Research Article

Comparative Study of Antioxidant Activities of *Nitraria retusa* and Quantification of Its Bioactive Components by GC/MS

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ABSTRACT

There is a growing interest in finding new antioxidant substances from natural sources. *Nitraria retusa* is a common medicinal plant in some Mediterranean countries. The present study deals with the evaluation of total phenolic and flavonoid contents, antioxidant activity and GC/MS profile of petroleum ether extracts of *N. retusa* collected from four different regions in Egypt (referred as A, B, C and D). The highest content of phenolic was found in the extract of region D (55±0.53 µg/g GAE, d.w). However, the extract of region C contains the highest flavonoid content (99±0.56 µg/g QUE, d.w) among all other extracts. To evaluate the antioxidant activities of the extracts, three *in vitro* tests were employed, i.e., radical scavenging activity (DPPH), total antioxidant activity and hydroxyl radical scavenging activity assays. The highest DPPH and total antioxidant activity was detected in the extract of region C however the lowest activity was observed in extract of region A. Using GC/MS analysis; some important compounds such as phenolic and terpenoids were identified. The present result revealed that *N. retusa* extracts contain variable patterns of phenolic, flavonoid, and various bioactive volatile compounds and it could be applied as a natural antioxidant source for pharmaceutical purposes.

Keywords: DPPH, Flavonoid, Free radicals, Phenolic, Terpenoids, Volatile compounds.

INTRODUCTION

The wild medicinal plants are rich source of phytochemicals, such as carotenoids, flavonoids and other phenolic compounds having high free-radical scavenging activity, which helps to reduce the risk of chronic diseases, such as cardiovascular disease, cancer, and age related neuronal degeneration.[1] Reactive oxygen species (ROS) cover a wide range of chemical components, including superoxide anion, hydrogen peroxide and hydroxyl radicals. However, the innate defense in human body may not be enough for severe or continued oxidative stress. Hence, exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS. Medicinal plants contain large amounts of antioxidant compounds, which can play an important role in adsorbing and neutralizing free radicals.[2]

The antioxidant and radical scavenging activities of medicinal plant extracts are correlated with their phenolic compound content.[3] Phenolic compounds are one of the most widely occurring groups of phytochemicals that exhibit antioxidants, antibacterial, antiviral, anti-cancer and anti-inflammatory properties.[4] Moreover, environmental stress, especially salt and drought stress might be responsible for the increase or decrease in the content of relevant natural products and may have a significant impact on the synthesis and accumulation of secondary plant products.[5] *Nitraria* L. species are belongs to family Nitrariaceae, in Egypt it represented by a monotypic species *Nitraria retusa* (Forssk.) Asch., it is widely spreads in deserts, salty sands, grows in marshes along the Red Sea coast and Sinai. The leaves, fruits and seeds of some species are often used in folklore medicine as an antispasmodic, antineuropathic, and anti-arrhythmic agent.[6] Furthermore, the ethyl acetate extract of *N. retusa* has a significant antioxidant and antigenotoxic activity in human chronic myelogenous leukemia cell line.[7] Previous chemical study on *N. retusa* have shown the presence of *O*-glycosides of flavones and flavonols compounds in addition to flavones *C*-glycosides which exhibits a notable activity in protecting against oxidative stress.[8] The aim of the present investigation was to evaluate the total phenolic, total flavonoid contents and antioxidant activities as well as GC/MS profile analysis in the petroleum ether extracts of *N. retusa* collected from four different regions in Egypt.

MATERIALS AND METHODS

Plant collection

Four samples of *N. retusa* were collected from different areas in Egypt. The samples collected were labeled as A, B, C, and D, and the location and collection date were as follows: A) Cairo-Ras Sudr road (35 km) North Sinai, B) Cairo-Ras Sudr road (30 km) North Sinai, C) El-Ain El-Sokhna-El-Suez road (66 km) beside the beach and D) Wadi Rishrash, two hours from Cairo along the Korymat road. Samples were identified by Dr. S.R. Hussein in the Herbarium of Phytochemistry and Plant Systematic Dept., National Research Centre - Egypt.

Preparation of plant crude extracts

The shade dried aerial parts of *N. retusa*, were ground to powder using a mechanical grinder. Ten grams of each ground plant materials were shaken separately in 200 ml of petroleum ether (60-80°C) at room temperature on an

orbital shaker (Heidolph Unimax 2010) for 72 h. Extracts were filtered, and each filtrate was concentrated to dryness under reduced pressures at 40°C using a rotary evaporator (Heidolph-Germany). The dried petroleum ether crude extracts were re-dissolved in methanol for further analysis.[9]

Determination of total phenolic (TP) content

Total phenolic content in all extracts was determined using Folin-Ciocalteu reagent method as described by Slinkard and Singleton.[10] Briefly, 300 µl of each plant extract was made up to 3 ml with methanol then mixed thoroughly with 0.5 ml of Folin-Ciocalteu reagent. After 5 minutes, 2 ml of 5% sodium carbonate was added to the mixture. The mixture was allowed to stand in darkness for 60 min at 30°C. The absorbance was determined using spectrophotometer (Unicam UV300) at 650 nm. The TP was expressed as µg of gallic acid equivalents (GAE) per g of dry weight.

Determination of total flavonoid (TF) content

A modified method of Chang *et al.*[11] was used to determine total flavonoid content in *N. retusa* extracts. 300 µl from each extract was separately mixed with 1.7 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M sodium acetate and 2.8 ml of distilled water. After standing for 30 min at room temperature, the absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. The TF was expressed as µg of quercetin equivalents (QUE) per g of dry weight.

Antioxidant activity assays

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of *N. retusa* extracts was determined according to our previously published procedure.[3] One ml of DPPH radical solution (0.1 mM) in methanol was mixed with 3 ml of petroleum ether extracts at various concentrations (0.5, 1.0 and 1.5 mg/ml). Discoloration was measured at 517 nm after 30 min using spectrophotometer. BHT was used as positive control. Measurements were taken in triplicate. The ability to scavenge the DPPH· radical was calculated using the following equation:

DPPH· scavenging activity (%) = $[A_{DPPH} - A_S / A_{DPPH}] \times 100$

Where, A_{DPPH} is the absorbance of the DPPH solution and A_S is the absorbance of the solution when the sample extract is added. The extract concentration providing 50% inhibition of radical scavenging activity (IC_{50}) was calculated and expressed as mg/ml.

Total antioxidant activity

The total antioxidant activity of *N. retusa* extracts was carried out following the method of Pan *et al.*[12], one ml of each extract at different concentrations (0.5, 1.0 and 1.5 mg/ml) was mixed with 3 ml reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min, after cooling at room temperature; the absorbance was measured at 695 nm using spectrophotometer against blank (The reaction mixture without the sample). Gallic acid was used as positive control. The total antioxidant activity was expressed as absorbance of the sample.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of *N. retusa* extracts was assayed by the method of Smirnoff and Cumbes.[13] One ml of each extract at different concentrations (0.5, 1.0 and 1.5 mg/ml) was mixed with 1ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide and 0.3 ml of 20 mM sodium salicylate. The reaction mixture was incubated in a water bath for one hour at 37°C, after incubation the absorbance of the mixture was measured at 562 nm. Gallic acid was used as positive control.

The scavenging activity of hydroxyl radical was calculated as follows:

Scavenging activity (%) = $[1- (A_1-A_2) / A_0] \times 100$

Where, A_0 is absorbance of the control (without extract) and A_1 is the absorbance in the presence of the extract and A_2 is the absorbance without sodium salicylate.

Gas chromatography/mass spectrometry (GC/MS) analysis

The analysis of *N. retusa* extracts were performed using a Thermo Scientific capillary gas chromatography (model Trace GC ULTRA) directly coupled to ISQ Single Quadruple MS and equipped with TG-5MS non polar 5% phenyl methylpolysiloxane capillary column (30 m × 0.25 mm ID × 0.25 µm). The operating condition of GC oven temperature was maintained as: initial temperature 40°C for 3 min, programmed rate 5°C/min up to final temperature 280°C with isotherm for 5 min.

For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. 1 µl of each extract was injected automatically in the splitless mode. The quantification of the components was based on the total number of fragments (total ion count) of the metabolites as detected by the mass spectrometer. The identification of the chemical components was carried out based on the retention time of each component (R_t) compared with those of the Wiley 9 and NIST 08 mass spectra libraries.[14]

Statistical analysis

All data are presented as means ± SE, the mean values were calculated based on the data taken from at least three independent experiments conducted on separate days using freshly prepared reagents.

RESULTS AND DISCUSSION

Total phenolic and total flavonoid contents

The aerial parts of different *N. retusa* extracts exhibited different variations in their contents of both total phenolic and total flavonoid depending upon the collected regions from where they were collected (Figure 1). In totality, the plant collected from region D gave the highest content of total phenolic (55±0.53 µg/g GAE, d.w). Whereas, the extract of *N. retusa* collected from region C gave the highest content of total flavonoid (99±0.56 µg/g QUE, d.w).

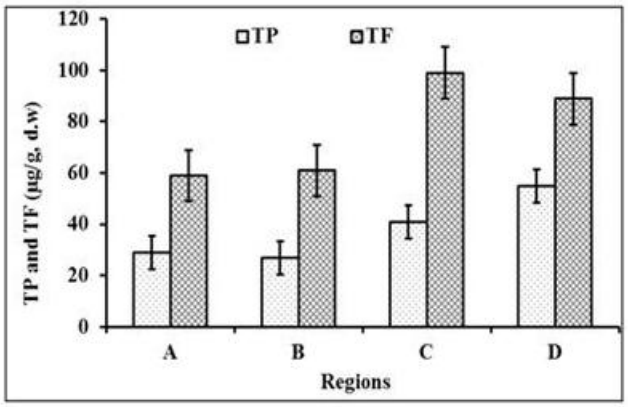

Figure 1: Total phenolic (TP) and total flavonoid (TF) contents (µg/g, d.w) of petroleum ether extracts of *N. retusa* collected from four different regions (A, B, C and D). (n= 3, value = mean ± SE).

The impact of the collected region on the total phenolic and total flavonoid contents is supported by the fact that variety of diverse factors such as climatic conditions, biotic and abiotic stresses may affect the production of secondary metabolites in plants.[5] The author explained this point as follows; under environmental stress, a strong oversupply of reduction equivalents is generated in order to prevent damage by oxygen radicals, $NADPH+H^+$ is re-oxidized by photorespiration cycle. Yet, the high concentration of reduction equivalents also leads to a stronger rate of synthesis of highly reduced compounds, i.e. isoprenoids, phenols or alkaloids. In view of that, the plant collected from region D where the desert climate gave the highest content of total phenolic content due to limited water supply and much higher light intensities, this kind of stress cause production of high amounts of secondary metabolites.[15] It is well known that, the levels and types of phenolic and flavonoid vary significantly, depending on not only plant species but also solvent nature.

Antioxidant activities

DPPH free radical scavenging activity

Figure 2 revealed that the radical-scavenging activities of all *N. retusa* extracts increased with increasing concentration (dose dependent manner). The antioxidant activity values of *N. retusa* extracts collected from regions C, B, D and A was 40.9, 33.8, 19.6 and 15.9 %, respectively at 1.0 mg/ml concentration. Generally lower IC_{50} indicating the higher antioxidant activity of the extract. The values of IC_{50} were in the ascending order BHT > extract of region C > extract of region B > extract of region A > extract of region D with values of 0.1, 1.2, 1.4, 2.9 and 3.9 mg/ml respectively. These results indicated that *N. retusa* extract collected from region C exhibited the highest DPPH radical scavenging activity compared to extracts of *N. retusa* collected from other regions, but it still gave low DPPH radical scavenging activity compared to the synthetic antioxidant compound (BHT) which used as positive control.

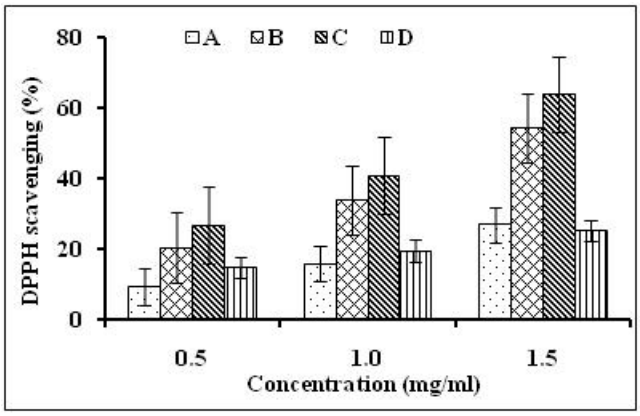

Figure 2: DPPH scavenging activity of petroleum ether extracts of *N. retusa* collected from four different regions (A, B, C and D). (n= 3, value= mean ± SE).

In this study the DPPH scavenging activity of *N. retusa* may be attributed to the presence of some phenolic compounds such as eugenol and butylated hydroxytoluene besides to other component other than phenolic compounds such as stigmasterol and hexadecanoic acid which has been identified in the *N. retusa* petroleum ether extracts by GC/MS as presented in Table (1). The antioxidant activity and DPPH scavenging activity of eugenol, butylated hydroxytoluene, stigmasterol and hexadecanoic acid have been reported.[16-19]

The global antioxidant property of a plant extract is generally considered as the result of the combined activity of a wide range of compounds such as phenolics, terpenoids, peptides, organic acids and other components.

Total antioxidant activity

The absorbance of petroleum ether extracts ranged from 0.027 to 0.138 among all extracts as shown in Figure 3. The total antioxidant capacity of *N. retusa* extracts increased with increasing the concentration of samples. However, petroleum ether extract collected from region C exhibited the highest total antioxidant activity (0.138) at 1.5 mg/ml concentration, while this activity is lower than that exhibited by gallic acid (used as positive control) which gave absorbance (0.171) even at 100 µg/ml concentration.

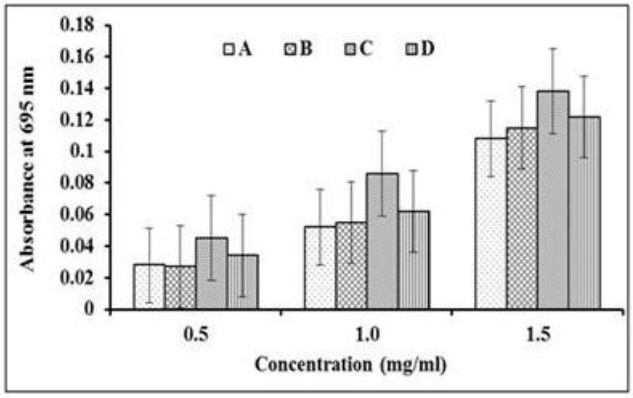

Figure 3: Total antioxidant activity of petroleum ether extracts of *N. retusa* collected from four different regions (A, B, C and D) (n= 3, value= mean ± SE).

Total antioxidant capacity of *N. retusa* extracts at 1.5 mg/ml concentration exhibited the following order: C>D>B>A. In this concern, the total antioxidant activity of methanolic extract of *N. retusa* fruits was evaluated by Hegazy *et al.*[20] and they found that *N. retusa* fruits exhibited total antioxidant activity ranged from 0.16 to 0.25 absorbance at 695 nm. In the present study the highest total antioxidant activity of *N. retusa* petroleum ether extract collected from region C might be attributed to its highest content of total phenolic and flavonoid contents as presented in Figure 1. The antioxidant activity of plant materials is well correlated with the content of their phenolic compound.[21] Indeed, the total antioxidant activity of *N. retusa* may be also attributed to the presence of eugenol in petroleum ether extracts which has been identified by GC/MS (Table 1).

Hydroxyl radical scavenging activity

Scavenging ability of *N. retusa* petroleum ether extracts collected from four different regions (A, B, C and D) at different concentrations (0.5-1.5 mg/ml) on hydroxyl radical is given in Figure 4. The percentage of inhibitions was increased with increasing the concentrations of the extracts. As shown from Figure 4, a region dependent of hydroxyl radical scavenging activity was clearly demonstrated. *N. retusa* petroleum ether extract collected from region D exhibited the highest scavenging activity (27.01 %) at concentration 1.5 mg/ml compared to the other regions at the same concentration. But, it was still lower than that of gallic acid which gave 29.16 % of hydroxyl radical scavenging activity at 400 µg/ml concentration. The hydroxyl radical scavenging activity of *N. retusa* petroleum ether extracts could be attributed to the presence of flavonoids and other phenolic compounds especially in plant collected from region D which contains moderate amounts of total phenolic and flavonoid as presented in Figure 1.

Also the antioxidant activity may be due to the presence of α-Eudesmol (belong to sesquiterpene group) which has been identified only in the GC/MS profile of region D (Table 1).

Moreover, the hydroxyl radical scavenging activity of *N. retusa* might be attributed to the inhibition of hydroxyl radical generation by chelating Fe^{2+} ions. Phenolic compounds were able to form complexes with Fe^{3+}, this general chelating ability of phenolic is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule.[22,23] Furthermore, phenolic compounds are reported to quench oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical.[24]

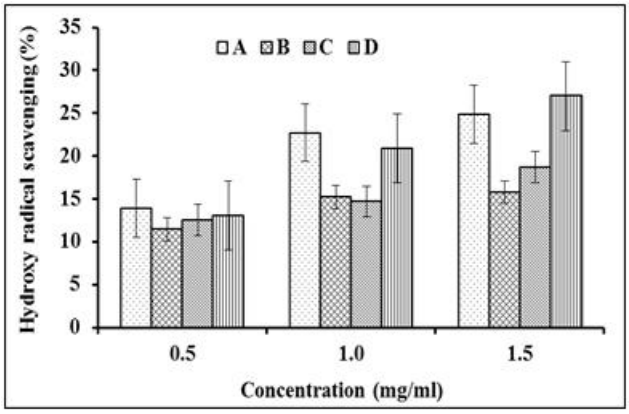

Figure 4: Hydroxyl radical scavenging activity of petroleum ether extracts of *N. retusa* collected from four different regions (A, B, C and D). (n= 3, value= mean ± SE).

GC/MS analysis

The GC/MS spectral results and comparison of results with library search successfully enabled the identification of the total 34 compounds in *N. retusa* extracts (16 from A, 16 from B, 12 from C and 20 from D) as presented in Table 1. Eugenol was one of the major compounds present in *N. retusa* extracts, it represents as (47.57%) in the extract of region A, (3.9%) of the extract of region B and (0.57%) of the extract of region C while it is not detected in region D extract, it is the principle chemical constituent of clove oil and has been used to cure dental problems and is used as antiseptic and analgesic.[25]

However, another important component identified by gas chromatography/mass spectrometry was germacrene D (a sesquiterpene hydrocarbon) which is presented only in the extract of region (A) and represents (13.52%) of the total extract. It has antimicrobial and insecticidal properties.[26] Additionally, Butylated Hydroxytoluene (BHT) was found in all extracts; it represented in extract of regions (C) and (D) at the same concentration (3%) while in extract of region A it was presented as (0.91%) and in B extract as (2.26%). The antioxidant, anticancer and antiviral activity of BHT is well characterized.[16,27] Hexadecanoic acid is a saturated fatty acid which present in all extracts of *N. retusa* but the largest concentration presented was (4.51%) in region D. It has antioxidant and anti-inflammatory activities.[28] Hentriacontane is presented in the extract of region A only by (25.08%) while tetratetracontane found in the extract of regions B, C and D by (71.84%), (65.37%) and (57.65%), respectively.

Hentriacontane (C31) was found to have the highest antitumor activity.[29] In the extract of region D, yomogi alcohol was detected at 0.38%, the yomogi alcohol was isolated from different Artemisia species and has antioxidant, antifungal; and insecticidal activities as stated by Özek *et al.*[30] Additionally, 1, 5-epoxysalvial-4(14)-ene (0.33%) present in the extract of region D only, and has not been reported previously in *N. retusa* in Egypt. Moreover, the anti-neurogenic inflammation action of α-eudesmol (identified in region D only) was reported by Asakura *et al.*[31] Methyl jasmonate, fatty acid-derived cyclopentanones, also was identified in the extract of region D (0.38%) only (Table 1). The anticancer effect of methyl jasmonate against several cell lines [32] and anti-inflammatory effect [33] were documented. This study explores the goodness of wild *N. retusa* collected from Egypt which has a commendable sense of purpose and can be advised as a phyto pharmaceutical importance.

Table 1: Chemical composition of petroleum ether extracts of *N. retusa* collected from four different regions (A, B, C and D).

NO	Compounds	R_t [a]	Relative area %			
			A	B	C	D
1	Yomogi alcohol	12.1	-	-	-	0.38
2	Nonanal	15.3	-	-	0.25	-
3	2,4-Dimethyl-5,6-dihydro-2-Hpyran	15.5	-	-	-	2.29
4	2,7-Dimethyl-2,7-octanediol	19.4	-	-	-	2.19
5	2,4-Decadinal	21.5	0.11	0.12	-	-
6	Eugenol	22.9	47.57	3.9	0.57	-
7	Caryophyllene	24.3	-	1.31	-	-
8	Germacrene D	24.4	13.52	-	-	-
9	α-Humulene	25.2	1.81	-	-	-
10	5-Hydroxy- 2-Decenoic acid lactone	25.7	-	-	-	0.9
11	Butylated Hydroxytoluene	26.5	0.91	2.26	3.06	3.01
12	Aceteugenol	26.9	3.29	-	-	-
13	1,5-Epoxysalvial-4-(14)-ene	27.9	-	-	-	0.33
14	Caryophyllene oxide	28.3	0.32	-	-	-
15	Methyl jasmonate	29.7	-	-	-	0.38
16	α-Eudesmol	29.9	-	-	-	0.45
17	Hexahydrofarnesyl acetone	33.8	0.14	0.2	0.41	0.98
18	Farnesyl acetone	35.3	0.12	0.21	0.21	1.41
19	Hexadecanoic acid, methyl ester	35.4	0.09	1.76	0.35	0.54
20	Hexadecanoic acid	36.3	0.56	1.06	2.46	4.51
21	Linoleic acid, methyl ester	38.6	-	1.02	-	0.38
22	Phytol	39.0	0.21	0.98	0.78	0.86
23	Linoleic acid	39.5	0.11	-	0.59	0.55
24	Methyl stearate	39.2	-	0.15	-	-
25	Stigmasterol	46.2	-	0.31	-	-
26	α-Sitosterol	49.15	-	0.77	-	-
27	1,5-Dimethyl-6-(1,5-dimethylhexyl)-15,16-epoxy18-decane-13-oneoxatetracyclo-[9.6.1.0(2,10).0(5,9)]-oct	49.16	-	-	1.24	-
28	8,9:14,15-Dibenzo-2,4,6,16,18,20-docosahexaene-10,12-diynedial	49.19	-	-	-	2.21
29	2,3 : 4,5-bis-(1',2'[2.2]-Paracyclophane-1',9'dieno)-cyclopent-2-enone	49.31	-	-	-	3.64
30	Nonacosane	51.2	-	5.6	10.89	5.52
31	Tricaprylin	52.0	0.08	-	-	-
32	Triacontane	52.7	0.34	0.79	-	0.82
33	Tetratetracontane	54.75	25.08	-	-	-
34	Hentriacontane	54.77	-	71.84	65.37	57.65

Note: Relative proportions of the extract constitutes were expressed as percentages; [a]Retention time.

CONCLUSION

The antioxidant potential of petroleum ether extract of *N. retusa* collected from different regions may be attributed to the presence of good amounts of bioactive compounds (phenolics, flavonoids and terpenoids) that may serve as effective antioxidants. The GC/MS analysis revealed that eugenol and butylated hydroxytoluene were found in *N. retusa* extracts as the major compounds. The findings suggest that *N. retusa* could be a potential source of natural antioxidant that could have great importance as therapeutic agent. Nonetheless, further *in vivo* studies

and isolation of the compounds responsible for antioxidant activity are needed.

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