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Assessment of the cytotoxic effect of aerial parts of *Gazania rigens* hexane extract on HRT-18 and MCF-7 cell line and chemical composition analysis using GC/MS and LC/MS

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Abstract

Gazania rigens are cultivated for their vividly colored flowerheads. The herb, belonging to the Asteraceae family, displays various classes of secondary metabolites, including terpenes, phenols and fatty acid derivatives. Antioxidant, hepatoprotective, nephroprotective, and antimicrobial activities of the herb have been evaluated and have proven to benefit humans clinically specially in case of cancer affected people. Breast cancer is the second most frequent reason for female mortality, and the third most frequent reason in the world is colorectal cancer. A previous study stated the *in vitro* anticancer effect of the genus *Gazania rigens* on the MCF-7 breast cancer cell. N-hexane extract of *Gazania rigens* aerial parts was prepared by maceration, cold extraction methods and subjected to GC/MS and LC-MS analysis to characterize its constituents. In GC/MS, the total number of identified compounds were 2-pentadecanone, 6,10,14-trimethyl, hexadecanoic acid methyl ester, 11-octadecenoic acid methyl ester, methyl stearate hexanedioic acid, and bis(2-ethylhexyl) ester. LC/MS analysis revealed and confirmed the presence of lupeol. Based on the result, n-hexane extract was evaluated for its *in vitro* anticancer effect against the MCF-7 and HRT-18 cell lines using an MTT assay. Results from the MTT assay showed there is a significant cytotoxic effect for hexane extract against both cell line HRT-18 and MCF-7, the IC_{50} value for HRT-18 was 102.2±10 ug/ml, while the IC_{50} value for MCF-7 was 121.2±12 ug/ml.

Keywords

Gazania; HRT-18 cell line; MCF-7 cell line; cytotoxic

Introduction

Cancer is a complex illness that is typically incurable and remains as one of the leading causes of death globally (1). Breast cancer is the second most frequent reason for female mortality (3), and metastases from breast cancer primarily affect the central nervous system, bone, lungs, liver, and chest wall (4). There is now no known cure for cancer. Hence, the need for a reliable and secure therapy for the cancer is important and has been considered as a main goal to decrease mortality rate. The poor solubility, the low stability of the drugs, the low proportion that the tissues absorb from the drug, and the tumor treatment resistance often limits the effectiveness of carcinoma medications (5). Additionally, a high percentage of side effects are linked to antineoplastic medications, which increase the risk of toxicity (6). Consequently, a great deal of the side effects connected to the chemotherapeutic drugs that are already available drive the search for new, extremely potent, and readily absorbable drugs. There has been a surge in pharmacological research using plant extracts in an effort to yield safer and more effective chemotherapeutic drugs that are derived from plants (7). There are more than 32,000 identified flowering plant species in more than 1,900 genera within the Asterales order, which make up the family Asteraceae. Genus *Gazania*, which belongs to the family Asteraceae of flowering plants, is native to South Africa. *Gazania* species are cultivated for their vividly coloured flowerheads, which arise in late spring and frequently bloom from summer to autumn. *Gazania rigens* is a plant belonging to Asteraceae family. *Gazania rigens* are indigenous to South Africa and Mozambique. In folk medicine, gazania has been used to treat toothaches and miscarriages. It has also been used in purgative medicines. The biological effects of *Gazania* have been part of a few reported investigations, which involve antioxidant and hepatoprotective properties of *G. nivea* (8) and the antibacterial activity of *G. rigens* (9). Reports also have shown that the *Gazania rigens* plant possesses anti-microbial, antioxidant, and hepatoprotective properties (9, 10). The isolated compounds from *G. rigens* include phenolic compounds (Gallic acid, 3,5- di-O-caffeoylquinic acid) and flavonoids (Rutin, Apigenin, Luteolin) (10, 11). Thus, study on HRT-18 and MCF-7 cell lines anticancer properties along with isolation and identification of some pharmacological active compound in n-hexane extract of aerial parts of *G. rigens* cultivated in Iraq, was conducted. *G. rigens* was selected as plant because there is no study for its anti-cancer effect but there is study for other species like *G. linearis.* We selected breast and colon cancer cell because they are most common cancers worldwide (2).

Materials and Methods

Plant materials

In November 2023, *G. rigens* aerial parts (stems, leaves, flowers) were gathered from Iraq, the Baghdad city at latitude 33°18′00.00″ North, longitude 44°24′00.00″ East. Prof. Dr. Sukyna Abass of the University of Baghdad's Department of Biology and College of Sciences confirmed and identified the plant. After cleaning, the parts were allowed to air dry before being processed using a machine grinder to a powder. The hexane extract was prepared using maceration (cold method). Firstly, the dried powder was weighed and then extraction was done by cold method maceration using n-hexane as a solvent for 24 hrs. Then the sample was filtered and rotary evaporator was used to dry the residue, then weighed again (12).

Initial testing of plant extracts

Chemical tests were used to check the presence of sterols, steroids, terpenoids, coumarin, flavonoid, phenolic compounds (13). The Salkowski test was for checking the presence of terpenoids. It was performed by taking 1 mL of sample in a test tube and dissolving it in 1 mL chloroform. An equal volume of concentrated sulphuric acid was added to it and the presence of red color indicated the presence of terpenoids. Keller-Kiallian's test: The Keller-Kiallian's test was performed by adding 1ml of the extracts in 1ml of glacial acetic acid, 2-3 drops of ferric chloride and 2ml of concentrated sulphuric acid was added carefully along the walls of the test tube, a reddish-brown ring indicated the presence of cardiac glycoside.

Identification and characterization of isolated lupeol by Liquid chromatography–mass spectrometry (LC–MS)

The analytical LC/MS had been carried out in the Jordan University of Science and Technology at Irbid, Jordan. The following conditions for liquid chromatography were employed: column: GL-Science-C18- 250mm x 4.6 (5um particle size) – Japan; oven temperature: 35 °C; injection volume: 10 um; flow rate: 1 ml/min; run duration: 25 min. The mass parameter was as follows: software AB-Siecx-OS, ionization mode ESI Positive, scan range (50-800 mz), ion source voltage 5500V, and LCMSMS-Q-TOF model X500 QTOF.

By Gas chromatography-mass spectrometry (GC/MS) analysis

The n-hexane extract of the *G. rigens* was analyzed using Gas Chromatograph. Agilent Technologies (7820A) Gas, GC Mass Spectrometer (5977E) USA having 30 m length, 250 µm inner diameter, and 0.25 µm film thickness were employed in the HP-5ms Ultra Inert column. The injection volume was 1µl, and the scan range was m/z 25–1000. The following parameters were used in the analysis: splitless injection type, carrier gas: helium 99.99%, pressure: 11.933 psi, temperatures of the injector are 250 °C, the inlet line is 250 °C, and the aux heaters are 300 °C.

Determination of the cytotoxicity

To assess the cytotoxic activity of n-hexane extract against Breast cancer and colon cancer, HRT-18 and MCF-7 cells were employed as models and work conducted in triplicate.

Maintenance of cell cultures

A humidified atmosphere was maintained at 37°C for the cell lines culture in MEM (US Biological, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Capricorn-Scientific, Germany), 100 IU penicillin, and 100 µg streptomycin (Capricorn-Scientific, Germany). For the experiments, exponentially growing cells were employed (14, 15).

Cytotoxicity Assays

A 96-well microplate (NEST Biotech, China) was seeded with 10,000 cells per well, and the cells were then cultured at 37 °C for 72 hours or until monolayer confluence was reached. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Elabscience, China) was utilized to examine cytotoxicity. A range of concentrations (1000, 500, 250, 125, 62, and 31 ug) were applied to the cells. After 72h of infection, MTT dye solution 28 µL of (2 mg/ml) was added to each well. Three hours were spent on the incubation process. Each well received 100 µl of DMSO, which was then incubated for 15 minutes. The optical density was determined with a microplate reader at 492 nm (14, 16). Cytotoxicity % was measured by the following equation: Cytotoxicity % = (OD Control – OD sample)/OD Control × 100, where OD control is the mean optical density of untreated wells, and OD Sample is the optical density of treated wells (17).

Statistical analysis

Using GraphPad Prism 8 and Tukey's ANOVA multiple comparison test, the collected data were statistically examined. The values were shown as the triple measurements' mean ± standard deviation.

Results and Discussion

Initial testing of plant extracts

This screening was carried out to provide an overview of the terpenoids, sterols, cardiac glycoside contained in the *G. rigens* aerial part extract. The components contained in the extract were analyzed by their compounds by color test (qualitative) with several reagents like the Salkowski test is commonly used to detect the presence of terpenoids and sterols in plant extracts, and Keller-Kiallian's test to detect the presence of cardiac glycosides, both Salkowski test and Keller-Kiallian's test gave a positive result in n-hexane extract.

GC/MS analysis

Data in Fig. 1 shows identified 20 peaks GC/MS chromatogram. Identified through comparison of the mass spectrum fragmentation, peak retention duration, peak area (%), height (%), known compounds listed in the National Institute of Standards and Technology (NIST) collection. According to the results, five chemicals were found in the n -hexane extraction.

The phytoconstituents in the n-hexane extract of *G. rigens* were found to be 2-pentadecanone, 6,10,14-trimethyl, hexadecanoic acid, methyl ester, 11-octadecenoic acid, methyl ester, methyl stearate, and hexanedioic acid, bis(2-ethylhexyl) ester. Table 1 displays the structure, molecular weight, peak area, and Chemical Abstracts Service of the five phytocompounds found in n-hexane extracts.

Identification of isolated compound by LCMS/MS spectroscopy

Recognition of peaks of lupeol isolated from *G. rigens* was done by examining mass spectra of standard data as shown in Fig. 2 and literature. From the full scan mass spectra of the isolated lupeol, and Fig. 3 show mass fragments of lupeol, some of the fragments were 218 m/z ($C_{16}H_{26}$), 203 m/z ($C_{15}H_{23}$) these fragments were closely similar to that reported in literature for lupeol (18, 19).

Cytotoxic effect

In this study, the cytotoxic effect of *G. rigens* n-hexane extract versus tumor cells was assessed using the MCF-7 and HRT-18 cell lines. The outcomes show that there is a highly significant cytotoxic action against the cell lines HRT-18 colon cancer and MCF-7 that represent breast cancer as shown in Fig. 4. The data below demonstrates the capacity of n-hexane extract to significantly suppress the proliferation of colon cancer HRT-18 and breast cancer MCF-7 cell lines in a concentration-dependent way as shown in Fig. 5. The IC_{50} of n-hexane value was 102.2 µg/ml for HRT-18; for MCF-7, the IC_{50}=121.2±12µg/ml. The data demonstrated the capacity of n-hexane extract to significantly suppress

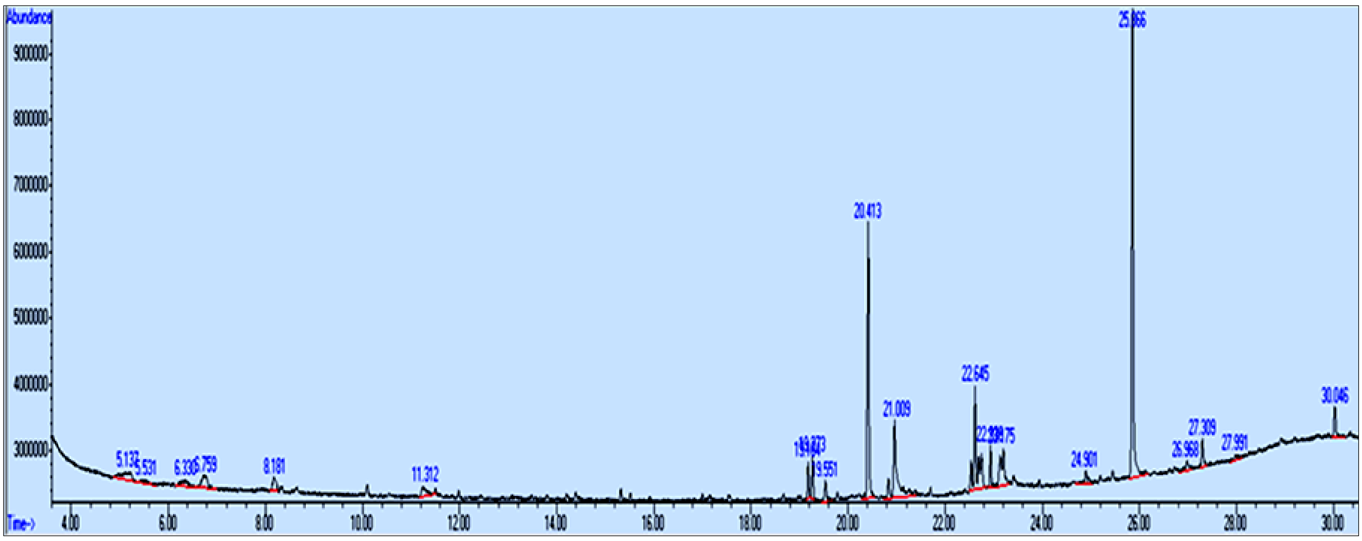

Fig. 1. GC/MS chromatogram of the *Gazania rigens* n-hexane extract.

Table 1. GC/MS analysis of hexane extract.

No	CAS	Name of the compound	Molecular formula	Molecular weight	Peak area (%)	Retention Time (min)
1	502-69-2	2-Pentadecanone, 6,10,14-trimethyl	C18H36O	268.4778	2.05	19.270
2	112-39-0	Hexadecanoic acid, methyl ester	C17H34O2	270.4507	15.10	20.413
3	52380-33-3	11-Octadecenoic acid, methyl ester	C19H36O2	296.4879	11.55	22.646
4	112-61-8	Methyl stearate	C19H38O2	298.5038	2.03	22.931
5	103-23-1	Hexanedioic acid, bis(2-ethylhexyl) ester	C22H42O4	370.5665	29.68	25.866

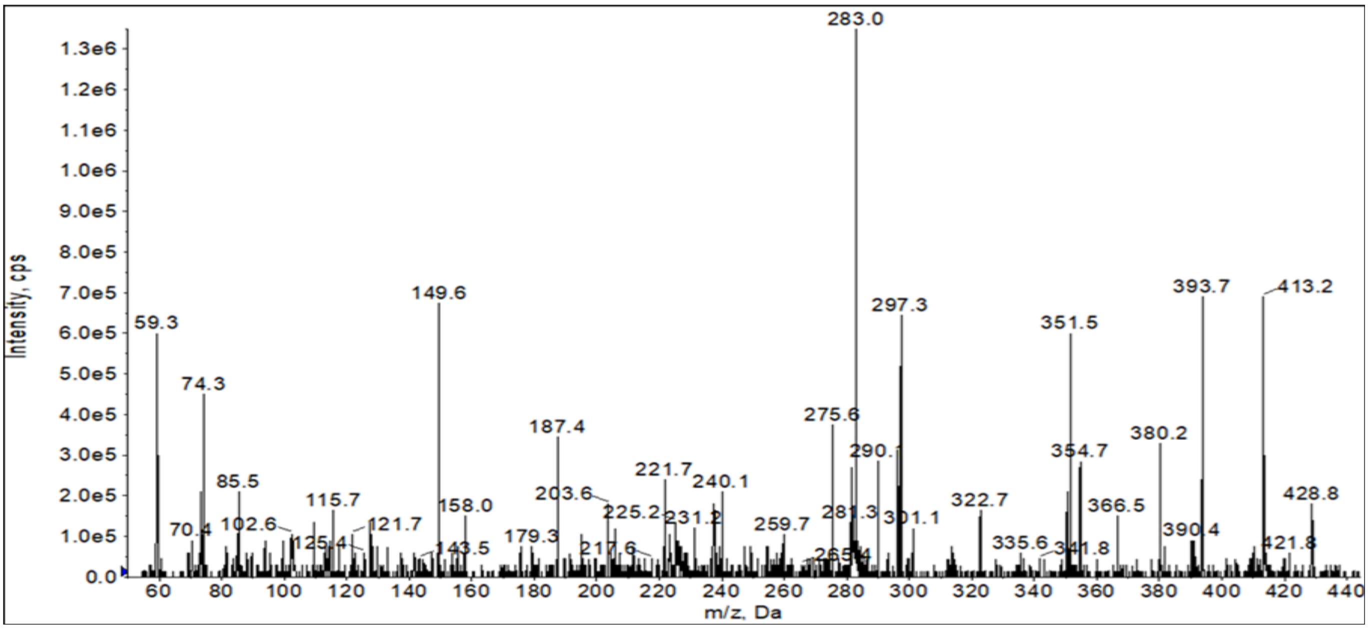

Fig. 2. LC-MS of lupeol.

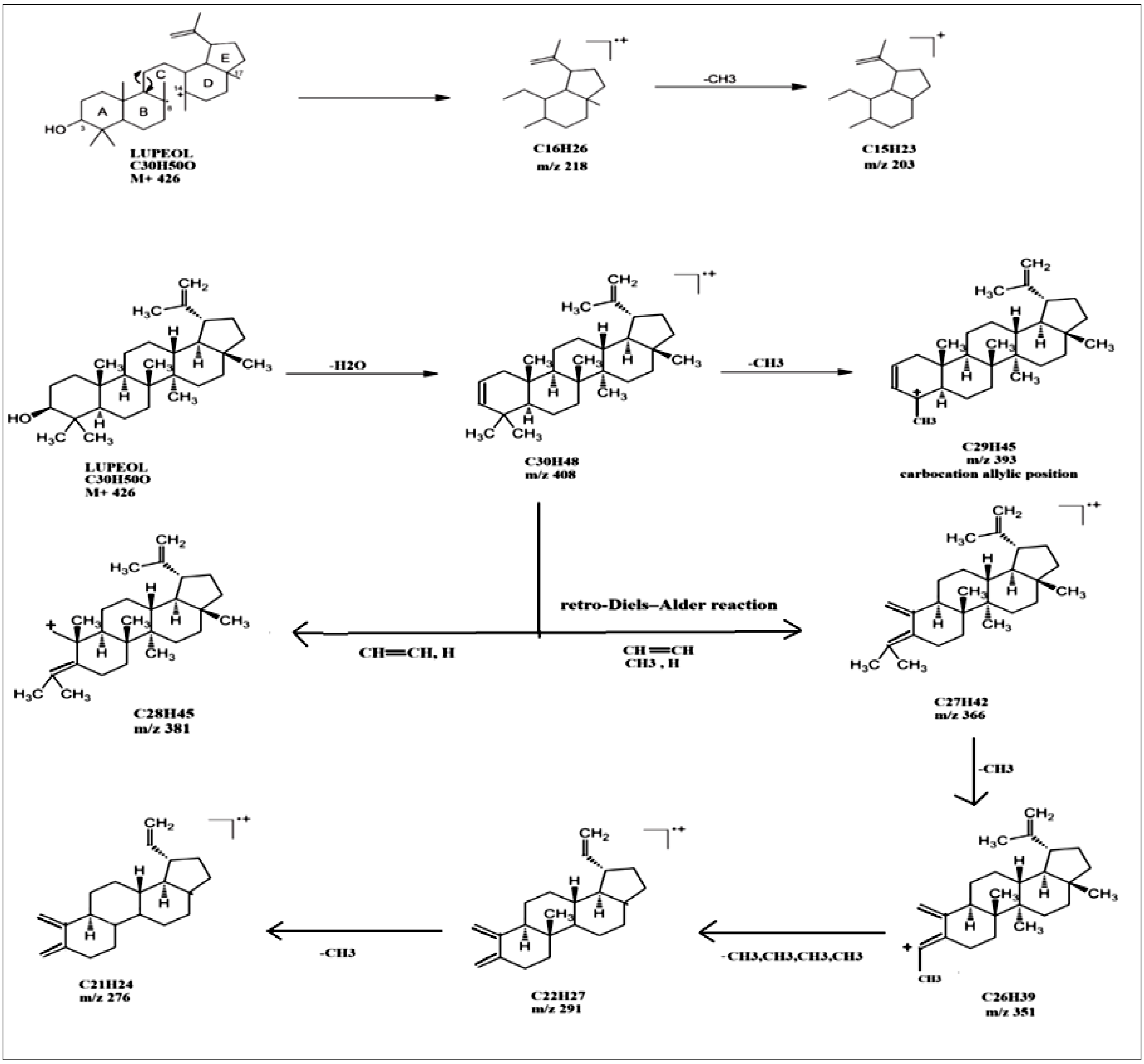

Fig. 3. Mass Fragment of lupeol.

the proliferation of colon cancer HRT-18 and breast cancer. The IC_{50} of n-hexane value was 102.2 μg/ml for HRT-18;

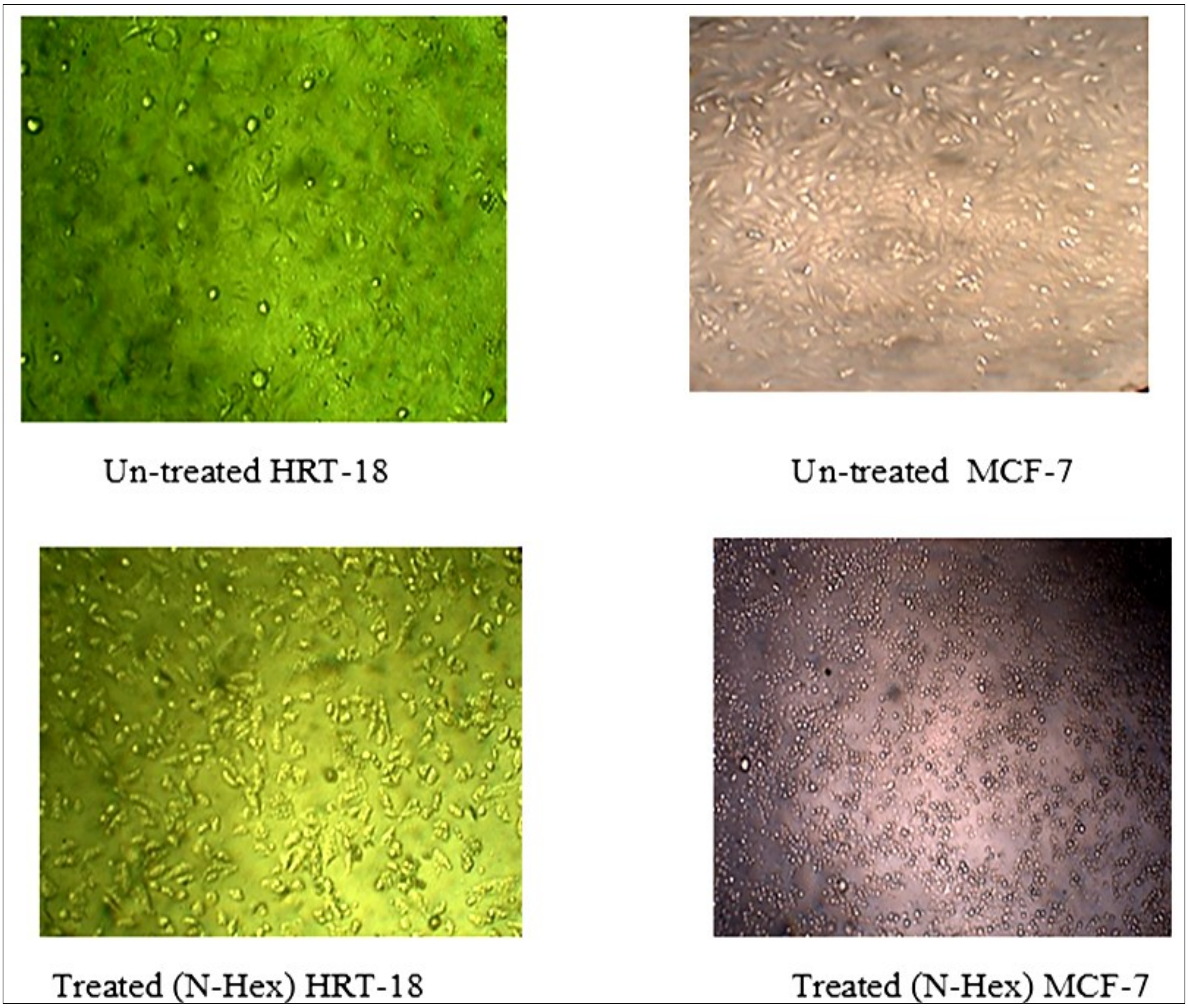

Fig. 4. Morphological changes in MCF-7 and HRT-18 cell lines before and after being treated with *Gazania rigens* n-hexane extract.

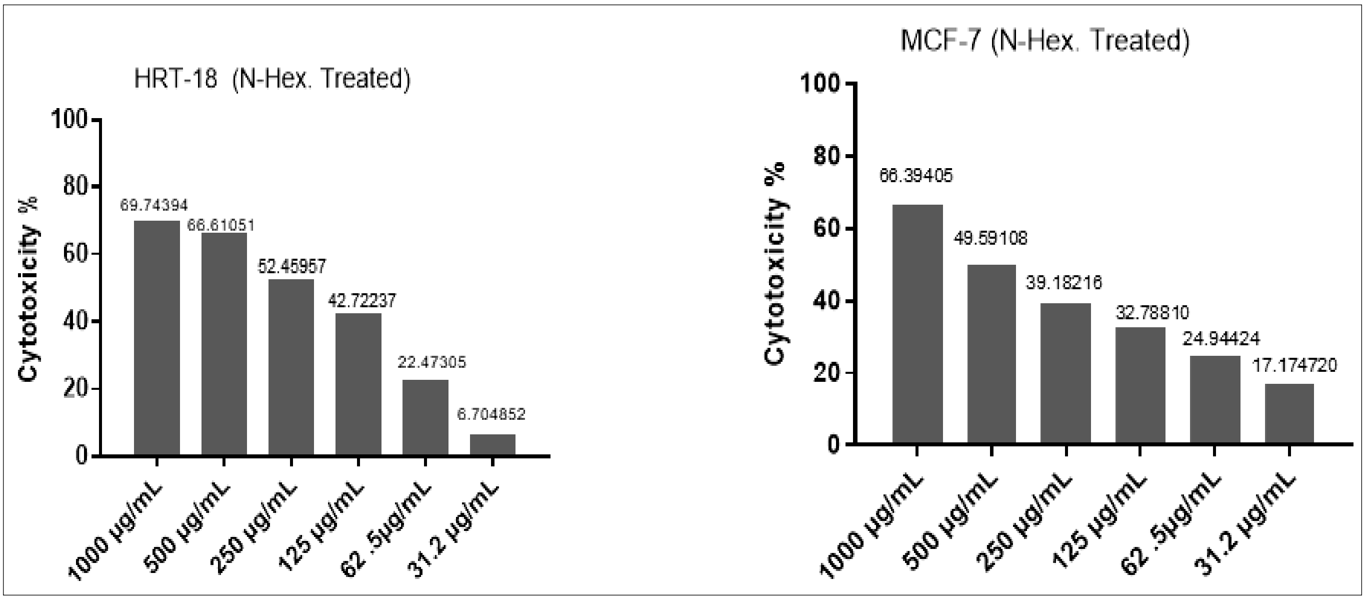

Fig. 5. Cytotoxic effect of *Gazania rigens* n-hexane extract on MCF-7 and HRT-18 in a concentration-dependent way.

for MCF-7, the IC_{50}=121.2±12µg/ml. The presence of terpenoids, fatty acids in n-hexane extract, may be the cause of their antiproliferative properties The terpenoids are able to inhibit tumor cell growth by inhibiting multiple cancer-specific targets, including the proteasome, NF-κB, and antiapoptotic protein Bcl-2 (20, 21). Lupeol suppresses EGFR/STAT3 activity, activates the mitochondrial-mediated apoptosis pathway, inhibits the Akt/PKB pathway and promotes mitochondrial hyperfission which causes cancer cells to die (22). This mechanism of action of terpenoid and lupeol may play an important role in the folk medicine use of *G. rigens* to treat toothaches and miscarriages (9). In comparison of IC_{50} of *G. rigens* n-hexane extract with a previous study done on the same extract using same MTT assay method but different plant species, IC_{50} for *G. linearis* in MCF-7 cell line was 2.43×107 ug/ml, which has higher concentration of IC_{50} by about 200,495 times than *G. rigens* n-hexane fraction IC_{50} concentration (23).

Conclusion

The plant is a rich source of terpenoids and fatty acids. The obtained data demonstrated the presence of terpenoids, fatty acids in cold method hexane extract. The present study shows that cold hexane extract *G. rigens* exhibits a significant cytotoxic effect against the colon cancer (HRT-18) cell line and breast cancer (MCF-7) cell line, this work highlights the importance of terpenoids in cancer treatment and throws a light on it as a natural rich source of terpenoid, which may be used as a supplement in the future as for cancer patient.

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Authors' contributions

The authors conceived and planned the experiments and carried out the sample preparation, extraction process, identification, isolation and structure elucidation. The authors also wrote the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

AI Declaration

None

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