ACTA MICROBIOLOGICA BULGARICA

Investigation of Antioxidant and Antiviral Properties of Geraniol

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Abstract

Geraniol is an acyclic monoterpene alcohol with characteristic rose-like odour. It is an important constituent of Bulgarian *Rosa alba* L. and *Rosa damascena* Mill. essential oils. The purpose of the present study was to investigate antioxidant ability as well to reveal the potential for antiviral activity of geraniol against the replication of viruses belonging to different taxonomic groups and representing important human pathogens. Geraniol significantly depressed the effect of oxidation - it showed good ability to capture 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and to inhibit lipid peroxidation in a egg liposomal suspension. Geraniol showed low cytotoxicity toward HEp-2 cells. It was tested *in vitro* for its activity against viruses representing important human pathogens assigned to different taxonomic groups: coxsackievirus B1 (CV-B1) from the *Picornaviridae* family, respiratory syncytial virus (RSV) from the *Paramyxoviridae* family, and influenza virus A/Aichi/68/H3N2 from the *Orthomyxoviridae* family. *In vitro* antiviral effect was examined by the virus cytopathic effect inhibition assay. Geraniol showed antiviral activity only against CVB1 - the ratio of selective index is 3.9. The investigated biological properties of geraniol, including good antioxidant and antiviral activities against some virus families, together with negligible toxicity, warrant further studies to explore the feasibility of formulating geraniol-containing consumer products with health promoting properties.

Key words: geraniol, antioxidant activity, antiviral properties

Резюме

Гераниол е ацикличен монотерпенов алкохол с характерен мирис на роза. Той е важна съставна част от етеричните масла на българската *Rosa alba* L. и *Rosa damascena* Mill. Целта на настоящото проучване е да се изследва антиоксидантната способност, както и да се разкрие потенциала за антивирусна активност на гераниол срещу репликацията на вируси, принадлежащи към различни таксономични групи, които са важни човешки патогени. Гераниол показа добра способност да улавя 2,2-дифенил-1-пикрилхидразил (DPPH) радикали и да инхибира липидната пероксидация в моделна система от яйчени липозоми. Гераниол демонстрира ниска цитотоксичност към HEp-2 клетки. *In vitro* беше тествана неговата активност срещу вируси, които са важни човешки патогени, принадлежащи към различни таксономични групи: Коксаки B1 вирус (CV-B1) от семейство *Picornaviridae*, респираторен синцитиален вирус (RSV) от семейство *Paramyxoviridae* и грипен вирус A/Aichi/68/H3N2 от семейство *Orthomyxoviridae*. Антивирусният ефект беше изследван *in vitro* в постановка на многоциклов ЦПЕ (цитопатичен ефект)-инхибиращ тест. Гераниол показа антивирусно действие само срещу CVB1 - селективният индекс е 3.9. Изследваните биологични свойства на гераниол, сред които са добрата антиоксидантна и антивирусна активност срещу някои вирусни семейства, заедно с незначителната токсичност, налагат провеждането на допълнителни изследвания, за да се проучи приложимостта на гераниол-съдържащите продукти с добри здравословни показатели.

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Introduction

Compounds from natural plants are important sources of drugs against a wide variety of diseases. Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is an acyclic monoterpene alcohol with the chemical formula $C_{10}H_{18}O$. The product referred to as "geraniol" is a mixture of the two cis-trans isomers (Fig. 1) properly named geraniol (*trans*) and nerol (*cis*). Geraniol has characteristic rose-like odour and the taste (at 10 parts per million) is described as sweet floral rose-like, citrus, with fruity, waxy nuances (Burdock, 2010). It is an important constituent of essential oil of ginger, lemon, lime, lavender, nutmeg, orange, rose, etc., an acyclic monoterpenoid, and the main component of oil of rose, e.g. Bulgarian *Rosa alba* L. and *Rosa damascena* Mill. (Mileva *et al.*, 2014). Geraniol is a fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps, and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. Its use worldwide is approximately greater than 1 000 metric tones per annum (Lapczynski *et al*., 2008). In addition, geraniol exhibits various biochemical and pharmacological properties. Researchers have shown geraniol to be an effective plant-based insect repellent (Barnard and Xue, 2004) and its potential as an antimicrobial agent has been highlighted in several studies (Bard *et al.,* 1988). Geraniol exerts in vitro and in vivo antitumor activity against murine leukemia, hepatoma and melanoma cells (Burke *et al*., 1997; Yu *et al.,* 1995 a, b). Geraniol is reported to prevent cancer (Carnesecchi *et al.,* 2004).

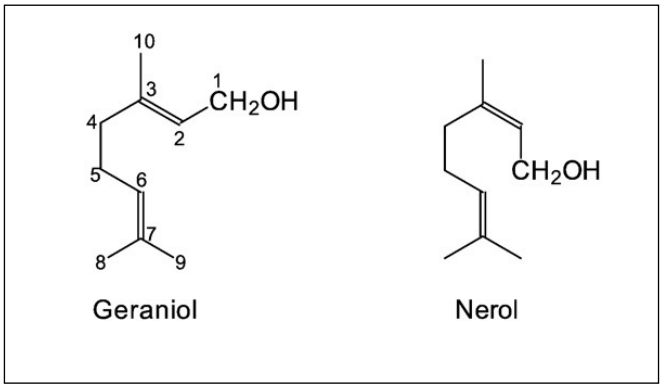

Fig. 1. Chemical structure of geraniol and nerol

The purpose of the present study was to investigate antioxidant activity as well to reveal the potential for antiviral activity of geraniol, against the replication of viruses, belonging to different taxonomic groups and representing important human pathogens.

Materials and Methods

Chemicals Used

All chemicals, standards, solvents, and culture media of high purity (>99%) were purchased from Sigma-Aldrich Chemie GmbH, Merck (Germany), and Givaudan (Switzerland).

DPPH Test

Hydrogen atoms and electron-donating potential of geraniol was measured from the bleaching of the purple-colored ethanol solution of DPPH. The compound was dissolved in ethanol to a concentration of 100 $mg.mL^{-1}$ stock solutions for the following dilutions. DPPH assay was measured, as follows: freshly prepared ethanolic solution of DPPH (100 mM) was incubated with tested substance in the concentration of 1 to 0.1×10^{-5} $mg.mL^{-1}$; after incubation for 30 min in the dark, at room temperature, the optical density (OD) was monitored spectrophotometrically at wavelength (λ) of 517 nm. Inhibition of DPPH in percentage (I, %) was calculated as given below:

$$I\ (\%) = [(OD\ control - OD\ sample)/ (OD\ control)] \times 100$$

IC_{50} was defined as the quantity of substance necessary to decrease the initial DPPH by 50%. All activities were compared against 2,6-di-tert-butyl-4-methylphenol (BHT) and ascorbic acid, as well popular antioxidants. Data were obtained from the plotted graph scavenging activity of each sample. Lower IC_{50} value means higher antiradical activity. Each experiment was performed in triplicate and data were presented as a mean of the three values (Singh *et al.,* 2008).

Extraction of Liposomal Suspension

We used a liposomal suspension obtained from phospholipids of egg yolk as lipid rich media, extracted according to Folch *et al.* (1957). After evaporation under vacuum, the chloroform fraction was dissolved in 50 mM potassium-sodium phosphate buffer pH 7.4 (Sigma Chemicals Company Ltd) to a final concentration of 2 mg $lipid.mL^{-1}$, and vortexed for 10 min. Ultrasonic sonication was performed in Branson ultrasonic bath for 30 min.

Antioxidant Activities in Liposomal Suspension

Antioxidant activities in liposomal suspension were measured by formation of endogenous lipid peroxidation products, reacting with 2-thiobarbituric acid (TBARS), and detected spectrophotometrically (λ = 532 nm) by the method of Bishayee and Balasubramanian (1971), adapted by Mileva *et*

al. (2000). Briefly, each sample in the test tube contains 1.8 ml liposomal suspension with concentration of 2 mg lipid.mL^{-1}, and 100 µL methanol solutions of compounds to achieve concentrations of 0.01, 0.1, and 1 mg.mL^{-1}, prepared immediately before use. The samples were vigorously stirred and, after pre-incubation for 10 min at 37°C, the induction of lipid peroxidation was initiated by adding of 50 µl Fe^{2+} and 50 µl ascorbic acid to a final concentration of 1 mmol.L^{-1}. After incubation for 30 min at 37°C, the reaction was stopped with 0.5 ml of 15 % trichloroacetic acid and 0.5 ml of 0.67 % thiobarbituric acid. The samples were heated at 100°C for 20 min and cooled in ice. 5 ml of n-butanol was added to each tube - it was vigorously stirred and centrifuged at 1200 × g for 10 min. The amount of TBARS generated in the system was determined from the upper organic layer. The ratio of the absorption at 560 nm for the sample, containing tested substances in different concentration and the same absorption for the controls (without tested substances) in percentage was called antioxidant activity (AOA). The experiments were performed in triplicate.

$$AOA\ (\%) = Es/Ec \times 100\%$$

where Es were content of TBARS, formed in samples, containing tested substances, and Ec were TBARS of the controls (without tested substances). All experiments were performed in triplicate and data were presented as a mean of the three values. As positive control served BHT.

Cells and Viruses

Coxsackievirus B1 (CV–B1) (strain Connecticut) from the *Enterovirus* genus of the *Picornaviridae* virus family, human respiratory syncytial virus A2 (HRSV-A2) from the *Paramyxoviridae* family, were grown in the Hep-2 cell line. Cells and viruses were from the cell culture collection of the *Stephan Angeloff* Institute of the Bulgarian Academy of Sciences, Sofia, Bulgaria. Cell lines were grown in a humidified atmosphere at 37°C and 5% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Grand Island, NY, USA), in a growth medium containing 5% fetal bovine serum and supplemented with antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamycin). When harvesting viruses and performing antiviral assays, maintenance medium was used, in which serum was reduced to 0.5%. Viruses themselves were grown in a humidified atmosphere at 37°C and 5% carbon dioxide.

Cellular Toxicity

Monolayer cell cultures in 96-well plates (Cellstar®, Greiner Bio-one, GmbH, Frickenhausen, Germany) were inoculated with 0.1 mL/well maintenance medium containing different concentrations of the samples in 0.5 lg intervals. On the 48th hour after incubation, they were subjected to the neutral red uptake procedure (Borenfreund E, and J.A. Puerner, 1985), and the 50% cytotoxic concentration (CC_{50}) was calculated. Briefly, after removal of the maintenance medium, which contained the test compound, cells were washed and 0.1 mL fresh maintenance medium, supplemented with 0.005% neutral red dye (Fluka Chemie AG, Buchs, Switzerland), was added to each well and cells were incubated at 37°C for 3 hours. Afterwards, cells were washed once with PBS and 0.15 mL/well desorb solution (1% glacial acetic acid, 49% ethanol, 50% distilled water) was added. After 10 min of mild shaking, the optical density (OD) of each well was read at 540 nm in a microplate reader (Organon Teknika reader 530, Oss, Netehrlands). The CC_{50} value was defined as the concentration of each sample that reduced the absorbance of the treated cells by 50% when compared to the untreated control. The CC_{50} values were determined by regression analysis.

Antiviral Activity

The virus cytopathic effect (CPE) inhibition assay was used for evaluating the antiviral effects of the samples. Monolayer cells in 96-well plates were inoculated with 0.1 mL virus suspension containing 100 $CCID_{50}$ ($CCID_{50}$ is the 50% Cell Culture Infectious Dose which was previously determined by the standard virus titration assay in the respective cell culture). After one hour for virus adsorption (two hours in the case of HRSV-A2), excessive virus was discarded, and cells were inoculated with 0.1 mL of maintenance medium containing different non-toxic concentrations (in 0.5 lg intervals) of the test samples. Then cells were further incubated in a humidified atmosphere at 37°C and 5% carbon dioxide. The CPE was scored daily till the appearance of its maximum in the virus control wells (with no compound in the maintenance medium), that happened usually in 48 hours. Then viable cells were stained according to the neutral red uptake procedure and the percentage of CPE inhibition for each concentration of the test sample was calculated using the following formula:

$$\%\ CPE = [OD\ test\ sample - OD\ virus\ control]/[OD\ toxicity\ control - OD\ virus\ control] \times 100.$$

Table 1. DPPH scavenging activities and AOA in egg liposomal suspension of geraniol and reference standards ascorbic acid and butylated hydroxyl toluene (BHT).

N	Compounds	DPPH IC_{50} [$\mu g.L^{-1}$]	AOA (%) 1 $mg.mL^{-1}$	AOA (%) 10 $mg.mL^{-1}$	AOA (%) 100 $mg.mL^{-1}$]
1	Geraniol	9.45 ± 0.34	31.13 ± 1.34	24.51 ± 0.34	22.33 ± 0.34
2	BHT	4.03 ±0.24	34 ± 2.11	27.62 ± 3.11	21.30 ± 1.87
3	Ascorbic acid	3.12 ± 0.37	NT*	NT*	NT*

*NT – non tested; Results are expressed as average ± SD (n=3).

The concentrations that inhibited 50% of the virus-induced CPE, and the 50% inhibitory concentrations (IC_{50}), were determined by regression analysis. The selectivity index (SI) was calculated as the ratio between CC_{50} and IC_{50} ($SI = CC_{50}/IC_{50}$).

Results and Discussion

Antioxidant Properties

Antioxidant defence system of cells comprises of endogenous antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione, ascorbic acid, uric acid, etc., which act either independently, or cooperatively, (or even synergistically) against free radicals (Vandana *et al.,* 2006). These antioxidants protect against the deleterious effects of reactive oxygen species by scavenging them, converting them to non-toxic compounds, or chelating the ions required for their activation. Cells suffer because of deterioration at physiological processes during oxidative stress, when the antioxidant defence system becomes inadequate to neutralize the excess reactive oxygen species (ROS) produced. The supplementation of exogenous antioxidants has been found to be effective in restoring the homeostatic disturbances due to oxidative stress. This supports the role of natural antioxidants in achieving strong immune system as well as healthy aging (Han *et al*., 2005). This study has been held to explore the antioxidant effect of geraniol in pro-oxidant conditions, as compared with the reference standards ascorbic acid and butylated hydroxyl toluene.

The DPPH assay usually involves a hydrogen atom transfer reaction (Li *et al*. 2009). DPPH radical scavenging test is a sensitive antioxidant assay and depends on substrate polarity. The presence of multiple hydroxyl functions could be considered as an option for the hydrogen donation and/or radical scavenging activity.

Antiradical activities of tested substances against stable DPPH radical expressed in IC_{50} [$\mu g.L^{-1}$] showed notable values (Table 1). A lower IC_{50} value indicates a greater antioxidant activity. Most active was BHT, the same activity demonstrated ascorbic acid, and least active was geraniol. As a rule, the antioxidant properties of the plant extracts cannot be attributed to activities of single constituents. Their scavenging activity could be explained by the combination of effects with one another. Ruberto and Baratta (1999) demonstrated that the most radical scavenging activities of natural extracts are mainly due to the cumulative effect of ingredients as polyphenols, as well as nerol, eugenol and geraniol; within their structure has been observed polar-bonded hydrogen. Undoubtedly, DPPH radical has little relevance to present in biological systems as well in living organisms, but this study is indicative of the capacity of geraniol to scavenge free radicals, and will refer to hydrogen atom or electron donation ability, independently of any enzymatic activity.

AOA of tested compounds in Fe^{2+}/ascorbic acid-induced oxidation of egg liposomes are expressed as percentage of inhibition of oxidation process in comparison to control sample (without tested substances). Geraniol significantly depressed the effect of oxidation. It exhibited a protective capacity against Fe^{2+}/ascorbic acid-induced lipid peroxidation in liposomes in a concentration-dependent manner.

The damaging reactions of free radicals are widely implicated in the etiology of numerous oxidative stress-related diseases (Piaru *et al*., 2010). These typically electrophilic reactive moieties interact with lipids, proteins, and nucleic acids, and cause oxidative damages (Deighton *et al,*. 2010). Lipid peroxidation is one of the effects induced by free radicals, and it can occur in lipid system due to the presence of structures rich in highly peroxidizable, polyunsaturated fatty acids. The presence of antioxidants in the fraction will minimize the oxidation of these structures due to the inhibition of the chain reaction of lipid peroxidation (Sherry *et al.,* 2013). Antioxidant power of natural products is an expression of their capacity to defend from the action of free radicals as well as to prevent degen-

eration from oxidants (Deighton *et al*., 2000, Piaru *et al*., 2010, Sherry *et al.,* 2013).

Yu *et al*. (1995 a) reported that geraniol suppressed lipopolysaccharide-induced nitric oxide and prostaglandin E2 production at a system of RAW 264.7 macrophages in a dose-dependent manner. The inhibitory efficacy of geraniol was concomitant with decreases in protein and mRNA expression levels of inducible nitric oxide synthase (iNOS)

Although peroxidation in model membranes may be very different from peroxidation in cell membranes, the results obtained in the former membranes may be used to advance understanding of peroxidation in biological membranes (Schnitzer *et al.,* 2007).

Antiviral Test

The *in vitro* antimicrobial activity of eugenol against various pathogens has been reported earlier; very little is known about its activity and mode of action against viruses, which are important human pathogens assigned to different taxonomic groups: coxsackievirus B1 (CV-B1) from the *Picornaviridae* family, respiratory syncytial virus (RSV) from the *Paramyxoviridae* family, and influenza virus A/Aichi/68/H3N2 from the *Orthomyxoviridae* family. Therefore, the mode of antiviral action of eugenol against those viruses *in vitro* was evaluated in the present study. The results obtained demonstrated that geraniol was showing low cytotoxicity in a Hep2 cell system (Table 2). In the same model system, CC_{50} of geraniol is lower than that of disoxaril, used as reference substance for the study of antiviral effect of CVB1. Our research on antiviral screening of geraniol showed that there is no antiviral effect against influenza virus A/Aichi/68/H3N2, as well as against RSV. The scientific literature also lacks data published on this subject.

Pronounced antiviral effect was observed against the representative of the *Picornaviridae* family - coxsackievirus B1. IC_{50} of geraniol is 48 μg/mL, about 30% lower than oxoglaucine and about three times lower than disoxaril.

The antiviral activities of monoterpene alcohols (including linalool, nerol, citronellol, and geraniol) probably are due to their solubility in the phospholipid bilayer of cell membranes and increased permeability of cells (Knobloch *et al.,* 1989; Devi *et al.,* 2010).

These results suggest that geraniol exhibits anti-coxsackievirus B1 activity, supporting its therapeutic potential for virus-associated disorders.

In conclusion, geraniol is abundant and occurs in a large number of plants. This molecule is widely used as a fragrance chemical in both cosmetic and household products. Several studies have confirmed the pharmacological properties of this acyclic monoterpene alcohol. Geraniol - with its good chemopreventive activity - may present a new class of antiviral agent, and this renders a great opportunity for further investigation. The investigated biological properties of geraniol, including antiviral activities against some virus families, together with negligible toxicity, warrant further studies to explore the feasibility of formulating geraniol-containing consumer products with health promoting properties.

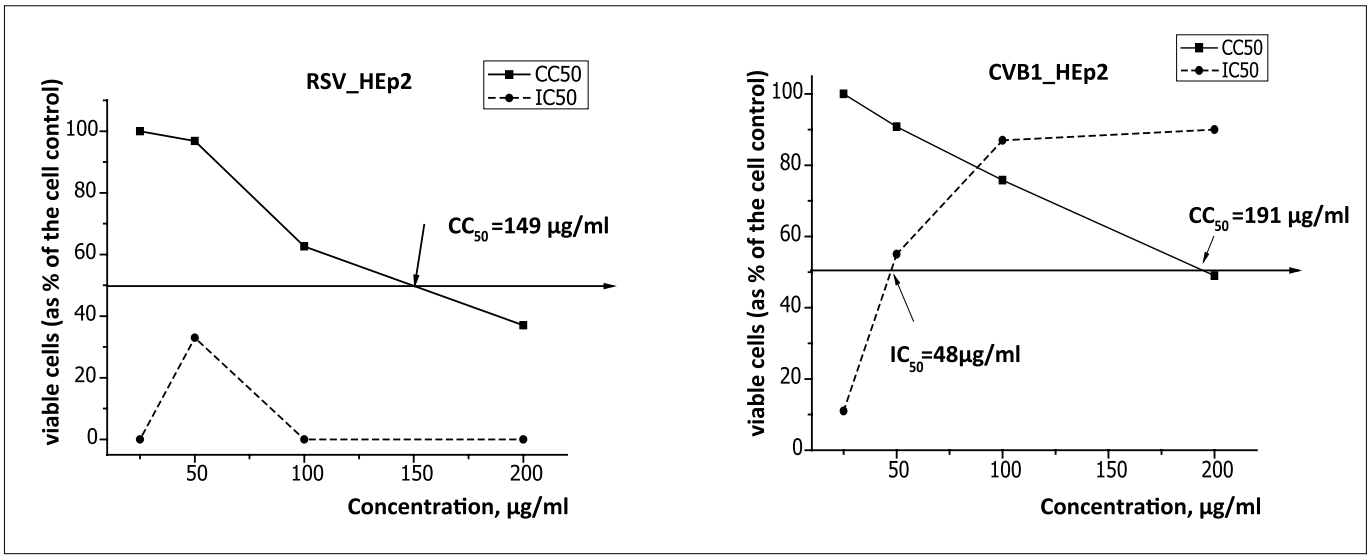

Fig. 2. Antiviral activity of geraniol against CVB1 and RSV virus in HEp2 cells. Data are present as CC_{50} – percentage viable HEp2 cells, and IC_{50} – percentage protection.

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