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PROTECTIVE INFLUENCE OF *HIBISCUS SABDARIFFA*, AN EDIBLE MEDICINAL PLANT, ON TISSUE LIPID PEROXIDATION AND ANTIOXIDANT STATUS IN HYPERAMMONEMIC RATS

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Abstract

The present study was undertaken to examine the protective influence of the alcoholic leaf extract of *Hibiscus sabdariffa* (Linn) Malvaceae (an indigenous edible medicinal plant used in Ayurvedic and traditional Medicine in India, China and Thailand) on oxidative stress during ammonium chloride induced hyperammonemia by measuring the extent of oxidative damage as well as antioxidant status. The levels of tissue (liver and kidney) lipid peroxides and the antioxidants; superoxide dismutase, catalase, reduced glutathione and glutathione peroxidase were studied in hyperammonemic rats. Hyperammonemia was induced by daily intraperitoneal injections of ammonium chloride at a dose of 100mg/kg body weight for 45 days. Decreased levels of tissue lipid peroxidation accompanied with increased antioxidant levels in hyperammonemic rats were observed during oral administration of HSEt (250mg/kg body weight), which clearly shows the antioxidant property of HSEt. The study of induction of the antioxidant status is considered to be a reliable marker for evaluating the antiperoxidative effect of the medicinal plant. Our present findings show the protective role of HSEt against lipid peroxidation and suggest that HSEt possesses antioxidant potential that may be used for therapeutic purposes. The exact mechanism of action of the extract still has to be investigated and the isolation of its active constituents remains to be done.

Keywords: *Hibiscus sabdariffa*; hyperammonemia; oxidative stress; antioxidants; lipid peroxidation.

Introduction

Reactive oxygen species (ROS) are an important part of the defense mechanism against infection, but excessive generation of free radicals may damage tissues. The formation of lipid peroxides by the action of free radicals on unsaturated fatty acids has been implicated in the pathogenesis of various diseases (Steinberg et al., 1989). Epidemiological and experimental studies have shown that ROS-induced lipid peroxidation occurs during hyperammonemia (Lena and Subramanian, 2004; Kosenko et al., 1997, 2000). The extent of oxidative damage caused by

ROS can be exacerbated by a decreased efficiency of antioxidant defense mechanisms (Mat'és et al., 1999). The level of lipid peroxidation in cells is controlled by various cellular defense mechanisms of enzymatic and non-enzymatic scavenger systems (Simmons 1972; Halliwell and Gutteridge, 1994). The levels of these defense mechanisms are altered in hyperammonemia (Lena and Subramanian, 2004) and therefore, the ineffective scavenging of free radicals plays a crucial role in determining tissue injury. Measurement of lipid peroxidation and antioxidants is therefore valuable in assessing hyperammonemia because they reflect the bioavailability of antioxidants as well as their increased utilization to scavenge lipid peroxidation products.

Hyperammonemia is a major contributing factor to neurological abnormalities observed in hepatic encephalopathy and in congenital defects of ammonia detoxication. Ammonia affects both excitatory and inhibitory synaptic transmission in the mammalian brain by a variety of mechanisms (Monfort and Felipo, 2005). Ammonia toxicity occurs partly via oxidative stress, which leads to lipid peroxidation and free radical generation. This causes hepatic dysfunction and failure, which is a primary cause of neurological disorders and alterations in the function of the central nervous system associated with hyperammonemia, such as, hepatic encephalopathies, Reye's syndrome, irritability, somnolence, vomiting, seizures, and derangement of cerebral function, coma and death (Hilgier et al., 1994; Kosenko et al., 1997; Lena and Subramanian, 2004; Majeed, 2005; Rodrigo et al., 2004; Tream, 1994; Saez et al., 1999; Mathias et al., 2001; Murthy et al., 2001).

Nowadays, the use of complementary/alternative medicine and especially the consumption of botanicals have been increasing rapidly worldwide, mostly because of the supposedly less frequent side effects reported when compared to modern Western medicine (Hu et al., 2003). *Hibiscus sabdariffa* (Linn) (family Malvaceae), is an annual dicotyledonous herbaceous shrub popularly known as 'Gongura' in Hindi or 'Pulichha Keerai' in Tamil. This plant is well known in Asia and Africa and is commonly used to make jellies, jams and beverages. In the Ayurvedic literature of India, different parts of this plant have been recommended as a remedy for various ailments like hypertension, pyrexia, liver disorders, and as antidotes to poisoning chemicals (acids, alkali, pesticides) and venomous mushrooms (Chifundera et al., 1994). A number of active principles from this plant include anthocyanins, flavonols, protocatechuic acid (PCA) (Lewis and Neelakantan 1965; Osman et al., 1975; Seca et al., 2000; 2001) that have been identified as contributors to the observed medicinal effect of this plant. Among them, anthocyanin and PCA have been found to have antioxidant activity, and to offer protection against atherosclerosis and cancer (Meyer et al., 1997; Satue-Gracia et al., 1997). Anthocyanins were also found to have many times more activity than common antioxidants such as ascorbate (Wang et al., 1997). It is a well-documented fact that most medicinal plants are enriched with phenolic compounds and bioflavonoids that have excellent antioxidant properties (Shirwaikar et al., 2003). Overall, there is now increasing evidence that antioxidants in the human dietary supplements are of major benefit for health and well-being.

In the preliminary phytochemical screening, the ethanolic extract of HSEt gave positive tests for glycosides, anthocyanins, polyphenols and flavones (Trease and Evan 1959). To our best of knowledge, no other biochemical investigations have been carried out on tissue lipid peroxidation and antioxidant status in experimental hyperammonemia. Since the study of the protective effects of plant-based antioxidants may be beneficial in evolving strategies for the treatment of hyperammonemia, the present investigation was undertaken to study the protective effect of the HSEt on tissue lipid peroxidation and antioxidant status in ammonium chloride-induced hyperammonemic rats.

Materials and Methods

Plant material and Extract preparation

The mature green leaves of *Hibiscus sabdarifa* were collected from Chidambaram, Cuddalore District, Tamil Nadu, India. The plant was identified and authenticated at the Herbarium of Botany Directorate in Annamalai University. A voucher specimen (No. 3648) was deposited in the Botany Department of Annamalai University. The shade-dried and powdered leaves of *Hibiscus subdariffa* (500 gm) were subjected to extraction with 70% ethanol (1.5 l) under reflux for 8 h and the resulting solution concentrated to a semi solid mass under reduced pressure (Rotavapor apparatus, Buchi Labortechnik AG, Switzerland). The yield was about 24% (w/ w) of the starting crude material (Srinivasan et al., 2001; Essa et al., 2005). The residual extract was dissolved in sterile water and used in the investigation.

Animals

Adult male albino Wistar rats, weighing 180-200 g bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, were used. The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, a temperature of $22 \pm 2^\circ\text{C}$ and humidity of 45-64%. The Animals were fed with a standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. All animal experiments were approved by the ethical committee (Vide. No. 273/2004), Annamalai University and were in accordance with the guidelines of the National Institute of Nutrition (NIN), Indian Council of Medical Research (ICMR), Hyderabad, India. Ammonium chloride was purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used in the study were of analytical grade.

Experimental Design

Hyperammonemia was induced in Wistar rats by daily intraperitoneal injections of ammonium chloride at a dose of 100 mg/kg body weight for 8 weeks (Essa et al., 2005). In the experiment, a total of 32 rats were used. The rats were divided into 4 groups of 8 rats each. Group 1: Normal untreated rats. Group 2: Normal rats treated with HSEt orally (250 mg/kg body weight) (Odigie et al., 2003). Group 3: Ammonium chloride (100 mg/kg body weight) treated rats. Group 4: Rats treated with ammonium chloride (100 mg/kg) + HSEt (250 mg/kg). At the end of 8 weeks, blood samples were taken for ammonia (Wolheim, 1984) and plasma urea (Varley et al., 1998) determination. After the estimation of blood ammonia and plasma urea, the animals were killed by decapitation. Liver and kidney were dissected out, washed in ice cold saline, patted dry and weighed and 10% tissue homogenate was prepared to measure TBARS (thiobarbituric acid reactive substances) (Niehaus and Samuelsson 1968), HP (hydroperoxides) (Jiang et al., 1992), CD (conjugated diene) (Klein, 1979), SOD (superoxide dismutase) (Kakkar et al., 1984), CAT (catalase) (Sinha 1972) and GSH (reduced glutathione) (Ellman 1959) and GPx (glutathione peroxidase) activity (Rotruck et al., 1973).

Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) and the groups were compared using Duncan's Multiple Range Test (DMRT).

Results

Table 1 shows the levels of blood ammonia and plasma urea of control and experimental groups. The levels of ammonia and urea were significantly higher in ammonium chloride-treated rats. Rats treated with ammonium chloride and HSEt showed significantly low levels of ammonia and urea when compared with the corresponding ammonium chloride group. Rats treated with HSEt alone showed no significant differences in levels of ammonia and urea when compared with control rats.

Table 1: Effect of HSEt on changes in the blood ammonia and plasma urea of normal and experimental rats

Group	Blood ammonia (µmol/L)	Plasma Urea (mg/dl)
Normal	88.01 ± 6.70 ^a	10.79 ± 0.82 ^a
Normal + HSEt (250 mg/kg)	82.30 ± 6.27 ^a	11.60 ± 0.88 ^a
Ammonium chloride (100 mg/kg)	342.67 ± 26.09 ^b	22.18 ± 1.69 ^b
Ammonium chloride (100 mg/kg) + HSEt (250 mg/kg)	163.70 ± 12.46 ^c	13.00 ± 0.99 ^c

ANOVA followed by Duncan's multiple range test

Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$

The levels of TBARS, HP and CD were significantly higher and the levels of SOD, CAT, GPx and GSH were significantly lower in the liver of ammonium chloride treated rats (Table 2). Rats treated with ammonium chloride and HSEt showed significantly low levels of TBARS, HP and CD and significantly elevated levels of SOD, CAT, GPx and GSH when compared with the corresponding ammonium chloride group. Rats treated with HSEt alone showed no significant differences in levels of TBARS, HP, CD, SOD, CAT, GPx and GSH when compared with control rats (Table 2).

Table 3 shows the levels of TBARS, HP and CD were significantly higher and the levels of SOD, CAT, GPx and GSH were significantly lower in the kidneys of ammonium chloride

treated rats. Rats treated with ammonium chloride and HSEt showed significantly low levels of TBARS, HP and CD, and significantly elevated levels of SOD, CAT, GPx and GSH when compared with the corresponding ammonium chloride group. Rats treated with HSEt alone showed no significant differences in levels of TBARS, HP, CD, SOD, CAT, GPx and GSH when compared with control rats (Table 3).

Discussion

A relationship between oxidative stress and hyperammonemia has been well established and evidences point out that ammonium (acetate / chloride) salts induce hyperammonemia partly via oxidative stress-mediated lipid peroxidation. (Kosenko et al., 1997; Lena and Subramanian 2004; 2003; Dakshayani et al., 2002 a; b; Velvizhi et al., 2002 a; b; Vidya and Subramanian 2003).

In our present study, the levels of ammonia and urea were significantly increased in group 3 rats, which might exhibit hyperammonemic condition in the rats treated with ammonium chloride (Lena and Subramanian 2004; 2003). Maximum reduction in ammonia and urea levels were observed following treatment with the alcoholic leaf extract of *Hibiscus sabdariffa*, which may be due to the significant antihyperammonemic activity of HSEt. This is probably indicative of the antioxidant efficacy of the plant and the presence of natural antioxidants. It has also been reported that natural antioxidants, phenolic compounds and flavonoids have the ability to remove excess ammonia and urea and to offer protection against hyperammonemia (Essa et al., 2005). Our present findings corroborate these reports.

Marked elevations in the concentration of TBARS, HP and CD were observed in liver and kidney tissue of hyperammonemic rats. Increases in lipid peroxide concentrations in hyperammonemic animals have been observed (Kosenko et al., 1997; Lena and Subramanian 2004; 2003; Dakshayani et al., 2002 a; b; Velvizhi et al., 2002 a; b; Vidya and Subramanian, 2003), which may be due to the liver damage caused by ammonia-induced free radical generation. Free radicals initiate lipid peroxidation, which is considered to be deleterious for cell membranes and has been implicated in a number of pathological situations. Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes. Its products (lipid radicals and lipid peroxide) are harmful to the cells in the body and are associated with atherosclerosis and brain damage (Baynes, 1995). Reports have shown that excess ammonia induces nitric oxide synthase, which leads to enhanced production of nitric oxide, leading to oxidative stress and liver damage (Kosenko et al., 2000; Schliess et al., 2002).

The administration of HSEt significantly decreased the levels of TBARS, HP and CD in group 4 rats. This may be due to the free radical scavenging property of HSEt. Previous reports have shown that HSEt is an effective free radical scavenger (Amin and Hamza, 2005; Liu et al., 2002). In addition, the alcoholic extract of *H. sabdariffa* flowers and calyses revealed marked nitric oxide scavenging activity suggesting potent antioxidant property (Odigie et al., 2003; Obiefuna et al., 1993; Amin and Hamza, 2005; Liu et al., 2002; Wang et al., 2000; Adegunloye et al., 1996). Oxidative stress mediated lipid peroxidation is one of the characteristic features of hyperammonemia (Lena and Subramanian, 2004). Reactive oxygen species (ROS) formed continuously during cellular metabolism are normally prevented from being formed or are scavenged by a host of antioxidants (Datta et al., 2000; Halliwell and Gutteridge, 1999; Sen, 1995).

Table 2: Effect of HSEt on the levels of TBARS, HP, CD, SOD, CAT, GPX and GSH in liver tissue of normal and hyperammonemic rats

Group	SOD (U ^A)	CAT (U ^B)	GPx (U ^C)	GSH (mg/100 g tissue)	TBARS (mM/100 g tissue)	HP (mM/100 g tissue)	CD (mM/100 g tissue)
Normal	9.41 ± 0.67 ^a	83.21 ± 6.89 ^a	9.55 ± 0.67 ^a	49.66 ± 3.63 ^a	0.87 ± 0.09 ^a	63.50 ± 5.25 ^a	65.18 ± 3.23 ^a
Normal + HSEt (250 mg/kg)	9.51 ± 0.71 ^a	87.52 ± 6.66 ^a	9.46 ± 0.74 ^a	50.23 ± 3.29 ^a	0.82 ± 0.06 ^a	66.33 ± 5.89 ^a	63.77 ± 3.21 ^a
Ammonium chloride (100 mg/kg)	3.81 ± 0.31 ^b	41.99 ± 3.85 ^b	5.19 ± 0.32 ^b	25.69 _b ± 2.49	2.19 ± 0.17 ^b	97.27 ± 7.56 ^b	99.26 ± 5.35 ^b
Ammonium chloride + HSEt	6.57 ± 0.39 ^c	72.24 ± 4.98 ^c	7.85 ± 0.39 ^c	43.37 ± 3.13 ^c	1.19 ± 0.12 ^c	75.61 ± 5.34 ^c	73.05 ± 3.66 ^c

ANOVA followed by Duncan's multiple range test. Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$

A-amount of enzyme required to inhibit 50% of NBT reduction/mg protein. B-Micromoles of H₂O₂ consumed/min/mg protein.

C-Micromoles of GSH utilized/g protein. SOD – Super oxide dismutase; CAT – Catalase; TBARS- Thiobarbituric acid and reactive substances;

HP- Hydroperoxides; CD- Conjugated dienes; GPX- Glutathione peroxidase; GSH- Reduced glutathione

Table 3: Effect of HSEt on the levels of TBARS, HP, CD, SOD, CAT, GPX and GSH in kidney tissue of normal and hyperammonemic rats

Group	SOD (U ^A)	CAT (U ^B)	GPx (U ^C)	GSH (mg/100 g tissue)	TBARS (mM/100 g tissue)	HP(mM/100 g tissue)	CD (mM/100 g tissue)
Normal	2.87 ± 0.19 ^a	2.07 ± 0.14 ^a	7.12 ± 0.49 ^a	33.99 ± 2.73 ^a	1.52 ± 0.09 ^a	54.36 ± 4.14 ^a	16.12 ± 0.39 ^a
Normal + HSEt (250 mg/kg)	2.89 ± 0.22 ^a	2.05 ± 0.16 ^a	7.26 ± 0.39 ^a	34.15 ± 2.41 ^a	1.51 ± 0.12 ^a	54.75 ± 4.17 ^a	15.99 ± 0.37 ^a
Ammonium chloride (100 mg/kg)	1.75 ± 0.16 ^b	1.49 ± 0.10 ^b	4.24 ± 0.31 ^b	20.97 ± 1.71 ^b	2.18 ± 0.07 ^b	74.20 ± 5.65 ^b	26.48 ± 0.87 ^b
Ammonium chloride + HSEt	2.55 ± 0.19 ^c	1.74 ± 0.09 ^c	6.47 ± 0.37 ^c	28.91 ± 1.61 ^c	1.31 ± 0.06 ^c	63.10 ± 4.80 ^c	20.01 ± 0.01 ^c

ANOVA followed by Duncan's multiple range test. Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$. A-amount of enzyme required to inhibit 50% of NBT reduction/mg protein. B-Micromoles of H₂O₂ consumed/min/mg protein. C-Micromoles of GSH utilized/g protein. SOD – Super oxide dismutase; CAT – Catalase; TBARS- Thiobarbituric acid and reactive substances; HP- Hydroperoxides; CD- Conjugated dienes; GPX- Glutathione peroxidase; GSH- Reduced glutathione

SOD and CAT play key roles in the detoxification of superoxide anion and hydrogen peroxide respectively, thereby protecting against ROS-induced damage (Eaton, 1991; Fridovich, 1995). GSH, a physiologically important non-protein thiol, in conjunction with GPx, is involved in protection against free radicals, peroxides, and other toxic compounds (Cotgreave et al., 1998; Ketterer, 1998; Rahman and Macnee, 2000). This might have decreased levels of antioxidants such as SOD, CAT, GPx and GSH in group 3 rats.

Elevated levels of enzymatic and non-enzymatic antioxidants in ammonium chloride and HSEt-treated rats may be due to the presence of phenolic phytochemicals and bioflavonoids (like anthocyanins, glycosides, PCA, hydroxycitric acid, etc.) (Lewis and Neelakantan, 1965; Osman et al., 1975) in HSEt which offers possible role in reducing the oxidative stress by inducing cellular antioxidant enzymes. Flavanoids and polyphenolic compounds are potent free radical scavengers and are known to modulate the activities of various enzyme systems due to their interaction with various biomolecules (Devipriya and Shyamaladevi, 1999). Most phenolic phytochemicals that have positive effects on health are believed to function by countering the effects of reactive oxygen species generated during cellular metabolism. Phenolic phytochemicals due to their phenolic ring and hydroxyl substituents can function as effective antioxidants due to their ability to quench free electrons. It is therefore believed that dietary phenolic antioxidants can scavenge harmful free radicals and thus inhibit their oxidative reactions with vital biological molecules (Rice-Evans et al., 1996) and prevent the development of many physiological conditions, which can manifest into disease. Previous reports show that the calyx extract of *Hibiscus sabdariffa* decreased lipid peroxidation and cell damage and overall, *Hibiscus sabdariffa* is a good source of antioxidants (Tsai et al., 2002; Tseng et al., 1996; Duh and Yed 1997). Hence, the possible mechanism by which the HSEt modulates oxidant-antioxidant imbalance in hyperammonemic conditions could be attributed to the presence of natural antioxidants and its free radical scavenging properties.

Medicinal plants continue to provide valuable therapeutic agents, both in modern medicine and in traditional systems. The doubts about the efficacy and safety of antihyperammonemic agents have prompted a search for safe and effective drugs as alternatives in the treatment of hyperammonemia. The present investigation shows that HSEt possesses an antioxidant activity, which may be attributed to its protective action on lipid peroxidation and to the enhancing effect on cellular antioxidant defense contributing to the protection against oxidative damage in ammonium chloride induced hyperammonemia. The exact mechanism of action of the extract still has to be investigated and the isolation of the active constituents remains to be done.

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