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Research Article

Hepatoprotective effect of *Semecarpus anacardium*in rats: a molecular approach

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Abstract

This investigation was studied to verify the possible hepatoprotective effects of *Semecarpus anacardium* nut milk extract (SAE) against ammonium chloride (NH_4Cl) - induced experimental hyperammonemia. Experimental hyperammonemia was induced in adult male Wistar rats (180–200g) by intra-peritoneal injections of NH_4Cl (100mg/kg b.wt). Results of oral administration of SAE (150mg/kg b.wt) on blood ammonia, nitric oxide, Na^+/K^+-ATPase in brain and expression of liver urea cycle enzymes of normal and experimental animals were evaluated. NH_4Cl induced rat's demonstrated considerable increase in the level of blood ammonia. Significant decrease in the levels of nitric oxide, Na^+/K^+-ATPase activity and reduced expression of liver urea cycle enzymes were also detected. Treatment of SAE normalized the above said changes in hyperammonemic rats. This study tenders a support for antihyperammonemic, and hepatoprotective potential of SAE in resistance to oxidative stress and cellular damages induced by NH_4Cl; cytoprotective actions and presence of active phytochemical constituents and antioxidants could be accountable for antihyperammonemic effects of SAE.

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Keywords: Hyperammonemia, *Semecarpus anacardium*, Nitric oxide, Urea cycle enzymes.

Introduction

Hyperammonemia is characterized by raised ammonia levels in blood, owing to defective liver functions ensuing insufficient ammonia detoxification. Ammonia is neurotoxic while accruing in surplus and hyperammonemia is primarily accountable for the neurological modifications found in liver diseases and hepatic encephalopathy[1]. Antiepileptic drugs such as valproate and salicylate cause hyperammonemia in mammalian systems[2,3]. Ammonia toxicity results in free radical production that causes oxidative stress mediated tissue damages[4-6] and raised ammonia concentration in brain cause lethal effects on neural cells[7]. The screening and appraisal of drugs for their anti-hyperammonemic activity is pursuing till date, essentially from traditional medicinal plants and natural products.

Semecarpus anacardium, commonly known as 'marking nut' has high priority and applicability in indigenous system of medicine against various diseases. *Semecarpus anacardium* nut milk extract (SAE)potentiated the efficacy of commonly used anti-cancer drugs like mitomycin, fluorouracil and methotrexate[8]. The major active components of *Semecarpus anacardium* nuts are shown in Table 1[9-12]. SAE was subjected to investigation against hepatocellular carcinoma[13], mammary carcinoma[14] and rheumatoid arthritis[8] in experimental mammals for its beneficial effects. Further, SAE is known to offer hepatoprotective[13], anti-inflammatory[15], antiatherogenic[16], cardio protective[17] and antiglycemic properties[18].

Based on the literature survey the anti-hyperammonemic effect of SAE was not studied. Therefore, we hypothesized that SAE could be effective in treating hyperammonemia; furthermore, its actions at molecular level in the modulation or expression level of urea cycle

enzymes have not been investigated till date and in this perspective the influence of SAE on blood ammonia, brain nitric oxide (NO), activity of Na^+/K^+-ATPase in brain and expression of liver urea cycle enzymeshave been investigated in an rodent model system.

Materials and Methods

Experimental animals

All the experiments were carried out in male albino Wistar rats (180–200g), obtained from Central Animal House, Faculty of Medicine, Annamalai University, Tamil Nadu, India. They were housed in polypropylene cages (47×34×20 cm) lined with husk, renewed every 24 h, kept under 12:12 h light/dark cycle at 23±2°C and had free access to drinking water and food. The rats were fed with standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Animal Ethical Committee of AnnamalaiUniversity (Approval no. 537; dated 20/03/2008).

Chemicals

Ammonium chloride was purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used in the study were of analytical grade.

Semecarpusanacardium nut milk extract (SAE)

The preparation contains purified nuts of *Semecarpus anacardium*, cow's milk and ghee. The SAE was prepared by boiling the nuts (200g) with 500 ml of milk. After decanting the decoction, 500 ml of milk was added to the boiled nuts and the mixture was again boiled for 15 minutes. The decoction was recovered and the process was repeated again with the milk. All the three portions of milk nut decoction were mixed with ghee and boiled till dehydrated, then filtered and stored[19].

Induction of experimental hyperammonemia

Hyperammonemia was induced in Wistar rats by intraperitoneal injections of ammonium chloride at a dose of 100 mg/kg body weight (b.wt) thrice in a week for 8 consecutive weeks[6,20].

Experimental design

The rats (180– 200 g) were divided into 5 groups of 6 rats each. Group I rats were administered with olive oil (as vehicle) 0.5 ml each; group II rats were administered with SAE (150 mg/kg b.wt. dissolved in 0.5 ml olive oil) orally by using an intragastric tube (thrice a week for 8 consecutive weeks)[8]; group III rats were injected intraperitoneally with NH_4Cl (100 mg/kg b.wt); group IV rats were given NH_4Cl + SAE (thrice a week for 8 consecutive weeks) and SAE control and group V rats (SAE control) were orally administered with milk and ghee extract without SA nuts (thrice a week for 8 consecutive weeks respectively).

At the end of 8th week, the rats were made to fast overnight and sacrificed by cervical dislocation. Blood samples were collected; plasma and serum were separated by centrifugation. Liver and brain tissues were excised immediately and rinsed in ice-chilled normal saline; 500 mg of the tissues were homogenized in 5.0 ml of 0.1 M Tris-HCl buffer (pH, 7.4). The homogenate was centrifuged and supernatant was used for the estimation of biochemical indices.

Biochemical analysis

Blood ammonia was determined by enzymatic spectrophotometric assay of Wolheim[21]. NO in the brain was estimated by the method of Green et al[22]. Activity of brain Na^+/K^+-ATPase was estimated by the method of Bonting[23].

Expression of urea cycle enzymes

Expression of urea cycle enzymes (such as carbamylphosphatesynthetase I (CPS-I), ornithine transcarboxylase (OTC), argininosuccinatesynthetase (AS), argininosuccinatelyase (AL) and arginase (AR)) in liver were investigated by western blotting.

The rat liver tissue (100 mg) was homogenized in lysis buffer [135 mMNaCl, 20 mMTris, 2 mM EDTA and 1 mM phenyl methyl sulfonyl fluoride (PMSF-pH 7.4)] and the volume was made up to 1 ml using the same buffer. The homogenates were centrifuged (15 min, 10,000 rpm at 4 °C) and the protein content of the supernatant was determined with BSA as standard by the method of Lowry et al[24]. Aliquots of supernatant (50 µg total protein) were boiled for 5 min in sample buffer [0.2MTris–HCl buffer, 10% glycerol, 2% SDS, 0.02% β-mercaptoethanol]. Proteins were separated by Tris-Glycine-SDS discontinuous 12% polyacrylamide gel electrophoresis, and electroblotted onto polyvinylidenefluoride (PVDF) membrane (Sigma

chemicals, Mumbai) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, USA).

After protein transfer, the membrane was incubated for 2 h at room temperature in blocking buffer [TBS, pH 7.5 containing 0.05% Tween 20, 5% BSA] with gentle shaking (50 rpm) on a rotary shaker. After blocking, the membrane was rinsed for 5 min in antiserum buffer (5% milk powder in 1X PBS+ 0.1% Tween 20), then incubated for 16 h at 4 °C with primary antibody [rabbit polyclonal anti-rat CPS-I antiserum (1:1000), rabbit polyclonal anti-human OTC antiserum (1:1000), rabbit polyclonal anti-rat AS antiserum (1:1000), rabbit polyclonal anti-rat AL antiserum (1:1000), rabbit polyclonal anti-human AR antiserum (1:1000)] with blocking buffer. After incubation with primary antibody, the unbound primary antibody was removed with 2-3 washings with antiserum buffer. The membrane was incubated for 1 hour at room temp in goat anti-rabbit IgG alkaline phosphatase conjugate (Bangalore Genei Pvt. Ltd., Bangalore, India) diluted (1:5000) in antiserum buffer. The unbound secondary antibody was removed with 2-3 washings with PBST (1X PBS+ 0.1% Tween 20). Specific binding was detected using 5-bromo-4-chloro-3-indolyl Phosphate /nitroblue tetrazolium (BCIP/NBT) as substrates. Membrane with bands was scanned with a scanner (HP Scanjet 3500 c) and the intensity of each bands were quantified using the Biorad Quantity on 1-D Analysis Software (BioRad).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using SPSS software package 9.05. Results were expressed as mean ± SD from 6 rats in each group. *P* values <0.05 were considered as significant.

Results

Rats induced with NH_4Cl showed a significant increase in the levels of blood ammonia and brain nitric oxide (Figure 1&2). Oral treatment with SAE to NH_4Cl-induced rats significantly decreased the levels of blood ammonia and brain nitric oxide. Activity of brainNa^+/K^+-ATPase was found to be significantly increased in NH_4Cl-induced rats, when compared to controls. The levels were found to be significantly reduced in hyperammonemic rats treated with SAE (Figure 3).

NH_4Cl rats revealed a significant decrease in the expression of CPS-I, OTC, AS, AL, and AR in liver tissue (Figure 4). Oral administration of SAE to NH_4Cl-induced rats significantly increased the expression of urea cycle enzymes in liver.

Discussion

Ammonia is removed from liver either in the form of urea in periportal hepatocytes and/or as glutamine in perivenoushepatocytes[25]. Increased levels of circulatory ammonia might indicate hyperammonemic condition in rats treated with NH_4Cl, which may be owing to liver damage caused by ammonia intoxication[5,6,20]. The reduction in the level of ammonia during SAE treatment in hyperammonemic rats indicated the significant anti-hyperammonemicactivity of SAE. It has also been reported that the effect of SAE to improve the components of immune system in adjuvant arthritics, with marked free radical scavenging activity, suggesting a potent antioxidant property[26].

In our study, NH_4Cl treated experimental rats revealed increased activity of Na^+/K^+-ATPase and elevated level of brain NO; this could be due to excessive activation of *N*-Methyl-*D*-aspartate (NMDA) receptors by ammonia intoxication, which could lead to neuronal degeneration and cell death[27]. The excessive activation of NMDA receptors allows the entry of excessive Ca^{2+} and Na^+ concentration in the postsynaptic neuron[28]. To maintain Na^+ homeostasis, Na^+ entering through the channel is extruded from the neuron by Na^+/K^+-ATPase, which consumes ATP. Kosenko et al[29] reported that acute ammonia intoxication could lead to increased activity of Na^+/K^+-ATPase in brain. In the postsynaptic neuron, Ca^{2+} binds to calmodulin and activates nNOS, increasing the formation of NO and thus contributing to the neurotoxic process. Activation of NMDA receptors could lead to increased production of superoxide radical[28]. Superoxide and NO have the ability to generate hydroxyl radicals, leading to oxidative stress and could cause tissue damage[27,30]. SAE has remarkable reduction in nitrate/nitrite level, which can be attributed to antioxidant constituents present in it[8].Previous studies reported that SAE could offer neuroprotective effect in Alzheimer's disease models[31].This effect could be attributed to the presence of flavonoids in the SAE. The inhibition of lipid peroxidation may be due to the free radical scavenging property of flavonoids, which can scavenge singlet O_2, terminating peroxides by their low redox potential[32] and thus SAE could reduce the brain damage caused by ammonia[8,30].

Table 1: Active phyto constituents present in SAE

S.No	Major active components of SAE
1	anacardic acid
2	semicarpol
3	bhilawanol
4	monolefin I
5	dilefin II
6	bhilawanol-A
7	bhilawanol-B
8	biflavone A
9	biflavone A1
10	biflavone A2
11	biflavone B
12	biflavone C
13	tetrahydroamentoflavone
14	tetrahydrobustaflavone
15	jeediflavanone
16	semicarpuflavanone
17	gulluflavanone
18	nallaflavanone
19	semecarpetin
20	anacarduflavanone
21	catechol

Figure 1

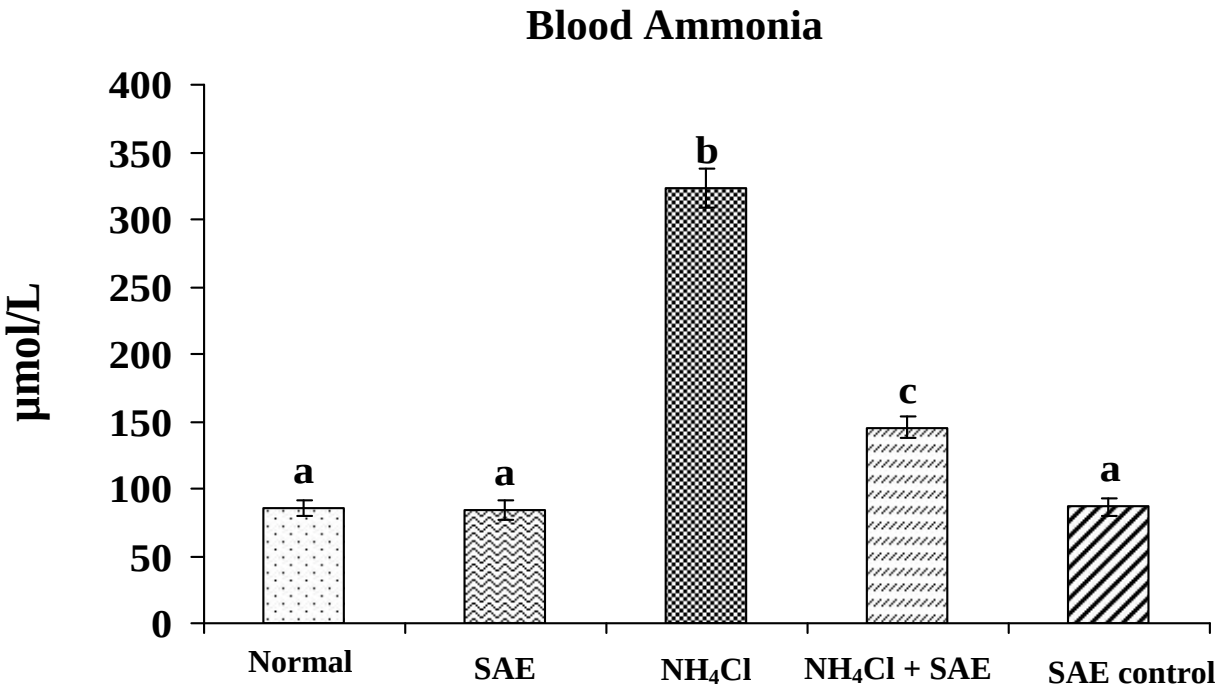


Figure 2

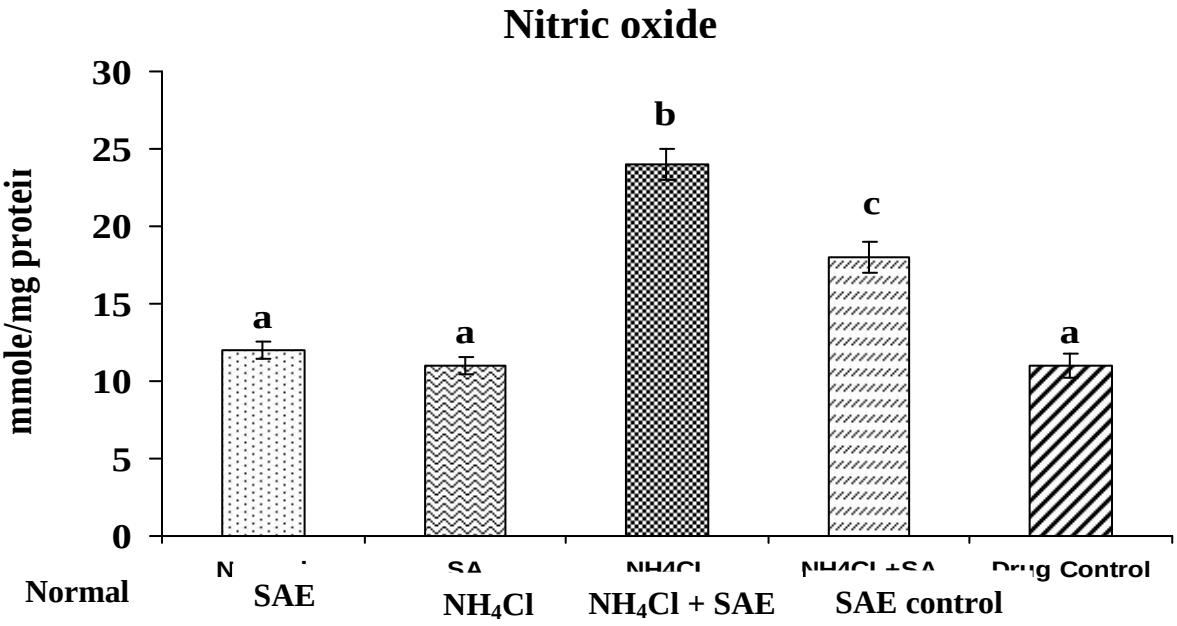


Figure 3

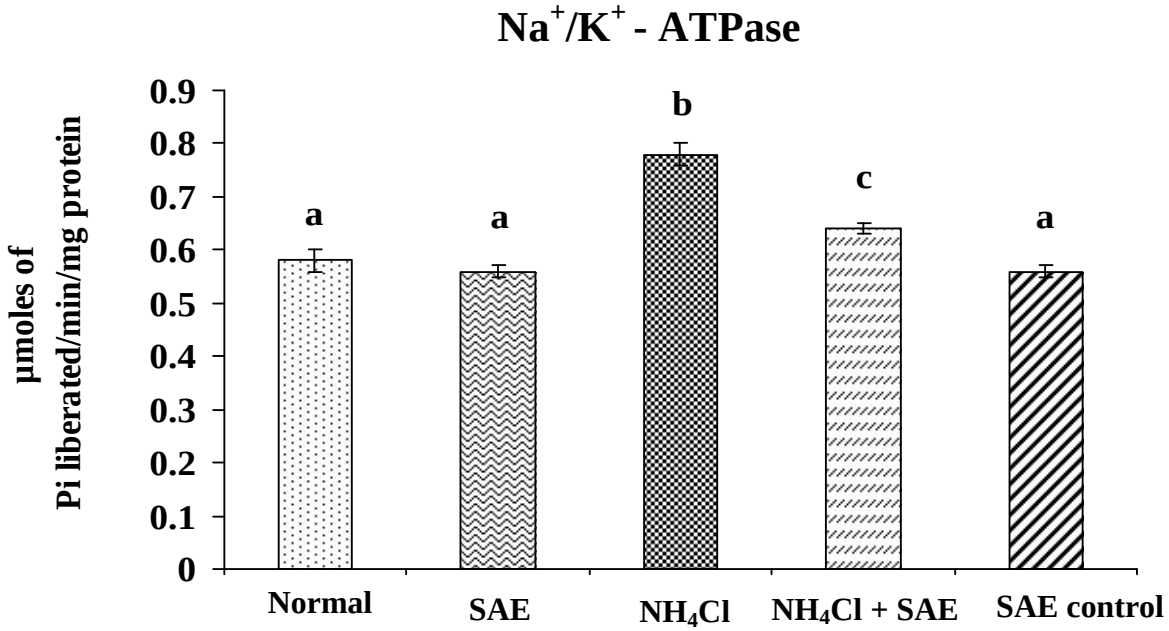
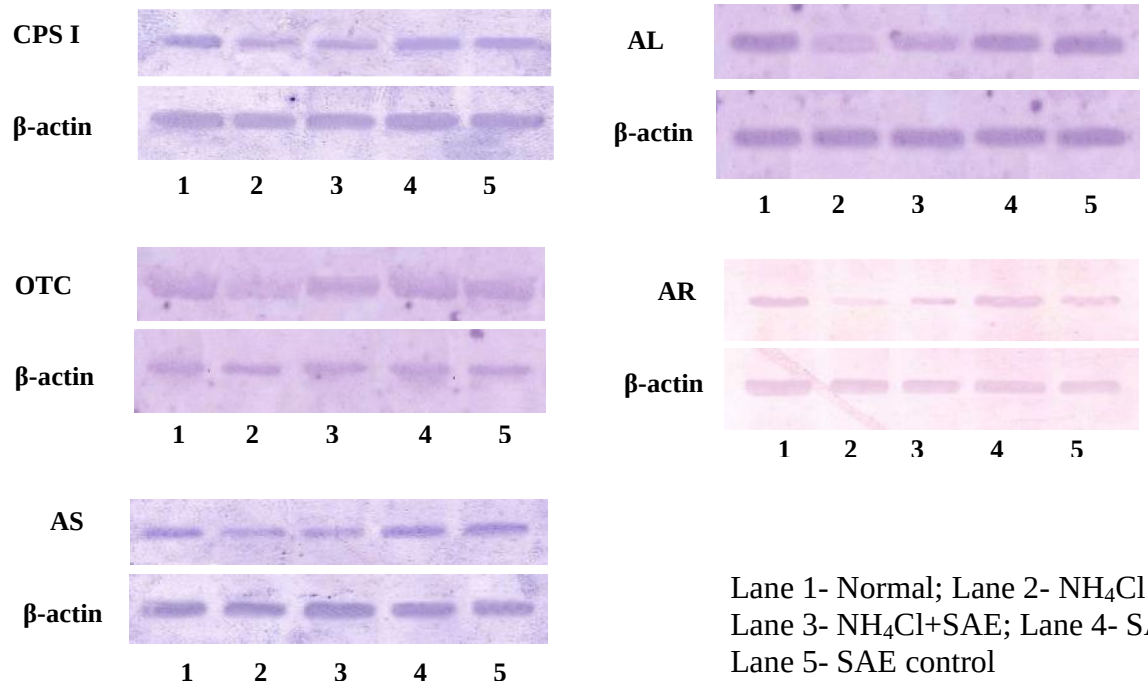


Figure 1-3 Changes in the levels of (figure 1) blood ammonia, (figure 2) brain nitric oxide (figure 3)and brain Na^+/K^+-ATPase activity in normal and experimental rats. Each value is mean ± SD for six rats in each group Values not sharing a common superscripts (a, b and c) differ significantly at $P \leq 0.05$ (DMRT).

Figure 4 (A)



(B)

Lane 1- Normal; Lane 2- NH_4Cl
Lane 3- NH_4Cl+SAE; Lane 4- SAE
Lane 5- SAE control

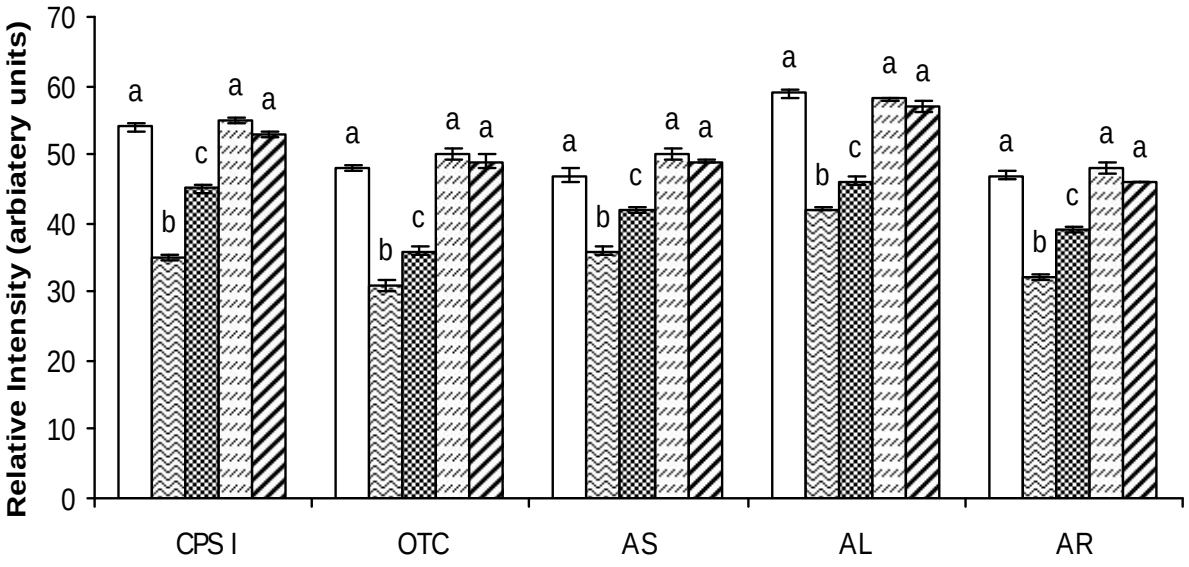


Figure 4 Immunoblot expression of liver urea cycle enzymes [CPS I, OTC, AS, AL and AR] in normal and experimental groups of rats.**(A)** Lane 1, lane 2, lane 3, lane 4 and lane 5 correspond to immunoblot expression in normal, NH_4Cl, NH_4Cl+SAE, SAE and SAE control alone treated rats respectively. The expression of β actin acts as positive control. **(B)** Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity arbitrary unit.

Hyperammonemia is a major element in the pathogenesis of hepatic encephalopathy and in other brain metabolic disorders; particularly in association with inherited urea cycle enzyme deficiencies[36]. In the present study, hyperammonemic rats showed decreased expression of urea cycle enzymes (CPS-I, OTC, AS, AL and AR), which might be due to increased free radical generation and subsequently owing to liver damage. SAE supplementation in NH_4Cl treated rats was able to improve the expression of these urea cycle enzymes to near normal. These effects can be attributed to the phytoactive constituents (flavonoids, polyphenols, sterols and glycosides) present in SAE.

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