PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 12(1): 1-13
https://doi.org/10.14719/pst.5134

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RESEARCH ARTICLE

Anticarcinogenic properties of the desert truffle (*Terfezia boudieri*) and its host plant (*Helianthemum aegyptiacum* (L.) Mill.)

Asmaa Ahmed Zaghloul[1], Hediat Mohamed Hamad Salama[1], Amr M Abd El_gawad[2], Mervt Almostafa[3], Khaled Abdelaal[4*], Marwa A Yassin[1]

[1]Department of Botany and Microbiology, Faculty of Science, Zagazig University, Zagazig 44519, Egypt

[2]Department of Soil Fertility and Microbiology, Desert Research Center, Cairo 11753, Egypt

[3]Department of Chemistry, College of Science, King Faisal University, Al-Ahsa 31982, Saudi Arabia

[4]Agricultural Botany Department, EPCRS Excellence Center, Plant Pathology and Biotechnology Laboratory, Faculty of Agriculture, Kafr Elsheikh University, Kafr Elsheikh 33516, Egypt

*Email: khaled_elhaies@yahoo.com

OPEN ACCESS

ARTICLE HISTORY
Received: 18 September 2024
Accepted: 13 November 2024
Available online
Version 1.0 : 11 February 2025
Version 2.0 : 22 February 2025

Check for updates

Additional information
Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
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CITE THIS ARTICLE
Zaghloul AA, Salama HMH, El_gawad AMA, Almostafa M, Abdelaal K, Yassin MA. Anticarcinogenic properties of the desert truffle (*Terfezia boudieri*) and its host plant (*Helianthemum aegyptiacum* (L.) Mill.). Plant Science Today. 2025; 12(1): 1-13.https://doi.org/10.14719/pst.5134

Abstract

This study aimed to investigate the phytochemicals and cytotoxic properties of aqueous extracts of desert truffle, *Terfezia boudieri* Chatin and its host plant *Helianthemum aegyptiacum* (L.) Mill. The chemical composition of truffle is relatively higher than its host plants. Carbohydrate content showed the highest rate, but crude lipid showed the lowest rate in truffle and the host plant. Potassium is the highest concentration macro-element, and iron is the highest concentration of micro-element in the truffle and host plant. Compared to the several extracts tested, ethyl acetate extracts of the desert truffle, *T. boudieri* and host plant *H. aegyptiacum* (L.) Mill. gave the highest cytotoxic activity against five tested cancer cell lines (the human eye carcinoma cell line MP38, the human central nervous system cell line SF-268, the colorectal carcinoma cell line HCT116, the prostate cancer cell line DU-145 and the breast cancer cell line MDA). The active substances of truffles are more effective than the active substances of host plants in terms of cell mortality rate and nuclear condensation of cancer cells. Human eye cancer cells MP38 treated with truffle ethyl acetate extract showed a greater cell mortality rate than those treated with host plant ethyl acetate extract. Thus, it could be concluded that desert truffles have distinctive metabolites with powerful biological activities, such as antiproliferative activities, compared to the host plant's corresponding metabolites.

Keywords

cytotoxic effect; host plant; mortality rate; nuclear condensation; phytochemical properties; truffle

Introduction

The desert truffle of the genera *Terfezia* spp. are hypogeal ascomycetes that naturally inhibit the Mediterranean countries (1, 2), in the form of mycorrhizal associations on the roots of different species of the genus *Helianthemum* (3). This truffle grows in various soil types with different characteristics, particularly in association with plant species adapted to their soil, such as the family Cistaceae, mainly the genus *Helianthemum* (4-6). Truffles mainly belong to the order Pezizales, which grows and is

distributed in the Mediterranean basin's arid and semiarid climate regions (2, 7). Among mycorrhizal desert truffles fungi, the well-known edible fungi of *Terfezia* are white and dark brown (8), which usually inhibits the annual or perennial herbaceous *Helianthemum* spp. (9, 10).

Truffle biochemical composition encompasses multiple nutritional and medicinal benefits. Truffles are rich in essential nutritional products, including carbohydrates, proteins, fats, minerals, lipids, and amino acids (11). Furthermore, they contain high concentrations of phytosterols, phenolics, terpenoids, and polysaccharides, all of which have antibacterial, antioxidant, anticancer properties, hepatoprotective, anti-inflammatory and immunomodulatory properties (10). In recent decades, researchers have paid great attention to the bioactive compounds derived from diverse truffle species and their potential in nutritional and medicinal applications (12). Cancer is the most prevalent and deadly disease in the modern period (13). Since most known cancer medicines have side effects and different cancers respond differently to treatment, new approaches or substances must be found. Plant-based medicines have good potential as a primary source of chemotherapeutic drugs. Plants were originally the source of several commonly used chemotherapeutic drugs, including vinca alkaloids for leukemia, paclitaxel for breast cancer, and flavopiridol for colorectal cancer (14). In addition, herbal products are still used as primary healthcare products in most third-world countries (15). Accordingly, plants as herbal medicines are good sources for searching for antitumor compounds (16).

Cell mortality rate means cytolysis occurs and then digests the dying cell. For a long time, it was thought that the primary mechanism of cell death was the activation of endonucleases and specific proteases (17). Cell mortality rate is one of the most researched subjects among cell biologists. Since the underlying process of cell mortality is essential to the etiology of many diseases, it is crucial to understand it. Certain diseases, such as degenerative ones, are caused by excessive cell death; cancerous disorders, on the other hand, are caused by insufficient cell death (18). The cell mortality model consists of three steps: a pro-mitochondrial phase during which signal transduction cascades or damage pathways are activated; the mitochondrial stage, in which mitochondrial membrane function is lost; the post-mitochondrial stage, where proteins released from the mitochondria cause the activation of proteases and catabolic nucleases and the appearance of DNA fragments (17).

It is considered that desert truffles are a source of medicinal substances with anti-inflammatory, immunosuppressive, antimutagenic, anticarcinogenic, antioxidant, and antiradical properties (19). The juice of the truffle (*Terfezia claveryi*) was used to treat trachoma patients, showing great promise for treatment methods in contrast with currently available synthetic antibiotics (20). Truffles have been recognized for their potential antimicrobial activities, especially against multidrug-resistant bacteria and fungi (21, 22). Truffles have been reported to possess anti-inflammatory, immunosuppressive and anticarcinogenic properties (23). Thus, this study aimed to investigate the phytochemical, cytotoxic properties, and mortality rate of aqueous extracts of desert truffle *T. boudieri* Chatin inhabiting the host plant *H. aegyptiacum* (L.) Mill.

Materials and Methods

The area of study is located on the Mediterranean northern coast. Samples of *T. boudieri* Chatin and host plant *H. aegyptiacum* (L.) Mill. were collected from Ras El-Hekma in January 2020 and identified in the Zagazig University Herbarium, where they are kept (Fig. 1).

Extracts preparation of truffle and their host plant

The truffle *T. boudieri* and the young leaves of the host plant *H. aegyptiacum* (L.) Mill. were air-dried and transported to the laboratory for extraction. The truffle samples were dried in an oven at 35–40°C and then ground to a fine powder using a blender. The truffle *T. boudieri* and the host plant *H. aegyptiacum* (L.) Mill. were turned into fine powder using a mortar and preserved in well-stopped bottles for phytochemical analysis. The experiments were repeated twice, and the mean values were determined. 100 grams of the powdered samples were extracted using hexane, ethyl acetate, ethanol, and

Fig. 1. The desert truffle *T. boudieri* and its host plant *H. aegyptiacum* (L.) Mill. growing under natural conditions.

methanol, in succession, using a soxhlet apparatus for approximately 6 hours. The solvents were then evaporated under reduced pressure using a sotary evaporator apparatus. The extracts were left to air dry until complete dryness. The hexane extract was dissolved in hexane, the ethyl acetate extract was dissolved in ethyl acetate, the ethanol extract was dissolved in ethanol, and the methanol extract was dissolved in methanol (24). To prepare the water extraction, 100 grams of the powdered samples were boiled in 500 mL of distilled water in a water bath at 70°C for 15 minutes and then filtered. The filtrate was dried using a freeze-dryer apparatus, and the extract was dissolved in water (25).

The proximate compositions

The proximate compositions of the truffle *T. boudieri* and the host plant *H. aegyptiacum* (L.) Mill. which include moisture, dry matter, crude protein, crude lipid, crude ash and organic matter, were determined using the Latimer (26) methodology. The total amount of nitrogen (N) was determined using the Kjeldahl method. N×6.25 was used to compute the crude protein. Crude fat was determined using the soxhlet extraction method, where complete ash was burned at 550°C in addition to a solvent. The formula for calculating organic matter is % dry matter - ash. crude fiber can be subtracted from the percentage of the proximate components to determine the carbohydrate content (26).

Element analysis

After being re-dried overnight at 105°C, the truffle *T. boudieri*, and the host plant *H. aegyptiacum* (L.) Mill. were crushed with a crusher and pestle. Using a mixture of HNO_3: H_2SO_4: H_2O_2 (10:1:1, 12 mL for 1 g sample), the truffle and host plant samples were digested for ten to fifteen minutes at 100°C. After cooling, add 50 mL of deionized water, and the mixture is filtered. To determine all materials, use all cleaned glassware with deionized water. While amounts of Fe, Zn, Mn, Cu, Mg, P, and Co were determined by atomic absorption spectrometer, amounts of K, Ca, and Na were determined by atomic emission spectrometer (26).

Determination of some secondary metabolisms

Chemical tests were carried out on the aqueous extract and the powdered specimens using a standard procedure to identify the constituents (27).

Total phenolic content

2 g of dried powder samples in 10 mL of 80% ethanol were centrifuged. The residue was dissolved in 3 mL distilled water, and then 0.5 mL of Folin-Ciocalteau reagent and 2 mL of 20% $NaCO_3$ solution were added to each tube. Incubation for 1 minute was cooled, and the optical density of color was measured at 650 nm using a UV-Vis spectrophotometer (UNICO, Japan). Catechol (100 mg/ 100 mL distilled water) was used as standard (28).

Total alkaloids determination

After mixing 3 mL of each extract with 1 mL of methanol in a test tube, the mixture was heated for 20 minutes, filtered, cooled, and Wagner's alkaloidal reagent was added to detect the presence of alkaloids by precipitates. 5 grams of the sample was mixed with 200 mL of 10% acetic acid in ethanol. The mixture was then filtered, and ammonium hydroxide was used until precipitation was complete. The resulting alkaloid residue was dried and weighed (29).

Total flavonoids content

For flavonoid indication, 3 mL of an extract was mixed with 1 mL of 10% NaOH, which appears yellow if present. We extracted 10 grams of samples using 100 mL of 80% methanol. Next, we filtered the extracts and transferred them into a crucible. The extracts were evaporated to dryness over a water bath and weighed until reaching a constant weight (28).

Total saponins

2 g powder samples were boiled in 20 mL of water, filtrated and shaken vigorously for a stable froth. Then, frothing was mixed with a few drops of olive oil and shaken vigorously to form the emulsion. For determination, 20 g of each sample was mixed with 100 mL ethanol (20%) at 55°C for 4 hours, filtrated, and the residue was re-extracted with 200 mL ethanol (20%). The extracts were reduced to 40 mL at a 90°C water bath. The concentrate was separated into a funnel, 20 mL of diethyl ether was added shaken vigorously, and then 60 mL of n-butanol and 20 mL of NaCl (5%) were added. After heating, the samples were dried in the oven until constant weight (30).

Total tannins

In a test tube, 0.5 g of dried powder samples were boiled in 20 mL of water, then filtrated, then ferric chloride (0.1%), and then observed for brownish-green or blue-black coloration. For tannins determination, 1 g of plant material was extracted in 50 mL of methanol and centrifuged. 5 mL of vanillin hydrochloride reagent was added to the supernatant (1 mL) and then measured at 500 nm the optical density of the color that appeared after 20 minutes. Tannic acid was used as a standard (28).

Cancer cell lines

The cancer cell lines used in the experiments were the human eye carcinoma cell line MP38, the human central nervous system cell line SF-268, the colorectal carcinoma cell line HCT116 (CCL-247 | ATCC), the prostate cancer cell line DU-145 (HTB-81 | ATCC) and the breast cancer cell line MDA (MB-231/ATCC) were collected from Creative Egyptian Biotechnologists (CEB), Egypt. These cell lines were maintained in RPMI 1640 medium and Dulbecco's modified Eagle's medium (CEB, Egypt).

Cell lines maintenance

The cell lines (SF-268, and HCT116) were maintained in DMEM medium Dulbecco's modified Eagle's medium (CEB, Egypt). The DMEM medium contained 10% fetal bovine serum (FBS) (Sigma, Egypt), 4% glucose (Sigma, Egypt), and sodium pyruvate (Sigma, Egypt). Cell lines were incubated at 37°C in 10 CO_2 (Sigma, Egypt) with 2% sodium bicarbonate buffer system (Sigma, Egypt), which helps maintain physiological pH levels and 95% humidity.

The cell lines (MP38, MDA, and DU-145) were maintained in RPMI 1640 medium (CEB, Egypt). The RPMI 1640 medium contained 10 fetal bovine serum (FBS) (Sigma, Egypt), 1% glutathione (Sigma, Egypt), 1% biotin (Sigma, Egypt), 1% vitamin B12 (Sigma, Egypt), 1% para-aminobenzoic acid (PABA) (Sigma, Egypt), 30% vitamins inositol (Sigma, Egypt), and 25 choline (Sigma, Egypt). Cell lines were incubated at 37°C in 10% CO_2 (Sigma, Egypt) with a 2% sodium bicarbonate buffer system (Sigma, Egypt), which helps maintain physiological pH levels and 95% humidity.

Cytotoxicity analyses

The MTT protocol assessed the cytotoxicity of the different fungal and plant extracts. The 96-well plate was seeded at 1.5×10^4 cell lines per well in 100 µL in fresh culture medium and incubated at 37°C in 5% CO_2 for 16 hours. After being incubated for 48 hours, the cell lines reached a concentration of 100 µg/mL of extracts. The MTT solution (5 mg/mL in sterile PBS-phosphate buffered saline) was added and incubated for 4 hours at 37°C in 5% CO_2. After incubation, the wells were solubilized with 100 µL DSMO, and the fluorescence intensity was measured at 570 nm by Freedom evo TECAN. Each plate contained the samples and control. DMSO (1% v/v) was used as blank. Butanoic acid and 5-fluorouracil used to treat cancer as standard reference control for MP38, SF-268, HCT116, DU-145 and MDA cell lines (31).

Mitochondrial membrane potential

A major cause of cell mortality is the decrease in mitochondrial membrane potential. Mitochondrial membrane potential changes in MP38 cells treated with ethyl acetate extracts of truffle and host plant were detected by Rhodamine 123 fluorescence retention. Cell lines were transplanted in individual 96-well plates and incubated overnight. Cell lines were treated with ethyl acetate truffle and host plant extracts at 100 µg/mL concentration, each one individually at 16 hours, and then fixed for 30 minutes using 4% paraformaldehyde. DMSO (0.1%) (No. C6295- Sigma-Aldrich) were used as blank. Butanoic acid (100 µg/ mL) were used as control. Ultimately, mitochondria were stained with Rhodamine 123 for 30 minutes in incubation, then imaged by fluorescence and transmitted light application at high magnification using an EVOS fluorescence microscope-digital inverted microscope (32).

Nuclear condensation assay

The effect of ethyl acetate truffle and host plant extracts on nuclear chromatin condensation in hypersensitive cell line MP38 was measured using DAPI (4′,6-diamidino-2-phenylindole) staining solution, a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA. The cell line was treated with ethyl acetate truffle and host plant extracts (100 µg), both of them alone, and analyzed at 16 hours. DMSO (0.1%) was used as blank and butanoic acid (100 µg/mL) as control. Then samples were fixed for 20 minutes using 4% paraformaldehyde. Then, the DAPI solution (1 µg stain/mL in phosphate-buffered saline) was added after 20 minutes, and then nuclear morphology was examined by fluorescence microscopy. The cell line with shrunken or fragmented brightly colored nuclei was considered cell mortality. The number of cell lines was counted in randomly selected fields for each well. The cell line was photographed by fluorescence and transmitted light application under magnification 20x using an EVOS fluorescence digital inverted microscope (33).

Statistical analysis

Variations of analytical and experimental data are expressed as mean ± SD. Analysis of variance and significant difference among means were tested by one-way ANOVA, and considered significant at $P < 0.05$. The SPSS statistical 15.0 package was utilized to perform statistical analysis as a statistical hypothesis test (34).

Results

Phytochemical analysis

Determination of proximate constituents in truffle and its host plant: The results of the estimation of the moisture, dry matter, total ash content, crude fiber, crude lipid content, total nitrogen content, crude protein, organic matter content, and total carbohydrates are presented in Table 1.

The results showed that the truffle had a dry matter value of 27.26% compared to 24.16% in the host plant. The moisture content varied from 66.21% in the truffle to 63.99% in the host plant. The ash content showed variation in the truffle and the host plant with 14.44% and 11.32%, respectively. The crude fiber content showed a relatively wide range of variation, ranging from 10.24% in the truffle to 8.12% in the host plant. The truffle had a higher crude lipid content of 2.49% compared to 2.01% in the host plant. The total nitrogen content of the truffle and the host plant showed a little variation at 5.64% in the truffle and 4.73% in the host plant. The crude protein content of the truffle and the host plant exhibited a wide range variation ranging from 34.21% in the truffle to 29.25 in the host plant. The truffle had a higher organic matter content of 26.46% compared to 22.06% in the host plant. The total carbohydrate content showed variation in

Table 1. Proximate constituents in *T. boudieri* (black truffle) and *H. aegyptiacum* (L.) Mill. host plant

Nutrients (%)	Truffle *T. boudieri*	Host plant *H. aegyptiacum* (L.) Mill.
Dry matter	27.26*± 1.63	24.16*± 1.39
Moisture	66.21*± 2.51	63.99*± 2.45
Ash	14.44*± 0.15	11.32*± 0.13
Fiber	10.24*± 0.32	8.12*± 0.27
Lipid	2.49*± 0.11	2.01*± 0.11
Total Nitrogen	5.64*± 0.10	4.73*± 0.51
Protein	34.21*± 1.41	29.25*± 1. 25
Organic matter	26.46*± 1.12	22.06*± 1.01
Carbohydrates	48.56*± 2.91	35.11*± 2.11

*Values are the means of three replicates ±SD

the truffle, with 48.56% higher than 35.11% in the host plant (Table 1).

Elemental analysis of truffle and the host plant: The concentrations of the macro (Na^+, K^+, Ca^{++}, Mg^{++}, P) and micro-elements (Fe, Cu, Mn, Zn, and Co) are estimated in the truffle and the host plant, as shown in Table 2.

The results showed that potassium is the highest concentration macro-element in the truffle and the host plant, with 10.78 and 8.89 mg/g dry weight, respectively. Phosphorus recorded a high concentration in truffles with 3.29 mg/g dry weight and in the host plant with 2.81 mg/g dry weight, respectively. The calcium concentration ranged from 1.98 mg/g dry weight in the truffle to 1.28 mg/g dry weight in the host plant. Magnesium showed a narrow range value in truffle and host plants ranging from 1.68 to 1.12 mg/g dry weight, respectively. Sodium is the lowest concentration recorded in the truffle with 0.68 mg/g dry weight and in the host plant with 0.51 mg/g dry weight (Table 2).

Iron concentration is the highest concentration of micro-element in the truffle and host plant with 3.21 and 2.91 mg/g dry weight, respectively. Zinc was recorded to have high concentrations in the truffle and the host plant, with 1.99 and 1.25 mg/g dry weight, respectively. Cobalt and manganese concentrations showed a narrow range of variation that ranged from 0.98 and 0.45 mg/g dry weight in truffle, to 0.37 and 0.31 mg/g dry weight in the host plant, respectively. Copper is the lowest concentration of micro-element was recorded in the truffle with 0.11 mg/g dry weight and in the host plant with 0.09 mg/g dry weight. This showed that mineral elements are higher in desert truffles than in their host plant (Table 2).

Quantitative analysis of some Secondary metabolites: The comparative study of truffle *T. boudieri* and the host plant *H. aegyptiacum* (L.) Mill. extracts revealed that they were high in phenolics, saponins, and tannins composition. However, truffle exhibited the highest significant value compared to the host plant (Table 3).

Table 2. Mineral elements concentration of *T. boudieri* (black truffle) and *H. aegyptiacum* (L.) Mill. host plant

	Elements (mg/ g dry weight)	Truffle *T. boudieri*	Host plant *H. aegyptiacum* (L.) Mill.
Macro-elements (mg/ g dry weight)	Na^+	0.68*± 0.15	0.51*± 0.13
	K^+	10.78*± 1.91	8.89*± 1.51
	Ca^{++}	1.98*± 0.43	1.28*± 0.41
	Mg^{++}	1.68*± 0.61	1.12*± 0.40
	P	3.29*± 0.52	2.81*± 0.32
Micro-elements (mg/ g dry weight)	Fe	3.21*± 0.38	2.91*± 0.28
	Cu	0.11*± 0.02	0.09*± 0.02
	Mn	0.45*± 0.03	0.31*± 0.02
	Zn	1.99*± 0.11	1.25*± 0.05
	Co	0.98*± 0.08	0.37*± 0.03

*Values are the means of three replicates ±SD

Table 3. Concentration of secondary compound in *T. boudieri* (black truffle) and *H. aegyptiacum* (L.) Mill. host plant

Secondary compound (mg/g DW)	Truffle *T. boudieri*	Host plant *H. aegyptiacum* (L.) Mill.
Phenolics	2.12*± 0.19	1.98*± 0.11
Alkaloids	1.39*± 0.13	1.01*± 0.81
Flavonoids	1.67*± 0.17	1.11*± 0.02
Saponins	2.21*± 0.14	2.04*± 0.14
Tannins	1.84*± 0.09	1.56*± 0.08

*Values are the means of three replicates ±SD

The results showed that saponins have the highest concentration with 2.21 mg/g dry weight in truffle, with 2.04 mg/g dry weight in the host plant, followed by phenolics with 2.12 mg/g dry weight in truffle, and 1.98 mg/g dry weight in the host plant. Tannins were recorded with 1.84 mg/g dry weight in truffle and 1.56 mg/g in the host plant. Flavonoids recorded 1.67 mg/g dry weight in the truffle but 1.11 mg/g dry weight in the host plant. Alkaloids recorded the lowest value in the truffle and their host plant, with 1.39 and 1.01, respectively (Table 3).

Cytotoxic effect of the desert truffle and its host plant

The study assessed the cytotoxic potential of desert truffle, specifically *T. boudieri,* along with its host plant *H. aegyptiacum* (L.) Mill. The effect of the *T. boudieri* and *H. aegyptiacum* (L.) Mill. extracts on 5 types of cancer cell lines (MP38, SF-268, HCT116, DU-145 and MDA) were presented in Table 4.

The results showed that *T. boudieri* recorded that the ethyl acetate extract as the preferred solvent significantly inhibited the human eye carcinoma cell line (MP38). The most powerful extract was recorded with ethyl acetate with IC_{50} values of 280.8 ± 10.4 µg/mL, followed by ethanol extract with an IC_{50} value of 209.3 ± 9.11 µg/mL. Additionally, the methanol and hexane extracts demonstrated a moderate level of cytotoxicity against MP38, with corresponding IC_{50} values of 196.3 ± 11.1 and 193.5 ± 11.9 µg/mL.

The methanol extract inhibited the human central nervous system cell line of SF-268 with an IC_{50} of 201.1 ± 11.5 µg/mL, followed by ethyl acetate extract with an IC_{50} value of 181.2 ± 10.9 µg/mL. Moderate cytotoxicity against SF-268 has been observed with ethanol and hexane with an IC_{50} value 179.3 ± 7.8 and 165.5 ± 6.1 µg/mL, respectively. Ethyl acetate extract showed inhibition to the human colorectal carcinoma cell line of HCT116 with an IC_{50} value of 215.3 ± 9.5 µg/mL, followed by methanol extract with an IC_{50} value of 192.8 ± 9.5 µg/mL. The moderate cytotoxicity against HCT116 has been assessed for ethanol and hexane with an IC_{50} value of 180.3 ± 7.2 and 149.4 ± 6.5 µg/mL, respectively.

Also, ethyl acetate extract inhibited the human prostate cancer cell line of DU-145 with an IC_{50} value of 213.2 ± 9.3 µg/mL, followed by methanol extract with an IC_{50} value of 189.2 ± 8.9 µg/mL. The moderate cytotoxicity against DU-145 was found with ethanol and hexane extracts, recording IC_{50} values of 162.8 ± 6.9 and 130.2 ± 7.1 µg/mL, respectively.

Table 4. Cytotoxic activities of different extracts of *T. boudieri* (black truffle) and *H. aegyptiacum* (L.) Mill. host plant

	Cells	Hexane	Ethyl acetate	Ethanol	Methanol	Water
T. boudieri	MP38	193.5*± 11.3 A	280.8*± 10.4 K	209.3*± 9.11 U	196.3*± 11.1 E	328.5*± 11.9 O
	SF-268	165.5*± 6.1 B	181.2*± 10.4 L	179.3*± 7.8 V	201.1*± 11.5 F	342.4*± 11.5 P
	HCT116	149.4*± 6.5 C	215.3*± 9.5 M	180.3*± 7.2 W	192.8*± 9.5 G	415.9*± 11.7 Q
	DU-145	130.2*± 7.1 D	213.2*± 9.3 N	162.8*± 6.9 X	189.2*± 8.9 H	309.1*± 11.4 R
	MDA	139.2*± 7.9 E	279.2*± 11.2 O	195.8*± 10.5 Y	172.6*± 9.4 I	301.2*± 11.3 S
***H. aegyptiacum* (L.) Mill.**	MP38	130.8*± 7.5 F	199.2*± 10.3 P	160.1*± 6.2 Z	162.3*± 6.9 J	272.5*± 11.5 T
	SF-268	125.1*± 8.1 G	140.5*± 9.1 Q	145.3*± 9.2 A	151.3*± 2.5 K	281.4*± 11.2 U
	HCT116	115.3*± 9.9 H	170.1*± 10.5 R	153.1*± 8.5 B	132.1*± 8.1 L	379.1*± 11.5 V
	DU-145	90.2*± 6.1 I	181.2*± 3.3 S	121.9*± 7.2 C	149.3*± 9.3 M	265.1*± 10.1 W
	MDA	101.6*± 8.5 J	193.9*± 8.1 T	161.9*± 6.8 D	142.2*± 9.1 N	250.5*± 10.3 X

*Values are the means of three replicates ±SD, and also (a, b, c, d, etc. is the significant letters).

Eventually, ethyl acetate inhibited the human breast cancer cell line of MDA with an IC_{50} of 279.2 ± 11.2 µg/mL, followed by ethanol extract with an IC_{50} of 195.8 ± 10.5 µg/mL. The moderate cytotoxicity against MDA was methanol and hexane with an IC_{50} value of 172.6 ± 9.4 and 139.2 ± 7.9 µg/mL, respectively. It is noteworthy that the water extract showed the weakest inhibition effect with an IC_{50} of 301.2 ± 11.3 and 309.1 ± 11.4 µg/mL toward the MDA and DU-145 cell lines, respectively, than 328.5 ± 11.9, 342.4 ± 11.5 and 415.9 ± 11.7 toward the MP38, SF-268 and HCT116 µg/mL cell lines, respectively.

H. aegyptiacum (L.) Mill. recorded that the ethyl acetate` extract significantly inhibits the human eye carcinoma cell line (MP38). It is the strongest extract with IC_{50} values of 199.2 ± 10.3 µg/mL, followed by methanol extract with IC_{50} values of 162.3 ± 6.9 µg/mL. However, ethanol and hexane extracts showed moderate cytotoxicity against MP38 with an IC_{50} value of 160.1 ± 6.2 and 130.8 ± 7.5 µg/mL, respectively. Eventually, methanol inhibited the human (CNS) central nervous system cell line of SF-268 with an IC_{50} of 151.3 ± 2.5 µg/mL, followed by ethanol with an IC_{50} of 145.3 ± 9.2 µg/mL. The moderate cytotoxicity against SF-268 was ethyl acetate and hexane with an IC_{50} value of 140.5 ± 9.1 and 125.1 ± 8.1 µg/mL, respectively.

As the ethyl acetate extract inhibited the human colorectal carcinoma cell line of HCT116, the human Prostate cancer cell line of DU-145, and the breast cancer cell line MDA with an IC_{50} of 170.1 ± 10.5, 181.2 ± 3.3 and 193.9 ± 8.1 µg/mL, followed by ethanol extract with IC_{50} value of 153.1 ± 8.5 and 161.9 ± 6.8 µg/mL toward HCT116 and MDA, but hexane extract with IC_{50} of 15.3± 1.9 µg/mL toward HCT116, but methanol extract inhibited DU-145 with an IC_{50} of 149.3 ± 9.3. Methanol had a moderate cytotoxicity against HCT116 and MDA with IC_{50} values 132.1 ± 8.1 and 142.2 ± 9.1 µg/mL, respectively. However, ethanol has a moderate cytotoxicity against DU-145 with an IC_{50} of 121.9 ± 7.2 µg/mL. Also, Hexane has moderate cytotoxicity against HCT116, DU-145, and MDA cell lines with an IC_{50} value of 115.3 ± 9.9, 90.2 ± 6.1, and 101.6 ± 8.5 µg/mL. Eventually, the water extract showed the weakest inhibition effect with IC_{50} values of 379.1 ± 11.5, 281.4 ± 11.2, 272.5 ± 11.5, 265.1 ± 10.1, and 250.5 ± 10.3 µg/mL toward the HCT116, SF-268, MP38, DU-145, and MDA cell lines, respectively (Table 4).

Effect of truffle and host plant on the mitochondrial membrane

These results indicated that the cell mortality in MP38 cancer eye cell lines was targeted by Rhodamine 123 mitochondrial specific fluorescent dye when processed by ethyl acetate extract from truffle and host plants. The impact of ethyl acetate extract from desert truffle and its host plant on the mitochondrial membrane was evaluated.

The ethyl acetate extract of truffle has a significant inhibitory effect on the mitochondrial membrane of MP38 cancer cells, more than the ethyl acetate extract of the host plant. When cell death occurs, inducing the cell to a manifest permeability transition via mitochondria. Cell mortality rate refers to the loss of the mitochondrial membrane potential; subsequently, a decrease in the mitochondrial membrane potential leads to dying cancer cells. The decrease of mitochondrial membrane potential in treated MP38 human cancer cells with truffle and host plant extract was measured using Rhodamine 123. After 16 hours, the ethyl acetate extract of truffle reduced the mitochondrial membrane potential of MP38 cells more than the host plant ethyl acetate extract at 100µg/mL (Fig. 2).

Fluorescence intensity decreased with increased cell mortality rate, cells losing mitochondrial membrane potential, and dead cancer cells. A strong fluorescence intensity in the untreated cells (controlled cells) indicated the invader growth and propagation of the cells. Fluorescent signals were observed in the cells treated with ethyl acetate extracts of truffle and host plant (Fig. 2). The cells treated with truffle ethyl acetate extracts displayed fewer fluorescence signals than those treated with host plant ethyl acetate extracts, as shown in Fig. 2.

These results indicate that the ethyl acetate extract of truffle is more effective at inducing cancer cell death and this process via the mitochondrial pathway than the

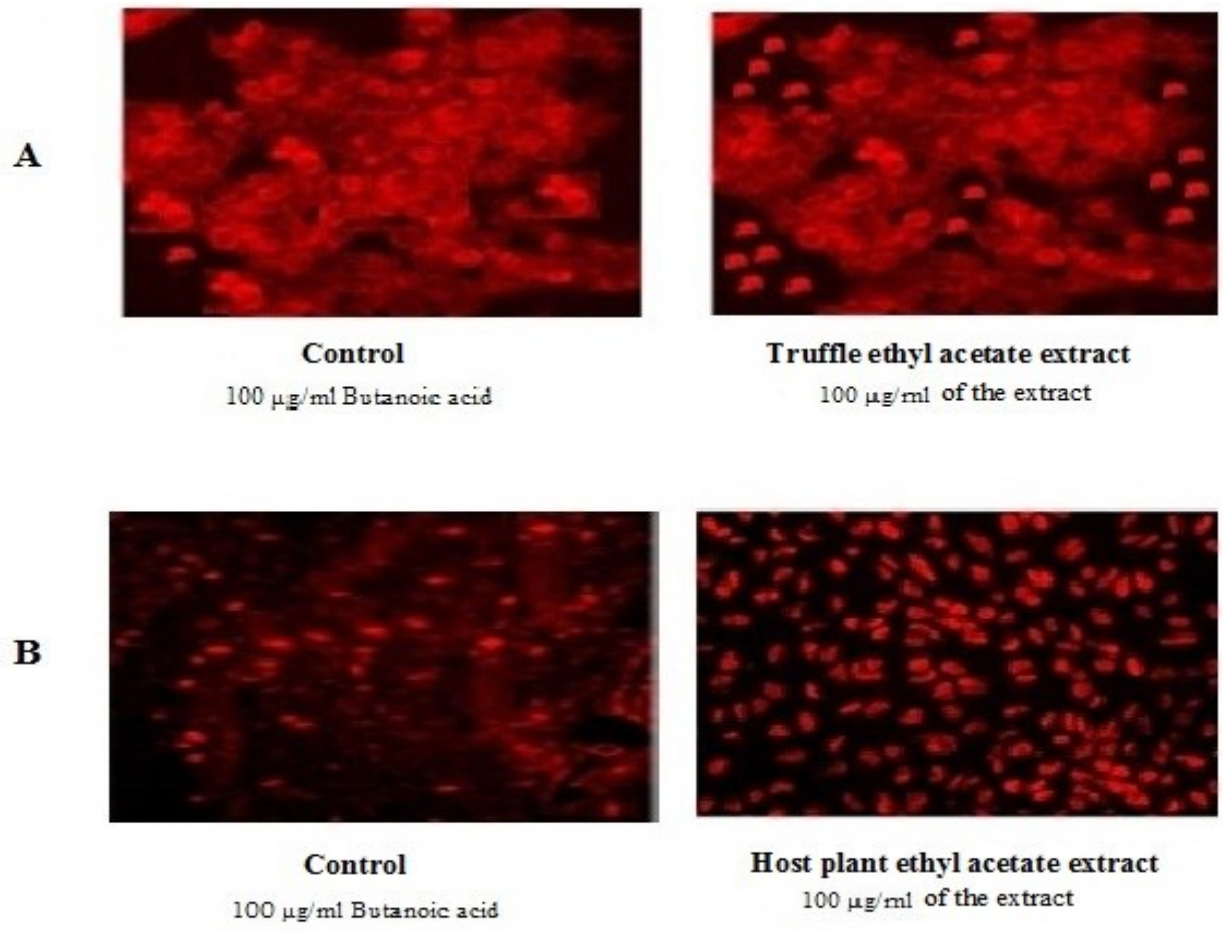

Fig. 2. (A) Effect of ethyl acetate extracts of truffle on the mitochondrial membrane in MP38 cancer eye cells using Rhod 123 stain. **(B)** Effect of ethyl acetate extracts of host plant on the mitochondrial membrane in MP38 cancer eye cells using Rhod 123 stain. Untreated control (Butanolic acid at 100 µg/mL) each experience individually.

ethyl acetate extract of the host plant. This suggests that the medicinal properties present in truffles have stronger effects on cancer cells than on the host plant.

Nuclear condensation by 4', 6-diamidino-2-phenylindole (DAPI) stain

Cell mortality rate means cell destruction mechanism. It occurs firstly due to the loss of mitochondrial membrane potential caused by tumor necrosis factor. As a result, DNA fragmentation occurs, leading to changes in the cell that eventually cause cell death. The presence of DNA fragments is an indication of the death of cancer cell lines.

This study showed that 4', 6-diamidino-2-phenylindole (DAPI) stain is a DNA-binding dye that accumulates in mitochondria with different membrane potential. This result showed a significant increase in cell death when exposed to ethyl acetate extracts of truffles and host plants at 100µg/mL for 16 hours each. The treated cells showed condensed and fragmented nuclei formed clusters with a crescent shape, shown in Fig. 3.

The human carcinoma cells MP38 treated with ethyl acetate extract of truffle have higher potential cell death activity than others treated with ethyl acetate extract of the host plant, compared to control. The ethyl acetate extract from the truffle appeared to increase DNA fragmentation more than the ethyl acetate extract of the host plant (Fig. 3). This refers to the extract of truffle being more effective in eliminating cancer cells than extract from host plants. According to the findings, the active extract from truffle is more effective in inhibiting cancer cells through the cell mortality rate than the extract from the host plant. The active substances in truffle have a significant role in inducing nuclear condensation and causing the death of cancer cells, more so than the active substances present in the host plant.

Discussion

Truffle contains various nutritionally valuable compounds and has a unique aroma. Phytochemical compounds of truffle and their host plant vary from species to species and area to area. Carbohydrates and proteins are generally the most abundant nutrients in truffles but relative lower in host plants, besides minerals, crude fibers, amino acids, fatty acids and crude lipids account for a significant portion of truffle composition (35, 36). It is indicated from a study that *T. boudieri* collected from Libya contains proteins, carbohydrates, crude fibers, ash, lipids, starch, reducing and non-reducing sugars, fatty acids, amino acids, mineral compounds, and very few toxic compounds (37).

Our results are similar to those of another study on the main components of *T. boudieri* (11). The experimental sample possessed 46–48 g/100 g/dry weight

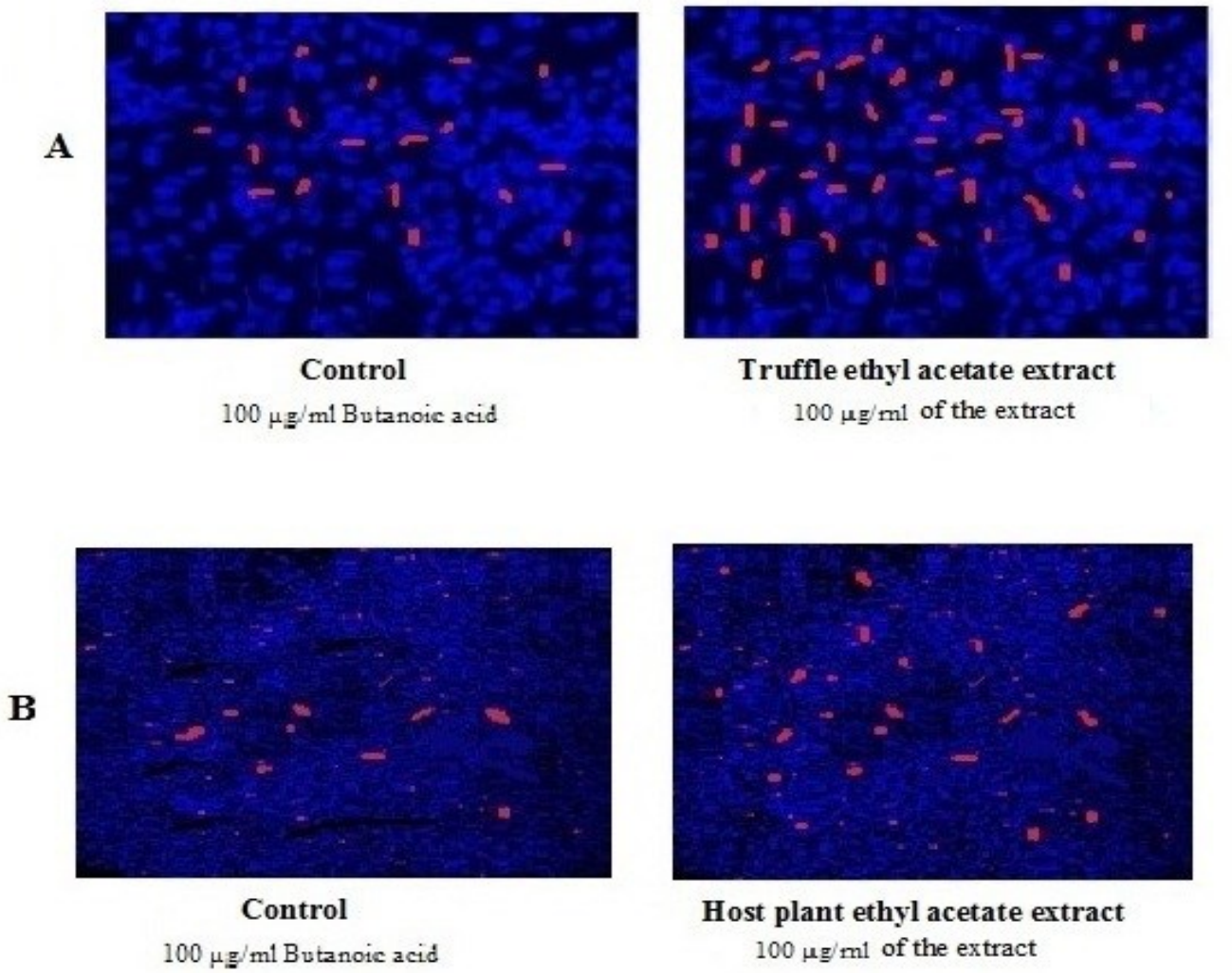

Fig. 3. (A) Effect of ethyl acetate extract of truffle on nuclei of MP38 cancer eye cells using DAPI stain. **(B)** Effect of ethyl acetate extract of host plant on nuclei of MP38 cancer eye cells using DAPI stain. Untreated control (Butanoic acid at 100 µg/mL) each experience individually.

of total carbohydrates, 32–35 g/100 g/ dry weight of crude protein, 14–15 g/100 g/ dry weight of crude ash, and 2.8–3.2 g/100 g/ dry weight of crude lipid, and this is in agreement with previously conducted investigations (38, 39). Our results also agree with another study on *T. boudieri,* which detected the presence of essential macro and micro elements in substantial quantities (40). Our results agree with the results of the study conducted on black truffles and show that the presence of a relatively high level of carbohydrates above 30% of dry weight and particularly abundant potassium, phosphorus, iron, calcium and other metals are the factors characterizing black truffles (41).

In contrast, our results are dissimilar to the results of this study conducted on *T. boudieri* Chatin collected from Turkey in which the amounts of dry matter (89.75%), fat (3.45%), and organic matter (81.95%) of the dry weight were higher than the present study. However, moisture (10.25%), crude ash (7.80%) and protein (20.13%) of the dry weight were lower than in the present study (42). It has been shown in an investigation that the mineral contents of K, Fe, Zn, Mn, and Cu were 63.8, 14.55, 42.5, 15.8, and 30.1 mg/kg (dry weight) higher than the present study, but Ca, Na are 0.27, 0.2 mg/kg (dry weight) that lower than the present study. Limited concentrations of toxic elements such as Pb, Ni, Cd, and Co have been detected (42).

A further study revealed that *T. boudieri* sourced from Saudi Arabia exhibited protein contents of 4.57% and 7.95% (43). The protein content of Egypt *T. boudieri* identified in this study (34.21%) is markedly more than that documented for Libya *T. boudieri* in prior research. These differences may be due to the origin, habitat, and state of maturity of the truffles analyzed (11). The mineral contents documented in the study (43) align with findings from another investigation (37), indicating that Mn, Cu, Na, Ca, K, Fe, Mg, P, and Co are present at levels of 0.39, 0.09, 0.60, 1.41, 9.63, 2.44, 1.61, 2.85, and 0.76 mg/kg (dry weight), which are lower than those observed in the current study.

A separate investigation on black truffles yielded lower results than our findings in ash content (8.3%) and crude protein (23.3%), although greater in carbohydrate (66.2%) and crude fiber (27.9%) while showing comparable levels in crude fat (2.2%) (44). A separate study indicated lower findings than ours and the aforementioned investigations, revealing that black truffles comprise 53–76% water, 9% protein, 7% carbohydrates, and 8% minerals (45). It's worth mentioning that the chemical composition of truffles may differ between the same species in different regions. Several environmental factors, including soil types, temperature fluctuations, and the amount of rain and time, likely influence the diversity of

the chemical composition (40).

Another study on the Iraqi *T. claveryi* species showed a protein content of 17.6% (46). Moreover, *T. claveryi* from Saudi Arabia contained (16%) protein (28% carbohydrate) and 78% total moisture (47). While another desert truffle genera *Tuber* exhibited lower protein (7–20 g/100 g dry weight) and ash content (4.5–5.1 g/100 g dry weight) but higher carbohydrate (56–50 g/100 g dry weight) and fat content (3.2–6.9 g/100 g dry weight) (11, 39). The dry matters of three Chinese truffle species, *Tuber latisporum, Tuber subglobosum* and *Tuber pseudohimalayense*, were composed of 74–79 g/100 g total carbohydrates, 11–15 g/100 g crude protein, 8.1–8.8 g/100 g crude ash, 2.2–2.5 g/100 g crude fat, 24–50 g/100 g total sugars, 96–265 mg/100 g monounsaturated fatty acid (39, 48). Chinese truffles and diverse truffle species including *Tuber aestivum, Tuber borchii, Tuber magnatum,* and *Tuber melanosporum* contain high levels of unsaturated fatty acid*,* about 60% of their total fatty acid content (48). The crude lipids contain essential fatty acids responsible for most truffle species' vital aroma-active compounds (48). The crude lipid content in the truffle primarily consisted of linoleic and oleic acid; it was devoid of heavy elements such as Cd, Hg, and cyanide (39). Crude lipid content is high in three Chinese truffles *Tuber latisporum, Tuber subglobosum,* and *Tuber pseudohimalayense*. Truffles are believed to have potential nutritional and medicinal benefits (49).

Minerals are the necessary dietary components for various metabolic processes and regulating water and salt balance (36). Mineral elements are present in truffles in both substantial and diverse amounts. Major minerals found in desert truffles, including Mg, K, P, and Ca, were abundant (36). Truffles are a source of amino acids, especially sulfur-containing ones, which are in lower amounts in host plant (50).

The minerals and enzymes play a role in nutrient acquisition and symbiotic fungus-host interaction, helping truffles to penetrate and colonize the roots of the host plants, and also, the plant partner supplies carbohydrates and fats to the roots in exchange for nutrients (36).

Our findings align with another report indicating that *H. aegyptiacum* (L.) Mill. serves as the host plant for *T. boudieri*, which extracts nutrients through mycorrhizal mycelium. *H. aegyptiacum* (L.) Mill. contains approximately 36 g/100 g dry weight of total carbohydrates, 31 g/100 g dry weight of crude protein, 12 g/100 g dry weight of crude ash, and 2.1 g/100 g dry weight of crude lipid (50).

Our results agreed with the results of the comparative study that the extracts from *H. lippii* (L.) serve as the host plant for *T. nivea* (Desf.) Trappe (51). The white truffle *T. nivea* contains a higher percentage of carbohydrates, protein, organic matter, and lipid (53.87%, 40.14%, 32.98%, and 6.99%, respectively) compared to its host plant *H. lippii* (L), which contains these materials in lower proportions (47.51%, 35.87%, 35.87%, and 5.51%, respectively) (51).

The concentrations of the macro-elements (Na^+, K^+, Ca^{++}, Mg^{++}, P) in the white truffle *T. nivea* about 1.84 mg/ g dry weight, 15.14 mg/ g dry weight, 3.85 mg/ g dry weight, 4.83 mg/ g dry weight, and 5.58 mg/ g dry weight, respectively and it's higher than there found in its host plant *H. lippii* (1.24 mg/ g dry weight, 11.14 mg/ g dry weight, 2.58 mg/ g dry weight, 3.21 mg/ g dry weight, and 3.98 mg/ g dry weight, respectively (51).The concentrations of micro-elements (Fe, Cu, Mn, Zn, and Co) in the white truffle *T. nivea* (4.25 mg/ g dry weight, 2.28 mg/ g dry weight, 1.11 mg/ g dry weight, 3.21 mg/ g dry weight, and 1.58 mg/ g dry weight, respectively and it's higher than there found in its host plant *H. lippii* (2.98 mg/ g dry weight, 1.59 mg/ g dry weight, 0.74 mg/ g dry weight, 2.41, and 0.99 mg/ g dry weight), respectively (51).

Also, our result agreed with the comparative study exhibited highly significant secondary compounds such as phenolics, saponins, alkaloids, flavonoids, and tannins in the white truffle *T. nivea* compared to the host plant *H. lippii* (51). The white truffle *T. nivea* contains the secondary compounds (6.54 mg/g dry weight, 4.65 mg/g dry weight, 4.25 mg/g dry weight, 3.58 mg/g dry weight, and 3.12 mg/g dry weight, respectively higher than it's found in the host plant *H. lippii* (5.48 mg/g dry weight, 3.28 mg/g dry weight, 3.14 mg/g dry weight, 2.54 mg/g dry weight, and 2.17 mg/g dry weight), respectively (51). Our findings are consistent with another study on the genera of desert truffles (52). *Tuber magnatum* and *Tuber aestivum* demonstrated a total phenolic content of approximately 1.31 g gallic acid equivalents (GAE) per 100 g of fresh mass, surpassing the host plants. They also contained elevated levels of tannins and other bioactive compounds, including saponins, flavonoids, steroids, and glycosides, recognized for their antimicrobial properties (52). It has been reported that the methanol extracts of three Chinese truffle species comprised a high number of flavonoids (74.52 mg GAE/g extract) and tannins (19.78 mg catechin equivalent (CE)/g extract) (52).

Furthermore, they demonstrated that the phenolic content ranged from 450 to 735 mg of GAE per 100 g, and the flavonoid content ranged from 611 to 1355 mg of rutin per 100 g, which may be utilized for the treatment of diseases (11).

Truffles contain significant amounts of carbohydrates and lipids, which play essential roles in various biological processes and cancer cell division-preventing abilities, including strengthening the immune system's activity against tumors. Carbohydrate extract from *T. boudieri* possessed an anticancer effect (10). Previous investigations have examined the anticancer properties of truffles. In vitro studies indicate that methanol extracts from *Tuber magnatum* and *Tuber aestivum* exhibit substantial cytotoxic effects against cancer cell lines HT-29, MCF-7, and HeLa, as well as activity of the water extract against breast adenocarcinoma (MCF-7) (53).

Our result agreed with the result of another study that the extracts from the black desert truffle, *T. boudieri*, possess anticancer properties (54). Our findings

corroborate those that established the significance *in vitro* through the MTT assay. They demonstrate the cytotoxic effects of hexane and ethyl acetate extracts of *T. boudieri* on various cancer cell lines, including the human brain carcinoma cell line (U-87 MG), with IC50 values of 50.3 µg/mL DW of extract (10).

Our findings concur with those documented in a prior work regarding *T. claveryi*, which assessed the cytotoxic activity of the hexane extract (55). The results showed that *T. claveryi* had the strongest cytotoxic effect on the U-87 MG human brain carcinoma cell line, with an IC50 value of 50.3±5.2 µg/mL, followed by the ethyl acetate extract. The ethanol and methanol extract also exhibited moderate cytotoxicity against the U-87 MG cell line, with IC50 values of 109.25±9.51 and 136.2±7.4 µg/mL, respectively. Moreover, our research found that the hexane extract of *T. boudieri* also had a significant cytotoxic effect on the human colorectal carcinoma cell line HCT116. On the other hand, the hexane extract of *H. aegyptiacum* (L.) Mill. exhibited only moderate cytotoxicity against the human central nervous system cell line SF-268 and the prostate cancer cell line DU-145.

Another study reported that methanol extract has moderate cytotoxic activity from *T. claveryi* toward the colon adenocarcinoma cell line (HT-29) (55). Similarly, we found that methanol extract from *T. boudieri* and *H. aegyptiacum* (L.) Mill has moderate cytotoxic activity toward the colorectal carcinoma cell line HCT116. It was also reported that gold nanoparticles (AuNPs) from *Tirmania nivea* extract show the highest cytotoxicity against the prostate cancer cell line (VCaP) by a percentage of 74.6 % using an MTT assay (56).

Our result agreed with this study, which showed that the hexane extract of *T. boudieri* induces the reduction of mitochondrial membrane potential and nuclei condensation in U-87 MG cells (10). This played a role in inhibiting cancer cell growth mechanisms and stimulating cancer cell death. It has been reported that cell death is a process in which mitochondria play a crucial role. When there are significant changes in the integrity of their membrane, the mitochondria act as the control center for cell death. This disruption in the membrane potential leads to the loss of mitochondrial function, and as a result, DNA fragments are released between the membranes through the outer membrane. This process enhances the death of cancer cells (32, 51).

Further, our result agreed with the results of a previously conducted study which demonstrated the extracts from the black desert truffle, *T. boudieri*, inhibit the viability of colon cancer cell lines (HCT-116 and Caco-2) based on MTT assays (54). This effect is attributed to the influence of *T. boudieri* extract on the mitochondrial pathway of apoptosis and its impact on cellular proliferation and the cell cycle.

A study was performed on *T. boudieri*, a black desert truffle from the Mediterranean region (57). Researchers treated human colon cancer cells HCT-116 with a water extract of *T. boudieri*, utilizing the MTT cell viability assay and crystal violet assay. A prior investigation indicated that HCT-116 cells treated with the water extract demonstrated a dose-dependent antiproliferative impact (54). As the concentration of the applied water extract increased, the viability of cancer cells decreased, yielding an IC50 of 6.6 mg/mL (57). The viability of cancer cells was further diminished in those treated with a combination of capecitabine and the extract, as opposed to treatment with capecitabine alone.

Our results agree with another study that used rhodamine 123 to measure the decrease in mitochondrial membrane potential in *T. claveryi*-treated U-87 MG cell line cells (55). It was demonstrated that after 6 and 12 hours, 50 µg/mL of *T. claveryi* hexane extract decreased the mitochondrial membrane potential of U-87 MG cells. According to their findings, cells treated with *T. claveryi's* hexane extract showed decreased fluorescence signal, whereas intense fluorescence in untreated cells suggests the cells' rapid growth and proliferation. These results agree with the findings of another study indicating that truffles may have a significant role in inducing cell death via the mitochondrial pathway (11).

The fraction and DNA fragmentation system in cancer cell lines treated by truffle extractions showed a significantly higher cancer cell inhibition rate than those from untreated cancer cell lines (11). Moreover, the carbohydrates especially, heteropolysaccharides having lower molecular weight exhibited higher antitumor activity than those with higher molecular weight (55). In addition, the crude fatty acid content in truffles contributed to the anticancer activities, especially oleic acid in fatty acids, which could prevent metastasis of cancer cells and induce cancer cell death (11).

Our result agreed with a previously conducted experiment that detected significant DNA fragmentation, a hallmark of apoptosis, in colon cancer cell lines (HCT-116 and Caco-2) that were treated with *T. boudieri* water extract (54). Due to the extensive DNA fragmentation, the maximum percentage of viable cancer cell lines (HCT-116 and Caco-2) was approximately 4% after exposure to the *T. boudieri* water extract.

Our findings align with others investigating the morphological alterations in dying cells utilising Hoechst 33342 stain as a DNA-binding dye (55). The researchers found that when the cells were exposed to *T. boundri* and *T. claveryi's* hexane extract at a concentration of 50 µg/mL for 48 hours, the number of death cells increased significantly ($p < 0.05$). These results agreed with ours in that they showed that both *T. boundri* and *T. claveryi's* hexane extract had an increased death cell activity effect on the cancer cell line U-87 MG, suggesting that the death cell pathway plays a crucial role in both *T. boundri* and *T. claveryi* extracts' ability to limit cancer cell growth (11, 55).

Conclusion

This study aimed to determine the phytochemical and cytotoxic activity of desert truffle, *T. boudieri*, and its

host plant, *H. aegyptiacum* (L.) Mill. shows the dynamic range of biological activities by cell death and DAPI staining, which exhibited changes in cell morphology and selectively accumulated in mitochondria with different membrane potentials. These results demonstrated the ethyl acetate extracts of the black truffle, *T. boudieri*, and its host plant, *H. aegyptiacum* (L.) Mill. has the highest cytotoxic activity against human eye carcinoma, central nervous system, colorectal carcinoma, prostate, and breast cancer cell lines. Notably, the active substances of *T. boudieri* are more effective than those in *H. aegyptiacum* (L.) Mill. to induce a higher level of cytotoxicity, cell mortality rate, and nuclear condensation of cancer cells.

Acknowledgement

WE would like to express my deepest sincerest gratitude to Dr. Osama Abd Ellatief, Environmental Consultant at the Ministry of Environment, and General Organization for Studies and Research and Technology, for their support during research. Also, we acknowledge the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia.

Authors' Contributions

AAZ, MA and KA conceptualized the idea for the study, designed the study and reviewed the manuscript. KA reviewed and participated in editing of the manuscript. AAZ and KA helped in the statistical analysis of the study. HMHS, AMAE, MA, KA, MAY were involved in investigation and supervision. MA, KA participated in the revision and editing of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

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

Ethical issues: None

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