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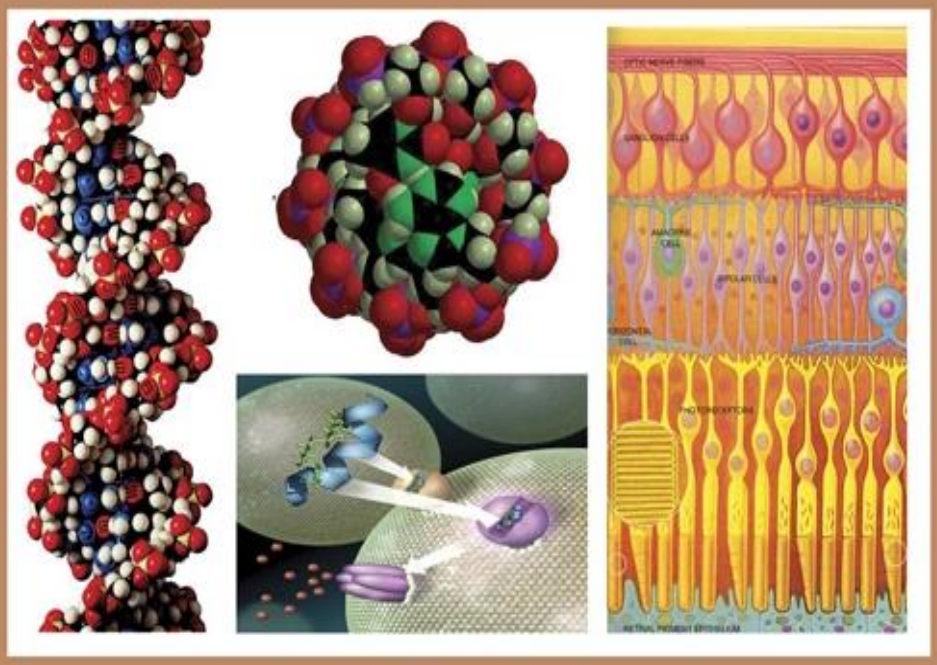

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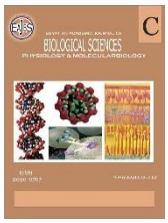

Syzygium cumini (L) Extract Ameliorates Aluminium Chloride-Induced Acute Hepatic and Renal Toxicity in Rats

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ABSTRACT

Aluminium (Al) is the most common metallic element associated with pathogenesis in humans and animals. This study was conducted to evaluate the ability of *Syzygium cumini* (L.) leaves extract in attenuating the acute toxicity of aluminium-induced hepatic and renal toxicity in rats. Male rats were received oral administration of $AlCl_3$ (150mg/kg) followed by oral administration of *S. cumini* (250mg/kg) leaves extract for 14 days. Aluminium concentration, MDA, NO, glutathione, oxidative enzymes (catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase) was measured in liver and kidney homogenate. In addition, we demonstrate the level of serum and liver AST, ALT, ALP and serum urea and creatinine. Compared to the control group, the animal that received Al showed a significant elevation in the tissue level of Al, MDA, NO and reduction in the level of glutathione and antioxidant enzymes. It also showed a marked increase in serum liver function enzymes, creatinine and urea. The combined treatment with *S. cumini* leaves extract caused restoration of the oxidative levels and upregulation in antioxidant enzymes as compared to Al-intoxicated rats. In addition, the administration of *S. cumini* extracts in rats after Al administration caused a decrease in cytokines TNF-α and IL- 6, Bax & caspase-3 expression, in addition, to an increase in antiapoptotic marker Bcl_2 in liver and kidney homogenate. In conclusion, our study revealed that the treatment with *S. cumini* leaves extracts ameliorates hepatic and renal cells toxicity produced by Al exposure by elevating the antioxidant enzymes, antiapoptotic and anti-inflammatory activity.

INTRODUCTION

Acute or chronic exposure to hazardous or environmental toxicants leads to series of severe health problems, including impairment, permanent organ failure and even death. In general, when chemicals and other toxicants are absorbed through the body, they travel through the systems and affect particular organs mainly the liver and kidney because of their active role in the metabolism and elimination of toxins.

Aluminium (Al) is the most common metallic element in the earth and may enter the human body by ingestion of food containing Al such as yellow cheese, herbs, and spices and through toothpaste, cosmetics and Al containers.

In addition, Al is used in a wide range in products of pharmaceuticals such as (antacids, antidiarrheal drugs and allergy immunotherapy injections) (Karempour Malekshah. *et al.,* 2005). It is also added to drinking water for purification purposes (Yokel, 2000). Due to its activity, Al is naturally found in combination with other elements like sulphate and chloride (Verstraeten *et al.,* 2008).

Several studies investigated that Al accumulates in the liver more than the brain and other organs (Kumar and Gill. 2009). Its accumulation is mainly in hepatocyte macrophages and lysosomes. Thus, Al in lysosomes can be eliminated from the liver through bile excretion (Tetsuji and Kiyokazu, 2005). Overexposure to Al by high consumption of Al-containing products, lead to an increase in Al concentration in the body and excess accumulation in hepatocytes in addition to enlargement of the bile duct and liver fatty degeneration (Bogdanović *et al.,* 2008)

Inside the body, Al has the ability to react with water resulted in the formation of compounds that easily react with oxygen to produce Al superoxide which in turn causes a decrease in mitochondrial Fe and generates oxygen free radicals leading to oxidative damage and apoptosis (Willhite *et al.,* 2014).

Syzygium cumini (L.) is one of the most important herbal medicine that has been used in a wide range of food spices, preservatives and many medical proposes (Ahmed *et al*., 2019). The *S. cumini* leaves and flowers are rich in kaempferol, quercetin, myricetin, isoquercetin (quercetin-3-glucoside), myricetin-3-L-arabinoside, quercetin-3-D-galactoside, dihydromyricetin, oleanolic acid, acetyl oleanolic acid, eugenol-triterpenoid A and eugenoltriterpenoid B (Ayyanar and Subash-Babu, 2012). Several studies reported the beneficial effect of eugenol (*in-vitro and in-vivo*) as antiproliferative, anti-inflammatory, cytotoxic and antioxidant compounds (Salem *et al.,* 2014 and Said and Abd Rabo, 2017).

Hence, we investigated the therapeutic effect of *S. cumini* extract on $AlCl_3$ induced hepatic and renal toxicity in rats.

MATERIALS AND METHODS

1-Aluminum Chloride ($AlCl_3$) was purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

2-*S. cumini* Leaves Extract Aqueous Extract Preparation:

Dried *S. cumini* leaves were specified by the Department of Botany, Faculty of Science, Helwan University and grinded to (2 mm mesh size). 10 g crude powder was mixed in 50 ml of distilled water, and left-over night. Then, it is filtered and stored at 4°C till use (Ahmed *et al*., 2019).

3-Animals:

To investigate the therapeutic effect of S. cumini extract on $AlCl_3$ induced toxicity in rats, 40 adult male Wister rats (weighing 150-170g) are used. The animals were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). The rats were housed in polypropylene cages and maintained at room temperature (22 ± 3 °C) on a 12-h light/12-h dark cycle throughout the experiment. The rats were provided with water and a balanced diet ad libitum. They allowed adapting to the housing conditions for one week before starting the experiment. All protocols and animal handling approved by the Committee on Research Ethics for Laboratory Animal Care at the Department of Zoology, Faculty of Science, Helwan University, and were in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, 8th edition (NIH Publication No. 85-23 revised 1985).

4-Experimental Design:

Animals were randomly divided

into four groups each of ten rats. The first one served as a control group (Ctrl) and received distilled water orally. The second group received oral administration of $AlCl_3$ (150 mg/kg b.wt.) (Deng *et al.,* 2000). The aqueous extract *S. cumini* leaves (250 mg/kg b.wt.) (Modi *et al.,* 2010) was administered to rats of the 3rd group. In addition, the 4th group was received oral administration of $AlCl_3$ and after one hour, they treated orally by an aqueous extract of S. cumini leaves. After 14 days of treatment, all animals were sacrificed by sudden decapitation. Blood was collected and serum was separated by centrifugation at 3000 r.p.m for 15 min. Liver and kidney were immediately dissected after decapitation, weighed, washed and parts of them were quickly stored in formalin buffer 10% for histological investigations and the rest was homogenized in ice-cold 10 mM phosphate buffer (pH 7.4) to produce a 10% (w/v) homogenate for biochemical analysis.

Estimation of Liver and Kidney Aluminium Concentration:

Aluminium concentration in the liver and kidney tissues was determined by using an atomic absorption spectrophotometer at 228.8 nm according to the method described by Kubaszewski *et al.* (2014).

Biochemical Assays:

1-Liver function tests:

The activity of Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by using Randox laboratories kits (UK) and alkaline phosphatase (ALP) assayed using a kit from Quimica Clinica Aplicada (Amosta／Tarragona, Spain) in both serum and liver homogenate according to the manufacturer instructions.

2-Kidney Function Tests:

Serum Creatinine and urea were determined by using Randox laboratories kits (UK) according to the manufacturer's instructions.

3-Oxidative Stress Marker:

The level of malondialdehyde (MDA) was measured as a lipid peroxidation (LPO) marker in the tissue using the method described by Ohkawa *et al.* (1979). A mixture consisting of distilled water, 0.67% thiobarbituric acid and 0.22% sulfuric acid was added to 500 µl of supernatant. The formed mixture was boiled at 95 °C for 30 min and then cooled at room temperature and centrifuged for 15 min at 1000g. The supernatant was estimated spectrophotometrically at 540 nm. The obtained data are expressed as nanomoles MDA per milligram of protein. Nitric oxide (NO) level was measured using the Griess reagent according to Green *et al.* (1982). 100 µl of supernatant was mixed for 10 min with Griess reagent at room temperature. The formed reddish purple azo dye was measured spectrophotometrically at 540 nm.

For the estimation of 8-hydroxy-2-deoxyguanosine (8-OHdG), the isolation and hydrolysis of liver and kidney DNA were performed using the method of Lodovici *et al.* (1997).

4-Estimation of Antioxidants:

Glutathione (GSH) was estimated by the reduction of Elman's reagent [5,5′ dithiobis (2- nitrobenzoic acid); DTNB] with GSH to produce a yellow compound. The reduced chromogen is directly proportional to the GSH concentration, and its absorbance can be measured at 405 nm. Catalase (CAT) activity was estimated according to Aebi, (1984). The activity of superoxide dismutase (SOD) was assayed using the method of Nishikimi *et al.* (1972). Furthermore, glutathione peroxidase activity was measured using the method of Paglia and Valentine (1967). GPx activity was estimated in terms of the decrease in NADH per min using a reaction coupled with glutathione reductase (GR). The decrease in absorbance at 340 nm was recorded, and GPx activity was expressed as U/mg protein. In addition, GR activity was measured by quantifying glutathione-dependent oxidation of NADPH at 340 nm

and expressed as U/mg protein.

Inflammatory Markers In Liver And Kidney Tissues:

The concentration of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were determined by using commercial ELISA kits (R&D System, Minneapolis, MN, USA) according to the manufacturer procedures.

Estimation of Apoptotic Markers In Tissue:

Liver and kidney homogenates were made in lysis buffer and analyzed using a colorimetric caspase-3 assay kit (Sigma-Aldrich Co. USA) according to the manufacturer's instructions. B cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax) levels were measured in the tissue homogenate by ELISA kits, (LifeSpan BioSciences, Inc., Seattle, WA, USA). The procedure was performed according to the instructions of the manufacturer. Levels were expressed as ng/ mg tissue protein.

Statistical Analysis:

Statistical Package for the Social Sciences (SPSS) was used for data analysis. The results were expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Duncan's test was applied for determining the significance. The acceptable level of significance was established at $p < 0.05$.

RESULTS

Oral administration of $AlCl_3$ (150mg/kg) resulted in a significant increase in Al concentration in both liver and kidney tissues. This increase in Al concentration was inhibited by the treatment with *S. cumini* extract (**Fig. 1**).

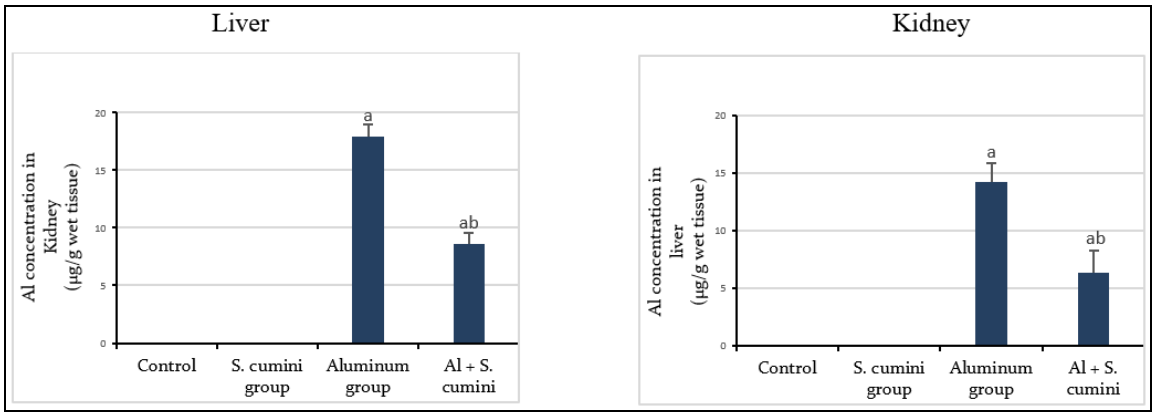

Fig.1: Bioaccumulation of Al in Hepatic and renal tissue in response to *S. cumini* and/or $AlCl_3$ treatment. Results are displayed as the mean ± SE (n = 8). (a) $p < .05$ versus the control group; (b) $p < .05$ versus the $AlCl_3$-treated group.

As compared to the control group, our results recorded a marked elevation ($p<0.05$) in serum AST, ALT and ALP in rats exposed to $AlCl_3$, while it showed a significant reduction in the levels of these enzymes in liver tissue homogenate (Fig. 2). The combination of treatment between $AlCl_3$ and *S. cumini* leaves extract showed restoration in these enzyme levels nearer to the control levels ($p<0.05$). Moreover, we determined the efficacy of the kidney by estimate the level of creatinine and urea in serum. Administration of $AlCl_3$ for 14 days caused significant raise ($p<0.05$) in creatinine and urea level in comparison with the control level. However, as compared to $AlCl_3$ intoxicated rats the treatment with *S. cumini* leaves extract showed a decline in the levels of serum creatinine and urea significantly (Fig. 3).

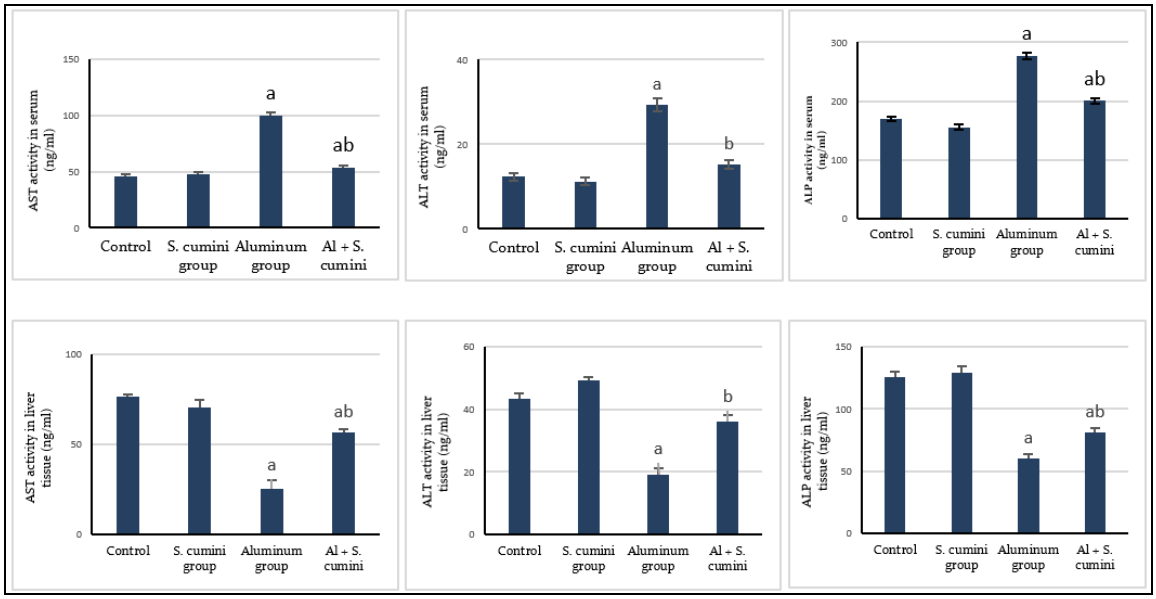

Fig. 2: The effect of *S. cumini* extract treatment on serum and tissue liver function marker in $AlCl_3$ induced acute hepatic toxicity. Results are displayed as the mean ± SE (n = 8). (a) $p < 0.05$ versus the control group; (b) $p < 0.05$ versus the $AlCl_3$-treated group.

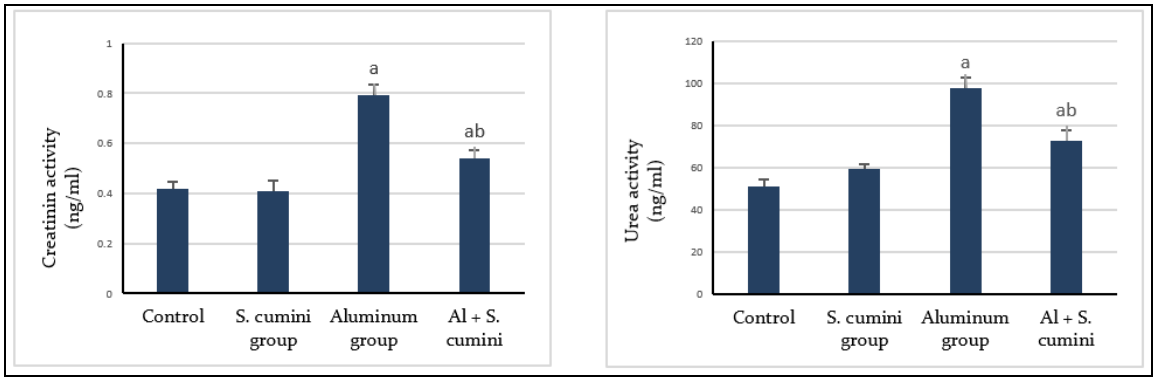

Fig. 3: The effect of *S. cumini* extract treatment on serum kidney function marker in $AlCl_3$ induced acute renal toxicity. Results are displayed as the mean ± SE (n = 8). (a) $p < 0.05$ versus the control group; (b) $p < 0.05$ versus the $AlCl_3$-treated group.

To demonstrate the anti-inflammatory effect of *S. cumini* extract, the level of (TNF-α and IL-6) were quantified in liver and kidney tissue using ELISA. Our present results recorded a significant increase in anti-inflammatory marker (TNF-α and IL-6) in $AlCl_3$-intoxicated rats as compared to control group. The elevation in liver and kidney inflammation was ameliorated due to *S. cumini* leaves extract treatment in animals intoxicated with Al (Fig. 4).

In addition, oral administration of $AlCl_3$ resulted in a significant increase ($p<0.05$) in the level of MDA and NO in liver and kidney tissue homogenate. Similarly, the 8-OHdG level was increased significantly in tissue homogenate as compared to the control group (Fig. 5). In addition, the content of GSH significantly reduced as compared to the control group thus, indicated disturbance in the oxidative balance in studied organs (Fig. 6). The treatment with *S. cumini* leaves extract in rats exposed to Al toxicity showed amelioration in these enzymes levels as compared to $AlCl_3$ treated rats.

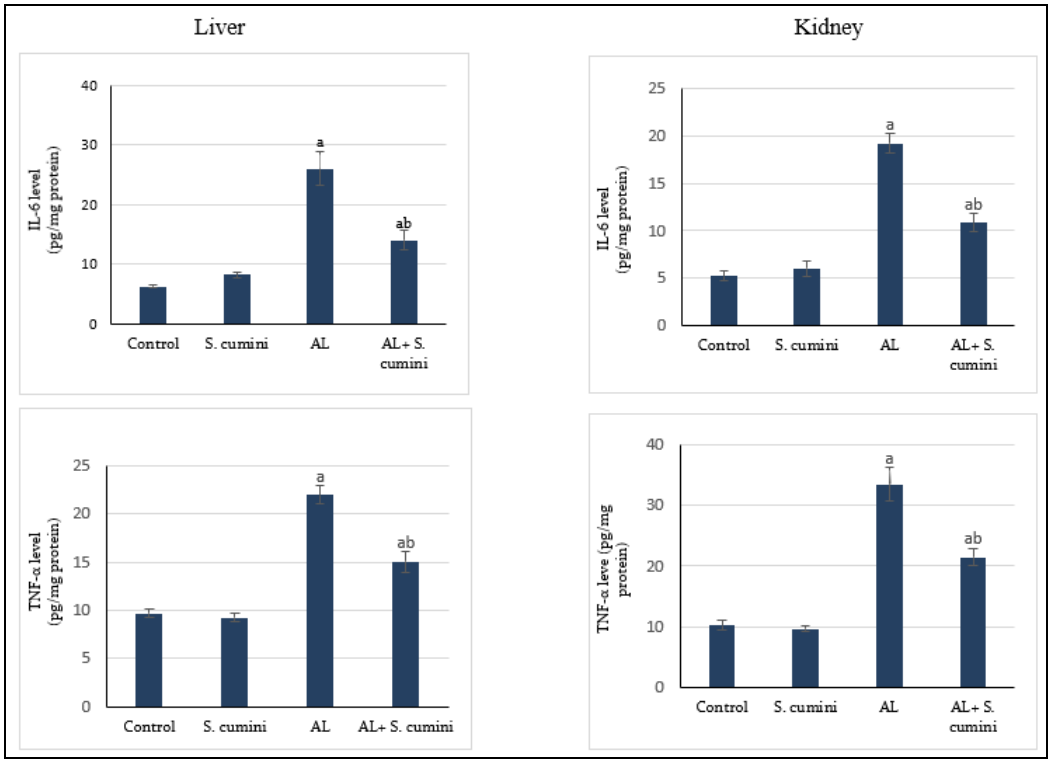

Fig.4: level of proinflammatory cytokines in the liver and kidney tissue in response to *S. cumini* extract treatment in $AlCl_3$ induced toxicity. Results are displayed as the mean ± SE (n = 8). (a) $p < 0.05$ versus the control group; (b) $p < 0.05$ versus the $AlCl_3$-treated group.

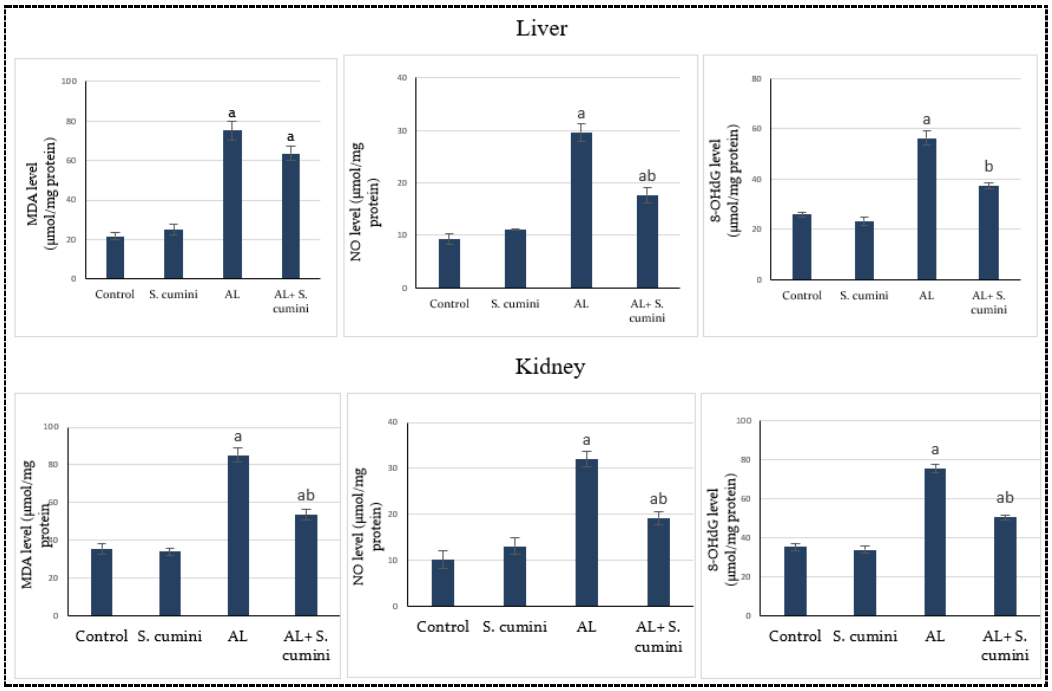

Fig 5: Variation of non-enzymatic oxidative stress marker in the liver and kidney tissue in response to *S. cumini* extract treatment in $AlCl_3$ induced toxicity. Results are displayed as the mean ± SE (n = 8). (a) $p < 0.05$ versus the control group; (b) $p < 0.05$ versus the $AlCl_3$-treated group

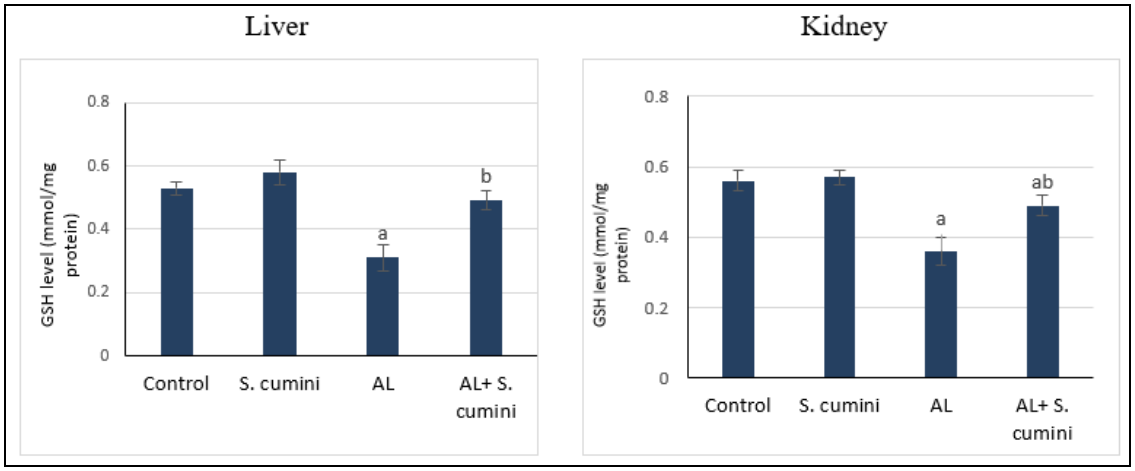

Fig. 6: Variation of glutathione (GSH) marker in liver and kidney tissue in response to *S. cumini* extract treatment in $AlCl_3$ induced toxicity. Results are displayed as the mean ± SE (n = 8). (a) $p < 0.05$ versus the control group; (b) $p < 0.05$ versus the $AlCl_3$-treated group.

The present study recorded changes in the levels of the antioxidant system (SOD, CAT, GPx and GR) in liver and kidney tissue homogenate in studied animals (Fig. 7). We recorded a marked decline ($p<0.05$) in the activities of SOD, CAT, GPx and GR enzymes in rats exposed to $AlCl_3$ orally as compared to control animals, however, comparing to $AlCl_3$-intoxicated rats' treatment with *S. cumini* leaves extract raises the levels of these antioxidant enzymes.

Our finding recorded a marked upregulation in Bax and caspase-3 expression, however, the level of antiapoptotic protein (Bcl_2) was downregulated in the liver and kidney of animals intoxicated with $AlCl_3$ as compared to the control group. On the other hand, treatments with *S. cumini* leaves extract in rats after being treated with Al significantly restored the changes in the expression of studied pro and antiapoptotic markers (Fig. 8).

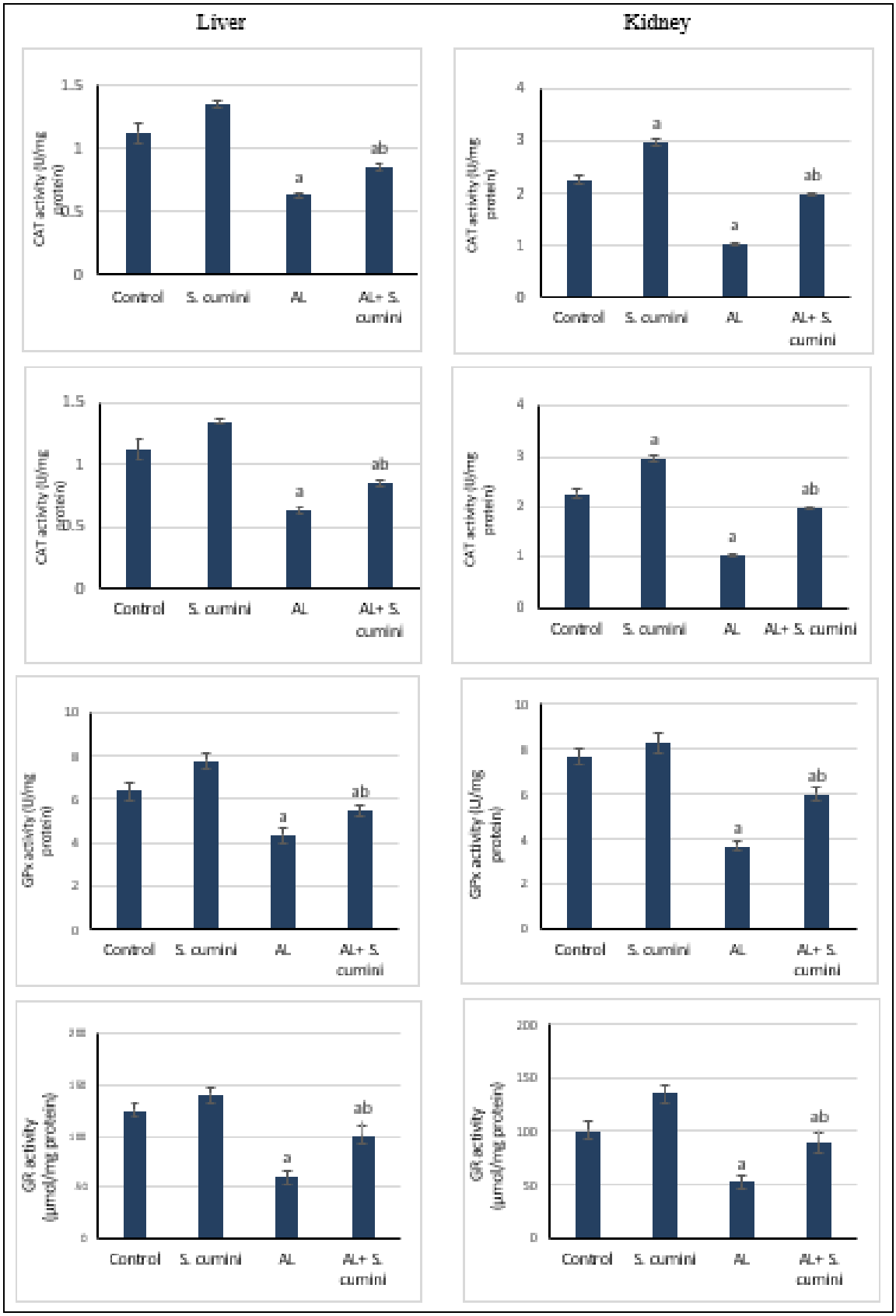

Fig. 7: The effect of S. cumini extract on antioxidant enzyme activities (SOD, CAT, GPx, GR) in hepatic and renal tissues in $AlCl_3$ treated rats. The enzyme activity values are presented as mean ± SD (n = 8), (a) $P < 0.05$ versus the control rats, (b) $P < 0.05$ versus the $AlCl_3$-treated rats.

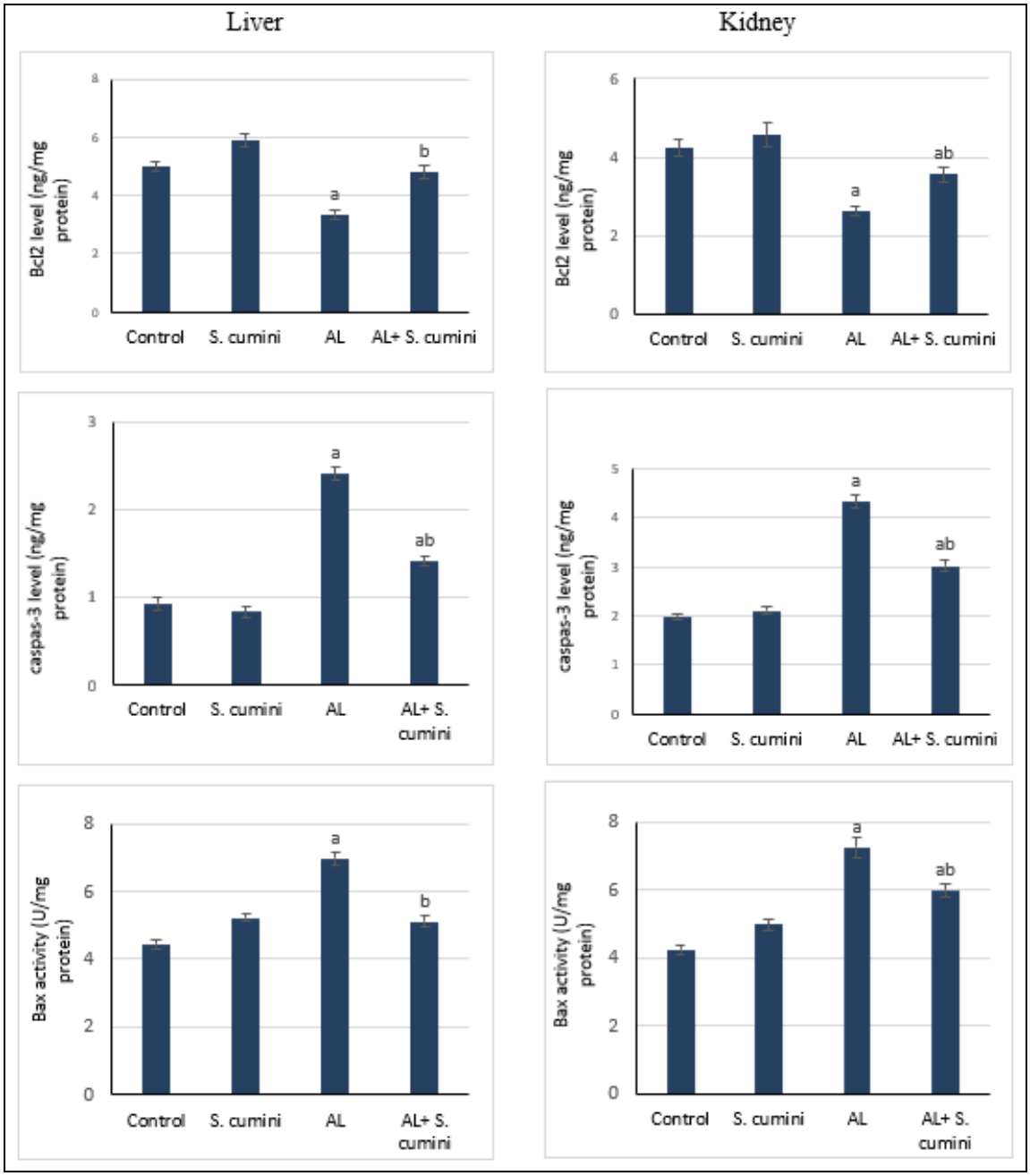

Fig. 8: The effect of S. cumini extract on apoptotic (caspas-3 and Bax) and anti-apoptotic (Bcl_2) enzyme activities in hepatic and renal tissues in $AlCl_3$ treated rats. The enzyme activity values are presented as mean ± SD (n = 8), (a) $P < 0.05$ versus the control rats, (b) $P < 0.05$ versus the $AlCl_3$-treated rats.

DISCUSSION

The use of natural extract including *S. cumini* has been proposed to have a beneficial effect against many health problems related to the environmental toxicant. Many studies proved that *S. cumini* has a large number of phytochemicals, these components reported for various pharmacological actions (Hasanuzzaman *et al.*, 2016). Thus, it is difficult to restrict the effect of *S. cumini* extract on any of the specific active ingredients. It proposed that the extract should be used in its crude form as it used traditionally (Ahmed *et al.*, 2019).

In the present study, we planned to test the effect of *S. cumini* extract on toxicity produced by $AlCl_3$ induced hepatic and renal injury in the rat.

Al is a toxic compound that causes

a harmful effect to the human and animals. It is rapidly accumulated in liver and kidney cells after injection (Ibraheem *et al.,* 2016). Liver and kidney tissue showed an increase in their Al metal ion content in rats intoxicated with $AlCl_3$ in our present study. This accumulation may be due to that Al ions cause defects in Fe^{+3} carrying protein in the cell membrane, which causes membrane distraction and permeability and hence disruption in membrane receptor function which facilitates the ion accumulation (Sumathi *et al.,* 2013). The accumulation of Al in the liver and kidney was previously observed by Abubakar *et al.,* 2003; Ibraheem *et al.,* 2016; and Aly *et al.,* 2018.

Oral administration of *S. cumini* extract was efficient in reducing the amount of Al from the liver and kidney tissue in our present results. The phenolic constituents of *S. cumini* extract have the ability to cut off metal ion, because they have OH^- group forming ligation with Al^{+3} (Kaur and Bansa, 2020).

Several studies showed that Al accumulation induces liver damage (Bogdanović *et al.,* 2008 and Agarwal and Gupta, 2010). The liver damage was evaluated by measuring the level of liver transaminases (AST and ALT) and ALP enzymes. Our finding showed that the exposure to Al caused a significant decline in the level of transaminases (AST and ALT) and ALP in liver tissue homogenate while it recorded a marked increase in these enzymes in serum. This is possibly due to the toxicity of Al ion on liver parenchymal cells or the permeability of the membrane, which in turn cause leakage of these enzymes from the liver cytosol into the blood (Ahmed and Hammad, 2018). Comparably, several investigations in animals proved that Al had a toxic effect on renal tissue (Sanai *et al.,* 1991 and Al Kahtani, 2010). In agreement with these investigations, our results recorded significant elevation in the marker of kidney function (urea and creatinine) that elucidated kidney dysfunction. The kidney plays a major role in the detoxification of blood from metal ions including Al through excreting them to the urine, which helps in preventing its accumulation in the body (Stoehr *et al.,* 2006). Overexposure to Al ion causes its accumulation in kidney tissue, nephrotoxicity, and renal cell dysfunction.

Treatment with *S. cumini* extract restored the disturbance in liver and kidney functions through the inhibition of the ion that induced alternation in membrane permeability and fluidity (Shruti *et al.,* 2020). High levels of flavonoids and eugenole in the *S. cumini* extract are able to reduce xenobiotic-induced hepatotoxicity and preserved the hepatocellular membrane, which resulted in a decrease in serum transaminases in mice intoxicated with CCl_4 (Islam *et al.,* 2015). The present result showed amelioration in the levels of liver enzymes (AST, ALT and ALP) in both serum and tissue homogenate in animals treated with *S. cumini* extract after being exposed to Al. Similarly, the oral administration of *S. cumini* extract in rats intoxicated with Al recorded a marked reduction in serum creatinine and urea, which may be due to its constituents of flavonoids that acts as a chelator for Al ion and maintain the structural integrity of renal cells (Behera *et al.,* 2019).

Various Al salts administration can allow an inflammatory cascade in the brain (Prema *et al.,* 2017 and Ahmad Rather *et al.,* 2018). In the present study, Al injection for 14 days increased the production of proinflammatory cytokines specially TNF-α (trigger for other cytokines) and IL-6 (interface of inflammatory and immune response) which are the cause of damage in tissue especially liver and kidney. TNF- α is a transmembrane protein/cytokine that is apparent as a response to pathogen invasion in macrophages. It is also, used as an inflammatory interface of both local and systemic inflammation (Tracey, 2002). TNF- α is playing a major role in the

production of IL-6 and other mediators important in extending the inflammatory response and tissue damage (Aly *et al.,* 2018). Yang *et al.,* 2017, proved that the inflammatory cytokines have a stimulating effect on the accumulation of neutrophils to increase the injury of inflammation in the liver tissue. The present results are also in harmony with liu *et al.,* 2016 and Ahmed and Hammad, 2018, who found an increase in TNF- α and IL-6 in the liver and kidney of rats treated with $AlCl_3$.

In the present study, the administration of *S. cumini* extract reduced the elevation in the inflammatory cytokines (TNF- α and IL-6) in both liver and kidney tissues. The mechanism by which the *S. cumini* extract repair the damage produced by Al and ameliorate the studied cytokines may be due to its potent anti-inflammatory effect (Modi *et al.,* 2010). Said and abd Rabo, 2017, has attributed the ability of *S. cumini* extract in reducing apoptosis and proinflammatory cytokines production to its eugenol content which has an effect on Al potency in inducing toxicity and regulates the generation of inflammatory cytokines in the rat brain.

Al toxicity is mainly due to the production of reactive oxygen species, which in turn cause oxidative damage (Ahmed and Hammad, 2018). The ability of Al in the production of ROS was confirmed by measuring the level of NO, MDA and 8-OHdG in addition to the determination of the activity of antioxidant enzymes (GSH, GPx, GR, CAT and SOD) in liver and kidney homogenate. Our results revealed that the exposure to Al caused elevation in the level of NO, MDA and 8-OHdG suggesting the involvement of free radicle and ROS induced cell damage as a result of Al toxicity (Wen-Yi *et al.,* 2012). 8-OHdG is a critical biomarker for DNA oxidative damage (Al Omairi *et al.,* 2018). A positive correlation between Al exposure and 8-OHdG concentration in humans and animals has been reported (Valavanidis *et al.,* 2009 and Samir and Rashad, 2018). In addition, $AlCl_3$ causes depletion in serum, hepatic and renal antioxidant enzymes (GSH, GPx, GR, CAT and SOD) in the present study. Our results are similar to previous studies that illustrated that the exposure to Al ion promoted oxidative stress and consequently caused membrane damage leading to depletion in the components of the antioxidant system (Ahmed and Hammad, 2018). *S. cumini* extract administration in animals intoxicated with Al in the present study resulted in a significant inhibition in NO, MDA and 8-OHdG and marked elevation in the antioxidant enzymes (GSH, GPx, GR, CAT and SOD). The *S. cumini* extract caused a reduction in oxidative stress in diabetic rats ((Behera *et al.,* 2019). França *et al.* (2019), concluded that *S. cumini* has a major role in enhancing the secretion of the antioxidant system which in turn abrogates the oxidative stress by scavenging the free radicle due to its flavonoids constituents. Moreover, eugenol ameliorates the level of LPO and enhance the antioxidant system in the brain of Al-induced neurotoxicity in rat.

Apoptosis is one of the mechanisms participating in Al-induced cytotoxicity (Singla and Dhawan, 2015). As previously mentioned, there are a wide variety of factors and situations that help in the enhancement of apoptosis such as inflammation, oxidative stress and mitochondrial damage (Rong and Distelhorst, 2008). Apoptosis is regulated by Bcl_2 gene and enhanced by the Bax gene. Also, caspase-3 is activated in apoptosis in most body cells and has the ability to initiate and achievement of apoptosis (Akhtar *et al.,* 2015). Our study showed that Al treatment resulted in marked upregulation in both apoptotic Bax and caspase-3 genes and down-regulation in Bcl_2 anti-apoptotic gene in kidney and liver tissue. The increase in apoptotic genes (Bax and caspase-3) is indicating that Al enhances cell death in rat liver and kidney. These results are similar to

Hassanin *et al.*, 2017, who recorded a marked increase in caspase-3, Bax and Bax/Bcl_2 ratio in rat brain intoxicated with Al. The elevation in Bax and caspase-3 genes may be due to the capability of Al in deactivating the normal mitochondrial metabolic function causing apoptosis (Vasudevaraju *et al.*, 2008). Cytochrome C is released from mitochondria because of Bax protein enhancement, which caused stimulation to caspase-3 protein activity (Banerjee *et al.*, 2006). Rats treated with *S. cumini* extract suppressed apoptosis in the kidney and liver tissue via decreasing the expression of Bax and caspase-3 and increasing the level of Bcl_2 expression, which indicates that *S. cumini* extract elevates the hepatocyte and renal cells vitality by inhibiting the apoptotic proteins.

In conclusion, our results proved the therapeutic effect of *S. cumini* extract against Al-mediated hepatic and renal toxicity via its efficiency as anti-inflammatory, antioxidant and antiapoptotic activities. Thus, the use of *S. cumini* extract could be beneficial for Al-induced hepatic and renal toxicity.

Ethical Approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed. We respected the welfare of animals and excluded situations when animals were in pain.

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