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RESEARCH ARTICLE

Mutagenicity of *Hypericum lysimachioides* extracts

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Abstract

Hypericum (Hypericaceae) species are extensively used in several fields such as traditional medicine, food and crop protection. Despite its usage in many fields, the identification of the genotoxic potential of this herb is still incomplete. In this study, we evaluated genotoxic effects of the petroleum ether, hexane, ethyl acetate, and methanol extract of *Hypericum lysimachioides* Boiss. var. *lysimachioides* by Ames *Salmonella*/microsome test and SOS chromotest. The mutagenic activity of *Hypericum lysimachioides* var. *lysimachioides* extracts was investigated by using *Salmonella typhimurium* strains *TA98* and *TA100* and also the SOS chromotest with *Escherichia coli* PQ37 strain, with or without S9 metabolic activation. In this initial report we demonstrated that all extracts of *H. lysimachioides* var. *lysimachioides* showed significant mutagenic activity on both strains of *Salmonella* either with or without S9 mixture. No mutagenicity was found in the SOS chromotest either with or without S9 mixture. These results indicate a significant mutagenicity of the petroleum ether, hexane, ethyl acetate and methanol extracts of *Hypericum lysimachioides* var. *lysimachioides in vitro*. It can be suggested that quercetin and flavonol or their synergistic effects may be main mutagenic agents in the photopharmaceuticals *Hypericum lysimachioides* var. *lysimachioides* extract.

Keywords: *Hypericum lysimachioides; Hypericum extracts; mutagenicity; Salmonella microsome test; SOS chromotest*

Introduction

Environmental substances such as synthetic chemicals and daily foods contain a high number of genotoxic agents. Recently, some authors have reported that various degenerative diseases, such as cancer, neurodegenerative, reproductive diseases and cardiovascular disorders, are associated with exposure to these genotoxic substances (Aruoma, 1994; De Flora et al., 1996). These substances consist of many compounds, whose origins have proved to have considerable pharmacological activity. There are undesirable properties of some compounds, such as mutagenicity, carcinogenicity and toxicity. Also, the medicinal use of some drugs of vegetable origin is only possible after modification of their chemical structures, frequently resulting in string pharmacological activity and lower toxicity and mutagenicity (Simoes et al., 1999).

In many parts of the world, thousands of species of endemic higher plants are used in traditional medicine. Also, there are many studies showing considerable interest in mutagenicity and antimutagenicity of medical plants used by traditional herbalists. The plants used commonly in traditional medicine have been considered to be benign and not to have any side effects due to their long use and being natural in origin. This concept is largely circumstantial and it is important to determine toxicology of plant extracts, especially those which are used frequently over long periods (Elgorashi et al., 2002; Popat et al., 2001).

Hypericum (Hypericaceae) is one of the plants used traditionally in medicine, crop protection, and flavoring, as well as fragrance in food (Isman et al., 2001; Daferera et al., 2003). This genus is represented in the flora of Turkey by 77 species (Robson, 1975). The leaves of *Hypericum* species, when held to the light, reveal translucent dots, giving the impression that the leaf is perforated. Therefore, this species known as “binbirdelikotu” or “kantaron” in Turkish (Baytop, 1984; Saya et al., 2001).

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Chemical compositions of these extracts are well documented, and they include hypericin, hyperforin, quercetin, quercitrin, xanthon, pyrone, and phloroglucinol (Umek et al., 1999; Verotta et al., 2000; Constantine & Karchesy, 1998; Chung et al., 1999; Decosterd et al., 1991; Ferraz et al., 2001; Kikuchi et al., 1985; Trifuniviç et al., 1998).

Hypericin, a major bioactive component of *Hypericum* species, is widely used in neurological disorders and depression (Okpanyi et al., 1990). Recently, hypericin has been shown to have antitumor activity (Vandenbogaerde & Witte, 1995; Kamuhabwa et al., 2000), highly active against HIV (Takahashi et al., 1989; Meruelo et al., 1988), and exhibits a number of pharmacological effects. Antimicrobial and antioxidant activities of *Hypericum lysimachioides* Boiss. var. *lysimachioides, Hypericum triquetrifolium* Turra. and *Hypericum scabroides* Robson & Poulter were also reported (Toker et al., 2006; Hakimoğlu et al., 2007; Kızıl et al., 2008).

Quercetin is another component of *Hypericum* which was determined to be mutagenic in numerous studies. In one study of quercetin it was found to be mutagenic without S9 mix activation, and with S9 mix it increased significantly (Bjeldanesi & Chang, 1997). Quercetin was also indicated to have comutagenic activity on 2-acetylaminofluorene (AAF) (Ogawa et al., 1987). The 3-*O*-acetyl ester of flavonol, quercetin, was mutagenic with S9 mix. Three quercetin glycosides were mutagenic after pre-incubation with hesperidinase, a crude extract of *Aspergillus niger* (Nagao et al., 1981). Furthermore, quercetin enhanced the mutagenicity of the tricyclic aromatic amines (aminofluorene, aminoanthracene, and aminophenanthrene) and their acetamides by 1.2–5.9-fold (Adam et al., 1990).

Animals fed with *H. perforatum* flowers for 2 weeks showed significant signs of toxicity, including erythema, edema of the portion of the body exposed to light, alopecia, and changes in blood chemistry. Mutagenic activity in a *Salmonella*/microsome test was attributed to flavonols in one study and to quercitin in another, but other genotoxicity assays were negative. Adverse reactions to *Hypericum* extract in the clinical treatment of depression include skin rashes and itching, dizziness, constipation, fatigue, anxiety, and tiredness (Bjeldanesi & Chang, 1997; Okpanyi et al., 1989). Ethanol, chloroform, and hexane extracts of *H. perforatum* significantly reduced growth in all three cell lines (Schmitt et al., 2006). The hexane fraction of *Hypericum ascyron* L. and the *n*-BuOH fraction of *Hypericum japonicum* Thunb. both showed appreciable antibacterial activities with minimum inhibitory concentration (Mu et al., 2006).

The bacterial tests are commonly employed as an initial screening for genotoxic activity. Two of these bacterial tests are *Salmonella*/microsome (Ames test) and SOS chromotest, which have been recommended by various workers as a valid indicator of mutagenicity/genotoxicity of various substances present in the environment. The *Salmonella*/microsome mutagenicity test is an *in vitro* test detecting point mutation on *Salmonella typhimurium* strains. The SOS chromotest shows primary DNA damage on *Escherichia coli* PQ37.

Although *Hypericum lysimachioides* Bois var. *lysimachioides* usage is widespread, the identification of the genotoxic potential of this herb is still incomplete. Therefore, this study was performed to investigate the mutagenic activity of the petroleum ether, ethyl acetate, hexane and methanol extracts of *H. lysimachioides* var. *lysimachioides* by using the *Salmonella*/microsome assay and SOS chromotest, either with or without metabolic activation.

Materials and methods

The strains *S. typhimurium TA98* and *TA100* were purchased from the Salmonella Genetic Stock Centre (University of Calgary, Canada), and their genetic backgrounds were controlled as described (Ames et al., 1973; Levin et al., 1982) every 6 months.

The strains *S. typhimurium TA98* and *TA100* were used with and without addition of rat hepatic metabolic fraction S9 mix (Maron & Ames, 1983).

The bacteria strain *E. coli* PQ37 was obtained from M. Hofnung (Institute Pasteur, Paris). Its genotype was controlled as described by Quillardet and Hofnung (1985).

Collection of plant material

H. lysimachioides var. *lysimachioides* was collected from the south-eastern Anatolia region in June 2005. A voucher specimen has been deposited in the Herbarium of the Department of Biology, Faculty of Science and Arts, Dicle University (DUF-2514). It was identified by A. Selçuk Ertekin from the same institution.

Preparation of extracts

Aerial parts (stems, leaves and flowers) were dried for 10 days at room temperature. The dried and finely powdered bark material was extracted by adding solvents in increasing order of polarity, namely petroleum ether, hexane, ethyl acetate, and methanol. The obtained extracts were kept in dark glass bottles at 4°C until use.

Aliquots were prepared from the dried crude extracts and dissolved in 10% DMSO to give an initial concentration of 1, 0.5 and 0.05 mg/mL for mutagenic screening by using the Ames test as described by Maron and Ames

(1983) and SOS chromotest as described by Quillardet and Hofnung (1985).

Salmonella mutagenicity assay

The potential mutagenic effects of *Hypericum* extracts were evaluated on two *S. typhimurium* strains *TA98* and *TA100*. *S. typhimurium TA98* and *TA100* were used to identify reverse mutations from histidine dependence to histidine independence by the plate incorporation method of Ames et al. (1973). The plate incorporation assay was performed according to Maron and Ames (1983), by adding 0.1 mL of the overnight bacterial culture and 0.1 mL of crude *Hypericum* extracts at different concentrations to the test tubes. In the case of metabolic activation, 0.5 mL S9 mixture was supplemented. After incubation of the culture for 24 h at 37°C, 2.5 mL of top agar was added to each tube and then plated on minimal agar. Histidin revertants were counted after 72 h of incubation at 37°C on a colony counter.

The S9 mixture is mammalian liver tissue that is used in the *Salmonella*/microsome test to provide a first approximation of mammalian metabolism. S9 mix, used routinely for general mutagenesis screening, was prepared from livers of male rat according to Maron and Ames (1983). All steps were performed at 4°C with cold and sterile solutions and glassware. S9 fraction was distributed in aliquots in small sterile tubes and stored at -80°C. The crude *Hypericum* extracts were prepared freshly for each experiment by dissolving them in DMSO. The positive controls used were 1 μg/plate NaN_3 for *TA100*, 1.5 μg/plate 2-AF for *TA98*. Compounds were tested by using five concentrations depending on the samples tested. Three plates each in two separate experiments were used for each concentration tested and for positive and negative controls.

SOS chromotest

The genotype of *E. coli* PQ37 strain was described by Quillardet and Hofnung (1985). In this assay, the *lacZ* gene of the tester strain *E. coli* PQ37 is fused to the bacterial *sfiA* SOS operon. Thus, *lacZ* is concomitantly expressed in the case of bacterial SOS response; photometrically determined β-galactosidase induction is indicative of the extent of SOS induction and bacterial genotoxicity. Bacterial alkaline phosphatase activity serves to determine the range of bacterial cytotoxicity; the ratio of bg/ap activity is termed as induction factor IF and indicates the extent of SOS induction of a compound tested.

This assay was performed according to Quillardet and Hofnung (1985) and following adaptation of Marzin et al. (1986). In brief, 100 μL of a 13 h overnight culture of *E. coli* PQ37 was diluted with 5.1 mL La-medium (L-medium + ampicillin) and incubated until a transmission of 40% at 600 nm was reached (corresponding to 200×10^6 cfu/mL). This culture was diluted as 1:40 with L-medium, resulting in 5×10^6 cfu/mL. Test substances and cultures were incubated for 2.5 h at 37°C, and after incubation, the mixtures were divided into two series: One for β-galactosidase activity measurement (an induction assay) and the other for alkaline phosphatase activity (a control of protein synthesis). β-galactosidase activity was determined after addition of 2.7 mL of B buffer and 600 μL ONPG (ortho-nitrophenyl-β-D-galactopyranoside) as substrate. Constitutive alkaline phosphatase activity was determined after addition of 2.7 mL of P buffer and 600 μL PNPP (*p*-nitrophenyl phosphate) as substrate. All mixtures were incubated at 37°C for 25 min in water bath, and then photometrical determinations were performed at 405 nm.

Calculations of test results

1. The means and standard deviations were calculated. Data obtained in improved *Salmonella*/microsome test were subjected to ANOVAs. Data obtained in tests were calculated and expressed in percentage frequency and the significance at different dose levels was tested by application of ANOVA test. Differences between the groups were considered to be statistically significant at $P < 0.05$. All data were analyzed by using SPSS 13.0.
2. SOS chromotest: Responses of the SOS chromotest were calculated as ratios of β-galactosidase and alkaline phosphatase activities. The genotoxic activity for concentration *c* may be expressed in the ratio $R_c = \beta/ap$, where β represents β-galactosidase, where ap represents alkaline phosphatase activity. The induction factor (IF) for a compound at concentration *c* is defined as $I = R_c/R_o$, in which Ro is the spontaneous ratio measured in the blank test (solvent control). Experiments were performed with and without the metabolic activation mixture for each extract tested. In order to ensure the validity of the assay, a positive control was included in each experiment. The positive control used was 4-NQO (1 μg/mL) without S9 mix and B[a]P (30 μg/mL) with S9 mix. Compounds were tested by using four concentrations, depending on the extracts tested.

Results

The petroleum ether extract of *H. lysimachioides* (3.67 g) was obtained as a yellow powder, the hexane extract

(1.31 g), as a yellowish powder, the ethyl acetate extract (2.87 g) as a brown powder, and the methanol extract (24.12 g) as a purple powder. The results of the *Hypericum* extracts are shown in Tables 1 and 2. Different concentrations of extracts were evaluated when the assay was carried out with *Salmonella*, both in the presence and absence of metabolic activation mixture. The revertant clones obtained at each concentration and treatments were scored manually in the *Salmonella*/microsome test.

Acute observation, under a magnifying glass, of the bacterial lawn in each culture dish was used to check for possible toxicity of the treatment. Spontaneous reversion of the tester strains to histidin independence was measured routinely in mutagenicity experiments, and expressed as the number of spontaneous revertants per plate.

All tested *Hypericum* extracts were seen to have positive results with *S. typhimurium TA98* and *TA100*, both in the presence and absence of S9 mix. The mean number of revertants of the substances tested, the reference mutagens and the negative control with their corresponding standard deviations were determined. Each sample was assayed with three replicate plates at each concentration (Table 1). The results show their mean values and standard deviations (SD) of them. The concentration of the bacterial suspension was 0.8-2.4 × 10^9 cfu/mL. Criteria for a positive test result were as follows: at least double in the number of spontaneous revertants (factor > 2) and a dose-response effect compared to the spontaneous mutation frequency.

As illustrated in Table 1, all *Hypericum* extracts were found to have a mutagenic activity in both strains of *S. typhimurium TA98* and *TA100*, in the absence or presence of S9 mix. The petroleum ether, hexane and methanol extracts of *H. lysimachioides* var. *lysimachioides* were shown to have a significant mutagenic activity in *S. typhimurium TA100*, in the presence of S9 mix.

The bacterial SOS chromotest with *E. coli* PQ37 was used for the assessment of genotoxicity. Responses with positive control included in each experiment of the SOS chromotest were calculated as discussed in the Material and method section. As shown in Table 2, *Hypericum* extracts did not induce the SOS response in the *E. coli* PQ37 strain with or without S9 mix. The results of samples of *Hypericum* extracts in SOS chromotest are presented in Table 2.

Table 1. *Salmonella* microsome test results of petroleum ether, ethyl acetate, hexane and methanol extracts of *H. lysimachoides* var. l*ysimachoides* on *S. typhimurium* strains.

Extract		Number of *His*+ revertants per plate (mean ± SD)			
		TA98		*TA100*	
Concentration tested μg/plate		-S9	+S9	-S9	+S9
Control		52.4 ± 4.5	101 ± 8.4	201 ± 17.8	328.8 ± 33.7
DMSO		64 ± 12.2	68.3 ± 18.3	163.3 ± 22.3	169.3 ± 16.2
Sodium azide		-	-	1786 ± 452	1412 ± 339.9
2-AF		81 ± 13	388 ± 16	-	-
Petroleum ether	5	309.3 ± 33.6	324 ± 19.5*	1391.5 ± 212.8	2750.3 ± 362.4**
	15	332 ± 14	341.6 ± 4.4**	1437.3 ± 190	2580.3 ± 339.2**
	30	333.8 ± 38	318.5 ± 24*	1408.5 ± 183.5	2760 ± 452**
	45	346.8 ± 30.3	309.7 ± 39.6*	1349.5 ± 256.7	2672.6 ± 447.7**
	60	204 ± 31.8[a]	188 ± 43.2[a]*	970.6 ± 177.9[a]	1050 ± 396.4[a]
Ethyl acetate	5	412.2 ± 102	340.5 ± 20.2*	1835.6 ± 344.7	1556.1 ± 585.6*
	15	379.8 ± 78.3	371 ± 76**	1756.8 ± 287.9	1295 ± 267.5**
	30	397 ± 94	373.1 ± 27.9**	1822.8 ± 261.3	1323 ± 134.9*
	45	270.6 ± 48.2	357 ± 41.8*	1758.6 ± 287.1	1484 ± 144.2**
	60	159.3 ± 56.7[a]	184.5 ± 84.5[a]*	884.3 ± 413.2[a]	971.5 ± 170.2[a]*
Hexane	0.1	1324.6 ± 181.5	1192 ± 262.6**	2765.7 ± 681.9	4162.2 ± 739**
	1	1381 ± 214	1206.4 ± 181.6**	2658 ± 589.7	4236.9 ± 875,6*
	10	1418.5 ± 350.5	1267.5 ± 220.9**	2875.8 ± 763	4344.3 ± 627.4**
	25	1350.9 ± 290.8	1272.8 ± 179.8**	2675.4 ± 833.2	4124 ± 1042**
	50	850 ± 463[a]	735 ± 237[a]**	1354 ± 818.3[a]	2382.1 ± 1081.8[a]**
Methanol	0.1	363 ± 48.2	615.6 ± 99.2**	1382.3 ± 238.6	5511 ± 657.9**
	1	444.3 ± 91.4	610 ± 171.3**	1408 ± 364.3	5641 ± 784.4**
	10	466.1 ± 53.4	607.6 ± 100.7**	1381 ± 257.9	6386.8 ± 726*
	25	335 ± 79.9	604.7 ± 63.8**	1403.5 ± 134.1	5888 ± 521.7**
	50	239.4 ± 83.3[a]	297.5 ± 138.5[a]**	815.6 ± 229.2[a]	28592.1 ± 679[a]*

Values are means ± SD of three replicates.
[a]Toxicity.
*P < 0.05 (ANOVA).
**P < 0.01 (ANOVA).

Table 2. The SOS response induction of petroleum ether, ethyl acetate, hexane and methanol extracts of *H. lysimachoides* var. l*ysimachoides* in *E. coli* PQ37.

Extract	Concentration tested (μg/assay)	Induction factory* S9-	Induction factory* S9+
0.20(4NQO)		3.28 ± 0.55	3.32 ± 0.88
0.10(4NQO)		3.58 ± 0.92	3.64 ± 0.90
Petroleum ether	0	1 ± 0	1 ± 0
	1	0.97 ± 0.11	1.04 ± 0.03
	10	1.17 ± 0.19	1.02 ± 0.04
	20	1.21 ± 0.05	1 ± 0.02
	40	1.38 ± 0.06	1.18 ± 0.24
	60	1.53 ± 0.07	1.23 ± 0.30
	80	1.85 ± 0.05	1.05 ± 0.05
	100	0.85 ± 0.03[a]	0.94 ± 0.02
Ethyl acetate	0	1 ± 0	1 ± 0
	1	0.99 ± 0.04	1.11 ± 0.03
	10	1 ± 0.07	1 ± 0.02
	20	1.17 ± 0.05	1.07 ± 0.05
	40	1.5 ± 0.02	1.02 ± 0.30
	60	1.39 ± 0.03	1.13 ± 0.02
	80	1.66 ± 0.04	1.06 ± 0.05
	100	0.9 ± 0.04[a]	0.91 ± 0.01
Hexane	0	1 ± 0	1 ± 0
	1	0.99 ± 0.04	0.97 ± 0.01
	10	1 ± 0.02	1.03 ± 0.02
	20	1.17 ± 0.03	1.12 ± 0.04
	40	1.5 ± 0.06	1.15 ± 0.02
	60	1.39 ± 0.05	1.09 ± 0.01
	80	1.66 ± 0.04	1.06 ± 0.03
	100	0.71 ± 0.03[a]	0.97 ± 0.02
Methanol	0	1 ± 0	1 ± 0
	1	0.99 ± 0.04	0.98 ± 0.04
	10	1 ± 0.05	1.01 ± 0.01
	20	1.17 ± 0.05	1.07 ± 0.02
	40	1.5 ± 0.6	1.15 ± 0.05
	60	1.39 ± 0.5	1.19 ± 0.03
	80	1.66 ± 0.04	1.06 ± 0.04
	100	0.81 ± 0.5[a]	0.9 ± 0.05

Each of β-galactosidase and alkaline phosphatase units is average of eight data which were obtained from four experiments.
[a]Toxic or bacteriostatic effect

Discussion

The studied plants are orally or topically used in folk medicine for the treatment of various diseases; however, there are no reports on the toxicity of *H. lysimachioides* var. *lysimachioides* in the literature. In this initial report, we demonstrated that the evaluated extracts induced frameshift mutations on *S. typhimurium TA98,* base pair substitution on *S. typhimurium TA100,* but did not induce oxidative DNA damage in the strain *E. coli* PQ37.

The *Salmonella*/microsome mutagenicity test is commonly employed as an initial screening for genotoxic activity, and in particular for point and frame shift mutation inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests (OECD, 1977).

SOS chromotest is a bacterial test for detecting DNA-damaging agents. It is a colorimetric assay based on the detection of primary damage to DNA as measured through the SOS DNA repair system. In this test system, damage of the DNA results in the activation of the SOS system (Quillardet & Hofnung, 1985).

In our study we have investigated the mutagenic effect of *Hypericum* extracts which were employed to detect the induction of base pair mutations as well as frame shift mutations in the *S. typhimurium* strains *TA98* and *TA100* and primary DNA damage in *E. coli* PQ37.

As it can be seen in Table 1, different concentrations tested in the *Salmonella*/microsome test were found to be significantly ($p < 0.01$, $p < 0.05$) mutagenic both in the presence and absence of S9 mix. The petroleum ether, hexane and methanol extracts of *H. lysimachioides* var. *lysimachioides* were found to be more effective on *S. typhimurium TA100* than *TA98* in the presence of S9 mix. Therefore, these extracts can be assumed to be strong mutagens, causing base pair substitution.

Positive results from the *Salmonella*/microsome test indicate that a substance induces point mutations by base substitutions or frame shifts in the genome of either *S. typhimurium* and/or *E.coli*. Negative results, however, indicate that, under the test conditions, the test substance is not mutagenic in the tested species.

As indicated in Table 2, *Hypericum* extracts did not induce SOS response in *E. coli* PQ37 strain with or without of S9 mix. The reason for the lower sensitivity of the SOS chromotest may be due to relatively short-term contact with the genotoxic agents (or extracts make the presence of SOS inhibiting compounds possible). It is likely that *Hypericum* extracts make the presence of SOS inhibiting compounds possible.

However, plant extracts exhibiting a positive response and hence a mutagenic effect need to be extensively investigated to determine their possible genotoxicity to humans, as their safe use in traditional medicine is questionable. Screening is required to identify and eradicate the use of all mutagenic plants, since numerous studies have shown that the proportion of carcinogens identified as mutagens by the Ames test ranges from about 50% to 90% (Zeiger, 2001). Most of the mutagenicity was exhibited in the presence of metabolic activation in this study.

The short-term bacterial tests used in this study are reliable, quick and easy. These tests are used to screen for possible carcinogens; however, a positive result does not necessarily indicate the substance as being a carcinogen. Also, if a substance screened does not suggest a

mutagenic response, it does not necessarily confirm that it is not mutagenic or carcinogenic. It confirms that the substance is not mutagenic to the particular bacterial strain used for the genetic endpoint tested.

From this study and earlier results, we suggest that quercetin and flavanol or their synergistic effect can be the main mutagenic factor (Nagao et al., 1981; Ogawa et al., 1987; Adam et al., 1990; Bjeldanesi & Chang, 1997). The positive results obtained from the *Salmonella*/microsome test might be checked through further experiments, such as animal tests. These investigations support the view that results from short-term bacterial tests are of very limited transferability to human.

In conclusion, the results from the present study suggest that *H. lysimachioides* var. *lysimachioides* extracts are mutagenic in the *Salmonella*/microsome test but do not induce the SOS response in *E. coli* PQ37 strain. The genotoxic activity observed in the *Salmonella*/microsome test might result from several mechanisms, and short-term bacterial test systems are not capable of detecting all possible mechanisms. We think that a test battery consisting of at least both bacteria and mammalian assays should be used in both *in vitro* and *in vivo* assays. To test for carcinogenicity, a two-year carcinogenicity test is required to be performed by testing the effect of the mutagenic sample in mice and rats (male and female) (Zeiger, 2001).

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