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Effect of Aqueous Extract of Fenugreek (*Trigonella foenum-graecum* L.) On Selected Biochemical and Oxidative Stress Biomarkers in Rats Intoxicated with Carbon Tetrachloride

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ABSTRACT
The objective of the current study was to investigate the protective effects of aqueous extract of fenugreek (*Trigonella foenum-graecum* L.) against carbon tetrachloride (CCl_4), induced hepatotoxicity in rats. Therefore, twenty rats were fed standard diet and divided into four groups. Rats in the first and second groups were injected i/p with paraffin oil and received either tap water (control 1) or aqueous extract of fenugreek (control 2), respectively. Rats in the third and fourth groups were injected i/p with CCl_4 and received either tap water or aqueous extract of fenugreek, respectively. At the end of the experiment (5 weeks), blood and liver samples were collected. Sera were used for selected biochemical analysis and liver tissues were used for estimation of selected oxidative stress biomarkers. The present findings revealed that, CCl_4 elevated serum transaminases, hepatic lipid peroxidation and hepatic enzymatic antioxidants activities. Oral fenugreek aqueous extract ameliorated the detrimental effects of CCl_4 and corrected all examined biomarkers toward the control values. The present study concluded that aqueous extract of fenugreek plays a protective role against CCl_4-induced liver damages in rats. These protective effects were in the form of improvement of serum transaminases, attenuation of hepatic lipid peroxidation and activation of hepatic antioxidant enzymes.

Key words: Carbon tetrachloride, fenugreek, antioxidants, biochemistry, serum, liver

INTRODUCTION

Carbon tetrachloride (CCl_4) is a highly toxic chemical agent, the most famous drug used to induce liver damage experimentally. The CCl_4 elevated serum enzyme activities of liver and some biochemical parameters in rats (Althnaian *et al*., 2013). Histological sectioning of liver tissues indicated that CCl_4 induced fibrosis, cirrhosis and hepatocarcinom (Junnila *et al*., 2000). It induced infiltration of great amount of mononuclear cells, necrotic cells and few fibroblasts in liver of CCl_4 treated rats (Althnaian *et al*., 2013). The toxic effect of CCl_4 is attributed to trichloromethyl radical produced during oxidative stress (Stoyanovsky and Cederbaum, 1999). Once the liver became injured, its efficient treatment with famous chemical drugs is limited (Lee *et al*., 2007). Therefore, interest concerned the use of alternative medicines for the treatment of hepatic disease has been arisen. Natural products received great attention as potentially antitoxic and antioxidants agents (Lee *et al*., 2007). Fenugreek (*Trigonella foenum-graecum*) is one of the oldest medicinal plants, dating back to Hippocrates and ancient Egyptian times (Jensen, 1992). The leaves and seeds are used to prepare extracts or powder for medicinal uses (Muralidhara *et al*., 1999). The antihyperlipidemic properties of oral fenugreek

seed powder has been suggested in rats (Shrivastava *et al*., 2009; Elmahdi *et al*., 2014), in rabbits (Al-Habori *et al*., 1998) and human (Awal *et al*., 1999). The mechanism of action of hypocholesterolemic effect of Fenugreek has been demonstrated by Sharma (1984, 1986). Authors explained that, fenugreek administration increased excretion of bile acids and neutral sterols in feces, thus depleting the cholesterol stores in the body of experimental rats.

As mentioned, the previous researches were focused on hypolipidemic and hypocholesterolemic effect of Fenugreek. However, publications regarding the antitoxic effects of fenugreek (*Trigonella foenum-graecum* L.) are scarce. Due to increasing interest in alternative/herbal medicine for the prevention and treatment of various illnesses and because fenugreek is used daily by many people, this study was undertaken to evaluate the protective effect of fenugreek (*Trigonella foenum-graecum* L.) aqueous extract against CCl_4-induced liver toxicity in rats.

MATERIALS AND METHODS

Chemicals and kits: Commercial diagnostic kits for Glucose (EP37L-660), total proteins (EP56-660), albumin (EP03-570), TAG (EP59-660), cholesterol (EP24-660), ALT (EP07-500), AST (EP15-500), ALP (EP04L-660), ACP (EP02-295), BUN (EP20-420), uric acid (EP61-620), creatinine (EP33K-660), calcium (EP22-660), phosphorus (EP46-660), magnesium (EP50-660), chloride (EP27-500) were purchased from United Diagnostic Industry, UDI, Dammam, Saudi Arabia. Paraffin oil, carbon tetrachloride (Spectrosol® BHD chemicals Ltd., pool, England) and other chemicals and solvents used in the study were of highest grade and commercially available.

Fenugreek (*Trigonella foenum-graecum* L.) aqueous extract: Fenugreek aqueous extract made according to Noor (2008) by soaking 10 g of fenugreek seeds in 250 mL of boiling de-ionized water for 1 h. The extract left for the whole night and then filtered and completed to 250 mL by de-ionized water (4%; Noor, 2008). Fenugreek aqueous extract (4%) was orally administered to the experimental group for 5 weeks after CCl_4-intoxication as a sole source of drinking water.

Animals and treatment: A total of 20 Albino rats (200-250 g) was obtained from Laboratory House of College of Veterinary Medicine and Animal Resources, King Faisal University, Al-Ahsa, Saudi Arabia and acclimated for 10 days before starting the experiment. All animals were housed in standard cages (5 rats/cage), fed with standard laboratory diet and tap water *ad libitum*. The experimental animals were housed in air-conditioned rooms at 21-23°C and 60-65% of relative humidity and kept on a 12 h light/12 h dark cycle. The animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by ethics of scientific research committee of King Faisal University, Saudi Arabia.

Induction of hepatotoxicity by CCl_4: Liver toxicity was induced by the intraperitoneal injection of CCl_4 (1 mL kg^{-1} b.wt.), 1:1 diluted with paraffin oil, for two successive days of the experiment (Althnaian *et al*., 2013).

Experimental groups and protocol: The rats were divided randomly into 4 groups comprising 5 rats in each group and fed the same diet throughout the experimental period (5 weeks). Group 1: Control rats fed only with basal diet and tap water and injected i/p with paraffin oil, this group served as control 1. Group 2: Rats fed normal basal diet, injected i/p with Paraffin oil and treated with fenugreek aqueous extract (4%; Noor, 2008) as their sole source of drinking water, this group served as control 2. Group 3: Rats fed basal diet and water and intoxicated with Ccl_4 (1 mL kg^{-1} b.wt.), 1:1 diluted with paraffin oil on first two days of the experiment (Althnaian *et al*., 2013; El-Bahr, 2014). Group 4: Rats fed basal diet and intoxicated with CCl_4 (1 mL kg^{-1} b.wt.), 1:1 diluted with paraffin oil on first two days of the experiment and then treated with fenugreek aqueous extract (4%) as their sole source of drinking water.

Samples collection: At the end of the experiment (5 weeks), the overnight fasted rats were anesthetized with diethyl ether. Blood samples were collected by cardiac puncture before incision of the abdomen; 5 mL of blood samples were collected in plain tubes, serum was collected and frozen at -30°C until the time of analysis of serum glucose, total proteins, albumin, triglyceride, total cholesterol, ALT, AST, ALP, ACP, BUN, uric acid, creatinine, calcium, phosphorus, magnesium and chloride using commercial assay kits according to the manufacture instruction. The liver tissues were removed and liver fragments were immediately frozen and stored at -80°C for biochemical analysis of selected antioxidant enzymes, namely catalase (CAT) and glutathione peroxidase (GPX).

Analysis of selected biochemical parameters: Serum glucose, total proteins, albumin, TAG, cholesterol, ALT, AST, ALP, ACP, BUN, uric acid, creatinine, calcium, phosphorus, magnesium and chloride levels were determined by using commercial kits (United Diagnostic Industry, UDI, Dammam, Saudi Arabia) on ELIPSE full automated chemistry analyzer (Rome, Italy). Concentration of the biochemical constituents was calculated according to the manufacture instruction.

Analysis of hepatic lipid peroxidation and selected hepatic Antioxidant enzymes: The concentration of TBARS (μM; Cayman Chemical Company, USA, Catalog No. 10009055) and the activities of CAT (nmol min^{-1} g^{-1} tissue; Cayman Chemical Company, USA, Catalog No. 707002) and GPX (nmol min^{-1} g^{-1} tissue; Cayman Chemical Company, USA, Catalog No. 703102) were determined by ELISA reader (Absorbance Microplate Reader ELx 800TM BioTek®, USA). Results were calculated according to the manufacture instructions.

Statistical analysis: All data is presented as the Mean±Standard error of the mean using one way analysis of variance (ANOVA). All tests were performed using a statistical analysis system program (SAS., 2002).

RESULTS

The activities of AST, ALT and ALP were estimated in serum samples as liver function biomarkers. These results are given in Table 1. The CCl_4 treatment markedly affected the liver specific enzymes. A significant increase ($p<0.05$) of serum AST, ALT and ALP activities was observed in untreated CCl_4 intoxicated rats (group 3) compared to controls (group 1 and 2). This result suggests that these hepatic biomarkers are elevated in the serum due to release of the enzymes from damaged liver. However, a significant decrease ($p<0.05$) was observed in the respective serum activities in rats intoxicated with CCl_4 and treated with aqueous extract of fenugreek (group 4) compared to untreated CCl_4 intoxicated rats (group 3). In the other hand, the activities of ACP showed insignificant changes ($p>0.05$) in all treated groups. The levels of serum kidney biomarkers of rats in all groups are presented in Table 2. Serum kidney biomarkers (BUN, uric acid and creatinine) levels remained unchanged in all treated group ($p>0.05$). The profiles of serum protein, lipid and glucose are presented in Table 3. A significant decrease ($p<0.05$) in serum glucose, total proteins and albumin ($p<0.05$) was observed in untreated CCl_4 intoxicated rats (group 3) compared to controls (group 1 and 2). However, administration of aqueous extract of fenugreek corrected these values towards the control values in CCl_4 intoxicated rats. A significant increase ($p<0.05$) in serum cholesterol and triglyceride was observed in untreated CCl_4 intoxicated rats (group 3) compared to controls (group 1 and 2). However, administration of aqueous extract of fenugreek corrected these values towards the control values in CCl_4 intoxicated rats. A significant change was not observed in calcium, phosphorus, magnesium and chloride levels of control and all treated groups ($p>0.05$) throughout the experimental period. Results in Table 5 shows that the TBARS level was significantly ($p<0.05$) increased in the liver of CCl_4 intoxicated rats compare to the controls. Treatment of CCl_4 intoxicated rats with aqueous extract of fenugreek caused significant ($p<0.05$) decrease in TBARS level of liver tissue compared to that in liver from untreated CCl_4 intoxicated rats. Administration of aqueous extract of fenugreek only did not affect significantly ($p>0.05$) the level of TABARS in rat liver compare to the control. The results that presented in the same table (Table 5) indicated that, all the antioxidant enzyme activities were reduced significantly ($p<0.05$) in CCl_4 intoxicated rats compare to the control. However, all activities of these enzymes showed a significant ($p<0.05$) recovery in response to administration of aqueous extract of fenugreek to CCl_4 intoxicated rats compared to untreated CCl_4

Table 1: Effects of CCl_4 and fenugreek water extract on serum liver function biomarkers in rats

	Parameters			
Groups	AST (IU L^{-1})	ALT (IU L^{-1})	ALP (IU L^{-1})	ACP (IU L^{-1})
C	115.6±5.0	28.9±0.1	159.2±4.30	7.4±0.1
FG	126.9±6.0	25.6±1.4	186.4±26.0	6.7±1.2
CCl_4	135.3±3.7*	49.1±4.0*	368.9±10.0*	6.9±1.0
FG+CCl_4	113.9±4.1**	37.1±4.7**	259.8±5.40**	7.6±1.0

Each value represents the Mean±Standard Error of Means (SEM) of 5 rats. *Significantly different from control group ($p<0.05$). **Significantly different from CCl_4 group ($p<0.05$). C: Control, FG: Fenugreek, CCl_4: Carbon tetrachloride, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphates, ACP: Acid phosphatase

Table 2: Effects of CCl_4 and fenugreek water extract on kidney biomarkers in rats

	Parameters		
Groups	BUN (mg dL^{-1})	Uric acid (mg dL^{-1})	Creatinine (mg dL^{-1})
C	10.8±0.1	1.6±0.2	0.4±0.05
FG	11.7±0.9	1.2±0.6	0.4±0.05
CCl_4	11.6±1.1	1.8±0.7	0.4±0.01
FG+CCl_4	11.0±1.2	1.7±0.4	0.4±0.02

Each value represents the Mean±Standard Error of Means (SEM) of 5 rats. C: Control, FG: Fenugreek, CCl_4: Carbon tetrachloride, BUN: Blood urea nitrogen

Table 3: Effects of CCl_4 and fenugreek water extract on protein, lipids and glucose profiles in rats

	Parameters				
Groups	Glucose (mg dL^{-1})	Total protein (g dL^{-1})	Albumin (g dL^{-1})	TAG (mg dL^{-1})	Cholesterol (g dL^{-1})
C	319.0±1.9	6.0±1.0	3.6±0.1	56.7±4.0	45.1±3.0
FG	316.5±7.1	6.2±1.3	3.8±0.2	51.7±3.0	46.1±2.0
CCl_4	230.0±0.9*	4.6±1.2*	2.1±0.2*	70.0±7.0*	52.0±0.6*
FG+CCl_4	310.8±6.1**	6.1±1.3**	3.0±0.4**	46.8±5.0**	41.0±5.4**

Each value represents the Mean±Standard Error of Means (SEM) of 5 rats. *Significantly different from control group ($p<0.05$). **Significantly different from CCl_4 group ($p<0.05$). C: Control, FG: Fenugreek, CCl_4: Carbon tetrachloride, TAG: Triacylglycerol

Table 4: Effects of CCl_4 and fenugreek water extract on minerals profile in rats

	Parameters			
Groups	Calcium (mg dL^{-1})	Phosphorus (mg dL^{-1})	Magnesium (mg dL^{-1})	Chloride (mEq L^{-1})
C	10.0±0.2	2.1±1.1	4.6±0.5	55.5±1.3
FG	11.4±2.4	2.9±0.2	4.9±0.5	54.5±1.2
CCl_4	11.0±1.1	2.6±0.1	4.1±0.2	55.6±1.7
FG+CCl_4	10.4±1.3	2.7±0.1	4.5±0.1	54.8±1.4

Each value represents the Mean±Standard Error of Means (SEM) of 5 rats. C: Control, FG: Fenugreek, CCl_4: Carbon tetrachloride

Table 5: Effects of CCl_4 and fenugreek water extract on lipid peroxidation and oxidative stress biomarkers in rats

	Parameters		
Groups	TBARS (μM g^{-1} tissue)	CAT (nmol min^{-1} g^{-1} tissue)	GPX (nmol min^{-1} g^{-1} tissue)
C	29.0±0.5	28.0±0.1	300.2±0.6
FG	28.9±0.1	27.6±0.4	294.4±6.0
CCl_4	35.0±0.1*	25.4±0.4*	270.7±8.0*
FG+CCl_4	30.2±0.1**	26.5±0.4**	290.8±6.4**

Each value represents the Mean±Standard Error of Means (SEM) of 5 rats. *Significantly different from control group ($p<0.05$). **Significantly different from CCl_4 group ($p<0.05$). C: Control, FG: Fenugreek, CCl_4: Carbon tetrachloride, TBARS: Thiobarbituric acid reactive substance

intoxicated rats. The activities of hepatic antioxidant enzymes of rats treated only with aqueous extract of fenugreek were comparable to that of the control (Table 5).

DISCUSSION

In the present study serum hepatic biomarkers, AST and ALT activities were greatly increased in untreated CCl_4 intoxicated rats (group 3) compared to controls (group 1 and 2; Table 1). The increased serum levels of hepatic transaminases biomarkers have been attributed to the liver injury, because these enzymes are place in cytoplasmic area of the cell and are released into circulation in case of cellular damage (Brent and Rumack, 1993; Recknagel *et al*., 1989). CCl_4 induced the increase of serum ALT and AST levels which source from cell membrane and mitochondrial damages in liver cells (Zimmerman *et al*., 1965). Many publications reported that, hepatic transaminases enzymes activities (ALT and AST) were significantly elevated after CCl_4 treatment (Arici and Cetin, 2011; Althnaian *et al*., 2013; Das, 2014; El-Bahr, 2014). The hepatotoxic effects of CCl_4 was attributed to lipid peroxidation caused by its active metabolite CCl_3 which abstract hydrogen from fatty acids, initiating the lipid peroxidation and subsequent liver damage (Park *et al*., 2005).

On the other hand, current findings indicated that, the increase in serum AST and ALT activities induced in untreated CCl_4 intoxicated rats (group 3) have been significantly corrected towards normal control values when these rats treated with aqueous extract of fenugreek (group 4; Table 1). This finding implies that, fenugreek aqueous extract challenge to protect liver tissue from CCl_4 injury. The reversal of increased serum enzymes in CCl_4-induced liver damage by fenugreek aqueous extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew and Joice, 1987). Several studies have provided a considerable support for evidencing the protective effects of fenugreek aqueous extract on liver damage (Khalil, 2004; Eidi *et al*., 2007; Sakr and Abo-El-Yazid, 2012; Das, 2014).

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. Fenugreek aqueous extract decreased CCl_4 induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells (Palanivel *et al*., 2008). It has been demonstrated that, the protective effect of fenugreek aqueous extract against CCl_4-induced oxidative stress in rats was due to its antioxidant properties (Sakr and Abo-El-Yazid, 2012). The antioxidant activity of fenugreek could be attributed to the presence of flavonoids which act as scavengers of reactive oxygen species (Belguith-Hadriche *et al*., 2010; Chatterjee *et al*., 2009; Sakr and Abo-El-Yazid, 2012). Moreover, 10 different flavonoids namely 5,7,3-trihydroxy-5-methoxylisoflavon, biochanin A, formononetin, irilone, tricine, daidzein, calycosin, orientin-2-O-p-trans-coumarate, vitexin-2-O-p-trans-coumarate and tricin-7-O-beta-D-glucopyranoside have been isolated from fenugreek seeds (Wang *et al*., 2010).

As discussed below, the antioxidant activity of fenugreek aqueous extract has been approved in the current study as reflected on significant reduction of lipid peroxidation biomarker (TBARS) and significant elevation of antioxidant enzymes activities (CAT and GPX) in liver of CCl_4 intoxicated rats. As in the current experiment, previous experimental studies have shown that $CC1_4$ increased significantly serum ALP levels (Khan and Al-Zohairy, 2011; Althnaian *et al*., 2013; Table 1) and decreased total protein (Fahim *et al*., 1999; Khan and Al-Zohairy, 2011; Althnaian *et al*., 2013) and albumin (Fahim *et al*., 1999; Khan and Al-Zohairy, 2011; Althnaian *et al*., 2013) levels (Table 3). However, there is a controversy about the effect of $CC1_4$ on serum creatinine level. While some investigator (Cruz *et al*., 1993) found a decrease in serum creatinine in CCl_4 toxicity, parallel to the present study others (Palaparthy *et al*., 200; Wirth *et al*., 1997; Khan and Al-Zohairy, 2011; Althnaian *et al*., 2013) found no significant changes (Table 2). The restoration of total protein and albumin in serum of rats intoxicated with CCl_4 and treated with fenugreek aqueous extract (group 4) compared to untreated CCl_4 intoxicated rats (group 3; Table 3) might attributed to the antioxidant properities of fenugreek which improves organ functions (Devasena and Menon, 2002; Thirunavukkarasu *et al*., 2003; Khalil, 2004).

In addition current study, reported that calcium, phosphorus, chloride and magnesium values in $CC1_4$ administrated rats were not statistically different from control values (Table 4; Ogeturk *et al*., 2004).

In the present study, serum glucose was reduced in CCl_4-treated rats and returned to normal values by administration of fenugreek aqueous extract (Table 3).

Studies have demonstrated decreased hepatic glycogen content after treatment with CCl_4, reflecting decreased gluconeogenesis by the liver (Muriel *et al*., 2001). It has been known that, hypoglycemia is main feature of carbon tetrachloride toxicity (Mion *et al*., 1996). The same author reported that liver cirrhotic rats exhibited hypoglycemia. The normoglycemic effect of fenugreek extract may be due to the major existence of 4-isohydroxy leucine which stimulates insulin secretion from pancreas (Al-Habori and Raman, 1998). The normoglycemic effect of fenugreek extract may be mediated also through stimulating insulin synthesis and/or increasing secretion β pancreatic cells of Langerhans (Puri *et al*., 2002).

The results of the present study have also established that the CCl_4 treatment affected the lipid metabolism of liver (triglyceride and cholesterol levels). This is evidenced from the present observations that, CCl_4 caused a significant increase in the levels of lipid parameters (Table 3). Muller *et al.* (1974) stated that CCl_4 intoxication is similar the hepatitis in case of the triglycerides catabolism. This situation could be also attributed to the reduction of lipase activity which could lead to decrease in triglyceride hydrolysis (Jahn *et al*., 1985). On the other hand, it can be assumed that hypercholesterolemia in CCl_4 intoxicated rats has resulted in damage of hepatic parenchymal cells that lead to disturbance of lipid metabolism in liver (Havel, 1986). When CCl_4 intoxicated rats treated with fenugreek aqueous extract (group 4), a significant decline in above mentioned parameters (triacylglycerol and cholesterol) has been observed (Table 3) compared with untreated CCl_4-intoxicated rats (group 3). This might be because fenugreek contains lecithin which dissolve cholesterol and contains lipotropic (fat dissolving) substances which dissolves deposits of fat, prevents fatty accumulation and water retention (Blumenthal *et al*., 1998). Hypocholesterolemic effect of fenugreek has been attributed to increased conversion of hepatic cholesterol to bile salts due to loss of complexes of these substances in the feces with fenugreek fiber and saponins (Al-Habori and Raman, 1998).

The significant increase of TBARS in liver of untreated CCl_4 intoxicated rats (group 3; Table 5) suggests enhanced peroxidation leading to tissue damage and the failure of the antioxidant mechanisms in preventing of excessive free radicals (Romero-Alvira and Roche, 1996). This confirmed by the results of the current study which reported a significant decrease of activities of antioxidant enzymes (CAT and GPX) in CCl_4 intoxicated rats (group 3) compare to controls (group 1 and 2) which impose in elevation of TBARS levels (Table 5). These findings were found to be in agreement with other previous studies in rats liver intoxicated with CCl_4 (Yousef, 2004; Aranda *et al*., 2010; Al-Fartosi *et al*., 2012; Bona *et al*., 2012; Pirincciogluet *al*., 2012; El-Bahr, 2014). The significant decrease of TBARS in liver of rats intoxicated with CCl_4 and treated with fenugreek aqueous extract (group 4) compared to untreated CCl_4 intoxicated rats (group 3) suggests the protective effect of fenugreek aqueous extract against CCl_4-induced oxidative stress.

The protective effect of fenugreek aqueous extract was augmented by increasing the activities of antioxidant enzymes (CAT and GPX) when administered to CCl_4 intoxicated rats (Table 5). Consistent to the current findings, stimulation of antioxidant enzymes activities by fenugreek has been documented previously (Anuradha and Ravikumar, 2001; Sakr and Abo-El-Yazid, 2012). Flavonoid contents of fenugreek may be responsible for the antioxidant enzyme activation and subsequent scavenging of free radical species (Belguith-Hadriche *et al*., 2010; Chatterjee *et al*., 2009; Sakr and Abo-El-Yazid, 2012).

CONCLUSION

Administration of CCl_4 to rats induced elevation of hepatic transaminases, lipid peroxidation and reduction of antioxidant enzymes activities. Oral fenugreek aqueous extract ameliorated the detrimental effects of CCl_4 and corrected all examined biomarkers toward the control values. The ameliorative effects of fenugreek aqueous extract were in the form of improvement of serum transaminases, attenuation of hepatic lipid peroxidation and activation of hepatic antioxidant enzymes.

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