ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2021; 26(2): 2396-2405
doi: 10.25083/rbl/26.2/2396.2405

Received for publication, August, 2, 2019
Accepted, November, 2, 2019

Original paper

Evaluation of cytotoxicity, nutritional and anti-oxidative status of lyophilized plant extracts used in dietary supplements

BIANCA-MARIA TIHAUAN[1], IOANA-CRISTINA MARINAS[2,3], CORALIA BLEOTU[1,6], GEORGIANA DOLETE[1,4], TATIANA ONISEI[5], FLOAREA SERBANCEA[5], CRISTINA MATEESCU[5], MANUELA RĂSCOL[5]

[1]Research and Development Department of SC Sanimed International Impex SRL, Șos. București-Giurgiu (DN5), No. 6, PO code 087040, Jud. Giurgiu, Romania
[2]Research Institute of the University of Bucharest-ICUB, Splaiul Independentei Nr. 95, Sector 5, Bucharest, Romania
[3]Physico-Chemistry Laboratory, National Institute of Research & Development for Food Bioresources – IBA Bucharest, 5 Băneasa Ancuța, 020323, Bucharest, Romania
[4]Scientific Research Assistant, Department of Science and Engineering of Oxide Materials and Nanomaterials, University Politehnica of Bucharest, Str. Gheorghe POLIZU, nr. 1-7, sector 1, 011061, București, Romania
[5]Research and Development Department, The National Institute for Research and Development in Food Bioresources, Str. Dinu Vintilă, No. 6, PO code 021102, Sector 2, Bucharest, Romania
[6]Stefan S. Nicolau Institute of Virology, Mihai Bravu 285, Bucharest, 030304, Romania

Abstract

Immune functions are indispensable in defending the body against pathogens and thus play a vital role in maintaining health. Immune functions are disturbed by malnutrition, aging, physical and mental stress or unwanted lifestyle. Therefore, the intake of immuno-modulatory dietary supplements is considered effective in improving immune functions and reducing the incidence of immunological disorders.

The main focus of this study was to evaluate the antioxidative status of lyophilized plant extracts, such as rosehip (*Rosa canina*), sea buckthorn (*Hippophae rhamnoides*), echinacea (*Echinacea angustifolia*) and bilberries (Vaccinium myrtillus), their cytotoxicity and nutritional status, in order to effectively utilize them as ingredients in a dietary supplement with immunomodulatory properties.

Keywords Dietary supplements, plant extracts, antioxidative, cytotoxicity.

To cite this article: TIHAUAN BM, MARINAS IC, BARBOI G, DOLETE G, ONISEI T, SERBANCEA F, MATEESCU C, RĂSCOL M. Evaluation of cytotoxicity, nutritional and antioxidative status of lyophilized plant extracts used in dietary supplements. *Rom Biotechnol Lett*. 2021; 26(2): 2396-2405. DOI: 10.25083/rbl/26.2/2396.2405

✉ *Corresponding author: Bianca-Maria TIHAUAN, MSc., Research and Development Department of SC Sanimed International Impex SRL, Șos. București-Giurgiu (DN5), No. 6, PO code 087040, Jud. Giurgiu, Romania, Telephone: +40733229834
E-mail: bianca.tihauan@sanimed.ro

Introduction

The importance of a well-functioning and well-balanced immune system for maintaining health has become extremely evident in recent decades. The attention given to diet and nutrition contributes to the consolidation of a balanced lifestyle, its lack affecting the functioning of different immune parameters (ALBERS, 2013). Dietary supplements today include vitamins, minerals, herbs and botanicals, amino acids, enzymes and many other products. Supplements can be found in a variety of forms: traditional tablets, capsules and powders, as well as energy drinks. In general, supplements can help restore the body's metabolic balance while maintaining its caloric balance, but cannot take the place of meals or foods that are vital for a healthy diet.

Immune functions are indispensable in defending the body against pathogens and thus play a vital role in maintaining health. However, immune functions are disturbed by malnutrition, aging, physical and mental stress or unwanted lifestyle. Therefore, the intake of immunomodulatory dietary supplements is considered effective in improving immune functions and reducing the incidence of immunological disorders (ALBERS, 2013; WICHERS, 2009).

The importance of plant extracts in dietary supplements is sustained by intensive concerns about the phytochemical and pharmacological study of herbs and herbal remedies, for which the therapeutic properties such as the immunomodulatory ones have been demonstrated (DHAR, 2013; LEWICKA, 2019). The approaches are complex, from *in vitro* experimental studies to clinical studies that underpin the use of phyto-complexes and minerals for prophylactic or curative purposes in food supplements.

One of the many important contributors to an efficient dietary supplement formula are the natural antioxidants. It can be defined as molecules that prevent the destruction of cells under the action of free radicals, thus having an extremely important role in the proper functioning of the immune system. In all living systems, cells require adequate levels of antioxidant protection to avoid the destructive effect of excessive ROS release. Antioxidants have the ability to modulate host sensitivity or resistance to infectious pathogens.

Bilberries (*Vaccinium myrtillus* L.) are one of the richest natural sources of anthocyanins. These polyphenolic components give bilberry its blue/black color and high antioxidant content, and they are believed to be the key bioactives responsible for the many reported health benefits of bilberry and other berry fruits. Although bilberries are promoted most commonly for improving vision, it has been reported to lower blood glucose, to have anti-inflammatory and lipid-lowering effects, and to promote antioxidant defense and lower oxidative stress. Therefore, studies sustain that bilberries have an immense potential value in treating and preventing conditions associated with inflammation, dyslipidemia, hyperglycemia or increased oxidative stress, cardiovascular disease (CVD), cancer, diabetes, and dementia and other age-related diseases. There are also reports on bilberries antimicrobial activity (CHU, 2011; KOLEHMAINEN, 2012; MOŽE, 2011; SHI, 2017).

Sea buckthorn *(Hippophae rhamnoides* L.) possesses strong antioxidant properties so it is used to improve blood pressure and lipids, to prevent and control cardiovascular symptoms (e.g. angina), to reduce free radicals levels and prevent atheroma. Both *Hippophae rhamnoides* leaves and flowers are used in arthritis, gastrointestinal ulcers, gout, and rashes. Its fruits are also used to prevent infection and boost immune function. There are reports of its administration as an expectorant in treating common cold, asthma and pneumonia; as an aid to improve vision and prevent nyctalopia. It also helps to heal wounds/injuries from burns, acne, skin ulcers; it may help to improve eczema skin lesions and dermatitis symptoms. Finally, it seems to participate in body's protection against radiance effects (UV, X-rays, and radioactivity) (DHAR, 2013; GEETHA, 2002, 2005).

A number of studies revealed that alkamides are involved in the immunomodulatory properties of *Echinacea* extracts *in vitro* and *in vivo*. Additionally, caffeic acid is found in some species of *Echinacea* and could be applied toward authentication and quality control of the plant extracts. The polysaccharides play an important role in the anti-inflammatory effect of *Echinacea* preparations. The immunostimulant activity of the plant or its preparations is caused by three mechanisms: phagocytosis activation, fibroblast stimulation, and the enhancement of respiratory activity that results in augmentation of leukocyte mobility. There are numerous *in vivo* studies on the immunomodulatory and anti-inflammatory effects of *Echinacea* that suggest that innate immunity is enhanced by administration of the plant and that the immune system is strengthened against pathogenic infections through activation of the neutrophils, macrophages, polymorphonuclear leukocytes (PMN), and natural killer (NK) cells (ALBERS, 2013; DHAR, 2013).

Rosehip (*Rosa canina)* is rich in beneficial compounds that can contribute to the maintenance of human health. Since *R. canina*, especially its hip, is rich in vitamin C and phenolic compounds, it has been used for its antioxidant properties to prevent or even treat various ailments. The beneficial effects of the consumption of R. canina however, are not limited to its antioxidant properties. It is indeed valued for its antibacterial and antidiabetic properties too. Moreover, it has been approved in a variety of researches that *R. canina* is capable of reducing cardiovascular diseases through decreasing blood pressure and LDL without having any side effect. It is also an efficient plant against osteoarthritis and rheumatoid arthritis, especially with regard to its anti-inflammatory properties and its capacity to reduce pain (BHAVE, 2017; PATEL, 2013; SAABY, 2011; SELAHVARZIAN, 2018).

All in all, the immune functions are indispensable in defending the body, therefore, the intake of immunomodulatory dietary supplements is considered effective in improving immune functions and reducing the incidence

of immunological disorders. Therefore our research focuses on evaluating four plant extracts that can be included into a dietary supplement as enhancers for boosting the immune system.

Materials and Methods

We evaluated the antioxidative status of fine ground dried fruits of rosehip (*Rosa canina)*, sea buckthorn (*Hippophae rhamnoides)* and bilberries (*Vaccinium myrtillus)* as well as the lyophilized total extract of echinacea (*Echinacea angustifolia)*, their cytotoxicity and nutritional status. All samples were commercially acquired (in powder form) from authorized merchants. They had as country origin mainly UE member states, such as Poland (sea buckthorn fruits), Germany (bilberries fruits), Bulgaria (rosehip fruits), but also China (echinacea extract).

Samples were prepared by aqueous extraction with a conventional heating technique.

The extraction procedure: 5g from each sample were introduced into a 100 mL beaker with 50 mL of distilled water for 1:10 sample to solvent ratio. This mixture was mechanically stirred until the mixture was well homogenized, then the mixture was heated to extraction temperature (i.e. 35-40°C) and rigorous stirring was continued throughout the extraction time (i.e. 5-150 minutes). The extracts were then carefully filtered using a vacuum pump filter.

Evaluation of the antioxidative status of plant extracts

a. Total phenolic content

The total phenolic content was determined using the Folin-Ciocâlteu method and is based on the oxidation of the phenolic compounds using a molybdowolframate ($Na2WO_4$ / Na_2MoO_4). From this reaction results $O^{2\bullet}$, which reacts with molybdate, resulting in the (Mo^{4+}) ion (blue), whose absorbance is observed spectrophotometrically at λ= 765 nm. The total phenol (TP) content was achieved by homogenizing 50 µL sample or standard (gallic acid) with 50 µL Folin-Ciocâlteu reagent and 500 µL 7% sodium carbonate solution. The absorbance was measured after 60 minutes and compared to a control sample (containing distilled water) using the FlaxStation microplate spectrophotometer from Molecular Devices. The absorbance of the samples (standard/ actual sample) was determined at λ = 765 nm. The calibration curve was drawn with standard gallic acid solutions of concentrations between 2 and 20 µg/mL. The equation of the calibration curve was y = 0.0608x – 0.0253 (R2 = 0.999) The TP content was expressed in equivalents of mg gallic acid in one mL of extract.

b. ABTS radical cation decolorization assay

The ABTS radical neutralization method for the 10% aqueous extracts obtained from the species *Hippophae rhamnoides* L., *Rosa canina* L., *Echinacea angustifolia* L. and *Vaccinium myrtillus* L. was determined by the ABTS radical cation discoloration test. The $ABTS^{\cdot+}$ solution was obtained through the reaction of 7 mM ABTS in water and 2.45 mM potassium persulfate (Re. R., PEL. et al, 1999), stored in the dark at room temperature for 12-16 hours before use. The ABTS + solution was then diluted with 96% ethanol to obtain an absorbance of 0.700 ± 0.04 at 734 nm. After adding 20 µl of the sample in 180 µl of diluted $ABTS^{\cdot+}$ solution, the absorbance was measured after 30 min (RE. R, PEL. et al, 1999). Also, distilled water was used as a control. All measurements were performed three times. The results were expressed as µM Trolox / ml extract. Trolox was used as a standard substance, the calibration curve was made with the concentration range of 0-40 µM, that represented final concentrations (THAIPONG K. et al, 2006), starting from a stock solution of 1 mM Trolox.

c. The CUPRAC method

The CUPRAC method is based on the reduction of a copper complex named neocuproine (Cu (II) -Nc) by a complex of antioxidants in cuprous form (Cu (I) -Nc). The CUPRAC method for testing total antioxidant capacity (TAC) utilizes the chelation cation bis (2,9-dimethyl-1,10-phenanthroline: neocuproine) as a chromogenic oxidizing agent, which is reduced in the presence of antioxidants in [Cu (I) -Nc] and shows maximum absorption at λ= 450 nm (KARASAKAL A, 2015).

The antioxidant method of copper ion reduction (CUPRAC) was performed according to a method adapted by Meng et al. (2011), as follows: 0.1 ml of standard extracts/solutions of different concentrations were mixed with 0.3 ml $CuSO_4$ (5 mM), neocuproine 0.3 ml (3.75 mM) and 2, 8 ml of distilled water, reaching a final volume of 3.5 ml. After 30 minutes, the absorbance was measured at 450 nm. The standard Trolox solutions required for the calibration curve were made from a stock solution of 5 mM, and the concentrations taken into consideration were: 2.5 mM, 2 mM, 1.5 mM, 1mM, 0.5 mM, 0.25 mM.

d. The DPPH method

The measurement of DPPH radical capture activity was performed by a method adapted by Madhu (2013). The reaction mixture consisted of adding 100 µl of sample/ standard and 100 µl of 0.3 mM DPPH radical solution in ethanol. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced, varying in color from purple to light yellow. The absorbance was determined at λ = 517 nm after 30 min of reaction using a UV-VIS spectrophotometer. Ethanol was used as a control. The concentrations used for Trolox were: 0.4 mM, 0.2 mM, 0.15 mM, 0.1 mM, 0.075 mM, 0.05mM.

***In vitro* cytotoxicity assessment of standardized plant extracts** was carried out using the CellTiter-Glo® (Promega) kit for testing cell proliferation. The kit provides a homogeneous method to determine the number of viable cells in culture by quantitating the amount of ATP present, which indicates the presence of metabolically active cells. 10^4 cells were seeded in each well of a 96-well plate (960,000 total), and at 24 hours the treatment was performed. At 24 hours after treatment, the effect was evaluated using the Promega kit.

The homogeneous assay procedure involves addition of a single reagent directly to cells cultured in serum-supplemented medium. Cell washing, removal of

medium and multiple pipetting steps are not required. The luciferase reaction for this assay is shown in Figure 1. The results in cell lysis and generation of a luminescent signal that are proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture. The CellTiter-Glo Assay relies on the properties of a proprietary thermostable luciferase, which generates a stable "glow-type" luminescent signal.

For the test performed, the samples were labeled as presented in Table 1.

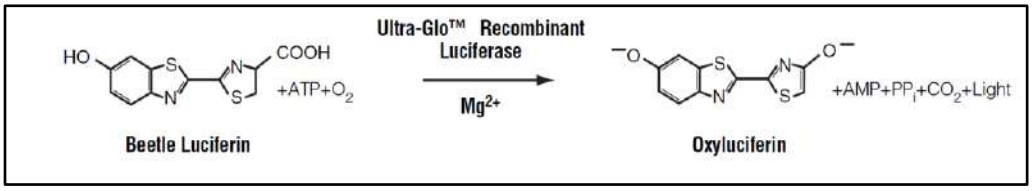

Figure 1. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg2+, ATP, which is contributed by viable cells, and molecular oxygen.

Table 1. Sample codes

Samples	Sample codes
1_Rosehip extract in 1% acetic acid	**EAAM**
2_Echinaceea extract in 1% acetic acid	**EAAE**
3_Blueberries extract in 1% acetic acid	**EAAA**
4_Sea buckthorn extract n 1% acetic acid	**EAAC**
5_Aqueous extract of rosehip	**EM**
6_Aqueous extract of echinacea	**EE**
7_Aqueous extract of blueberries	**EA**
8_Aqueous extract of sea buckthorn	**EC**

For the microscopic evaluation of cytotoxicity using a ZEISS Primovert microscope, 105 cells were seeded into a 24 well plate, and treatment was performed at 24 hours. The effect of the treatment was evaluated after 24 hours by microscope visualization (phase contrast photo) and by staining the samples with fluorescein diacetate (FDA) and propidium iodide (PI).

Evaluation of the nutritional status and physico-chemical properties

a. Determination of total proteins

Total protein content was determined using the Kjeldahl method. Following the breakdown of proteins and other nitrogen compounds, ammonium ions are released, which further combine with sulfuric acid to form ammonium bisulphate. By strong alkalization, the ammonium bisulphate from the mineralized sample, releases ammonia, which is distilled and captured in an acid solution. Knowing the amount of acid needed to neutralize the distilled ammonia, it was calculated the amount of nitrogen in the sample to be analyzed.

b. Determination of extractible and total fats

Total fats were determined using the Soxhlet method. Lipids determination is based on their solubility in organic solvents: acetone, ethyl ether, petroleum ether and was made using Soxhlet extractor. The Soxhlet method is based on a continuous periodic extraction, using a well-determined solvent volume. The solvent used in the extraction goes through a closed cycle. The total product obtained after removing the solvents represents the gross fat. This method is applied to the analysis of all foodstuffs/food supplements being included.

c. Determination of total glucids

For the nutritional declaration, the values of sugars, which according to the EU Regulation 1169/2011, are defined as the sum of the mono and disaccharides present in the food, must be taken into account. These were determined by the chemical method provided in SR 91/2007. The available carbohydrates were calculated as follows:

- *Carbohydrates available (g /100g) = Total carbohydrates (g /100g) – total dietary fiber (g / 100g)*
- *Total carbs (g / 100g) = dry matter (g / 100g) – [protein (g /100g) + lipids (g /100g) + total ash (g / 100g)]*

The carbohydrate value was expressed in g/100g edible part of the food.

d. Determination of ash contents

The total mineral substances (ash) represent the residue obtained after calcination of the sample at 525 ± 25°C, until the constant weight is reached.

e. Determination of minerals content

Minerals were determined using the inductively coupled plasma mass spectrometry method (ICP-MS) applied after wet hydrolysis of the sample in a microwave field. All samples were prepared and analyzed in duplicate. For the hydrolysis of the samples, the Advanced Microwave Digestion System ETHOS EASY (Milestone) – microwave oven, was used.

Results and Discussions

Evaluation of the antioxidative status of plant extracts

It is well known that, in general, phenolic compounds have the role of neutralizing reactive oxygen radicals (ROS) (VAYA, 1997; SENGUL, 2009). Natural antioxidants can be defined as molecules that prevent the destruction of cells under the action of free radicals. In all living systems, cells require adequate levels of antioxidant protection in order to avoid the destructive effect of excessive ROS release. During inflammatory processes, neutrophils and macrophages release free superoxide and peroxide radicals, which are essential for defense against invaders. Antioxidants are absolutely necessary to control the reactions that involve free radicals release. Antioxidants commonly included in the diet, vitamin E, vitamin C, β-carotene, selenium, copper, iron and zinc optimize the functionality of the immune system and play an important protective role against bacterial, viral or parasitic infections. Antioxidants have the ability to modulate host sensitivity or resistance to infectious pathogens (PUERTOLLANO, 2011).

The antioxidant activity of the extracts cannot be taken into account if it is based on a single antioxidant test model. In practice, several *in vitro* testing procedures are performed to evaluate antioxidant activities. Another aspect is that antioxidant activity assay models vary in different aspects. Therefore, it is difficult to compare the results obtained by different methods. To some extent, the comparison between different *in vitro* methods was made by Badarinath et al. (2010). Therefore, the choice of method is made in a critical way. In general, *in vitro* antioxidant assays using free radical neutralization are relatively simple to perform. Of the free radical neutralization methods, the DPPH method is additionally fast, simple (i.e., does not require several steps and reagents) and is cheap compared to other test models. But, there are some shortcomings that limit its application. DPPH can only be dissolved in organic media (especially in alcohols), not in aqueous media, which is an important limitation in interpreting the role of hydrophilic antioxidants. Although widely used for the measurement and comparison of the antioxidant status of phenolic compounds, the assessment of antioxidant capacity by modifying the absorbance of DPPH must be carefully deduced because the absorbance of the DPPH radical at 517 nm after reducing the reaction with an antioxidant can be influenced by light, oxygen and solvent type. It was concluded that over a certain limit of solvent water content, the antioxidant capacity decreased, because a part of DPPH coagulates and is not readily accessible to the reaction with antioxidants (KARADAG A. et al, 2009).

On the other hand, the ABTS bleaching test is applicable for both hydrophilic and (ALAM N. et al, 2013) lipophilic antioxidants. A major disadvantage of this method is the comparison between the antioxidant capacity obtained at a final point and the kinetic behavior of the samples, when considering both the concentration and the time required for radical depletion, they can lead to differences. For example, BHA has a higher antioxidant capacity than the ferulic acid when results are obtained at an endpoint, but has a lower value indicating slower kinetics than ferulic acid. The fact that the ABTS radical used in the TEAC analyzes is not found in biological systems and is not similar to the radicals found in these systems is also a problem (KARADAG A. et al, 2009).

The advantages of the CUPRAC method over other similar tests are: (**a)** the CUPRAC reagent is fast enough to oxidize thiol-type antioxidants; (**b**) the reagent is more stable and more accessible than other chromogenic reagents (e.g. ABTS, DPPH); (**c**) absorption curves versus the concentration curve are linear over a wide range, unlike those other methods that give polynomial curves; (**d**) the redox reaction the produces colored species is carried out at buffer pH 7; (**e**) the method may measure both hydrophilic and lipophilic antioxidants. CUPRAC analysis is complete within minutes for ascorbic acid, uric acid, gallic acid and quercetin, but requires 30-60 minutes for more complex molecules, but presents similar problems with a complex mixture of antioxidants in choosing an adequate reaction time (KARADAG A. et al, 2009).

To determine the total polyphenol content, the Folin-Ciocâlteu method used gallic acid as a standard.

To evaluate the antioxidant activity, calibration curves were performed for each method using the standard Trolox solution (Figs. 4, 5, 6), and the results were expressed in μM Trolox / ml extract. A good linearity is found on a concentration range between 0 and 40 μM Trolox for the TEAC method, 2.5-0 mM Trolox for the CUPRAC method and 0.4-0 mM Trolox for DPPH, the correlation coefficient being: R2 = 0.9963 for ABTS, R2 = 0.9957 for the CUPRAC method and R2 = 0.9978 for the DPPH method.

Halliwell et al. (2007) defined antioxidants as "any substance that delays, prevents, or removes oxidant injury to a target molecule." In the same year Khlebnikov et al. (2007) defined antioxidants as any escaping substance that neutralizes ROS or acts indirectly to regulate antioxidant defense or inhibit ROS production. Another property that a compound considered antioxidant should have is the ability, after removal of the radical, to form a new radical that is stable by intramolecular hydrogen bonding for further oxidation. During human evolution, endogenous defenses gradually improved to maintain a balance between free radicals and oxidative stress. Antioxidant activity can be effective in different ways: as inhibitors of free radical oxidation reactions (preventive oxidants) by inhibiting the formation of free lipid radicals; by stopping the propagation of the reaction in the self-oxidation chain; as singlet oxygen neutralizing agents; through synergy with other antioxidants; as reducing agents that convert hydroperoxides into stable compounds; as metal chelators that transform metal pro-oxidants (iron and copper derivatives) into stable products; and finally as inhibitors of pro-oxidative enzymes (CAROCHO M. et al, 2013).

The human antioxidant system is divided into two major groups, enzyme antioxidants and non-enzyme oxidants.

As for enzyme antioxidants, they are divided into primary and secondary enzyme defense. The primary defense is composed of three enzymes that prevent the formation or neutralization of free radicals: glutathione peroxidase, which yields two electrons to reduce peroxides by the formation of selenols; catalase, which converts hydrogen peroxide into water and molecular oxygen and has one of the highest rates of rotation known to man, allowing only one catalase molecule to convert 6 billion hydrogen peroxide molecules; and finally, superoxide dismutase converts superoxide anions into hydrogen peroxide as a reduction for catalase (RAHMAN, 2007). Enzyme secondary defense includes glutathione reductase and glucose-6-phosphate dehydrogenase. Glutation reductase reduces glutathione (antioxidant) from its oxidized form to its reduced form, recycling it to continue neutralizing more free radicals. Glucose-6-phosphate regenerates NADPH (coenzyme used in anabolic reactions), creating a reducing environment. These two enzymes do not directly neutralize free radicals, but have supporting roles for other endogenous antioxidants (CAROCHO M.et al, 2013).

Exogenous antioxidants are mainly derived from medicinal and food plants, such as fruits, vegetables, cereals, mushrooms, beverages, flowers, spices and traditional herbs. In addition, industries that process agricultural by-products are also potential important sources of natural antioxidants. The main classes of compounds with antioxidant action are mainly polyphenols (phenolic acids, flavonoids, anthocyanins and lignans), carotenoids (xanthophylls and carotene) and vitamins (vitamins E and C). In general, these natural antioxidants, especially polyphenols and carotenoids, exhibit a wide range of biological effects, such as anti-inflammatory, antibacterial, antiviral, anti-aging and anticancer (XU D.P. et al, 2017).

In this context, a series of aqueous extracts were studied, noting that the extract with the best antioxidant activity is the one obtained from *V. myrtillus* by the methods CUPRAC, ABTS and DPPH, probably due to the high content of phenolic compounds and implicitly by the anthocyanins content. The results obtained for the antioxidant activity by different methods are presented in Table 2.

Table. 2. Antioxidant activity of the extracts taken and the total content of phenolic compounds

Sample	Total phenolic content (mg gallic acid/ ml of extract)	TEAC (µM Trolox/ ml of extract) ± DS	DPPH (µM Trolox/ ml of extract) ± DS	CUPRAC (µM Trolox/ ml of extract) ± DS
R. canina	97.91 ± 0.44	783.64 ± 27.45	1188.12 ± 19.94	2106.44 ± 9.7
H. rhamnoides	54.27 ± 0.50	600 ± 29.39	916.85 ± 19.02	2084.03 ± 68.78
E. angustifolia	33.05 ± 0.25	390.91 ± 17.28	453.39 ± 13.75	1425.77±27.01
V. myrtillus	64.14 ± 0.34	837.58 ± 16.89	6752.98 ± 25.78	4268.91±29.11

In vitro cytotoxicity assessment of standardized plant extracts

The assessment of toxicity of any given substance that is intended to be exposed to living organisms relies on *in vitro* evaluation of dose response. (TIHAUAN, 2020) The dose–response relationship, or exposure–response relationship, describes the magnitude of the response of an organism, as a function of exposure (or doses) to a stimulus or stressor (usually a chemical) after a certain exposure time.

The toxicity curve (Fig. 2) obtained for the lyophilized plant extracts, such as rosehip (*Rosa canina)*, sea buckthorn (*Hippophae rhamnoides)*, echinacea (*Echinacea angustifolia)* and bilberries (*Vaccinium myrtillus)*, shows that the concentration are indirectly proportional with the percentage of viable cells.

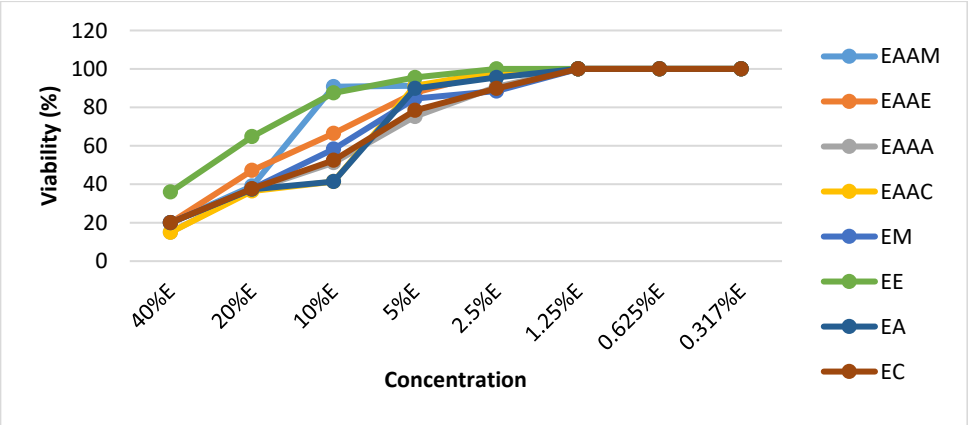

Figure 2. Assessment of cell viability in the presence of plant extracts at different concentrations

Using the direct counting method, samples stained with fluorescein diacetate (FDA) – excitation at 488 nm, were observed in fluorescent microscopy. Cells that appear green in fluorescent light are considered viable cells (Fig. 3). Samples stained with propidium iodide (PI) – excitation at 546 nm, were observed in fluorescence microscopy. The cells that appear red under fluorescent light are considered non-viable (dead) cells".

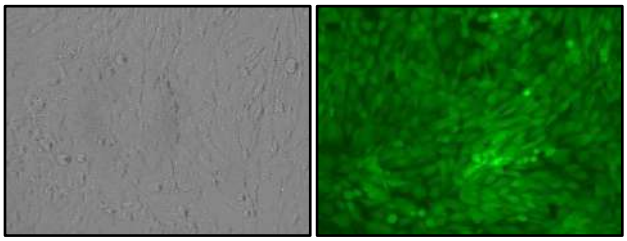

Figure 3. Viable cells MG63 (200x)

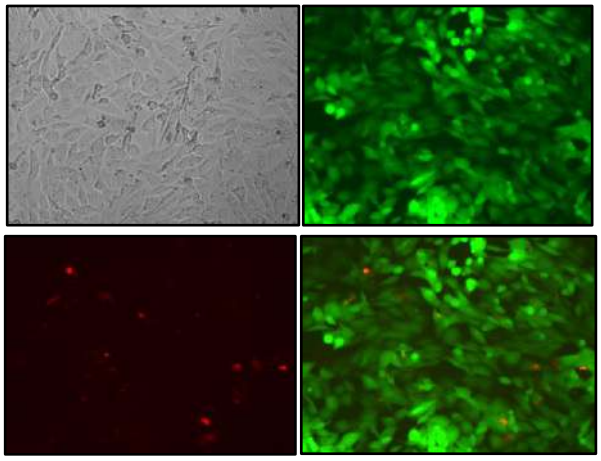

Figure 4. The appearance of cells in the presence of Rosehip extract – conc. 10% aqueous extract – 200x

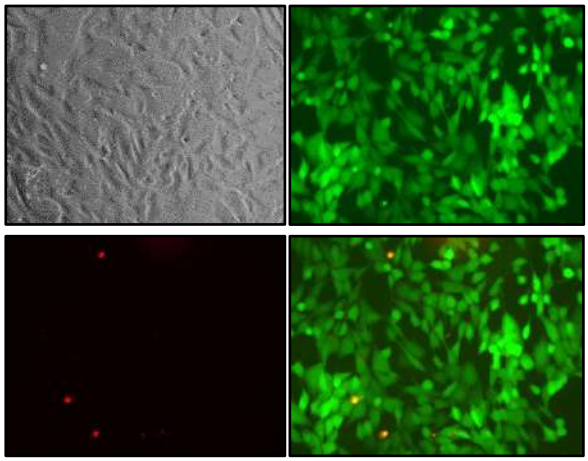

Figure 5. Appearance of cells in the presence of Echinacea extract – conc. 10% aqueous extract – 200x

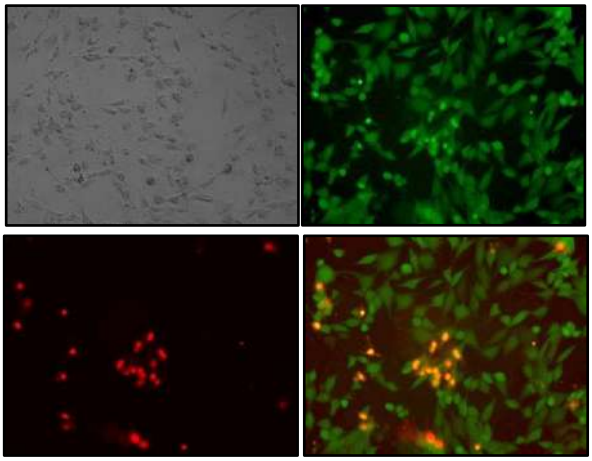

Figure 6. Appearance of cells in the presence of Sea buckthorn extract – conc. 10% aqueous extract – 200x;

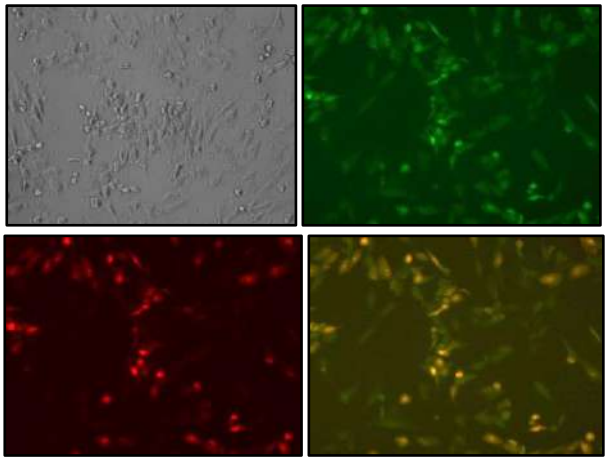

Figure 7. Appearance of cells in the presence of Bilberries extract – conc. 10% aqueous extract – 200x

As seen in Fig. 4, for rosehip extract and in figure 5 for the echinacea extract, the percentage of viable cells at a 10% concentration is considerably high, as for the sea buckthorn extract (Fig. 6) and the bilberries extract (Fig. 7) the viability percentage decreases, probably due to the accumulation of anthocyanins or tannins.

Evaluation of the nutritional status and physico-chemical properties

The sea buckthorn samples proved to be quite rich in minerals such as Calcium (22,646 mg/100g) and Copper (0,472 mg/100g), total lipids (22,4%) and glucids (58,0%), having a high energetic value of 484 kcal/100g, while bilberries samples (Table 3) showed a diminished protein content (3,77%), total lipids (4,82%) and micronutrients (1,5% ash, 3 times less Calcium than in buckthorn fruits).

Rosehip samples had a low content of lipids (3, 41%) and proteins (5, 25%), but were very rich in glucids (82, 51%), registering a remarkable energetic value (382 kcal/100g). As compared to all other samples, rosehips seemed to be the most balanced sample in terms of mineral content, being rich in Calcium (36, 05 mg/100g) and having 10 times more Manganese than all other tested samples (1,143 mg/100g).

Table 3. Nutritional content of analyzed samples

Samples	Total proteins %	Total Lipids %	Total glucids %	Energetic value Kcal/100g	Ash %
Sea buckthorn	12,7	22,4	58,0	484	2,24
Blueberries	4,82	1,97	70,0	317	1,5
Echinacea	12,56	0,84	57,4	287	21,62
Rosehips	5,25	3,41	82,51	382	2,50

Surprisingly, the echinacea extract had the poorest content of macronutrients (0, 84% lipids; 2, 01% proteins), low mineral values, except for Zinc (Table 4), but a very high content of total ash (21, 62%) which could not be correlated with none of the analyzed minerals. This could indicate a high level of inorganic impurity of tested samples.

Table 4. Mineral content of analyzed samples

Samples	Mineral content (mg/100g)				
	Cu	Zn	Mn	Fe	Ca
Sea buckthorn	0,472	0,464	0,007	1,66	22,646
Blueberries	0,098	0,289	0,018	0,42	7,017
Echinacea	0,093	1,534	0,007	0,27	10,461
Rosehips	0,102	0,455	1,143	1,41	36,056

Regarding the safety assessment, none of the samples was microbiologically contaminated. The analyzed dried fruits and plant extracts showed no bacterial alteration, absence of pathogenic bacteria, molds and yeasts, all the samples complied at this point with food safety requirements.

Conclusions

Of the tested aqueous extracts, a higher phenolic content was obtained for the extract obtained from *R. canina*. By testing the antioxidant activity it was concluded that the best activity by all the methods was obtained for the extract of *V. myrtillus* even though it has a lower concentration of phenolic compounds. The three methods correlate as a trend but the values are very different. The higher values obtained by the CUPRAC method may be due to the concomitant measurement of both hydrophilic and lipophilic antioxidants.

As for the cytotoxicity assays, an increased percentage of dead cells was observed in the case of aqueous extracts of sea buckhorn and blueberries. The obtained result may be due to the accumulation of anthocyanins or tannins. Therefore, it requires an additional step for eliminating its seemingly negative effects, or using lower concentrations in the final product.

The nutritional assays showed the most valuable samples from a nutritional point of view were sea buckthorn and rosehips, while the most balanced samples in terms of mineral content were rosehips, which had high levels of Calcium (36, 05 mg/100g) and ten times more Manganese than all other tested samples (1,143 mg/100g), being rich in glucids (82, 51%), which ensured a high energetic value (382 kcal/100g).

Acknowledgments

This work was supported by a European funding programme POC-A1-A1.2.3-G-2015, ID_40_404, MySMIS code 105509, "*Exploiting expertise in agro-food research by transferring knowledge to the private environment in order to obtain safe and nutritionally optimized food products*" acronym EXPERTAL, Financing Agreement no. 57 / 05.09.20170, Subsidiary Contract Type D no. 22 of 10.10. 2017 IBA – Sanimed International Impex SRL and by the Operational Programme Human Capital of the Ministry of European Funds through the Financial Agreement 51668/09.07.2019, SMIS code 124705.

Conflict of Interest

The authors have no conflict of interest to declare.

References

1. ALBERS, R., BOURDET-SICARD, R., BRAUN, D., CALDER, P.C., HERZ, U., LAMBERT, C., … SACK, U. "Monitoring immune modulation by nutrition in the general population: Identifying and substantiating effects on human health." *British Journal of Nutrition*. 2013, 110 (SUPPL. 2). https://doi.org/10.1017/S0007114513001505
2. BHAVE, A., SCHULZOVA, V., CHMELAROVA, H., MRNKA, L., & HAJSLOVA, J. "Assessment of rosehips based on the content of their biologically active compounds." *Journal of Food and Drug Analysis*. 2017, 25(3), 681-690. https://doi.org/10.1016/j.jfda.2016.12.019
3. CHU, W., CHEUNG, S.C. M., LAU, R.A.W., & BENZIE, I.F.F. "Bilberry (Vaccinium myrtillus L.).". 2011.

4. DHAR, P., TAYADE, A.B., KUMAR, J., CHAURASIA, O.P., SRIVASTAVA, R.B., & SINGH, S.B. "Nutritional profile of phytococktail from trans-Himalayan plants." *PLoS ONE*. 2013, 8(12). https://doi.org/10.1371/journal.pone.0083008
5. GEETHA, S., SAI RAM, M., SINGH, V., ILAVAZHAGAN, G., & SAWHNEY, R.C. "Anti-oxidant and immunomodulatory properties of seabuckthorn (Hippophae rhamnoides) – An in vitro study." *Journal of Ethnopharmacology*. 2002, 79(3), 373-378. https://doi.org/10.1016/S0378-8741(01)00406-8
6. GEETHA, S., SINGH, V., RAM, M. S., ILAVAZHAGAN, G., BANERJEE, P.K., & SAWHNEY, R.C. "Immunomodulatory effects of seabuckthorn (Hippophae rhamnoides L.) against chromium (VI) induced immunosuppression." *Molecular and Cellular Biochemistry*. 2005, 278(1-2), 101-109. https://doi.org/10.1007/s11010-005-7095-9
7. KOLEHMAINEN, M., MYKKÄNEN, O., KIRJAVAINEN, P.V., LEPPÄNEN, T., MOILANEN, E., ADRIAENS, M., … TÖRRÖNEN, R. "Bilberries reduce low-grade inflammation in individuals with features of metabolic syndrome." *Molecular Nutrition and Food Research*. 2012, 56(10), 1501-1510. https://doi.org/10.1002/mnfr.201200195
8. LEWICKA, A., SZYMAŃSKI, Ł., RUSIECKA, K., KUCZA, A., JAKUBCZYK, A., ZDANOWSKI, R., & LEWICKI, S. "Supplementation of Plants with Immunomodulatory Properties during Pregnancy and Lactation–Maternal and Offspring Health Effects." *Nutrients*. 2019, 11(8), 1958. https://doi.org/10.3390/nu11081958
9. MOŽE, Š., POLAK, T., GAŠPERLIN, L., KORON, D., VANZO, A., POKLAR ULRIH, N., & ABRAM, V. "Phenolics in slovenian bilberries (Vaccinium myrtillus L.) and blueberries (Vaccinium corymbosum L.)." *Journal of Agricultural and Food Chemistry*. 2011, 59(13), 6998-7004. https://doi.org/10.1021/jf200765n
10. PATEL, S. (, August). "Rose hips as complementary and alternative medicine: Overview of the present status and prospects." *Mediterranean Journal of Nutrition and Metabolism*. 2013. https://doi.org/10.1007/s12349-012-0118-7
11. SAABY, L., MOESBY, L., HANSEN, E.W., & CHRISTENSEN, S.B. "Isolation of immunomodulatory triterpene acids from a standardized rose hip powder (Rosa canina L.)." *Phytotherapy Research*. 2011, 25(2), 195-201. https://doi.org/10.1002/ptr.3241
12. SELAHVARZIAN, A., ALIZADEH, A., BAHARVAND, P.A., & ELDAHSHAN, O.A. "Medicinal Properties of Rosa canina L. Review Article Medicinal Properties of Rosa canina L.". 2018, (June). https://doi.org/10.22087/hmj.v0i0.615
13. SHI, D., XU, M., REN, M., PAN, E., LUO, C., ZHANG, W., & TANG, Q. "Immunomodulatory Effect of Flavonoids of Blueberry (Vaccinium corymbosum L.) Leaves via the NF- κ B Signal Pathway in LPS-Stimulated RAW 264.7 Cells." *Journal of Immunology Research*. 2017, 2017. https://doi.org/10.1155/2017/5476903
14. WICHERS, H. (September). "Immunomodulation by food: Promising concept for mitigating allergic disease?" *Analytical and Bioanalytical Chemistry*. 2009. https://doi.org/10.1007/s00216-009-2838-1
15. RE R., PELLEGRINI N., PROTEGGENTE A., PANNALA A., YANG M., RICE-EVANS C., 1999. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay, *Free Radical Biology & Medicine*, 26(9/10): 1231-1237.
16. THAIPONG K., BOONPRAKOB U., CROSBY K., CISNETOS-ZEVALLOS L., BYRNE D.H., 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts, *Journal of Food Composition and Analysis*, 19:669-675.
17. KARASAKAL A., 2015. Evaluation of Antioxidant activities of Brassica napus's seeds by CUPRAC, ABTS/ Persulphate and DMPD methods, *Marmara Pharmaceutical Journal* 19: 153-158.
18. APAK R., ÇEKIÇ D.S., AYŞEM U., ÇELIK S.E.K., MUSTAFA B. & BEKDEŞER B., ZIYA C., SENER S., ÖNEM A.N., ERÇAĞ E. 2018. Novel Spectroscopic and Electrochemical Sensors and Nanoprobes for the Characterization of Food and Biological Antioxidants. *Sensors*. 18(1): 186.
19. MENG J., FANG Y., ZHANG A., CHEN S., XU T., REN Z., HAN G., LIU J., LI H., ZHANG Z., WANG H., 2011. Phenolic content and antioxidant capacity of Chinese raisins produced in Xinjiang Province, *Food Research International* 44: 2830-2836.
20. MADHU K., 2013. Phytochemical Screening And Antioxidant Activity Of In Vitro Grown Plants Clitoria Ternatea L., Using Dpph Assay, *Asian Journal of Pharmaceutical and Clinical Research*, 6(2):38-42.
21. VAYA J., BELINKY P.A., AVIRAM M., 1997. Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation, *Free Radic Biol Med.*, 23(2): 302-313.
22. SENGUL M., YILDIZ H., GUNGOR N., CETIN B., ESER Z., ERCISLI S., 2009. Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants, Pak J Pharm Sci., 22(1):102-106.
23. PUERTOLLANO M.A., PUERTOLLANO E., DE CIENFUEGOS G.Á., DE PABLO M.A., 2011. Dietary antioxidants: immunity and host defense, *Curr Top Med Chem.*, 11(14):1752-1766.
24. BADARINATH, A.V., RAO, K.M., CHETTY, C.M.S., RAMKANTH, V., RAJAN, T.V.S., GNANAPRAKASH, K., 2010. A review on in-vitro antioxidant methods: comparisons, correlations and considerations. *Int. J. PharmTech Res*. 2 (2), 1276-1285.
25. KARADAG A., OZCELIK B., SANER S., 2009. Review of Methods to Determine Antioxidant Capacities, *Food Anal. Methods*, 2:41-60.
26. ALAM N., BRISTI N.J., RAFIQUZZAMAN, 2013. Review on in vivo and in vitro methods evaluation of antioxidant activity, *Saudi Pharmaceutical Journal* 21: 143-152.
27. HALLIWELL, B., 2007. Biochemistry of oxidative stress. *Biochem. Soc. Trans*. 35, 1147-1150.
28. KHLEBNIKOV, A.I., SCHEPETKIN, I.A., DOMINA, N.G., KIRPOTINA, L.N., QUINN, M.T., 2007. Improved quantitative structure–activity relationship models to predict antioxidant activity of flavonoids in chemical,

enzymatic, and cellular systems. *Bioorg. Med. Chem*. 15, 1749-1770.
29. CAROCHO M., FERREIRA I.C.F.R., 2013. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives, *Food and Chemical Toxicology*, 51:15-25.
30. RAHMAN, K., 2007. Studies on free radicals, antioxidants and co-factors. *Clin. Inverv. Aging* 2, 219-236.
31. CAROCHO M., FERREIRA I.C.F.R., 2013. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives, *Food and Chemical Toxicology*, 51:15-25.
32. XU D.P., LI Y., MENG X., ZHOU T., ZHOU Y., ZHENG J., ZHANG J.J., LI H.B., 2017. Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources, *Int J Mol Sci*., 18(1): 96.
33. ALBERS, R., BOURDET-SICARD, R., BRAUN, D., CALDER, P. C., HERZ, U., LAMBERT, C., … SACK, U. "Monitoring immune modulation by nutrition in the general population: Identifying and substantiating effects on human health." *British Journal of Nutrition*. 2013, 110 (SUPPL. 2). https://doi.org/10.1017/S0007114513001505
34. BHAVE, A., SCHULZOVA, V., CHMELAROVA, H., MRNKA, L., & HAJSLOVA, J. "Assessment of rosehips based on the content of their biologically active compounds." *Journal of Food and Drug Analysis*. 2017, 25(3), 681-690. https://doi.org/10.1016/j.jfda.2016.12.019
35. CHU, W., CHEUNG, S.C.M., LAU, R.A.W., & BENZIE, I.F.F. "Bilberry (Vaccinium myrtillus L.).". 2011.
36. DHAR, P., TAYADE, A.B., KUMAR, J., CHAURASIA, O.P., SRIVASTAVA, R.B., & SINGH, S. B. "Nutritional profile of phytococktail from trans-Himalayan plants." *PLoS ONE*. 2013, 8(12). https://doi.org/10.1371/journal.pone.0083008
37. GEETHA, S., SAI RAM, M., SINGH, V., ILAVAZHAGAN, G., & SAWHNEY, R.C. "Anti-oxidant and immunomodulatory properties of seabuckthorn (Hippophae rhamnoides) – An in vitro study." *Journal of Ethnopharmacology*. 2002, 79(3), 373-378. https://doi.org/10.1016/S0378-8741(01)00406-8
38. GEETHA, S., SINGH, V., RAM, M.S., ILAVAZHAGAN, G., BANERJEE, P.K., & SAWHNEY, R.C. "Immunomodulatory effects of seabuckthorn (Hippophae rhamnoides L.) against chromium (VI) induced immunosuppression." *Molecular and Cellular Biochemistry*. 2005, 278(1-2), 101-109. https://doi.org/10.1007/s11010-005-7095-9
39. KOLEHMAINEN, M., MYKKÄNEN, O., KIRJAVAINEN, P.V., LEPPÄNEN, T., MOILANEN, E., ADRIAENS, M., … TÖRRÖNEN, R. "Bilberries reduce low-grade inflammation in individuals with features of metabolic syndrome." *Molecular Nutrition and Food Research*. 2012, 56(10), 1501-1510. https://doi.org/10.1002/mnfr.201200195
40. LEWICKA, A., SZYMAŃSKI, Ł., RUSIECKA, K., KUCZA, A., JAKUBCZYK, A., ZDANOWSKI, R., & LEWICKI, S. "Supplementation of Plants with Immunomodulatory Properties during Pregnancy and Lactation–Maternal and Offspring Health Effects." *Nutrients*. 2019, 11(8), 1958. https://doi.org/10.3390/nu11081958
41. MOŽE, Š., POLAK, T., GAŠPERLIN, L., KORON, D., VANZO, A., POKLAR ULRIH, N., & ABRAM, V. "Phenolics in slovenian bilberries (Vaccinium myrtillus L.) and blueberries (Vaccinium corymbosum L.)." *Journal of Agricultural and Food Chemistry*. 2011, 59(13), 6998-7004. https://doi.org/10.1021/jf200765n
42. PATEL, S. (August). "Rose hips as complementary and alternative medicine: Overview of the present status and prospects." *Mediterranean Journal of Nutrition and Metabolism*. 2013. https://doi.org/10.1007/s12349-012-0118-7
43. SAABY, L., MOESBY, L., HANSEN, E.W., & CHRISTENSEN, S.B. "Isolation of immunomodulatory triterpene acids from a standardized rose hip powder (Rosa canina L.)." *Phytotherapy Research*. 2011, 25(2), 195–201. https://doi.org/10.1002/ptr.3241
44. SELAHVARZIAN, A., ALIZADEH, A., BAHARVAND, P.A., & ELDAHSHAN, O.A. "Medicinal Properties of Rosa canina L. *Review Article Medicinal Properties of Rosa canina L*.". 2018, (June). https://doi.org/10.22087/hmj.v0i0.615
45. SHI, D., XU, M., REN, M., PAN, E., LUO, C., ZHANG, W., & TANG, Q. "Immunomodulatory Effect of Flavonoids of Blueberry (Vaccinium corymbosum L.) Leaves via the NF- κ B Signal Pathway in LPS-Stimulated RAW 264.7 Cells." *Journal of Immunology Research*. 2017, 2017. https://doi.org/10.1155/2017/5476903
46. WICHERS, H. (September). "Immunomodulation by food: Promising concept for mitigating allergic disease?" *Analytical and Bioanalytical Chemistry*. 2009. https://doi.org/10.1007/s00216-009-2838-1
47. TIHĂUAN B-M, BERCA LM, ADASCALULUI M, SANMARTIN AM, NICA S, CIMPONERIU D, DUȚĂ D. Experimental in vitro cytotoxicity evaluation of plant bioactive compounds and phytoagents: a review. *Rom Biotechnol Lett*. 2020; 25(4): 1832-1842. DOI: 10.25083/rbl/25.4/1832.1842