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EFFECTS OF *ERYNGIUM PLANUM* AND *ERYNGIUM CAMPESTRE* EXTRACTS ON LIGATURE-INDUCED RAT PERIODONTITIS

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Eryngium planum and *Eryngium campestre* were used as remedies in several inflammatory disorders while local treatment was used for dental conditions like periodontitis, dental caries and toothache. The aim of the study was to study the anti-inflammatory effect of topical *Eryngium planum* and *Eryngium campestre* treatment in a rat model of periodontitis. The 20% tincture were prepared by maceration with ethanol. The sterols content was assessed by a HPLC-MS method. The *in vivo* anti-inflammatory effects were evaluated on a rat ligature-induced periodontitis by the histopathological examination of periodontal biopsy and serum total nitrites and nitrates, total oxidative status, total antioxidant response and oxidative stress index. The tested hydroalcoholic extracts contained β-sitosterol, stigmasterol and cholesterol. The results provide evidence for the hypothesis that topical treatment with *Eryngium planum and E. campestre* extract in experimental rat periodontitis exerted anti-inflammatory activity by reducing the leucocytes infiltration and the nitro-oxidative stress.

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Keywords: Eryngium planum, *Eryngium campestre*, sterols, periodontitis, NO.

1. Introduction

Periodontitis is a chronic inflammatory disease affecting the gums, periodontal ligament and alveolar bone [1]. Microorganisms from the dental plaque, along with excessive and aggressive immune response against these microorganisms, are considered to cause periodontitis [2]. Therefore, the purposes of periodontal treatment are to remove subgingival microorganisms and to modulate host response. One of the pathogenetic mechanisms in periodontitis is the oxidative stress [1].

The genus Eryngium L. (Sea Holly) belongs to the subfamily Saniculoideae of the Apiaceae, and is represented by 317 taxa widespread throughout Central Asia, America, Central and Southeast Europe [4]. Some Eryngium species (*E. foetidum* L., *E. maritimum* L., *E. campestre* L. and *E. creticum* Lam.) have been used in traditional medicine worldwide. Earlier reports on ethanol extracts of several Eryngium species (*E. billardieri* Delar., *E. campestre*, *E. caucasicum* [5], *E. creticum, E. davisii, E. foetidum* [6], *E. isauricum, E. kotschyi, E. maritimum* [7], and *E. trisectum)* have demonstrated their anti-inflammatory and antinociceptive activity [8, 9]. To date, *Eryngium* extracts or isolated compounds have shown *in vitro* and *in vivo* activities such as

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cytotoxicity against various human tumors, anti-inflammatory, anti-snake and scorpion venoms, antibacterial, antifungal, antimalarial, antioxidant, and antihyperglycemic effects [10]. In Romanian folk medicine the decoction or infusion of *Eryngium* species were used locally as remedies for dental diseases (periodontitis, dental caries, and tooth aches) [11].

The pharmacological effects of Eryngium species were correlated to the high triterpenoid saponin content [4], the presence of flavonoids [12, 13], phenolic acids [14] coumarin derivatives [15], acetylenes [16, 17], rosmarinic acid and chlorogenic acid, known as antioxidants [18, 19]. In some *Eryngium* species was revealed the presence of sterols: *β*-sitosterol, stigmasterol, campesterol, brassicasterol, 3*α*-cholesterol, clerosterol, avenasterol, and $\Delta^{5,24}$-stigmastadienol in *E. foetidum* and brassicasterol in *E. agavifolium* [20]. It has been confirmed that polyhydroxylated triterpenoid saponins with ester functions resembling to escine from the seeds of horse chestnut (*Aesculus hippocastanum* L.) were proved to possess anti-inflammatory properties. Saponin mixtures isolated from *E. planum* administrated i.v. prevented inflammatory responses in rats but oral administration had no anti-inflammatory effect [21]. An *E. campestre* extract was able to inhibit cytokine-stimulated, iNOS-dependent synthesis of nitric oxide in murine cells. In other studies it was confirmed that polar sterols from *E. foetidum* and *E. agavifolium* strongly reduced myeloperoxidase activity in the inflamed tissue of the acute model [22].

The aims of the study were to analyze sterols in *Eryngium planum* (plain eryngo) and *E. campestre* (field eryngo) aerial parts tinctures and to evaluate their anti-inflammatory effects in experimental rat periodontitis.

2. Materials and methods

2.1. Plant material

The aerial parts of *Eryngium planum* (plain eryngo) and *E. campestre* (field eryngo) were collected from its natural habitats while flourishing (July, 2012) from wild flora in Jucu region, Romania. Plant materials were identified by PhD. Professor Mircea Tămaş, from the Department of Botany, University of Medicine and Pharmacy, Cluj-Napoca, Romania, and the voucher specimens (no. E 201, E 202) were deposited in the Herbarium of the Botany Department.

2.2. Preparation of plant extracts

Dried and grounded plant material (100 g) was macerated with $70^{\%}$ ethanol (500 mL) for 10 days at room temperature in order to afford a 20% tincture from each sample [23].

Sterols analysis

Tinctures sterols were identified and quantified by a reversed phase high-performance liquid chromatography coupled with MS detection (LC-MS) [24, 25]. *Eryngium planum*`s aerial parts (S1), *E. campestre*`s aerial parts (S2) 20% tinctures were analyzed after a prior dilution 1:10 with acetonitrile before injection.

The main difference comparing to the prior method was in relation to the chromatographic column and the flow rate. Thus, the new method had the advantage of shortening the time required for the separation of the sterols from 30 minutes to 5 minutes, without interfering the separation and the resolution.

The chromatographic system consisted in a LC/MS system: HP 1100 Series binary pump, an autosampler HP 1100 Series, thermostat HP 1100 Series, detector UV HP 1100 Series (Hewlett Packard Avondale, PA, USA) coupled with an Agilent 1100 MSD Ion Trap VL mass detector.

Compounds were separated on a reversed-phased Zorbax SB-C18 analytical column (Agilent, Santa Clara, CA, USA), 100 mm x 3.0 mm i.d., 5 μm particle, and temperature 35^0C. Isocratic elution of the mobile phase consisting in a mixture of methanol: acetonitrile 30:70 (v/v) was applied. The flow rate was 1 mL/min and injection volume was 10 μL. All solvents used were filtered through 0.5 ml Sartorius filters and degassed with ultrasounds. Detection was performed at 205 nm (only for the chromatographic profile) and by MS. The time required for chromatography

of one sample was 5 min. Quantitative determinations were performed using the external standard method.

The MS was equipped with a Turbo-Ionspray (ESI - electrospray ionisation) interface, negative ion mode. ESI settings were: negative ionization, ions source APCI (atmospheric pressure chemical ionization) at 360^{o}C, gas: nitrogen, flow rate 6 L/min, nebulizer: nitrogen, pressure 50 psi, capillary voltage -2000 V. MS/MS detection using multiple reaction monitoring (MRM) of specific daughter ions was used for each sterol.

Compounds identification was verified according to theirs retention time and mass spectra with those of standards on the HPLC–MS system.

2.3. In Vivo Anti-inflammatory effects

Ligature-induced periodontitis

To induce periodontitis, male Wistar rats (each weighing from 200 to 300 g) were first anesthetized by intraperitoneal injection with ketamine (90 mg/kg) and xylazine (15 mg/kg). A cotton ligature (4/0) was placed around the cervixes of both sides (right and left) of mandibular first molars and maxillary second molars in each animal (four ligatures /animal). The ligature was knotted on the vestibular side, so that it remained subgingival on the palatinal side. Sham-operated rats had the ligature removed immediately after the procedure. After 14 days rats were randomly allocated into the following groups (n = 8): group I - negative control of sham-operated rats (CONTROL) + saline (0.5 mL i.p.); group II – ligature-induced periodontitis (PER)*;* group III – ligature-induced periodontitis (PER) + *E. planum* tincture; group IV – ligature-induced periodontitis (PER) + *E. campestre* tincture. Tinctures of *E. planum* and *E. campestre* were diluted with glycerin (v/v) on a steam bath at 40^{o}C. Glycerin was used as a vehicle because of its adherence properties to the tissues and well tolerance. It is also the basic media of toothpaste maintaining smoothness and viscosity. A dose of 500 mg/kg/day from each Eryngium tincture applied topically for 7 days was tested. Rats were fed with a hard pelleted diet for the duration of the study. After 21 days rats were anesthetized by a combination of ketamine (50 mg/kg b.w.) and xylazine (20 mg/kg b.w.) and blood was withdrawn by retro-orbital puncture [26]. At completion the study rats were euthanized by cervical dislocation, and gingival biopsies were performed. For the leukocytes count blood was harvested on EDTA 0,1% while for the nitro-oxidative stress tests it was collected without anticoagulant [27]. Serum was separated and stored in -80^{o}C until analyzed. Experiments were performed in triplicate. The study design and experimental protocol was approved by the Ethics Committee of "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania.

Acute Phase Bone Marrow Response evaluation

Acute phase bone marrow response, as part of the systemic acute phase response, is induced by the early production of pro-inflammatory cytokines IL-1, IL-6, and TNF-alfa, resulting in leukocytosis and neutrophilia. It was assessed by determining the total leukocyte count (WBC) and leukocyte count expressed as percentage. The WBC was performed with an optical microscope (Olympus), using a Bürcker–Turk counting-chamber. Differential leukocyte count was carried out on May-Grünwald-Giemsa stained smears [28].

Serum nitro-oxidative stress evaluation

Nitrite is the only stable end-product of the auto-oxidation of NO in aqueous solution. A high correlation between endogenous NO production and nitrite/ nitrate levels was established. Therefore, measurement of these provides a reliable and quantitative estimate of NO synthesis *in vivo.* The Griess reaction was used as an indirect assay to determine the total serum nitrite and nitrate (NOx) as a measure of the degree of NO production. Briefly, after serum deproteinization by methanol/diethylether (3/1, v/v) VCl_3 was added for reduction of nitrate to nitrite, followed by addition of the Griess reagents. After 30 min incubation at 37ºC, absorbance was determined at 540 nm. Serum NOx level was expressed in nitrite μmol/L [29].

Serum total oxidant status (TOS) was measured using a colorimetric method. The assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in

acidic medium and the measurement of the ferric ion by xylenol orange. The assay is calibrated with hydrogen peroxide (H_2O_2) and the results are expressed in μmol H_2O_2 Equiv./L [30].

Serum total antioxidant response (TAR) was measured using a colorimetric method. In this method the hydroxyl radical is produced by the Fenton reaction, and the rate of the reactions was monitored by following the absorbance of colored dianisidyl radicals. Upon addition of a serum sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction medium are suppressed by the antioxidant components of the serum, preventing the color change. The assay is calibrated with Trolox and results are expressed as mmol Trolox Equiv./L [30].

The ratio of the total oxidative status to the total antioxidant response gave the oxidative stress index (OSI), an indicator of the degree of oxidative stress. OSI was calculated with the following formula [31]:

$$\text{OSI (Arbitrary Unit)} = \text{TOS}(\mu\text{mol } H_2O_2 \text{ Equiv./L}) / \text{ TAC(mmol Trolox Equiv./L)}$$

Histopathological evaluation

For light microscopy (LM) samples were fixed in 10% buffered formalin (pH=7) for 24 hours, embedded in paraffin wax and cut into 3-5 μm sections. The hematoxylin-eosin (HE) stained slides were evaluated using a light microscope (Olympus BX51 microscope with Olympus SP 350 digital camera). Cell B" basic imaging software (Olympus) was used for semi automat counting of cells. Neutrophils and mononuclear cells were counted in 5 randomly selected microscopic high-power fields (×400) (35.450 μm^2 each) and a semi-quantitative scoring of the stained sections was employed. Measurements were performed 3 times in representative sections. Scoring was performed by a veterinary pathologist (T.M.A.) who was blinded for the treatment [32].

Statistical analysis

All results were expressed as the mean±S.E.M. Statistical comparisons between two independent groups were performed using the Student's *t*-test. Pearson's and Spearman's correlation analyses were used to calculate statistical relationships between parameters. A p-value <0.05 was considered as statistically significant. Analyses were performed using SPSS 16.0 for Windows (SPSS Inc, USA).

3. Results

3.1. Sterols analysis

Chromatogram of the sterols standards mixture (cholesterol, stigmasterol and β-sitosterol) by UV detection at 205 nm is shown in fig. 1.

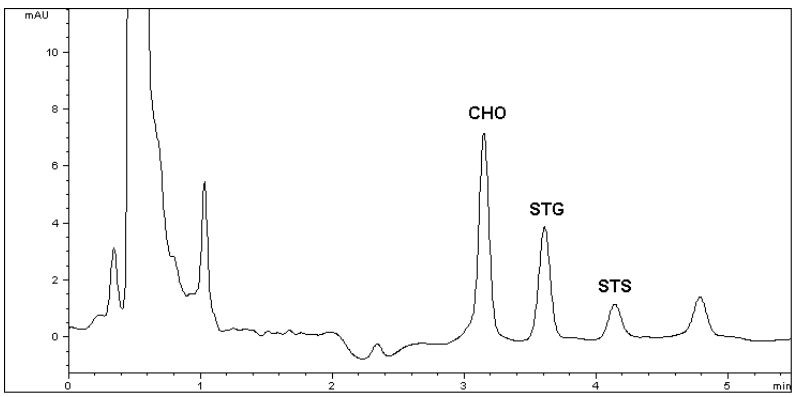

Fig. 1. HPLC chromatogram of the sterols standards mixture (CHO = cholesterol, STG = stigmasterol, STS = β-sitosterol)

MS analysis mode and the specific ions from the mass spectrum of the 3 sterols standards are shown in Table 1.

Because in the ionization conditions all the sterols lose a water molecule, the detected ions by the spectrometer are presented always in the same form $[M\text{-}H_2O+H]^+$. MS detection identified parent-ions of the sterols which are ions formed after ionization of the sample, before an eventual fragmentation. Every sterol was identified on the basis on a specific expected ion (Table 1).

Table 1. Specific ions of sterols standards monitored in the screening method

Sterol standard	**M**	**M-H_2O**	**M-H_2O+H^+**
Cholesterol	386	368	369
β-Sitosterol	414	396	397
Stigmasterol	412	394	395

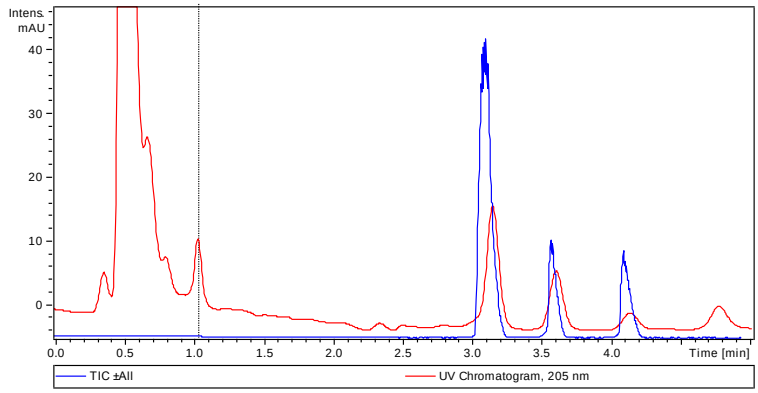

Fig. 2 HPLC chromatogram of the total ionic content (TIC) of the sterols mixture regardless of the molecular weight (TIC, blue) and in UV (red). The order of elution is: cholesterol, β-stigmasterol and sitosterol.

Specific chromatograms for each sterol were extracted: ion with m/z 369 for cholesterol, m/z 395 for stigmasterol and m/z 397 for β-sitosterol).

Chromatograms of the specific ions and also the mass spectrums (full-scan) are shown in fig. 3-5.

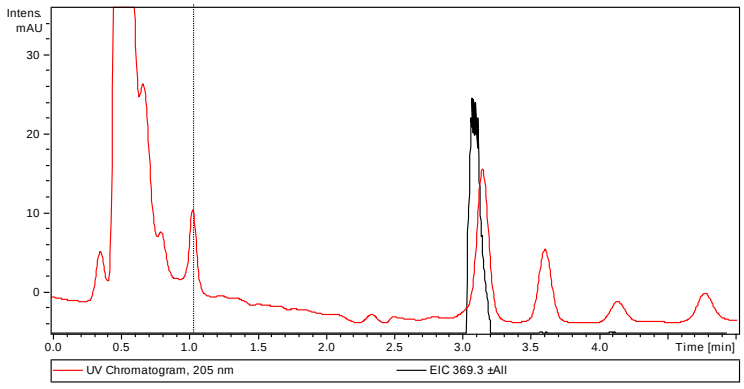

Fig. 3a Chromatogram of the specific ion for cholesterol with m/z 369 (black) and UV chromatogram at 205 nm (red). Retention time for cholesterol was 3.1 min.

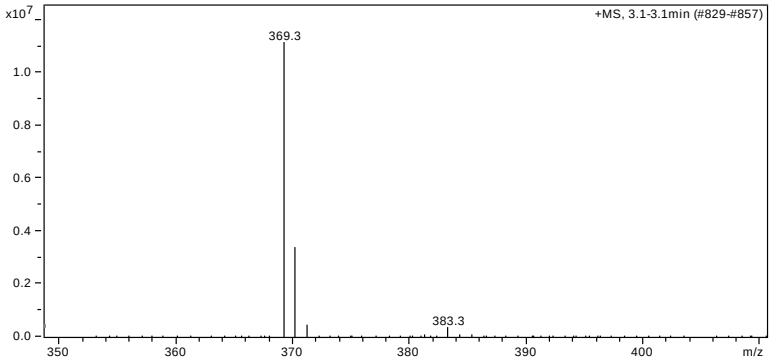

Fig. 3b The full-scan spectrum of cholesterol on the basis the chromatogram from Fig. 3a was built

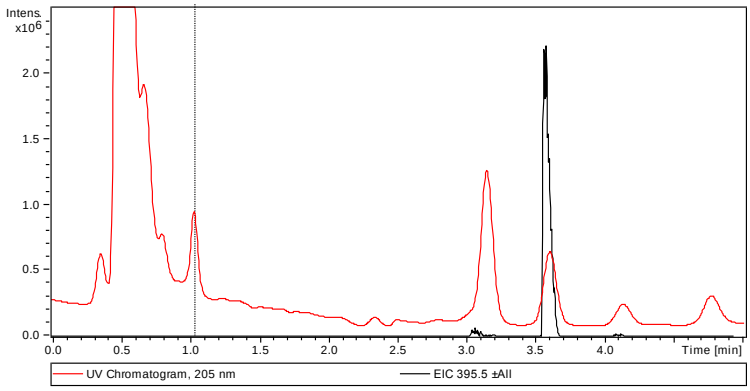

Fig. 4a Chromatogram of the specific ion for stigmasterol with m/z 395 (black) and UV chromatogram at 205 nm (red). Retention time for stigmasterol was 3.6 min.

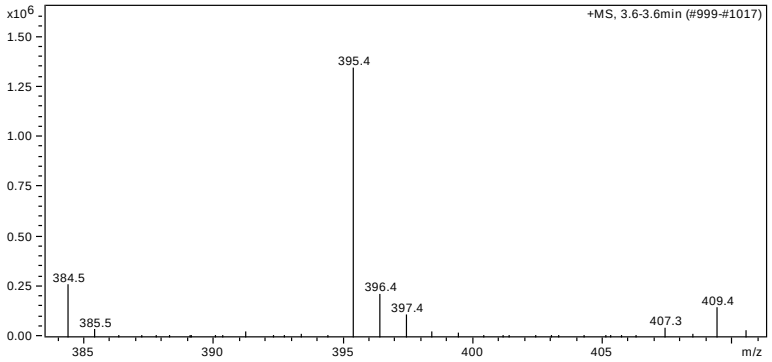

Fig. 4b The full-scan spectrum of stigmasterol on the basis the chromatogram from Fig. 4a was built

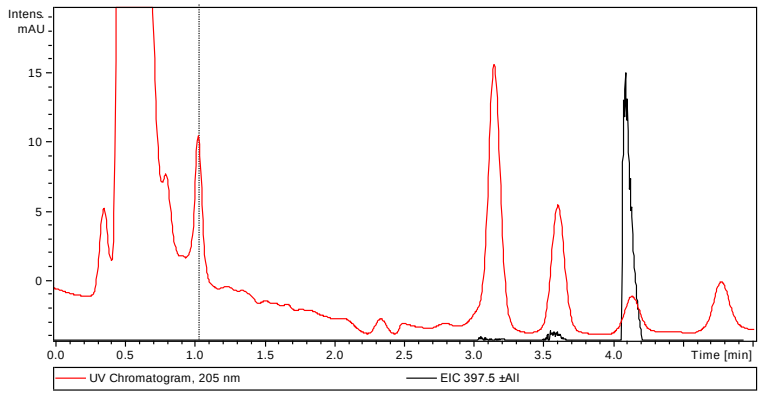

Fig. 5a Chromatogram of the specific ion for sitosterol with m/z 397 (black) and UV chromatogram at 205 nm (red). Retention time for β-sitosterol was 4.1 min.

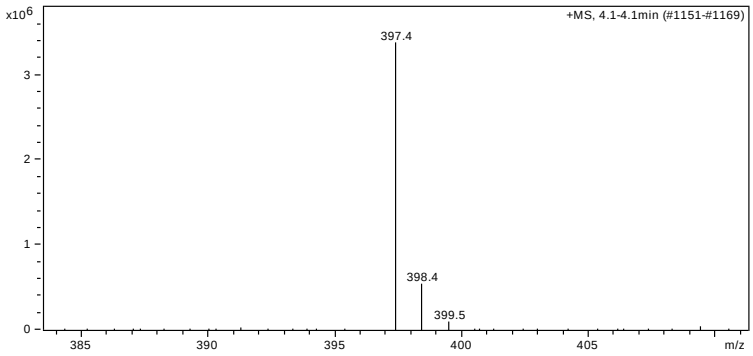

Fig. 5b The full-scan spectrum of β-sitosterol on the basis the chromatogram from Fig. 5a was built

For quantification of the three sterols in tinctures, the extracted chromatograms were built by considering the intensity of the major ions from the mass spectrums (Table 2).

Table 2. Ions of the MS spectrum of sterols on which quantification was achieved

No.	Sterol	Specific ions for identification
Ion [M-H_2O+H^+] > Spectrum ions		
1	Cholesterol	369> 215; 233; 243; 259; 273; 287
2	Stigmasterol	395> 255; 297; 283; 311; 241; 201
3	β-Sitosterol	397> 160.9; 174.9; 188.9; 202.9; 214.9; 243; 257; 287.1; 315.2

Calibration curves of the sterols were built by dissolving the sterols standards in chloroform (1 mg/mL), followed by successive dilutions in acetonitrile. All calibration curves showed a linear correlation coefficient $r^2 > 0.99$ and a satisfactory level of precision (RSD of 3% or lower).

The calibration curves for each sterol used for the quantification of sterols in plant tinctures are shown in fig. 6-8.

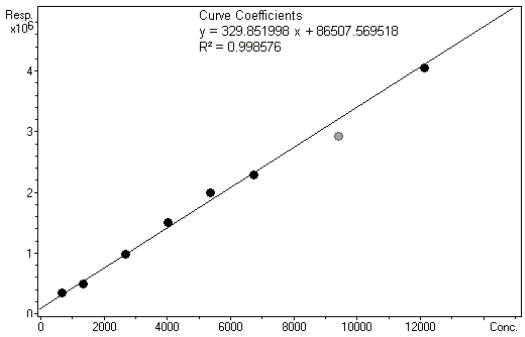

Fig.6. Calibration curve for cholesterol

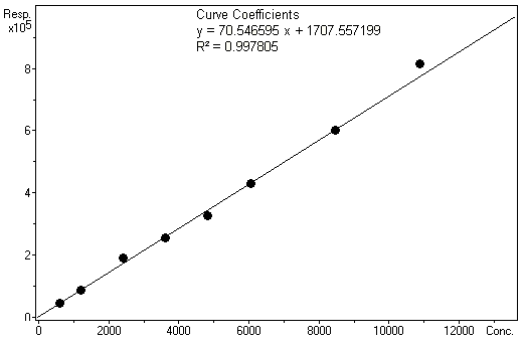

Fig.7. Calibration curve for stigmasterol

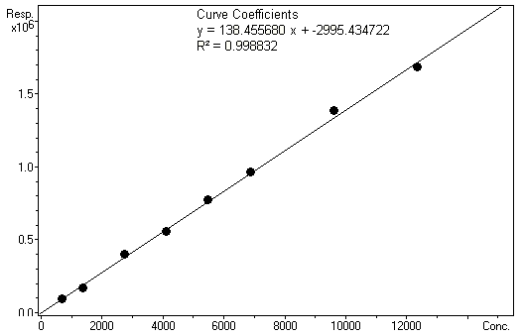

Fig. 8. Calibration curve for β-sitosterol

The content in sterols (μg/mL) in *Eryngium*`s tinctures is presented in Table 3.

Table 3. Concentration of sterols in Eryngium tinctures (μg/mL)

Samples	Sitosterol (μg/mL)	Stigmasterol (μg/mL)	Cholesterol (μg/mL)
S1	25.131	25.952	2.867
S2	6.709	32.217	1.001

3.2. In Vivo Anti-inflammatory effects

Results regarding the effect of tested tinctures in a ligature-induce acute model of periodontitis are shown in fig 9 and fig. 10. Ligature-induced periodontitis increased the nitro-oxidative stress by increasing neutrophils ($p<0.01$), monocytes ($p<0.05$), TOS ($p<0.01$) and NOx ($p<0.01$), associated with TAR decrease ($p<0.01$) (fig. 9-10).

E. planum and *E. campestre* lowered significantly total leukocytes count ($p< 0.01$) due to the reduction of neutrophils and monocytes. *E campestre* had a better inhibitory effect ($p<0.01$) than *E. planum*.

E campestre and *E. planum* ($p>0.05$) extracts had no significant effect on TOS.

TAR was significantly increased by *E campestre* ($p<0.01$) and *E. planum* ($p<0.05$) extracts. TAR raised significantly at group treated with *E. planum* tincture (by 94.5%), while *E. campestre* increased TAR modestly (by 16%).

NO synthesis was also decreased by *E campestre* ($p<0.05$) and *E. planum* ($p<0.05$) extracts. NOx level was slightly reduced by 20% by *E. planum* tincture and by 12% by *E. campestre* when comparing to periodontitis control group.

OSI was lowered by *E campestre* ($p<0.01$) and *E. planum* ($p<0.05$) extracts. OSI was lowered by 46% by *E. planum* tincture and by 37.5% by *E. campestre* tincture.

OSI decrease was correlated with TAR increase ($r=0.62$) and NOx reduction ($r=0.071$).

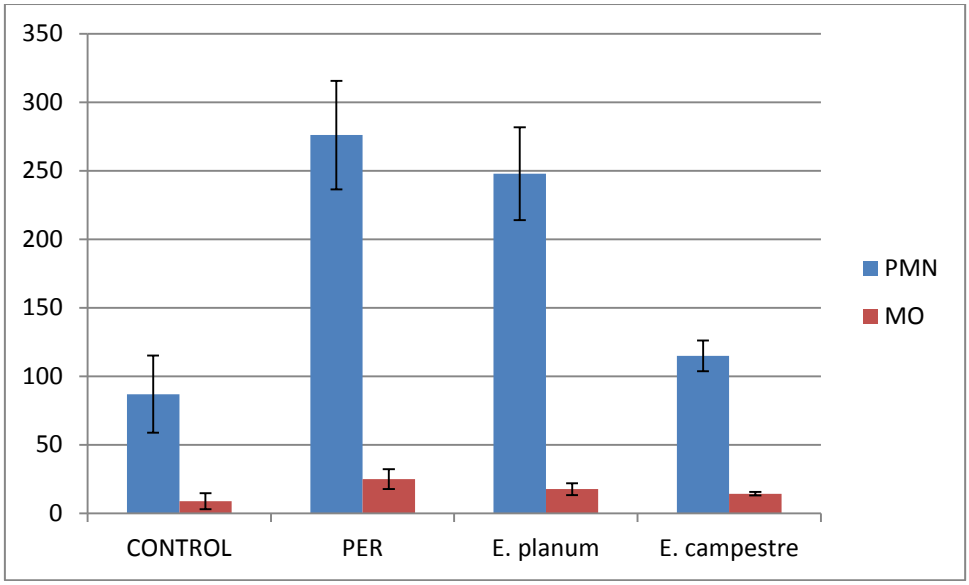

Fig. 9. E planum and E. campestre effect on neutrophils (PMN) and monocytes (MO) from the biopsies

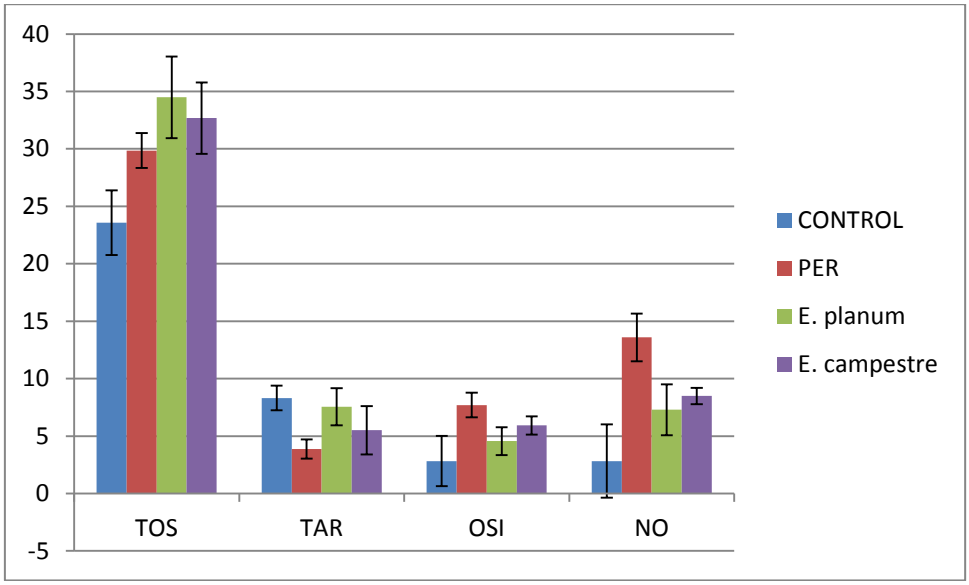

Fig. 10. E planum and E. campestre effect on TOS, TAR, OSI and NO

Histological examination of the gingivomucosal tissue biopsies of periodontitis rats showed edema, tissue injury, as well as infiltration of the tissue with inflammatory cells (fig. 11 a-c).

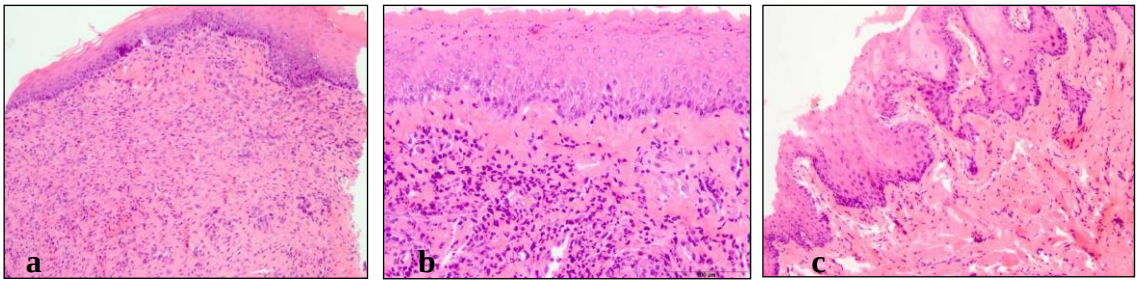

Fig. 11. Histopathological examination of the biopsies:
***a.** PER group - fibrovascular proliferation, congestion and intense inflammatory infiltrate with mononuclears and neutrophils, HE x 10.*
***b.** group E. planum – moderate inflammatory infiltrate with neutrophils and macrophages, HE x 20.*
***c**. group E. campestre – discrete inflammatory infiltrate with neutrophils and macrophages, HE x 10.*

4. Discussion

E. planum and *E. campestre* phytochemical analysis revealed significant concentrations of sterols and anti-inflammatory effects.

Both tested *Eryngium* tinctures contained high amounts of sitosterol and stigmasterol, while cholesterol was detected in small amounts. Stigmasterol was the major sterol. Generally, the role of plant sterols is based on lowering the LDL-cholesterol levels, a well known risk factor for heart disease. Previous studies demonstrated that stigmasterol exerts also topical anti-inflammatory activity while other phytosterols inhibit 12-O-tetradecanoylphorbol acetate-induced oedema [33]. The antiexudative effects of *Eryngium sp*. suggested their inhibitory effect on the vascular response associated to the acute stage of inflammation [34, 35]. Also, phytosterols (sitosterol, stigmasterol, and campesterol) are responsible for antioxidant effects in some diseases [36]. That is why *E. planum* and *E campestre* sterols analysis was associated to the anti-inflammatory study.

In acute inflammation systemic distribution of the inflammatory cytokines activate bone marrow acute phase response and increase phagocytes proliferation and their release in the blood. Inflammatory mediators activate also a vascular response that will allow leukocytes to migrate into the inflamed tissue and in order to form the inflammatory infiltrate. Periodontitis severity and progression was evaluated systemically by counting blood leukocytes and locally by analyzing on biopsy inflammatory infiltrate [37]. *E. planum* and *E. campestre* extracts showed a very significant inhibition of the bone marrow acute phase response by reducing the phagocytic leukocytes count and by lowering neutrophils and monocytes infiltrates. These effects were stronger in *E. campestre* treated group.

Activated phagocytes destroy the substrate by using oxygen dependent mechanisms that involve NO and reactive oxygen species formation (ROS). Formation of NO from L-arginine is catalyzed by a family of nitric oxide synthase (NOS) isoenzymes. Activation of the immune system can result in expression of inducible NOS (iNOS) in numerous cell types (macrophages, neutrophils, hepatocytes). NO reacts with ROS, leading to peroxinitrite formation and increases oxidative stress. Excessive NO and ROS synthesis are involved in the pathological progression of inflammatory diseases and NO, as an effector molecule may be a useful marker. Low NO synthesis also regulates neutrophils functions: inhibits degranulation, leukotriene production, superoxide anion generation and chemotactic movement in activated neutrophils and regulates leukocyte recruitment into the inflammatory focus. Many of these mechanisms were correlated to antioxidant mechanisms of NO. Therefore, treatments that reduce induced NO synthesis may potentiate the antioxidant effects. *E. planum* and *E. campestre* tinctures proved to have antioxidant effect by reducing NOx and increasing TAR, even when TOS was not significantly influenced. Although almost all organisms possess antioxidant defense and repair systems that protect them against oxidative damage, these systems are insufficient to prevent the damage entirely. Therefore, antioxidant supplements containing *E. planum* and *E. campestre* may be useful to reduce oxidative damage.

5. Conclusions

Inflammation is a protective physiological response and it is a self-limiting process. However, if this fails, inflammation becomes chronic. In chronic inflammations excessive production of NO and ROS cause nitro-oxidative stress and may injury the health tissue and further amplify the inflammatory response [38]. That is the reason of trying to reduce NO and ROS synthesis as a host modulatory therapy in periodontitis. In conclusion, this study suggests a potential therapeutic application of *E. planum* and *E. campestre* extracts for treatment of the active inflammatory periodontal disease. The anti-inflammatory activities of the extracts were supported by inhibition of phagocytes and nitro-oxidative stress reduction. The effect is probably related to a synergic activity of the detected sterols, triterpenoid saponins, and polyphenolic compounds [39].

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