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VITEX AGNUS-CASTUS ESSENTIAL OIL AFFECTS THYROID C CELLS AND BONE METABOLISM IN MIDDLE-AGED MALE RATS

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Ageing in men is accompanied by an increased occurrence of osteoporosis, but traditional hormonal replacement therapy elevates the risk of developing endocrine cancer. Vitex agnus-castus L. (Vac) essential oil is commonly used as an alternative therapy for ageing symptoms in both men and women. It is known that calcitonin (CT), thyroid C cell hormone, inhibits bone resorption. The purpose of this experimental study was to investigate the influence of Vac essential oil administration on the immunohistomorphometric features of thyroid C cells and bone metabolism in 16-month-old male Wistar rats. The first group of animals (n=8) was treated subcutaneously (s.c.) with 60 mg/kg of Vac essential oil once a day for 3 weeks. Control animals (n=8) received sterile olive oil s.c. by the same schedule. After Vac treatment significant increases ($p<0.05$) were found in the volume of C cells (by 10%) and serum CT level (by 27%) compared with the controls. Serum osteocalcin (OC) and calcium (Ca^{2+}) levels were 31% and 8% lower ($p<0.05$) respectively, in comparison with the control group. These are the first experimental results suggesting that Vac essential oil stimulates thyroid C cells activity and decreases bone turnover in middle-aged male rats.

Key words: bone, essential oil, thyroid C cells, Vitex agnus-castus

INTRODUCTION

The age-related decline in sex steroids levels and other hormonal changes contributes to bone loss in both men and women. These changes may lead to osteoporosis, a bone disease characterized by structural deterioration of bone tissue due to a disturbed balance between bone formation and bone resorption. However, in contrast to postmenopausal osteoporosis in women, age related bone loss in men and therapeutical strategies have been poorly studied. Treatment of osteoporosis includes hormone replacement therapy, as well as application of bisphosphonates, calcium (Ca^{2+}) and calcitonin (CT). The effects of sex steroids on bone tissue are mainly mediated through estrogen and androgen

receptors present in osteoblast and osteoclast cells (Colvard *et al*., 1989; Kusec *et al*., 1998; Van Der Eerden *et al*., 2002).

The calcitropic hormone CT secreted by thyroid C cells, may inhibit osteoclast bone-resorbing activity by binding to CT receptors (Nicholson *et al*., 1986; Chambers and Magnus, 1982). Thus, CT has been widely used for treating osteoporosis (Silverman, 2003). Therefore, it is important to examine the CT secreting activity of thyroid C cells in conditions of sex steroids deficiency and after applying therapeutics for prevention and treatment of osteoporosis. Previous human and animal studies have suggested that sex steroid deficiency after gonadectomy affected thyroid C cell structure and reduced the synthesis and release of CT in both sexes (Isaia *et al*., 1989; Sakai *et al*., 2000; Filipović *et al*., 2003, 2007). On the other hand treatment with estrogen or testosterone, stimulated thyroid C cells and increased trabecular bone mass in rats (Filipović *et al*., 2003; Takano-Yamamoto and Rodan, 1990; Sekulić *et al*., 2006; Pantelić *et al*., 2010). However, in addition to protective effects on bone, sex hormone therapy has some adverse effects, such as increased risk of breast or prostate cancer (Grey and Reid, 2005; Cranney *et al*., 2002; Loughlin and Richie, 1997). Therefore, it is important to develop treatments for preventing bone loss without side effects. Filipović *et al*. (2010) suggested that the phytoestrogen, daidzein, increased tibial trabecular bone mass and decreased bone turnover in orchidectomized middle-aged rats. Other authors have shown that strategies and alternative approaches to prevent and treat osteoporosis may include phytoestrogens and also aromatic plant compounds with estrogenic action (Draper *et al*., 1997; Tobe *et al*., 1997; Khalil *et al*., 2005; Soung *et al*., 2006).

The therapeutical potential of *Vitex agnus-castus* L. *(Vac)* is known since ancient times, especially in treating ageing symptoms in women (Lucks *et al*., 2002), while the *Vac* products prophylactic effects on bone tissue in males were also observed (Jarry *et al*., 2003; Liu *et al*., 2004; Sehmisch *et al*., 2009). Biological activity of *Vac* essential oil is mostly attributed to various monoterpenes and sesquiterpenes, in addition to some other non-terpene components (Edris, 2007). These compounds are typical lipophiles which can pass through cell membranes or accumulate in the hydrophobic membrane core (Dolder *et al*., 2006; Bakkali *et al*., 2008). Some studies revealed an inhibitory role of terpenes on bone resorption in rats, while all available data suggest that monoterpenes have antiosteoporotic properties, but the exact mechanism of action has not been elucidated (Dolder *et al*., 2006; Mühlbauer *et al*., 2003; Spichiger *et al*., 2006). Considering the possible side effects of hormone therapy and efforts to find the best alternative treatment for osteoporosis, the aim of this study was to analyze the effects of *Vac* essential oil on calcitonin secreting thyroid C cells and bone turnover in middle-aged male rats, an adequate “for the human” ageing animal model.

MATERIALS AND METHODS

Experimental animals

In the present study, we used 16-month-old male Wistar rats, bred at the Institute for Biological Research, Belgrade. They were maintained in a 12/12 h

light-dark cycle at 22±2°C under soy-free conditions. The animals were fed a soy-free diet prepared in cooperation with the Department of Food, School of Veterinary Medicine, and ISHRA PKB (Belgrade, Serbia), according to Picherit *et al*. (2000) with corn oil as the fat source (Table 1). Casein and crystalline cellulose were purchased from Alfa Aesar, Johnson Mattehey Gmbh &Co. KG (Karlsruhe, Germany). DL-methionine was obtained from Sigma Chemical Company, St. Louise, Missouri, USA. All other ingredients were from ISHRA PKB. Food and water were available *ad libitum*. The experimental protocols were approved by the Local Animal Care Committee in conformity with the recommendations provided in European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS no. 123. Appendix A.) The animals were divided into two groups. Rats in the first group were injected s.c. with essential oil from ripe fruit of *Vac* (60 mg *Vac*/kg of b.w.) once a day for 3 weeks. The second group was treated s.c. with sterile olive oil by the same schedule, and served as the control. Animals were decapitated 24 h after the last injection. Blood was collected from individual animals and serum was stored at -70°C until required for biochemical determinations.

Table 1. The diet composition

Ingredient	Per 100 g of diet
Casein	23.3 g
Cornstarch	45 g
Sucrose	20 g
Corn oil	5.2 g
Fiber (crystalline cellulose)	3.7g
Vitamin/mineral mix (Ca-phosphate deficient)	1.5 g
Calcium phosphate dibasic	1.8 g
Calcium carbonate	1.0 g
DL-methionine	1.5 g

Plant material and essential oil preparation

Ripe *Vac* berries were identified and collected, during June-October in 2007. by Prof. Dr. Dragan Grubišić (Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia) in Igalo, Montenegro. A voucher specimen (No VAC23987) has been deposited at the Institute for Biological Research "Siniša Stanković". Material was dried at room temperature. *Vac* essential oil was prepared by hydrodistillation in a Clevenger type apparatus for 3h. The yield of the oil was 0.72%. The obtained essential oil was stored at +4°C until further tests.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). Qualitative and quantitative analyses of the oil were performed using GC and GC/MS. The GC analysis of the oil was carried out on a GC HP-5890 II

apparatus, equipped with split-splitless injector, attached to HP-5 column (25 m x 0.32 mm, 0.52 μm film thickness) and fitted to a flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 mL/min, split ratio 1:30, injector temperature was 250^{o}C, detector temperature 300^{o}C, while column temperature was linearly programmed from 40^o-240^{o}C (at rate of 4^o/min). The same analytical conditions were employed for GC/MS analysis, where HP G 1800C Series II GCD system equipped with HP-5MS column (30 m x 0.25 mm, 0.25 μm film thickness) was used. Transfer line was heated at 260^{o}C. Mass spectra were acquired in electron impact (EI) mode (70 eV), in m/z range 40-400. Electron impact identification of individual constituents was made by comparison of their retention times with those of analytical standards of available terpenoids, and by computer searching, matching mass spectra with those held in Wiley 275 library of mass spectra. Confirmation was done using the calibrated AMDIS programme for determination of experimental values for retention indices of recorded constituents and comparing them with those from literature (Adams, 2007). For quantification purpose, area percent data obtained by FID were used. The contents of particular compounds were calculated from the GC peak areas, using the normalization method. According to the analyses, the following compounds were prevalent in the *Vac* essential oil: 1,8-cineole (16.3%), sabinene (13.4%), α-pinene (9.4%), *trans*-β-farnesene (9.3%), limonene (6.8%), α-terpinyl acetate (4.6%), caryophyllene oxide (4.6%), *trans*-β-caryophyllene (4.1%) and cis-β-farnesen (0.7%) (Table 2), (Stojković *et al*., 2011).

Table 2. Chemical composition of essential oil from ripe fruits of *Vitex agnus-castus*

Vitex agnus-castus essential oil	Ripe fruits
Constituents	%ID
α-pinene	9.4
sabinene	13.4
limonene	6.8
1,8-cineole	16.3
Trans-β-farnesene	9.3
α-terpinyl acetate	4.6
Caryophyllene oxide	4.6
Trans-β-caryophyllene	4.1
Cis-β-farnesen	0.7

Immunohistochemistry

Thyroid glands were fixed in Bouinžs solution at room temperature for 48 h and embedded in paraplast. The glands were cut into 5 μm sections. We used a peroxidase-antiperoxidase (PAP), (Sternberger *et al*., 1970) method for localization of CT in the C cells. Sections were incubated with the primary antibody (rabbit anti-human CT, Dakopatts, Copenhagen, Denmark) diluted 1:500 in phosphate-buffered saline, as previously described (Sekulić *et al*., 1998).

Morphometry

Sections of thyroid gland with immunostained C cells were streologically analyzed by simple point counting according to Weibel (1979). The mean volumes of the cytoplasm (Vc; μm^3) and nuclei (Vn; μm^3) of the thyroid C cells and volume density (Vv; %) of their nuclear profiles were determined using the multipurpose M_{42} test system, with 50 test areas under a light microscope at 1000x magnification.

Bone histomorphometrical analysis

After fixation in Bouin's solution, proximal right tibia were decalcified with 20% disodium salt of ethylenediaminetetraacetic acid. The samples were routinely processed and sectioned longitudinally followed by the Azan staining method, as previously described (Filipović *et al*., 2007). Histomorphometric measurements were made using an ImageJ public-domain-image processing program, starting from the distal epiphyseal growth plate at 1 mm. All parameters were expressed as recommended by the American Society for Bone and Mineral Research nomenclature (Parfitt *et al*., 1987). These data were used to calculate cancellous bone area (B.Ar) and cancellous bone perimeter (B.Pm) (Evans *et al*., 1994). Trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were derived from B.Ar and B.Pm (Parfitt *et al*., 1983; Chappard *et al*., 1999) as previously described (Filipović *et al*., 2007).

Biochemical analysis

Serum CT was determined by an immunochemiluminometric assay (ICMA; Nichols, USA), using a mouse monoclonal antihuman CT antibody marked with acridium ester (AE). The luminescence was quantified with a semiautomated MLA 1 hemiluminescence analyzer (Ciba-Corning). Serum OC level were determined by electrochemiluminescence, using a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Serum Ca^{2+} and phosphorus (P) levels, as well as urinary Ca^{2+} level were measured on a Hitachi 912 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

All results were expressed as mean ± SEM. Differences between the groups were assessed by one–way analysis of variance (ANOVA), followed by the multiple range test of Duncan (Pharmacological Calculation System, 1986). Values of $p<0.05$ were considered statistically significant. Student's t–test was used to evaluate differences in urinary Ca content and serum Ca and P levels between treated and control animals.

RESULTS

Effects of Vac administration on thyroid C cells and cancellous bone in middle-aged male rats

The thyroid C cells in control rats were located in the middle of the thyroid lobes, grouped in clusters between the follicles, with cytoplasm strongly

immunostained for CT (Figure 1A). After *Vac* administration, these cells were larger and their cytoplasm was less intensely immunostained for CT in comparison to the control group (Figure 1B).

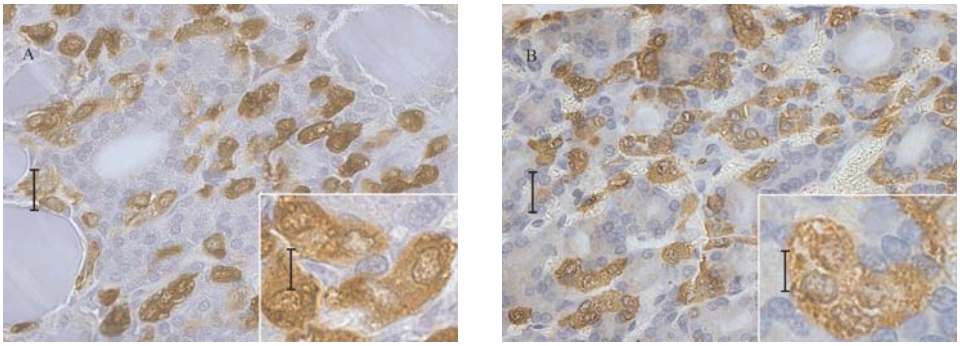

Figure 1. (A) Thyroid C cells with strongly CT immunostained cytoplasm in a control middle age male rat. (B) Large C cells with light cytoplasm in a rat treated with *Vitex agnus-castus* essential oil (*Vac*). Immunoperoxidase staining specific for calcitonin. Scale bar 20 μm; inset (bar= 8 μm)

Stereological analysis showed a significant 10% increase in Vc ($p<0.05$), while Vn and Vv were not significantly changed in comparison with the control values (Figure 2A-C).

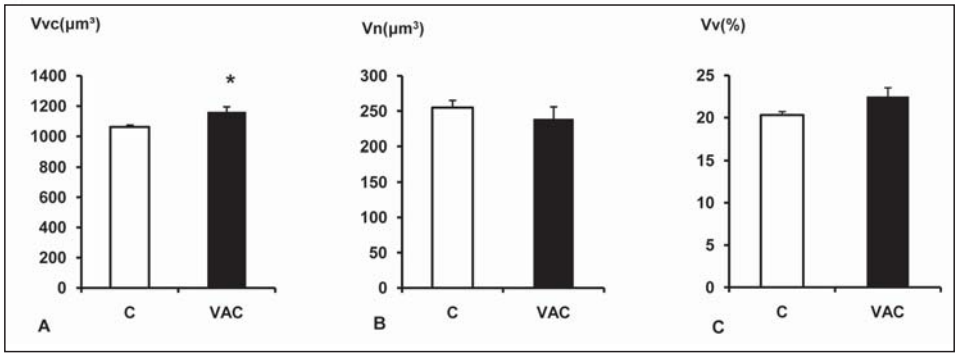

Figure 2. (A) Cellular (Vc) volume (μm^3) of thyroid C cells. (B) Nuclear (Vn) volume (μm^3) of C cells. (C) Volume density (Vv, %) of C cells. Results are shown as the maen ± SEM. *$p<0.05$ C control rats, *Vac*-rats treated with *Vitex agnus-castus* essential oil

Histological analyses of trabecular bone (Figure 3A-B) and histomorphometrical parameters from the proximal tibiae showed no statistically significant differences when compared to the control (Table 3).

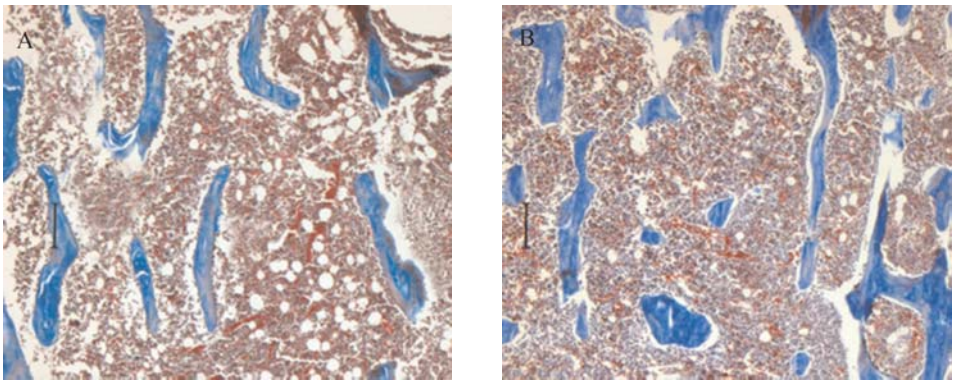

Figure 3. Trabecular bone microarchitecture in the metaphyseal region of the proximal tibia in (A) control (C) animals and (B) animals treated with essential oil of *Vitex agnus castus* (*Vac*). 5 μm sections from the center of the proximal tibia; Azan method stain (scale bar 160 μm)

Table 3. Cancellous bone histomorphometry

	B.Ar (%)	Tb.Th (μm)	Tb.N (mm)	Tb.Sp (μm)
C	9.75±1.91	44.40±4.38	2.23±0.65	424.0±131.52
Vac	7.11±1.54	50.47±7.12	1.57±0.19	599.4± 79.33

Cancellous bone area (B.Ar); Trabecular thickness (Tb.Th); Trabecular number (Tb.N); Trabecular separation (Tb.Sp)

Effects of Vac administration on serum and urine parameters in middle-aged male rats. After *Vac* administration, serum CT level increased by 27% ($p<0.05$), while serum OC concentration was 31% ($p<0.05$) lower than values for the control rats (Figure 4A-B). Serum Ca level (Figure 4C) had decreased by 8% ($p<0.05$) in

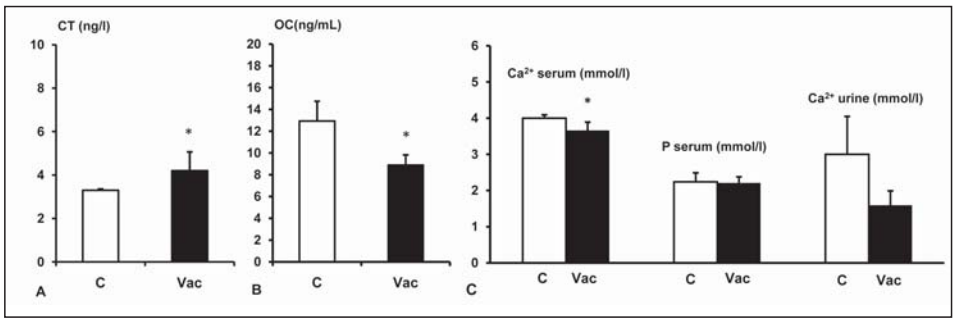

Figure 4. (A) Serum calcitonin (CT) concentration (ng/L); (B) Serum osteocalcin (OC) concentration (ng/L); (C) Serum calcium (Ca^{2+}) concentration (mmol/L); serum phosphorus (P) concentration (mmol/L); urine Ca^{2+} concentration (mmol/L). All values are mean ± SEM. *$p<0.05$ versus C. C-control rats, *Vac*-rats treated with *Vitex agnus-castus* essential oil

animals treated with *Vac* in comparison to the control group. No significant decreases in serum P or urinary Ca concentration were detected after *Vac* treatment in relation to control animals (Figure 4C).

DISCUSSION

In contrast to postmenopausal osteoporosis, studies on age-related male osteoporosis and therapeutical strategies to treat it are rare. The studies in ageing male rats showed decline in serum testosterone levels (Wang, 2008), which has a negative impact on bone mass and structure (Ke *et al*., 2001). Estrogen has been shown to be effective in the prevention of osteoporosis in male animals (Vandenput *et al*., 2002), but it is not useful for men because of the unwelcome side effects. Non-estrogenic therapies for treatment of osteoporosis include CT, a hormone secreted by thyroid C cells that regulates Ca^{2+} homeostasis and bone remodeling. Because of the adverse side effects of estrogen therapy, plant-derived estrogenic substances (phytoestrogens) have played an important role in the treatment of osteoporosis (Adlercreutz *et al*., 1998). Recently, it was shown that a *Vac* extract, used in the treatment of menopausal syndrome, had osteoprotective effects in male rats (Sehmisch *et al*., 2009).

In order to contribute to the search for alternatives to conventional hormone therapy, we have investigated the effects of *Vac* essential oil on the structure and function of thyroid C cells and bone metabolism in middle-aged male rats. Treatment of these rats with Vac essential oil reduced CT immunoreactivity of C cells, while significantly increasing their volume and rising serum CT level in comparison with the controls. These data indicate a stimulatory effect of *Vac* on thyroid C cells activity and they are the first experimental data illustrating the impact of *Vac* essential oil on structure and function of thyroid CT producing C cells. The mechanism by which this oil affects these cells and CT secretion is unknown. It is possible that some components of essential oils have estrogen activity. Tabanca *et al*. (2004) indicated that essential oils from diverse plant species and different plant parts vary in estrogenic potency. Although most essential oils containing considerable amounts of anethone were highly estrogenic, other constituents may also contribute towards the estrogenic activity. These authors suggested that high concentration in essential oil of *Pimpinella* has the potential to interact with estrogen receptors. Some compounds, such as limonene, sabinene, cis-β-farnesene, were detected in *Vac* essential oil. Moreover, the bicyclic monoterpenoid 4-methylbenzylidenecamphor, an organic derivative of camphor, is able to interact directly with estrogen receptors α and β (Mueller *et al*., 2003). Estrogen receptors were detected in thyroid C cells (Naveh-Many *et al*., 1992; Blechet *et al*., 2007). Previously, we have shown that both estradiol and the phytoestrogen daidzein stimulated CT secreting activity of thyroid C cells (Filipović *et al*., 2003; 2010). Bearing in mind the possible estrogenic activity of essential oils, the mechanism of action on thyroid C cells might include the potential of certain oil components to interact with estrogen receptors.

Generally, essential oils are very complex natural mixtures, mainly consisting of terpenes, terpenoids and the other low molecular weight aromatic

and aliphatic compounds. As typical lipophiles, they can pass through the cytoplasmic membranes or accumulate in the hydrophobic membrane core (Dolder *et al*., 2006; Bakkali *et al*., 2008). Monoterpenes have been suggested to impair the membrane potential and increase intracellular Ca^{2+} in plant cells (Maffei *et al*., 2001; Mucciarelli *et al*., 2001). In eukaryotic cells, essential oils can provoke depolarization of mitochondrial membranes by decreasing the membrane potential and affecting Ca^{2+} channels function (Richter and Schlegel, 1993; Novgorodov and Gudz, 1996; Vercesi *et al*., 1997). The triterpene, escin, also increased endothelial cell permeability to Ca^{2+} (Omar and Horacio, 2007). These changes may cause an increase in cytosolic Ca^{2+} concentration, which is a regulatory factor for many cellular processes, including secretion. It is known that increased intracellular Ca^{2+} resulting from high extracellular Ca^{2+} levels, induces CT secretion from C cells (Freichel *et al*., 1966; McGehee *et al*., 1997). These data suggest a serious role for essential oils constituents in Ca^{2+} mediated processes, so we can assume that the *Vac* terpenes had a definite role in CT secretion in the present study. However, it is necessary to conduct further researches to clarify the possible effects of essential oils on CT secretion.

Our analysis of trabecular bone microstructure, showed the absence of significant differences in bone histomorphometrical parameters between control and *Vac* treated rats. However, *Vac* administration significantly decreased serum OC and Ca^{2+} concentrations. Urinary Ca^{2+} level was also slightly lower. OC produced by osteoblasts, is a marker of bone formation. The reduction of serum OC level after *Vac* administration, observed here indicates diminished bone turnover, i.e. less formation and less resorption, which may have a protective effect on bone. Other studies also demonstrated that essential oils and their monoterpene components affect bone metabolism. The mechanism by which monoterpenes affect bone may involve direct effects on bone cells. It has been suggested that these compounds and their metabolites can inhibit osteoclastogenesis and bone resorption (Dolder *et al*., 2006; Mühlbauer *et al*., 2003; Spichiger *et al*., 2006). Also, some monoterpenes may have a wide range of effects on osteoblasts and stimulate bone formation (Dolder *et al*., 2006; Yen *et al*., 2007). The results obtained in our study indicate that *Vac* essential oil stimulates the morphofunctional features of CT producing thyroid C cells. Based on these findings, we think that, in addition to the direct effects on bone cells, some components of plant essential oils may act indirectly on bone metabolism. These indirect effects can be achieved by influencing calciotropic hormones such as CT.

In summary, our findings suggest that the essential oil of *Vac* stimulates thyroid C cells activity, which is accompanied by decreased bone turnover in middle-aged male rats. These are the first experimental data on the effect of these oils on thyroid C cells and suggest that CT is a possible mediator in the indirect effects of plant essential oil components on bone metabolism.

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UTICAJ ESENCIJALNOG ULJA BILJKE *VITEX AGNUS-CASTUS* NA C ĆELIJE ŠTITASTE ŽLEZDE I KOŠTANO TKIVO MUŽJAKA PACOVA SREDNJEG ŽIVOTNOG DOBA

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SADRŽAJ

Proces starenja kod muškog pola povećava verovatnoću nastanka osteoporoze, međutim primena hormonske terapije nosi sa sobom rizik razvoja određenih malignih tumora. Esencijalno ulje biljke *Vitex agnus-castus* (*Vac*) koristi se za ublažavanje simptoma starenja kod oba pola. Takođe, poznato je da kalcitonin, hormon C ćelija štitaste žlezde, inhibira proces resorpcije koštanog tkiva. Svrha ove eksperimentalne studije podrazumevala je ispitivanje uticaja esencijalnog ulja *Vac* na imunohistomorfometrijske karateristike C ćelija štitaste žlezde i dinamiku metabolizma koštanog tkiva 16 meseci starih mužjaka pacova Wistar soja. Prva eksperimentalna grupa životinja je tretirana subkutano sa 60mg/kg telesne mase esencijalnog ulja *Vac*, jednom dnevno u toku tri sedmice. Kontrolna grupa životinja je tretirana sterilnim maslinovim uljem po istom obrascu. Nakon tretmana *Vac*-om utvrđeno je statistički značajno povećanje ($p<0.05$) volumena C ćelija za 10%, dok je nivo kalcitonina u serumu povećan ($p<0.05$) za 27%, u poređenju sa odgovarajućim kontrolnim vrednostima. Nivoi osteokalcina i kalcijuma u serumu su statistički značajno sniženi ($p<0.05$) za 31% odnosno 8%, kod životinja tretiranih *Vac*-om u poređenju sa kontrolama. Ovo su prvi rezultati koji sugerišu da esencijalno ulje *Vac* stimuliše aktivnost C ćelija štitaste žlezde i smanjuje stepen resorpcije koštanog tkiva kod mužjaka pacova srednjeg životnog doba.