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Original Article

Inhibition of protein glycation by essential oils of branchlets and fruits of *Juniperus communis* subsp. *hemisphaerica*

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Abstract

Oxidative stress and protein glycation play pivotal roles in the pathophysiology of diabetes mellitus and its vascular complications. The present study aimed to investigate the anti-glycation properties of essential oils obtained from different parts of *Juniperus communis* subsp. *hemisphaerica*. The branchlets of male tree (BMT) and branchlets of female (BFT) tree, and fruits of *J. communis* subsp. *hemisphaerica* were extracted using steam distillation method. The oils were phytochemically analyzed using gas chromatography-mass spectrometry. Anti-glycation properties were evaluated using hemoglobin and insulin glycation assays. Overall, 18 volatile components were identified in the *J. communis* subsp. *hemisphaerica* oils, amounting to 82.1%, 100.0% and 96.4% of the BMT, BFT and fruit oils, respectively. Promising inhibitory activity was observed from all concentrations of the tested oils in the hemoglobin and insulin glycation assays. The inhibitory activities peaked to 89.9% (BFT oil; 200 μg mL^{-1}) and 81.0% (BFT oil; 600 μg mL^{-1}) in the hemoglobin and insulin glycation assays, respectively. The evidence from this study suggests that essential oils obtained from the fruits and branchlets of *J. communis* subsp. *hemisphaerica* possess anti-glycation properties. These activities may find implication for the prevention and treatment of diabetic complications.

Keywords: *Juniperus communis* subsp. *hemisphaerica*; Cupressaceae; Protein glycation; Diabetes mellitus; Volatile oil

INTRODUCTION

Development and progression of both macrovascular (cardio- and peripheral vascular disease, and stroke) and microvascular (retinopathy, nephropathy and neuropathy) complications of diabetes mellitus (DM) has been shown to be closely associated with increased oxidative burden. Upon glucose oxidation, a variety of pro-oxidant species including superoxide, hydroxyl and peroxynitrite radicals are formed. Another important consequence of oxidative stress in DM is the formation of advanced glycation end products (AGEs) (1). AGEs are implicated in the pathogenesis of numerous disorders and significantly contribute to the generation of reactive oxygen species (ROS) (2,3).

Modern scientific research has unveiled a variety of biological and pharmacological activities for essential oils (4-6). One of the most appealing biological properties of essential oils is their antioxidant potential which has been supported by a plethora of scientific research (7). Such an activity might have preventive effects against protein glycation and thereby diabetic macro- and microvascular complications.

Juniperus communis L. subsp. *hemisphaerica* (Presl) Nyman (Cupressaceae) is an evergreen and dioecious shrub which grows widely in Europe, Caucasus, Turkey and Iran. (8). This

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plant has a number of medicinal properties and has been reported to have antimicrobial, anti-rheumatic, analgesic and contraceptive effects (9,10).

Although a number of studies have previously reported the antioxidant activity of *J. communis* subsp. *hemisphaerica* volatile oils (11-15), only a few have specifically focused on *J. communis* subsp. *hemisphaerica* (13). Besides, there has been no investigation of the anti-glycation properties of this plant whilst other closely related plants namely *J. foetidissima*, *J. oblonga* and *J. Sabina* have been recently reported to possess considerable anti-glycation properties (16-18).

Therefore, in an ongoing attempt to find bioactive natural products, we aimed to investigate the anti-glycation properties of essential oils obtained from different parts of *J. communis* subsp. *hemisphaerica*.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Merck (Germany) apart from bovine insulin (Novo Nordisk, Denmark).

Plant material

The branchlets and fruits of *J. communis* subsp. *hemisphaerica* were collected from the area between Gadook and Veresk, at a height of 1900 m, located in Firoozkooh, Mazandaran province, north of Iran. The plant was identified by Dr. M. Assadi from the National Botanical Garden (Tehran, Iran). Voucher specimens of the plants were deposited in the herbarium TARI (no. 72897). The plant materials were stored at -20 °C before use.

Essential oil isolation

Steam distillation method (4 h) was applied for the extraction of essential oils (19). Extracted oils were dried over anhydrous sodium sulfate and their yields were then calculated.

Specific gravities of essential oils were determined using a sensitive scale (Scaitec, Germany). In addition, refractive index (Abbe refractometer) and specific rotation (DIP-310 digital polarimeter, Electrothermal 2200, UK) were measured for each oil.

Gas chromatography-mass spectrometry analysis

The gas chromatography-mass spectrometry (GC-MS) apparatus consisted of a Hewlett Packard 6890 gas chromatograph equipped with a fused-silica column (DB-5, 30 m×0.25 mm i.d., film thickness 0.25 μm; Agilent Technologies HP), and interfaced with a quadruple mass selective detector (HP 6890). The operating conditions were: oven temperature 60 to 275 °C at 4 °C min^{-1}; injecton mode: split injection, with helium as the carrier gas, flow rate of 2 ml min^{-1}, 70 eV ion source, 1000 μA ionization current, and scan range of 30-300 atomic mass unit (AMU). The oil components were identified from their retention indices (RI) obtained with reference to *n*-alkane series (Sigma, UK) on DB-5 column and comparing mass spectra with those of authentic samples, composition of their mass spectra and fragmentation patters reported in literature (20) and computer matching with MS-data bank (Wiley Library). In order to prepare a stock solution, 100 μL of essential oil was added to 250 μL of Tween 20 (0.2%) and diluted with deionized water to a final volume of 50 ml. The obtained mixture was shaken in an ultrasonic bath (SU3 THE, Japan) for 15 min to obtain the stock emulsion (2000 μg mL^{-1}).

Hemoglobin and insulin glycation in vitro assays

Hemoglobin used for this study was extracted from blood that was obtained from a local blood bank. Pooled blood (100 ml) was mixed with 5 g/dl EDTA solution (3 ml), followed by centrifugation at 3000 rpm and removal of clear plasma and the buffy coat layers. The red blood cells (RBCs) were washed with cold normal saline three times. Then, 0.5 ml of washed RBC was resuspended in 2 ml of phosphate buffer (0.1 M, pH 7.4) and 2 ml of CCl_4 and centrifuged at 2800 rpm for 5 min. The upper layers (containing hemoglobin) were transferred into new tubes and hemoglobin concentrations were measured by Drabkin method (21). A dilution with final concentration of 5 mg/100 ml was prepared from each hemoglobin solution. Human insulin (100 IU/ml) was prepared in phosphate buffer 0.01 M (pH 7.4). Three concentrations (200, 400 and 600 μg ml^{-1}) of each oil were tested in this assay. All experiments were performed in triplicate.

The rates of hemoglobin and insulin glycation in the presence and absence of different concentrations of examined oils (200, 400 and 600 μg mL^{-1}) were measured after 48 h of incubation at room temperature, as described previously (22). All experiments were performed in triplicate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. Btetween group comparisons were conducted with one-way ANOVA followed by Tukey-Kramer post-hoc multiple comparison testing. A two-sided *p*-value of <0.05 was considered as statistically significant.

RESULTS

Phytochemical analysis

Some physical characteristics of the analyzed oils (yield%, refractive index, specific gravity and specific rotation) are presented in Table 1. Overall, 18 volatile components were identified in the *J. communis* subsp. *hemisphaerica* oils, amounting to 82.1%, 100.0% and 96.4% of the branchlets of male tree (BMT), branchlets of female tree (BFT) and fruit oils, respectively. The major constituents (occurring at frequencies ≥ 10%) of *J. communis* subsp. *hemisphaerica* BMT oil were α-pinene (24.9%), sabinene (19.8%) and δ-2-Carene (10.7%).

In the BFT oil, the main volatile constituents were α-pinene (26.7%) and sabinene (34.0%). As for the fruit oil, α-pinene (22.6%), sabinene (13.1%), β-pinene (29.5%) and limonene (11.0%) were found to be the most frequent components.

All three oils were dominated by monoterpene hydrocarbons equivalent to 70.0%, 88.1% and 88.2% of the BMT, BFT and fruit oils, respectively. Chemical composition and relative frequencies of the identified volatile components are presented in Table 2.

Table 1. Characteristics of essential oils obtained from different parts of *J. communis* subsp. *hemisphaerica.*

	BMT oil	**BFT oil**	**Fruit oil**
Appearance	Transparent yellow	Transparent yellow	Pale yellow
Yield (v/w %)	0.60	0.63	1.27
Specific gravity (g/ml)	0.89	0.86	0.88
Refractive index	1.47	1.47	1.47
Specific rotation	+ 39.7	+ 46.5	+ 33.3

BMT: branchlets of male tree; BFT: branchlets of female tree.

Table 2. Chemical composition (%) of the branchlet and fruit oils of *J. communis* subsp. *hemisphaerica.*

Component	**RRI**[a]	**RA (%)**[b]		
		BMT oil	**BFT oil**	**Fruit oil**
α-Thujene	935	1.7	3.6	4.3
α-Pinene	942	24.9	26.7	22.6
Sabinene	985	19.8	34.0	29.5
β-Pinene	990	4.3	6.1	13.1
δ-2-Carene	1010	10.7	6.2	1.8
α-Terpinene	1022	0.8	1.1	0.8
Limonene	1050	5.4	5.2	11.0
γ-Terpinene	1065	0.7	2.7	1.9
Isoterpinolene	1088	1.7	2.5	3.2
Terpinen-4-ol	1158	0.8	2.2	1.5
Undecanone	1295	0.6	0.3	-
α-Cubebene	1350	0.8	0.5	-
α-Copaene	1385	-	-	0.2
E-Caryophyllene	1435	0.8	0.4	0.3
α-Humulene	1465	0.7	0.5	0.4
Germacrene D	1492	6.3	6.2	5.2
β-Bisabolene	1510	0.4	-	-
γ-Cadinene	1535	1.7	1.6	0.6
Monoterpene hydrocarbons		70.0	88.1	88.2

Table 2. (Continued)

Component	RRI[a]	RA (%)[b]		
		BMT oil	BFT oil	Fruit oil
Oxygenated Monoterpenes		0.8	2.2	1.5
Sesquiterpene hydrocarbons		10.7	9.2	6.7
Oxygenated sesquiterpenes		-	-	-
Miscellaneous		0.6	0.3	-
Total identified		82.1	100.0	96.4

BMT: branchlets of male tree; BFT: branchlets of female tree. [a]RRI: relative retention indices as determined on a DB-5 column using the homologous series of *n*-alkanes. [b]RA: relative area (peak area relative to total peak area).

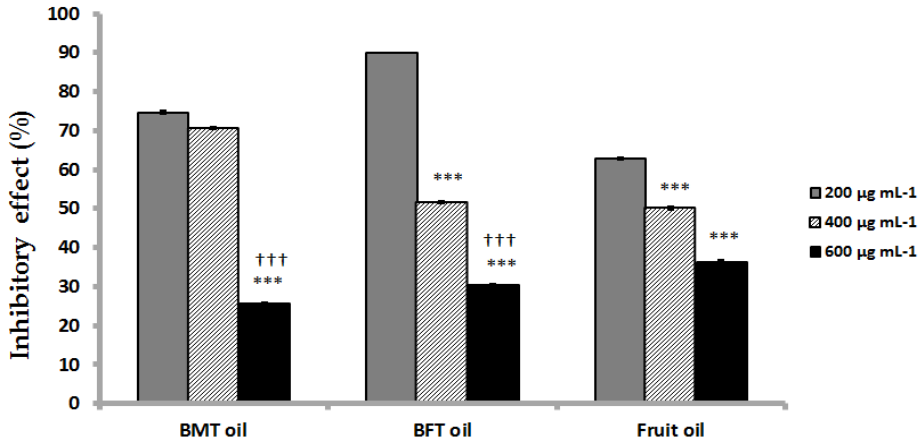

Fig. 1. Inhibitory effects of *J. communis* subsp. *hemisphaerica* essential oils against hemoglobin glycation. BMT: branchlets of male tree; BFT: branchlets of female tree. Each calculated %inhibitory effect has been normalized its specific control solution. *p*-values refer to the comparison of %inhibitory effects between different concentrations of each oil **p*<0.05; ***p*<0.01; ****p*<0.001: compared to the 200 µg ml^{-1} group; †*p*<0.05; †††*p*<0.001: compared to the 400 µg ml^{-1} group.

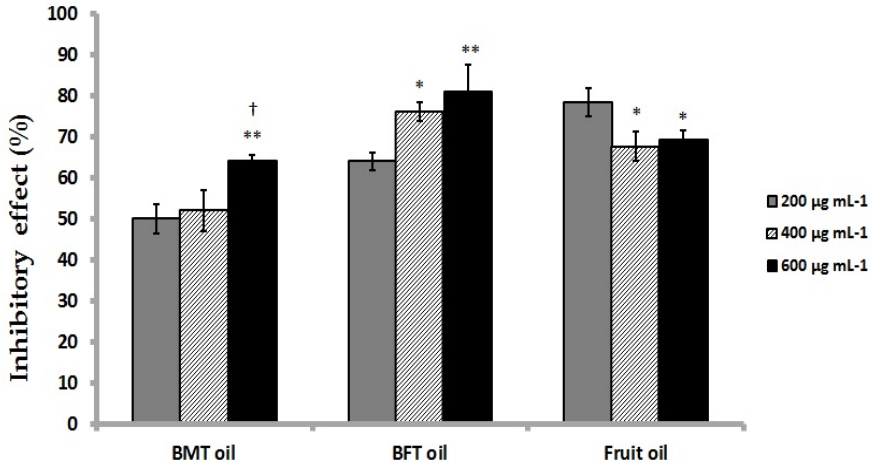

Fig. 2. Inhibitory effects of *J. communis* subsp. *hemisphaerica* essential oils against insulin glycation. BMT: branchlets of male tree; BFT: branchlets of female tree. Each calculated %inhibitory effect has been normalized its specific control solution. *p*-values refer to the comparison of %inhibitory effects between different concentrations of each oil:**p*<0.05; ***p*<0.01; ****p*<0.001: compared to the 200 µg ml^{-1} group; †*p*<0.05; †††*p*<0.001: compared to the 400 µg ml^{-1} group.

Hemoglobin glycation

Branchlets of male tree, branchlets of female tree and fruit oils of *J. communis* subsp. *hemisphaerica* could inhibit hemoglobin glycation at all tested concentrations i.d. 200, 400 and 600 μg ml^{-1}. The highest hemoglobin glycation inhibitory activity for all tested oils was observed at the concentration of 200 μg ml^{-1}.

The inhibitory activity of the tested oils against hemoglobin glaciation was found to be inversely associated with oil concentration. Overall, the highest inhibitory activity was observed with BFT oil (89.9%) followed by BMT (74.7%) and fruit (62.8%) oils. The anti-hemoglobin glycation effects of *J. communis* subsp. *hemisphaerica* oils at different concentrations are shown in Fig. 1.

Insulin glycation

All tested oils showed ≥ 50% inhibitory activity against insulin glycation. The highest activity of BMT and BFT oils was observed at 600 μg ml^{-1} amounting to 64.0% and 81.0% inhibition, respectively.

In contrast, fruit oil exerted its highest activity at 200 μg ml^{-1} concentration (78.4%). Overall, no regular association between oil concentration and anti-glycation effects was observed in the assays. The inhibitory effects of *J. communis* subsp. *hemisphaerica* oils at different concentrations are shown in Fig. 2.

DISCUSSION

Considering the unequivocal role of AGEs in the development and progression of diabetic complications (23,24), there is currently a significant research focus on finding effective inhibitors to be exploited for the treatment of DM. Aside from DM, AGEs are also implicated in a wide variety of other disorders including Alzheimer's disease, stroke, cardiovascular disease and many other inflammatory disorders (25). In order to find AGE inhibitors, natural products are appealing candidates because of their well-documented antioxidant activity, which could be exerted through different mechanisms, potential safety compared to chemically synthesized agents, and availability.

This study produced results which corroborate the findings of previous work with respect to the antioxidant properties of *J. communis* oils (11-15). However, the most interesting finding of the present study was the promising inhibitory activity of *J. communis* subsp. *hemisphaerica* oils against hemoglobin and insulin glycation. To the best of our knowledge, this is the first report on the anti-glycation activities of *J. communis* subsp. *hemisphaerica* varieties. Recently, we have reported the same strong anti-glycation properties from 3 closely related *Juniperus* species.

The peak inhibitory effects of *J. foetidissima* against hemoglobin and insulin glycation were 95% and 100%, respectively, both exerted by fruit oil (18). *J. oblonga* also inhibits hemoglobin and insulin glycation, with the highest inhibitory effect reported from BMT oil amounting to about 90% (16). As for *J. Sabina,* glycation of hemoglobin and insulin were reduced and the greatest effects were reported from BFT (80%) and BMT (90%) oils (17). We have also determined the antiglycation properties of the essential oils from *Cupressus sempervirens*, another member of the cupressaceae family.

The peak inhibitory activity of *C. sempervirens* against hemoglobin and insulin glycation were observed from fruit oil, amounting to about 60% and 80%, respectively (26). In a previous work by Dearlove and colleagues, extracts obtained from 24 herbs and species were screened for their inhibitory activity against albumin glycation. The results indicated a potent activity of spice extracts that was correlated with their phenolic content (27). Similar findings have also been reported by other studies (28).

Several investigations have demonstrated the inhibition of fructose- and glucose-mediated albumin glycation by isolated phenolic compounds such as quercetin, kaempferol, catechin, epicatechin and gallic acid (28,29). While the anti-glycation properties of phenolics, in particular flavonoids, is clear (30), the present findings adds additional evidence regarding the activity of volatile terpenoids.

The process of AGE formation is irreversible and is strongly associated with the oxidative stress. Pro-oxidant species such as hydrogen peroxide and superoxide radicals are produced during the course of glycation and are capable of exacerbating protein glycation through autoxidation of sugars (31). Moreover, binding of AGEs to the receptor for advanced glycation end products (RAGE), stimulate ROS formation (32).

In the present study, α-pinene, sabinene, β-pinene, limonene and δ-2-carene were the main constituents of *J. communis* subsp. *hemisphaerica* oils. This is in agreement with the two previous reports on the same plant apart from δ-2-carene for which previous studies failed to detect significant quantities (9,13). In previous investigations, the frequency of sabinene were reported to be 16.4-21.9% (BMT oil), 19.5-20.3% (BFT oil) and 23.8-25.1% (fruit oil) while α-pinene was found to occur at 12.1-15.6%, 13.6-15.8% and 13.6-33.3% frequencies in the BMT, BFT and fruit oil, respectively (9,13). Such compositional differences are common due to the impact of geographic, climatic and seasonal conditions as well as variations in the harvest period, extraction method and part of the source plant being extracted (33).

Previous reports on the biological activities of *J. communis* subsp. *hemisphaerica* have been scant. In a previous report by Emami and coworkers, BMT, BFT and fruit oils of *J. communis* subsp. *hemisphaerica* were tested for their antioxidant activity using DPPH and deoxyribose degradation assays (13). In the DPPH test, strong radical scavenging activities were observed from the oils, in particular BMT oil which was attributed to the high content of γ-terpinene in the respective oils. *J. communis* subsp. *hemisphaerica* oils also showed degrees of antioxidant activity in the deoxyribose degradation assay, though their effects were not remarkable probably due to the low content of β-pinene (13). In another survey on Iranian conifers, methanol extracts obtained from leaves and fruits of *J. communis* subsp. *hemisphaerica* were screened for their antioxidant capacity using ferric thiocyanate (FTC) method and thiobarbituric acid (TBA) assays. The findings of the latter study implied that BMT, BFT and fruit extracts had > 80% activity in the FTC and > 60% in the TBA method. In both assays, the highest antioxidant activity was observed from the fruit oil (14). The antioxidant capacity of hydrocarbon terpenoids has been suggested to be mainly the result of reaction between chain carrying peroxyl radicals (HOO•) with lineloyl peroxyl radicals. This reaction causes termination of the oxidation chain reaction and is a plausible mechanism for the inhibition of oxidative stress and subsequent formation of AGEs (34).

CONCLUSION

The most obvious finding to emerge from this study is the effective inhibition of protein glycation by *J. communis* subsp. *hemisphaerica* oils. In the light of the present findings, further work needs to be done to confirm the observed anti-glycation properties in *in-vivo* models of DM, and also to establish whether these anti-glycation and antioxidant properties could be of therapeutic utility for DM and its complications. Finally, it remains to be determined that which phytochemicals play the main role in the anti-glycation properties of this plant and the exact mechanism by which such effects are exerted.

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