Agronomy Research **21**(3), 1293–1303, 2023
https://doi.org/10.15159/AR.23.070

Isolation and study of a bioactive extract enriched with anthocyanin from red grape pomace (Cabernet Sauvignon)

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Received: February 6th, 2023; Accepted: May 7th, 2023; Published: July 25th, 2023

Abstract. Grape pomace is a natural product rich in dietary fibers, polyphenols and anthocyanidins. By their chemical composition, secondary products from grape processing are valuable raw materials for obtaining a variety of new products. Recently, in food biotechnology, fermented and unfermented grape pomace have undergone a deeper study of the role of additives in dry powders or extracts. The quality and biological value of natural food products are determined by their chemical composition and a whole complex of integral organoleptic properties that depend on this composition. Natural anthocyanin dyes not only give color to vegetable raw materials, but also have a well-known physiological activity, in particular, coloring and antioxidant. Purified natural dyes have recently been increasingly used to improve the consumer properties of food products, in biologically active additives, in pharmaceutical preparations for the treatment and prevention of various diseases. At the same time, the composition of anthocyanins, even for the same variety of plant raw materials, is complex and variable; it depends on climatic conditions, on the maturity of berries, root crops, and the quality of agricultural work. Anthocyanins easily undergo a number of transformations depending on the conditions of extraction and analysis.
The originality and novelty of the isolation and production of biologically active extracts with antiradical properties from by-products of local wineries in ecologically safe areas of southern Kazakhstan lies in the fact that natural environmental conditions: high temperatures and low humidity which contribute to the formation of biologically active substances with increased concentrations. Current research was aimed at the deep study of extracts from grape pomace rich - anthocyanins.
Red grape pomaces of *Vitis vinifera* L. Cabernet Sauvignon were extracted using a solvent-based (SE) method with concentrations of 70% v/v and solid/liquid ratios of 1:10, followed by incubation at three different temperatures of 30 °C, 40 °C, and 50 °C for 1 h, 2 h, 3 h, and 4 h. All solvent extracts showed higher amounts of anthocyanin pigments. The maximum yield was obtained by using the optimal time of extraction (2 h at 50 °C), with the highest total anthocyanin

recovery obtained by means of 70% ethanol. The anti-radical and toxic effects of the obtained extract (anthocyanin) were studied.

Key words: isolation, antioxidants, anthocyanins, extract, red grape pomace, antiradical activity.

INTRODUCTION

The use of agro-waste and food-industry processing residues in order to reduce their environmental impact and implement a new management strategy for the agricultural waste in a circular bio- based economy instead of chemical ones is an actual direction of modern medicine, pharmacology and cosmetology (Monari et al., 2020). Biologically active substances in plant extracts and products of their processing are promising for these purposes. The activity of extracts is largely due to the presence of certain bioactive compounds in them. These biologically active substances have a diverse composition and belong to different classes of chemical compounds (flavonoids, phenolic carboxylic acids and anthocyanins) It is the presence of these components that causes antimicrobial, fungicidal, antioxidant, etc. properties of plant extracts. The efficient use of agro-industrial and food by-products, residues and wastes offers a new perspective on a wide range of benefits in addition to their employment as feeds or in the biogas production. Innovative bio refinery extraction cascades may in fact allow the recovery of bioactive molecules and fibers from such poorly exploited biomass streams, thus finding applications in several industrial fields, and meeting the increasing demand from consumers for sustainable products and Resematerials (Tassoni et al., 2020).

Grapes are one of the largest fruit crops in the world, with a huge annual production of more than 80 million metric tons (FAO STAT, 2022). The main by-products of winemaking are collected during combing (stems), crushing of grapes and pressing (skin, seeds and sediment). Grape pomace consists mainly of the peel, stems and seeds and accounts for about 20% of the weight of grapes processed into wine. Thus, any useful production from these by-products can represent an interesting progress in maintaining ecological balance, as well as the economic value of the traditional use of by-products, mainly grape pomace from the technology of red and white vines (Garcia-Lomillo et al., 2017).

Grape pomace is a natural product rich in dietary fibers and polyphenols. By their chemical composition, secondary products from grape processing are valuable raw materials for obtaining a variety of new products. Recently, in food biotechnology, fermented and unfermented grape pomaces have undergone a deeper study of the role of additives in dry powders or extracts (Fontana et al., 2013; Teixeira et al., 2014). Grape pomace phytochemicals are responsible for multiple benefits involved in the prevention of degenerative processes through their integration into functional foods, nutraceuticals, and cosmetics. Hence, the most relevant activities attributed to bioactive phytochemicals from winery by-products are antioxidant, antimicrobial, anti-inflammatory and anticancer ones (Teixeira et al., 2014; Kalli et al., 2018), some of these also which have proven *in vivo*, such as the antioxidant and anti-hypertensive effects observed in rats (Rasines-Perea et al., 2018). For this reason, grape pomace is exploitable in the food, feed, cosmetic and pharmaceutical industries.

Several publications reported the optimization of red and white pomace extraction techniques in order to obtain phenolic fractions showing potentially beneficial biological

activities (Ferri et al., 2016; Ferri et al., 2017). Most of the published studies used conventional solvent extraction for polyphenol recovery, while in recent years the need for more green technologies moved research towards other methodologies, such as pressurised liquid extraction (PLE), hydrolysis by cell wall polysaccharide degrading enzyme mixtures (Ferri et al., 2016; Ferri et al., 2017) or supercritical fluid extraction (Mustafa et al., 2011; Muhlack et al., 2018). Meanwhile, nowadays there is widely used possibility of using microwaves to obtain extracts from berry press residues and jelly products with bioactive characteristics (Sepelevs et al., 2020). Recent research, was dedicated to a recycling method of an industrial potato processing by-product. There are several stages: collected peel homogenisation in the solvent media, initial phenolic compound and carbohydrate extraction, concentration of acquired extracts (recovery of the solvent for further reuse), and following encapsulation through spray-drying. The proposed method showed was very effective in terms of technology development (Sepelevs et al., 2020).

The idea of the isolation and production of biologically active extracts with antiradical properties from by-products of wineries in ecologically safe areas of the Turkestan region (South Kazakhstan) lies in the fact that natural environmental conditions: high temperatures and low humidity contribute to the formation of biologically active substances with increased concentrations. For example, the studied extracts obtained from grape pomace of unique grape varieties are rich in natural pigments - anthocyanins.

Anthocyanins are coloring substances of berries, fruits and flower petals, as well as leaves of some plants. Anthocyanins are widely distributed in the plant world. Their main representatives are the following aglycones: cyanidin, delphinidine, petunidin, malvinidine and pelargonidine (Harborne et al., 1998). Anthocyanins are naturally occuring pigments belonging to the group of flavonoids, a subclass of the polyphenol family. They are common components of the human diet, as they are present in many foods, fruits and vegetables, especially in berries, red wine vine making by-products (Lee et al., 2005). There were more studies conducted on effect of processing and storage on changes and stability of colors of anthocyanins in foods such as fruits and also for their use as natural colorants. Besides, the interest in anthocyanins is still growing owing to their strong antioxidant activity against many chronic diseases and the numerous studies about their medicinal, therapeutic, and nutritional values that have been conducted (Martín et al., 2017). There are pieces of evidence regarding the positive association of their intake with healthy biological effects. They act as antioxidants both in the foodstuffs in which they are found and in the organism that take in foods rich in anthocyanins. Many efforts have been carried out to develop new analytical techniques for identification and quantification of anthocyanins in plant materials, as well as their effects in vivo and in vitro (Elejalde et al., 2021). In study of antioxidant content of dark colored berries: Blackberries (*Rubus caesius* L.), elderberries (*Sambucus nigra* L.), highbush blueberries (*Vaccinium corymbosum* L.) and black currants (*Ribes nigrum* L.) were analyzed their content of phenolic compounds, including anthocyanins and comparing their content in these berries. Berries are a good source of vitamins, minerals and antioxidants in our diet. The results of the study confirmed that the dark colour berries are rich in antioxidants because of the high content of phenols, flavonoids and anthocyanins (Ozola & Dūma, 2020). With this in mind, it is possible to apply the knowledge and results obtained on the antiradical activity of red grape pomace extract.

MATERIALS AND METHODS

Materials

Red grape pomaces from by-products of wineries in ecologically safe areas of Southern Kazakhstan:Cabernet Sauvignonwere provided by Limited Liability Partnership Shato Silk Alley (Shymkent, Kazakhstan). Pomace was only softly pressed and collected right after wine production, and contained berry skins, seeds, petioles and stalks. The pomace dry weight (DW) was about 30.0% of the fresh weight (FW) and was determined by weighing aliquots of 3 g FW placed at 80 °C for 48 h.

The polymer used as matrix for composites was the commercial ion exchange resin Purosorb PAD 400 (Purolite, China).

Solvent-based extraction (SE) of phenols

Cabernet Sauvignon pomace was ground in a kitchen blender and stored at -20 °C until further use. One type of solvents - ethanol were tested at aqueous solution concentrations 70%. Each one of the solvent aqueous solutions was added to sample aliquots at solid/liquid ratios (S/L) of 1:10 (3 g FW + 30 mL solvent), incubated at 30 °C, 40 °C, 50 °C in a shaking water bath for 2 h and centrifuged (5 min, 4,500 g) to separate the liquid extract from fiber residue. Different incubation times (1 h, 2 h, 3 h, 4 h) were also tested. Water controls were used to detect the lowest level of extractable phenols in each test condition.

Liquid extracts' spectrophotometric analysis

Red grape pomaces from by-products: Cabernet Sauvignon extracts were characterized for total contents of anthocyanins by spectrophotometric assays: (Ferri et al., 2009). For each assay, a dose-response calibration curve was plotted by using a specific standard compound. The results were expressed as g of standard equivalents per kg of pomace DW. Anthocyanin results were converted from absorbance to malvidin-3-glucoside (MALV) equivalents (Considine & Frankish, 2014).

Antiradical activity

Red grape pomaces from by-products: Cabernet Sauvignon extracts were studied by technique of spectrophotometric measurement of the kinetics of the reduction of molecules of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPHG) by antioxidants was used. The studied compounds were dissolved in water at a concentration of 1 mg mL^{-1}. From the prepared solution, 100, 200, 300, 400 and 500 were added to 3 mL of the DFPG solution.

Toxicity

Assessment of acute toxicity of the studied samples at intragastric admission was carried out by the fixed dose method according to OECD (2002), Test No. 420: Acute Oral Toxicity - Fixed Dose Procedure, in which a group of animals (white mongrel mice) of the same sex, using a step-by-step procedure, fixed doses of 5, 50, 300 and 2,000 mg per kg are given.

RESULTS AND DISCUSSION

Optimization of anthocyanin pigment extraction

Solvent-based extraction(SE), was applied to Cabernet Sauvignon grape pomace and total anthocyanin pigment contents were assessed (Table 1).The SE methodology was used in all of the experiments, with ethanol, one of the most studied solvents for anthocyanin pigment extraction, at concentrations of 70% v/v and solid/liquid ratios of 1:10, and at three different incubation temperatures: 30 °C, 40 °C, 50 °C for 1 h, 2 h, 3 h and 4 h. All solvent extracts showed higher amounts of anthocyanins pigments. The maximum yield was obtained by using optimal time of extraction - 2 h and 50 °C (Table 1). Also extraction with using temperature higher 50 °C showed the destruction anthocyanins pigments.

Table 1. Solvent-based extraction (SE) is showed to mixed grape pomace of Cabernet Sauvignon for further determination of anthocyanin concentration by spectrophotometric method

Temperature of extraction 30 °C													
1 h	pH1.0	A510	0.3833	A700	0.1457	pH 4.5	A510	0.2307	A700	0.1013	0.11	25	47.68
2 h		A510	0.3394	A700	0.1166		A510	0.1166	A700	0.0922	0.20	25	87.42
3 h		A510	0.2693	A700	0.1315		A510	0.3598	A700	0.1759	-0.05	25	-20.31
4 h		A510	0.2862	A700	0.1445		A510	0.348	A700	0.1535	-0.05	25	-23.27
Temperature of extraction 40 °C													
1 h	pH 1.0	A510	0.3209	A700	0.1079	pH 4.5	A510	0.2674	A700	0.1183	0.06	25	28.16
2 h		A510	0.2997	A700	0.1002		A510	0.2578	A700	0.1113	0.05	25	23.35
3 h		A510	0.3356	A700	0.2911		A510	0.1084	A700	0.1183	0.05	25	23.97
4 h		A510	0.324	A700	0.2759		A510	0.1126	A700	0.1122	0.05	25	21.02
Temperature of extraction50 °C													
1 h	pH 1.0	A510	0.4782	A700	0.1527	pH 4.5	A510	0.3583	A700	0.1583	0.13	25	55.30
2 h		A510	0.4107	A700	0.1078		A510	0.2745	A700	0.1167	0.15	25	63.93
3 h		A510	0.2192	A700	0.0912		A510	0.2161	A700	0.0942	0.01	25	2.69
4 h		A510		A700			A510		A700		0.00	25	0.00

Total anthocyanins pigments were quantified and data expressed as TA: Total anthocyanin pigment (mg kg^{-1}), A: The absorbance value of the diluted GP extracts, MW: Molecular weight of Malvidin-3-O-glucoside (493.5), ε: Molar absorptivity (28,000), l: Path length, DF: Dilution factor is defined as: total volume of solution per aliquot volume

As to the effect of increased incubation time (up to 4 h) on anthocyanin pigment extraction, the data did not indicate a univocal trend. Previous data from a combined enzymatic plus ethanol extraction on both red and white grape pomace showed that the amount of phenols decreased with increasing incubation time (Ferri et al., 2016). In order to reduce processing energy costs, incubation temperature and time are major factors during the extraction optimization. Therefore, 50 °C for 2 hours seemed to be the right balance between processing energy cost and high anthocyanin pigment recovery.

Preparation of extracts with a given pH

When studying the dependence of the absorption spectra of anthocyanin extracts on pH, aliquot volumes of extracts were transferred to a beaker and the pH value was adjusted to the required value by adding 0.1–1.0 M solutions of sodium hydroxide or

hydrochloric acid. The solution was brought to the mark in a measuring flask with distilled water and the pH of the resulting solution was determined again.

A combined glass electrode ESC-10601/7 and an ionometer pH-150M were used in the work.

Recording of electronic spectra

SF 56 and Shimadzu UV-2550 spectrophotometers were used in the work. The spectra were recorded in quartz cuvettes with an optical path length of 0.1 ' 1.0 cm relative to water-alcohol mixtures.

Determination of anthocyanin concentration by spectrophotometric method

Preparation of working solutions.

Solution A: 0.025 M X1, pH 1.0

The KS1 suspension weighing 0.465 g was dissolved in 240 mL of distilled water in a beaker. The pH value was adjusted to 1.0 with a solution of concentrated hydrochloric acid, adding it drop by drop. The resulting solution was transferred to a 250 mL volumetric flask and brought to the label with distilled water, followed by pH control.

Solution B: 0.4 M CH3COONa, pH 4.5

A sample of CH3COONa·3H2O weighing 13.6 g was dissolved in 240 mL of distilled water in a beaker. The pH was adjusted to 4.5 with a solution of concentrated hydrochloric acid, adding it drop by drop. Transferred the resulting solution was placed in a 250 mL volumetric flask and brought to the label with distilled water, re-controlling the pH.

Aliquot volumes of the analyzed extract of Va anthocyanins (with preliminary selection of dilution) were transferred to Vk volumetric flasks and brought to the label, respectively, with solutions A and B.

Determination of optical density of solutions and calculation of anthocyanin concentration

The optical density of the prepared solutions was measured using a spectrophotometer Varyan Cary 50. The spectra were recorded in quartz cuvettes with an optical path length of 0.1 ' 1.0 cm relative to water-alcohol mixtures.

The concentration of anthocyanins (mol L^{-1}) was calculated by the formula:

$$c = n \cdot \{A_{max}(pH = 1) - A_{700}(pH = 1)] - [A_{max}(pH = 4.5) - A_{700}(pH = 4.5)\}/(\varepsilon_{st} \cdot l),$$

where $A_{max}(pH = 1)$, $A_{max}(pH = 4.5)$, $A_{700}(pH = 1)$ and $A_{700}(pH = 4.5)$ – the optical density of solutions at the maximum absorption for samples with pH = 1 and pH = 4.5, respectively; n – the degree of dilution of the initial solution; est – extinction coefficient, 26,900 L mol^{-1} cm^{-1} in case of conversion to cyanidin-3-glucoside chloride (Cabrita et al., 2000); l – the length of the optical paths. The content of anthocyanins in plant raw materials was expressed in g/100 g of the starting material:

$$m = c \cdot (100 \div m) \cdot M, \quad (1)$$

where M = 484.8 g/mol is the molar mass of cyaniding-3-glucoside chloride; m is the weight of the anthocyanin source sample, g

$$A = (A510\ nm - A700\ nm\ at\ pH\ 1) - (A510\ nm - A700\ nm\ at\ pH\ 4.5) \quad (2)$$

$$A = (A510 - A700)pH1.0 - (A520 - A700)pH4.5 \quad (3)$$

$$TA\ mg\ kg^{-1} = A*493{,}5*DF*1{,}000/\ 28{,}000*L \quad (4)$$

Spectrophotometric characterisation of the extract

Spectrophotometric techniques were used to characterize the total amount anthocyanin pigment and antioxidant activity of the previously selected SE samples (Fig. 1). In addition to total anthocyanin pigment previously reported in Table 1, the content of the most relevant amount of extracted anthocyanin pigment was determined (Fig. 1).

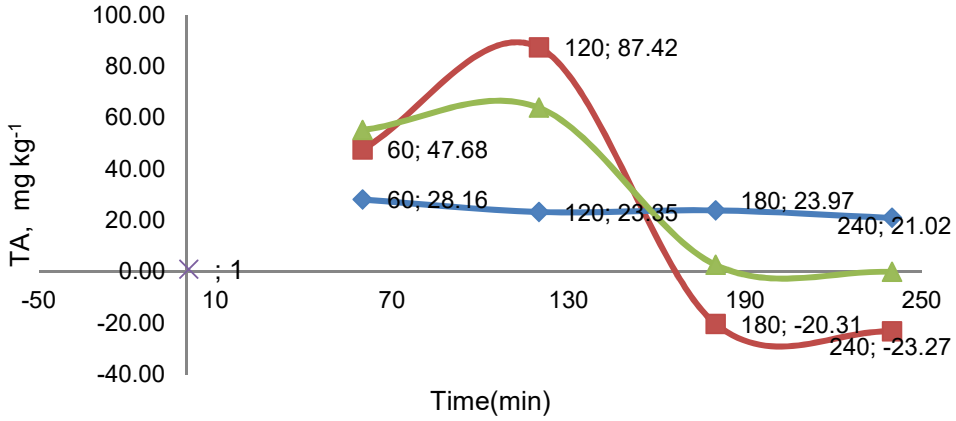

Figure 1. Comparison of the dependence of TA (Total anthocyanin pigment (mg kg^{-1})) mass on temperature.

Sorption of anthocyanins on the polymer used as a matrix for composites was the commercial ion exchange resin Purosorb PAD 400 (Purolite, China).

The obtained extracts of enriched anthocyanins and solvent were carried out through a column filled with the above polymer to obtain a highly concentrated liquid extract with a coloring effect.

It should be noted that in order to completely remove the solvent from the samples, it is necessary to remove the solvent on a vacuum evoparator. To control the stability of anthocyanins in the prepared dry compositions, liquid extractis dried to powder. It is stored in the chamber of a household refrigerator at a temperature of 4 °C.

Antiradical activity

Recently, there has been increased interest in studying the role of reactive oxygen species in the pathogenesis of various diseases. On the one hand, reactive oxygen species are formed during natural physiological processes and is necessary for maintaining the body's immune system, transmitting cellular signals and synthesizing hormones. On the other hand, oxidative stress caused by high concentrations of pro-oxidants can lead to damage to proteins, cell membranes and nucleic acids. The most pronounced consequences of free radical cell damage in cardiovascular, bronchopulmonary and oncological diseases. The required level of reactive oxygen species aremaintained by the body's antioxidant system. However, in some cases this system does not work, which leads to the emergence of a pathological process. Thus, the work investigated the antiradical activity of some plant samples.

When extract is added to the alcohol solution of stable radical 2,2-diphenyl-1-picrylhydrazyl, the transition of free radical molecules into a non-radical form occurs, while the intensely purple solution of stable radical 2,2-diphenyl-1-picrylhydrazyl discolors. Fig. 2 shows the kinetics of the change in the optical density of the stable radical 2,2-diphenyl-1-picrylhydrazyl solution with the addition of the three compounds studied by us.

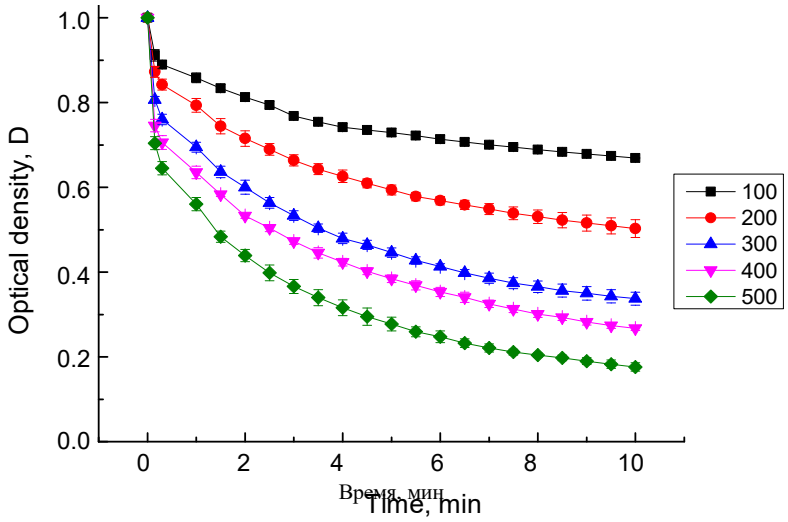

Figure 2. The change in the optical density of the alcohol solution of DPH with respect to the control when adding the compounds under study, depending on time.

To compare the reactive oxygen species of studied extract-Anthocyanin, a concentration of 100 µl for extract was selected from a prepared solution of 1 mg of the substance in 1 mL of water. Analyzing the results obtained, it can be concluded that when the studied sample are added to the alcohol solution, there is a sharp decrease in the optical density of the stable radical 2,2-diphenyl-1-picrylhydrazyl solution, which indicates their high antiradical ability.

The solid line is based on nonlinear regression. The concentration of DFPG is 0.1 mM. Measurements were carried out at 20 °C immediately after the addition of the studied drugs. The concentration of the studied samples is 100, 200, 300, 400 and 500 µl (from the prepared initial solution of 1 mg mL^{-1}) in 3 mL of the DPH solution.

According to experimental data extract has a high ability to quench free radicals. To quantify the antiradical activity, a stable radical 2,2-diphenyl-1-picrylhydrazyl (DPH) was used, as well as the parameter t50 - the time required for the studied drugs to reduce the initial radical concentration by 50% at a sample concentration of 500 µl. In the reaction of DFPG with extracts t50 at 20 °C is for extract - 256 ± 12, therefore.

Toxicity

The study of acute toxicity of the drug was carried out on 10 healthy white mongrel mice, females weighing 20 ± 2.0 g, 5 animals in each group, quarantined for at least 14 days in the vivarium of the Laboratory of Pharmacology and screening of biologically active substances. Mice of the experimental groups were injected once, intragastrically,

with an extract- Anthocyanin sample at a dose of 2,000 mg kg^{-1}, and mice of the control group were injected with distilled water. The animals were monitored hourly during the first day of the experiment in the laboratory, while the following indicators of the functional state of the animals were recorded: general condition, possible seizures, survival and death. Further, daily, for 2 weeks in vivarium conditions in animals of all groups, observations were made on the general condition and activity, behavioral characteristics, frequency and depth of respiratory movements, the condition of the hair and skin, the position of the tail, the amount and consistency of fecal masses, the frequency of urination, changes in body weight, etc. indicators. All experimental animals were kept in the same conditions and on a common diet with free access to water and food. At the end of the experiment, the average lethal dose (LD-50), was calculated and the toxicity class was determined.

When extract- Anthocyanin was administered to mice once intragastrically at a dose of 2,000 mg kg^{-1}, the animals experienced increased respiration 2 out of 5. After 10–15 minutes, washing, narrowing of the eyes and bunching were observed in all experimental animals. The mice returned to normal within 1–3 hours, no animal deaths were recorded (0/5). The results obtained are shown in Table 2.

Table 2. Indicators of acute toxicity during intragastric administration sample No. 3 to mice

Samples	Type of animal/ intake	Dose, mg kg^{-1}	Number of dead/ number of animals in the group	Lethal $Dose_{50}$, mg kg^{-1} type of toxicity
Anthocyanin	Miceoral	2,000	0/5	> 2,001 (V class)
Control	Mice/ oral	0.5 mL	0/5	

During the entire study period (14 days), the animals were observed after administration of Anthocyanin. Observation of experimental animals according to the studied indicators did not reveal deviations in the condition of the coat and skin, the position of the tail, the consistency of feces, urination from animals of the control group.

The average weight gain of mice treated with Anthocyanin at a dose of 2,000 mg kg^{-1} was 27.2%, which is 1.4 times higher than in the control group (19.6%), but no statistical difference was achieved. The results obtained are presented in Table 3.

Table 3. Average value of body weight gain in mice with intragastric intake of Anthocyanin (M ± m; n = 5)

Samples	Dose, mg kg^{-1}	Body weight, g begin	7 days	14 days	Weight gain, %
Control	0.4 mL	20.5 ± 1.2	23 ± 1.4	25.5 ± 2.3	19.6
Anthocyanin	2,000	20 ± 0.4	23.5 ± 0.8	27.5 ± 1.3	27.2

$P > 0.05$ – relative to the control.

Thus, the study of acute toxicity with intragastric administration in accordance with the modified OECD classification showed that Anthocyanin corresponds to the V class of toxicity of chemicals (Practically non-toxic), LD50 2,000 mg kg^{-1}.

Conclusion: Anthocyanin corresponds to chemical toxicity class V (Practically non-toxic), LD50 2,000 mg kg^{-1}. The average weight gain in mice receiving sample No. 3 was 1.4 times higher than in the control group.

CONCLUSIONS

Red grape pomaces of Cabernet Sauvignonwas subjected to solvent-based (SE) extraction method with the concentrations - 70% v/v and solid/liquid ratios S/L; 1:10, and at three different temperature incubation at 30 °C, 40 °C, 50 °C for 1 h, 2 h, 3 h and 4 h. All solvent extracts showed higher amounts of anthocyanins pigments. The maximum yield was obtained by using optimal time of extraction - 2 h and 50 °C suitable for treatment of pomace with highest total anthocyanins recovery obtained by means of 70% ethanol. In view of an industrial scale up of this by-product valorisation, the selection of the best treatment could depend on the targeted compounds as well as on the environmental and economic impact of the extraction process.

Taking their composition and colouring effect into consideration, the recovered anthocyanidins extracts could find several different applications, e.g. as food supplements, bioactive pharmaceutical components, additives for cosmetics and harmaceuticals. In this regard, extract has high antiradical activity it is important to study the possibility of correcting disorders that occur in oncological, diabetic and other non-infectious patients and to consider it as a promising drug.

Finally, the current study demonstrated the feasibility of full recovery for anti-radical activity and effective valorization of grape pomace by-product. In future perspective, the potential of such wine making residues as an enrichment for various products as a natural pigment antiradical activity.

ACKNOWLEDGEMENTS. The research was supported by financial support of the Ministry of Education and Science of the Republic of Kazakhstan, under the project AP09563292 'Development of technology for the extraction of biologically active substances from plant raw materials with a multi-enzyme preparation, followed by enrichment of fruit juices and fermented beverages'.

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