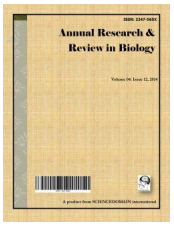

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Pathological Overview and Antioxidant Status of Thyme on H_2O_2-induced Spleen Tissues Damage

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Authors' contributions

This work was carried out in collaboration between both authors. Author GF designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GF and LA managed the analyses of the study. Author GF managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Objectives: To study the preventive effect of *Thymus algeriensis* essential oil (TAS) against hydrogen peroxide (H_2O_2)-induced spleen toxicity in rats.
Materials and Methods: Rats were treated with Hydrophobic fractions of *Thymus algeriensis* (180 mg/kg body weight, n=6), H_2O_2 (0.1, 1 mmol/L body weight, n=6) and the exposure to both drugs orally for 15 days. Histological examination was performed and the levels of biochemical parameters and lipid peroxides were determined.
Results: In spleen tissue protein, catalase, superoxide dismutase, and glutathione (GST, GPx and GSH) levels were increased significantly (P<0.05) in the essential oil pretreated rats when compared to H_2O_2. TAS decreased the intracellular malondialdehyde (MDA) levels in spleen tissues. Vascular congestion was seen in spleen of high dose H_2O_2-treated rats and normal architecture of tissues was observed in other groups.
Conclusion: The biochemical parameters and histopathology examination support the cytoprotective effect of Thyme which could be attributed to terpenes.

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Keywords: H_2O_2; *Thymus algeriensis*; *spleen; MDA; antioxidant enzymes; non-enzymatic antioxidants.*

ABBREVIATIONS

CAT : Catalase
GPx : Glutathione peroxidise
GSH : Reduced glutathione
GST : Glutathione s transferase
MDA : Malondialdehyde
ROS : Reactive oxygen species
SOD : Superoxide dismutase
TBARS : Thiobarbituric acid reactive substances

1. INTRODUCTION

A number of environmental stressors and stimulants induce reactive oxygen species (ROS) generation inside a cell. However, built‑in endogenous defense system (antioxidant enzymes) neutralizes the ROS, and normal cells perform their function uninterrupted [1]. Oxidative stress, characterized by a cellular imbalance in the production and elimination of ROS, plays an important role in the pathogenesis [2] of various spleen disorders. It can induce radical mediated damage to cellular biomembranes resulting in lipid peroxidation, which converts unsaturated lipids into polar lipid hydroperoxides [3].

The spleen is the largest secondary lymphoid organ and contains one-fourth of the total lymphocytes and it plays an important role in maintaining immune homeostasis [4]. The toxicity of spleen tissue by any kind of chemicals will damage all the immune system. Therefore, the search for anti-toxicity drugs is an important task, which could have a positive effect on patients after radiation therapy. Natural products have the advantages of low toxicity, wide effects and so on. They can be applied to many target organs [5] and can reduce tissue damage. Therefore, it is important to search for high efficiency, low toxicity, and protective agents from natural products.

Protection against free radicals can be enhanced by ample intake of dietary antioxidants. Substantial evidence indicates that foods containing antioxidant nutrients may be of major importance in disease prevention [6]. The therapeutic efficacy of many natural plants has already been described by practitioners of traditional medicine for several disorders [7]. *Thymus hirtus sp.algeriensis* belongs to Lamiaceae family. This specie is widely distributed in the Mediterranean countries. It has been recorded in traditional medicine of the city of Gafsa, Tunisia. A toxicological assessment of hydrophobic fraction of *T. algeriensis* has been undertaken, which found *T. algeriensis* has good edible-safety properties that means it can be widely used as a natural food plant-essential oil. Previous reports indicated that T. *algeriensis* essential oil (EO) could be used as free radical scavengers, with anti-oxidation and anti-cytotoxicity efficiency [8,9]. Most of EOs have been first identified and used for the treatment of inflammatory and oxidative diseases [10].

To the best of our knowledge, this study is the first to investigate the protective effects of TAS on spleen in rats after oxidation induced by H_2O_2 and to elucidate this mechanism by underlying the antioxidant status and the histological overview.

The aim of this report is to outline the toxicity elicited by H_2O_2 to rat tissues and the effect of TAS essential oil as a potential agent for protecting the spleen, and this was assessed by antioxidant and non-antioxidant enzyme. Furthermore, the effectiveness of TAS was carried out by histopathology changes.

2. MATERIALS AND METHODS

2.1 Reagents

All reagents, including H_2O_2, 2,4-dinitrochlorobenzene (CDNB), Ellman's reagent, thiobarbituric acid (TBA), Tris-HCl buffer, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), bovine serum albumin (BSA), and GSH were purchased from Merck (Nottingham, UK), Sigma (St. Louis, MO, USA), and Fluka Chemie (Buchs, Switzerland).

2.2 Extraction and Analysis of Hydrophobic Fraction of *T. algeriensis* by Gaz Chromatography-Mass Spectrometry (GC/MS)

Two hundred fifty g of aerial parts of *T.algeriensis* collected from the Mount Orbata, Gafsa-Tunisia, were extracted with 500 mL distilled water. The procedure was conducted in a Clevenger apparatus for 6 hours. The essential oil extracted from *T. algeriensis* was preserved at 4°C in the dark, ready for *in vivo* experimental procedures. GC/MS analysis, used to profile

Thymus essential oil (Fig. 1), was carried out on an Agilent model 5975 C. One microliter of samples, diluted in 10% hexane, was subjected to the apparatus. Gaz chromatography analysis was carried out on a model 7890 A gaz chromatograph, with a flame ionization detector (FID) and a split ratio of 1:50 using a fused silica capillary column, HP5-MS (30 m × 250 µm i.d., 0.25 µm film thickness). Temperature for analysis was 250°C, and helium was the carrier gaz, with a flow rate 0.8 ml/min.

2.3 Animals and Experimental Procedures

A total of 36 6–8 week old male Sprague Dawley rats (165 ± 4 g) were provided by the animal laboratory of Pasteur Institute of Tunis, Tunisia (Ethic# LNSP/Pro 152012). Rats were maintained for a week at 20 ± 25 °C with relative humidity of 55% ± 10% under a cycle of 12 h light/dark. Rats were allowed ad libitum to access to tap water and food pellets. Rats were randomly divided into six groups with six rats housed per cage and received chemicals with an oral dose for 15 consecutive days: control group, Hydrogen peroxide low dose (LD H_2O_2) (0.1 mmol/L), Hydrogen peroxide high dose (HD H_2O_2) (1 mmol/L), TAS (180 mg/kg, body weight/day dissolved in normal saline), TAS combined with Hydrogen peroxide (TAS + LD H_2O_2) (180 mg/kg, body weight/day and 0.1 mmol/L, respectively), and TAS combined with Hydrogen peroxide (TAS + HD H_2O_2) (180 mg/kg, body weight/day and 1 mmol/L, respectively). In these two latter groups receiving hydrogen peroxide and essential oil, rats were treated with TAS 1 h prior to H_2O_2 administration in animals. After experiments, rats were killed by cervical dislocation and the spleen collected from different groups was dissected. A portion of the spleen tissue were fixed in buffered formaldehyde (10%) for histological process. A part of the spleen were extracted in phosphate buffer (0.1 mol/L, pH = 7.4) with rotary homogenizer. After centrifugation (8000 × g for 15 min), spleen samples obtained from all groups were used to evaluate the antioxidant enzyme (GSH, GPx, GST, CAT, and SOD) and MDA levels.

2.4 Protein Determination

Total protein content of spleen samples was estimated by the method of Lowry et al. [11]. Assays were done in triplicate.

2.5 MDA Estimation

TBARS assay was carried out to evaluate lipid peroxide content of spleen tissues. Thiobarbituric acid (TBA) reaction was described by Ohkawa [12] and the lipid peroxide concentration was expressed as nmol MDA/mg protein. Assays were done in triplicate.

2.6 Biochemical Parameters

Enzymatic and non-enzymatic activities were assessed as described previously. CAT was assayed according to the method of Takahara et al. [13]. SOD was determined by the method of Marklund et al. [14]. GPx was estimated according to the method of Hafeman et al. [15]. GST was assayed using the method of Habig et al. [16]. GSH content were determined by using the method of Sedlak and Lindsay [17]. Assays were done in triplicate.

2.7 Histology Assessment

Histopathological overview was carry out using spleen tissues from different groups. Ten percent of neutral buffered formalin solution was used to fix tissue samples rinsed with saline solution (0.9%). After that, spleen sections (4 µm thickness) were prepared and embedded in paraffin blocks and tissues were stained with hematoxylin and eosin (H&E) stains and photographed with light microscopy (Nikon Optiphot 2, Tokyo, Japan). It was carried out from Service of Anatomo-Pathology of Menzel Bourguiba, Bizerte, Tunisia. Damage scoring system was assessed as follow: 0: no damage, 3: mild, 5: moderate, and 10: severe damage.

2.8 Statistical Analysis

Values are presented as mean ± SEM (n=3 independent experiments for each bar). Statistical significance between treated groups and control group was tested using one-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparison or Dunnett's multiple range test. Differences were considered to be statistically significant when $P < 0.05$.

3. RESULTS

3.1 Behavior and Body Weight

During the experimental procedures, no death was observed and rats treated with essential oil

shown a normal behaviour in comparison to the control group, whereas, for H_2O_2-treated rats, they showed decreased physical activity, drinking water and eating food than control group.

There was a significant (P < 0.05) difference between the control rats and treated-groups in body weight gain. In this report, it was found that there was body weight loss of toxic rats when compared to the control group. After 2 weeks of experiment, body weights of H_2O_2-treated rats were significantly lower than that of rats treated with TAS (Fig. 2).

3.2 Total Proteins in Spleen Tissues

As shown in Fig. 3A, we noted a significant decrease in the amount of protein of spleen tissue in H_2O_2-treated group. Whereas, TAS significantly increased total amount of protein.

3.3 TBARS and Enzymatic Antioxidant Levels

Fig. 3B shows that rats treated with TAS showed lower lipid peroxidation levels as compared to the group administered with H_2O_2. Furthermore, a dose-dependent decrease (P<0.05) in the cellular SOD, GST, CAT and GPx content following exposure of the spleen to H_2O_2 was observed suggesting oxidative stress. Exposure to TAS reversed H_2O_2-induced alterations of antioxidant defense enzyme activities. In addition, increased levels of GSH content was observed in TAS-treated rats and was significantly (P<0.05) higher that H_2O_2-treated groups (Fig. 3C, D, E, F, G).

3.4 Microscopical Examinations

Exposure of rat to the stress of H_2O_2 induced spleen atrophy evidenced by vascular congestion in H_2O_2-treated group (Fig. 3Hi). The H_2O_2-induced histopathological alterations were decreased with TAS administration. Notably, histopathological analysis showed that TAS markedly alleviated vascular congestion in the spleen tissues and normal morphological appearances were detected. The semi-quantitative analysis of histologic injury showed a significant decrease in the score damage of the spleen tissue of experimental group treated with both H_2O_2 and TAS when compared to H_2O_2 group (Fig. 3Hii).

4. DISCUSSION

Spleen is the largest lymphoid tissue, bean shaped organ for filtering blood. It plays an important role in the body such as formation of blood and removal of the old and ineffective cells and allows only young active cells to pass into circulation. It is also involved in the iron metabolism and reacts against infection [6].

The present report has shown that H_2O_2 induces lipid peroxidation and decreases the levels of enzymatic and non-enzymatic status in the spleen tissues. The loss in body weight in H_2O_2-

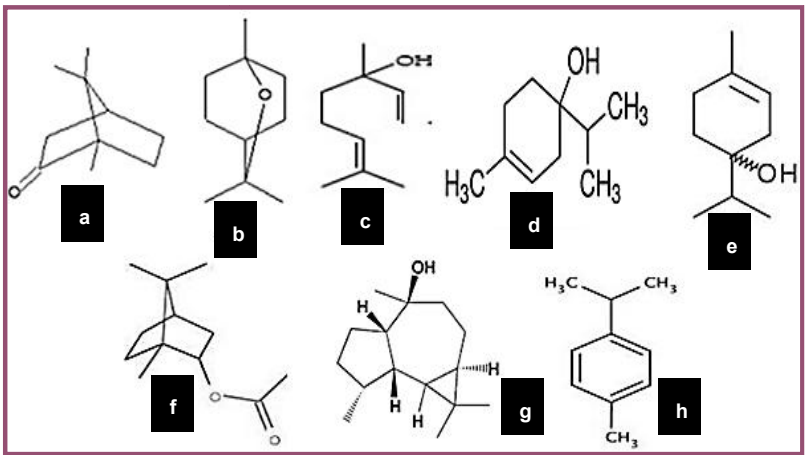

Fig. 1. The chemical structure of Campher, (a) 1,8-Cineol, (b) Linalol, (c) 4-Carvomenthenol, (d) Terpinen-4-ol, (e) Bornyl acetate, (f) Viridiflorol, (g) p-cymen, (h) Terpene present in *T. algeriensis*

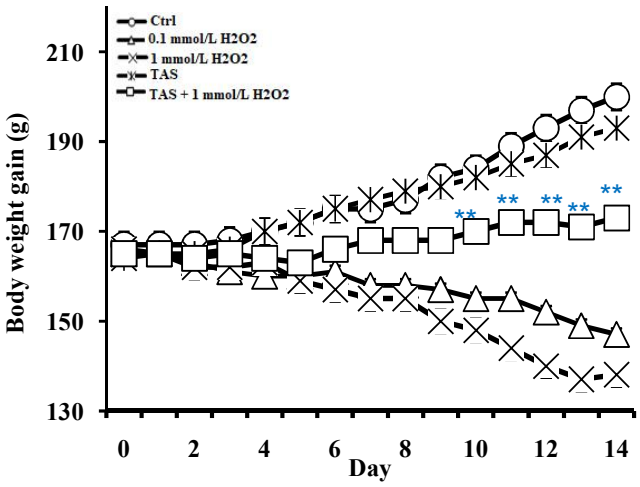

Fig. 2. Effect of TAS on body weight in rats. Ctrl: Control, TAS: *Thymus algeriensis*
Mean values of 3 independent experiments have been plotted
***Significant Value was at P<0.05*

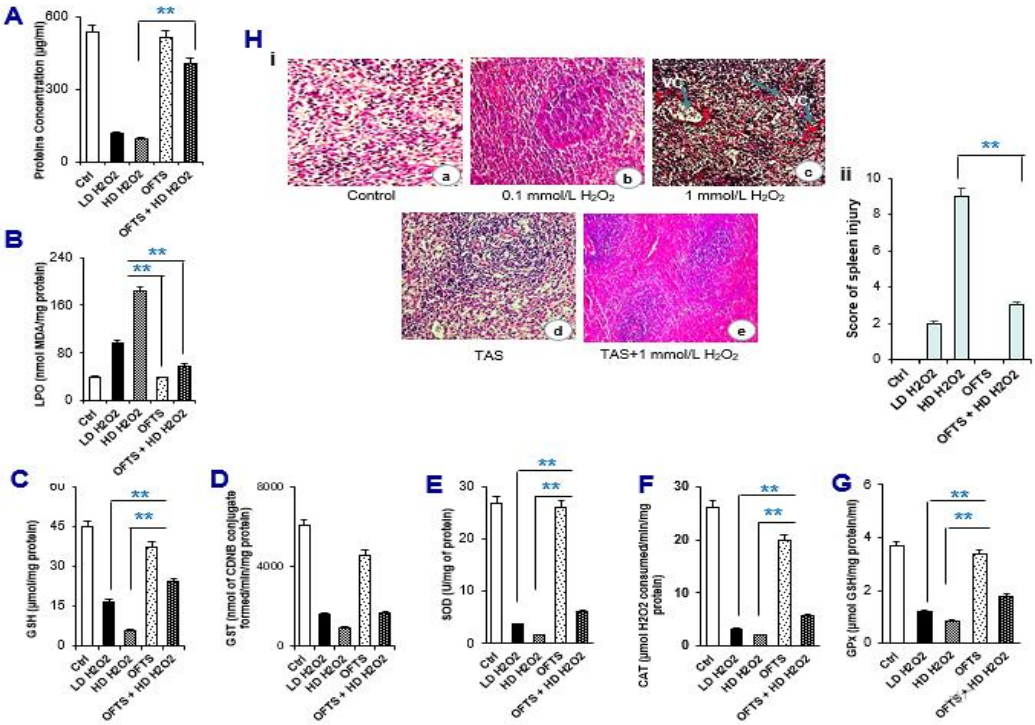

Fig. 3. A. Variations of Lipid peroxidation. B. Protein content of spleen tissues. C, D, E, F, G. Variations of GSH, GST, SOD, CAT, and GPX activities, respectively, in spleen of different groups after treatment with essential oil and hydrogen peroxide. H. Photomicrograph of the spleen muscle tissues of normal control (a) and experimental group (b, c, d, e) of rats (i) and semi-quantitative analyses of H&E staining results (ii). Arrows were used to indicate Vascular congestion (VC). Magnification: A, C, D, and E, ×40; B, ×20
*Data are represented as x ± s (n=6); **Significant Value was at P<0.05*

treated rats is mainly due to increased muscular wasting on loss of tissue protein. Our data demonstrated that pre-treatment of TAS orally significantly reduced the lipid peroxidation and

increased the levels of GST, CAT, GPx, SOD, and GSH. In accordance with our finding, another report showed that GSH decreased to less than half of the control level at 2 and 5 h after incubation with H_2O_2 [18].

These results indicate the protective effect of TAS on H_2O_2-induced spleen toxicity by scavenging free radicals. The depletion of cellular GSH may be due to disturbance of Ca^{2+} influx and to the lipid peroxidation. The H_2O_2 downregulation of GSH may be more important for apoptosis than H_2O_2 induction of lipid peroxidation, and the H_2O_2 induced changes in redox status of the cell may be among the original events which lead up to other biochemical changes [19]. MDA is correlated to pathological conditions or stress including aging [20]. The excess MDA produced as a result of tissue injury can combine with free amino groups of proteins (MDA reacts mainly with Lys residues by Michael addition), producing MDA-modified protein adducts. Modification of proteins by MDA could conceivably alter their biological properties [21].

Pathological examinations of spleen following both exposure to H_2O_2 and TAS showed reduced spleen cytoxicity compared to experimental group treated only with H_2O_2, where we noticed potential toxic reactions appeared with spleen infiltration and vascular congestion. In another report, H_2O_2 affected the mitochondrial function (negatively) and apoptosis (positively) of the human spleen cells in a dose- and time-dependent manner. The apoptotic rates were significantly different between different groups (Total: 55.01±9.11%, 44.07±9.00%, 30.20±6.75% and 9.97±1.68% for 100, 50, 25 µmol/L and control group respectively) [22]. Intriguingly, Han et al. [22] showed that a transient H_2O_2 insult (5-min exposure to 400 µM H_2O_2) did not cause cell death in its presence but triggered a delayed time-dependent increase in apoptosis after H_2O_2 had been withdrawn.

5. CONCLUSIONS

This work elucidates the therapeutic effects of terpenes extracted from *Thymus algeriensis* in spleen toxicity. This specie has a new application and protect spleen tissues from oxidation induced by hydrogen peroxide and appears to have anti-inflammatory potential which have often been linked to their ability to act as antioxidants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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