Rhizophora Mucronata Lam. (Mangrove) Bark Extract Prevents Ethanol-induced Liver Injury, Oxidative Stress and Apoptosis in Swiss Albino Mice

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Abstract

The bark extract of *Rhizophora mucronata* (BERM) was recently reported for its prominent *in vitro* protective effects against liver cell line toxicity caused by various toxicants, including ethanol. Here, we aimed to verify the *in vivo* hepatoprotective effects of BERM against ethanol intoxication. An oral administration of different concentrations (100, 200, and 400 mg/kg) of BERM prior to high-dose ethanol via intraperitoneal injection was performed in mice. On the 7th day, liver and kidney sections were dissected out for histopathological examination. The ethanol intoxication caused large areas of liver necrosis while the kidneys were not affected. Pre-BERM administration decreased ethanol-induced liver injury, as compared to the mice treated with ethanol alone. In addition, the pre-BERM administration resulted in a decrement in the level of ethanol-induced oxidative stress, revealed by a concomitant increase of GSH and a decrease of MDA hepatic levels. The BERM extract also reversed the ethanol-induced liver injury and hepatotoxicity, characterized by the low detection of TNF-α gene expression level and fragmented DNA, respectively. Altogether, BERM extract exerts antioxidative activities and present promising hepatoprotective effects against ethanol intoxication. The identification of the related bioactive compounds will be of interest for future use at physiological concentrations in ethanol-intoxicated individuals.

1. Introduction

Ethanol, also called ethyl alcohol or alcohol, is considered one of the potent hepatotoxins capable of causing chronic liver damage (Julien et al., 2020). Liver diseases including alcoholic liver disorder (ALD) have been associated to chronic alcohol abuse leading to highest morbidity and mortality worldwide (Asrani et al., 2019; Crabb et al., 2020). The time and dosage contingent intake of alcohol increase the risk of ALD (Marugame et al., 2007). ALD progression is revealed by a series of liver diseases, which begins from fatty liver to swelling and noxious cells such as steatohepatitis, cholecystitis, and cirrhosis to ultimately develop into hepatocellular carcinoma (HCC) (Morgan et al., 2004). To overcome any side effects caused by toxicants or conventional chemotherapeutic drugs causing liver cell damage (hepatotoxicity) and tissue injury, cost-effective plant and plant-based preparations posing no side effects could be valuable for the treatment of liver disorders (Mondal et al., 2014; Anwanwan et al., 2020).

Ethanol consumption followed by its metabolism results in high toxic levels of acetaldehyde via alcohol dehydrogenase, which generates oxidative stress (Yin et al., 1999; Zima et al., 2001; Zhou et al., 2003). Through its highly reactive nature, acetaldehyde interacts with cellular proteins, lipids, and deoxyribonucleic acid (DNA) leading to the production of adducts and reactive species, which subsequently cause elevated hepatotoxicity and severe liver injury. Acetaldehydes mainly cause the formation of protein adducts that are toxic and highly immunogenic (Niemelä, 2001). Consequently, acetaldehyde-adducted proteins and alcohol-induced oxidative stress increase the synthesis and release of tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine mainly secreted by the macrophages and demonstrated to contribute to liver injury and damage (Sapkota et al., 2016; ). In addition, one of the factors playing major roles in the alcohol toxicity is the oxidative stress caused by excessive generation
of reactive oxygen species (ROS) (Bailey and Cunningham, 1998). Under normal physiological situations, the liver oxidative stress is regulated by the hepatic enzymatic (i.e., glutathione reductase) and non-enzymatic (i.e., reduced glutathione (GSH) and malondialdehyde (MDA)) antioxidant systems to maintain the cellular redox homeostasis. An excessive consumption of alcohol impairs the hepatic antioxidant system and results in lipid peroxidation, indicated by MDA, and in GSH deficiency (Chen et al., 2016; Pérez-Hernández et al., 2017).

Consumption of ethanol leads to another major consequence leading to cell fate towards programmed cell death (i.e., apoptosis), a complex process characterized by DNA fragmentation, which is occurred not only in the liver (Nanji, 1998) but also in other tissues including brain (Slomiany et al., 1997), salivary gland (Zhang et al., 1998) and gastric mucosa (Piotrowski et al., 1997). Furthermore, ethanol toxicity also interferes with the electron transport chain that provokes mitochondrial dysfunction, apoptosis, cellular damage, and ultimately necrosis, a form of a premature cell death caused by autolysis and occurring in response to injury (Hoek et al., 2002; Manzo-Avalos et al., 2010; Songet al., 2014; Wang et al., 2016).

Based on a previous report, using human hepatocarcinoma cell line HepG2, the plant parts of Rhizophora mucronata Lam. (R. mucronata), also known as Mangrove, including leaves, roots, flowers, bark and fruits were shown to have promising therapeutic values and to be capable of neutralizing various toxicants, including ethanol intoxication (Jairaman et al., 2019). However, no studies have explored the in vivo biological protective impact of the bark extract of R. mucronata (BERM) against ethanol-induced liver injury and hepatotoxicity. Thus, in this present study, BERM was tested for its hepatoprotective properties against ethanol intoxication in Swiss albino mice. This in vivo assessment was made in an attempt to find a novel and safe hepatoprotective drug against ethanol-induced liver injury. This study primarily focused on the evaluation of liver injury biomarkers, including the measurements of non-enzymatic antioxidant components, MDA and GSH, using oxidative stress-related biochemical assays; the monitoring of TNF-α gene expression level using real time-polymerase chain reaction (RT-PCR) technique; and the evaluation of cell damage based on apoptotic status using terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay. Hepatoprotective flavonoid milk thistle seeds-derived Silybum marianum (Silymarin), well-known for its anti-oxidative properties, was used as a positive control throughout this study.

2. Materials And Methods

2.1. Sample Collection and Extract Preparation

During January 2018, the barks of R. mucronata Lam. were collected from Pichavaram Mangrove forest and were authenticated by Prof. P. Jayaraman with the specimen no: PARC/2018/3854 at Plant Anatomy Research Centre, West Tambaram, Chennai, India for future reference. The barks were dried in shade for 15 days, roughly powdered and kept in containers, which were impermeable to air and later used for further study.
The pre-weighed 500 g of powdered bark of *R. mucronata* were brought in a tight glass container with lid and soaked with ethanol: water (3:1 *v/v*) weighing about 1500 mL. The container was sealed and kept for a period of 2 weeks with sporadic mixing and agitation. The extract was then filtered through Grade I Whatmann filter paper. In order to get the crude bark extract of *R. mucronata* (BERM), the filtrate was evaporated at room temperature and stored in refrigeration at 4°C for further use.

### 2.3. Animal Procurement and Maintenance

The animals were procured from Biogen Laboratory Animal Facility (Bangalore, Karnataka, India). For the present study, healthy male Swiss C57/BL/6 Albino mouse strains (*n* = 36) aged between 8 and 10 weeks, weighing approximately 25 to 30 g, were obtained. The animal experiments were conducted in accordance with the ethical norms and guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (New Delhi, India) and was also approved by the Institutional Animal Ethical Committee (IAEC) of Saveetha Medical College (SU/CLAR/RD/002/2018).

The mice were shifted 10 days before the start of the experiment to the laboratory conditions for acclimation. The mice were kept in plastic cages and were marked on the tail for the identification of each individual. Throughout the experiment, the mice were fed with ADILAID Hemster pellets vegetable (Mumbai, India) and drank potable water *ad libitum*, except during the short fasting period where the supply of food was still in ad-lib but potable water was not supplied 2 h before the treatment.

### 2.4. Animal Study Design and Sample Preparation

The experiment was designed as per the previously published protocol (Padmanabhan and Jangle, 2014). The 36 Swiss Albino mice were sorted into 6 groups consisting of 6 animals in each group. **Group 1**: standard control group. For 6 days, the mice were orally administered with distilled water (5 mL/kg body weight (b.w.)). **Group 2**: ethanol-induced liver injury group. The mice were administered with ethanol (cat. #64-17-1, Sigma-Aldrich Corp.) alone. **Group 3**: silymarin (cat. #S0292, Sigma-Aldrich Corp.) + ethanol group. The mice were orally administered with a single dose of silymarin (50 mg/kg b.w.) prior to ethanol administration. **Group 4**: 100 BERM + ethanol group. The mice were orally administered with a single dose of 100 mg/kg b.w. BERM prior to ethanol administration. **Group 5**: 200 BERM + ethanol group. The mice were orally administered with a single dose of 200 mg/kg b.w. BERM prior to ethanol administration. **Group 6**: 400 BERM + ethanol group. The mice were orally administered with a single dose of 400 mg/kg b.w. BERM prior to ethanol administration. Except the untreated mice in Group 1, all the treated mice were given ethanol (5 mL/kg b.w. of 25% *v/v* ethanol), via intraperitoneal (i.p.) injection for 6 days, after half-hour oral administration of the plant extract.

On day 7, the mice were euthanized by applying pressure to the neck and dislocating the spinal cord. Kidneys and liver were removed, thoroughly rinsed with regular brine, then dried with tissue paper. The upper left lobe of the liver was cut with sterile scissors and wrapped in the aluminum foil, and kept at −70°C before processing RT-PCR for TNF-α gene expression level monitoring and TUNEL assay. The remaining part of the liver was mixed evenly to get the homogenate, which was dissolved in 0.2 M phosphate buffer (pH 7.4). By using a tissue homogenizer (MC Dalal & Co., Chennai, India), 10%
homogenized liver tissue was made. After centrifugation at 2075 ×g for 15 min, the supernatant was utilized for the detection of MDA and GSH.

### 2.5. Histopathological Analysis

The liver and kidneys were first fixed in 10% formalin then they were dehydrated using gradual ethanol (50–100%), rinsed in xylene, and were impregnated in paraffin wax. The tissue sections (5-6 µm thickness) were generated using rotary microtome and later stained with haematoxylin and eosin (HE) dye for histopathological examination.

### 2.6. Oxidative Stress-Related Non-Enzymatic Assays

#### 2.6.1. Estimation of Reduced Glutathione

Reduced GSH was measured in the homogenized supernatant as described in (Moron et al., 1979). Briefly, in order to precipitate the proteins, 125 µL of 25% of trichloroacetic acid (TCA) were added to 0.5 mL of supernatant. The test tubes were cooled on ice for 5 min and the supernatant was then diluted with 0.6 mL of 5% TCA and centrifuged for 10 min at 9000 ×g. To the 0.3 mL of the aliquot, 0.7 mL of 0.2 M sodium phosphate buffer (pH 8.0) were added to make it up to 1 mL. Further, to the tubes freshly prepared, the 5-5′-dithio-bis(2-nitrobenzoic acid) (DTNB) solution (2.0 mL) was added. After 10 min, the formed yellow color produced by the presence of 2-nitro-5-thiobenzoic acid, generated from the reduced glutathione GSH and DTNB reaction, was read at 412 nm using a spectrophotometer (Lovibond, ACD Company, New Delhi, India). Similarly, standards were also included to measure the content of GSH.

#### 2.6.2 Estimation of Malondialdehyde

The measurement of MDA detected in the homogenized supernatant was carried out as described in (Högberg et al., 1974). Briefly, in a total volume of 2 mL, 0.2 mL of supernatant, 0.03 M Tris-HCl buffer (pH 7.4) and 0.2 mM sodium pyrophosphate were added. The mixture was incubated for 20 min at 37°C. The reaction was stopped by the addition of 1 mL of 10% TCA, after which 1.5 mL of the organic compound 2-thiobarbituric acid (TBA) were added and the mixture was heated. The pink-colored product formed revealing the presence of MDA due to the oxidation of fatty acids, was measured using spectrophotometer at an absorption of 535 nm.

### 2.7. RNA Extraction and RT-PCR

Total ribonucleic acid (RNA) was isolated utilizing ONE STEP-RNA Reagent (Biobasic Inc.) from untreated and treated liver tissue homogenates. Concentration and quality of RNA samples were assessed using ultra-violet (UV) spectrophotometry. Easy Script Plus™ Reverse Transcriptase (Tinzyme, New Delhi, Indian) was used for the reverse transcription of high-quality RNA extracts. Briefly, 0.5 µg total RNA, 2 µL oligo dT and 0.5 µg/mL random hexamer primers in diethyl pyrocarbonate (DEPC)-treated water were inoculated for 5 min at 65°C and instantly cooled down on ice. After the addition of 4 µL dithiothreitol (10 mM), 2 µL dNTP (10 mM) and 8 µL First Strand buffer, the temperature of the solution was lowered to 55°C and completed with 200 U Superscript II® (MiRXES, Heal Force Company, Shanghai, China). The solution was
then incubated at 55°C for 60 min then at 85°C for 15 min thus generating complementary DNA (cDNA). TNF-α and internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were amplified by PCR using selected primer pairs of sequences as follows: \textit{TNF-α}, 5′-CCGAGGCAGTCAGATCATCTT-3′ \textit{(forward)}, 5′-AGCTGCCCTCAGCTTGA-3′ \textit{(reverse)}; \textit{GAPDH}, 5′-GCAAGTTCAACGGCAGTCAAG-3′ \textit{(forward)}, 5′-ACATACTCAGCACCAGCATCACC-3′ \textit{(reverse)}. PCRs were performed in duplicate for each sample. To verify whether the results were confined to one single amplified product, all the reactions were assessed through dissociation curve analysis. Each of the amplicons had different melting temperatures, which were 83°C and 84°C for TNF-α and GAPDH, respectively. Pfaffl's mathematical model was used to calculate the relative quantification of TNF-α transcripts (Pfaffl, 2001). In a total volume of 25 mL, 1.5% agarose and 1X Tris-acetate-EDTA (TAE) were prepared and cascaded onto a gel tray. The loading dye was blended with the PCR product. Along with the 1 kilobase pair (kbp) DNA ladder used as a reference; the mixture sample was loaded to each well. The gel was run at 50 volt (V) for 90 min and then PCR products were visualized by ethidium bromide staining and analyzed using Gel Pro Analyzer software (version 4.0.). The quantity of TNF-α transcript was related to GAPDH.

### 2.8. Terminal deoxynucleotidyl transferase (TdT)-Mediated dUTP Nick-End Labelling (TUNEL) Assay

DNA fragmentation analyses were carried out in paraffin-impregnated liver tissues using a terminal deoxynucleotidyl TUNEL reaction conforming to manufacturer's instructions. TUNEL reaction mixture (250 µL) was formulated using: TdT (25 µL) diluted in the nucleotide mixture (225 µL). Nucleotide solution excluding TdT was considered as negative control in all experiments. After lysing the cells and the DNA strands were decondensed, the slides containing the paraffin-impregnated liver tissues were washed twice with PBS. The TUNEL reaction mixture (25 µL) was added to each slide and a coverslip was placed on the top for mounting. Later, the slides were incubated in a dark and highly moist chamber for 60 min at 37°C. The coverslips were then taken off and the slides were rinsed three times with PBS. Slides were developed with diaminobenzidine substrate, counterstained with HE dye, and scrutinized for confirmation of programmed cell death (i.e., apoptosis), revealed by DNA fragmentation. The count of brown apoptotic cells was normalized to total cells as visualized by HE staining. The apoptotic index was calculated by dividing the number of apoptotic cells by the total number of cells in random fields.

### 2.9. Statistical Analysis

Sigma Plot-13 software (version 14.0, Systat Software, CA, USA) was used to carry out statistical analysis. The results are expressed as the mean ± standard error of mean (SEM). One-way ANOVA with Dunnett’s comparison post-hoc test was used for evaluating the significance of difference. If \( p < 0.001 \), the data were considered statistically significant.

### 3. Results
3.1. BERM Prevents Ethanol Intoxication-Induced Liver Injury

Histopathological observations were made in gross liver and kidney tissues, dissected from all the studied mouse groups. In the group treated with ethanol alone, spotty necrosis was visualized in the liver tissue while the sections of kidney appeared to be normal, as seen in the Figure 1. The mouse groups, which received treatment with either silymarin (the positive control) or BERM extract (100 mg and 200 mg) prior to ethanol administration showed a gradual improvement towards normal tissue architecture indicated by the absence of spotty necrosis induced by ethanol, as compared to the control (Group 1), the untreated tissues (Figure 1). The oral treatment with BERM prior to the administration of the toxic ethanol for 7 consecutive days showed an obvious decrease in toxicant liver tissue injury, as compared with ethanol-induced liver injury (Figure 1).

Based on histopathological microsection examination of the liver and kidney tissues collected from the 6 groups of mice, different tissue characteristics were observed, however both tissues showed necrotic cells and fibrosis due to ethanol intoxication, as seen in the Figure 2. The liver and kidney tissues collected from ethanol-administered groups pretreated with BERM presented normal tissue architecture, as compared with the normal untreated group, the negative control (Figure 2).

3.2. BERM Decreases Ethanol-Induced MDA Levels and Enhances Ethanol-decreased GSH Levels in Liver Tissues

As a biomarker of ethanol-induced oxidative stress and of liperoxidation (Galicia-Moreno et al., 2016), the content of MDA was measured in the supernatants of tissue homogenates. The administration of alcohol significantly increased the level of MDA produced in liver tissues, as seen in the Figure 3. The oral pre-treatment with silymarin followed by the administration of ethanol resulted in the significant decrease in ethanol-induced MDA production, as compared with hepatic tissue MDA levels detected in the ethanol study group (Figure 3). A significant gradual reduction in ethanol-induced MDA production was observed by the increased concentrations of the oral pre-treatment with BERM extracts (100, 200 and 400 mg), as compared to the ethanol group (Figure 3). The decrease in ethanol-induced MDA production by the pre-treatment with 400 mg BERM was similar to the decrease in ethanol-induced MDA production in liver tissues caused by the pre-treatment with silymarin (Figure 3).

GSH levels was found to be the least measured in the toxic ethanol study group and the highest in the silymarin study group, as seen in the Figure 3. A significant gradual increase in ethanol-induced GSH production was observed by the increased concentrations of the oral pre-treatment with BERM extracts (100, 200 and 400 mg), as compared to the ethanol group (Figure 3). A significant difference was still observed between the increased level of GSH content detected in ethanol-induced liver injury pre-treated with 400 mg BERM and the increased level of GSH detected in ethanol-induced liver injury pre-treated with silymarin (Figure 3).
3.3. BERM Reduces Ethanol-Induced TNF-α Gene Expression Level

Chronic ethanol consumption also leads to the increase in the gene expression level of TNF-α, a pro-inflammatory cytokine used as a biomarker of liver injury (Yin et al., 1999). Amplified by RT-PCR then visualized using gel electrophoresis, the liver treatment with ethanol significantly up-regulated TNF-α mRNA expression level, as compared to TNF-α mRNA expression level monitored in the untreated control liver tissue, as seen in the Figure 4. The pre-treatment with either BERM or silymarin significantly decreased ethanol-induced TNF-α mRNA expression level, as compared to TNF-α mRNA expression level monitored in the toxic alcoholic liver tissue (Figure 4).

3.4. BERM Inhibits Apoptosis Due to Ethanol-Induced Liver Injury

Ethanol intoxication-induced liver injury leads to premature and programmed cell death, including apoptosis (Wang et al., 2016). Known as a hallmark of apoptosis, nuclear DNA fragmentation was assessed using TUNEL assay under light microscope. Mainly observed in the toxic ethanolic liver tissue, the shrunken cells of which nuclei was stained brown were identified as TUNEL-positive apoptotic cells, as compared with the healthy cells that were observed in the untreated control liver tissue, as seen in the Figure 5A. Fewer TUNEL-positive apoptotic cells were spotted in the ethanol-induced liver injury after pre-treatment with either BERM or silymarin, as compared to TUNEL-positive apoptotic cell number observed in the toxic ethanolic liver tissue (Figure 5A). The apoptotic index was significantly decreased in ethanol-induced liver injury in the mice group pre-treated with BERM, as compared with the mice treated with ethanol alone, as seen in the Figure 5B. The pre-treatment with silymarin decreased at a higher extent the apoptotic index induced by ethanolic intoxication alone than the one determined following to pre-treatment with BERM (Figure 5B).

4. Discussion

Liver and kidneys are both vital organs that are crucial for xenobiotic and drug elimination from our body (Galicia-Moreno et al., 2016). In contrast to the kidneys, the liver tissue, consisted of a mass of cells tunneled through with bile ducts and blood vessels, is mainly involved in the detoxification of viral infection, prolonged drug therapy, various toxicants (i.e., CCl₄ and environmental pollutants, industrial chemicals, etc.), and chronic alcoholism (Zima et al., 2001; Ndoe et al., 2015; Unsal et al., 2020). Their metabolism and detoxification can generate a myriad of oxidative stress-related intermediate and end-products leading to hepatotoxicity, characterized by the hepatocyte death, capable of causing liver damage, liver injury and eventually liver diseases (Li et al., 2015). These harmful free radicals and reactive oxygen species may impair the prominent hepatic enzymatic and non-enzymatic antioxidant systems, and thus decrease the detoxification capability of the liver (He et al, 2017). So far, conventional medical therapy of liver failure or liver diseases such as drug-based treatment or even post-
transplantation medication is still not denuded from side effects, requesting the urgent needs to discover new plant and plant-based formulations as safe medication therapies. These last decades, numerous studies have reported hepatoprotective activities of various natural products extracted from plants (Jiménez-Arellanes et al., 2016), including silymarin reaching clinical trials (Gillessen and Schmidt, 2020). We previously evaluated the \textit{in vitro} hepatoprotective activities of BERM based on the reduction of the cytotoxicity in HepG\textsubscript{2} cell line exposed to the combined BERM and toxicants (\textit{i.e.}, CCl\textsubscript{4}, ethanol and paracetamol) treatment (Padmanabhan and Jangle, 2014). In this present study, we demonstrated the hepatoprotective activities of BERM in ethanol-intoxicated mice associated with a decrease of ethanol-induced MDA production, enhancement of ethanol-decreased GSH production, and with the concomitant reduction of ethanol-induced hepatotoxicity, revealed by the down-regulation of TNF-\alpha gene expression level and by the quasi-disappearance of fibrosis and apoptotic hepatocytes.

Oxidative stress has been mainly associated with the pathological process of ethanol-induced liver injury (Phaniendra et al., 2015). The production of MDA, commonly known as oxidative stress marker and as a marker of lipid peroxidation, was considerably enhanced in the ethanol study group in comparison with the untreated control group. The MDA overproduction due to ethanol-induced liver damage aligned with previous research studies (Chang et al., 2021). Another study reported that oxidative stress in brain due to ethanol consumption also elevated MDA levels (Das et al., 2007).

A crucial non-enzymatic antioxidant pertaining to oxidative stress is GSH, which removes H\textsubscript{2}O\textsubscript{2} radicals and reacts directly with certain ROS (\textit{e.g.}, the hydroxyl radical) and nullify its toxic effects. In the present study, the ethanol toxicity group exhibited decreased levels of GSH in comparison with the control and treated groups, resulted in reduced synthesis of GSH, as previously reported (Husain et al., 2001). Observed in rats subjected to alcohol and tobacco smoke exposure, the generation of oxidative stress was also stated to decrease GSH levels in liver (Ignatowicz et al., 2013), which agreed with our present findings.

Oxygen radicals generated by the ethanol intoxication-induced injury play an important role in the stimulation of inflammation through up-regulation of inflammatory cytokines such as TNF-\alpha (Gutierrez-Ruiz et al., 2001). TNF-\alpha is a key pro-inflammatory cytokine which induces the secretion of enzymes and other cytokines in various cells and tissues. In this present study, the prolonged exposure to ethanol leads to increased level of TNF-\alpha gene in the toxic study group. Similar results were obtained by Nowak and Relja who demonstrated that NF-\kappaB signaling pathway was activated during alcoholic liver disease, which resulted in the increased of gene expression levels of pro-inflammatory cytokines and chemokines (Nowak and Relja, 2020). In humans, chronic alcohol consumption is associated with increase in the production of serum pro-inflammatory cytokines (\textit{e.g.}, TNF-\alpha, IL-1, IL-6, IL-8) (McClain and Cohen, 1989, McClain et al., 1999). Thus, the correlation between oxidative stress and inflammation within the course of alcoholic liver injury is indisputable. Moreover, improper metabolism of ROS ends up in the expression of hypoxia-inducible factor-1 alpha that may also increase TNF-\alpha secretion, resulting in associate immune reaction that intensifies the liver injury (Wilson et al., 2014). In addition to play a major role in inflammation, TNF-\alpha bound to its receptor, which initiates programmed death pathways such as
apoptosis through activation of downstream kinases and proteases, including caspases (Fouad et al., 2019). A deeper investigation of the reverse effect of ethanol intoxication inducing apoptosis-related molecular mechanisms, including caspase-dependent (extrinsic) and mitochondria-dependent (intrinsic) pathways, contributing to hepatoprotective activities of BERM would be of interest.

The TUNEL assay was carried out for the detection of apoptotic cells that undergo massive DNA fragmentation during the final stages of apoptosis. The DNA damage may be incurred due to ethanol-induced oxidative stress exposed to the hepatocytes causing production of ROS and of TNF-α-induced cell death, which in turn lead to hepatic injury (Wang et al., 2016). The current study showed that ethanol intoxication towards mouse hepatocytes increased the number of apoptotic cells that was determined using TUNEL assay and observed with light microscopy, which agrees with previous studies using human alcoholic hepatitis specimens (Zhao et al., 1997; Natori et al., 2001). However, in this present study when pre-treated with either silymarin or BERM, a considerable decrease in the number of ethanol-induced apoptotic cells was noticed, confirming the in vivo hepatoprotective effect of BERM from alcohol intoxication.

5. Conclusion

There is growing interest in the discovery of new antioxidant plant-based bioactive compounds that can reverse the deleterious effects of toxicants, including ethanol overdose. In this present study, BERM at the concentration of 400 mg/kg showed the highest protective effect from ethanol intoxication-induced liver damage in mice and were revealed to be comparable with the hepatoprotective standard herbal drug, silymarin. The hepatic MDA levels were subsequently found to be low, indicating the decrease in the oxidative stress levels, and the increase in hepatic GSH levels clearly reflected the hepatoprotective effect of BERM due to the presence of antioxidants. Thus, the recently reported purification and isolation of unidentified bioactives compounds from BERM along with those characterized BERM-derived hepatoprotective agents, including daidzein, epicatechin, hesperidin, diosmin, and quercetin (Saha et al., 2019; Chitra et al., 2020), will pave the way for the development of an alternative and cost-effective hepatoprotective agents against toxic liver disorders.

Declarations

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors do not have any conflict of interest to declare.

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**Authors’ contribution**

SAMY and SMP conceived the study. ZIA, SMN and SAMY conducted the study. CJ, AV, SKS, VR, YN, GM, RP and SR performed, collected the data, and reviewed the manuscript. CJ, ZIA, SMP, SAMY and SMN wrote and reviewed the manuscript.

**Abbreviations**

ALD, alcoholic liver disorder; BERM, bark extract of *Rhizophora mucronata*; b.w., body weight; cDNA, complementary DNA; DEPC, diethyl pyrocarbonate; DNA, deoxyribonucleic acid; DTNB, 5'-dithio-bis(2-nitrobenzoic acid); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; HCC, hepatocellular carcinoma; HE, haematoxylin and eosin; i.p., intraperitoneal; kbp, kilobase pair; MDA, malondialdehyde; RNA, ribonucleic acid; ROS, reactive oxygen species; RT-PCR, real time-polymerase chain reaction; SEM, standard error of mean; TAE, Tris-acetate-EDTA; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid; TNF-α, tumor necrosis factor-alpha; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling; UV, ultra-violet;

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Figures

Figure 1
BERM pre-administration prevented ethanol-induced liver injury in mice. Representative gross sections of liver and kidney tissues of each mouse from 6 groups of mice after (5 mL of 25% ethanol/kg b.w.) ethanol treatment via i.p. injection following to either oral pre-administration with silymarin tested at 50 mg (Group 4) or oral pre-administration with BERM tested as 100 mg (Group 3), 200 mg (Group 4) and 400 mg (Group 5), as compared to healthy untreated control mice (Group 1) and mice administered with ethanol alone (Group 2). Spotty necrosis as indicator of tissue damage was only observed in the liver tissue of ethanol-treated mice.

**Figure 2**

BERM pre-administration prevented mild ethanol-induced kidney injury and severe ethanol-induced liver injury in mice. Histopathological analysis of representative microsections from kidney and liver tissues extracted from untreated (Group 1: Control) and treated mice (Group 2-6). From the Group 2, a mild ethanol-induced injury was observed in the kidney tissues as indicated by the arrows, pointing glomeruli damage (GL-D) and tubule damage (T-D) while a severe ethanol-induced injury was observed in the liver tissues as indicated by the arrows, pointing central vein damage (CV-D) and bile duct damage (BD-D). From the Group 3, the oral pre-administration with silymarin prevented both ethanol-induced kidney and liver injuries as indicated by normal tissue architectures (glomeruli-normal, GL-NL; tubule-normal, T-NL; central vein-normal, CV-NL; bile duct-normal, BD-NL; hepatosis-normal, H-NL). From the Group 4-6, the oral
pre-administration with BERM (100-400 mg) prevented mild ethanol-induced kidney injury and severe ethanol-induced injury as indicated by the absence of histopathologic alterations (No histopathologic alterations, N-H) and areas showing hepatosis regeneration (H-R), as compared with the Group 1 presenting no damage (ND) of the kidney and liver tissues.

![Figure 3](image)

**Figure 3**

Effect of BERM pre-administration on hepatic tissue GSH and MDA levels following to ethanol-induced liver injury. The bar graphs show hepatic tissue levels of GSH and MDA measured using colorimetric methods involving specific substrates such as DNTB and TBA solutions, respectively. Refer to methods section for more information. Alphabets a, b and ab, clearly indicate that they are statistically significant with the control (untreated) mouse group.
Figure 4

BERM pre-administration decreased ethanol-induced TNF-α mRNA expression levels. (A) Representative gel electrophoresis showing mRNA expression levels of TNF-α and GAPDH, the internal control, determined by polymerase chain reaction analysis in the control mouse group, ethanol treated group, BERM or silymarin administration prior to ethanol injection. (B) The bar graph shows the expression levels of TNF-α mRNA related to GAPDH. The results are presented as the mean ± SEM of three independent experiments.
Figure 5

BERM pre-administration decreased ethanol-induced apoptosis. (A) Representative photomicrographs showing apoptotic DNA fragments containing digoxigenin-labeled nucleotides, revealed using TUNEL assay. (B) The bar shows the percentage TUNEL-positive apoptotic cells. The results are presented as the mean ± SEM based on three independent experiments.

Supplementary Files

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