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**Title** ANTISTEATOTIC AND ANTIOXIDANT ACTIVITIES OF THYMBRA SPICATA EXTRACTS IN HEPATIC AND ENDOTHELIAL CELLS AS IN VITRO MODELS OF NON-ALCOHOLIC FATTY LIVER DISEASE

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# ANTISTEATOTIC AND ANTIOXIDANT ACTIVITIES OF *THYMBRA SPICATA* EXTRACTS IN HEPATIC AND ENDOTHELIAL CELLS AS *IN VITRO* MODELS OF NON-ALCOHOLIC FATTY LIVER DISEASE

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**KEYWORDS:** Non-alcoholic fatty liver disease; *Thymbra spicata*; phenolic compounds; steatosis; oxidative stress.

**ABSTRACT**

***Purpose.*** *Thymbra spicata,* a plant of traditional medicine in Lebanon, is rich in polyphenols. Our aim was to verify if *T. spicata* extracts may represent possible nutraceuticals for treatment of the non-alcoholic fatty liver disease (NAFLD), a common liver disease for whom no ideal pharmacological treatments are available.

***Methods.*** Two different extracts from *T. spicata* aerial parts were prepared using water (TW) or ethanol (TE) as solvent. Both extracts were tested for their lipid-lowering and antioxidant effects on cultured hepatic and endothelial cells treated to mimic *in vitro* the NAFLD. Different parameters were evaluated being related to lipid accumulation, and oxidative stress.

***Results.*** Both the total phenolic and the total flavonoid contents were higher in the ethanolic extract. Rosmarinic acid was the most abundant polyphenol in TW, while TE was richer in monoterpenic phenols and flavonoids, carvacrol especially. Our findings demonstrate that both extracts ameliorated lipid accumulation, oxidative stress and inflammation in these models. However, the aqueous extract was more effective to reduce hepatic steatosis and to accelerate the wound repair, while the ethanolic extract had higher anti-oxidant potential.

***Conclusions.*** *T. spicata* extracts could be a promising natural product for improving hepatic fat mobilization and preventing endothelium damages in NAFLD.

## 1. INTRODUCTION

The number of studies regarding the functional molecules in food which may modulate metabolic processes resulting in health benefits and promotion of well-being has greatly increased [1]. Phenolic compounds (PC) are the most numerous and widely distributed group of functional molecules [2]. Although the antioxidant activity is the most relevant biological function of PC which protect cells against many kinds of "reactive oxygen species" (ROS), other biological actions; such as the hepatoprotective potential, have been reported for some of them [3].

Plants are a source of natural bioactive compounds that might serve as leads for the development of novel drugs. In Mediterranean area, thyme-like plants such as *Thymbra spicata* are widely diffused; their dry or fresh leaves and flowers are used in salad and tea infusion, and its components find applications in food industry for flavouring and as preservation agents because of their antimicrobial and antifungal activity [4,5]. *T. spicata* is an aromatic plant belonging to the Lamiaceae family; it is reach of PC including phenolic acids (rosmarinic acid), phenolic monoterpenoids (carvacrol, thymol) and flavonoids (both glycosides and aglycones) [6,7]. Leaves of this plant seem to play anti-hypercholesterolaemic, anti-oxidant and anti-steatotic activities in HFD-fed mice [8].

Excess fat intake is associated with body weight gain, which can lead to obesity and other related metabolic disorders including the non-alcoholic fatty liver disease (NAFLD), the most important chronic liver disease in Western countries [9]. Hepatic steatosis, consisting of excess accumulation of triglycerides (TGs) in the liver, is the hallmark of NAFLD, which is also associated to increased production of ROS leading to oxidative stress which contributes to the disease progression [10]. NAFLD may range from simple steatosis to steatohepatitis (NASH) with inflammatory fibrosis, cirrhosis and hepatocellular carcinoma [11]. NAFLD is a multisistemic disease where both hepatocytes and endothelial cells participate in its progression; indeed, metabolic disorders cause endothelial dysfunction which in turn worsen metabolic alterations.

To date, no ideal pharmacological treatment is available for NAFLD, due to potential adverse effects of conventional medical therapies. Therefore, a growing interest in identifying agents for treatment and/or prevention of NAFLD progression has been addressed on studying complementary therapies that employ both natural and safe products such as herbal medicine [12].

Our study focused on investigating the beneficial effects of *T. spicata* extracts being rich of bioactive phenolic compounds. We used rat hepatoma FaO cells exposed to a mixture of oleate/palmitate that represent a reliable *in vitro* model for hepatic steatosis widely employed in previous studies of our group [13,14]. We used also human

endothelial HECV cells exposed to hydrogen peroxide, as an *in vitro* model of endothelium damage, which is typically observed in metabolic syndrome [11]. On these *in vitro* models we assessed the anti-steatotic and anti-oxidant activities of two different extracts from *T. spicata* aerial parts.

## 2. MATERIALS AND METHODS

### *2.1 Chemicals*

All chemicals, unless otherwise indicated, were supplied by Sigma-Aldrich Corp. (Milan, Italy).

### *2.2 Collection of plant material and extraction*

Aerial parts of *Thymbra spicata* were collected from flowering plants growing wild in *"Maarakeh"* south Lebanon (33° 16′ 35.59″ N and 35° 19′ 02.89″). The plant was verified by botanists from the Lebanese University. The aerial parts were left to dry for three weeks at room temperature in the shade. Then, the grinded materials were extracted with two different solvents, ethanol (100%) or distilled water using standard procedures [15]. For aqueous extraction, 15 grams of dried material were dissolved in 250 ml of distilled water, and placed on stirrer for 3 hours at room temperature; and for the last 10 min at 60°C. For ethanolic extraction, 25 grams of dried material were dissolved in 300 ml of pure ethanol and placed on stirrer overnight at room temperature. At the end, both solvents were evaporated in a Rotavac vario power unit (Heidolph Instruments, Schwabach, Germany), and then freeze-dried in Alpha 1-4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany). Then, the dry fractions were stored at 4°C until use.

### *2.3 Total Phenol Quantification*

The total phenol content (TPC) was estimated according to the *Folin-Ciocalteu* method [16]. Briefly, 100 µl aliquots of each extract (1 mg/ml) were mixed with 0.5 ml of *Folin–Ciocalteau* reagent. After 5 min, 1.5 ml of $Na_2CO_3$ 2% (w/v) were added, and the sample was incubated in the dark at room temperature for 30 min. The controls contained all the reaction reagents except the extract. The absorbance was measured at 760 nm using an U-2900 UV-Vis spectrophotometer 200 V (Hitachi High-Technologies, Japan) and compared to a gallic acid calibration curve. The results were expressed in mg of Gallic Acid Equivalents (GAE) per g of dry weight of plant powders.

### *2.4 Total Flavonoid Quantification*

Total flavonoid content (TFC) was estimated by aluminium chloride colorimetric method according to Quettier-deleu et al [17]. Briefly, aliquots of 1 ml of extracts (0.5 mg/ml) were mixed with 1 ml of 2% (w/v) methanolic $AlCl_3$ solution. After incubation at room temperature in darkness for 15 min, the absorbance of all samples was determined at 415 nm using UV-Vis spectrophotometer and compared to a quercetin calibration curve. The blank contained all the reaction reagents except the extract. The results were expressed in mg of Quercetin Equivalent (QE) per g of dry weight of plant powders.

### *2.5 Chromatographic Analyses*

***2.5.1 GC–MS analysis*** Analytical gas chromatography (GC) was carried out using a GC FOCUS SERIES (Thermo Fisher Scientific, Milan-Italy) fitted with a GsBP-5MS capillary column (30 m×0.25 mm), 0.25 um film thickness. Helium was the carrier gas (0.7 ml/min). Column temperature was initially kept at 40°C for 5 min, then gradually increased to 85°C at 5°C/min rate, held for 20 min and finally raised to 300°C at 10°C/min. Aliquots (1 µl) of the diluted samples (1/100 v/v, in methanol) were injected at 250°C, in the splitless mode. The mass spectroscopy (MS) analyses were performed with a ISQ-MASS SPECTROMETER (Thermo Fisher Scientific), the source temperature was 310°C, the transfer-line temperature 320°C, the ionization voltage was 70 eV, and the mass range was 50–400 amu in the full scan mode. The identification and quantification of the different constituents in the extracts were based on the comparison of: (i) their retention indices (RIs), determined relatively to the retention time of n-alkanes (C8–C24) on capillary columns, compared with those reported in the literature or with those measured for pure compounds; (ii) their mass spectra with those listed in the commercial mass spectral libraries NIST, Wiley 275. The relative percentages of the components were calculated based on the GC-FID peak areas without using correction factors.

***2.5.2 HPLC-MS analysis.*** High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) was performed using an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies, Santa Clara, CA, USA). Separation of extracts was performed on a Jupiter C18 column 1 mm × 150 mm with 3.5 µm particle size (Phenomenex, USA). As eluents we used water (eluent A) and MeOH (eluent B), both added with 0.1% formic acid. The gradient employed was: 15% eluent B for 5 min, linear to 100% eluent B in 35 min, and finally hold at 100% eluent B for another 5 min. The flow rate was set to 50 µL/min with a column temperature of 30°C. The injection volume was 8 µL. Ions were detected in the positive and negative ion mode, in the 100–800*m*/*z* range, and ion charged control with a target ion value of 100,000 and an

accumulation time of 300 ms. A capillary voltage of 3300 V, nebulizer pressure of 20 psi, drying gas of 8 L/min, dry temperature of 325°C, and 2 rolling averages (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using an amplitude optimized time by time for each compound. From the chromatograms, the percentage of PC for each extract was calculated on the basis of the peak area.

### *2.6 Radical Scavenging Activity*

The free radical scavenging capacity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [18]. Briefly, 1 ml aliquots of extract at different concentrations were mixed with 1ml of DPPH solution (0.15 mM in ethanol) and incubated in darkness at room temperature for 30 min. Ascorbic acid was used as positive control. At the end, the absorbance at 517 nm was measured by UV-Vis spectrophotometer. Control sample contained all the reaction reagents except the extract. The percentage inhibition values were calculated using the following equation:

$$\% \text{ Scavenging activity} = [(\text{Abs control} - \text{Abs sample})]/ (\text{Abs control})] \times 100$$

and the IC50 values were estimated by a nonlinear regression algorithm (SigmaPlot 2001 version 7.0).

### *2.7 Cell culture and treatments*

FaO cells (European Collection of Authenticated Cell Cultures, Sigma-Aldrich) are a rat hepatoma cell line maintaining hepatocyte-specific markers. Cells were grown in a humidified atmosphere with 5% $CO_2$ at 37°C in Coon's modified Ham's F12 medium supplemented with L-Glutamine and 10% foetal bovine serum (FBS) [11]. HECV cells (Cell Bank and Culture-GMP-IST-Genoa, Italy) are a human endothelial cell line isolated from umbilical vein; they were grown at 37°C with 5% CO2 in in Dulbecco's modified Eagle's medium High Glucose (D-MEM) supplemented with L-Glutamine and 10% FBS. For treatments, cells were grown until 80% confluence, then incubated overnight in serum-free medium with 0.25% bovine serum albumin (BSA). To mimic *in vitro* the effect of a high fat diet, FaO cells were treated for 3 h with a mixture of oleate/palmitate at a final concentration of 0.75 mM (2:1 molar ratio). To induce oxidative stress, HECV cells were incubated for 24 h with H2O2 (20nM). Thereafter, cells were incubated for 24h with either water or ethanol extracts at different concentrations (0.15, 1.5 and 15 μg/ml). As a positive control we used pure carvacrol (Sigma-Aldrich Corp.) at increasing concentrations (1, 10 and 100 μM, corresponding of 0.15, 1.5 and 15 μg/ml).

### *2.8 Protein quantification*

The protein content was determined by the Bradford assay using BSA as a standard [19].

### *2.9 Quantification of triglycerides*

At indicate times, FaO cells were scraped, centrifuged and lysed and lipids were extracted in chloroform/methanol (2:1), then chloroform was evaporated [20]. Then, TG content was determined using the 'Triglycerides liquid' kit (Sentinel diagnostic, Milan, Italy). Spectrophotometric reading was performed with UV-VIS spectrophotometer.Values were normalized for the protein content. Data are expressed as percent TG content relative to controls.

### *2.10 ROS production and lipid peroxidation*

The oxidation of the cell-permeant 2'-7' dichlorofluorescin diacetate (DCF-DA, Fluka, Germany) to 2'-7'dichlorofluorescein (DCF) allowed to quantify *in situ* the production of $H_2O_2$ and other ROS [21]. Stock solution of DCF-DA (10 mM in DMSO) was prepared and stored at -20°C in the dark. At the end of treatment, cells were scraped and gently spun down (600xg for 10 min at 4°C). After washing, cells were loaded with 10 mM DCF-DA in PBS for 30 min at 37°C in the dark. Then, cells were centrifuged, suspended in PBS and the fluorescence was measured fluorometrically (lex=495 nm; lem=525 nm). All measurements were performed in a LS50B fluorimeter (Perkin Elmer, USA) at 25°C using a water-thermostated cuvette holder.

Lipid peroxidation was determined spectrophotometrically through the thiobarbituric acid reactive substances (TBARS) assay which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) [22]. Briefly, 1 vol. of cell suspension was incubated for 45 min at 95°C with 2 vol. of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl). Then, 1 vol. of N-butanol was added and the organic phase was read at 532 nm in a UV-VIS spectrophotometer at 25°C using Peltier-thermostated cuvette holder. The MDA level was expressed as pmol MDA/mL/mg protein.

### *2.11 Oil-Red O staining*

Neutral lipids were visualized using the selective Oil-RedO (ORO) dye [23]. Briefly, after fixing in 4% paraformaldehyde, cells were washed three times with PBS, stained for 20 min with 0.3% ORO solution prepared from a stock 0.5% in isopropanol and diluted in water. After washing with distilled water, slides were examined by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

### *2.12 Nitrite/Nitrate (NOx) Levels*

NO production was measured by spectrophotometric measurement of the end products, nitrites and nitrates, using the Griess reaction [24]. After treatments, nitrite accumulation (μmol $NaNO_2$/mg sample protein) was calculated against a standard curve of sodium nitrite ($NaNO_2$). All spectrophotometric analyses were carried out at 25°C recording absorbance at 540 nm.

### *2.13 Western blotting*

Protein levels of NF-kB p65 were assessed by Western blot analysis. Briefly, the cellular pellet was suspended in 400 μL ice-cold Buffer A (20 mM Tris HCl pH 7.8, 50 mM KCl, 10 μg/mL Leupeptin, 0.1 mM Dithiothreitol-DTT, 1 mM phenylmethanesulfonyl fluoride-PMSF); then 400 μL Buffer B (Buffer A plus 1.2% Nonindet P40) was added. The suspension was vortex-mixed for 10 sec; after centrifugation the nuclear pellet was washed and resuspended in 100 μL Buffer B, mixed thoroughly in ice for 15 min and finally centrifuged. The supernatant containing the nuclear extracts was collected and the protein content was measured. About 30 μg proteins were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [25]. Membrane was blocked in 5% fat-free milk/PBST (pH 7.4) and probed using rabbit anti-human NF-kB p65 antibody (SC-109; Santa Cruz Biotechnology, DBA, Milan, Italy). Membranes were incubated overnight at 4°C with primary antibody in PBST buffer (PBS with 0.1% Tween 20) washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) in PBST for 1h at room temperature [11]. Immune complexes were visualized using an enhanced chemiluminescence Western blotting analysis system (Bio-Rad ChemiDoc XRS System). Films were digitized and band optical densities were quantified against the actin band using a computerized imaging system and expressed as Relative Optical Density (ROD, arbitrary units). ROD of each band was expressed as percentage respect to control.

### *2.14 Wound Healing assay*

The migration of HECV was evaluated using the wound healing assay [26]. The cells were seeded on 35×10 mm tissue culture dishes and incubated until confluence was reached, the cell monolayer was scraped with a p100 pipet tip making two crossing straight lines to create a ‘‘scratch’’. Then, five views on the cross were photographed using a observed by F20 Tecnai electron microscope (Philips, Eindhoven, The Netherlands), and representative images were captured with an Eagle CCD camera and iTEM software at. After scratching, medium was replaced with fresh medium in the absence or presence of each plant extract at 1.5 μg/ml concentration. Set of images were acquired at 0, 3, 6 and 24 h. To determine the migration of HECV, the images were analysed using ImageJ free software

(http://imagej.nih.gov/ij/). Percentage of the closed area was measured and compared with the value obtained before treatment. An increase of the percentage of closed area indicated the migration of cells. Data are means ± S.D. of at least three independent experiments.

### *2.15 Statistical analysis*

Data are means ± S.D. of at least three independent experiments. Statistical analysis was performed using ANOVA with Tukey's post-test (GraphPad Software, Inc., San Diego, CA, USA).

## 3. RESULTS

### *3.1 Phenol and flavonoid contents and radical scavenging activity of the extracts*

Total phenol (TPC) and total flavonoid (TFC) contents were assessed in the two extracts from *T. spicata* leaves by spectophotometric assays (Table 1). The ethanolic extract (TE) showed TPC higher than the aqueous one (TW) (250±6.9 *vs* 150±3.7 mgGAE/g of dry extract, respectively). Also the TFC was higher in TE than in TW (9.04±1.2 *vs* 4.21±1.06 mgQE/g of dry extract respectively)

The free radical scavenging capacity was evaluated by the DPPH assay. Both the extracts showed a concentration-dependent scavenging potential (data not shown). The antioxidant activity was similar for the two extracts as indicated by the similar IC50 values (24.5 µg/ml ±1.09 for TE, and 25.8 µg/ml ±1.28 for TW) (Table 1).

### *3.2 Chemical characterization of the aqueous and ethanolic extracts of T. spicata*

The phenolic components of both extracts were identified using HPLC coupled with MS/MS in negative ionization mode; representative HPLC chromatograms are shown in Figure 1A supplementary data. We identified eighteen different polyphenols in TW, and thirteen in TE (Table 2). In both extracts, the most abundant classes of components were monoterpenoic phenols, polyphenolic acids, flavonoids and their derivatives. We observed some differences between them; rosmarinc acid (RA) was the most abundant PC in TW (38.60%), followed by salvalonic acid I (10.17%) and rutin (7.17 %), while carvacrol (CVL) was the most abundant PC in TE (36.84%), followed by thymusin (20.25%) and eriodictyol derivate (9.45%). Both extracts were rich in flavonoids such as apigenin, luteolin, quercetin and eriodictyol under form of aglycones or other derivatives (especially flavonoid glycosides).

We assessed the main volatile compounds in the extracts by GC-MS analysis; representative chromatograms are shown in Figure 1B supplemantary data. As summarized in Table 3, in the aqueous extract, only nine volatile

compounds were identified including four phenols, with p-cresol 2,2 methylenebis(6-tert butyl) being the most abundant one (31.5%). The ethanolic extract contained a larger number (twenty-six) of volatile compounds with carvacrol being the most abundant component (68.8%).

### *3.3 T. spicata extracts counteract fat-dependent steatosis and oxidative stress in hepatic cells*

TO assess if the extracts affected viability of FaO cells, the MTT assay was carried on FaO cells treated for 24 h with increasing concentrations of TE or TW (0.15, 1.5 and 15 μg/ml) or with identical concentrations of pure carvacrol (CVL) and rosmarinic acid (RA) for comparison. In all cases, no significant changes in viability were observed (data not shown).

To mimic *in vitro* what is occurring in the liver during high fat feeding and/or obesity, FaO cells were overloaded of lipids by exposure to oleate/palmitate mixture (0.75 mM). Figure 1A shows that treatment with FAs induced a moderate steatosis (SS) quantified by the increase in the intracellular TG content (+155% compared to controls; $p \leq 0.0001$). When SS cells were treated for 24h with different concentrations of TW or TE a significant reduction in TG accumulation was observed, but the effect was not in dose dependent manner. In details, TW treatment at the doses of 0.15, 1.5 and 15 μg/ml led to a decrease in TG content of about -114%, -113%, and -104%, respectively ($p \leq 0.05$) compared to steatotic cells. At the same doses, TE led to a lower reduction in the TG content (-88%, -57% and – 67 % respectively; $p \leq 0.05$) compared to steatotic cells.

Cytosolic LDs were visualized and analysed by ORO staining and optical microscopy (Fig. 1B). Both the number and size of LDs increased markedly in steatotic cells compared to controls (from 1.3 to 5.3 LDs/cell, and from 0.9 to 2.65 μm of diameter). LD size and number changed upon incubation with extracts; LD size decreased to 1.52 μm with TW and to 1.65 μm with TE ($p \leq 0.001$, $p \leq 0.0001$) at the intermediate dose (1.5 μg/ml). The number of LDs/cell decreased significantly only with TE (4.3 LDs/cell for the intermediate dose; $p \leq 0.05$) (Fig.1C).

The oxidative stress was assessed by measuring lipid peroxidation by TBARS assay. The MDA level (Fig. 2) increased in steatotic cells (+86% compared to controls; $p \leq 0.0001$), and decreased when steatotic cells were exposed to both extracts. In details, TW treatment at the doses of 0.15, 1.5 and 15 μg/ml led to a decrease in MDA level with respect to steatotic cells of about -70%, -68% ($p \leq 0.01$) and -75% ($p \leq 0.001$), respectively. At the same doses, TE decreased the MDA level of about -110%, -110% and -102% ($p \leq 0.0001$). In control cells, MDA level was not affected by extracts (data not shown).

### *3.4 T. spicata extracts rescue the radical-dependent oxidative stress and dysfunction in endothelial cells*

For experiments with HECV cells we chose the intermediate concentration of 1.5 μg/ml, after assessing that it did not affect cell viability (data not shown). HECV cells exposed to 20 μM $H_2O_2$ for 24 h showed a marked increase in MDA level (+43% with respect to controls; $p \leq 0.01$) (Fig. 3A). Treatment of $H_2O_2$-insulted cells with TW or TE for 24 h partially counteracted this effect leading to a decrease in MDA level of about -37% ($p \leq 0.01$) for TW, and of -58% ($p \leq 0.0001$) for TE as compared to $H_2O_2$-insulted cells. The intracellular production of ROS was also visualized *in situ* by fluorescence microscopy of DCF-stained cells Compared to controls, higher and diffuse DCF fluorescence was observed in $H_2O_2$-insulted cells, and it was reduced after treatment with TW or TE for 24 h (Fig. 3B). When these changes were quantified by fluorimetric analysis we observed a significant DCF decrease, of similar extent, in $H_2O_2$-insulted cells treated with TW (-58%; $p \leq 0.05$) or with TE (-62%; $p \leq 0.05$) (Fig. 3C). Both MDA level and DCF signal were not affected by extracts in control cells.

The effect of extracts on NO production, a classical marker of inflammation, was assessed in HECV cells. In $H_2O_2$-insulted cells we observed a stimulation of NO release of +35% with respect of control ($p \leq 0.01$) (Fig. 4A). Both extracts were able to counteract this effect; TW and TE treatments decreased NO release compared to $H_2O_2$-insulted cells (about -57% and -43%; $p \leq 0.001$ and $p \leq 0.01$, respectively). NF-κB activation is a central mediator of inflammatory response in oxidative stress conditions. $H_2O_2$-insulted cells showed an increase in NF-κB p65 level with respect to control (+41%; $p \leq 0.01$); this increase was counteracted by both extracts (-44% for TW, and -77% for TE; with respect to stressed cells; $p \leq 0.001$ and $p \leq 0.0001$) (Fig. 4B).

We verified if the extracts were able to modulate the HECV migration ability by T-scratch assay. When the scratched confluent cell layer was incubated for 24 h in media containing TW or TE we observed an average acceleration of the wound repair compared to control (Fig. 4C). No significant differences in cell migration rate were observed at short times after the scratch (3 and 6 h), whereas at a longer time (24 h), the control cells reduced the wound width of about 40% compared to time 0. Both extracts significantly stimulated cell migration resulting in a wound width smaller to that of controls at the same time, 32% for TW and 23 % for TE.

## 4. DISCUSSION

Medicinal plants are known for the beneficial properties of their bioactive compounds. The Mediterranean diet consisting of many vegetal foods rich in phenolic compounds is one of the factors responsible for the reduced incidence of metabolic and cardiovascular (CVD) disorders and mortality. Here, we have shown that extracts from

*T. spicata*, an aromatic plant of the east Mediterranean area, may counteract the excess hepatic accumulation of triglycerides (antisteatotic activity) and the oxidative stress (hepatopretection activity) in NAFLD models.

*T. spicata* is widely used as a healthy plant in Lebanon where it is known as "Za'tar". The leaves are rich in antioxidants such as the phenolic compounds, which are receiving growing attention among nutritionists and food scientists as possible therapy in the prevention of degenerative diseases, particularly metabolic and CVD diseases [27]. NAFLD is the most common hepatic disorder in developed countries, often associated to overnutrition and overweight [28]. Currently, the main goal for NAFLD therapy is to reverse or prevent the progression of simple steatosis to liver fibrosis, and PC are possible therapeutic interventions, because their hepatoprotective potential against liver damages promoted by the oxidative reactions triggering lipid peroxidation [29,30].

In the present study, we tested two different extracts of *T. spicata,* as the extraction solvent (water or ethanol) is important for the final composition. The water extraction is consistent with the traditional preparation of tea infusion of *T. spicata* leaves, whereas the ethanol extraction is typical of pharmacological studies. As expected, the number of volatile species was larger in the ethanolic extract than in the aqueous one (twenty-six *vs* nine), indeed the abundancy of extracted molecules is one of the reasons to prefer alcohol extracts in pharmacology.

We, firstly, reported a detailed composition analysis of the extracts from *T. spicata* aerial parts which were prepared using water or ethanol as extraction solvents. Monoterpenic phenols and flavonoids were more abundant in the ethanolic than in the aqueous extract. According to previous reports [7], rosmarinic acid was the most abundant PC in the aqueous extract. RA, a common polyphenol of the Lamiaceae family [31], shows low toxicity and widespread biological properties, including antioxidant and anti-inflammatory effects [32], protective activity against stress–induced pathologies such as ischemia–reperfusion [33], and liver injuries induced by diabetes [34] or drugs [35]. TW contained other components which may play synergic action with RA, such as the salvalonic acids (SAs), polyphenols with antioxidant, cardioprotective and anti-inflammatory potential [36,37], rutin, vicinin 2 and other flavone derivatives, which are known for their health benefits [38,39]. Carvacrol was the most abundant component in the ethanolic extract; this monoterpene is a common component of essential oils from aromatic plants and spices, and it is known for its antibacterial, antifungal, anti-inflammatory, hepatoprotective and anti-carcinogenic activities [40,41]. TE was also rich in thymusin, a flavonoid with antiallergic [42] and antidiabetic [43] activities, and of *p-cymene-2,3-diol,* a semivolatile compound being characterized by an additional hydroxyl group with respect to carvacrol [44]. Moreover, *p-cymene-2,3-diol* was also present in dimeric form, the 3,4,3′,4′-tetrahydroxy-5,5′-

diisopropyl-2,2′- dimethylbiphenyl, which was already found in leaves of *Thymus vulgaris* [45]. Based on these data, we might assume that the beneficial activity of TW could depend mainly on the rosmarinic acid, and that of TH on carvacrol, as they are the predominant agents in each extract.

In the liver, excess TG are stored in lipid droplets which are the hallmark of NAFLD. For the purpose of exploring and testing potential drug candidates, we employed an experimental model consisting of rat hepatoma FaO cells exposed to a mixture of oleate/palmitate which represent a reliable *in vitro* model for hepatic steatosis widely employed in previous studies of our group [11,13]. The moderate steatosis induced by FAs was reduced by both *T. spicata* extracts. However, the aqueous extract was most effective to counteract hepatic steatosis *in vitro* with respect to the ethanolic extract at all the tested doses (about -110% *vs* -60% of TG accumulation, respectively, compared to steatotic cells). The lipid-lowering activity of the extracts was depending on their action on lipid droplets: both the number and size of LDs increased markedly in steatotic cells compared to controls, and decreased of a similar value upon incubation with both extracts.

Excess fat accumulation in hepatic cells is accompanied by increased oxidative stress. As markers of oxidative stress we assessed both the intracellular ROS production, and the level of lipid peroxidation. Both these indices were significantly increased in steatotic cells compared to control hepatocytes. Incubation of steatotic cells with TW or TE led a significant reduction in the level of ROS and of lipid peroxidation. However, the ethanolic extract was most effective as anti-oxidant with respect to the aqueous extract at all the tested doses.

Endothelium is a crucial blood–tissue interface controlling energy supply; it is the first rate-limiting step in the utilization of long-chain FAs as fuels, and it is altered in NAFLD. Endothelial cells play regulatory functions through releasing various factors including nitric oxide and ROS. In the liver, the endothelial cells of sinusoids act in hepatic fibrosis development by sustaining wound healing response and inflammation [46]. HECV cells exposed to hydrogen peroxide can mimic what happens *in vivo* in atherosclerosis, as endothelial damage is typically observed in metabolic syndrome [11]. The oxidative stress induced in HECV cells by exposure to $H_2O_2$ was partially counteracted by both extracts. The ROS production was visualized *in situ* by fluorescence microscopy and fluorimetric analysis and it resulted significantly decreased by both TW and TE. The decrease in lipid peroxidation was larger for TE (-58%) than for TW (37%) compared to $H_2O_2$-insulted cells. Also the increase in NO release, a key indicator of endotheliium inflammation and oxidative damage, was counteracted by both extracts (-57% for TW and -43% for TE). NF-κB is a master transcription factor in the activated by oxidative stress. We observed an

increase in NF-κB p65 level in $H_2O_2$-insulted cells which was counteracted by both extracts but TE played a larger effect (-77%) compared to TW (-44%). The extracts were also assessed for their effects on endothelial cell migration. We observed that both extracts were able to accelerate the wound repair, however TW was more effective than TE (-32% *vs* -23% of wound width, respectively). Taken together these data indicate that both the *T. spicata* extracts might ameliorate oxidative damage and inflammatory response in endothelial cells by inhibiting oxidative/nitrosative stress pathway and reducing the contents of intracellular NO and ROS. However, each extract showed some specificity in its antioxidant activity, with TE showing a higher antioxidant potential, likely depending on the panel of PC contained.

We can conclude that both extracts from *T. spicata* aerial parts are efficacious lipid-lowering and antioxidant agents for steatotic hepatocytes and activated endotheliocytes, representing reliable *in vitro* models of NAFLD. Interestingly, the aqueous extract was more effective as lipid-lowering and wound-repair agent, likely depending on its PC profile consisting of a high content of rosmarinic acid. By contrast, the ethanolic extract played a more marked anti-oxidant activity, both in hepatic and endothelial cells, possibly depending on its abundance of carvacrol. Moreover, the results suggest that both the extracts and their single components, carvacrol and rosmarinic acid especially, act directly on hepatic and endothelial cells, although *in vivo* their effects may also be secondary to the anti-inflammatory/antioxidant responses in other tissues/organs.

In conclusion, the beneficial effects of the two extracts of *T. spicata* leave extracts which contain a different panel of compounds clearly indicate that this edible plant possesses a potential therapeutic benefit which explains its traditional use in Lebanon. Therefore, we suggest that this plant may have a great relevance as healthy and dietary supplement in the prevention and treatment of diseases involving free radicals and oxidants such as NFALD.

## 5. Conclusion


## 6. ACKNOWLEDGEMENTS AND FUNDING

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**Table 1:** Total phenol content (TPC), total flavonoid content (TFC) and DPPH radical scavenging activity of *T. spicata* extracts.

| | **TPC mgGAE/g dry extract** | **TFC mgQE/g dry extract** | **IC50 (DPPH) µg/mL** |
|---|---|---|---|
| **Ethanolic Extract (TE)** | 250±6.9 | 9.04±1.2 | 24.5±1.09 |
| **Aquesous Extract (TW)** | 150±3.7 | 4.21±1.06 | 28.5±1.28 |

**Table 2:** Phenolic compounds identified in *T. spicata* extracts by using HPLC-MS/MS in the negative ionization mode.

**ETHANOLIC EXTRACT**

| a | RT (min) | Measured m/z | MS/MS fragments | Proposed compound | Percentage area (%) |
|---|---|---|---|---|---|
| 1 | 14.1 | 593 | 575 503 473 383 353 | Vicinin 2 | 2.56 |
| 2 | 14.5 | 303 | 285 177 125 | Dihydroquercetin (taxifolin) | 2.38 |
| 3 | 17.1 | 417 | 371 287 263 | Eriodictyol derivate | 9.45 |
| 4 | 18.5 | 609 | 301 | Rutin | 1.3 |
| 5 | 19.1 | 359 | 223 197 179 161 133 | Rosmarinc acid | 4.2 |
| 6 | 19.7 | 287 | 269 151 135 107 | Eriodictyol | 6.8 |
| 7 | 21.5 | 329 | 314 | Thymusin | 20.25 |
| 8 | 22.8 | 269 | 201 181 149 | Luteolin | 7.95 |
| 9 | 23 | 285 | 257 243 151 | Apiginin | 0.75 |
| 10 | 23.3 | 343 | 328 313 300 285 | Unknown | 1.24 |
| 11 | 24 | 165 | 149 | p-cymene-2,3-diol | 2.82 |
| 12 | 24.3 | 343 | 328 313 | Cirsilineol | 1.76 |
| 13 | 25.7 | - | - | Carvacrol* | 36.84 |
| 14 | 27.3 | 329 | 314 299 286 271 | 3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′-dimethylbiphenyl | 1.64 |

[a] Compounds are listed according to their elution order in the reverse phase HPLC; * detected with standard authentic compound.

**AQUEOUS EXTRACT**

| a | RT (min) | Measured m/z | MS/MS fragments | Proposed compound | Percentage area (%) |
|---|---|---|---|---|---|
| 1 | 8.1 | 305 | 225 | Gallocatechin | 3.6 |
| 2 | 12.2 | 387 | 369 225 207 163 | Tuberonic acid glucoside | 0.5 |
| 3 | 14 | 593 | 575 503 473 383 353 | Vicenin 2 | 3.76 |
| 4 | 15 | 637 | 461 351 285 | Luteolin-O-diglucuronide | 4.81 |
| 5 | 15.4 | 537 | 493 339 | Salvalonic acid I | 10.17 |
| 6 | 15.7 | 477 | 397 373 343 301 | Quercetin-glucuronide | 2.78 |
| 7 | 16.3 | 595 | 473 429 287 | Eriodictyol-rutinoside | 1.25 |
| 8 | 16.5 | 623 | 433 287 | Luteolin-glucuronide-hexoside | 1.73 |
| 9 | 17 | 717 | 537 519 475 365 339 | Salvalonic acid E/B | 5.85 |
| 10 | 17.4 | 461 | 285 | Luteolin 7-O-glucuronide | 4.16 |
| 11 | 17.6 | 593 | 285 | Luteolin-O-rutioside | 2.49 |
| 12 | 17.9 | 441 | 418 405 373 305 225 175 | Unknown | 2.05 |
| 13 | 18.1 | 521 | 359 179 161 | Rosmarinic acid-glucoside | 1.26 |
| 14 | 18.5 | 609 | 301 | Rutin | 7.17 |
| 15 | 19.1 | 359 | 223 197 179 161 133 | Rosmarinic acid | 38.6 |
| 16 | 19.7 | 549 | 387 | Tuberonic acid derivate | 4.37 |
| 17 | 19.8 | 607 | 559 427 299 284 | Methyl kaempferol O-rutinoside | 3.27 |
| 18 | 21.6 | 491 | 443 311 267 | Salvanolic acid C | 1.73 |
| 19 | 25.7 | - | - | Carvacrol* | 0.8 |

[a] Compounds are listed according to their elution order in the reverse phase HPLC; * detected with standard authentic compound.

**Table 3:** Chemical composition of volatile compounds of *T. Spicata* ethanolic extracts detected by GC-MS. Compounds are numbered in order of percentage.

| Compounds of TE | RT(min) | Percentage (%) |
|---|---|---|
| Carvacrol | 32.32 | 68.8 |
| p-Cresol. 2,2-methylenebis(6-tert-butyl) | 47.38 | 5.7 |
| Pyrocatechol, 4-tert-butyl- | 36.38 | 2.75 |
| Methyl isosetearate | 44.74 | 2.7 |
| Palmitic acid methyl ester | 42.8 | 2.6 |
| Phenol,2,4-di-tert-butyl- | 37.69 | 2.35 |
| 2-Hexadecanol | 35.3 | 1.5 |
| caryophyllene | 35.87 | 1.5 |
| Caryophyllene oxide | 38.88 | 1.18 |
| Dimethyloxybutane | 4.01 | 1.15 |
| Azulene, 7-isopropyl- 1,4 dimethyl- | 40.63 | 1.1 |
| Hexa hydro farnesol | 33.14 | 0.9 |
| p-Cymene | 10.38 | 0.85 |
| Cedrol | 38.35 | 0.8 |
| p cymen 7 ol | 31.92 | 0.8 |
| Thymol | 31.79 | 0.7 |
| Spathunlenol | 38.78 | 0.68 |
| Oleic Acid | 20.52 | 0.4 |
| Ocimene | 11.72 | 0.35 |
| 5-Octadecenal | 13.7 | 0.27 |
| Z-11-Tetradecenoic acid | 7.57 | 0.25 |
| E-9-Tetradecenoic acid | 17.76 | 0.2 |
| Tran 13 octadeonic | 34.75 | 0.18 |
| cis-Vaccenic acid | 38.57 | 0.18 |
| 6-Octadeoenoic acid | 25.11 | t |
| à-N-Normethadol | 36.15 | t |

| **Compounds of TW** | **RT(min)** | **Percentage (%)** |
|---|---|---|
| *p-Crersol 2,2 methylenebis(6-tert butyl)* | 47.37 | 31.5 |
| *Palmitic acid methyl ester* | 42.8 | 10.8 |
| *Carvacrol* | 32.26 | 8.5 |
| *Azulene, 7-isopropyl- 1,4 dimethyl-* | 40.63 | 6.8 |
| *Ethyl iso-allocholate* | 44.74 | 6.6 |
| *Phenol,2,4-di-tert-butyl-* | 37.7 | 5.0 |
| *Hydroquinon, 2-6-di-tert-butyl* | 13.75 | 3 |
| *à-N-Normethadol* | 41.32 | 1.4 |
| *2-hexadecanol* | 31.11 | 1.2 |

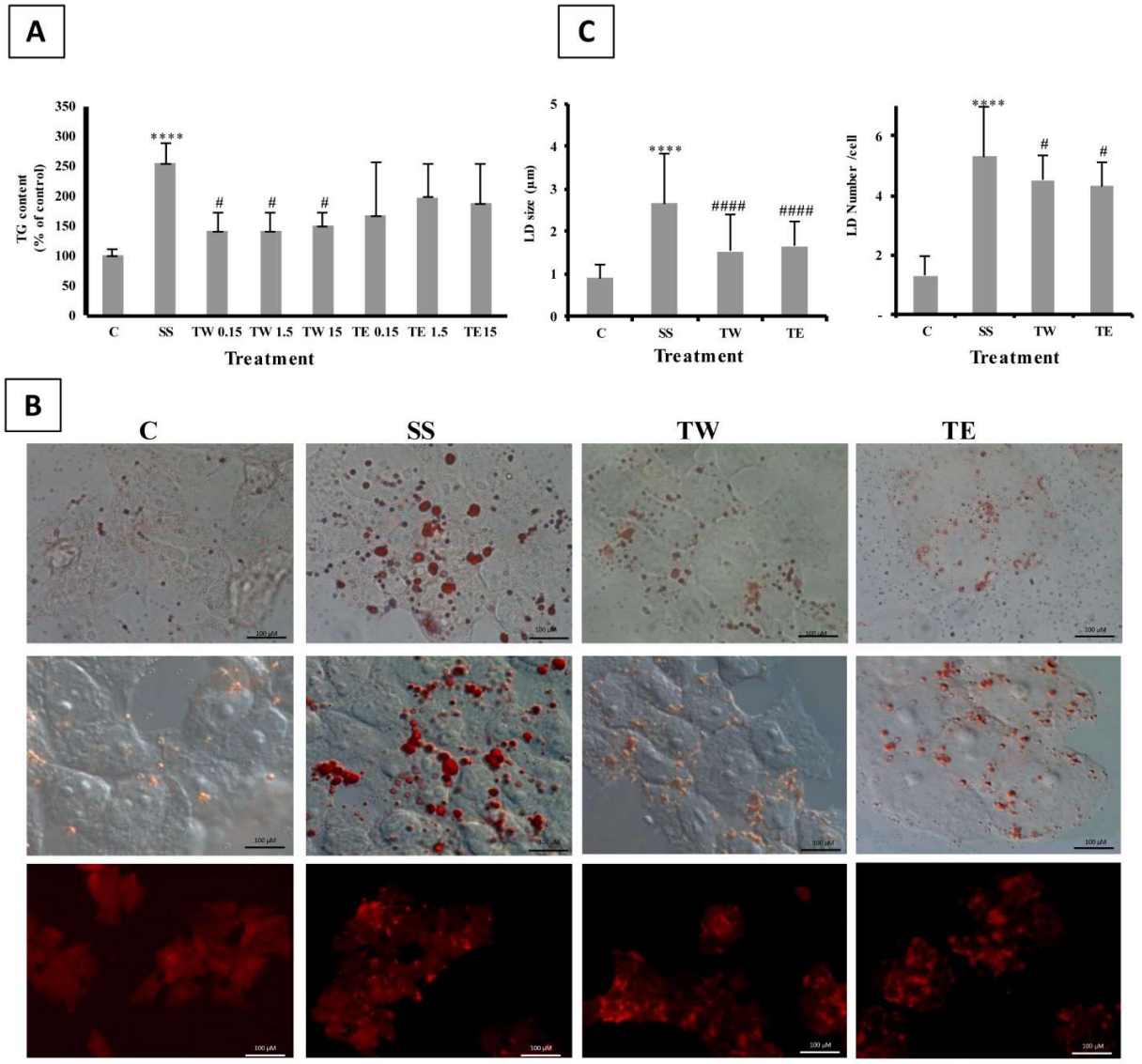


**Figure 1: Effects of *T. spicata* extracts on lipid accumulation in hepatic cells.**

**(A)** Triglyceride content was quantified by spectrophotometric assay in control and steatotic FaO cells incubated with FA mixture (SS) and then treated with *T. spicata* extracts (TE and TW) at different concentrations (0.15, 1.5 and 15 µg/ml) for 24h. Results are expressed as percent TG content relative to control and normalized for total proteins. **(B)** Neutral lipid accumulation was assessed i*n situ* in ORO-stained control and steatotic FaO cells (SS) and extracts-treated FaO cells (1.5 µg/ml). Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany), magnification 100x; Bar: 100µm **(C)** Average size and number of Lipid Droplets per cell were calculated using ImageJ free software (http://imagej.nih.gov/ij/). Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* SS ****p≤0.0001 and SS *vs* different concentration of extracts #p≤0.05**.**

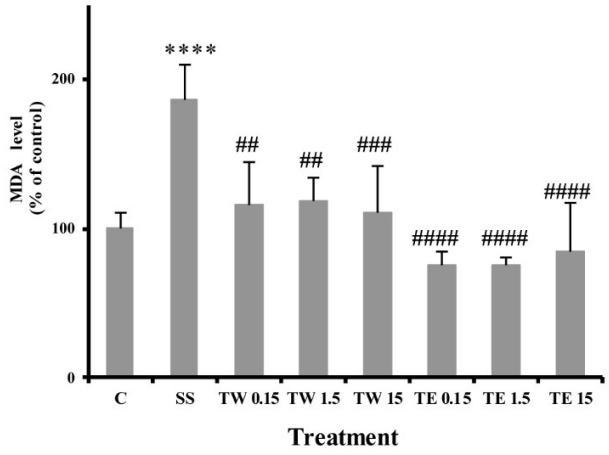


**Figure 2**: **Effects of *T. spicata* extracts on lipid peroxidation in hepatic cells**

**(A)** Intracellular MDA level was quantified by TBARS assay as pmol MDA/mL x mg of sample protein. Values are reported as % of control and are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* SS ****p≤0.0001 and SS *vs* different concentration of extracts #### p≤0.0001, ### p≤0.001, ##p≤0.01, #p≤0.05.

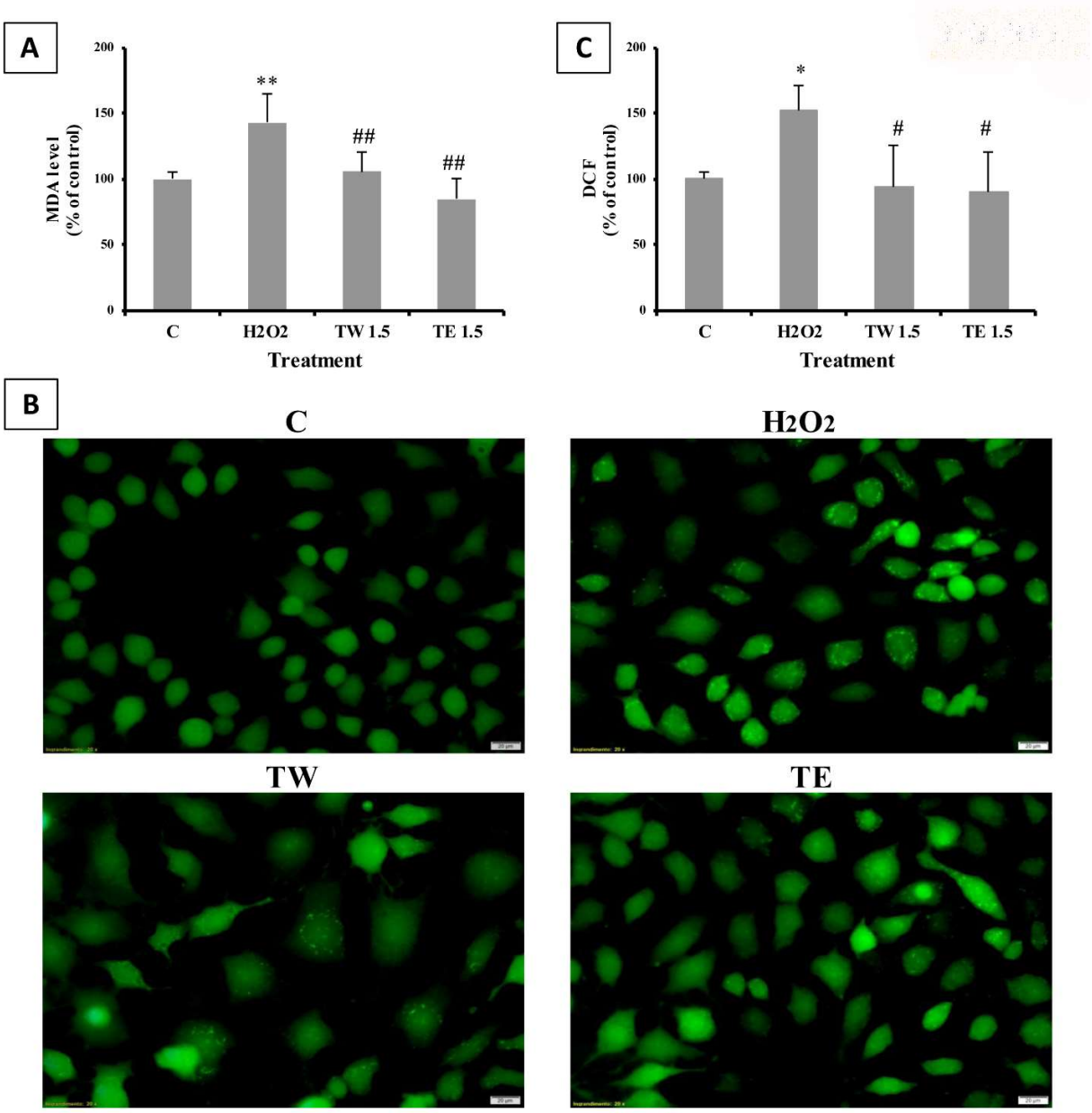


**Figure 3: Effects of *T. spicata* extracts on lipid peroxidation and ROS production in endothelial cells**.

HECV cells treated with $H_2O_2$ (20µM) for 24h to induce oxidative stress were then treated with both extracts at the intermediate concentration (1.5 µg/ml) for 24h. **(A)** Intracellular MDA level was quantified by TBARS assay; **(B)** The intracellular ROS production was visualized *in situ* by fluorescence microscopy of DCF-stained cells. Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany), magnification 20x (Bar: 20µm); **(C)** DCF fluorescence was quantified by spectrofluorimeter assay of DCF-stained cells. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* $H_2O_2$ insulted cells **p≤0.01, *p≤0.05 and $H_2O_2$ insulted cells *vs* different concentration of extracts, ### p≤0.001, ##p≤0.01, #p≤0.05.

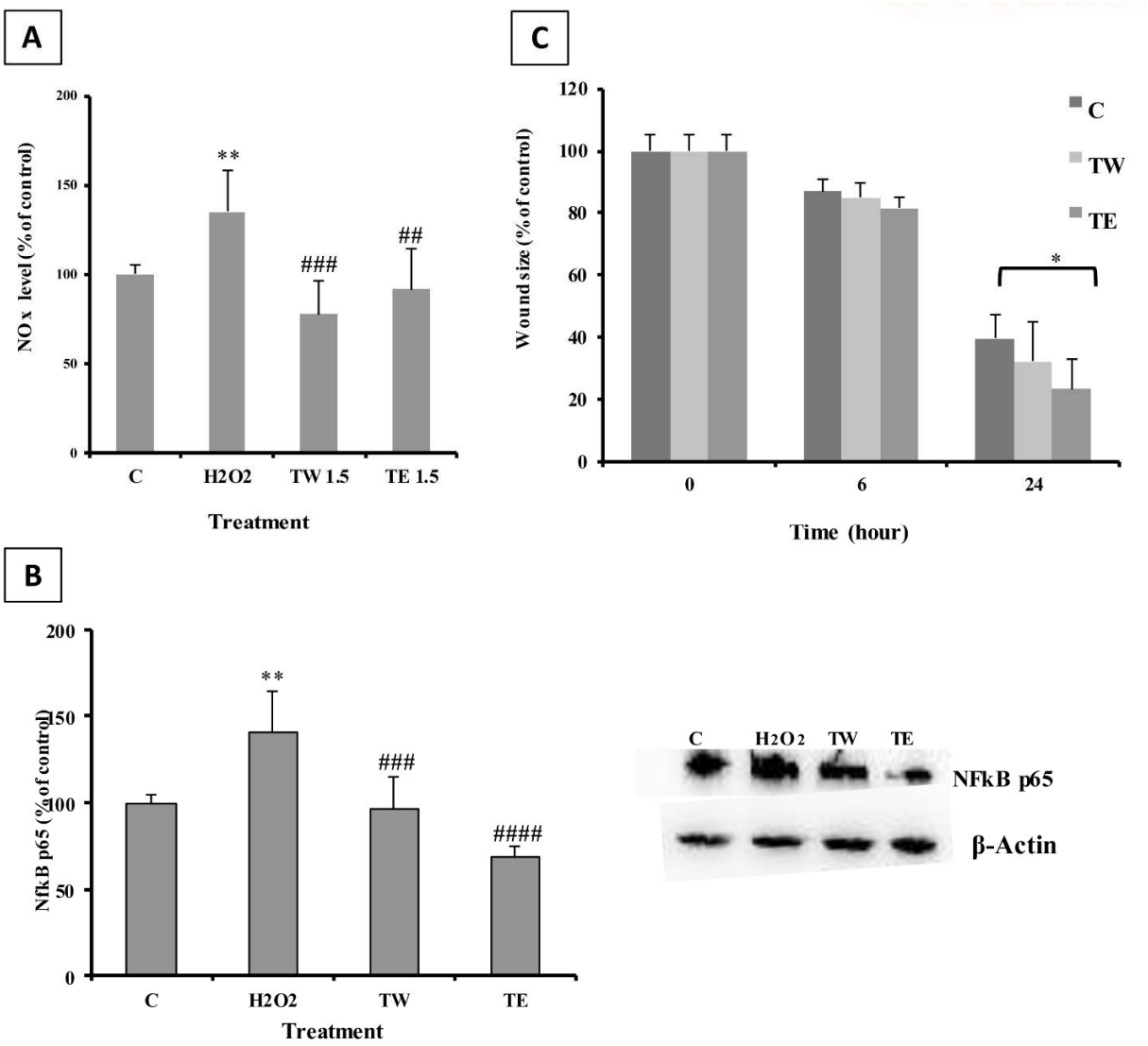


**Figure 4: Effect of *T. spicata* extracts on oxidative stress-induced dysfunction in endothelial cells**

HECV cells treated with $H_2O_2$ (20µM) for 24h were treated with both extracts at the intermediate concentration (1.5 µg/ml) for 24h **(A)** Nitric oxide production was quantified in the medium of HECV cells as µmol $NaNO_2$/mg sample protein by Griess reaction. Values are expressed as % of control **(B)** Densitometric analysis of nuclear NF-kB/p65 was evaluated by Western blotting; β-actin was the housekeeping gene for normalization; data are expressed as percentage values with respect to controls **(C)** Cell migration measured by the T scratch assay photographed at 0, 6 and 24 h incubation with both extracts at1.5 µg/ml, graphs representing the percentage of the closed area as compared to time=0. T scratch assay representative images are also shown. Values are mean ± S.D from at least three independent experiments. Significant differences are denoted by symbols C *vs* TE treatment *p≤0.05.

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