Chemical Constituents of *Pinus densiflora* Oil and its Antimicrobial and Cytotoxic and Necrotic Responses

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Pinus densiflora **oil chemical constituents and its biological properties were studied in this work. Volatile oil was identified using UPLC MS/MS analysis. Antimicrobial activity of the oil was investigated against fungi, bacteria (G+ve, G-ve), and yeasts measuring the inhibition zones. Sustainability of pine oil treated Hep G2 cells were assessed by MTT assay, flow cytometric analysis of cell cycle progression was carried out using 0.4, 0.8, 1.2 and 1.6 % (v/v) of pine oil for 3h. UPLC MS/MS analysis showed that the major constituents of the oils were monoterpene hydrocarbons and phenolic flowed by sesquiterpenes. β-pinene and α-pinene were the major hydrocarbons constituents. Antimicrobial assay showed that crude *Pinus* volatile oil has an antimicrobial activity at 60 µl of the crude oil comparable to 40 µl while the activity was very low at 20 µl for all tested microorganisms and this may related its monoterpene hydrocarbons and phenolic contents. Exposure of Hep G2 cells to pin oil for 24 h resulted in significant cytotoxicity as a concentration dependent, decrease in survival of cells were obtained. A significant increase in the proportions of apoptotic/necrotic Hep G2 cells in sub-G1 phase. No change in cell cycle progression was indicated. Results expressed the possibility of using *Pinus* essential oils as antimicrobial agents. However, Pine oil treatment was found extremely toxic.**

Key words: *Pinus densiflora* oil, Chemical constituents, Antimicrobial activity.

Volatile oils are largely used in various fields: perfumery, cosmetics, pharmaceuticals, synthesis. Historically essential oils have been used for thousands of years to promote health[1,2]. According to the composition of essential oils the genus *Pinus* is divided into two groups[3]. First group includes the species rich in monoterpene hydrocarbons (α-β-pinene, limonene, β-caryophylene, germacrene D, D-3-carene) and second is rich in the oxygenate monoterpenes (borneol, bornyl acetate). During the last year most of the phytochemical studies were related to aromatherapy, a branch of herbology. α- and β-pinene together with a mixture of limonene and β-phellandreneare the major volatile constituents[4]; other monoterpene hydrocarbons (myrcene, camphene, sabinene, terpinolene and bornyl acetate) were identified in traces. Enantiomers of four monoterpene hydrocarbons (α- and β-pinene, camphene and limonene) in the *pinus* essential oil were separated identified and quantified[5]. The authors reported the occurrence of both enantiomers of camphene α- and β-pinene and the (–) isomer of limonene. (–Limonene and α-pinene as sum of the (–) and (+) isomers dominated in the needle essential oil[11]. A literature survey revealed no studies on the biological effects of *pinus* volatiles.

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The chemistry and the biological effects of essential oils of some *pinus* species have been intensively studied[6,7,8,3,9,10]. The antibacterial activity of many essential oils over a wide range of microorganisms has been studied extensively both in vitro and applied to foods [11,12]. It is well known that the antimicrobial activity of essential oils is related to their chemical composition, mainly the phenolic components[13]. *Pinus* is one of the many trees that are known for their medicinal properties[14] as well as for their economic importance[15]. Few studies were achieved to determinate the bioactive compounds of *Pinus* species[16,17]. This work was carried out in order to investigate the chemical composition, antimicrobial activities, and cytotoxicity and necrotic responses of *Pinus densiflora* volatile oil.

MATERIALS AND METHODS

Extraction of essential oil

The air-dried and grained plant was extracted using water-distillation for 5 hours using a Clevenger apparatus. The obtained essential oil was dried with anhydrous sodium sulfate and stored at 4°C before analyzed.

UPLC MS/MS Analysis of volatile oil

Chemical constituents for the extracted volatile oil was performed using a Waters Alliance system UPLC / USA, auto-sampler and a waters 2996 diode array detector. UV spectra between 200-900 nm were collected, extracting 274 nm for chromatograms. BEH C18 (4.6 × 250 mm, 5 µm) column was used. The mobile phase was an isocratic combination of Methanol: Formic acid (80:20) with a flow rate of 0.5 ml/min. Injection volume for all samples and standard solutions was 10 µl.

Antimicrobial activity

Volatile oil extract was tested for its antimicrobial activities against[19] microorganisms including 5 Gram positive (*Micrococcus roseus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtils*, *Bacillus cereus*), 5 Gram-negative bacteria (*Escherichia coli*, *Erwiniacarotovora*, *Serratiamarcines*, *Pseudomonas fluoresceus*, *Salmonella typhimurium*), 5 fungi (*Fusariumsolani*, *Fusariumoxysporum*, *Rhizoctoniasolani*, *Aspergillusniger*, *Penicillium sp.*), and 4 yeasts (*Saccharomyces cerevisiae*, *Saccharomyces roxii*, *Candida utilis*, *Candida albicans*). Antimicrobial activity was determined by measuring the inhibition zones (mm) using agar diffusion method[18]. Due to the immiscibility of pine volatile oil with water; emulsion was prepared using 0.2% agar solution[17,19]. 400, 800 and 1200 µL of EO were added to 1600, 1200 and 800 µL of 0.2% agar solution, respectively. A total volume (2mL) of each dilution was added to 18 mL of cultural medium potato dextrose agar (PDA). Final concentrations of 20, 40, and 60µL/mL were obtained. The results were measured after 5 days of incubation at 25±2 °C.

Cell culture

The human hepatocellular carcinoma cell line (Hep G2) exhibiting growth with no contact inhibition were grown in DMEM, supplemented with high glucose, 10 % FBS and antibiotic-antimycotic solution (100x, 1 ml/100 ml of medium) at 37 °C in a 5% CO_2 atmosphere with 95% humidity. Cell viability was assessed by visual microscopic inspection for exclusion of trypan blue. Batches of Hep G2 cells exhibiting less than 95% cell viability were not used.

Tetrazolium bromide salt (MTT) assay

Viability of pine oil treated Hep G2 cells was assessed by use of the MTT assay according to[20, 21]. Cells (1×10^4) were allowed to adhere for 24 h in 96-well plates, and then exposed to concentrations of pine oil ranging from 0.4 to 1.6 % (v/v). Subsequently, 10 µl of 5 mg MTT/ml stock in PBS was added to each well and incubated at 37°C for 4 h, after which the medium was removed and 200 µl of DMSO added to each well and mixed gently. The plate was then kept on a rocker shaker for 10 min at room temperature and the purple color developed was read at 550 nm by use of a multi-well micro plate reader (Multiskan Ex, Thermo Scientific, Finland). The solvent control (1.6% v/v of ethanol and methanol) was also run under identical conditions.

Flow cytometric analysis for the progression of cell cycle

Hep G2 cells treated with 1.6% of solvent control or cells treated with 0.4 to 1.6 % (v/v) of pine oil for 3h were harvested and centrifuged at 1000 rpm for 4 min. Pellets were resuspended in 500 µl of PBS. Cells were fixed with equal volume of chilled 70 % ice-cold ethanol, and incubated at 4 °C for 1 h. After two successive washes with PBS

at 1000 rpm for 4 min, cell pellets were resuspended in PBS and stained with 50 µg propiodium iodide (PI)/ml containing 0.1% Triton X-100 and 0.5 mg/ml RNAase A for 1 h at 30 °C in dark. Fluorescence of the PI was measured by flow cytometry by use of a Beckman Coulter flow cytometer (Coulter Epics XL/Xl-MCL, Miami, USA) through a FL-4 filter (585 nm) and 10,000 events were acquired [22,23]. Data were analyzed by Coulter Epics XL/XL-MCL, System II Software, Version 3.0. Cell debris was characterized by a low FSC/SSC was excluded from the analysis.

RESULTSAND DISCUSSION

UPLC/SM/ MS

The chromatographic profile of hydro-distilled *Pinus densiflora* oil data, plotted using logarithmic scale in order to illustrate the percentages of minor and major components, revealed that the major component monoterpenoids (70.9%) followed by Sesquiter penoids (23.2%), Hydrocarbons monoterpens were 60.7% while Alcohols monoterpens were 10.2%

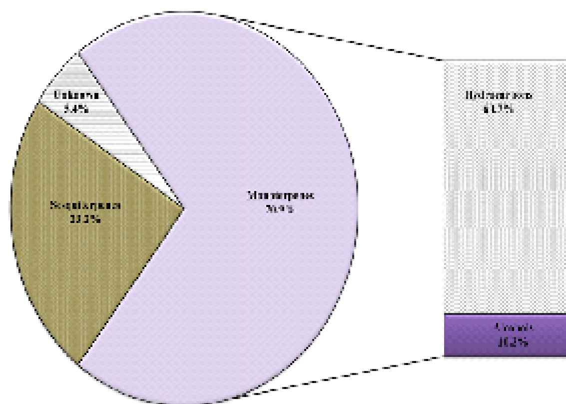

Fig. 1. Percentage of different terpene classes of Pine essential oil, obtained usingUPLC, isocratic combination of Methanol: Formic acid (80:20) were used as a mobile phase, and plotted using pie in order to illustrate the percentages of minor and major classes, showing that monoterpenes are the major constituent of pine oil followed by sesquiterpenes

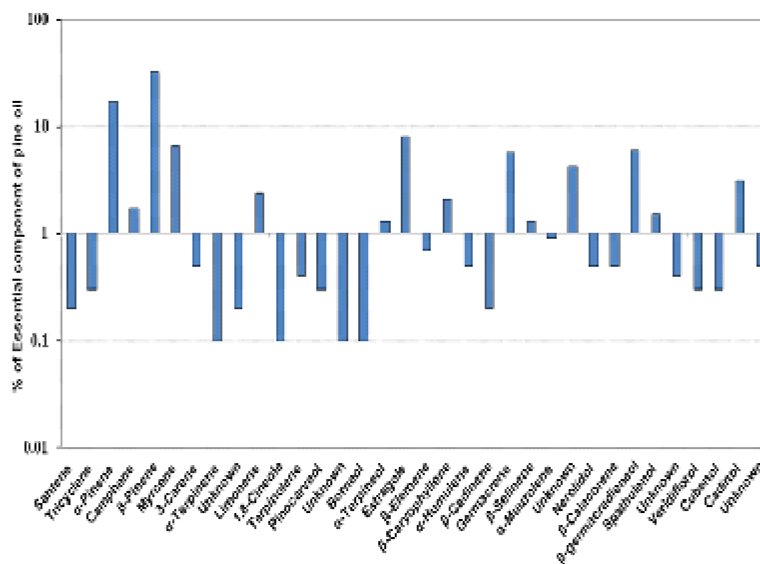

Fig. 2. Percentage of different component profile of Pine oil,obtained using UPLC, isocratic combination of Methanol: Formic acid (80:20) as a mobile phase, and plotted using logarithmic scale in order to illustrate the percentages of minor and major components

(Figure 1). More than 29 compounds were identified, the major constituents of this oil were β-pinine followed by α- pinine and myrecene (monoterpenoids hydrocarbons) 32%, 17%, 6.5% respectively (Fig. 2). Estragole (Alcohols monoterpens) were 8% (Fig. 2). Smaller amounts of other alcohols such as α-terpineol, 1, 8-Cineole, terpinolene were also detected (Figure 2). Other monoterpene hydrocarbons (Santene, Tricyclene, 3-Carene, α-Terpinene) were identified in traces. These data are in agreement with those obtained by[8] who reported that the one of the main compounds of pine oil was α-pinene with 73.2% of monoterpenes and 21.2% of sesquiterpenes. According to our data *Pinus densiflora* belongs to the first group which is includes the pine species who rich in monoterpene hydro carbons[3].

Microbiological study

The obtained data are listed in figures (3, 4, 5, and 6). The resulted data could be generalized as the inhibitory effect of pine essential oil increased with increasing its volume from 20 to 60 µl despite of the type of the tested microorganism. Also the different types of organisms showed variable responses towards pine oil. Most strains of fungi, gram positive, negative bacteria and yeast showed high sensitivity and inhibited to great extent at the highest concentration (60 µl). On the other hand some strains showed high resistance and survived at low concentration of the tested

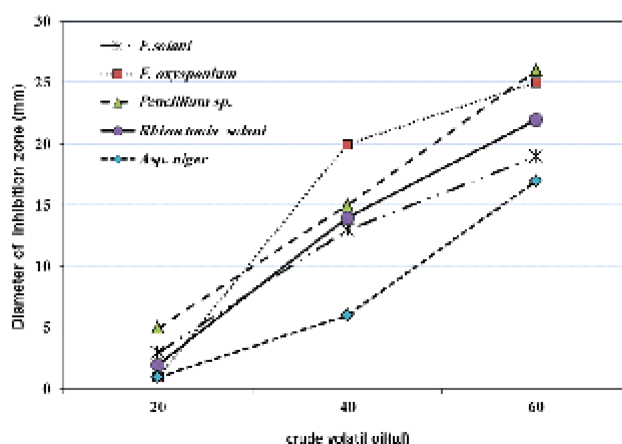

Fig. 3. Antifungal activity of Pine crude volatile oil, determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud pine oil, incubated for 5 days at 25 ± 2°C

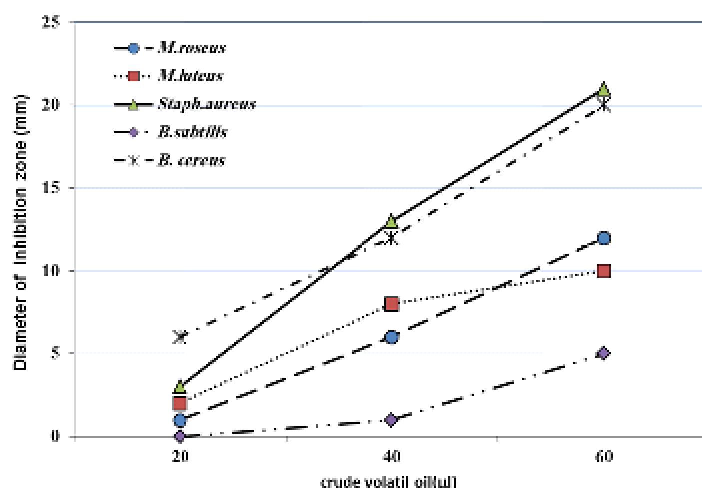

Fig. 4. Antimicrobial activity of Pine crude volatile oil on Gram positive bacteria,determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud pine oil, incubated for 5 days at 25 ± 2 °C

essential oil (20 µl). Dealing with antifungal activity: data in Figure (3) reveal the marked inhibitory effect pine oil especially at 60 µl against all tested strains. *pencillium sp*. And *F. oxysponum* showed the relatively highest sensitivity (26 mm, 25 mm respectively) in 60 µl treatment. The other remained types, *Rhizoctoniasolan, F.solani,* and *Asp. Niger*, were exhibited relative resistance, as their inhibition zone did not exceed than 22, 19 and 17 mm respectively. Regarding to G+ve, data revealed that both *Staph aureus and B. cereus* the highest sensitivity towards pine oil (21 mm, 20 mm respectively) in 60 µl treatment compared to the other tested strains (figure 4). Pine essential oil had low inhibitory effect against *B .subtilis* (G+ve), *E. coli* (G-ve) and *Candida ablicans* (yeast) as shown in (Figs. 4, 5, and 6). At close, the above-mentioned data clearly indicate the marked inhibitory effect of pine crude oil against several human and plant pathogens and nonpathogenic microorganisms. However our data are in good consonance with those reported by[24,25]. Considering with the mode of antibacterial action[12] explained that essential oils comprise large number of components and, in turn their mode of action involves several targets in bacterial cell. The hydrophobicity of essential oils enables them to partition in the lipids of cell membrane and mitochondria rending them permeable and leading to leakage of cell contents i.e. exert bactericidal or at least bacteriostatic properties.

Monoterpenes such as β-pinine, α-pinine, and myrecene are playing the most important role as antimicrobial agents[26,27]. Also, it may play a role in denaturing elicitor proteins produced by these pathogenic and nonpathogenic

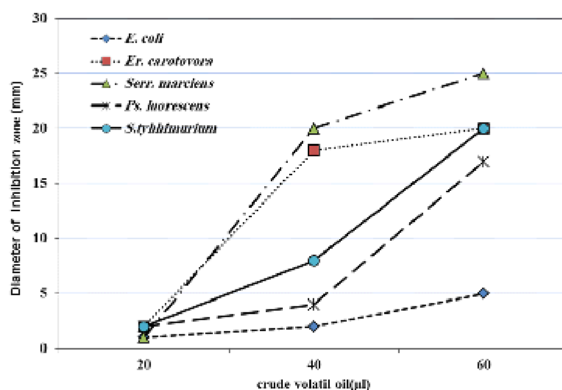

Fig. 5. Antimicrobial activity of Pine crude volatile oil on Gram -negative bacteria, determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud pine oil, incubated for 5 days at 25 ± 2 °C

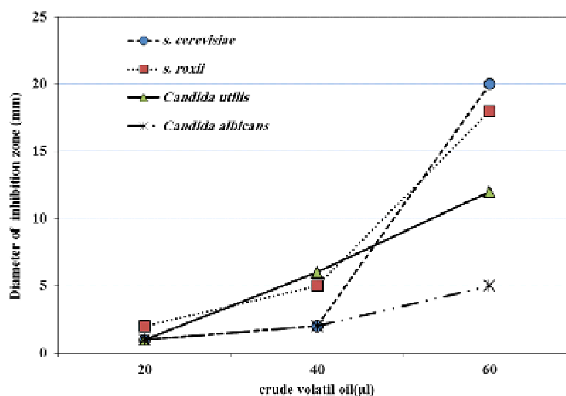

Fig. 6. Antimicrobial activity of Pine crude volatile oil on yeasts, determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud pine oil, incubated for 5 days at 25 ± 2 °C

microorganisms and preventing the cell death program induction in plant cells inducing the interior enzymes of phytoalexin synthesis in[28-30]. Minor classes of essential oils (alcohols, ketones, and esters) may have a synergistic effect with the major classes (Hydrocarbons and sesquiterpenes) of the oil as antibiotics against microbial diseases, their roles should be considered as well.

Pine oil induced cytotoxicity in Hep G2 cells

Exposure of Hep G2 cells to pin oil for 24 h resulted in significant cytotoxicity with a concentration dependent decrease in survival of cells. Pin oil has exhibited a strong cytotoxicity at all tested concentrations. The percent decline in cell survival was determined to be 74.6, 84.1, 82.6 and 85.1 % at 0.4, 0.8, 1.2 and 1.6 %, respectively (Fig. 7).

Pin oil induced apoptosis/necrosis in Hep G2cells

Cell cycle analysis of PI-stained pin oil treated cells did not indicated any change in the cell cycle progression.

Pine oil treatment was found extremely toxic, a significant increase in the proportions of apoptotic/necrotic Hep G2 cells in sub-G_1 phase

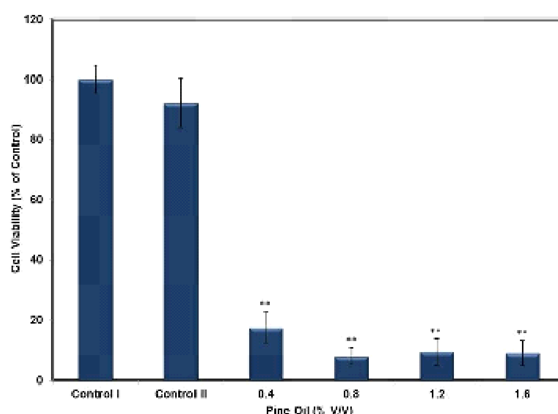

Fig. 7. Cytotoxicity assessment in Hep G2 cells showing the percent cell viability after 24 h of pine oil exposure using MTT assay, respectively. Each histogram represents the mean ± S.D. values obtained from three independent experiments. **p<0.01 vs control II. Control I: untreated control, control II: ethanol:methanol (1:1) as solvent control

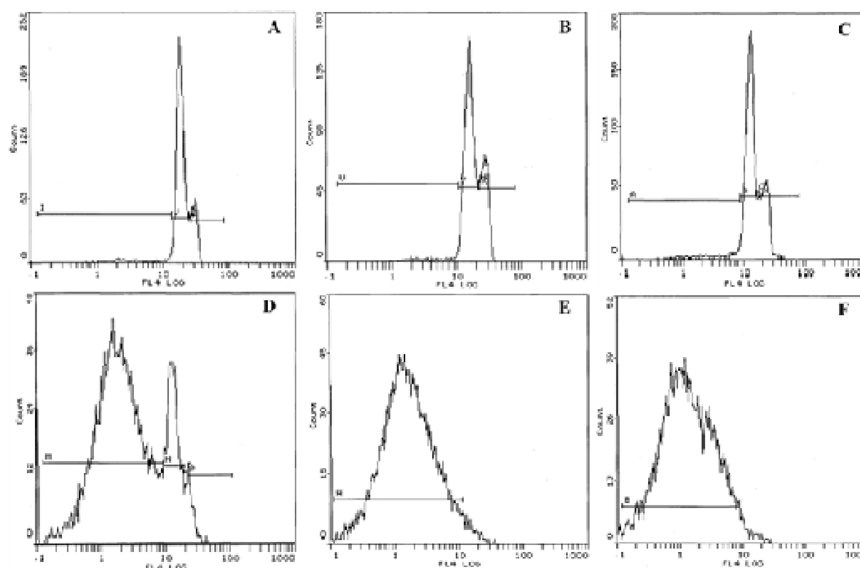

Fig. 8. Representative flow cytometric images exhibiting changes in the progression of normal cell cycle in Hep G2 cells after 3 h of pine oil treatment. Sub panels are represented as (A): untreated control; (B): solvent control (1.6%) ethanol:methanol (1:1) ratio; (C): 0.4%; (D): 0.8%; (E): 1.2% and (F): 1.6%

determined were 78.5 ± 1.8 %, 99.2 ± 0.4 % and 99.3 ± 0.6 % exposed to 0.8, 1.2 and 1.6 %, respectively. The proportion of apoptotic/necrotic cells in the solvent control population was 4.3 ± 0.2 %. The treatment concentrations of 1.2 and 1.6 % of pine oil have completely abolished the other phases of cell cycle (G1, S and G2M) (Fig. 8 and 9).

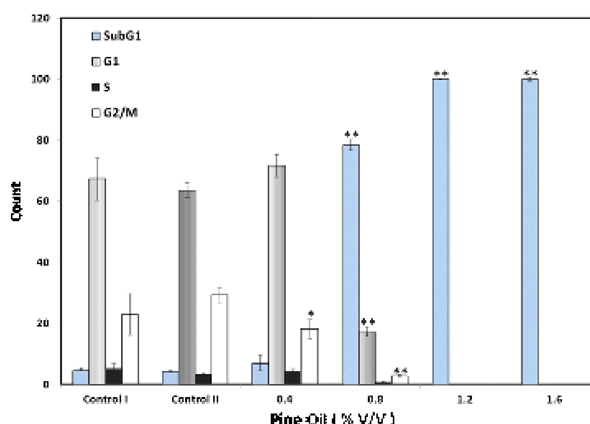

Fig. 9. Effect of pine oil on cell cycle progression in Hep G2 cells treated for 3 h. Each histogram represents mean ± SD values of different phases of cell cycle obtained from three independent experiments done in triplicate tubes. G1, S, G2/M represents the percentage of cells present in normal phases of cell cycle, SubG1 represents percentage of cells undergone apoptosis/necrosis.Representative flow cytometric image from single experiment exhibiting changes in the progression of normal cell cycle in Hep G2 cells after 3 h of compound A treatment. *p<0.05, **p<0.01 vs control II

CONCLUSION

The UPLC/SM/ MSanalysis showed that the major constituents of the oils were monoterpene hydrocarbons and phenolic flowed by sesquiterpenes. β- pinene and α- pinene were the major hydrocarbons constituents. The antimicrobial assay showed that crude volatile oil extracted from *Pinus densiflora* possess marked inhibitory effect against several human and plant pathogens and nonpathogenic microorganisms. Antimicrobial activity of *Pinus* essential oil is mainly related to their monoterpene hydrocarbons and phenolic contents. Exposure of Hep G2 cells to pin oil for 24 h resulted in significant cytotoxicity as a concentration dependent, decrease in survival of cells were obtained. A significant increase in the proportions of apoptotic/necrotic Hep G2 cells in sub-G1 phase. No change in cell cycle progression was indicated. Results expressed the possibility of using *Pinus* essential oils as antimicrobial agents. However, Pine oil treatment was found extremely toxic.

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