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Review Paper

Antioxidant Activity of Dihydroxyfumaric Acid and its Derivatives: an Analysis by the $ABTS^{+\cdot}$ Decolorization Assay

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Abstract: Dihydroxyfumaric acid has a chemical structure very similar to that of ascorbic acid. Due to this fact a range of derivatives were synthesized and their antioxidant activity was assayed using the $ABTS^{\bullet+}$ decolorization assay. It was shown that the most powerful $ABTS^{\bullet+}$ scavenger is compound **1** – dihydroxyfumaric acid, followed by its dimethyl ester - compound **4** and the anilide - compound **5**. The latter compounds are followed by mono- and bis- benzimidazole derivatives of dihydroxyfumaric acid - compounds **10** and **11** which show the similar scavenging activity. These results are quite significant, knowing the fact that the utilization of dihydroxyfumaric acid in chemical, pharmaceutical and cosmetic applications is limited by its ability to prone to decarboxylation in acidic media. Thus, new derivatives having the active antioxidant structure and stabilized by side chains open new perspectives from the practical standpoint.

The role of dihydroxyfumaric acid in biological systems

The role of many hydroxy acids, such as maleic, lactic, glycolic, citric, tartaric, in living organisms and plant metabolism is generally recognized [1]. The interest in plant metabolites as sources of antioxidants appeared a long time ago. One of the leaders, due to its potential, in the series of natural reductons is the dihydroxyfumaric acid **1** [2].

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For the first time, it was chemically obtained in 1894 by Fenton, as a product of tartaric acid oxidation by hydrogen peroxide, in the presence of Fe(II) [3,4]. Dihydroxyfumaric acid is present in nature as trans- and cis- isomers. The trans- isomer is called the dihydroxyfumaric acid, and the cis- isomer is called the dihydroxymaleic acid. Fenton suggested that the dihydroxyfumaric acid mainly exists in its cis- form. Therefore in all scientific works before 1950's, the acid is referred to as dihydroxymaleic. At the beginning of the 20th century it was proved [5] that these forms are chemically identical. In 1953 Hartree [6] showed that in crystalline form, as well as in solution, only

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the trans- isomer exists. It should also be mentioned that dihydroxyfumaric acid in solution exists in equilibrium of two tautomeric forms. (scheme 1):
In solution 80 % of the acid usually exists in its enolic form, and the other 20% exists in its keto-form [7].

Similarly to tartaric acid, dihydroxyfumaric acid plays an important role in nature. The first proofs of its biological significance appeared in 1915 when Neuberg [8] observed the fermentation of DHF by yeast. In 1938 research teams Banga and Szent-Gyiirgyi and Banga and Philippot extracted an enzyme from plants, which they called dihydroxyfumaric acid oxidase; later the oxidation product was proved to be diketosuccinic acid [9]. In 1940 Theorell discovered an enzyme in some plants, which oxidized dihydroxyfumaric acid with oxygen uptake, and the results of his studies allowed him to conclude that enzyme was peroxidase [10].

It was shown that the active centers of dihydroxyfumaric acid oxidase and peroxidase are the coordination compounds of iron and copper [11]. As it was previously mentioned, these ferments catalyze the transformation of DHF into diketosuccinic acid **2**, according to scheme 2.

Therefore, it was suggested [11] that in the system oxygen + DHF-oxidase, the role of DHF is similar to that of ascorbic acid in the ascorbate-oxidase system which afforded α-diketone **3** (scheme 3).

From these schemes it may be observed that the dihydroxyfumaric acid bears some similarities with the ascorbic acid. Therefore, in biological oxidation, it may play a similar role to that of ascorbate, i.e. intermediate hydrogen carrier from substrates to oxygen.

Later the oxidase function of peroxidase was shown towards other compounds, such as: glutathione, hydro- and naphtoquinone, fluoroglycine and others. It was proved that the necessary condition for the oxydase reaction is the presence of cofactors – manganese ions and various phenolic compounds. Further information on certain enzymatic reactions of dihydroxyfumaric and diketosuccinic acids in plant tissues was obtained by Stafford, Magaldi, and Vennesland [12] in 1954.

For the first time the role of dihydroxyfumaric acid in animal metabolism was evidenced in 1934. It was found that the content of glycogen was increased in muscle on incubation with DHF [13]. Later was discovered a sequence of enzyme reactions as a pathway for glyconeogenesis, based on the observation the formation of a pentose (or a pentose phosphate) in addition of DHF and glyceraldehydes (or fructose-l,6-diphosphate and aldolase as a source of glyceraldehyde-3-phosphate) in rabbit muscle extract was suggested. The sequence of reactions leading from DHF to 3-ketopentose is given in scheme 4, as follows [14].

It is well known that di– and tricarboxylic organic acids play an important role in plant and animal metabolism. Due to the fact that these compounds are products of carbohydrates transformations, they participate in the biosynthesis of alkaloids, glycosides, amino acids and other biologically active compounds [15]. The dihydroxyfumaric acid is linked with the cycle of di- and tricarboxylic acids, and also the dihydroxyfumaric acid is linked with the glyoxalic cycle via tartaric acid transformation cycle.

Without going into details, it should be mentioned that the main function of these

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cycles is that they represent the final collective path in oxidation of carbohydrates, lipids and proteins. Thus during metabolism processes glucose, fatty acids and amino acids are transformed either into acetyl-CoA, or in intermediate compounds of cycles mentioned above.

The dihydroxyfumaric acid is formed from tartaric acid by dehydrogenation, in the presence of NAD, tartaric acid dehydrogenase, and bivalent iron.

The dihydroxyfumaric acid is involved in metabolism during grapes ripening. It is although found in grapes in small amounts. It serves as a catalyst for redox reactions. Dihydroxyfumaric acid is easily oxidized by DHF oxidase . Therefore, grapes contain the products of its disintegration: mesoxalic acid, glycolic acid, oxalic acid and glyoxalic acid.

Dihydroxyfumaric acid is very important in winemaking and in food industry. It is well known that organic acids contribute to the formation of acidity of wines – one of the major important wine characteristics. Although its content in wine is small, DHF plays an important role in reduction processes occurring in wine. It was observed that DHF improves wine taste and flavor, leaving out turbidity and inhibiting catechol and phenol oxidation [14].

Derivatives of the dihyroxyfumaric acid

Ascorbic acid, bears two hydroxyl groups at vicinal carbons, linked by a double bond. Its structure is very similar to DHF structure. Due to this fact dihydroxyfumaric acid **1** is a good radical scavenger and has high antioxidant properties. Indeed dihydroxyfumarate proved to be a very efficient inhibitor of N-nitrosamines formation in model systems [17], *in vitro* [18] and *in vivo* [19].

It was also found [19] that the sodium salt of DHF decreases the MetHb speed formation during oxidation of HBO_2 with nitrites. This process occurs by decreasing the acceleration factor with increasing reduction concentration. Research in the endogenous formation of N'-nitrosonornicotine (one of the most abundant strong carcinogens in unburned tobacco and cigarette smoke) in rats demonstrated efficient inhibition (86%) of this process by dihydroxyfumaric acid [19]. Studies regarding the formation of N'-nitrosoamines in meat products (smoked, fried, dried, salted) demonstrate that the sodium salt of dihydroxyfumaric acid significantly decreases the concentration of nitrites and nitrates in meat products [20].

However, in the literature there is very little data concerning the kinetic investigation of reactions between dihydroxyfumaric acid **1** and free radicals due to the very high reaction rates. A perspective direction of obtaining new compounds with the properties mentioned above is the synthetic transformation of known metabolites.

We developed a new method for the synthesis of the dimethyl ether **4** of dihydroxyfumaric acid and its aminated derivatives **5-11** [22]. From the range of obtained derivatives, the following compounds together with dihydroxyfumaric acid **1**, for the ABTS decolorization assay were chosen (Fig.1).:

Experimental part

Chemicals

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Trolox (2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), dihydroxyfumaric acid, L-cysteine and ascorbic acid were purchased from Sigma Aldrich. Dihydroxyfumaric acid derivatives, namely: dimethyl 2,3-dihydroxyfumarate, 2,3-dihydroxy-N^1,N^4-bis(2-hydroxyethyl)fumaramide, 2,3-dihydroxy-N^1,N^4-bis(2-hydroxypropyl)fumaramide, (E)-methyl 2,3-dihydroxy-4-oxo-4-(phenylamino)but-2-enoate, 2,3-dihydroxy-N^1,N^4-bis(1-hydroxy-2-methylpropan-2-yl)fumaramide, dimethyl (2E)-2-hydroxy-3-(tetrahydro-2H-pyran-2-yloxy)but-2-enedioate, (E)-3-(1H-benzo[d]imidazol-2-yl)-2,3-dihydroxyacrylic acid, (E)-1,2-di(1H-benzo[d]imidazol-2-yl)ethene-1,2-diol, N^1,N^4-bis(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)-2,3-dihydroxyfumaramide, (*E*)-3-(1H-benzo[*d*]imidazol-2-yl)-2,3-dihydroxyacrylic acid and (E)-1,2-di(1H-benzo[d]imidazol-2-yl)ethene-1,2-diol were obtained by us, as described in [22]. Ethanol 95% was of pharmaceutical grade and was used without further purification.

The TEAC method used in the $ABTS^{+\cdot}$ scavenging assay

The TEAC method (Trolox Equivalent Antioxidant Capacity) for the determination of the antioxidant activity reflects the ability of antioxidants to donate hydrogen atoms for the scavenging of the radical cation $ABTS^{\bullet+}$. It has absorption peaks at 645, 734 and 815 nm, in comparison to Trolox, a hydrosoluble analogue of vitamin E. TEAC is defined as the concentration of Trolox solution (mmol/L or mg/L) with an antioxidant potential equivalent to 1 mM of studied compound.

The $ABTS^{+\cdot}$ radical-scavenging activity of the studied compounds was determined by a procedure reported in [23] with some modifications. The $ABTS^{+\cdot}$ solution was prepared by mixing 8 μM of ABTS salt with 3 μM of potassium persulfate in 25 ml of distilled water. The solution was held at room temperature in the dark place for 16 h before use. The $ABTS^{+\cdot}$ solution was diluted with 95% ethanol, in order to obtain an absorbance between 0.8 and 0.9 at 734 nm. For each analysis new $ABTS^{+\cdot}$ solution was prepared. Antioxidant or standard solutions, 20 μl, were mixed with 1 ml of diluted $ABTS^{+\cdot}$ solution and incubated at 30 ºC. The absorbance at 734 nm was observed at 1 min, 4 min and 6 min. Ethanol (95%) was used as a blank.

The calibration graph was drawn against Trolox standard (concentrations varied between 0–15 μM and were diluted in ethanol 95%), optical density was measured at 734 nm exactly at 1 min and 6 min after mixing. All determinations were performed in triplicate. The dose-response curve showing the TAA of the Trolox solution is depicted in Fig (3).

In the $ABTS^{\bullet+}$ decolorization assay, the antioxidant capacity is defined as the difference between the absorption of this radical cation (λ=734 nm) at t = 0 min and t = 1 min divided by the absorption of the initial solution. The correction is performed using the blank solution. Therefore, the total antioxidant activity (TAA) is determined according to equation 1:

$$\mathbf{TAA}=\left(\frac{T0_a - T1_a}{T0_a}\right)-\left(\frac{T0_b - T1_b}{T0_b}\right) \qquad \mathbf{(1)}$$

where $T0_a$ and $T1_a$ are the optical absorbencies of the tested solution at 0 and 1 min, and the $T0_b$ and $T1_b$ are the optical

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absorbencies of the blank solution at 0 and 1 min. For the total antioxidant activity, the calibration graph was drawn for Trolox (Tx) allowing the determination of the antioxidant capacity in Trolox equivalents (TEAC).

Results and discussion

The method described above gives a measure of the antioxidant activity of dihydroxyfumaric acid and a range of its derivatives through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. Figure 2 illustrates the duration of interaction for specific antioxidants on the diminution of the absorbance for the ABTS radical cation at 734 nm for Trolox, the standard reference compound. Trolox was compared with dihydroxyfumaric acid and its studied derivatives: dimethyl 2,3-dihydroxyfumarate (**4**), (*E*)-methyl 2,3-dihydroxy-4-oxo-4-(phenylamino)but-2-enoate (**5**), 2,3-dihydroxy-N^1,N^4-di(pyridin-2-yl)fumaramide (**6**), 2,3-dihydroxy-N^1,N^4-bis(2-hydroxyethyl)fumaramide (**7**), 2,3-dihydroxy-N^1,N^4-bis(2-hydroxypropyl)fumaramide (**8**), 2,3-dihydroxy-N^1,N^4-bis(1-hydroxy-2-methylpropan-2-yl)fumaramide (**9**), (*E*)-3-(1H-benzo[*d*]imidazol-2-yl)-2,3-dihydroxyacrylic acid (**10**), (E)-1,2-di(1H-benzo[d]imidazol-2-yl)ethene-1,2-diol (**11**). The results demonstrate that the reaction with $ABTS^{\bullet+}$ completes by 2 min, except for dihydroxyfumaric acid **1** that shows a further small inhibitory effect up to 2 min reaction, as seen in Fig. (3).

The extent of inhibition on the $ABTS^{\bullet+}$ absorbance is plotted as a function of concentration in order to determine the TEAC, that can be assessed as a function of time. The dose-response curve was obtained by analysis for the range of antioxidant concentrations at the end of the reaction. The total antioxidant activity percentage was plotted as a function of antioxidant concentration, shown in Fig. 4.

The concentration of antioxidant gives the same percentage inhibition of absorbance for the radical cation at 734 nm as 1 mM Trolox. It was calculated in terms of the Trolox equivalent antioxidant activity at the end of the reaction. This gives the TEAC at the end of the reaction for the studied concentrations of tested compounds which are given in Table 1.

Fig. 4 and Tab. 1 show that the most powerful $ABTS^{\bullet+}$ scavenger is compound **1** – dihydroxyfumaric acid, followed by its dimethyl ester - compound **4** and the anilide - compound **5**. The latter compounds are followed by mono- and bis- benzimidazole derivatives of dihydroxyfumaric acid - compounds **10** and **11** which show the similar scavenging activity. Compound **9** exhibits a scavenging activity equal to 30% from that of compound **1**, followed by compounds **8** and **7** with a slightly lower activity. Compound **6** has the lowest $ABTS^{\bullet+}$ scavenging activity, 20 folds lower that of compound **1**.

Obtained results show that the reaction between tested antioxidants and the ABTS radical is completed in less than 2 min.

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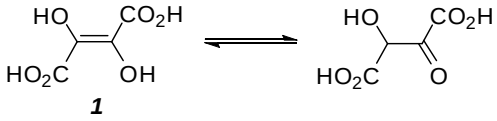
Thus, compound **1** quenches more than 60% of the radical, while its dimethyl ester **4** and the anilide **5** are able to quench only 30% of the radical. Thus we can say that these derivatives are half as strong antioxidants as the dihydroxyfumaric acid. Although dihydroxyfumaric acid proved to be the most potent scavenger in this assay, it should be noted that its utilization in chemical, pharmaceutical and cosmetic applications is limited by the fact that it's prone to decarboxylation in acidic media. The existence of this decarboxylation process encouraged us to find synthetic ways to stabilize the molecule by changing the –COOH groups with the others ones, which are more stable. Another advantage of these derivatives is their ability to add new groups with important biological activity instead of the initial –COOH groups, thus enhancing the importance of the obtained molecule. For example, it was noted that benzimidazole inhibits the growth of certain yeasts and bacteria that attracted considerable attention to this derivative. Such heterocyclic systems can be modified not only by changing the nature and the number of the connecting atoms but by changing the nature of the substituents in the benzimidazole nucleus as well. Thus, compounds **10** and **11** tested in this research become even more interesting for further studies.

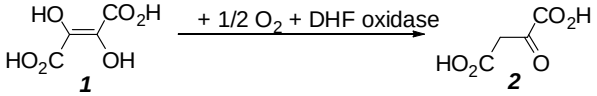
Obtained ester derivative and nitrogen containing derivatives of dihydroxyfumaric acid **1** may open new possibilities in organic synthesis: selection of appropriate combinations for substituents may allow adjusting the polarity in wide ranges, the solvating ability, catalytic properties, and thereby influence the depth and selectivity of the reaction.

Therefore, further investigations of dihydroxyfumaric acid derivatives are a scientific direction with potential significant results in the continuous search for biologically active compounds in biology and medicine.



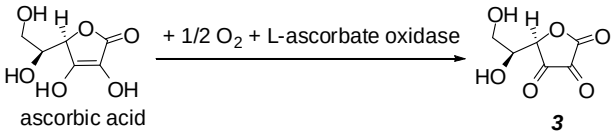
1

Scheme 1



1 *2*

Scheme 2



ascorbic acid *3*

Scheme 3

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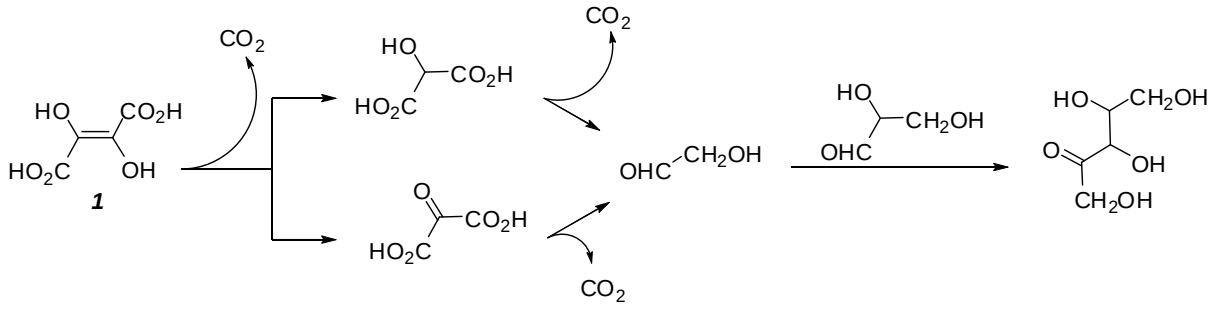
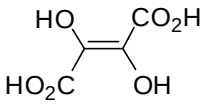
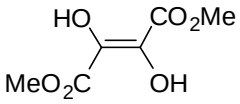


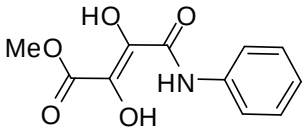
Scheme 4



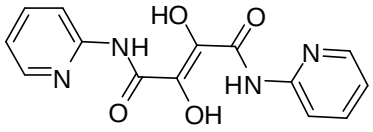
dihydroxyfumaric acid **1**



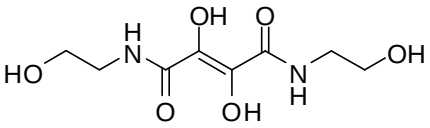
dimethyl 2,3-dihydroxyfumarate **4**



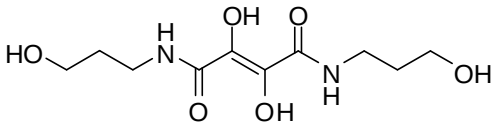
(*E*)-methyl 2,3-dihydroxy-4-oxo-4-(phenylamino)but-2-enoate **5**



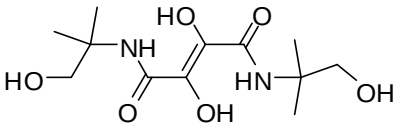
2,3-dihydroxy-N^1,N^4-di(pyridin-2-yl)fumaramide **6**



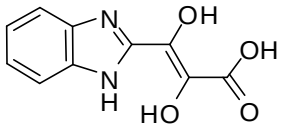
2,3-dihydroxy-N^1,N^4-bis(2-hydroxyethyl)fumaramide **7**



2,3-dihydroxy-N^1,N^4-bis(2-hydroxypropyl)fumaramide **8**



2,3-dihydroxy-N^1,N^4-bis(1-hydroxy-2-methylpropan-2-yl)fumaramide **9**



(*E*)-3-(1H-benzo[*d*]imidazol-2-yl)-2,3-dihydroxyacrylic acid **10**

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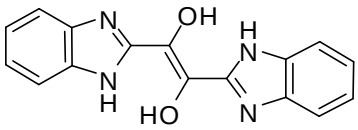

(E)-1,2-di(1H-benzo[d]imidazol-2-yl)ethene-1,2-diol **11**

Fig. (1). Compounds tested for the $ABTS^{\bullet+}$ scavenging activity.

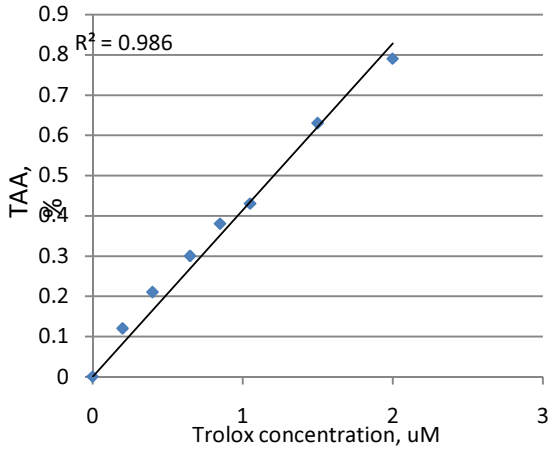

Fig. (2). The TAA of Trolox solution.

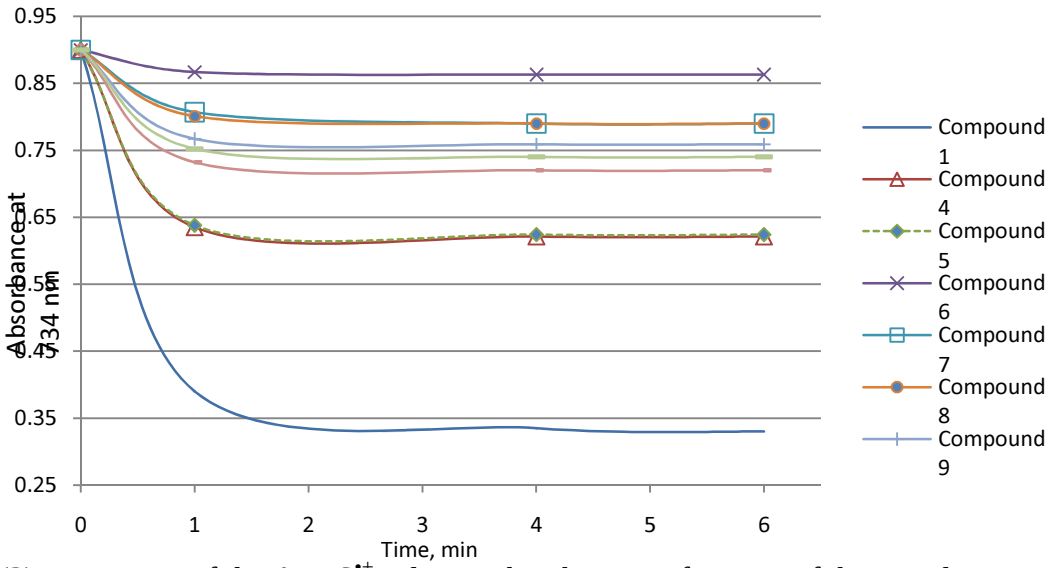

Fig. (3). Decreeing of the $ABTS^{\bullet+}$ solution absorbance as function of the tested compounds nature, for the highest tested concentration of 25 μM.

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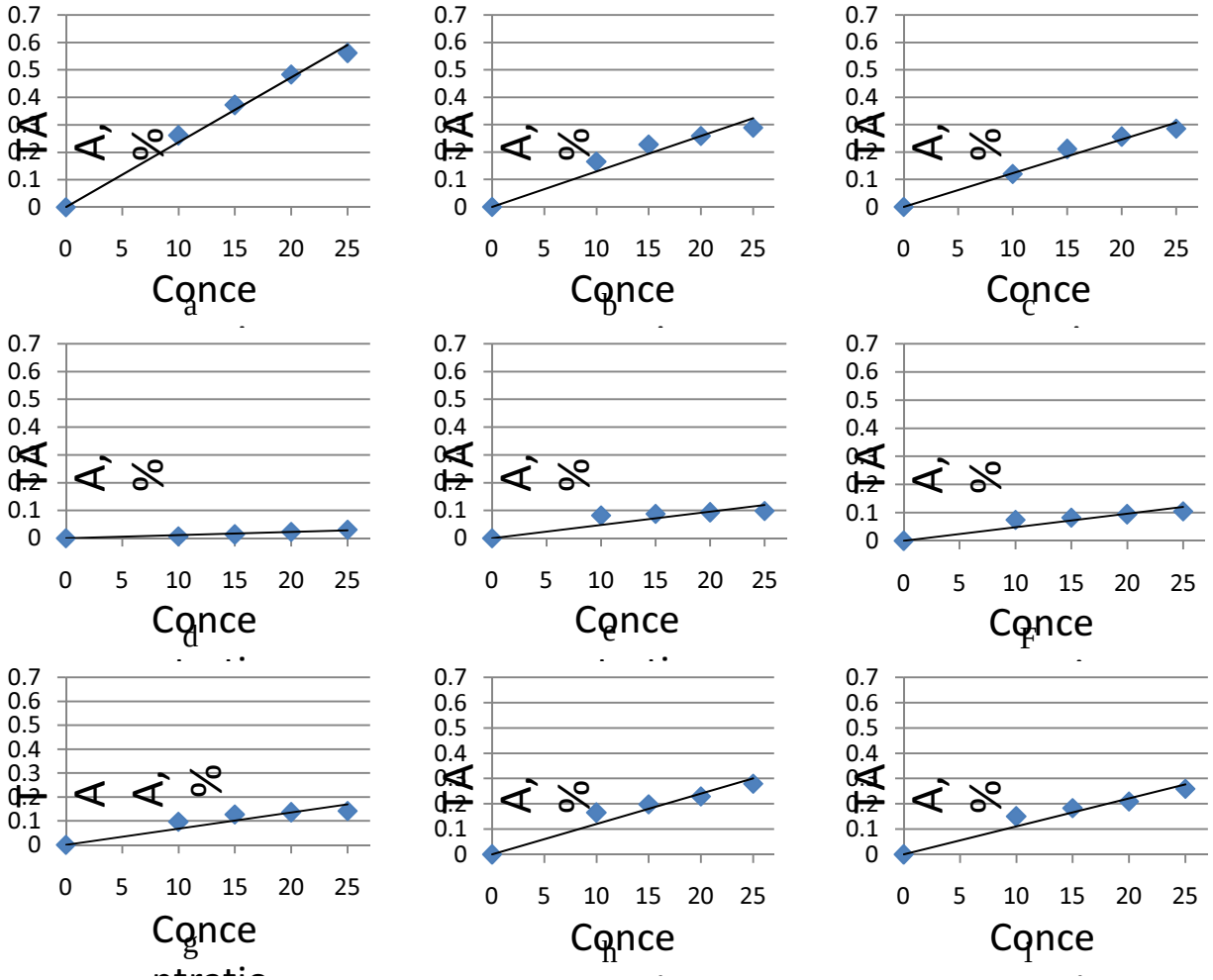

Fig. (4). Total antioxidant activity percentage as a function of the tested compound concentration, for the following compounds: a - dihydroxyfumaric acid, b - dimethyl 2,3-dihydroxyfumarate (**4**), c - (*E*)-methyl 2,3-dihydroxy-4-oxo-4-(phenylamino)but-2-enoate (**5**), d - 2,3-dihydroxy-N^1,N^4-di(pyridin-2-yl)fumaramide (**6**), e - 2,3-dihydroxy-N^1,N^4-bis(2-hydroxyethyl)fumaramide (**7**), f - 2,3-dihydroxy-N^1,N^4-bis(2-hydroxypropyl)fumaramide (**8**), g - 2,3-dihydroxy-N^1,N^4-bis(1-hydroxy-2-methylpropan-2-yl)fumaramide (**9**), h - (*E*)-3-(1H-benzo[*d*]imidazol-2-yl)-2,3-dihydroxyacrylic acid (**10**), i - (E)-1,2-di(1H-benzo[d]imidazol-2-yl)ethene-1,2-diol (**11**).

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Table 1. TEAC index for the studied concentrations of tested compounds.

Compound	Concentration of tested compound, µM	TEAC, µM Trolox
1	10	0,627±0.1
	15	0.894±0.1
	20	1.161±0.1
	25	1.348±0.012
4	10	0.395±0.01
	15	0.547±0.01
	20	0.622±0.02
	25	0.695±0.02
5	10	0.414±0.01
	15	0.507±0.01
	20	0.617±0.012
	25	0.686±0.012
6	10	0.016±0.001
	15	0.032±0.001
	20	0.056±0.001
	25	0.075±0.001
7	10	0.198±0.001
	15	0.211±0.005
	20	0.225±0.005
	25	0.235±0.005
8	10	0.179±0.002
	15	0.200±0.005
	20	0.227±0.005
	25	0.251±0.005
9	10	0.232±0.005
	15	0.305±0.01
	20	0.326±0.01
	25	0.342±0.01
10	10	0.396±0.01
	15	0.475±0.01
	20	0.552±0.01
	25	0.672±0.012
11	10	0.36±0.01
	15	0.44±0.01
	20	0.504±0.01
	25	0.624±0.012

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References

[1] Q. Ashton Acton; Hydroxy Acids—Advances in Research and Application; Scholarly Editions: Atlanta, Georgia, **2012**.

[2] Gh. Duca, B. Gaina, O. Kovaleva, V. Kovalev, M. Gonta; Ecologically clean wine-making industry (in Russian); Shtiintsa: Chisinau, **2004**, I, 120.

[3] Fenton H.J. The formation and properties of a new organic acid. *Brit. Assoc. Advance Sci. Rep*., **1895**, 65, 663.

[4] W. H. Koppenol, Free Rad. Biol. And Med., **1993**, 15:6, 645-651.

[5] H. J. Fenton, W.A. Wilks, J. Chem. Soc., **1912**, 101, 1570-1582.

[6] The Chemistry of Metal Enolates; Edited by Jacob Zabicky; John Wiley &Sons: England, **2009**, 212.

[7] N. Secara, Chem. J. Mold., **2008**, 3: 1, 127.

[8] C. Neuberg, E. Schwenk, Biochem. J., **1915,** 71, 126-132.

[9] D.M. Needham; Machina Carnis: The Biochemistry of Muscular Contraction in Its Historical Development, Syndics of the Cambridge University Press : London, **1971.**

[10] K-U. Rehman, M. Yaqub, M. A. Sheikh, M. Arshad, Int. J. Agri.Biol. **1999**,1:3, 170-173.

[11] Gh. Duca, "Catalysis of oxidation of fumaric and dihydroxyfumaric acids", PhD thesis, State University of Moldova, Chisinau,**1979**.

[12] H.A. Stafford, A. Magaldi, B. Vennesland, Science, **1954**,120(3111), 265-266.

[13] A. Stepanow, A. Kusin, Ber. Dtsch. Chem. Ges., **1934**, 67, 723-726.

[14] K. Fukunaga, J. of Biochem., **1960**, 6, 47.

[15] A. Sychev, Gh. Duca, "Sadovod. Vinograd. Vinodel. Mold." (in Russian), Stiinta, Chisinau, **1985**, I, 165.

[16] Nelson, Eric L., Richard L. "Contact Lens Preserving Solution Containing Ascorbic Acid Or Salts Thereof", United States Patent 4490389, 25 December, **1984**.

[17] M. Gonta, M. Duca, D. Porubin, N. Voloc. In: Scientific annals of the State University of Moldova (in Romanian); State University of Moldova, Chisinau, **2003**, 419-425.

[18] D. Porubin, Chemistry Journal of Moldova, **2007**, 2, 3-7.

[19] D. Porubin, S. S. Hecht, Zh. Li, M. Gonta, I. Stepanov, Journal of Agricultural and Food Chemistry, **2007**, 55, 7199-7204.

[20] M. Gonta, "Inhibition of haemoglobin oxidation by dihydroxyfumaric acid and its sodium salt", In: Scientific annals of the State University of Moldova (in Romanian), State University of Moldova, Chishinau, **2006**, 123-125.

[21] M. Gonta, "Inhibition of polycatechins oxidation using the dihydroxyfumaric acid", Dr.Sc. Thesis; State University of Moldova, Chisinau, **2008**.

[22] N. Secara, Gh. Duca, L. Vlad, F. Macaev, Chem. J. Mold., **2010**, 5 (2), 59-67

[23] N. J. Miller, C. A. Rice-Evans, Free Radic Res., **1997**, 26(6), 594.