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RESEARCH ARTICLE

Apoptotic, antioxidant and antiradical effects of majdine and isomajdine from *Vinca herbacea* Waldst. and kit

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Abstract
In the present study, apoptotic, antioxidant and antiradical effects of majdine and isomajdine from *Vinca herbacea* Waldst. and Kit were studied. For testing the possible apoptotic effects of majdine and isomajdine from *V. herbacea*, DNA fragmentation assay was conducted on the rat brain cortical tissue homogenates, *in vitro*. Also their possible effects on mitochondrial activity were tested by using the same tissue samples of rats. In addition, the antioxidant activity of isomajdine and majdine was determined using various *in vitro* antioxidant assays, including 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical scavenging and *N*,*N*-dimethyl-p-phenylenediamine ($DMPD^{\cdot+}$) radical scavenging, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing abilities and ferrous ions (Fe^{2+}) chelating activity. On the other hand, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as reference antioxidants.

Keywords: Antioxidant activity, radical scavenging, apoptotic effect, isomajdine, majdine

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Introduction

Antioxidants are substances that delay the oxidation process, inhibit the polymerisation chain, initiated by free radicals and other subsequent oxidising reactions. Thereby, they help prevent cancer, heart disease, diabetes mellitus, neurodegenerative and inflammatory diseases[1]. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone are the most commonly used synthetic antioxidants[2]. Although, many synthetic antioxidants are promising for various human ailments, pro-oxidant or cytotoxic natures at higher concentration limit their long term use. In addition, these are suspected to be responsible for liver damage and carcinogenesis. Therefore, new interest has been developed to search natural and safe antioxidative agents[3].

Many antioxidant compounds, naturally occurring in plant sources, have been identified as free radical or active oxygen scavengers. Recently, interest has considerably increased in finding naturally occurring antioxidants for use in foods or medical materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods[3].

Vinca herbacea Waldst. and Kit. is in the Apocynaceae family, which is composed of dicots, usually herbs that have two either opposite or whorled entire leaves[4]. This plant has many alkaloid kinds with high amount[12]. Vincoline an alkaloid previously isolated from *V. herbacea* has been isolated and its structure elucidated[5]. The chemical structures of majdine and isomajdine isolated from *V. herbacea* Waldst. and Kit were given in Figure 1.

Apoptosis is a form of cell death in which a cell undergoes an internally controlled transition from

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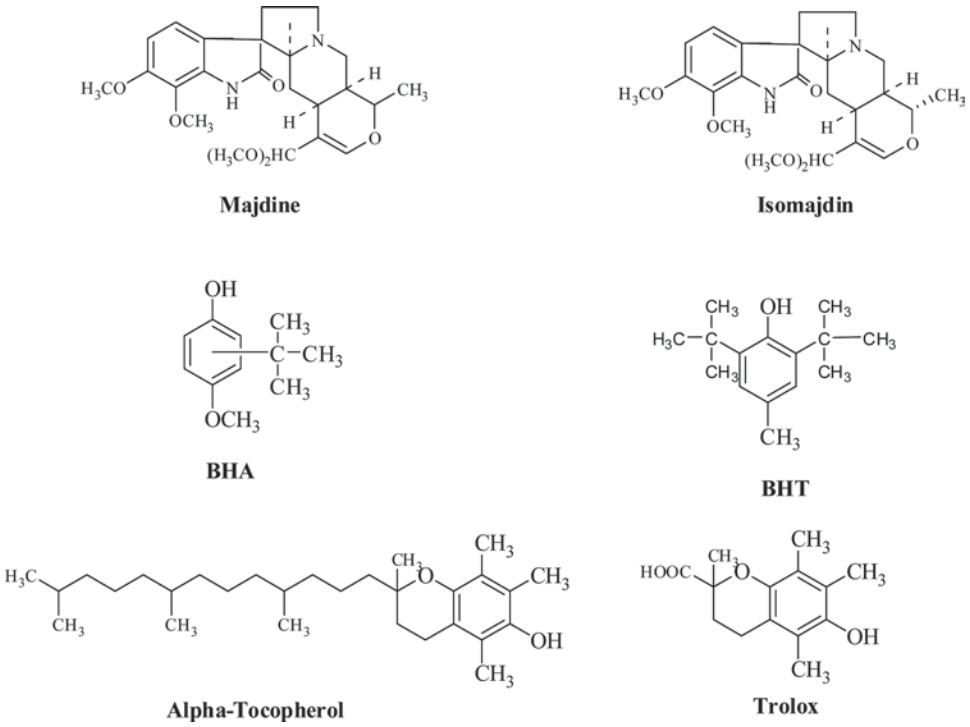

Figure 1. Chemical structures of standard antioxidants (BHA, BHT, α-tocopherol and trolox), majdine and isomajdine from *Vinca herbacea* Waldst. and Kit.

an intact metabolically active state into a number of shrunken remnants retaining their membrane bound integrity. Moreover, direct exposure of cells to various oxidants causes multiple intracellular alterations, particularly inflammation. Therefore, the presence of apoptosis directly linked with antioxidants in living organisms[6].

The aim of the present study was to investigate apoptotic and antioxidant activities of isomajdine and majdine isolated from *V. herbacea* by using different antioxidant tests including $ABTS^{\bullet+}$ scavenging, $DMPD^{\bullet+}$ scavenging, ferric ions (Fe^{3+}) reducing ability by Fe^{3+}–Fe^{2+} transformation, cupric ions (Cu^{2+}) reducing ability by CUPRAC method and ferrous ions (Fe^{2+}) chelating activity. An important goal of this research was to compare these compounds to most commonly used synthetic antioxidants such as BHA, BHT, α-tocopherol, and trolox.

Materials and methods

Chemicals

N,*N*-dimethyl-p-phenylenediamine (DMPD), neocuproine (2,9-dimethyl-1,10-phenanthroline), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), α-tocopherol and trolox were obtained from Sigma (Sigma–Aldrich GmbH, Steinheim, Germany). All other chemicals used, were of analytical grade, and obtained from either Sigma–Aldrich or Merck.

Plant materials

The roots of *V. Herbacea* Waldst. and Kit (Fam. Apocynaceae) were collected in the East of Georgia in a phase of flowering (2005). The plant was identified in the department of Pharmacobotany I. Kutateladze Institute of Pharmacochemistry [Herbarium #9 (120) Tbilisi, Georgia].

Isolation of majdine and isomajdine

The air-dried plant material (10 kg) was basified with ammoniac (25%) and extracted with chloroform (3 × 50 L) at room temperature. The combined extracts were concentrated in vacuum to a 5 L and fractioned with gradient pH with citrate–phosphate buffer. Crude alkaloids obtained at pH: 3.6 (30 g) from methanol was considered as isomajdine (2.3 g) and at pH: 3.0 (30 g) from methanol was considered as majdine (4.6 g). It was proved that isolated compounds are majdine and isomajdine by thin layer chromatography, than NMR spectra has been performed. Isomajdine and majdine were dissolved in DMSO (20%) and final concentrations of 5, 10, 25, 50, 100 and 200 μM for both compounds were prepared. Cortical homogenates were incubated 24 h with H_2O_2 (1 mM), DMSO (20%) and majdine or isomajdine solutions.

Determination of antioxidant effects of isomajdine and majdine

Fe^{3+} reducing power assay

Reducing power of majdine and isomajdine were measured by direct reduction of $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$, and were determined by again, measuring absorbance

resulted from the formation of the Perl's Prussian blue complex following the addition of excess ferric ions (Fe^{3+}). For determination of the ferric reducing antioxidant power of isomajdine and majdine, the method of Oyaizu was used[7]. This method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess, relative to the antioxidants. Different concentrations of isomajdine and majdine (10–30 μg/mL) in 0.75 mL of distilled water were mixed with 1.25 mL of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The mixture was incubated at 50°C for 20 min. After 30 min of incubation, the reaction mixture was acidified with 1.25 mL of trichloroacetic acid (10%). Finally, 0.5 mL of $FeCl_3$ (0.1%) was added to this solution, and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates grater reduction capability[8].

Cupric ions (Cu^{2+}) reducing-CUPRAC assay

In order to determine the cupric ions (Cu^{2+}) reducing ability of isomajdine and majdine, the method proposed by Apak et al[9]. For this reason, 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution (7.5×10^{-3} M) and 0.25 mL CH_3COONH_4 buffer solution (1 M) were added to a test tube, followed by mixing with different concentrations of isomajdine and majdine (10–30 μg/mL). Then, total volumes were adjusted to 2 mL with distilled water, and mixed well. The tubes were stoppered and kept at room temperature. Absorbance was measured at 450 nm against a reagent blank, 30 min later. Increased absorbance of the reaction mixture indicates increased reduction capability[10].

Chelating activity on ferrous ions (Fe^{2+})

Ferrous ions (Fe^{2+}) chelating activity of isomajdine and majdine was measured by inhibiting the formation of Fe^{2+}–ferrozine complex after the treatment of test material with Fe^{2+}, following the method of Dinis and co-workers[11]. Fe^{2+}-chelating ability of isomajdine and majdine was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, different concentrations of isomajdine and majdine (10–30 μg/mL) in 0.4 mL methanol were added to a solution of 0.6 mM $FeCl_2$ (0.1 mL). The reactions were initiated by the addition of 5 mM ferrozine (0.1 mL) dissolved in methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated by using the formula given below:

$$\text{Bounded ferrous ions (\%)} = \left(1 - \frac{\lambda_{562-S}}{\lambda_{562-C}}\right) \times 100$$

where λ_{562}-C is the absorbance of control and λ_{562}-S is the absorbance in the presence of amandine, isomajdine or standards. The control contains only $FeCl_2$ and ferrozine[12].

$ABTS^{\bullet+}$ scavenging activity

In this method, an antioxidant is added to a pre-formed ABTS radical solution, and after a fixed time period, the remaining $ABTS^{\bullet+}$ is quantified spectrophotometrically at 734 nm[12]. The $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 6 h. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of isomajdine and majdine solution in ethanol at different concentrations (10–30 μg/mL). The absorbance was recorded 30 min after mixing, and the percentage of radical scavenging was calculated for each concentration relative to a blank, containing no scavenger. The extent of decolourisation is calculated as the percentage reduction of absorbance. The $ABTS^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r^2: 0.9899):

$$\text{Absorbance } (\lambda_{734}) = 2.5905 \times [ABTS^{\bullet+}]$$

The scavenging capability of test compounds was calculated using the following equation:

$$\text{ABTS}^{\bullet+}\text{ scavenging effect (\%)} = \left(1 - \frac{\lambda_{734-S}}{\lambda_{734-C}}\right) \times 100$$

where $\lambda_{734\text{-}C}$ is absorbance of a control lacking any radical scavenger and $\lambda_{734\text{-}S}$ is the absorbance of the remaining $ABTS^{\bullet+}$ in the presence of scavenger[13].

$DMPD^{\bullet+}$ scavenging activity

The second antiradical capacity of isomajdine and majdine were analysed by $DMPD^{\bullet+}$ assay. DMPD radical scavenging ability of isomajdine and majdine were performed according to Fogliano and co-workers[14]. In the presence of Fe^{3+}, a coloured DMPD radical cation is generated; antioxidant compounds able to transfer a hydrogen atom to $DMPD^{\bullet+}$ cause a decolourisation of the solution measured by the decrease in the absorbance at 505 nm. DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 mL of deionised water, and 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.25), and the coloured radical cation ($DMPD^{\bullet+}$) was obtained by adding 0.2 mL of a solution of 0.05 M ferric chloride ($FeCl_3$). The absorbance of this solution, which is prepared daily, is constant up to 12 h at room temperature. Different concentrations of standard antioxidants or isomajdine and majdine (10–30 μg/mL) were added in test tubes, and the total volumes were adjusted to 0.5 mL with distilled water. Ten minutes later, the absorbance was measured at 505 nm. One millilitre of $DMPD^{\bullet+}$ solution was directly added to the reaction mixture, and its absorbance was measured at 505 nm. The buffer solution

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was used as a blank sample. The $DMPD^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r^2: 0.9993):

$$\text{Absorbance}\ (\lambda_{505}) = 0.0088 \times [DMPD^{\bullet+}]$$

The scavenging capability of $ABTS^{\bullet+}$ radical was calculated using the following equation:

$$\text{DMPD}^{\bullet+}\text{scavenging effect (\%)} = \left(1 - \frac{\lambda_{505-S}}{\lambda_{505-C}}\right) \times 100$$

where in $\lambda_{505\text{-}C}$ is the initial concentration of the $DMPD^{\bullet+}$ and $\lambda_{505\text{-}S}$ is absorbance of the remaining concentration of $DMPD^{\bullet+}$ in the presence of isomajdine and majdine.

Determination of apoptotic effects of isomajdine and majdine

Animals

A total of 6 mature (180–220 g) male Sprague-Dawley rats were used in the present study. The procedure was suitable for the Animal Care and Ethics Committee of Abant Izzet Baysal University, Medical Faculty. Animals were anaesthetised under the ether vapour, decapitated and the temporal cortical tissues were dissected out and homogenised in 5 mL of Dulbecco's Minimal Essential Medium (DMEM, Sigma) solution, by using ultraturrax. Then, the homogenised cortical tissues were aliquoted equally into 10 eppendorf tubes with glycine (20%) and kept at–80°C until the pharmacological procedure.

DNA fragmentation assay

The DNA fragmentation assay allows determining the amount of DNA that is degraded upon treatment of cells with the following doses of majdine and isomajdine as well as reference compound of this test, hydrogen peroxide (H_2O_2). The dishes were kept at 37°C in humidified 95% air and 5% CO_2, with H_2O_2, DMSO, isomajdine or majdine for 24 h, in DMEM. After that period, homogenates were centrifuged at 3000 rpm for 3 min; pellets were washed twice with fresh Hank's Balanced Salt Solution (HBSS). Than the pellets were lysed in a buffer containing 0.1 M NaCl, 10 mM Tris (pH: 8.0), 1 mM EDTA (pH: 8.0), 1% SDS and 2% proteinase K for additional 24 h at 50°C. Lysates were vortexed and cleared by centrifugation at 10000 ×*g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analysed electrophoretically on 1.5% agarose gels containing 0.1 μg/mL ethidium bromide at 100 V for 50 min.

WST-1 assay

For testing mitochondrial damage, cell proliferation reagent of WST-1 (Roche) was used. Briefly, the test was based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. After incubating the cortical tissue homogenates with H_2O_2, DMSO, isomajdine or majdine for 24 h, in Dulbecco's Modified Eagle Medium (DMEM), at 37°C in humidified 95% air and 5% CO_2 in the doses which described above. They were centrifuged at 3000 rpm for 3 min, after that the supernatant was removed. Pellets were washed twice with fresh HBSS solution. HBSS solution of 180 μL and WST-1 reagent of 20 μL were added into the each tube and homogenates were left in same conditions as described above for additionally 4 h. At the end of that period, tubes were centrifuged at 2000 ×*g* for 4 min, 50 μL of supernatant from each tube was transferred into the 96 well plates. By using a microplate reader, mitochondrial activity was measured at 480 nm wavelength while the reference wavelength set up at 690 nm.

Statistical analysis

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation and analysed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of variance ANOVA was performed by procedures. Significant differences between means were determined by Duncan's Multiple Range tests, and $p < 0.05$ was regarded as significant, and $p < 0.01$ was very significant.

Results

Reducing power reflects the electron donating capacity of bioactive compounds and is associated with antioxidant activity. Isomajdine and majdine had effective reducing power determined by using the potassium ferricyanide reduction and cupric ions reducing methods, when compared to the standards. For the measurements of the reductive ability of isomajdine and majdine, Fe^{3+}–Fe^{2+} transformation was investigated in the presence of isomajdine and majdine using the method of Oyaizu[7]. As can be seen from Figure 2A, isomajdine (r^2: 0.921) and majdine (r^2: 0.979) demonstrated effective Fe^{3+} reducing ability with these differences being statistically significant ($p < 0.01$). The reducing power of isomajdine, majdine, BHA, BHT, α-tocopherol and trolox increased steadily with increasing concentration of samples. Reducing power of isomajdine, majdine and standard compounds was as follows: BHA > trolox ≈ BHT > α-tocopherol > majdine > isomajdine (at the concentration of 30 μg/mL). These results demonstrated that isomajdine and majdine had marked ferric ions (Fe^{3+}) reducing ability and had electron donor properties for neutralising free radicals by forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

A cupric ion (Cu^{2+}) reducing ability of isomajdine and majdine is shown in Figure 2B. A correlation was observed between the cupric ions reducing ability and isomajdine (r^2: 0.994) or majdine concentrations (r^2: 0.9767). Cu^{2+} reducing capability of isomajdine and majdine by CUPRAC method was found to be concentration- dependent (10–30 μg/mL). Cu^{2+} reducing powers

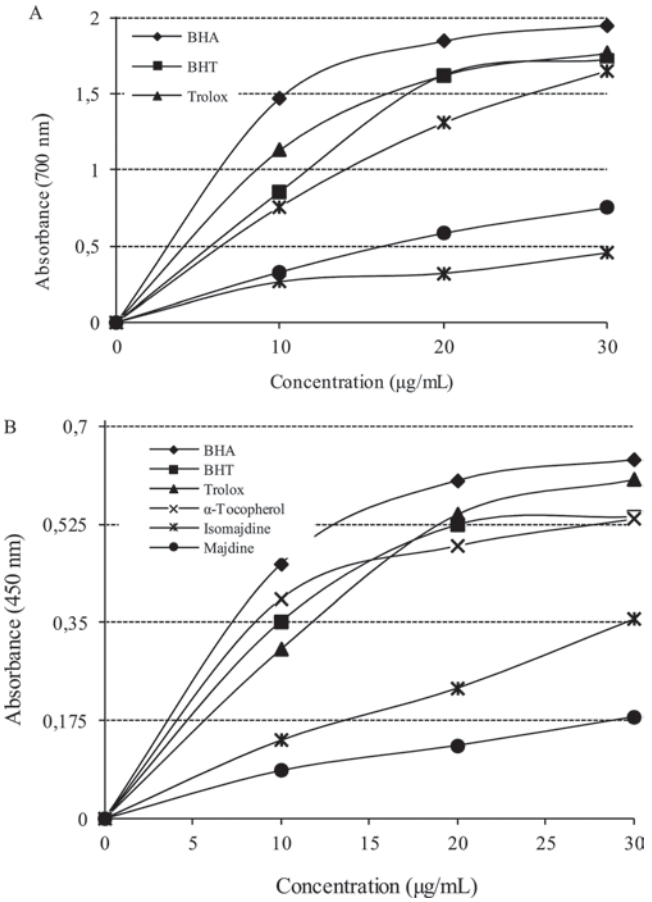

Figure 2. (A) Fe^{3+}-Fe^{2+} reductive potential of different concentrations (10–30 g/mL) of majdine (r^2:0.933), isomajdine (r^2:0.933) and reference antioxidants BHA, BHT, α-tocopherol and trolox using spectrophotometric detection of the Fe^{+3}-Fe^{+2} transformation. (B) Cupric ions (Cu^{2+}) reducing ability (Cuprac method) of different concentrations (10–30 g/mL) of majdine (r^2: 0.9330), isomajdine (r^2: 0.9330), BHA, BHT, α-tocopherol and trolox using spectrophotometric detection of the Cu^{+2}-Cu^{+} transformation. (BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene).

of isomajdine, majdine and standard compounds at the same concentration (30 µg/mL) were as follows: BHA > trolox α BHT > α-tocopherol > isomajdine > majdine.

All the tested compounds exhibited effective $ABTS^{\bullet+}$ radical cation scavenging activity. As seen in Table 1, isomajdine and majdine have an effective $ABTS^{\bullet+}$ radical scavenging activity. EC_{50} value for isomajdine and majdine in this assay were 34.11 and 57.69 µg/mL, respectively. On the other hand, EC_{50} values for BHA, BHT, α-tocopherol and trolox were found to be 4.65, 52.81, 73.15 and 62.32 µg/mL, respectively. The scavenging effect of isomajdine, majdine and standards on $ABTS^{\bullet+}$ decreased in the following order: BHA > isomajdine > BHT > majdine > trolox > α-tocopherol (98.1, 97.8, 96.9, 86.3, 79.6 and 55.9%, respectively) at the same concentration (30 µg/mL).

As shown in Table 1, isomajdine and majdine were an effective $DMPD^{\bullet+}$ radical scavenger in a concentration-dependent manner (10–30 µg/mL). EC_{50} for isomajdine and majdine were found 32.9 and 71.24 µg/mL. This value was found as 63.25 µg/mL for BHA and 14.89 µg/mL for trolox. There was a significant decrease ($p < 0.05$) in the concentration of $DMPD^{\bullet+}$ due to the scavenging capacity at all isomajdine and majdine concentrations.

Table 1. Comparison of IC_{50} values of $ABTS^{\bullet+}$ scavenging, $DMPD^{\bullet+}$ scavenging and ferrous ion (Fe^{2+}) chelating activity of majdine, isomajdine and standard antioxidant compounds such as BHA, BHT, α-tocopherol and trolox (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene, $ABTS^{\bullet+}$:2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals, $DMPD^{\bullet+}$: *N*,*N*-dimethyl-p-phenylenediamine radicals).

Compounds	$ABTS^{\bullet+}$ scavenging	$DMPD^{\bullet+}$ scavenging	Metal chelating
BHA	4.65	63.25	34.31
BHT	52.81	—*	13.72
Trolox	62.32	14.89	16.17
α-Tocopherol	73.15	—*	16.31
Isomajdine	34.11	32.09	10.05
Majdine	57.69	71.24	14.01

*It was reported the main drawback of the $DMPD^{\bullet+}$ method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants such as α-tocopherol or BHT were used. Hence, these standard antioxidant compounds were not used in this antiradical assay[28,37].

The data obtained from Table 1 reveal that isomajdine and majdine possess a marked capacity for iron binding, suggesting that their main action as a peroxidation inhibitor may be related to their iron binding capacity. In this assay, isomajdine and majdine interfered with the formation of the ferrous–ferrozine complex. It suggests that isomajdine and majdine have chelating activity and are able to capture ferrous ion before ferrozine. Isomajdine and majdine had strong chelating effect on ferrous ions (Fe^{2+}). As can be seen in Table 1, the ferrous ion chelating effect of isomajdine and majdine were compared to that of BHA, BHT, α-tocopherol and trolox. IC_{50} values of ferrous ion chelating capacity of BHA, BHT, α-tocopherol and trolox were found to be 34.31, 13.72, 16.31 and 16.17 µg/mL, respectively. On the other hand, IC_{50} values of ferrous ion chelating capacity of isomajdine and majdine were found to be 10.05 and 14.01, respectively. These results show that the ferrous ion chelating effect of isomajdine and majdine were statistically similar to that of BHT, trolox and α-tocopherol ($p > 0.05$) but higher than that of BHA ($p < 0.01$).

According to the results of DNA fragmentation and WST-1 assays, isomajdine and majdine induce apoptosis more potently than H_2O_2. As can be seen in Figures 2 and 3, H_2O_2 in 1 mM concentration, for 24 h, induces DNA fragmentation, in respect to controls. Isomajdine, especially in higher concentrations such as 200 and 100 µM, showed same DNA fragmentation profile with H_2O_2 and this effect maintained until 10 and 5 µM concentrations. Despite the fact that DNA fragmentation effect of majdine was not as strong as isomajdine, almost all doses of majdine slightly induced DNA fragmentation, in respect to controls. It is obvious that 10 and 5 µM concentrations

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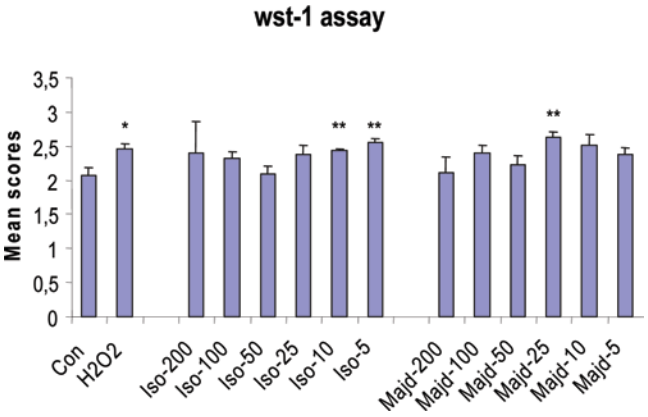

Figure 3. WST-1 assay of the groups. [H_2O_2: 1 mM of hydrogen peroxide. Iso: isomajdine and majd: majdine in μM concentrations. *$p < 0.05$, **$p < 0.01$ with respect to control group (Con), WST-1: water-soluble tetrazolium salt].

of isomajdine decreased DNA fragmentation in respect to higher concentrations of it and H_2O_2.

Controversially to our expectations in the ANOVA and the independent samples *t*-test, H_2O_2, isomajdine and majdine were found to induce mitochondrial activity, as a result of WST-1 assay. All of the groups tested, including H_2O_2, induce formazan formation from tetrazolium salt, more than the control group. During the pre-apoptotic period, the consumption and demand of ATP increase for intracellular chromatin condensation, formation of apoptotic vacuoles, etc. The highest mitochondrial activity was found in the groups of isomajdine's 5 and 10 μM and majdine's 25 μM concentrations (Figure 3).

Discussion

It was suggested that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with antioxidant activity[15]. Antioxidants can be reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other. The presence of reductants, such as antioxidant substances in the samples, causes the reduction of the Fe^{3+}–ferricyanide complex to the ferrous form. Fe^{2+} formed can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. Antioxidants can exert their effects *via* reducing and inactivating the oxidants. The reducing capacity of a compound can be measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. There are a number of assays designed to measure overall antioxidant activity, or reducing potential, as an indication of a host's total capacity to withstand free radical stress[16]. The ferric ion reducing antioxidant power assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[8].

In the present study, we also used the CUPRAC assay which is based on reduction of Cu^{2+} to Cu^{+} by antioxidants. CUPRAC chromogenic redox reaction is carried out at a pH (7.0) close to the physiological pH, and the method is capable of measuring thiol-type antioxidants such as glutathione and non-protein thiols unlike the widely applied FRAP test, which is non-responsive to -SH group antioxidants[8].

Elemental species, such as ferrous iron (Fe^{2+}), can facilitate the production of ROS within living organisms, and the ability of substances to chelate iron can be valuable for antioxidant property. Ferrous ions are the most effective pro-oxidants in food systems; the good chelating effect would be beneficial, and removal of free iron ion from circulation could be a promising approach to prevent oxidative stress-induced diseases. Iron, in nature, can be found as either ferrous (Fe^{2+}) or ferric ion (Fe^{3+}), with the latter form predominant in foods. Ferrous chelation may render important antioxidative effects by retarding metal-catalysed oxidation[17].

Ferrous ion chelating activities of isomajdine, majdine, BHA, BHT, α-tocopherol and trolox are shown in Table 1. The chelation of ferrous ions by isomajdine and majdine or standards was determined according to the method of Dinis et al[11]. An effective ferrous ion chelator affords protection against oxidative damage by removing iron that may otherwise participate in HO• generation *via* the Fenton type reactions. Ferric ions (Fe^{3+}) also produce radicals from peroxides although the rate is 10-fold less than that of ferrous ion[18]. Minimising ferrous ion may afford protection against oxidative damage by inhibiting production of ROS and molecular damage. Also, ferrozine can quantitatively form complexes with Fe^{2+} in this method. In the presence of chelating agents, complex formation is disrupted, resulting in a reduction in the red colour of the complex. Measurement of colour reduction therefore allows estimation of the metal chelating activity of the coexisting chelator. Lower absorbance indicates higher metal chelating activity[19].

One measurement method of the metal-chelating activity of an antioxidant is based on the absorbance measurement of Fe^{2+}–ferrozine complex after prior treatment of a ferrous ion solution with test material. Ferrozine forms a complex with free Fe^{2+}, but not with Fe^{2+} bound to other chelators; thus, a decrease in the amount of ferrozine–Fe^{2+} complex formed after treatment indicates the presence of antioxidant chelators[19]. The ferrozine–Fe^{2+} complex produces a red chromophore with absorbance that can be measured at λ_{562} nm. A significant drawback of this complexation reaction in measuring the presence of antioxidant chelator is that the reaction is affected by both the

antioxidant-Fe^{2+} and ferrozine–Fe^{2+} complex formation constants, and the competition between the two chelators for binding to iron. Thus, a weak antioxidant iron chelator would be seriously underestimated in quantitative determination. Nonetheless, this reaction serves as a convenient assay to access iron chelating activity of antioxidants[19].

The antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby form a stable end-product which does not initiate or propagate further oxidation of lipids. Assays based upon the use of $ABTS^{\bullet+}$ and $DMPD^{\bullet+}$ radicals are among the most popular spectrophotometric methods for determination of the antioxidant capacity of foods, beverages and vegetable extracts. Both chromogens and radical compounds can directly react with antioxidants. $ABTS^{\bullet+}$ are more reactive radicals, which involve H atom transfer and the reactions with $ABTS^{\bullet+}$ radicals involve an electron transfer process. Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of pure substances, aqueous mixtures and beverages[20]. A more appropriate format for the assay is a decolourisation technique in which the radical is generated directly in a stable form prior to the reaction with putative antioxidants.

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\bullet+}$, which has a characteristic long-wavelength absorption spectrum showing absorption at 734 nm. Bleaching of a pre-formed solution of the blue-green radical cation $ABTS^{\bullet+}$ has been extensively used to evaluate the antioxidant capacity of complex mixtures and individual compounds. The reaction of the pre-formed radical with free radical scavengers can be easily monitored by following the decay of the sample absorbance at 734 nm[20].

The principle of the $DMPD^{\bullet+}$ assay is that DMPD can form a stable and coloured radical cation ($DMPD^{\bullet+}$) at acidic pH and in the presence of a suitable oxidant solution. The UV–visible spectrum of $DMPD^{\bullet+}$ shows a maximum absorbance at 505 nm. Antioxidant compounds, which are able to transfer a hydrogen atom to $DMPD^{\bullet+}$, quench the colour and produce a decolourisation of the solution. This reaction is rapid, and the end point, which is stable, is taken as a measure of the antioxidative efficiency. Therefore, this assay reflects the ability of radical hydrogen-donors to scavenge the single electron from DMPD radicals[21].

Preliminary experiments show that the choice of oxidant solution and the ratio between the concentration of $DMPD^{\bullet+}$ and the concentration of the oxidative compound are crucial for the effectiveness of the method. In fact, the formation of radical cation is very slow and results in a continuous increase of the absorbance. The best results were obtained with $FeCl_3$, which gives a stable coloured solution up to a final concentration of 0.1 mM. Moreover, this method ensures low cost and highly reproducible analysis[22].

DMPD assay is particularly suitable for hydrophilic antioxidants, but is less sensitive to hydrophobic bioactive compounds[14], the opposite of the other two tests. In contrast to the ABTS procedure, the $DMPD^{\bullet+}$ method guarantees a very stable end point. This is particularly important when a large-scale screening is required. It was reported that the main drawback of the $DMPD^{\bullet+}$ method is that its sensitivity and reproducibility dramatically decreases when hydrophobic antioxidants such as α-tocopherol or BHT were used. Hence, these standard antioxidant compounds were not used in this antiradical assay[22].

Phytochemicals have been known to possess a number of physiological benefits, including antioxidant, anti-inflammatory, antiapoptotic and anti-carcinogenic activities[8,23]. Moreover, direct exposure of cells to various oxidants causes multiple intracellular alterations, particularly inflammation. The response of a cell to an increase in oxidants or inflammatory stimuli often involves the activation of numerous intracellular signalling pathways. These cytosolic pathways can, in turn, regulate a host of transcriptional changes that allow the cell to respond appropriately to oxidative stress. The evidence suggests that certain transcription factors can directly or indirectly alter their activities in the activation of numerous intracellular signalling pathways, depending on cellular redox conditions[24].

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes and death. These changes include loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation[25]. Increase in mitochondrial activity, instead of mitochondrial damage and inactivity in WST-1 results gives strong clues that apoptosis works on the non-mitochondrial pathway. It is possible that cytoplasmic proteins such as granzyme B, JNK or NFB activation may be responsible for the non-mitochondrial pathway of apoptosis. Another possibility is that H_2O_2, majdine and isomajdine induce nuclear p53 directly, which upregulates proapoptotic genes such as Bax, Bad, Fas, TRAIL, Apaf-1 and/or AIP1. Increased mitochondrial activity in respect to control group could be explained as being a compensatory activation of antiapoptotic pathways *via* mitochondria. In our study, both majdine and isomajdine induced apoptosis *via* non-mitochondrial pathways[25].

As a conclusion, according to those results, it can be speculated that majdine and isomajdine had effective antioxidant and antiradical activity in different *in vitro* antioxidant assays including $ABTS^{\bullet+}$ and $DMPD^{\bullet+}$ radical scavenging, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing abilities and ferrous ions (Fe^{2+}) chelating activity. The both compounds have phenolic groups. It is well-known that the phenolic compounds were demonstrated antioxidant effect and powerful reducing ability. Also, majdine and isomajdine induce apoptosis *via* non-mitochondrial pathways.

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Declaration of interest

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