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ANTIOXIDANT CONTENT IN ROMANIAN TRADITIONAL DISTILLED ALCOHOLIC BEVERAGES

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ABSTRACT. Reported here is a first detailed analysis of the antioxidant activity in traditional fruit-derived distilled alcoholic beverages from Romania ("țuica"). A distinctly highest activity, paralleled by Folin-Ciocalteu data and UV-vis absorbances, was seen in the most common type of such brandy, made of plums; other samples included brandies made of apples, pears, fruit mixtures, wine, and cereals. In fact, the values seen for the plum brandy were, even before maturation with wood, similar to those of commercially-available wood-maturated whisky. Increases of up to one order of magnitude in antioxidant activity were seen in all brandies upon maturation with various types of wood – with mulberry tree by far the most efficient, followed by oak and cherry – and with lowest values seen for acacia. Attempts to identify anticancer activity in concentrated extracts prepared from plum brandy, failed. Copper electron paramagnetic (EPR) signals are shown for the first time in such brandy samples.

Keywords: *brandy; antioxidant; polyphenol; hemoglobin; EPR*

INTRODUCTION

The beneficial effects on human health and disease prevention (cardiovascular, neurodegenerative, colon cancer) of moderately consumed wine and brandy are correlated with their antioxidant capacity, especially due to the higher ability of the polyphenolic compounds to quench free radical species [1-14].

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Polyphenols and antioxidant capacity are almost absent in the case of vodka, gin and rum. However, whisky and brandy have an intermediate antioxidant capacity between red and white wine. In this case, polyphenols come from the wood of the storage container during the maturation process [15,16]. Numerous studies show that storage of wine or other alcoholic beverage, especially in oak barrels, may lead to significant changes in the polyphenolic composition, due to the transformation of native compounds as well as to the hydrolysis of tannins or lignin from the woods [7,17-25]. Recently, new technologies have been developed in order to simplify the aging process and to decrease the cost of preservation of brandy in wood barrels. Thus, one potential alternative technology consists in adding of wood pieces to the beverage kept in stainless steel tanks [26,27].

Although there are numerous studies regarding the antioxidant capacities of some distilled alcoholic beverages (cognac, liquor, whisky) [9,28,29], limited data have been obtained in the case of traditionally-produced brandies in Romania (tuica or palinca). As opposed to the use of cereals in other European countries (Scotland, Ireland, Northern Europe), traditional Romanian distilled beverages are based on processing of various autochthonic of fruits such as plums, apples, pears, cherries, apricots, berries. The distillation of the fermented fruit pulp is typically performed in cooper stills, with open fire, followed by a maturation phase in oak barrels [30].

According to the alcoholic concentration (37 vol % cf. current legislation), Romanian fruit brandies are classified as superior distilled drinks palinca (the name is a translation of the similar Hungarian product, palinka), obtained by a mixture of fruits, or slibovita, obtained by distillation and redistillation of plum marc, and inferior assortments known as spirits. The tuica, other traditional beverage obtained typically exclusively from plums, is produced at various alcohol concentrations, depending on region [31,32].

The purpose of this study was to investigate the relationship between polyphenolic content of various types of traditional distilled Romanian beverages and the antioxidant capacities under the influence of different types of wood used during the maturation process. Herein, besides traditional methods used to investigate antioxidant capacities of food and drinks (ABTS, DPPH) [16,33,34], a new physiologically relevant method based on hemoglobin ascorbate peroxidase activities is applied.

RESULTS AND DISCUSSION

General features

There are only a few data regarding the quality of Romanian distilled fruit beverages, mainly focused on technical analyses such as relative density, alcohol concentration, total and volatile acidity, pH, total dry extract, total polyphenol index and volatile compounds [31,32,35]. However, a detailed analysis of the antioxidant character has not been reported. By contrast, it is known that several kinds of polyphenolic compounds such as ellagic acid, gallic acid and lyoniresinol could be detected in whisky; nevertheless, a majority of them come from oak barrels, due to the higher concentration of alcohol, in the maturation process [36]. Here, some traditional and new methods for the characterization of antioxidant capacities were applied for the analysis of 5 types of Romanian brandy; additionally, the effect of maturation in the presence of different species of wood was also examined.

All studied brandy samples have an alcoholic concentration between 47 and 52 % very close to the commercial distilled beverage, whisky. Copper is the only detected metal in all samples, having a concentration between 0.1627-0.377 ppm; this is an acceptable level according to the current legislation (5 ppm legal limit (Monitorul oficial al Romaniei, part I, Nr. 268.11 VI 1999)). The metals found in alcoholic beverage may have different sources including raw materials, brewing, process type and equipment, bottling, aging/storage and adulteration [37]. The presence of Cu in the studied alcoholic distilled beverage was detected here for the first time, in our knowledge, using not only elemental analysis but also EPR spectroscopy (Supplementary Information, Figure S1).

UV-vis spectra

All analyzed brandy samples display UV-vis absorbance between 200 and 320 nm (Figure 1 A) - a region specific for the absorption of aromatic and unsaturated compounds. The difference in the absorption intensity of spectra suggests the diversity of the compounds found in these brandies. The highest intensity was obtained for plum brandy and the lowest for wine and corn ones. Considering that antioxidants present here are organic compounds, presumably polyphenolic compounds, one would expect a good correlation between the intensity of the spectra and the antioxidant activity of the samples. Compared to other distilled beverages (Fig. 1 B), the spectrum of the Romanian tuica has a visible difference in the intensity of absorbance at 250 nm. At 275 nm, the tuica appears to be more similar to whisky and tequila; in fact, below 300 nm, its absorbance (and, hence, its organic content – implying antioxidant activity) are the highest among all samples tested.

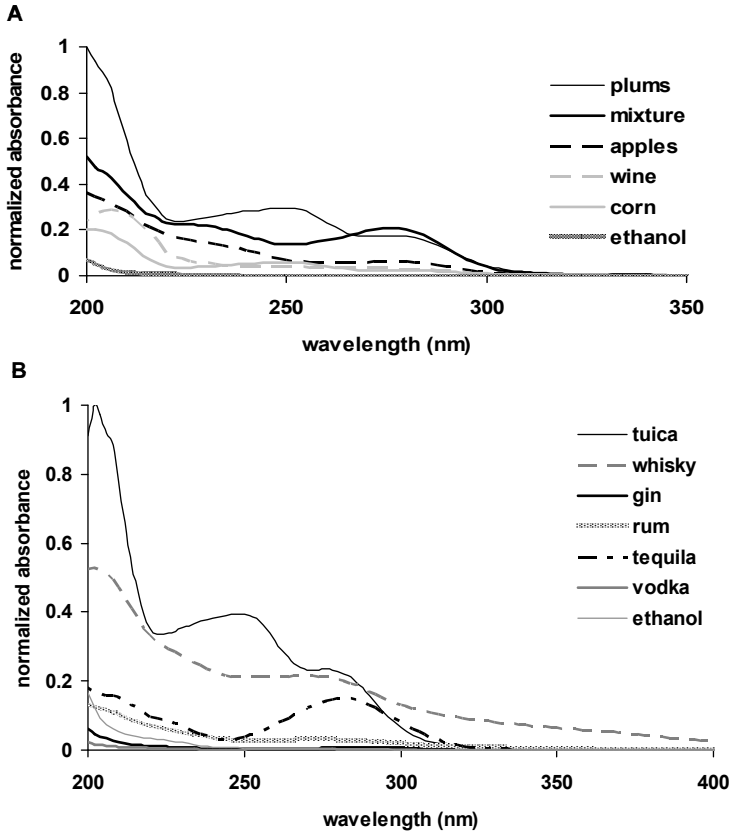

Figure 1. UV-vis spectra of Romanian brandy samples (A) and other commercial distilled beverage (B).

Chromatographic analyses

The attempts to detect antioxidant compounds from tested Romanian brandies by TLC were, unfortunately, unsuccessful even in the case of concentrated samples. This may suggest that part of the responsible compounds for antioxidant activity were also volatile (as seen in the following sections, chemical analysis does reveal a certain polyphenolic content). On the other hand, the chromatogram obtained for the maturated brandies (Fig. 2) indicates specific and obvious differences in the type and content of compounds: the highest amounts were in mulberry tree (1), cherry (3) and oak (2) samples. It must be specified that in the case of maturated brandies the sample pre-concentration is not necessary.

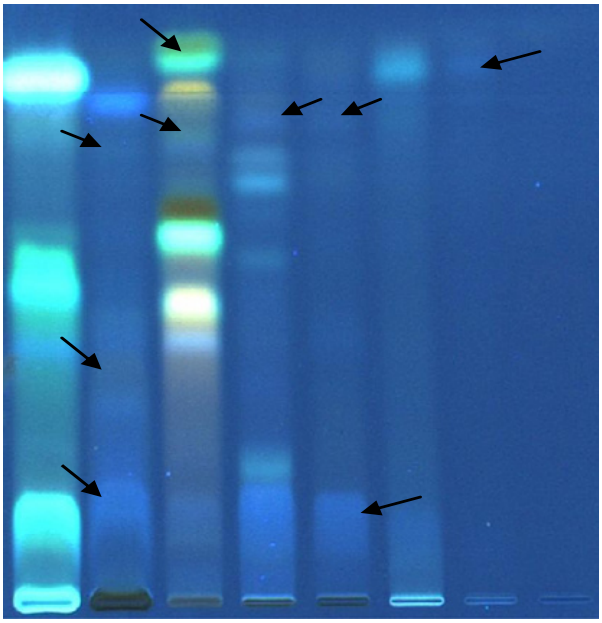

Figure 2. TLC separation of maturated brandies on silica gel 60 F_{254} with ethyl acetate: methanol: water (7.7: 1.3: 1, v/v/v) as mobile phase. Derivatization with NP/PEG, UV 366nm. Control sample is represented by plums brandy before maturation, obtained by concentration of initial extracts 20 times by evaporation, lyophilization and suspension of the residue in ethanol.

The separation shows that the largest number of separated compounds are found in the brandy maturated in mulberry, followed by brandies maturated in oak and cherry. Furthermore, a distinction regarding the number of separated compounds and their concentration can be done. The specific compounds presented only in the particular brandy are marked with arrow on the chromatogram. All these characteristics of TLC separation allow the differentiation of analyzed samples according to the wood and can be used as fingerprinting of maturated brandies.

DPPH and Folin-Ciocalteu reagent based assays

Figure 3 reveals a close correlation between the DPPH assay, which is widely used for biologically relevant antioxidant capacity evaluation, due to its simplicity and low cost, and total polyphenolic content, determined by Folin-Ciocalteu assay. Both methods are based on electron transfer mechanisms and are suitable for aqueous systems.

The simple end-time measurement of DPPH percentage bleached used for the analyses of the 5 Romanian types of brandy indicates that the highest polyphenolic contents appears to be in the case of the plum brandy. No significant differences were obtained between the apple brandy and the mixed sample (apple + plum mixture); the lowest concentration of polyphenols was found in the corn brandy. Also, kinetic measurements were employed to compare a plum brandy sample (not maturated with wood) and commercial whisky. Similar results were obtained measuring the area under the curve (described in Methods section) between plum brandy (553±5) and whisky (551±1) and a slight difference was shown in the case of end point experiments (0.048±0.006 for the plum brandy and 0.075±0.001 for whisky). Taking into account that plum brandy samples had not been maturated yet with wood, the antioxidants in this brandy originate mainly from consumable plant materials, not from wood hydrolysis – which may in principle be considered an advantage over other European distilled beverages such as whisky or vodka.

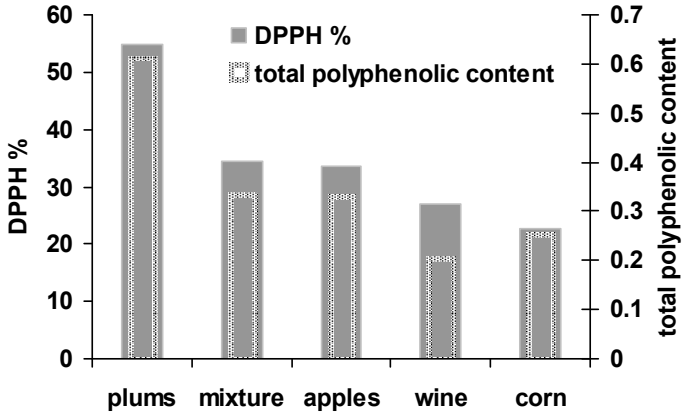

Figure 3. DPPH based assay and Folin-Ciocalteu data for the 5 types of Romanian brandy.

TEAC assay

In Fig. 3 the antioxidant capacity of the aqueous and ethanolic extracts of the brandies is analyzed, using the simplest and cheaper test TEAC, which is based on the ability of antioxidants to react directly with ABTS$^{\bullet}$ radical. The extracts, obtained after lyophilization and resuspension in 20-time smaller volumes of solvent (either water or alcohol) would be expected to contain a higher concentration of antioxidants (10-20 times) than the original brandy samples.

Kinetic measurements were performed measuring both the difference of the absorbance, which is directly correlated with the antioxidant capacities of the samples and area under the curve, which is inversely correlated with the antioxidant capacities. Thus, for the first case, (Fig. 4 A) the ethanolic extract displayed measurable amounts (in line with DPPH and Folin-Ciocalteu assays on the intact brandy), much larger than the water extracts.

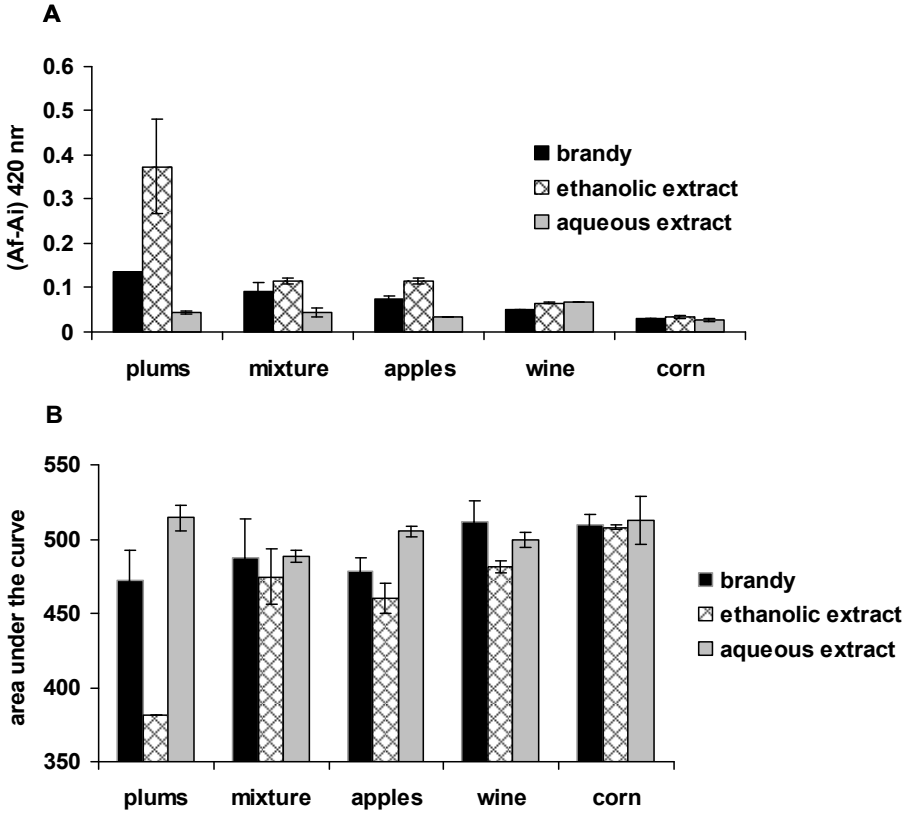

Figure 4. ABTS assay reflected in total change in absorbance (A) and area under the curve (B), obtained for aqueous and ethanolic extract of 5 types of Romanian brandy.

A good correlation could be observed between the difference of absorbance and the second parameter used for the characterization of antioxidant capacities - area under the curve shown in Figure 4 B. Although one may have expected a higher antioxidant capacity of the ethanolic extract, due to the increased concentration of original solution (20 times), this could not be observed - most probably due to loss of antioxidant content during the

lyophilization process. Indeed, one must consider that since these brandies are obtained via distillation, the majority of the compounds would also be volatile (of the type illustrated in Table S3 in Supporting Information) – or at least predisposed to being carried by vapors of the volatile compounds. Reference [35] does in fact list a few compounds that, while volatile, may also be targets for free radicals and hence act as antioxidants.

The characteristic parameters of the kinetic curve, fitted using a bi-exponential function, are described in Tab. S1 (Supporting Information). Thus, two concomitant processes of different rates occur during this reaction, most probably due to the structure of the antioxidants, reflected in a different rate consuming of $ABTS^{\bullet}$ radicals.

Kinetics data of the consumption of ABTS radical by polyphenols, obtained by EPR measurements fitted using a mono-exponential function (due to the different time scales between this experiment and UV-vis experiment), show a good correlation with UV-vis experiments for plums and corns brandy (Figure 5).

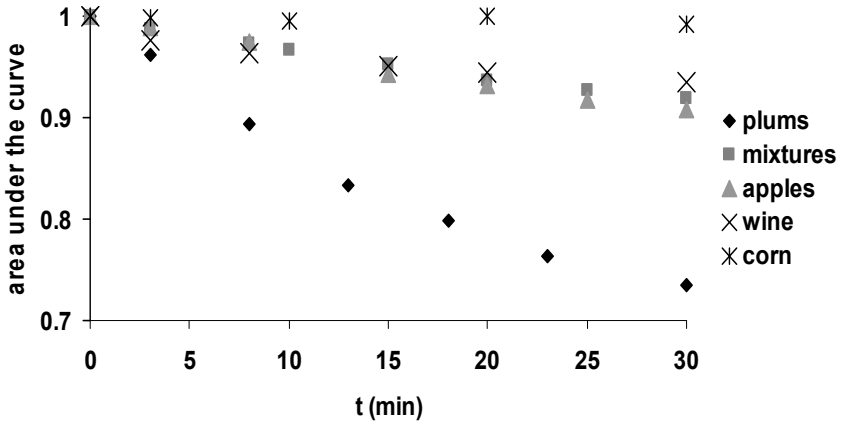

Figure 5. Kinetic of the consumption of $ABTS^{\cdot+}$ radical during an EPR experiment for 5 type of Romanian brandy. Area under the curve was normalized and corresponded to the integration of each EPR spectrum.

Effects of maturation

UV-vis spectra of maturated brandies with wood show an increase in the intensity of absorbance, especially in the case of mulberry tree, compared with original sample (Figure 6). A good similarity can be observed for oak and cherry spectra. One would expect a correlation between the UV-vis characteristics and the antioxidant capacity.

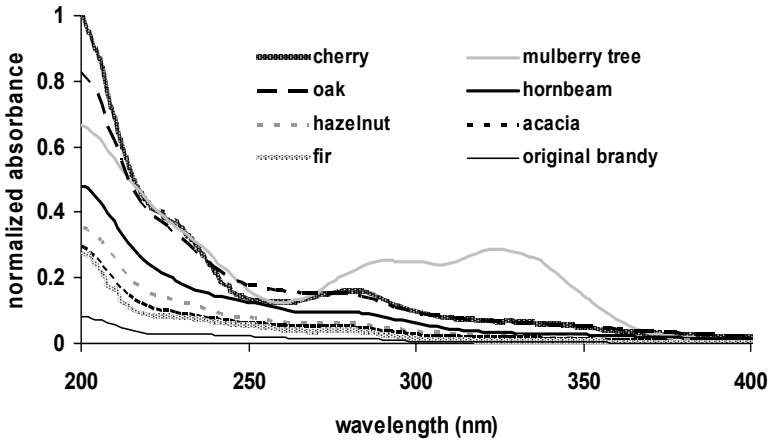

Figure 6. UV-vis spectra of the maturated brandies.

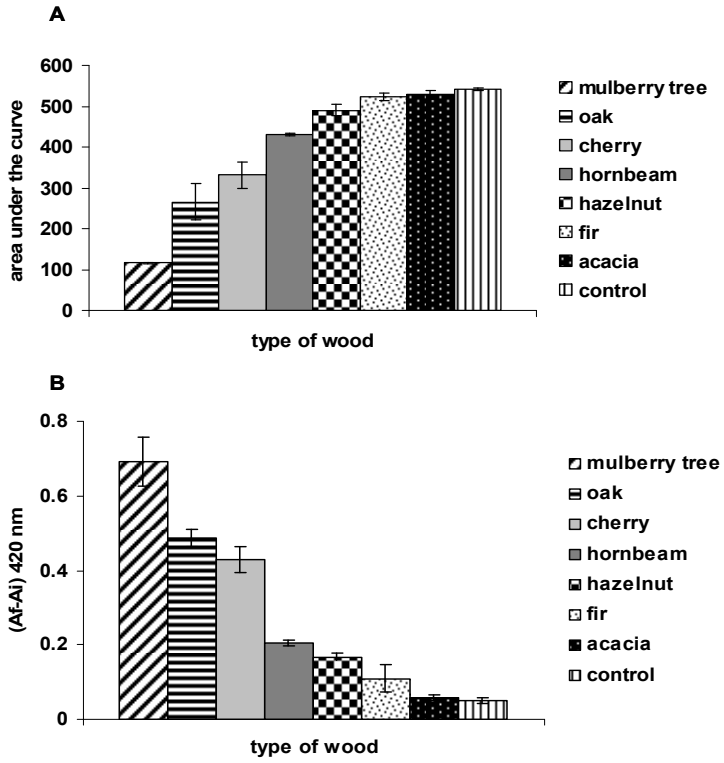

Figure 7. The effect of different wood used in the maturation process of plums brandy, reflected in the ABTS parameters: total change in absorbance (A) and area under the curve (B).

Indeed, a significant improvement of the antioxidant capacity (compared to original samples) was achieved after the maturation of brandies using different pieces of wood species. The results presented in Fig. 7 (kinetic parameters fitted using a bi-exponential function are presented in Supplementary Information, Table S2) show that the best improvement, in agreement with the chromatogram shown in Figure 2 and UV-vis spectra shown in Figure 6, was obtained for mulberry tree followed by oak. Acacia and fir woods proved to have the lowest influence on this process.

HAPX assay

A new physiologically relevant method proposed for the evaluation of antioxidant capacities is based on the ascorbate peroxidase activity of hemoglobin, as described in detail in [38-44]. Under some natural (physical effort) or pathological conditions such as haemolytic anaemias, subarachnoid

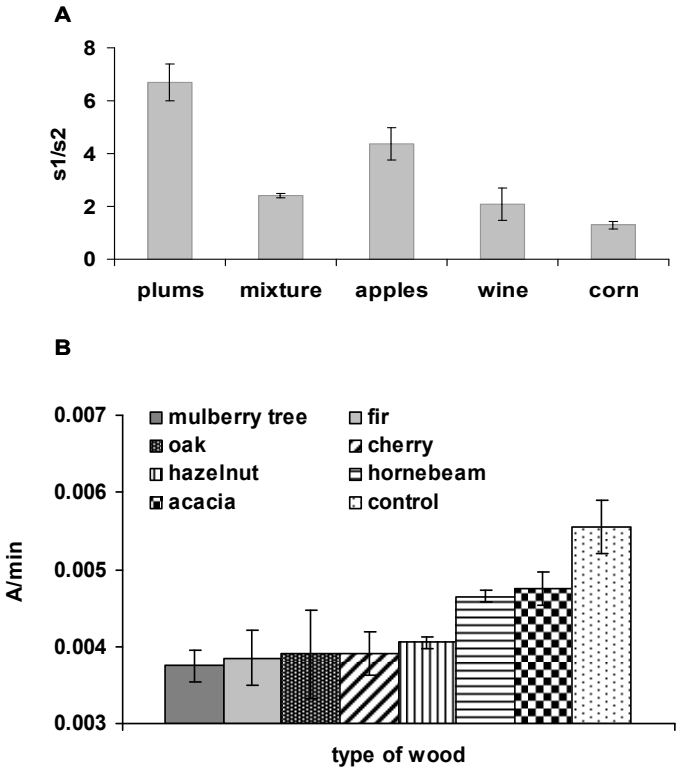

Figure 8. A. HAPX results reflected in the change of slope at 290 nm, for the Romanian analyzed brandies. S1/s1 represents the ratio between slope 1 and slope 2. B. HAPX assay results reflected in the change of slope at 290 nm, for the plums brandy maturated with different types of wood.

haemorrage and rhabdomyolysis, globins can initiate free radical chemistry, in particular by interaction of the ferric form of hemoglobin (met) with peroxide yielding two strong oxidants: ferryl iron and a protein-bound free radical. Ascobate has the ability to reduce plasma methhemglobin, ferryl hemoglobin and globin radicals. Other antioxidants compounds present in the sample can also interact with these highly oxidant species during the catalytic cycles of these reaction; these easily could be observed in the inhibition of ascorbate hemoglobin dependent oxidation [45].

HAPX applied for Romanian brandy shows a good correlation with traditional methods used for evaluation of antioxidant capacity (ABTS, DPPH) (Figure 8 A). Plums sample was proved to have the best antioxidant capacity, and corn brandy the lowest. The results obtained using this method for the maturated samples are shown in Figure 8 B. With some few exceptions, a good correlation can be observed between the results obtained here and in the ABTS test. Thus, the higher antioxidant capacity was achieved for the brandy maturated in the presence of a piece of mulberry tree species, followed, unlike on the ABTS results, by the fir wood, oak and cherry. The lowest antioxidant capacity was observed, as in the ABTS experiments, for the acacia wood.

Cell culture tests

The antioxidant contents of various foodstuffs is often cited as indicative of health-promoting effects; alcoholic beverages make no exception, as reviewed in the Introduction. Biological tests were in this context performed on 20-fold concentrated extracts of the plum brandy, following methodologies described elsewhere [46]. Unfortunately, no anticancer effect was detected – nor was any other protective or toxic effect noticed.

CONCLUSIONS

Reported here is a first detailed analysis of the antioxidant activity in traditional fruit-derived distilled alcoholic beverages from Romania (“țuica”). By far, the highest activity is seen in the most common type of such brandy, made of plums. In fact, these values are, even before maturation with wood, similar to those of commercially-available wood-maturated whisky. Increases of up to one order of magnitude in antioxidant activity were seen in all brandies upon maturation with various types of wood – with mulberry tree by far the most efficient, followed by oak. Attempts to identify anticancer activity in concentrated extracts prepared from plum brandy were not successful. Copper electron paramagnetic (EPR) signals are shown for the first time in such brandy samples.

EXPERIMENTAL SECTION

5 types of brandy (from plums, mixture of plums and apples, apples, wine and corn) produced simultaneously with a similar alcoholic concentration were collected directly from a producer originating from the Bistrita-Nasaud county in Romania. From the same region, 7 pieces of different species of wood were collected from mulberry tree, oak, hazelnut, acacia, fir, cherry and hornbeam. Whisky (commercial brand, 40 vol % alcohol) was obtained from a local store.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), DPPH, and ascorbic acid were purchased from Sigma-Aldrich (Germany). The Folin-Ciocalteu reagent (standard solution 2 N, 1: 10 dilution) was obtained from Merck KGaA (Germany). Bovine hemoglobin, purified according to the Antonini and Brunori procedure [47], was oxidized to met hemoglobin (metHb) by ferricyanide treatment.

Silica gel $60F_{254}$ thin layer chromatographic (TLC) plates (10x10cm) were purchased from Merck, Darmstadt (Germany). NP (Natural Product) was prepared by dissolution of 1 g of diphenylboronic acid aminoethyl esther in 200 mL ethyl acetate; PEG solution was prepared by dissolution of 10 g polyethylene glycol 400 (Macrogol) in 200 mL dichloromethane.

Brandy aging (maturation) occurred in the presence of a piece of wood (3 cm^3), in plastic containers, at 4ºC, during 24 hours. Then, the samples were decanted and analyzed. Selected samples of brandies were concentrated on a water bath at 40^{o}C under mild vacuum (water suction), until the final volume of sample was decreased 20-fold. The obtained extracts were lyophilized and then, the residues were suspended in ethanol and water, respectively.

Thin layer chromatography

20 μL of each concentrated brandy samples and those obtained by maturation with different types of wood were applied as 8mm bands with a rate of 30nL/s to the TLC plate using a semi-automatic applicator device (Linomat V, Camag). The plates were developed in normal chromatographic twin trough chamber (Camag) pre-saturated for 30 minutes with mobile phase - ethyl acetate: methanol: water (7.7: 1.3: 1, v/v/v). The dried developed plate was heated at 1000C for 3 min, dipped while still hot in the NP solution, dried in cold air and then immersed in the PEG solution. The plates dipping were performed using an immersion device (Camag). The detection was performed under UV light (254 nm and 366 nm) and under visible light, before and after the dipping of the plates in the NP/PEG solutions and the documentation of the plates was performed using a TLC vizualizer device (Digistore 2 - Camag).

The total polyphenolic content was determined using the Folin-Ciocalteu assay, described in [45]. For each sample, 1.5 mL Folin-Ciocalteu reagent were added to 1 mL brandy, and samples were incubates in the dark for 5 minutes. Then, 1.2 mL sodium carbonate (0.7 M) were added and samples were incubated in the dark for 2 h, at which point the solution turned deep blue to various degrees, depending the sample. The absorbance of the blue samples was recorded at 760 nm, on a Cary 50 UV-vis spectrometer (Varian).

DPPH assay

A stock solution of 0.09 mg/mL was prepared. 1 mL of DPPH solution was added to the 2 mL brandy and the samples were incubated for 15 minutes at room temperature, followed by measurement of the absorbance at 517 nm. Besides the end-point experiment, kinetics measurements were also performed for 30 minutes, at 517 nm. Typical decay curves were obtained for every sample, allowing one to calculate the total change in absorbance (ΔA), which is directly correlated with antioxidant capacities of the samples, as well as the calculation of the area under the curve, which is inverse correlated with the antioxidant capacities.

Trolox equivalent antioxidant capacities (TEAC) assay

The ABTS radical was enzymatically obtained by 2 hours of incubation of 2 mM reduced ABTS solution in 5 mM sodium acetate pH 5.5, with 50 nM horseradish peroxidase and 1.3 mM hydrogen peroxide. The radical was separated from the enzyme using a 10 kDa cut-off amicon filter. For this assay, kinetic measurements were performed. In a quartz cuvette, 50 µl ABTS radical was added to the 900 µl PBS buffer and 10 µl brandy. The decrease of the ABTS radical absorbance was monitored at 420 nm. As in the DPPH methods, the evaluation of the antioxidant capacities of the sample can be obtained using the total change in absorbance as well as via the calculation of the area under the curve.

The inhibition of hemoglobin ascorbate peroxidase activity (HAPX) assay was conducted as in [45]. For this experiment, kinetic measurements were performed. Thus, in a quartz cuvette, 14 µl metHb of 1mM, 13 µl peroxide of 60 mM and 10 µl were added to 952 sodium acetate buffer, pH 5.5 - after which the absorbance changes were monitored at 290 nm. After absorbance stabilization, the reaction was triggered by the addition of 50 µl ascorbate of 50 mM. The kinetic profile for ascorbic acid degradation after the addition of met-Hb, was linear for least 100 s. For the evaluation of the hemoglobin ascorbate peroxidase activity, the slope of the linear decrease was calculated (corresponding to the oxidation of ascorbate).

EPR measurements were performed as described in [46].

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