

**VITEX AGNUS-CASTUS L. ESSENTIAL OIL INCREASES
HUMAN ERYTHROCYTE MEMBRANE FLUIDITY**ESENCIJALNO ULJE BILJKE *VITEX AGNUS-CASTUS L.*
POVEĆAVA FLUIDNOST ERITROCITNE MEMBRANEVladimir Ajdžanović¹, Ivan Spasojević², Jasmina Pantelić¹, Branka Šošić-Jurjević¹,
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Summary: Erythrocyte membrane fluidity is related to their rheologic behavior, the dynamic quality of erythrocytes, which is tempted in hypertension and atherosclerosis. An increased risk of these and other cardiovascular diseases occurs in ageing women. Menopause-related conditions are often treated with hormone replacement therapy that may increase the risk of malignancies. *Vitex agnus-castus L.* essential oil contains various organic compounds (monoterpenes, sesquiterpenes and terpenoids), and is increasingly used as an alternative therapy for menopausal symptoms. These components of the oil may be incorporated into cell membranes, thereby changing the membrane fluidity. The aim of this study was to determine the effects of *Vitex agnus-castus* essential oil on human erythrocyte membrane fluidity at graded depths. We used Electron Paramagnetic Resonance spectroscopy and fatty acid spin probes (5-doxyl stearic acid and 12-doxyl stearic acid), whose spectra depend on membrane fluidity. After treatment with *Vitex agnus-castus* essential oil the erythrocytes had a significant ($p=0.029$) and reversible increase in membrane fluidity in the deeper hydrophobic membrane regions, with no change ($p>0.05$) in fluidity near the membrane's hydrophilic surface. These results document increased fluidity of the human erythrocyte membrane by *Vitex agnus-castus* essential oil, and this action may be useful in patients with menopause-related hypertension and other cardiovascular conditions.

Keywords: erythrocytes, membrane fluidity, Vac essential oil, EPR

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Kratak sadržaj: Fluidnost membrane eritrocita uslovljava njihove reološke karakteristike, svojevrsne dinamičke kvalitete, izmenjene u hipertenziji i aterosklerozi. Rizik od navedenih i drugih kardiovaskularnih oboljenja se kod žena povećava sa starenjem. Tegobe koje prate menopauzu se obično ublažavaju supstitucionom hormonskom terapijom, koja može povećati rizik od pojave malignih oboljenja. Esencijalno ulje biljke *Vitex agnus-castus L.* sadrži različita organska jedinjenja (monoterpene, seskviterpene i terpenoide) i sve češće se koristi kao alternativno terapijsko sredstvo za simptome menopauze. Navedene komponente esencijalnog ulja mogu se ugraditi u ćelijske membrane i menjati njihovu fluidnost. Cilj studije bio je da se utvrde efekti esencijalnog ulja biljke *Vitex agnus-castus* na fluidnost membrane eritrocita po njenoj dubini. Korišćena je spektroskopija elektronske paramagnetne rezonance i masnokiselinske spinske probe (5-doksil stearinska i 12-doksil stearinska kiselina), čiji spektri zavise od fluidnosti membrane. Nakon tretmana eritrocita esencijalnim uljem biljke *Vitex agnus-castus* utvrđeno je značajno ($p=0,029$) i reverzibilno povećanje fluidnosti njihove membrane u dubljim, hidrofobnim regionima, dok u površinskim, hidrofilnim regionima eritrocitne membrane nisu utvrđene značajne promene ($p>0,05$). Ovi rezultati ukazuju na povećanje fluidnosti eritrocitne membrane nakon tretmana esencijalnim uljem biljke *Vitex agnus-castus*, što može biti koristan efekat pri tretmanu pacijentkinja sa hipertenzijom i drugim kardiovaskularnim oboljenjima u menopauzi.

Ključne reči: eritrociti, fluidnost membrane, Vac esencijalno ulje, EPR

List of Abbreviations: Vac, *Vitex agnus-castus*; EPR, Electron Paramagnetic Resonance; GC, gas chromatography; GC/MS, gas chromatography-mass spectrometry; FID, flame ionization detector; EI, electron impact; DS, doxyl stearic acid; S, order parameter.

Introduction

Hormone replacement therapy is a common therapeutic approach to treat symptoms in ageing women. Estrogens have reduced the morbidity related to coronary heart diseases in postmenopausal women (1) but estrogen replacement therapy has also increased the risk of breast cancer (2) and the frequency of thromboembolic events (3). Considering the potential risks of estrogen replacement in menopausal women, increasing attention is being focused on alternative, plant-based therapy for menopausal symptoms, including those related to the cardiovascular system. Soybean isoflavones (isolated or as a part of complex formulas) are often exploited for this purpose (4) although they may have disruptive effects on the endocrine system, potentially resulting in cell transformation and cancer (5). Also, essential oils from aromatic plants, which may have useful therapeutic potential, are increasingly used to treat a wide spectrum of age-dependent diseases (6). Germane to this, essential oil from both the berry and the leaf of the chaste tree (*Vitex agnus-castus* L., Verbenaceae; Vac), native to the Mediterranean and Central Asia, has been found to be effective in the treatment of ageing symptoms in women (7).

Serious cardiovascular risks related to menopause are mainly due to estrogen deficiency, and include, *inter alia*, changes in lipid profiles, obesity, hypertension and atherosclerosis (8, 9). It has been suggested that cell membrane alterations are responsible for certain blood pressure disorders (10). The fluidity of the erythrocyte membrane is an important determinant that modulates their rheologic behavior and is a main feature defining blood viscosity (11); this property is related to hypertensive and atherosclerotic disease. For example, the membrane fluidity of erythrocytes is reduced in both spontaneously hypertensive rats and patients with essential hypertension, when compared to normotensive controls (12, 13). Also, the fatty acid profile of the erythrocyte membrane defines its physicochemical properties, i.e., fluidity/viscosity (14, 15). Because lipid profiles are altered in menopause, the possibility arises for changes in the erythrocyte membrane properties to occur that may be at least partially responsible for increased cardiovascular risks. Therefore, therapeutic agents capable of increasing the fluidity of the erythrocyte membrane could be beneficial for treating hypertension and other cardiovascular complications associated with menopause.

The aim of this study was to determine if Vac essential oil (a mixture of various monoterpenes, sesquiterpenes and terpenoids (Figure 1)), modulates erythrocyte membrane fluidity in a manner that would be favorable for use in the treatment of menopausal symptoms. Namely, such information could be important to determine the utility of Vac essential oil in

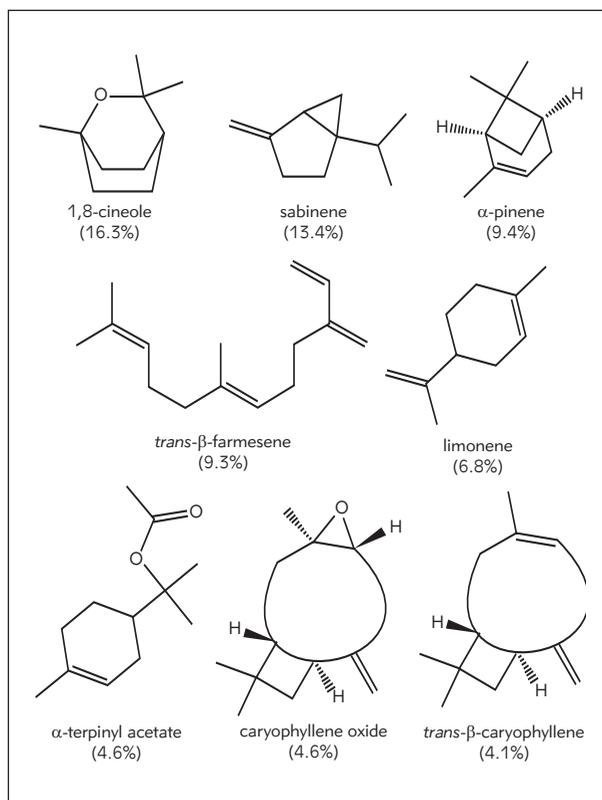


Figure 1 The chemical structures of organic compounds prevalent in the Vac essential oil. The proportions of major components are shown as percentages.

hypertension and atherosclerosis, that frequently occur in elderly women.

In order to evaluate the essential oil effects, we used Electron Paramagnetic Resonance (EPR) spectroscopy and lipophilic spin-probes which intercalate the membrane and whose EPR spectra are affected by membrane fluidity. Namely, EPR measurements provide the calculation of the order parameter (S) which is reciprocally proportional to the membrane fluidity. This method revealed a specific and significant effect of the essential oil on the human erythrocyte membrane that is compatible with potentially beneficial effects for menopausal women.

Materials and Methods

Plant material and essential oil preparation

Ripe Vac berries were identified and collected from June through October 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 3 h. The yield of the oil was 0.72%. The obtained essential oil was stored at +4 °C until testing.

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 a split-splitless injector, attached to an HP-5 column (25 m × 0.32 mm, 0.52 µm film thickness) and fitted to a flame ionization detector (FID). Carrier gas flow rate (H₂) was 1 mL/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while column temperature was linearly programmed from 40 to 240 °C (at a rate of 4°/min). The same analytical conditions were employed for the GC/MS analysis, where the HP G 1800C Series II GCD system equipped with an HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness) was used. The transfer line was heated at 260 °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 the available terpenoids, and by computer searching, matching mass spectra with those filed in the Wiley 275 library of mass spectra. Confirmation was obtained using the calibrated AMDIS program for the determination of experimental values for retention indices of the recorded constituents and by comparing them with values from the literature (16). For quantification purposes, area percent data obtained by FID were used. The content of particular compounds was 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%) and trans-β-caryophyllene (4.1%) (Figure 1) (17).

Erythrocyte sample preparation

Fresh blood was obtained from four healthy volunteers between the ages of 30 and 35 years, using tubes containing 0.072 mL of 7.5% K₃EDTA as the anticoagulant per 3 mL of blood (Vacuette EDTA; Greiner Bio-One, Kremsmünster, Austria). The erythrocytes (erythrocyte membranes) were spin-labeled as described previously (18). Fresh blood erythrocytes were washed three times with isotonic phosphate-buffered saline (PBS; NaCl 8.8 g/L, Na₂HPO₄ 1.2 g/L, NaH₂PO₄ 0.43 g/L, pH 7.4) by centrifugation at 2500 g for 10 min at 4 °C. The hematocrit for fresh blood was ~ 40%, and all samples were adjusted to the same hematocrit before incubation. Aliquots of

Vac essential oil were added to the washed erythrocytes to obtain the final ratio of 0.7 µL/mL. This concentration was selected in order to mimic *in vivo* levels in the blood of menopausal women exposed to dermal application of Vac essential oil (19). The control samples contained erythrocytes untreated with the oil. All samples were gently shaken at 37 °C for 20 minutes, and coagulation was absent during the procedure. To determine the reversibility of potential changes in the fluidity of the erythrocyte membrane, a part of each sample was taken and gently washed three times with PBS.

EPR measurements

Ethanol solutions of the fatty acid spin-probes 5-doxyl stearic acid (5-DS) and 12-doxyl stearic acid (12-DS) (2-(3-carboxypentyl)-2-tridecyl-4,4-dimethylloxazolidine-3-oxyl and 2-(10-carboxydecyl)-2-hexyl-4,4-dimethylloxazolidine-3-oxyl; (Molecular Probes, Junction City, OR, USA)) were applied to the walls of tubes. EPR with 5-DS provides information about the fluidity near the hydrophilic membrane surface, while 12-DS has a nitroxide (EPR active) structure farther down the lipid acid chain (at carbon 12 from the carboxyl group), thus providing information on the fluidity of deeper membrane regions (20). The amount of DS to be used was calculated to obtain the optimal spin-label/membrane-lipid ratio of approximately 1:100 (21). After the ethanol was completely evaporated, the sample was added and gently mixed. Evaporation of ethanol was necessary to prevent its potential effect on erythrocyte membrane fluidity. Samples were placed in Teflon tubes with a wall thickness of 0.025 mm and an internal diameter of 0.6 mm (Zeus Industries, Raritan, NJ) and inserted into quartz capillaries. The incubation and EPR measurements were performed in air. EPR spectra were recorded using a Varian E104-A EPR spectrometer (Palo Alto, CA, USA) operating at X-band (9.1 GHz) and adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 100 G; scan time, 4 min; time constant, 0.25 s. The temperature was controlled at 20 °C during the measurements. Spectra were recorded and analyzed using EW software (Scientific Software Inc., Bloomington, IL). The order parameter (S), which is reciprocally proportional to fluidity, was calculated as described previously (22). All procedures related to the use of human blood in this study conformed to the recommendation provided in the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Statistical analysis

The data are presented as means ± standard deviation (SD) for at least five separate experiments.

Significance differences were calculated by the non-parametric two-tailed Mann-Whitney test. Means were considered significantly different at $p < 0.05$.

Results

We used two spin-probes, 5-DS and 12-DS, for this study in order to explore the effects of Vac essential oil on the erythrocyte membrane at graded depths. Figure 2 shows the characteristic EPR spectra of un-

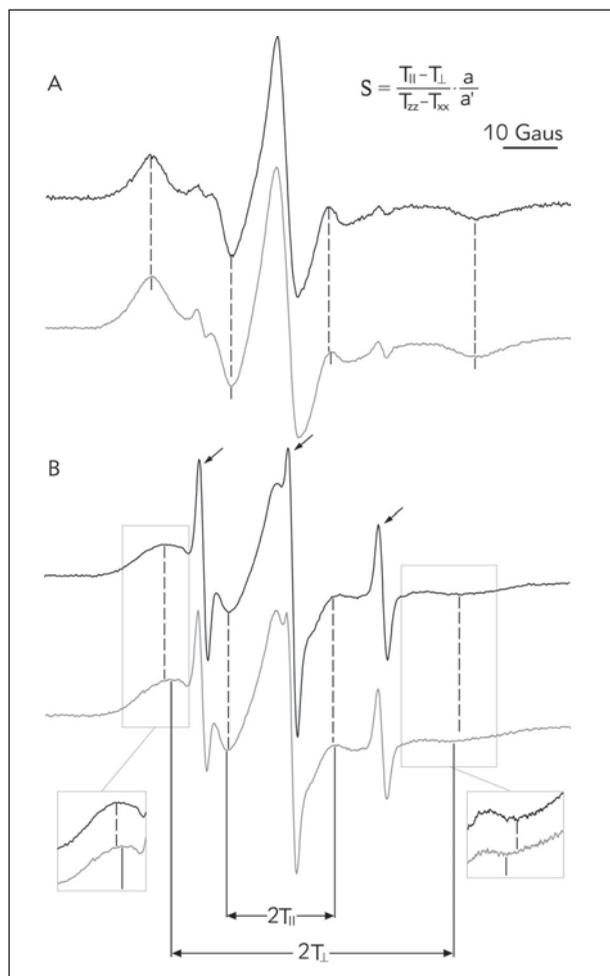


Figure 2 Characteristic EPR spectra of A) erythrocytes labeled with 5-DS without (control; dark trace) or with Vac essential oil (0.7 $\mu\text{L}/\text{mL}$; pale trace). B) Erythrocytes labeled with 12-DS without (control; dark trace) or with Vac essential oil (0.7 $\mu\text{L}/\text{mL}$; pale trace); S, order parameter; $2T_{II}$, outer hyperfine splitting; $2T_{\perp}$ subscript, inner hyperfine splitting; a, isotropic hyperfine coupling constant in crystal [$a = 1/3(T_{xx} + T_{yy} + T_{zz})$]; a' , isotropic hyperfine coupling constant in membrane [$a' = 1/3(T_{II} + 2T_{\perp})$]; T_{xx} , T_{yy} , T_{zz} hyperfine constants (for 5-DS they were taken to be $T_{xx} = T_{yy} = 6.1$ G, $T_{zz} = 32.4$ G; for 12-DS $T_{xx} = 6.26$ G, $T_{yy} = 5.81$ G, $T_{zz} = 33.46$ G) (22). Dashed lines marking parameters in control spectra are shown in order to stress the differences between controls and treated samples. Three narrow lines originate from the DSs in the solution (arrows) (18, 22).

Table I Order parameters (S) obtained by using 5-DS and 12-DS in human erythrocytes exposed to the Vac essential oil

Samples	5-DS (S)	Statistical significance	12-DS (S)	Statistical significance
Control	0.746 \pm 0.004	–	0.609 \pm 0.007	–
Vac essential oil	0.736 \pm 0.012	n.s.	0.585 \pm 0.006	p = 0.029
Control + wash	0.735 \pm 0.009	–	0.631 \pm 0.005**	–
Vac essential oil + wash	0.739 \pm 0.006	n.s.*	0.626 \pm 0.007**	n.s.*

* – Non-significant;

** – The order parameter of washed erythrocytes (both control and pre-treated with Vac essential oil) determined using 12-DS was significantly ($p < 0.05$) higher in comparison with corresponding unwashed samples. Data are shown as means \pm S. D.

treated erythrocytes or erythrocytes treated with Vac essential oil, labeled with 5-DS (near the hydrophilic membrane surface, Figure 2A) or 12-DS (in the deeper membrane regions, Figure 2B). The calculated order parameters (S) of the erythrocyte membrane labeled with 5-DS and 12-DS are summarized in Table I. Considering that the order parameter is reciprocally proportional to the membrane fluidity, it was determined that Vac essential oil significantly ($p = 0.029$) increased membrane fluidity in the deeper, hydrophobic membrane regions, without affecting ($p > 0.05$) fluidity near the membrane hydrophilic surface. The observed changes of fluidity in the deeper membrane regions were reversible, as the order parameter of the membranes of erythrocytes that were treated with Vac essential oil and then washed had a value similar ($p > 0.05$) to the order parameter obtained in washed controls. Although not part of our study design, we noted that even gentle washing decreased erythrocyte membrane fluidity when compared to unwashed erythrocytes. This observation should be considered in studies designed to evaluate erythrocyte membrane properties.

Discussion

Advanced age in females implies an altered physiologic status characterized by estrogen deficiency that is accompanied by unfavorable changes in lipid metabolism and increased cardiovascular morbidity and mortality (9). Estrogen replacement therapy ameliorates post-menopausal symptoms, but serious side effects occur, including an increased risk of breast cancer, especially when estrogens are com-

bined with progestational agents (2), an increased rate of venous thromboembolic events (3) and significantly higher rates of cholecystitis (23). Such deleterious effects have limited the use of estrogen therapy. As an alternative, soybean isoflavones have been assessed as potential therapy for various cardiovascular issues and ageing symptoms. However, these also have drawbacks (5, 24–26). Recently, emphasis has been placed on therapeutically useful essential oils from aromatic plants (6). One of these, Vac essential oil, has shown promising effects in the treatment of ageing symptoms in women (7). Cardiovascular conditions related to female ageing include hypertension and atherosclerosis, whose pathophysiology is linked to changes in erythrocyte membrane fluidity (11) and blood viscosity (27), while an increase of erythrocytes' elastic deformability is the therapeutic imperative.

In the present study, Vac essential oil had differing effects on erythrocyte membrane fluidity depending on depth. Namely, membrane fluidity was increased ($p=0.029$) in the deeper, hydrophobic portions of the membrane whereas it was unchanged ($p>0.05$) near the hydrophilic surface of the erythrocyte. We exposed erythrocytes to low concentrations of Vac essential oil in order to mimic *in vivo* levels present in the blood of menopausal women exposed to dermal application of the oil. Hence, these results obtained *in vitro* are comparable to *in vivo* conditions, and indicate that the application of Vac essential oil may be expected to increase erythrocyte membrane fluidity in the blood of treated subjects. Since the fluidity of the erythrocyte membrane strongly affects their rheologic behavior/deformability which relate to blood pressure (11–13), Vac essential oil could be efficacious when hypertension and other cardiovascular problems related to menopause are present.

The main constituents of Vac essential oil are various monoterpenes, sesquiterpenes and terpenoids. Most are small cyclic molecules without polar groups (see Figure 1), and therefore hydrophobic. The current data imply that compounds in Vac essential oil are preferentially positioned in the hydrophobic core of the membrane. Observed changes after oil treatment were reversible, indicating that Vac oil constituents affecting membrane fluidity are not covalently bound to membrane lipids/proteins but, rather, are intercalated into the membrane. Further,

they dissociate from the membrane when they are removed from the medium. This is in accordance with previous results showing the accumulation of monoterpenes in the cell membrane as well as rapid release after removing from the media (28). Anjos and co-workers (29) have shown, using EPR with 5-DS, that 1,8-cineole, a main constituent of Vac, modifies the properties of cell membranes by rupturing the hydrogen-bonded network at the membrane–water interface, but not by changing membrane fluidity near the surface. Similar results were obtained for limonene and other related compounds (30). The ability to increase the susceptibility of the membrane to the penetration of various compounds seems to be a general characteristic of cyclic terpenes (31). To date, several constituents of Vac essential oil – 1,8-cineole, α -pinene, limonene (31), have been used for increasing percutaneous permeation and to facilitate the passage of drugs through cellular membranes (32). Judging from the present results, this property of cyclic terpenes could be, in addition to the previously proposed mechanism, explained by the decreased level of order within the hydrophobic core of the membrane. Germane to this idea, Vac essential oil could represent a useful addition to facilitate percutaneous absorption of other agents.

The presented results demonstrate a specific and reversible increase of deep erythrocyte membrane fluidity after Vac essential oil application, thus raising the possibility of potential benefit in menopausal women and the various cardiovascular conditions common to this specific age. In addition, the membrane effects of various organic compounds in Vac essential oil that improve percutaneous absorption may be applicable to other areas of potential therapeutic value.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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