Antioxidant Activity of some Nutraceuticals Based on Romanian Black and Red Fruits Mixed Extracts

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Abstract

With content rich in polyphenols, carotenoids vitamins, minerals and many other bioactive compounds, fruits extracts from Morus nigra L. – black mulberry (blackberry) from Moraceae family, Cerasus avium (syn. Prunus avium) Moench. - bitter cherry from Rosaceae family, Cornus mas L. – cornelian cherry from Cornaceae family, may be useful as a supplementary treatment and especially in preventing many diseases which arise from action of oxidative stress. The aim of this paper is to obtain selected syrups with an increased antioxidant activity based on a mixture of fluids extracts from Morus nigra L. , Cerasus avium (syn. Prunus avium) Moench. and Cornus mas L. indigenous Romanian fruits. Fluid extracts were obtained using different extraction methods (maceration, reflux) and solvents (ethylic alcohol 40% and 70%). The obtained fluid extracts were mixed in different molar ratio and analysed for their physical-chemical properties, total polyphenols assay (Folin–Ciocalteu assay) and total antioxidative capacity by photochemiluminescence method (ACL, Analytik Jena AG procedure). The selected mixed fluid extracts with greatest content of polyphenols and antioxidant activity were used for syrups obtaining. The new obtained syrups were analysed for their physical-chemical properties (appearance, pH and relative density), polyphenols content and total antioxidative capacity. Preliminary results emphasize that syrups with highest antioxidant activity correlated with the polyphenols content would represent a possible new stimulating nutraceuticals that could be used in oxidative stress associated to different diseases.

Keywords: *nutraceutical, black and red fruits, antioxidant activity*

1. Introduction

Numerous studies have proven that vegetal compounds originated from plants or fruits possess a high spectrum of biological activity. However, polyphenolic extracts (e. g. flavonoids, anthocyanin, tannins etc.), despite having excellent *in vitro* bioactivity, demonstrate less or no *in vivo* actions due to their improper molecular size, resulting in a poor absorption and bioavailability. Moreover, the efficacy of natural polyphenols depends on preserving the stability, bioactivity and bioavailability of the active compounds. Up to now, the development of pharmaceutical formulations has remained restricted to individual chemical drugs, even the properties obtained by using an optimal mixture of bio-active compounds could be strongly influenced by synergism appeared in the system [1 – 5]. With content rich in vitamins, minerals, polyphenols, carotenoids and many other compounds, extracts from *Morus nigra* L. , *Cerasus avium* (syn. *Prunus avium*) Moench. and *Cornus mas* L. fruits, may be useful as an adjunct in the treatment and especially in preventing many diseases which arise from action of oxidative stress. *Morus nigra* L. , *Cerasus avium* (syn. *Prunus avium*) Moench. and *Cornus mas* L. fruits, as different vegetable products are often used in folk medicine most due to their content in polyphenolic acids with great antioxidative capacity mainly useful in anti – inflammatory diseases, hypoglycemic activities, tonic action due to their synergistic action [6 -8].

Cerasus avium (Moench.), bitter cherry, contains a variety of active principles, flavonoids, saponosids, carotenoids, terpinoids, volatils oils, with different farmacological actions - healing, emollient, antiinflamatory, antibacterial. The *Cerasus avium* fruits contain fibers, water, antocyanins, vitamins (nicotinamide, pantotenic acid, piridoxine, riboflavine, tiamine, small amounts of biotine, folic acid, C, E vitamin, caroten, minerals, oligoelements (zinc copper, manganese, cobalt), fluorine, glucids pectins, proteins, lipids and tannins [9, 10]. Organic acids are represented

by malic acid, citric acid, clorogenic acid, ferrulic acid, caffeic acid, p-cumaric acid and oxalic acid [10]. Due to its flavonoid, C and E vitamins, oligoelements content, known for their antioxidant activity, the raw material expressed as *Cerasus avium* fruits will be studied to establish its antioxidative capacity and therapeutically potential related to this [9, 11].

*Morus nigra (*L.), black mulberry, has been used in popular medicine as an analgesic, diuretic, antitussive, sedative, anxiolytic and hypotensive, in addition to its uses in the treatment of a variety of ailments, including inflammatory disorders. There are many studies involving the chemical composition and evaluation of biological and pharmacological properties of *Morus nigra*. This genus contains a variety of phenols compounds including flavonoids, and a variety of Diels-Alder adduct compounds. Recent investigations revealed that the fruits and leaves of mulberry plants contained many bioactive components, such as alkaloids, anthocyanins, and isoprenylated flavonoids stilbenes, 2-arylbenzopyrans, coumarins, chromones, xanthones [12]. Black mulberry fruits are rich in alkaloid components including 1-deoxynojirimycin, which is known as one of the most potent glycosidase inhibitors that decreases blood sugar levels [13, 14].

Cornus mas (L.), cornelian cherry, present a medium biomass which vary between 5 and 7g (depends on varieties), representing a significant source for food industry, because of active principles content, being used in juices production. The fresh fruits consumed in small quantities are recommended because of increased intake of minerals and active principles, tonic and refreshing action; the sugar content vary depending on varieties – the average is between 7,5% and 14%. If the fruits are consumed in big quantities, may occur some unpleasant adverse digestive, nausea, intestinal fermentation [15 - 18].

The aim of this paper is to obtain selected nutraceuticals type syrups with increased antioxidant activity based on a mixture of fluid extracts from *Morus nigra* L. , *Cerasus avium* (syn. *Prunus avium*) Moench. and *Cornus mas* L. fresh fruits, indigene species. Fluid extracts were obtained using two extraction methods, maceration and refluxing, in solvents (ethyl alcohol 40% and 70% concentrations) [19]. The obtained hidroalcoholic extracts were mixed in different ratio and analysed for their physico-chemical properties, polyphenols assay (Folin–Ciocalteu, HPLC assay) [20, 21] and antioxidative capacity by photochemiluminescence method (ACL, Analytik Jena AG procedure) [22, 23]. The obtained results emphasize that syrups with highest antioxidant activity correlated with the polyphenols content would represent a possible new nutritional supplements used in associated oxidative stress dysfunctions.

2. Material and Methods

2.1 Syrups obtaining and analyzing methods

For the fluid extracts obtaining were selected the follows raw material: *Cornus mas* L. , cornelian cherry fresh fruits (notate C), *Morus nigra* L. , black mulberry fresh fruits (notate M), *Cerasus avium* Moench. , bitter cherry fresh fruits (notate C).

The methods used for fluid extracts obtaining were *maceration in solvent (*fresh vegetal product: ethyl alcohol 40%, respectively fresh vegetal product: ethyl alcohol 70% in 1: 10 ratio), stir and allow soaking in the dark for 10 days in a constant temperature and stirred periodically and respectively *refluxing* in ethyl alcohol for 2h of fresh vegetal product. After 10 days, the extract was filtered, brought to a concentration of 10% with a 100 mL volumetric flask and stored at 4°C. Fluid vegetal extracts assay were *pH*, *relative density* and *total phenols compounds by Folin Ciocalteu method.* Syrups codification and preparation method based on CMC fluid extracts mixture is presented in Tables I – II:

Table I. Samples codification

Syrup Code	Content in C: M: C fluid extracts mixture / extract preparation method
S1	C: M: C (1: 1: 1) ratio / maceration in 40% alcohol
S2	C: M: C (2,5: 1: 1) ratio / maceration in 70% alcohol
S3	C: M: C (1: 1: 2,5) ratio / reflux in 40% alcohol
S4	C: M: C (1: 2,5: 1) ratio / reflux in 70 % alcohol
S5	C: M: C (1: 2,5: 1) ratio / maceration in 70% alcohol

Table II. Syrups formula based on CMC fluid extracts (S1 – S5)

Ingredients	Quantities (g) Syrups S1 - S4	Quantities (g) Syrup S5
Simple syrup 64%	55	-
Glycerol	15	-
Sodium carboxymethylcellulose	-	1
Saccharinummatricum	-	0. 2
C: M: C fluid extracts (1: 1: 1) ratio	30	5
C: M: C fluid extracts (2,5: 1: 1) ratio	30	-
C: M: C fluid extracts (1: 1: 2,5) ratio	30	-
C: M: C fluid extracts (1: 2,5: 1) ratio	30	-
C: M: C fluid extracts (1: 2,5: 1) ratio	-	30
Preservative solution ad.	-	100

The control tests for syrups based on CMC fluid extracts quality were: *appearance, pH, relative density, initial and after 30 days of obtaining.*

2.2 HPLC phenolic compounds assay from syrups based on CMC fluid extracts mixture

For HPLC analysis, the working solution based on syrups S1 – S5, were obtained from 0. 5 g sample diluted with 5 mL methyl alcohol. For the separation, identification and quantification of phenols compounds an adapted standardized HPLC method for total polyphenols determination described by USP 30-NF25 monograph was used.

Apparatus used: HPLC Agilent 1200 quaternary pump, DAD, thermostat, degassing system, autosampler.

Conditions: C18 type chromatographic column, 250 mm 4. 6 mm; 5 mm (Zorbax XDB or equivalent); Mobile phase: solution A - 0. 1% phosphoric acid, solution B - acetonitrile; Gradient elution is presented in Table III; Temperature: 35°C; Flow rate: 1. 5 mL / min; detection: UV - 310 nm; Injection volume: 20; Analysis time: 22 minutes; Reference substances (70% solution in methanol): E-resveratrol = 37 mg / mL, Z - resveratrol = 0. 22 mg / mL (obtained by the solution of trans-resveratrol from UV radiation λ = 254 nm for 12 h) acid, caffeic = 0. 36 mg / mL, chlorogenic acid = 0. 37 mg / mL, cinnamic acid = 0. 58 mg / mL, vanillin 0. 42 mg / mL = 0. 39 mg gallic acid / mL, ferulic acid = 0. 50 mg / mL, the reference substances were injected 6 times (20 µL).

Table III. HPLC - gradient elution

No.	Time, min.	Solution A, %	Solution B, %
	0-13	90	10
	13	78	22
	13	78	22
	14	60	40
	17	60	40
	17,5	90	10
	22	90	10

Retention times corresponding to reference substances are presented in Table IV. To simplify measurements worked their mixture.

Table IV. Retention time for phenolic compounds (reference substances)

No.	Phenolic compound	Retention time ± SD
1.	gallic acid	0,990 ± 0,025
2.	3 -methyl galic acid	2,606± 0,008
3.	chlorogenic acid	3,501 ± 0,015
4.	caffeic acid	4,598 ± 0,036
5.	vaniline	6,919 ± 0,051
6.	p-coumaric acid	7,187± 0,019
7.	ferulic acid	8,565± 0,058
8.	*E* - resveratrol	14,467 ± 0,017
9.	ellagic acid	15,303± 0,027
10.	*Z* - resveratrol	15,751 ± 0,058
11.	cinammic acid	15,867 ± 0,007

Identification and quantitative determination of the active principles of the test solution was performed by comparing chromatograms of standard mixture and the analyzed solution (Fig. 1).

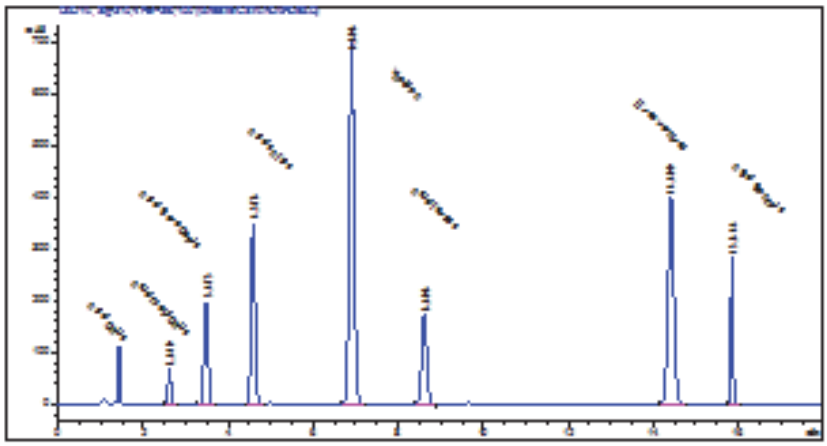

Fig. 1. HPLC chromatogram of the standards mixture

The reproducibility of the method was determined by the square of correlation coefficients (Table V).

Table V. Square of the correlation coefficient of the calibration curves

No.	Phenolic compound	r^2
1.	trans resveratrol	0. 99965
2.	cis resveratrol	0. 99729
3.	clhorogenic acid	0. 99999
4.	caffeic acid	0. 99619
5.	cinammic acid	0. 99845
6.	vaniline	0. 99691
7.	galic acid	0. 99537
8.	ferulic acid	0. 99863
9.	ellagic acid	0. 99885
10.	p - coumaric acid	0. 99798
11.	3 - methyl galic acid	0. 99563

2.3 Antioxidative activity by photochemiluminescence method

Apparatus used: photochemiluminometer Photochem, Analytik Jena AG, Germany.

Sample preparations for stock solution: 5 g of syrups S1 – S5 based on CMC fluid extracts were diluted with 5 mL methyl alcohol p. a. (Reagent 1 of Analytik Jena procedure). From each syrup stock solution, were taken 5 µL, respectively 10 µL working volume, according with Antioxidative Capacity in Lipid-soluble substances (ACL) procedure of Analytik Jena AG.

The total antioxidative capacity of the samples were quantified by comparison with the standard substance Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) vitamin E derivative and is given in Trolox equivalent antioxidant activity (TEAC). Due to the difficulties in measuring individual antioxidant components of a complex vegetal mixture, Trolox equivalency (nmol/sample) is used as a benchmark for the antioxidant capacity of such a mixture. Calibration curve of Trolox standard is presented in Fig. 2.

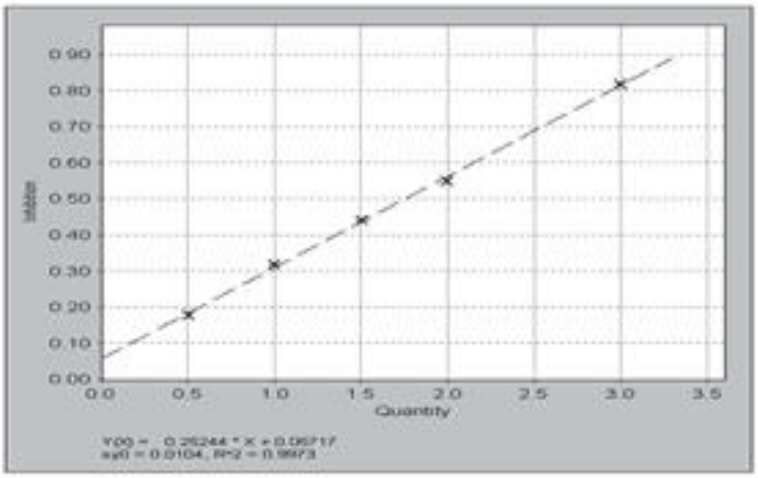

Fig. 2. Trolox standard calibration curve

3. Results And Discussions

The values of the determined parameters did not significantly modified, proving a good compatibility between the selected formula and CMC fluid extracts (Table VI).

HPLC assay results syrups S1 – S5 based on CMC fluid extracts are presented in figures 3 - 7 and polyphenolic compounds (PC) quantification is presented in Table VII.

Table VI. CMC fluid extracts quality control results

Fluid extract type	*p*H	Relative density	Folin Ciocalteu assay, mg/100 g vegetal product
C	4. 65 – 5. 0	0. 9423 – 1. 058	671. 23
M	5. 24 -5. 51	1. 011- 1. 0253	560. 68
C	5. 35 – 5. 69	1. 0023 – 1. 0321	384. 70

Table VII. Physical–chemical results for syrups based on CMC fluid extracts

Parameter	Syrups (S1 – S4)	Syrup S5
Appearance	Clear liquid, viscous, brownish color, sweet taste, characteristic smell	Clear liquid, slightly viscous, brown color, characteristic smell
*p*H	5. 6 – 6. 2	5. 5 – 6. 00
Relative density	1. 2443 – 1. 259	1. 037

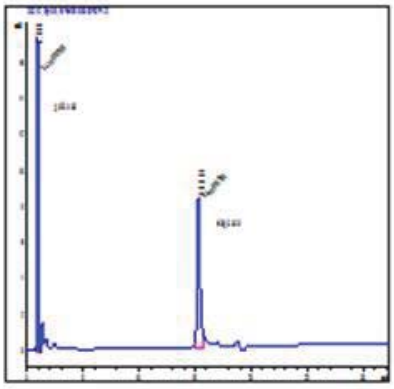

Fig. 3. Syrup 1 - HPLC analysis

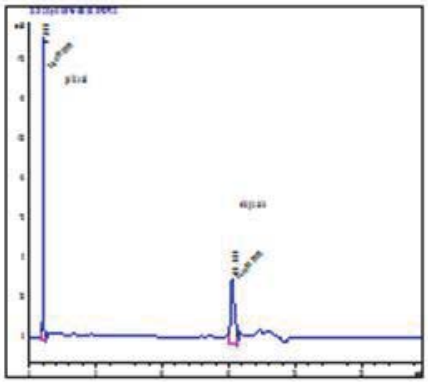

Fig. 4. Syrup 2 - HPLC analysis

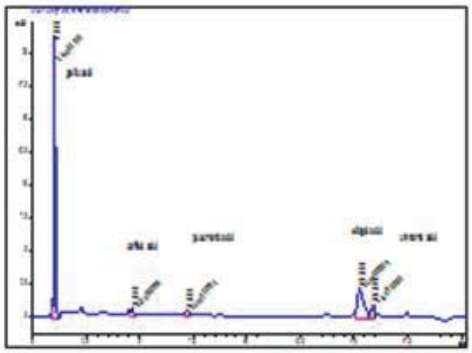

Fig. 5. Syrup 3 - HPLC analysis

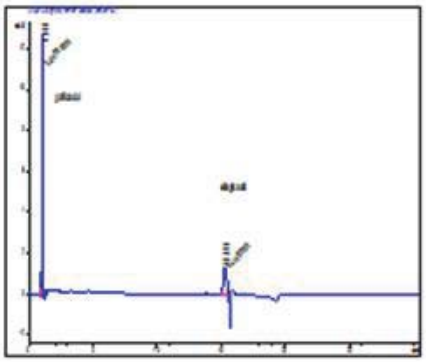

Fig. 6. Syrup 4 - HPLC analysis

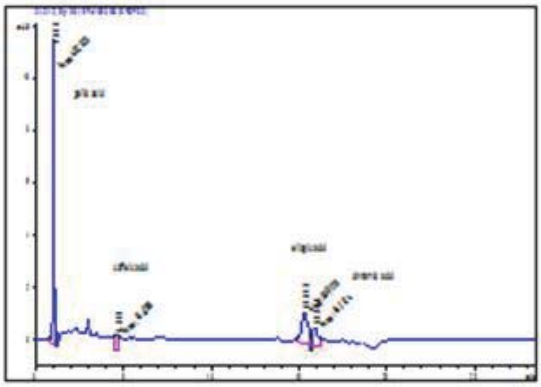

Fig. 7. Syrup 5 - HPLC analysis

Table VIII. Polyphenols compounds of syrups S1 – S5 by HPLC quantification [mg/ 100 g vegetal product] ± SD

Syrup type	Caffeic acid / SD		p-coumaric acid / SD		Cinnamic acid / SD		3-methyl gallic acid / SD		chlorogenic acid / SD		Gallic acid / SD		Ellagic acid / SD	
S1	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	51. 4857	1. 1850	177. 9698	1. 6066
S2	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	0. 0000	19. 6796	1. 2566	97. 3875	1. 5039
S3	0. 1555	0. 0040	0. 2430	0. 0023	4. 5555	0. 5388	0. 0000	0. 0000	0. 0000	0. 0000	23. 3022	1. 6880	51. 6166	1. 1110
S4	0. 0000	0. 0000	0. 0000	0. 0000	2. 7460	0. 5916	0. 0000	0. 0000	0. 0000	0. 0000	12. 0663	1. 6357	17. 6518	1. 1035
S5	0. 2691	0. 0145	0. 0000	0. 0000	7. 2961	0. 2238	0. 0000	0. 0000	0. 0000	0. 0000	13. 1290	1. 6431	34. 8489	0. 1437

The obtained results (Table VIII) emphasize the presence of the follows polyphenols compounds:

- caffeic acid present in S3 - S5
- p-coumaric acid present in S3
- cinammic acid present in S3 – S5
- gallic acid present in S1 - S5
- ellagic acid present in S1 – S5

In all syrups, gallic acid and ellagic acids were present in high concentration; small amounts of other acids were quantified.

3.1 Total antioxidative capacity results for syrups S1 - S5 based on CMC fluid extracts mixture

The obtained results regarding the total antioxidative capacity of CMC fluid extracts separately and syrups S1– S5 based on CMC fluid extracts mixture (stock solutions), are presented in Table IX, as average of two registered values for each sample.

Table IX. Total antioxidative capacity for CMC fluid extracts and syrups S1– S5 based on CMC mixture

No.	Sample type	Working volume sample (µL)	Inhibition Max. value	Quantity (TEAC) (nmol equiv. Trolox/ volume sample)
1	cornelian cherry extract (C)	5	0. 643	3. 729
2	black mulberry extract (M)	5	0,506	2,732
3	bitter cherry extract (C)	5	0,629	3,415
4	Syrup 1	5	0,161	0,650
5	Syrup 1	10	0,430	3,270
6	Syrup 2	5	0,331	1,898
7	Syrup 2	10	0,435	3,372
8	Syrup 3	5	0,297	1,573
9	Syrup 3	10	0,489	4,628
10	Syrup 4	5	0,443	3,518
11	Syrup 4	10	0,547	6,501
12	Syrup 5	5	0,503	5,064
13	Syrup 5	10	0,504	5,823

- at working volume sample (5 µL) according to the procedure, it was observed a low TEAC for all the analyzed samples, with values between 0,650 – 5. 064 nmol equiv. Trolox / volume sample.
- the most decreased total antioxidative capacity for sample Syrup 1 (0,650 nmol equiv. Trolox / volume sample), was registered.
- at working solution volume (10 µL) according to the procedure, it was observed a high TEAC for all the analyzed samples, between 3,270 – 6,501 nmol equiv. Trolox / volume sample.
- at working solution volumes 5 and 10 µL according to the procedure total antioxidative capacity, for Syrup 5, very close values were registered (5. 064 nmol equiv. Trolox / 5 µL volume sample and 5. 823 nmol equiv. Trolox / 10 µL volume sample).
- the most increased total antioxidative capacity for sample Syrup 4 (6,501 nmol equiv. Trolox / 10 µL volume sample), was registered.

4. Conclusions

- the new phytopharmaceuticals type syrups based on CMC fluid extracts after obtaining, did not have quality parameters modifications during 30 days of observation;
- syrups based on CMC fluid extracts contain most of the important polyphenols compounds, especially gallic acid and ellagic acids present in all analyzed samples;
- the vegetal fluid extracts present a decreased antioxidant activity compared with the one of the new syrups at the same working volume;
- the increased total antioxidative capacity for Syrup 4 (6,501 nmol equiv. Trolox / 10 µL volume sample) and Syrup 5 (5. 823 nmol equiv. Trolox / 10 µL volume sample) was registered;
- CMC fluids extracts used for obtaining Syrup 4 and Syrup 5 were extracted under maceration and refluxing with alcohol 70%, mixed in 1: 2,5: 1 CMC ratio;
- for Syrup 5, a syrup based on sodium carboxymethylcellulose, the big value for total antioxidative capacity is correlated with a rich polyphenolic content (caffeic, cinammic, gallic and ellagic acids), in this case, nature of base used for syrups obtaining (sucrose, sodium carboxymethylcellulose) interfered with antioxidative capacity;
- the new obtained syrups in variants S4 and S5, could be used for their antioxidative potential as a possible new nutritional supplements in associated oxidative stress dysfunctions.

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