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The *In Vitro* Antioxidant and Anti-Proliferative Effect and Induction of Phase II Enzymes by a Mistletoe (*Viscum Album*) Extract

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Abstract. The increasing interest for phytochemical antioxidants, found in many medicinal plants, is demonstrated experimentally by epidemiological and *in vitro* studies which show their positive effects on human health. Mistletoe contains therapeutically active ingredients which incubated with several human cancer cell lines revealed cytotoxicity for the cancer cells, but no evidence is available for their chemoprotective properties. We investigated *in vitro* the potential of extracts from *Viscum album* growing on *Mallus domestica* (VAM) to modulate the activity of phase II detoxifying enzymes using the gluthatione S-transferase (GST) and quinone oxidoreductase (QR) assays and to inhibition of adherent epithelial human ovarian tumor cells A2780 proliferation using the MTT assay. We found that different concentrations of VaM extract had inhibitory effects on the growth of adherent epithelial human ovarian tumor cells A2780 (IC_{50} = 120.62 µg/ml, after 48 h treatment). Also, this extract is a good antioxidant source (total phenol content of 91.51 mg gallic acid equivalents /g dry weight), having a radical scavenging ability and a reducing potential proportional to the dose used (from 0.312 to 5 mg/ml) *in vitro*. At 120 µg/ml, this extract increased, significantly, the activity of GST and QR, as well the level of GSH.

Keywords: *Viscum album*, gluthatione S-transferase (GST), quinone oxidoreductase (QR), DPPH, FRAP, MTT.

INTRODUCTION

Viscum album is a medicinal plant used for many years as a traditional remedy in preventive medicine and in complementary cancer therapies. European mistletoe (*Viscum album L.*) is an evergreen, semiparasitic plant, normally found growing on a variety of trees, especially pine, poplar, apple trees, locus trees etc. The phytochemical profile of mistletoe depends on the host trees of this plant (Vicas et al., 2011, Luczkiewicz *et al*., 2001). The main bioactive compounds found in mistletoe are lectins (glycoproteins with effects on cell-proliferation) and viscotoxin (a small protein molecule of 5 kDa) (Edlund *et al*., 2000; Romagnoli *et al*., 2000). Alkaloid concentration is usually low, and dependent on the host tree type (Peng *et al.,* 2005). It is considered that, flavonols like quercetin, kampherol and their methyl derivatives, and flavonone, naringenin are the antioxidant compounds in mistletoe (Haas *et al.,* 2003). Among the phenolic acids presents in mistletoe, digallic and *o*-coumaric acid in the free or glycosilated forms (Luczkiewicz *et al.,* 2001) are also considered to be compounds with antioxidant activity.

The human body is exposed to a various xenobiotics in one's life, from food components to environmental toxins to pharmaceuticals, and has developed complex enzymatic mechanisms to detoxify these substances.

The non-reactive compounds (xenobiotics) could be biotransformed in two phases: *functionalization*, which use oxygen to form a reactive site and *conjugation*, which results in addition of a water-soluble group to the reactive site. These two steps, functionalization and conjugation, are termed Phase I and Phase II detoxification, respectively. The result is the biotransformation of a lipophilic compound, not able to be excreted in urine, to a water soluble compound able to be removed in urine. Therefore, detoxification is not one reaction, but rather a process that involves multiple reactions and multiple players (**Fig. 1**).

A major protective mechanism against oxidizing substances capable of damaging DNA integrity and initiating carcinogenesis is the ***induction*** of phase II detoxification and antioxidant enzymes by chemopreventive agents.

The families of enzymes induced by chemopreventive agents have been classified into several categories (Giudice and Montella, 2006):

- transferases (GSTs), UDP-glucuronosyltransferases (UDP-GTs), NAD(P)H:quinine oxidoreductase 1 (NQO1), epoxidehydrolase (EH), aflotoxin B1, aldehyde reductase (AFAR), heme oxygenase 1 (HO-1), ferritin)
- antioxidants and their modulating enzymes (gamma-glutamylcysteine synthetase (γ-GCS), superoxide dismutase (SOD), catalase (CAT), gluthathione reductase (GR), thioredoxin reductase (TR), peroxiredoxins (Prxs), gluthathione S-conjugate efflux pumps, nicotinamide adenine dinucleotide phosphate (NADPH) and cofactors-generating enzymes)
- molecular chaperones/proteasome systems
- DNA repair enzymes
- Anti-inflammatory response proteins (HO-1, ferritin, leucokotriene B_4 dehydrogenase)

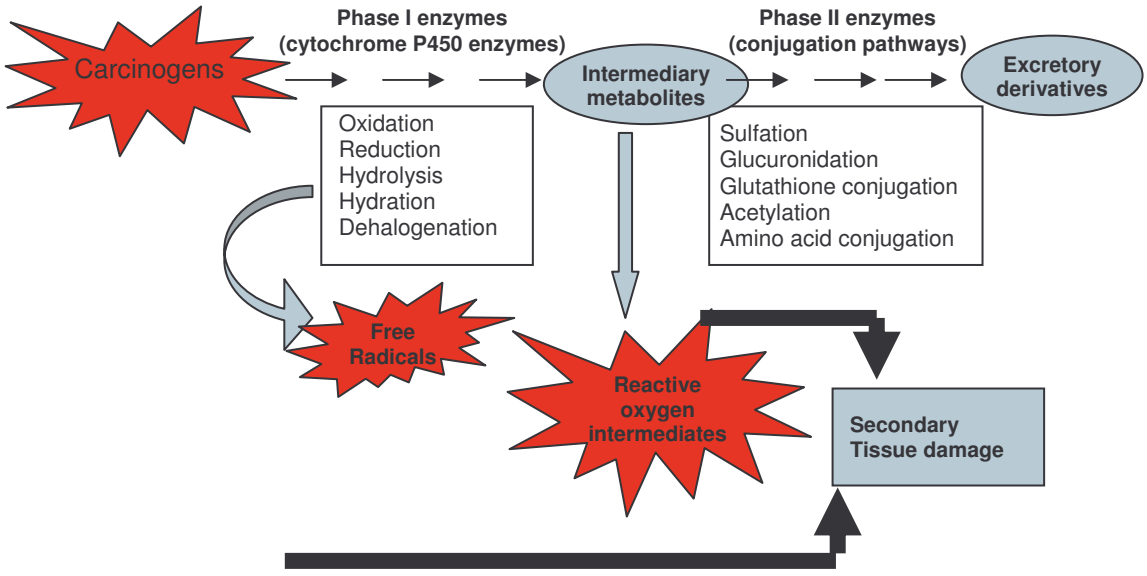

Fig. 1. The phase I- and phase II- enzyme systems and their relevance for detoxification.

One of the most promising areas in cancer research is chemoprevention. The induction of phase II enzymes, such as glutathione S-transferase (GST) and quinone reductase (QR) is considered a major mechanism of protection against the initiation of carcinogenesis and chemical stress. GSTs are ubiquitous multifunctional enzymes, which play a key role in cellular detoxification and catalyzes the conjugation of electrophilic compounds with glutathione, resulting in the products more water-soluble that are generally more hydrophilic and less cytotoxic, that are metabolized further to mercapturic acid and then excreted. GSTs

are only part of a complex detoxification system because multiple enzymes are involved in the redox cycling of GSH.QR functions as an inducible protective device against quinone toxicity by reducing quinones to relatively stable hydroquinones. These resulting hydroquinones can be conjugated and excreted through mercapturic acid pathways.The aim of our work is to investigate the *in vitro* potential of an extract obtained specifically from *Viscum album* growing on *Mallus domestica* (VaM) and its ability to induce phase II detoxifying enzymes. The gluthatione S-transferase (GST) and quinone oxidoreductase (QR) assays as well the inhibition of adherent epithelial human ovarian tumor cells A2780 proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were applied.

MATERIALS AND METHODS

Misletoe samples and phenolic compounds extraction

The leaves and stems of *V. album*, were harvested from *Malus domestica* (VaM) in February 2010 on the North-West of Romania country (Borod – Gheghie region). The dried leaves and stems were kept for 3 days, at 60^0C, grounded and stored in sealed plastic bag until the subsequent analysis. The dried material was treated with chloroform (1:4, g/v) for 24 hours in order to remove lipophilic compunds. After removal of chloroform, phenolic acids were extracted with 70% ethanol solution added to a final volum ten fold higher than sample weight, at room temperature, in the dark, for 24h. Ethanol was removed using a IKA Distilling Rotary Evaporator and then, the aqueous extract was freezed and liophylized with a Laboratory Freeze-Dryer Martin Christ. For the determination of antioxidant activity, liophylized extract was dissolved in methanol at different concentration (0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.062 mg/ml and 0.031 mg/ml).

Determination of antioxidant activities

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity was determined using the method proposed by Brand-Williams *et al.,* (1995). Briefly, a volume of 150 µl mistletoe extract at different concentration (between 0.031 to 0.5 mg/ml) and 1400 µl DPPH methanolic solution (80 µM) were added and the decrease in the absorbance of the resulting solution was monitored at 515 nm after 30 min. The percentage of scavenging effect of mistletoe extract against DPPH radicals, was calculated using the following equation:

$$\textbf{DPPH scavenging effect (\%) = [(A}_0 \textbf{ - A s) x 100] / A}_0$$

Where, A_0 is absorbance of the blank, and A_S is absorbance of the samples at 515 nm. As positive antioxidant control we used Trolox (the concentration between 50 and 500 µM), and also, the results were expressed as µmol Trolox per 1 g of extract's dry weight. The discoloration of DPPH was plotted against the sample concentration in order to calculate the IC_{50} value, which is the amount of sample necessary to decrease the absorbance of DPPH by 50%.

The Ferric reducing antioxidant power (FRAP) assay was applied according to the method of Benzie and Strain (1996) with some modifications. The FRAP assay consists in the ferric tripyridyltriazine (Fe(III)-TPTZ) complex reduction to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by an antioxidant at low pH. The stock solutions included: 300 mM acetate buffer; 270 mg $FeCl_3 \cdot 6\ H_2O$ dissolved in 50 ml distillated water; 150 mg TPTZ and 150 µl HCl, dissolved in 50 ml distillated water. The working FRAP solution was freshly prepared by mixing 50 ml acetate buffer, 5 ml $FeCl_3 \cdot 6\ H_2O$ solution and 5 ml TPTZ solution. Mistletoe extracts (100 µl) were allow to react with 500 µl FRAP solution and 2 ml distilled

water, for 1 h, in dark. The final colored product (ferrous tripyridyltriazine complex) was quantified by VIS absorption at 595 nm, and IC_{50} of VaM was calculated. As positive antioxidant control we used Trolox and obtained a standard linear curve, between 50 and 500 µmol/l Trolox. The FRAP value was obtained using the following equation based on the calibration curve: y=0.0157 x + 0.0549 (R^2=0.9981), where x was the absorbance and y was the µmol Trolox equivalent.

Determination of total phenolics

The total phenolic content was determined by the Folin-Ciocalteu method (Singleton et al., 1999). Mistletoe extract (100 µl) was mixed with 1750 µl distillated water, 200 µl Folin-Ciocalteu reagent (dilution 1:10, v/v) and 1000 µl of 15% Na_2CO_3 solution, and the mixture incubated at room temperature, in the dark, for 2 hours. The absorbance was measured at 765 nm using a spectrophotometer Schimadzu mini UV-Vis. The calibration curve was linear for the range of concentrations between 0.1-0.5 mg/ml gallic acid. Total phenolic content of mistletoe extract was expressed as mg gallic acid equivalents (GAE)/g dry weight, using the following equation based on the calibration curve: y=1.9735x + 0,0261 (R^2=0.9928), where x was the absorbance and y was the gallic acid equivalent (mg/ml).

Cell culture

Adherent epithelial human ovarian tumor cells A2780 were maintained in RPMI medium supplemented with 10% fetal serum, at 37◦C, 5% CO2, 90% humidity. For proliferation A2780, 1 x 10^4 cells/well were seeded in 96 well plates. For other experiments cells were seeded in 6 well plates 5 x 10^5 cells/well. After reaching 90% confluence, the growth medium was removed and replaced with fresh medium for untreated cells or with medium containing mistletoe extract for treated cells.

To evaluate the respiration capacity and viability of cells, we aplied the MTT assay. Cells were incubated for 24, 48 and 72 hours with different concentrations (0, 10, 20, 40, 80 and 160 µg/ml) of mistletoe extract (VaM). The tetrazolium salt was used for assaying the quantification of living metabolically active cells. 200 µl MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)(MTT) in HBSS buffer were added to each well. MTT reagent is metabolized by mitochondrial dehydrogenase to formazan. After 2 h of incubation the MTT reagent was removed and the formazan particles were solubilized with 200 µl DMSO. The end-product was measured at 550 nm with a microplate plate reader HT BioTek Synergy (BioTek Instruments, USA). Viability was expressed as the percentage of actively proliferative cells.

Determination of phase II enzymes in A2780 cells

GST assay. The activity of gluthatione S-Transferase from human ovarian tumor cells A2780, after exposure at different concentration of VaM extract was measured with the Cayman Chemical Glutathione S-Transferase Assay Kit. The principle of assay consist in the measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample.

QR assay. The activity of quinone reductase was measured according to Lind at al. (1990). The activity of quinone reductase was determined using $NADP^+$/menadione as substrates. The spectrophotometric assays were based on absorption at 550 nm and all samples were measured in triplicate. One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1µmol of substrate to product per minute at 37^0C. The activity of QR was expressed as U/mg protein.

Glutathione assay. The GSH assay was performed using an optimized enzymatic recycling method with glutathione reductase (Sigma, St. Louis, USA). Results are expressed as nmoles GSH/mg protein in cell pellet.

Protein determination for enzymes assay. The cells were centrifuged at 1000 rpm, the PBS was removed and the pellet was homogenized in phosphate buffer 100 mM, containing 2mM EDTA. Then the homogenate was centrifuged at 10000 rpm, for 15 minutes. The supernatant was used to determine protein concentration using the bicinchoninic acid assay kit instructions (Sigma, St. Louis, USA).

RESULTS AND DISCUSSION

Antioxidant activity

Antioxidant properties of mistletoe extracts that are growing on *Malus domestica* were determined by two methods, DPPH and FRAP (Tab.1 and 2)

Table 1 include data which show the scavenging effect of different concentrations of VaM extract, as determined by DPPH assay, measured as percentage of inhibition (%), as Trolox eauivalents. The IC_{50} value, expressed as percentage, which represent the quantity of VaM extract which decrease by 50% the absorbance of DPPH, is also shown. We found a dose-dependent antioxidant activity of this extract, higher than Trolox equivalent at 2.5 and 5 mg/ml. The IC_{50} value was low (102.54 μg/ml) demontrating that this extract was powerful antioxidant.

Tab. 1

The scavenging effect of different concentrations of VaM extract, as determined by DPPH assay, measured as percentage of inhibition (%), as Trolox eauivalents and as IC_{50} values.

Concentration of VaM extract (mg/ml)	% DPPH	μmol Trolox/g dry weight	IC_{50} (μg/ml)
5	26.97	1.58 ± 0.001	102.54
2.5	18.49	1.02 ± 0.019	
1.250	9.67	0.44 ± 0.039	
0.625	8.82	0.28 ± 0.001	
0.312	7.21	0.38 ± 0.018	
TROLOX	-	-	0.13

Other authors (Oluwaseun and Ganiyu, 2008) who investigated the antioxidant properties of methanol extracts of *V. album* isolated from cocoa and cashew trees in the South Western part of Nigeria, found the scavenging ability of methanol extracts to be dose-dependent (0-10 mg/ml). The free radical scavenging ability of the *V. album* extract from cocoa tree performs better than that from cashew tree, a fact that is in agreement with the total phenol content of the two extracts (182 mg /100 g, and 160 mg /100 g, respectively).

By FRAP assay it was measured the antioxidant, reducing potential of the VaM extract reacting with a ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex and producing a colored complex of ferrous tripyridyltriazine (Fe^{2+}–TPTZ). The antioxidant capacity was expressed in μmol Trolox/g dry weight and were significantly dependent on the extract doses, as shown in Tab. 2.

Tab. 2

The antioxidant capacity of VaM, measured by FRAP assay. The values are expressed in μmol Trolox/g dry weight.

Concentration of VaM extract (mg/ml)	µmol Trolox/g dry weight
5	1962.02 ± 0.002
2.5	1048.28 ± 0.001
1.250	566.11 ± 0.000
0.625	313.11 ± 0.005
0.312	155.37 ± 0.000

Vicas et al., 2009, investigated by the same FRAP assay the antioxidant capacity of aqueous extracts of fresh leaves and stems from *V. album.* There were noticed differences between the antioxidant activity of mistletoe extract from leaves and stems. Among the selected plants, crude aqueous extract of *V. album* leaves grown on *Mallus domestica* exhibited the highest antioxidant activity.

Phenolic compounds are known to have antioxidant activity and their activity is due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Zheng and Wang, 2001, Bhoyar et al., 2011). Phenolic content in the mistletoe extract, as determined by Folin-Ciocâlteu method was 91.51 mg gallic acid equivalents /g dry weight. Acoording to HPLC analysis of mistletoe hosted by *Mallus domestica* (VaM), Vicas et al., 2011 identified seven phenolic acids in leaves, ferulic acid being the main compound in leaves and stems (7.81 µg/g dry matter, and 6.88 µg/g dry matter, respectively) of VaM samples.

Antiproliferative effect on cells A2780 by mistletoe extract

The anti-proliferative effects of VaM extract on A2780 cells were investigated using MTT assay. The results obtained are presented in the Fig.2. The A2780 cell viability was inhibited to 25.51% and 63.47 % by 160 µg/ml mistletoe extract treatment after 24 hours respectively, 48 hours. The IC_{50} (50% inhibitory concentration) was obtained for 261.70 µg/ml, 120.62 µg/ml and 120.10 µg/ml extract concentration after 24 hours, 48 hours, respectively 72 hours, as presented in Table 3.

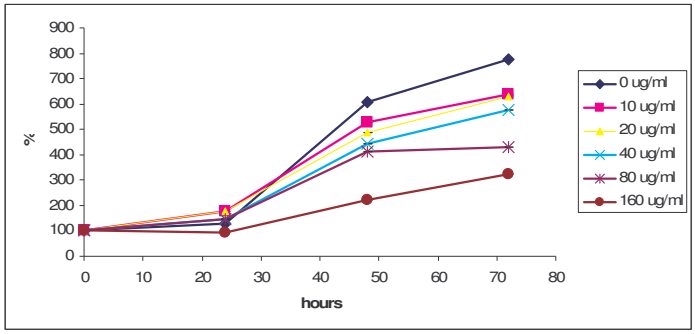

Fig.2. The antiproliferative effects of VaM extract on A2780 cells, at different concentrations, using the MTT assay.

Tab. 3

The IC_{50} of VaM extract on A2780 cells at different time of exposure

VaM	IC_{50} (µg/ml)		
	24 hours	48 hours	72 hours
	261.70	120.62	120.10

Recently, the cytotoxic and apoptosis-inducing effects of an aqueous mistletoe extract dose-dependent in leukemic Jurkat cells were reported. The IC_{50} of mistletoe extract on Jurkat cells was 35.67 μg/mL (Sabová et al., 2010). Sadeghi-Aliabadi et al., 2006, evaluated the cytotoxic effects of Iranian mistletoe extracts (growing on *Carpinus betulus*) on the following cancer cell lines: Hela, KB, MDA-MB-468, K562 and MCF using colorimetric MTT assay. Their results showed that plant juice was the most cytotoxic fraction on all cancer cells tested (IC_{50}=0.0316 mg). The IC_{50} of hydroalcoholic and methanolic extracts were 0.1 and 0.316 mg, respectively. Rugina et al., 2010, used A2780 cells for treatment with mistletoe extract for 24 h and assayed for cell viability. A2780 cell viability was inhibited to 87% by 50 μg/ml mistletoe extract treatment, and to 79% at 110 μg/ml extract concentration. Cytotoxic concentration of 200 μg/ml of mistletoe extract reduced A2780 cell viability with 60%. The IC_{50} (50% inhibitory concentration) was obtained for 170 μg/ml extract concentration. Hashimoto et al., 2007, investigated the anti-cancer mechanism of benalu extract (a parasitic plant to tea and coffee plants) on human leukemia Jurkat T cell. The treatment with the extract inhibited the cell proliferation in a dose and time dependent manner.

Induction on phase II enzymes

The modulation of phase II enzymes by the different extracts or drugs can be study both, *in vitro*, using different cell lines, or *in vivo*, used animal experiment. *In vitro,* the following cell lines were used: mouse liver cells (Hepa-1c1c7) (Bolling et al., 2007; Awika et al., 2009; Yang and Liu, 2009), human hepatoma (HepG2) (Brandon et al., 2006; Westerink and Schoonen, 2007; Syng-ai et al., 2011), ovarian carcinoma (IGROV-1) (Brandon et al., 2006), colon carcinoma (CaCo-2, LS180) cell lines(Brandon et al., 2006), Human breast tumor cell lines, MDAMB and MCF-7 cells (Syng-ai et al., 2004), rat hepatocytes (Syng-ai et al., 2004)

The effect of VaM extract on the GST and QR activities in adherent epithelial human ovarian tumor cells A2780 was analyzed. GST activity in control (the cells without of treatment with mistletoe) was 6.98 ± 0.17 nmol/min/mg protein (Fig.3 A).

A slight increase of GST activity (7.42 ± 0.17 nmol/min/mg protein) was observed after 48 h of treatment with 60 μg/ml VaM extract compared to untreated cells. Instead, the extract concentration of 120 μg/ml, increased the GST activity to 9.66 ± 0.20 nmol/min/mg protein. Interestingly, a higher amount of extract (240 μg/ml) induced an inhibitory effect on GST enzyme activity (6.50 nmol/min/mg protein).

The QR activity of untreated A2780 cells was 0.88 ± 0.56 U/mg protein. Administration of 60 μg/ml of VaM extract induced a drastic decrease of QR activity (0.43 ± 0.39 U/mg protein), but for the other two extract concentrations tested (120 and 240 μg/ml) resulted an increase of enzyme activity (1.35 ± 0.00, respectively 1.01 ± 0.41 U/mg protein) (Fig.3 B).

In addition, we determined the level of GSH in human ovarian tumor cells without and with the treatment with mistletoe extract. The highest concentration of VaM extract (240 μg/ml) increased the level of GSH (65.98 ± 0.353 nmol GST/mg protein) compared to untreated cells (Fig.3C).Yang and Liu, 2009, shown that the phytochemicals present in grapes (especially the grape variety Cabernet Franc) have potent QR induction (CD = 0.6 ± 0.1 mg/ml) and antiproliferative activity toward Hepa1c1c7 cells (EC_{50} = 8.7 ± 2.4 mg/ml).

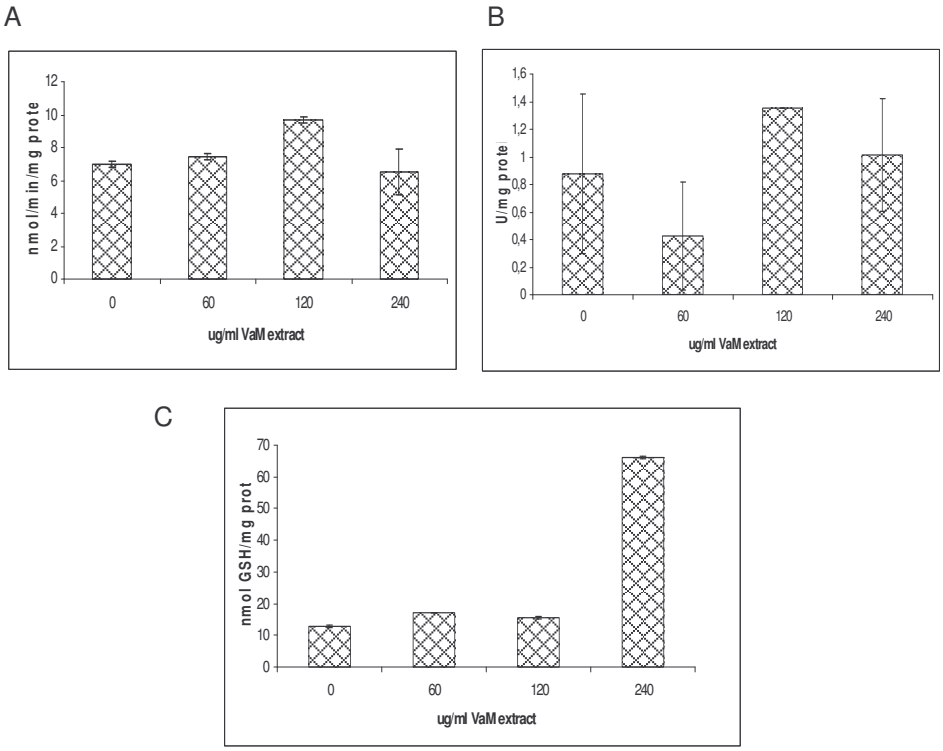

Fig. 3. The effects of VaM extracts on GST activity (A), QR activity (B) and the level of GSH in adherent epithelial human ovarian tumor cells A2780. Data shown are mean values, with bars indicating the SD of the mean (n = 3)

Treatment of Hepa 1c1c7 cells with medicinal plant *Thunbergia laurifolia* Lindl. (Acanthaceae)extracts resulted in a dose-dependent increase in QR specif c activity for all extracts. Acetone extract (92 µg GAE/mL) increased QR activity 2.8-fold, while ethanol (120 µg GAE/mL) and water (1000 µg GAE/mL) extracts increased QR activity by 1.35- and 1.56-fold, respectively (Oonsivilai et al., 2007).

CONCLUSION

We show that different concentrations of an extract from mistletoe growing on *Mallus domestica* had inhibitory effects on the growth of adherent epithelial human ovarian tumor cells A2780 (IC_{50} = 120.62 µg/ml, after 48 h treatment). Also, this extract is a good antioxidant source (total phenol content of 91.51 mg gallic acid equivalents /g dry weight), having a radical scavenging ability and a reducing potential proportional to the dose used (from 0.312 to 5 mg/ml) *in vitro*. At 120 µg/ml, this extract increased, significantly, the activity of GST and QR, as well the level of GSH.Further studies are needed to demonstrate the specific induction of phase II detoxifying enzymes activity by mistletoe extract, using different *in vitro* cell models.

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