or to compounds with no antioxidant activity. The enrichment in compounds of interest can be accomplished by a bioactivity-guided fractionation of the crude extract.

There was a significant gender and cultivar-dependency on the bioactivity of the extracts. The extracts from Galhosa and hermaphrodites exhibited a strong cytotoxic activity, with the lowest $IC_{50}$ values (Table 3). The maximal degree of inhibition for the 24-h period of incubation was highest for Gasparinha (85.3%), Galhosa (85.2%) and Costela/Canela (75.0%), followed by the hermaphrodites (71.6%) and Aida (70.4%), (Table 3).

Numerous studies have shown that antioxidant micronutrients present in food, such as phenolics, can inhibit carcinogenesis by affecting the molecular events in the initiation, promotion or progression states [26]. In this work, positive correlations were found between the total amount of phenolic compounds identified by HPLC, gallic acid and the cytotoxic activity on HeLa cells (data not shown). Moreover, under the experimental conditions and concentrations tested, theophylline, the main compound detected in the extracts, exerted a cytotoxic activity which was both time- and concentration-dependent (Fig. 2). When applied during 24 h, it had no effect on HeLa cells viability (Fig. 2). However, treatment with concentrations higher than 100 $\mu g\ ml^{-1}$ for 48 and 72 h resulted in a significant reduction of cell viability (Fig 2). These results suggest the contribution of the phenolic compounds and theophylline to the cytotoxic activity of the samples towards HeLa cells.

In this work, it was evaluated the Mulata extract effect on ROS production after challenging HeLa cells with $H_2O_2$ to produce the oxidative stress. As shown in Fig 3, the intracellular ROS accumulation resulting from $H_2O_2$ exposure was significantly increased in the presence of the extract (207.5±1.6%, $P<0.05$), compared with cells in the presence of $H_2O_2$ without extract (110.7±9.8%, $P<0.05$; Fig. 3). This may be related with the induction of early apoptosis. ROS can act as anticancer, namely by the promotion of cell-cycle stasis, senescence, apoptosis and necrosis, or as pro-cancer, stimulating cell proliferation, causing DNA mutations, and promoting genetic instability [27]. Additional studies are needed to clarify the mechanism of action of methanol extracts from carob germ flour, namely through the assessment of its effect on cell-cycle progression, its ability to activate caspases, or mitochondrial and DNA damages.

## Conclusions

This work shows that carob germ flour is a source of compounds with antioxidant and *in vitro* cytotoxic activities, and that those activities are strongly dependent on the cultivar. The female cultivar Galhosa revealed to be the best source of bioactive compounds. Additional experiments are needed in order to identify and characterize the bioactive compounds present in the extracts, namely through the bioguided fractionation and isolation of pure compounds.

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