ISSN : 0974 - 7508 Volume 11 Issue 3

Natural Products

An Indian Journal

Full Paper

NPAIJ, 11(3), 2015 [081-089]

Antioxidant and inhibition of xanthine oxidase potentials of fluidized bed extracts from leaves of the ash tree

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ABSTRACT

The aim of the study was to determine both the antioxidant and inhibition potentials of the activity of xanthine oxidase by ethanol and methanol extracts in a fluidized bed of leaves of the ash tree. In traditional medicine, this plant is well known for its anti-inflammatory properties, especially against gout. The best results were obtained with methanol extract in the case of evaluation of antioxidant potential, and with ethanolic extract in reference to the inhibition activity of xanthine oxidase. The values calculated for different methods of valuation *in vitro* were correlated with the phytocontent of the two lyophilized extracts.

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KEYWORDS

Ash leaves;
Phytocontent;
AAPH;
Phytopharmacological value.

INTRODUCTION

The common ash tree (*Fraxinus excelsior*) is a common tree found in forests and, especially, in the rural plains of Romania. It belongs to the *Oleaceae* family. Owing to its abundance and rapid growth it is extensively used in the furniture industry. The bark and, especially, leaves are used for therapeutic purposes. In traditional medicine, it is known for its anti-inflammatory, antirheumatic, healing, and diuretic properties. The leaves are mainly used for making tea, or in one's bath water (for external use only). In recent years, capsules containing dried chopped leaves, recommended for their anti-inflammatory effect, anti-diabetes, and cardiovascular protective effect have appeared on the market.

Leaves of the ash tree contain terpenes, flavonoids (sophoretin, cvercetrol), organic acids (malic, ursolic, and tannic acids) and vitamin C. Recent research has shown that extracts made with hexane showed strong antimicrobial effects and even antioxidant capacity. They are associated with the presence of phenols and flavonoids[1]. The scavenging capacity of DPPH radical is dependent on concentration[2]. Thus, the antioxidant properties of the ash leaves are a new finding in the validation of empirical knowledge about the therapeutic properties of the ash. The objective of this study was to investigate the influence of solvents (ethanol and methanol) on antioxidant activity and xanthine oxidase inhibition. Alcoholic extracts were obtained by extraction in a fluidized bed. Furthermore, we investigated the influence of the extracted solvent on phytochemical composition, and its correlation with the determined antioxidant activity.

MATERIALS AND METHODS

Chemicals

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All chemicals and reagents were purchased from Sigma, Aldrich GmbH, Sternheim, Germany. All other unlabelled chemicals and reagents were of analytical grade.

Plant material and fluidized bed extraction process

The dried leaves of *F. excelsior* were provided by SC Stef Mar SRL, Valcea, România. A quantity consisting of 100 g of the powdered leaves and 150 mL ethanol/methanol 70% was used to generate an extract using a fluidized bed extractor (fexIKA 200, IKA Labortechnik), after two extraction cycles (Figure 1). The extract was freeze-dried (Martin Christ Christ Alpha 1-2 LD) to obtain the dry extract. The alcohol extracts were concentrated in a rotary evaporator (Buchi R 210) with vacuum controler at 50 ^{0}C, 175 mbar and 200 rpm. The elected concentrated solution was freeze-dried in a Martin Christ Christ Alpha 1-2 LD, to obtain the solid substance. The dried fractions were then re-dissolved in 80% ethanol to yield solutions containing 0.2–1.0 mg of extract per mL[3].

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

A quantity consisting of 0.8 mL of 0.2 mM DPPH solution was mixed with a 0.2 mL different concentration of the extracts (0.2-1 mg/mL). The mixture was shaken and left to stand for 30 min. The absorbance was measured at 517 nm using a Helios λ spectrophotometer. The DPPH radical scavenging activity (%) was calculated with the following equation: 1 – (As/Ac)×100, where As is the absorbance in the presence of sample and Ac is the absorbance in the absence of sample. Tert-Butylhydroquinone (TBHQ) was used as standard. The EC_{50} value (mg extract/mL), being the effective concentration at which the DPPH scavenging effect being 50%, was obtained[4].

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging assay

Radical scavenging assay ABTS radical cations are produced by reacting ABTS (7 mM) and potassium persulfate (2.45 mM) on incubating the mixture at room temperature in darkness for 16 – 24 h. The solution thus obtained was further diluted with ethanol to give an absorbance of 1.000. Different concentrations of the extracts (0.2-1 mg/mL), 50 µl, were added to 950 µl of the ABTS working solution to give a final volume of 1 ml. The absorbance was recorded immediately at 734 nm with the Helios λ spectrophotometer. The percentage of inhibition was calculated with the following equation: % inhibition = [(Absorbance of control –Absorbance of test

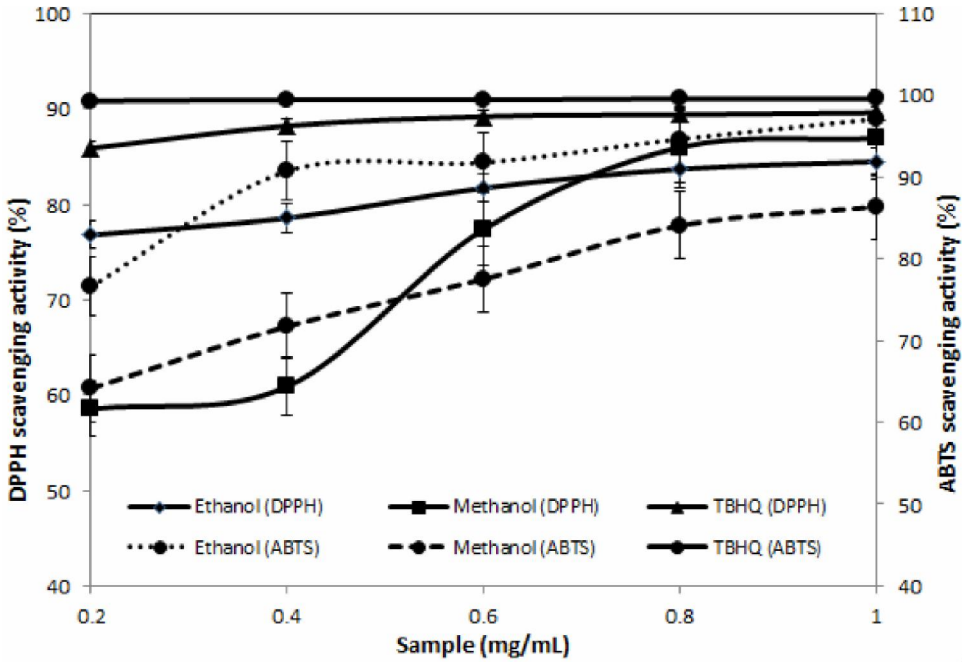

Figure 1 : DPPH and ABTS radical scavenging activity of leaves of the ash tree extracts

sample)/Absorbance control] x100. TBHQ was used as standard. The EC_{50} value (mg extract/mL), being the effective concentration at which the ABTS scavenging effect being 50%, was obtained[5].

Determination of hydroxyl radical scavenging activity

Quantities consisting of 0.2 mL of 0.1 mM $FeSO_4$/ 0.1 mM EDTA·2Na, 0.2 mL 2-deoxyribose (10 mM), 0.2 mL sample (different concentration 0.2–1 mg/ mL), and 1.2 mL phosphate buffer (0.1 M; pH 7.4) were mixed. After the addition of 0.2 mL H_2O_2 (10 mM), the mixture was incubated at 37 °C for 4 h, and the reaction stopped by addition of a 1 mL trichloroacetic acid (2.8%) solution. Thiobarbituric acid/50 mM NaOH (1%; 1 mL) was then added and the mixtures heated at 100 °C for 10 min, followed by rapid cooling and measurement of OD_{532}[6].

Superoxide radical scavenging assay

The reaction mixture contained the same volume of 120 μM PMS (phenazine methosulfate), 936 μM NADH, freeze-dried extract, and 300 μM NBT, in a total volume of 1 mL of 100 mM phosphate buffer (pH 7.4). After 5 min of incubation at ambient temperature, absorbance of the resulting solution was measured at 560 nm. The superoxide radical activity was calculated as: scavenging effect (%) = (1 - absorbance of sample/absorbance of control) × 100. Ascorbic acid was used for comparison. EC_{50} value (milligram extract/mL) is the effective concentration at which superoxide radicals were scavenged by 50%[7].

Ferrous ion chelating assay

1 mL of the sample (0.2–1 mg/mL) was mixed with 3.7 mL of ultrapure water, following which the mixture was reacted with ferrous chloride (2 mmol/ L, 0.1 mL) and ferrozine (5 mmol/L, 0.2 mL) for 20 min. The absorbance at 562 nm was determined spectrophotometrically. EDTA was used as positive control. The chelating activity on the ferrous ion was calculated using the equation below: Chelating Activity (%) = [(Ab-As)/Ab]×100, where Ab is the absorbance of the blank without the extract or EDTA and As is the absorbance in the presence of the extract or EDTA[8].

Inhibition of human erythrocyte hemolysis

The capacity to inhibit human erythrocyte hemolysis was based on the method described by BARROS et al.[9]. Blood was obtained by harvesting from the author. Blood tubes were immediately centrifuged at 3000 rpm for 10 min in a cooled Heidolff 320R centrifuge, with cooling at 9 °C. The sediment was washed three times with 0.9% NaCl, and the reunited final sediments were brought into a 10% solution in 7.4 phosphate buffer. The reaction mixture consisted of 0.1 mL of 10% human erythrocytes suspension, 0.2 mL of 200 mL 2,2 '-azo-bis (2-amidinopropane) dihydrochloride, and 0.1 mL sample of extract (0.2–1 mg/mL). Test tubes were maintained at 37 °C for 3 h. For dilution, 8 mL phosphate buffer pH 7.4 was added and each sample was centrifuged at 3000 rpm for 10 min. Finally, absorbance was read at 540 nm, and the inhibition of human erythrocyte hemolysis was calculated following the equation: $[(A_C–A_S)/A_C]\times100$, where A_C represents the absorbance of the control sample without extract, and A_S is the absorbance of the sample containing the extract. TBHQ was used as standard. The EC_{50} value (mg extract/mL), the effective concentration at which the inhibition of human erythrocyte hemolysis being 50%, was obtained[10].

Anti-inflammatory activity

In vitro anti-inflammatory activity was performed similary to previous studies[11].

Determination of phytochemical content

Total phenolic content

The amount of total phenolic compounds was determined colorimetrically with Folin-Ciocalteu reagent. Gallic acid was used as the reference standard and the results (total phenolic content) were expressed as gallic acid equivalents (GAE) in grams per gram extract[12].

Total flavonoids content

Into 0.5 mL of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid content was calculated as quercetin equivalents (mg/g) using the calibration curve[13].

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Determination of proanthocyanidins content

A volume of 0.5 mL of 0.1 mg/mL of each extract solution was mixed with 3.0 mL of 4% vanillin in methanol and 1.5 mL hydrochloric acid, the mixture was allowed to stand for 15 min at room temperature, and the absorbance was measured at 500 nm. Total proanthocyanidin contents were expressed as catechin (mg/g) using the following equation of the curve: $Y = 0.5825x$, $R^2 = 0.9277$, where x is the absorbance and Y is the catechin equivalent[14].

Determination of saponin content

One gram of each extract was mixed with 20 mL of 20% ethanol. The mixture was heated at 55 °C for 4 h with continuous stirring, and at the end it was filtered under vaccum. The mixtures were concentrated in a rotary evaporator (Buchi R 210) with vacuum controller, to 4 ml at 90 °C. The concentrate was mixed with 2 mL diethyl ether. The ether layer was discarded. Then, 6 ml of n-butanol was added and the extracts were washed with 1 mL of 0.9% sodium chloride. The sample was dried in the Memmert oven to a constant weight. The saponin content was calculated according to the equation: amount of saponin (mg/g) = weight of residue/weight of sample[13].

Determination of alkaloids content

A quantity consisting of 0.5 g of the extract was mixed with 20 ml of 10% acetic acid in ethanol and allowed to stand for 4 h. This was filtered and the extract was concentrated in a waterbath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The precipitate was isolated and washed with ammonium hydroxide and then filtered under vaccum. The alkaloid content was calculated according to the equation: amount of saponin (mg/g) = weight of residue/weight of sample[13,15].

Ascorbic acid content

Determination of ascorbic acid was performed using the method described by Barros et al[9].

Statistical analysis

All the assays for fermentation and antioxidant activity were assessed in triplicate, and the results were expressed as mean ±SD values of the three sets of observations ($p < 0.05$). The mean values and standard deviation were calculated using the EXCEL program from Microsoft Office 2010 package.

RESULTS AND DISCUSSION

Following extraction in a fluidized bed, this resulted in an extraction yield (in reference to the quantity, in grams, of resulted lyophilized extract) of 18.81 ± 0.33% for ethanol and 18.23 ± 0.47% for methanol. These results are confirmed by previous studies, to the detriment of other solvents, such as chloroform and petroleum ether[16]. Comparing the results with those obtained after simple extraction, it was shown that the resulting extraction yield following extraction in a fluidized bed increases by approximately 60%[17].

DPPH and ABTS radicals' scavenging activity

As shown in Figure 1, methanol extract showed the highest DPPH scavenging activity, while ethanolic extract showed the highest ABTS scavenging activity. The comparative analysis showed that differences between the two extracts, in reference to scavenging activity of these two free radicals were, on average, 6%. In the determinations performed, the color of the reaction mixtures had faded due to the scavenging activity of DPPH and ABTS radicals, and was directly correlated with the concentration of the samples. Following the comparative analysis, it was noted that the high capacity of inhibition of free radicals generated by the two types of extracts, the process takes place through the donation of a hydrogen atom[18,19]. In comparison with the standard (TBHQ), the value of EC_{50} was roughly similar, ranging between 0.07 and 0.1 mg/mL (unpublished data). The results obtained by analyzing the antioxidant potential of ethanol extract confirm earlier studies regarding the fact that DPPH scavenging activity shows lower values when compared with ABTS scavenging properties. In this case, the differences were approximately 13%, being caused by the polarity of the solvent that caused differences in phytochemical composition. Thus, the evaluation

method that uses ABTS radical is preferred because it is much faster and is appropriate for the evaluation of extracts of natural products[20]. These records were confirmed by the good value of the ethanolic extract for DPPH and ABTS assays that was 0.781. Instead, the value of R^2 corresponding to the two methods of analysis of methanolic extract of ash tree leaves was 0.9313.

Chelating abilities and inhibition of human erytrocyte hemolysis

The results obtained so far have indicated that the phenolic fraction of the phytochemical composition of an extract is responsible for the inhibition of the processes favoring lipid peroxidation, but also for the inhibition of peroxidation of membrane phospholipids. Although the mechanism of action has not been fully deciphered, there are studies that consider it as being associated with a series of biophysical processes acting on the reactions generated by free radicals[21]. Oxidative stress acting on human erythrocytes is determined by the generation of peroxyl radicals, resulting in membrane lipid peroxidation, a process that is completed by hemolysis[22]. AAPH-induced oxidation *in vitro* determining erythrocyte hemolysis, correlated with iron capacity of chelation is shown in Figure 2. Methanol extract of ash tree leaves showed a higher capacity of inhibition of biological processes that favor lipid peroxidation. If we compare the value of EC_{50}, the difference when compared with the ethanolic extract of ash tree leaves is ≈50% for the effect of chelation of iron. Instead, the direct action manifested by the oxidative process on membrane lipids was found to have a value between 1.0 and 1.3 mg/mL, of $EC_{50.}$ Compared with TBHQ value which was ≈10 times lower, shows an accurate picture of pharmacological potential represented by existing active principles in the extract from the leaves of *Fraxinus excelsior*. The effect on the oxidation process of membrane lipids was also demonstrated by calculating R^2, which was 0.8236 for the ethanolic extract of ash tree leaves and 0.9462 for the methanolic extract of ash tree leaves.

Superoxide and hydroxyl radicals' scavenging activity

The two species of free radicals are some of the most reactive, being extremely harmful, with direct action on key molecules at the cellular level. Deterioration at the level of tissues represents the major cause of diseases caused by it, and is manifested by dysfunction of the affected organ. The most severe pathology is cancer, when genetic material is affected, which is found most often in the action of hydroxyl radicals[23]. Figure 3 shows the scavenging activities for the two species of free radicals, not resulting in a significant difference of 1 mg/mL for

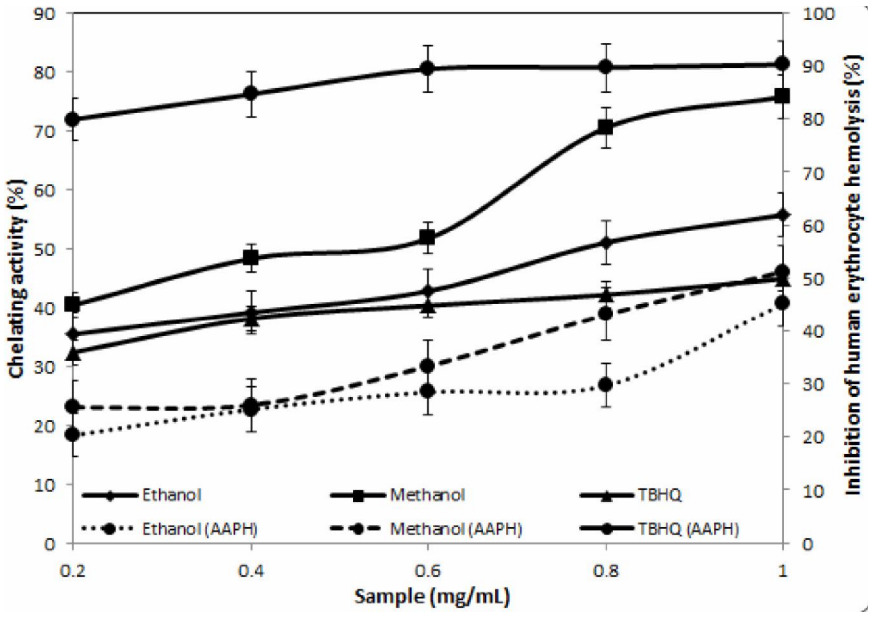

Figure 2 : Chelating effect and inhibition of erythrocyte hemolysis of leaves of the ash tree extracts

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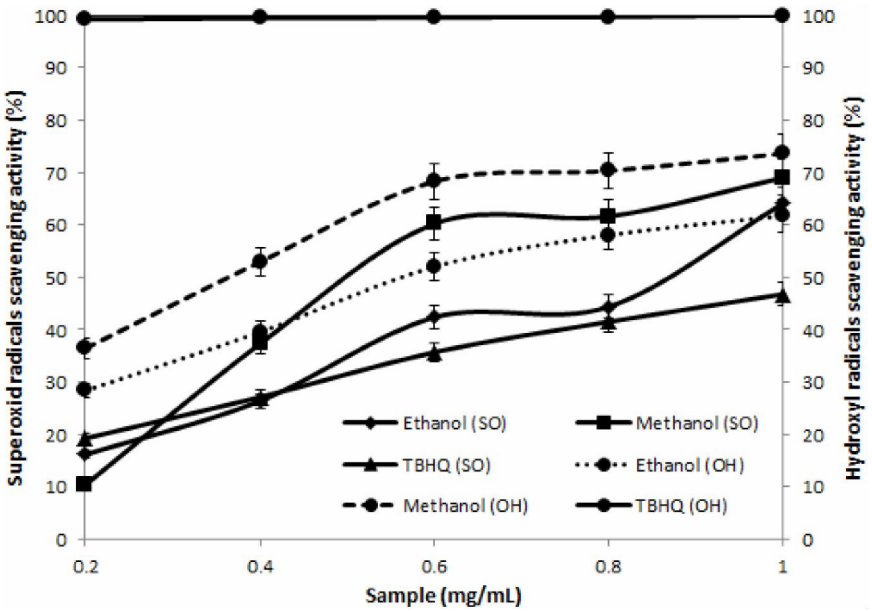

Figure 3 : Superoxide and hydroxyl radicals scavenging activity of leaves of the ash tree extracts

the two activities determined, ethanolic extract of ash tree leaves, and of ≈ 8% for methanolic extract of ash tree leaves. This difference was also observed for EC_{50} values, which fell, on average, by 60% for methanol extract of ash tree leaves. Hydroxyl scavenging activity showed an average of 0.42 mg/mL, compared with 0.67 mg/mL for superoxide radicals scavenging activity (methanol extract of ash tree leaves). Generally, the ethanolic extract of ash tree leaves had the same tendency, with an average value for EC_{50} of 0.63 mg/mL (hydroxyl scavenging activity) and 0.86 mg/mL (superoxide scavenging activity). For superoxide radicals, a significant difference is noted compared with the standard whose EC_{50} exceeds 1 mg/mL. The correlation coefficient expressed as R^2 value was 0.9079 (ethanolic extract of ash tree leaves), and 0.9982 (methanol extract of ash tree leaves), having a strong correlation that demonstrates a high capacity to combat oxidative damage expressed by the action of the two species of free radicals.

Xanthine oxidase inhibition activity

The infusion of ash leaves, as well as the gemotherapeutic extract of ash tree buds, are known in phytotherapy as a natural remedy of hyperuremia that leads to gout. By its anti-inflammatory action at the level of joints, it is considered to be an effective remedy against the accumulation of uric acid. According to the website http://medlive.hotnews.ro hyperuremia is a risk factor for atherosclerosis. Epidemiological studies have shown an association between hyperuricemia and coronary heart disease, because the increased value of serum uric acid is a direct indicator of oxidative stress[19,24]. In Figure 4, an increase of the inhibition activity of xanthine oxidase that was proportional to the increase of extract of ash tree leaves concentration was noted. For a concentration of 1 mg/mL of the ethanolic extract of ash tree leaves, the value obtained was ≈17.3% higher than that of the methanolic extract of ash tree leaves but, on average, 6% lower than that of standard (allopurinol). From previous studies, it was confirmed that the inhibitory effect exercised over xanthine oxidase corresponds to the level of flavonoids, which is the main component responsible for this inflammatory response[25].

Phytochemical analysis

Phenolic compounds are secondary compounds, which are present in extracts from medicinal plants, or with antioxidant potential. The biological activity of the extract analyzed is of prime importance. Generally, their action is manifested by the power of their scavenging activity against different free radicals, which is found in the form of various properties that determine the biological value of the extract analyzed. Figure 5 shows, in parallel, the total

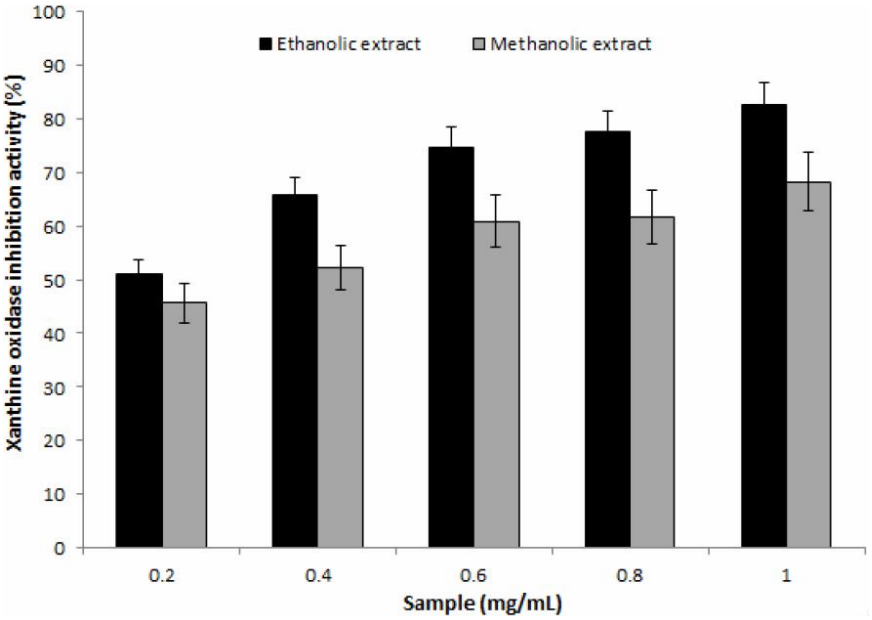

Figure 4 : Xanthine oxidase inhibition activity of leaves of the ash tree extracts

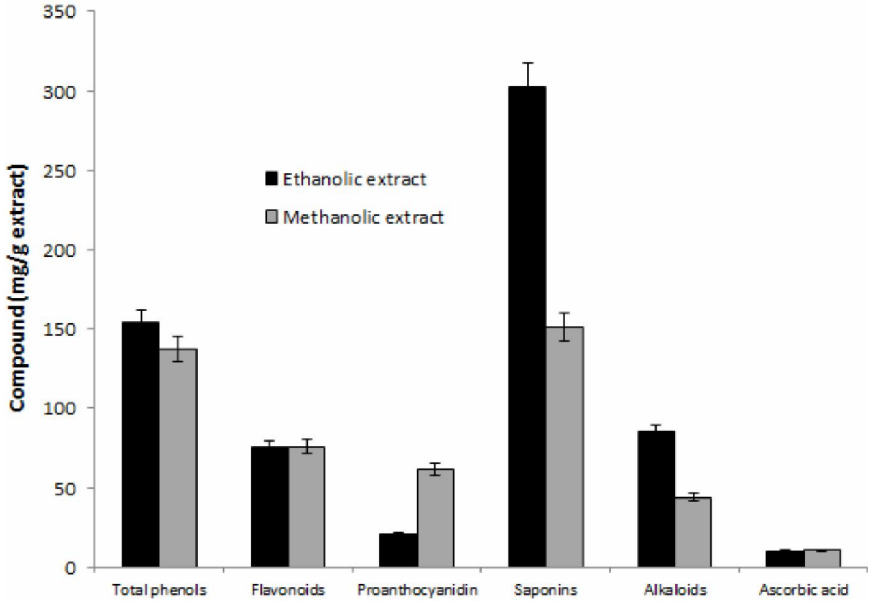

Figure 5 : Phytochemical analysis of leaves of the ash tree extracts

quantity of phenols in the two extracts. A difference of ≈12.7% was noted in favor of the ethanolic extract of ash tree leaves (154 mg gallic acid/g extract). Among phenolic compounds, the group considered as predominant in most researches, that is, flavonoids, is responsible *in vitro* for the antioxidant properties higher than those of ascorbic acid, or α-tocopherol[26]. For these compounds, the resultant quantity obtained consequent to extraction in a fluidized bed, presented similar values (p <0.05). Proanthocyanidins were the second group of phenolic compounds that were determined. Differences were significant in this case, with methanol extract of ash tree leaves having in its composition a three times higher amount of proanthocyanidins (p <0.03). The differences correspond to data in the literature showing the importance of these compounds in the inhibition of reactive oxygen species, especially of the *peroxyl radical*[27].

Other components that were identified as possessing biologically active action are represented by alkaloids and saponins in large quantities, which

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can cause allergies and even toxicity. The importance of saponins has been shown in animal studies where they had an effect of reducing cholesterol and triglycerides. In the study performed, saponins represented the main phytocomponent determined. Ethanolic extract of ash tree leaves showed a 50% higher amount when compared with the methanolic extract of ash tree leaves (150.9 ± 2.67 mg/g extract). Instead, the level of alkaloids was ≈70% lower than that of the saponins. The difference between the two extracts of ash tree leaves was approximately 48%, in favor of the ethanol extract of ash tree leaves (85 ± 0.87 mg/g extract). Nevertheless, biological importance has been shown to be higher, being demonstrated in vitro by a significant antioxidant activity, and protection against AAPH action[28].

Another compound found in small quantities was ascorbic acid which has significant antioxidant properties. The difference between the two extracts of ash tree leaves is small, only 2.35% in favor of the methanol extract of ash tree leaves (102 mg/g extract). This small amount can be interpreted by the fact that the stages of the extraction process in a fluidized bed involve the completion of several steps during which temperature can exceed 50^0C, resulting in a degradation of this compound.

In most studies, polyphenolic acids and flavonoids represent the main compounds identified, which are responsible for the scavenging properties against free radicals and the inhibition of factors favoring inflammatory processes. It has been identified as a positive correlation of these compounds with most *in vitro* tests performed. In the case of extraction in a fluidized bed, it has been identified, by contrast, as a doubling of the amount of saponins compared with that of phenolic compounds. This demonstrates the importance of the extraction process in changing the ratio of bioactive molecules of a phytoextract, which is directly correlated with the properties performed *in vitro*. In the study, a determination coefficient was calculated (R^2) corresponding with the scavenging activity of ABTS (0.6964), hydroxyl radicals (0.8109) and inhibition of xanthine oxidase (0.7911) for the ethanolic extract of ash tree leaves, in reference to the phenolic component. The highest correlation coefficient was calculated in relation to the inhibition of erythrocyte hemolysis (0.9563) and the capacity of chelation (0.9458). The correlation coefficient corresponding to methanol extract of ash tree leaves maintained the same tendency, but the values determined were 5%–10% lower. These results showed the different degrees of involvement of these two classes of biomolecules in exercising the antioxidant response. The values obtained do not necessarily correlate directly with the quantity of a certain phytocomponent. Depending on the method used, the expression of phytopharmacological value is analyzed separately, because the action determined is an accumulation of all potential active components.

CONCLUSIONS

In conclusion, the determination of antioxidant and anti-inflammatory potential, correlated with the determination of phytochemical content, demonstrated the biological value of the extract of ash tree leaves in a fluidized bed of ash leaves. The extracts support traditional knowledge related to the pharmacological value of ash leaves. These extracts of ash tree leaves do, therefore, constitute an important ingredient in the manufacture of functional supplements with therapeutic effects against acute inflammatory processes that lead to atherosclerosis.

ACKNOWLEDGMENTS

This work was partially supported by the project PNCDI II CNCSIS—Human Resources, Theme 9/ 2010. Vegetal material was provided by the student, Andreea Mihaela (Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine, Bucharest), for his university graduation thesis.

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