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EFFICACY OF DRUMSTICK (*Moringa oleifera*) LEAF-WATER EXTRACT ON THE MODULATION OF OXIDATIVE STRESS THROUGH EXPRESSION OF SUPEROXIDE DISMUTASE AND CATALASE GENES IN HIGH FAT-FED RATS

Bij Suthar, Hinal Patel* and V.H. Patel

Laboratory of Foods and Nutrition, P.G. Department of Home Science, Sardar Patel University, Vallabh Vidhyanagar, Anand – 388 120, Gujarat (India)
*e mail: *patelvh2004@yahoo.co.in*

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ABSTRACT

Obesity-related kidney diseases require an effective approach to avert high fat-diet induced renal damage through oxidative stress. The aim of present study was to evaluate antioxidative strength and protective effects of drumstick (*Moringa oleifera*) leaf-water extract (DLWE) on kidney in Wistar rats. In this study rats were either fed with control diet or control diet with DLWE or high-fat diet or high-fat diet with DLWE for 16 weeks. The body and kidney weight, oxidative status (lipid peroxidation, ferric reducing antioxidant power, non-enzymatic and enzymatic antioxidants) and gene expression of superoxide dismutase and catalase were assessed. High fat-diet fed rats showed changes in body and kidney weights, increase in lipid peroxidation and decrease in ferric reducing antioxidant power, vitamin C, glutathione, superoxide dismutase and catalase indicating a higher oxidative stress index. At molecular level, high fat-diet caused a significant reduction in gene expression of superoxide dismutase and catalase which was overcome by DLWE supplementation. The oral supplementation of DLWE restored oxidative stress in high fat-diet fed rats. The diet restored lipid peroxidation and kidney antioxidants to control group level. It seems that DLWE has therapeutic potential, so be developed as a supplement to prevent obesity and related oxidative stress.

Keywords: Antioxidant, drumstick, gene expression, obesity, oxidative stress

INTRODUCTION

Obesity is a major health problem in the world and has become widespread in developing countries also (Rutkowski *et al.*, 2006). Many recent studies suggest a relationship between obesity and renal dysfunction (Glastras *et al*., 2016). Obesity is not only a predictor for the progression to end stage renal failure but also an independent risk for cardiovascular mortality (Keith *et al.*, 2004). Although the mechanism of chronic kidney diseases (CKD) associated with obesity is multifactorial, an abnormal inflammatory response and oxidative stress in kidney plays a crucial role. Oxidative stress has commonly been identified in obesity-related renal diseases and may be the mechanism underlying the initiation or progression of renal injury in obesity (Darouich *et al.*, 2011). Oxidative stress is caused by an imbalance between increased production of reactive oxygen species (ROS) and/or

reduced antioxidant activity, leading to oxidative damage to cells or tissue including lipids, proteins and nucleic acids. Analysis of oxidative markers in obesity subjects indicates that oxidative damage is associated with increased body mass index (BMI) and body fat (Piva *et al.*, 2011). Oxidative stress in obesity may be due to increased oxygen consumption in mitochondrial respiration, diminished antioxidant capacity, fatty acid oxidation, lipid oxidation and cell injury causing increased rates of free radical formation (Brown *et al.*, 2009). Earlier studies suggest that oxidative stress trigger, at an early age, the onset of kidney lesions and functional impairment in Zucker obese fa/fa rats, a good model of obesity-related renal disease, in absence of hyperglycaemia, hypertension, and inflammation (Poirier *et al.*, 2000).

ROS play a crucial role in mediating renal injury (Jaimes *et al.*, 2010). ROS are highly reactive molecules that oxidize lipids and proteins, cause cellular injury, promote glomerular and renal tubule injury and associated proteinuria (Habibi *et al.*, 2011). Therefore, the attenuation of inflammation and oxidative stress in kidney may be a potential treatment for nephropathy associated with obesity. ROS play key role in the pathogenesis of renal injury such as glomerulosclerosis and tubulointerstitial fibrosis, therefore, the approaches to reduce oxidative stress by antioxidants supplementation, nutritional and surgical interventions may have renoprotective effects. Garcinia reportedly protects against obesity-induced nephropathy by attenuating the oxidative stress through reduced lipid peroxidation and oxidized low-density lipoprotiens. Chander *et al.* (2004) reported that nephropathy is associated with oxidative stress and supplementation with antioxidant improves kidney damage by ameliorating proteinuria and renal focal and segmental sclerosis. Other phyto-antioxidants like grape seed pro-anthocyanidin extract can protect against nephrotoxicity effects induced by cisplatin and gentamicin (Safa *et al.*, 2010) and reverse experimental myoglobinuric acute renal failure (Stefanovic *et al.*, 2000). Quercetin, a flavonoid that exhibits antioxidant properties in many diseases, could also protect the rat kidney against lead-induced injury and improve renal function (Liu *et al.*, 2010).

Drumstick (*Moringa oleifera* Lam.) is a cruciferous plant that belongs to family Moringaceae. *M. oleifera* is commonly called horseradish tree or drumstick tree by locals. *M. oleifera* is consumed not only for its nutritional values but also for medical benefits (Anwar *et al.*, 2007). *M. oleifera* leaves are rich in β-carotene, vitamin C, vitamin E, polyphenols and natural antioxidants (Mahmood *et al.*, 2010), and provide a variety of biological functions including anti-inflammatory, anti-cancer, hepato-protective and neuroprotective effects (Abdull *et al.*, 2014). Reports reveal its enormous therapeutic value such as anti-diabetes, anti-rheumatoid arthritis, anti-atherosclerosis, anti-infertility, pain relief, anti-depression and diuretic and thyroid regulation (Banji *et al.*, 2012). Usually, natural compounds rich in polyphenolic compounds have strong antioxidant properties and can decrease oxidative damage in tissues by scavenging free radical (Neidzwiecki *et al.*, 2016). The methanolic extract of *M. oleifera* leaves contains chlorogenic acid, rutin, quercetin glucoside and kaempferol rhamno-glucoside (Atawodi *et al.*, 2010). As a medicinal plant, *M. oleifera* extracts from both mature and tender leaves exhibit strong antioxidant activity against free radicals and prevent oxidative damage due to the enrichment of polyphenols. In view of above, the present study was aimed to assess the impact of drumstick leaf-extract on renal oxidative stress in rats induced by feeding high fat diet.

MATERIALS AND METHODS

Collection and preparation of plant extract

The leaves of *M. oleifera* were collected after proper identification and authentication by the Dr. Hemant Patel, Chief Scientist, College of Horticulture, AAU, Anand (Gujarat). The fresh leaves were oven-dried at 50°C, crushed in a pestle and mortar and pulverized into fine powder using electric blender. The powder was sieved through a 2 mm mesh and used for the preparation of water extract. The extract was prepared using Soxhlet method. The supernatants were pooled and reduced to a known volume using rotary vacuum evaporator.

Dose selection

In the present study, 200 mg kg^{-1} body weight dose of *M. oleifera* leaf extract was selected on the basis of previous reports of acute toxicity study performed using the single dose of orally administered 2 g kg^{-1} methanolic extracts of *M. oleifera* (leaf) which shows no signs of toxicity in rats (Adedapo *et al.*, 2009).

Animals

The present study was carried out on 24 male Wistar rats weighing 105-145 g. During the study the animals were kept in plastic cages with *ad libitum* access to water. The animals were exposed to 12:12 h light dark cycles, room temperature of 25±2°C and humidity of 55-60%. The animals were randomly divided into four equal groups, each group consist of six animals *viz*., group I received the control diet, group II received control diet with DLWE, group III received high fat diet (HFD), and group IV received HFD with DLWE. The above diets were fed for 16 weeks period. Both control and high fat diets contained 20.8% protein and 41.2% carbohydrates, however fat content in control was 5.6% and in HDF 23.6%. The control diet (10% energy from fat) and HFD (45% energy from fat) were purchased from VRK Nutritional Solutions, Pune (India). The experimental protocol [SPU/VH/2016(01)] was approved by the Institutional Animal Ethics Committee of BRD School of Biosciences, Sardar Patel University, Vallabh Vidyanagar (Ethical Committee: 337/PO/ReBi/S/01/ CPCSEA). The study was conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India.

Sample collection

At the end of experimental period, all rats were sacrificed using mild anaesthesia. Kidney of each animal was weighed and homogenized separately with a tissue homogenizer (Remi) using phosphate buffer saline (50 mM, pH 7.4). The crude tissue homogenate was centrifuged at 8,000 rpm for 20 min in refrigerated centrifuge (Remi) and the resultant supernatant was used for the different estimations.

Biochemical analysis

Standard methods were used for the estimation of FRAP [ferric reducing antioxidant power] (Benzie *et al.*, 1996), lipid peroxidation (Ohkawa *et al.*, 1979), vitamin C (Roe and Kuether, 1955), vitamin E (Desai, 1984), glutathione (Ellman, 1959), catalase (Aebi, 1983), SOD [superoxide dismutase] (Marklund and Marklund, 1974) and GST [glutathione-S-transferase] (Habig *et al.*, 1974). Protein content in tissue homogenate was measured as per the method of Lowry *et al*. (1951).

Total RNA isolation and real-time RT-PCR of antioxidant enzymes in kidney

The frozen kidney tissue (50 mg) was homogenized after the addition of 1 mL trizol (Sigma Aldrich) using a tissue homogenizer (Remi). Total RNA was extracted from tissue homogenate via chloroform extraction followed by nucleic acid precipitation with isopropanol. The pellet was washed with 75% ethanol and resuspended in DEPC (diethyl pyrocarbonate)-treated Milli Q water. The purity of RNA was judged on the basis of absorbance ratio at 260:280 nm. The samples with acceptable purity (ratio ≥1.8) were quantified and used for reverse transcription. RNA (5 µg) were treated with DNase to remove any possible DNA contamination. The cDNA (complementary deoxyribonucleic acid) was prepared as per the manufacturer's instructions given in a high capacity cDNA reverse transcription kit (Invitrogen, USA). The polymerase chain reaction (PCR) conditions and primers sequence for different genes used for RT-PCR were as under:

Genes	Sequences	Annealing temp. (ºC)	Cycles (No.)	Amplicon size (bp)
ß-Actin	FP: ACGGTCAGGTCATCACTATCG RP: GGCATAGAGGTCTTTACGGATG	60	30	155
SOD-1 [Cu-Zn]	FP: GCAGAAGGCAAGCGGTGAAC RP: TAGCAGGACAGCAGATGAGT	58	28	387
CAT	FP: GCGAATGGAGAGGCAGTGTAC RP: GAGTGACGTTGTCTTCATTAGCACTG	60	28	670

The expression of differential mRNA was quantified by real time PCR (Light Cycler® 480II, Roche). SYBR green master mix I (Roche, Switzerland) containing SYBR green PCR buffer, dNTP mix including SYBR green I, ROX (passive reference dye) was used for gene expression analysis. The amplification was carried out in a final reaction volume of 20 µL containing 10 µL SYBR green master mix I, 1 µL each gene specific primer and 2 µL cDNA template. The PCR protocol was designed for 45 cycles. PCR products were electrophoresed in 1.5% agarose gel stained with ethidium bromide and visualized under gel documentation system (UVP, Cambridge, UK).

Statistical analysis

Data presented are the mean ± standard deviation (SD) of four readings. The statistical analysis was performed using statistical software SPSS V.20 (SPSS Institute INC, Cary, NC). Analyses of variance were performed using ANOVA. Significant difference ($p<0.05$) between the means were determined using Duncan's test.

RESULTS AND DISCUSSION

Recent research has suggested that obesity is an independent risk for development and progression of CKD (Deji *et al.,* 2009). Obesity could induce renal damage which may culminate in end-stage renal disease. Drumstick leaf-water extract (DLWE) reportedly reduces the risk of many chronic disorders associated with obesity such as nonalcoholic fatty liver disease, type 2 diabetes, and cardiac hypertrophy (Charytoniuk *et al.*, 2017). However, whether DLWE could exert protective effects on oxidative stress induced by obesity is still unclear. In present study, we tried to induced obesity and its associated oxidative stress by feeding HFD in rats and evaluated the effect of DLWE on it.

Growth performance and kidney weight of animals

The final body weight of G-I and G-IV were significantly ($p \leq 0.05$) higher as compared to G-I and G-II groups (Table 1). The maximum increase (93%) in body weight was found in G-III where animals were fed with high fat diet. These results are in conformity with Noeman *et al.* (2011), Das *et al.* (2015) and Wicks *et al*. (2016) who reported significant increase in body weight of high fat diet-fed rats/mice as compared to the control group. Bais *et al*. (2014) found protective effect of methanolic extract of *M. oleifera* leaves against obesity in Wistar albino rats. Huang *et al.* (2016) have reported significant decrease in body weight of male SD rats after supplementation of cocoa, coffee, green tea and garcinia complex for 4 weeks as compared to the high fat diet fed rats.

The kidney weight increased significantly ($p \leq 0.05$) in G-III as compared to G-I group. In present study, the supplementation of DLWE for 16 weeks showed a protective effect against fat deposition in body. There was no significant ($p \leq 0.05$) effect of DLWE on kidney weight in G-IV. Metwally *et al.* (2016) have reported decreased weight in female Wistar rats fed with diets supplemented with ethanolic extract of *M. oleifera* @ 600 mg kg^{-1} as compared to the control group.

Table 1: Effect of DLWE administration on body weight and kidney weights of rats

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Kidney weight (g)
G – I	124.20 ± 5.08^{a}	206.70 ± 23.81^{a}	82.50 ± 23.56^{a}	0.99 ± 0.08^{a}
G – II	125.00 ± 4.80^{a}	$222.63 \pm 16.28^{a,b}$	$97.63 \pm 15.26^{a,b}$	1.01 ± 0.09^{a}
G – III	126.88 ± 7.43^{a}	$245.82 \pm 20.08^{b,c}$	$118.87 \pm 19.71^{b,c}$	1.10 ± 0.05^{b}
G – IV	127.26 ± 5.66^{a}	255.45 ± 20.30^{d}	128.23 ± 17.14^{c}	0.98 ± 0.07^{a}
F value	0.383	7.121*	6.958*	3.121*

*indicates significant difference at $p< 0.05$. G-1, control; G-2, control with Drumstick leaves extract; G-3, high fat; G-4, high fat with Drumstick leaves extract. The values given are mean ± SD; n = 6.
Different superscripts of alphabets within column are significantly different from each other.

Effect of DLWE on lipid peroxidation and antioxidant defense system of kidney tissue

Increased adiposity seems to be the driver for increased kidney cellular oxidative stress. To determine whether cellular reactive oxygen species production is associated with obesity in kidney, we analyzed lipid peroxidation as an oxidative stress marker and kidney non-enzymatic [GSH (glutathione), vitamin C, vitamin E] and enzymatic antioxidant [superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST)] along with gene expression of SOD and CAT. The increase in obesity-associated oxidative stress reportedly is due to the presence of excessive adipose tissue accumulation (Wicks *et al.*, 2016). High fat diet induced obesity can cause increase in lipid peroxidation (LPO) and induces cell injury. LPO is the result of interaction between free radicals and membrane lipids. LPO is a molecular mechanism involved in obesity-induced toxicity (Noeman *et al.*, 2011). The end products of LPO in cell membrane are thiobarbituric acid-reactive substances (TBARS) like malonaldehyde (MDA), which is used as a marker of tissue oxidative stress (Pessayre *et al.*, 2002). In present study LPO showed significant ($p \leq 0.05$) increase in G-III as compared to G-I group. Increased LPO was significantly ($p \leq 0.05$) protected by the supplementation of DLWE (Table 2). High fat induced oxidative stress may trigger LPO and LPO product formation which, in turn, causes cyclic development of ROS. Oxidative stress has commonly been identified in obesity related renal diseases and may be the mechanism underlining the initiation of renal injury in obesity. Many studies reported that LPO increased significantly in kidney tissue in the form of melonaldehyde in obese rats or mice as compared to the control group (Noeman *et al.*, 2011; Wicks *et al.*, 2016). Das *et al*. (2013) observed similar effect of *M. oleifera* leaf-extract and quercetin on melonaldehyde content, the end product of LPO. They reported 71 and 74% reduction in LPO by *M. oleifera* leaf-extract and quercetin, respectively. Dominguez-Avila *et al*. (2015) showed that high fat diet + whole pecan fed group had lowest TBARS value (the end product of LPO) as compared to the control group. DLWE showed a reduction in LPO and other oxygen or nitrogen species radicals induced oxidative damage (Mantena *et al.*, 2009). The stress developed by HFD lead to the exhaustion of antioxidant status by lowering the enzymatic- and non-enzymatic-antioxidants in kidney tissue. The FRAP value indicates the total antioxidant power and provides antioxidant power of sample. The higher FRAP value in samples revealed its better power to neutralize free radicals (Table 2). The mean value of FRAP was significantly ($p \leq 0.05$) decreased in G-III group as compared to G-I group. The supplementation of DLWE resulted in significantly higher FRAP values among their treated groups (G-II & G-IV) and when compared to their respective control (G-I & G-III). This confirms the reduction and neutralization of increased oxidative stress by phenolic compounds (such as tannins, sterols, terpenoids, flavnoids, saponins, anthraquinones, alkaloids, etc.) and reducing sugar present along with anticancerous agents like quercitin, isoquercitin, kemfericitin, glucinolates, isothiocynates,

Table 2: Effect of DLWE supplementation on LPO and FRAP levels; enzymatic antioxidants (SOD, CAT & GST); non-enzymatic antioxidants (vitamin C, vitamin E & GSH) on kidney in control and experimental groups of Wistar rats

Parameters	G-I	G-II	G-III	G-IV	F-value
LPO (µg 100 g^{-1})	$132.33 \pm 14.85^{a,b}$	$141.16 \pm 17.09^{b,c}$	161.33 ± 12.70^{c}	115.33 ± 31.16^{a}	5.345*
FRAP (mE Trolox 100 g^{-1})	110.92 ± 14.77^{b}	149.45 ± 8.44^{c}	84.48 ± 22.07^{a}	119.66 ± 18.66^{b}	15.295*
SOD (IU mg^{-1} protein)	37.03 ± 4.19^{b}	$32.41 \pm 7.27^{a,b}$	28.56 ± 4.34^{a}	$33.31 \pm 2.90^{a,b}$	2.965
CAT (IU mg^{-1} protein)	10.55 ± 2.57^{b}	$8.75 \pm 0.98^{a,b}$	6.79 ± 1.35^{a}	7.82 ± 2.02^{a}	4.526*
GST (IU mg^{-1} protein)	626.61 ± 116.6^{b}	690.85 ± 113.1^{b}	471.08 ± 46.10^{a}	659.86 ± 120.1^{b}	5.323*
Vitamin C (mg 100 g^{-1})	14.66 ± 3.25^{c}	11.11 ± 2.06^{b}	8.05 ± 0.94^{a}	$9.10 \pm 0.99^{a,b}$	12.169*
Vitamin E (mg 100 g^{-1})	467.35 ± 53.20^{a}	470.01 ± 67.17^{a}	411.45 ± 41.75^{a}	462.33 ± 48.64^{a}	1.613
GSH (mg 100 g^{-1})	39.49 ± 5.68^{c}	$31.78 \pm 9.52^{b,c}$	22.03 ± 5.58^{a}	$24.57 \pm 5.84^{a,b}$	7.859*

Values are mean ± SD; n = 6.

Different superscripts of alphabets within column are significantly different from each other.

*indicates significant difference at $p<0.05$. G-1, control; G-2, control with drumstick leaves extract; G-3, high fat; G-4, high fat with Drumstick leaves extract.

glycoside compounds and glycerol-1-9-octadeconate (Berkovich *et al.*, 2013). Thus kidney antioxidant machinery remains same as or affected less in DLWE supplemented groups. Das *et al.* (2013) reported similar result in liver of high fat fed mice. In present study, FRAP depicted a negative but significant correlation with LPO.

Vitamin C directly interacts with a wide range of free radicals, terminating the chain reaction initiated by free radicals via electron transfer as well as by participating in the regeneration of vitamin E (Afroz *et al.*, 2016). Ming *et al*. (2008) reported a reduction of vitamin C in kidney in HFD fed animals. Vitamin E has been reported the most important soluble lipid and chain breaking antioxidant which protects cell membranes from oxidation by reacting with lipid radicals produced in LPO chain reaction (Traber and Atkinson., 2007). The GSH is a powerful antioxidant in cells that can directly scavenge free radicals or act as a substrate for GSH during the detoxification of hydrogen peroxide and lipid peroxide. SOD converts the superoxides to hydrogen peroxides (H_2O_2) which catalases further covert to harmless water and oxygen.

The non-enzymatic (GSH and vitamin C) and enzymatic antioxidants in kidney tissue was significantly reduced in the animals fed with high fat diet (G-III) as compared to the control animals (G-I) (Table 2). Administration of DLWE markedly increased non-enzymatic and enzymatic antioxidants (G-IV) as compared to the animals of high fat fed group. Noeman *et al*. (2011) studied antioxidative markers in kidney of rats fed with HFD and found that in renal tissue GST and CAT enzymes showed a significant decrease in obese rats. Ming *et al.* (2008) found significant decrease in the activities of SOD, CAT, GSH, vitamin C and vitamin E in kidney of HFD fed group animals as compared to the control group. Mukthamba and Srinivasan (2016) found that the supplementation of fenugreek seeds and garlic to the rats with high fat diet caused significant decrease in GST, SOD and CAT in kidney by 40.7, 24.3 and 28.3%, respectively, in comparison to control group. We found 45.0, 11 and 44.21% reduction in vitamin C, vitamin E and GSH, respectively, in HFD fed animals. Similarly, SOD, CAT and GST were reduced by 35.0, 22.8 and 24.8%, respectively. The reduction in SOD and CAT confirm the oxidative stress by high fat-diet and higher SOD and CAT in high fat-fed animals along with DLWE supplementation ensures this catalytic conversion of superoxides.

In present study, the gene expression of SOD and CAT in kidney of rats which have the capacity to scavenge free radical in body were studied. It was evaluated in light-wise if the administration of high-fat diet and/DLWE group modulates the expression of SOD and CAT. The gene expression studies of SOD and CAT revealed a significant decrease in gene expression of both SOD and CAT in rats fed with high fat diet (Fig. 1). There was increase in gene expression of SOD and CAT in kidney of animals fed with HFD plus DLWE. The reports reveal that the balance between the rate of production and activity of SOD and CAT determines the concentration of free radicals in body. Yida *et al*. (2015) found a decrease in gene expression of SOD 1 in liver of rats fed with high fat diet. The antioxidant properties of DLWE may possibly be due to the bioactive compounds particularly phenolics present in DLWE which may neutralize free radicals form in response to high fat diet. In conclusion, DLWE alleviated kidney oxidative stress in high fat diet induced obesity in rats. Therefore, DLWE may serve as a potential Nutraceutical for obesity induced oxidative stress.

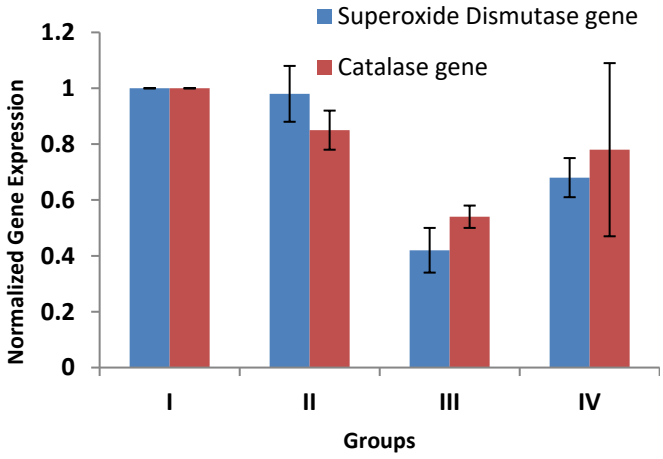

Fig. 1: Effect of DLWE supplementation on gene expression in kidney of rats

Conflict of Interest: There is no potential conflict of interest.

Ethical statement: The experimental protocol (SPU/VH/2016(01)) was approved by the Institutional Animal Ethics Committee of BRD School of Biosciences, Sardar Patel University, Vallabh Vidyanagar (ethical committee: 337/PO/ReBi/S/01/ CPCSEA) and conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India.

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