Ruta graveolens mitigates ammonium chloride-induced hyperammonemia by modulating antioxidant status and pro-inflammatory cytokines

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Abstract: Hyperammonemia is a major contributing factor to neurological abnormalities observed in hepatic encephalopathy. The present study was designed to evaluate the possible protective effects of *Ruta graveolens* against ammonium chloride (AC)-induced hyperammonemia in rats. Hyperammonemia was induced by daily intraperitoneal injections of AC at dose of 100 mg/kg body weight for 8 weeks. The biochemical results showed that administration of AC induced significant increase in blood ammonia and urea levels. In addition, hyperammonemia induced oxidative stress in the liver and brain as evident from the increased lipid peroxidation (LPO), declined glutathione (GSH) content and glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities. Moreover, hyperammonemia was associated with a significant increase in serum tumor necrosis factor alpha (TNF-α). Concomitant administration of *Ruta graveolens* efficiently alleviated the altered biochemical parameters. In conclusion, *Ruta graveolens* showed a marked protective effect against AC-induced hyperammonemia in rats through its antioxidant and anti-inflammatory efficacies.
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1.Introduction

Hepatic encephalopathy (HE) is a medical phenomenon characterized by psychomotor, intellectual and cognitive abnormalities with emotional/affective and behavioral disturbances (Av, 2007; Cichoż-Lach and Michalak, 2013). It can be the result of acute liver failure, portosystemic bypass without hepatocellular disease or liver cirrhosis, and portal hypertension or portosystemic shunts (Av, 2007). It has been also reported that HE is a broad spectrum of neuropsychiatric manifestations usually affecting individuals with end-stage liver disease (Dbouk and McGuire, 2006).

Although the underlying pathologic mechanisms of HE are not fully understood, Jones and Mullen (2012) stated that elevated plasma and central nervous system (CNS) ammonia levels are considered a key factor in this syndrome's pathogenesis. Childrens with congenital urea cycle enzyme defect have the high serum ammonia concentration, which-if left untreated-leads to the development of severe neurological symptoms, seizures, and coma, and at those who survived to mental retardation and paralysis of the brain (Felipo and Butterworth, 2002). Multiple studies reported that elevated ammonia influences mechanisms leading to impaired blood-brain barrier (BBB), changes in neurotransmission, pro-inflammatory cytokines, oxidative stress, abnormalities in GABA-ergic or benzodiazepine pathways, impaired energy metabolism of the brain, and impaired cerebral blood flow (Montgomery and Bajaj, 2011; Srivastava *et al*., 2011; Li *et al*., 2012; Skowron´ska and Albrecht, 2012). In addition, the studies of Lena and Subramanian (2004), Rodrigo *et al*. (2004) and Majeed (2005) demonstrated that ammonia toxicity occurs partly via oxidative stress leading to hepatic dysfunction and failure, which is a primary cause of neurological disorders and alterations in the function of the CNS associated with hyperammonemia. Moreover, a study conducted by Shawcross *et al.* (2004) in patients with liver cirrhosis demonstrated that inflammation and inflammatory mediators may significantly modulate ammonia influence on the CNS.

Medicinal plants play a major role in managing human diseases and numerous important modern drugs have been developed from molecules originally isolated from natural sources (Lee, 2000; Balunas and Kinghorn, 2005). *Ruta graveolens* L., (*Rutaceae*), commonly known as rue, is known as a medicinal plant since ancient times and currently used for treatment of various disorders such as aching pain, eye problems, rheumatism, and dermatitis (Miguel, 2003). Raghav *et al*. (2006) stated that *R. graveolens* is a native of the Mediterranean region and now being cultivated throughout Europe and many Asian countries. *R. graveolens* extracts and essential oil are important areas in drug development with numerous

pharmacological activities in many countries (Asgarpanah and Khoshkam, 2012). In addition, *R. graveolens* has been shown to possess a wide range of pharmacological properties such as antioxidant (Ahmed *et al*., 2010), anti-inflammatory (Ratheesh and Helen, 2007), antidiabetic (Ahmed *et al*., 2010; Toserkani *et al*., 2011), antibacterial, antifungal (Meepagala *et al*., 2005), antiandrogenic (Khouri and El-Akawi, 2005) and insecticidal activity (Barbosa *et al*., 2011). Based on the traditional uses of *R. graveolens*, the current investigation was undertaken to evaluate the effects of *R. graveolens* aqueous extract on oxidant-antioxidant status and the pro-inflammatory cytokine, TNF-α, in ammonium chloride-induced hyperammonemic rats.

2. Materials and methods:

Experimental animals:

White male albino rats weighing about 150-180 g were used in the current study. They were obtained from the animal house of the National Research Center, El-Giza, Egypt. They were kept under observation for about 15 days before the onset of the experiment to exclude any intercurrent infection. The chosen animals were housed in plastic well aerated cages at normal atmospheric temperature and normal 12-hour light/dark cycle. Moreover, they had free access to water and were supplied daily with standard diet of known composition *ad libitum*. All animal procedures were in accordance with the recommendations of the Canadian Committee for Care and Use of Animals (Canadian Council on Animal Care, 1993).

Preparation of plant extract:

R. graveolens (sadab) was obtained from Experimental Station of Medical Plants (ESMP), Faculty of Pharmacy, Cairo University, Egypt. Its leaves were air dried and then powdered with an electric grinder. The infusion (water extract) was prepared according to the method described by Swanston-Flatt *et al*. (1995). Powdered plant material was added to boiling water and infused for 15 minutes. The infusion was filtered and the filtrate was freshly used.

Experimental design:

Hyperammonemia was induced in male Wistar rats by daily intraperitoneal (i.p.) injection of ammonium chloride (AC) at dose of 100 mg/kg body weight for 8 weeks (Mahmoud, 2012). The rats were divided into 3 groups. Group 1: normal untreated rats; Group 2 (AC): rats treated with AC (100 mg/kg b.wt.) and Group 3: rats concurrently treated with AC (100 mg/kg b.wt.) and 100 mg/kg b.wt. *R. graveolens*.

Biochemical assays:

At the end of 8 weeks, the animals were killed by decapitation under ether anesthesia. Blood samples were taken for the determination of ammonia (Wolheim, 1984) and urea (Varley *et al*., 1998) using reagent kits purchased from Spinreact (Spain).

Serum levels of the pro-inflammatory cytokine, TNF-α, was determined by specific ELISA kits (R&D Systems, USA) according to the manufacturer's instructions. The concentration of TNF-α was determined spectrophotometrically at 450 nm. Standard plot was constructed by using standard cytokine and the concentrations for unknown samples were calculated from the standard plot.

Liver and brain lipid peroxidation, reduced glutathione (GSH), and superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured according to the methods of Preuss *et al*. (1998), Beutler *et al*. (1963), Marklund and Marklund (1974) and Kar and Mishra (1976), respectively.

Statistical analysis:

Statistical analysis was performed using SPSS v.16. Results were articulated as mean ± standard error (SE) and all statistical comparisons were made by means of one-way ANOVA test followed by Duncan's multiple range test post hoc analysis. A P value <0.05 was considered significant.

3. Results:

Data describing the effect of *R. graveolens* on blood ammonia and urea concentrations were represented in Table 1. AC-administered rats showed a significant elevation in ammonia (P<0.001) and urea (P<0.01) levels when compared to normal control rats. Concurrent administration of *R. graveolens* along with AC produced a potential alleviation of the altered ammonia (P<0.001) and urea (P<0.05) levels.

Table 1: Effect of *R. graveolens* on blood ammonia and urea levels of hyperammonemic rats.

Parameter / Group	Ammonia (μmol/L)	Urea (mg/dl)
Normal	65.89 ± 5.25^{b}	17.12 ± 1.64^{b}
AC	290.51 ± 7.63^{a}	54.59 ± 3.96^{a}
AC + *R. graveolens*	106.80 ± 7.82^{b}	23.20 ± 1.76^{b}
F-Prob.	P<0.001	P<0.01

Data are expressed as Mean ± SE. Means which share different superscript symbol(s) are significantly different.

The effect of AC administration on serum TNF-α was represented in Figure 1. The recorded data showed a significantly elevated serum TNF-α concentration (P<0.01) in hyperammonemic rats when compared to the normal control ones. On the other hand, *R. graveolens* supplementation significantly (P<0.05) decreased the elevated serum TNF-α.

Liver and brain lipid peroxidation, estimated as nmol MDA/100 mg tissue, showed a significant (P<0.01, P<0.05) increase in hyperammonemic rats when compared to the normal control group. Supplementation of 100 mg/kg *R. graveolens* markedly ameliorated the elevated MDA levels (Figs. 2a & b).

Conversely, AC administration produced a significant (P<0.01, P<0.05) decrease in GSH content in the liver and brain of hyperammonemic rats. On the other hand, *R. graveolens* co-administration significantly increased liver and brain GSH content when compared to rats received AC only as depicted in Figures 3a & b.

Similarly, activities of the antioxidant enzymes, SOD and GPx showed a significant decrease in hyperammonemic rats (Fig. 4a,b and 5a,b). Along concurrent administration with AC, *R. graveolens* significantly increased the activities of liver and brain SOD (P<0.001) and GPx (P<0.01).

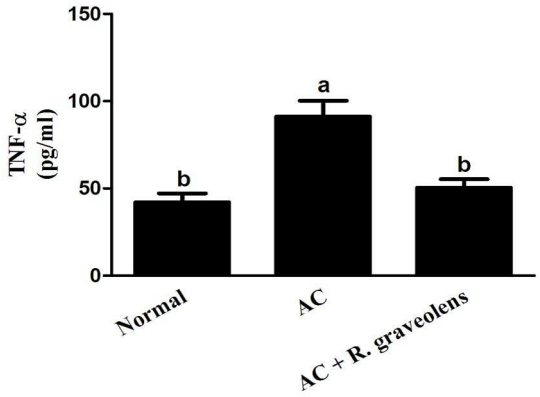

Figure 1: Effect of *R. graveolens* on serum TNF-α of hyperammonemic rats. Data are expressed as Mean ± SE. Means which share different superscript symbol(s) are significantly different.

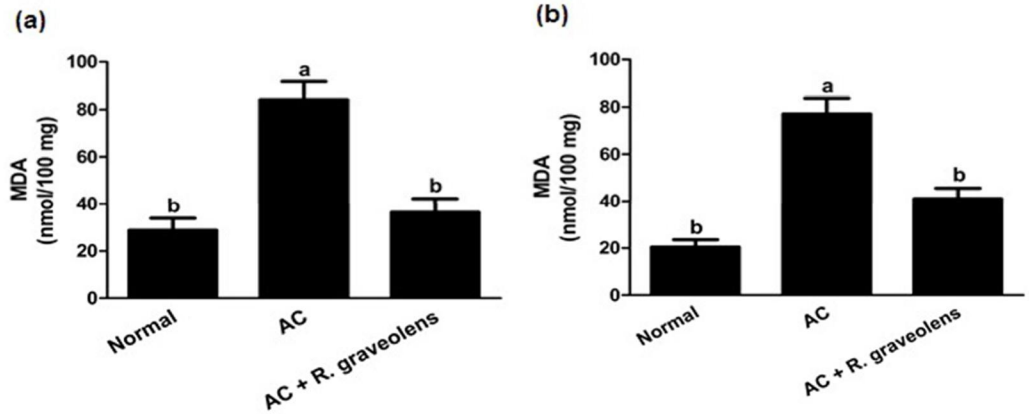

Figure 2: Effect of *R. graveolens* on liver (a) and brain (b) lipid peroxidation of hyperammonemic rats. Data are expressed as Mean ± SE. Means which share different superscript symbol(s) are significantly different.

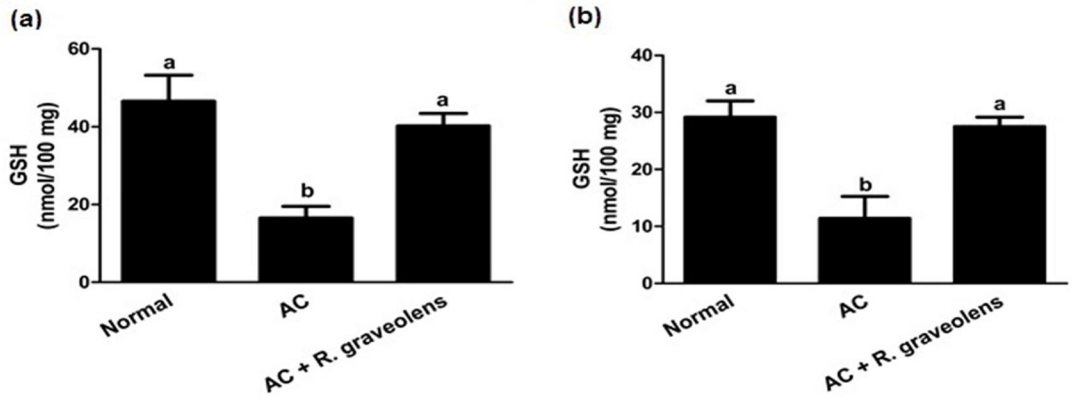

Figure 3: Effect of *R. graveolens* on liver (a) and brain (b) GSH content of hyperammonemic rats. Data are expressed as Mean ± SE. Means which share different superscript symbol(s) are significantly different.

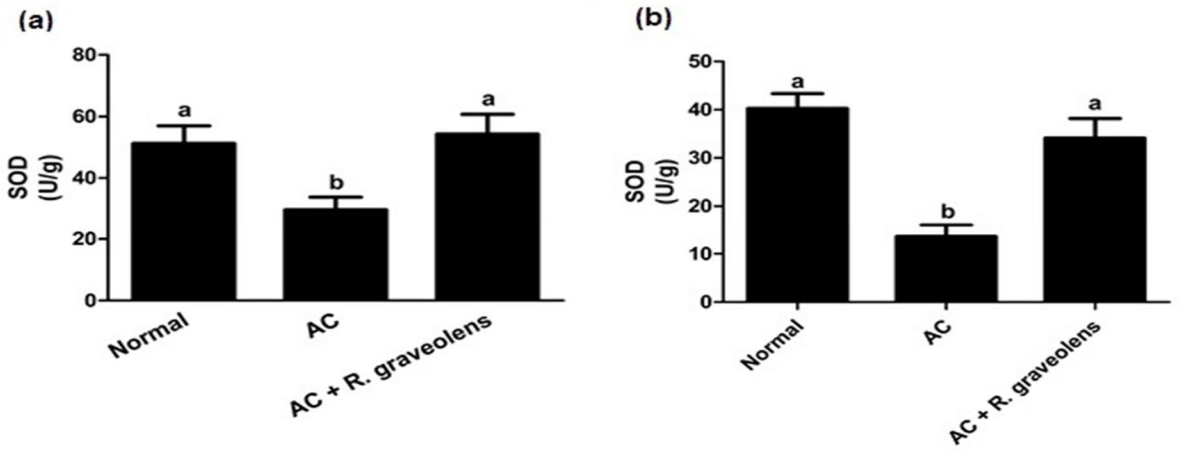

Figure 4: Effect of *R. graveolens* on liver (a) and brain (b) SOD activity of hyperammonemic rats. Data are expressed as Mean ± SE. Means which share different superscript symbol(s) are significantly different.

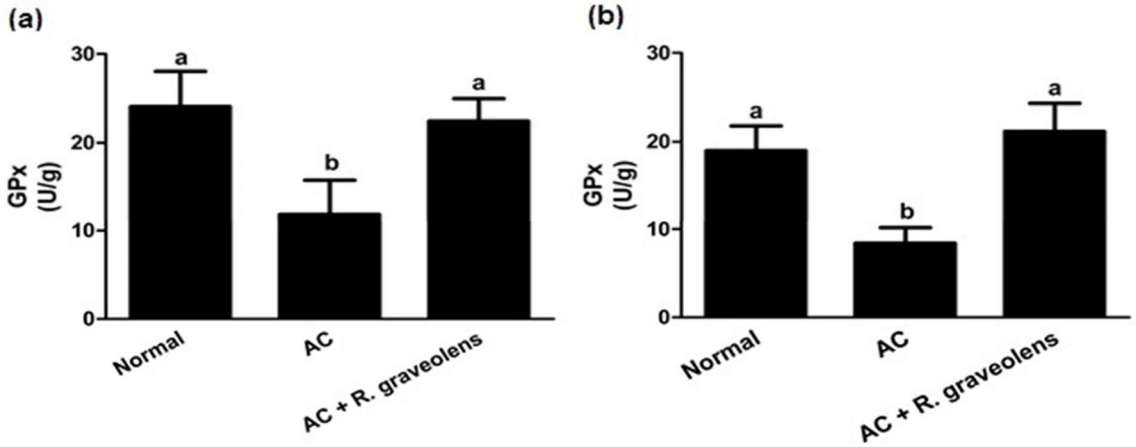

Figure 5: Effect of *R. graveolens* on liver (a) and brain (b) GPx activity of hyperammonemic rats. Data are expressed as Mean ± SE. Means which share different superscript symbol(s) are significantly different.

4. Discussion:

Ammonia is a substrate for a number of enzymatic reactions in the brain and is also a product of some other reactions (Felipo and Butterworth, 2002). Hyperammonemia is a major contributing factor to neurological abnormalities observed in HE and congenital defects of ammonia detoxification. Ammonia affects both excitatory and inhibitory synaptic transmission in the mammalian brain through a variety of mechanisms (Monfort and Felipo, 2005). The serious adverse effects, toxicity and reappearance of symptoms after discontinuation (Srinivasan *et al*., 2001) are the common disadvantages of the currently available conventional or synthetic antihyperammonemic agents. Therefore, there is a growing need to find protective agents against hyperammonemia from traditional medicinal plants.

The current results showed that AC administration produced a significant elevation of ammonia and urea levels. These findings are in agreement with the studies of Mahmoud (2012) and Essa and Subramanian (2007) who demonstrated that increased levels of circulatory ammonia and urea indicate hyperammonemic condition in AC supplemented rats, which may be due to liver damage caused by ammonia intoxication. On the other hand, concurrent administration of *R. graveolens* aqueous extract protected against AC-induced hyperammonemia. We assume that the anti-hyperammonemic effect of *R. graveolens* might be attributed to its rich content of phenolic compounds. In this regard, multiple investigations have revealed that plant extracts containing phenolic compounds and flavonoids offer ammonia detoxification by removing excess ammonia, urea, uric acid and creatinine during various disease conditions, such as hyperammonemia, and nephrotoxicity (Nakamura *et al*., 2001; Shirwaikar *et al*., 2003). In addition, Mahmoud (2012) reported that rutin, the major

constituent of *R. graveolens*, protected against AC-induced hyperammonemia in rats.

Concerning the role of inflammation, the study of Shawcross *et al.* (2007) demonstrated that inflammation is an important factor determining the presence and severity of neuropsychological dysfunction in minimal hepatic encephalopathy caused by ammonia, that is, more significant in more severe inflammation. In addition, the peripheral immune system has been found to communicate with the brain in response to infection and inflammation. This response results in neutrophil adhesion, migration across the BBB and release of chemokines, pro-inflammatory cytokines, proteases and reactive oxygen species (ROS) as well as inflammatory gene transcription (Shawcross *et al.*, 2010). Our data revealed that hyperammonemia was accompanied with a significant increase of the circulatory TNF-α levels. Through its anti-inflammatory effect (Ratheesh and Helen, 2007), *R. graveolens* supplementation efficiently protected against hyperammonemia associated TNF-α elevation. The anti-inflammatory efficacy of *R. graveolens* may be due to the presence of the broad range of flavonoids present in the plant extract especially rutin and quercetin.

Studies have shown that there is a close relationship between hyperammonemia and oxidative stress (Essa and Subramanian, 2007; Mahmoud, 2012). Oxidative stress mediated lipid peroxidation is one of the characteristic features of hyperammonemia (Lena and Subramanian, 2004). In addition, multiple studies have addressed the relationship between oxidative stress and hyperammonemia and pointed out that ammonium (acetate/chloride) salts induce hyperammonemia partly via oxidative stress-mediated lipid peroxidation (Vidhya and Subramanian, 2003; Lena and Subramanian, 2003, 2004; Mahmoud, 2012). Moreover, Studies in patients with portosystemic anastomosis showed a disproportionately high level of ammonia in some regions of the brain such as cerebral cortex, which may impair the integrity of astrocytes (Butterworth *et al.*, 1988).

In the present study, marked elevation in brain and liver lipid peroxidation levels, accompanied with declined activity of the antioxidant defense system has been observed. In accordance, previous studies demonstrated the elevation of lipid peroxides in hyperammonemic animals (Velvizhi *et al.*, 2002a,b; Vidhya and Subramanian, 2003; Essa and Subramanian, 2006; Mahmoud, 2012) which may be due to the liver damage caused by ammonia-induced free radical generation (Rehman *et al.*, 2003; Mahmoud, 2012). These findings are being supported by the considerable liver damage induced in hyperammonemic rats as reported by Mahmoud (2012). In addition, a study by Sinke *et al.* (2008) on ammonia-induced astrocyte swelling demonstrated that ammonia-induced oxidative stress activated nuclear factor κB (NF-κB), followed by increased inducible nitric oxide synthase (iNOS) protein expression and the subsequent generation of nitric oxide (NO), leading to cellular damage. Concurrent administration of *R. graveolens* potentially alleviated the altered antioxidant defense mechanisms with subsequent decreased lipid peroxidation levels. The protective effects of *R. graveolens* may be attributed to the potent free radical scavenging activity of the contained flavonoids and polyphenolic compounds. In this regard, a recent study by Mahmoud (2012) demonstrated that rutin, the major active constituent of *R. graveolens*, markedly decreased lipid peroxidation, increased glutathione concentration and ameliorated the antioxidant enzyme activities in liver and brain of AC-induced hyperammonemic rats. Moreover, the antioxidant efficacy of *R. graveolens* has been reported in multiple studies (Ratheesh and Helen, 2007; Ratheesh *et al.*, 2009; Ahmed *et al.*, 2010).

In conclusion, the current study suggests that administration of *R. graveolens* protected against the deleterious effects of hyperammonemia by mechanisms related to its antioxidant and anti-inflammatory efficacies.

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