ORIGINAL ARTICLE

ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECTS OF *MAHONIA AQUIFOLIUM* LEAVES AND BARK EXTRACTS

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Abstract

Oxidative stress and inflammation are interlinked processes that seem to play an important role in aging. The present work aimed to test the antioxidant and anti-inflammatory activity of ethanolic *Mahonia aquifolium* leaves and bark extracts in an experimental acute inflammation. Six polyphenols and four alkaloids were measured by HPLC. The radical scavenging activity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) test. Inflammation was induced in rat with turpentine oil. Anti-inflammatory activity was evaluated with serum nitric oxide (NOx) and tumour necrosis factor alpha (TNF-alpha), and oxidative stress with total oxidative status (TOS), total antioxidant reactivity (TAR), oxidative stress index (OSI), 3-nitrotyrosine (3NT), malondialdehyde (MDA) and total thiols (SH). Extracts were administrated orally (100%, 50%, 25%) for seven days prior to inflammation. The effects were compared to diclofenac. The most abundant polyphenol was chlorogenic acid, and alkaloids were identified only in the bark extract. The DPPH assay showed good results, except for the bark extract. All extracts decreased NOx, TOS, 3NT, and increased SH. TNF-alpha was reduced, and TAR was increased only by the leaves extract. MDA was not influenced. Our findings suggest that *M. aquifolium* leaves and bark extracts have anti-inflammatory and antioxidant effects that support the use in primary prevention of the "inflammaging" process.

Rezumat

Lucrarea a urmărit evaluarea activității antioxidante și antiinflamatorii a extractelor etanolice de frunze și scoarță de *Mahonia aquifolium* într-un model experimental de inflamație acută. Șase polifenoli și patru alcaloizi au fost cuantificați printr-o metodă HPLC. Activitatea antioxidantă a fost măsurată prin testul 1,1-difenill-2-picrilhidrazil (DPPH). Inflamația a fost indusă la șobolan cu ulei de terebentină. Activitatea antiinflamatorie a fost evaluată prin măsurarea nitriților și nitraților din ser, a TNF-alfa și a stresului oxidativ total, reactivitatea totală antioxidantă, indicele de stres oxidativ, 3-nitrotirozina, malondialdehida și tiolii totali. Extractele au fost administrate pe cale orală (100%, 50%, 25%) timp de șapte zile înainte de inflamație. Efectele au fost comparate cu cele ale diclofenacului. În cantitate mare a fost regăsit acidul clorogenic, iar alcaloizii au fost identificați numai în extractul de scoarță. Testul DPPH a reliefat acțiunea antioxidantă pentru frunze. Constatările noastre indică faptul că frunzele și extractele din scoarță de *M. aquifolium* au efecte antiinflamatorii și antioxidante.

Keywords: *Mahonia aquifolium*, anti-inflammatory, antioxidant, polyphenols, alkaloids

Introduction

Aging is a complex process that leads to gradual decrease of organ function. Oxidative stress and inflammation seem to play an important role in the biology of aging [46].

The immune cells produce oxidants and inflammatory mediators necessary for the destruction of pathogens

and tumour cells [6]. The aerobic organisms also generate enzymes and non-enzymatic antioxidant compounds as an antioxidant defences. A loss of the equilibrium between the oxidants and the anti-oxidants will cause oxidative stress and damage of cell components, including proteins, lipids and DNA. In the same time, excessive anti-inflammatory defence induces an inflammatory stress. Moreover, it is currently accepted that oxidation and inflammation are interlinked processes, because overproduction of reactive oxygen species (ROS) can induce an inflammatory response, and inflammatory mediators can induce oxidative stress [6].

The low-grade inflammatory status associated with aging, known as "inflammaging", was proved by the increased plasma levels of pro-inflammatory cytokines, acute phase reactants, and soluble cytokine receptors. However, it is not clear whether this imbalance is a cause or the consequence of aging [49].

Phytochemical studies demonstrated that medicinal plants are a rich source of antioxidant compounds such as phenolics, flavonoids, quinones, vitamins, coumarins and alkaloids [58]. A healthy lifestyle with a controlled diet rich in plant derived antioxidant bioactive nutrients may be useful in primary prevention of oxidative stress induced diseases and aging [19].

The *Berberidaceae* family contains nine genera and 590 species native to the northern hemisphere and South America. The genus *Mahonia* Nuttall is the second largest genus in the *Berberidaceae* family, and contains approximately 70 species that are native to Eastern Asia, North America, and Central America [13]. *Mahonia* plants have been widely used in traditional medicine for a long time. It was proved to have antioxidant, anti-inflammatory [60], antifungal [3], antimicrobial [30], antiproliferative [26], hepato-protective and analgesic effects [26].

Mahonia aquifolium (Pursh) Nutt. (*M. aquifolium*) is one of the most abundant plants of this genus and a cultivated medicinal plant [26]. It has been used to treat fever, diarrhoea, dyspepsia, gout, rheumatic ailments, renal and biliary diseases and particularly for chronic relapsing dermatoses [18, 26, 40]. The phytochemical studies of the *Mahonia* species have focused on alkaloids, such as berberine, palmatine and jatrorrhizine, regarded as the major constituents [23].

The amount of bioactive compounds varies with cultivar, soil composition, climate, geographic origin and cultivation practices or exposure to diseases [47]. The phytochemical profile of every plant extracts differs from every other plant, but it also differs depending upon the particular organ of a given plant [62]. It has been also reported that the extraction method can affect the phytochemical profile of the extracts [45]. Therefore, more phytochemical and pharmacological studies on *Mahonia* species are still needed in order to identify the mechanisms underlying the biological activities.

The present work aimed to perform a phytochemical analysis and to investigate the antioxidant potential and the anti-inflammatory activity of ethanolic *M. aquifolium* leaves and bark extracts in an experimental acute inflammation model.

Materials and Methods

Plant material

Fresh *Mahonia aquifolium* (Pursh) Nutt. leaves and bark were purchased from the "Alexandru Borza" Botanical Garden "Babeş-Bolyai" University, Cluj-Napoca, Romania, between April and June 2015 and extracted with 70% ethanol (Merck, Germany) in the Mycology Laboratory of "Babeş-Bolyai" University, Cluj-Napoca, Romania, by a modified Squibb repercolation method, producing the following extracts of *M. aquifolium*: leaves extract 1:1.2 (g:mL) (ML) and bark extract 1:1.5 (g:mL) (MB) [43]. The plants were taxonomically identified, authenticated and voucher specimens (No. 665978) were deposited in the Herbarium of "Alexandru Borza" Botanical Garden, "Babeş-Bolyai" University, Cluj-Napoca, Romania.

High-performance liquid chromatography (HPLC) analysis of polyphenols and alkaloids

A high-performance liquid chromatography with a diode-array detector (HPLC-DAD) was used to separate and quantitatively determinate the compounds of interest. In the first chromatographic approach, the assays were performed on an Agilent 1200 HPLC system (Waldbronn, Germany) equipped with an on-line vacuum degasser, quaternary pump, temperature controlled sample tray, automatic injector, a column thermostat compartment and a DAD detector. The chromatographic separations were run on a Nucleosil 100 C18 column (240 mm x 4.6 mm, 5 µm particle size) from Macherey-Nagel (Düren, Germany). The injection volume was 5 µL (0.2 µm filtered extract), the column temperature was set to 25°C and the flow rate was 1.2 mL/min. Several preliminary tests were employed for method optimization by varying the experimental conditions. The optimum method consisted of a gradient elution using solvent A, 10 mM ammonium acetate pH 5 and solvent B as acetonitrile. The gradient was as follows: 0 - 15 min from 8 to 30% B, 15 - 25 min isocratic at 30% B, 25 - 35 min from 30% to 85% B, 35 - 38 min from 85% to 95% B, 38 - 39 min isocratic at 95% B and 39 - 39.1 min back to 8% B where was kept until 40 min. As standards, there were chlorogenic acid, p-coumaric acid, ferulic acid, rutin, isoquercitrin, quercetin, berbamine, jatrorrhizine, palmatine and berberine, all of analytical grade purity for different commercial available sources (Sigma, Germany). Calibration curve was constructed for each compound at 11, 22, 44, 88, 175, 340 µg/mL using the area of the peak by integration employed by the Agilent soft. The UV-Vis

detection of the compounds has been accomplished using the DAD detector that measured the entire spectrum in 210 - 700 nm region, every 1 s and the chromatograms were monitored at 220, 280, 340 and 425 nm. The identification of the compounds was employed by both chromatographic retention time (with a 0.3 s as tolerance) and spectral similarities (higher than 99.9% was considered as positive) which were done by the built-in soft. The chromatograms were exported and the graphs were developed in Excel.

In vitro antioxidant effects

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was used for the evaluation of the antioxidant capacity of the investigated extracts. Briefly, in 3 mL of each diluted extract 1mL of methanolic solution of DPPH 0.1 mM was added. Blanks were included by replacing the extracts volumes with methanol/water. The mixtures were kept in the dark at room temperature for 30 min and the absorbance was measured at 517 nm against blank. The following equation was used to determine the percentage of the radical scavenging activity of each extract:

The percentage of radical scavenging activity was calculated as follows: (AA%) = [(OD control - OD sample)/OD control] x 100, where OD control is the absorbance of methanolic solution of DPPH (containing all reagents except the sample) and OD sample is the absorbance of DPPH radical and sample extract. The percentage of DPPH consumption was converted to Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid) equivalents using a calibration curve (R^2 = 0.985) of Trolox standard solutions (0.5 - 5 µg/mL). The IC_{50} value, which means the concentration of sample required to scavenge 50% of DPPH free radicals, was calculated [7].

Experimental design

The experiments were carried out on male Albino Wistar rats, weighing 200 - 250 g, that were bred in the Animal Facility of "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. The animals were housed in standard polypropylene cages (five *per* cage) under controlled conditions (12 h light/dark cycle, at an average temperature of 21 - 22°C), and had free access to standard pellet diet ("Cantacuzino" Institute, Bucharest, Romania) and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania. The animals were randomly assigned to nine groups (n = 5). The tested extracts were *M. aquifolium* leaves (ML) and bark (MB). Each extract was administrated orally by gavage (1 mL/animal) in three dilutions (100%, 50%, 25%) for seven days. Rats from the negative control group (CONTROL) and from the positive inflammation group (INFLAM) tap water (1 mL/animal) received by gavage for seven days. An anti-inflammatory control treated for seven days with diclofenac (10 mg/kg body-weight (bw)) (DICLO) was also used [4, 48]. In the 8th day, inflammation was induced by i.m. injection of turpentine oil (6 mL/kg bw) in the animals treated with the extracts, in INFLAM and DICLO groups [17]. CONTROL animals were injected intramuscularly (i.m.) with 0.9% saline. Twenty-four hours after the inflammation induction, the rats were anesthetized using a combination of 60 mg/kg bw ketamine and 15 mg/kg bw xylazine [17], and blood was with-drawn by retro-orbital puncture, and serum was stored at -80°C until use. The experiments were performed in triplicate. All the animals were used only once and they were killed by cervical dislocation immediately after the assay.

Anti-inflammatory effect evaluation

The anti-inflammatory effects of the plant extracts were determined by measuring the inhibition of nitric oxide (NO) production and tumour necrosis factor alpha (TNF-alpha).

The Griess reaction was used to indirectly determine the NO synthesis (NOx). Serum proteins were removed by extraction with a 3:1 (v:v) solution of methanol/diethyl ether [20]. The sample methanol/ diethyl ether ratio was 1:9 (v:v) . In brief, 100 µL of 8 mg/mL vanadium (III) chloride (VCl_3) was added to 100 µL of filtered and extracted serum supernatant in order to reduce nitrate to nitrite, followed by the addition of the Griess reagents, 50 µL of sulphanil-amide (SULF) (2%) and 50 µL of N-(1-Naphthyl)-ethylenediamine dihydrochloride (NEDD) (0.1%). After 30 min incubation at 37°C, the sample absorbance was read at 540 nm. The concentration of serum NOx was determined using a sodium nitrite-based curve, and expressed as nitrite µmol/L [8].

Serum TNF-alpha was measured using rat ELISA kit (MBS175904) that applies the quantitative sandwich enzyme immunoassay technique.

Antioxidative effect evaluation

The total serum oxidative status (TOS) was measured using a colorimetric assay [15]. This assay measures the oxidation of ferrous ion to ferric ion in the presence of various reactive oxygen species in an acidic medium. The ferric ions were detected by reaction with xylenol orange. Assay measurements were standardized using hydrogen peroxide (H_2O_2) as the oxidative species, and the assay results are expressed in µmol H_2O_2 Equiv/L.

The total antioxidant response (TAR) was measured in serum using a colorimetric assay [16]. In this assay, the rate of hydroxyl radical production by the Fenton reaction was monitored by following the changes in the absorbance of coloured dianisidyl radicals. Upon addition of a serum sample, the hydroxyl radical initiated oxidative reactions are suppressed by the anti-oxidants present in the serum. Inhibition of

dianisidyl oxidation prevents the subsequent colour change, thereby effectively measuring the total antioxidant capacity of the serum. This assay was calibrated using Trolox and results are expressed as mmol Trolox Equiv/L.

The ratio of the TOS to the TAR represents the oxidative stress index (OSI), an indicator of the degree of oxidative stress [22]: OSI (Arbitrary Unit) = TOS (mol H_2O_2 Equiv/L)/TAR (mmol Trolox Equiv/L) [9].

The 3-nitrotyrosine (3NT) was measured using rat ELISA kit (MBS732683) that applies the quantitative sandwich enzyme immunoassay technique.

The malondialdehyde (MDA) was assessed as a lipid peroxidation marker, using thiobarbituric acid, as previously described [12]. Briefly, 150 µL of serum was reacted with 125 µL 10% trichloroacetic acid (TCA), 125 µL 5 mM ethylenediaminetetraacetic acid, 125 µL 8% sodium dodecyl sulphate and 10 µL 0.5 µg/mL butylated hydroxytoluene. After vortexing vigorously for 30 s, the mixture was incubated for 10 min at room temperature. Than 500 µL 0.6% thiobarbituric acid was added, and the mixture was heated at 95ºC for 30 min. After cooling to room temperature, the mixture was centrifuged at 10,000 xg for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was generated with a 1,1,3,3-tetraethoxypropane standard (0.3 - 10 nmol/mL). Serum MDA concentration was expressed as nmol/mL of serum.

The total thiols (SH) were estimated using Ellman's reagent [25]. In a final volume of 4.0 mL we added 0.2 mL serum and 0.6 mL of 20 mM tris-HCl buffer pH 8.2, followed by 0.04 mL of 10 mM 5,5'-di-thiobis(2-nitrobenzoic acid) (DTNB) in absolute methanol and 3.16 mL of absolute methanol. The tubes were capped and colour was developed for 15 min at room temperature. The tubes were then centrifuged at 3,000 xg for 20 min. Supernatant absorbance was measured at 412 nm. To create a standard curve, solutions of glutathione (GSH) concentration, ranging from 0.25 to 2 mM GSH, were used. Serum SH concentration was expressed as mmol GSH/mL.

All of the spectroscopic measurements were performed using a Jasco V-530 UV-Vis spectrophotometer (Jasco International Co., Ltd., Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± standard deviation (SD), averaged over at least three independent experiments for normally distributed data. Otherwise, the median and first quartile (Q1) and third quartile (Q3) were reported. Comparisons among groups, in all studied parameters, were analysed by using one-way analysis of variance (ANOVA) test and Bonferroni-Holm *post-hoc* test. A $p < 0.05$ was considered statistically significant. Correlations among data were calculated using Pearson's correlation coefficient (r). All analyses were performed using SPSS version 16 (SPSS Inc, Chicago, IL, USA).

Results and Discussion

Phytochemical analysis

Six polyphenols (chlorogenic acid, p-coumaric acid, ferulic acid, rutin, isoquercitrin, quercetin), and four alkaloids (berbamine, jatrorrhizine, palmatine, berberine) were measured (Table I, Figure 1). In the ethanolic extracts of *M. aquifolium*, three hydroxy-cinnamic acid derivates, namely chlorogenic acid, ferulic acid and p-coumaric acid were identified and quantified (Table I). The most abundant phenolic acid was chlorogenic acid. ML had a higher content of chlorogenic acid (5049 ± 25 µg/mL) than MB (18.1 ± 4 µg/mL). p-Coumaric acid had a higher concentration in MB (5.5 ± 0.2 µg/mL) than in ML (4.3 ± 1.0µg/mL). Ferulic acid was more abundant in ML (11.8 ± 1.9 µg/mL) than in MB (9.2 ± 0.0 µg/mL). Two flavonoid glycosides, rutin and isoquercitrin were found in all studied extracts. Rutin was found in a higher concentration in ML (371 ± 18 µg/mL) and then in MB (268 ± 57 µg/mL). Isoquercitrin was most abundant in ML (217 ± 7.6 µg/mL) and then in MB (47.5 ± 3.2 µg/mL). In all the samples, quercetin was under the limit of detection (LOD).

Table I

Elution time, analytic parameters and found concentrations of the determined compounds in the five studied samples

No	Compounds	$t_{elution}$ (min)	R^2	LOD (µg/mL)	LOQ (µg/mL)	Sample1 MB (µg/mL)	Sample2 ML (µg/mL)
1	chlorogenic ac.	5.41	0.9991	3.2	9.8	18.1 ± 4	5049 ± 25
2	p-coumaric ac.	9.17	0.9999	1.3	4.0	5.5 ± 0.2	4.3 ± 1.0
3	ferulic ac.	10.07	0.9998	1.4	4.2	9.2 ± 0.0	11.8 ± 1.9
4	rutin	14.55	0.9996	2.7	8.1	268 ± 57	371 ± 18
5	isoquercitrin	15.34	0.9995	1.7	5.2	47.5 ± 3.2	217 ± 7.6
6	quercetin	24.1	0.9949	13.7	41.6	< LOD	< LOD
7	berbamine	25.5	0.9997	2.4	7.3	139.7	< LOD
8	jatrorrhizine	30.57	0.9994	2.7	8.3	1902.1	< LOD
9	palmatine	34.96	0.9998	1.7	5.2	427.9	< LOD
10	berberine	36.08	0.9996	2.1	6.4	1294.4	< LOD

M. aquifolium: ML – leaves, MB – bark; LOD – limit of detection, LOQ – limit of quantification, R^2 – coefficient of determination for the calibration curves (at six levels of concentrations).

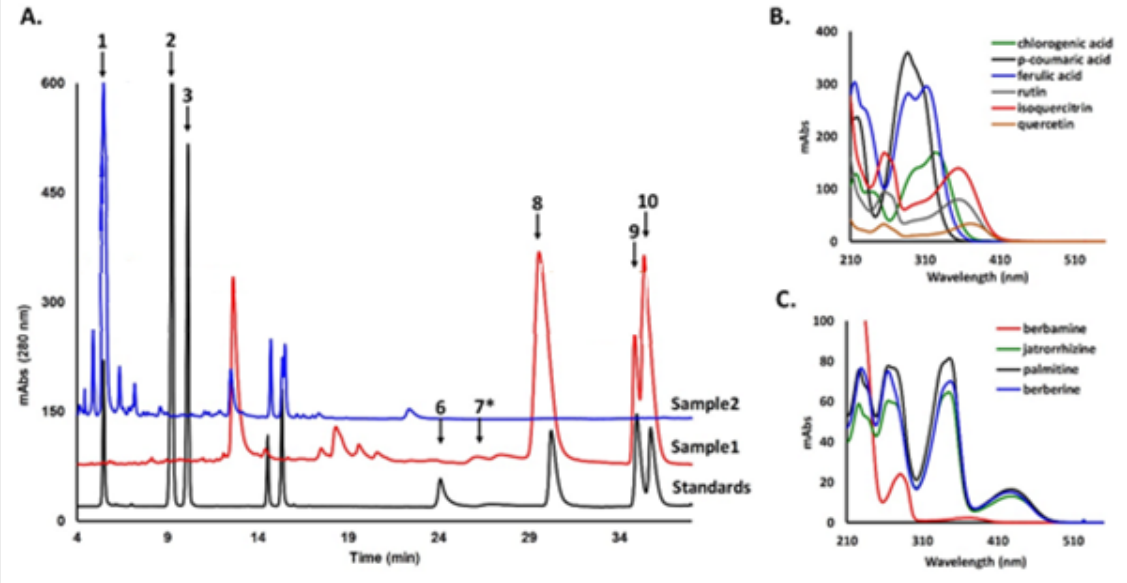

Figure 1.

A. Chromatograms at 280 nm of the studied samples. The ten standards are indicated by arrows and numbers. Berbamine (7*) is barely visible in 280 nm chromatogram, but it is much better detected and quantified separately from 220 nm chromatogram. B. HPLC-DAD registered absorption molecular spectra in UV-Vis domain for the polyphenolic standards at 350 µg/mL C. HPLC-DAD registered absorption molecular spectra in UV-Vis domain for the alkaloids standards at 350 µg/mL

1 – chlorogenic acid, 2 – p-coumaric acid, 3 – ferulic acid, 4 – rutin, 5 – isoquercitrin, 6 – quercetin, 7 – berbamine, 8 – jatrorrhizine, 9 – palmatine, 10 – berberine, Sample 1 – *Mahonia aquifolium* bark, Sample 2 – *Mahonia aquifolium* leaves

The tested alkaloids were identified in concentrations above LOD only in MB, respectively jatrorrhizine 1902.1 µg/mL, berbamine 139.7 µg/mL, palmatine 427.9 µg/mL, and berberine 1294.4 µg/mL.

In vitro antioxidant activity

The ethanolic extracts of *M. aquifolium* showed good DPPH radical scavenging activity as its absorbance increases with the concentration at 517nm (Table II). Trolox IC_{50} was 11.2 µg/mL. An IC_{50} between 50 - 100 µg/mL proves a good anti-oxidant activity, and between 100 - 200 µg/mL there is a weak antioxidant activity. So ML IC_{50} (72.33 µg/mL), MB IC_{50} (77.12 µg/mL) had a good antioxidant activity.

Table II

DPPH radical scavenging activity of *M. aquifolium* extracts

ML		MB	
µg/mL	AA%	µg/mL	AA%
500	92.27	375	90.17
312.37	85.9	250	82.91
250	79.31	125	71.39
208.25	52.84	62.5	60

M. aquifolium: ML – leaves, MB – bark; AA% – percentage of radical scavenging activity

In vivo anti-inflammatory and antioxidant effects

Inflammation increased NOx synthesis significantly ($p < 0.01$) and diclofenac caused a good reduction ($p < 0.01$). Compared to the inflammation group, ML extract reduced NOx significantly ($p < 0.01$), ML25 being the most efficient. ML effects were comparable to that of diclofenac ($p > 0.05$). MB50 and MB25 had a small inhibitory effect on NOx ($p < 0.05$) and this effect was comparable with that of diclofenac ($p > 0.05$) (Table III).

Table III

In vivo anti-inflammatory effects of *M. aquifolium* extracts

	NOx (µmol/L)	TNF
CONTROL	48.489 ± 12.975	116.634 ± 0.678
INFLAM	75.234 ± 12.136**	140.594 ± 15.960***
DICLO	52.318 ± 5.389**	127.228 ± 10.332
ML100%	51.641 ± 4.320**	121.782 ± 15.815
ML50%	50.050 ± 7.722**	121.535 ± 6.572*
ML25%	47.900 ± 3.536***	123.515 ± 27.804
MB100%	66.515 ± 11.663	128.327 ± 1.360
MB50%	65.926 ± 9.435	150.297 ± 11.260
MB25%	68.548 ± 11.401	154.703 ± 11.377

3NT – 3-nitrotyrosine; *M. aquifolium*: ML – leaves, MB – bark; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Inflammation was associated with an important increase of TNF-alpha ($p < 0.01$). From the tested extracts only ML50 reduced TNF-alpha ($p < 0.05$). The rest of the extracts had no significant effect on TNF-alpha ($p > 0.05$) (Table III).

TOS analysis showed that inflammation caused an important increase ($p < 0.01$) of oxidative stress and diclofenac a significant reduction ($p < 0.001$). Compared to the inflammation group, only ML 100 had a significant inhibitory effect ($p < 0.05$), and it was smaller than diclofenac's effect ($p < 0.05$). ML100

and ML50 reduced TOS ($p < 0.01$), but the effect was smaller than that of diclofenac ($p < 0.05$). MB extract had better inhibitory effect on TOS at higher dilutions, MB50 ($p < 0.05$) and MB25 ($p < 0.01$), but it was weaker that diclofenac ($p > 0.05$) (Table IV).

Table IV

In vivo antioxidant effects of *M. aquifolium* extracts

	TOS (μmol H_2O_2 Equiv./L)	TAR (mmol Trolox Equiv./L)	OSI	3NT	MDA (nmol MDA/L)	SH (mmol GSH/L)
CONTROL	29.588 ± 1.85	1.090 ± 0.001	27.139 ± 1.676	0.348 ± 0.063	4.316 ± 0.898	0.679 ± 0.095
INFLAM	41.583 ± 6.55**	1.088 ± 0.0007*	38.208 ± 6.009**	0.674 ± 0.180**	7.628 ± 0.626***	0.425 ± 0.065***
DICLO	24.920 ± 3.02***	1.089 ± 0.0004*	22.879 ± 2.77***	0.298 ± 0.024***	5.496 ± 0.724***	0.563 ± 0.056**
ML100%	32.378 ± 4.28*	1.090 ± 0.001**	29.702 ± 3.930*	0.314 ± 0.043**	6.958 ± 0.486	0.631 ± 0.031**
ML50%	34.256 ± 5.51	1.091 ± 0.0009***	31.394 ± 5.048*	0.293 ± 0.038***	6.947 ± 0.567	0.660 ± 0.068**
ML25%	34.438 ± 8.18	1.092 ± 0.0010***	31.544 ± 7.511*	0.285 ± 0.030***	7.348 ± 0.298	0.626 ± 0.120**
MB100%	33.917 ± 6.23	1.088 ± 0.0012	31.163 ± 5.697	0.304 ± 0.070**	7.259 ± 0.504	0.731 ± 0.156**
MB50%	31.648 ± 6.75*	1.088 ± 0.0005	29.092 ± 6.205*	0.413 ± 0.103*	6.698 ± 0.624*	0.630 ± 0.104**
MB25%	29.588 ± 2.15**	1.088 ± 0.0002	27.192 ± 1.976**	0.397 ± 0.238	6.136 ± 0.456**	0.697 ± 0.132**

TOS – total oxidative status; TAR – total antioxidant reactivity; OSI – oxidative stress index; 3NT – 3-nitrithyrosine; MDA – malondialdehyde; SH – total thiols; *M. aquifolium*: ML – leaves, MB – bark; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Experimental inflammation reduced TAR ($p < 0.05$), and diclofenac caused a small increase of TAR ($p < 0.05$). ML increased significantly TAR ($p < 0.001$) and the effect was better than diclofenac ($p < 0.01$). MB extracts had no significant effects on TAR ($p > 0.05$) (Table IV).

Inflammation increased OSI ($p < 0.01$) and diclofenac reduced OSI ($p < 0.01$). ML100 extract decreased OSI ($p < 0.05$), but the effect was smaller than that of diclofenac ($p < 0.05$). MB extracts were good OSI inhibitors, respectively MB100 ($p < 0.001$), MB50 ($p < 0.05$) and MB25 ($p < 0.01$). Compared to diclofenac MB had a smaller activity on OSI ($p < 0.05$) (Table IV).

Inflammation caused an important increase of 3NT ($p < 0.01$) and diclofenac reduced 3NT significantly ($p < 0.001$). All ML dilutions, respectively ML100 ($p < 0.01$), ML50 and ML25 ($p < 0.001$), decreased 3NT. From the MB extracts only MB100 ($p < 0.01$) and MB50 ($p < 0.05$) had an important inhibitory activity on 3NT (Table IV).

Inflammation caused an increase MDA production ($p < 0.001$), and diclofenac an important decrease of MDA ($p < 0.001$). All *M. aquifolium* extracts had no significant effect on MDA ($p > 0.05$) (Table IV).

SH was reduced by the inflammation ($p < 0.001$) and diclofenac treatment increased it ($p < 0.01$). All *M. aquifolium* extracts increased SH ($p < 0.01$ - 0.001), the best being the higher concentrations. Compared to diclofenac, *M. aquifolium* extracts had better stimulatory activity on SH (Table IV).

The study of biological effects of plant extracts, including the phytochemical analysis, is a topic that focuses the attention of numerous scientists. The present study first analysed the phytochemical composition of the ethanolic *M. aquifolium* leaves and bark extracts, and then evaluated whether the extracts had anti-inflammatory and antioxidant activity in the rat turpentine oil induced-inflammation model.

These phytochemical analyses suggested the possible anti-inflammatory and antioxidative effects of the *M. aquifolium* extracts. In the ethanolic *M. aquifolium* extracts, three hydroxycinnamic acid derivates were measured. In all tested extracts, the principal component was chlorogenic acid. The higher content of chlorogenic acid was in the ML. Chlorogenic acid is a polyphenolic compound that has antioxidant and anti-inflammatory effects. The antioxidant effects of chlorogenic acid are due to the hydroxyl groups contained in the aromatic ring [51]. The anti-inflammatory effects of chlorogenic acid are due to the suppressive action on the NFkB signalling pathway, and reduction of IL-6, IL-8 and TNF-alpha pro-inflammatory cytokines [42].

Ferulic acid and p-coumaric acid were found in lower concentrations. Ferulic acid was higher in ML, and p-coumaric acid in MB. Ferulic acid possesses anti-inflammatory, antioxidant [2, 32, 54], anti-apoptotic, anti-carcinogenic, antidiabetic, hepatoprotective, cardio-protective properties [21] and antidepressant effects [34]. The p-coumaric acid has antioxidant and anti-apoptotic [36, 53] effects, due to its antioxidant capacity it has an immunomodulatory effect, and it also reduces serum glucose levels and improves lipid metabolism [1, 39]. The anti-cancer effects of p-coumaric acid and ferulic acid are believed to be due to the antioxidant properties [50]. The higher levels of the three measured hydroxycinnamic acid derivates correlated with a better antioxidant activity evaluated by DDPH for MF, compared to ML and the other extracts.

From the three measured flavonoid glycosides, only rutin and isoquercitrin were found in all *M. aquifolium* extracts. Quercetin was under the LOD. Rutin is a flavonoid with antioxidant [28], anti-inflammatory and antiapoptotic [35] properties. Antioxidant activity seemed to be mediated by the stimulation of the antioxidant enzymes activity [55, 61]. Rutin anti-inflammatory effects consisted of pro-inflammatory cytokines reduction. The high rutin concentration in ML and MB correlated with the good DPPH scavenging activity. Isoquercitrin can both behave as antioxidants

in an indirect (i.e., Fe^{2+}-chelating) and direct manner to scavenge ROS [31]. The higher level of isoquercitrin in ML can explain the good antioxidant effect of ML.

The isoquinoline alkaloids are the major subclass of alkaloids of the genus *Mahonia*. The identified alkaloids belong to three major classes: protoberberines, aporphines and bis-benzyl-isoquinolines. Berberine is the most widely distributed alkaloid in *Mahonia* species but other protoberberines, including palmatine, jatrorrhizine, berbamine, columbamine and coptisine were also found in these species [23].

The analysis of *M. aquifolium* extracts determined the presence of berberine, palmatine, jatrorrhizine and berbamine only in the MB extract. *M. aquifolium* leafs, fruits and flowers extracts contained only trace amounts of this alkaloids that were under LOD.

In the MB extract jatrorrhizine and berberine were found in the highest quantities, palmatine and berbamine having much smaller concentration. Jatrorrhizine has antioxidant, antifungal, antibacterial, antiplasmodial, anti-amoeba, antimutagenic [38], anti-apoptotic [27], antitumoural [33] effects, it is a mono-amine oxidase (MAO) inhibitor [37] and reduces cholesterol [59]. Berberine has antioxidant effect [10], has anti-inflammatory properties due to the inhibition of IL-1 and TNF-alpha, and exerts antibacterial, anti-viral and antifungal activity. These data may explain the good *in vitro* antioxidant activity of MB evaluate by DPPH.

The present study evaluated whether *M. aquifolium* extracts had anti-inflammatory and antioxidant activities in an experimental inflammation. The experimental inflammation was induced in rats by injecting turpentine oil, which is like carrageenan a non-antigenic inflammatory stimulus [52] that activates inflammatory cytokines and NO release. High rats' serum levels of TNF-alpha and NOx were markers of the inflammatory response. Treatment with *M. aquifolium* extract ML50 reduced TNF-alpha. NOx was decreased by the *M. aquifolium* ML and MB extracts. NO is a ubiquitous modulator of physiological to pathophysiological biological processes. There are three isoforms of nitric oxide synthase (NOS). The constitutive isoforms, endothelial and neuronal NOS (eNOS/NOS3 and nNOS/NOS1), produce low cellular levels of NO. Inducible NOS (iNOS/NOS2) upon induction can produce sustained NO fluxes in the micromolar range for days, being able to produce NO over four orders of magnitude [56]. NO acts as a "double-edged sword", cytostatic and cytotoxic. The balance between these effects of NO may lie in the tissue concentration of NO produced [29]. Low levels of NO induce normal physiological signalling, lead to activation of soluble guanylate cyclase (sGC) and antioxidant reactions. At the intermediate level, NO stimulate anti-inflammatory and immunosuppressive responses, anti-apoptotic, pro-growth and angiogenic factors, favouring wound-healing responses. In high concentration, NO is anti-proliferative, induces cell cycle delay, and down regulates nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB). Prolonged NO increase can induce apoptosis, and the highest levels of NO lead to formation of reactive nitrogen species (RNS) [56]. In previous studies, inhibition of NO synthesis was an important mechanism of the anti-inflammatory effects of some plant extracts [57]. In the current study, for ML and MRF extracts NOx reduction was comparable with that induced by diclofenac. Furthermore, NOx decrease was not correlated with the phytochemical analysis or DPPH test of the extracts, but it correlated with the other inflammation marker as TNF-alpha.

Many studies have indicated that polyphenols in herbs possess anti-inflammatory activities manifested through antioxidant activity. Systemic oxidative stress evaluation by measuring stable markers in the circulation is a useful way to evaluate plant extracts effects [24]. Biomarkers of oxidative stress can be classified as molecules that are modified by interactions with ROS, (e.g. DNA, lipids, proteins and carbohydrates) and molecules of the antioxidant system that change in response to increased redox stress. Various methods have been developed for measuring the total oxidant status (TOS) [16]. In our study, serum TOS, of which the main components are hydrogen peroxide and lipid hydroperoxide, was higher in the inflammation group. *M. aquifolium* ML and MB extracts lowered serum TOS. For ML the effect may be correlated with chlorogenic acid and flavonoid glycosides levels.

The antioxidant mechanisms are expressed in terms of its ability to eliminate free radicals (free radical scavengers), metal chelation and synergism with other antioxidants. There are two groups of methods used for the determination of the total antioxidant capacity: those based on single electron transfer monitored spectrophotometrically by a colour change due to free radical reduction, and those based on hydrogen atom transfer measured by elimination of peroxyl radicals [5].

Serum TAR [16], of which the main components are thiol groups and vitamin C, was lower in rats with inflammation. Treatments with *M. aquifolium* extracts increased TAR only for ML. In these extracts chlorogenic acid, ferulic acid, rutin, and isoquercitrin were found. All these phenolic compounds have antioxidant activities. MB did not influence TAR, suggesting that the evaluated alkaloids do not improve the antioxidant activity.

Oxidative stress index provides a global assessment of the oxidant/antioxidant balance of the organism. It was reduced by the MF and MB extracts. Correlation analysis showed that in MB it was induced by the identified phenols and alkaloids.

Lipids are susceptible targets of oxidation because of their molecular structure abundant with reactive double bonds. One of the most well studied markers of lipid peroxidation is malondialdehyde (MDA). MDA is generated *in vivo via* peroxidation of poly-unsaturated fatty acids [24]. After *M. aquifoliun* extracts administration, MDA decreased only in the MB groups, and was negatively correlated with TAR. These results demonstrated that lipoperoxides are not the main constituents with antioxidant activities.

Nitric oxide (NO) has also an important role in oxidative stress because it is the primary substrate of the original reactive oxygen species (ROS), superoxide (O^{2-}). The reaction rate of NO with O^{2-}, is 3 to 4 times faster than its catalysis by Cu, Zn-SOD and 2 to 4 orders of magnitude faster than its reaction with macromolecules such as aminoacids, proteins, lipids and DNA [56]. This reaction gives rise to peroxy-nitrite ($ONOO^-$), which triggers nitration, nitrosation and is also a strong oxidant [11]. NO can interact in direct equimolar concentrations with superoxide to form ONOO-. The greater availability of superoxide may favour ONOO- production and toxicity. Thus, superoxide may be an important rate limiting factor influencing the protective *versus* toxic effects of NO. Protein tyrosine nitration is mediated by RNS, and it has been used as indicators of oxidative stress (free 3-nitrotyrosine) [44].

All *M. aquifolium* tested extracts lowered 3NT. This effect may be linked to levels of the phenolic compounds, because other studies showed that natural phenols are efficient scavengers of nitrogen dioxide (NO_2.), a peroxynitrite ($ONOO^-$) intermediate [41]. Furthermore, *M. aquifolium* extracts had effects comparable to diclofenac on 3NT. For both diclofenac and *M. aquifolium* extracts, this effect was due to iNOS inhibition and NOx production reduction after treatment.

Thiols are sulphur containing compounds that can scavenge free radicals through enzymatic or non-enzymatic pathways. The main plasma SH pool are protein thiols, albumin thiols and to a lesser degree low-molecular weight thiols. In protein structure, thiol groups of sulphur-containing aminoacids such as cysteine and methionine are primary targets of ROS. In human cells the most abundant thiol is glutathione [14]. Serum SH increased after all treatments with *M. aquifolium* extracts. Through this mechanism ethanol *M. aquifolium* extracts may be an important antioxidant treatment option.

Conclusions

Taken together, these findings show that *M. aquifolium* leaves and bark ethanol extracts are good anti-inflammatory and antioxidant candidates. The efficiency varies with plant organ used for extract preparation, and is dependent of the phytochemical composition. *M. aquifolium* ethanolic extracts may be considered for therapeutic interventions in order to prevent the "inflammaging" process and thereby to reduce the incidence of age-associated diseases.

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Conflict of interest

None of the authors has any conflict of interest that could affect the performance of the work or the interpretation of the data.

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