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Chemical defenses in the tree *Ziziphus mistol* against the leaf-cutting ant *Acromyrmex striatus*

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ABSTRACT. *Ziziphus mistol* Griseb. (Rhamnaceae) trees are avoided by the leaf-cutting ant *Acromyrmex striatus* Roger (Formicidae, Attini) in a semi-arid Chaco forest of Santiago del Estero Province, Argentina. In order to find out the chemical fraction responsible of ant rejection, we analyzed putative chemical defenses (tannins, phenolics and saponins) in young and mature leaves of *Ziziphus mistol* and carried out field bioassays to test both polar and non-polar leaf extracts for ant-repellence. We included into our bioassays two flavonoid compounds, namely quercetin and its glycoside rutin, which are common in *Ziziphus mistol* leaves, and commercial quebracho tannin. Condensed tannins and total phenols were significantly in higher concentrations in mature than in young leaves. We were unable to detect hydrolyzable tannins on both young and mature leaves. Saponins were only detected in young leaves. The non-polar extract was significantly repellent, whereas the polar extract was not significantly attractive. Tannin, quercetin and rutin did not exhibit significant attractiveness or repellence at the tested concentrations. Our results suggest that some unidentified constituent(s) of the non-polar fraction of the foliar extract, likely of terpenoid nature, accounts for the rejection of the entire *Ziziphus mistol* leaves by *Acromyrmex striatus* ants.

RESUMEN. Defensas químicas en el árbol *Ziziphus mistol* contra la hormiga cortadora de hojas *Acromyrmex striatus*: Las hojas de *Ziziphus mistol* Griseb. (Rhamnaceae) no son consumidas por la hormiga cortadora de hojas *Acromyrmex striatus* Roger (Formicidae, Attini) en un bosque chaqueño semiárido de Santiago del Estero, Argentina. Para determinar cuál es el componente químico responsable de ese comportamiento de las hormigas, se analizaron las defensas químicas putativas (taninos, fenólicos y saponinas) en hojas jóvenes y maduras de *Ziziphus mistol* y se llevaron a cabo bioensayos a campo en los que se probaron las propiedades repelentes de las fracciones polar y no polar de un extracto foliar. En los bioensayos se incluyeron dos flavonoides presentes en las hojas de *Ziziphus mistol*, quercetina y su glicósido rutin, y extracto comercial de quebracho colorado, una fuente de taninos. Los niveles de taninos condensados y fenoles totales fueron significativamente mayores en las hojas maduras que en las jóvenes. No se detectaron taninos hidrolizables en ningún tipo de hoja. La presencia de saponinas solo fue registrada en las hojas jóvenes. El extracto clorofórmico (no polar) fue significativamente repelente, mientras que el extracto metanólico (polar) no fue significativamente atractivo. Ni los taninos ni los flavonoides mostraron una atracción o una repelencia significativas a las concentraciones ensayadas. Nuestros resultados sugieren que algún constituyente no identificado de la fracción no polar del extracto foliar, posiblemente de naturaleza terpenoide, daría cuenta del rechazo de las hojas de *Ziziphus mistol* por la hormiga *Acromyrmex striatus*.

INTRODUCTION

Leaf-cutting ants belonging to the fungus-growing tribe Attini are extremely polyphagous herbivores of the Neotropics. They harvest vegetable matter on which a symbiotic fungus is grown for food, mainly for the larvae. Through this association with the fungus, the ants are able to cope with a diverse array of allelochemicals present in the plants they cut, and probably this is crucial for the success of these ants, which are agricultural pests in many countries.

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Despite their broad diet preferences, leaf-cutting ants seldom or never cut some plant species they found in nature. Although several factors influencing ants choice have been suggested to explain this fact [e.g., nutritional value of the tissues (Berish 1986); mechanical defenses of the plant, including leaf toughness, trichome density, and latex (Stradling 1978; Howard 1988; Nichols-Orians & Schultz 1989); moisture content of leaves (Bowers & Porter 1981); presence of aggressive ant species on the host-plant (Koptur 1984; Folgarait et al. 1994)], plant secondary chemistry seems to be central (Hubbell & Wiemer 1983; Hubbell et al. 1984; Howard 1987, 1988; Nichols-Orians & Schultz 1990). Leaves of avoided species might contain high concentrations of secondary compounds that are toxic to the ants, their fungus, or both; or secondary compounds that can reduce the digestibility of the plant material by the ant or its fungus, such as tannins.

As shown by a diet study (Farji Brener & Protomastro 1992) and a "pick-up" assay (Pelotto, unpubl. data), the leaf-cutting ant *Acromyrmex striatus* Roger (Formicidae, Attini) avoids leaves of *Ziziphus mistol* Griseb. (Rhamnaceae) trees in a semi-arid Chaco forest of Santiago del Estero Province, Argentina. This deciduous tree species is "apparent" (sensu Feeny 1976) to herbivores, owing to its high abundance in the area (about 30% total basal area; Lopez de Casenave et al. 1995); so a heavy investment into plant defenses is predictable. Moreover, estimates of *Acromyrmex striatus* nest density in the study site are so high as 24 nests/ha (Bolkovic & Pelotto, unpubl. data), representing a strong herbivore pressure.

With this evidence in mind, we analyzed putative chemical defenses (tannins, phenolics and saponins) in young and mature leaves of *Ziziphus mistol* and tested both polar and non-polar leaf extracts for ant-repellence to find out the chemical fraction responsible of ant rejection. Additionally, we included into our bioassays two flavonoid compounds, namely quercetin and its glycoside rutin, which are present in *Ziziphus mistol* leaves (Pelotto & Del Pero Martínez 1993a), and commercial quebracho tannin.

METHODS

Study area

The study was conducted in an old-growth forest of the Copo National Park (25°55'S; 62°05'W), Santiago del Estero, Argentina. Vegetation is a xerophytic woodland dominated by *Schinopsis quebracho-colorado*, *Aspidosperma quebracho-blanco* and *Ziziphus mistol* in the tree stratum, and *Acacia praecox*, *Celtis pallida* and *Capparis retusa* in the shrub stratum. Climate is semi-arid, markedly seasonal with a cold dry period (April-September) and a warm wet season (October-March). For a more detailed description of the site see Lopez de Casenave et al. (1995).

Leaf collection

Ten adult trees (>1.10 m height) of *Ziziphus mistol* were selected for leaf collection. Mature and young leaves were collected in March and October 1991, respectively. Leaves were oven-dried at <45 °C in the field within 12 h post-collection. Young and mature leaves were discriminated by position along branch, colour and toughness, and only healthy leaves were included. Tree damage by herbivores or fungus either prior to or during sampling was not evident.

Chemical analysis

For each individual, a sample of leaf-powder (250 mg) was exhaustively extracted in 80% methanol under reflux. On methanolic extracts we determined condensed tannin content by BuOH/HCl (PRO) assay (Swain & Hillis 1959) for proanthocyanidins and vanillin (VAN) assay (Price et al. 1978) for catechin-based tannins, and total phenols (FD) by Folin-Denis assay (Kritzman & Chet 1980). Calibration curves were constructed with commercial quebracho colorado extract, a mixture of catechin and epichatechin 1:1, and tannic acid as standards, respectively. Lectures were done by triplicate. The actual concentrations of phenolic compounds in the leaves were estimated in terms of equivalent concentrations of the above standards and were expressed as mg/g dry mass.

Hydrolyzable tannins were qualitatively investigated on the crude extracts after acid hydrolysis of an extract aliquot by chromatography. Acid hydrolysis was performed with 2N HCl heating at 100 °C for 45 min. Ethereal extract of the hydrolyzate was co-chromatographed on TLC cellulose/forestal with authentic samples of gallic and ellagic acids. Detection of gallic and/or ellagic acid with spray of Folin-Denis reagent plus ammoniac would be indicative of hydrolyzable tannins presence (Harborne l985).

Saponins were evaluated by its haemolytic capability following the procedure proposed by Jones and Elliott (1969). Crude saponin extracts were prepared by soaking 750 mg of *Ziziphus mistol* leaves in powder, representing a pool of five individuals (150 mg each one), in 10 mL of physiological solution for 120 min. After filtration extracts were refrigerated at 3 °C until used. A 2% erythrocyte solution was prepared by centrifuging human blood at 5.9 g for 5 min, washing the collected red blood cells twice and adding 20 mL to 980 mL of physiological solution. To evaluate each plant extract, a series of 10 tubes with 1 mL of the erythrocyte solution each one was used. One mL of the plant extract was added to tube 1, mixed, and 1 mL transferred to tube 3. The contents of tube 3 were mixed and transferred, as above, through tubes 5, 7 and 9. One mL of a diluted plant extract (1.3 mL plant extract + 0.7 mL physiological solution) was placed in tube 2, mixed, and transferred in the same manner through tubes 4, 6, 8 and 10. The volume of 2% erythrocyte solution/volume of undiluted plant extract was 1.00/1.00, 1.00/0.65, 1.50/0.50, 1.50/0.33, 1.75/0.25, 1.75/0.16, 1.88/0.13, 1.88/0.08, 1.94/0.06, 1.94/0.04 in tubes 1 to 10, respectively. The tubes were incubated at 37 °C for 30 min, and then allowed to remain at room temperature for 15–20 min before reading. We recorded the lowest concentration of plant extract causing complete haemolysis, defined as absence of erythrocyte sedimentation at the bottom of the test tube. An haemolysis index was calculated as the quotient between the volume of 2% erythrocyte solution and the volume of undiluted plant extract in the first tube where complete haemolysis occurred. This value is indicative of saponin content. Each assay was repeated twice.

Means of chemical analysis were compared using a Two-tailed t-test for equal variances (FD assay) or a t'-test for unequal variances when samples were heteroscedastic (PRO and VAN assays; Snedecor & Cochran 1980).

Bioassay

To test for repellence we ran a field bioassay similar to that designed by Hubbell et al. (1983). In our case, two colonies growing >4 km apart in the study site were assessed. The bioassay consisted of a comparison of pickup rates of control and extract-treated flakes of pressed oat offered to the ants on a foraging arena. Sixty control and 60 test positions were randomly distributed on a grid built on acetate. Each time a new clean (free of trail pheromone) grid was used. A sample of 1 g of pressed oat flakes was soaked in 1 mL of solvent alone (control flakes) or in 1 mL of extract (test flakes) for 1 min, and air-dried. Equal numbers (60) of control and test flakes were then offered to the ants on the acetate plate. We counted the number of test and control flakes taken at the point when half of the control or test flakes had been removed, whichever came first. The significance of the difference d in the number of control and test flakes removed was assessed by a modified binomial test. The null hypothesis was that there was no differential repellence or attractiveness of test oat flakes vs. control flakes. The P values associated with difference d in number of control and treated flakes removed are: $d < 13$, not significant; $d = 13$, $P < 0.06$; $14 \leq d \leq 16$, $P < 0.05$; $17 \leq d \leq 18$, $P < 0.01$; $19 \leq d \leq 20$, $P < 0.005$; $d = 20$, $P < 0.001$; $22 \leq d \leq 23$, $P < 0.0005$; and $d \geq 25$, $P < 0.0001$ (Hubbell et al. 1984).

Ziziphus mistol extracts were prepared as follows: 15 g (dry weight) of mature leaves were extracted successively with chloroform (nonpolar extract) and then with 80% methanol (polar extract). After concentration in vacuum, the residues were taken into 15 mL of chloroform or 80% methanol, respectively.

Four different concentrations of commercial quercetin and rutin were tested: 1%, 0.5%, 0.1% and 0.01% (w/v). Pure methanol was used as solvent. Quebracho tannin was tested using 40%, 10%, 1% and 0.1% (w/v) aqueous solutions.

RESULTS

The results of the phytochemical screening of the *Ziziphus mistol* leaves are summarized in Table 1. Condensed tannins and total phenols were significantly in higher concentrations in mature than in young leaves. On the contrary, presence of saponins was only detected in young leaves. We were unable to detect hydrolyzable tannins on both young and mature leaves.

Condensed tannins were the principal phenolics in mature leaves as suggested by the significant correlation between their concentrations (PRO-FD: $r_s = 0.77$, $P = 0.009$; VAN-FD: $r_s = 0.79$, $P = 0.006$; Spearman rank correlations), whereas simple phenols, maybe tannin precursors, predominated among phenolics in young leaves as showed by the absence of correlation between tannin and total phenols measurements (PRO-FD: $r_s = -0.31$, $P = 0.38$; VAN-FD: $r_s = 0.24$, $P = 0.51$; Spearman rank correlations).

Table 2 shows the results of the bioassay for repellence of the chloroform and methanol extracts, as well as of the flavonoid and quebracho tannin solutions. As our bioassay measured repellence ("control" minus "treated"), positive entries in the Table 2 are repellent extracts (solutions), and negative entries are attractive extracts (solutions). We used a two-tailed test because we had no a priori expectation of repellence or attractiveness of the extracts or solutions to the ants.

The non-polar extract was significantly repellent ($P < 0.06$) for both colonies, whereas the polar extract was not significantly attractive. Quebracho tannin, quercetin, and rutin did not exhibit significant attractiveness or repellence at the tested concentrations. In several cases, the effect of a given concentration depended on colony, tending to be attractive for one colony and repellent for the other. Within a colony, no relationship was evident between attractiveness or repellence and concentration.

Table 1. Results of the chemical assays performed on young ($n = 10$) and mature ($n = 10$) leaves of *Ziziphus mistol* from a semi-arid Chaco forest at Copo National Park. Data are means, with CV (%) in brackets. nd: not detected. PRO: condensed tannins (proanthocyanidins), mg/g leaf dry weight as commercial quebracho colorado equivalents. VAN: condensed tannins (catechin-based tannins), mg/g leaf dry weight as catechin and epichatechin 1:1 equivalents. FD: total phenols, mg/g leaf dry weight as tannic acid equivalents. HT: hydrolyzable tannins. HI: saponins, haemolysis index. Means were compared by a Two-tailed t-test for equal variances (FD assay) or a t'-test for unequal variances (PRO and VAN assays). *: $P < 0.05$; **: $P < 0.001$.

Tabla 1. Resultados de los análisis químicos realizados a hojas jóvenes ($n = 10$) y maduras ($n = 10$) de *Ziziphus mistol* del bosque chaqueño semiárido del Parque Nacional Copo. Los datos son promedios, con el CV (%) entre paréntesis. nd: no se detectó. PRO: taninos condensados (proantocianidinas), mg/g peso seco en equivalentes de extracto comercial de quebracho colorado. VAN: taninos condensados (taninos basados en la catequina), mg/g peso seco en equivalentes de una mezcla de catequina y epicatequina 1:1. FD: fenoles totales, mg/g peso seco en equivalentes de ácido tánico. HT: taninos hidrolizables. HI: saponinas, índice de hemólisis. Los promedios se compararon con una Prueba t de dos colas para muestras con igualdad de varianza (ensayo FD) o una Prueba t' de dos colas para muestras con heterogeneidad de varianza (ensayos PRO y VAN). *: $P < 0.05$; **: $P < 0.001$.

	Young leaves	Mature leaves	*P*
PRO	643.61 (23.27)	862.68 (32.79)	*
VAN	176.39 (37.79)	378.59 (33.54)	**
FD	28.43 (27.87)	49.36 (16.40)	**
HT	nd	nd	
HI	7	nd	

DISCUSSION

Plant defenses

Ziziphus mistol leaves exhibited a bimodal phenological pattern of defense allocation characterized by saponins picking up early to decline and be replaced by condensed tannins with ageing. Bimodality in phenological pattern of leaf defense appears to be a general phenomenon (Dement & Mooney 1974; Cooper-Driver et al. 1977; Coley 1983; Hatcher 1990), and may be explained under both the classical theories of apparency (Feeny 1976) and optimal defense (Rhoades 1979), and the more recent and comprehensive growth-differentiation balance model (Herms & Mattson 1992). Younger, rapidly expanding leaves have lower probabilities of being attacked and depend on low concentrations of highly potent,

Table 2. Results of the repellence tests of *Ziziphus mistol* polar and non-polar extracts, commercial quebracho tannin and the flavonoids quercetin and rutin, at a series of concentrations (%), on *Acromyrmex striatus* from a semi-arid Chaco forest at Copo National Park. Positive *d*-values are indicative of repellence while negative values indicate attractiveness. *P*- values associated with *d* are listed in the corresponding column. ns: not significant.

Tabla 2. Resultados de los ensayos de repelencia de los extractos polar y no polar de *Ziziphus mistol* y de soluciones de distintas concentraciones (%) de extracto comercial de quebracho y de los flavonoides quercetina y rutin, sobre *Acromyrmex striatus* del bosque chaqueño semiárido del Parque Nacional Copo. Valores positivos de *d* indican repelencia, mientras que los negativos indican atracción. Los valores de probabilidad *P* asociados con *d* están indicados en la columna correspondiente. ns: no significativo.

	Colony 1		Colony 2	
	d	*P*	*d*	*P*
Ziziphus mistol				
polar extract	-7	ns	-9	ns
non-polar extract	13	0.0595	23	<0.0005
Comm. quebracho tannin				
0.1%	9	ns	9	ns
1%	6	ns	-3	ns
10%	6	ns	-8	ns
40%	12	ns	7	ns
Quercetin				
0.01%	-1	ns	1	ns
0.1%	-12	ns	7	ns
0.5%	6	ns	1	ns
1%	-4	ns	10	ns
Rutin				
0.01%	2	ns	-1	ns
0.1%	-9	ns	7	ns
0.5%	-4	ns	2	ns
1%	3	ns	8	ns

low-molecular-weight toxins and deterrents (e.g., saponins, terpenoids, alkaloids, cyanogenetic glycosides and glucosinolates). These "qualitative" secondary metabolites (sensu Feeny 1976) are effective at low concentrations and are removed only temporarily from the plant's primary metabolic resource pool. This is compatible with the physiological trade-off between growth and defense that limits secondary metabolism during periods of intense growth, such as leaf flushing, when the demand of limited plant resources is high. As leaf maturation proceeds, concentrations of qualitative defenses generally decline as a result of dilution as leaves expand and/or the cycling of low-molecular-weight secondary metabolites into primary metabolism, and simultaneously, leaves are protected by increasing concentrations of high-molecular-weight polymers such as lignin and tannins ("quantitative" defenses) and structural defenses. Fully expanded leaves that have ceased to grow can divert resources into the synthesis of defenses to counter the higher risk of herbivory, since they are a more predictable resource to herbivores.

Chemicals and ant deterrence

The results of our bioassays suggest that some unidentified constituent of the non-polar fraction of the *Ziziphus mistol* extract account for the rejection of the entire leaves by *Acromyrmex striatus*. Although further research is necessary for the isolation of the specific active compound(s), its terpenoid nature can be suspected on the base of two reasons. First, terpenoids have been found in *Ziziphus mistol* leaves (Mendiondo et al. 1973). Secondly, leaf-cutting ant deterrence is well documented for a variety of terpenoids: mono-, sesqui- and diterpenes (Hubbell et al. 1983; Hubert & Wiemer 1985; Howard et al. 1988; Barnola et al. 1994), triterpenoids (Okunade & Wiemer 1985b; Hammond et al. 1990; Sugayama & Salatino 1995), sesquiterpene lactones (Okunade & Wiemer 1985a). Terpenoids are known to have toxic or inhibitory effects on both ants and fungus (Hubert & Wiemer 1985; Howard et al. 1988). Therefore, harvest of plant material containing these compounds, either for use as fungus substrate or as a source of ant nutrition, may have serious effects on the viability of both symbiotic partners. Thus, leaf terpenoids would operate as a selective force bringing to plant avoidance by ants.

Our results are consistent with the finding that ant deterrence has only been well established for either crude non-polar plant extracts (Hubbell et al. 1984; Howard 1987) or compounds purified from them, including metabolites other than terpenoids (e.g., cinnamic acid derivatives; Capron & Wiemer 1996). On the contrary, no clear relationship have been demonstrated between polar plant extracts or their constituents, such as phenolics or tannins, and ant repellence. In the present work, neither the methanolic extract of

Ziziphus mistol leaves nor the flavonoid and quebracho tannin solutions were active on ants. The assayed methanolic extract was a mixture of polar compounds such as condensed tannins, flavonoids and saponins (Pelotto & Del Pero de Martínez 1993a, and this study); the effects on ants of these individual compound classes will be discussed in the mentioned order.

Tannins have been involved in ant avoidance of plants or its parts on a qualitative base mainly, but there are some studies that assess the relationship between tannin concentration and ant acceptability (Nichols-Orians & Schultz 1990; Nichols-Orians 1991a, 1991c, 1991d; Pelotto & Del Pero Martínez 1993b). In this line, it has been shown that ant deterrence as well as growth inhibition of the attine fungus by condensed tannins are concentration dependent and increase with tannin levels (Nichols-Orians 1991a, 1991b, 1991c, 1991d; Powell & Stradling 1991). Working with *Atta cephalotes*, Nichols-Orians (1991a, 1991c, 1991d) has suggested that condensed tannins would be only effective at higher concentrations. Although we tested quebracho tannin at a higher dose than she did (30% w/v), we failed to demonstrate ant deterrence by these compounds. Moreover, our tannin-rich methanolic extract did not discourage ant foraging. Our data do not provide support for the idea that higher levels of tannins in the leaves prevent ant consumption.

This study is, to our knowledge, the first to test flavonoids for ant repellence. As showed by a number of studies, flavonoids may have stimulant or antifeedant effects on other herbivores (Harborne 1997 and references therein). However, leaf-cutting ants seem to be insensitive to these ubiquitous secondary compounds. Notwithstanding, a wider structural array of aglycones and glycosides should be tested for a more conclusive result.

Saponins could explain ant deterrence of young *Ziziphus mistol* leaves because some evidence exists to show that they are capable of deterring and possibly killing leaf-cutting ants (Febvay & Kermarrec 1986), but mature leaves lacking saponins are equally avoided (Pelotto, unpubl. data). Moreover, the polar extract containing these compounds was ineffective to arrest ant foraging, and this result arises as the main reason to deny a defensive role for saponins against leaf-cutting ants in *Ziziphus mistol*.

This study provides an explanation to why *Ziziphus mistol* is not represented in the *Acromyrmex striatus* diet (Farji Brener & Protomastro 1992). However, when we offered disks of young and mature leaves in a mixture with other five plant species and a control to three colonies, foragers picked-up some *Ziziphus mistol* disks (Pelotto, unpubl. data); but once in the nest, workers rejected most of them next to the nest entrances before 24 h (Pelotto, pers. obs.). To explain this ant behavior, we can suggest the following arguments. In our bioassays, the ants are much more likely to come into immediate contact with the repellent(s), since that non-polar extract was surface-applied; whereas, in the entire leaves, repellent(s) would be sequestered into leaf tissues and may be less concentrated at the leaf surface. In a pick-up assay, the ants do not experience the prolonged contact with leaf sap that they must experience when they are cutting the leaf, and as a consequence, ants could fail to detect deterrents. In the nest, gardening ants (minor workers) chew and cut the plant material into small pieces to supply the fungus gardens with it. At this moment, workers may detect the harmful compounds and reject materials which contain them. Taking into account the above mentioned, one might conclude that the pick-up assay overestimates the true palatability of *Ziziphus mistol*, and the same would also be expected for other less acceptable species.

Conclusions

Leaf-cutting ants may avoid *Ziziphus mistol* plants either because the leaf chemistry is harmful to the ants and/or the ants have learned not to cut leaves containing substances toxic to their mutualistic fungus. According to our results, the presence of some unknown lipidic compound(s) in the leaves seems to be sufficient to explain deterrence of leaf-cutting ants at all. Nevertheless, other antiherbivore plant defenses have been recorded in this species such as tannins, saponins, leaf toughness and nectivorous ants associated with homopterans (Folgarait et al. 1994 and this study). Interactions between different defenses can occur in nature. For instance, the presence of certain tannins could inhibit fungal enzymes responsible for detoxifying toxic terpenoids, enhancing their toxicity (Nichols-Orians 1991b). Anyway, our results represent a starting point for future investigations on *Ziziphus mistol* chemical constituents as a source of natural pesticides for ant control.

Finally, regarding the question about how plant resistance to ant attack has evolved, two hypotheses can be suggested. One involves directly leaf-cutting ant herbivory as a selective factor leading to ant deterrence. The other has been proposed by Hubbell et al. (1983) and suggests that defense against ant attack may be largely an incidental benefit of selection for fungal resistance. In the battle against fungi, plants would synthesize compounds that are also toxic to the attine fungus, affecting ants that depend on it. Both hypotheses are waiting for testing in *Ziziphus mistol*.

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