

Full Length Research Paper

Antioxidant and antimutagenic activities of *Viscum album* fruit ethanolic extract in human lymphocytes

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Polyphenolic compounds are widely distributed in plants and known to be excellent antioxidants *in vitro*. They have the capacity to reduce free-radical formation by scavenging free-radicals. In this study we have evaluated the antioxidant and antimutagenic potencies of polyphenolic compounds of *Viscum album* against trichloroethylene (TCE)-induced oxidative and genotoxic damage. *V. album* extract (VAE 0.5 µg/ml) protected human lymphocytes against TCE. In chromosomal aberration (CA) analysis, no significant increase in total aberrations were found after treatment with TCE and all VAE concentrations. The mitotic index (MI) showed significant increase in 0.5 µg/ml VAE samples when compared with TCE-treated (2 µM) group. VAE (0.5 µg/ml) reduced the levels of malondialdehyde (MDA) significantly whereas VAE (1.0 and 2.0 µg/ml) samples increased MDA concentrations significantly. We have also shown that the various DNA effects of TCE treatment seem to be DNA damages, but not mutations as TCE treated profiles were reverted back to the control like profiles by most probably DNA repair mechanisms in VAE 0.5 µg/ml treated group.

Key words: *Viscum album*, polyphenolic compounds, antioxidant, DNA instability, lipid peroxidation.

INTRODUCTION

There is an increasing interest in plant polyphenolic compounds because of their potential roles as cancer chemopreventive agents and chronic disease protectors. Their beneficial effects are considered to be mainly due to their antioxidant and chelating activities. One of the major groups of plant extracts are the polyphenolic compounds which occur widely in food, such as fruit, vegetables and cereals (Hashim et al., 2005).

Viscum album L. (Mistletoe) is a highly specialized angiosperm of the family Loranthaceae, which is well known as a broad host range hemi-parasite of a variety of different gymnosperms and angiosperms (Deeni and

Sadiq, 2002). It is of great economic importance due to the major damages it causes to its host which leads to economic losses. Mistletoes have been used both in traditional and supplementary medicine in the treatment and management of many diseases such as diabetes mellitus, stroke, hypertension, chronic cramps, stomach problems, heart palpitations, difficulties in breathing and hot flushing in menopause for many years (Orhan et al., 2005; Oluwaseun and Ganiyu, 2008). A number of biological effects such as anticancer, antimicrobial, immunomodulatory and apoptosis inducing activities have been reported (Table 1) (Cetin and Ozcelik, 2007). Mistletoe extracts could also reduce harmful and mutagenic effects of reactive oxygen species (ROS) generated during radiotherapy and chemotherapy (Onay-Ucar et al., 2006).

Although several biological activities of this plant have been reported in many studies, to our knowledge no study examined its direct antioxidant and antimutagenic activity in human lymphocyte culture. The aim of this study was to determine antimutagenic and antioxidant activities of ethanolic extracts of *V. album* against TCE which is a widely used mutagenic and oxidative agent

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Abbreviations: TCE, Trichloroethylene; VAE, *Viscum album* extract; CA, chromosomal aberration; MI, mitotic index; MDA, malondialdehyde; ROS, reactive oxygen species; RAPD, randomly amplified polymorphic DNA; DMSO, dimethyl sulfoxide; LPO, lipid peroxidation.

Table 1. Commercial *V. album* extract and their activities.

Extract	Activities
Iscador ^R	Anticancer Cytotoxic Immunomodulatory Anti-HIV Antineoplastic
Helixor ^R	Anticancer Cytotoxic Immunostimulant Immunomodulatory
Plenosol ^R	Immunomodulatory
Iserol ^R	Anticancer Immunomodulatory
Vyserol ^R	Immunomodulatory
Eurixor ^R	Immunomodulatory

(Hu et al., 2008). We have also aimed to assess the DNA effects of *V. album* extract (VAE) using changes in random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) profiles of the experiment groups to further integrate in genomic instability and carcinogenesis work.

MATERIALS AND METHODS

Plant materials

V. album fruits were harvested from Isparta in May 2009. Fresh fruit were picked and washed using tap and distilled water. Then they were cut into small pieces, weighed and used immediately, or stored at -20°C until use.

Extraction of polyphenolic compounds

Polyphenolic compounds in *V. album* fruit were extracted with 80% ethanol. 2.5 g of the fruit material was put in stoppered flasks placed in an ultrasonic bath first with 10 ml solvent for 60 min, then with 10 ml solvent for 45 min, and finally with 5 ml more solvent for 15 min. The overall extraction taking 120 min. The three extracts were combined and brought to a final volume of 25 ml with 80% ethanol. The ethanol was removed from vacuum rotary at 40°C until dryness. Then it was dissolved with 5 ml 80% ethanol. The extract was filtered through a GF/PET (glass fiber/polyethyleneterephthalate) 1.0/0.45µm microfilter, to be used in further steps. Polyphenolic compounds of *V. album* are identified using MS analysis (Cebovic et al., 2008; Sengul et al., 2009).

Lymphocyte cultures and cell harvesting for MI and CA

The chemicals (TCE, n-butanol, pyridine, thiobarbituric acid, tetraethoxypropane and dimethyl sulfoxide (DMSO)) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Peripheral blood lymphocytes were taken from 2 non-smoking healthy

males (age-28 and 35). Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood to RPMI-1640 chromosome medium supplemented with 15% heat-inactivated fetal calf serum, 100 IU/ml streptomycin, 100 IU/ml penicillin and 1% L-glutamine. Lymphocytes were stimulated to divide by 1% phytohaemagglutinin. TCE and *V. album* extracts (VAE) were dissolved in DMSO. TCE (2 µM) and VAE (0.5 and 1.0 and 2.0 µg/ml) were added to the culture just before incubation. Used concentrations of TCE and VAE were adopted from Hu et al. (2008) and Ipek et al. (2003) respectively. The experiments for each donor were performed with 5 groups as follows:

Group 1: Control (DMSO); group 2: 2µM TCE; group 3: 2µM TCE + VAE 0.5 µg/ml; group 4: 2µM TCE + VAE 1.0 µg/ml; group 5: 2µM TCE + VAE 2.0 µg/ml.

For CA analysis, the cultures were incubated at 37°C for 72 h. To arrest the cells at metaphase, 0.1 mg/ml of colcemide was added to the culture 3 h before harvesting. The cultures were centrifuged at 800 g for 10 min to get supernatant for enzyme analysis. The pellet was treated for 30 min with hypotonic solution (0.075 M KCl) and fixed in a 1:3 mixture of acetic acid: methanol (vol/vol). Metaphase chromosomes were stained with Giemsa technique. The mitotic index was calculated from the number of metaphases in 1000 cells analyzed per culture for each dose group and donor for CA assay (Lamberti et al., 1983). The analysis of cells with CAs was performed for 30 metaphases for each culture. The CAs were classified according to the International System for Human Cytogenetic Nomenclature (ISCN) (Pazy-Mino et al., 2002). Gaps were not evaluated as a CA according to Mace et al. (1978).

For each well-spread slide containing 44 - 46 chromosomes in each cell were scored, and the values obtained were calculated as CAs per cell.

DNA isolation and RAPD profiling

PCR based RAPD analysis were used to confirm the expected DNA effects of various dilutions of VAE on human lymphocyte culture DNA. DNA from lymphocyte culture cells were extracted using Qiagen's QIAamp DNA Blood Mini Kit as described by the manufacturer.

Optimisations of DNA concentration, primer choice, annealing temperature and repeatability confirmation were done according to previous review literature by Atienzar and Jha (2006). DNA profiles were generated in RAPD reactions performed in a reaction volume of 25 ml containing 1 x PCR buffer (NH₄)₂SO₄, 0.2 mM from each dNTP (2 mM dNTP mix), 2.5 µM of the 10-mer oligonucleotide with sequence: OPB18-5'- CCACAGCAGT-3', 10 - 50 ng of genomic DNA and 0.5 units of *Taq* DNA polymerase. Amplification was performed in a Techne TC-512 Gradient thermal cycler programmed for 3 min at 95°C (initial denaturation of template DNA) followed by 45 cycles of 1 min at 94°C (denaturation), 1 min at 50°C (annealing temperature), 2 min at 72°C (extension), and 5 min at 72°C (final extension step).

Amplification products were run on 2% agarose gels stained with ethidium bromide and visualization was done under UV-light; the banding profiles were documented with GelDoc 2000 (BioRAD) and Quantityone programme.

Determination of lipid peroxidation

Lipid peroxidation (LPO) was measured in terms of changes in MDA concentration. MDA levels in cell culture supernatants were determined spectrophotometrically according to the method described by Ohkawa et al. (1979). A mixture of 8.1% sodium dodecyl sulphate, 20% acetic acid and 0.9% thiobarbituric acid was

added to 0.2 ml of sample, and distilled water was added to the mixture to bring the total volume up to 4 ml. This mixture was incubated at 95°C for 1 h. The tubes were cooled under cold water. 1 ml distilled water with 5 ml n-butanol/pyridine (15:1, v/v) was added on to the cooled sample and vortexed. The samples were centrifuged at 4000 × g for 10 min. The supernatants were collected and absorbances were measured at 532 nm. 1, 1, 3, 3-tetraethoxypropane was used as the standard of the assay. Lipid peroxidation levels were expressed as μmol/l MDA. Spectrophotometrical measurements were performed with BIO-TEK microplate reader Power Wave XS (BioTek Instrument, USA).

Statistical analysis

For MI and CA statistical analysis, t-test was used. For MDA statistical analysis, Mann-Whitney U-test was used. Results were expressed as mean ± SD. For these procedures, statistical package for the social sciences (SPSS) 15.0 version for Windows (SPSS Inc, Chicago, Illinois, USA) was used.

RESULTS

The results of the MI and CA are given in Table 2 and Figure 1. Table 2 lists the effects of VAE (0.5, 1.0 and 2.0 μg/ml) on the mitotic index and the number of chromosomal aberrations in human lymphocyte cell cultures treated with TCE and different concentrations of VAE. In CA analysis, no statistically significant increase in total aberrations were found after treatment with TCE and VAE concentrations decreased in MI values of TCE-treated group, 2 μM TCE + 1.0 and 2.0 μg/ml VAE samples when compared with control ($p < 0.001$) was statistically significant. Whereas the MI showed a statistically significant increase in 2 μM TCE + 0.5 μg/ml VAE samples when compared with TCE-treated group ($p < 0.001$) (Figure 1 and Table 2)

The level of MDA decreased in 2 μM TCE + 0.5 μg/ml VAE samples (donor 1 and 2) when compared with control and TCE-treated group. The concentration of MDA showed a statistically significant increase in 2 μM TCE + 1.0 and 2.0 μg/ml VAE samples when compared with control and TCE-treated group ($p < 0.001$). When compared with 2 μM TCE + 0.5 μg/ml VAE, there was a statistically significant increase in the concentration of MDA (Table 3 and Figure 2) in the 2 μM TCE + 1.0 and 2.0 μg/ml VAE samples ($p < 0.05$).

Various DNA effects as a result of either DNA damages or mutations can be seen on RAPD profiles as loss of bands, appearance of bands, decrease in band intensity and increase in band intensity. Each DNA was amplified in triplicates from the same mastermix to confirm the repeatability and show the extend of variation (Uzonur et al., 2004a, 2004b). The DNA effects on RAPD profiles are prominent for group 2 (2 μM TCE), lanes 7 - 9 and group 4 (2 μM TCE + VAE 1.0 μg/ml) DNA RAPD profiles, lanes 1 - 3, whereas for group 3 (2 μM TCE + VAE 0.5 μg/ml), lanes 4 - 6 and group 1 (Control) DNA, lanes 10 - 12 have stable RAPD profiles.

Table 2. Antigenotoxic effects of phenolic compounds from VAE against TCE in number of CA and the MI in human lymphocytes (mean ± SD).

Treatment	Metaphases scored	CA (mean ± SD)	MI (mean ± SD)
Control (DMSO)	30	0.30 ± 0.15	9.25 ± 0.30
2 μMTCE	30	0.48 ± 0.22	4.91 ± 0.27 ^a
2 μMTCE + 0.5 VAE	30	0.20 ± 0.20	9.83 ± 0.40 ^b
2 μMTCE + 1.0 VAE	30	0.56 ± 0.24	3.85 ± 0.33 ^{acd}
2 μMTCE + 2.0 VAE	30	0.48 ± 0.22	3.63 ± 0.41 ^{acd}

^a $p < 0.001$ compare with control; ^b $p < 0.001$ compare with 2μM TCE; ^c $p < 0.05$ compare with 2μMTCE; ^d $p < 0.001$ 2μM.

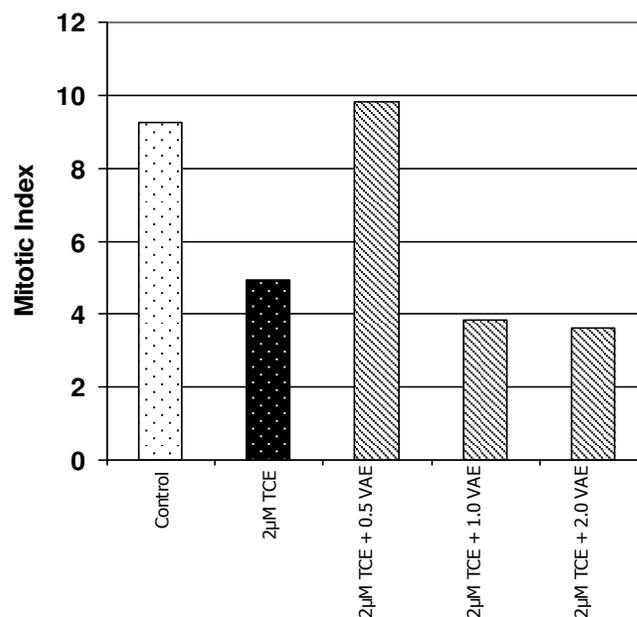


Figure 1. Antigenotoxic effects of phenolic compounds from VAE against TCE in number of CA and the MI in human lymphocytes (mean ± SD).

Table 3. Effects of phenolic compounds from VAE against TCE on level of MDA in human lymphocyte culture.

Treatment	MDA (Mean ± SD) (μmol/l)
Control (DMSO)	10.10 ± 0.82
2 μMTCE	92.62 ± 2.49 ^a
2 μMTCE + 0.5 VAE	6.91 ± 0.73 ^{ab}
2 μMTCE + 1.0 VAE	139.89 ± 1.87 ^{abc}
2 μMTCE + 2.0 VAE	156.00 ± 0.93 ^{abcd}

^a $p < 0.001$ compare with control; ^b $p < 0.001$ compare with TCE; ^c $p < 0.001$ 2μM TCE + 0.5μg/ml VAE; ^d $p < 0.05$ 2μM TCE + 1.0μg/ml VAE.

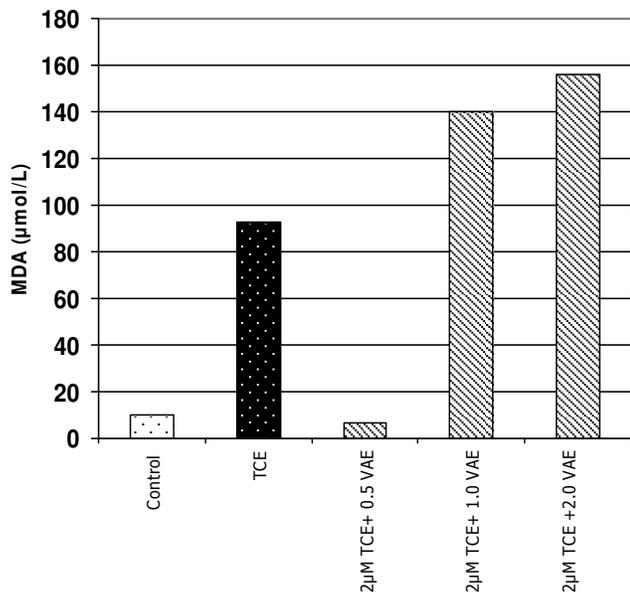


Figure 2. Effects of phenolic compounds from VAE against TCE on level of MDA in human lymphocyte culture.

DISCUSSION

Damages caused by free radical-induced oxidative stress is the major causative agent of many disorders including cancer, tissue injury, neurodegenerative diseases, aging and rheumatoid arthritis. In recent years, antioxidants derived from natural resources, mainly from plants, have been intensively used to prevent oxidative damages, because of some advantages over synthetic ones; they can be obtained easily and economically and have slight or negligible side effects (Onay-Ucar et al., 2006).

In recent years, phenolic compounds have attracted the interest of researchers because they show promise of being powerful antioxidants that can protect the human body from free radicals, the formation of which is associated with the natural metabolism of aerobic cells (Halliwell, 1996; Oluwaseun and Ganiyu, 2008). It has been reported that *V. album* has a high antioxidant activity (82.23%) and totalphenolic content (42.29mg/g) (Sengul et al., 2009).

The present study indicates that phenolic compounds of *V. album* have protective effect against lipid peroxidation. We have also shown that the antioxidant capacity of the extract could differ depending on concentration of VAE. Our results were supported by many previous work such as Janakat and Al-Thnaibat (2007) reported that LPO was reduced by methanolic and boiled aqueous extracts of *V. album*. It has been reported that methanolic extract of *V. album* has antioxidant capacity (Onay-Ucar et al., 2006; Avci et al., 2006; Ioana et al., 2007; Janakat and Al-Thnaibat, 2007; Oluwaseun and Ganiyu, 2008; Sengul et al., 2009).

The RAPD-PCR analysis results of the present study confirmed and support the CA and MI analysis results (Figure 3). The most similar results to the control group

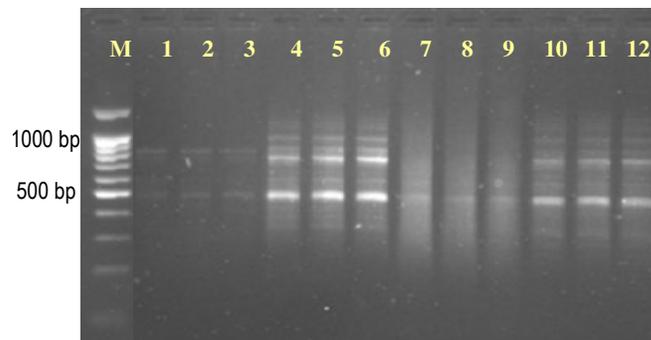


Figure 3. The result of RAPD-PCR analysis as RAPD-PCR amplification profiles of groups 4, 3, 2 and 1 DNA in triplicates respectively to show the extend of DNA variation.

are the *V. album* (2 µM TCE + VAE 0.5 µg/ml) treated sample DNA which is an indication for DNA repairing effect of VAE in 0.5 µg/ml concentration, whereas in higher concentrations the DNA effects are highly significant as genomic instability. The various DNA effects seem to be DNA damages, but not mutations as TCE treated profiles are reverted back to the control like profiles by DNA repair mechanisms in 2 µM TCE + 0.5 µg/ml VAE treated sample.

The molecular and cytogenetic analysis of the effects of VAE on TCE treated lymphocyte cultures in this work is promising for unraveling the extract's probable antioxidant, anti-mutagenic and DNA repair mechanism inducing properties at concentrations that should be further investigated.

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