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The analysis of the composition and antioxidant properties of freeze-dried chokeberry, strawberry, blackberry and selected raspberry fruits

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Abstract: Context: Anthocyanins are a group of organic compounds believed to show anti-inflammatory, anti-proliferative, antioxidant, pro-apoptotic effects. For these reasons, anthocyanins may potentially be lied in the cancer chemoprevention as well as in providing a metabolic support during oncological treatment.
Objective: The aim of this study is to compare the antioxidant activity and analyze the content of bioactive substances in selected freeze-dried fruit species in order to determine the optimal source of anthocyanins for subsequent clinical trials involving patients undergoing treatment for colon cancer.
Materials & Methods: The study employed freeze-dried fruits of chokeberry, blackberry, strawberry and raspberries: black, yellow and red. The total antioxidant activity was determined using the FRAP, the DPPH method and total polyphenol by UV-VIS spectrophotometer.
Results: Chokeberry exhibited the highest antioxidant activity, as measured by the FRAP method, followed by black raspberry, blackberry, strawberry, yellow and red raspberry. The highest content of polyphenols was found in chokeberry and black raspberry, while the other fruits had relatively lower levels. Black raspberry fruits contained the highest concentration of anthocyanins. The highest concentration of vitamin C was detected in yellow raspberry fruits.
Discussion and Conclusion: Based on biochemical analysis and considering organoleptic properties, particularly taste and the potential for good tolerance of the formulation by patients, freeze-dried fruits of black raspberry and strawberry were selected for clinical study on the use of anthocyanins in the nutritional and metabolic support of patients undergoing treatment for colorectal cancer.

Keywords: anthocyanins, antioxidant activity, biological activity, colon cancer, cancer prevention, functional food.

Introduction

While the world's attention is primarily directed towards the issue of obesity, paradoxically, an even greater problem is arising from the shadows, namely malnutrition. The global health community has been slow to acknowledge double burden of malnutrition (DBM) — the coexistence of undernutrition (i.e., micronutrient deficiencies, underweight, childhood stunting and wasting) and overweight/obesity and diet-related non communicable diseases [1]. In the meantime, it was stated that malnutrition is an independent risk factor of several diseases, affects clinical outcomes of treatment, impairs the quality of life, body function and autonomy therefore it constitutes as a serious social problem [2]. Identifying patients at risk of malnutrition early in the process of treatment is a crucial task for clinicians. It is widely recognized that adequate and timely implementation of nutritional support is associated with reduced length of hospital stay, reduced incidence of severe complications, reduced post-procedural mortality, improved cost-effectiveness and very significant improvement of quality of life [2–4].

On the other hand, over past few years our perception about food has significantly changed. "Functional food" is a term used to describe the foods that based on the scientific evidence may improve general condition of the organism, provide metabolic support, can be used in chemoprevention to decrease risk of some serious diseases (e.g., cancer) or even cure some illnesses [5]. Given the above, efforts of scientific communities are focused on the search of ingredients that will significantly improve the quality of food thus creating superfoods.

Numerous research studies have delved into the favorable effects of berries on overall wellness and the relationship between distinct berry varieties and diverse health conditions. There is evidence, that blueberries have a preventive effect towards cardiovascular diseases and development of cancers [6]. Blackcurrants are extensively examined due to their anti-inflammatory, immune-modulatory and antioxidant properties [7–9]. Among many compounds found in fruits and vegetables anthocyanins remain very promising group of substances believed to display a wide range of biological effects like modulating different pathways and processes in our body. Anthocyanins is a group of polyphenols present mostly in all tissue layers of fruits, vegetables and flowers and are responsible for their sensory properties such as red to blue color. They are water soluble therefore accumulated in vacuoles. For plants they play a vital role in pollination and by absorbing light they protect them from UV-radiation. Anthocyanins are glycosylated forms of anthocyanidins which are synthetized by plants via phenylpropanoid pathway. Current studies show that anthocyanins display antioxidant, anti-inflammatory, antiproliferative, glucose regulating and neuroprotective activities [10].

The primary factor that led us to undertake this study is the fact that antioxidant activity of anthocyanins may have tangible clinical implications. They can quench reactive radical species by single electron transfer reaction and through hydrogen atom absorption from phenolic groups. The key structures that enable anthocyanins, to suppress radical species are flavylium skeleton and phenolic hydroxyl groups in the positions 3' and 4' that undergo the process of oxidation and as a result stabilize one-electron oxidation products [11]. There are papers that focused on assessing antioxidant potential of anthocyanins derived from various plant species [11–14].

The second aspect that contributed to the emergence of this study is the highly diverse methodology employed in previous human studies on anthocyanins, encompassing variations in administration methods, formulations, and a lack of establishing a correlation between the administered dose of anthocyanin and the defined biological effect. Therefore, it is impossible to draw final conclusion and make recommendations.

The aim of this study was to compare the antioxidant activity as well as analyze the content of biologically active substances in selected species of freeze-dried fruits in order to determine the optimal source of anthocyanins for subsequent clinical trials involving patients undergoing treatment for colon cancer.

Materials and Methods

In the analysis we used freeze-dried extracts of chokeberries, strawberries, blackberries, and raspberries: red, yellow, and black. The substances from the fruit were extracted with a methanol--acetone mixture, which was then centrifuged. The clear supernatant was used after appropriate dilution to perform individual measurements. All measurements were conducted using the JASCO V-530 spectrophotometer. The thawed fruits were crushed and centrifuged, and the obtained juice was used for the determination of vitamin C and total anthocyanins content.

Polyphenols

The total polyphenol content in the tested samples was determined by using the method developed by Singleton and Rossi, with slight modifications. The reagent used in the analysis was Folin-Ciocalteau, which is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). As a result of the ongoing reaction, phenols undergo oxidation while simultaneously reducing the components of the Folin-Ciocalteau reagent to tungsten oxides (W_8O_{23}) and molybdenum oxides (Mo_8O_{23}). The reaction takes place in an alkaline environment, and its product exhibits maximum absorption at a wavelength of 760 nm. The concentration of polyphenols in the tested sample is proportional to the absorbance value measured at this wavelength. The contents of the cuvettes were mixed and stored in a dark room at room temperature. Absorbance measurements were taken in cuvettes with an optical path length of 1 cm at wavelengths of $\lambda = 760$ nm and $\lambda = 725$ nm, 30 minutes after adding the tested sample to the remaining substrates. The measurements were performed three times. Calculations were based on calibration curves. The resulting value, expressed as the average, was reported as gallic acid equivalent (g GA/L). A blank sample was prepared analogously, but instead of the sample, 100 μL of deionized water was added.

Antioxidant activity

The determination of total antioxidant activity was carried out using two methods: the FRAP method (Ferric Reducing–Antioxidant Power) and the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical reduction method.

The FRAP method was first introduced by Benzie and Strain. Slight modifications were made in the analysis. This method is based on the reduction of Fe3+ ions, which form a complex with tripyridyltriazine (Fe3+-TPTZ), to Fe2+ ions (Fe2+-TPTZ) under the influence of antioxidant compounds present in the tested sample. This reaction takes place at acidic pH. A strong blue color appears, originating from the iron II complex, which absorbs radiation at a wavelength of 593 nm. The reducing power of the tested sample increases with the increase in absorbance. The contents of the cuvettes were thoroughly mixed and then placed in a water bath and heated at a temperature of 37°C. Absorbance at a wavelength of $\lambda = 593$ nm was measured in cuvettes with an optical path length of 1 cm, 15 and 30 minutes after adding the sample to the remaining reagents. The measurements were performed three times, and for each of the tested samples, the average absorbance value and standard deviation were calculated. The FRAP value was reported in millimoles of Fe2+/L.

The DPPH radical reduction method is based on the use of the DPPH reagent (1,1-diphenyl-2-picrylhydrazyl), which is a stable free radical that reaches maximum absorption at a wavelength of λ = 515 nm. The radical forms a blue-colored alcoholic solution. DPPH undergoes reduction during the reaction with an antioxidant compound, causing the previously formed color to disappear. The remaining amount of oxidized DPPH in the solution is proportional to the decrease in absorbance. The contents of the cuvettes were mixed and stored in a dark room at room temperature. Absorbance measurements were taken in cuvettes with an optical path length of 1 cm at a wavelength of λ = 515 nm, 15 and 30 minutes after combining both substrates. The measurements were performed three times, and for each of the tested samples, the average absorbance value and standard deviation were calculated.

Vitamin C

Vitamin C determinations were carried out using the iodometric method and the end point was determined when the color changed in the presence of starch mucilage. The measurement results were calculated based on the standard curve prepared from the l-ascorbic acid standard. All measurements were made three times.

Total anthocyanins

The test consists in differentiating the color of the tested juice under the influence of changing pH (1.0 and 4.5) measured at two wavelengths λ = 520 and 700 nm. The concentration of anthocyanins was calculated based on the formula:

$$(A \times 449.2 \times \text{dilution factor} \times 1000)/26900$$

$$\text{where } A= (A_{520nm} - A_{700nm}) \text{ pH } 1.0 - (A_{520nm} - A_{700nm}) \text{ pH } 4.5$$

Vitamin C and total anthocyanins levels were measured only for blackberry, red, black and yellow raspberry. Strawberry and chokeberry fruit were purchased in freeze-dried form. Therefore, it was not possible to determine the total anthocyanins in them using the described method.

Results

The obtained results are presented in the graphs below. The first graph (Fig. 1) shows the content of total polyphenols. Chokeberry contains the highest concentration of this parameter.

It is almost twice as high as the next fruit. Second in line is black raspberry. Blackberry has a slightly lower concentration. The lowest contents of total polyphenols were recorded for yellow and red raspberries. They were more than four times lower than in the case of chokeberry. When it comes to antioxidant activity, chokeberry achieves the highest activity (Fig. 2). However, comparing its activity with black raspberry, it is higher only by about 30% and 35–40% in relation to blackberry. In the case of other fruits, the antioxidant activity is more than two times lower than in chokeberry. An interesting observation was recorded for the antioxidant activity measured by the DPPH test (Fig. 3). In this case, also the highest activity was shown by chokeberry. However, the second place was taken by strawberry. Raspberries and blackberries had more than two times less activity than chokeberry. This proves the chemical difference of antioxidant substances present in strawberries, raspberries and blackberries.

Dark-colored fruits are a rich source of anthocyanins. This is also confirmed by our measurements. However, the highest content of total anthocyanins was confirmed for black raspberries, followed by blackberries (Fig. 4). Blackberries look even darker, but the color saturation for this fruit is the highest on the surface. Black raspberry, in turn, is saturated with anthocyanin dye uniformly for the whole fruit. Its result is more than twice as high as for blackberries. In relation to blackberry and red and yellow raspberries, these values are more than five times higher. Unfortunately, for the reasons mentioned earlier, it was not possible to determine the content of anthocyanins in freeze-dried samples of chokeberry and strawberry. The content of vitamin C was a surprise for us (Fig. 5). Its level was the highest for yellow and red raspberries. Black raspberries and blackberries had over two times lower content of this vitamin than yellow raspberries.

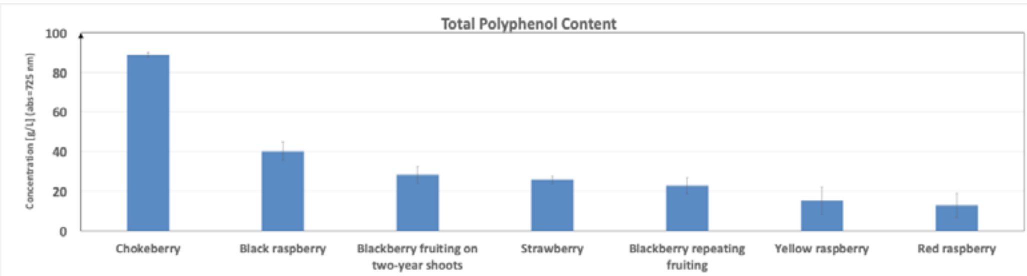

Fig. 1. Comparison of Polyphenol Concentration in the Analyzed Samples of Freeze-Dried Fruits.

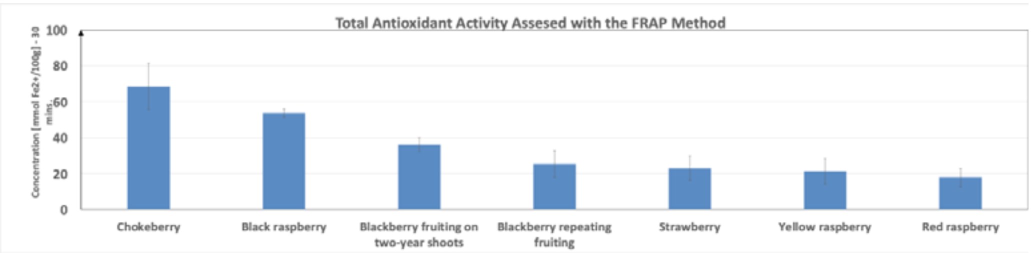

Fig. 2. Comparison of Antioxidant Activity Determined by the FRAP Method in Selected Samples of Freeze-Dried Fruits.

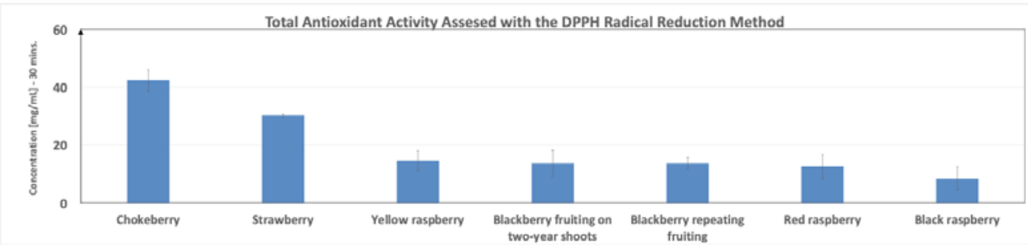

Fig. 3. Comparison of Antioxidant Activity Determined by the DPPH Radical Reduction Method in Selected Samples of Freeze-Dried Fruits.

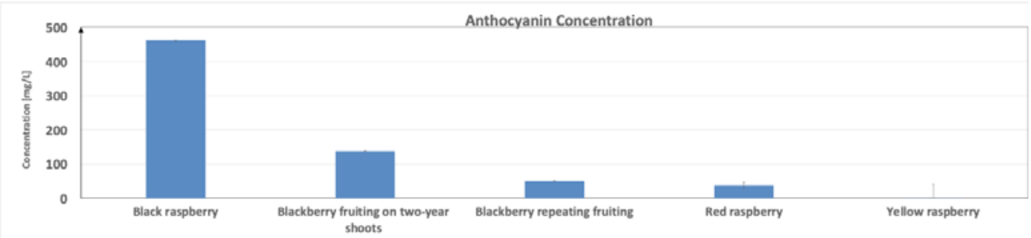

Fig. 4. Comparison of Anthocyanin Concentration in Selected Fruit Samples.

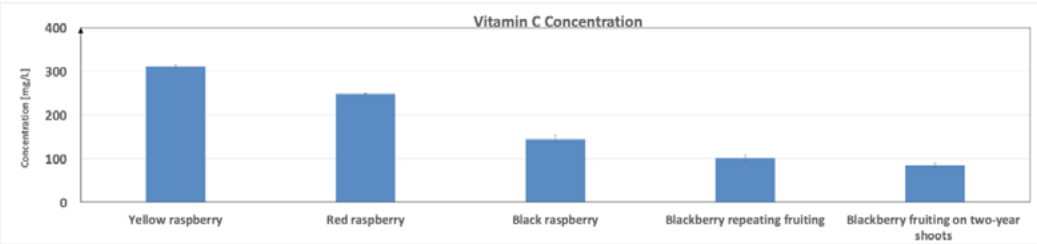

Fig. 5. Comparison of Vitamin C Concentration in Selected Fruit Samples.

Discussion

This research presents detailed comparison of biologically active components of freeze-dried chokeberry, strawberry, blackberry and selected raspberry fruits. We selected those species among others because they are popular and widely available in Polish market. Our results partially correspond to what has been described earlier in literature. According to previous studies the most efficient source of anthocyanins seems to be elderberries and chokeberries with reported anthocyanins concentration from 1400 to 1800 mg per 100 g of product [15]. Other plant species rich in anthocyanins with levels ranging from about 100 to even 700 mg per 100 g of fresh product are berries such as blueberries, blackberries, strawberries, redcurrant, blackcurrant and raspberries [10, 15, 16]. Furthermore, purple corn, cherries, plums, pomegranate, eggplant, wine, grapes, and red/purple vegetables can contain up to 300 mg of anthocyanins/100 g of product. In our study we indicated that black raspberries serve as a rich source of polyphenols including anthocyanins, with their mean concentration of approximately 462 g/l. Although chokeberries showed best antioxidative properties among examined fruits due to their tart taste they were not well tolerated by the testers. This poses a significant obstacle to their further utilization as it increases the risk of non-compliance with consumption recommendations. However, it is crucial to remember that concentration of anthocyanins in foods does not reflect their bioavailability which is determined by their chemical structure and the presence of functional groups [6]. Dietary anthocyanins need to be available in the circulation and tissues to exert an effect in the human body. Anthocyanin presence in plasma and urine after dietary intake suggest bioavailability in general is poor (<1%) but actually it is their metabolites that are suspected to be bioactive molecules. The small intestine is the main site of anthocyanin absorption. Once anthocyanins cross the wall of the enterocyte, they are modified by xenobiotic metabolism, which ultimately increases their hydrophilicity, bioavailability and facilitates elimination from the body through the bile and urine. The diminished level of oxygen in the intestine is an interesting fact because of the higher stability of anthocyanin structures under low oxygen levels. This could explain a notable amount of anthocyanins detected in the colon lumen.

From the perspective of the authors, the most captivating aspect of anthocyanins is their potential application in chemoprevention and their inclusion as a therapeutic component in the process of colon cancer treatment. In colon cancer rodent models all the preclinical studies, to a greater or lesser extent, suggest a chemopreventive effect of anthocyanins [17]. Other authors in their study on rats proved that anthocyanins can reduce colon carcinogenesis associated with acute inflammation, as well as modify the motility of human colon cancer cells which may reduce their ability to metastasize [18]. The in vitro studies with the human adenocarcinoma cell lines (HT-29) revealed that the dried peel powders of anthocyanins inhibited cell proliferation, arrested cell cycle, and raised apoptosis induction in a dose-dependent manner. Moreover, it was shown that they can inhibit the cell viability of HT-29 colon cancer cells and are potent antioxidants [19]. Another interesting and worth mentioning property of anthocyanins is the fact that to some extent they can modulate the colonic microbiota composition. It was found that administration of anthocyanins-rich extract can increase the level of Lactobacillus and Akkermansia in feces in rats as well as in human [6, 20].

There are relatively few in vivo human experimental studies assessing the effect of anthocyanin consumption on the risk of developing colorectal cancer as well as on possible modification of the natural course of the disease. Moreover, due to the varied methodology and the lack of a fixed dose of the daily supply of anthocyanins the results of these studies are often contradictory. As a consequence, it is difficult to draw final conclusions and make recommendations. In one cohort study, in the group of postmenopausal women, total anthocyanins intake was significantly inversely associated with rectal cancer incidence [21]. In the secondary analysis of a randomized dietary intervention trial (The Polyp Prevention Trial) high intake of flavanols was associated with a decreased risk of advanced adenoma recurrence (OR: 0.24; 95% CI: 0.11–0.53) [22]. In the intervention study involving patients diagnosed with colorectal cancer and supplemented with anthocyanins, proliferation in tumor tissue collected from all participants was decreased by 7% compared with preintervention values (determined by immunohistochemistry of Ki-67) [23]. On the other hand, in the largest prospective study relating habitual intake of various subclasses of flavonoids to risk of colon cancer occurrence no association was observed [24]. Finally, a meta-analysis published in 2018, including three cohort studies and four case-control studies, with approximately 611,709 total members, suggested that high anthocyanin intake can reduce colorectal cancer risk [25]. To our knowledge in only one study, it was reported that extracts of black raspberries exhibit a favorable impact on colon cancer in patients particularly by modulating the Wnt pathway [6]. In the literature review published in 2020 authors conclude that more interventional human studies, with a large number of volunteers and taking the EFSA (European Food Safety Authority) recommendations into account, are required to confirm the encouraging data obtained from the preclinical research. To date, some clinical data has demonstrated an inverse relation between anthocyanin consumption and colon cancer incidence [17].

Taking into consideration the above, we focused to compose an anthocyanin-rich formulation that would be optimal for conducting further clinical research involving patients diagnosed with colorectal cancer. It should be noted that in our geographical region, we have access to fresh products rich in polyphenols, including anthocyanins, only for a short period of the year. Therefore, we considered freeze-dried fruits more convenient to reach the objective. In the analysis of the obtained results, we aimed to examine not only the biochemical properties and the presence of biologically active components but also the sensory attributes, possibility to establish a convenient form of administration, and availability of the raw material in the Polish market. Among the analyzed species, dried black raspberries caught our particular attention. They exhibited relatively high concentration of polyphenols, including anthocyanins, along with promising antioxidant activity confirmed by two measurement techniques employed in the study. Additionally, these fruits provided pleasant sensory experiences, as affirmed by an independent group of testers. All these characteristics led us to select black raspberries for further experimental investigations involving patients. The tolerance of black raspberry extracts has been extensively described in the literature, no negative effects of anthocyanin derivatives have been reported, even at high doses. Hence, they remain an appealing option for nutraceutical ingredients, chemoprevention, and disease treatment. Likewise, chokeberries demonstrated encouraging biochemical properties, showing good antioxidant potential and high anthocyanin content in the tested material. However, based on the testers' feedback, chokeberries were found to have significantly inferior sensory attributes, leading to their exclusion from further clinical research. On the other hand, despite slightly inferior biochemical parameters compared to chokeberries, strawberries received very positive evaluations in terms of taste and tolerance when combined with black raspberries. Therefore, they were also chosen for further investigation. One surprising finding of the study was that yellow and red raspberries exhibited the highest content of vitamin C.

Determining the daily intake of anthocyanins that could induce the desired anti-inflammatory effect, modulate the body's response, and provide appropriate metabolic support for patients diagnosed with colorectal cancer proved to be a significant challenge. A literature review revealed considerable variations in the amount of anthocyanins used in previous clinical studies, with doses ranging from approximately 10 mg/day [26, 27] to as high as 2 g/day [23, 28]. Furthermore, the forms of anthocyanin administration in previous studies with colorectal cancer patients varied widely, ranging from free choice meals to powdered forms and liquid extracts [17]. These studies also varied in terms of the duration of observation/intervention, ranging from 7 days [29] to as long as 6 months [30]. Consequently, it was decided that the most suitable form of anthocyanin administration for the purposes of our study, considering the target population, would be orally soluble tablets. This formulation was obtained by compressing freeze-dried black raspberry and strawberry fruits under appropriate pressure. In this way, we obtained a formulation that corresponds to the consumption of approximately 100 grams of fresh fruits per day, containing around 600–700 mg of anthocyanins. We are confident that these achieved parameters will prove to be optimal, with the tablets being convenient and palatable for use, and will allow for a reliable verification of the anti-inflammatory and antioxidant effects of anthocyanins among patients with colorectal cancer.

The unique value of our study lies in the fact that the theoretically anticipated and subsequently measured and confirmed antioxidant properties of a specific fruit sample will now be put into practice to assess whether similar results and clinical responses can be expected in the complex human body. Of course, this study has certain limitations. Firstly, as mentioned above, the selection of the studied species was limited to native Polish ones, which means that in other parts of the world, products with better parameters may be found. Additionally, freeze-dried fruits, due to the stability of certain compounds, may differ in their properties from fresh fruits.

Variability of plant material is also a big problem. The content of bioactive components in this material may be affected by weather, harvest time, soil composition. These factors make it difficult to standardize plant material. For this reason, when planning an experiment, all raw materials should be properly homogenized to ensure their homogeneity.

Improvement of nutritional status and implementation of metabolic support in patients diagnosed with colon cancer can directly contribute to better performance status and treatment outcomes. Anthocyanins are group of biologically active substances whose potential utilization in chemoprevention and as a supportive measure in the treatment of colon cancer is not fully defined. In this study we determined the content of biologically active compounds (polyphenols, anthocyanins, vitamin C) and assessed the antioxidant potential of selected freeze-dried fruit species. Based on biochemical analysis and taking into consideration organoleptic properties especially taste and potential good tolerability by patients, freeze-dried black raspberry and strawberry fruits were selected for further clinical studies on the use of anthocyanins in nutritional and metabolic support of patients undergoing treatment for colorectal cancer.

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