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# Effect of Extracts of Bilberries (*Vaccinium myrtillus* L.) on Amyloglucosidase and *α*-Glucosidase Activity

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**Background:** *Vaccinium myrtillus* L. is a species belonging to the genus *Vaccinium* of the family *Ericaceae*. Bilberries have drawn attention due to the multiple benefits for the human health, including antioxidant, anti-inflammatory, anticancer, anti-neurodegenerative, and cardioprotective effects. Recently, bilberries were shown to inhibit the activity of carbohydrate-hydrolysing enzymes that can help reduce the intensity of the metabolic syndrome and prevent type 2 diabetes.

**Aim:** In this study, we investigated the *α*-glucosidase and amyloglucosidase inhibitory activities of polyphenol-rich extracts from fruit of *Vaccinium myrtillus* L. from different regions in Bulgaria.

**Materials and methods:** The total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu method. With HPLC analysis, phenolic acid composition of extracts was assessed. Enzymatic inhibitory activities were determined according to the methodology by Borooah et al. (1961), and Dewi et al. (2007). Amyloglucosidase assay and *α*-glucosidase assay were used to measure the inhibition potential of bilberries' extracts.

**Results:** Phenolic compound content ranged from 1299.60 mg to 510.88 mg GAE/100 g for organic extracts and from 453.63 mg to 290.83 mg GAE/100 g for aqueous extracts. Based on qualitative HPLC analyses, gallic acid and chlorogenic acid were found to be among the major phenolic acids present in bilberries. Methanol and aqueous extracts there were found to be effective inhibitors of *α*-glucosidase with an $IC_{50}$ value of 20 μg GAE/ml and 55 μg GAE/ml, respectively.

**Conclusion:** The inhibitory activity of bilberries' extracts towards *α*-glucosidase offers the patients with type 2 diabetes the opportunity to manage their own glycaemic levels with a diet.

## BACKGROUND

*Vaccinium myrtillus*is L. is a species belonging to the genus *Vaccinium* of the family *Ericaceae*. Bilberries have raised the interest of researchers because of the multiple beneficial effects the plant possesses for the health of people, including antioxidant, anti-inflammatory, anticancer, anti-neurodegenerative, and cardioprotective effects.[1,2] Recently, bilberry fruit were shown to inhibit the activity of carbohydrate-hydrolysing enzymes such as *α*-amylase and *α*-glucosidase, enzymes that can help reduce the intensity of the metabolic syndrome and prevent obesity and type 2 diabetes.[3,4] In addition, postprandial hyperglycaemia (PPHG) or hyperinsulinaemia are independent risk factors for the development of macrovascular complications of diabetes mellitus. Amyloglucosidase and *α*-glucosidase are enzymes that catalyze the final step in the digestive process of carbohydrates and, hence, their inhibitors could retard the use of dietary carbohydrates to suppress PPHG. Although several drugs targeted at carbohydrate-hydrolysing enzymes are in clinical use, it is necessary to have a large inhibitor pool as diabetic patients can develop resistance to current regimens.

The phytochemicals present in bilberry fruit implicated for the above biological effects are phenolic compounds, including anthocyanins, mainly in the glycosylated forms of cyanidin-, delphinidin- and malvidin-3-*O*-glycosides[5,6], flavonols (such as quercetin, kaempferol and myricetin), flavan-3-ols - catechins, stilbenoids and phenolic acid derivatives.[7-10] The composition and content of phenolic compounds in bilberries vary widely depending on the season and the location where they are present.[11]

In this study, we investigated the $\alpha$-glucosidase and amyloglucosidase inhibitory activities of polyphenol-rich extracts from fruit of *Vaccinium myrtillus* L. from different regions in Bulgaria.

## MATERIALS AND METHODS

### 1. Plant material

Fruit of *Vaccinium myrtillus* L. were picked from the following Bulgarian regions: the Balkan Mountains, Rila, the Rhodope Mountains (Yundola and Tsigov Chark areas) in August and September 2015.

### 2. Enzymes, substrates and chemicals

*Alfa*-glucosidase (EC 3.2.1.20) - 19.3 U/mg from *Saccharomyces cerevisiae* (a product of Sigma-Aldrich Chemie), fungal amyloglucosidase (E.C. 3.2.1.3) - 35 U/mg from *Aspergillus niger* was obtained from Megazyme (Megazyme E-AMGFR), *p*-nitrophenyl-$\alpha$-D-glucopyranoside and *o*-nitrophenyl-$\alpha$-D-glucopyranoside (Merck), Folin-Ciocalteu's phenol reagent (Merck), hydrochloric acid, gallic acid, chlorogenic acid, caffeic acid, benzoic acid, (Sigma-Aldrich Chemie).

### 3. Preparation of phenol-rich extracts from *Vaccinium myrtillus* L. fruit

*3.1. Water extraction*

Whole bilberries (50 g) were blended with 200 ml of distilled water and then stirred for an hour at room temperature and filtered with nylon cloth.

*3.2. Extraction using an organic solvent*

Whole bilberries (50 g) were blended with 200 ml of extragent: acetone (80%) or methanol (80%), distilled water (19.5%) acidified with hydrochloric acid (0.5%) after which they were stirred for one hour at room temperature. Then they were filtered with nylon cloth.

The collected aqueous and organic extracts were concentrated using a rotary evaporator.

### 4. Total phenolic content (TPC)

TPC was determined spectrophotometrically using the Folin-Ciocalteu method.[12] The absorbancy was measured at 760 nm. Gallic acid was used as a standard and results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh berries. All experiments were conducted three times.

### 5. HPLC analysis of phenolic acids

Phenolic acid composition of extracts was assessed with an HPLC analysis using the chromatographic system VWR La Prep Σ (Knawer, Germany) which consists of LP 1100 HPLC pump, a LP 3104 UV absorbancy detector, a column Chromolith® Performance RP-18e (100 x 4.6 mm x 2 µm), Merck, Germany. The managerial chromatography system and data processing used EZChrome Elite, software of Agilent. Column temperature was maintained at 25°C. Mobile phase A was acetic acid and water (1:25 V/V) and mobile phase B was acetonitrile. The isocratic programme was as follows: 0 - 25 min, 80% A to 20% B. The injection volume was 20 µl, the mobile phase flow was 0.8 ml/min and the detection wavelength was 280 nm. The samples were determined by the retention time of gallic acid, chlorogenic acid, caffeic acid and benzoic acid standards.

### 6. Amyloglucosidase assay

The enzymatic activity was determined following the method described by Borooah et al.[13] One unit of enzyme (U) is defined as the amount of enzyme able to produce one µmol of *o*-nitrophenol per minute under the assay conditions (T = 40°C, pH 4.7).

### 7. $\alpha$-Glucosidase assay

One unit of enzymatic activity (U) is defined as the amount of enzyme which releases one µmol of *p*-nitrophenol per minute under the assay conditions (T = 37°C, pH 6.8). Measurements were performed on a 800 DU spectrophotometer (Beckman Coulter®, Brea, CA, the U.S.).

### 8. Inhibitory activity

The $\alpha$-glucosidase inhibitory activity was determined according to the procedure of Dewi et al.[14] An amyloglucosidase assay and an $\alpha$-glucosidase assay were used to measure inhibitory effect of the berry extracts. Inhibitors were added to a fixed total volume to obtain the concentration ranges required for individual experiments. Inhibitor-lacking controls were run where the control activity of each experiment was defined. The test samples were accompanied by blank samples containing all components except for the enzyme to account for the possible absorbancy of the berry extracts. All samples were run three times and values were presented as a percentage control activity ± standard errors.

## RESULTS

### 1. Total phenolic content

**Table 1** shows the total phenolic content of the three types of extracts of *Vaccinium myrtillus* L.

In different regions, the phenolic compounds content ranged from 1299.60 mg to 510.88 mg GAE/100 g for organic extracts and from 453.63 mg to 290.83 mg GAE/100 g for aqueous extracts. The extracts obtained with organic extragent have a higher content of total polyphenols in comparison with the aqueous extracts. The highest TPC values were found for the methanol extract from the Yundola area (1299.60 mg GAE/100 g) whereas the values for the aqueous extract from the Yundola area were 290.83 mg GAE/100 g.

### 2. Phenolic acids content

Phenolic compounds have antioxidant properties due to their ability of scavenging free radicals.[15] The gallic acid in the studied extracts ranged from 242.70 mg to 34.20 mg per 100 g of fresh berries. It depended on the type of extragent where the highest concentration of phenolic compounds was detected by extraction of methanol (data not shown).

**Fig. 1** presents different phenolic acids as a percentage compared to total polyphenols. We conducted HPLC analyses of bilberries' aqueous and organic extracts. Based on these qualitative HPLC analyses, gallic acid and chlorogenic acid were among the major compounds. The concentration of caffeic acid and benzoic acid is very low in all types of extracts **(Fig. 1)**.

### 3. Effect of bilberries' extracts on amyloglucosidase activity

**Fig. 2** shows the effect of the different type of extracts relative to that of amyloglucosidase activity.

Amyloglucosidase (AMG) is an enzyme able to produce glucose from starch by successively removing glucose units from the non-reducing end of amylose or amylopectin molecules of starch mainly by hydrolysis of the $\alpha$-1,4 glucosidic bond.[16]

Polyphenol-rich extracts from bilberry fruit from different regions in Bulgaria have shown a significant increase of amyloglucosidase activity **(Figs 2A, 2B)**.

**Table 1.** Total polyphenolic content in extracts expressed as gallic acid equivalents (mg GAE/100 g)

| Region | Total polyphenols (mg GAE/100 g) | | |
|---|---|---|---|
| | Water | Acetone | Methanol |
| Tsigov Chark | 453.63 ± 17.23 | 636.46 ± 22.90 | 675.60 ± 24.68 |
| Yundola | 290.83 ± 8.72 | 522.56 ± 15.16 | 1299.60 ± 32.46 |
| Rila | 363.39 ± 13.08 | 580.43 ± 18.81 | 618.40 ± 19.18 |
| The Balkan Mountains | 374.18 ± 11.97 | 510.88 ± 16.10 | 548.30 ± 16.75 |

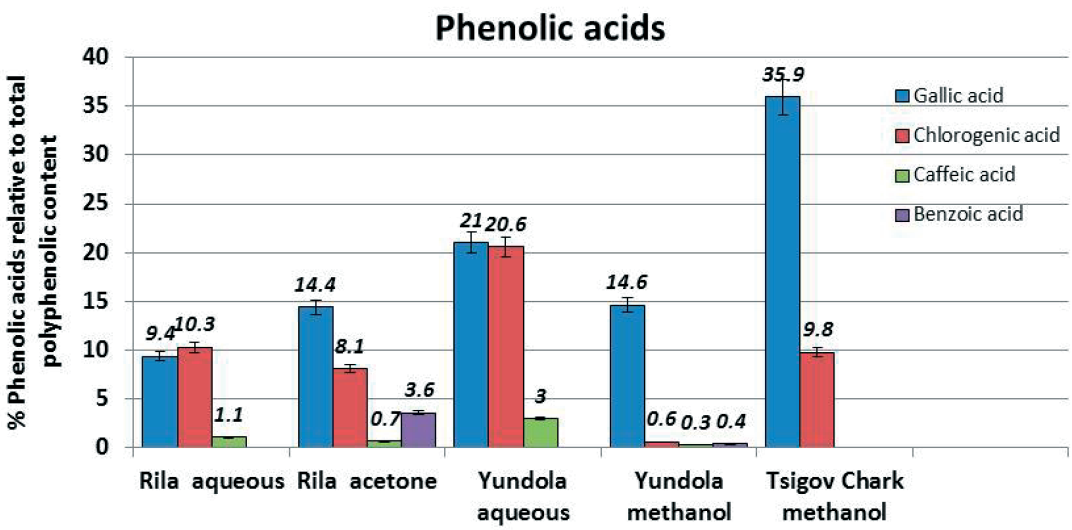


**Figure 1.** Phenolic acids as a concentration relative to total polyphenols.

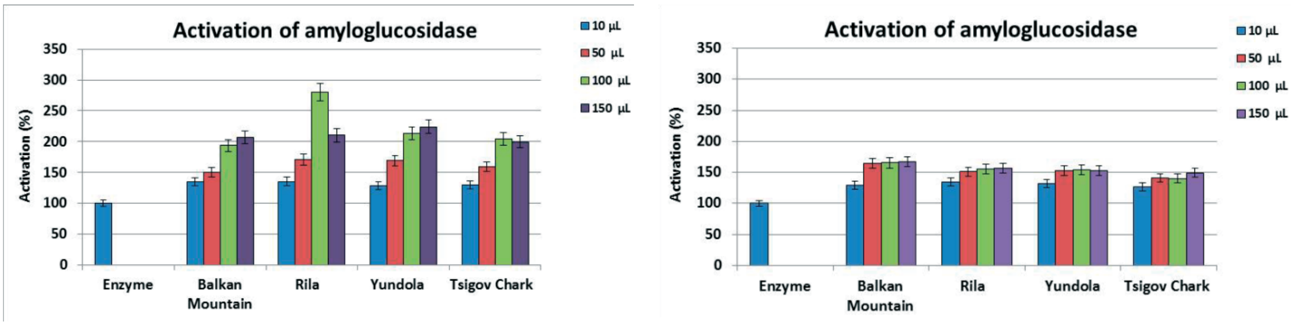


**Figure 2.** Effect of the aqueous (A) and acetone (B) extracts of bilberry fruit on the enzymatic activity of amyloglucosidase (%).

The aqueous extracts showed a higher degree of activation of enzymatic tests in comparison with acetone extracts. In the study, the effect of different concentrations of extracts from 10 µl to 150 µl has been studied and it has been found that the activation of amyloglucosidase reaches maximum concentration at 50 µl. The subsequent increase in the extract concentration to 150 µl did not affect the activity of the enzyme.

4. Effect of extracts of bilberries on $\alpha$-glucosidase activity from *Saccharomyces cerevisie*

The methanol and aqueous extracts of bilberry fruit were effective inhibitors of $\alpha$-glucosidase with an $IC_{50}$ value of 20 µg GAE/ml and 55 µg GAE/ml, respectively **(Figs 3A, 3B)**. The degree of inhibition of bilberries' extracts was established on $\alpha$-glucosidase. The most potent inhibitory capacity of the tested extracts shows that of methanol derived from berries picked in the Rhodope Mountains **(Fig. 3)**.

The methanol and aqueous extracts were characterised by the presence of 242.70 mg and 41.29 mg of gallic acid per 100 g of fresh fruit, respectively, and 66.34 mg and 40.43 mg of chlorogenic acid per 100 g of fresh berries.

On the other hand, the highest concentration of gallic acid relative to TPC is detected by a methanol extract: 35.9%. The aqueous extract has shown the right concentration of gallic and chlorogenic acid relative to TPC: 21% and 20.6%, respectively. Compared to the value of enzymatic inhibition, we have demonstrated that the presence of more gallic acid in the methanol extract increases the inhibitory activity.

## DISCUSSION

Experiments showed that phenolic acid-enriched extracts from bilberries can inhibit $\alpha$-glucosidase *in vitro* which is consistent with previous reports by McDougal et al.[3]

A decrease in activity of $\alpha$-glucosidase using inhibitors is considered one of the most effective ways to control type 2 diabetes.[17,18]

This coincides with data about inhibitory concentrations of other types of berries (blackcurrant and rowan) within 20-30 µg GAE/ml, down from

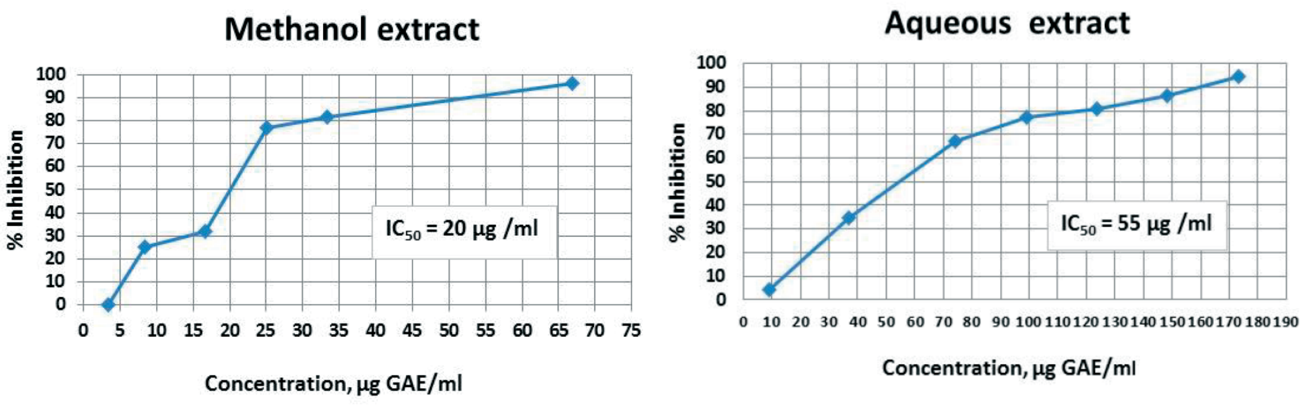


**Figure 3.** Inhibiting effect of the methanol and aqueous extracts of *Myrtilli* fructus on $\alpha$-glucosidase activity from *Saccharomyces cerevisiae*.

Boath et al.[19] The obtained methanol extract of bilberries is the highest total concentration of polyphenols (**Table 1**). There is a correlation between the amount of polyphenolic compounds and the presence of an inhibitory effect on $\alpha$-glucosidase. Acarbose inhibited $\alpha$-glucosidase in a dose-dependent manner to an $IC_{50}$ value of 40 μg/ml, a similar finding to that reported by Akkachaiyasit et al.[20]

Polyphenolic extracts from plants have been reported to be efficient inhibitors of intestinal $\alpha$-glucosidase/maltase activity $K_i$ values similar to acarbose which is used therapeutically to control type 2 diabetes mellitus.[21]

The inhibitory activity of chlorogenic acid derivatives found in plant tissues may explain the presence of varying extents of $\alpha$-glucosidase inhibition in plant extracts but it may also explain the more potent inhibitory activity of certain berry extracts such as bilberry.

Inhibition of $\alpha$-glucosidase by chlorogenic acid derivatives appears to be non-competitive which suggests binding to, or interaction with, the enzyme at a site other than the active site. It is possible that chlorogenic acid or its derivatives may also act to inhibit glucose uptake through inhibition of a specific transport mechanism which could also influence gastric hormone and insulin release. Therefore, inhibition of glucose production through inhibition of $\alpha$-glucosidase and glucose uptake could have synergistic effects on blood glucose control.[21]

## CONCLUSIONS

This study provided valuable data regarding the effect of different types of bilberries' extracts on the activity of amyloglucosidase and $\alpha$-glucosidase.

The yield of phenolic acids extracted was influenced by the concentration of alcohol in water.

In summary, the inhibitory activity of bilberries' extracts towards $\alpha$-glucosidase offers the patients with type 2 diabetes the opportunity to manage their own glycaemic control by means of a diet.

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# Влияние экстракта черники обыкновенной (*Vaccinium myrtillus L.*) на амилоглюкозидазную и α-глюкозидазную активность

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**Введение:** *Vaccinium myrtillus L.* является видом, принадлежащим к роду *Vaccinium* семейства *Ericaceae*. Черника вызывает интерес своими многочисленными свойствами, полезными для здоровья человека с точки зрения антиоксидантного, противовоспалительного, противоопухолевого, антинейродегенеративного и кардиопротективного воздействия. Последние исследования доказывают, что черника ингибирует активность углеводных-гидролиз ферментов, которые могут способствовать снижению интенсивности метаболического синдрома и предотвратить диабет 2 типа.

**Цель:** В данной работе нами была исследована ингибиторная активность амилоглюкозидазы и α-глюкозидазы насыщенных полифенолами экстрактов плодов *Vaccinium myrtillus L.* из различных регионов Болгарии.

**Методы:** Общее содержание фенолов было определено спектрофотометрически с использованием метода Folin-Ciocalteu. При анализе с использованием высокоэффективной жидкостной хроматографии - ВЭЖХ (HPLC) был измерен состав фенольных кислот. Ферментные ингибиторные активности были определены при помощи метода Borooah и сотр. (1961) и Dewi и сотр. (2007). Пробы амилоглюкозидазы и α-глюкозидазы были использованы, чтобы определить ингибиторный потенциал экстрактов черники.

**Результаты:** Содержание фенольных соединений варьировало от 1299.60 мг до 510.88 мг GAE/100 гр. для органических экстрактов и от 453.63 мг. до 290.83 мг. GAE/100 гр. для водных экстрактов. На основании ВЭЖХ (HPLC) анализа было установлено, что галловая кислота и хлорогеновая кислота являются основными кислотами, содержащимися в чернике. Метаноловые и водные экстракты оказались эффективными ингибиторами α-глюкозидазы с IC50 показателями соответственно 20 μg GAE/мл. и 55 μg GAE/мл.

**Заключение:** В заключение можно сказать, что ингибиторная активность экстрактов черники в отношении α-глюкозидазы обеспечивает возможность пациентам с диабетом 2 типа регулировать собственный уровень сахара при помощи диеты.