

Original Research Article

In Vitro Screening of α -Amylase Inhibition by Selected Terpenes from Essential Oils

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Received: 16 May 2014

Revised accepted: 11 August 2014

Abstract

Purpose: To assess some terpenes from herbal products for possible inhibitory effects on serum α -amylase in order to ascertain their potential usefulness in the prevention and/or treatment of diabetes Type 2.

Methods: Solutions of terpenes (citral, eukalyptol, β -pinene, myrcene, eugenol and terpineol) in deionized water were prepared by ultrasonic and manual mixing in four different concentrations ranging from 0.39 – 5.50 $\mu\text{mol cm}^{-3}$. Commercial sera (with normal-N and high-H enzyme activity) were used as a source of α -amylase. α -Amylase activity was determined by standard methods using an automated analyzer.

Results: All the selected terpenes at their maximal concentrations inhibited α -amylase in N-sera in the range 9.68 – 38.70 and 10.71 - 25.00 % for ultrasonic and manual mixing, respectively, while in H-sera, inhibition was in the range 17.10 - 21.05 and 13.58 – 25.92 % for ultrasonic and manual mixing, respectively. Regardless of the concentration of the inhibitor or the method of mixing, citral was the strongest inhibitor of α -amylase.

Conclusion: The selected terpenes, in their appropriate concentrations, influence α -amylase activity to varying degrees. Principal component and agglomerative hierarchical analysis reveal that the most significant factor in α -amylase inhibition is the mode of mixing the samples, rather than their concentrations.

Keywords: α -Amylase, β -Pinene, Citral, Diabetes, Essential oil, Eugenol, Eukalyptol, Myrcene, Terpenes, Terpineol

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. Thus, retardation of starch digestion by inhibition of enzymes such as α -amylase might play a key role in the control of diabetes. Some inhibitors currently in clinical use

are acarbose and miglitol. However, these synthetic hypoglycemic agents have their limitations, are non-specific, may fail to diminish diabetic complications and have several side effects [1]. Herbal medicines are becoming more important in the treatment of diabetes because they are free from side effects and are less expensive when compared to synthetic hypoglycemic agents [2]. In this respect,

traditional medicines possess great potential [3,4]. More and more plant products and their preparations have been used for therapeutic purposes [5-11].

There is increasing number of studies dealing with the influence of essential oils on α -amylase [12-15]. Since in the literature exists a lack of data dealing with inhibitory effect of pure essential oil components on the α -amylase [16], we chose common constituents of numerous and most frequently applied essential oils (citral, eucalyptol, β -pinene, myrcene, eugenol and terpineol) to investigate whether they have an effect on α -amylase activity. The selected terpenes are constituents of essential oils of many plants whose applications are widespread and well-known: citral (from citrus fruits, lemon myrtle, lemongrass, lemon tea-tree) [16,17], eucalyptol (L-eucalyptus, laurel, basil, wormwood, rosemary, sage), β -pinene (rosemary, pine, parsley, basil, yarrow, hops), myrcene (fennel, sage, ginger, hemp), eugenol (cloves) and terpineol (pine). To the best of our knowledge, this is the first study on the effect of selected terpenes on serum α -amylase activity for their potential benefits in the prevention/treatment of diabetes.

EXPERIMENTAL

Materials

As the enzyme α -amylase source, we used a commercial Olympus control sera 1, ODC0003 (sera with a normal enzyme activity N-sera) and Olympus control sera 2, ODC0004 (Olympus Life Science Research Europa GmbH, Sauerbruch str. 50, 81377 Munich, Germany) (sera with elevated enzyme activity H-sera). The authors determined the enzyme activity in the commercial sera according to the manufacturer's instructions (18). Abbott diagnostic analyzer (Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois, 60, 064-3500, USA) was used for all measurements of enzyme activities.

The dilution was necessary to confirm linear dependence between enzyme activity and its concentration in serum, as well as to be sure the inhibition is exclusively the result of use of the compounds in the concentrations at which they are usually administered. The citral, eucalyptol, β -pinene, myrcene, eugenol and terpineol used were 99.99 % pure (Fluka, USA), and their characteristics are shown in Table 1.

Potential inhibitors were prepared by mixing with deionized water in two ways: ultrasonic mixing to

give a homogenous emulsion and manual mixing resulting in a less homogenized emulsion of the test components. Ultrasonic mixing was performed over an ultrasonic bath at room temperature for 5 min.

Concentrations of the selected terpenes were chosen according to previous data [12] and checked experimentally starting from maximum solute concentration of terpenes, which did not lead to denaturation of sera over a period of 30 minutes (1:4, v/v), and then its dilution is carried out in the ratio 1:6, 1:8 and 1: 10 (v/v). Solute concentration in the reaction mixture of the selected terpenes, in the analyzer are shown in Table 2.

Enzyme assay

α -amylase activity under given conditions was measured on Abbott Diagnostics Chemistry Analyzer according to the manual obtained by the manufacturer [18].

Amylase hydrolyzes 2-chloro-4-nitrophenyl- α -D-maltotriose (CNPG3) to yield 2-chloro-4-nitrophenol (CPNP) and 2-chloro-4-nitrophenyl- α -D-maltosides (CNPG2), maltotriose and glucose. Amylase activity is proportional to the rate of 2-chloro-4-nitrophenol formation, whose absorbance is measured spectrophotometrically at 440 nm. Reference values for amylase activity, according to the applied test for adults up to 70 years, are from 25 to 125 U/l.

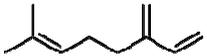
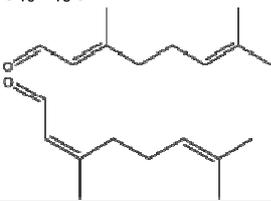
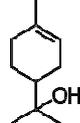
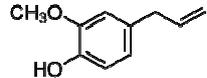
The analyzed samples were prepared by mixing 180 μ l of diluted serum (N or H) with 40 μ l of the potential inhibitor solution, incubated for 10 min and the enzyme activity determined as previously described above. The control was prepared in the same manner, except that instead of the inhibitor solution, deionized water was used. All experiments were carried out in triplicate on the same day. Inhibition was evaluated as in Eq 1.

Inhibition (%) = $\{(A_0 - A_i)/A_0\}100$ (1)
 where A_0 = activity of α -amylase in the absence of an inhibitor (control), and A_i = activity of α -amylase in the presence of an inhibitor (test terpenes).

Statistical analysis

For statistical analysis of the data, software Statistica 10.0 (StatSoft, Tulsa, Oklahoma, USA) was applied. Pattern recognition methods were applied to the data collection: principal component analysis (PCA) as an unsupervised classification method and agglomerative hierarchical analysis (AHA) as an unsupervised

Table 1: Chemical and physical characteristics of selected terpenoid compounds

Name	IUPAC nomenclature	Molecular and structural formula	Molecular weight (g mol ⁻¹)	Density (g cm ⁻³)
β-pinene	6,6-dimethyl-2-methylenebicyclo[3.1.1.]heptane	C ₁₀ H ₁₆ 	136.23	0.872
Myrcene	7-methyl-3-methylene-1,6-octadiene	C ₁₀ H ₁₆ 	136.23	0.794
Citral	3,7-dimethyl-2,6-octadienal E – isomer Z – isomer	C ₁₀ H ₁₆ O 	152.24	0.893
Eucalyptol	1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane	C ₁₀ H ₁₈ O 	154.25	0.9225
Terpineol	2-(4-methyl-1-cyclohex-3-enyl)propan-2-ol	C ₁₀ H ₁₈ O 	154.25	0.934
Eugenol	4-allyl-2-methoxyphenol	C ₁₀ H ₁₂ O ₂ 	164.20	1.060

learning method [19]. A probability level of $p < 0.05$ was considered statistically significant [20].

Table 2: Concentration of selected terpenes in reaction mixture ($\mu\text{mol}/\text{cm}^3$)

Name	C ₁	C ₂	C ₃	C ₄
β-pinene	1.54	1.16	0.77	0.39
Myrcene	4.88	3.66	2.44	1.22
Citral	4.99	3.74	2.50	1.25
Eucalyptol	4.78	3.59	2.39	1.20
Terpineol	5.16	3.87	2.58	1.29
Eugenol	5.50	4.13	2.75	1.38

RESULTS

The results of inhibition of serum amylase, expressed as percentage of inhibition are presented in Tables 3 and 4.

The inhibition in serum with normal activity of amylase, when inhibitors were prepared by ultrasonic mixing, ranged from 9.68 to 38.70 % (Table 3). The degree of inhibition for ultrasonically prepared samples was obtained for

all test compounds at corresponding inhibitor concentration C₁ (β-pinene, 1.54; myrcene, 4.88; eucalyptol, 4.78; terpineol, 5.16 and eugenol 5.50 $\mu\text{mol}/\text{cm}^3$), with the exception of citral (C₂ = 3.74 $\mu\text{mol}/\text{cm}^3$). The highest inhibition percentage at inhibitors' concentrations C₂, C₃ and C₄ had citral, while the lowest was recorded for eugenol. Inhibition by inhibitors prepared by manual mixing ranged from 10.71 to 25.00 %. Mean inhibitions at all inhibitor concentrations of β-pinene, myrcene and eucalyptol were not significantly different ($p < 0.05$). As in the case of ultrasonic mixing, the highest inhibition was shown by citral while the lowest was recorded for eugenol. The highest inhibition was demonstrated by all the inhibitors at concentration C₂, except eugenol which has proven as the best inhibitor at C₃ concentration.

The range of inhibition of α-amylase activity was 7.89 to 21.05 %, for samples prepared by ultrasonic mixing (Table 4). The highest inhibition was shown by all inhibitors at a concentration of C₁ for ultrasonically prepared samples, while all

the inhibitors showed maximum inhibition at concentration C₂ for manually mixed samples. Inhibition in H serum prepared by manual mixing ranged from 13.58 to 25.92 %, which are similar to the results for the experiment carried out in N serum. The lowest mean inhibition at all concentrations was highest for terpineol.

To understand the complex connection between samples, we used principal component analysis (PCA). PCA is designed to transform the original variables into new uncorrelated variables, called components, which are linear combinations of the original variables. After standardization, each parameter contributes equally to the data set variance and carries equal weight in principal

component calculation. The screen plot graph (Fig 1), a plot of eigen-value as a foundation of the Eigen-value number, was used to decide the number of principal components needed to be retained.

PC1–PC2 score plot for 96 samples of terpenes (pretreatment: standard normal variate and column centering) in serum with high α-amylase activity (H-mark;) and in serum with normal α-amylase activity (N-mark) was presented in Fig 2a, while PCA scatter plot of 96 samples of terpenes in four concentrations was shown in Fig 2b.

Table 3: Influence of selected inhibitors expressed as percentage inhibition prepared by ultrasonic and manual mixing, respectively, on the activity of α-amylase in N serum sample (corresponding inhibitor concentrations C₁, C₂, C₃ and C₄ are quoted in Table 2)

Inhibitor	Inhibitor's concentration	C ₁	C ₂	C ₃	C ₄
β-pinene*	Inhibition of α-amylase (%)	29.03 ± 0.02	16.13 ± 0.04	22.58 ± 0.07	22.58 ± 0.05
Myrcene*		32.26 ± 0.04	12.90 ± 0.08	25.81 ± 0.03	19.35 ± 0.10
Citral*		25.81 ± 0.13	38.70 ± 0.06	22.58 ± 0.09	22.58 ± 0.04
Eucalyptol*		29.03 ± 0.07	19.35 ± 0.08	22.58 ± 0.03	19.35 ± 0.03
Terpineol*		29.03 ± 0.02	9.68 ± 0.03	19.35 ± 0.06	25.81 ± 0.07
Eugenol*		25.81 ± 0.07	16.13 ± 0.11	16.13 ± 0.12	19.35 ± 0.09
β-pinene†		21.42 ± 0.07	21.42 ± 0.09	17.85 ± 0.06	21.42 ± 0.08
Myrcene†		17.85 ± 0.04	17.85 ± 0.06	17.85 ± 0.06	17.85 ± 0.09
Citral†		25.00 ± 0.03	25.00 ± 0.03	17.85 ± 0.11	17.85 ± 0.14
Eucalyptol†		14.28 ± 0.17	17.85 ± 0.02	17.85 ± 0.09	17.85 ± 0.05
Terpineol†		21.42 ± 0.08	21.42 ± 0.19	14.28 ± 0.06	14.28 ± 0.17
Eugenol†		10.71 ± 0.01	14.28 ± 0.09	17.85 ± 0.20	14.28 ± 0.05

Values are mean % inhibition ± SD (n = 3); *ultrasonic mixing; †manual mixing

Table 4: Influence of selected inhibitors expressed as percentage inhibition prepared by ultrasonic and manual mixing, respectively, on the activity of amylase in the H serum sample (corresponding inhibitor concentrations C₁, C₂, C₃ and C₄ are quoted in Table 2)

Inhibitor	Inhibitor's concentration	C ₁	C ₂	C ₃	C ₄
β-pinene*	Inhibition of α-amylase (%)	19.73 ± 0.09	13.15 ± 0.09	9.21 ± 0.06	15.78 ± 0.08
Myrcene*		21.05 ± 0.18	7.89 ± 0.02	15.78 ± 0.09	18.42 ± 0.01
Citral*		19.73 ± 0.04	13.15 ± 0.09	17.10 ± 0.14	11.84 ± 0.07
Eucalyptol*		18.42 ± 0.02	9.21 ± 0.02	15.78 ± 0.01	15.78 ± 0.05
Terpineol*		17.10 ± 0.13	13.15 ± 0.09	11.84 ± 0.08	19.73 ± 0.15
Eugenol*		18.42 ± 0.03	11.84 ± 0.04	15.78 ± 0.08	14.47 ± 0.09
β-pinene†		22.22 ± 0.18	23.45 ± 0.02	17.28 ± 0.01	17.28 ± 0.03
Myrcene†		20.98 ± 0.20	23.45 ± 0.19	17.28 ± 0.09	13.58 ± 0.05
Citral†		20.98 ± 0.04	25.92 ± 0.02	18.51 ± 0.10	20.98 ± 0.06
Eucalyptol†		17.28 ± 0.05	22.22 ± 0.08	18.51 ± 0.04	17.28 ± 0.21
Terpineol†		23.45 ± 0.06	25.92 ± 0.24	25.92 ± 0.07	23.45 ± 0.02
Eugenol†		18.51 ± 0.22	22.22 ± 0.09	14.81 ± 0.06	13.58 ± 0.04

Values are mean percentage inhibition ± SD (n =3); *ultrasonic mixing; †manual mixing

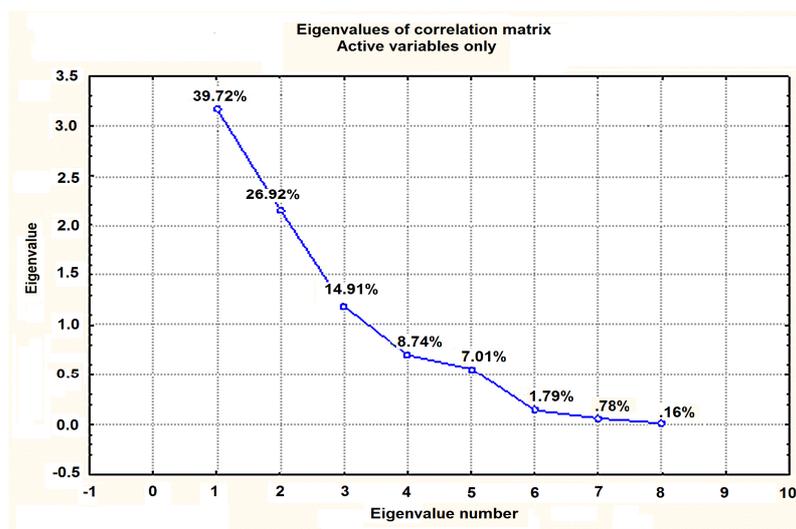


Fig 1: Eigen-values of the correlation matrix of selected terpenes based on their inhibition of amylase

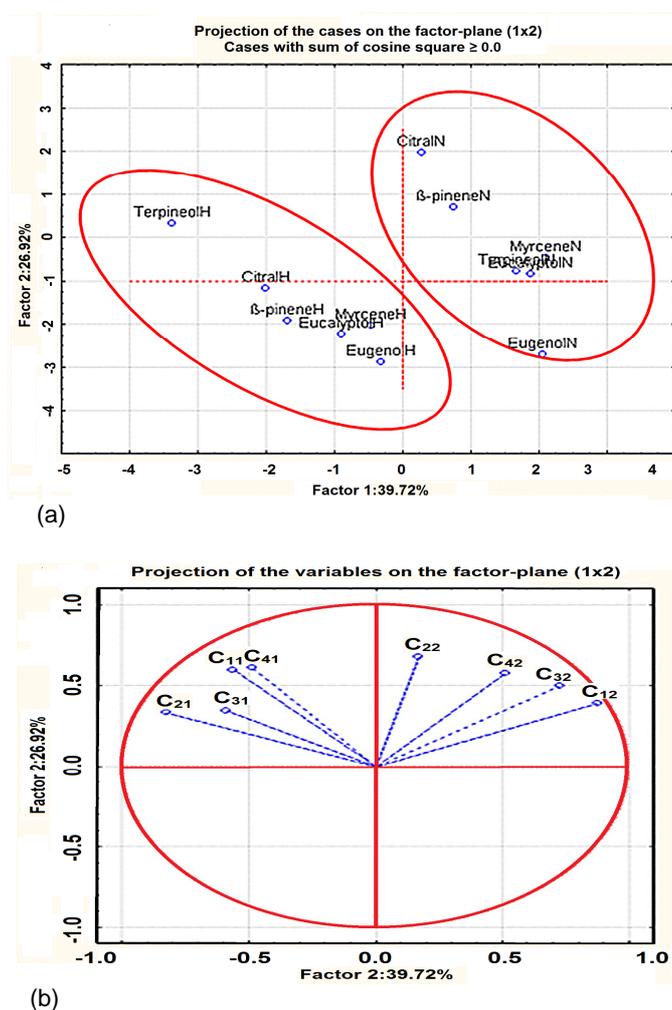


Fig 2: (a) PC1–PC2 score plot for 96 samples of terpenes (pretreatment: standard normal variate and column centering) (H-mark for high α -amylase activity; N-mark for normal α -amylase activity); (b) PC1–PC2 score plot for 96 samples of terpenes in four concentrations (pretreatment: standard normal variate and raw centering)

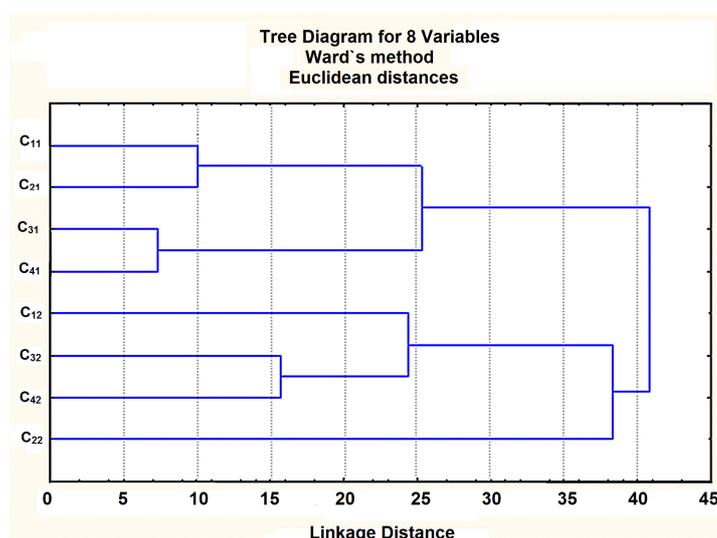
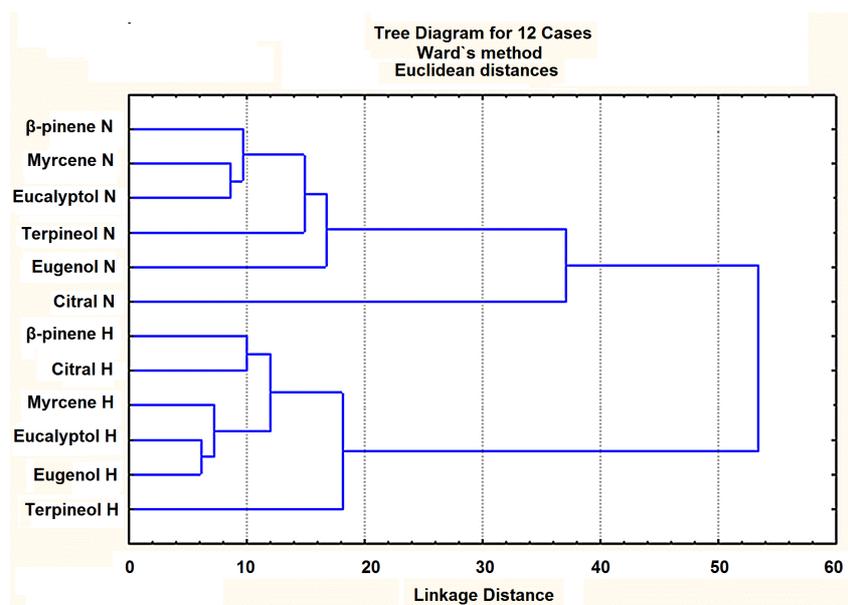


Fig 3: (a) Dendrogram obtained by hierarchical cluster analysis of inhibition percentage influenced by selected terpenes in N and H serum, respectively; (b) Dendrogram obtained by agglomerative hierarchical analysis using the concentration of terpenes and way of sample pretreatment (manual and ultrasonic)

In order to confirm the clustering results of principal component analysis, the agglomerative hierarchical analysis (AHA) technique also has been utilized. Several distance measures (i.e. Euclidean distance and the Pearson correlation coefficient) and linkage methods (i.e. single linkage, complete linkage, average linkage, weighted average linkage, centroid's method, median's method and Ward's method) were evaluated and compared (Fig 3a and Fig 3b).

Fig.3a illustrates hierarchical cluster analysis of inhibition percentage influenced by individual selected terpenes in N and H serum. Clustering

according to concentration of examined terpenes is presented in Fig 3b.

DISCUSSION

Solutions of selected terpenes were prepared in water (rather than organic solvents, which are completely miscible with terpenes) to prevent any possible inhibition/denaturation effect of the organic solvent on α -amylase. A higher degree of homogenization can be achieved by ultrasonic mixing, but in every day administration of essential oils, manual mixing is mostly applied, hence the influence of both mixing methods on enzyme activity was examined.

The inhibition range of selected terpenes was highest in N serum, with inhibitors prepared by ultrasonic mixing, and it is about two times higher in comparison with H serum. In N serum β -pinene, myrcene and eucalyptol exhibited the same or approximately the same percentage of inhibition, and citral showed the highest percentage of inhibition, whereas eugenol, showed the lowest percentage of inhibition.

Inhibitor solutions prepared by ultrasonic mixing showed better inhibition, reducing enzyme activity by 38 %, while the inhibitors prepared by manual mixing inhibited amylase by up to 25 %. Comparison of mean values of inhibition, for all concentration of inhibitor, showed that citral is the most potent inhibitor, while eugenol was the weakest, both in N and H sera. Citral has been shown to decrease hyperglycemia in diabetic rats serum [16]. This is in agreement with our results since citral was most effective in inhibiting α -amylase.

For the advanced statistical analysis of the inhibition of amylase caused by sort and concentration of selected terpenes, the obtained data were used in a Principal Component Analysis (PCA) approach. PCA was applied to the data set of 96 different samples after standardization (the mean of the values for each variable is subtracted from each variable value and the result is divided by the standard deviation of the values for each variable). Eigenvalues of selected terpenes correlation matrix defined the PC1 horizontal axis with 39.72% of the total variance while PC2 vertical axis had further 26.92% (Fig 1). So the first two components (PC1 and PC2) explained 66.64 % of the total variance.

In the two-dimensional coordinate system or the first and the second principal component, activity of amylase influenced by terpenes in N serum (β -pinene N, myrcene N; eucalyptol N, terpineol N, eugenol N, citral N) are located on the right half of the plot while activities affected by terpenes in high serum (β -pinene H, citral H, myrcene H, eucalyptol H, terpineol H, eugenol H) are situated on the left-hand side of the figure of the PC1 zero point (Fig 2a).

The most distinct sample was citral N. The average values of PC2 in case of citral N prepared by ultrasonic mixing is the largest, most likely due to the fact that the highest degree of inhibition ($38.70 \pm 0.06\%$) was produced by this terpene. PCA (Fig 2b) indicated that all the samples prepared by ultrasonic mixing (C_{11} , C_{21} , C_{31} , C_{41}) at all examined concentrations are

distinguished from those prepared by manual mixing (C_{12} , C_{22} , C_{32} , C_{42}).

Cluster analysis can classify the number of samples studied into a number of groups, according to the terpenes influence on activities of amylase by 'magnifying' their similarities. The dendrogram (Fig 3a) shows that the results of terpene inhibition are quite homogeneous and most of them also tend to be distributed in two homogeneous groups. According to the hierarchical cluster analysis, selected terpenes can be grouped as follows:

- I. cluster A: β -pinene N, myrcene N, eucalyptol N, terpineol N, eugenol N, citral N
- II. cluster B: β -pinene H, citral H, myrcene H; eucalyptol H, eugenol H, terpineol H

In cluster A, the greatest similarity was observed between samples of myrcene N and eucalyptol N, while cluster B was divided in three sub-groups (β -pinene H, citral H) (myrcene H, eucalyptol H, eugenol H) and terpineol H.

Application of PCA and AHA revealed that percentage of amylase inhibition is mainly caused by its activity in the serum, since the data for normal and high activity in serum were sorted in two different groups.

The cluster developed on the basis of the terpenes concentrations (Fig 3b) corresponds to the PCA analysis for the same data (Fig 2b). On the basis of AHA (Fig 3(a) and (b)), it can be concluded that inhibition of the amylase is more affected by way of sample preparation. The concentrations of studied inhibitors have less significant influence.

CONCLUSION

The findings of the *in vitro* study revealed that the of common constituents of essential oils inhibit α -amylase to a significant degree. The best inhibitor of the tested compounds was citral. The mode of terpene preparation before application in sera (ultrasonic or manual mixing) is more important factor in α -amylase inhibition than inhibitor concentration. Irrespective of the mode of preparation, the most effective concentration of the terpenes, ranges from $1.16 \mu\text{mol cm}^{-3}$ for β -pinene to $5.50 \mu\text{mol cm}^{-3}$ for eugenol. However, the additional studies are necessary in order to fully assess the potential of these terpenes in control of blood sugar levels in diabetics. Also, it is ultimately important to carry

out experiments *in vivo* to confirm their efficacy in the prevention and treatment of diabetes.

ACKNOWLEDGEMENT

Part of the experiments of this study was done in the biochemical laboratory of the Health Center in Nis, Serbia and the authors express their sincere gratitude to Dr Radmila Vučković, former head of the laboratory. We also thank Dr Jelena Milovanović, Spec. Med. Biochemistry, Ljiljana Dimitrijević, Senior Laboratory Technician, and Svetlana Mladenović, Laboratory Technician. The authors are very grateful to the Ministry of Education and Science for funding this work through the grants from Program of Basic Research (172047 and 172051).

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