Nigella sativa Essential Oil Constituents and its Antimicrobial, Cytotoxic and Necrotic Replies

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***Nigella sativa* essential oil (EO) chemical constituents and its biological properties were studied in this work. EO was identified using UPLC MS/MS analysis. Antimicrobial activity of the EO was investigated against fungi, bacteria (G+ve, G-ve), and yeasts quantifying the inhibition zones. Tolerate ability of EO treated Hep G2 cells were assessed by MTT assay, flow cytometric analysis of cell cycle progression was conducted using 0.4, 0.8, 1.2 and 1.6 % (v/v) of *Nigella sativa* EOfor 3h. UPLC MS/MS analysis revealed that the EO major constituents were monoterpenes (hydrocarbons and phenolics) flowed by Phenylpropanoids and sesquiterpenes. p-Cymene followed by γ-Terpinene, α-Phellandrene, and α-Thujene were the major hydrocarbons constituents. Antimicrobial inhibitory effect of *Nigella sativa* EO increased at 20, 40, and 60 μl/ml for all tested microorganisms which may correlated with its monoterpene hydrocarbons and phenolic contents. Exposure of Hep G2 cells to *Nigella sativa* EOfor 24 h caused a substantial cytotoxicity as a concentration dependent, while survival of cells were lessened. 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 conveyed the ability of using *Nigella sativa* EO as antimicrobial agents.**

Key words: *Nigella sativa*, Essential oil, Biological activity.

Homeopathic plants are used in herbal medicines regarding to its safetyin comparison torecent allopathic medications[1].Volatile oils are largely used in various fields: perfumery, cosmetics, pharmaceuticals, synthesis. Historically essential oils were used as a health promotion [2,3,4]. *Nigella sativa* (black cumin) is a commonly used as a homeopathic plant;as its seeds are extensively used in curing of altered illnesses and disorders[1,5,6]. Wide studies; have been carried out on *Nigella sativa* (*N. sativa*) for its biological activities and healing prospective; revealed that it retain inclusive scale of actions as antidiuretic, antihypertensive, anti-diabetic, anticancer,immune modulatory, analgesic, antimicrobial, anthelmintic, analgesics, anti-inflammatory, spasmolytic, bronchodilator, gastro protective, hepatoprotective, renal protective,and antioxidant properties[6,7]. Seeds of *N. sativa* (black seeds) are commonly used in curing of various illnessessuch as bronchitis, asthma, diarrhea, rheumatism, skin ailments, appetite stimulant, and in stimulating milk production in nursing mothers[7].

Numerous active composites are insulated, identified from different diversities of *N. sativa*[8]. Former studies revealed that monoterpenes (p-cymene, α-thujene, γ-terpinene, carvacrol, α-pinene and β-pinene)are the central constituents of the black cumin essential oil[8]. Furthermore, *N. sativa* seeds contain some other compounds such ascarvone, limonene, citronellolin trace quantities[9,10,11]. The antimicrobial activities of many

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essential oils over a wide range of microorganisms has been studied extensively both in vitro and applied to foods [12,13].Antimicrobial activities of essential oils (EO) are correlated with their chemical composition, mainly the aliphatic and phenolic constituents[14]. Dissimilar crude extracts of *N. sativa* EO were verified for antimicrobial efficiency contrary to different bacterial segregates (gram negative and gram positive) and exhibited an encouraging consequence[15,16]. The essential oil of black cumin hasa solid antifungal consequence against diverse strains of *Candida albicans*[16]. *Nigella Sativa* EO have antibacterial and antifungal activities against *Staphylococcus Aerus*, *Proteus Vulgaris*, *Escherichia Coli*, *Candida Albicans*[17,18,19]. EO of *N. sativa* seeds retains antimicrobial activity counter to numerous medication resistant pathogenic bacteria[20]. Toxicological studies that were carried out on *N. sativa* seeds showed that no toxic effects for *N. sativa* fixed oil when given orally to experimental animals[21, 22].However, no much studies conducted on its essential oil.In this work we investigate the constituents, antimicrobial activities, cytotoxicity and necrotic retorts of *Nigella sativa* essential oil (EO).

MATERIALS AND METHODS

Extraction of essential oil

The air-dried and grained*Nigella sativa* plant was extracted using water-distillation for 5 hours using a Clevenger apparatus. The obtained EO was dried via anhydrous sodium sulfate then stored at 4°C before analyzed.

UPLC MS/MS Analysis of volatile oil

Chemical constituents for the extracted *Nigella sativa* EO 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

Nigella sativa EO extract was tested for its antimicrobial activities against 16 microorganisms including 4 Gram positive (*Micrococcus roseus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtils*), 4 Gram-negative bacteria (*Escherichia coli*, *Erwinia carotovora*, *Serratia marcines*, *Pseudomonas fluoresceus*), 4 fungi (*Fusarium solani*, *Fusarium oxysporum*, *Penicillium sp.*, *Rhizoctonia solani*), 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[23] using Nunc® petri dishes (diam. × H 150 mm × 25 mm).Due to the immiscibility of *Nigella sativa* EO with water;emulsion was prepared using 0.2% agar solution[24,25]. 800, 1600, and 2400 µL of EO were added to 3200, 2400 and 1600 µL of 0.2% agar solution, respectively. A total volume (4mL) of each dilution was added to 36 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) revealing growth withouta contact inhibition was grown in DMEM, complemented 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. Viability of cell was gaged by optical microscopic examination for exclusion of trypan blue. Batches of Hep G2 cells revealing fewer than 95% cell viability were omitted.

Tetrazolium Bromide (MTT) Assay

Viability of *Nigella sativa* EO Hep G2 treated cells was gaged using the MTT assay according to[26,27].Cells (1 x 10^4) were endorsed for 24 h in 96-well platesto adhere, exposed to *Nigella sativa* EO concentrations from 0.4 to 1.6 % (v/v). Subsequently, 10 µl from 5 mg MTT/ml stock in PBS were added to each well, and incubated at 37°C for 4 h, then the medium was removed and 200 µl of DMSO were added to each well and mixed gently. The plates were shacked for 10 min at room temperature until the purple color was developed .The obtained color was read at 550 nm using a multi-well microplate 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 as a control. However,treated cells with 0.4 to 1.6 % (v/v) of *Nigella sativa* EO for 3h were collected and centrifuged (1000 rpm for 4 min). Pellets were re-suspended in 500 µl of PBS, fixed with identicalamount of 70 % chilled ethanol (ice- cold), and then incubated at 4 °C for 1 h. After two consecutive washes with PBS (centrifuged 1000 rpm for 4 min), pellets were re-suspended in PBS and stained using 50µg/mlPI (propiodium iodide) 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 determined via flow cytometry using Beckman Coulter Flow Cytometer (Coulter Epics, USA) using a FL-4 filter (585 nm) and 10,000 events were acquired[28,29]. Data were examined using Coulter Epics XL/XL-MCL, System II Software (Version 3.0).

RESULTS AND DISCUSSION

UPLC/SM/ MS

The chromatographic profile of hydro-distilled *Nigella sativa* EO data, plotted using logarithmic scale in order to illustrate the percentages of minor and major components, revealed that the major component was monoterpenoids (93%) followed by Phenyl propanoids (4%), Hydrocarbons monoterpenes were 77% while Alcohols monoterpenes were 16% (Figure 1).The major constituents of *Nigella sativa* EO were p-Cymene followed by γ-Terpinene,α-Phellandrene, and α-Thujene (All monoterpenoids hydrocarbons) 45.31 %, 8.6 %,7.61 %, 5.14% respectively (Figure 2). However, trans-4-Methoxythujane, Carvacrol(Alcohols monoterpens) were4.47 %, 3.83%respectively (Figure 2). Phenyl propanoids such as Myristicin (2.46%), and Apiol (1.29 %) in addition to smaller amounts of othermonoterpenes and sesquiterpenes were also detected (Figure 2). Thymol (Alcohols monoterpens) and β-Bisabolene (Sesquiterpenes) (Figure 2).These data are in agreement with those obtained byAnna*et al.*[8]who reported thatthat monoterpenes(p-cymene, α-thujene, γ-terpinene, carvacrol, α-pinene and β-pinene) are the central constituents of the *N. sativa* EO followed by Phenyl propanoids and sesquiterpenes.Moreover, *N. sativa* EO contain some other compounds such as carvone, limonene, citronellol in trace quantities[9,10,11].

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 *Nigella sativa* EO increased with increasing its concentration from 20 up to 60µl/ml despite of the type of the tested microorganism. Also the different types of

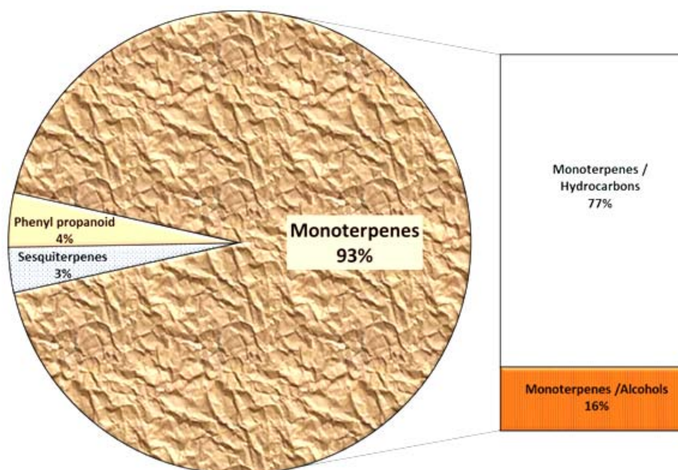

Fig. 1. Percentage of various terpene classes of *Nigella sativa*, obtained usingUPLC, isocratic combination of Methanol: Formic acid (80:20) were used as a mobile phase, and plotted using pie in order to illustrate the percentages of minor and major classes, showing that monoterpenes are the major constituent of *Nigella sativa* essentially hydrocarbons.

organisms showed variable responses towards *N. sativa* EO. Most strains of fungi, gram positive, negative bacteria and yeast showed high sensitivity and inhibited to great extent at the highest concentration (60µl/ml). Dealing with antifungal activity: data in Figure (3) reveal the marked inhibitory effect *N. sativa* EO especially at 60µl/ml against all tested strains. *pencillium sp.* showed the relatively highest sensitivity in all EO treatments but *F. oxysponum* exhibited relatively

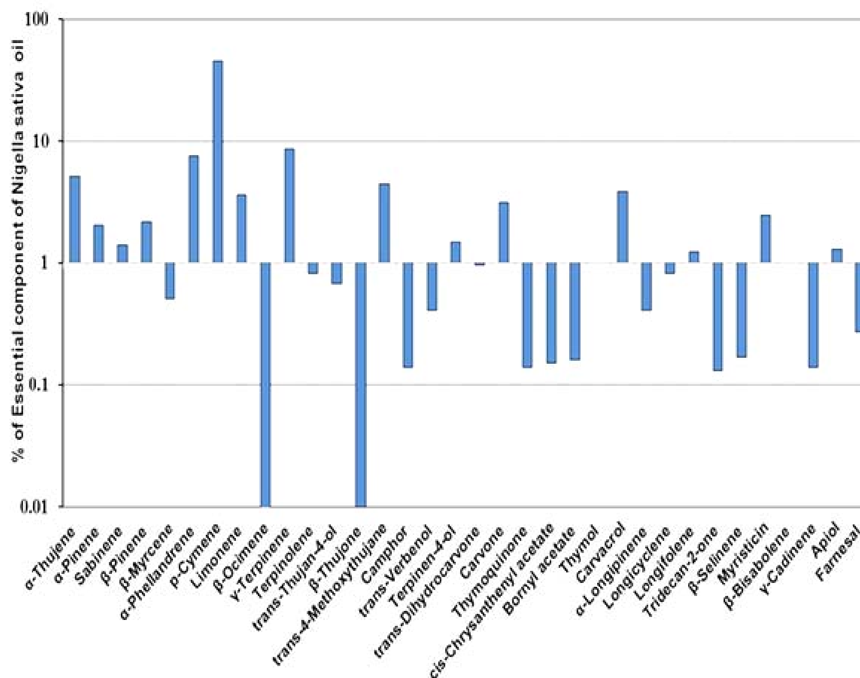

Fig. 2. Percentage of different component profile of *Nigella sativa*,obtained using UPLC, isocratic combination of Methanol: Formic acid (80:20) as a mobile phase, and plotted using logarithmic scale in order to illustrate the percentages of minor and major components.

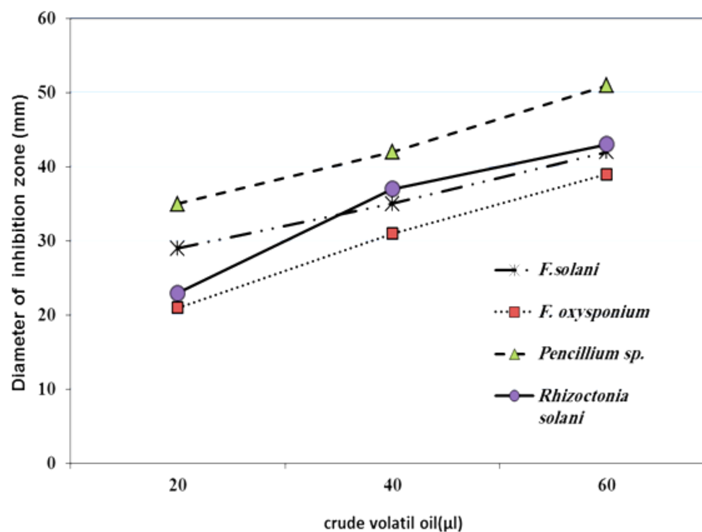

Fig. 3. Antifungal activity of *Nigella sativa* crude volatile oil, determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud volatile oil, incubated for 5 days at 25 ± 2 °C.

low sensitivity in comparison to the remained two types (*Rhizoctonia solan*). Regarding to G+ve, data revealed *Staph.aureus* has highest sensitivity towards all *N. sativa* EO treatments compared to the other tested strains. However, *M. roseus* showed relatively more resistance compared with the other types (figure 4). *N. sativa* EO hasa relatively low inhibitory effect against *Serratia marcines*(G-ve)and high inhibitory effect against *E. coli* (G-ve) (figures 5). *Saccharomyces cerevisiae* has highest sensitivity at 60µl/ml mean while *Saccharomyces roxii* was more sensitive at both 20 and 60µl/ml treatments compared with the other yeasts(figure 6). At close, the above-mentioned data clearly indicate the marked inhibitory effect of *N. sativa* EO against several human and plant pathogens and nonpathogenic microorganisms. However our data are in good

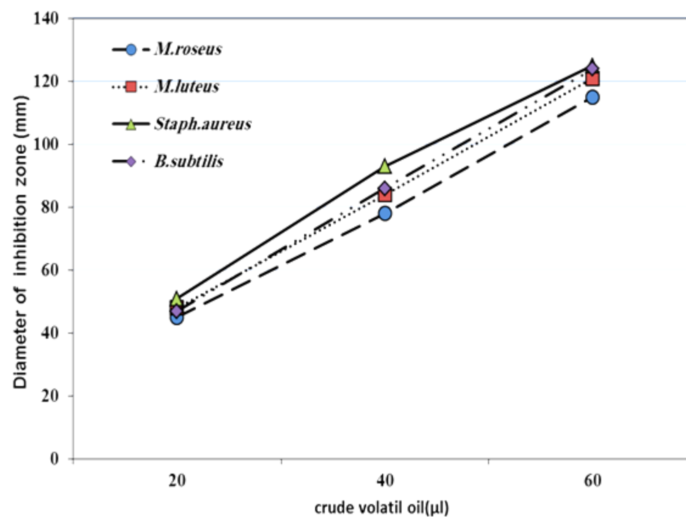

Fig. 4. Antimicrobial activity of *Nigella sativa* crude volatile oil on Gram positive bacteria,determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud volatile oil, incubated for 5 days at 25 ± 2 °C.

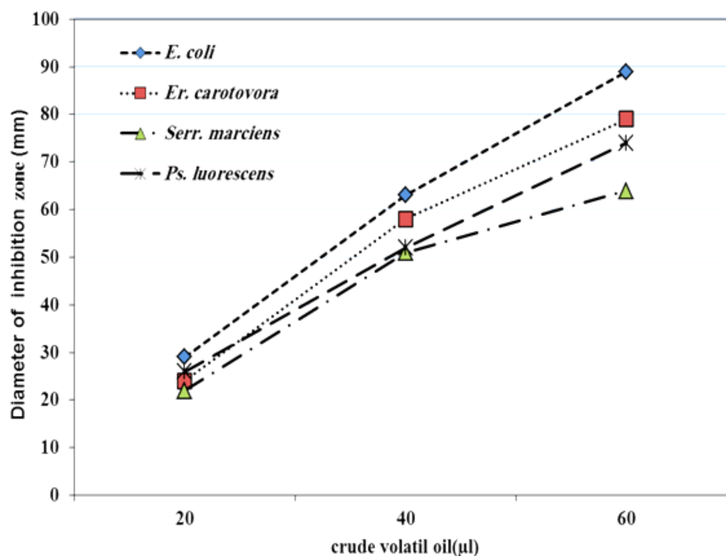

Fig. 5. Antimicrobial activity of *Nigella sativa* crude volatile oil on Gram -negative bacteria, determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud volatile oil, incubated for 5 days at 25 ± 2 °C.

consonance with those reported by[15,16,17,18,19,20]. Considering with the mode of antibacterial action[30] explained that essential oils comprise large number of components and their action involves numerous targets in bacterial cell. EO hydrophobicity enables them to probing in cell membrane and mitochondria lipids and rending them permeable and leakage of cell contents is occurs i.e. exert bactericidal or at least bacteriostatic properties.Monoterpenesare playing the most important role as anti-microbial agents[31, 32]. Minor classes of EO (Phenyl propanoids, ketones, and esters) may have a

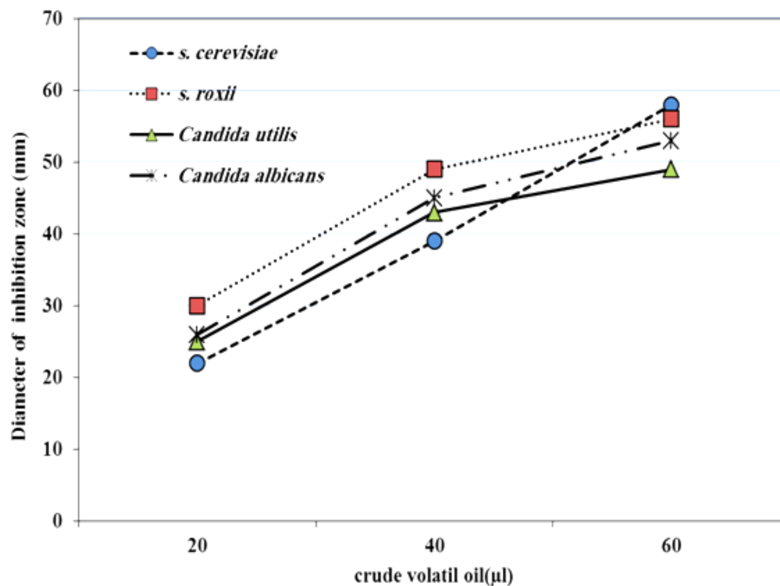

Fig. 6. Antimicrobial activity of *Nigella sativa* crude volatile oil on yeasts, determined by measuring the inhibition zones (mm) using agar diffusion method, using 20, 40, and 60µL/mL as a final concentration of crud volatile oil, incubated for 5 days at 25 ± 2 °C.

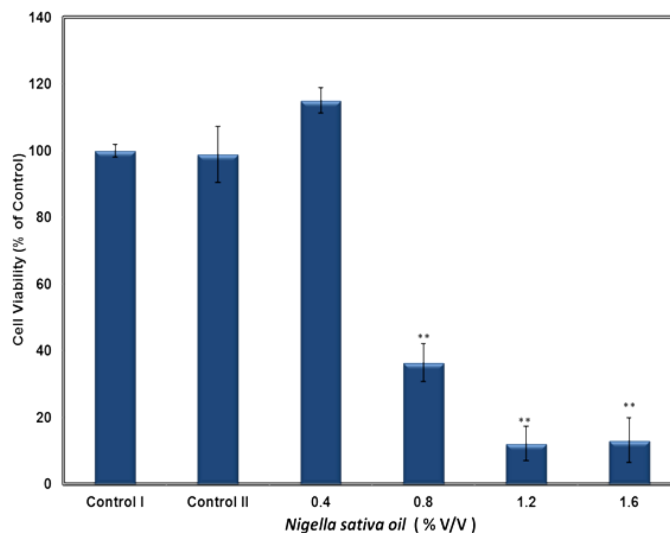

Fig. 7. Cytotoxicity assessment in Hep G2 cells showing the percent cell viability after 24 h of *Nigella sativa* oil exposure using MTT assay, respectively. Each histogram represents the mean ± S.D. values obtained from three independent experiments. **$p<0.01$ vs control II. Control I: untreated control,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

synergistic effect with the major classes (Hydrocarbons and alcohols) of the oil as antibiotics against microbial diseases, their roles should be considered as well.

Cytotoxicity induction in Hep G2 cells via *Nigella sativa* EO

Disclosure of Hep G2 cells to *Nigella sativa* EO for 24 h resulted ina significant cytotoxicity as a concentration dependent. *Nigella sativa* EO has exhibited a strong cytotoxicity at all tested concentrations. Compared to the solvent control, a significant decline in cell survival were determined to be 62.4, 86.7 and 85.7 % at the concentrations 0.8, 1.2 and 1.6 %, respectively (Fig. 7).

***Nigella sativa* EO induced apoptosis/necrosis in Hep G2 cells**

Cell cycle examination of PI-stained revealed that an increase in sub-G1 peak with concomitant reduction in G1 and G2/M phases was observed with *Nigella sativa* essential oil treatment. A significant increase in the proportions of apoptotic/necrotic Hep G2 cells in sub-G1 phase determined were 24.5 ± 7.3 %, 44.6 ± 2.2 % and 60.0 ± 4.5 % exposed to 0.8, 1.2 and 1.6 % of *Nigella sativa* essential oil essential oil, respectively. The proportion of apoptotic/necrotic cells in the solvent control population was 4.5 ± 0.5 %. Significantly low percent of cells appeared in the G1 and G2/M peaks in Hep G2 cells exposed to 1.6 % *Nigella sativa* essential oil (33.1 ± 4.0% and 4.8 ± 1.2) as compared to 67.4 ± 0.4 % and 23.9 ± 1.0 %, respectively in solvent control cells. (Fig. 8 and 9).

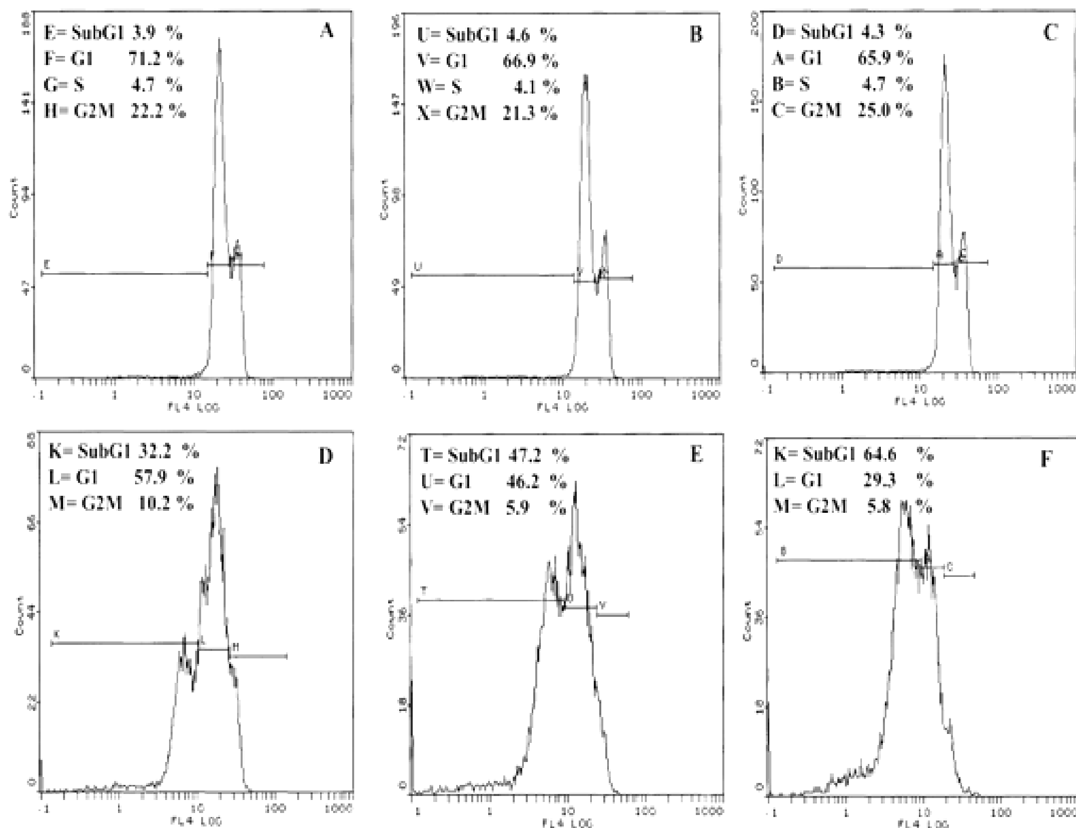

Fig. 8. Representative flow cytometric images exhibiting changes in the progression of normal cell cycle in Hep G2 cells after 3 h of *Nigella sativa* oil treatment. Sub panels are represented as (A): untreated control; (B): solvent control (1.6%) ethanol:methanol (1:1) ratio; (C): 0.4%; (D): 0.8%; (E):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

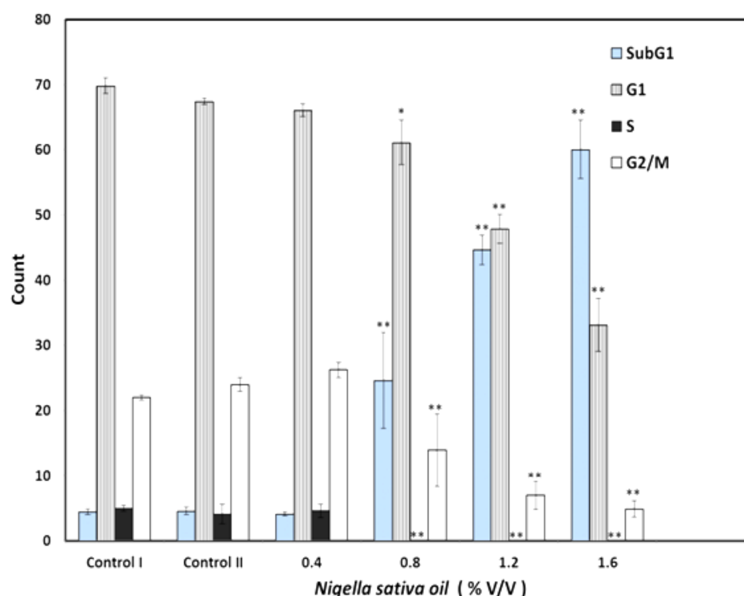

Fig. 9. Effect of *Nigella sativa* oil on cell cycle progression in Hep G2 cells treated for 3 h. Each histogram represents mean ± SD values of different phases of cell cycle obtained from three independent experiments done in triplicate tubes. G1, S, G2/M represents the percentage of cells present in 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

UPLC/SM/ MS data displayed that the major constituents of the *Nigella sativa* EO were monoterpenoids (meanly hydrocarbons and alcohols) followed by Phenyl propanoids. @-Cymene followed by γ-Terpinene, α-Phellandrene, and α-Thujenewere the major hydrocarbons constituents.The antimicrobial assay showed that crude volatile oil extracted from *Nigella sativa* possess marked inhibitory effect against several human and plant pathogens and nonpathogenic microorganisms. Antimicrobial activity of *Nigella sativa* essential oil is mainly related to their monoterpenes hydrocarbons and phenolic contents. Treatment of Hep G2 cells to *Nigella sativa* essential oil for 24 h caused a significant cytotoxicity as a concentration dependent, and a decrease in survival of cells were obtained. Cell cycle examination of PI-stained revealed that an increase in sub-G1 peak with concomitant reduction in G1 and G2/M phases.A significant increase in the proportions of apoptotic/necrotic Hep G2 cells in sub-G1 phase.Results expressed the capability of using *Nigella sativa* essential oils as antimicrobial product.

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