Protective Role of Rosemary Ethanolic Extract on Thioacetamide Induced Hepatic Encephalopathy: Biochemical and Molecular Studies

Alshaimaa M. Said1,2, Rania M. Waheed2, Olla A. Khalifa3

1Biochemistry Department, Faculty of Veterinary Medicine, Benha University, 13736 Moshtohor, Toukh, Qaliobiya, Egypt
2Forensic Medicine and Toxicology Department, Educational Hospital, Faculty of Veterinary Medicine, Benha University, 13736 Moshtohor, Toukh, Qaliobiya, Egypt
3Animal Wealth Development Department, Faculty of Veterinary Medicine, Benha University, 13736 Moshtohor, Toukh, Qaliobiya, Egypt

Abstract

Background: Hepatic encephalopathy (HE) is a major health concern worldwide with a significant morbidity and mortality in addition to the economic burden. Oxidative stress, induced from exposure to various pollutants, with the resultant inflammation contributed to the pathogenesis of HE. This study was conducted to evaluate the antioxidant and anti-inflammatory effects of rosemary ethanolic extract on thioacetamide (TAA) induced HE. Methods: Rats used in this study were divided into three groups: control group, TAA group received intraperitoneal (i.p) injection of TAA twice weekly for 12 weeks, rosemary treated group received i.p injection of TAA twice weekly and oral daily dose of rosemary ethanolic extract for 12 weeks. Results: The results revealed an obvious state of oxidative stress in liver and brain as evidenced by elevated malondialdehyde (MDA) and oxidized glutathione (GSSG) levels and lowered reduced glutathione (GSH) and total antioxidant capacity (TAC). Additionally, the concentration and gene expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) indicating a significant inflammatory response. On the other hand, co-treatment with rosemary extract exhibited a significant improvement antioxidant capacities and inflammatory status of liver and brain. Moreover, the matrix metalloproteinase-1 (MMP-1) and paraoxonase-1 (PON-1) as fibrotic markers were analyzed proposing a possible anti-fibrotic activity of rosemary. Conclusion: it can be concluded that rosemary extract provide a powerful ameliorating effect against TAA induced HE owing to the antioxidant, anti-inflammatory and possible anti-fibrotic activities of its constituents.

Keywords: Hepatic encephalopathy, interleukin-10, interleukin-6, matrix metalloproteinase-1, rosemary, thioacetamide.

INTRODUCTION

Hepatic encephalopathy is a profound systemic consequence of liver insufficiency with neuropsychiatric symptoms and in severe cases coma and death developed [1]. Mortality rate of HE ranging from 50-90% of patients allow the world [2]. Normally, liver is able to detoxify endogenous and exogenous ammonia into urea. Once liver fails, hyperammonemia occurs and ammonia passes blood brain barrier [3]. Glutamine synthase catalyze synthesis of glutamine which accumulates inside astrocytes leading to cerebral edema and intracranial hypertension with subsequent alterations in cerebral functions [4].

Frequent unavoidable exposure to environmental pollutants, which are metabolized and detoxified in liver, results in a state of oxidative stress, hepatic, renal and even neurotoxic effects. Thioacetamide (TAA) is a sulfur containing compound widely used in technical applications and industries as leather, textile and paper production [5]. Single dose of TAA used in several experimental studies to induce acute liver injury while exposure to small doses of TAA used to develop chronic liver failure followed by HE [6].

Various side effects of pharmaceutical preparations used in treatment of liver insufficiency triggers the need for searching new safe and effective therapeutic agents. Since oxidative stress has been implicated in acute and chronic liver injury the natural antioxidants are acceptable candidates for protection and management of liver injury and its subsequent neurologic effects [7].

Rosmarinus officinalis, known as Rosemary and belong to Lamiaceae family, cultivated now in many parts of the world after being restricted to the Mediterranean region only [8]. It is widely used as flavoring agent and natural food preservative due to its...
potent antioxidant constituents [9]. Moreover, rosemary has been used for long time in folk medicine for treatment of various diseases as peptic ulcer, gastrointestinal tract disturbances, biliary colic, renal colic, bronchial asthma, headache and epilepsy [10]. Several research studies revealed the active constituents of rosemary leaves. The dark green needle like leaves contain phenolic diterpenes as carnosol, carnosic acid, rosmanol and phenolic acids as rosmarinic acid. These active compounds constitute a wide variety of biological activities as antioxidant, anti-inflammatory and anticarcinogenic agents [11].

The aim of the present experimental study was to assess the antioxidant and anti-inflammatory effects of rosemary ethanolic extract on TAA induced hepatic encephalopathy in rats. Oxidative stress was assessed by measuring liver and brain levels of malondialdehyde (MDA), oxidized glutathione (GSSG), reduced glutathione (GSH) and total antioxidant capacity (TAC) in addition to hepatic matrix metalloproteinase-1 (MMP-1) and paraoxonase-1 (PON1) as fibrotic markers. Moreover, the concentration and gene expression of TNF-α, IL-6 and IL-10 were determined in liver and brain homogenate.

MATERIAL AND METHODS

Chemicals

Thioacetamide was purchased from Sigma–Aldrich, St. Louis, MO, USA.

Preparation of Rosemary Ethanolic Extract

Rosemary dry leaves were purchased from local market in Cairo, Egypt and grinded to fine powder. The plant extract was prepared by addition of ethanol 70% to the fine powder in a firmly closed jar and let for 3-5 days at room temperature with vigorous shaking twice a day. The mixture was filtered using filter paper and a rotatory evaporator was used to evaporate the alcohol and obtain the pure extract. The residues were re-extracted by the same method in order to obtain the whole constituents of the plant.

Animals

All experiments were approved by the Ethical Committee of Faculty of Veterinary Medicine, Benha University. Thirty male Wistar albino rat (150 ± 20 g) were supplied by the animal house of Faculty of Veterinary Medicine, Benha University, Egypt. They were acclimatized in our animal facility for one week under controlled environmental conditions before the start of experiment. Fresh daily supplies of food and tape water were served ad libitum.

Experimental Protocol

Rats were randomly divided into three groups (10 animals per group) and treated for 12 consecutive weeks. Control group (1st group): received saline i.p. twice weekly and considered as control group. Thioacetamide group (2nd group) received TAA (200 mg/kg, i.p.) twice weekly to provoke HE [12]. Rosemary treated group (3rd group) received TAA (200 mg/kg, i.p.) twice weekly and oral daily dose of rosemary ethanolic extract (200 mg/kg) for 12 weeks [13]. Tissue samples were collected twice during the experiment with six weeks interval.

The animals were fasted overnight and euthanized with diethyl ether. After sacrifice, liver and brain were quickly removed, washed three times with cold physiological saline and homogenized using phosphate buffered saline at 4 °C producing a 20% homogenate. In addition to homogenates, representative liver and brain specimens were kept in RNA later storage solution (Sigma–Aldrich) for RNA extraction once at the end of experiment. Tissue homogenates and samples for RNA extraction were kept at 80 °C till analyses.

Assessment of Oxidative Stress Markers

The malondialdehyde (MDA), oxidized glutathione (GSSG), reduced glutathione (GSH) and total antioxidant capacity (TAC) were assayed as indices for oxidative stress in liver and brain using appropriate commercial kits (Biodiagnostic, Cairo, Egypt).

Assessment of inflammatory markers

Liver and brain tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 concentrations were assayed using ELISA kits based on TNF-α, IL-6 and IL-10 monoclonal antibodies and converted to the TNF-α, IL-6 and IL-10 levels expressed as picograms per milligram tissue.

Quantitative real time polymerase chain reaction (RT-PCR) analyses of TNF-α, IL-6 and IL-10 gene expressions:

Total RNA was extracted from the samples using the RNeasy Mini Kit (Qiagen). For One-Step RT-PCR procedures cDNA was synthesized from (0.5 μg) RNA with the Invitrogen SuperScript™ II Reverse Transcriptase (Fisher Scientific, England, UK). Quantitative real time PCR amplifications were performed with 2X SYBR Green RT-PCR reaction mix, in the thermocycler Rotor-Gene Q (Qiagen), under the following cycle conditions: cDNA synthesis 10 minutes at 50 °C, Reverse transcriptase inactivation five minutes at 95 °C, PCR cycling and detection (30 to 45 cycles) 10 seconds at 95 °C followed by 30 seconds at 55 °C to 60 °C. Transcript quantities were normalized by GAPDH as a housekeeping gene. The following primers (Sigma–Aldrich) were used for cDNA amplification:

GAPDH forward: CAATGAATACGGCTACAGCAAC and GAPDH reverse: AGGGAGATGCTCAGTGTTGG.© 2019 |Published by Scholars Middle East Publishers, Dubai, United Arab Emirates
TNF-α forward: ATAACTGACCCACTTCCCA and TNF-α reverse: GAGGCCATTTGGGAACCTTCT.

IL-6 forward: GACTCCAGCCAATGCTCTCTG and IL-6 reverse: TGGTCTGTTGCGGTGATCCTC.

IL-10 forward: ATAACTGCACCATTTCTCTACCAGT.

Assessment of Hepatic Fibrosis
Liver matrix metalloproteinase-1 (MMP-1) and paroxonase-1 (PON-1) concentrations were assayed using ELISA kits based on monoclonal antibodies against MMP-1 and PON-1 and converted to the MMP-1 and PON-1 concentration expressed as nanograms per gram tissue.

Statistical Analysis
The values were expressed as the mean ± standard error of the mean. A significant difference was used at the 0.05 probability level. One way analysis of variance and the least significant difference test were used to differentiate the differences between treatments using the Statistical Package for Social science Software (Version 18, SPSS Inc., Chicago, IL, USA).

RESULTS
Assessment of Oxidative Stress
MDA, GSSG and GSH concentrations and TAC were taken as markers of oxidative status in liver and brain tissues. TAA significantly increased MDA and GSSG concentrations and decreased GSH content and TAC compared to the control group. Treatment with rosemary extract significantly reduced MDA, GSSG and enhanced GSH content and TAC compared to the TAA group. It is worthy to note that rosemary extract reversed such changes showing no significant differences from the control group at the end of experiment in both organs (Table 1 & 2).

Assessment of Inflammatory Markers
The concentrations of TNF-α, IL-6 and IL-10 in hepatic and cerebral tissues were analyzed immunoenzymatically at the end of experiment. The mean concentrations of TNF-α and IL-6 were significantly higher in the group that was given TAA compared to controls while the anti-inflammatory cytokine IL-10 was significantly decreased. On the other hand, co-treatment with rosemary extract significantly improved these changes when compared to TAA group.

The abovementioned results were confirmed by the gene expression analyses of these cytokines. As shown in (Table-3), TAA treated rats showed significant up-regulation of TNF-α and IL-6 genes meanwhile, IL-10 gene expression was significantly down regulated compared to the control value. On the contrary, treatment with rosemary extract markedly but not completely restored these alterations when compared to TAA group.

Table-1: Effect of rosemary ethanolic extract on antioxidant and fibrotic markers in liver of TAA injected rats

| Liver | MDA (nmol/g) | GSH (ng/g) | GSSG (Pg/g) | TAC (nmol/g) | PON-1 (ng/g) | MMP-1 (Pg/g) |
|-------|--------------|------------|-------------|--------------|--------------|--------------|
| 1st group | 156.00 ± 13.45 | 326.67 ± 19.10 | 97.67 ± 11.22 | 236.00 ± 15.72 | 247.33 ± 15.98 | 220.33 ± 13.78 |
| 2nd group | 358.00 ± 25.54 | 108.00 ± 15.72 | 283.00 ± 17.21 | 107.67 ± 12.17 | 128.00 ± 13.45 | 354.00 ± 23.81 |
| 3rd group | 244.00 ± 24.84 | 220.33 ± 15.51 | 204.00 ± 13.75 | 178.00 ± 13.00 | 192.33 ± 15.93 | 289.33 ± 11.32 |
| 1st group | 135.33 ± 15.41 | 308.00 ± 22.50 | 138.33 ± 18.81 | 252.00 ± 12.74 | 278.67 ± 14.52 | 181.67 ± 13.86 |
| 2nd group | 335.33 ± 24.63 | 156.33 ± 13.80 | 283.67 ± 20.21 | 123.67 ± 12.68 | 154.00 ± 12.10 | 308.33 ± 13.30 |
| 3rd group | 178.67 ± 19.41 | 265.00 ± 19.86 | 156.67 ± 16.90 | 215.33 ± 14.68 | 261.33 ± 15.62 | 208.33 ± 10.48 |

Mean values with different superscript letters in the same column are significantly different at (P ≤ 0.05).

Table-2: Effect of rosemary ethanolic extract on antioxidant markers in brain of TAA injected rats:

| Brain | MDA (nmol/g) | GSH (ng/g) | GSSG (Pg/g) | TAC (nmol/g) |
|-------|--------------|------------|-------------|--------------|
| 1st group | 130.33 ± 12.35 | 265.33 ± 13.84 | 110.33 ± 8.35 | 229.33 ± 15.21 |
| 2nd group | 355.33 ± 32.99 | 125.00 ± 12.66 | 259.00 ± 15.13 | 90.67 ± 10.71 |
| 3rd group | 235.67 ± 20.21 | 168.67 ± 12.73 | 141.33 ± 12.25 | 140.33 ± 11.86 |
| 1st group | 135.00 ± 12.86 | 247.67 ± 11.39 | 124.33 ± 11.78 | 237.00 ± 16.37 |
| 2nd group | 332.00 ± 24.06 | 141.00 ± 13.75 | 225.00 ± 12.49 | 127.33 ± 12.03 |
| 3rd group | 172.67 ± 13.62 | 223.33 ± 15.88 | 162.00 ± 16.26 | 203.67 ± 14.25 |

Mean values with different superscript letters in the same column are significantly different at (P ≤ 0.05).

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The oxidative stress contributed to the pathogenesis of HE since the increase in hepatic TNF-α, IL-6 and IL-10 in liver and brain were analyzed. TAA injection induced a marked increase in the concentration and gene expression of TNF-α and IL-6 as inflammatory cytokines with a significant decrease in the anti-inflammatory IL-10. These results were in line with the previous study of Algandaby [22] who found a marked increase in hepatic TNF-α and IL-1β with up-regulation to nuclear factor-κβ (NF-κβ) and cyclo-oxygenase-2 (COX-2) expressions. The author explained these

Assessment of Liver Fibrosis

MMP-1 and PON-1 were immunoenzymatically measured to evaluate the impact of rosemary ethanolic extract on the extent of liver fibrosis. As shown in (Table 1), TAA injection showed significant increase in MMP-1 and decrease in PON-1 in comparison to the control group. However, co-treatment with rosemary extract was able to demonstrate a clear attenuation to those alterations when compared to the TAA group. Interestingly, at the end of experiment no statistically significant differences were found in both parameters between rosemary treated group and control group.

DISCUSSION

Oxidative stress and inflammation are proposed as possible mechanisms for the pathogenesis of hepatic encephalopathy. Therefore, the current experiment aimed at evaluating the potential protective effect of rosemary, a common spice known for its antioxidant property [13, 18]. This is consistent with the current results which revealed that co-treatment with rosemary extract decreased the hepatic and cerebral oxidative stress in TAA treated rats (Table 1 & 2). This effect was in agreement with the previous reports in which rosemary extract alleviated the hepatotoxic effect of carbon tetrachloride and azathioprine respectively. They attributed the hepatoprotective effect of rosemary to its antioxidant property [13,18].

Two possible mechanisms can explain the antioxidant activity of rosemary extract. Firstly, the bioactive constituents of rosemary extract act as scavengers for the free radical reactive oxygen species, via their hydroxyl groups, therefore preventing lipid peroxidation and preserving reduced glutathione [19]. Secondly, they preserve the antioxidant status either by stimulating the synthesis of antioxidant enzymes or increasing their activity [20]. Wijeratne and Cuppett [21] suggested that, carnosic acid and carnosol mainly contributed to the antioxidant properties of rosemary extract. In addition, the co-treatment with rosemary extract to rats injected with TAA exhibited a marked alleviation in the brain oxidative stress when compared to TAA group. This came in accordance with the previous studies that suggested the oxidative stress as one of the major mechanisms in the pathogenesis of HE [14]. So, attenuating the oxidative stress induced by TAA metabolites played an effective role in the improvement of brain antioxidant defenses.

In the further part of the experiment, the concentration and gene expression of TNF-α, IL-6 and IL-10 in liver and brain were analyzed. TAA injection induced a marked increase in the concentration and gene expression of TNF-α and IL-6 as inflammatory cytokines with a significant decrease in the anti-inflammatory IL-10. These results were in line with the previous study of Algandaby [22] who found a marked increase in hepatic TNF-α and IL-1β with up-regulation to nuclear factor-κβ (NF-κβ) and cyclo-oxygenase-2 (COX-2) expressions. The author explained these

| Liver | TNF-α (pg/mg) | IL-6 (pg/mg) | IL-10 (pg/mg) | TNF-α | IL-6 | IL-10 |
|-------|---------------|--------------|---------------|------|-----|------|
| 1<sup>st</sup> group | 85.89 ± 4.50<sup>a</sup> | 27.48 ± 3.60<sup>b</sup> | 15.49 ± 1.04<sup>c</sup> | 1.02 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>c</sup> | 1.02 ± 0.01<sup>b</sup> |
| 2<sup>nd</sup> group | 135.70 ± 4.89<sup>a</sup> | 61.52 ± 4.05<sup>a</sup> | 6.05 ± 0.55<sup>a</sup> | 3.36 ± 0.23<sup>b</sup> | 4.91 ± 0.33<sup>a</sup> | 0.47 ± 0.01<sup>c</sup> |
| 3<sup>rd</sup> group | 106.95 ± 7.58<sup>a</sup> | 40.77 ± 3.57<sup>a</sup> | 10.59 ± 1.10<sup>a</sup> | 1.85 ± 0.09<sup>b</sup> | 3.66 ± 0.32<sup>a</sup> | 2.42 ± 0.26<sup>a</sup> |
| Brain | | | | | | |
| 1<sup>st</sup> group | 106.30 ± 3.09<sup>b</sup> | 49.86 ± 3.81<sup>b</sup> | 32.95 ± 1.45<sup>b</sup> | 1.00 ± 0.01<sup>b</sup> | 1.00 ± 0.02<sup>b</sup> | 1.02 ± 0.01<sup>c</sup> |
| 2<sup>nd</sup> group | 224.43 ± 4.91<sup>b</sup> | 86.08 ± 3.82<sup>b</sup> | 13.29 ± 1.11<sup>b</sup> | 4.64 ± 0.33<sup>b</sup> | 17.04 ± 0.92<sup>b</sup> | 0.50 ± 0.02<sup>b</sup> |
| 3<sup>rd</sup> group | 121.13 ± 1.51<sup>b</sup> | 66.49 ± 4.49<sup>b</sup> | 22.50 ± 1.67<sup>b</sup> | 2.72 ± 0.18<sup>b</sup> | 4.66 ± 0.31<sup>b</sup> | 1.63 ± 0.21<sup>b</sup> |

Mean values with different superscript letters in the same column are significantly different at (P < 0.05).

1<sup>st</sup> group; control group. 2<sup>nd</sup> group; TAA group. 3<sup>rd</sup> group; Rosemary treated group.
results due to the oxidative stress elicited by oxidants like TAA. The ROS activate NF-κB which in turn stimulates the production of pro-inflammatory cytokines [23]. Additionally, NF-κB stimulates the production of COX-2 which also worsen the oxidative stress and inflammatory response [24]. The pro-inflammatory cytokines secreted from activated macrophage and kupffer cells could reach brain and stimulate activated astrocytes to secrete cytokines [25].

In the present study, treatment with rosemary ethanolic extract reduced the inflammatory response in liver and brain. This agreed with the previous investigation of Kuo et al., [26] who studied the anti-inflammatory activity of carnosic acid, a phenolic diterpenes of rosemary. They linked the anti-inflammatory activity of carnosic acid to the inhibition of NF-κB. Also, Mengoni et al., [27] attributed the anti-inflammatory activity of carnosic acid and carnosol of rosemary to the inhibition of COX-2 expression.

Pro-inflammatory cytokines released from kupffer cells in response to oxidative stress involved in fibrotic process through activation of hepatic stellate cells (HSCs) and accumulation of extracellular matrix (ECM) [28]. This was confirmed by the significant increase in MMP-1 in the current study. It is an endopeptidase that degrades ECM in order to maintain tissue homeostasis under normal physiological conditions [29]. The inflammation induced by TAA injection increased MMP-1 while co-treatment with rosemary extract effectively reduced the inflammatory response therefore, the observed decrease in MMP-1 is related to the antioxidant and anti-inflammatory activities of rosemary. Also, PON-1 was affected by rosemary extract, an antioxidant enzyme hydrolyzes phosphoorganic compounds and could be used as a marker of fibrosis [30]. Co-treatment with rosemary extract attenuated the marked decrease in the PON-1. Taken together, the modulation in the fibrotic markers, MMP-1 and PON-1, proposed a possible anti-fibrotic activity to rosemary extract which requires further investigation to understand the underlying mechanisms.

**CONCLUSION**

The findings of this study indicate that treatment with rosemary ethanolic extract was effective against TAA induced hepatic encephalopathy. This effect of rosemary may be attributed to the antioxidant and anti-inflammatory properties of one or more of its constituents. Further investigations are required to confirm the anti-fibrotic activity of rosemary and understand the underlying mechanism.

**Author Contribution**

Conceived & designed the experiments: Alshaimaa Said, Performed the experiments: Alshaimaa Said, Rania Waheed and Olla A. Khalifa, Analyzed the data: Alshaimaa Said, Wrote the paper: Alshaimaa Said, Rania M. Waheed and Olla A. Khalifa.

**Conflict of Interest**

This research did not receive any specific grant from any funding agency. The authors declare that there are no conflicts of interest.

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