Available online at www.journalijmrr.com

INTERNATIONAL JOURNAL OF MODERN RESEARCH AND REVIEWS

ISSN: 2347-8314

Int. J. Modn. Res. Revs.
Volume 4, Issue 11, pp 1414--1420,November, 2016

ORIGINAL ARTICLE

HEPATOPROTECTIVE EFFECT OF CHRYSIN ON AMMONIUM CHLORIDE INDUCED HYPERAMMONEMIA VIA ATTENUATING LIVER DAMAGE IN MALE ALBINO WISTAR RATS

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Article History: *Received 28th October ,2016, Accepted 17th November,2016, Published 18th November,2016*

ABSTRACT

The intention of this study was designed to elucidate the hepatoprotective effects of chrysin on ammonium chloride (NH_4Cl) induced hyperammonemia rats. For induction of experimental hyperammonemia, NH_4Cl was injected intraperitonially (*i.p*) at a dose of 100 mg/kg body weight (b.w.), meanwhile chrysin were administered orally at a dose of 100 mg/kg b.w. for thrice a week for eight consecutive weeks. The levels of blood ammonia, plasma urea, activities of liver marker enzymes and histological alterations in liver sections of normal and hyperammonemic rats were observed. Expressions of apoptotic markers include Bcl-2, Bax, p53 and caspase-3 were examined by means of immunohistochemical-staining analysis. Our results revealed that chrysin administration significantly attenuated the blood ammonia activities of liver marker enzymes and reinstated the plasma urea and histological alterations of liver. Furthermore, chrysin treatment potentially downregulated the expression patterns of pro-apoptotic markers such as P53, Bax and caspase-3, whereas significantly upregulated the expression patterns of anti-apoptotic marker (Bcl-2) in hyperammonemic rats. Chrysin could offer hepatoprotection by inhibiting the mitochondrial mediated apoptosis and recovering histological alterations produced by NH_4Cl induced hyperammonemia.

Keywords: Hyperammonemia, Liver damage, Apoptosis, Liver marker enzymes, Chrysin.

1.INTRODUCTION

Liver, key organ regulates many important metabolic functions (Wolf, 1999) and it is continuously exposed to xenobiotics which are capable of causing some degree of liver disease (Sturgill and Lambert, 1997). Liver failure is a life threatening disease, and the mortality rate is as high as 50-80%, which is primarily related to the complications associated with hyperammonemia especially in developing countries (Lau and Membreno, 2004). During liver failure, ammonia homeostasis are blocked, leads to an ammonia accumulation in the circulation, which increases the necrosis and apoptosis in hepatocytes (Pratap Mouli et al., 2015). Apoptosis (genetically controlled cell death), is essential for normal tissue homeostasis and it is involved in cell turnover in many tissues. It is a fundamental cellular mechanism utilized by multicellular organisms for the disposal of cells that are no longer needed or potentially detrimental (Suzanne and Steller, 2013). It can be initiated from signals coming from an exogenous source (extrinsic apoptosis) or from endogenous sources (intrinsic/mitochondrial apoptosis). These signals eventually induce the permeability of the mitochondrial outer membrane which causes the release of cytochrome c and other proteins (Susin et al., 2000).

Pathological investigation demonstrated that hyperammonemia induces liver cell damage by cellular apoptosis, but not by inflammation or necrosis. Excessive hyperammonemia may be implicated in the regulation of the developmental process of hepatocyte apoptosis and liver damage (Gao et al., 2015). Mitochondrial dysfunction triggered by ammonia intoxication, which could lead to increased formation of ROS, reflecting an oxidative stress condition (Montilla et al., 2001). Mitochondrial oxidative stress is known to be able to integrate various prosuicide stimuli. The mitochondrial apoptotic pathway is regulated by the Bcl-2 family proteins, consist of both anti-apoptotic (Bcl-2) and pro-apoptotic members (Bax) (Green and Reed, 1998). The anti-apoptotic members of proteins inhibited the release

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of cytochrome c whereas, the pro-apoptotic family proteins enhance the release of cytochrome c. When the cytochrome c is released, it will activate caspase-9 and the consequent caspase-3 activation.

Caspases are cysteine proteases playing key roles in the mechanism of apoptosis (Rupinder et al., 2007). Caspases-3 and -9, a pro-apoptotic protein, have been shown to be involved in ammonia-induced apoptosis (Yang et al., 2004). The mechanism of caspase-independent cell death involves protein p53, which acts as a transcription factor helps in the mechanism of apoptosis and cell cycle arrest (Riley et al., 2008). p53 directly upregulates the expression of proapoptotic proteins of the Bcl-2 family such as Bax and indirectly activates caspase-3. Nowadays, there is great attention to be focused on the use of natural compounds having antioxidative properties to enrich the effectiveness in reducing the organ toxicity (Khan et al., 2012). Chrysin (5,7-dihydroxyflavone), naturally exists in honey, propolis, and many plant extracts (Bischoff, 2008). Numerous studies have shown that chrysin has beneficial biological activities includes anti-estrogenic (Kao et al., 1998), anti-allergic (Pearce et al., 1984), anti-inflammatory (Fishkin and Winslow, 1997), antioxidant (Hecker et al., 1996), anticancer (Habtemariam, 1997), and anxiolytic properties (Wolfman, 1994). To validate this hypothesis, the current study was aimed to investigate the hyperammonemia induced liver damage via apoptotic signaling pathway.

2.MATERIALS AND METHODS

Chemicals

Chrysin and NH_4Cl were obtained from Sigma Chemical Company, St. Louis, MO, USA. All other salts were of analytical grade and purchased from Bangalore Genei, India.

Animal model and ethical statement

Male albino Wistar rats at 6-8 weeks of age were acquired from Biogen, Bangalore. In accordance with the ethical norms, this experiments was designed and performed with protocols approved by Animal Ethical Committee, Annamalai University & CPCSEA guidelines, New Delhi, India (Approval no. 1118; dated:16/04/2015).

Induction of experimental hyperammonemia

According to the previous reports (Vijayakumar, 2014), experimental hyperammonemia was induced in rats by *i.p*. injection of freshly prepared NH_4Cl (100 mg/kg b.w.) solution thrice a week for eight consecutive weeks.

Experimental design

The animals were separated into four groups, comprising a minimum of 6 rats in each group. Group 1: Rats received 1 ml of corn oil; Group 2: Rats received 1 ml of NH_4Cl (100 mg/kg b.w.) by *i.p*; Group 3: Rats received 1 ml of NH_4Cl and chrysin (100 mg/kg b.w.) by oral (Renuka et al., 2016). Group 4: Rats received chrysin (100 mg/kg b.w.) alone.

All the rats were fasted overnight and killed by cervical dislocation at the end of the experimental period (8^{th} week). The blood was collected; serum and plasma were separated by centrifugation. Liver tissue was excised and rinsed in ice-chilled normal saline. Blood samples and excised tissues were used for various estimations.

Biochemical estimation

Blood ammonia level was estimated by the method of Wolheim, (1984). Plasma urea level was determined by the diacetyl monoxime method of Varley et al., 1998. Activities of AST and ALT were assayed by the method of Reitman and Frankel, (1957). ALP and GGT was assayed by the method of King and Armstrong, (1951).

Histological studies

Liver tissue of all groups were washed, dehydrated in various concentrations of ethanol, cleared in xylene and fixed with paraffin wax. About 5-6 mm in thickness of liver tissue sections were sliced out, deparaffinized and then stained with H&E (Hematoxylin and Eosin) for visualizing under a light microscope (magnification 40X).

Immunohistochemical analysis

Liver tissue sections were rehydrated by the grade of ethanol to distilled H_2O. The activity of endogenous peroxidase was removed by adding H_2O_2 (3%) for 15 min. Tissue sections were incubated with blocking reagent (BlockTM) at room temperature for 15 min. Sections were incubated with the suitable primary antibody against p53, Bax, Bcl-2 and caspase-3 followed by accurately labeling with the secondary antibody with horseradish peroxidase. After the color intensity was reached, the slides were visualized with hematoxylin staining.

Statistical analysis

All the results are expressed as means ± SD and subjected to One-Way Analysis of Variance by using computer software SPSS (version 16.0) and the comparisons of significant groups were performed using the Duncan Multiple Range Test, at $p < 0.05$.

3.RESULTS

Effect of chrysin on the levels of blood ammonia, plasma urea and activities of liver marker enzymes

The results revealed that NH_4Cl induced hyperammonemia markedly increased the blood ammonia and the activities of liver marker enzymes (AST, ALT, ALP and GGT), whereas decresed the level of plasma urea as compared to normal rats. On the contrary, chrysin administration (100 mg/kg b.w.) to hyperammonemic rats reinstated the levels of blood ammonia, plasma urea and the activities of liver marker enzymes as compared with hyperammonemic rats. However, chrysin (100 mg/kg b.w.) alone treated rats did not show any difference in the the levels of blood ammonia, plasma urea and the activities of liver marker enzymes (Fig. 1& 2)

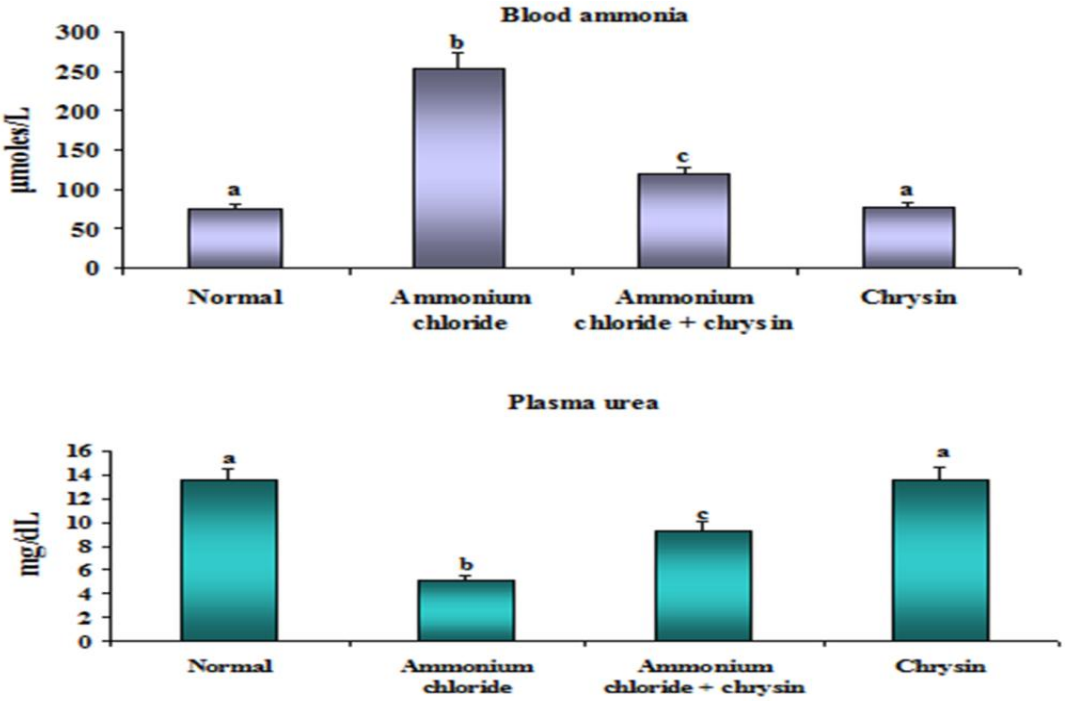

Figure 1 Effect of chrysin on the levels of blood ammonia and plasma urea in normal and experimental rats.

Values are expressed as mean ± SD for 6 rats in each group. One-way ANOVA repeated measures with Duncan's multiple rang test (DMRT) was used to calculate statistical significance. Values not sharing common superscript differ significantly at $P < 0.05$.

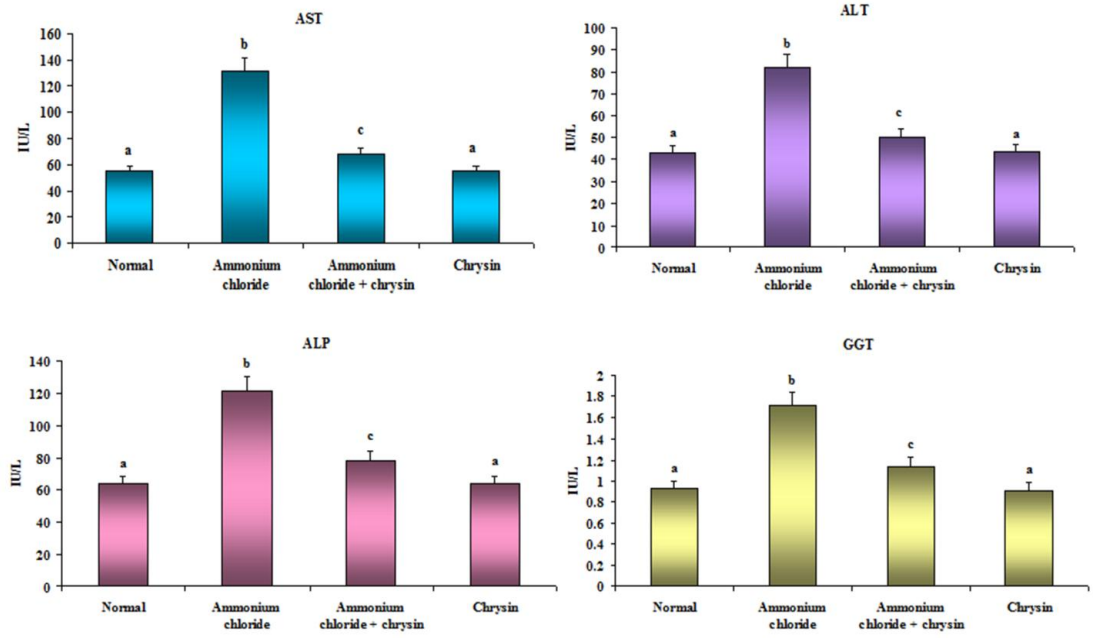

Figure 2 Effect of chrysin on the activities of liver marker enzymes in normal and experimental rats.

Values are expressed as mean ± SD for 6 rats in each group. One-way ANOVA repeated measures with Duncan's multiple rang test (DMRT) was used to calculate statistical significance. Values not sharing common superscript differ significantly at $P < 0.05$.

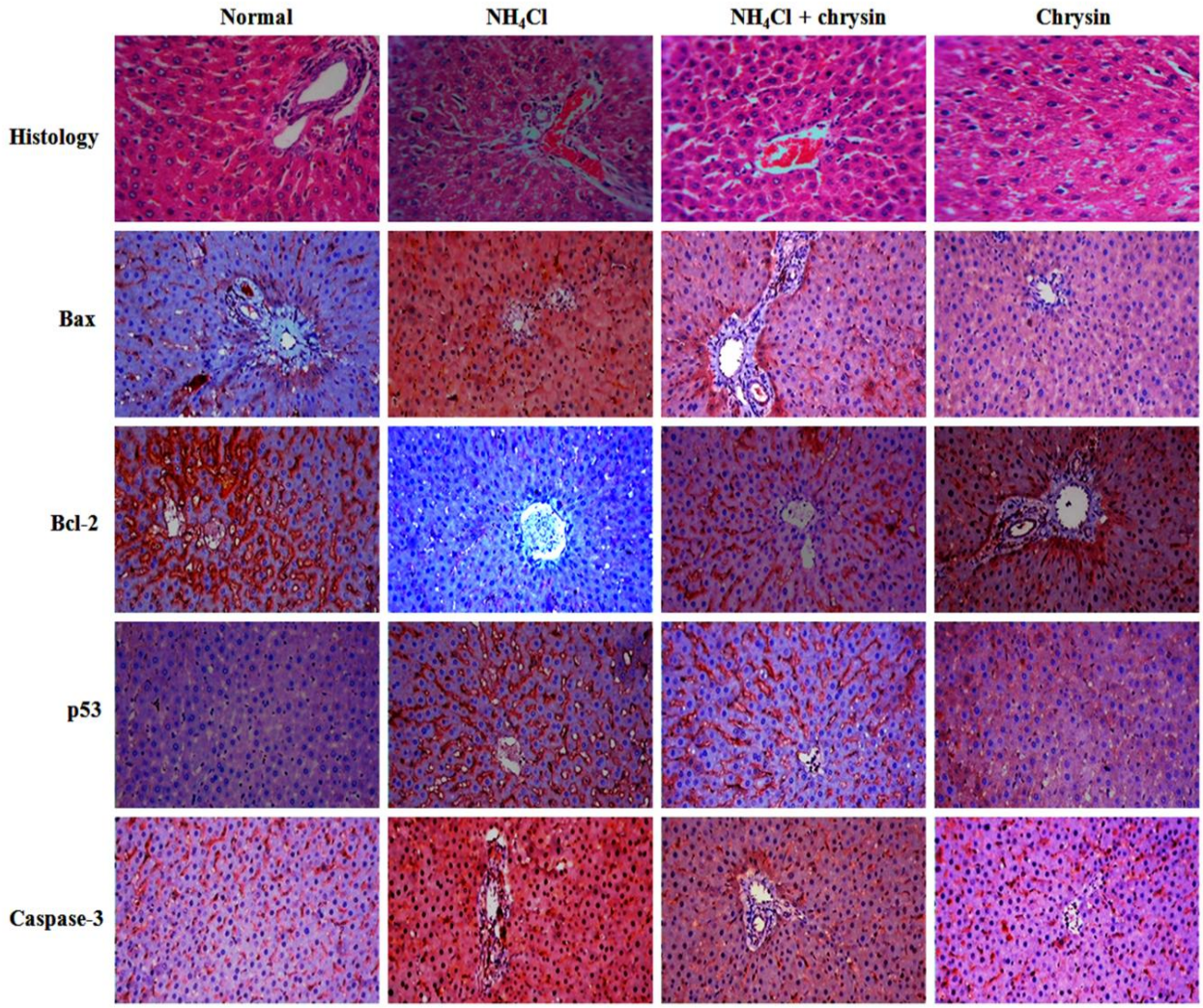

Figure 3. Histology and immunohistochemical expressions of Bax, Bcl-2, p53 and caspase-3 in liver sections of normal and experimental rats.

Effect of chrysin on histological alteration of liver

Normal rats illustrate that the apparently normal hepatic traids architecture with the central vein. Liver sections of hyperammonemic rats exhibited fibrosis, steatosis, tubular epithelial degeneration and fatty infilteration of mononuclear cells. Conversely, chrysin administration to hyperammonemic rats showed mild steatosis, epithelial degeneration and infiltered mononuclear cells. However, chrysin (100 mg/kg b.w.) alone treated rats did not show any pathological changes as compared with normal rats (Fig. 3)

Effect of chrysin on apoptotic markers

The results revealed that the induction of NH_4Cl might affect the liver cell indicating that altered expression pattern of apoptotic markers. We therefore assessed the NH_4Cl induced hyperammonemia mediated hepatic injury by immunohistochemical analysis. NH_4Cl induced hyperammonemic rats significantly increased the expression patterns of pro-apoptotic markers such as P53, Bax and caspase-3, whereas significantly decreased the expression patterns of anti-apoptotic marker (Bcl-2) as compared to normal rats On the contrary, chrysin treatment noticeably decreased the expression levels of P53, Bax and caspase-3 and increased the expression level of Bcl-2 as compared to hyperammonemic rats. However, chrysin alone treated rats does not alter the expression pattern of pro & anti-apoptotic markers (Fig 3).

4.DISCUSSION

Hyperammonemia is primarily caused by urea cycle disorders which are considered as the most common symptom of liver failure (Wright et al., 2011). It has been identified as a possible cause of hepatic encephalopathy subsequent to hepatic injury.

Liver contains all enzymes necessary for urea synthesis thereby eliminating the excess level of ammonia. Mahmoud

et al. (2014) reported that enhancement in the level of blood ammonia, subsequent decline in the urea synthesis indicates hyperammonemic condition which may be due to liver damage induced by ammonia intoxication. Flavonoid administration exerts antihyperammonemic effects by potentially decreased the level of blood ammonia and increased the level of urea synthesis, favoring reduction of urea cycle defects and hyperammonemic complications (Ramakrishnan et al., 2016a). Our findings are in agreement with previous reports, chrysin supplementation normalized the level of circulating ammonia and plasma urea which might indicate that it exerts defence mechanism against hyperammonemia by preventing the process of ammonia intoxication.

The cellular damage is closely correlated with the enzyme leakage in the circulation (Sallie, 1991). Enzymes include AST, ALT, ALP and GGT are the most important markers employed in the diagnosis of liver damage (Sehrawat and Sultana, 2006). Previous findings stated that ammonia administration leads to increased levels of serum liver marker enzymes indicates hepatic dysfunction. The increased activities of the liver marker enzymes might be due to the leakage of these enzymes from cytosol into the circulation resulting from liver damage during ammonia toxicity (Ramakrishnan et al., 2016b). This is an indicator of hepato-cellular damage due to liver dysfunction and disturbance in the biosynthesis of these enzymes, with alteration in the membrane permeability. Chrysin treatment prevented the ethanol-induced liver toxicity, as signified by a depleting the activities of serum liver marker enzyme, possibly by maintaining the hepato-cellular membrane integrity (Tahir and Sultana, 2012). Our findings corroborate with the previous reports.

Histological findings revealed that NH_4Cl induced hyperammonemic rats exhibited morphological alterations include severe necrosed hepatocytes, steatosis, and fatty infiltrated mononuclear inflammatory cells in liver tissue (Ramakrishnan et al., 2016b). Jayanthi et al., (2009) demonstrated that the liver sections of chrysin treated rats showed normal histology with portal triad. In agreement with the previous reports, it was found that chrysin treatment (100 mg/kg b.w.) normalized the NH_4Cl induced pathological alteration in the liver tissue thereby chrysin enhancing the process of ammonia detoxification.

Cell apoptosis is one of the major significant changes to arise following ammonia treatment in liver failure (Cruz et al., 2000). The cell proliferation and death is strongly regulated by the Bcl-2 family proteins and cell cycle enzymes. There are around 20 proteins in the Bcl-2 family and their function primarily acts as mediators of pro- or anti-apoptotic markers (Cande et al., 2002). Apoptosis was accompanied by a decrease in the expression of Bcl-2 protein and an increase in the expression of Bax protein, indicating that the significant role of these proteins in the regulation of apoptosis (Chipuk et al., 2010). Kumari and Kakkar, (2012) reported that acetaminophen induced hepatotoxicity down-regulated the expression of Bcl-2 and up-regulated the expression of Bax, both of which are key regulators of the apoptotic cascade. In this way, perturbing the balance between Bax and Bcl-2 in favour of Bcl-2, should be hepatoprotective. Our findings are in agreement with these previous reports. In the current study, it was demonstrated that chrysin supplementation effectively normalized the expression of Bcl-2 and Bax thereby enhances the ratio of Bcl-2/Bax more than 2 folds which could be probably by modulating the mechanism of oxidative stress and apoptosis (Ali et al., 2014).

p53, a nuclear phosphoprotein induced in response to cellular stress and functioning as a transcriptional transactivator in DNA repair and apoptosis. p53 is phosphorylated reversibly by phosphatases and protein kinases, which regulates the various cellular processes including apoptosis. It is suggested that p53 may induce apoptosis by stimulating the expression of Bax or repression of bcl-2 expression (Miyashita et al., 1994). Clark et al., (2008) demonstrated that MCLR administration *in vivo* / *in vitro* can both significantly increase the expression level of p53, suggested that overexpression of p53 plays a key role in the mechanism of apoptosis. Accordingly, current data revealed that NH_4Cl-induction caused significant enhancement in the expression of p53, whereas chrysin treatment does not alter the p53 expression by the inhibition of p53 hyper-phosphorylation, which in turn induce p53-dependent apoptosis (Yan et al., 1997)

The most important characteristic features of apoptotic cell death include numerous structural features as well as extensive caspase enzyme activation (Jaeschke and Lemasters, 2003). Caspases, cysteine protease family implicated in the commitment and execution of apoptotic cell death. Caspase-dependent apoptosis involved in the loss of mitochondrial membrane permeability, cytochrome-c release, and caspase-9-mediated activation of caspase-3 (key executioner of apoptosis) (Pallepati and Averill-Bates, 2011) Once cytochrome c is released, it will activate caspase-9 and the subsequent caspase-3 probably triggers apoptosis (Ding and Nam Ong, 2003). Rashid et al., (2014) reported that chrysin administration attenuated the apoptosis in the 5-fluorouracil induced renal tissue by inhibiting the activation of caspase-3 (apoptosis executioner). The results of the current study are in agreement with previous reports that chrysin treatment potentially decreased the NH_4Cl induced increased immunopositive staining of caspase-3 which could be due to the inhibition of cytochrome-c release (Miyashita et al., 1994).

5.CONCLUSION

The data revealed that hyperammonemia-induced liver damage in rats may be caused by urea cycle dysfunction and changes in the expression levels of apoptotic markers (P53, Bcl-2, Bax and caspase-3) by immunohistochemical analysis. Chrysin administration (100 mg/kg b.w.) attenuated the circulatory ammonia and mitochondria-mediated apoptotic pathway by inhibiting Bax translocation; enhancing Bcl-2 levels, preventing the release of cytochrome c into the cytosol and the subsequent conversion of caspase 9/3 into their active forms. Hence, our findings confirm that chrysin administration (100 mg/kg b.w.) offered hepatoprotection by the enhancement of urea synthesis and inhibition of mitochondrial mediated apoptosis caused by NH_4Cl induced hyperammonemia.

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