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Original Research Article

Chemical composition, *in vitro* antiradical and antimicrobial activities of Bulgarian *Rosa alba* L. essential oil against some oral pathogens

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Keywords

Rosa alba L. essential oil, GC/MC, antioxidant effect, antimicrobial activity

A B S T R A C T

Essential oil from *Rosa alba* L. - white oil-bearing rose, is produced quite recently only in Bulgaria. Because a few documentation of its phytochemical screening and biological activities, the purpose of this work was to investigate chemical composition, antiradical properties and antimicrobial activity of *Rosa alba* L. essential oil against oral pathogens A*ggregatibacter actinomycetemcomitans, Enterococcus faecalis, and Streptococcus mutans.* The oil was found to be rich in geraniol (18.28 %), followed by heneicosane (12.95 %), nonadecane (10.75%), and citronellol (9.00 %). *Rosa alba* L. essential oil scavenge DPPH radicals (IC_{50} = 2.1 µg.L^{-1}) similar to the referents standards ascorbic acid and butylated hydroxyl toluene, and demonstrated good ability to inhibit Fe^{2+}- induced lipid peroxidation of egg liposomes. Antimicrobial screening revealed that *Rosa alba* L. oil showed less capacity against Gram-positive *E. faecalis* as compare *S. mutans.* Most active was manifested against Gram-negative *A. Actinomycetemcomitan.* This findings indicate radical-scavenging, antioxidant and antimicrobial activities of *Rosa alba* L. essential oil, which probably due to its chemical composition and holds promise for it application as a novel pharmaceutical antioxidant and antibacterial agents.

Introduction

Currently there is great interest in plants essential oils because their multiple biological effects. They are a promising source of antioxidant and antimicrobial agents in the food and cosmetic industry to replace synthetic compounds with natural ones of plant origin.(Özkan et al. 2004, Naquvi et al. 2014, Jackie et al. 2014, .Kumar et al. 2014, Bharadwaj et al. 2014). The *Rosaceae* is a plant family within which several species have been demonstrated to possess multifunctional healing properties,

such as cooling, soothing, astringent, cardiotonic, antiinflammatory, skin protective and anti-aging effects, as well anti-HIV, antibacterial activities (Lawrence BM 1991, Basim E. and Basim H.(2003), Özkan et al. 2004, Pavlov et al. 2005, Boskabady et al. 2006, Jafari et al. 2008, Perumal et al. 2012). Most of these therapeutic activities are due to the essential oil. Traditionally, rose oil is used as a remedy for anxiety, depression and for the treatment of stress related condition (Naquvi et al. 2014). *Rosa damascena* Mill. has show as a potent antioxidant that has many therapeutic uses in addition to its perfuming effects (Jafari et al. 2008). Rose oil is a preferred ingredient in the perfumery and cosmetics products as a base component of many of the modern perfumes, creams, soaps, lotions, but it also finds application in the food industry as a flavour additive (Naquvi et al. 2014).

Rosa alba L., oil-bearing rose with white flowers is commonly known as "Bulgarian white rose". As ornamental plant, *Rosa alba* L. has a long history in Bulgarian folklore, holy believes, weddings rituals, and religious traditions (Degraf K. 2003). For industrial purpose in the past it was grown and processed mixed with *Rosa damascena* Mill. Currently, white rose is cultivated separately in the Roses Valley in Bulgaria only, and essential oil of *R. alba* L. is distilled separately too (Nedkov et al. 2009). About the chemical composition and biological activities of *R. alba* L. essential oil are scarce data (Dobreva and Kovacheva 2008, Nedkov et al. 2009, Gochev et al. 2010). A few studies were undertaken, but as far as we find before, no published data on the antioxidant as well antibacterial properties of *R. alba* L. oil against oral pathogens. Highly adherent Gram-negative pathogen *Aggregatibacte actinomycetemcomitan (A. actinomycetem comitans),* Gram-positive aerobic bacterium *Enterococcus faecalis (E. faecalis),* and Gram-positive microaerophilic bacterium *Streptococcus mutans (S. mutans),* which colonize the oral cavity, involved in dental infections including necrotizing ulcerative ginginvitis and periodontitis, periradicular abscesses, infected root canals etc. (van Houte J. 1994, Gomes et al. 2006, Fine et al. 2007). So, these bacteria cause dental disorders, recognized as to be one of the most important and dangerous problems in dental medicine.

In this sense, the purpose of this work was to investigate chemical composition, antioxidant properties and antimicrobial activity of *Rosa alba* L. essential oil and some it ingredients against clinically significant oral pathogens *A. actinomycetemcomitans, E. faecalis, and S. mutans*.

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 (Swiss).

Plants material and distillation of essential oil

As a raw material were used fresh petals of *Rosa alba* L., from plantation in experimental field of the Institute of Rose and Essential Oil Plants, in Kazanlak. The plant material was collected in the morning in May/Jun 2009, before the sunrise (6-8 a.m.), in a phase of flowering semi-blooming - blooming flowers. Rose oil was distilled immediately by water-steam distillation of semi-industrial processing line in Institute. Process parameters of the

distillation were as follows: a raw material for charge - 10 kg; hydro module 1:4, rate of 8-10% and call duration - 150 min. The aromatic water was re-distilled in the same apparatus. The essential oil of each charge is the sum of primary and secondary oil in their natural ratio Total oil is a mixture of distillates collected over 15 days - the time of the collection campaign of *Rosa alba* L. for 2009. Finally, it was dried with sodium sulfate, filtered and stored appropriately.

Chromatographic conditions

The quantitative and qualitative chromatographic analysis of *Rosa alba* L. essential oil was performed using Agilent 7890A/5975 GC-MS system, equipped with HP-5 apolar column (60 m x 0.25 mm x 0.25 μm). As a carrier gas helium with a constant flow rate of 1 mL/min was used. The splitless injection of 1 mL sample was performed. The parameters of the temperature program were as indicated in the standard described in international standard (ISO 9842).

The identification of compounds was performed by comparison of their relative retention indices and mass spectra with those of pure substances. Mass spectra also compared with these of National Institute of Standards and Technology (NIST) library database.

Examination of antioxidant properties

DPPH test

Hydrogen atoms and electron-donating potential of essential oils were measured from the bleaching of the purple-colored ethanol solution of DPPH. All compounds were dissolved in ethanol to a concentration of 100 $mg.mL^{-1}$ stock solutions for the follow dilutions. DPPH assay was measured as follow: freshly prepared ethanolic solution of DPPH (100 mM) was incubated with tested substances in the concentration of $1–0.1x10^{-5}$ $mg.mL^{-1}$, and the optical density (OD) monitored spectrophotometrically at λ517 nm after 30 min incubation in dark at room temperature. Inhibition of DPPH in percentage (I, %) was calculated as given below:

I (%) = [(OD control – OD sample)/(OD control)] X 100

IC_{50} was defined as the quantity of substance necessary to decrease the initial DPPH by 50%.

All activitis 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 of scavenging activity of each sample. Lower IC_{50} value means higher antiradical activity. Each experiment was carried out in triplicate and data were presented as a mean of the three values (Singh et al.).

Extraction of liposomal suspension

A liposomal suspension obtained from phospholipids of egg yolk as lipid rich media, extracted according to Folch et all. (1957). After evaporation under vacuum, the chloroform fraction was dissolved in 50 mM K-Na phosphate buffer pH 7.4 (Sigma Chemicals Company Ltd) to a final concentration of 2 mg $lipid.mL^{-1}$, and vortexed for a 10 min. Ultrasonic sonification was carried out 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[15], adapted from Mileva et al. (2000). Briefly, each sample in the test tube contents 1.8 ml liposomal suspension with concentration of 2 mg lipid.mL^{-1}, and 100 µL methanol solutions (1:10 v/v) of oil and pure compounds to achieve concentrations of 0.01, 0.1 and 1 mg.mL^{-1} prepared immediately before use.

The samples were vigorously stirring and after pre-incubation for 10 min at 37 ^{0}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 ^{0}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 ^{0}C for 20 min and cooling in ice. 5 ml of n-butanol was added to each tube, vigorously stirring and centrifugated at 1200 x g for 10 min. The amount of TBARS generated in the system was determined of 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 x 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 carried out in triplicate and data were presented as a mean of the three values. As positive control served BHT.

Antimicrobial activity

Microorganisms

Microorganisms were obtained as follows: *A. actinomycetemcomitans* strain 8324 and *S. mutans* strain 20523 from the collection DSMZ – Germany; *E. faecalis* strain *3360* from the Bulgarian national collection for microorganisms, and cell cultures and were stored in 10% glycerol containing micro-test tubes at -86 ^{0}C.

Antimicrobial assay

Antimicrobial activity of the *Rosa alba* L. essential oil and its major ingredients was evaluated using the microdilution broth method. *A. actinomycetemcomitans* was incubated in microaerophilic conditions (5% CO_2) at 37 ^{0}C for 48 h on Trypticase® Soy broth, which was supplemented by IsoVitaleX (BD 211876). *E. faecalis* was incubated in aerophilic conditions at 37 ^{0}C for 24 h on Trypticase® Soy broth; *S. mutans* was incubated in microaerophilic conditions (5% CO_2) at 37 ^{0}C for 24 h on Trypticase® Soy broth and supplemented with 0.5% yeast extract.

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of all samples was determined by the microdilution method described by Andrews J. (2001), using 96-well standard microtiter plates. Briefly, 50 µL of twofold serial dilutions of examined samples were added to 50 µL microbial suspension adjusted to yield approximately 1.0 x 10^5 CFU mL^{-1}. MIC was encountered as the lowest concentration of examined sample that inhibits the visible microbial growth after 24-48 h incubation at 37 ^{0}C at appropriate nutrient media. Antimicrobial activitity of *R. alba* L. essential oil and its ingredients were

compared against this of vancomycin as routinely used antibiotic for prevention of infections originating from the oral cavity.

Statistical analysis

All of the experiments were done in triplicate. The data were analysed with SPSS 11.0 software package and were recorded as means ± standard deviations.

Results and Discussion

Chromatographic profile of *Rosa alba* L. oil

Chromatographic composition of the essential oils of *R. alba* L. studied by GC-MC are presented on Table 1, in order of their elution from HP-5 apolar column.
Chromatographic profile is typical for representatives of this genotype (Nedkov et al., 2009). The main groups are monoterpenes (geraniol – 18.28%, nerol – 7.74%, citronellol – 9%), followed by hydrocarbons with high molecular weight (C_{15} – C_{31}) as nonadecane (10.75%), and heneicosane (12.95 %), trace amounts of phenylpropanoids as eugenol (0.02%) and methyeugenol (0.10 %), terpenoids such as neral and geranial (0.70 % and 0.90 %).

Rose oils comprise complex mixture of ingredients and their biological activities can be attributed to their different constituents (Bakkali et al. 2008). In order to verify which of the components of the oil are the best powerful antioxidant, as well as most active against set of dental important oral pathogens, the individual major components of oils were tested too.

Antiradical activities of *Rosa alba* L. oil

Antiradical properties of mentioned above substances against DPPH radical expressed in IC_{50} (Table 2) showed that the most active was *Rosa alba* L.essential oil, followed by eugenol, methyleugenol, nerol, geraniol, citronellol and citral.
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 of substrate polarity. As can see on chromatographic composition of the essential oil, most of components possess an ideal structural chemistry for free radical scavenging activity (Table 2). The presence of multiple hydroxyl functions could be considered as option for the hydrogen donation and/or radical scavenging activity.
Antiradical activities of both of essential oils against stable DPPH radical expressed in IC_{50} [$\mu g.L^{-1}$] showed notable values. A lower IC_{50} value indicates a greater antioxidant activity. Most active was *R. alba* L. oil – 2.1 $\mu g.L^{-1}$, followed by eugenol > BHT > methyleugenol ≈ ascorbic acid > citronellol and nerol. In a system applied we found that essential oil exhibited scavenging of DPPH radicals higher then eugenol and methyleugenol, as well then the benchmarks substances (BHT and ascorbic acid). As can see on Table 1, eugenol and methyleugenol are contained in small amounts in oil and probably have a little participation in its total radical scavenging properties.

The other pure compounds such as nerol, geraniol, citronellol and citral showed lower antioxidant activities as compare to essential oil. As a rule, the antioxidant properties of the plant extracts cannot be attributed of activities to single constituents. Their scavenging activity could be explained with the combination of effects with one another. Ruberto and Baratta (1999) demonstrated that most radical scavenging activities of essential oils are mainly due to the cumulate effect of ingredients nerol, eugenol and geraniol, within the structure has been

observed polar bounded hydrogen. In *R. alba* L. oil the amount of these components was total of 26.40 % (Table 1). According to these results we could assume that there is a relationship between the content of oxygenated monoterpenes and antiradical activity of *R. alba* L. essential oils. 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 the Bulgarian rose oil to scavenge free radicals, and will refer to hydrogen atom or electron donation ability, independently of any enzymatic activity.

AOA of essential oil in Fe^{2+}/ascorbic acid - induced oxidation of egg liposomes is show on Table 3. The results are expressed as percentage of inhibition of oxidation process in comparison to control sample (without tested substances). *R. alba* L. oil significantly depressed the effect of oxidation. It exhibited a protective capacity against Fe^{2+}/ascorbic acid-induced lipid peroxidation in liposomes. It has been established that nerol and geraniol inhibit the oxidation of liposomes in a concentration-dependent manner, but most active is citronellol. Eugenol and methyleugenol which showed the strongest radical-scavenging properties against DPPH, shows the less ability to inhibit liposomal iron/ascorbat - induced oxidation. Presumably, the inhibition of the oxidation in liposomal suspension under the influence of essential oil from *R. alba* L. was dependent on the content of citronellol, nerol and geraniol.

The damaging reactions of free radicals are widely implicated in the etiology of numerous oxidative stress related disease (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 be 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 its capability to defend from the action of the free radicals as well to prevent degeneration from oxidants (Deighton et al. 2000, Piaru et al. 2010, Sherry et al 2013). Although peroxidation in model membranes may be very different from peroxidation in cell membranes, the results obtained in model membranes may be used to advance understanding of peroxidation in biological membranes (Schnitzer et al. 2007).

Since the essential oils are volatile, unstable on light and temperature, they have the ability to degrade in presence of oxygen. Good approach in modern nanomedicine is the encapsolating of essential oils in nanoemulsions as well in liposomes. Liposomes can protect the fluidity of essential oils and decrease they stability at 4-5 ^{0}C for 6 months at least (Schnitzer et al. 2007).

Antimicrobial activity

A collection of three microorganisms was used, including the Gram-positive bacteria *E. faecalis* and *S. mutans* and Gram-negative ones *A. actinomycetemcomitan* are now considered to be one of the most important and dangerous stomatologic problems due to the difficulty of their control (Gomes et al. 2006). In the present study the result obtained from the microdilution method demonstrated that *Rosa alba* L. oil and its

major ingredients show low to high degree of antimicrobial activity against all tested oral pathogens (Table 3). The MIC values ($mg.mL^{-1}$) against *E. faecalis* shoved the lowest activity of tested compounds as follow: citral < *R. alba* L. oil and nerol < methyleugenol and geraniol < eugenol < citronellal < vancomycin. The MIC values against *S. mutans* were 1.1 for citral, citronellol, methyleugenol, and geraniol, and 0.82 for *R. alba* L. oil and nerol, the same value as vancomycine (0.83), but most active was eugenol – MIC = 0.17. Higher activity showed all of tested substances against *A. actinomycetemcomitans,* MIC of eugenol was 0.17, MIC of other pure compounds was about 0.2; MIC of *R. alba* L. oil was 0.45. Study of the MIC is the method most frequently used for screening of plant extracts for antimicrobial activity. Essential oils are poorly soluble in water and present a complex chemical composition; some of their constituents are volatile and have to be used in low concentration and low temperature. This causes many problems in studying their biological and pharmacological properties (Schnitzer et al. 2007, Thaweboon et al. 2009, Sherry et al. 2013.

Such antimicrobial agents essential oils target the bacterial cell wall and membrane, damage their permeability, and cause collapse of the normal cellular processes. When the disturbance of membrane integrity occurs, then its functions are compromised. The antimicrobial activity of the essential oils can be attributed to the contained monoterpenes that, due to their lipophilic character, act by disrupting the microbial cytoplasmic membrane, which thus loses its high impermeability for protons and bigger ions (Özkan et al. 2004, Cristiani et al. 2007, Gochev et al. 2010).

Essential oils and their chemical constituents displayed a broad spectrum and variable degree of antibacterial activities (Gochev et al. 2010, Cristiani et al. 2007). In fact, most researchers reported increasing tolerance and escalating level of drug′s and antibiotic′s resistance among pathogenic bacteria (Cristiani et al. 2007, Thaweboon et al. 2009, Rathera et al. 2012). Proper approach for prophylactic and therapy of bacterial infections is a combination of essential oils with conventional antibiotics and synthetic drugs because of synergism of this co-medication (Rathera et al. 2012)**.**

Table.1 Chemical composition of *Rosa alba* L. essential oil.

№	**Components**[a]	**Class**[b]	**RT, min**[c]	**KI**[d]	Relative percentage, %
1	Linalool	OM	11.12	867	1.45
2	β -phenylethyl alcohol	BC	11.66	1110	0.02
3	Citronellol	OM	14.90	1228	9.0
4	Nerol	OM	15.00	1228	7.74
5	Neral (Citral 1)	OM	15.33	1240	0.7
6	Geraniol	OM	15.79	1255	18.28
7	Geranial (Citral 2)	OM	16.21	16.21	0.9
8	Eugenol	BC	19.90	1280	0.02
9	Methyleugenol	BC	20.19	1401	0.1
10	Nonadecene	AH	33.43	1730	4.25
11	Nonadecane	AH	34.13	1900	10.75
12	Eicosane	AH	37.02	2000	1.37
13	Heneicosane	AH	41.04	2100	12.95
14	Tricosane	AH	46.24	2300	3.10

[a] Compounds are listed in order of their elution from HP-5 apolar column;
[b] Class of compounds; [c] Retention time, min; [d] KI- Kovats index on HP-5 apolar column in reference to *n*-alkenes

Table.2 DPPH (IC_{50}, $\mu g.L^{-1}$) scavenging activities and AOA (%) in egg liposomal suspension of different concentration of *Rosa alba* L. essential oil and some its ingredients.

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	Nerol	4.27 ± 0.11	40.73 ± 3.11	30.46 ± 0.11	20.84 ± 0.11
2	Citral	63 ± 4.27	98.01 ± 4.27	96.56 ± 4.27	95.52 ± 4.27
3	Eugenol	2.47 ± 0.12	87.9 ± 6.12	84.2 ± 0.12	81.3 ± 0.12
4	Citronellol	6.3 ± 0.27	32.41 ± 1.27	25.16 ± 0.27	12.08 ± 0.27
5	Methyleugenol	3.96 ± 0.18	92.80 ± 2.18	82.15 ± 0.18	76.34 ± 0.18
6	Geraniol	9.45 ± 0.34	31.13 ± 1.34	24.51 ± 0.34	22.33 ± 0.34
7	*R. alba* L. oil	2.1 ± 0.3	47.24 ± 3.03	41.23 ± 2.11	32.46 ± 2.21
8	BHT	2.5 ±0.14	34 ± 2.11	27.62 ± 3.11	21.30 ± 1.87
9	Ascorbic acid	3.96 ± 0.3	NT*	NT*	NT*

*NT – non tested

IC_{50} of DPPH scavenging activities were obtained from the plotted graph of each sample. BHT and ascorbic acid served as a positive control.

AOA - 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.

Results are expressed as average ± SD (n=3).

Table.3 Antibacterial activities against testes strains (MIC, $mg.mL^{-1}$) of *Rosa alba* L. essential oil and some its ingredients.

N	Compounds	*Enterococcus faecalis* MIC [$mg.mL^{-1}$]	*Streptococcus mutans,* MIC[$mg.mL^{-1}$]	A*ggregatibacter actinomycetemcomitans* MIC [$mg.mL^{-1}$]
1	Nerol	1.36	0.82	0.24
2	Citral	1.53	1.53	0.27
3	Eugenol	0.98	0.17	0.17
4	Citronellol	0.69	1.74	0.20
5	Methyleugenol	1.19	1.19	0.21
6	Geraniol	1.12	1.12	0.37
7	*R. alba* L. oil	1.36	0.82	0.45
8	Vancomicyn	0.12	0.30	1.8

Vancomycin as routinely used antibiotic served as reference.

In conclusions, *R. alba* L. essential oil indicates as main constituents oxygenated monoterpenes, and in particular geraniol. As a result of the present study, it was found to be effective in two different antioxidant experiments, including DPPH scavenging assay, and inhibition of liposomal oxidation. *R. alba* L. gives promise of practical application as a potential source of natural antimicrobial agent and antioxidant. Taken together, the above-mentioned results support the idea for using white rose oil in oral care products, or/and co-medication in conditions where inflammation, accompanied by oxidative processes due to the oral pathogen contamination are shown as a big problem.

Moreover, for more practical application, *R. alba* L. oil should be subjected to clinical trials to investigate it's *in vivo*

effects in animals and human. A clinical study may be required to confirm the validity of the results obtained.

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