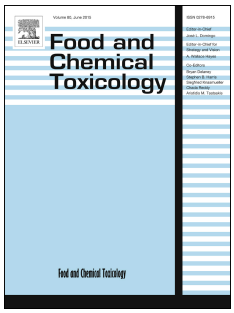
Accepted Manuscript



Chemical characterization, antioxidant, genotoxic and *in vitro* cytotoxic activity assessment of *Juniperus communis* var. saxatilis

Bojana Vasilijević, Jelena Knežević-Vukčević, Dragana Mitić-Ćulafić, Dejan Orčić, Marina Franciškoviċ, Tatjana Srdic-Rajic, Marina Jovanović, Biljana Nikolić

PII: S0278-6915(17)30792-5

DOI: 10.1016/j.fct.2017.12.044

Reference: FCT 9489

To appear in: *Food and Chemical Toxicology*

Received Date: 12 October 2017

Revised Date: 19 December 2017

Accepted Date: 21 December 2017

Please cite this article as: Vasilijević, B., Knežević-Vukčević, J., Mitić-Ćulafić, D., Orčić, D., Franciškoviċ, M., Srdic-Rajic, T., Jovanović, M., Nikolić, B., Chemical characterization, antioxidant, genotoxic and *in vitro* cytotoxic activity assessment of *Juniperus communis* var. saxatilis, *Food and Chemical Toxicology* (2018), doi: 10.1016/j.fct.2017.12.044.

CHEMICAL CHARACTERIZATION, ANTIOXIDANT, GENOTOXIC AND *in vitro* CYTOTOXIC ACTIVITY ASSESSMENT OF *Juniperus communis* var. saxatilis

Bojana Vasilijević[a*], Jelena Knežević-Vukčević[a], Dragana Mitić-Ćulafić[a], Dejan Orčić[b], Marina Frančišković[b], Tatjana Srdic-Rajic[c], Marina Jovanović[a] and Biljana Nikolić[a]

[a]*Chair of Microbiology, Center for Genotoxicology and Ecogenotoxicology, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia*

[b]*Chair of Biochemistry and Chemistry of Natural Products, Department for Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia*

[c]*Department of Experimental Oncology, Institute for Oncology and Radiology of Serbia, Pasterova 14, 11000, Belgrade, Serbia*

Corresponding author:

*Corresponding author: B. Vasilijević

Mailing address:

Department of Microbiology, Center for Genotoxicology and Ecogenotoxicology

University of Belgrade - Faculty of Biology

Studentski trg 16

11000 Belgrade

Serbia

tel/fax: +381 11 26 37 364

E-mail address: e-mail: bojanav@bio.bg.ac.rs orcid.org/0000-0002-9906-6522

E-mail addresses of authors: Jelena Knežević-Vukčević – jelenakv@bio.bg.ac.rs

Dragana Mitić-Ćulafić – mdragana@bio.bg.ac.rs

Dejan Orčić – dejan.orcic@dh.uns.ac.rs

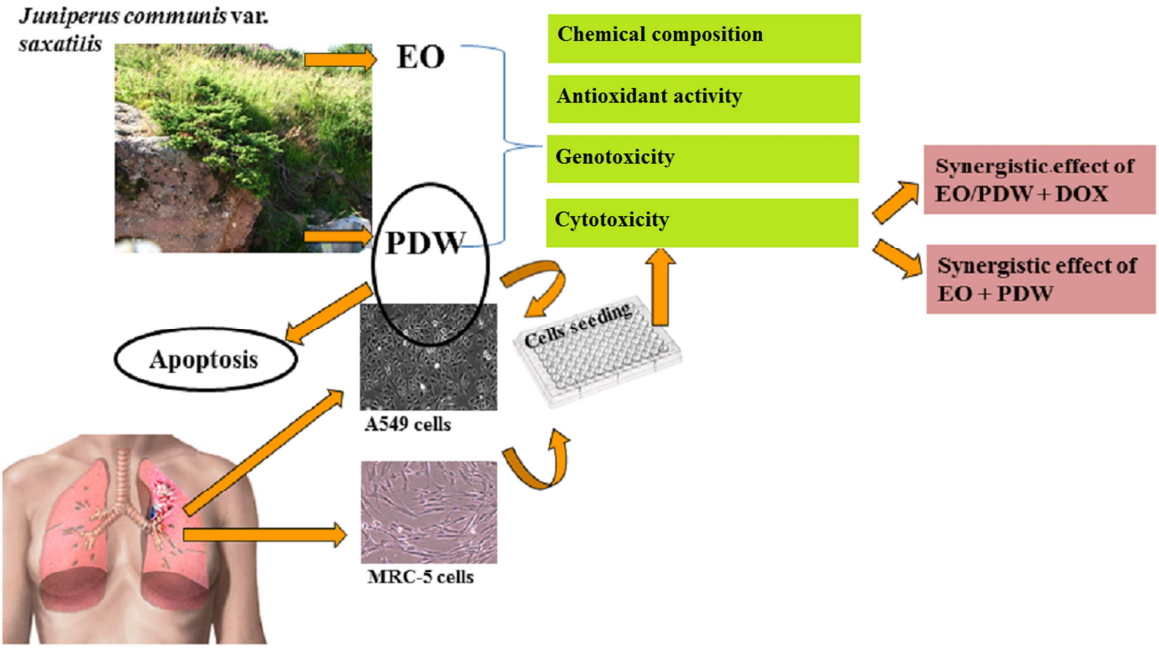
Marina Frančišković – marina.franciskovic@dh.uns.ac.rs

Tatjana Srdic-Rajic – tsrdic@gmail.com

Marina Jovanović – marina.rajic.jovanovic@gmail.com

Biljana Nikolić – biljanan@bio.bg.ac.rs

Abbreviations: DOX - doxorubicin; EO - essential oil; PDW - post-distillation waste ROS - reactive oxygen species



CHEMICAL CHARACTERIZATION, ANTIOXIDANT, GENOTOXIC AND *in vitro* CYTOTOXIC ACTIVITY ASSESSMENT OF *Juniperus communis* var. saxatilis

1. Introduction

Juniperus communis L. is a conifer plant widely distributed in the Northern Hemisphere. Its essential oil (EO), distilled dominantly from berries (female cones) is used as a flavoring in food and alcoholic beverage industry. Juniper berries are used as a spice for meat dishes in European cuisines and also give distinguishing flavor to alcoholic beverage gin (Lim, 2012). Specific type of brandy '*Klekovača*', containing juniper berries, is very popular alcoholic drink in Serbia, famous for unique aroma and known for appetite stimulation (Lesjak et al., 2013). *Juniperus* plants are also well-known in traditional medicine; needles and especially cones are widely used as folk remedies for digestive and gynecological disorders, cold and headache, and also known as potent diuretic agents. They are known as particularly useful against respiratory diseases, cough, bronchitis and asthma (Leporatti and Ivancheva, 2003; Tucakov, 1996). Numerous reports indicate antioxidant, anti-inflammatory and antimicrobial activities of different *Juniperus* species (Carpenter et al., 2012; Glišić et al., 2007; Lesjak et al., 2013; Orhan et al., 2011), justifying traditional use of *Juniperus* plants against respiratory disorders. In addition, nephroprotective and hepatoprotective effects of juniper leaves extract have been determined by Al-Attar et al. (2016, 2017). Furthermore, cytotoxic effects of some *Juniperus* species have been detected in different cancer cell lines, including lung cancer A549 cells (Barrero et al., 2004; Yaglioglu and Eser, 2017).

In this work we investigated *Juniperus communis* L. var. saxatilis Pall. (syn. *J. communis* subsp. *alpina* (Suter) Čelak, *J. sibirica* Burgsdorf, *J. nana* Willd, *J. intermedia* Schur.) wild-growing in Serbia. Despite its broad distribution and traditional use as a flavoring agent and plant remedy, only its antioxidative, antimicrobial and anti-inflammatory activities have been described so far (Cabral et al., 2012; Glišić et al., 2007; Lesjak et al., 2013; Marino et al., 2010; Miceli et al., 2009; Orhan et al., 2011). Taking into account that after EO distillation many active components can remain in post-distillation waste (PDW), we determined chemical composition, antioxidant, genotoxic and cytotoxic

properties of both EO and PDW. Antioxidant activity was measured by DPPH and TBA assays; FRAP assay was additionally performed for PDW. Cytotoxicity was evaluated in human lung adenocarcinoma epithelial cells (A549) and normal lung fibroblasts (MRC-5) by MTT assay, while genotoxicity was determined in comet assay in the same cell lines. In addition to EO and PDW applied individually, we examined cytotoxic effect of binary combinations of EO, PDW and conventional cytostatic doxorubicin (DOX). Finally, we determined the effect of EO and PDW on apoptosis and cell cycle arrest in A549 cells.

2. Material and method

2.1. Chemicals

Materials purchased from Sigma-Aldrich, USA, were: L-α-phosphatidylcholine (Cas. No. 8002-43-5), trichloroacetic acid (TCA, Cas. No. 76-03-9), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cas. No. 298-93-1), dimethyl sulfoxide (DMSO, Cas. No. 67-68-5), Dulbecco's modified Eagle's medium (DMEM); fetal bovine serum and annexin V-FITC/PI kit. Materials provided by Fluka Chemie GmbH, Switzerland, were: 2,2-diphenyl-1-picrylhydrazyl (DPPH, Cas. No. 1898-66-4) and 2-thiobarbituric acid (TBA, Cas. No. 504-17-6). Dulbecco's PBS, L-glutamine, Penicillin-Streptomycin, MEM non-essential amino acids were provided by Gibco, Thermo Fisher Scientific, USA, Folin-Ciocalteu reagent by Fisher Scientific, Leicestershire, UK, and Doxorubicin (Cas. No. 25316-40-9) by Actavis, S.C. Sindan-Pharma S.R.L., Romania.

2.2. Human cell lines

The human cell lines used in cytotoxicity assay were fetal lung fibroblasts MRC-5 (ECACC 84101801) and human lung adenocarcinoma epithelial cells A549 (ATCC CCL-185). Cells were cultured in DMEM supplemented with 10 % fetal bovine serum at 37 ˚C in 5 % CO_2. The cells were sub-cultured at 90 % confluence, using 0.1 % trypsin, every 2-3 days.

2.3. Plant material, essential oil and post-distillation waste preparation

Plant material (*Juniperus communis* L. var. saxatilis Pall.) was collected in July 2014, at Stara Planina Mountain, Serbia. Precise location of collected plant material is near the peak Babin Zub (UTM 34T FP 2 30). The voucher specimen (No. 16693) was prepared, identified in accordance with

Adams (2014) and Jovanović (1970) by Nemanja Rajčević (PhD, botanist), and deposited at the Herbarium of University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac" (BEOU Herbarium). Air-dried and finely ground plant material (300 g of seed cones) was added to 1200 mL of dH_2O and distilled in Clevenger-type apparatus for 4 h, followed by removal of recipient solvent (hexane) under reduced pressure. EO was dissolved in hexane (1:2000) for GC-MS analysis, in methanol for antioxidant activity assays, and in DMSO for MTT assay and flow-cytometric analysis. In order to prepare PDW extract, aqueous solution remained after distillation was evaporated in vacuum at 45 ˚C, dissolved in hot, distilled water (1 g mL^{-1}), exhaustively washed with petrol ether (fraction 40–60 ˚C) to remove non-polar compounds and dried under vacuum. For LC-MS/MS analysis dried PDW extract was dissolved in the mixture of 0.05 % aqueous formic acid and methanol (ratio 7:3) to obtain 2 % (w/v) stock solution. For DPPH and FRAP assays PDW extract was dissolved in 80 % aqueous methanol, while dH_2O was used as solvent for TBA and MTT assays, as well as for the flow-cytometric analysis.

2.4. Chemical analysis

Chemical characterization of EO was determined by an GC-MS method described by Lesjak et al. (2013) using Agilent Technologies 6890N gas chromatograph coupled with Agilent Technologies 5975B electron ionization MS detector and controlled by Agilent Technologies MSD ChemStation software (revision E01.01.335) combined with AMDIS (ver. 2.64) and NIST MS Search (ver. 2.0d). The content of 45 selected secondary biomolecules in PDW extract was determined by an LC-MS/MS method described by Orčić et al. (2014), using Agilent Technologies 1200 Series high-performance liquid chromatograph coupled with Agilent Technologies 6410A Triple-Quad tandem mass spectrometer with electrospray ion source, and controlled by Agilent Technologies MassHunter Workstation software (ver. B.03.01). The content of total flavonoids in PDW was determined according to the method described by Lesjak et al. (2011). The concentration of total flavonoids, expressed as milligrams of quercetin equivalents (QE) per gram of dry weight, was calculated according to the standard calibration curve.

2.5. Antioxidant activity

The DPPH radical neutralization effect of PDW and EO was determined by method previously described by Orčić et al. (2011). Briefly, 10µL of the dissolved EO and PDW (serial two-fold dilutions resulting in final concentrations ranging 230–1500 µg mL^{-1} and 0.52–66.67 µg mL^{-1}, respectively) was added to mixture of 100 µL of DPPH solution in methanol (66 µmol L^{-1}) and 190 µL of methanol. Both negative controls and corrections were included. Radical neutralization activity was estimated by measuring absorbance at 515 nm (Multiskan Spectrum, Thermo Scientific) and calculating the concentrations needed to decrease initial DPPH concentration by 50% (IC_{50}).

Extent of lipid peroxidation (LP) was determined by TBA assay, previously described by Mitić-Ćulafić et al. (2009). Commercial preparation of liposomes, l-α- phosphatidylcholine, was used as a model of biological membrane. Briefly, 60 µl of liposomes emulsion (1:10 in dH_2O), 10 µL of EO or PDW (in final concentrations ranging 14-1800 µg mL^{-1}, 5-640 µg mL^{-1}, respectively), 20 µl of 0.075 mol L^{-1} $FeSO_4$, 20 µl of 0.34 mol L^{-1} ascorbic acid, and 2900 µl of phosphate buffer (0,067mol L^{-1}, pH 7.4) were mixed. After incubation (1h at 37 ˚C), the reaction was terminated by adding 0.2 mL of EDTA solution (0.1 mol L^{-1}) and 2 mL of TBA solution (3.75 mg mL^{-1} in $HClO_4$/water mixture (1.3:100), and containing 0.15 g mL^{-1} TCA) to all samples (negative controls and corrections were also included). After that, samples were heated at 100 ˚C for 15 min, subsequently cooled and centrifuged (Medifuge, Heraeus Sepatech, Germany) at 1800*g* for 10 min. Inhibition of LP was estimated by measuring the absorbance at 532 nm (UV-6300 PC spectrophotometer, MRC Scientific instruments, Holon, Israel) and calculating the concentrations needed to decrease initial Fe^{2+}/ascorbate-induced LP for 50% (IC_{50}).

Reducing power of PDW was additionally determined in FRAP assay according to the method previously described by Orčić et al. (2011). Briefly, 10 µL of PDW or standard (ascorbic acid) was mixed with 300 µL of FRAP reagent (obtained by mixing 0.3 mol L^{-1} acetate buffer pH = 3.6, 20 mmol L^{-1} aqueous solution of $FeCl_3$, and 10 mol L^{-1} 2,4,6-tripyridil-*s*-triazine in 40 mmol L^{-1} HCl, in 10:1:1 ratio). Correction (absorbance of the untreated PDW) and control (absorbance of the FRAP reagent) were also measured. After 6 min of incubation, the absorbance at 593 nm was measured. The reducing power, expressed as mg of ascorbic acid equivalents (AA) per gram of dw, was calculated

according to the standard calibration curve. All reactions in antioxidant assays were carried out in triplicates.

2.6. Cytotoxicity and drug synergism analysis

The cytotoxic effect of EO, PDW and DOX was measured by MTT assay, as described by Nikolić et al. (2015). Cell viability was determined by measuring absorbance at 570 nm, using a micro-plate reading spectrophotometer (Multiskan FC, Thermo Scientific). For each test substance or its combinations, three independent experiments with eight replicates for every concentration were performed. To evaluate the nature of interaction between test substances in binary mixtures we used combination index (CI) analysis, providing quantitative definition for additive effect (CI=1), synergism (CI<1), and antagonism (CI>1) in drug combinations (Zhao et al., 2004). The CI was calculated for IC_{50} values of the mixtures, using the formula: $CI=D_1/Dx_1+D_2/Dx_2$, where D_1 is the concentration of the first test substance in the binary mixture; Dx_1 is the concentration of the first test substance alone; D_2 is the concentration of the second test substance in the binary mixture; Dx_2 is the concentration of the second test substance alone.

2.7. Apoptosis and cell cycle analysis

Apoptotic cell death and analysis of the cell cycle phase distribution were analyzed using a fluorescence activated sorting cells (FACS) Calibur Becton Dickinson flow cytometer and Cell Quest computer software. Apoptotic or necrotic cell death was assessed using the annexin V–fluorescein isothiocyanate /propidium iodide (annexin V-FITC/PI) kit, as described by Srdic-Rajic et al. (2016). Samples were prepared according to the manufacturer's instructions. Briefly, annexin V binds to the exposed phosphatidylserine of the early apoptotic cells, while PI labels the late apoptotic/necrotic cells, containing damaged membrane. The numbers of viable ($annexin^-PI^-$), early apoptotic ($annexin^+PI^-$) and late apoptotic/necrotic ($annexin^+PI^+$) cells were determined. Quantitative analysis of the proportion of cells in different cell cycle phases, including the hypodiploid cells with fragmented DNA (sub-G_0/G_1), was performed after staining with PI.

2.8. Comet assay

The genotoxic effect of EO, PDW and DOX was measured by Comet assay, as described by Nikolić et al. (2015). The comets were visualized using fluorescence microscope (Leica, DMLS, Austria) with an excitation filter 510-560 nm, barrier filter 590 nm, at 400x magnification. Image analysis software (Comet Assay IV, Perceptive Instruments, UK) was used for comet analysis. Fifty nuclei per experimental point in each of the three independent experiments were analyzed; the tail intensity was scored as a reflection of DNA damage.

2.9. Statistical analyses

The one-way ANOVA with Mann-Whitney *U* test was employed for the results of the comet assay, while for all the rest assays the one-way ANOVA with Tukey's post hoc test was used. The difference was considered significant when $p<0.05$.

3. Results and Discussion

The chemical characterization of EO by GC–MS analysis identified 93.95 % of total components and revealed 20 peaks exceeding 1 % and no single peak exceeding 25 % (Fig. 1). Unidentified 6.05 % of the EO belongs to the sesquiterpenes that could not be identified due to insufficient selectivity (existence of several compounds with similar spectra and retention), lack of corresponding spectra in used libraries, and/or insufficient quality of experimental spectra due to their low concentrations. Identified constituents are exclusively monoterpene and sesquiterpene hydrocarbons; all of them were unsaturated (1–3 double bonds), and all except β-myrcene were cyclic (64.2 % bicyclic, 24.8 % monocyclic, 3.6 % tricyclic. Among others, α-pinene (23.61 %), δ-cadinene (10.71 %), sabinene (9.53 %), α-muurolene (6.58 %) and γ-cadinene (5.87 %) were the most dominant (Table 1). The high abundance of α-pinene and sabinene is consistent with previous studies, while observed significant quantitative differences in terpenoid profiles could be attributed to intraspecific variability, which is generally high in *Juniperus* species (Adams, 2014).

Out of the 45 investigated secondary biomolecules, only 25 were detected in PDW. LC-MS/MS analysis identified 3.2 % of its total content (Table 2). Among the quantified constituents, rutin (12.2 mg g^{-1}) and quinic acid (11.1 mg g^{-1}) were the most abundant, followed by catechin (5.53 mg g^{-1}) and epicatechin (1.74 mg g^{-1}). The content of common phenolic acids was low, with total

hydroxybenzoic acids amounting up to 0.34 mg g^{-1} and total hydroxycinnamic acids up to 0.26 mg g^{-1}. Total content of flavonoids determined by two methods – LC-MS/MS and spectrophotometric – was in a good agreement (20.5 mg g^{-1} and 19.1 mg g^{-1}, respectively).

Antioxidant activity of EO and PDW was measured by DPPH and TBA assays, with BHT used as a positive control. Additionally, rutin and quercetin were used for comparison in DPPH assay. The results showed strong radical neutralization activity of PDW, which was close to that of BHT (IC_{50} values were 5.27 µg mL^{-1} and 4.9 µg mL^{-1}, respectively), but lower than the activity of rutin (IC_{50} value was 1.8 µg mL^{-1}) and especially of quercetin (IC_{50} value was 0.4 µg mL^{-1}). The activity of EO was multifold lower, with IC_{50} value determined at 1.88 mg mL^{-1}. The efficiency of lipid peroxidation inhibition was lower than the efficiency of DPPH radical neutralization. Obtained IC_{50} values were 0.54 mg mL^{-1}, 2.44 mg mL^{-1} and 20.35 µg mL^{-1} for PDW, EO and BHT, respectively. Additionally, FRAP test was used for PDW and it demonstrated moderate reducing capacity towards Fe^{3+}-TPTZ complex, with 78.77 mg of ascorbic acid equivalents per g of dry weight. Observed antioxidativity of PDW can be partially attributed to the dominant identified compounds rutin, catechin and epicatechin, since they are ubiquitous plant phenolics with well-known antioxidant activities (Azevedo et al., 2013; Cruz-González et al., 2016). Furthermore, strong antioxidant potential of PDW is in accordance with the literature data concerning antioxidant potential of *Juniperus* extracts (Lesjak et al., 2013; Orhan et al., 2011). However, low antioxidant effect of EO is not in line with current literature data (Lesjak et al., 2013).

Considering that lung cancer is one of the most common cancers with high mortality and frequent development of cytostatic resistance (Chang, 2011), in further work we examined the cytotoxic potential of EO and PDW against lung adenocarcinoma A549 cells and normal fetal lung fibroblasts MRC-5. The conventional cytostatic DOX, used to treat numerous malignances including lung cancer (Tacar et al., 2013), was used as a positive control. MTT assay revealed that both EO and PDW induced cytotoxicity in a dose-dependent manner. Although cytotoxicity of EO and especially of PDW was considerably lower comparing to DOX, preferable feature was that both substances exhibited higher selectivity towards cancer A549 cells (Supplementary Fig. 1). Taking into account

the EO composition, its cytotoxicity could be attributed to lipophilic terpenoid components which disrupt and permeabilize cell membranes, especially mitochondrial, leading to reactive oxygen species (ROS) release (Bakkali et al., 2008). Higher cytotoxicity of EO against A549 cells could be attributed to additional cytotoxic mechanisms, recognized specifically in cancer cells, such as topoisomerase inhibition, modulation of p53, bcl-2, AMPK and MAPK/ERK pathways and inhibition of isoprenylation of $p21^{ras}$. These mechanisms have been described for limonene, β-elemene, β-caryophyllene and α-humulene (Lesgards et al., 2014), constituting 12.45 % of *J. communis* EO. On the other hand, cytotoxicity of PDW could be assigned to its polyphenols. Although polyphenols are recognized as naturally occurring antioxidants, their high concentrations possess pro-oxidant properties and therefore they could induce cytotoxicity (Babich et al., 2011). Indeed, obtained results show that effective concentrations of PDW were extremely high, indicating that cytotoxicity could be attributed to pro-oxidative effect of its polyphenols.

In order to further investigate obtained anticancer activity of EO and PDW, their pro-apoptotic potential was monitored using flow cytometric analysis of A549 cells. Cells were initially treated with IC_{50} values of EO (69.4 µg mL^{-1}) or PDW (1.27 mg mL^{-1}) for 24 h. However, this concentration of PDW induced high percent of the late apoptotic and necrotic cells (data not shown) and subsequently we applied it in lower concentration, inducing only 30% lethality (0.3 mg mL^{-1}). As shown in Fig. 2, EO showed weak apoptotic effect, which could be seen as a slight increase of the cell numbers in early (annexin$^+$/PI$^-$) and late apoptotic phase (annexin$^+$/PI$^+$), but did not significantly affect the cell cycle progression. On the contrary, PDW induced significant increase in both early and late apoptotic cells and caused a cell cycle arrest in G_2/M phase that was associated with an increased number of apoptotic hypodiploid cells with fragmented DNA (sub-G_0/G_1 phase). This demonstrates that apoptotic potential of PDW could account for its cytotoxicity. Moreover, when we examined genotoxicity of EO and PDW in comet assay, no genotoxicity of EO was found, while PDW induced significant genotoxic effect, stronger in A549 cells (Fig. 3). This shows that pro-apoptotic effect of PDW could be mediated by its damaging effect on cellular DNA, which could be induced by its pro-oxidant activity. It is known that DNA damage initiates p53-mediated signaling cascades that can lead

to cell cycle arrest and apoptosis (Vogelstein et al., 2000). Literature data point that among identified PDW components rutin can induce DNA damage and modulate a wide range of intracellular signaling cascades leading to apoptosis of cancer cells (Perk et al., 2014; Marcarini et al., 2011).

Taking into account that clinical use of DOX is limited due to systemic toxicity, especially to liver, heart and kidney, a continuous search for auxiliary substances which could decrease its therapeutic doses is encouraged (Wang et al., 2012). For that reason, cytotoxic potential of binary combinations of EO/PDW with DOX was also monitored. While DOX was applied in concentrations sub-lethal for normal MRC-5 cells (0.312 µg mL^{-1} and 0.625µg mL^{-1}, resulting in about 70 % of cells survival), the concentrations of EO and PDW were variable, but also in the range inducing low cytotoxicity (survival was higher than 70 %). Comparison of results obtained in different cell lines indicated that both EO and PDW highly sensitized cancerous cell line to DOX, while the effect in normal cells was less pronounced (Figs. 4 and 5).

Cytotoxicity was also monitored for binary combinations of EO and PDW, both applied at concentrations which were sub-lethal for normal MRC-5 cells (survival was higher than 70 %). The results showed that increased cytotoxicity was obtained for all applied combinations in A549 cells, while in MRC-5 cells it was observed only at the highest tested concentration of PDW (Fig. 6). In Table 3 we summarized cytotoxic effect of tested compounds and their binary mixtures, specified by estimated IC_{50} values. It clearly shows that concentrations required to induce 50 % lethality in both cell lines were considerably lower in combinations than if substances were applied individually.

To monitor the mode of interactions between EO, PDW and DOX in binary mixtures, the combination index (CI) was calculated for the IC_{50} concentrations (Zhao et al., 2004). As shown in Table 4, all determined CI values were lower than 1 indicating synergism. Considering that DOX suppresses cell division by intercalating into DNA which inhibits topoisomerase II, but also by extensive production of ROS which lead to oxidative stress (Mizutani et al., 2005), we propose that pro-oxidative activity of PDW and ROS release caused by EO could increase DOX-induced oxidative stress and enhance its cytotoxic effect. Additionally, DNA damage caused by PDW could contribute

to DOX induced genotoxicity. Increased permeability of cell membranes caused by EO could also improve the uptake of DOX and its accumulation in nuclei, enhancing its cytotoxic effect (Ambrož et al., 2016). Stronger synergism obtained in A549 compared to MRC-5 cells is in line with previously reported data indicating that some EOs and polyphenols can activate additional pro-apoptotic pathways specific for cancer cells (Lesgards et al., 2014; Mahbubet al., 2015; Wang et al., 2004; Wang et al., 2012).

Conclusion

J. communis var. saxatilis possesses cytotoxic properties and increases anti-cancer effect of DOX in human lung adenocarcinoma A549 cell line. This indicates that combination with *J. communis* could decrease the chemotherapeutic doses of DOX, potentially reducing the side effects. Obtained results encourage further study of *Juniperus communis* var. saxatilis in order to evaluate its auxiliary potential in the treatment of lung and other cancers.

Acknowledgement

This work was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia, Project No. 172058. We thank Dragana Četojević-Simin, Oncology Institute of Vojvodina, Serbia, and Vera Simić, Medicines and Medical Devices Agency of Serbia, for providing MRC-5 and A549 cells, respectively; Ivica Dimkić, Faculty of Biology, University of Belgrade, for technical support.

References

Adams, R.P., 2014. Junipers of the world: the genus Juniperus, fourth ed. Trafford Publishing, Waco, USA.

Al-Attar, A.M., Alrobai, A.A., Almalki, D.A., 2016. Effect of *Olea oleaster* and *Juniperus procera* leaves extracts on thioacetamide induced hepatic cirrhosis in male albino mice. Saudi J. Biol. Sci. 23, 363-371.

Al-Attar, A.M., Alrobai, A.A., Almalki, D.A., 2017. Protective effect of olive and juniper leaves extracts on nephrotoxicity induced by thioacetamide in male mice. Saudi J. Biol. Sci. 24, 15–22.

Ambrož, M., Hanušová, V., Skarka, A., Boušová, I., Králová, V., Langhasová, L., Skálová, L., 2016. Essential oil from *Myrica rubra* leaves potentiated antiproliferative and prooxidative effect of doxorubicin and its accumulation in intestinal cancer cells. Planta Med. 82, 89-96.

Azevedo, M.I., Pereira, A.F., Nogueira, R.B., Rolim, F.E., Brito, G.A., Wong, D.V.T., Lima-Júnior, R.C., de Albuquerque Ribeiro, R., Vale, M.L., 2013. The antioxidant effects of the flavonoids rutin and quercetin inhibit oxaliplatin-induced chronic painful peripheral neuropathy. Mol. Pain 9, 53.

Babich, H., Schuck, A.G., Weisburg, J.H., Zuckerbraun, H.L., 2011. Research strategies in the study of the pro-oxidant nature of polyphenol nutraceuticals. J. Toxicol. Article ID 467305.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils–a review. Food Chem. Toxicol. 46, 446-475.

Barrero, A.F., Del Moral, J.F.Q., Herrador, M.M., Akssira, M., Bennamara, A., Akkad, S., Aitigri, M., 2004. Oxygenated diterpenes and other constituents from Moroccan *Juniperus phoenicea* and *Juniperus thurifera* var. africana. Phytochem. 65, 2507-2515.

Cabral, C., Francisco, V., Cavaleiro, C., Gonçalves, M.J., Cruz, M.T., Sales, F., Batista, M.T., Salgueiro, L., 2012. Essential oil of *Juniperus communis* subsp. *alpina* (Suter) Čelak needles: chemical composition, antifungal activity and cytotoxicity. Phytother. Res. 26, 1352-1357.

Carpenter, C.D., O'Neill, T., Picot, N., Johnson, J.A., Robichaud, G.A., Webster, D., Gray, C.A., 2012. Anti-mycobacterial natural products from the Canadian medicinal plant *Juniperus communis*. J. Ethnopharmacol. 143, 695-700.

Chang, A., 2011. Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. Lung cancer 71, 3-10.

Cruz-González, T., Cortez-Torres, E., Perez-Severiano, F., Espinosa, B., Guevara, J., Perez-Benitez, A., Melendez, F.J., Díaz, A., Ramírez, R.E., 2016. Antioxidative stress effect of epicatechin and catechin induced by Aβ 25–35 in rats and use of the electrostatic potential and the Fukui function as a tool to elucidate specific sites of interaction. Neuropeptides 59, 89-95.

Glišić, S.B., Milojević, S.Ž., Dimitrijević, S.I., Orlović, A.M., Skala, D.U., 2007. Antimicrobial activity of the essential oil and different fractions of *Juniperus communis* L. and a comparison with some commercial antibiotics. J. Serb. Chem. Soc. 72, 311-320.

Leporatti, M. L., Ivancheva, S., 2003. Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy. J. Ethnopharmacol. 87, 123-142.

Lesgards, J. F., Baldovini, N., Vidal, N., Pietri, S., 2014. Anticancer activities of essential oils constituents and synergy with conventional therapies: a review. Phytother. Res. 28, 1423-1446.

Lesjak, M.M., Beara, I.N., Orčić, D.Z., Anačkov, G.T., Balog, K.J., Franciškovič, M.M., Mimica-Dukić, N.M., 2011. *Juniperus sibirica* Burgsdorf. as a novel source of antioxidant and anti-inflammatory agents. Food Chem. 124, 850-856.

Lesjak, M.M., Beara, I.N., Orčić, D.Z., Ristić, J.D., Anačkov, G.T., Božin, B. N., Mimica-Dukić, N.M., 2013. Chemical characterisation and biological effects of *Juniperus foetidissima* Willd. 1806. LWT-Food Sci. Technol. 53, 530-539.

Lim, T.K., 2012. Edible Medicinal and Non-Medicinal Plants, Volume 1, Fruits. Springer, Dordrecht, Netherlands.

Jovanović, B., 1970. Rod *Juniperus* L. in: Josifović M (ed.), Flora SR Srbije, Vol. 1. SANU, Belgrade, Serbia, pp. 158–164.

Mahbub, A.A., Le Maitre, C.L., Haywood-Small, S.L., Cross, N.A., Jordan-Mahy, N., 2015. Polyphenols act synergistically with doxorubicin and etoposide in leukaemia cell lines. Cell Death Discov. 1, 15043.

Marcarini, J.C., Tsuboy, M.S.F., Luiz, R.C., Ribeiro, L.R., Hoffmann-Campo, C.B., Mantovani, M.S., 2011. Investigation of cytotoxic, apoptosis-inducing, genotoxic and protective effects of the flavonoid rutin in HTC hepatic cells. Exp. Toxicol. Pathol. 63, 459-465.

Marino, A., Bellinghieri, V., Nostro, A., Miceli, N., Taviano, M.F., Güvenç, A., Bisignano, G., 2010. In vitro effect of branch extracts of *Juniperus* species from Turkey on *Staphylococcus aureus* biofilm. FEMS Immunol. Med. Microbiol. 59, 470-476.

Miceli, N., Trovato, A., Dugo, P., Cacciola, F., Donato, P., Marino, A., Bellinghieri, V., La Barbera, T.M., Güvenç, A., Taviano, M. F., 2009. Comparative analysis of flavonoid profile, antioxidant and antimicrobial activity of the berries of *Juniperus communis* L. var. communis and *Juniperus communis* L. var. saxatilis Pall. from Turkey. J. Agric. Food Chem. 57, 6570-6577.

Mitić-Ćulafić, D., Žegura, B., Nikolić, B., Vuković-Gačić, B., Knežević-Vukčević, J., Filipič, M., 2009. Protective effect of linalool, myrcene and eucalyptol against t-butyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. Food Chem. Toxicol. 47, 260-266.

Mizutani, H., Tada-Oikawa, S., Hiraku, Y., Kojima, M., Kawanishi, S., 2005. Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide. Life Sci. 76, 1439-1453.

Nikolić, B., Jovanović, B., Mitić-Ćulafić, D., Vuković-Gačić, B., Knežević-Vukčević, J. 2015. Comparative study of genotoxic, antigenotoxic and cytotoxic activities of monoterpenes camphor, eucalyptol and thujone in bacteria and mammalian cells. Chem-Biol. Interact. 242, 263-271.

Orčić, D., Francišković, M., Bekvalac, K., Svirčev, E., Beara, I., Lesjak, M., Mimica-Dukić, N., 2014. Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection. Food Chem. 143, 48–53.

Orčić, D.Z., Mimica-Dukić, N.M., Francišković, M.M., Petrović, S.S., Jovin, E.Đ., 2011. Antioxidant activity relationship of phenolic compounds in *Hypericum perforatum* L. Chem. Cent. J. 5, 1-8.

Orhan, N., Orhan, I.E., Ergun, F., 2011. Insights into cholinesterase inhibitory and antioxidant activities of five *Juniperus* species. Food Chem. Toxicol. 49, 2305-2312.

Perk, A.A., Shatynska-Mytsyk, I., Gerçek, Y.C., Boztaş, K., Yazgan, M., Fayyaz, S., Farooqi, A.A., 2014. Rutin mediated targeting of signaling machinery in cancer cells. Cancer Cell Int. 14,124.

Srdic-Rajic, T., Nikolic, K., Cavic, M., Djokic, I., Gemovic, B., Perovic, V., Veljkovic, N., 2016. Rilmenidine suppresses proliferation and promotes apoptosis via the mitochondrial pathway in human leukemic K562 cells. Eur. J. Pharm. Sci. 81, 172-180.

Tacar, O., Sriamornsak, P., Dass, C.R., 2013. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J. Pharm. Pharmacol. 65, 157-170.

Tucakov, J., 1996. Curing with plants. Phytotherapy, sixth ed. Rad, Belgrade, Serbia. (*in Serbian*).

Vogelstein, B., Lane, D., Levine, A.J., 2000. Surfing the p53 network. Nature 408, 307-310.

Wang, G., Zhang, J., Liu, L., Sharma, S., Dong, Q., 2012. Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/Bcl-xl. PLoS One 7, e51764.

Wang, S., Konorev, E.A., Kotamraju, S., Joseph, J., Kalivendi, S., Kalyanaraman, B., 2004. Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms intermediacy of H2O2-and p53-dependent pathways. J. Biol. Chem. 279, 25535-25543.

Yaglioglu, A.S., Eser, F., 2017. Screening of some *Juniperus* extracts for the phenolic compounds and their antiproliferative activities. S. Afr. J. Bot. 113, 29-33.

Zhao, L., Wientjes, M.G., Au, J.L., 2004. Evaluation of combination chemotherapy integration of nonlinear regression, curve shift, isobologram, and combination index analyses. Clin. Cancer Res. 10, 7994-8004.

Table 1. Qualitative and semiquantitative composition of *Juniperus communis* var. saxatilis essential oil (EO)

Compound	Peak	**t_R [min]**	**LRI**	**Area%**
α-Thujene	1	5.014	925	0.90
α-Pinene	2	5.142	933	23.61
Sabinene	3	5.796	972	9.53
β-Pinene	4	5.872	976	1.10
β-Myrcene	5	6.047	987	1.36
Limonene	6	6.757	1029	2.07
γ-Terpinene	7	7.290	1060	0.91
4-Terpineol	8	9.436	1193	1.26
α-Copaene	9	12.089	1388	0.93
β-Elemene	10	12.242	1402	4.37
(sesquiterpene)	11	12.386	1414	0.55
β-Caryophyllene	12	12.584	1432	2.94
γ-Elemene	13	12.667	1440	1.31
Vidrene (thujopsene)	14	12.717	1445	2.66
α-Humulene	15	12.929	1465	3.08
cis-Muurola-4(14),5-diene	16	13.012	1473	1.15
(sesquiterpene)	17	13.113	1483	1.90
Germacrene D	18	13.187	1491	7.25
(co-eluting sesquiterpenes)	19	13.248	1497	1.95
α-Muurolene	20	13.328	1505	6.58
γ-Cadinene	21	13.477	1521	5.87
δ-Cadinene	22	13.537	1528	10.71
α-Cadinene	23	13.684	1544	1.83
(sesquiterpene)	24	13.755	1552	0.89
Germacrene B	25	13.906	1569	4.56
(sesquiterpene)	26	14.588	1654	0.76
	Yield (v/w)%			**0.94%**
	Total identified			**93.95%**
	Monoterpene hydrocarbons			40.7%
	Sesquiterpene hydrocarbons			59.3 % (6.05% unidentified)

Table 2. Quantitative composition of *Juniperus communis* var. saxatilis postdistillation waste (PDW)

Compound	Content [mg per g of dw][a]
Quercetin 3-O-rhamnosylglucoside (Rutin)	12.25 ± 0.37
Quinic acid	11.09 ± 1.11
Catechin	5.534 ± 0.553
Epicatechin	1.738 ± 0.174
Amentoflavone	0.392 ± 0.012
Umbilliferone	0.253 ± 0.025
Quercetin 3-*O*-glucoside (Isoquercitrin)	0.232 ± 0.007
Protocatechuic acid	0.145 ± 0.012
Apigenin 7-*O*-glucoside	0.140 ± 0.007
Quercetin 3-O-rhamnoside (Quercitrin)	0.139 ± 0.008
Cinnamic acid	0.097 ± 0.019
p-Hydroxybenzoic acid	0.093 ± 0.006
p-Coumaric acid	0.088 ± 0.008
Vanillic acid	0.060 ± 0.018
Ferulic acid	0.053 ± 0.005
Gallic acid	0.034 ± 0.030
Kaempferol 3-*O*-glucoside	0.021 ± 0.001
Gentisic acid	0.012 ± 0.001
5-*O*-Caffeoylquinic acid (Chlorogenic acid)	0.011 ± 0.001
Caffeic acid	<0.002[b]
Quercetin	<0.05
Luteolin	<0.1
Naringenin	<0.002

[a] Results are given as the concentration (mg per g of PDW dry weight) ± relative standard deviation of repeatability (as determined by method validation, Orčić et al, 2014).

[b] Concentrations above the limit of detection but below the limit of quantitation (according to the method validation, Orčić et al, 2014).

Table 3. IC_{50} concentrations (µg mL^{-1}) of EO, PDW and DOX, alone and in binary mixtures

	A549	**MRC-5**
EO	69.4	120
PDW	1270	2860
DOX	5.88	1.92
DOX + EO	0.312 + 7.5	0.312 + 69.8
DOX + EO	0.625 + 5.2	0.625 + 60.8
DOX + PDW	0.312 + 86	0.312 + 390
DOX + PDW	0.625 + 31	0.625 + 270
EO + PDW	15.62 + 190	15.62 + 970
EO + PDW	31.25 + 120	31.25 + 840

Table 4. Combination index calculated for the IC_{50} values of binary mixtures

A549		**MRC-5**	
Mixture content (µg mL^{-1})	CI	Mixture content (µg mL^{-1})	CI
PDW = 190 EO = 15.62	0.37	PDW = 970 EO = 15.62	0.47
PDW = 120 EO = 31.25	0.54	PDW = 840 EO = 31.25	0.55
EO = 7.5 DOX = 0.312	0.16	EO = 69.83 DOX = 0.312	0.75
EO = 5.2 DOX = 0.625	0.18	EO = 60.85 DOX = 0.625	0.84
PDW = 86 DOX = 0.312	0.12	PDW = 390 DOX = 0.312	0.30
PDW = 31 DOX = 0,625	0.13	PDW = 270 DOX = 0,625	0.42

CI < 1 indicate synergism; **CI = 1** indicate additive effect; **CI > 1** indicate antagonism.

Figure 1. GC–MS chromatogram of *Juniperus communis* var. *saxatilis* essential oil

Chemical characterization of EO: **1** – α-Thujene, **2** – α-Pinene, **3** – Sabinene, **4** – β-Pinene, **5** – β-Myrcene, **6** – Limonene, **7** – γ-Terpinene, **8** – 4-Terpineol, **9** – α-Copaene, **10** – β-Elemene, **11** – *(sesquiterpene)*, **12** – *β*-Caryophyllene, **13** – γ-Elemene, **14** – Vidrene (thujopsene), **15** – α-Humulene, **16** – *cis*-Muurola-4(14),5-diene, **17** – (*sesquiterpene*), **18** – Germacrene D, **19** – (*co-eluting sesquiterpenes*), **20** – α-Muurolene, **21** – γ-Cadinene, **22** – δ-Cadinene, **23** – α-Cadinene, **24** – (*sesquiterpene*), **25** – Germacrene B, **26** – (*sesquiterpene*).

Figure 2. Effect of *J. communis* EO, PDW and DOX on apoptosis and cell cycle distribution in lung adenocarcinoma A549 cells.

Cells were treated with EO (69.4 µg mL^{-1}) and PDW (300 µg mL^{-1}) alone for 24 h.
(A) Flow cytometry analysis using Annexin V-FITC/PI staining to discriminate the live cells ($Annexin^-/PI^-$), early apoptotic cells ($Annexin^+/PI^-$), necrosis or late apoptotic cells ($Annexin^+/PI^+$).
(B) Flow cytometry analysis using PI staining to discriminate alternations in cell cycle phase distribution;
M1 - cells with DNA content corresponding to sub-G_0/G_1; M2 - cells with DNA content corresponding to the G_0/G_1; M3 - cells with DNA content corresponding to S phase; M4 - cells with DNA content corresponding to G_2/M phases.

Figure 3. Genotoxicity of *J. communis* EO and PDW in A549 and MRC-5 cells in comet assay
Results are expressed as a percentage of DNA in the comet tail (TI).
Statistically significant difference comparing to solvent control: * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

Figure 4. Cytotoxic effect of binary combinations containing *J. communis* EO and DOX against lung adenocarcinoma (A549) and fetal lung fibroblast (MRC-5) cells in MTT assay.

Cells were treated with EO+DOX mixtures for 24h at 37°C in 5% CO_2 and 100% humidity. Experiments were performed in two independent experiments with eight replicates.
Effect of combinations containing 0.312 µg mL^{-1} of DOX and shown concentrations of EO against A549 cells (A) and MRC-5 cells (B).
Effect of combinations containing 0.625 µg mL^{-1} of DOX and shown concentrations of EO against A549 cells (C) and MRC-5 cells (D).
Statistically significant difference ($p<0.05$) comparing to solvent control (*), to DOX alone (a), and between binary combination (EO+DOX) and EO alone (b).

Figure 5. Cytotoxic effect of binary combinations containing *J. communis* PDW and DOX against lung adenocarcinoma (A549) and fetal lung fibroblast (MRC-5) cells in MTT assay.

Cells were treated with PDW+DOX mixtures for 24h at 37°C in 5% CO_2 and 100% humidity. Experiments were performed in two independent experiments with eight replicates.

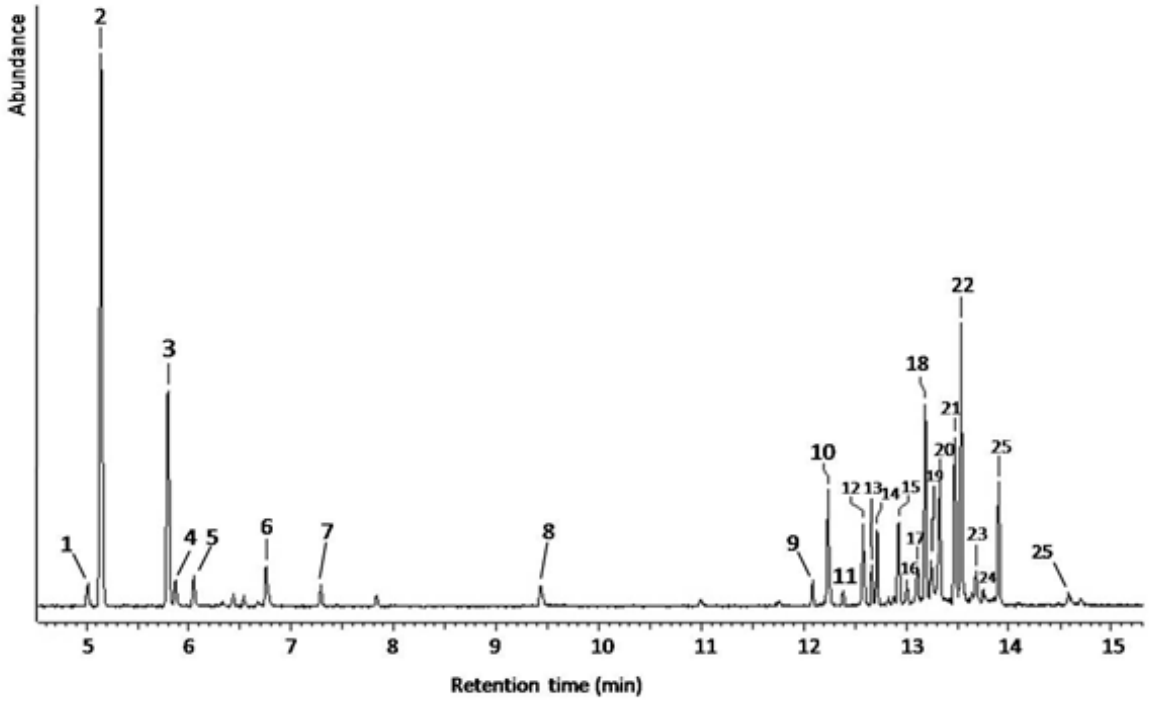
Effect of combinations containing 0.312 µg mL^{-1} of DOX and shown concentrations of PDW against A549 cells (A) and MRC-5 cells (B).

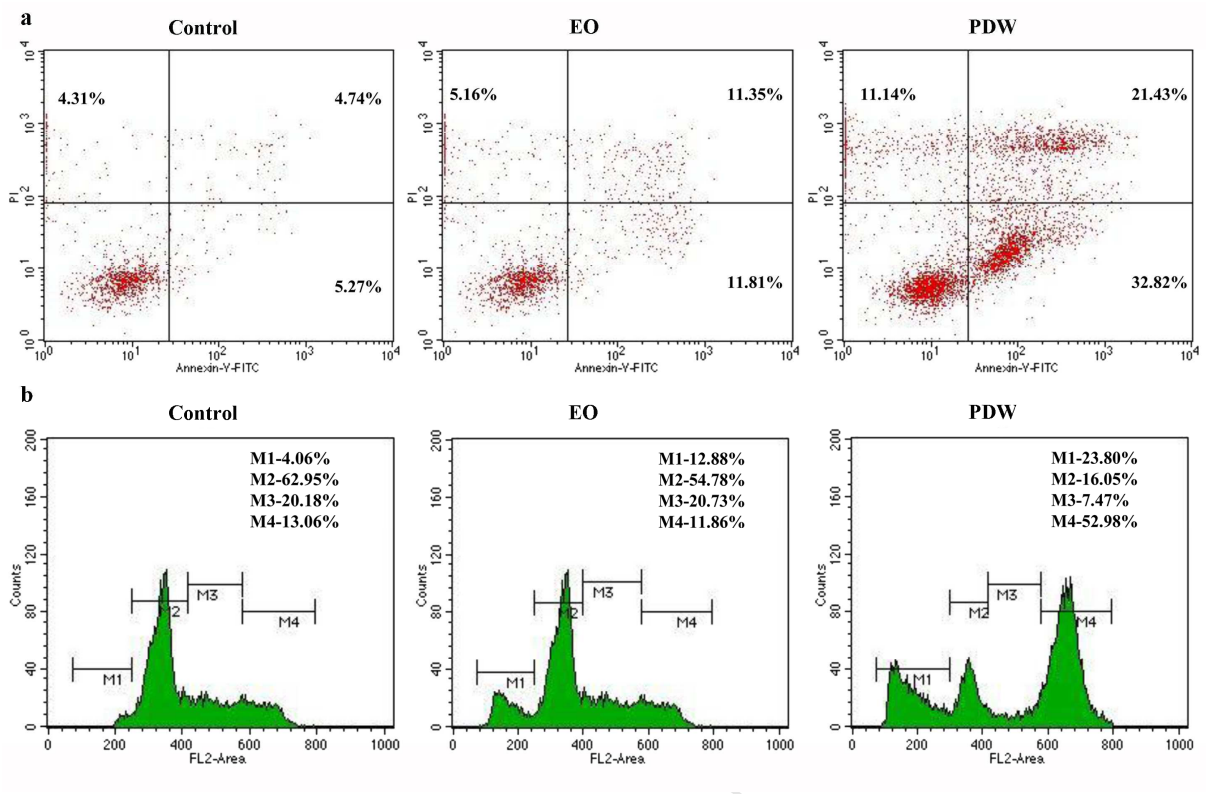
Effect of combinations containing 0.625 µg mL^{-1} of DOX and shown concentrations of PDW against A549 cells (C) and MRC-5 cells (D).

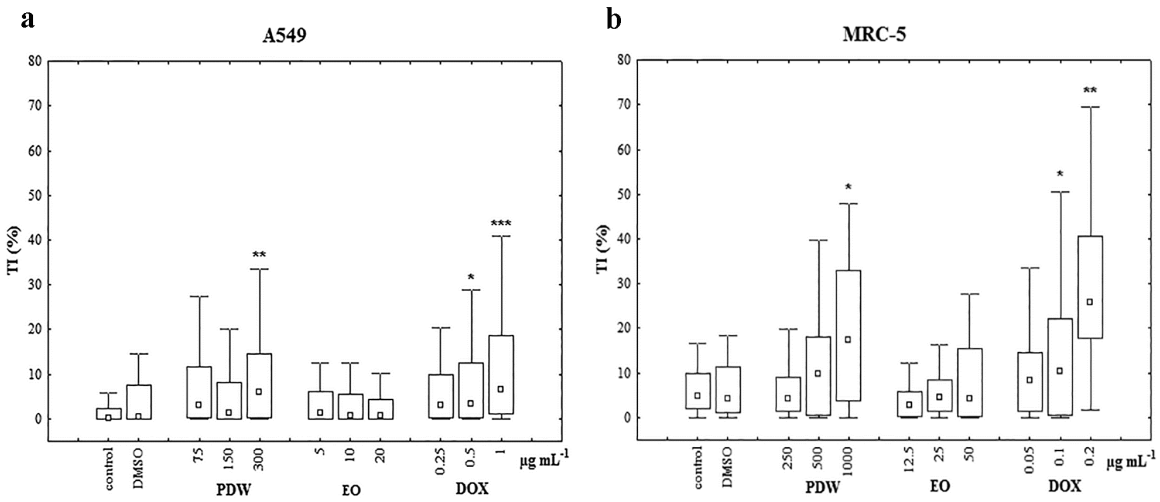
Statistically significant difference ($p<0.05$) comparing to solvent control (*), to DOX alone (a), and between binary combination (PDW+DOX) and PDW alone (b).

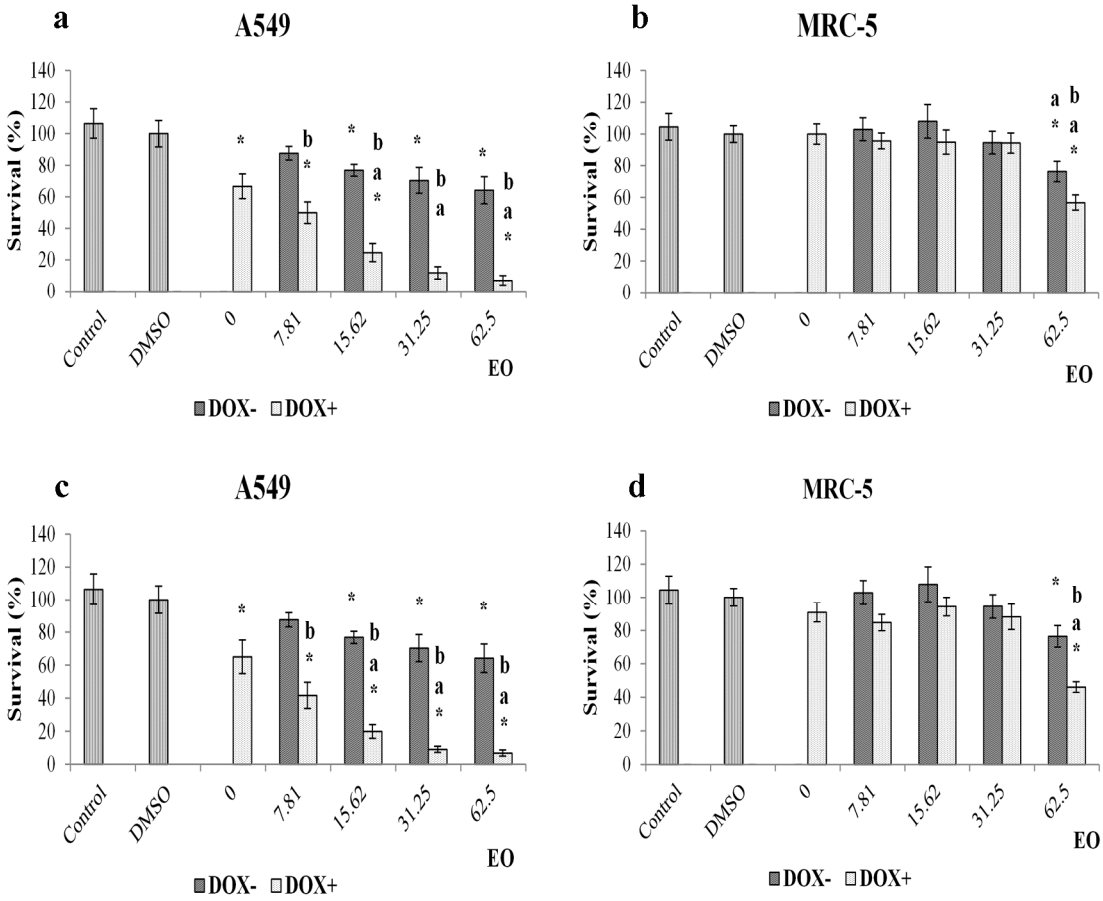
Figure 6. Cytotoxic effect of *J. communis* PDW and EO combinations against lung adenocarcinoma (A549) and fetal lung fibroblast (MRC-5) cells in MTT assay

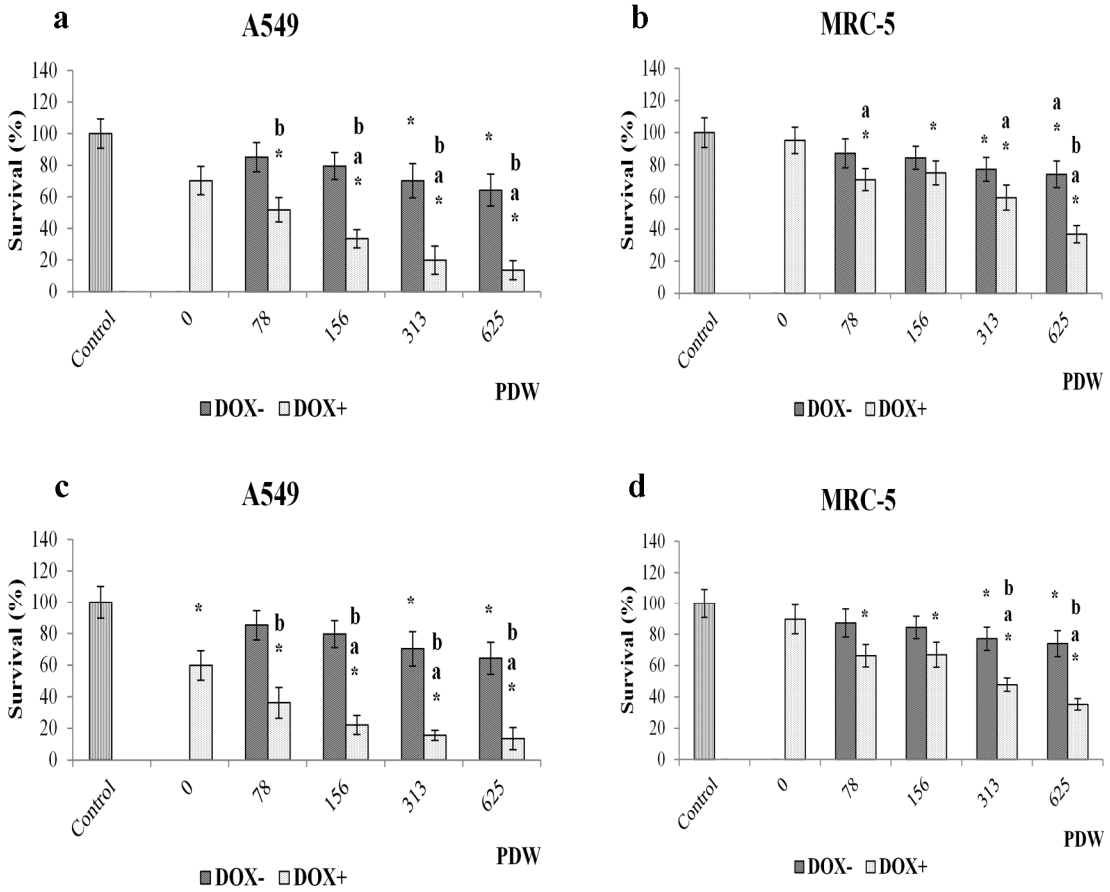
a,b - combinations containing 15.62 µg mL^{-1} EO. c,d - combinations containing 31.25 µg mL^{-1} EO. Experiments were performed three times, each with eight replicates. Statistically significant difference ($p<0.05$) comparing to solvent control (*), EO (a), and between binary combination (EO+PDW) and PDW alone (b).

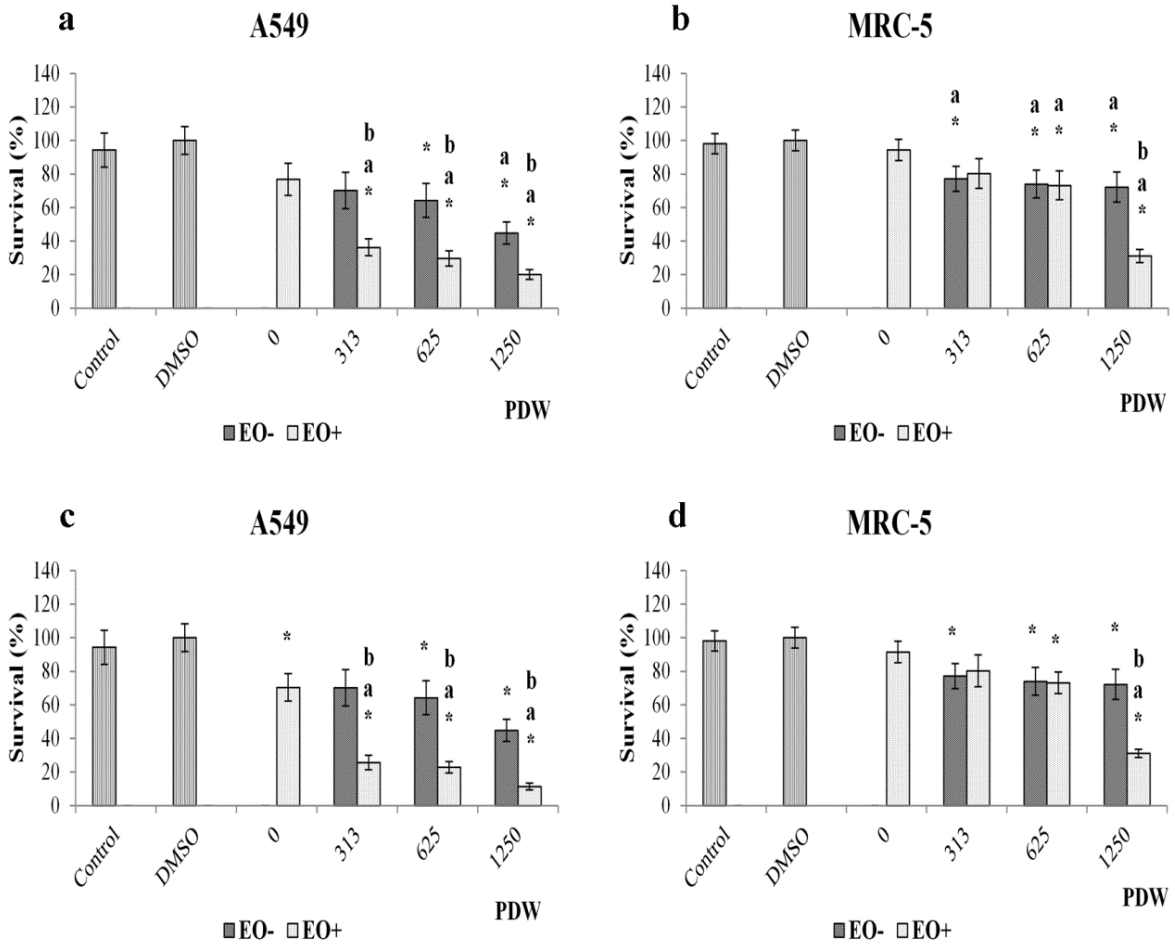











a
A549
b
MRC-5
c
A549
d
MRC-5
Survival (%)
140
120
100
80
60
40
20
0
Control
DMSO
0
313
625
1250
PDW
EO-
EO+

Highlights:

- Dominant constituents of essential oil (EO) of *Juniperus communis* was α-pinen.
- Dominant identified constituent of post-distillation waste (PDW) was rutin.
- Strong antioxidant, apoptotic and genotoxic effects were determined only for PDW.
- EO and PDW induced stronger cytotoxicity in cancer than in normal lung cells.
- Synergism in cytotoxicity of EO and PDW in combination with doxorubicin was found.