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THE EFFECT OF *TROPAEOLUM MAJUS* L. ON BACTERIAL INFECTIONS AND *IN VITRO* EFFICACY ON APOPTOSIS AND DNA LESIONS IN HYPEROSMOTIC STRESS

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Tropaeolum majus L. (*T. majus*) or nasturtium is a medicinal plant widespread in the areas with temperate climate, commonly used in culinary and in traditional medicine due to therapeutic properties. In the last few years, various effects of the flowers and leaves of this plant have been studied, but their benefits are not fully known. The aim of the study was to identify the phenolic compounds from *T. majus* edible flowers in relation with its antioxidant capacity and the antimicrobial activity against different bacteria and *Candida albicans*. In addition, the impact of natural extract on oxidative stress, inflammation and apoptosis was analysed on human umbilical vein endothelial cells (HUVECs) exposed to normotonic and hypertonic conditions. The major phenolic acids, identified by HPLC-RP with UV detection, were gallic acid, caffeic acid and p-coumaric and predominant flavonoids were quercetin, epicatechin and luteolin. The both fractions of *T. majus* were rich sources of polyphenols with marked antioxidant activity, evidenced by TEAC or DPPH methods. The extract exhibited a week antibacterial effect on some strains of streptococcus, without antifungal or antibacterial effect on gram negative bacteria. *T. majus* extract increased the p53 and Bcl-2 expressions and diminished the DNA lesions indicating the protective and antiapoptotic effects *in vitro,* on endothelial cells exposed to hyperosmotic stress. These experimental findings suggest that *T. majus* can exert some protection against bacterial infections and reduce apoptosis and DNA lesions in hypertonic conditions.

Key words: *Tropaeolum majus L., antioxidant, antimicrobial activity, endothelial cells, hyperosmotic stress, apoptosis*

INTRODUCTION

In recent years, consumers have increased their interest in the use of edible flowers for improving the presentation and smell of prepared food. Moreover, it has been proven that many edible flowers are a rich source of bioactive compounds, especially phenolic compounds such as flavonoids and anthocyanins, which have beneficial properties for human health (1). Numerous studies have demonstrated that the administration of plant extracts is important for counteracting the adverse side effects of synthesis medication or to enhance the medication's therapeutic effects (2, 3).

Tropaeolum majus L. has gained popularity lately in the last years as an important source of compounds with biological activity. *T. majus* or nasturtium is a medicinal plant widespread in the areas with temperate climate, commonly used as tea from plant leaves. In Bihor County it is found as a crop plant. The different parts of this plant are used for therapeutic purposes due to its expectorant and antitussive qualities, antiseptic and antitumor properties (4), antithrombotic (5) and also diuretics and hypothensive effects (6, 7). *T. majus* flowers were also widely used in culinary preparations, as part of salads, or for antidiabetic effect (8). There are several studies which have emphasized the antioxidant activity due to its content of polyphenols, flavonoids and ascorbic acid (9-12). In addition, the edible flowers of *T. majus* contain high levels of glucotropaeolin and a precursor of aromatic isothiocyanate with known growth-inhibiting properties on gram negative and gram-positive pathogenic bacteria (13), which can prevent the biofilm formation. The isothiocyanate could act synergistically with common antibiotics potentiating their inhibitory effect (14). Also, the isothiocyanates inhibited the metabolic activity of *Pseudomonas aeruginosa* (15) and killed the bacteria in biofilms by increasing the permeability of the bacterial membrane and induction of cell death (16, 17). Flowers and leaves of *T. majus* are considered an excellent dietary source of lutein, which reduces the risk of macular degeneration (18) and have also diuretic and antihypertensive activities (19, 20). The mechanism involved is related to the stimulation by isothiocyanates and flavonoids, from *T. majus,* of prostacyclin synthase enzyme activity and the release of renal prostaglandins (18).

The toxicity studies of hydroethanolic extract, obtained from *T. majus* leaves, on female and male mice, rats, and rabbits did not find the hematological, biochemical and histological alterations after 90 days of administration (21) and did not show genotoxicity in male rats (22). A subchronic toxicity study

confirmed the absence of harmful effect of the extract administered in different doses (75, 375 and 750 mg/kg), for 28 days (23) to Wistar rats.

The identification of new experimental models that can reproduce the pathology from clinical practice can offer new valences for natural compounds and may explain their protective effects. One such model *in vitro* can be osmotic stress, induced by exposure of endothelial cells to hypertonic conditions (24). The subjacent mechanisms imply the redox imbalance, inflammation and cell apoptosis (24). Thus, an increase of extracellular osmolarity induces vascular endothelial dysfunction due to the loss of water from the cell, triggering of the redox imbalance and the promotion of cell apoptosis (24, 25). In their adaptation effort the cells initiate protective mechanisms that ultimately lead to cell cycle arrest and apoptosis (26).

There were identified natural compounds which can modulate the effects of hypertonic medium on morphology and function of cells. Thus, *Equisetum arvense* L., administered in low doses, decreased the enhanced oxidative stress and apoptosis associated with hypertonic conditions while in high doses had prooxidant effects and induced apoptosis (27). Based on these data the purpose of the present study was to identify the active compounds from *T. majus* extract in relation with its antioxidant capacity and the antimicrobial activity against different bacteria and *Candida albicans*. The reference microbial strains and also the human clinical isolates from patients with infections or from the hospital environment were used. In addition, the impact of low dose of natural extract on oxidative stress and apoptosis was analyzed on human umbilical vein endothelial cells (HUVECs) exposed to normotonic and hypertonic conditions compared to luteolin, a compound identified in high levels in the hydrolized extract obtained from edible flowers of *T. majus*.

MATERIALS AND METHODS

Reagents

6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Folin-Ciocalteau's reagent, 1,1-diphenyl-2-pycrylhydrazyl (DPPH), 2,4,6-Tri(2-pyridyl)-s-triazine (FRAP), naringenin (> 95%) were purchased from Sigma Aldrich, USA. Gallic acid and sodium carbonate were obtained from Fluka, Switzerland. 2-thiobarbituric acid and Bradford reagent were from Merck KGaA(Darmstadt, Germany); absolute ethanol and n-butanol were purchased from Chimopar (Bucharest, Romania) and ELISA tests for the evaluation of caspases-8 and IL-6 were from R&D Systems (Minneapolis, MN, USA). Antibody against γH2AX (histone variant H2AX phosphoSer 139), p53 and Bcl-2 were acquired from Stressgen Bioreagents Corporation,Victoria, BC, Canada). All the chemicals used were of analytical grade.

Plant collection and sample preparation

To highlight the therapeutic effects of the *T. majus* flowers, we prepared a lyophilized extract of the plant's product. We also assessed the physico-chemical features in such a way as to allow the investigation results to be most eloquent. In order to obtain the extract, the edible wild *T. majus* flowers were harvested during July-September period, 2014, in Bihor county (Latitude*:* 47° 04' 19.92" N Longitude*:* 21° 55' 15.96" E), and were dried at 40°C temperature for 96 hours. Three different extraction methods were used to obtain phenol extracts from the plant's flowers. Because phenolics compounds may also be associated with other plant components such as carbohydrates and because to the fact that, many polyphenols are easily hydrolysed (28), we have prepared from lyophilized forms the unhydrolzed and the hydrolyzed extracts.

1. Preparation of T. majus extract for lyophylization

Dried flowers (10 g) were used for extraction with 70% aqueous ethanol (100 mL) using a magnetic mixer for 45 min and sonicated for 5 min. The sample was centrifuged and the alcohol was evaporated using a rotary evaporator. The dried extract was lyophilized. For all of the methods used for preparation of unhydrolyzed and hydrolysed extracts we followed the methods published by Pallag *et al*. (29).

2. Preparation of unhydrolyzed extract

Fifty mg of dry extract was dissolved in a mixture consist of 90% methanol and 0.5% acetic acid (2 mL). After addition of 2 mL of naringenin 10 mmol (standard intern) the sample was centrifuged and the supernatant obtained was evaporated on a rotary evaporator. Finally, the residue was dissolved in 1 mL dimethyl sulfoxide (DMSO) and transferred to a test tube.

3. Preparation of hydrolyzed extract

Fifty mg of lyophilized extract was dissolved in 4 mL 25% methanol and then was mixed with 2 mL of intern standard (naringenin 10 mmol) and 1 mL of HCl (6 M). Then, the sample was mixed in a water bath at 85 – 90°C for 2 hours and 2.5 mL of ethyl acetate was added. The extract was vacuum evaporated to dryness on a rotary evaporator and the residue was dissolved in 1 mL DMSO and stored. In order to physico-chemically characterize the lyophilized extract, we used the reversed phase-high performance liquid chromatography (RE-HPLC) method with UV detection. Due to the structure diversity of the active principles in the phenol and flavonoid groups, the lyophilized content was subjected to acid hydrolysis processes. Therefore, the bioactive compounds found in the *T. majus* flowers, responsible for the therapeutic effects, were emphasized.

RP-HPLC analysis

RP-HPLC was used to assay compositions of flavonoids and phenolic acids as previously published by Pallag *et al*. (29). The phenol standards used included 7 phenolic acids (gallic, caffeic, syringic, synapic, vanillic, p-coumaric, ferulic) and 7 flavonoids (catechin hydrate, rutinetrihydrate, naringenin, luteolin, quercetinedihydrate, epicatechin, myricetin). The standards were dissolved in a mobile phase as 1 mg/mL concentrations and then were filtered through a 0.45 µm membrane filter and immediately injected to the HPLC column. Analyses were performed in triplicate. The measurements were done using a Shimadzu SCL-POA HPLC system with LC-10ADVP pump equipped with SPD-10AVP Diode Array (UV) detector. The column type used was Kintex 5u RP C18 µm and the mobile phase consists of (A) 0.05% formic acid and (B) 0.05% acetonitrile (50:50 v/v). Identification of the flavonoids was achieved by comparison with retention times of standards, UV spectra and calculation of the absorbance ratios after coinjection of samples and standards.

Evaluation of antioxidant capacity

1. DPPH method

The antioxidant capacity of the extract was determined on the basis of its activity of the scavenging of the stable DPPH

radical using a slightly modified method of Brand Williams *et al*. (30). Variations of color (from dark violet to light yellow) were measured at 517 nm on a Shimadzu UV-VIS spectrophotometer. The solution of DPPH in methanol 6.10-5 M was freshly prepared daily before measurements. The samples were kept in the dark for 15 minutes at room temperature and then the absorbance was read. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula: % inhibition = [(AB – AA)/AB] × 100 where: AB = absorption of blank sample (t = 0 min), AA = absorption of test extract solution (after 15 min).

2. Ferric reducing antioxidant power (FRAP) method

FRAP method evaluates the antioxidant capacity of natural extract and is based on the reduction of ferric tripyridyltriazine complex (Fe(III) TPTZ) by a reductant, at an acid pH. The FRAP solution was prepared by mixing 50 mL acetate buffer, 5 mL $FeCl_3 \cdot 6H_2O$ solution and 5 mL TPTZ solution and Trolox was used as a standard solution. The calibration curve was made for concentrations between 0 and 300 µM, having a correlation coefficient R2 = 0.9956 and the regression equation (y = 0.0017x + 0.0848), where y represents absorbance detected at 595 nm. The results are expressed as µmol Trolox equivalents (TE)/100 g dry weight (DW) (31).

3. Cupric ions (Cu^{2+}) reducing - CUPRAC assay

The antioxidant activity of the extract was evaluated by cupric ions (Cu^{2+}) reducing capacity, method proposed by Apak *et al*. (32) and Karaman *et al*. (33). 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution (7.5 × 10–3 M) and 0.25 mL ammonium acetat buffer solution (1 M) were mixed with the plants extracts. Then, the total volume was adjusted to 2 mL with distilled water. Absorbance was measured at 450 nm, after 30 min, and Trolox was used as a standard solution. Increased absorbance of the reaction mixture indicates high reduction capability. The calibration curve was made for concentrations between 0 and 500 µM and the results were expressed as µmol Trolox equivalent/g DW.

Antimicrobial activity evaluation

Antimicrobial activity of *T. majus* flowers extract was determined by disk diffusion method according to CLSI 2016 methodology (34). The micro-organisms used included reference strains from American Type Culture Collection (ATCC): *Escherichia coli* (ATCC 25922), *Pseudomonas. aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumoniae* (ATCC 49619), *Candida albicans* ATCC 90029 and five clinical isolates of gram-positive bacteria: *Staphylococcus aureus* methicillin-sensible, *Staphylococcus epidermidis* methicillin-resistant, *Streptococcus pyogenes* (Group A Beta haemolytic Streptococcus (GABHS), *Streptococcus agalactiae* (Group B Beta haemolytic Streptococcus (GBBHS) and Group G Beta haemolytic Streptococcus (GGBHS). Appropriate culture media, Mueller-Hinton Agar (Oxoid) for staphylococci, *Escherichia coli* and *Pseudomonas aeruginosa* isolates, Mueller Hinton 2 agar and 5% sheep blood (BioMerieux) for streptococcal strains and Sabouraud Gentamicin Chloramphenicol 2 agar (BioMerieux) for *Candida albicans* were inoculated with the tested microorganisms suspension equivalent to a 0.5 McFarland standard. Sterile filter paper discs of 6 mm diameter (HiMedia Laboratories) impregnated with 20 µl of *T. majus* flowers extract were placed onto inoculated plates. The plates were incubated at 37°C overnight for bacteria and 24 hours at 37°C plus 24 hours at 25°C for *Candida albicans*. In order to assess the antibacterial activity of *T. majus* flowers extract, the inhibition zone diameters were measured in millimetres. Standard disks of Penicillin (10 U; Oxoid), Vancomycin (30 µg; Oxoid), Ofloxacin (5 µg; Oxoid), and Meropenem (10 µg; Oxoid) and Fluconazole (25 µg; BD BBL) were used as positive controls and disks impregnated with 20 ml of distilled water for negative control. To minimize errors, each test was carried out in triplicate.

In vitro experimental data

1. Cell culture

Commercial human umbilical vein endothelial cells (HUVECs) purchased from the European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK) were used. The cells were grown in RPMI medium, supplemented with 10% fetal calf serum (FCS), gentamicin 50 µg/ml, amphotericin 100 µg/ml (Biochrom AG, Berlin, Germany) in a humidified CO_2 atmosphere, at 37°C. Cell cultures in the 23rd to 26th passages were used. The surfaces markers of cells were analysed using flow cytometry (BD FACS Canto II flow cytometer, Becton Dickinson & Company, Franklin Lakes, NJ, USA) and ICAM-1, CD29, CD34, CD73, CD90, CD105 monoclonal antibodies (25). Cells seeded at a density of 104/cm² in cell culture Petri dishes (TPP) were settled for 24 hours in medium and then were exposed for 24 hours to 2.5 µg/ml *T. majus* extract and 1 µM/ml luteolin, as positive control; afterwards the cells were incubated for 24 hours, either in normotonic (137 mmol/l) or hypertonic conditions (200 mmol/l). Cells were collected and treated as previously described (27, 35). The protein level was determined by the Bradford method (Biorad, USA). All the *in vitro* experiments were conducted in triplicate.

2. Cell viability testing

In order to explore the cytotoxicity of *T. majus* extract HUVECs seeded, at a density of 10^4/well, in ELISA 96 wells micro titration flat bottom plaques (TPP, Switzerland), were treated with different concentrations of extract in medium ranging between 6.5 to 500 µg/ml, for 24 hours. The viability testes were done using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay as indicated by the producer. Untreated cultures exposed to medium were used as controls.

3. Oxidative stress assessment

Lipid peroxides were evaluated using 2-thiobarbituric acid (TBA) by Conti method (36) and were expressed as nmoles of malondialdehyde (MDA)/mg protein.

4. Inflammation and apoptosis evaluation

Inflammation was assessed by the measurement of IL-6 levels using ELISA immunoassay kit from R&D Systems, Inc (Minneapolis, USA). The readings were done at 450 nm with a correction wavelength set at 540 nm, by using an ELISA plate reader (Tecan, Austria). In order to assess the apoptosis the expression of p53, Bcl-2 and γH2AX and activity of caspase-8 were evaluated. The caspase-8 colorimetric assay kit was purchased from R&D Systems, Inc (USA) and the results were expressed as unit OD/mg protein. For Western blot analysis, lysates (20 µg protein/lane) were separated by electrophoresis as previously described (27). Samples were separated on SDS PAGE gels and then transferred to polyvinylidenedifluoride membranes, using Biorad Miniprotean system (BioRad). Blots were blocked for 1 hour at room temperature and then incubated with antibodies against p53, Bcl-2, γH2AX and β-actin 1:200. After washing, the blots were incubated with corresponding secondary HRP-linked

antibodies (1:1500) (Santa Cruz Biotechnology). Proteins were detected using Supersignal West Femto Chemiluminiscent substrate (Thermo Fisher Scientific, Rockford IL, USA) and were quantified using Image Lab analysis software (Bio-Rad, Hercules, CA, USA). β-actin was used as protein loading control.

Statistical analysis

The results obtained in the evaluation of *T. majus* antioxidant activity are generated within the one-way ANOVA and Tukey's Multiple comparison test ($P < 0.05$) by using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA). The statistical significance of the difference between *in vitro* groups was evaluated with two-way ANOVA, followed by Dunnett's Multiple test. All reported data were expressed as the mean of triplicate measurements ± standard deviation (SD) and a P value < 0.05 was considered statistically significant.

RESULTS

RP-HPLC analysis

In order to identify and quantify the active principles of the phenolic acid groups, flavonoid glycosides and aglycones from the lyophilized extract of *T. majus,* the HPLC-RP with UV detection were used. Chromatograms of the *T. majus* flowers were recorded at 270, 310 and 360 nm. Through this study we accomplished a more detailed analysis of the polyphenols, using a series of standard compounds, by investigation of the unhydrolyzed and hydrolyzed extracts. The chosen method allows a simultaneous analysis of different classes of polyphenols on one single passing through a column, in a short time interval. Each sample was analyzed before and after the acid hydrolysis in order to obtain more exact data regarding flavonoids and aglycones and to estimate the nature of the respective compounds. The phenolic acids and flavonoid compounds, identified in the analyzed samples, are presented in *Table 1* and were expressed as μg/g or mg/kg DW.

Five glycosides (epicatechin, luteolin, cathechin, rutin and miricetin) were emphasized before hydrolysis, and from the phenol derivates, six compounds: sinapic acid, p-coumaric acid, caffeic acid, ferulic acid, vanillic acid and syiringic acid. The dominant compound of the phenolic acids was the p-coumaric acid (3960.57 ± 12.45 μg/g DW). Regarding the flavonoids, the predominant compound in the extract's composition, before the hydrolysis, is cathechin (1125.66 ± 4.15 μg/g DW). The presence of sinapic acid (101.61 ± 1.23 μg/g DW), gallic acid (1581.79 ± 1.03 μg/g DW), naringenin (1098.44 ± 2.5.5 μg/g DW) and quercetine (425.04 ± 0.65 μg/g DW) in the hydrolysed extract was suggestive, being released from their corresponding glycosylated structures.

Evaluation of antioxidant capacity

To evaluate the antioxidant capacity, we determined the total polyphenol content and the flavonoids amount in the extract by using the Folin-Ciocalteu method (35). The total polyphenols content of the plant extract was expressed in milligram equivalents of gallic acid per gram of dry material (mg GAE/g DW), and the total amount of flavonoids in milligram equivalents of quercetine per gram of dry material (mg QE/g DW); the results are summarized in *Table 2*. The Folin-Ciocalteu method, generally used to assay the total phenol compound content, also measures the total reducing capacity of a sample. Total phenols generally correlate with redox status and antioxidant capacities as measured by the TEAC or DPPH methods. The antioxidant capacity of samples, measured by different complementary assays, is shown in *Table 3*. The antioxidant property of nasturtium flowers was tested by measuring their ability to scavenge DPPH. The highest DPPH inhibition was recorded in the case of *T. majus* flowers extract.

All analyses were mean of triplicate measurements ± SD. Means not sharing a common letter were significantly different at $P \leq 0.05$.

The antimicrobial activity

The aim of our study was to determine the antimicrobial activity of the *T. majus* extract, obtained from Bihor County, on

Table 1. The amount of flavonoids and phenolic acids in the edible flowers of *Tropaeoleum majus* L.

Bioactive compounds	**Group**	**R_T** (min)	**λ_{max}**	**Unhydrolyzed extract** (mg/kg)	**Hydrolyzed extract** (mg/kg)
Epicatechin	Flavanol	10.82	280	879.88 ± 0.05	2430.99 ± 1.05
Luteolin	Flavanol	18.27	345	409.44 ± 2.01	4379.88 ± 1.35
Cathechin	Flavanol	9.46	280	1125.66 ± 4.15	653.01 ± 2.03
Naringenin	Flavanone	19.99	285	0.00	1098.44 ± 2.55
Quercetin	Flavanol	18.3	260; 370	0.00	425.04 ± 0.65
Rutin	Flavanol	13.2	260 – 270; 360	81.97 ± 2.15	0.00
Myricetin	Flavanol	15.78	255; 370	33.63 ± 0.01	44.02 ± 0.08
Synapic acid	Hydroxycinnamic acid	13.47	235; 320	34.48 ± 0.15	101.61 ± 1.23
P-coumaric acid	Hydroxycinnamic acid	12.47	310	3960.57 ± 12.45	757.21 ± 7.08
Caffeic acid	Hydroxycinnamic acid	10.55	320	2695.25 ± 3.03	1552.14 ± 1.25
Ferulic acid	Hydroxycinnamic acid	13.51	320	409.24 ± 0.02	185.61 ± 603
Galic acid	Trihydroxybenzoic acid	4.45	270	0.00	1581.79 ± 1.03
Vanillic acid	Dihydroxybenzoic acid	10.4	259; 292	53.15 ± 2.02	283.27 ± 5.09
Syringic acid	Trihydroxybenzoic acid	11.1	274	1061.26 ± 0.03	895.25 ± 1.22

*All analyses were mean of triplicate measurements ± standard deviation. Means not sharing a common letter were significantly different at $P \leq 0.05$.

Table 2. The total polyphenols and flavonoids content.

Total bioactive compounds	**Lyophilized extract of *T. majus***
Content of total polyphenols (mg GAE/g DW)	552.69 ± 0.01
Total flavonoids mg QE/g DW	1.58 ± 7.61

*Data is expressed as mean ± standard deviation. The results are generated within the one-way ANOVA and Tukey's multiple comparison test ($P < 0.05$).

Table 3. Antioxidant activity of the *T. majus* flowers sample.

Sample	DPPH%	Cuprac (µmol Trolox equivalent/g DW)	FRAP (µmol Trolox equivalent/g DW)
T. majus flowers	84.5 ± 1.11	8.98 ± 5.98	56.69 ± 0.05

Data is expressed as mean ± standard deviation. Different letters in the same column indicate significant differences. The results are generated within the one-way ANOVA and Tukey's multiple comparison test ($P < 0.05$).

Table 4. Antibacterial activity of *Tropaeolum majus* L. flowers extract.

		Zone of growth inhibition (in mm diameter)									
Extract	**Conc**	**Staphylococcus aureus ATCC 25923**	**Streptococcus pneumoniae ATCC 49619**	**E. coli ATCC 25922**	**Pseudomonas aeruginosa ATCC 27853**	**Candida ATCC 90029**	**GABHS**	**GBBHS**	**GGBHS**	**Staphylococcus epidermidis**	**Staphylococcus aureus**
T. majus	100 mg/ml 200 µg/disk	6	8	6	6	6	8	6	8	6	6
Penicillin	10 U	30	26	Not tested	Not tested	Not tested	32	33	34	20	22
Vancomycin	30 µg	18	23	Not tested	Not tested	Not tested	22	20	23	Not tested	Not tested
Ofloxacin	5 µg	27	18	30	19	Not tested	19	20	18	16	24
Meropenem	10 µg	30	31	29	28	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Fluconazole	25 µg	Not tested	Not tested	Not tested	Not tested	32	Not tested	Not tested	Not tested	Not tested	Not tested
Distilled water		6	6	6	6	6	6	6	6	6	6

gram-positive and gram negative bacteria and also on *Candida albicans*. The results of the antimicrobial activity of *T. majus* flowers extract are shown in *Table 4*. In our study, *T. majus* flowers extract exhibited a weak antibacterial effect on *Streptococcus pneumoniae* ATCC 49619, *Streptococcus pyogenes* (Group A β-haemolytic Streptococcus) (GABHS) and Group G β-haemolytic Streptococcus (GGBHS). No antibacterial activity was obtained on staphylococci, gram negative bacteria or *Candida albicans.*

The influence of extract on oxidative stress, inflammation and apoptosis in HUVECs

The impact of osmotic stress response in conjunction with extract administration, on HUVECs, were evaluated at 24 hours with focus on cell viability, oxidative stress, inflammation, DNA lesions and apoptosis. Viability of HUVECs treated with *T. majus* extract was tested across a range of concentrations up to 500 µg/ml. The viability graphs did not show significant changes in cell toxicity at different doses ($P > 0.05$) (*Fig. 1*).

The hypertonic solution has a pro-oxidant activity as suggested by the increased levels of lipid peroxides in endothelial cells ($P < 0.0001$) (*Fig. 2a*). At a dose of 2.5 µg/ml, the *T. majus* extract did not change the malondialdehyde levels in HUVECs, both in the normotonic solution and in the hypertonic environment. Luteolin, in a concentration of 1 µg/ml, significantly decreased the lipid peroxidation compared to the control, both in normotonic and hypertonic conditions ($P < 0.001$) (*Fig. 2a*). The MDA values, obtained after treatment with luteolin, were comparable in the two conditions, suggesting a good protection of luteolin on cells exposed to hyperosmotic stress. In hyperosmotic stress, the misbalance between oxidants and antioxidants is related to the generation of prostaglandins and superoxide anion (37) and activation of xanthine oxidase, nitric oxide (NO) synthase and heme oxygenases. The antioxidant activity of the extract, demonstrated by *in vitro* analyses, was not confirmed, at this dose, in experiments performed on endothelial cells. This result can be explained by the using of low dose of extract or the too short incubation time. In fact, osmotic stress assume the activation of gene involved in

the synthesis and transport of osmolytes, in growth arrest and DNA damage (24); also increases the activation of p53, cytoskeleton remodeling occurs and are released proinflammatory cytokines (38).

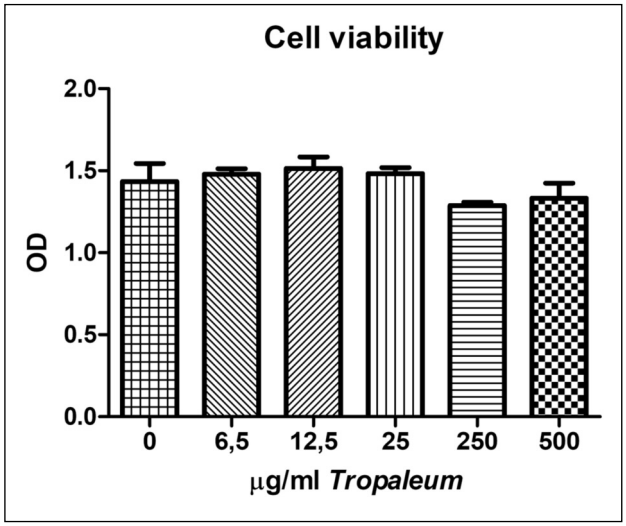

Fig. 1. Cell viability testing. *T. majus* extract was tested across a range of concentrations up to 500 μg/ml in HUVECs without to exhibit significant changes in cell viability (mean values ± standard deviation, n = 3).

In order to evaluate the effect of extract on inflammation, the levels of IL-6 were quantified. In the hypertonic conditions, the IL-6 concentrations increased significantly ($P < 0.01$) compared to normotonic environment (*Fig. 2b*), suggesting that osmotic stress is associated with nonspecific inflammation. The preadministration of *T. majus* and luteolin reduced the IL-6 secretion in endothelial cells exposed to hyperosmotic stress but without statistical significance ($P > 0.05$), compared to normotonic environment. In normotonic conditions the pattern was different; the two compounds used to protect the cells increased the IL-6 secretion but insignificantly ($P > 0.05$). To investigate whether the antioxidant and protective effects were related to the antiapoptotic property of *T. majus* the expression of p53, Bcl-2, γH2AX and activity of caspase-8 were evaluated (*Figs. 3* and *4*).

Western blot analysis revealed a considerably increase of p53 expressions in hypertonic environment compared to normotonic conditions ($P < 0.001$). Interestingly, the two compounds induced high expressions of p53 ($P < 0.001$), both in basic conditions and hypertonic solutions (*Fig. 4a*).

Thus, *T. majus* extract significantly amplified the increasing of p53 expressions induced by exposure to hypertonic conditions ($P < 0.001$). The values were higher compared to those obtained in normotonic conditions. Hypertonicity and pre-treatment with luteolin determined a significant rise of p53 protein expression ($P < 0.001$) compared to cells exposed to only osmotic stress. The p53 levels, in cells pretreated with luteolin, were comparable in the two types of conditions, but the differences between groups were not significant ($P > 0.05$). Bcl-2 is a protein family composed of antiapoptotic and proapoptotic members

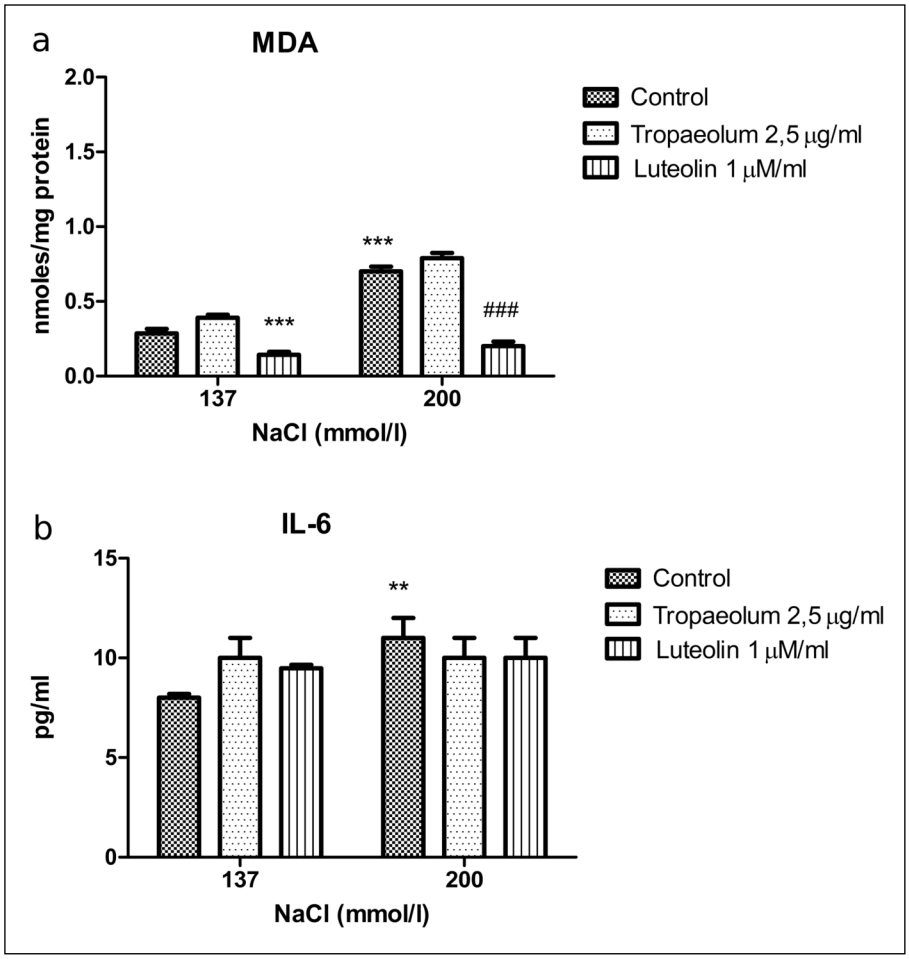

Fig. 2. Malondialdehyde and IL-6 levels in endothelial cells exposed to hyperosmotic stress and pre-treated with *T. majus* extract and luteolin. *a*) The MDA levels after exposure to hyperosmotic stress and pretreatment for 24 hours with *T. majus* extract and luteolin; b) IL-6 levels after exposure to hypertonic conditions and *T. majus* extract respectively luteolin. The statistical significance of the difference between treated and control groups was evaluated with two-way ANOVA, followed by Dunnett's Multiple test, , $^{###}P < 0.001$, treated versus untreated cells in hypertonic medium; $^{**}P < 0.01$, $^{***}P < 0.001$, hypertonic medium versus the parameters in normotonic conditions.

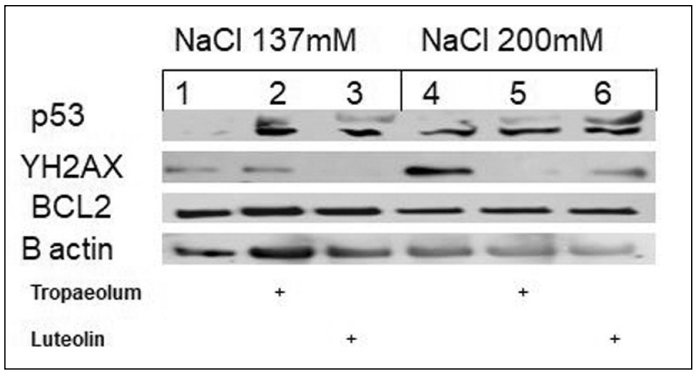

Fig. 3. The expression of p53, γH2AX, Bcl-2 and β-actin in endothelial cells exposed to hyperosmotic stress and pretreated with *T. majus* and luteolin. Comparative Western blot images showing expressions of p53, γH2AX, Bcl-2 and β-actin in HUVECs exposed to normotonic and hypertonic conditions and treated with the two compounds. Western blot images, 1 = cells in normotonic medium, 2 = cells in normotonic medium treated with *T. majus* extract, 3 = cells in normotonic medium treated with luteolin, 4 = cells in hypertonic medium, 5 = cells in hypertonic medium treated with *T. majus* extract, 6 = cells in hypertonic medium treated with luteolin.

that can control the release of mitochondrial apoptogenic factors and activation of caspases and therefore the apoptotic cell death or cell proliferation (39). The antiapoptotic members such as Bcl-2 and Bcl-XL prevent apoptosis by sequestering proforms of caspases and promote cellular survival by inhibiting the release of mitochondrial apoptogenic factors into cytoplasm. In our experiment we found a decreased of Bcl-2 expressions in hypertonic conditions ($P < 0.05$) compared to normotonic medium, an indirect proof for apoptosis as death cell mechanism (*Fig. 4b*). In cells exposed to normotonic conditions and pretreated with luteolin the Bcl-2 expressions significantly exceeded the values observed in untreated cells ($P < 0.001$) while *T. majus* did not exhibit protection. The Bcl-2 expression was significantly improved in hypertonic medium after *T. majus* pretreatment ($P < 0.001$) suggesting the antiapoptotic effect of polyphenols from the extract. In our experimental design, the exposure of cells to hypertonic medium did not induce apoptosis triggered by extrinsic mechanism compared to normotonic system ($P > 0.05$).

Caspase-8 activity, evaluated in the two conditions, had the same pattern of evolution (*Fig. 4c*). Thus, both in normotonic and hypertonic situations, the behaviour of caspase-8 activity was similar ($P > 0.05$), suggesting that the osmotic stress did not induce the increasing of enzyme activity. It is known that caspase-8 is activated after binding to the death receptor of the Fas/tumor necrosis factor receptor family and initiates the apoptotic signal either by directly cleaving and activating downstream caspases or by cleaving the Bcl-2 interacting protein (BID). BID after the translocation into the mitochondria, releases cytochrome c and activates in turn the caspases-9, 3, -7, and -2, -6, -8, -10 and consequently induce the cells' death (40-42). The preadministration of 2.5 μg/ml *T. majus* had a minor antiapoptotic effect while in the pretreatment with luteolin the activity of caspase-8 significantly decreased in endothelial cells exposed to normotonic conditions ($P < 0.001$) (*Fig. 3d*) and consequently diminished the apoptosis triggered by extrinsic mechanism. P53 is considered the 'guardian of genome", a transcription factor that binds to DNA and induces growth arrest for DNA repair or the elimination of a cell irreversible damaged by stress factors (41). In addition, p53 can induce apoptosis after translocation to mitochondria and binding to Bcl-2 family proteins with activation of cytochrome c release and caspases cascade (23, 43).

γH2AX is known as a marker of DNA damage and signifies the formation of DSBs (44). Practically, this protein is the first step in recruiting and localizing DNA repair proteins and can be induced after treatment with cytotoxic agents and environmental and physical damage. Western blot analysis revealed that the hyperosmotic conditions increased γH2AX levels compared to the normotonic medium ($P < 0.001$) (*Fig. 4d*). The pretreatment with *T. majus* reduced the formation of γH2AX and protected the cells from DNA damage induced by hyperosmotic stress ($P < 0.001$). Luteolin administration diminished the osmotic stress, induced DSBs formation, as shown by the significant decrease of γH2AX expression (*Fig. 3a*). In normotonic condition luteolin decreased significantly the nuclear γH2AX foci formation compared to the control group and group pretreated with *T. majus* extract ($P < 0.001$).

DISCUSSION

In our study, the extract exhibited a week antibacterial effect on some strains of streptococcus and increased the p53 and Bcl-2 expressions in parallel with decreasing of DNA lesions, indicating the protective effects on endothelial cells exposed to hyperosmotic stress. The antioxidant activity of extract, measured by different methods, revealed that the *T majus* edible flowers are a promising source of efficient antioxidants. It is known that DPPH radicals is widely used to evaluate the antioxidant properties of natural products. Therefore, a good activity in these tests also suggests that anthocyanins and phenolics present in nasturtium flowers play an important role as antioxidants. The studies below confirm these observations. Thus, Navarro-Gonzales *et al*. compared the profile of phenolic compounds from *T. majus* with those from *Tagetes erecta* and *Spilanthes Oleracea* and found that total phenolic compounds were higher in *T. erecta* than *S. oleracea* and *T. majus,* in correlation with an increased antioxidant capacity (45). Leaves of *T. majus* and *Amaranthus hybridus* L. presented the highest rates of antioxidant activity and an increased amount of total polyphenols compared to *Amaranthus viridis* L., *Basella alba* L., *Eryngium campestre* L., *Hibiscus sabdariffa* L., *Lactuca canadensis* L., *Rumex acetosa* L. *Stachys byzantina* K. Koch and *Xanthosoma sagittifolium* L. (46).

There are few studies on the antimicrobial activity of *T. majus* extract and they show contradictory results. Zanetti *et al*. (47) and Butnariu *et al*. (14) supported an antibacterial effect on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* while other studies showed no antibacterial activity of *T. majus* extract (48, 49). Generally, the antimicrobial action of plant extracts is due to phenolic compounds, especially flavonoids (50). In addition, the glucosinolates identified in the plant of the nasturtium family and released after hydrolysis, can exert antibacterial activity (51). Isothiocyanates belong to the glucosinolates and represent the compounds with the most studied antibacterial effect. They are efficient against gram-positive and gram-negative bacteria (15) and their action involves different mechanisms: preventing biofilm formation, inhibiting bacterial metabolic activity and growth (16), reducing the adhesion of bacteria and increasing the membrane permeabilization and, consequently, inducing cell death (17). According to other authors,

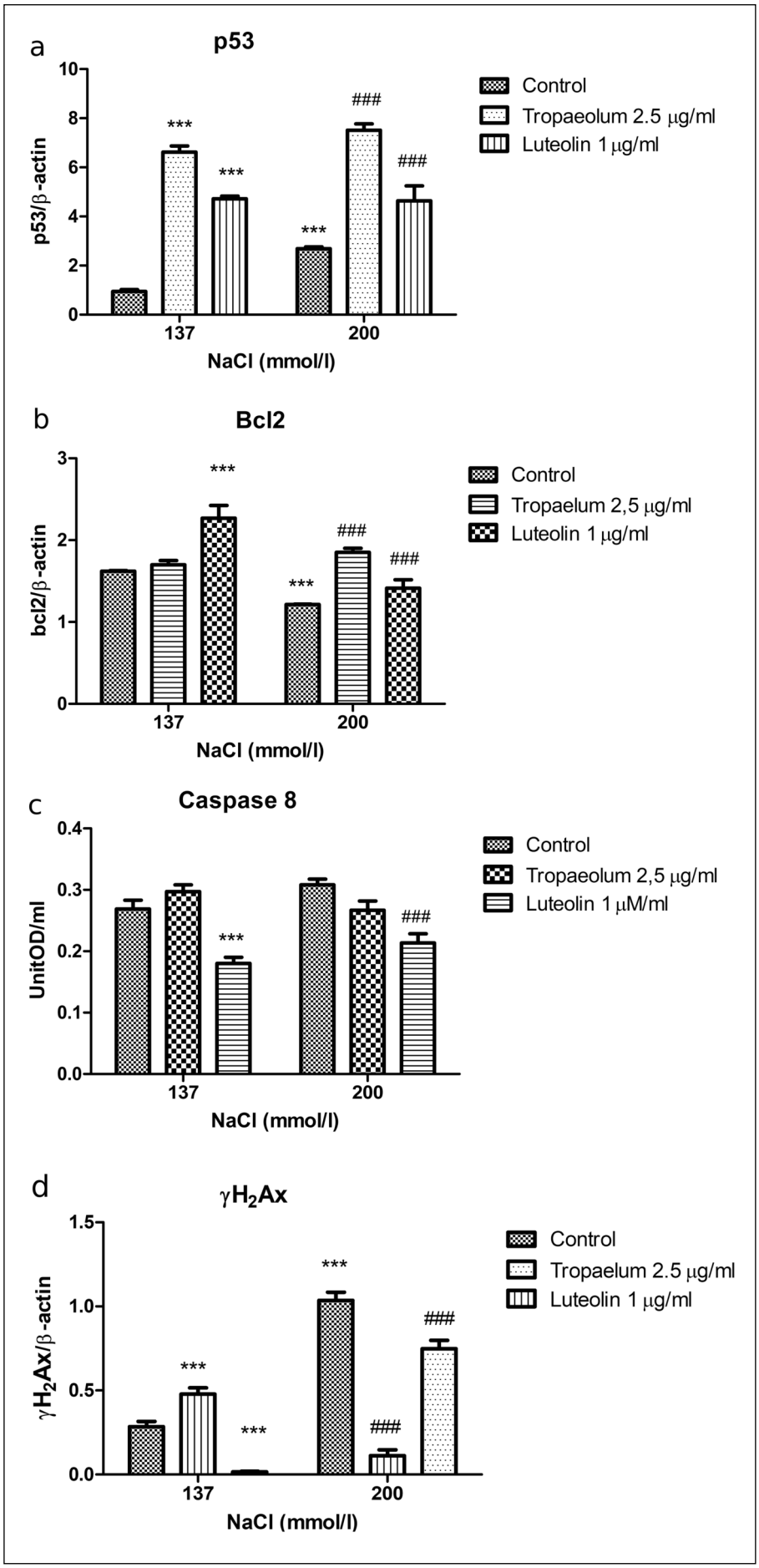

Fig. 4. Graphical representation of the caspase-8 activity and quantitative western blot results for HUVECs in normotonic and hypertonic conditions. Image analysis of Western blot bands' was done by densitometry and the, results were normalised to β-actin. Statistical analysis for the ratio of p53 protein (a), Bcl-2 (b), caspase-8 (c), γH2AX (d) and β-actin expressions in the two conditions. Two-way ANOVA, followed by Dunnett's Multiple test was used to evaluate the statistical significance of differences in the mean values of the measured parameters. Each bar represents mean ± standard deviation (n = 3); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, treated versus untreated cells in hypertonic medium; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, hypertonic medium versus the parameters in normotonic conditions.

the antimicrobial ways of isothiocyanates action are not very well established. It seems that isothiocyanates inhibit respiratory enzymes, induce heat-shock protein response and oxidative stress in bacteria (52). Most likely, the leaves from *T. majus* possess a high concentration of bioactive compounds with antibacterial and antifungal activities while the flowers have only cathechin and a few phenol derivates with antimicrobial effect. Therefore, additional studies should be performed with *T. majus* leaves and its major flavonoid, isoqercitrin, to fully evaluate the antimicrobial activity of this plant.

In hyperosmotic stress are involved several mechanisms: redox imbalance, inflammation, cytoskeleton remodeling and DNA damage and finally p53 activation and cell apoptosis. In normal cells, p53 is maintained at a low level but in the presence of osmotic stress p53 showed a biphasic response: increased in parallel with tonicity of the medium and then decreased when the extracellular tonicity reaches 700 mOsm (38). In our study, the low levels of p53 in normotonic conditions were explained probably by the involvement of MDM2 which mediated its proteasomal degradation (53). In cells exposed to osmotic stress, the p53 expression increased possibly due to the reduction of degradation and increase of p53 phosphorylation at serine/threonine residues (54, 55). It seems that p53 exerted protection and determined the cell cycle delay only at moderate osmolarities (500-600 mOsm) while only in severe situations induced apoptosis and cell death (56, 57). The literature data are in agreement with our results which evidenced a slight increase of caspase-8 activity in the hyperosmotic medium but insignificantly statistically. It seems that there are other mechanisms involved in the apoptosis of cells exposed to severe osmotic stress. Reinehr and Haussinger (58) proposed as mechanisms the implication of increased trafficking of death receptors to membrane and activation of caspase-3 without involvement of p53. Several *in vitro* and *in vivo* studies have shown that polyphenols from plants and fruits induce overexpression of wild-type of p53. Extensive data provide evidence that genistein, luteolin, quercetin, epigalocatechin gallate, curcumin and resveratrol can induce the expression of wild-type of p53 which leads consequently to cells cycle arrest and increase Bax expression associated with apoptosis (59, 60). In our study *T. majus* and luteolin increased the expression of p53, both in normotonic and in hypertonic medium, suggesting the protective role in detection and repair of DNA damage before generation of double stranded breaks lesions. The enhancing of p53 expression does not evolve in parallel with the increase in caspase-8 activity and is associated with an improved expression of Bcl-2, especially after *T. majus* treatment ($P < 0.001$), and also with low levels of double stranded breaks (DBSs). Mechanisms for polyphenols action probably involved the decrease of inflammation and activity of caspase-8 and reduced Bcl-2 degradation, phenomena which may explain the endothelial cell survival. Other authors demonstrated that polyphenols induced vasorelaxation by PI3K/Akt- and Ca^{2+}-eNOS-NO signalling pathway activation in the endothelial cells and subsequent stimulation of the NO-sGC-cGMP in the vascular smooth muscle cells (61). The polyphenols from different fruits proved the anti-adhesive properties and beneficial effect in cardiovascular diseases (62) or can exerted antiproliferative and protective activity against oxidative stress (63). In normotonic situations luteolin showed a good protection and increased Bcl-2 levels ($P < 0.001$), effect that disappears in the hypertonic environment. In these conditions *T. majus* exhibited an antiapoptotic effect demonstrated by high values of Bcl-2 in endothelial cells ($P < 0.001$).

In summary, the experimental evidence acquired in this study supports the conclusion that *T. majus* edible flowers are a promising source of efficient antioxidants. The extract exerted a low antibacterial activity and protected *in vitro* the endothelial cells exposed to hyperosmotic stress in different ways: decrease the apoptosis triggered by extrinsic stimuli, maintaining the balance of antiapoptotic/proapoptotic signalling proteins in the apoptotic cell death and limit the formation of DBSs, protecting thus the DNA integrity. In addition, *T. majus* extract reduced to a lesser extent the nonspecific inflammation determined by hypertonic conditions. However, further complex studies with multiple doses or exposures times are necessary to prove its clinical potency in infections and conditions associated with hypertonic stress.

Abbreviations: γH2AX, H2A histone family, member X; Bax, Bcl-2-like protein 4; BID, Bcl-2 interacting protein; Bcl-2, B-cell lymphoma 2; DSBs, double stranded breaks; DNA, deoxyribonucleic acid; DPPH, 1,1-diphenyl-2-pycrylhydrazyl; DW, dry weight; Fas, CD95 receptor; FRAP, 2,4,6-Tri(2-pyridyl)-s-triazine; HUVECs, human umbilical vein endothelial cells; IL, interleukin; DA, malondialdehyde; MDM2, murine double minute 2; NO, nitric oxide; RE-HPLC, reversed phase-high performance liquid chromatography;

Authors' contributions: Conceived and designed the experiments: T Jurca, D. Olteanu and G.A. Filip. Performed the experiments: D. Olteanu, I. Baldea, E. Marian, L. Vicas, O. Micle and A. Pallag. Analyzed the data: G.A. Filip, M. Muresan and S. Clichici. Contributed reagents/materials/analysis tools: T. Jurca, D. Olteanu, I. Baldea, G.A. Filip, CS, MO, MM. Wrote the paper: T. Jurca, D. Olteanu, G.A. Filip and M. Muresan.

T. Jurca, I. Baldea and D. Olteanu these authors contributed equally to this work

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