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Phytochemical Contents of *Salvia grossheimii* SOSN. Species Extract and Its Protective Effect on Alcohol-Induced Fatty Liver in Rats

Seyed Morteza Hosseini [1], Mostafa Asadbegy [1,*], Roya Karamian [2] and Siamak Yari [2]

[1] Medicine, Quran and Hadith Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2] Department of Biology, Faculty of Science, Bu-Ali Sina University, Hamedan, Iran

[*] *Corresponding author*: Medicine, Quran and Hadith Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98-9122499473; +98-2187554428, Fax: +98-2188068923, Email: mostafaasadbegy@yahoo.com

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Abstract

Background: Researchers are interested in finding new agents with natural sources to cure oxidant-induced diseases.
Objectives: The study aimed at determining the antioxidant potential and protective effect of *S. grossheimii* extract against alcohol-induced fatty liver.
Methods: This experimental study was performed in Bu-Ali Sina University, Hamedan, Iran. In 2016 - 2017. The sample size was determined to include 22 male Wistar rats (150 - 200 g) using Morgan's table. In total, 18 rats were divided into three different groups to receive (1) 1 mL water daily (control), (2) 1 mL alcohol daily (alcohol group), and (3) 1 mL extract (500 mg/kg) and alcohol daily (alcohol + extract). Tissue and blood samples were obtained to determine the protective effect of *S. grossheimii* extract against alcohol-induced fatty liver by histological and biochemical examinations. The antioxidant activity of the extract was also assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.
Results: The extract possessed stronger antiradical activity (IC50: 0.102 $\pm$ 0.002 mg/mL) than Vitamin C (IC50: 0.162 $\pm$ 0.009 mg/mL). The histological studies found liver tissue injury in group 2 and biochemical examinations indicated significantly lower ($P < 0.05$) total protein content (0.205 $\pm$ 0.002 mg/g.W.t) and superoxide dismutase (42.11 $\pm$ 0.18 U protein/min) enzyme tissue activity than group 1 (TP: 0.236 $\pm$ 0.003 mg/g.W.t and SOD: 62.22 $\pm$ 0.90). In addition, the levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were significantly higher ($P < 0.05$) than group 1. Also, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) serum enzyme levels (314.33 IU/L) were significantly higher ($P < 0.05$) in alcohol-treated animals than in group 1 (152.33 IU/L). However, these injuries were remarkably lower ($P < 0.05$) in animals treated by the extract (group 3).
Conclusions: The results demonstrated the strong pharmaceutical activity of *S. grossheimii* extract to apply as a new antioxidant agent, especially for the treatment of fatty liver.

Keywords: Antioxidants, Fatty Liver, Hydrogen Peroxide, Malondialdehyde, Oxidative Stress, Phytochemicals, Rats, *Salvia grossheimii* SOSN, Wistar

1. Background

Alcohol drinks are being used widely in the world. Chronic alcohol consumption can induce oxidative stress and affect several organs of which, the liver is the primary target leading to social problems (Figure 1) (1-3). Alcohol-induced hepatotoxicity can cause the generation of free radicals such as reactive oxygen species (ROS), which in association with the cytochrome P_{450} (CYP2E1) enzyme can affect lipid metabolisms such as triglyceride (TG) and malondialdehyde (MDA) as oxidative stress markers (1-4). Other studies suggested a mechanism for alcohol-induced hepatotoxicity via changing gene expression related to cytokines as inflammatory factors such as Interleukin 6 (IL-6), Interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) (5-7). However, there are no agents or drugs to protect the liver and decrease the speed of alcohol-induced hepatotoxicity progression (8).

Phytochemicals as antioxidant agents are plant-based compounds that act as free radical scavengers to prevent oxidative stress injuries in the body (Figure 1) (6, 7, 9). Several studies showed that alcohol hepatotoxicity can be decreased by using antioxidants including vitamins A, C, and E (6), pentoxifylline as an inhibitor of TNF-α, anti-inflammatory and other cytokines (7), glutathione, epigallocatechin-3-gallate (6), N-acetyl-L-cysteine (7), methanolic and aqueous extracts of *Acorus calamus*, *Vitis vinifera*, and *Trigonella foenum graecum* (6, 10), and

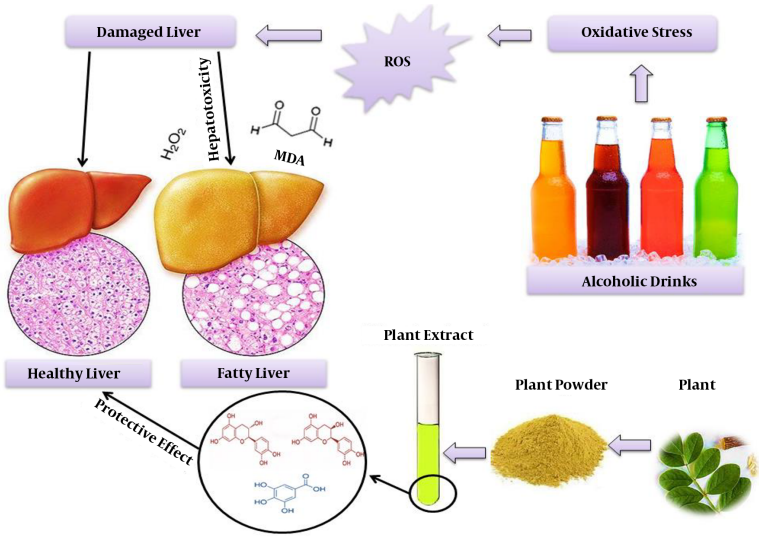

Figure 1. Graphical abstract. The schematic diagram representing the biological activity of *Salvia grossheimii*

quercetin (11).

The genus *Salvia* L. has about 900 species, 58 of which are found in Iran (12, 13). The results of recent studies showed that the chemical constituents of *Salvia* species mainly included phenolic compounds, sterols, and terpenoids. These metabolites showed high pharmaceutical potential such as antimicrobial and antiradical activities (14, 15). In this study, the degree of liver tissue damage and the protective potential of the extract against fatty liver were observed by histological and biochemical examinations, including H_2O_2, MDA, AST, ALT, and SOD in tissue and serum.

2. Objectives

This experimental study aimed to determine the antioxidant potential of *S. grossheimii* extract and its protective effect against changes in histological and biochemical factors of the liver following alcohol consumption.

3. Methods

3.1. Plant Material and Extract Preparation

The *S. grossheimii* plant was collected randomly from Qazvin province, Iran, on 25 Feb 2016. The voucher specimens were deposited at the Bu-Ali Sina University Herbarium (BASU), Hamedan, Iran. The aerial parts of the plant were powdered and then extracted in absolute methanol by a Soxhlet apparatus. In the end, the extract was dried by a rotary evaporator (Lab Tech, Ev 311, Italy).

3.2. Determination of Total Phenol and Flavonoid Contents

Briefly, 0.5 mL of the plant extract (1:10 g/mL) or a standard (gallic acid) was mixed with 5 mL of Folin Ciocalteu reagent (1:10 in distilled water) and 4 mL of 1 M Na_2CO_3 (16). For the determination of flavonoid content, 0.5 mL of the extract (1:10 g/mL) or a standard (quercetin) was mixed with 1.5 mL of methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1M KCH_3COO, and 2.8 mL of distilled water (17). The total phenol and flavonoid contents of samples were determined at 765 and 415 nm, respectively, by a double-beam Perkin Elmer UV/visible spectrophotometer (USA).

3.3. Biological Activity

3.3.1. Antiradical Capacity

The extract was dissolved in absolute methanol (0.2 - 1 mg/mL) and mixed with a DPPH solution (0.3 mM). The absorbance of samples was measured at 517 nm, and the antiradical potential (AA% and IC_{50}) was calculated (18):

$$AA\ (\%) = [1\,(A_s - A_b)\,/A_c\,] \times 100 \quad (1)$$

In the above formula, as is the absorbance of the extract (2.5 mL) and 1 ml DPPH, Ab is the absorbance of the reference (containing the extract and methanol), and Ac is the absorbance of the control sample (containing DPPH and methanol). Ascorbic acid was employed as control and IC_{50} was calculated.

3.3.2. Animals and Experimental Design

This experimental study was performed in Iran in 2016 - 2017. A group of 22 male Wistar rats (150 - 200 g) were obtained from the Animal House of Bu Ali Sina University, Hamadan, Iran and they were used and housed in an air-conditioned room (22 ± 2°C and 12:12 h light: dark cycle). The sample size was determined based on Morgan's table. In this study, animals were treated through intragastric administration. Rats were divided into three different groups (each containing six animals), as follows: group 1 as the control group received 1 mL water daily, group 2 (alcohol group) received 1 mL alcohol (40%) daily, and group 3 received 1 mL extract (500 mg/kg) and alcohol daily (alcohol + extract) at 4 h intervals. In the end, all rats were anesthetized and sacrificed. Then, the liver tissues of animals were excised and transferred to formalin (10%) for histological studies. The study was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences with the code of IR.BMSU.REC.1396.694.

3.3.3. Histological Analyses

The livers were fixed in formalin (10%) for 24 h. Then, the tissues were dehydrated with alcohol and embedded in paraffin. Sections from the liver tissue of about 4-5 μm were stained by hematoxylin-eosin. The prepared sections were photographed by a microscope (BX-51 Olympus, Nagano, Japan) and assessed for some factors such as fatty changes (known as microvesicular steatosis) and inflammatory cells.

3.3.4. Biochemical Analyses

3.3.4.1. Tissue Enzyme Extraction

Briefly, 0.2 g of frozen liver tissue (-70°C) was powdered (in liquid nitrogen) and then homogenized in 1.5 mL extraction buffer (including 50 mM Phosphate Buffer Saline (PBS), pH: 7.8, 0.1 mM EDTA, and 0.3% Polyvinylpolypyrrolidone (PVPP)). After centrifugation (4°C, 12000 rpm, 15 min), the supernatant was used to analyze the enzyme activity.

3.3.4.2. Determination of Tissue Total Protein Content

The total protein content was assessed by Bradford's assay using bovine serum albumin as the standard (19).

3.3.4.3. Determination of Superoxide Dismutase Activity

Briefly, 1.5 mL reaction mixture contained PBS (50 mM, pH = 7.8), EDTA (0.1 mM), nitro blue tetrazolium (NBT, 75 mM), riboflavin (2 mM), methionine (13 mM), and the enzyme extract. Samples were incubated for 25 min in light (30-watt bulb). The blank contained a reaction mixture without light exposure (20). The absorbance of samples was recorded at 560 nm. The SOD enzyme activity was calculated using the following formula:

$$Activity\ (U\ protein/\ min) = (A_s - A_c)/A_c \times 100 \quad (2)$$

Here, as is the absorbance of the sample containing reaction mixture reference with enzyme extract (100 μL) and Ac is the absorbance of the control (reaction mixture reference).

3.3.4.4. Determination of Liver Enzymatic Markers in Plasma

Briefly, blood samples were taken from the heart of rats and plasma was obtained by centrifugation. The plasma levels of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) (liver enzymes) were examined by commercial kits (Pars Azmon, Iran).

3.3.4.5. Measurement of Lipid Peroxidation

Briefly, 0.2 g liver tissue was extracted in 5 mL (w/v) trichloroacetic acid (TCA, 0.1%) solution and centrifuged at 10000 rpm for 10 min (21). Then, 4 mL TCA solution (20%) containing thiobarbituric acid (TBA, 0.5%) was added to 1 mL of the extract. The reaction mixture was heated in a water bath at 95°C for 15 min, and then, the reaction stopped immediately by cooling down samples in an ice water bath. The samples were centrifuged again (10000 rpm, 10 min), and the absorbance was read at 532 nm.

3.3.4.6. Determination of H_2O_2 Content

The H_2O_2 level was assessed in a way similar to the MDA assay in the same centrifugation condition (10000 rpm, 10 min) (22). Then, 0.5 mL phosphate buffer (pH = 7.0) and 1 mL KI were added to the extract (0.5 mL), and the absorbance was read at 390 nm.

3.4. Statistical Analysis

Analyses were performed through Simple Random Sample by SPSS Statistics for Windows, version 16.0 (SPSS Corp., Chicago, Ill, USA) using the Duncan method (P value < 0.5 and ± SD). All apparatuses were calibrated to generate precise data. Also, all experimental steps were conducted by a professional investigator. In this research, a numerical scale was used to measure the variables.

4. Results

4.1. Total Phenolic Contents and Antiradical Activity

In this study, the analysis of secondary metabolites of *S. grossheimii* leaf and stem (aerial parts) extracts showed a high total phenol content (4 to 11 mg/g DW). Also, the extracts were found to be rich in flavonoids with remarkable differences in two parts ($P < 0.05$) (Table 1). The results showed that the antiradical activity of both extracts was more than that of ascorbic acid as a standard antioxidant. The free radical scavenging potential was as follows: stem extract (IC_{50}: 0.102 ± 0.002 mg/mL) ≥ leaf extract (IC_{50}: 0.106 ± 0.003 mg/mL) > ascorbic acid (IC_{50}: 0.162 ± 0.002 mg/mL) (Table 1).

Table 1. Total Phenol and Flavonoid Content and Antioxidant Activity of Extracts[a, b]

Sample	Total Phenol Content, mg/g.D.W	Total Flavonoid Content, mg/g.D.W	DPPH Free Radical Scavenging	
			IC_{50}, mg/mL	Mean, %
S. grossheimii				
Leaf	10.11 ± 1.32^{A}	3.42 ± 0.51^{A}	0.102 ± 0.002^{A}	94.88
Stem	4.22 ± 0.77^{B}	4.44 ± 0.23^{B}	0.106 ± 0.003^{B}	90.11
Ascorbic acid	-	-	0.162 ± 0.009^{C}	86.23

[a]Values are expressed as mean ± SD.
[b]Values in each column with different superscripts are significantly different ($P < 0.05$).

4.2. Hepatotoxicity Assay

4.2.1. Histological Examination

The liver sections from various studied groups were used to assess the histopathological changes (Figure 2). The results showed normal liver cells (white arrows in Figure 2A) in the control group, while microvesicular steatosis and inflammatory cells (blue and yellow arrows, respectively, in Figure 2B) were observed in the alcohol group. The microvesicular steatosis was detected because of fatty changes in hepatocytes. The use of the extract, along with alcohol, showed the potential of hepatocytes recovery with minimal microvesicular steatosis (Figure 2C). These results indicated that treatment by the extract protected against alcohol-induced hepatotoxicity and fatty liver in rats.

4.2.2. Biochemical Assays of Liver Tissue

The total protein content and SOD enzyme activity were significantly lower ($P < 0.05$) in group 2 than in group 3 and group 1 (control group). However, group 1 and group 2 showed no significant differences in the total protein content and SOD activity ($P < 0.05$). Other results indicated that group 2 had significantly higher ($P < 0.05$) H_2O_2 and MDA levels than groups 1 and 3 (Table 2). Also, serum biochemical analysis showed that ALT, AST, and ALP levels were higher in group 2, which could be because of hepatotoxicity. These higher levels were due to the hepatocyte damage induced by alcohol that confirms our histological studies. The administration of the extract prevented the alcohol-induced elevation of tissue MDA and H_2O_2 content and serum ALT, AST, and ALP levels. However, it caused a decrease in SOD and total protein tissue levels in studied groups.

Experiments were performed in triplicate and expressed as mean ± SD. Values in each column with different superscripts are significantly different ($P < 0.05$).

5. Discussion

The application of polyphenols, especially flavonoids, in the treatment of liver damage induced by alcohol has been extensively studied (3-16). A wide spectrum of polyphenols has shown pharmaceutical effects on hepatotoxicity induced by different toxins in animal models. Recent studies indicated that the underlying mechanism mainly involves enhancing the antioxidant defense enzymes by mediating nuclear factor expression such as the CYP2E1 enzyme and alleviating tissue inflammation (1-4). As a natural flavonoid, quercetin has a remarkable protection property against liver free radical-induced damages via antioxidant and anti-inflammation defense system. The main mechanism was the reduction in hepatic inflammatory cytokines such as IL-6 and IL-1β through a chain of reactions (23). Puerarin, as another natural flavonoid has the ability to reduce the ROS content, improving the antioxidant defense enzyme system and regulating hepatic lipid metabolism gene expression (24). Dieckol, as a polyphenol was found to act against liver free radical-induced injury in animals by mediating apoptosis-regulating genes (25-27).

Drug-induced hepatotoxicity is a remarkable clinical issue. As an example, acetaminophen is a known intrinsic hepatotoxic agent at high doses. Baicalin as an important flavonoid can decrease drug-induced hepatotoxicity by reducing effector gene expression in tissue inflammation. In this study, the leaf extract had higher total phenol content; however, the studied extracts were not significantly different ($P < 0.05$) in total flavonoid content (Table 1). In other studies, among antiradical agents, plant phenolic compounds were more potent than ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E) on a molar basis as antioxidant standards (28). In agreement with other studies, our study showed that the antiradical potential of both stem and leaf extracts was higher than that of vitamin C (Table 1).

Blood alcohol level increases after the consumption of ethanol, leading to changes in the behavior of animals. The liver is the first and the most important organ that detoxifies xenobiotics with the property of liver damage induction such as alcohol (29). The basic mechanisms of alcohol-induced hepatotoxicity include histological damages, including liver cell steatosis and necrosis, as well as changes in the level of serum factors related to liver function, including Triglycerides (TG), MDA, H_2O_2, and GSH.

Table 2. Results of Biochemical Assay of Liver Tissue in Different Groups of Rats[a, b]

Factors	Ethanol	Ethanol + Extract	Control
Protein, mg/g.W.t	0.205 ± 0.002^{A}	0.234 ± 0.006^{B}	0.236 ± 0.003^{B}
ALT, IU/L	146 ± 0.7^{A}	133 ± 1.0^{B}	100 ± 0.09^{C}
AST, IU/L	76 ± 4.3^{A}	49 ± 1.5^{B}	32 ± 1.4^{C}
ALP, IU/L	721 ± 10.6^{A}	611 ± 9.2^{B}	325 ± 5.4^{C}
SOD, U protein/min	42.11 ± 0.18^{A}	65.46 ± 0.46^{B}	62.22 ± 0.90^{B}
MDA, mg/g.W.t	0.122 ± 0.004^{A}	0.085 ± 0.003^{B}	0.076 ± 0.009^{B}
H_2O_2, μM/g.W.t	0.67 ± 0.07^{A}	0.18 ± 0.08^{B}	0.16 ± 0.01^{B}

[a] Values are expressed as mean ± SD.
[b] Values in each column with different superscripts are significantly different ($P < 0.05$).

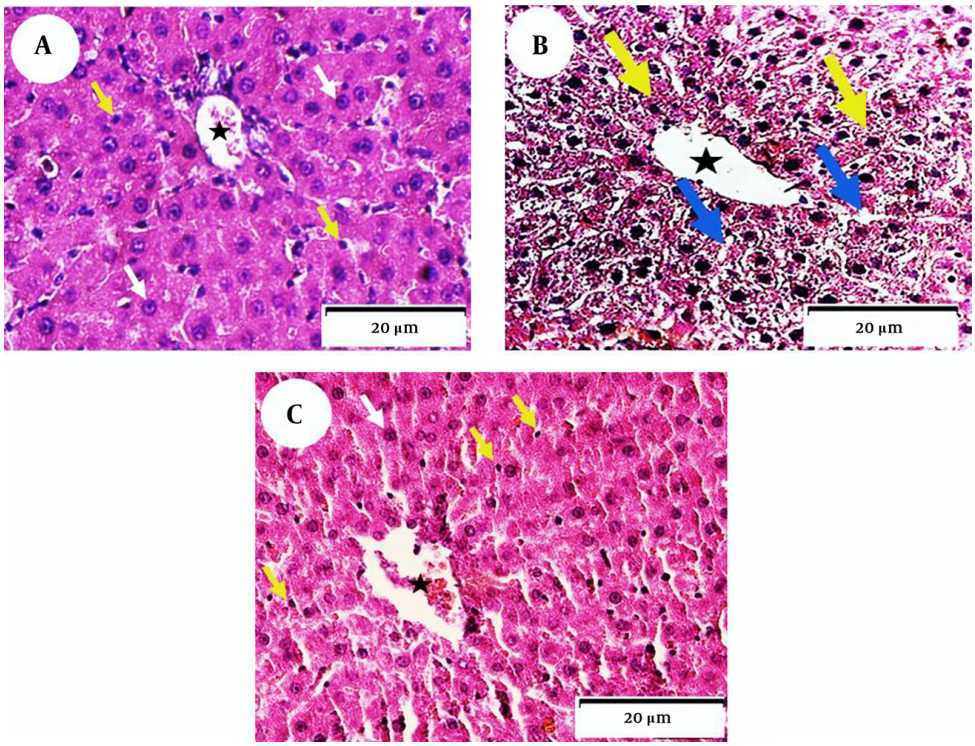

Figure 2. Liver histological changes after treatment. A, Control group; B, ethanol group showing central venous (star) and inflammatory cells (yellow arrows) and microvesicular steatosis (blue arrows); C, ethanol + plant extract group showing inflammatory cells (yellow arrows) and normal hepatocytes (white arrows)

Also, serum levels of ALT, ALP, and AST are very important factors used to detect liver disease (30). Previous studies showed that plant extracts such as green tea extract normalized these changes in blood alcohol levels and biochemical and histological liver factors (31-34).

In our study, the antioxidant system of alcohol-treated rats (group 2) was severely impaired, causing a high content of tissue MDA and H_2O_2 (Table 2). The high amounts of these compounds were because of a significant decrease in the antioxidant enzyme system. The low amount of SOD as an antioxidant enzyme causes a high risk of liver cell injury. In this study, the level of SOD was higher in group 3 than in group 2 (Table 2). As a protective action against injuries induced by a toxic substance like alcohol, antioxidant agents can recover hepatocytes to normal conditions and make them able to increase the SOD enzyme level (32-34). Our results also proved the protective activity of plant extracts against alcohol-induced hepatotoxicity (Figure 2 and Table 2). The histological study of rats showed that the studied extract had protective effects against hepatotoxicity (Figure 2). Overall, biochemical findings were supported by observing liver sections histopathologically. As

a result, in agreement with previous studies of plant extracts, *Salvia grossheimii* methanolic extract showed pharmaceutical properties and could prevent the progress of fatty liver induced by oxidative stress.

5.1. Conclusions

Although our study had limitations, the results demonstrated that *Salvia grossheimii* methanolic extract contained important metabolites with pharmaceutical activities. Therefore, as a plant therapeutic agent, it can help prevent the progression of various diseases such as fatty liver induced by oxidative stress.

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Footnotes

Authors' Contribution: Study concept and design: Seyed Morteza Hosseini, Mostafa Asadbegy, Roya Karamian and Siamak Yari. Conducting the experiments: Mostafa Asadbegy. Data analysis and interpretation of results: Mostafa Asadbegy and Siamak Yari. Drafting of the manuscript: Mostafa Asadbegy, Seyed Morteza Hosseini, and Siamak Yari. Supervising of the study: Seyed Morteza Hosseini and Roya Karamian. All authors revised the final manuscript.

Conflict of Interests: Authors had no conflicts of interest.

Ethical Approval: The Ethics Committee of Baqiyatallah University of Medical Sciences approved the study with the code of IR.BMSU.REC.1396.694.

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