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FREE RADICAL-SCAVENGING AND ANTIMUTAGENIC POTENTIAL OF ACETONE, CHLOROFORM AND METHANOL EXTRACTS OF LEAF OF *ARGEMONE MEXICANA*.

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ABSTRACT

Antioxidant and antimutagenic potency of acetone, chloroform and methanol extracts of *Argemone mexicana* leaves were investigated by employing nitroblue tetrazolium and Ames assay. Maximum superoxide anion radicals scavenging responses noticed were 84.0, 81.0 and 75.0 % in acetone, methanol and chloroform extracts at a dose of 200µg/ml. Inhibitory potential was compared with the standard (L-ascorbic acid). IC_{50} value of acetone extract of *A. mexicana* leaves was double to that of L-ascorbic acid showing maximum inhibitory effect. Methanol extract showed maximum (antimutagenic effect) inhibition i.e. 135.4% at doses of $1.00x10^3$ in pre-incubation and 85.40 % at doses of 0.50×10^3µg/ml in co-incubation mode, respectively. Chloroform extract showed moderate inhibition i.e. 40.66 and 66.66% in co-incubation and less i.e. -17.83 and 34.00 % in pre-incubation mode at above concentrations. These results indicate that leaves of *A. mexicana* have antioxidant as well as antimutagenic activity. Further studies are in progress to evaluate the effect of leaves extracts by other antioxidant and antimutagenic assays and to identify the factors responsible for these activities.

KEYWORDS

Antioxidant, antimutagenic, *Argemone mexicana,* sodium azide

INTRODUCTION

Argemone mexicana belongs to family Papaveraceae, commonly known as Bharband. It is an erect prickly annual herb with milky latex. It grows wild and is troublesome weed, recently used in reclamation of usar. Leaves are yellow with latex, exstipulate, alternate, sessile, simple, glaucous deeply dissected with spiny teeth Seeds yield nauseous, bitter, non-edible oil, used in coetaneous troubles. It is cathartic; presence of argemone oil in edible mustard oil is probably responsible for outbreaks and epidemic dropsy. Mixed with drying oils such as linseed oil, it may be used in the paint industry. It is also used for soap making.

Reactive oxygen species (ROS) cause breakage of important bimolecular, important cross links like DNA-DNA and DNA-protein, sister chromatid exchanges, in addition to base modification, which have been related with coronary heart diseases, carcinogenesis and many other health problems associated with the old age[1,2,3].As the free radicals play major role in causing the diseases, the supply of antioxidants, in the diet is of great importance for a healthy life[4].Foods of plant origin and medicinal plants have been suggested as natural sources of antioxidants[5,6,7,8].

Environmental mutagens are a threat to public health and cancer has become the number one cause of death in the world though the infectious diseases are now more or less under control[9]. Chemical carcinogenesis was of great interest in scientific investigation. But little attention had been paid to the substances in the environment/ in diet that may protect against chemical mutagens or carcinogen acting as inhibitor in the carcinogenesis process. These chemicals are present in plants which may act as anticarcinogens or antimutagens by blocking ultimate carcinogen electrophiles in a nucleophilic chemical reaction to form innocuous products. A continuous input of these could serve as buffer against DNA damage. A wide array of phenolic substances particularly those present in dietary and medicinal plants have been reported to possess both antimutagenic and anticarcinogenic activities[10,11,12,13,14].

Keeping in mind the medicinal value of *Argemone mexicana*, the present investigation was planned to study the antioxidant and antimutagenic effect of acetone, choloroform and methanol extract using nitroblue tetrazolium (NBT) superoxide scavenging and *Salmonella* microsomes assay.

MATERIAL AND METHODS

Chemicals

Ascorbic acid, ntroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), dimethyl sulfoxide (DMSO), histidine and phenazine methosulfate (PMS) were procured from Hi-media Laboratory Pvt. Ltd. Bombay.

Leaves

The leaves of *Argemone mexicana* were collected in the month of March from the plant growing in Mohindergarh district (North India). It was washed with tap water (twice), dried in oven at 40 ^{o}C for 24 h and ground to fine powder.

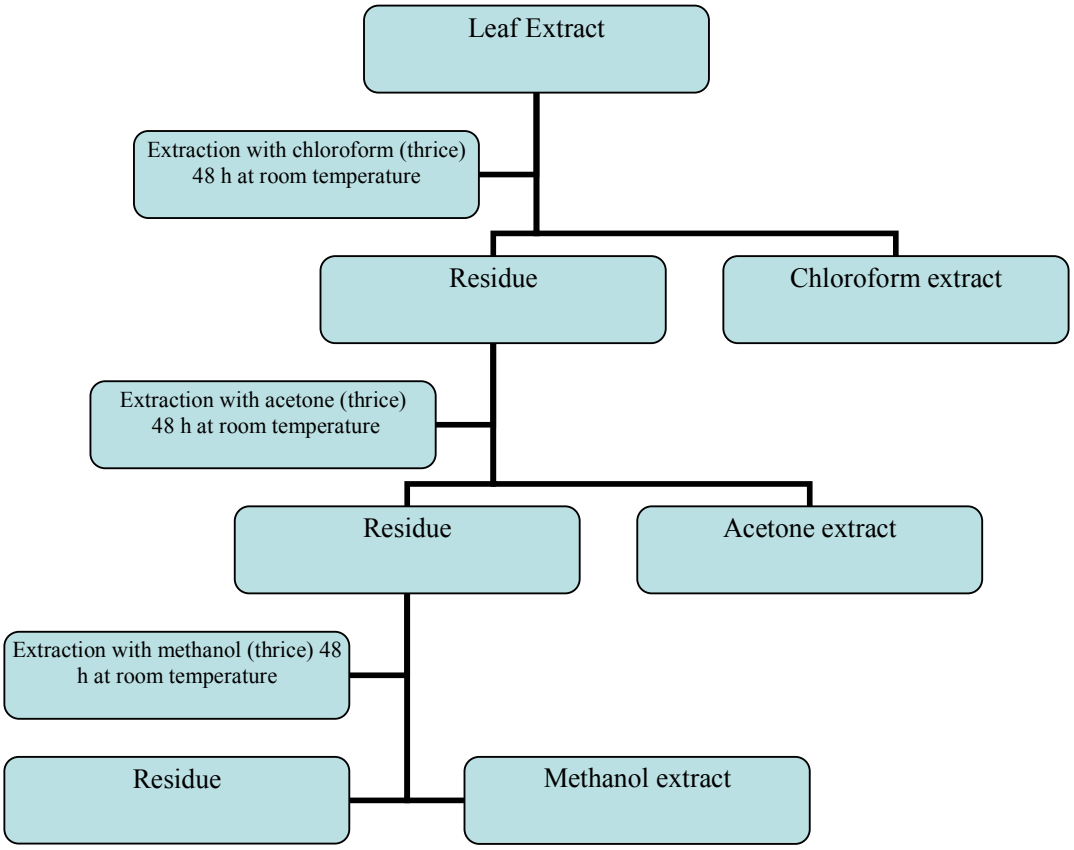
Preparation of extract

To 500 g of leaves powder, 1500 ml each of the solvent viz. chloroform, acetone and methanol were added serially (Flow chart). After filtering through the several layers of mesclin cloth, filterate in different solvents was recovered. The respective solvent from the filterate was evaporated under vacuum at a temperature below 50 ^{o}C.

Extracts were re-dissolved in dimethyl sulfoxide (DMSO) and various concentrations were used for antioxidant and antimutagenic activities.

Culture

Salmonella typhimurium TA100 tester strain (base substitution tester) used in the anitmutagenic study was a kind gift from Dr. (Mrs) Saroj Arora, Department of Botanical and Environment Sciences, Guru Nanak Dev University, Amritsar.



Flow chart: Extraction by maceration of leaf powder of *Argemone mexicana* by inceasing polarity.

Antioxidant testing assays

Nitroblue tetrazolium (NBT) superoxide scavenging assay

The superoxide anion radical scavenging activity was assayed as described by Liu and Ng[15]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide anions radicals were generated in 3.0 ml of Tris-HCl buffer (16 µM, pH 8.0)

containing 750 µl of NBT (50 µM) solution, 750 µl of NADH (78 µM) solution and 300 µl of different concentrations (25-175 µg/ml) of extracts. The reaction was initiated by adding 750 µl of PMS (10 µM) to the mixture.

After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in a spectrophotometer against blank. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. L-Ascorbic acid was used as a positive control. The antioxidant activity of test samples was evaluated by calculating the percent inhibition of superoxide anion radicals by applying the following formula:

$$\% \text{ Inhibition} = \frac{A0-A1}{A0} \times 100$$

Where

A0: is the absorbance of the control (blank, without extract)

A1: is the absorbance in the presence of the extract.

The IC_{50} values (the concentration required to inhibit superoxide radical formation by 50%) of plant extracts were determined and compared with L-Ascorbic acid, used as positive control.

Antimutagenicity test

The plate incorporation assay, as suggested by Maron and Ames[16] was used in the present investigation to check the inhibitory activity of acetone, chloroform and methanol extracts. To check the antimutagenicity of the extracts in TA100 tester strain of *Salmonella typhimurium*, two modes of experimentation were followed viz. co-incubation and pre-incubation. During co-incubation, to 2 ml of top agar containing 0.5 ml histidine, 0.1 ml of fresh overnight grown *Salmonella* culture of tester strain (TA100), 0.1 ml of sodium azide and 0.1 ml of extract were added. A pre-incubation procedure was also followed where equal volume of positive mutagen and extract were mixed in sterile capped tube and allowed to stand for 30 min at 37 ^{o} C, 0.2 ml of this was added to 2 ml of soft agar with 0.1 ml of bacterial culture.

After pouring the soft agar on minimal agar plates, the plates were titled, rotated and placed on a leveled surface to harden for half an hour and then incubated at 37 ^{o}C for 48 h. Similar experiments were also designed for other concentrations and extracts. A set of positive control (where only mutagen was added), negative control (only extract) and spontaneous reversion (only bacterial inoculum) was added in each experiment. After 48 h of incubation, the numbers of revertant colonies were counted against a background lawn. The concentrations of various extracts used for antimutagenicity assay were 1.00×10^3, 0.5×10^3 and 0.10×10^3 µg/ 0.1 ml/ plate. The test samples were testing against direct acting mutagen i.e. sodium azide (2.5 µg/0.1ml/plate) in TA100 tester strain of *Salmonella typhimurium*. All the test samples were dissolved in DMSO. Sodium azide was dissolved in water. In each case, no over toxicity was observed and the numbers of spontaneous revertants were identical to the DMSO vehicle control. Non toxic concentrations were determined to be those without statistically significant difference in the number of the spontaneous revertant colonies, size of colonies and intensity of the background lawn as compared to the control where no extract was added. Each concentration was tested in triplicate and the entire experiment was repeated thrice.

The inhibitory activity is expressed as percentage decrease of reverse mutation:

$$\text{Percent inhibition} = \frac{[X-Y]}{[X-Z]} \times 100$$

Percent mutagenic activity was calculated as:

$$\text{Percent of control} = \frac{Y}{X} \times 100$$

Where

X: is the number of histidine revertants induced by mutagen alone (sodium azide).
Y: is the number of histidine revertants induced by mutagen in the presence of extract.
Z: is the number of revertants induced in negative control.

Statistical analysis

All the experiments were repeated three times. Results are reported as mean ± SE (not shown in graphs). IC_{50} values were also calculated.

RESULTS AND DISCUSSION

NBT (superoxide scavenging) assay

The acetone, chloroform and methanol extracts of leaves of *Argemone mexicana* quenched NBT superoxide anion radicals in a dose dependant manner because as the concentration of extracts increased, the NBT superoxide radicals quenching activity was also increased. The degree of discoloration indicates the superoxide scavenging potential of the extracts. Fig. 1 shows the superoxide scavenging activity of acetone, methanol and chloroform extracts of leaves of *Argemone mexicana* with L-ascorbic acid as control. The order of effectiveness of the extracts was: acetone extract (84.0 %) > methanol extract (81.0 %) > chloroform extract (75.0 %) at 200 µg/ml.

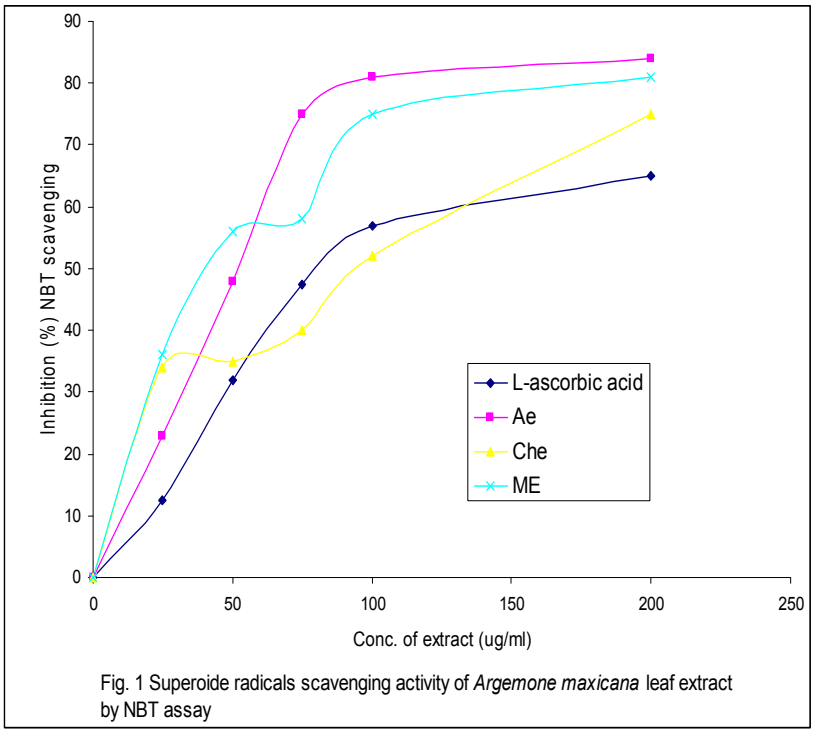


Fig. 1 Superoide radicals scavenging activity of *Argemone maxicana* leaf extract by NBT assay

Table.1 shows that the IC_{50} value for the acetone extract was found to be 40.0 µg/ml whereas for methanol and chloroform, the values were 52.0 µg/ml and 78.0 µg/ml, respectively which are less

than L-ascorbic acid i.e. 81.0 µg/ml. Maximum superoxide radicals were inhibited by all the three extract of *Argemone mexicana* at a dose of 200 µg/ml. The superoxide scavenging activity of *A. mexicana* leaf extracts was remarkably higher than that of L-ascorbic acid.

Table 1

IC_{50} values of acetone, chloroform and methanol extract of leaves of Argemone mexicana in antioxidant system.

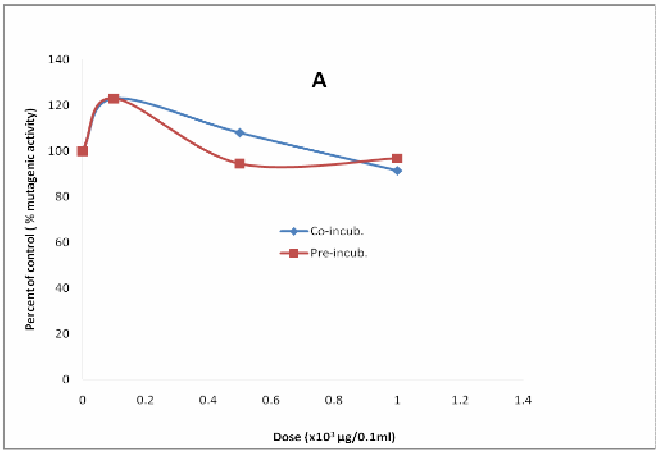
S. No.	Assay	Extract/standard	IC_{50}value (µg/ml)
1	NBT superoxide scavenging assay	Ascorbic acid	81
		Acetone	40
		Chloroform	78
		Methanol	52

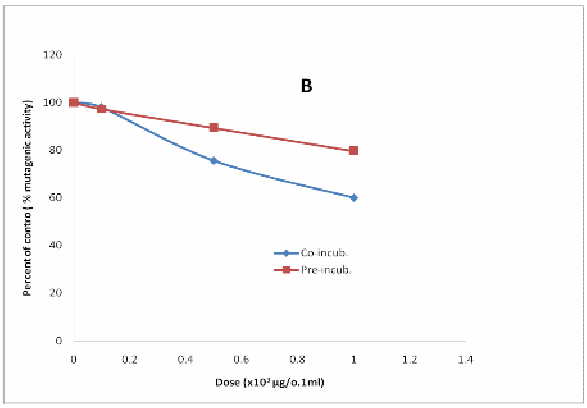
In group cellular oxidation reactions, superoxide radicals are normally formed first and their effect can further magnified because of production of other kinds of free radicals and oxidizing agents[15]. Besides these, xanthine oxidase is one of the main enzymatic sources of these reactive oxygen species *in vivo*. In the current study, IC_{50} value of acetone extract of leaves of *A. mexicana* is just double to that of L-ascorbic acid showing the maximum inhibitory effect followed by methanol and chloroform extracts. It is noteworthy that the superoxide scavenging activity of *A. mexicana* leaf extracts is superior to that of L-ascorbic acid. IC_{50} value of acetone extract of leaves of *A. mexicana* (40 µg/ml) is larger than that of *Paeonia suffruticosa* (50 µg/ml)[15] but less than *Poligonum aviculare* L. (0.8µg/ml)[17]and *Polygonium cuspidatum* (3.2µg/ml)[17].

Antioxidants are also known to block the free radical chain reaction of auto-oxidation by donating the hydrogen of the phenolic hydroxyl thereby giving rise to a stable end product which does not initiate the further oxidation of lipids[18]. The data showed that acetone, methanol and chloroform extracts are free radical scavenger and may act as primary antioxidants, which may react with the free radical by donating hydrogen. Maximum inhibition was observed in higher concentrations of acetone extract of leaves followed by methanol and chloroform (Fig.1). Similar work was carried out by Singh *et al.*[19] on *Acacia auriculiformis* A. cunn. and found that ethyl acetate fraction of bark extract has more DPPH scavenging as well as reducing power as compared to water fraction and crude extract. In this study it is expected that NBT radicals may get stabilized by accepting the hydrogen donated by hydroxyl group present in phenolic compounds[19]. These results indicate that *A. mexicana* leaf is an important source of scavenger of superoxide radicals.

Antimutagenicity of various extracts of Argemone mexicana

The results of antimutagenic activity of different extracts are shown in the Fig.2 and Table 2





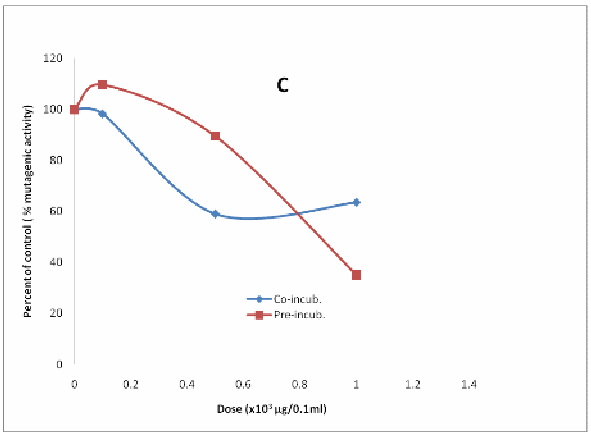


Figure. 2

Effect of acetone (A), chloroform (B) and methanol (C) extracts of leaves of Argemone mexicana on sodium azide induced mutagenicity in TA 100 tester strain of Salmonella typhimurium.

$$\% \text{ of control} = \frac{\text{His+ revertant/plate with mutagen and antimutagen}}{\text{His+ revertant/plate with mutagen}} \times 100$$

The inhibitory effect observed at different concentrations, i.e. 0.10 x 10^3, 0.50 x 10^3 and 1.00 x 10^3 in both co-incubation and pre-incubation assay was found to be dose dependent. Among the different extracts, methanol extract of leaves of *A. mexicana* exhibited maximum inhibition i.e. 3.7, 85.4 and 76.0 % followed by chloroform extract i.e. 3.33, 40.66 and 66.66 % respectively, at above concentrations in co-incubation mode. While results obtained with acetone extracts were insignificant. Similarly in pre-incubation mode of experimentation, methanol extract exhibited a strong antimutagenic activity (135.4 %) and was ranked in the strong activity group. Methanol extract showed maximum inhibitory effect i.e. -20.2, 21.8 and 135.4 % followed by chloroform extract i.e. 4.66, 17.83 and 34.0 % respectively. Similar research work was carried out by Bala *et al.*[20] on wood apple and found that overall effect of chloroform extract was negligible. Similarly the results obtained with acetone extract were found insignificant in this study also. The maximum inhibitory effect was observed in pre-incubation studies (135.4 %) while other observations of pre-incubation mode were not so satisfactory as compared to co-incubation mode (Table 2).

Table 2

Effect of acetone, chloroform and methanol extracts of leaves of Argemone mexicana on mutagenicity of sodium azide in TA100 tester strain of Salmonella typhimurium.

Concentration of extract (µg/0.1ml)	TA100 tester strain of *Salmonella typhimurium*					
	Acetone extract (AE)		Chloroform extract (ChE)		Methanol extract (ME)	
	Sp = 1000 ± 35.36 Sp + AE= 820 ± 14.14 Sa = 2000± 141.44 (2.5 x 10^3 µg/0.1ml)		Sp = 1000± 70.72 Sp+ChE = 800± 21.21 Sa = 2000 ±106.08 (2.5 x 10^3 µg/0.1ml)		Sp = 1000 ± 91.93 Sp+ME = 1040 ±14.14 Sa = 2000 ± 70.72 (2.5 x 10^3 µg/0.1ml)	
Co-incubation	**Mean ±SE**	**% inhibition**	**Mean ± SE**	**% inhibition**	**Mean ± SE**	**% inhibition**
0.10 x 10^3	2460±14.14	-38.0	1960±28.28	3.33	1964±2.82	3.7
0.50 x 10^3	2160±28.28	-13.0	1512±22.68	40.66	1180±42.43	85.4
1.00 x 10^3	1828±12.98	14.0	1200±14.14	66.66	1270±49.50	76.0
Pre-incubation						
0.10 x 10^3	2462±15.55	-39.1	1944±59.40	4.66	2194±52.33	-20.2
0.50 x 10^3	1888± 302.6	9.4	1786±74.96	17.83	1790±63.64	21.8
1.00 x 10^3	1936+181.04	5.4	1592+6506	34.00	700+56.56	135.4

Data shown are mean ± SE of three repeated experiments
Sp – Spontaneous reversion, Sa -- Sodium azide

The results of this experiments demonstrated that both the methanol and chloroform extracts contained some antimutagenic substances which inhibits the mutagenisity of sodium azide. Antimutagen present in the extracts may interact with the specific enzyme systems, which are necessary for activation of mutagens[21]. Tea extract showed strong antimutagenic action against five indirect mutagens in TA98 and TA100 tester strains of *S. typhimurium* reported by Yen and Chen[22]. Polyphenols which are the important

constituents may be one of the factor for the antimutagenicity as they have been reported as antimutagens in number of systems[23, 24, 25, 26].The enhanced antimutagenic activity of methanol extract may be due to its oligomeric nature. Gali *et al*.[27] suggested that oligomeric hydrolisable tannins have more antitumour promoting activities than monomeric hydrolisable tannins. But the exact mechanism by which the antimutagen present in the acetone and chloroform extracts inhibited the mutagenicity is not known. Further studies are underway to confirm these results with other antioxidants and antimutagenic assays and to isolate and characterize the bioactive compounds responsible for the antioxidant and antimutagenic activities in these extracts.

CONCLUSION

From the present investigation it is concluded that that leaf of *A. mexicana* have antioxidant as well as antimutagenic activity. Both the methanol and chloroform extracts contained some antimutagenic substances which inhibits the mutagenisity of sodium azide. Antimutagen present in the extracts may interact with the specific enzyme systems, which are necessary for activation of mutagens. Further studies are in progress to evaluate the effect of leaf extracts by other antioxidant and antimutagenic assays and to identify the factors responsible for these activities.

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