

BIOACTIVITY OF THE ESSENTIAL OIL FROM BERRIES OF VITEX AGNUS CASTUS IN MIDDLE AGED MALE RATS

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Pharmaceutical preparations of *Vitex agnus castus* L. (VAC) were shown to contain several bioactive compounds, which were capable to interfere with action of specific human hormones, thus exerting putative beneficial therapeutic effects. The goal of this study was to examine the effects of the essential oil from berries of VAC on the motor behavior of male rat, in relation to the possible dopaminergic actions of the active components. Middle-aged male Wistar rats were treated with VAC essential oil for 3 weeks (60 mg/kg/day *s.c*) and submitted to the open field test. Their brain preparations were used for *in vitro* binding assays at dopamine receptors and immunohistochemical/morphometric analyses of pituitary lactotrophs. Chronic application of VAC essential oil significantly reduced rat motor activity, although it did not influence *in vitro* binding parameters at nigrostriatal D₁ and D₂ dopamine receptors. However, the relative volume density of prolactin-immunopositive cells *per* pituitary unit was significantly decreased by 36%, which reflected down-regulation of their function following the treatment. These findings suggest that the reduction in rat motor activity, registered after chronic administration of VAC essential oil, is probably induced by non-dopaminergic mechanism(s)

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1. Introduction

In recent history, the use of plants as medicines has involved the identification and isolation of bioactive compounds. Drug discovery from medicinal plants has evolved to combine numerous fields of inquiry and various methods of analysis. The process of investigation typically includes preparation of extracts/essential oils of the plant species with known biological activity, followed by submission of these extracted materials to biological screening in pharmacologically relevant assays. Positive results may stimulate further stages of isolation and characterization of the active compound(s) through bioassay-guided fractionation [1].

Vitex agnus castus L. (VAC; Family: Verbenaceae) is a tree or a shrub widely distributed in the Middle East and Southern Europe. This medicinal plant has been utilized in medicine for centuries to treat variety of gynecologic conditions [2]. Nowadays, because of their minor side-effects, the extracts of the fruits (berries) of VAC is one of the most popular botanical dietary

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supplements for the treatment of premenstrual syndrome (PMS) [3]. Recent research suggests that VAC might also be a valuable tool for the treatment of male osteoporosis, benign prostate hyperplasia and prostate cancer [4]. The possible bioactive compounds of VAC berries were identified as flavonoids, tannins, iridoids, diterpenoids and components of essential oil [3]. Many of the therapeutic effects of VAC are assigned to the indirect actions of its active compounds on various hormones, especially on prolactin and progesterone [2, 3]. The proposed mechanism for decrease of prolactin level is identified as the stimulation of D₂ dopamine receptors (DAR) located at anterior pituitary by VAC dopaminergic components [5]. The active principles were identified in the lipophilic fraction of VAC extracts, where the diterpenes (rotundifurane and clerodadienols) were acknowledged as D₂ receptor agonists with prolactin suppressive activity [5]. These discoveries directed attention to the possible contribution of the volatile components in the pharmacological activity of VAC. It was suggested that the components of VAC essential oils might contribute to the hormonal activity of VAC herbal preparations. Several human studies supported this premise by recording the beneficial effects of these essential oils on a variety of menstrual and menopausal complaints [6]. Knowing that the traditional application of VAC and its folk names, Chasteberry and Monk's pepper, came from the belief that it sedated sexual libido in men [7], we performed literature surveys which showed scarcity of the VAC behavioral studies in men. Therefore, this study was conceived to examine the effects of the chronic treatment with VAC essential oil on the motor behavior of the middle-aged male rats. The purpose of this study was to evaluate the influence of a putative dopaminergic component of VAC essential oil on the open-field activity of rats. The impact of the chronic treatment with VAC essential oil on the radioligand binding at the striatal D₁ and D₂ DAR was estimated by *in vitro* binding assays. The changes in immunohistochemical and morphometric parameters of pituitary lactotrophs were also examined.

2. Experimental

2.1. Animals and treatment

Male Wistar rats were housed in the unit for experimental animals at the Institute for Biological Research "Siniša Stanković" in Belgrade, Serbia. They were kept individually under constant laboratory conditions - room temperature (22±2°C) and lighting (12L: 12D). 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 [8] with corn oil as the fat source. The diet contained *per* 100 g: 20.3 g casein; 65 g carbohydrate (45 g cornstarch + 20 g sucrose); 5.2 g corn oil; 3.7 g of fiber (crystal cellulose); 1.5 g vitamin/mineral mix (Ca₃(PO₄)₂ deficient); 1.8 g CaHPO₄; 1 g CaCO₃; 1.5 g DL-Methionine. Casein and crystal cellulose were originated from Alfa Aesar, Johnson Matthey GmbH & Co.KG, Karlsruhe, Germany; carbohydrate, oil, vitamin/mineral mix, CaCO₃, Ca₃(PO₄)₂ from INSHRA PKB, Belgrade, Serbia; and DL-Methionine from Sigma Chemical Company, St. Louis, MO, USA. Food and water were available *ad libitum*. The maintenance of animals and experimental protocols were in accordance with the Official Institutional Guide for Experimental Work on Animals, adjusted to the European Communities Council Directive (86/609).

At the age of 15-16 months, the rats (720±40 g) were randomly divided in two groups of 6 animals each: rats in the first group were injected (0.3 ml/kg b. weight *s.c.*) with the essential oil from ripe fruit of VAC (60 mg/kg) diluted in sterile olive oil, once a day for 3 weeks; the control group received only a vehicle by the same schedule. Each animal was submitted to the behavioral test immediately after the last injection, at the 21st day. After 60 min spent in an open-field apparatus, the animals were decapitated with light diethyl-ether anesthesia and their brains were removed on ice. Dissected *corpora striata* were weighed and membranes with dopamine D₁ and D₂ binding sites were prepared according to standard procedures [9].

2.2. Plant materials

Ripe fruits, i.e. berries of VAC (*Agni casti fructus*) were collected by Prof. Dr. D. 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. Essential oil was prepared by hydrodistillation with the Clevenger system, and its chemical composition further determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MC). The contents of particular compounds were calculated from the GC peak areas using the normalization method [10], and the most abundant components (>1%) are presented in Tab 1.

2.3. Open field test

The motor behaviour of animals was automatically registered in the open field test by video camera and analyzed by ANY-maze software (Stoelting Co., Wood Dale, IL, USA). Each rat was placed separately in a plexiglas cage (41 cm × 41 cm × 41 cm) immediately after the last (21-st) injection of VAC or saline, and they are allowed to accommodate with a new environment for the next 15 min. The locomotion of four animals was simultaneously monitored during the subsequent 45 min. There are two registered parameters of the locomotory activity: (1) the percentage of time when animals are in ambulation, and (2) the total head distance travelled during 45 min. The second parameter reflects both ambulation and stereotype head movements.

Behavioral test was performed between 10 a.m. and 1 p.m. Plexiglas cages were placed in the light- and sound-attenuated room, with artificially regulated ventilation, illumination and temperature, to eliminate any interaction of animals with the environment during the experimental session. The floor of each cage was washed with water and dried before placing new animals.

2.4. Saturation binding assay

In vitro binding assays were performed by standard pharmacological procedures [9]. The samples in duplicate, which contained synaptosomal membranes and different concentrations of the radioligands (³H]SCH23390, 91 Ci/mmol, Amersham, USA, for D₁ and [³H]raclopride, 80 Ci/mmol, American Radiolabeled Chemicals, for D₂ receptors), were incubated (37°C, 10 min) in Tris buffer, pH = 7.5 [120 mM NaCl (Merck, Germany), 5 mM MgCl₂ (Alkaloid Skopje, Macedonia), 2 mM CaCl₂ (Merck, Germany), 1 mmol/l MgCl₂ (Merck, Germany) and 50 mM Tris-HCl (ICN Pharmaceuticals, USA)]. To determine nonspecific binding, 1 μM butaclamol (Sigma Chemical, USA) was used. The binding was stopped by adding ice-cold buffer. After vacuum filtration (Whatman GF-B filters) and thorough rinsing with the buffer, radioactivity remained on filters was measured using liquid scintillation counter (LKB RACKBETA 1219). Results are presented as mean ± S.E.M. of six independent trials performed in duplicate (Table 2.).

2.5. Immunohistochemical and morphometric analyses of pituitary lactotrophs

Pituitaries were excised, fixed in Bouin's solution and further processed according to routine protocols for immunohistochemistry and light microscopy. The series of seven pituitary sections (5 μm thick) from the dorsal, middle and ventral pituitary tissue level were stained by peroxidase-anti-peroxidase immunohistochemical method [11], using primary rabbit antisera directed against the rat prolactin (donation from dr A.F. Parlow, National Hormone and Pituitary Program, Harbor-UCLA Medical Center, CA, USA). In brief, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min. Reduction of non-specific background staining was achieved by incubation with normal porcine serum (Dakopatts, Glostrup, Denmark) diluted 1:10, for 45 min. The following sequence of antisera was then applied: first, the rabbit “anti-rat prolactin” (donation from dr A.F. Parlow, National Hormone and Pituitary Program, Harbor-UCLA Medical Center, CA, USA) was applied for pituitaries at a dilution ratio of 1:500, at room temperature for 1h; second, swine “anti-rabbit IgG” (Dakopatts, Glostrup,

Denmark) was applied at a dilution ratio of 1:500 for 45 min; finally, the rabbit "peroxidase-antiperoxidase" complex (Dakopatts, Glostrup, Denmark) was used at a dilution ratio of 1:100, for 45 min. All washes and dilutions were performed using 0.1 M PBS (pH 7.4). Binding sites were then visualized by 0.05% 3,3-diaminobenzidine tetrachloride (DAB) and 0.03% hydrogen peroxide in 0.2M TRIS-HCl buffer (pH 7.4; Serva, Heidelberg, Germany). The sections were counterstained with hematoxylin and mounted in Canada balsam (Molar Chemicals KFT, Budapest, Hungary). For the control pituitary sections, the primary antibody was substituted with PBS (pH 7.4).

Morphometric analyses of pituitaries were carried out using a point-counting method [12]. Four to five transversal sections from the anterior, central and posterior parts of the pituitary were analyzed using the M_{42} multipurpose test grid inserted into the ocular of a Zeiss light microscope (Jena, Germany). Counting was carried out until the whole section had been covered and 50 test fields were counted per animal at total magnification of x1000. The relative volumes of the prolactin-immunopositive cells (V_c ; μm^3) and their nuclei (V_n ; μm^3) were calculated according to Weibel and Gomez [13]. The volume density of prolactin-immunoreactive cells was expressed as a percentage of total pituitary cell volume in pituitary unit volume.

2.6. Statistical analysis

Statistical analysis of the results from an open-field test, saturation binding assays and morphometric analyses of pituitaries were made by the software (GraphPad Prism v.5.01, San Diego, CA, USA). All data are expressed as mean \pm SEM. Normal distribution of the data was assessed using Kolmogorov–Smirnov test. Two-way Student's t-test was used to evaluate the statistical significance of the differences between the groups chronically treated with VAC essential oil and with vehicle.

Table 1. The most abundant components (>1%) of the essential oil from ripe fruits of VAC. Adapted from ref [10] with the permission of the publisher.

Component	Class of	% of total oil
1,8-Cineole (Eucalyptol)	monoterpenes	16.3
Sabinene	monoterpenes	13.4
α -Pinene	monoterpenes	9.4
trans- β -Farnesene	sesquiterpenes	9.3
Limonene	monoterpenes	6.8
α -Terpinyl acetate	monoterpenes	4.6
Caryophyllene oxide	sesquiterpenes	4.6
trans- β -Caryophyllene	monoterpenes	4.1
Terpinen-4-ol	monoterpenes	2.7
α -Terpineol	monoterpenes	2.5
p-Cymene	monoterpenes	2.3
(E,Z)-Geranyl linalool	diterpenes	2.1
Sclarene	diterpenes	2.0
β -Myrcene	monoterpenes	1.9
Abietatriene	diterpenes	1.2

3. Results

3.1. Motor behaviour

The effects of 3-week-treatment with VAC essential oil on rat motor activity was evaluated by open field test (Fig. 1). Both registered parameters of rat activity, the percentage of ambulatory periods (Fig. 1a) and the total head distance traveled during 45 min (Fig. 1b), were significantly reduced ($p < 0.05$) by 54% and 46%, respectively. These data suggest attenuation of the motor activities of middle-aged male rats following chronic VAC treatment.

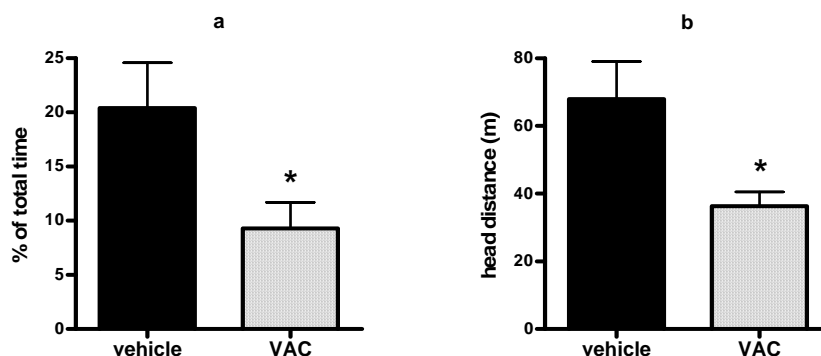


Fig. 1. Effects of the chronic treatment with VAC essential oil vs. vehicle (olive oil) on the (a) proportion of ambulatory active periods and (b) the distance traveled by head of animals during 45 min, in middle-aged male rats. The bars represent means \pm S.E.M. ($n = 6$). *, $p < 0.05$ by Student's two-tailed t -test.

3.2. Saturation binding assay

Striatal synaptosomal preparations were isolated subsequently to the open-field test from the rats treated with VAC essential oil or vehicle. They were used for the saturation binding assays on DAR. Binding affinities of the specific radioligands at D_1 and D_2 DAR are presented by the dissociation constants (K_d); B_{max} values are the estimated tissue densities of the receptors (Tab. 2). Statistical analysis of the results with the two-way Student's t -test did not distinguish significant differences of these binding parameters between the group of animals treated with VAC essential oil and vehicle.

Table 2. The effects of the chronic treatment with VAC essential oil/vehicle on *in vitro* binding parameters at the striatal dopamine D_1 and D_2 receptors. Values are means \pm S.E.M., $n=6$.

	K_d (nM)		B_{max} (fmol/mg)	
	Vehicle	VAC	Vehicle	VAC
D_1	5.1 ± 1.0	6.7 ± 1.0	58.3 ± 11.3	71.6 ± 12.7
D_2	3.2 ± 0.5	2.5 ± 0.1	26.5 ± 1.0	22.5 ± 2.4

3.3. Immunohistochemical and morphometric analyses of pituitary lactotrophs

The pituitary preparations exhibit lactotroph cells in close proximity to numerous capillaries of the pituitary *pars distalis* (Fig. 2a, b). These cells fluctuate in size and appearance, while the prolactin-immunopositive granules are uniformly distributed throughout their cytoplasm in control animals (Fig. 2a). At rats treated with VAC essential oil (Fig. 2b), pituitary lactotrophs are less

numerous and smaller in size, though their location and irregular shape remain the same as in the controls. Morphometric analysis demonstrates that VAC-treated rats have 14% and 36% lower ($p < 0.05$) relative cellular and volume density of prolactin-immunopositive cells, respectively, than the control rats (Fig. 3).

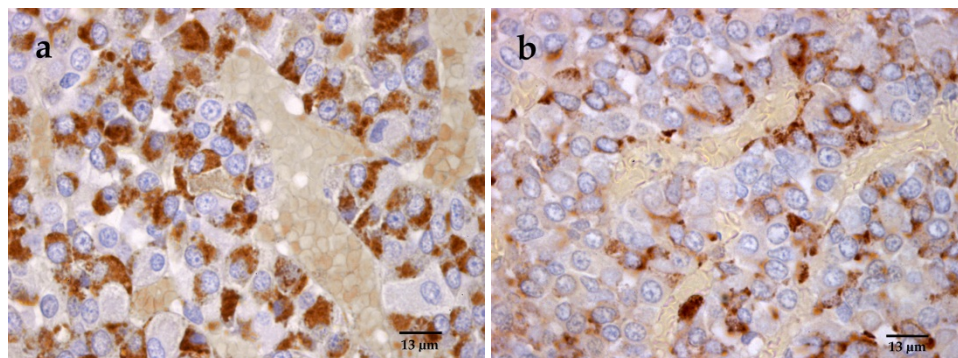


Fig. 2. Immunoreactive prolactin cells in the pars distalis of the pituitary gland from middle-aged male rats chronically treated with vehicle (a) and VAC essential oil (b).

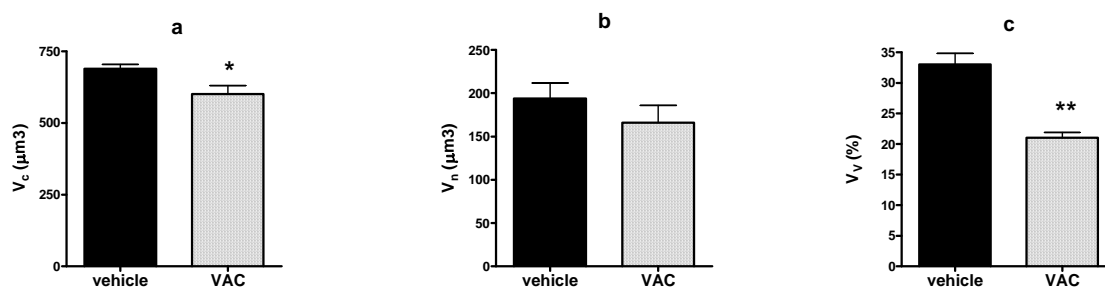


Fig. 3. The cellular (V_c ; a) and nuclear (V_n ; b) volume, and the relative volume density (V_v ; c) of immunoreactive prolactin cells in the pituitaries of the middle-aged male rats chronically treated with VAC essential oil or vehicle. The bars represent means \pm SEM ($n = 6$). * $p < 0.05$ and ** $p < 0.01$ vs. control group (Student's t -test).

4. Discussion

This study reveals that chronic application of VAC essential oil may induce an apparent reduction in the locomotor activity of adult male rats. This action is partially analogous to the traditional usage of this herb for the sedation of male sexual activities. Moreover, several studies notified that VAC preparations in women alleviated symptoms of PMS, including depression, restlessness, emotional irritability and reactivity [14]. The effects of VAC have also been investigated in men, where low doses of the extract were reported to increase, and the high doses decreased serum prolactin concentrations [15]. The suggested mechanism is prolactin-suppressive effect of VAC, which is attained by selective stimulation of dopamine D₂-receptors at the lactotroph cells [5a, 16]. This action is assigned to the VAC diterpenes including clerodadienols, which were found to be almost identical in their prolactin-suppressive properties as dopamine itself [5a, 16]. However, a majority of these studies emphasized diterpenes as the most pharmacologically active VAC compounds and a question arises if the VAC essential oil would have any therapeutic action similar to the total extracts of VAC. Namely, because of their high boiling points, diterpenes may hardly pass over during steam distillation, so they would be only in trace amounts at best in an essential oil. The essential oil used in our study was prepared from VAC berries [10]. It is composed mainly of monoterpenes, with a sparse content of diterpenes (<10%), and trace of clerodadienols (Table 1). Our interest to investigate the effects of this oil was

derived from clinical studies showing a real potential of VAC essential oils to address many common menstrual and menopausal complaints (e.g. calming emotional swings, coping with some of the cognitive changes during menopause) [6]. These data reinstated the question of the active VAC compounds and their mechanisms of action.

Our findings that VAC essential oil may induce silencing of the motor activities of male rats in the open-field test cannot be associated with other data, since there is a lack of animal studies investigating VAC effects on male behaviors and activities. Without any suggestions for the possible mechanism of this calming action we decided to correlate the behavioral effects of the VAC essential oil with the influence of this chronic treatment on the central dopaminergic system and on the activity of the pituitary lactotroph cells. The applied concept to track the changes in the rat nigrostriatal dopaminergic system was based on the registered dopaminergic activity of VAC preparations [5a] and a massive evidence regarding the essential participation of this system in the regulation of the total motor activity [17]. Several studies in rodents indicated that stimulation of cerebral dopamine receptors with high doses of dopamine receptor agonists increased locomotor activity and induced stereotype behaviors, whereas low doses decreased locomotor activity [18]. The hyperactivity was thought to be due to the agonist actions on postsynaptic receptors whilst the motor reduction has been attributed to the stimulation of D₂ autoreceptors [17b, 19]. The chronic application of dopamine agonists typically lead to the down-regulation of the affected local DAR, which may be detected by changes of *in vitro* binding parameters in binding assays [20].

The results of our study revealed insignificant changes of the *in vitro* binding parameters at rat striatal D₂ receptors after the treatment (Tab 2). Thereby, it is suggested that the components of VAC essential oil did not exert the registered behavioral effects by the proposed dopaminergic mechanism. However, it was found that this chronic treatment reduced relative volume and volume density of prolactin-immunopositive cells (Figs 2, 3). A possible dopaminergic action of VAC essential oil on the pituitary lactotrophs may not be excluded, knowing that such morphometric changes of pituitary lactotrophs were found to be associated with the depletion of serum prolactin level [21] and with the level of dopaminergic stimulation [22]. After all, the exact mechanism of the locomotor inhibition in middle aged male rats, which was induced by the chronic treatment with VAC essential oil, remains indistinct. Hereupon, further studies are required to distinguish this mechanism and the active VAC compounds.

5. Conclusions

In conclusion, the registered attenuation of motor activity in middle aged male rats, after their chronic treatment with VAC essential oil, suggests that this essential oil contains active compounds that may be pharmaceutically applicable for both man and woman. Furthermore, it is suggested that the observed reduction in rat motor activity is probably induced by the non-dopaminergic mechanism(s), which remains to be elucidated afterwards.

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