PRELIMINARY RESEARCH REGARDING THE CYTOTOXICITY AND ANTIOXIDANT ACTIVITY OF ARBUTUS UNEDO L. LEAVES

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Abstract

The aim of the study was the selection of optimum extraction procedure of Arbutus unedo L. leaves in order to obtain dry extracts with high content of polyphenols and arbutin. The antioxidant activity, cytotoxicity and genotoxicity of the extracts were also evaluated. Two types of extracts were obtained using refluxation and sonication, respectively. The total polyphenolic compounds were assessed spectrophotometrically with the Folin-Ciocalteu method. Arbutin was detected and quantified by HPLC-DAD. The extracts’ antioxidant activity was investigated using ferric reducing activity and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. The results were expressed as antioxidant activity index (AAI) for DPPH method and as EC50 (μg/mL) for both methods. Cytotoxicity/genotoxicity was verified by the Trigittum (vegetal cell) and Artemia tests (animal cell). The highest content of arbutin, polyphenolic compounds and the highest DPPH free radical scavenging activity were found for the extract obtained by refluxation (32.84 g% polyphenols expressed as tannic acid, 1.92 g% arbutin, AAI = 6.45, EC50 = 43.78 mg/mL). The extract containing the highest concentration in polyphenols is a very strong antioxidant as stated by antioxidant index. Despite the genotoxic effect of both extracts at high concentrations, low concentrations showed no cytotoxicity either on the plant cell or on the animal cell.

Rezumat

Scopul studiului a fost selectarea metodei optime de obținere a unor extracte uscate cu conținut ridicat de polifenoli și arbutină din frunzele de Arbutus unedo. Au fost evaluate activitatea antioxidantă, citotoxicitatea și genotoxicitatea acestor extracte. Două extracte au fost obținute utilizând refluixarea și respectiv sonorarea. Totalul compușilor polifenolici a fost evaluat spectrofotometric prin metoda Folin-Ciocalteu. Arbutina a fost detectată și cuantificată prin HPLC-DAD. Activitatea antioxidantă a fost investigată folosind două metode (DPPH și reducerea ferului). Rezultatele au fost exprimate prin intermediul EC50 (μg/mL) pentru ambele metode. Pentru metoda DPPH s-a calculat și indicele de activitate antioxidantă (AAI). Citotoxicitatea/genotoxicitatea a fost verificată prin testele Trigittum (celulă vegetală) și Artemia (celula animală). Cel mai mare conținut de arbutină, compuși polifenolici și cea mai mare activitate de eliminare a radicalilor liberi DPPH au fost găsite pentru extractul obținut prin refluixare (32.84 g% polifenoli exprimat ca acid tanic, 1.92 g% arbutină, AAI = 6.45, EC50 = 43.78 mg/mL). Extractul care conține cea mai mare concentrație în polifenoli este un antioxidant foarte puternic, după cum indică indicele antioxidant. În ciuda efectului genotoxic al ambelor extracte la concentrații mari, concentrațiile scăzute nu au prezentat citotoxicitate nici asupra celulei vegetale, nici asupra celulei animale.

Keywords: Arbutus, polyphenols, arbutin, antioxidant, cytotoxicity

Introduction

Arbutus unedo L. (strawberry tree) is a species of woody shrub type. It is widespread in the Mediterranean where it grows spontaneously and in many other parts of Europe as a cultivated plant. Currently it is a great interest for seeking micro-propagation species using adult plants in order to acclimation them [11, 12]. The species is known mainly for decorative looks, given that flowers and fruits with different degrees of maturity coexist. The fruits are used for making alcoholic beverages, jams, marmalades or it is freshly consumed (in a lower manner) [35]. Traditional medicine recommended the leaves and fruit of the genus Arbutus as antiseptic, diuretic, laxative and antihypertensive remedy [24, 33].

The chemical composition of the whole plants or various organs was studied in order to exploit its therapeutic properties, because the species products have lower economic importance [22]. Fruits are the most studied organs of the plant. The chemical composition of the leaf is divers, previous studies showing the presence of the following active principles: flavones (quercitrin, isoquercitrin, hyperoside), phenol-
carboxylic acids (chlorogenic acid), procyanidin dimers and other proanthocyanidins, tannins (catechins, gallo-
catechin, ethyl gallate), arbutin (only in leaves, up to 
1.21 g%), sterols, triterpenes, essential oils, fatty acids, 
organic acids and vitamins [22, 27].
 Previous investigations on the leaves of these species are 
modest, but pharmacological research has shown that 
Arbutus unedo extracts have antimicrobial (Candida 
albicans, Enterococcus faecalis), antiparasitic (Trichomonas 
vaginalis) and antihypertensive properties [1, 9, 24].
The search for antimicrobial agents is an urgent need 
emerged from the increased antibiotic microbial 
resistance [38]. In this regard, new sources of substances 
with antimicrobial activity may be found in plants where 
compounds with different antimicrobial mechanism 
may coexist (inhibiting microbial growth, inducing 
cellular membrane perturbations, interfering with certain 
microbial metabolic processes, modulating signal 
transduction or gene expression pathways) [25].
 Recent work shows that a high level of phenolic 
compounds depends on various and environmental or 
extractions factors [3, 4] and is correlated with anti-
inflammatory and antimicrobial activities [10]. In the 
case of Arbutus leaves, Malheiro et al. showed that 
the extracts with the highest antimicrobial activity are 
the richest in phenolic compounds [20]. Another 
phytochemical property that can contribute to the 
antimicrobial activity of strawberry tree leaves is arbutin 
and its’ metabolite hydroquinone [16]. Therefore, in 
order to obtain standardized extracts with antimicrobial 
activity, we have focused on the content of phenolic 
compounds and arbutin.
 Currently, the best source of arbutin is Arctostaphylos 
 uva-ursi, but the plant is protected by law in Romania, 
being considered a natural monument. Hydroquinone 
(the active part of the arbutin) may have genotoxic and 
carcinogenic effects [21], although it was estimated 
that at the usual doses of arbutin commonly used by 
humans it is unlikely to be unsafe [6]; in this context we 
considered useful to evaluate the cytotoxic/genoto-
xic potential of the extracts.

Materials and Methods

Chemicals and Materials
All solvents and reagents were purchased from Carl 
Roth (Germany) and Sigma-Aldrich (Germany).
Arbutin, DPPH and ABTS were purchased from Carl 
Roth (Germany).
Plant material
The leaves of Arbutus unedo were collected in main-
land Greece (Nikaia) during September 2018 and 
October 2018. A voucher specimen (no. 230) was 
deposited at the Herbarium of the Department of 
Pharmacognosy, Faculty of Pharmacy, “Carol Davila” 
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Romania. The herbal products were naturally dried in 
shade and stored in controlled laboratory conditions.

Sample preparation
Microscopic determination. For the microscopic 
analysis, NaOH (5% w/v), carmine alum, green iodine 
and acetic orcein (for phytobiological testing) were 
used. Microscopic preparations were obtained from 
fresh biological material (leaves) by cross sectioning; 
superficial preparations were also analysed. Micro-
graphs were obtained with a Nikon microscope 
Labophot 2.

Spectrophotometric and chromatographic determination.
Two extraction procedures were used: refluxation 
(heating at 60°C using a reflux condenser and a water 
bath) and ultrasonication at room temperature (with an 
Elmasonic S15H ultrasonic bath). For both procedures, 
5 g of powdered leaves were heated/ultrasonicated 
for 30 minutes, with 100 mL of hydroalcoholic mixture 
(water:ethanol = 50:50 v/v). After cooling and cotton 
filtration, the solutions were filled up to 100 mL 
with solvent in a volumetric flask. The extractive 
solutions were codified as AU1 (obtained by heating) 
and AU2 (obtained by ultrasonication).

In vitro genotoxicity and cytotoxicity analysis. The 
genotoxic/cytotoxic potential was tested in vitro on 
Trichium aestivum L. seeds coming from organic crops 
(Râmnicu Sărat, Buzău, Romania) and on Artemia 
salina from Grate Salt Lake (USA). For the in vitro 
genotoxicity and cytotoxicity analysis, the solutions 
were brought to dry residue in a water bath and the 
residue was suspended in distilled water.

Analytical Methods
Total phenolics content assay
Determination of the total phenolic (TP) compounds. 
Determination of the total phenolic compounds, 
expressed as tannic acid equivalents, was performed 
according to Folin-Ciocâlteu method [17]. The blue 
complex (obtained from the oxidation of polyphenols 
via Folin-Ciocâlteu reagent) is assessed by absorbance 
at 725 nm, against a reference solution of tannic acid. 
The results are expressed as tannic equivalents/100 g 
dry extract, using a standard curve, linear between 
2.1 - 12.12 µg/mL (Y = 0.0605X + 0.0533, r² = 0.999, 
where Y = absorbance, and X = concentration).

Determination of the antioxidant activity using DPPH 
assay. (1,1-diphenyl-2-picrylhydrazil) was employed 
according to Brand-Williams (1995) with slight 
modifications [19]. The reaction is based on the 
reduction of purple radical DPPH (1,1-diphenyl-2-
 picrylhydrazil) to yellow 1,1-diphenyl-2-picryl-
hydrazine. Briefly, 2 mL of 120 µM DPPH was added 
to 2 mL of the extract solution at different concentrations 
(0.020 - 217 mg of extracts). The flasks were shaken 
energetically and kept in the dark, at room temperature 
for 30 minutes. The absorbance was measured against 
blank (absolute ethanol) at the maximum absorbance 
of DPPH (λ = 516 nm) using a spectrophotometer 
Jasco V-530, Germany. The assays were carried out in 
triplicate and the results were expressed as mg/mL 
extact that inhibits with 50% the DPPH absorbance
The Chrompass software from make (USA) were purchased from gradient

7 - me reducing power assay

for (v/v); the elution started at 90% to 78% phosphoric mobile phase: acetonitrile and phosphoric acid 0.1%

Nucleosil

The experimental conditions were the following: RP Pharmacopoeia method was the one provided by the European pump, column thermostat MD

a HPLC

In order to identify and quantify the content of arbutin HPLC analysis was established using a correlation between the blue concentrations (mg/mL).

curve plotted between absorbance and test solutions

concentration providing 0.5 of absorbance. It was (mg/mL) that represents the analysed dry extract

The antioxidant activity was also expressed as EC50 (mg/mL) [32].

Polyphenols are major contributors to the antioxidant activity of plants [23]. Therefore, we considered that a correlation between DPPH inhibition and the polyphenols content of the extracts is welcomed.

Reducing power assay. The reducing power assay was determined according to Oyaizu, with slight modification by Jayanthi [15]. Antioxidant compounds reduce potassium ferricyanide (Fe3+) to ferrocyanide (Fe2+), which reacts with ferric chloride to form ferric ferrous complex. The ferric ferrous complex absorbance is directly proportional with the extract reducing power. Briefly, 2.5 mL of extract solutions were mixed with phosphate buffer pH 6.6 (2.5 mL) and potassium ferricyanide (2.5 mL). The mixture was kept at 50°C for 20 minutes. After cooling, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 minutes. The upper layer was mixed with 2.5 mL fresh distilled water and 0.5 mL of 1% ferric chloride. The absorbance was measured against controls (prepared with the same ingredients and procedure, except the extracts) at the 700 nm wavelength. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations.

The antioxidant activity was also expressed as EC50 (mg/mL) that represents the analysed dry extract concentration providing 0.5 of absorbance. It was determined graphically from the linear regression curve plotted between absorbance and test solutions concentrations (mg/mL).

The TP influence on the reducing power of the extracts was established using a correlation between the blue complex absorbance and their polyphenolic content.

HPLC analysis

In order to identify and quantify the content of arbutin a HPLC-DAD method was used, on a Jasco HPLC MD-2015 equipped with degasser, binary gradient pump, column thermostat and UV detector. The HPLC method was the one provided by the European Pharmacopoeia for Echinacea angustifolia radix. The experimental conditions were the following: RP Nucleosil – C18 25 x 0.4 mm i.d, 5 µm column; the mobile phase: acetonitrile and phosphoric acid 0.1% (v/v); the elution started at 90% to 78% phosphoric acid 0.1% for 13 minute, then decreased from 78% to 60% in 1 minute, followed by an isocratic flow with 60% phosphoric acid 0.1% for another 6 min; system flow rate: 1.5 mL/min; injection volume: 20 µL; detection: 218 nm. The chromatographic data were processed using the Chrompass software from Jasco, Japan. The retention time of the arbutin (370 µg/mL) was obtained from 5 consecutive injections of the reference solution. Phytobiological analysis

The effect of the extractive solutions AU1 and AU2 was determined using the Triticum Bioassay (method of Constantinescu) [7]. The method is based on determining the highest dilution of active substance influencing the elongation of the root and the caryokinetic film, depending on the duration of action. Five concentrations of each extractive solution (3.33, 2.50, 1.67, 0.33 and 0.03%) were tested and distilled water was used as a negative control. An index of inhibition was also calculated based on median values. The comparison of root length was performed 72 hours from baseline. EC50 was determined by nonlinear regression modelling of the Efi versus log C (concentration of the tested extract solution). Artemia franciscana nauplii toxicity

The acute toxicity of the different samples can be verified using Artemia organisms [14]. The brine shrimp lethality test used is briefly described. Artificial sea water was prepared by dissolving a commercial salt mixture (CoralMarine, Grotech) in distilled water (33.4 g/L). Artemia franciscana (Kellogg, 1906) cysts, derived from Grate Salt Lake (USA) were purchased from Ocean Star International (USA), repackaged by S.K. Trading (Thailand). Hatching was initiated roughly 48 hours before the test initiation, by immersing about 2 grams of cysts in 500 mL artificial marine water at 25°C, under continuous aeration. The hatched organisms (nauplii) were used within a 10 hour interval from hatching. The nauplii were separated from cyst remainders and concentrated by placing them in a 100 mL crystallizing dish and using a light source positioned towards its bottom. From the crystallizing dish nauplii were transferred with a Pasteur pipette in a smaller crystallizing dish (10 mL), from which they were collected for testing.

Statistical analysis

Origin 6.1 is the statistical software used to calculate the average, standard deviation (± sxn) and the determination factors (r²) for mathematical correlations (P.T. with DPPH/Reducing power assay). The results of the genotoxicity and cytotoxicity tests were interpreted statistically using the Kruskal-Wallis test, because after checking their distribution by the D’Agostino & Pearson and Shapiro-Wilk tests an abnormal distribution has been observed. The Dunn’s post-hoc test was used for multiple comparisons between different concentrations and the control group. GraphPad Prism 5 was used to calculate the EC50 and for statistical interpretation.
Results and Discussion

The macroscopic characters and the microscopic analysis confirmed the identity of the species *Arbutus unedo* L. in agreement with the literature data [12]. The following anatomical features were observed: glandular hairs on the young leaves; mature leaf with spiny edge; dorsiventral structure with a double palisade layer; partially sclerenchymatous pericycle (in the median rib). The anomocytic stomata, prismatic calcium oxalate crystals and drops of oil were observed in the superficial preparations.

The chromatographic and spectrophotometric results are found in Table I. The arbutin calibration curve in the 0.5 - 5.0 mg/mL range had good linearity ($r^2$ = 0.994, n = 5). The polyphenolic and arbutin content is higher for AU1 than for AU2 (Table I). Thus, the arbutin content of the extracts increases with the temperature of extraction. Positive influence of an increased temperature maintained for short time on arbutin extraction was also reported for other herbal products [29, 32]. Similar statement may be made for polyphenolic compounds as well [37]. The ultrasonic process can increase mass transfer between raw material and solvent [31], but in our case, the influence of high temperature, is more effective for the extraction of TP. Our results (32.84 and 10.44 g% tannic acid, for refluxation and sonication extracts, respectively) are higher than ones found by Parissi et al. (1.9 g% tannic acid) [26], but other comparisons with scientific reports are difficult because most scientific papers express TP content of *Arbutus unedo* leaves as gallic acid equivalents [13].

| Sample | g% total polyphenolic compounds expressed as tannic acid | DPPH EC50 (mg/mL) | DPPH AAI | Reducing power assay (EC50) | g% arbutin |
|--------|--------------------------------------------------------|-------------------|----------|----------------------------|-----------|
| AU 1   | 32.84 ± 2.45                                          | 3.72 ± 0.36       | p < 0.05 | 57.41 ± 0.56               | 1.92      |
| AU 2   | 10.44 ± 3.61                                          | 22.28 ± 0.28      | 1.05     | 43.78 ± 1.09               | 0.52      |
| Gallic acid | -                                                   | 15.51 ± 0.32     | -        | 88.6 ± 0.02                | -         |

The DPPH technique is often used to evaluate the antioxidant properties of plant extracts. The EC50 values are inversely correlated with the antioxidant activity of the extracts (Table I). The antiradical activity decrease in the following order: AU1 > Gallic acid > AU2. The fact that an extract (AU1) may have a higher activity than the standard has also been reported by other authors. Djabou et al. found that an ethyl acetate extract of *Arbutus sp.* exhibits a higher reduction of the free radicals than ascorbic acid and catechin [8].

Another way to express the antioxidant activity of the extracts is by means of AAI (antioxidant activity index). Due to the fact that the EC50 values depend on the initial free radical concentration, the AAI was used to classify the antioxidant activity of the extracts [32]. According to Scherer R et al., the antioxidants are divided in: weak (AAI < 0.5), moderate (AAI < 0.5 - 1.0), strong (AAI < 1.0 - 2.0) and very strong (AAI > 2.0) [32]. As such, AU1 is a very strong antioxidant and AU2 and gallic acid are strong antioxidants.

The ferric reducing assay evaluate the ability of compounds to act as antioxidants in the system, by reducing the Fe$^{3+}$ to its more active Fe$^{2+}$ [29, 30]. As such, the antioxidant capacity is evaluated by the formation of Fe$^{2+}$ in the Perl’s Prussian complex, whose absorbance is assessed at 700 nm [29]. The antiradical activity expressed as EC50 is found in Table I. In this case, AU2 has a higher antioxidant activity than AU1, in an opposite manner to the results obtained in the DPPH method. A possible explanation resides in the chemical composition of the extracts.

As, by ultrasonication, thermolabile compounds (like ascorbic acid) are protected, they may reinforce the polyphenol activity to give higher reduction of the ferric salts. Therefore, the antioxidant activity of the extract depends not only on polyphenols, but also on other compounds. Another possible explanation is related to the fact that low temperature extraction procedures might give lower quantities of polyphenols, but with higher antiradical activity than heating procedure methods [5].

As it was expected, for DPPH method the determination factor $r^2$ between the EC50 and the polyphenolic content has a high value ($r^2$ = 0.932), which indicate that phenolic compounds are responsible for the antioxidant activity. Another influence might be due to the higher content of arbutin obtained by refluxation. For the ferric reducing assay, the $r^2$ factor has lower value indicating that other compounds are involved in the antiradical activity of the extracts.

**Phytobiological analysis**

By analysing data from the third day obtained from by calculating of inhibition indices for both extractive solutions, it was found that, as expected, at high concentrations (3.33 - 1.66%), the inhibitory effect is strong (greater than 95% for both solutions – p < 0.001). For the concentration of 0.33%, the two extracts also showed a high, statistically significant inhibitory effect, (greater than 85% for both solutions – p < 0.001). For the lowest of the tested concentrations (0.03%) the inhibitory effect (41.89% for AU1 and 21.49% for AU2), it was proved to be statistically insignificant (p > 0.05). For the solution produced by sonication (AU2), the determined inhibitory effect has half of
the value of the one determined for the solution obtained by refluxing at the lowest concentrations (Figures 1, 2 and 3). In addition, EC50 for AU2 and for AU1 is presented in Table II.

| Parameters                          | AU 1 (mg/100 mL) | AU 2 (mg/100 mL) |
|-------------------------------------|------------------|------------------|
| EC50                                | 41.89            | 82.93            |
| Standard error of EC50              | 0.15             | 0.09             |
| Confidence interval for EC50 (α = 0.05) | 3.27 - 4.20     | 4.12 - 4.72     |
| Goodness of Fit ($r^2$)             | 0.9786           | 0.9956           |

The semi-logarithmic graphs showing the variation of the inhibitory effect is presented in Figure 1.

![Semi-logarithmic graph showing the variation of the inhibitory effect as a function of concentration (log)](image)

The microscopic examination of the preparations has shown mitoinhibitory effects (Figure 4), micronuclei (Figure 5) and disorganization of nuclear material (Figure 7) the 3.3 - 0.3% concentration interval, and changes in the mitotic film like tropokynesis at the lowest concentration (0.03%) (Figure 6). Cytotoxicity and genotoxicity seen in the presence of high concentrations might be caused by the polyphenols and arbutin [36].

![Mitoinhibition (ob. 100x)](image)
and water were found to be extremely toxic. The difference in effects between plant extracts was more pronounced at high concentrations. Low concentrations showed no toxicity on *Artemia franciscana* (for AU1, LC50 higher than 9000 μg/mL), indicating that the extract may be considered “practically non-toxic”.

**Conclusions**

The hydroethanolic extract obtained by refluxation has the highest content of arbutin, polyphenolic compounds and is classified as a very strong antioxidant, according to the antioxidant activity index. The antioxidant activity depends on the chemical composition of the extracts that varies with the extraction procedure. The ferric reducing activity is higher for the extract obtained by ultrasonication. Both analysed extracts showed induced genotoxic effects on plant cells at high concentrations. Low concentrations showed no toxicity on *Artemia franciscana*.

**Conflict of interest**

The authors declare no conflict of interest.

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