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Obtaining and characterizing modified tannins by physical-chemical methods+

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Alexandru Gonta[1*], Tudor Lupascu[1],
Nina Timbaliuc[1], Aurelia Meghea[2]

[1]*Institute of Chemistry,*
Academy of Sciences of Moldova,
MD 2028, Chisinau, Republic of Moldova

[2]*Faculty of Applied Chemistry and Material Science,*
"Politehnica" University of Bucharest,
Bucharest 011061, Romania

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Abstract: After oxidation of tannins (Tannins 1-3) extracted from grape seeds, Tanoxil compounds with increased solubility and enhanced antioxidant activities were synthesized. In this research, $ABTS^{+\bullet}$ assay and a chemiluminescence method were used for antioxidant measurement of hydrophilic compounds (Tanoxils 1-3).

It was observed that the antioxidant activity (AA, %) is very similar for the three samples, while at the same time AA is quite high (93.16% -96.48%). The percentage of inhibition by $ABTS^{+\bullet}$ is higher for Tanoxil 2 (96.4%) as compared to Tannin 2 compound (14.34%). Moreover, the total content (T_{CF}) of carboxyl and phenolic groups was investigated. T_{CF} values, determined for Tanoxil products, revealed a double (Tanoxil 1) or triple (Tanoxil 2) increase as compared to the value of the T_{CF} of Tannin 1. Tanoxil products represent an interest for future research as they have a high AA (96.4%) and the content of acidic groups is significant (T_{CF} 0.191 meq g^{-1}).

Keywords: *Chemiluminescence • Antioxidant • Polyphenols • Tannins • $ABTS^{+\bullet}$*

1. Introduction

Processing grapes in the wine industry is considered to have minimal impact on the environment. However, just one wine factory could process more than three thousand tons of grapes annually. This means an ecological pollution equivalent to the emission of pollutants by a city with a population of 37,000, according to the national Institute of Ecology and Geography Environmental Statistics, Chisinau, Moldova.

Numerous practical and epidemiological studies have confirmed that the micronutrients, specifically the antioxidants existing in aliments, are able to inhibit carcinogenesis through their influence at the molecular level, during the initiation, promotion and progression stages [1,2].

In recent times the most studied compounds were the polyphenols which are constituents of the plants. The antioxidant activity of the polyphenols is controlled by the presence of the hydroxyl groups in the B ring in the 3' and 4' positions and to a lesser degree by the presence of the hydroxyl group from the C ring in the 4' position [3,4].

The flavonols, especially catechin, quercetin, kaempferol and their glycosides are elements of black and green teas and red wine [5]. Diets rich in vegetables and fruits, especially in grapes, protect against heart disease and various forms of cancer [6,7].

These protective upshots were attributed, to a great extent, to the antioxidants that include flavonoids as well as carotenoids and vitamin C.

Certain natural colorants can be important nutritional antioxidants and their presence in food can also reduce the risk of cancer and heart disease [8,9]. It is known that natural colorants, such as carotenoids, anthocyanidins and curcuminoids, display antioxidant, anti-inflammatory,

* *E-mail: alexandru.gonta@chem.asm.md*

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anti-viral, anti-cancerous properties and anti-tumor effects [10,11].

Among various vegetables, grape seeds are a rich source of so called enotannins – condensed tannins which represent a wide variety of natural substances with polyphenol structure, remarkable due to the high content of proanthocyanidins with enhanced antioxidant properties, that could inhibit free radicals [12,13]. Grapes and wine contain high concentrations of antioxidants. Based on the study of the overall antioxidant activity of red wine, it was concluded that 54.76% is determined by the contribution of catechin and epicatechin, which form approximately 63.54% of the phenolic constituents (191 and 82 mg L^{-1}, respectively) [4].

One of the causes of health imbalance in our body at biological, biochemical and genetic levels is the formation of free radicals and oxidative stress [14].

Polyphenols are efficient scavengers of free radicals in various *in vitro* systems. The capacity of the compounds to scavenge the radicals is partially determined by the reduction potential of one electron, which is a measure of antioxidants reactivity, as donors of hydrogen or oxygen [4].

At the moment, efficient medicinal compounds on the basis of tannin compounds are rare, mostly due to their insolubility in aqueous or alcoholic solutions. The capacity of phenolic (tannin) extracts to disinfect and rapidly treat wounds is based on two fundamental phenomena - antimicrobial and antioxidant activities, determined by a series of mechanisms.

In order to increase their therapeutic efficiency and to improve their physical-chemical properties of enotannins, structural modifications of these polyphenols have been studied. For increasing solubility in water, depolimerization reactions of macromolecular chains of enotannins have been assessed. Finally, new hydrophilic compounds have been synthesized [15].

The degree of polymerization has a great influence upon the inhibitory features of the polyphenols. The polyphenolic fractions extracted from grapes with various degrees of polymerization had different antioxidant/antiradical and antiproliferative effects [3]. The polyphenol solutions extracted from grapes were divided with RP-HPLC into two fractions having different degrees of polymerization [5]. The antioxidant/antiradical activity determined *via* DPPH test was higher for the flavanols fraction from grapes, composed of small monomers and oligomers, as compared to the fraction that included catechin polymer units, which have a greater molecular mass [16].

Additionally, the extracts obtained from aromatic and medicinal plants (Sage, Lavender, Calendula, Echinacea, *etc.*) were investigated, through the application of $DPPH^{\cdot}$ test and $ABTS^{+\cdot}$ method [17]. Stable radicals, such as 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$) radical and $ABTS^{+\bullet}$ cation-radical (2,2- azino-bis-3- ethylbenzthiazoline-6-sulphonic acid) are used for the assessment of flavonoids *in vitro* antioxidant activity. The use of the Trolox equivalent antioxidant activity test (TEAA) demonstrated that the catechin and theaflavin are more efficient in $ABTS^{+\bullet}$ cation-radical reduction than vitamins E and C [18].The antioxidant activities are correlated with antibacterial properties of the compounds investigated [19]. In order to obtain compounds with enhanced antiradical activities, the scavenging activity of free radicals by antioxidants was analyzed.

This work presents a study of the physical-chemical properties of the Tanoxil compounds obtained after oxidation, by means of antioxidant and potentiometric determinations.

For the analysis of antioxidant properties of compounds obtained, the $ABTS^{+\cdot}$ method and chemiluminescence methods have been employed. Both the chemiluminescence method and the $ABTS^{+}$ radical assay are fast, reliable and comparable tests often used for analysis and determination of percentage of inhibition of free radicals by antioxidants [20].

The potentiometric technique „Boehm titrations" is a widely used method which has been assessed in our research for investigation and fast data acquisition of total carboxylic and phenolic content of Tanoxil products [21].

2. Experimental procedure

2.1. Chemicals

Catechin hydrate (±)-Cat, Luminol (3-Aminophthalhydrazide), Tris-Edta buffer solution (pH 8.0) BioUltra and $ABTS^{+}$ (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were purchased from Sigma-Aldrich. All reagents were of HPLC analytical grade, ≥96%. Hydrogen Peroxide solution (35% H_2O_2), NaOH 1 M solution and 33% HCl solution were purchased from a local chemical supplier. Tannin products extracted from Red grapes (Tan1 and Tan3) and white grapes (Tan2) were purchased from a local wine merchant.

2.2. Synthesis of Tanoxil compounds

New oxidized flavonoid compounds have been obtained according to the procedure of depolymerization reaction described by Lupascu *et al.* [15], with slight modifications. Tanoxil samples were prepared as follows: 0.5 g of Tan1 (the same amount for Tan2 and

Tan3) was subjected to oxidation of the polymer chain. All samples were left to dry in oven at 60.-.80^{0}C. As a result 0.35 g (0.38 g of and 0.34 g) of Tnx1 (Tnx2 and Tnx3, respectively) were obtained.

2.3. Antioxidant scavenging ABTS$^{+\bullet}$ assay

To determine the antioxidant activities of flavonoids, radical ABTS$^+$ was employed in this assay according to the protocol of Re Roberta *et al*. [22]. The ABTS$^{+\bullet}$ was obtained by mixing ABTS$^+$ stock solution (7 mM) and ammonium persulphate solution (2.45 mM, final volume concentration), and left in the dark for 12-16 hours, at room temperature. The solution of cation radical ABTS was diluted with 70% ethanol solution to a final absorbance of 0,700 (±0.02) measured at 734 nm. After adding 40 µL of antioxidant to 4 mL of ABTS$^{+\bullet}$ solution, it should produce an inhibition of free radicals of 20-80%. The absorbance data was taken at the Jenway Spectrophotometer 6505, exactly 1 minute after initial mixing, up to six minutes.

2.4. Determination of total carboxylic acids and phenolic groups content

For the analysis of T_{CF}, the Boehm titration method has been used with slight parameter changes [21]. Approximately 1 g of 0.1% sample solution was transferred to a 150 mL flask, and then 25 mL bidistilled water was added and agitated with a magnetic stirrer until it was completely dissolved. Furthermore, 25 mL of 0.05 mol L^{-1} NaOH solution was added to the sample solution and the excess of NaOH was titrated with 50 mM of HCl solution. Exactly at pH 4.5 the volume of consumed HCl solution was taken for further calculation of T_{CF}, expressed as meqiv 1 g^{-1} of sample solution and calculated according to the relation: T_{CF}= *([NaOH] * V_1 – [HCl] * V_2) m^{-1}*, where V_1 is the volume of the NaOH solution titrated, V_2 is the volume of HCl solution titrant and *m* is the mass in g of the sample solution tested. Measurements were carried out at a Titrator "Titroline 6000" instrument.

2.5. Chemiluminescence measurement

The oxidative cleavage reaction of Luminol compound by free radicals was used to measure the polyphenols antioxidant activity. In this assay, H_2O_2 solution (100 µM) was used as a source of free radicals, which in slightly alkaline pH solution produces hydroxyl, singlet oxygen and superoxide radicals [23]. Therefore, inhibition percentage of free radicals produced in the Luminol/Tris-EDTA CL generation system by different concentrations of antioxidants, between 0.01% - 0.25% (w/w), have been determined. Finally, the emission of light was triggered by adding 50 µL of aqueous hydrogen peroxide (C_{per}, 50 µM) to 200 µL of Luminol solution (C_{Lum}, 0.1 mM), 650 µL Tris-EDTA solution (pH 8.0) and 100 µL aqueous or alcohol solution of flavonoids. The final mixture volume of 1 mL was agitated for 10 seconds, prior to inserting in the reactor tube. Emitted light was recorded on a Promega Glomax Luminometer instrument over 15 seconds, after initial mixing of peroxide solution. The antioxidant activity was according to the relation: AA (%) = $((I_0 - I)/I_0) \times 100$, where I_0 and I are relative light emissions of blank and sample solutions after 15 seconds, respectively.

2.6. Data analysis

The research results of antioxidant activity and T_{CF} measurements were expressed as a mean ± standard deviation of three replicates.

3. Results and discussion

3.1. Obtaining of Tanoxil compounds

It is known that tannin products are insoluble in water, which minimizes their use as medicinal products. In this research new hydrophilic compounds Tanoxil (Tnx1, Tnx2 and Tnx3) were obtained. According to Kulcitki *et al.* in the process of tannin oxidation the molecular chain is depolymerized and a new product (Enoxil) which is water soluble is obtained [24]. This product possesses high antioxidant activity and amplified therapeutic properties [25,26].

Soluble products were synthesized from Tannin products with slight modifications, such as concentration of oxidant and time of oxidative cleavage of the tannin macromolecular structure. Moreover, oxidation reactions happen very fast and intermediate flavonoid compounds are transformed to final products, with no possibility to recover them.

The aim of this research is to determine to what extent the antioxidant activity and total organic carboxylic groups after formation of oxidized compound could be increased with optimized physical-chemical parameters compared to Enoxil research.

3.2. Chemiluminescence method

Chemiluminescence is a fast and reliable method for antioxidant activity determinations [27,28]. This method involves the transformation of Luminol's chemical structure to an excited state which in the process of transition to a lower energy state emits photons, which can be recorded with a chemiluminometer instrument. The role of Luminol in chemiluminescence reaction is to enhance the ultraweak light emitted by free radicals [27].

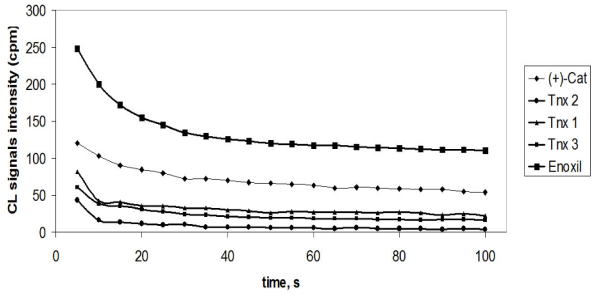

Figure 1. Kinetic curves of chemiluminescent signals intensity decrease in the presence of antioxidants (A) 0.1% (+)-Cat solution, (B) 0.1% Tnx 1 (C) 0.1% Tnx 2 (D) 0.1% Tnx 3, (E) 0.1% Enoxil solutions.

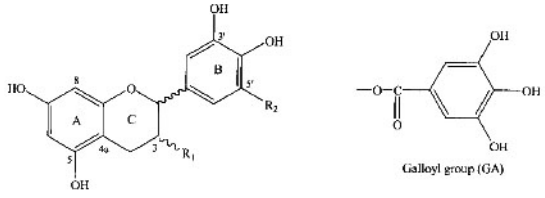

Figure 2. Chemical structure of the catechins present in tea and wine. Catechin-(R_1 - OH, R_2 - H), gallocatechin-(R_1 - OH, R_2 - OH), catechingallate (R_1 - GA, R_2 - H), gallocatechingallate (R_1 - GA, R_2 - OH).

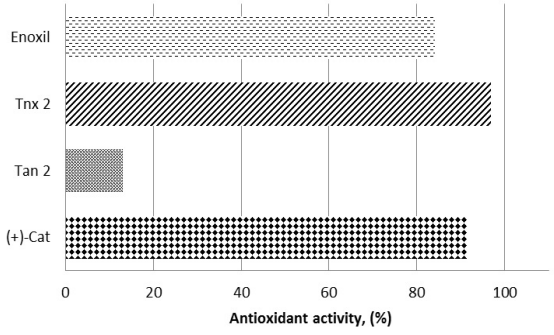

Figure 3. Antioxidant activities of compounds (A) 0.1%, Enoxil solution (B) 0.1% Tnx2 solution (C) 0.1% Tan2 solution and (D) (+)-Cat solution.

Polyphenolic compounds interfere in the propagation reactions of hydroxyl radicals through a complex inhibition mechanism.

The antioxidant activity of polyphenols is determined by the presence of hydroxyl groups in the 3' and 4' positions of the B ring. Flavonols, especially catechins, quercetin and kaempferol, are formulated in cosmetic, pharmaceutical and dairy products by means of antioxidant defense and maintaining the stability of the main compounds [29].

For the estimation of the antiradical activity of Tanoxil compounds, a CL generation system, Luminol/Tris-EDTA/H_2O_2 was employed. The dependence of CL light intensities on time (s) in the presence of Tanoxil (sample) and in the absence (blank), are shown in Fig. 1. All Tanoxil samples reduce the light emission of Luminol, while manifesting antioxidant activity (AA, 94%-96%).

According to the results, Tanoxil compounds inhibit the major quantity of free radicals which is due to the OH groups attached to the polyphenol rings. The studies carried out *in vitro* outlined the antioxidant potential of the polyphenols as a parameter that determines the scavenging capacity of free radicals, for example super oxide radicals ($O_2^{-\bullet}$), the singlet oxygen ($^1O_2$), the hydroxyl radical ($^\bullet OH$), peroxyl radical ($HO_2^\bullet$), nitrogen monoxide ($^\bullet ON$) and peroxynitrite ($ONOO^-$), which in turn, are the causes of various pathologies [7]. The chemical structures that contribute to the antioxidant activity of polyphenols, including the dihydroxy- or trihydroxy-neighbouring structure, can chelate the ions of metal, forming the complexes and preventing the creation of free radicals. This structure also allows the delocalization of the electrons, offering a higher reactivity of free radical destruction [30].

It is noticed that Tanoxils had higher antioxidant capacity compared to the standard (+)-Cat antioxidant, which is one of the major compounds of tannins, shown in Fig. 2. Moreover Enoxil product antioxidant activity was lower than of Tanoxil compounds (Fig. 3). Therefore, confirming that Tanoxil product is a mixture of compounds which manifest their antioxidant activity by a synergism mechanism between oxidized structures of catechin molecules and other compounds such as carboxylic esters, aldehyde and ketone compounds [25]. The antiradical activities for initial tannins are almost the same or with insignificant differences compared to the blank, as shown in Fig. 3.

The oxidative cleavage by OH radicals could not be inhibited by aqueous solution of Tannins 1-3 because they possess antioxidant activities in the range (10.23-14.5%) which is mainly due to the insolubility of tannin macromolecular compounds in water.

3.3. $ABTS^{+\bullet}$ assay of phenolic compounds

$ABTS^{+\bullet}$ assay is a sensitive method for the estimation of water soluble antioxidants. Inhibition of the $ABTS^{+\bullet}$ after the interaction with antioxidants will result in a decrease of dark green $ABTS^+$ solution color with maximum absorbance at 734 nm, which is represented by line charts in the Fig. 5.

After one minute, the antioxidant activities in the presence of aqueous solution of initial tannins, Tnx 1-3 and in the absence of antioxidants were measured. The percentage of inhibition (PI%) of $ABTS^{+\bullet}$ after the addition of 0.1% Tnx 1, 0.1% Tnx 2 and 0.1% Tnx 3 are indicated in Table 1. The line charts 5(D) of Fig. 5.

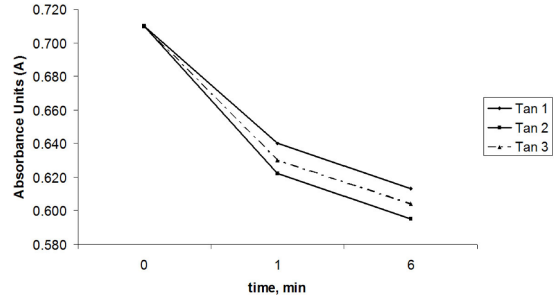

Figure 4. Absorbance values after reduction of $ABTS^{\bullet+}$ with (A) 0.1% Tan 1, (B) 0.1% Tan 2 and (C) 0.1% of Tan 3 solution.

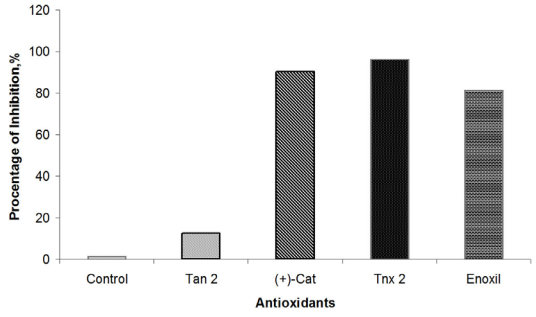

Figure 5. PI% of antioxidants after addition of (A) 30μL of H_2O, (B) 30μL of 0.1% Tan 2, (C) 30μL of 0.1% (+)-Cat (D) 30μL of 0.1% Tnx 2 and (E) 30μL of 0.1% Enoxil, to 3mL of $ABTS^{\bullet+}$ ethanol solution.

Table 1. Parameters of $ABTS^{\bullet+}$ PI%, AA% by chemiluminescence and T_{CF} content of polyphenolic compounds.

Sample	$ABTS^{\bullet+}$, PI (%)	CL, AA (%)	TCF, meq g^{-1}
(+)-Cat	91.23±0.23*	91.51±0.32*	-
Enoxil	83.45±0.14*	84.11±0.20*	0.152±0.018*
Tan 1	9.86±0.41*	10.23±0.35*	0.066±0.031*
Tan 2	12.38±0.35*	13.02±0.28*	0.085±0.021*
Tan 3	11.75±0.68*	11.34±0.44*	0.072±0.041*
Tnx 1	94.08±0.12*	94.44±0.37*	0.134±0.070*
Tnx 2	96.23±0.89*	96.93±0.19*	0.191±0.040*
Tnx 3	95.43±0.74*	95.09±0.17*	0.156±0.029*

**Values represent mean± standard error of the means of three independent determinations (n=3)*

indicate that the PI% of Tnx 2 (96.23%) is higher than PI% of Tan 2(12.38%) 5(B), PI% of (+)-Cat standard (90.2%) 5(C), PI% of Enoxil (81.22%) 5(E) and PI% of control (1.27%) 5(A), respectively.

Therefore, the line charts 4(A), 4(B) and 4(C) in Fig. 4, indicate a decrease of $ABTS^{\bullet+}$ solution color for Tan samples 1-3. Moreover, it can be postulated that Tan 1-3 compounds have very low antioxidant activities.

3.4. Total content of carboxylic and phenolic groups

In order to measure the T_{CF}, the Boehm titration method has been assessed. For the optimization of the method, the time of titration, pH and concentration of HCl and NaOH have been adjusted.

In the process of tannin oxidation, carboxylic phenolic, ketone, aldehydes and ester groups are formed [24].

The antibacterial activity depends on the bioavailability of the drug tested, pH and hydrophilicity [25]. One way to increase solubility in water is the presence of carboxylic groups, which are polar groups and could change the polarity to acid medium.

The results of the T_{CF}, determined by acidic-base titrations are shown in Table 1.The polarity of tannin compounds has been changed which is confirmed by T_{CF} value of Tnx1 (0.134 meq g^{-1}), which represents a twice increase in contrast to T_{CF} of Tan1 value shown in Table 1. Moreover, T_{CF} value of Tnx2 (0.191 meq g^{-1}) certify that new products with polar groups were obtained.

4. Conclusions

This research highlights the importance of obtaining compounds with enhanced antioxidant activities, using valuable secondary wine material. Additionally this research has presented the characterization of the antioxidant properties of the oxidized tannins products by a chemiluminescence method and $ABTS^{\bullet+}$ assay. After oxidation reaction new compounds with higher antioxidant activities than enotannins have been obtained. Based on our investigations, we have proved that the process of depolymerization was achieved and new products have been synthesized. The tannin oxidation reaction, antioxidant activities and T_{CF} determinations further confirmed the higher antiradical properties of Tanoxils and the presence of carboxylic and phenolic groups in their structure, in contrast to (+)-catechin and Enoxil. Similar findings may contribute to understanding to what extent these antioxidants could be used in the formulation of pharmaceutical and cosmetic products, as they are of natural origin and perform antioxidant defense by a synergism mechanism.

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