Environmental and Experimental Botany 65 (2009) 330–337

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Naphthoquinones as allelochemical triggers of programmed cell death

Petr Babula[a,*], Vojtěch Adam[b], René Kizek[b], Zdeněk Sladký[a], Ladislav Havel[c]

[a] Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Palackeho 1-3, CZ-612 42 Brno, Czech Republic
[b] Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[c] Department of Plant Biology, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic

ARTICLE INFO

Article history:
Received 9 September 2008
Received in revised form 23 October 2008
Accepted 9 November 2008

Keywords:
Programmed cell death
Juglone
Plumbagin
Tobacco BY-2

ABSTRACT

Juglone and plumbagin are plant bioactive derivatives of 1,4-naphthoquinone occurring in plants, whereas lots of these plants belong to invasive species. Clarifying of action of juglone and plumbagin applied on plant cell model represented by tobacco BY-2 cells was the basic aim of this work. It was shown that naphthoquinones are able to induce various structural, functional and enzymatic changes leading to processes of apoptic-like cell death. Using dihydroethidium as fluorescent probe the mechanism of naphthoquinones action was explained. They are able to generate reactive oxygen species, which play important role in processes of programmed cell death. Disruption of mitochondrial respiratory chain was detected too. This study shown that mechanism of naphthoquinones action to plant cells is very complex and predestine them to be very effective compounds in plant competition fight.

1. Introduction

The term alellopathy denotes the production of specific biomolecules (alellochemicals) by one plant that can induce suffering in, or give benefit to, another plant. However, it is most commonly used in the former sense—an interaction in which one plant causes suffering to another plant. There are several ways in which an alellopathic plant can release its protective chemicals: (a) volatilization—alellochemicals can release specific compounds in the form of gas through stomata in their leaves, (b) exudation—beneficial chemicals are released into the soil through roots, (c) leaching—protective chemicals are stored in the leaves after their dropping they decompose and release chemicals.

The allelopathic events are usually caused by secondary metabolites. There are hundreds of them in the plant kingdom (Einhellig, 1995; Barkosky and Einhellig, 2003). The compounds exhibit a wide range of mechanisms of action, from affects on DNA (alkaloids), phytohormone activity, ion uptake, and water balance (phenolics) as well as photosynthetic and mitochondrial function (quinones). Interpretations of mechanisms of action are complicated by the fact that individual compounds can have multiple phytotoxic effects (Einhellig, 1995).

One group of such allelophatic compounds are naphthoquinones, oxygen-derivatives of naphthalene. They are widespread in nature as secondary metabolites of micro-organisms and fungi as well as plants. They have been detected in many vascular plant families (*cf. Droseraceae, Juglandaceae, Nepenthaceae* and *Plumbaginaceae* (Binder et al., 1989; Crouch et al., 1990; Lin et al., 2003). The most widespread naphthoquinones originated from shikimic acid are juglone (5-hydroxy-1,4-naphthoquinone, amber-colored compound isolated from numerous *Juglans* spp.) and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, orange-colored compound isolated from *Plumbago, Drosera, Dionaea* and *Nepenthes*). The interest of many investigators in these compounds is due to their broad-range of activities as antibacterial, fungicidal, antiparasitic and insecticidal effects (Babula et al., 2009; Lee and Lee, 2008). They have inhibitory effect on insect larval development and sedative or toxic effect on fish and animals (Higa et al., 1998) besides that they have also cytostatic and anticarcinogenic properties. These properties emerge due to their ability to act as potent inhibitors of electron transport (Vennerstrom and Eaton, 1988), as uncouplers of oxidative phosphorylation (Ferraz et al., 2001), as intercalating agents in the DNA double helix, as bioreductive alkylating agents of biomolecules, and as producers of reactive oxygen radicals by redox cycling under aerobic conditions (Monks et al., 1992).

Plumbagin and especially juglone are widely studied for their allelopathic activities. Massey (1925) hypothesized that inhibitory effect of black walnut (*Juglans nigra* L.) on growth of some associated species is caused by a compound exuded by roots (Massey, 1925) and this compound was determined by Davis (1928) as juglone (Davis, 1928). Juglone is stored in plant tissues in vacuoles as hydrojuglone-β-D-glucopyranoside, which can be decomposed by enzyme hydrojuglone-β-D-glucopyranosid-β-glucosidase to juglone (Duroux et al., 1998). Juglone, as well as plumbagin, is able to be released to soil and surrounding trough necrotic plant tissues decomposition, may persist in the soil for weeks and months and can be attributed to alellopathy. Growth and germination inhibition at micromolar concentration has been

* Corresponding author.
E-mail address: babulap@vfu.cz (P. Babula).

0098-8472/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.envexpbot.2008.11.007

reported for numerous plant species (Marion Meyer et al., 2007; Terzi, 2008; Topal et al., 2007). Mechanisms of plant growth depression by juglone bases in reducing of H^+-ATP-ase activity, which was shown at corn and soybean root microsomal fraction treated with juglone in concentration from 0 to 1000 μM. Other effects are inhibition of p-hydroxyphenylpyruvate dioxygenase, the crucial enzyme of plastoquinone synthesis, decrement photosynthesis in leaf tissues and transpiration and stomatal conductance inhibition (Hejl and Koster, 2004). Important fact is ability of naphthoquinones to generate active oxygen species that may play a critical role in the protection of plants against pathogens. This work was aimed on investigating the way of cell death observed in cultures of tobacco BY-2 cells exposed to various doses of juglone as well as plumbagin.

2. Materials and methods

2.1. Plant material

Nicotina tabacum L. cv. Bright Yellow-2 suspension-cultured cells (BY-2) were grown in liquid medium according Murashige and Skoog (1962), modified by Nagata et al. (1992) with constant shaking (Kühner Shaker LT-W, Adolf Kühner AG, Switzerland, 130 rpm) at 27 °C in the dark in 250 ml Erlenmeyer flasks.

Cells in exponential growth phase were exposed to naphthoquinones plumbagin and juglone for various times under culture conditions. Naphthoquinones were added to the cell suspension at final concentrations between 0 and 500 μg l^{-1}. Control and treated cells were collected in the time interval of 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h, respectively.

2.2. Cytological observation

The viability of the cells was measured by the addition of fluorescein diacetate (FDA, Sigma–Aldrich Chem. Corp., USA) and propidium iodide (PI, Sigma–Aldrich Chem. Corp.). The sample of cell suspend culture was supplemented to volume of 50 μl and incubated 5 min at room temperature with FDA (2.4 μmol l^{-1}) and PI (30 μmol l^{-1}). PI is the a nucleic acid stain that can only penetrate cells with damaged or leaking cell membranes, so PI-positive staining will only be observed in dying or dead cells. The percentage of viable and death cells was evaluated by counting using the fluorescent microscope (Olympus AX 70) equipped with broad spectrum UV excitation, from each series was evaluated 10 random fields in the microscope and the viability was determined in triplicates. The cell density was determined using Fuchs-Rosenthal haemocytometer by cell counting at white light (Olympus AX 70) at 0–240 h after the beginning of treatment; from each series was evaluated 10 random fields in the microscope and the cell number was determined in triplicates.

For nuclei observation, cells (4 ml) were treated by adding 4 ml PEM-buffer (100 mM PIPES, 10 mM EGTA, 10 mM $MgCl_2$, pH 6.9) containing formaldehyde (4%, w/w). Hoechst 33258 (Sigma–Aldrich Chem. Corp.) was used. One thousand nuclei in each preparation were observed using the fluorescent microscope (Olympus AX 70) equipped with broad spectrum UV excitation and the numbers of each morphological change were expressed as a percentage of the total cells.

To determine reactive oxygen (ROS) production and intracellular O_2^- levels oxidative fluorescence probe dihydroethidium (Sigma–Aldrich Chem. Corp.) in the combination with MitoTracker Green FM (Molecular Probes, Inc., USA) was used. The cells have been incubated with MitoTracker Green FM (75 nmol l^{-1}) for 40 min at the room temperature and dark. After this time, dihydroethidium (10 μmol l^{-1}) was added and the incubation was continued for next 20 min under the same conditions. Then the cells were twice washed by fresh cultivation medium; fluorescence was monitored by using the Olympus AX 70 fluorescent microscope.

2.3. Isolation of genomic DNA and electrophoresis of DNA

Total genomic DNA was isolated from control as well as treated cells using CTAB method. The frozen tobacco cells (100–500 mg) were mechanically disrupted with a mortar and pestle in the presence of liquid nitrogen and then lysed by buffer (2% cetyltrimethylamonium bromide—CTAB, 2% polyvinylpyrrolidone, 100 mM Tris–HCl, 25 mM EDTA and 2 M NaCl, pH 8.0). After thawing the mixture was incubated for 40 min at 65 °C and treated with extraction mixture (chloroform:phenol 9:1, w/w) and DNA was precipitated by adding 2-propanol (1:1, v/v). After centrifugation (500 × *g*, 25 °C, 5 min), washing (70% ethanol, w/w) and dissolving in TE buffer (1 mM EDTA, 10 mM Tris, pH 8,0) the solution was incubated with RNAase (at the final concentration 40 μg ml^{-1}) for 10 min at 65 °C. Electrophoresis was carried out on 1% agarose gel containing ethidium bromide at the final concentration 0.1 μg ml^{-1}. 1 μg DNA samples in 5 times diluted buffer (50% glycerol and 0.04% bromophenol blue) were electrophoresed at constant voltage (70 V). The gels were observed with a Grab-IT 1.3 (UVP, USA).

2.4. MTT and TTC assays

The dehydrogenase and oxidoreductase activity was evaluated by measuring the mitochondrial-dependent reduction of 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich Chem. Corp.) and 1,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich Chem. Corp.) to formazan. At the each point of treatment, the cells were twice washed (0.05 M Na_2HPO_4/KH_2PO_4 buffer, pH 7.4) and incubated (0.6% (w/v) MTT/TTC in 0.05 M Na_2HPO_4/KH_2PO_4 buffer, pH 7.4 with 0.05% (v/v) Tween 80) for 60 min at 28 °C. Cells were than lysed by addition of the same volume of propane-2-ol + 0.1 mM HCl. The amount of formazan produced was proportional to absorbance measured at 570 (MTT) or 490 (TTC) nm. Dehydrogenase and oxidoreductase activity are expressed as a percentage of the activity of control tobacco cells, which were taken as 100%.

3. Results

3.1. Effect of naphthoquinones on growth and viability of tobacco BY-2 cells

As it is shown in Fig. 1, the exposure of tobacco BY-2 cell to naphthoquinones plumbagin and juglone for 0–240 h resulted in a dose-dependent growth deceleration and cell viability reduction compared with control cells.

The presence of both naphthoquinones decreased cell number in culture at concentration dependent manner. The plumbagin had a stronger influence in all concentration than juglone except the highest used concentrations (50 and 500 μg l^{-1}). The maximum of the cell number was recorded 48 h after juglone and plumbagin exposure. The total amount of tobacco BY-2 cells was enhanced at the lowest juglone concentration (1 μg l^{-1}) during 240 h cultivation. Compared with control BY-2 cells, plumbagin showed strong cell growth inhibition at all used concentrations. After 240 h lasted treatment of BY-2 tobacco cells with naphthoquinones in the concentration 500 μg l^{-1} the amount of tobacco cell reached only 9.4% (juglone) and 13% (plumbagin) of untreated control cell number.

The experiment provided evidence of cytotoxicity of plumbagin as well as juglone above 50 μg l^{-1}, but the toxicity of plumbagin is more significant (Fig. 2A and B). At the concentration 50 and

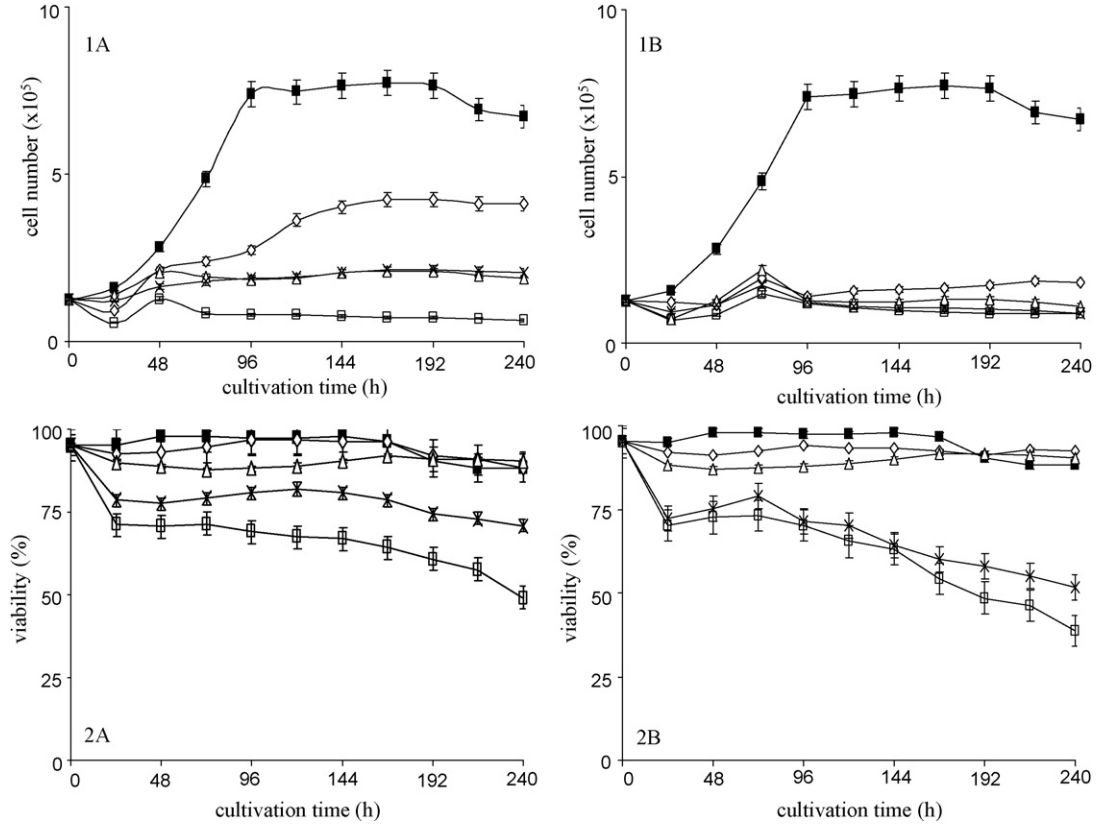

Fig. 1. Time-dependent response of tobacco BY-2 cells treated with naphthoquinones on cell number (1) and BY-2 cells viability (2). Control tobacco BY-2 cells (■); BY-2 cells treated with naphthoquinones juglone (1A, 2A) and plumbagin (1B, 2B) at the concentration 1 μg l^{-1} (◊), 5 μg l^{-1} (△), 50 μg l^{-1} (×) and 500 μg l^{-1} (□). Each point represents the mean of three independent experiments.

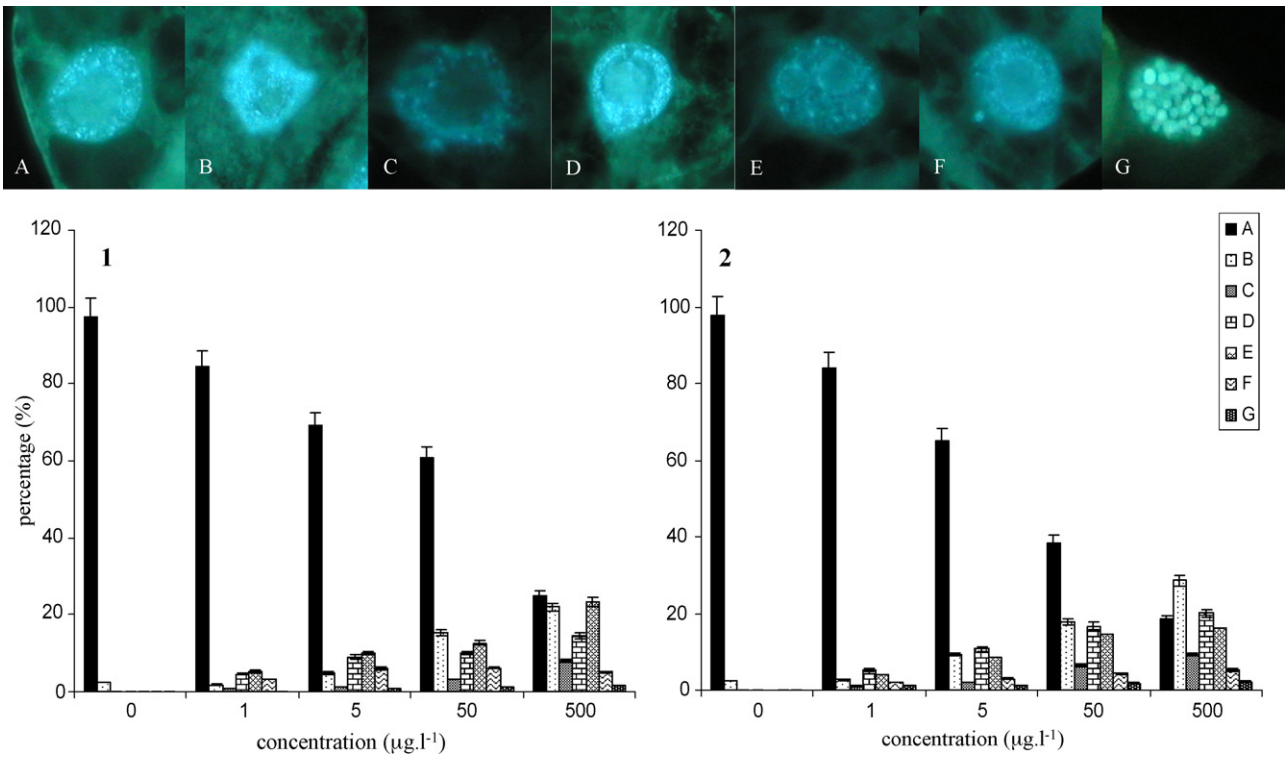

Fig. 2. Dose-dependent response of tobacco BY-2 cells treated with juglone (1) and plumbagin (2). The figure shows the percentage of various types of morphological changes in nuclei after 48 h of treatment. The diagrams represent three independent experiments, where 1000 nuclei have been analysed. A, Normal nucleus; B, irregular nucleus; C, stretched nucleus; D, pre-apoptic nucleus with hyperchromasia at the periphery; E, pre-apoptic nucleus with chromatin condensation around nucleolus; F, micronucleus formation; G, apoptic nucleus.

500 μg l^{-1} cell viability decreased with time of treatment: within 24 h after the addition of 500 μg l^{-1} naphthoquinone, 29% of cells are dead in the case of juglone and more than 30% in the case of plumbagin whereas 240 h after treatment, 51% (juglone) and more than 61% (plumbagin) of the cells were dead. Low concentrations of juglone and plumbagin (1 and 5 μg l^{-1}) had no significant influence on cell viability.

3.2. Characterization of structural changes in tobacco BY-2 cells treated with naphthoquinones

The study of morphological structural changes was performed on control tobacco BY-2 cells and at 48 h after exposure of 0–500 μg l^{-1} juglone and plumbagin, respectively. The BY-2 cells underwent various structural nuclear changes related to the concentration of juglone and plumbagin. These changes included chromatin condensation, nucleolar architecture and "apoptic-like nuclei" formation, resembling those observed during apoptosis induction in animal as well as human cells, described, but not quantified previously (Babula et al., 2006). Empty cells with completely lysed nuclei were not examined.

Six prominent nuclear structural changes were determined and quantified 48 h after treatment (Fig. 2). These changes were not very significant at low concentrations of juglone and plumbagin, but with increasing amount of these naphthoquinones the changes were more and more apparent. The most numerous events at the concentration of 500 μg l^{-1} were represented by irregular nuclei (juglone nearly 22%, plumbagin 28.5%), nuclei with chromatin condensation around nucleoli (juglone 23.52%, plumbagin 16.23%), nuclei with hyperchromasia at the periphery (14.51% juglone and 20% plumbagin) and stretched nuclei (juglone 8.24%, plumbagin 9.34%). Micronuclei (juglone 5.09%, plumbagin 5.21%) and "apoptic-like" nuclei (juglone 1.57%, plumbagin 2.15%) were also observed.

The cells presenting appearance of micronuclei, apoptic cells and cells with irregular nuclei are still alive, the cells with stretched nuclei or with chromatin condensation around nucleoli were dead within 48 h, as observed with double staining with fluorescein diacetate and propidium iodide. The first dead cells were observed 12 h after the start of treatment.

3.3. Characterization of mitotic changes

The changes in frequency of particular phases of mitosis as well as several chromosomal disorders 48 h after addition of juglone and plumbagin to the tobacco BY-2 cell suspension culture were observed (Fig. 3). The representation of mitotic phases was more or less well-balanced (29% prophase, 19.4% metaphase, 25.8% anaphase and 22.6% telophase), less common "chromosomes extra metaphase mitotic spindle" were observed too (3.2%). Metaphase unambiguously predominated after application of both naphthoquinones, frequency of anaphase and telophase was decreased with naphthoquinones concentration. Chromosomal disorders including "extra chromosomes" and "chromosomal bond" were determined at low concentrations (from 1 μg l^{-1}, the most expressive at 500 μg l^{-1}). The most numerous chromosomal disorders were chromosomes out of metaphasic mitotic spindle (juglone at concentration 500 μg l^{-1} 19.04% and nearly 12.5% for plumbagin at

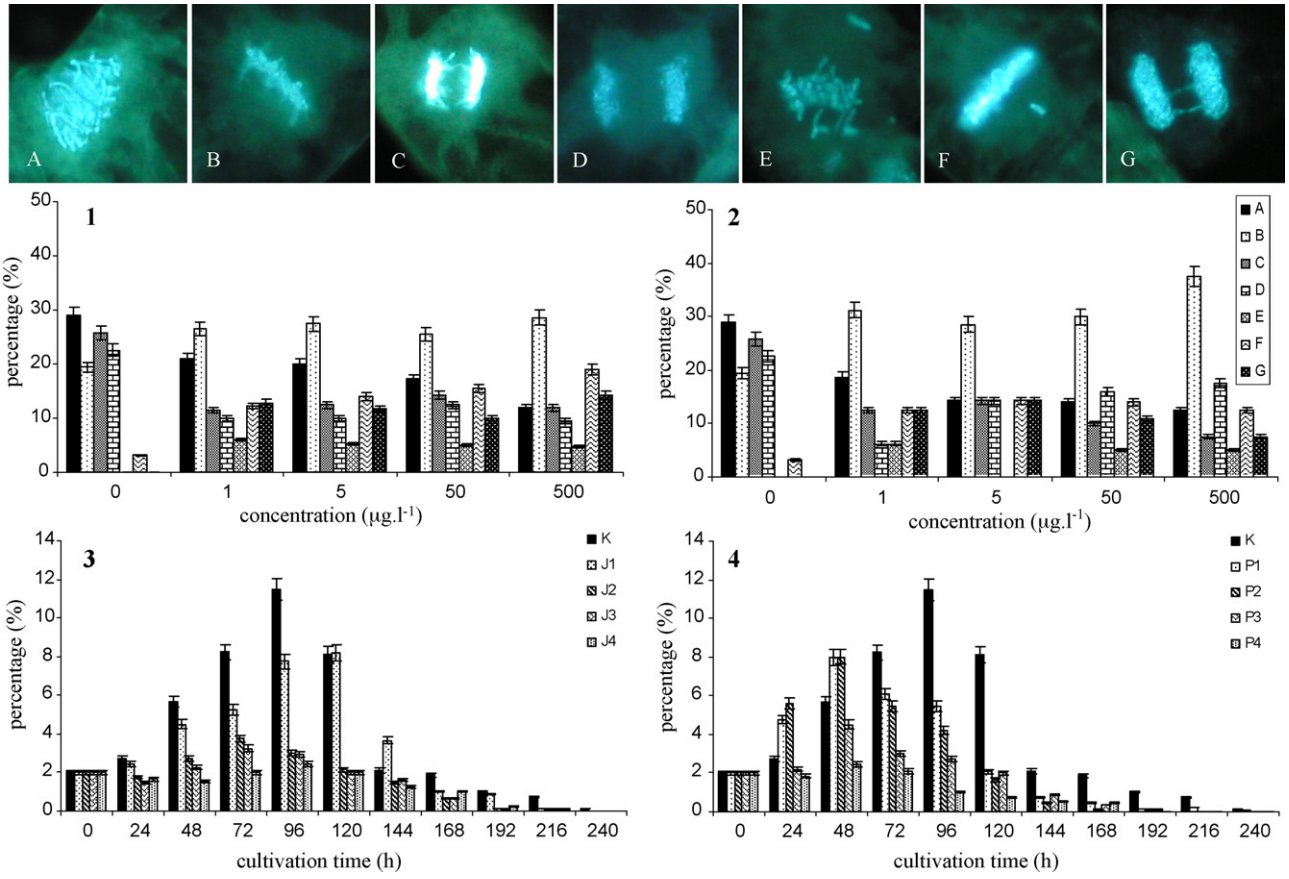

Fig. 3. (1 and 2) phases of mitosis (A–D) and chromosomal disorders during mitosis (E, F, and G) of tobacco BY-2 cells after 48 h treatment with juglone (1) and plumbagin (2); (A) prophase. (B) Metaphase. (C) Anaphase. (D) Telophase. (3 and 4) Time response diagram showing the dependence of mitotic index of BY-2 cells on cultivation time; cells were treated with juglone (3) and plumbagin (4).

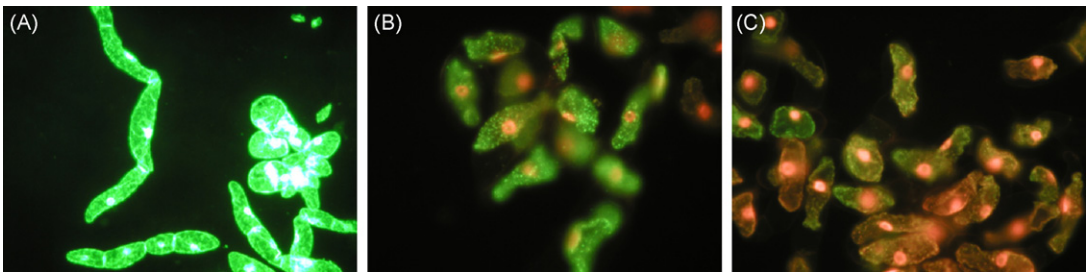

Fig. 4. Effect of naphthoquinone plumbagin on ROS production using double MitoTracker Green FM, dihydroethidium staining in tobacco BY-2 cells untreated cells (A) and BY-2 cells treated with plumbagin at the concentration 50 $\mu g\,l^{-1}$ (A) and 500 $\mu g\,l^{-1}$ 24 h after treatment. Increasing in dihydroethidium staining is evident. Magnification is 200×.

the same concentration), less "chromosome out of chromosomal make-up in prophase" (juglone 4.76%, plumbagin 5%) and "chromosomal bonds" in anaphase (4 G) (7.5% juglone and 14.3% plumbagin).

Mitotic index was determined during whole experiment in 24 h intervals. The mitotic index of the control gradually increased and the maximum reached 96 h after sub cultivation (11.49%); then it gradually decreased and 168 h after sub cultivation was lower than 2%. The decrease of mitotic index was observed after application of both naphthoquinones at all applied concentrations with an exception in plumbagin where 24 and 48 h after sub cultivation at concentrations 1 and 5 $\mu g\,l^{-1}$. The maximal mitotic index was observed after 120 h in cell culture treated with juglone concentration 1 $\mu g\,l^{-1}$—it was higher than mitotic index of untreated BY-2 cells. Maxima at other juglone concentrations (from 5 to 500 $\mu g\,l^{-1}$) were reached 72 h after application. Maxima of mitotic indexes after plumbagin treatment were observed at all concentrations 48 h after application. Mitotic index was most reduced by higher naphthoquinone concentrations (50 and 500 $\mu g\,l^{-1}$).

3.4. Production of ROS

Production of reactive oxygen species was monitored in tobacco BY-2 cells exposed to naphthoquinones using the superoxide-sensitive fluorophore dihydroethidium that is oxidized by superoxide anions into fluorescent ethidium. BY-2 cells were incubated with MitoTracker Green FM for 40 min at the room temperature and dark. After this time, dihydroethidium was added and incubation was continued for next 20 min under the same conditions. Both naphthoquinones induced increases in dihydroethidium staining in the concentration-dependent doses at time course (not shown). Representative results of dihydroethidium staining of control BY-2 cells and BY-2 cells treated by juglone and plumbagin at

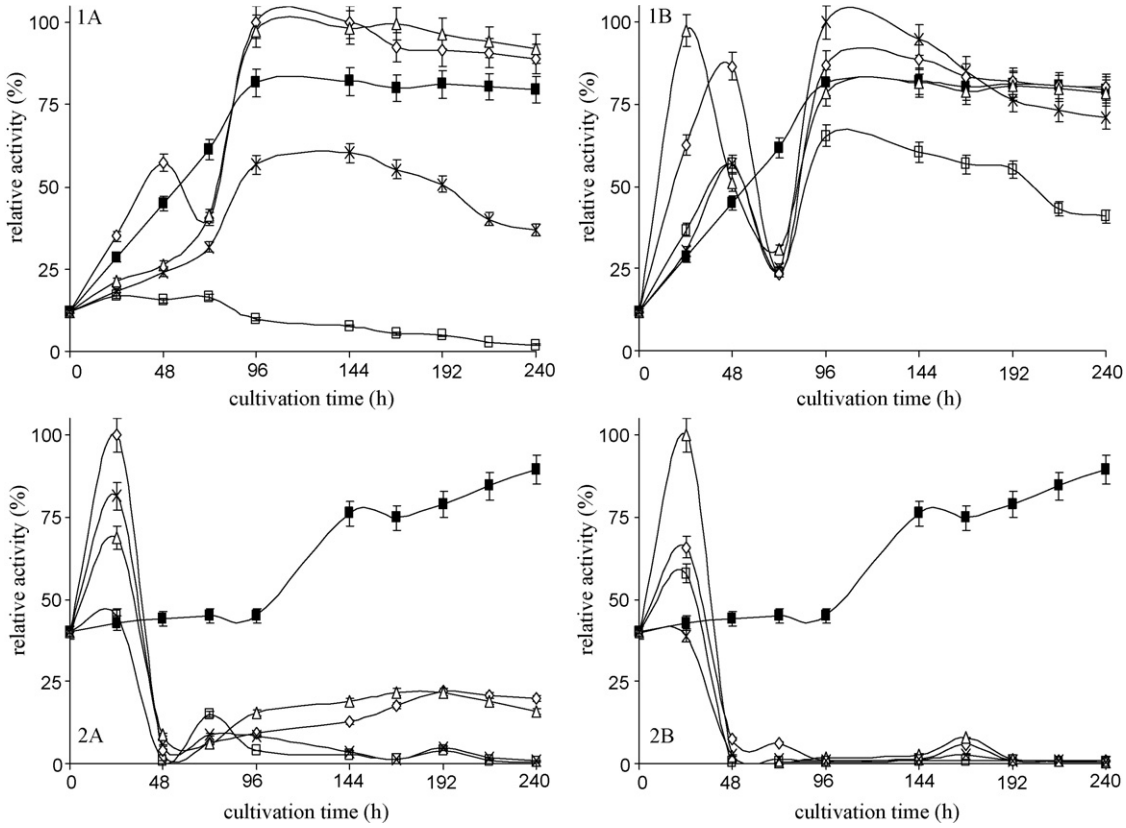

Fig. 5. Time dependent response of tobacco BY-2 cells treated with naphthoquinones juglone and plumbagin on dehydrogenase (1) and oxidoreductase (2) activity. 100% represent the highest value of the experiment. Control tobacco BY-2 cells (■); BY-2 cells treated with naphthoquinones juglone (1A, 2A) and plumbagin (1B, 2B) at the concentration 1 $\mu g\,l^{-1}$ (◊), 5 $\mu g\,l^{-1}$ (△), 50 $\mu g\,l^{-1}$ (×) and 500 $\mu g\,l^{-1}$ (□). Each point represents the mean of three independent experiments.

the concentration 50 and 500 μg l^{-1} 24 h after treatment are shown in Fig. 4.

3.5. MTT and TTC assays

MTT assay as well as TTC assay revealed that untreated cells exhibited continual increase of dehydrogenase (maximum 96 h after sub cultivation) and oxidoreductase activities (maximum after 240 h of sub cultivation) (Fig. 5). Juglone increased dehydrogenase activity at the concentration 1 μg l^{-1} 24 and 48 h after treatment and at the concentrations 1 and 5 μg l^{-1} 96 h after treatment in comparison with untreated cells, when dehydrogenase activity of this control cells was significantly lower. The lowest percentage was confirmed by the highest concentration of juglone. Plumbagin increased dehydrogenase activity to 48 after treatment at all used concentrations; this elevation was followed about rapid descent with the minimum at 72 h after treatment.

Oxidoreductase activity was enhanced after juglone as well as plumbagin application. The highest point of this activity was obtained 24 h after application, and then quickly decreased. The second maxima of oxidoreductase activity of cells treated by plumbagin were observed 168 h after treatment, but these points did not reach enzyme activity of control tobacco BY-2 cells. Oxidoreductase activity was reduced to a minimum 48 h after application and 192 h after plumbagin treatment was not determined.

3.6. DNA fragmentation in tobacco BY-2 cells treated with juglone and plumbagin

The integrity of DNA was monitored by electrophoresis in 1% agarose gel. Fragmentation of DNA from tobacco BY-2 cells treated with naphthoquinones juglone and plumbagin was not observable. This fact could correspond to the low appearance of "apoptic-like bodies" in only 1.57% of tobacco cells treated with juglone and 2.15% of tobacco cells treated with plumbagin.

4. Discussion

Two studied naphthoquinones in this work, juglone and plumbagin, play important role in ecological interactions as protective compounds. They can protect plants against insectual pests (herbivores) as well as fungal pathogens. However, what functions do naphthoquinones have in the relations between plants, respectively? Necrotic plant tissues can release naphthoquinones to the surroundings and than the naphthoquinones can affect other plants. Previous essays demonstrated that naphthoquinones, especially juglone, affect growth and differentiating processes of various plant species, including herbs, shrubs, deciduous trees (Rietveld, 1983) and coniferous seedlings (Funk et al., 1979). Significant reductions in relative growth rates of shoots and roots of hydroponically grown corn and soybean after 3 days exposure to juglone at concentration 10 and 100 μM were demonstrated (Jose and Gillispie, 1998). The mechanism of effect of naphthoquinones on animal cell models is well known—includes inhibition of purine and pyrimidine bases synthesis (Elangovan et al., 1994), inhibition of dihydroorotate dehydrogenase, enzyme, that catalyses conversion of dihydroorotate to orotate (Jones, 1980; Angermuller and Loffler, 1995), interactions with DNA and topoisomerases I and II inhibition (Fujii et al., 1992; Vanni et al., 1998; Krishnan and Bastow, 2000, 2001; Ting et al., 2003) and inhibition of electron transport of respiratory chain resulting in cycline-dependent kinase activation and apoptosis induction (Srinivas et al., 2004a,b). Juglone disrupts electron transport function in isolated mitochondria and chloroplasts and affects processes of photosynthesis and respiration (Hejl and Koster, 1993). Reduction of transpiration and stomatal conductance after juglone treatment was observed too (Jose and Gillispie, 1998). Later explorations modified these views—juglone does not damage processes proceed in chloroplasts, but reduces water uptake by the roots. Reduction of H^+-ATP-ase activity in corn and soybean root microsomal fraction by increasing amount of juglone in concentration from 0 to 1000 μM was investigated too (Hejl and Koster, 2004). Detailed essays of naphthoquinones effects on plant cell models are absent. Because of these facts, this work was aimed at investigating of naphthoquinones effects in cultures of plant BY-2 cells. Plant cells were treated using different naphthoquinones concentrations (1, 5, 50 and 500 μg l^{-1}) with the aim on investigation of growth parameters, viability and enzymatic changes of BY-2 cells during 240 h cultivation in 24 h intervals. In this study, we determined and especially quantified changes in cell and nuclear architecture, mitosis and DNA fragmentations.

It was shown that naphthoquinones applied to BY-2 tobacco cells induced various anomalous cell processes that were closely dependent on the amount and type of applied naphthoquinone. Already low levels of applied naphthoquinones significantly influenced growth curves of tobacco BY-2 cells without important effect on cell viability – it means that these compounds can interfere with processes of mitosis and slow the number of "new" cells – so they retard cell cycle of BY-2 cells. Studies on animal cell models demonstrated mitotic abnormalities, such as decrease in the percentage of mitotic figures with accumulation of cells in metaphase—the authors concluded that juglone inhibits the entering of cells to mitosis (Okada et al., 1967; Santhakumari et al., 1980). These data concur with our results—in our study the accumulation of cells in metaphase and decrease of other mitotic phases (especially anaphase and telophase) as well as mitotic indexes were demonstrated too. In higher concentrations of applied naphthoquinones (50 and 500 μg l^{-1}) the cell viability was significantly decreased as well as number of cells; amount of cells in metaphase was increased. Mitotic indexes were decreased by both naphthoquinones excepting plumbagin in the concentrations 1 and 5 μg l^{-1}, where cells in mitosis were markedly accumulated in metaphase. Mitotic abnormalities were determined too, such as chromosomes out of metaphasic mitotic spindle and chromosomal bonds in anaphase according to naphthoquinones levels with the maximum at the concentration 500 μg l^{-1}. Cells in mitosis were still alive—this fact means that chromosomes out of mitotic spindle formed micronuclei that were observed too, particularly in longer period after application.

Programmed cell death (PCD) in plants as physiological cell death process of unwanted cells (Ellis et al., 1991), plays important role in a cell and tissue homeostasis and specialization, tissue sculpting and diseases. Damaged cells or cells unable to function correctly can undergo PCD. Cell death can occur in the cortex of root and stem base in response to hypoxia (Armstrong, 1979) and in plant–pathogen interactions (Dangl et al., 1996). The observation of various cell death processes dependent on the amount of applied juglone or plumbagin was the most considerable result in our experiments. All major hallmarks of apoptosis following the treatment of tobacco BY-2 cells with juglone as well as plumbagin were observed. Nuclei reported characteristic marks of chromatin condensation, namely how in the peripheries of the nuclei, so around nucleoli. Well perceptible "apoptic-like bodies" were identified using epifluorescence microscopy. Necrotic cells with distinctive cell disorganization were not observed. Similar results were obtained experimentally in tobacco BY-2 cells treated with hydrogen peroxide, camptothecin, okaidic and salicylic acid (OïBrien et al., 1998). Very important it is assignment that changes in architecture of chromatin and chromatin condensation originated by naphthoquinones juglone and plumbagin are very analogous to chromatin changes in studies, where BY-2 cells were treated by hydrogen peroxide (Houot et al., 2001). The only

significant difference is absence of cleavage of nuclear DNA into nucleosomal fragments. This may be caused by low percentage of cell in the state of apoptosis induced by naphthoquinones (only near 2%, while after application of 12.5 mM hydrogen peroxide about 20%). Mechanism of incurrence of PCD by naphthoquinones stays question. Some works demonstrated that hypersensitive cell death is a process associated with the accumulation of O_2^- and H_2O_2 leading to the protein kinase-mediated cell death process that is similar to PCD (Mehdy, 1994; Van Breusegem and Dat, 2006); in many systems, sublethal oxidative stress induced by xenobiotics was found to be involved either directly or indirectly in PCD processes (Lamb and Dixon, 1997; Mylona et al., 2007). Ability of generating of reactive oxygen species (ROS) by naphthoquinones was recovered in bacterial cellular models (Imlay and Fridovich, 1992). The ability of juglone and plumbagin to generate ROS on plant cell model was investigated using fluorescent probe dihydroethidium in our study. Our study shows that ROS generating by investigated naphthoquinones is dependent on their concentration. This mechanism well explains the induction of PCD by naphthoquinones—it means that treatment of cultured tobacco BY-2 cells by juglone and plumbagin induces generation of ROS and subsequently initiation of steps leading to apoptic-like cell death processes. This theory is supported by studies on BY-2 cells using hydrogen peroxide, where very similar nuclear changes were observed. Ourselves finding morphological and biochemical events are associated with PCD during the hypersensitive response of tobacco plants infected with tobacco mosaic virus too; cleavage of nuclear DNA was observed, but in contrast, chromatin condensation and apoptic bodies were not observed (Mittler et al., 1997). The mechanism of PCD observed in our study may be very similar to PCD described in animal cell models that includes nuclear condensation and especially DNA fragmentation into small fragments. We did not observe fragmentation of genomic DNA from tobacco BY-2 cells treated with juglone and plumbagin because of very low level of "apoptic-like" cells.

Changes in enzymatic activities of dehydrogenases and oxidoreductases of BY-2 cells treated by naphthoquinones were investigated too. These methods are based on reduction of tetrazolium salts to intensely colored formazans (Nineham, 1955). Tetrazolium salts are able to accept electrons of respiratory chain and are also found to be able to uncouple phosphorylation from electron transport (Palmer and Kalina, 1967), but they can serve as substrates to elucidate damage to the mitochondrial electron transport of cells (Musser and Oseroff, 1994). Inhibition of MTT by different compounds was explained by their entrance to complex I, inhibition of TTC by entrance to complex IV of mitochondrial electron transport chain (Musser and Oseroff, 1994). The former experiments on animal cell models demonstrate that naphthoquinones can perform as acceptors of electrons (Muto et al., 1987; Srinivas et al., 2004a,b) and our experiments on plant cell model confirm this fact. This fact means that juglone as well as plumbagin are compounds capable to pass through cell walls and participate in various places of mitochondrial electron transport, where operate as acceptors of electrons and disrupt electron transport. Very intense increase of oxidoreductase activity and less markedly increase of dehydrogenase activity of BY-2 cells 24 h after treatment following by expressive depression is very interesting. These data can be explained by 1. Disruption of mitochondrial electron transport and after it release of electrons that are able to reduce TTC as substrate, or 2. Metabolize of naphthoquinones by oxidoreductases.

Ascertained data demonstrate very heterogeneous and complex effect of naphthoquinones on plant cells. They affect processes of mitosis with accumulation of mitotic cells in metaphase as well as they cause consequential disorders of mitosis. Mitochondria are the next place of naphthoquinone activities: they disrupt electron transport in various places of respiratory chain. Very important fact is the ability of naphthoquinones to generate reactive oxygen species leading to PCD processes. However, how can naphthoquinones affect plants? They may be released from necrotic plant parts containing naphthoquinones and after it they can be reuptake by roots of other plant species, where can start sequence of processes leading to negative affection of plant condition or plant death. These interactions are undoubtedly an important factor in determining species distribution and abundance within some plant communities. Allelopathic interactions are also thought to be an important factor in the success of many invasive plants, for specific examples, see species of genera *Impatiens* (Macias et al., 2008). Due to these naphthoquinones juglone and plumbagin properties, they can be very effective compounds of plant competition fight.

5. Conclusion

Naphthoquinones, the natural occurring phenolic compounds derived from naphthalene, were tested for cytotoxic properties using plant cell suspension culture of tobacco BY-2 as. Our results demonstrate that cytotoxic effect of naphthoquinones plumbagin and juglone is caused by their ability to generate reactive oxygen species (ROS) as well as DNA damage and disturbance of mitosis. All these plumbagin and juglone features lead to programmed cell death that was observable as chromatine condensation as well as apoptic nuclei. Our work confirms the utilization of tobacco BY-2 suspension culture as suitable model for cytotoxic properties of various compounds testing.

Acknowledgment

This work was supported by grant 1M06030.

References

Angermuller, S., Loffler, M., 1995. Location of dihydroorotate oxidase in myocardium and kidney cortex of the rat. An electron microscopic study using the cerium technique. Histochemistry 103, 287–292.
Armstrong, W., 1979. Aeration in higher plants. Adv. Bot. Res. 7, 225–332.
Babula, P., Huska, D., Hanustiak, P., Baloun, J., Krizkova, S., Adam, V., Havel, L., Zemlicka, M., Horna, A., Beklova, M., Kizek, R., 2006. Flow injection analysis coupled with carbon electrodes as the tool for analysis of napthoquinones with respect to their content and functions in biological samples. Sensors 6, 1466–1482.
Babula, P., Adam, V., Havel, L., Kizel, R., 2009. Noteworthy secondary metabolites naphthoquinones—their occurrence, pharmacological properties and analysis. Curr. Pharm. Anal. 5 (1).
Barkosky, R.R., Einhellig, F.A., 2003. Allelopathic interference of plant-water relationships by parahydroxybenzoic acid. Bot. Bull. 44, 53–58.
Binder, R.G., Benson, M.E., Flath, R.A., 1989. Eight 1,4-naphthoquinones from juglans. Phytochemistry 28, 2799–2801.
Crouch, I.J., Finnie, J.F., vanStaden, J., 1990. Studies on the isolation of plumbagin from *in vitro* and *in vivo* grown Drosera species. Plant Cell Tiss. Org. Cult. 21, 79–82.
Dangl, J.L., Dietrich, R.A., Richberg, M.H., 1996. Death donït have no mercy: cell death programs in plant–microbe interactions. Plant Cell 8, 1793–1807.
Davis, E.F., 1928. Toxic principle of Fuglans nigra as identified with synthetic juglone, and its toxic effect on tomato and alfalfa plants. Am. J. Bot. 15, 620.
Duroux, L., Delmotte, F.M., Lancelin, J.M., Keravis, G., Jay-Allemand, C., 1998. Insight into naphthoquinone metabolism: beta-glucosidase-catalysed hydrolysis of hydrojuglone beta-D-glucopyranoside. Biochem. J. 333, 275–283.
Einhellig, F.A., 1995. Mechanism of action of allelochemicals in allelopathy. Allelopathy ACS Symp. Series 582, 96–106.
Elangovan, V., Ramamoorthy, N., Balasubramanian, S., Sekar, N., Govindsamy, S., 1994. Studies of antiproliferative effect of some naturally occurring bioflavonoidal compounds against human carcinoma of larinx and sarcoma—180 cell lines. Ind. J. Pharm. 26, 266–269.
Ellis, R.E., Yuan, J., Horvitz, H.R., 1991. The mechanisms and functions of cell death. Ann. Rev. Cell Biol. 7, 663–698.
Ferraz, P.A.L., de Abreu, F.C., Pinto, A.V., Glezer, V., Tonholo, J., Goulart, M.O.F., 2001. Electrochemical aspects of the reduction of biologically active 2-hydroxy-3-alkyl-1,4-naphthoquinones. J. Electroanal. Chem. 507, 275–286.
Fujii, N., Yamashita, Y., Arima, Y., Nagashima, M., Nakano, H., 1992. Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. Antimicrob. Agents Chemother. 36, 2589–2594.
Funk, D.T., Case, P.J., Rietveld, W.J., Phares, R.E., 1979. Effects of juglone on the growth of coniferous seedling. Forest Sci. 25, 452–454.
Hejl, A.M., Koster, K.L., 1993. Effect of juglone on growth, photosynthesis, and respiration. J. Chem. Ecol. 19, 559–568.

Hejl, A.M., Koster, K.L., 2004. Juglone disrupts root plasma membrane H+-ATPase Activity and impairs water uptake, root respiration, and growth in soybean (*Glycine max*) and corn (*Zea mays*). J. Chem. Ecol. 30, 453–471.
Higa, M., Ogihara, K., Yogi, S., 1998. Bioactive naphthoquinone derivates from Diospyros maritima Blume. Chem. Pharm. Bull. 46, 1189–1193.
Houot, V., Etienne, P., Petitot, A.S., Barbier, S., Blein, J.P., Suty, L., 2001. Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner. J. Exp. Bot. 52, 1721–1730.
Imlay, J., Fridovich, I., 1992. Exogenous quinones directly inhibit the respiratory NADH dehydrogenase in *Escherichia coli*. Arch. Biochem. Biophys. 296, 337–346.
Jones, M.E., 1980. Pyrimidine nucleotide biosynthesis in animals: genes, enzymes and regulation of UMP biosynthesis. Ann. Rev. Biochem. 49, 253–279.
Jose, S., Gillispie, A.R., 1998. Allelopathy in black walnut (*Juglans nigra* L.) alley cropping. II. Effect of juglone on hydroponically grown corn (*Zea mays* L.) and soybean (*Glycine max* L.) growth and physiology. Plant Soil Environ. 203, 199–205.
Krishnan, P., Bastow, K.F., 2000. Novel mechanisms of DNA topoisomerase II inhibition by pyranonaphthoquinone derivatives—eleutherin, alpha lapachone, and beta lapachone. Biochem. Pharm. 60, 1367–1379.
Krishnan, P., Bastow, K.F., 2001. Novel mechanism of cellular DNA topoisomerase II inhibition by the pyranonaphthoquinone derivatives alpha-lapachone and beta-lapachone. Cancer Chem. Pharm. 47, 187–198.
Lamb, C., Dixon, R.A., 1997. Die and let li-programmed cell death in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 251–275.
Lee, C.H., Lee, H.S., 2008. Acaricidal activity and function of mite indicator using plumbagin and its derivates isolated from Diospyros kaki Thunb. roots (Ebenaceae). J. Microbiol. Biotech. 18, 314–321.
Lin, L.C., Yang, L.L., Chou, C.J., 2003. Cytotoxic naphthoquinones and plumbagic acid glucosides from Plumbago zeylanica. Phytochemistry 62, 619–622.
Macias, F.A., Oliveros-Bastidas, A., Marin, D., Carrera, C., Chinchilla, N., Molinillo, J.M.G., 2008. Plant biocommunicators: their phytotoxicity, degradation studies and potential use as herbicide models. Phytochem. Rev. 7, 179–194.
Marion Meyer, J.J., Van der Kooy, F., Joubert, A., 2007. Identification of plumbagin epoxide as a germination inhibitory compound through a rapid bioassay on TLC. South Afr. J. Bot. 73, 654–656.
Massey, A.B., 1925. Antagonism of the walnuts (*Juglans nigra* L. and *Juglans cinerea* L.) in certain plant associations. Phytopathology 15, 773.
Mehdy, M.C., 1994. Active oxygen species in plant defense against pathogens. Plant Physiol. 105, 467–472.
Mittler, R., Simon, L., Lam, E., 1997. Pathogen-induced programmed cell death. J. Cell Sci. 110, 1333–1344.
Monks, T.J., Hanzlik, R.P., Cohen, G.M., Ross, D., Graham, D.G., 1992. Quinone chemistry and toxicity. Toxicol. Appl. Pharm. 112, 2–16.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Phys. Plant Pathol. 15, 473–497.
Musser, D.A., Oseroff, A.R., 1994. The use of tetrazolium salts to determine sites of damage to the mitochondrial electron transport chain in intact cells following in vitro photodynamic therapy with Photofrin II. Photochem. Photobiol. 59, 621–626.
Muto, N., Inouye, K., Inada, A., Nakanishi, T., Tan, L., 1987. Inhibition of cytochrome P-450-linked monooxygenase systems by naphthoquinones. Biochem. Biophys. Res. Commun. 146, 487–494.
Mylona, P.V., Polidoros, A.N., Scandalios, J.G., 2007. Antioxidant gene responses to ROS-generating xenobiotics in developing and germinated scutella of maize. J. Exp. Bot. 58, 1301–1312.
Nagata, T., Nemoto, Y., Hasetawa, S., 1992. Tobacco BY-2 cell-line as the HeLa-cell in the cell biology of higher plants. Int. Rev. Cytol. Surv. Cell Biol. 132, 1–30.
Nineham, A., 1955. The chemistry of formazans and tetrazolium salts. Chem. Rev. 55, 355–483.
OïBrien, I., Baguley, B., Murray, B., Morris, B., Ferguson, I., 1998. Early stages of the apoptic pathway in plant cells are reversible. Plant J. 13, 803–814.
Okada, T.A., Roberts, E., Brodie, A.F., 1967. Mitotic abnormalities produced by juglone in Ehrlich ascities tumor cells. Proc. Soc. Exp. Biol. Med. 126, 583–588.
Palmer, J.M., Kalina, M., 1967. Some effect of tetrazolium salt on the metabolism of mitochondria isolated from Jerusalem artichoke tubers. Plant 78, 358–365.
Rietveld, W.J., 1983. Allelopathic effects of juglone on germination and growth of several herbaceous and woody species. J. Chem. Ecol. 9, 295–307.
Santhakumari, G., Saralamma, P., Radkakrishnan, N., 1980. Effects of plumbagin on cell growth and mitosis. Ind. J. Exp. Biol. 18, 215–218.
Srinivas, G., Annab, L.A., Gopinath, G., Banerji, A., Srinivas, P., 2004a. Antisense blocking of BRCA1 enhances sensitivity to plumbagin but not tamoxifen in BG-1 ovarian cancer cells. Mol. Carcinog. 39, 15–25.
Srinivas, P., Gopinath, G., Banerji, A., Dinakar, A., Srinivas, G., 2004b. Plumbagin induces reactive oxygen species, which mediate apoptosis in human cervical cancer cells. Mol. Carcinog. 40, 201–211.
Terzi, I., 2008. Allelopathic effects of juglone and decomposed walnut leaf juice on muskmelon and cucumber seed germination and seedling growth. African J. Biotech. 7, 1870–1874.
Ting, C.Y., Hsu, C.T., Hsu, H.T., Su, J.S., Chen, T.Y., Tarn, W.Y., Kuo, Y.H., Whang-Peng, J., Liu, L.F., Hwang, J.L., 2003. Isodiospyrin as a novel human DNA topoisomerase I inhibitor. Biochem. Pharmacol. 66, 1981–1991.
Topal, S., Kocacaliskan, I., Arslan, O., Tel, A.Z., 2007. Herbicidal effects of juglone as an allelochemical. Phyton Ann. Rei Bot. 46, 259–269.
Van Breusegem, F., Dat, J.F., 2006. Reactive oxygen species in plant cell death. Plant Physiol. 141, 384–390.
Vanni, A., Fiore, M., de Salvia, R., Cundari, E., Ricordy, R., Ceccarelli, R., Degrassi, F., 1998. DNA damage and cytotoxicity induced by beta-lapachone: relation to poly(ADP-ribose) polymerase inhibition. Mutat. Res. 401, 55–63.
Vennerstrom, J.L., Eaton, J.W., 1988. Oxidants, oxidant drugs and malaria. J. Med. Chem. 31, 1269–1277.