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In vitro cytotoxic activities of selected Saudi medicinal plants against human malignant melanoma cells (A375) and the isolation of their active principles

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Abstract

Introduction: natural products are known to be a continuous source of potential anti-cancer agents due to their chemical and biological diversity. This study aimed to evaluate the *in vitro* cytotoxic properties of medicinal plants and their mechanisms of action in human malignant melanoma cells. Methodology: The study investigated the effect of the cytotoxic extracts on cell cycle, caspase-3/7, apoptosis induction using Annexin V-FITC/PI staining, morphological changes and lactate dehydrogenase activity and 2D cell migration studies. Results: There were 9 extracts considered to be promising (GI_{50} < 30µg/mL); *Haplophyllum tuberculatum* (0.45µg/mL), *Plicosepalus curviflorus* (4µg/mL), *Capparis decidua* (10µg/mL), *Acacia nilotica* (11µg/mL), *Aizoon canariense* (14µg/mL), *Carissa edulis* (15µg/mL), *Pulicaria schimperi* (19µg/mL) *Cyperus rotundus* (20µg/mL), *Osteospermum vaillantii* (21µg/mL). Cell cycle arrest at S phase was detected in cells treated with *C. decidua*, *C. edulis, H. tuberculatum, P. curviflorus* and *P. schimperi.* Cellular exposure *to A. canariense* resulted in G2/M arrest whereas *A. nilotica*, *C. rotundus* and *O. vaillantii* elevated the sub-G1 population. Caspase-3/7 was activated by *C. decidua, C. edulis, C. rotundus, H. tuberculatum, P. schimperi* and *O. vaillantii*. Most of the cytotoxic effects were accompanied by externalization of phosphatidylserine and morphological abnormalities like cell shrinkage and chromatin condensation. Lupeol was isolated from *C. decidua*, justicidin A, B, tuberculatin and tuberculatin acetate from *H. tuberculatum* and ursolic acid and its acetate from *C. edulis* as the anti-melanoma principles. Conclusion: The bio-guided isolation of plants extracts led to the identifications of anti-melanoma constituents belonging to different classes including lignans, lignan glycosides, triterpenes and flavonoids.

Keywords: melanoma; cancer; natural products; extracts; apoptosis; caspase; cell cycle.

1. INTRODUCTION

Skin cancer is classified into non-melanoma skin cancer (NMSC) and melanoma. Although NMSC is the most common form, melanoma is the most aggressive and is also the major cause of mortality [1, 2]. The death cases caused by malignant melanoma are higher than other cancer types and it represents currently the 5th most common cancer among men and women in the USA [1, 3]. Also, its incidence has increased by 88% in men and 66% in women by 2020 in the UK [4].

The discovery of anti-cancer agents from nature is one of the most promising strategy to defeat certain types of tumors, it requires continuous screening of natural sources including terrestrial plants seaweeds and marine organisms [5-7] . The flora of Saudi Arabia is characterized by high biodiversity due to the climatic and geographic variations. In the last decade it was subjected to extensive screenings aimed to discover anti-cancer agents elucidating the precise mechanism of action including as apoptosis inducer, cell cycle arrest and anti-migratory effect [8].

1.1 Phytochemistry, traditional uses and reported biological activities of the selected plants

In this study, we have selected seven wild Saudi medicinal plants and two from commercial sources. These plants were chosen because they are common and abundant, either in nature or commercially, as this facilitates the isolation of sufficient quantities of potentially bioactive compounds. The literature review below summarizes briefly the traditional uses and biological activities of these plants as well as listing the phytochemicals that have been isolated from each one.

1.1.1. Acacia nilotica (Fabaceae family)

This plant contains phenols (gallic acid), terpenes (niloticane), alkaloids, flavonoids (kaempferol), tannins, saponins, acetyleugenol and coumarin (umbelliferone) [9, 10]. It has been traditionally used for bacterial, fungal, viral and amebic infections, vitiligo, congestion, hypertension, haemorrhoids, menstrual problems, and cancers (mouth, bone and skin) [11]. Biological and pharmacological studies have also revealed the plant's anti-viral, anti-tuberculosis, anti–inflammatory, anti-platelet aggregating and anti-proliferative (breast, lung and uveal melanoma cancer cells) properties. It is rich in gallic acid, digallic acid, methyl gallate, catechin, catechin 5-O-gallate and gallocatechin 5-O-gallate [12-14].

1.1.2. Aizoon canariense (Aizoaceae family)

This plant is used traditionally for jaundice, hepatitis, wound healing and cancer [15, 16]. Various properties have been documented, such as anti-microbial, antioxidant, and anticholinesterase activity, and it has been found to be cytotoxic against breast and colon cancer cells [17, 18]. The cytotoxic activity has been attributed to its adenine-based alkaloid content, which exists in addition to other chemical classes such as coumarins, saponins, tannins, flavonoids, steroids, adenosine derivatives, triterpenes and fatty acids like protocatechuic acid [17, 19].

1.1.3. Capparis decidua (Capparidaceae family)

C. decidua contains a number of alkaloids (capparisinine), phenols (phydroxybenzoic acid), flavonoids (isorhamnetin) sterols, terpenes, glycosides and capparisesterpenolide [20, 21]. Those classes are linked to various biological activities, such as CNS depressant, anti-convulsant, hypolipidemic, anti-microbial, anti-hypertensive, anti-diabetic, analgesic, anti-platelet, hepatoprotective, and antioxidant activity. It is also cytotoxic against prostate cancer cells due to the presence of stachydrine [20-22]. Some of these activities are known in traditional herbal medicine, where the plant is used as a remedy for rheumatoid, kidney, liver and cardiac problems, cancer, asthma, dysentery, cholera, and intermittent fever [20].

1.1.4. Carissa edulis (Apocynaceae family)

This plant is reported to be widely used in folk medicine for tumours, epilepsy, malaria, pyrosis with heartburn, arthritis, fever, hernia and headache [23, 24]. Terpenes (carissone), phenols (chlorogenic acid), lignans (nortrachelogenin), coumarins (scopoletin) and flavonoids are the most reported chemical classes in *C. edulis* [25]. Those compounds have different medicinal properties, showing analgesic, hypoglycemic, antiplasmodial, hepatoprotective and anti-hyperlipidemic activity. They have also demonstrated cytotoxic activity on leukaemia, brain, lung and breast cancer cells due to the presence of apiosylated phenolics, coumarin-secoiridoid and lignans, among them bisdesmoside and monodesmoside [25-27].

1.1.5. Cyperus rotundus (Cyperaceae family)

This plant is used in traditional treatments for cancer, gastrointestinal disorders, food poisoning, irritation of bowel, fevers, wounds, malaria, cough, bronchitis, deficient lactation, loss of memory, dysuria, infertility and menstrual disorders. It is known for its diverse chemical profile, containing sesquiterpenes (patchoulane, rotundane and caryophyllene types), quinones, flavonoids, saponins, alkaloids, coumarins, essential oils and steroids. A wide range of pharmacological activities are reported such as anti-platelet aggregation, anti-inflammatory, antioxidant, anti-diabetic, anti-microbial, anti-allergic, neuroprotective, analgesic, and anti-convulsant activity; it is cytotoxic against leukaemia and ovarian cancer cells, where isolated sesquiterpene 6-acetoxy cyperene induced caspase-dependent apoptosis [28, 29].

1.1.6. Haplophyllum tuberculatum (Rutaceae family)

Chemical studies show that this plant contain alkaloids (tuberine), lignans (justicidin A), essential oil (β-caryophyllene), and flavonoids [30, 31]. This diverse chemical profile is linked to various medicinal uses: it is anti-infective against bacteria, fungi, *Plasmodium* and leishmania, also shows acaricidal and molluscicidal properties, and is cytotoxic to leukemia, multiple myeloma and bladder cell lines [31-33]. It is used in traditional medicine as a remedy for cancer, malaria, rheumatoid arthritis, headache, gynecological problems, and as a treatment for the nervous system and infertility [31].

1.1.7. Osteospermum vaillantii (Asteraceae family)

This plant belongs to the family Asteraceae, which is potentially a strong source of anticancer agents [5]. Its traditional uses are listed as a remedy for fever, stomach and liver disorders. Chemical compounds that have been isolated from this plant are triterpene and saponin glycosides such as osteosaponin 1 and 2 [34, 35]. The methanol extract of *O. vaillantii* has shown a promising activity against the ovarian cancer cell line A2780 [36].

1.1.8. Plicosepalus curviflorus (Loranthaceae family)

Various phytochemical studies of crude extracts of the plant showed the presence of flavonoids (plicosepalin A and curviflorin), flavane gallates, sterols, and terpenes like lupeol [37, 38]. Its value in folk medicine is documented for cancer, tonsillitis and otitis media. It shows promising antioxidant, antimicrobial, anti-diabetic, and antihepatotoxic activity, and has been shown to be cytotoxic against five cancer cell lines MCF-7, HepG-2, HCT-116, Hep-2, HeLa due to pentahydroxyflavane-5-O-gallate [39, 40].

1.1.9. Pulicaria schimperi (Asteraceae family)

Polysaccharides, polyphenols, and flavonoids such as chlorogenic acid are among the major classes found in this plant [41]. It is not reported in the literature as a remedy for cancer, but other species of the genus Pulicaria, such as *Pulicaria undulata*, have been shown to be a good source of anticancer agents due to the presence of sesquiterpene lactones [42].

None of the selected plants have been tested against melanoma cell lines, with exception of *A. nilotica*, which was tested against uveal melanoma [14]. Since the plants are commonly found and easily obtainable, we can isolate bioactive compounds with potential anti-melanoma activity on an industrial scale in future research. The objective of this study is to screen medicinal plants to detect potential anti-melanoma activity, therefore we have chosen plants that are used in cancer treatments in traditional herbal medicine, as well as those which have already shown potential as anticancer agents against different carcinogenic targets.

2. MATERIALS AND METHODS

2.1. Chemical

Sulforhodamine B (SRB, 95%), trichloroacetic acid (99%), Trizma base (99.9%), propidium iodide (94%) and Ribonuclease A (RNase) (≥70 Kunitz units/mg protein) were purshased from Sigma Aldrich (St Louis, MO, USA). TrypLE Express (1X), phosphate-buffered saline (PBS, pH 7.4) and trypan blue (0.4%) were purchased from Thermo Fisher Scientific Inc., (Waltham, MA, USA)

2.2. Plant materials

As reported in Table 1. six plants were collected from Makkah Al-Mukkaramah region, Saudi Arabia, and one plant from Riyadh region, Saudi Arabia, both at the flowering stage and two plants were obtained from commercial sources (Table 1). Plants were identified by Prof. Ammar Bader, Pharmacy college, Umm Al-Qura University, Makkah, with exception of *H. tuberculatum* which was supplied and identified by Prof. Adnan Al-Rehaily, Pharmacy College, King Saud University, Riyadh, thereafter the voucher specimens were deposited in Pharmacognosy department at Umm Al-Qura University.

2.3. Preparation of plant extracts

The air-dried materials of different parts of the plants were ground to powder and extracted with different solvents; hexane (95%), chloroform (99.2%) and methanol (99.8%). The three extracts were filtered and the crude materials were obtained after evaporating the solvents to dryness under reduced pressure at 40°C [43]. A stock solution was prepared in DMSO (100%) at a concentration of 50mg/mL and lastly filter sterilized using a 0.2 μm filter before testing on the cell line.

2.4. Bioguided isolation

2.4.1. *Haplophyllum tuberculatum*

The chloroform extract was subjected to vacuum liquid chromatography (VLC) using silica gel eluted with hexane and an increasing polar gradient of 10% increments of ethyl acetate, to afford 11 fractions. Fraction 8, eluted with 30% hexane, was purified by prep-then layer chromatography (TLC) normal silica developed using 60% hexane: 40% ethyl acetate to afford two active compounds, justicidin A (6.90 mg) and justicidin B (7.30 mg). Methanol extract of the same plant was also fractionated using hydrophilic interaction chromatography (HILLIC) column on normal silica gel and eluted with 95% acetonitrile: 5% H2O, 2 drops of acetic acid; 90% acetonitrile: 10% H2O, 2 drops of acetic acid; and 80% acetonitrile: 20% H2O, 2 drops of acetic acid. Fraction 2 was subjected to reverse phase prep-TLC developed by 50% acetonitrile: 50% H2O, yielding 2 compounds tuberculatin (23.05 mg) and tuberculatin acetate (13.90 mg).

2.4.2. *Capparis decidua*

Chloroform extract of *C. decidua* underwent VLC using silica gel as the separation method with 100% hexane up to 100% ethyl acetate yielding 14 fractions. Fraction 4, eluted with 70% hexane, was one of the most active fractions and purified by prep-TLC normal silica developed with 80% hexane: 20% ethyl acetate, yielding 1 active compound (lupeol, 27.91 mg).

2.4.3. *Carissa edulis*

Hexane extract was subjected to VLC silica gel eluting with a combination of hexane and ethyl acetate affording 11 fractions. Fraction 9, eluted with 80% hexane, underwent column using normal

silica eluted with the isocratic solvent system of 80% hexane: 20% ethyl acetate, to yield two active compounds (ursolic acid, 22.30 mg and ursolic acid acetate 16.27 mg). Chloroform extract was separated using SPE in normal phase mode, eluted with hexane and 10% increment ethyl acetate and washed with methanol (30%) to obtain 16 fractions. Fraction 15, eluted with 25% methanol, was separated by Sephadex column eluted with methanol, giving quercetin (3.47 mg).

2.5. Cell cultures

A375 and B16-F10 cells were used in this work to evaluate the cytotoxicity and antimigratory properties of the extracts, respectively. They were purchased from Sigma and were obtained from the American Type Culture Collection (ATCC number: CRL-1619™). Both cell lines were maintained in Dulbecco's Modified Eagle Media (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) and supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin antibiotic, consisting of 10,000 units of penicillin and 10,000 µg of streptomycin (Gibco) per mL.

2.6. Sulforhodamine B (SRB) assay

This assay determined the ability of the extract to inhibit cellular growth by measuring the cell density, thereby estimate cell number. This assay was performed according to a previously described method [44]. A375 cells were seeded at density of 10,000 cells/well in a 96-well plate (Thermo Scientific) and left overnight to attach at 37°C. Afterwards, cells were treated with a serial dilution of the plant extracts (200, 100, 50, 25, 12.5, 6.25 µg/mL) at several time points. Upon the completion of the incubation period, the cells were fixed with trichloroacetic acid solution for one hour at 4°C. After washing with water, cellular protein was stained with SRB solution and left at room temperature for one hour. followed by washing the plate four times with 1% acetic acid and flicked to remove unbound dye. Then, Tris base buffer solution was added to each well and the absorbance was measured at 510 nm. Cell growth was calculated using the following equation:

$$\%Cell\ growth = \frac{Absorbance\ (sample) - Absorbance\ (blank)}{Absorbance\ (vehicle\ control) - Absorbance\ (blank)} \times 100$$

2.7. Alamar Blue (AB) assay

AB assay was conducted to investigate the effect of the extracts on mitochondrial viability where alamar blue dye is converted to a highly fluorescent substance by metabolically active mitochondria in a live cell and the assay was performed according to the manufacturer's instructions (Bio-Rad, CA, USA). A375 cells were treated in the same conditions as section 2.6. Upon the completion of the incubation period, 100 µL of diluted alamar blue reagent was added and incubated for 2 h at 37°C. The fluorescence intensity (FI) was measured at 560 nm excitation and 590 nm emission and the percentage of cell viability was calculates using the following equation:

$$\%Viable\ cells = \frac{FI\ (sample) - FI\ (blank)}{FI\ (vehicle\ control) - FI\ (blank)} \times 100$$

2.8. Cell cycle analysis by flow cytometry

Distribution of cell population at different stages of cells cycle was studied by flow cytometry analysis after A375 cells exposure to cytotoxic extracts and the method as described by Li *et al.* was followed [45, 46]. Synchronized cells were seeded at 500,000 cells/well in a 6-well plate and that was followed by their exposure to the GI_{50} concentrations of the extracts and incubated for 48 h at 37°C. After cell detachment, cells were fixed with cold 70% ethanol and left for 18h at 4°C. Later, the cells were washed, treated with 100 µg/mL of RNase and stained with propidium iodide stain. The data acquisition was performed using MACSQuant Analyzer® and assessed by MACSQuantify™ software (Miltenyi Biotech, Gladbach, Germany).

2.9. Evaluation of apoptosis induction by flow cytometry

Phosphatidylserine translocation to the outer leaflet of the membrane was evaluated as it is one of the early signs of apoptotic death and carried out according to the manufacturer's instructions (Annexin V-FITC Kit, Miltenyi Biotec, Gladbach, Germany). Around 250,000 cells were seeded overnight at 37°C in a 12- well plate. The cells were exposed to the GI_{50} concentrations of the extracts for 24h and then harvested, washed with PBS. Following washing with the binding buffer, cells were incubated with binding buffer containing Annexin V-FITC for 15 min. Afterwards, they were washed with binding buffer and stained with propidium iodide prior data acquisition. The analysis was performed using The MACSQuant® Analyzer and assessed by MACSQuantify™ software (Miltenyi Biotech, Gladbach, Germany).

2.10. Determination of morphological changes in cells

The morphological changes induced by the treatments were studied to complement the quantitative results of the SRB assay with qualitative data. Cellular morphology was studied according to a previously described method [47] with slight modifications. Briefly, A375 cells were plated (10,000 cells) in a sterile chamber slide system (Lab-Tek™ II Chamber Slide™ System, Nunc™, Roskilde, Denmark). Following A375 exposure to the cytotoxic extracts, the media was replaced with PBS and cells were imaged from six random fields at 20X and 40X using phase contrast inverted microscope (EVOS cell imaging system, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Fluorescence images were taken for the treated cells by using the ReadyProbes® Cell Viability Imaging Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA)., following manufacturer's recommendations. A375 cells were exposed to the same conditions mentioned in section 2.10.1. After 48 h incubation, both dyes were added and incubated for 15 mins, and images were taken from six random fields at 20X and 40X using EVOS cell imaging system.

2.11. Assessment of caspase activity

Luminescent caspase-Glo 3/7 assay was carried out on exposed cells to the cytotoxic extract according to the manufacturer's instructions (Promega, Madison, WI, USA). The cells were seeded at a density of 10,000 cells/ well in a white multi-well plate and incubated overnight at 37°C. After treating cells with GI_{50} concentrations of the extract for 48h, 100 µL of Caspase-Glo® 3/7 reagent was added to each well, mixed gently and incubated at the room temperature for 1h. This was followed by measuring the enzymatic activity of caspase through luminescent signal using microplate reader (Infinite® M200, Tecan, Mannedorf, Switzerland).

2.12. Evaluation of LDH release

Plasma membrane damage is one of the signs of cellular death, we assessed its integrity by evaluating the leakage of one of the cytosolic enzymes, lactate dehydrogenase (LDH) using pierce LDH cytotoxicity assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and following manufacturer's protocol. In brief, 10,000 cells were plated in a 96-well plate overnight, treated with the cytotoxic extracts and incubated for 48 h. Later, 10µL of lysis buffer was added to wells of maximum LDH activity control and water to the spontaneous LDH activity control, and incubated at 37°C for 45 min. Later, 50 µL of the supernatant of each well was mixed with 50 µL of the reaction mixture and incubated for 30 min at room temperature in the dark. The absorbance was measured at 490 nm and 680 nm and LDH activity was determined by subtracting the 680nm absorbance value (background) from the 490nm absorbance before calculation of % Cytotoxicity [(LDH at 490nm) - (LDH at 680nm)] as follow:

$$\%\text{Cytotoxicity} = \frac{Extract-treated\ LDH\ activity\ - \text{Spontaneous LDH activity}}{Maximum\ LDH\ activity\ - Spontaneous\ LDH\ activity} \times 100$$

2.13. 2D cell migration studies

This assay was carried out as described previously with slight modifications using the B16-F10 cell line as it is a highly aggressive cell line to close the gap within a limited time [48]. Around 250,000 cells were plated on a 6-well plate to produce a nearly confluent cell monolayer and later a scratch was made using a sterile 200 µL plastic pipette tip in the monolayer and wells were washed with the media to remove the detached cells. Free serum medium containing DMSO, the extracts were added and incubated for 24h. Later, cells were fixed with 3.7% formaldehyde and representative images for each well were taken with EVOS cell imaging system. The images were analysed using the TScratch software (ETH Zurich, Switzerland).

2.14. Statistical analysis

Results are expressed as mean ± standard deviation (SD) from at least three independent experiments. All data were analysed using unpaired two-tailed Student's t-test with a p-value of <0.05

considered as significant to find the statistical significance between treated groups and controls using InStat v.3 (GraphPad, San Diego, CA, USA).

3. RESULTS

3.1. Structural elucidation of isolated compounds

The chemical structure of the isolated compounds was identified using spectroscopic techniques such as NMR including 1H-NMR, 13C-NMR and 2D spectra, high resolution ESI-MS and IR. Their structures is shown in Figure 1 and their data are shown as follows:

Justicidin A, appeared as a pale powder from *H. tuberculatum*, HR ESI-MS gave $[M+H]^+$ ion at m/z 395.1132 ($C_{22}H_{18}O_7$). ^{1}H-NMR (500 MHz, CDC_{l3}) δ 7.54 (1H, s, H5), 7.05 (1H, s, H8), 6.96 (1H, d, H5'), 6.82 (1H, d, H2'), 6.80 (1H, dd, H6'), 6.09, 6.05 (2H, d, H2'), 5.55 (2H, s, H12), 4.13 (3H, s, OMeH4), 4.07 (3H, s, OMeH6), 3.82 (3H, s, OMeH7). ^{13}C-NMR δ 169.79 (C11), 151.73 (C6), 150.45 (C7), 147.94 (C4), 147.66 (C4'), 147.58 (C3'), 134.56 (C1), 130.78 (C9), 128.67 (C1'), 126.12 (C10), 124.60 (C3), 123.78 (C6'), 119.48 (C2), 110.93 (C2'), 108.38 (C5'), 106.28 (C8), 101.40 (O-CH_2-O), 100.72 (C5), 66.82 (C12), 59.84 (OMe4), 56.32 (OMe6), 56.01 (OMe7). This compound was identified as justicidin A and this was in agreement with those published in literature [49].

Justicidin B, was obtained as pale yellow compound from *H. tuberculatum* with a molecular formula $C_{21}H_{16}O_6$ established by HR ESI-MS with $[M+H]^+$ ion at m/z 365.1039. ^{1}H-NMR (500 MHz, CDC_{l3}) δ 7.71 (1H, s, H4), 7.19 (1H, s, H8), 7.11 (1H, s, H5), 6.98 (1H, d, H5'), 6.87 (1H, d, H2'), 6.84 (1H, dd, H6'), 6.10, 6.06 (2H, d, O-CH_2-O), 5.39 (2H, s, H12), 4.06 (3H, s, OMe-H7), 3.82 (3H, s, OMe-H6). ^{13}C-NMR δ 170.18 (C11), 151.96 (C7), 150.22 (C6), 147.75 (C3'), 147.71 (C4'), 139.83 (C1), 139.70 (C10), 133.33 (C3), 129.02 (C2), 128.56 (C1'), 123.65 (C6'), 118.69 (C9), 118.46 (C4), 110.75 (C2'), 108.42 (C5'), 106.16 (C8), 105.98 (C5), 101.45 (O-CH_2-O), 68.24 (C12), 56.27 (OMe7), 56.03 (OMe6). By comparing the literature data, this compound was described as justicidin B [49].

Tuberculatin, was observed as light yellow materials from *H. tuberculatum* giving $[M+H]^+$ ion by HR ESI-MS at m/z 513.1391 ($C_{26}H_{24}O_{11}$). ^{1}H-NMR (500 MHz, CD_3OD) δ 7.66 (1H, s, H5), 7.04 (1H, s, H8), 6.99 (1H, d, H5'), 6.76 (1H, d, H2'), 6.75 (1H, dd, H6'), 6.07, 6.05 (2H, s, O-CH_2-O), 5.56, 5.52 (2H, d, H12), 5.53 (1H, d, H1''), 4.54 (1H, d, H2''), 4.36, 3.98 (2H, d, H4''), 4.03 (3H, s, OMeH6), 3.73 (3H, s, OMeH7), 3.72, 3.71 (2H, s, H5''). ^{13}C-NMR δ 137.04 (C1), 119.94 (C2), 130.35 (C3), 146.14 (C4), 101.95 (C5), 153.10 (C6), 151.50 (C7), 107.09 (C8), 131.70 (C9), 128.41 (C10), 172.57 (C11), 68.94 (C12), 129.76 (C1'), 111.66 (C2'), 148.92 (C3'), 148.92 (C4'), 109.24 (C5'), 124.82 (C6'), 102.71 (O-CH_2-O), 56.79 (OMe6), 56.22 (OMe7), 112.65 (C1''), 78.76 (C2''), 80.51 (C3''), 75.93 (C4''), 64.05 (C5''). Based on this data and in comparison to the published data, this compound was recognized as tuberculatin [49].

Tuberculatin acetate, was obtained as a pale powder from *H. tuberculatum* and exhibited a molecular formula $C_{28}H_{26}O_{12}$ by HR ESI-MS, to give the $[M+H]^+$ ion at m/z 555. ^{1}H-NMR (500 MHz,

CD_3OD) δ 7.71 (H, s, H5), 7.09 (H, s, H8), 6.98 (1H, d, H5'), 6.81 (1H, d, H2'), 6.80 (1H, dd, H6'), 6.07, 6.05 (2H, s, O-CH_2-O), 5.57, 5.52 (2H, d, H12), 5.52 (1H, d, H1''), 4.47 (1H, d, H2''), 4.34, 4.01 (2H, d, H4''), 4.30, 4.28 (2H, s, H5''), 4.03 (3H, s, OMeH6), 3.75 (3H, s, OMeH7), 2.13 (3H, s, -C=O-CH3). ^{13}C-NMR δ 172.63 (C=O-), 172.19 (C11), 153.51 (C6), 151.91 (C7), 149.10 (C3'), 149.10 (C4'), 146.49 (C4), 137.21 (C1), 131.97 (C9), 130.63 (C3), 130.09 (C1'), 128.48 (C10), 124.87 (C6'), 120.21 (C2), 112.87 (C1''), 111.91 (C2'), 109.09 (C5'), 107.29 (C8), 102.71 (O-CH_2-O), 101.98 (C5), 79.42 (C2''), 78.62 (C3''), 76.06 (C4''), 68.83 (C12), 66.80 (C5''), 56.63 (OMe6), 56.15 (OMe7), 20.76 (C=O-CH_3). These data demonstrated that this compound was tuberculatin acetate.

Lupeol, appeared as a white amorphous powder from *C. decidua* and HR ESI-MS established the molecular formula as $C_{30}H_{50}O$ the giving the $[M+H]^+$ ion at m/z 427.3930. ^{1}H-NMR (500 MHz, pyridine-*d*5) δ 4.90, 4.75 (2H, m, H29), 3.48 (1H, dd, H3), 2.50 (1H, m, H19), 1.99, 1.44 (2H, m, H21), 1.88, 1.65 (2H, m, H2), 1.80, 1.18 2H, m, H12), 1.76 (3H, s, H30), 1.69, 0.99 (2H, m, H1), 1.68, 1.01 (2H, m, H15), 1.59, 1.43 (2H, m, H10), 1.52, 1.48 (2H, m, H16), 1.48, 1.38 (2H, m, H7), 1.43 (1H, m, H18), 1.41, 1.25 (2H, m, H22), 1.41 (1H, m, H13),1.36 (1H, m, H9), 1.26 (3H, s, H23), 1.19, 1.01 (2H, m, H11), 1.07 (3H, s, H24), 1.05 (3H, s, H26), 0.99 (3H, s, H27), 0.90 (3H, s, H25), 0.84 (3H, s, H28), 0.79 (1H, m, H5). ^{13}C-NMR δ 151.45 (C20), 110.33 (C29), 78.50 (C3), 56.25 (C5), 51.15 (C9), 48.99 (C18), 48.65 (C19), 43.60 (C17), 43.43 (C14), 41.51 (C8), 40.61 (C22), 39.94 (C4), 39.68 (C1), 38.70 (C13), 37.88 (C10), 36.19 (C16), 35.09 (C7), 30.52 (C21), 29.08 (C23), 28.73 (C2), 28.17 (C15), 25.95 (C12), 21.51 (C11), 19.85 (C30), 19.20 (C6), 18.58 (C28), 16.86 (C25), 16.78 (C24), 16.59 (C26), 14.68 (C27). By reviewing the published data, this compound was identified as lupeol [50].

Ursolic acid, was obtained as a white amorphous solid from *C. edulis* with molecular formula by HR ESI-MS ($C_{30}H_{48}O_3$) and $[M+H]^+$ ion at m/z 457.3674. ^{1}H-NMR (500 MHz, pyridine-*d*5) δ 5.52 (1H, t, H12), 3.48 (1H, dd, H3), 2.68 (1H, d, H18), 2.36, 1.20 (2H, m, H15), 2.15, 2.04 (2H, m, H16), 2.01 (2H, m, H22), 1.96 (2H, m, H11), 1.84 (2H, m, H2), 1.66 (1H, m, H9), 1.60, 1.06 (2H, m, H1), 1.60, 1.41 (2H, m, H6), 1.58, 1.36 (2H, m, H7), 1.53 (1H, m, H19), 1.49 (2H, m, H21), 1.27 (3H, s, H23), 1.25 (3H, s, H27), 1.08 (3H, s, H26), 1.05 (1H, m, H20), 1.05 (3H, s, H24), 1.03 (3H, d, H29), 0.98 (3H, d, H30), 0.91 (3H, s, H25), 0.89 (1H, m, H5). ^{13}C-NMR δ 180.29 (C28), 139.67 (C13), 126.05 (C12), 78.52 (C3), 56.22 (C5), 53.95 (C18), 48.45 (C9), 48.45 (C17), 42.90 (C14), 40.37 (C8), 39.89 (C19), 39.80 (C20), 39.79 (C4), 39.47 (C1), 37.85 (C22), 37.68 (C10), 33.98 (C7), 31.47 (C21), 29.21 (C23), 29.09 (C15), 28.54 (C2), 25.32 (C16), 24.31 (C27), 24.03 (C11), 21.81 (C30), 19.18 (C6), 17.92 (C29), 17.86 (C26), 16.98 (C24), 16.08 (C25). Based on the data, this compound was identified as ursolic acid and in a good agreement with the literature [51].

Ursolic acid acetate, was obtained as a white amorphous solid from *C. edulis* with molecular formula by HR ESI-MS ($C_{32}H_{50}O_4$) and $[M+H]^+$ ion at m/z 499.3775. ^{1}H-NMR (500 MHz, pyridine-*d*5) δ 5.49 (1H, t, H12), 4.72 (1H, dd, H3), 2.67 (1H, d, H18), 2.34, 1.22 (2H, m, H15), 2.14, 2.02 (2H, m, H16), 2.00 ((2H, m, H22), 1.90 (2H, dd, H11), 1.70 (2H, m, H2), 1.57 (1H, t, H9), 1.49, 0.91 (2H,

m, H1), 1.49, 1.34 (2H, m, H6), 1.51, 1.30 (2H, m, H7), 1.53 (1H, m, H19), 1.49, 1.34 (2H, m, H21), 0.92 (3H, s, H23), 1.24 (3H, s, H27), 1.03 (3H, s, H26), 1.06 (1H, m, H20), 0.89 (3H, s, H24), 1.03 (3H, d, H29), 0.98 (3H, d, H30), 0.83 (3H, s, H25), 0.84 (1H, m, H5), 2.07 (3H, s, C=O-CH_3). ^{13}C-NMR δ 180.3 (C28), 139.7 (C13), 125.7 (C12), 81.10 (C3), 55.90 (C5), 53.90 (C18), 48.20 (C9), 48.40 (C17), 42.80 (C14), 40.20 (C8), 39.80 (C19), 39.80 (C20), 38.20 (C4), 38.60 (C1), 37.80 (C22), 37.4 (C10), 33.70 (C7), 31.40 (C21), 28.50 (C23), 29.00 (C15), 24.20 (C2), 25.30 (C16), 24.20 (C27), 23.90 (C11), 21.80 (C30), 18.80 (C6), 17.90 (C29), 17.70 (C26), 17.30 (C24), 15.90 (C25), 171.0 (C=O), 21.50 (O-CH_3). The compound was identified as ursolic acid acetate and data were in agreement with those in the literature [52], which has not been reported in this plant before.

Quercetin, was obtained as a yellow powder from *C. edulis* showing a molecular weight ($C_{15}H_{10}O_7$) by HR ESI-MS giving $[M+H]^+$ ion at m/z 303.0419. 1H-NMR (500 MHz, CD_3OD) δ 7.63 (1H, d, H2'), 7.53 (1H, dd, H6'), 6.78 (1H, d, H5'), 6.28 (1H, d, H2), 6.07 (1H, d, H6). 13C-NMR δ 177.42 (C4), 165.67 (C7), 162.60 (C5), 158.13 (C9), 148.86 (C2), 148.06 (C4'), 146.31 (C3'), 137.33 (C3), 124.22 (C1'), 121.75 (C6'), 116.30 (C5'), 116.06 (C2'), 104.59 (C10), 99.31 (C6), 94.48 (C8). The data was in a good agreement with those for quercetin in the literature [53].

3.2. Cytotoxicity of crude extracts

Three crude extracts from each plant obtained from cold sequential extraction were tested using SRB and AB assays over a range of concentrations (200, 100, 50, 25, 12.5, 6.25 µg/mL) at different time points (24h, 48, 72h). The cytotoxic effects were investigated and the GI_{50} (The average growth inhibition of 50%) was determined. Table 1 summarizes the GI_{50} values of the tested plant extracts. According to the criteria of the American National Cancer Institute, crude plant extracts which show an GI_{50} of less than 100 µg/mL are considered to be cytotoxic selected for further studies, whereas the most promising ones are those with an GI_{50} lower than 30 µg/mL [54, 55].

Table 1. Voucher specimen, site of collection and GI_{50} concentrations (µg/mL) of the hexane, chloroform and methanol plant extracts determined for A375 cells, as assessed by the SRB and AB assays at 48 hours. Each result was obtained in three independent experiments run in triplicate.

Plant species Family / Used part	**Site of collection, Voucher specimen**	**Extract**	**Sample code**	**GI_{50} (µg/mL)**	
				SRB assay	**AB assay**
Acacia nilotica L. (Delile) Fabaceae/ Aerial parts	Commercial source	Hexane	ANH	>200	>200
	UQU-UCL-2016/1	Chloroform	ANC	121±4	104±11
		Methanol	ANM	11±6	25±7
Aizoon canariense L. Aizoaceae/ Aerial parts	Taif UQU-UCL-2016/2	Hexane	ACH	>200	>200
		Chloroform	ACC	14±6	22±7
		Methanol	ACM	>200	>200
Capparis decidua (Forssk.) Edgew Capparidaceae/ Aerial parts	Taif UQU-UCL-2016/3	Hexane	CDH	0.05±6	12±14
		Chloroform	CDC	18.±3	50±12
		Methanol	CDM	>200	>200
Carissa edulis (Forssk.) Vahl Apocynaceae/ Leaves	Wadi Thee Ghazal-Taif UQU-UCL-2016/4	Hexane	CEH	17±6	50±7
		Chloroform	CEC	14±6	49±14
		Methanol	CEM	>200	153±9
Cyperus rotundus L. Cyperaceae/ Aerial parts	Commercial source UQU-UCL-2016/5	Hexane	CRH	163±4	102±10
		Chloroform	CRC	20±3	42±9
		Methanol	CRM	>200	>200
Haplophyllum tuberculatum Juss. Rutaceae/ Aerial parts	Majmaah-Riyadh 15932	Hexane	HTH	73±4	80±15
		Chloroform	HTC	0.5±0.04	1.3±7
		Methanol	HTM	1.7±0.68	8±13
Osteospermum vaillantii (Decne.) Norl Asteraceae/ Flowering aerial parts	Al-Hada UQU-UCL-2016/6	Hexane	OVH	>200	74±14
		Chloroform	OVC	21±7	45±13
		Methanol	OVM	>200	>200
Plicosepalus curviflorus (Benth. ex Oliv.) Tiegh Loranthaceae/ Flowering aerial parts	Al-Waheet-Taif UQU-UCL-2016/7	Hexane	PCH	4±0.8	11±9
		Chloroform	PCC	>200	>200
		Methanol	PCM	>200	>200
Pulicaria schimperi DC. Asteraceae /Flowering aerial parts	Makkah Al-Mukarramah UQU-UCL-2016/8	Hexane	PIH	>200	151±9
		Chloroform	PIC	19±5	15±12
		Methanol	PIM	>200	>200

3.3. Effects of the extracts on cell cycle distribution

To gain insight into the effects of the cytotoxic extracts on cell cycle distribution, synchronized A375 cells were evaluated for their DNA content by flow cytometry analysis. Cells were treated with media (living cells control), DMSO in media, positive control (paclitaxel) and GI_{50} concentrations of the extracts. After 48h exposure, the percentage of cell population in each phase changed significantly compared to untreated cells, as presented in Figure 2. The percentage of cells at G1 phase was significantly reduced in *A. canariense*-treated cells, to 45.74% ($p < 0.0001$), with a corresponding increase in the percentage of sub-G1 fraction (14.49%, $p < 0.0001$). Interestingly the cell population was also blocked in S and G2/M phases and markedly elevated reaching 27.09% ($p = 0.0025$) and 11.82% ($p = 0.0126$) respectively. The methanol extract of *A. nilotica*, chloroform extract of *O. vaillantii* and *C. rotundus* resulted in a significant accumulation of cells at sub-G1 peak around 3.74% ($p = 0.0069$), 4.37% ($p = 0.0115$) and 9.22% ($p = 0.0217$) respectively.

Notably, cell cycle arrest at S phase was observed in treated cells with the chloroform extract of *C. decidua* giving 26.17% ($p = 0.0029$) and of *H. tuberculatum* with a level of 41.49% ($p = 0.0001$) accompanied by an elevation of sub-G1 population of 9.64% ($p = 0.0090$) and 10.37% ($p = 0.029$) respectively. However, other extracts of the same plants caused different effects in which no significant increase in S phase population was detected, but cells exhibited an increase in sub-G1 fraction when treated with hexane extract of *C. decidua* (7.60%, $p = 0.0160$) and methanol extract of *H. tuberculatum* (5.14%, $p = 0.0220$). Also, the hexane and chloroform extracts of *C. edulis* had variable effects presented by similar arrest at S stage for both (23.61%, $p = 0.0263$ and 24.13%, $p = 0.0149$ respectively) whereas the chloroform extract also augmented sub-G1 fraction to 8.62% ($p = 0.005$). Cell cycle was blocked at S phase following treatment with chloroform extract of *P. curviflorus* reaching 26.02% ($p = 0.0113$) and *P. schimperi* gave 22.53% ($p = 0.0292$), accompanied by rises in the sub-G1 phase of 17.75% ($p = 0.0010$) and 6.14% ($p = 0.0119$) respectively.

3.4. Induction of apoptosis by the extracts.

Discrimination between necrotic and apoptotic death is required when the mechanism of action of the cytotoxic extracts is explored. One of the key assays is Annexin V-FITC/PI double staining to investigate the externalization of phosphatidylserine to the outer leaflet of the plasma membrane. As shown in Figure 3, the percentages of cells as unstained, single stain (Annexin V or PI) or double stained (both stains) populations were changed after 24h treatment with the extracts. Annexin V-positive /PI-negative (left lower quadrant) and Annexin V-positive /PI-positive population (left upper quadrant) contain early and late apoptotic cells which were significantly increased following cell exposure to chloroform extract of *C. rotundus* (9.29% ($p = 0.0362$), 14.04% ($p = 0.0173$)), hexane extracts of *C. decidua* (3.13% ($p = 0.0026$), 21.11% ($p = 0.0425$)) and *C. edulis* (2.80% ($p = 0.0059$), 11.12% ($p = 0.0165$)) as well as chloroform (3.48% ($p = 0.0107$), 18.43% ($p = 0.0021$)) and methanol extracts of *H.*

tuberculatum (3.48% (p = 0.0211), 27.30% (p = 0.0335)) compared to untreated cells at 0.8% and 4.02% respectively.

Notably, the percentage of cells in all quadrants was significantly changed in cells treated with chloroform extracts *of C. decidua, A. canariense*, and *P. schimperi*, whereby the percentage of early apoptotic cells reached 6.39% (p = 0.0051), 2.56% (p = 0.0452) and 4.71% (p = 0.0029) respectively, and higher Annexin V-positive /PI-positive population (late apoptosis) was detected for the same extracts, reaching 32.10% (p < 0.0001), 20.54% (p = 0.0142) and 29.12% (p = 0.0256) respectively. Interestingly, there was also an increment of necrotic population after treatment with *C. decidua* (12.13%, p = 0.0020), *A. canariense* (14.95%, p = 0.0451), and *P. schimperi* up to 13.25% (p = 0.0036) accompanied by a reduction in unaffected population.

Figure 3 allows the identification of the elevated Annexin V-negative /PI-positive populations (necrotic cells) because of the treatment with chloroform extract of *O. vaillantii* up to 35.64% (p = 0.0050) and *C. edulis* up to 9.25% (p = 0.0180). Regarding the hexane extract of *P. curviflorus*, approximately 23.63% (p = 0.0070) of cells were observed in the late apoptosis quadrant as a high level compared to untreated cells 4.02%.

3.5. Effect of the extracts on A375 cell morphology

A375 cells exposed for 48h to the GI_{50} concentrations of the cytotoxic extracts were morphologically examined using phase contrast inverted microscope and stained with Hoechst 33342 and NucGreen® dead reagent double staining to be visualized by EVOS system. Due to morphological changes in nuclei, chromatin condensation was detected as shown in Figure 4 by the accumulation of Hoechst dye when cells were exposed to chloroform extracts *of A. canariense, C. decidua, H. tuberculatum, P. schimperi* and *O. vaillantii*. Several extracts led to the loss of integrity in the plasma membrane, as shown by the green dye, which stains dead cells with compromised plasma membranes.

Regarding the light micrographs in Figure 5., cells treated with chloroform extracts of *A. canariense, C. decidua, C. rotundus, O. vaillantii* and methanol extract of *H. tuberculatum* displayed cell shrinkage, rounding of shape, loss of cell-cell attachment and adherence, compared to the control cells. Furthermore, many of them exhibited the distinctive morphological changes of apoptosis such as membrane blebbing in cells treated with chloroform extract of *H. tuberculatum*, as well as apoptotic bodies occurring as a result of cell exposure to hexane extracts of *C. decidua* and *C. edulis*, and chloroform extract of *P. schimperi*. Notably, A375 cells tended to blast and lose their entire structure, appearing as floating and swollen cells following exposure to the chloroform extract of *C. edulis* and hexane extract of *P. curviflorus*.

3.6. Effect of the extracts on caspase-3/7 activity

Based on the results obtained so far, some of the extracts induced chromatin condensation, externalisation of phosphatidylserine, and DNA fragmentation, therefore we next explore the possibility

of the involvement of caspase in the mechanism of action. Figure 6 demonstrates the effect of the tested extracts on the caspase-3/7 induction in A375 cells.

There was a significant induction of caspase-3/7 activity following cells exposure to both extracts of *C. decidua* (hexane, 2.21 fold (p = 0.0067) and chloroform, 3.74 fold (p = 0.0118)) and *H. tuberculatum* (chloroform, 4.76 fold (p = 0.0020) and methanol, 4.47 fold (p = 0.0010)). Exposure to both cytotoxic extracts of *C. edulis* had different effects on caspase-3/7 activity in which the hexane extract caused a significant change up to 2.94 fold (p = 0.0018) whereas the chloroform did not. Furthermore, caspase-3/7 was activated in cells treated with chloroform extracts of *C. rotundus*, *O. vaillantii* and *P. schimperi* to elevate the levels to 2.44 fold (p = 0.0065) 2.11 fold (p = 0.0039) and 2.37 fold (p = 0.0396) respectively. It is worth mentioning that the hexane extract of *P. curviflorus*, chloroform extract of *A. canariense* and methanol extract of *A. nilotica* did not change the activity of caspase-3/7 significantly, suggesting that caspase was not involved in their mechanisms of action.

3.7. Detection of plasma membrane leakage (lactate dehydrogenase (LDH) assay)

To investigate whether the plasma membrane lost its integrity when treated with cytotoxic extracts, a LDH assay was carried out. A375 cells were treated for 48h with media alone (living cells control), DMSO in media, triton (positive control) or GI_{50} of the tested extracts. As shown in Figure 7, spontaneous LDH release in untreated cells was low but the level of the enzyme was significantly increased following cell exposure to hexane extract of *C. edulis* (15.75%, p = 0.0018), *P. curviflorus* (23.50%, p = 0.0013), chloroform extract of *C. decidua* (17.75%, p = 0.0163), *C. edulis* (18.25%, p = 0.0222), *H. tuberculatum* (23.75%, p = 0.0037), *O. vaillantii* (17.25%, p = 0.0117), methanol extract of *A. nilotica* (14.75%, p = 0.0258) and *H. tuberculatum* (8%, p = 0.0319).

3.7. Effect of the extracts on 2D cell migration

We investigated whether the plant extracts interfere with cancer cell movement (migration) using the well stablished 'scratch assay'. None of the extracts had any significant effect on the cell movement (data not shown)

4. DISCUSSION

Nine plants showed significant cytotoxicity against human melanoma cells without any effect on their migratory activity. Four cytotoxic lignans; justicidin A, justicidin B, tuberculatin and tuberculatin acetate were isolated from *H. tuberculatum*. In addition, ursolic acid acetate, ursolic acid and quercetin were obtained from *C. edulis*. Furthermore, bioguided isolation of the chloroform extract of *C. decidua* obtained lupeol for the first time as the active principle against A375 cells.

4.1. Effects of A. canariensis extracts and its cytotoxic principles

The chloroform was the most active of the *A. canariense* extracts with an GI_{50} = 14 µg/mL against A375 cells in a time-dependent fashion. Its anti-proliferative effect was associated with significant

disruption in all cell cycle stages, a reduction in G1 and an elevation of sub-G1, S and G2/M populations. Apoptosis was induced, as early and late apoptotic populations were markedly increased and interestingly, that was accompanied by a high level of necrotic population without a significant elevation of LDH level. In addition, morphological appearance was altered following cell exposure to the extract in which nuclear fragmentation was seen but caspase-3/7 was not activated, suggesting that that caspase did not act as the mediator of cell death. Based on these findings, we might conclude that some of the active compounds within the *A. canariense* chloroform extract are indeed able to induce caspase-independent apoptosis. The necrotic effect may be due to the presence of other compounds compromising the cell membrane integrity or it may be just a population in very late apoptosis.

'The cytotoxicity of *A. canariense* was reported to be 90 ug/mL in a brine shrimp lethality bioassay, however no activity has been reported against melanoma cell lines [56]. The IC_{50} concentrations of the methanol extract of the plant against breast cancer (MCF7), hepatocellular carcinoma cell (HEPG2) and cervical cancer cell lines (HELA) as 21, 24 and 25 µg/mL respectively [57]. Although the reported cytotoxic effect has been detected in the methanol extract, our findings showed the link between anti-melanoma activity and medium polar extract, chloroform one, with no evidence of cytotoxicity of the methanol extract against melanoma cells. This could either be because the active principles are medium polar candidates or because various methods of extraction are used in both studies. Moreover, in this study the extraction method relied on using a range of solvents with different polarities, whereas Almehdar *et al.* used only methanol solvent, therefore all compounds with a range of polarities were extracted together retaining cytotoxicity to methanol extract [57]. It has been mentioned that *A. canariense* is rich in fatty acids such as protocatechuic acid and cinnamic acid that are known as chemopreventive and anti-proliferative against melanoma cells, respectively [58, 59], which may contribute to the cytotoxicity of chloroform extract of the plant.

4.2. Effects of A. nilotica extracts and its cytotoxic principles

The methanol extract of *A. nilotica* retained the anti-proliferative activity against A375 cells with concentration- and time-dependent effects at GI_{50} = 11 µg/mL. Sub-G1 peak was an indication of cell apoptosis induced by the extract without the involvement of caspase-3/7 activity or the externalisation of phosphatidylserine. Also, there was an evidence of cytoplasmic membrane permealisation as LDH levels were elevated and the majority of cells became round-shaped and floating in the medium. The elevation of LDH may be ascribed to the occurrence of apoptosis followed by a disruption of the plasma membrane or the presence of other compounds causing necrotic effects.

The SRB results were comparable to those reported by Salem's group, in which gallocatechin 5-O-gallate isolated from the methanol extract was found to play a key role in the selectivity towards an uveal melanoma cell line [14]. It has been mentioned in other reports that bioguided isolation of ethanol extract (IC_{50} = 493 µg/mL in breast cancer cells (MCF-7) and 696 µg/mL in non-small lung cancer cells (A549)) resulted in identification of γ-sitosterol as the active principle, inducing cell cycle arrest at the

G2/M phase, decreasing the c-Myc expression in the tested cell lines, and playing a role in cell cycle progression and apoptosis [60]. Some authors have also documented the cytotoxicity of the water extract, of which gallic acid was found to be the bioactive compound on a mouse mammary tumour cell line (S115) at 40 µg/mL [61]. By observing GI_{50} values, *A. nilotica* seems to be more selective towards A375 cells (11 µg/mL) compared to MCF-7 (493 µg/mL), A549 (696 µg/mL) and S115 (40 µg/mL) or it may be that A375 cells are more selective to the plant compared to other cell lines. An *in vivo* study has been carried out for the methanol extract of *A. nilotica* in a lymphoma tumour model, revealing a reduction in the development of tumours in mice, increasing their life span and restoring the total white blood cell count and hemoglobin content [62]. Furthermore, an ethanol extract exhibited chemoprevention properties in a model of hepatocarcinogenesis via the reduction of liver injury and enhancement of anti-oxidant enzyme activity [63]. Our study provides additional evidence for the cytotoxic properties of *A. nilotica*, expanding it to melanoma cells.

Scientific evidence shown that the plant contains high level of phenolics, flavonoids such as gallic acid, ellagic acid, epicatechin, rutin, kaempferol and apigenin [64] as well as triterpenoids e.g. β-amyrin and betulin [65]. It is clear that phenolics and terpenes are the major classes of secondary metabolites in this plant, and both have been found to induce cell death in cancer models, e.g. gallocatechin 5-O-gallate, gallic acid and betulin. The possible mechanism of phenolics may be their ability to scavenge the reactive oxygen species (ROS) in which their production is augmented in cancer cells in order to promote their proliferation and differentiation [66]. In addition, terpenes have a considerable reputation as anti-cancer candidates such as betuline and lupeol [67], but these compounds are unlikely to exist in methanol extract which was the most cytotoxic extract in this study.

4.3. Effects of C. decidua extracts and its cytotoxic principles

In our study, both hexane and chloroform extracts of *C. decidua* suppressed the proliferation of melanoma cells at relatively low concentrations and in a time-dependent manner (GI_{50} = 10 and 18 µg/mL respectively), which was associated with an elevation of cells with low DNA content (sub-G1), and this effect on melanoma has not been explored previously. Both extracts induced caspase mediated apoptosis with translocation of phosphatidylserine to the outer membrane, as indicated by the high percentages of early and late apoptotic populations. However, the chloroform extract only afforded significant cell cycle arrest at S phase, accompanied by nuclear fragmentation. Moreover, signs of necrosis were indicated by the high level of Annexin V-negative/ PI-positive population and augmented LDH level. Bioguided isolation of the chloroform extract obtained lupeol from the plant for the first time as the active principle against A375 cells (GI_{50} = 65 µM). The cell cycle arrest at S phase in this study is related to lupeol, which has been reported by Saleem *et al*. to promote G1-S phase cell cycle arrest by suppressing the expression of cyclin D1, cyclin D2, and cdk2, with a selective growth inhibitory effect on melanoma cell lines (451Lu and WM35). It also induced apoptosis through the activation of caspase-3, induction of PARP cleavage and enhancement of the expression of p21 protein.

Lupeol has not only demonstrated anti-proliferative effects *in vitro*, but has also significantly reduced 451Lu tumour growth in athymic nude mice [68]. Taken all together, chloroform extract exerted a caspase-dependent apoptotic effect due to the presence of lupeol and a necrotic effect which is possibly related to the existence of other necrotic compounds in the extract.

Regarding the hexane extract, it induced caspase-mediated apoptosis and DNA fragmentation and β-sitosterol triacontenate, as a non-polar secondary metabolite, has been reported to be the active principle in *C. decidua* against human lung cancer cells (A549) [69]. This raises the possibility that the biological activity of hexane extract is due to its β-sitosterol triacontenate. Moreover, alkaloids may contribute to the cytotoxicity of *C. decidua*, as Rathee and colleagues have isolated stachydrine with influence on gene expression of CXCR3 and CXCR4 at mRNA and protein levels leading to the suppression, invasion and metastasis of a prostate cancer cell line [70].

4.4. Effects of C. edulis extracts and its cytotoxic principles

In this project, both hexane and chloroform extracts of *C. edulis* inhibited melanoma cell growth at different time points (GI_{50} = 17, 14 µg/mL respectively) and blocked the cell cycle at S stage. Caspase-3/7 activation with elevation of both the early and late stages of apoptosis were signs of apoptosis induction by both extracts, and also included high levels of LDH. Chloroform extract-treated cells presented additional features of cell necrosis, such as necrotic population (Annexin V-negative/ PI-positive) and typical necrotic morphological appearance, for instance cytoplasmic vacuoles, cell floating and cytoplasmic membrane permealisation, suggesting the existence of necrotic inducers in the extract. The apoptotic effect was in agreement with findings on the effect of n-butanol fractions of *C. edulis* on leukaemia cells (HL-60), causing DNA laddering, apoptotic body formation, an elevation hypodiploid sub-G1 fraction, activation of caspase-3, -6 and -9 leading to PARP cleavage, elevation of Bax and degradation of Bcl-2 [71]. Ursolic acid and ursolic acid acetate were yielded after bioguided isolation of the active hexane extract as mentioned earlier. Although the LDH level went up, this could be due to the existence of other necrotic compounds in the hexane extract.

Quercetin was obtained from the chloroform extract in this study, so this may raise the possibility of synergistic effects between quercetin and other constituents such as kaempferol and rutin, as both have been isolated from *C. edulis* [25]. Furthermore, previous study showed that the combination treatments of ursolic acid and quercetin act synergistically in migration capacity of melanoma cells [72]. A combined therapy of quercetin and kaempferol or rutin has reduced cell proliferation in a synergistic manner [73, 74]. The anti-proliferative activity of the chloroform extract might also be attributed to the cytotoxic lignans carinol, carissanol and nortrachelogenin as they were cytotoxic to breast and lung cancer cells [75].

Experimental studies conducted in this project have proved that *C. rotundus* induced growth inhibition of A375 cells in concentration- and time-dependent manners (GI_{50} = 20 µg/mL). The anti-proliferative effect was associated with pro-apoptotic activities as indicated by translocation of

phosphatidylserine, induction of caspase-3/7 activity and morphological changes such as cell shrinkage, rounding and loss of cell-cell attachment. This has also been highlighted in recent studies with essential oils on leukaemia cells, IC_{50} = 49 µg/mL, through apoptotic DNA fragmentation [76] and with a total oligomer flavonoid-enriched extract on erythroleukemia (K562) cells through apoptosis induction (IC_{50} = 70 µg/mL) [77].

Some active principles have been isolated and their mechanisms of action elucidated in the literature. Still, no reports on their anti-melanoma effects were found. Luteolin has been mentioned as the most active principle on erythroleukemia cell proliferation (IC_{50} = 25 µg/mL) with the highest anti-oxidant activities [77] which may correlate its anti-proliferative activity with free radical scavenging properties. In fact, the anti-cancer properties of some natural compounds have been linked to their ability to scavenge ROS, which are highly produced in tumour cells promoting their growth and increasing their metabolic activity [78]. Our results show the elevation of sub-G1 fraction and activation of caspase-3/7 in A375 cells and 6-acetoxy cyperene have been described to cause the same effect on ovarian cancer cells, hence it may responsible for the activity of *C. rotundus* on melanoma cells [29]. In addition, Tetsuro *et al*. have investigated the pro-apoptotic effect of cyperusphenols D isolated from the methanol extract of *C. rotundus* through nuclear fragmentation and PARP cleavage [79]. Also, another report has mentioned cytotoxicity of caryophyllene oxide against HeLa cell line [80], however there is controversy in the literature about this compound, which has been described as inactive on murine leukemia cell line (P388) [81]. These active principles isolated from the plant could individually or synergistically promote cell death in A375 cells.

4.5. Effects of H. tuberculatum extracts and its cytotoxic principles

Our data has shown that the chloroform extract of *H. tuberculatum* was the most cytotoxic among all tested extracts against melanoma cells (GI_{50} = 0.45 µg/mL) at several time points.

Its cytotoxicity was associated with cell cycle arrest at S phase and high percentage of low DNA content cells in A375 cells which is similar to the effect by Justicidin B derivatives [82]. Furthermore, it induced caspase mediated apoptosis and caused nuclear fragmentation. Justicidin A and B were afforded as a result of the bioguided isolation of the chloroform extract on melanoma cells. Not only did the chloroform extract demonstrate anti-proliferative effects, but the methanol extract also showed this ability, with an elevation of sub-G1 peak, activation of caspase-3/7 as well as cell shrinkage, rounding and poor adherence. Bioguided isolation obtained tuberculatin and tuberculatin acetate as the constituents play a crucial role in the cytotoxicity of the methanol extract. In both extracts, the level of LDH was elevated, indicating the disruption of plasma membrane after apoptosis incidence.

These results were in agreement with Varamini *et al.,* who described the anti-proliferative properties of the ethanol extract of *H. tuberculatum* against lymphoma cell lines (RAMOS), (U937) and a bladder carcinoma cell line (5637), IC_{50} = 25, 29, 23 µg/mL, respectively [32]. The difference in the IC_{50} concentrations may be attributed to the sensitivity of A375 to the tested extract compared to those

cell lines mentioned in literature. Kuete and colleagues have mentioned the same effect of the methanol extract on cell cycle distribution of leukemia cells accompanied by an alteration of the mitochondrial membrane potential. However, the caspases have not been activated on leukemia cells, whereas caspase-3/7 was highly induced in A375 cells [83].

4.6. Effects of O. vaillantii extracts and its cytotoxic principles

This is the first time that the cytotoxic effects of the chloroform extract of *O. vaillantii* have been explored and its anti-proliferative activity on A375 cells was detected (GI_{50} = 21 µg/mL) at different incubation periods of 24, 48 and 72h. Apoptosis was implicated in the mechanism of action indicated by caspase-3/7 activation, nuclear fragmentation and cells with low DNA content. However, both the necrotic population (Annexin V-negative/ PI-positive) and LDH level were increased in treated cells. These features may can be explained as there are apoptotic and necrotic inducers in the extract. This is not in agreement with Gouda's group, who observed no cytotoxicity of the methanol extract on breast cancer (MCF7), HeLa and ovarian cancer cell lines (A2780) [36] indicating that the plant cytotoxicity is more selective to A375 cells. Few saponin glycosides have been isolated from *O. vaillantii* but none of them is known for cytotoxic properties [34].

4.7. Effects of P. curviflorus extracts and its cytotoxic principles

P. curviflorus treatment efficiently potentiated both concentration- and time-dependent inhibition of A375 proliferation, causing 50% cell death at 4 µg/mL. In our effort to deepen the pharmacological properties of the hexane extract, we report for the first time the mechanism of action on melanoma cells. It blocked the cell cycle at the S phase manifested by the increase in the number of cells in S phase with no detectable caspase-3/7 activity. Although sub-G1 population was the highest among the tested extracts, the elevated LDH level and the morphological features of the treated cells including floated cytoplasmic vacuoles, as a feature of necrotic death [84], revealing that the cytotoxicity of the extract was due to the presence of apoptotic and necrotic compounds. Similarly, the methanol extract exerted cytotoxicity (IC_{50} = 5 µg/mL) against a human amniotic epithelial cell line (FL-cells) [85]. Fawzy *et al*. have also investigated the cytotoxicity of the methanol extract demonstrating high cytotoxicity against colon colorectal carcinoma cells (HCT-116), IC_{50} = 6 µg/mL. In the same report, pentahydroxyflavane-5-O-gallate (IC_{50} ranging from 11 to 38 µg/mL) and quercetin (IC_{50} ranging from 3.6 to 16 µg/mL) have been isolated as the responsible cytotoxic compounds [40]. However, these compounds are unlikely to contribute to the anti-melanoma activity since the hexane extract was the most active one and such polar compounds are more soluble in polar solvent such as methanol, which was inactive on A375 cells. In addition, lupeol and ursolic acid have been extracted from this plant.

4.8. Effects of P. schimperi extracts and its cytotoxic principles

Similarly, to our knowledge no studies have reported any biological activities of *P. schimperi*, which has not been so far explored in terms of active principles nor mechanism of action. Thus, it was

of interest to explore the cytotoxicity of this plant on A375 cells. Based on our findings, the chloroform extract was the most active and 19 µg/mL was the GI_{50} concentration inhibiting cell proliferation. The possible mechanism of action was caspase-mediated apoptosis since activating caspase-3/7, flipping of phosphatidylserine and sub-G1 population were observed in the treated cells. The extract was also able to arrest cell cycle at S phase with dramatic changes in the cell morphology, such as nuclear fragmentation, cell shrinkage, rounding and loss of cell-cell attachment. Another possible mechanism of cell death is necrosis, as the necrotic population (Annexin V-negative /PI-positive) was increased. Still, no reports on its phytochemical compositions were found which should therefore be further investigated to isolate the active principles against melanoma cells.

4.9. Overall effects of the selected plant extracts in the context of the Saudi biodiversity

Our work adds to the growing body of knowledge about plants from Saudi Arabia. A previous work on plants from the Western, South and Central areas area showed that some species are endowed with promising cytotoxic activities as shown here as well as other areas such as [57]. The plants here studied are also growing and used in parts of Latin America and Africa. Conditions in Saudi Arabia are to some extent similar to certain areas of in these continents where an estimated 4,500 plant species survive in harsh climates [86]. However, their properties may be largely changed as a result of its more extreme climate and lack of water availability in some geographical areas. This results in a wide range of plant with different biological activities as well as diversity of chemical constituents that are active against diseases such as cancer. This important diversity of flora in climatically interesting regions draws scientific attention towards investigative processes such as bio-guided isolation of native species and the extraction of active constituents to broaden chemical and pharmacological knowledge via *in vitro* and *in vivo* studies.

5. CONCLUDING REMARKS

This study has shown that nine medicinal plants here investigated demonstrated promising cytotoxicity (GI_{50} < 30 µg/mL) against human melanoma cells and should be considered as potential sources of new anti-melanoma drugs. The following anti-melanoma principles were found: lupeol was isolated from *C. decidua*; justicidins A and B, tuberculatin, and tuberculatin acetate from *H. tuberculatum*; and ursolic acid and ursolic acid acetate from *C. edulis*. Bioguided isolation of promising plants such as *O. vaillantii*, *P. curviflorus* and *P. schimperi* would reveal their bioactive principles and add greatly to an in-depth understanding of their mode of action.

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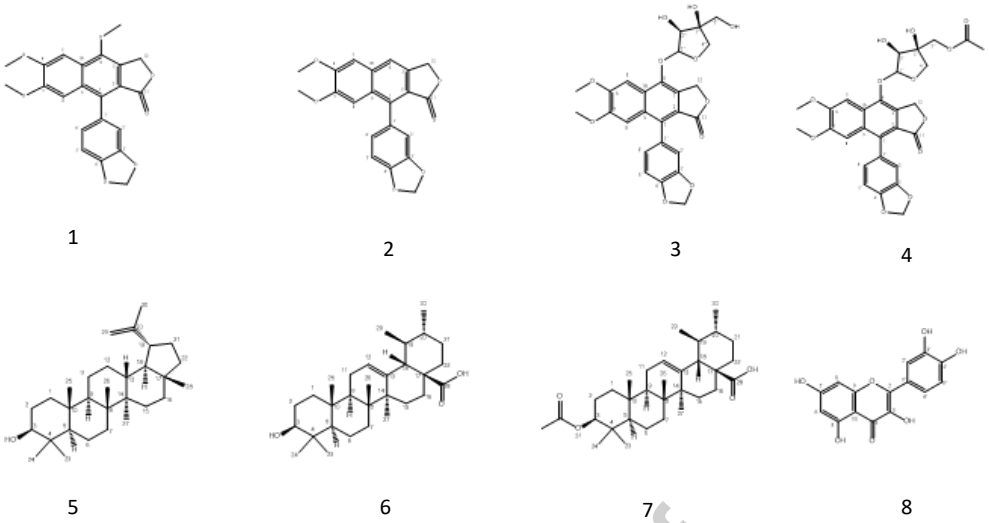

Figure 1

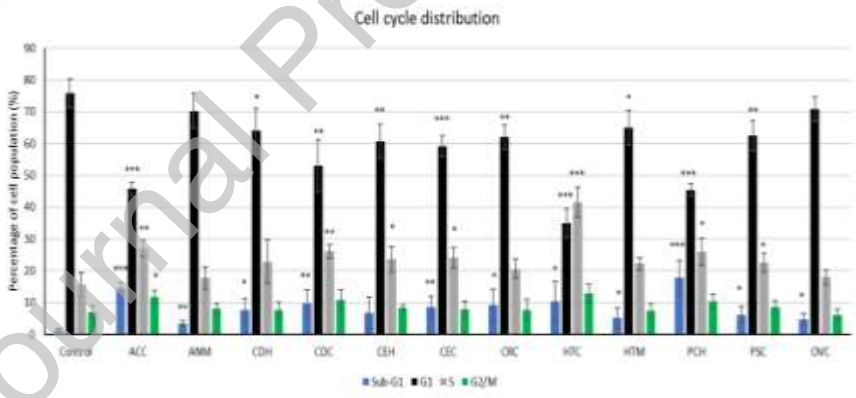

Figure 2

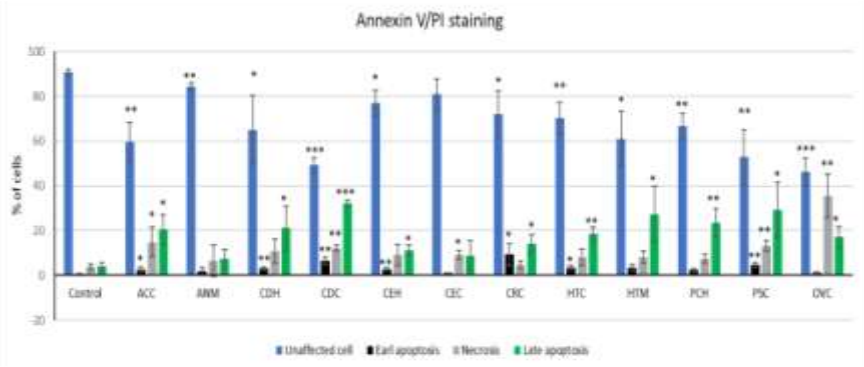

Figure 3

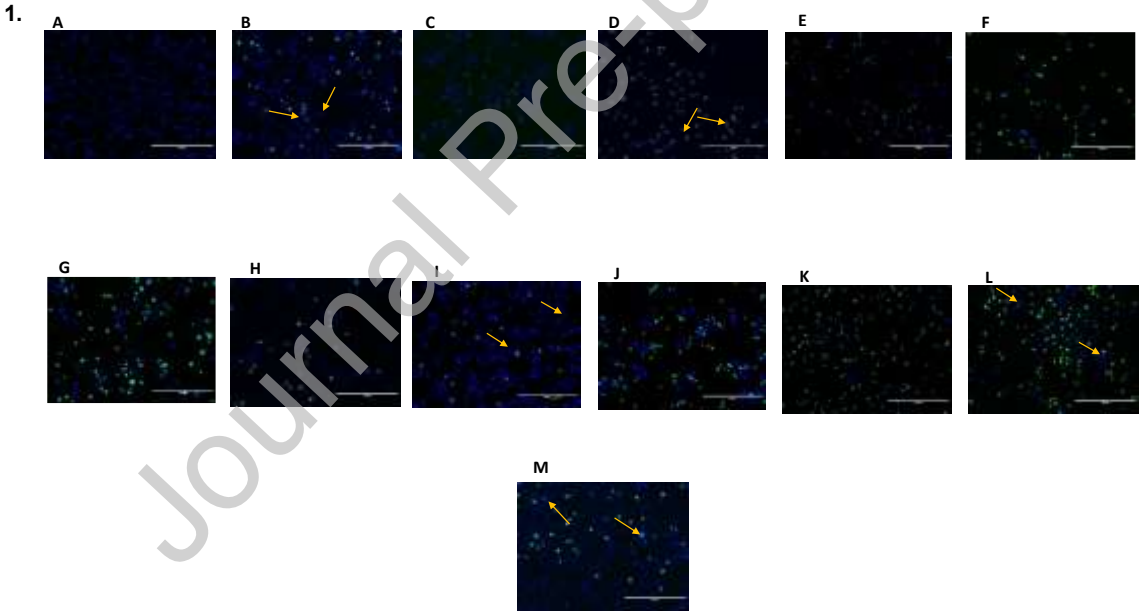

Figure 4

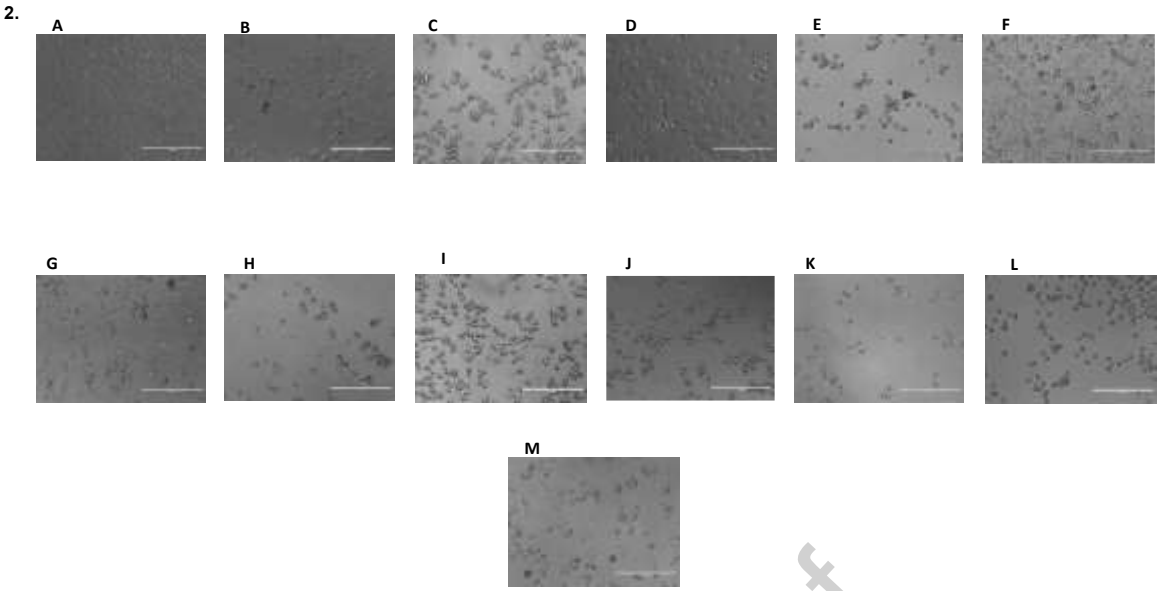

Figure 5

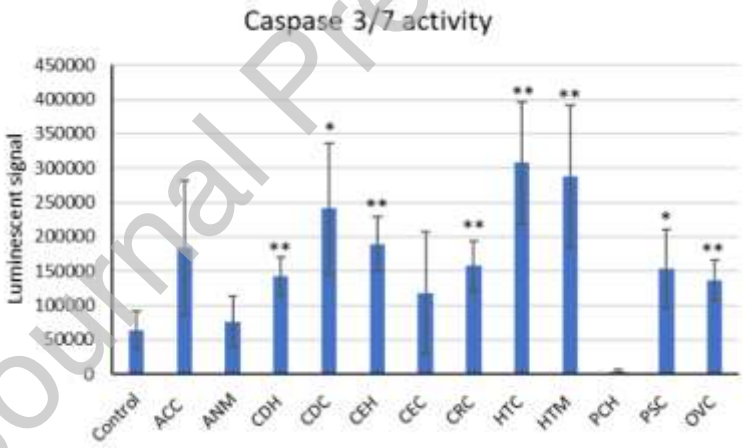

Figure 6

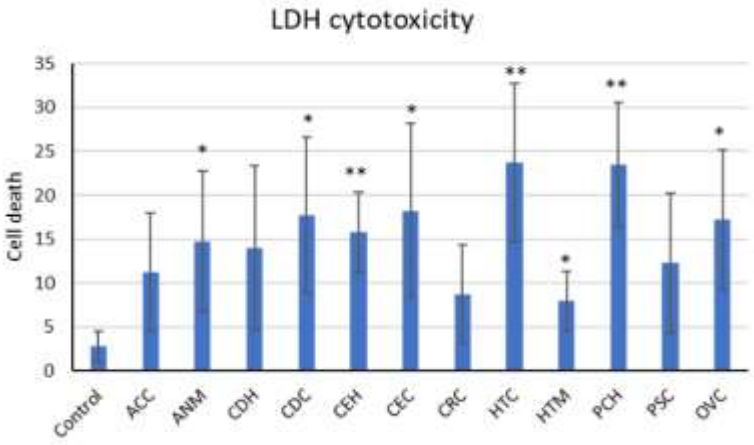

Figure 7