Biotechnology Reports 36 (2022) e00770

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Research Article

First report on isolation of *Mucor bainieri* from honeybees, *Apis mellifera*: Characterization and biological activities

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ARTICLE INFO

Keywords:
Fungi
Mucor bainieri
Honeybees
Apis mellifera
Antimicrobial activity
Antioxidant activity
Anticancer activity

ABSTRACT

Fungi are potential biocontrol agents and rich sources of secondary metabolites with demonstrated biological activities. This study aimed to isolate and identify fungi from surface-sterilized honeybees (*Apis mellifera*), as well as to evaluate their biological activities. One fungal isolate was obtained and identified morphologically and genetically as *Mucor bainieri* MK-Bee-2. Gas chromatography-mass spectroscopy (GC–MS) analysis of fungus crude extract, showed the existence of six major metabolites representing 92.48% of the total peak area. The crude extract of *Mucor bainieri* MK-Bee-2 was tested for antimicrobial, antioxidant, and antitumor activities. It demonstrated wide antimicrobial activities against human pathogenic Gram-positive and Gram-negative bacterial strains, as well as *Candida albicans*, with MIC values ranged from 62.5 to 250 μg/ml. The results revealed that the extract exhibited considerable antioxidant activities indicated by strong inhibition of both DPPH and ABTS free radicals. Additionally, the extract exhibited greater potential anticancer activity against both adenocarcinomic human non-small cell lung cancer cells (A549) [IC_{50} = 6.45 μg/ml], and immortal cell line hepatoma G2 (HepG2) human liver cancer cells [IC_{50} = 27.48 μg/ml] and higher selectivity in cancer cells than normal cell lines. Furthermore, the extract showed less cytotoxic activity against normal cells with higher IC_{50} values of 106.99 and 132.57 μg/ml against human lung fibroblast Wistar-38 (Wi-38) and oral epithelial cells (OEC), respectively. Taken together, the *Mucor bainieri* MK-Bee-2 extract comprises bioactive compounds as promising potential therapeutic candidates for the treatment of lung cancer. Strikingly, the extract sensitizes the lung cancer cells A549 to the ionizing radiation through the pro-apoptotic pathway as indicated by the annexin V flow cytometry analysis which showed that the extract reduced the apoptosis of lung cancer cells.

1. Introduction

Honeybees are extremely important to humanity and nature, particularly *Apis mellifera*, the most common species of honey bee found worldwide [1]. Honeybees are not only good pollinators for plants, but they are also important model organisms in scientific studies, particularly in the field of social evolution and studies of pesticide toxicity, as well as producing many beekeeping products like honey, wax, propolis, royal jelly, pollen and bee venom [2,3]. According to reports, honeybee workers interact with environmental microbes while foraging and returning pollen and nectar to the colony, resulting in microorganisms associated with *Apis mellifera* [4]. The microorganisms associated with bees and nests live in a saprophytic mode, but they can also have a mutualistic interaction with bees [5]. For instance, various helpful fungi are involved in several of honeybees' biochemical contributions, such as the transformation, improvement, and conservation of pollen stored as bee bread in comb cells [6,7]. Intriguingly, associated microorganisms create natural defense shield via synthesis of antimicrobial agents which in turn protect *Apis mellifera* colonies against harmful microbial diseases such as fungal disease chalkbrood (*Ascosphaera apis*) [8].

Secondary metabolites produced by associated microorganisms may include antimicrobial peptides, bacteriocins, surfactants, siderophores, protein hydrolyzing, and cell wall-destructing enzymes. These chemicals target key cellular components via various mechanisms of action as

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https://doi.org/10.1016/j.btre.2022.e00770
Received 31 July 2022; Received in revised form 16 September 2022; Accepted 16 October 2022
Available online 19 October 2022
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hole-formation, membrane dissolution or ion- sequestration. They interfere also with the cell structure and function of competing microorganisms by preventing surface adherence, biofilm development, and interrupting the quorum sensing system [9].

The associated microorganisms also secrete a variety of chemicals with anticarcinogenic and anti-inflammatory properties, and they play an important role in the host immune system's operation and the production of immunological responses [10]. There are some other metabolites produced by honey bee-related fungi, including short-chain fatty acids which serve as a primary source of energy for bees, and neuroactive substances for better brain function and behaviors [11]. Furthermore, the related microorganisms could provide nutritional benefits to their host honeybee by degrading pectin and lignin. The breakdown of these two key components of pollen cell walls gives access to pollen protein [12].

The honeybees-associated microorganisms include Bacteria (*Bacillus* sp., and Enterobacteriaceae), molds (*Aspergillus, Penicillium*, and some zygomycetes such as *Rhizopus* and *Mucor sp.*), and yeasts (*Torulopsis sp.*) which can be pathogenic or non-pathogenic, however, the majority of them are not harmful to honeybees [13,14]. Despite the wide range of active metabolites that fungi can produce for the honeybee, few studies have investigated fungi associated with the honeybee. Therefore, we aimed to isolate, identify, and characterize the honeybee (*Apis mellifera*)-associated fungal flora with metabolites having broad biological activities (antimicrobial, antioxidant, and antitumor activities).

2. Materials and methods

2.1. Sampling and isolation of fungi

Honeycombs containing honeybee (*Apis mellifera*) were obtained from an apiary located at Alenshaseyia, Aga, Dakahleya governorate, Egypt (30°50′25.1″N 31°19′34.7″E). Adult worker bees were collected alive and kept in sterile containers at 20 °C until the isolation procedure began. According to Ding et al. [15] and El-Sohaimy et al. [16], the selected bee workers were surface-sterilized using a quadruple surface-sterilization procedure in which they were submerged in 2% sodium hypochlorite for 3 min, then 70% ethanol for 60 s, followed by 5% NaCl sterile solution for 60 s, and lastly rinsed with sterile distilled water. In sterile conditions, honeybees were crushed and placed onto potato dextrose agar (PDA) and Rose-Bengal chloramphenicol agar (RBCA) (Merck, Germany) plates. The plates were incubated at 25 °C until hyphae emerged from the bee samples. The hyphal tips were picked up and reinoculated on new PDA and RBCA plates repeatedly until pure morphotype colonies were obtained. For further investigations, the pure isolate was cultured on PDA slants and maintained at 4 °C.

2.2. Morphological identification of the fungal isolate

Standardized methods for morphological characterization and species descriptions published by Samson et al. [17] were followed. The fungal isolate was cultured on a range of media including Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA), and Czapek yeast autolysate (CYA) agar (HiMedia, India). After 7 days of incubation at 25 °C, the culture characteristics including colony color, reverse pigmentations, texture, and appearance were observed and recorded. In addition, fungal isolate was cultured on creatine sucrose agar (CREA) to investigate whether fungus produces acid or base [18]. The fungal isolate was grown on PDA for 7 days before a piece of mycelium was stained with 0.1% lactophenol blue and subjected to microscopic examination using a light microscope (Optika, Italy) for observation of sporangiophores, sporangia, and sporangiospores [19–21]. For further characterization, a scanning electron microscope was used to examine the fine details of the fungal isolate. This investigation was done using scanning electron microscopy (JEOLTechnics Ltd, Japan) at the regional center for mycology and biotechnology- Al-Azhar University, Cairo, Egypt.

2.3. Molecular identification of the fungal isolation

Genetic identification of the fungal isolate was performed according to Elsehemy et al. [22] and Atalla et al. [23,24]. The Gene Jet genomic DNA purification Kit was used for DNA extraction (Thermo Fisher Scientific USA, K0791). Internal transcribed spacer (ITS) rDNA sequences were amplified using polymerase chain reaction (PCR) using forward and reverse primers of 5-GACTCCTTGGTCCGTGTT-3 and 5-TGAAATTGTTGAAAGGGAA-3, respectively. The PCR product was amplified using the BIO-RAD PCR System T100 thermal cycler (BIO--RAD Laboratories, Hercules, CA, USA). The PCR product was purified using the K0701 Gene JETTM PCR Purification Kit (Thermo Fisher Scientific USA) and sequenced using the ABI Prism 3730XL DNA analyzer (Applied BioSystems, Foster City, California, USA). The nucleotide sequence that resulted was deposited in GenBank and given an accession number. Then, they compared with the nucleotides of various ITS rDNA sequences which were already published on GenBank using the BLAST program to generate phylogenetic data.

2.4. Fermentation and preparation of the fungal crude extract

In one liter Erlenmeyer flasks, 200 ml of Potato dextrose broth medium (PDB) was inoculated with 6 discs (6 mm in diameter) of 7-day old culture of the fungal isolate. Under static conditions, the flasks were incubated for 28 days at 25 °C. After incubation, the fermentation culture was extracted using100% ethyl acetate as described by Kamat et al. [25] and Hashem et al. [26]. To get a total culture crude extract, the organic layer was collected and evaporated to dryness under reduced pressure at 40–45 °C using a rotary evaporator (Heidolph VV2001, Germany).

2.5. Gas chromatography-mass spectroscopy (GC–MS) analysis

The metabolites in the extracts of the fungal isolate were analyzed, quantified and identified by GC–MS, as described by Hamed et al. [27] and Zothanpuia et al. [28] with minor modifications. The fungal crude extract was dissolved in spectroscopy-grade methanol. The Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, Texas, USA) with a direct capillary column TG–5MS (length 30 m, thickness 0.25 μm, internal diameter 25 mm) was used for the GC–MS study. The column oven temperature was kept at 50 °C at the start, then increased at a rate of 5 °C/min to 230 °C and held for 2 min before being increased to the ultimate temperature of 290 °C and held for 2 min. The temperatures of the injector and MS transfer line were maintained at 250 °C and 260 °C, respectively. The sample (1 μl) was injected at 250 °C using helium as a carrier gas, split at a 1:30 ratio. The mass spectrometer was set to run in electron ionization (EI) mode at 70 eV and 200 °C, with a scan range of 40–1000 *m/z*. The observed compounds' spectra were compared to those of well-known compounds in the WILEY 09 (Wiley, New York, NY, USA) and NIST 11 (National Institute of Standards and Technology, Gaithersburg, MD, USA) libraries. The detected compounds' names, molecular weights, and chemical structures were also determined.

2.6. Antimicrobial activities of the fungal extract

Ten milligrams of ethyl acetate crude extract were dissolved in 1 ml of dimethyl sulfoxide (DMSO) to make a stock solution of fungal crude extract. Using Muller Hinton agar (HiMedia, India) for bacteria and Sabouraud Dextrose Agar (HiMedia, India) for *Candida albicans*, the antimicrobial activity of the prepared stock solution was evaluated using the agar diffusion technique [29,30]. The prepared agar plates were inoculated with 100 μl of overnight grown culture (10^6 CFU/ml) of *Staphylococcus aureus* ATCC 6538 (*S. aureus), Bacillus cereus* ATCC 10,

987 (*B. cereus), Escherichia coli* ATCC 8739 (*E. coli), Salmonella typhimurium* ATCC14028 (*S. typhimurium*), *Klebsiella pneumonia* ATCC 13,883 (*K. pneumonia)* and 48 h old culture of *Candida albicans* ATCC 10,231 (*C. albicans)*. Using a sterile cork borer, wells (8 mm in diameter) were cut in the inoculated agar media and 100 µl of crude extract stock solutions were transferred into each well. Ciprofloxacin (30 µg) was used as antibiotic control for bacterial strains while fluconazole (25 µg) was used as antifungal agent control for *Candida albicans* as well as DMSO which was used as a negative control. All plates were left for 2 h at 4 °C until the extract was diffused then incubated for 24 h at 37 °C in case of bacteria and 48 h at 28 °C for *Candida albicans*. The experiment was performed three times. After incubation, the inhibition zones were measured, and the mean values were calculated.

Minimum inhibitory concentration (MIC) of an antibacterial agent is stated in (µg/ml) and is defined as the lowest concentration of an antimicrobial agent that, under tightly regulated *in vitro* conditions, totally inhibits observable growth of the test strain [31]. The MIC values of crude extract of the fungal isolate were evaluated using a microdilution test in 96-well plates according to Sharaf et al. [32]. The above-mentioned standard bacterial and yeast strains were inoculated, with a cell suspension concentration of 10^6 CFU/ml, in Muller Hinton broth and Sabouraud dextrose broth media, respectively, and then 200 µl was loaded into wells. The fungal crude extract was diluted in twofold concentrations (7.8- 1000 µg/ml) and tested against bacterial and yeast strains. The differences in optical density were evaluated using the wells that contained a negative control (medium + tested stock solutions at the tested concentrations). The absorbance was measured at 630 nm after an 18-h incubation period at 37 °C.

2.7. Antioxidant activity of the fungal extract

The antioxidant activity of the crude extract of the fungal isolate was determined using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Merck, Darmstadt, Germany) techniques, as described by Lee et al. [33] and Elrefaey et al. [34] with minor changes.

For DPPH assay, DPPH (8 mg) was dissolved in 100 ml methanol to achieve 80 µg/ml solution concentration. In a 96-well microplate, 100 µl of DPPH reagent was combined with 100 µL of two-fold diluted fungal crude extract (1000–7.81 µg/ml) and incubated at room temperature in a dark place for 30 min to assess the scavenging activity. A standard antioxidant, ascorbic acid, was employed as a positive control. The absorbance was measured using ELISA reader (Mindray MR-96A, China) at 514 nm. The following formula was used to calculate the DPPH scavenging effect:

$$\text{Antioxidant activity} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

For ABTS assay, 5 ml of 7 mM ABTS was mixed with 88 µL of 140 mM potassium persulfate to make a fresh ABTS solution. After 16 h of incubation in the dark at room temperature to allow free radical production, the mixture was diluted with water at a ratio (1:44, v/v). In a 96-well microplate, 100 µL of ABTS reagent was combined with 100 µL of two-fold diluted fungal crude extract (1000–7.81 µg/ml) and incubated at room temperature in a dark place for 6 min to measure the scavenging activity. After incubation, absorbance was measured using ELISA reader (Mindray MR-96A, China) at wavelength 734 nm. The fungal extracts were compared to ascorbic acid (HiMedia, Mumbai, India) in terms of scavenging capacity. The antioxidant capacity% was calculated as follows:

$$\text{Antioxidant activity} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

The EC_{50} values (the concentration at which the extract exerts half of its maximal reaction "scavenging activity") were determined using an online tool freely provided by AAT Bioqest- EC_{50} calculator.

2.8. Cytotoxicity and antitumor activity of the fungal extract

Human lung carcinoma (A549) and hepatocellular carcinoma (HepG2), as cancer cell lines as well as human lung fibroblast (Wi-38) and oral epithelial cells (OEC), as normal cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). According to El-Didamony et al. [35], both normal and cancer cell lines were cultured in 96-well tissue culture microtiter plates (Nunc-Denmark) at a concentration of 1 X 10^5 cells/ml (100 µl/well) with RPMI-164 growth medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml) at 37 °C and 5% CO_2 for 24 h to develop a complete monolayer sheet. After a confluent sheet of cells was formed, the growth media was decanted, and the cells were treated with the test materials. The fungal crude extract was added to the cells at concentrations of 1000, 500, 250, 125, 62.5 and 31.25 µg/ml in a volume of 100 µl per well and equal volumes of media were added to cells as control. Plates were incubated at 37°C and 5% CO_2 atmospheric conditions for 24 h. After that, the media were removed, and plates were washed with phosphate-buffered saline (PBS) (pH ≈ 7.4, 0.137 M) and the cells were incubated with 50 µl/well of (3-(4, 5-Dimethylthiazol-2-yl)−2, 5-ditetrazolium bromide (MTT) (5 mg/ml) solution. After incubation for 4 h, DMSO solution was added at 0.05 ml/well. Finally, the absorbance of each well was read at 560 nm using ELIZA reader. The viability percent was calculated as follows:

$$\text{Viability\%} = \frac{\text{Mean OD Treated}}{\text{Mean OD Control}} \times 100$$

Where OD is optical density. The IC_{50} is the concentration of tested material required to induce a 50% inhibition of cell growth and the value was determined using an online tool freely provided by AAT Bioqest- IC_{50} calculator. Also, Morphological changes of treated cell lines were observed 24 h post-treatment using an inverted phase-contrast microscope (Helmut Hund GmbH, Wetzlar, Germany).

2.9. Radiation facility and irradiation procedures

The fungal crude extract was tested for inducible apoptotic cell death in the Human non-small cell lung cancer (A549) either individually or in combination with γ- irradiation. A549 cells were exposed to γ-irradiation (IR) at a single dose of 6 Gy, which was performed at the National Center for Radiation Research and Technology's (NCRRT), Cairo, Egypt using Canadian Gamma-cell-40 (Cs137), biological irradiator manufactured by Canada Ltd. Ottawa, Ontario, Canada. The unit has ventilation holes that align with ventilation parts through the main shield to provide a means for uniform irradiation at a dose rate of 0.403 Gy/min at the time of the experiment according to the guidelines of the Protection and Dosimetery Department.

2.10. Annexin V detection by flow cytometry analysis

Fluorochrome-labeled Annexin V was used to specifically target and identify apoptotic cells. Fluorochrome-labeled Annexin V retains its high affinity for phosphatidylserine (PS) and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC (Fluorescein isothiocyanate) Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. PS exposure on the outer leaflet of the plasma membrane was detected using the Annexin V-FITC/PI Apoptosis Detection Kit (BD pharmingin TM, BD Biosciences Co., USA; Number 51–66121E) according to the manufacturer's instructions by flow cytometry. In brief, A549 cells were treated with either IC_{50} of the fungal crude extract, radiation, or IC_{50} of crude extract combined with radiation for 24 h.

Untreated A549 cells were included as a control. Then, cells were rinsed with ice-cold PBS and then re-suspended in 500 µL of binding buffer. After full suspension, we added 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI). The cells were incubated for 5 min at 4 °C and then immediately analyzed on a FACSC-LSR (Becton, Dickinson and Company, San Jose, CA, USA) equipped with CellQuest (Becton, Dickinson and Company) software [36].

2.11. Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) by GraphPad Prism® version 8.00 (San Diego, CA, USA) software. The significant differences were considered statistically at $p < 0.05$. Data were presented as mean ± SEM.

3. Results

3.1. Identification of the fungal isolate

The isolated fungus was coded as MK-Bee-2. According to the macroscopic and microscopic investigations, the MK-Bee-2 isolate was identified at the genus level as *Mucor* sp. (Fig. 1). MK-Bee-2 colony showed overhasty growth with fluffy, wooly off-white mycelium on MEA, and whitish-gray on both CYA and SDA with the development of sporangia and sporangiospores. Reverse color ranged from gray on both CYA and PDA to pale beige on SDA after 7 days. On CREA, MK-Bee-2 showed restricted growth of white mycelia with also a negative result of acid production from creatine. Microscopic investigation of MK-Bee-2 exhibited two types of slightly encrusted sporangiophores up to 13 µm in diameter, one long erect unbranched with a length of 4670 µm, the other type is short repeatedly (up to seven times) helicoid sympodially branched up to 500 µm in length, septa are present at the base of branching. Ending with apical, spherical, multi-spored spiniculate sporangia (50–70 µm in diameter). Sporangia are supported with well-developed globose to sub-globose columellae up to 45 µm in diameter. Collarette is usually visible at the base of the columellae after rupturing of sporangia and sporangiospores dispersal. Sporangiospores are dull olive, -walled, few are rounded (4 to 5 µm), and the majority were ellipsoidal and 4–5.5 x 5–8 µm in size. ITS region was analyzed to determine the evolutionary relationships between examined isolate and the previously published sequences. Direct PCR amplification of ITS rDNA sequence from MK-Bee-2 isolate demonstrated excellent similarity between the morphology and ITS rDNA sequence data. Multiple sequence alignments were displayed to compare the ITS rDNA nucleotide sequence of MK-Bee-2 strain with other fungal species by using of NCBI GenBank databases. The Phylogenetic tree demonstrated that our fungal isolate MK-Bee-2 was located in a separate clade with *Mucor bainieri* CBS 293.63 with a sequence similarity value of 96.01% (Fig. 2). Accordingly, MK-Bee-2 was designated as *Mucor bainieri* MK-Bee-2 and deposited to NCBI database with accession number **MN966662**.

3.2. GC–MS analysis of the fungal extract

GC–MS analysis of *Mucor bainieri* MK-Bee-2 crude extract revealed the presence of nine various compounds with corresponding peaks at different retention times (Fig. 3). The molecular formula, molecular weight, peak area (PA) percent, retention time and biological activities of the crude extract of *Mucor bainieri* MK-Bee-2 are represented in Table (1). According to findings, the main compounds were 1-Dodecanamine, N, N-dimethyl (PA=47.67%), 1-Tetradecanamine, N,N-dimethyl (PA=17.00%), Chloromethyl benzene (PA=16.20%), N-Methyl-N-benzyl tetradecanamine (PA=6.63%), Oleic acid eicosyl ester

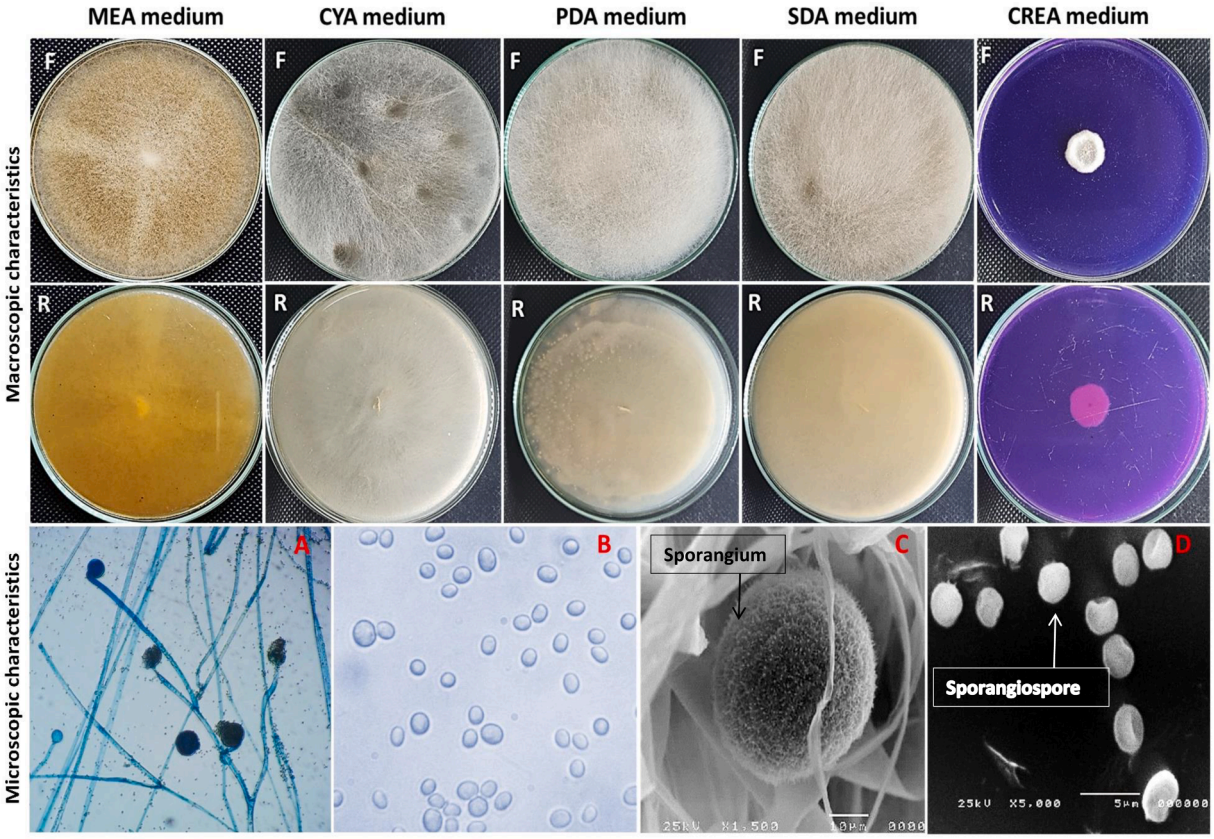

Fig. 1. Macroscopic culture characteristics (*F*= front and *R*= reverse) of *Mucor bainieri* MK-Bee-2 on different media after 7 days of incubation at 25 °C. Microscopic features of *Mucor bainieri* MK-Bee-2 mycelia (A) and spore (B) under the light microscope (200 X). Sporangium (C) and sporangiospores (D) of *Mucor bainieri* MK-Bee-2 under the scanning electron microscope (1500 and 5000 X, respectively).

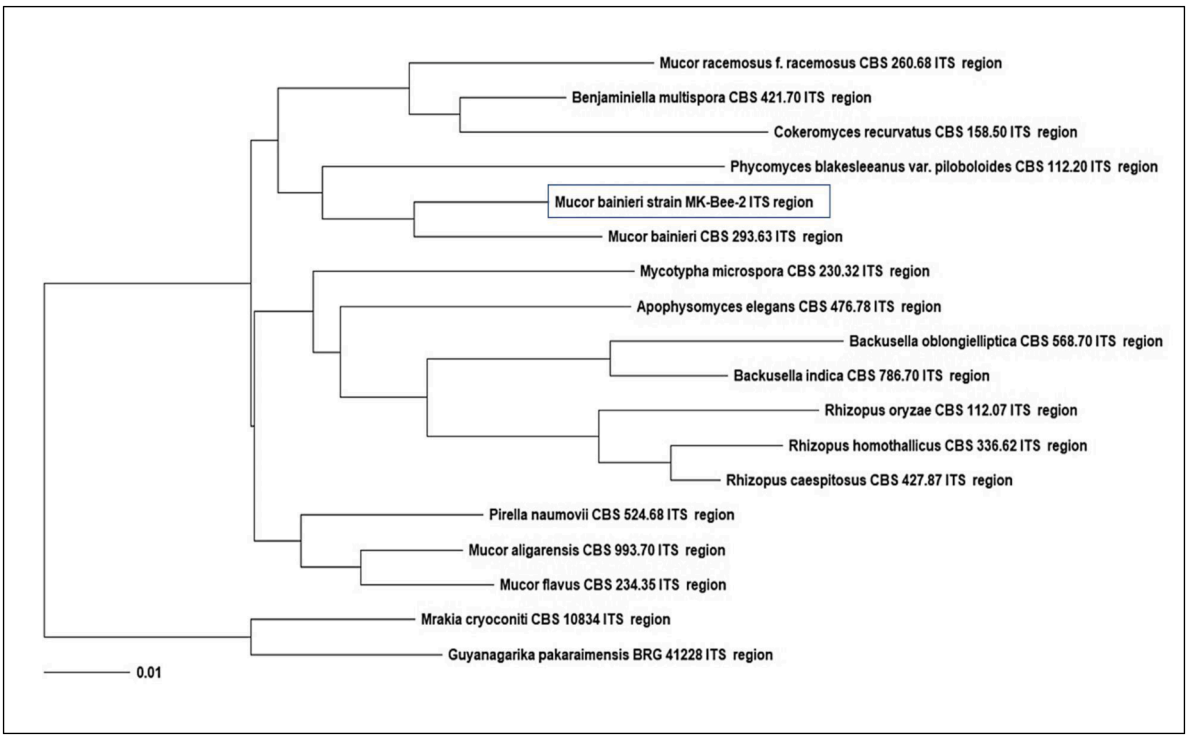

Fig. 2. Neighbor-joining phylogenetic tree of *Mucor bainieri* MK-Bee-2 comparisons to related fungi based on internal transcribed spacer (ITS) rDNA gene sequence.

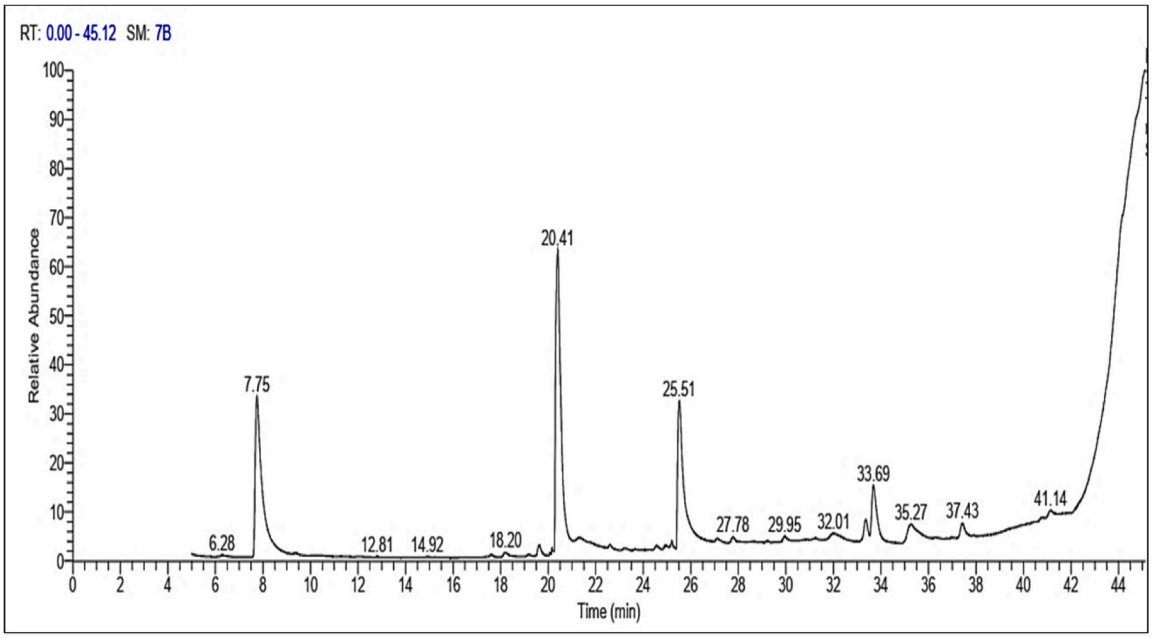

Fig. 3. GC–MS profile of *Mucor bainieri* MK-Bee2 crude extract.

(PA=2.72%), and 10-Octadecenoic acid methyl ester (PA=2.26%). All compounds of the extract were detected at a retention time of 7.75 to 44.10 min.

3.3. Biological activities of the fungal extract

3.3.1. Antimicrobial activity

Results shown in Fig. (4) and represented in Table (2) generally revealed that the crude extract of *M. bainieri* MK-Bee-2 exhibited activity against all tested microorganisms, whether Gram-positive bacteria, Gram-negative bacteria, or *Candida albicans*. In terms of the microorganisms that were affected by the fungal extract, *Candida albicans* was the most affected one (Inhibition zone = 31.1 ± 0.44 mm), followed by *Staphylococcus aureus* with an inhibition zone of 28.0 ± 0.57 mm. The crude extract was showed also a good impact on Gram-negative bacteria (*E. coli, Klebsiella pneumonia* and *S. typhimurium*) with inhibition zones ranged from 16 to 25 mm. Regarding control, Ciprofloxacin 30 µg was inhibited all examined bacteria, with zone of inhibition ranging from 25.66 ± 0.33 mm (*Bacillus cereus*) to 37.33 ± 0.33 mm (*S. typhimurium*). Fluconazole was also inhibited *Candida albicans* (diameter of the

Table 1
The detected compounds in the GC–MS profile of the fungal extract and related biological activities.

No.	Compound name	Retention time (min)	Molecular weight	Molecular formula	Peak area (%)	Biological activities
1	Chloromethyl benzene	7.75	126	C_7H_7Cl	16.20	Not reported
2	1-Chlorotetradecane	18.20	232	$C_{14}H_{29}Cl$	1.16	Antimicrobial (Kavitha and Uduman, 2017)
3	1-Dodecanamine, N,N-dimethyl-	20.41	213	$C_{14}H_{31}N$	47.67	Antibacterial, antifungal (Andersen, 1995), and anti-cancer (Liu et al. 2020)
4	1-Tetradecanamine, N,N-dimethyl	25.51	241	$C_{16}H_{35}N$	17.00	Antibacterial, antifungal (Santra and Banerjee 2020), and anti-cancer (Hussein et al. 2016)
5	10-Octadecenoic acid, methyl ester	33.36	296	$C_{19}H_{36}O_2$	2.26	Antioxidant and antimicrobial (Banaras and Shoaib 2017)
6	N-Methyl-N-benzyl tetradecanamine	33.69	317	$C_{22}H_{39}N$	6.63	Anti-ulcer agents (Al-Tameme et al. 2015)
7	9-octadecenoic acid (z)-	35.27	282	$C_{18}H_{34}O_2$	1.85	Anticancer, antiandrogenic, anti-inflammatory, dermatitigenic Anemiagenic, and Insectifuge (Adegoke et al. 2019)
8	Calcitriol	37.43	416	$C_{27}H_{44}O_3$	1.58	regulation of calcium homeostasis, reduce risk of cancer, hypertension, autoimmune diseases, and diabetes (Papoutsis et al. 2020)
9	Oleic acid, eicosyl ester	44.10	562	$C_{38}H_{74}O_2$	2.72	Insectifuge, anti-inflammatory, cancer preventive and hypocholesterolemic (Gurunathan et al. 2016)

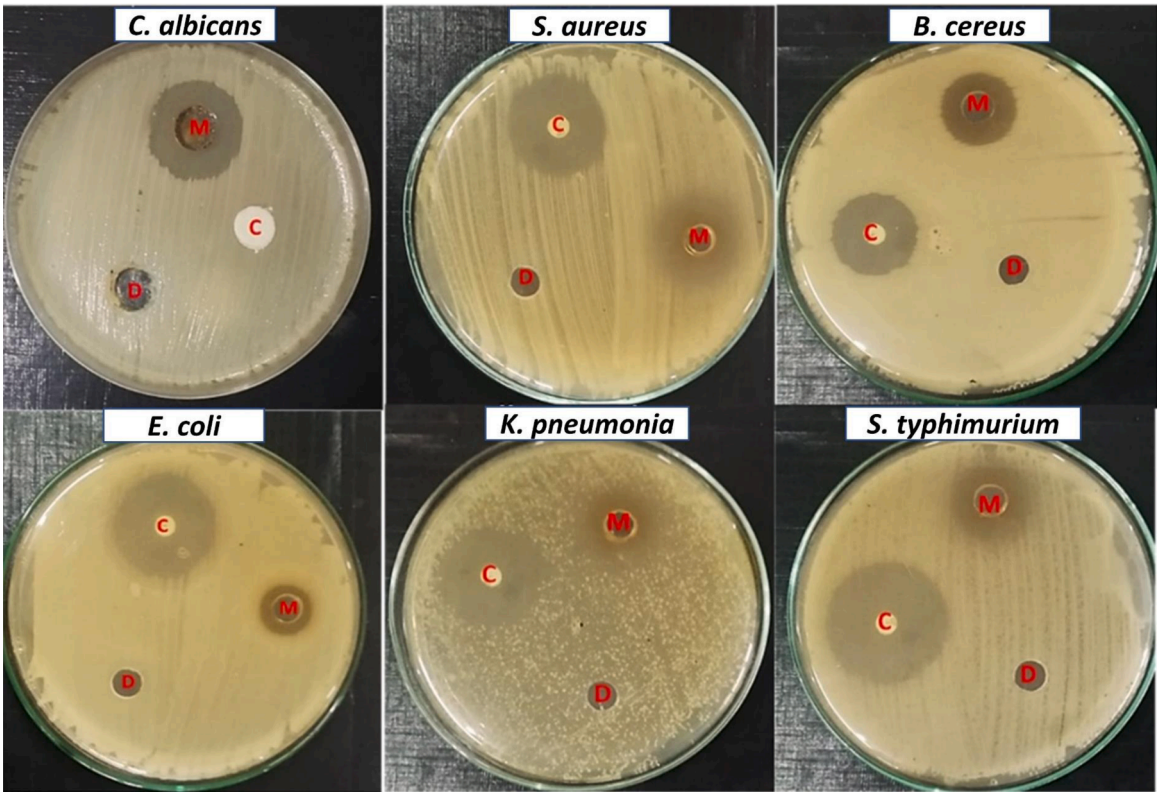

Fig. 4. Antimicrobial activity of *Mucor bainieri* MK-Bee2 extract against *Candida albicans* ATCC 10,231, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10,987, *Escherichia coli* ATCC 8739, *Klebsiella pneumonia* ATCC 13,883, *Salmonella typhimurium* ATCC 14,028. M; *Mucor bainieri* MK-Bee2 crude extract, C; antibiotic control and D; Solvent control (DMSO).

inhibition zone = 10 ± 0.57 mm). On the other hand, DMSO did not show any effect on the tested microorganisms, confirming that the inhibition zones observed with extract represent an exclusive effect of the metabolites included in the *M. bainieri* MK-Bee-2 crude extract. The MIC values of the extract were varied according to the tested microorganism, they ranged from 62.5 μg/ml (*Candida albicans*) to 250 μg/ml (*E. coli* and *Klebsiella pneumonia*) (Table 2).

3.3.2. Antioxidant activity of the fungal extract

DPPH and ABTS assays were used to assess the antioxidant activity of the *Mucor bainieri* MK-Bee-2 extract. Generally, the antioxidant capacity of this fungal extract was appeared to be high and in a concentration-dependent pattern at all concentrations, as its value increases with increasing concentration and vice versa. The high antioxidant activity of the crude extract of *Mucor bainieri* MK-Bee-2 was described graphically in Fig. (5). It scavenged the free radicals of DPPH with the inhibition of 97.34±0.22% (EC_{50} = 50.98 μg/ml) at 1000 μg/ml. Moreover, ascorbic acid which was used as a positive control exhibited a scavenging percentage value of 93.55±1.34% with EC_{50} of 26.25 μg/ml. At 1000 μg/ml, the crude extract of *Mucor bainieri* MK-Bee-2 scavenged the free radicals of ABTS with the inhibition of 91.8 ± 1.47% (EC_{50} = 48.06 μg/ml) compared to ascorbic acid which exhibited scavenging percentage value of 95.66±0.35% with EC_{50} of 27.4 μg/ml at the same concentration.

Table 2
Antimicrobial activity of the crude extracts of *M. bainieri* MK-Bee2.

Microorganism	Mean of inhibition zone diameter (mm ± SD)			MIC of the crude extract (µg/ml)
	M	C	D	
Candida albicans	31.1 ± 0.44	10.00± 0.57	0	62.5
Staphylococcus aureus	28.0 ± 0.57	31.33± 0.33	0	125
Bacillus cereus	23.8 ± 0.44	25.66± 0.33	0	125
E. coli	16.5 ± 0.28	30.00± 0.57	0	250
S. typhimurium	25.3 ± 0.33	37.33± 0.33	0	125
Klebsiella pneumonia	25.0 ± 0.57	30.66± 0.33	0	250

M: *Mucor bainieri* MK-Bee2 crude extract, C: Antibiotic control, D: Solvent control (DMSO).

3.3.3. Antitumor activity

To assess the cytotoxic effect of the crude extracts of *Mucor bainieri* MK-Bee-2 on cancer cells, human lung carcinoma cell line (A549) and hepatocellular carcinoma cell line (HepG2) were used and cell viability was tested by MTT assay. As shown in Fig. (6), the fungal crude extract inhibited cell proliferation (reduced the percentage of viable cells) of both lung A549 and liver HepG2 cancer cells compared to the control cells in a concentration-dependent manner. After treatment of cells for 24 h with the fungal extract, the growth of A549 and HepG2 cells was inhibited with IC_{50} values of 6.45 and 27.48 µg/ml, respectively Table (3). Also, our results demonstrated that the crude extract induced morphological changes including cell shrinkage, destruction of cell sheet cytoplasmic condensation, rupturing and detaching from the surface of tissue culture and irregularity of cell shape. Also, the viability of treated cells was decreased in a dose-dependent manner when compared to untreated cells that were well adherent, homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous cellular contents Fig. (7). Given the obvious strong activity of the fungal extract against A549 cells, these cells were continued for further examinations in this study

3.3.4. Cytotoxicity activity

The *in vitro* potential cytotoxic activity of the crude extracts of *Mucor bainieri* MK-Bee-2 was assessed against human lung fibroblast (Wi-38) and oral epithelial cells (OEC). Wi-38 and OEC cells were treated with *Mucor bainieri* MK-Bee-2 crude extract for 24 h and the cytotoxic effects were evaluated to check the safety of the fungal extract against normal cells. Data presented in Table (4) demonstrate that treatment of normal cells with *Mucor bainieri* MK-Bee-2 led to an increase in the IC_{50} for both Wi-38 and OEC normal cell lines (106.99 and 132.57 µg/ml) compared with the cancer cell line, respectively. Also, we evaluated changes in the morphology of Wi-38 and OEC cells using phase-contrast microscopy. The treated Wi-38 and OEC cells at high concentrations of (250–1000 µg/ml) showed rounded morphology and tended to detach from the substratum, cell shrinkage and irregularity in shape. However, at low concentrations (125–31.25 µg/ml) of the extract, the cells retained their normal morphological appearance Fig. (7).

3.3.5. Effect of fungal extract and γ- irradiation on the apoptotic induction of A549 cells

Because of the sensitivity of A549 cells to *Mucor bainieri* MK-Bee-2 extract, the apoptotic effects against this type of cells were further investigated. Annexin V–FITC/PI staining confirmed the apoptotic effects of *Mucor bainieri* MK-Bee-2 extract on A549 cells. As shown in Fig. (8A), early and late apoptotic cells, as well as normal cells, were detected after 24 h of treatment with the fungal extract and/or ionizing radiation. Apoptosis in the A549 group was significantly decreased compared to all the other groups. Treatment with extract, or **γ**- radiation

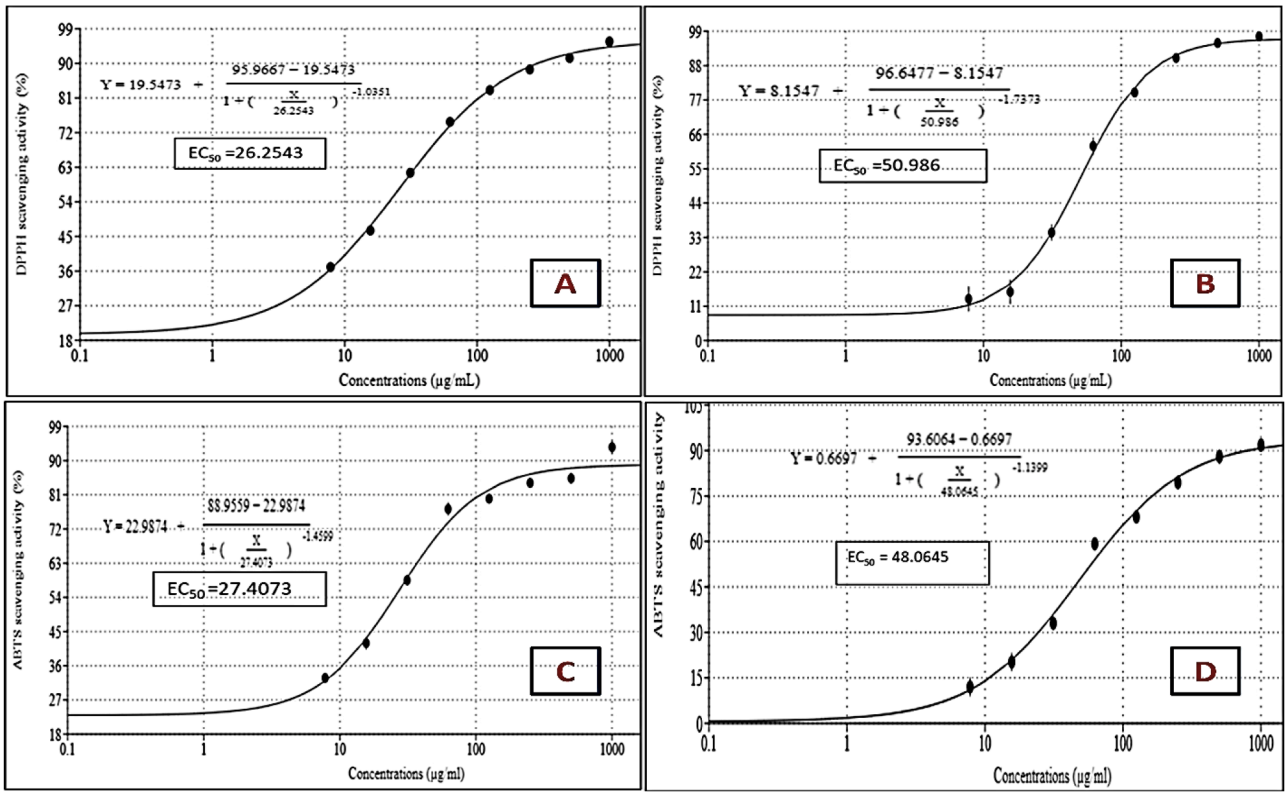

Fig. 5. Antioxidant scavenging activities of *Mucor bainieri* MK-Bee-2 extract and ascorbic acid. (A) ascorbic acid vs. DPPH, (B) MK-Bee-2 crude extract vs. DPPH, (C) ascorbic acid vs. ABTS, and (D) MK-Bee-2 crude extract vs ABTS.

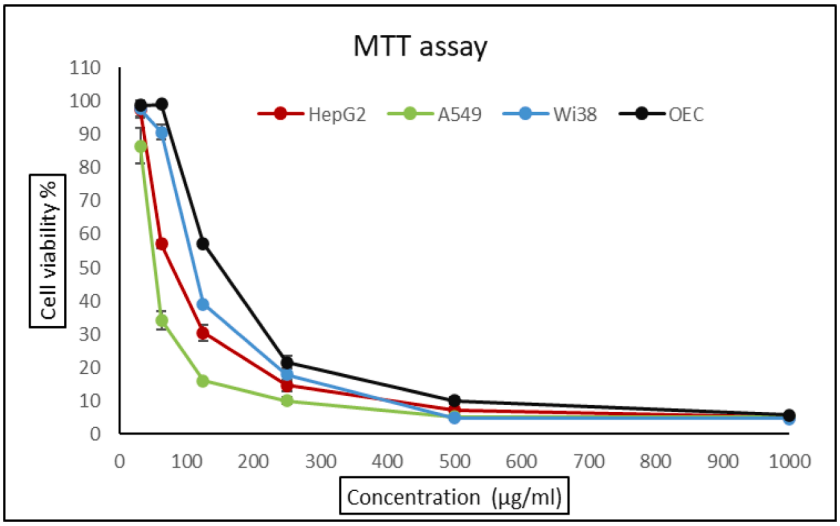

Fig. 6. Effect of crude extract of *Mucor bainieri* MK-Bee-2 on cell viability of liver (HepG2), lung (A549) cancer cell lines, normal human lung fibroblast (Wi-38) and normal oral epithelial (OEC) cells using MTT assay.

Table 3
IC_{50} value (µg/ml) of *Mucor bainieri* MK-Bee-2 crude extract on different cancer cell lines (A549 & HepG2) compared to normal cell lines (Wi-38 & OEC).

Crude extract	Normal cells		Cancer cells	
	Wi-38	OEC	A549	HePG2
Mucor bainieri MK-Bee-2	106.99	132.57	6.45	27.48

resulted in the appearance of two distinct populations: one presenting annexin V single positive expression, which was assigned for early apoptotic cells (lower right quadrant), and the other showing annexin V and PI double positive staining (upper right quadrant), that we have referred as late apoptotic cells. The exposure of A549 cells to the extract increased the ratio of total apoptotic cells to 15.1%. The untreated group exhibited high cell viability, with 7.2% total apoptosis. Concomitant treatment with radiation or extract + radiation elevated the total apoptotic cells percentage to 40 and 51.6%, respectively as shown in Fig. (8B). Therefore, the growth inhibition of A549 cells was related to the apoptosis-induction effect.

4. Discussion

Beneficial fungi such as *Penicillium, Cladosporium*, and some Zygomycetes including *Rhizopus* and *Mucor* play important roles in honeybee colonies (*Apis mellifera*). These fungi provide a natural defensive barrier to fight against possible microbial infections such as chalkbrood (*Ascosphaera apis*). In addition, they play a part in the production of bee bread, which is a pollen-based fungal fermentation product [7]. Moreover, these fungal species have been isolated from insects was indicated that insects are dependent on these fungal species to assimilate complex compounds such as cellulose, hemicelluloses, and lignin which represent the major components of the cell walls of woody plants. Furthermore, Insects require these fungi for a variety of metabolic processes, including the production of amino acids, vitamins, lipids, sterols, and pheromones, and chemical detoxification [37]. Many species of filamentous fungi have been isolated from the alimentary canal of worker honeybees and the most frequently found molds belonged to the genera of Penicillium and Aspergillus: commonly identified species include *P. frequentans, P. cyclopium, A. flavus,* and *A. niger* [38,39]. To the best of our knowledge, this is the first study to isolate, identify and characterize *Mucor bainieri* from healthy honeybees. *Mucor bainieri* had been identified as an endophytic fungus in plant *Begonia Plumier* located in the Atlantic Rain Forest [40]. Although Disayathanoowat et al. [41] have reported that *Mucor* as a fungal genus related to bees and bee products, *Mucor bainieri* has never been isolated from honeybees or even their products. Moreover, the biological activities of *Mucor bainieri* have never been documented in any early studies.

In general, much vivid information regarding the expected chemical compounds in unknown samples can be obtained by GC–MS. GC–MS analysis provides a typical spectrum output for each chemical compound in a given material. In recent years, GC–MS has been considered a major technology platform for characterizing secondary metabolites in both plant and non-plant species. Higher fungi have evolved a unique metabolic mechanism that can produce a range of functional secondary metabolites with varying chemical structures and bioactivity to survive harsh environments and complete the cell life cycle. So, they offer a comprehensive and interesting resource for innovative drug discovery [42,43]. In line with this hypothesis, our *Mucor bainieri* isolate has shown nine potentially active metabolites with biological properties including antimicrobial, antioxidant, and anticancer activities.

Different fungal genera are active producers for several metabolites with pharmaceutical importance; however, most of these fungal genera are included in Ascomycetous fungi, such as *Aspergillus* and *Penicillium*, and few genera in Zygomycetes and Basidiomycetous fungi [44]. So, the antimicrobial activity in the genus *Mucor* is relatively fewer than that of *Aspergilli*. For example, Noureen et al. [45] reported that out of nine screened *Mucor* isolates, only one isolate showed antibacterial activity against *Staphylococcus aureus, Bacillus cereus, E. coli* and *Pseudomonas aeruginosa* with inhibition zones ranged from 12 mm (against *Staphylococcus aureus*) to 7 mm (against *Bacillus cereus*). Herein, our fungal crude extract has shown a comparatively strong antimicrobial activity with lower MIC values(62.5 µg/ml) against the most common yeast pathogen *C. albicans* similar to that shown with silver nanoparticles [46]. Furthermore, based on the GC–MS analysis, the extract of *Mucor bainieri* MK-Bee2 includes five compounds with antimicrobial activities, accounted for 68.78% of the overall peak area percentage. This enrichment with antimicrobial active compounds in fungal extract might be related to the source of the fungal isolation (honeybees).

Reactive oxygen species (ROS) are developed in live cells as a result of the oxidation process, and they are extremely harmful due to their high redox potentiality. There is a balance between ROS generation and antioxidant capacity within healthy cells, but when this balance damaged, cells undergo oxidation processes resulting in oxidative stress [47]. Excessive oxidative stress generates uncontrolled ROS, which destroys cellular components including proteins, DNA, and lipids.

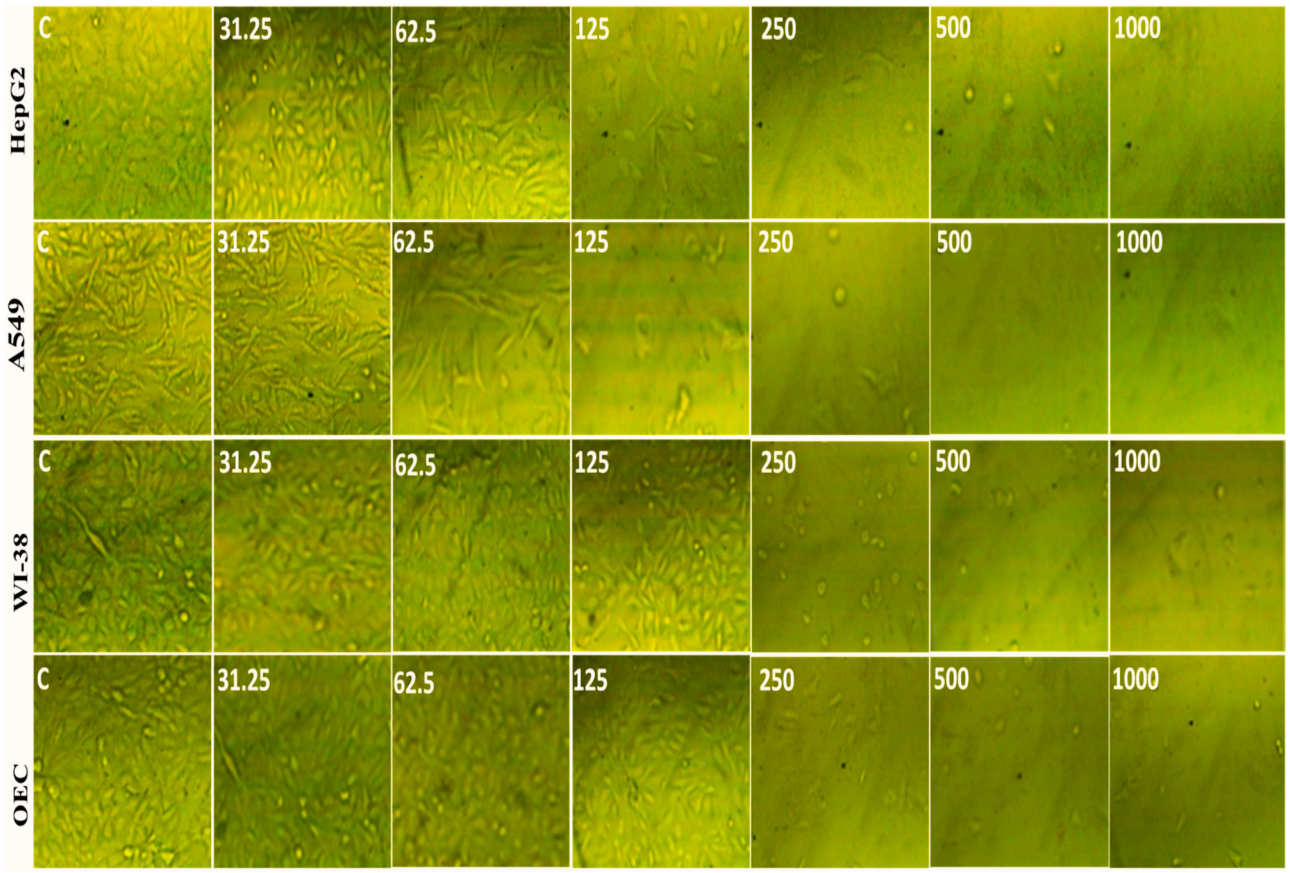

Fig. 7. *In vitro* anti-proliferative morphological effects of crude extract of *Mucor bainieri* MK-Bee-2 at twofold concentrations (31.25 −1000 μg/ml) against human hepatocellular carcinoma (HepG2), human lung carcinoma (A549), human lung fibroblast (Wi-38), and oral epithelial cells (OEC) (×10 magnification). The experiments were repeated three times and data were presented ±SD.

However, antioxidant efficiency for any substance refers to the ability of the substance to minimize the detrimental effects of oxidative stress by preventing or scavenging uncontrolled free radical production. The antioxidant activity can be assessed by a variety of assays which could affect the outcome. As a result, one assay would not be indicative enough for determining the antioxidant activity of the fungal crude extract. So, we used two different techniques to assess the antioxidant capacity of the fungal crude extract, which may yield more trustworthy results [48]. Based on the antioxidant activity results, we can conclude that the crude extract of *Mucor bainieri* MK-Bee2 exhibited a promising ability to scavenge free radicals of DPPH and ABTS. This ability may be correlated to the presence of various bioactive compounds in this fungal crude extract, such as 10-Octadecenoic acid methyl ester, 9-Octadecenoic acid (z)-, Oleic acid eicosyl ester, and 9-octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yL) methyl ester cis- which belong fatty acid esters. The various active groups in the chemical structure of these bioactive compounds, such as hydroxyl groups and unsaturated bonds may offer the fungal extract high ability to scavenge free radicals and inhibit excessive oxidation processes [49]. Previous research has suggested that fungi may be a good source of antioxidants. For example, Hameed et al. [50] reported three *Mucor circinelloides* strains with antioxidant activities due to the presence of phenolic compounds, condensed tannins and flavonoids.

Previous studies reported that fungal crude extracts contain several metabolites that possess different biological activities, including anticancer properties [51–54]. In the present study, we found that the fungal extract exhibited high anticancer activity with higher potency against human lung carcinoma (A549), followed by hepatocellular carcinoma (HepG2) cancer cells, with the lowest impact on normal cells including human lung fibroblast (Wi-38) and oral epithelial cells (OEC). The fungal crude extract exhibited a cytotoxic effect against normal cells, Wi-38 and OEC cells, with an IC_{50} of 106.99 and 132.57 μg/ml, respectively. But, same extract has resulted higher cytotoxic effect with much lower IC_{50} of 6.45 and 27.48 μg/ml against A549 and HepG2 cancer cell lines, respectively. The demonstrated IC_{50} values in the current study indicate that this extract can be considered less toxic to human normal cells and it will preferentially destroy the A549 and HepG2 cancer cells.

The high incidence of cancer and the high cost of its treatment are important factors driving the search for new and effective chemotherapeutic substances with multi-target activity and safe for normal cells. Our results demonstrated that the crude extract of *Mucor bainieri* MK-Bee2 significantly inhibited cell proliferation of all tested cancer cells and had the strongest inhibitory effect against A549 cells in a concentration-dependent manner. Annexin V is an intracellular protein that can specifically bind to phosphatidylserine (PS) in a concentration-dependent manner. Normally, PS is only found on the inner leaflet of the plasma membrane in healthy cells. Externalization or translocation of PS from the intracellular to the external plasma membrane leaflet (extracellular side) occurs in early apoptosis and precedes other apoptotic events such as loss of membrane integrity and DNA fragmentation. Morphological observations and Annexin-V/PI flow cytometric analysis further evidenced the apoptosis-inducing effect of the crude extract. Our study is supported by the result of Wang et al., [55], who indicated that exopolysaccharide promoted SGC-7901 cell death via apoptosis. Therefore, crude extract from *Mucor bainieri* MK-Bee2 can be developed as a potential antitumor agent according to the studies of El Bakary et al., [56,57] on honey bee venom pure extract; they found that extract might represent a potential therapeutic strategy for solid tumor. More interesting was the observation that the crude extract, along with

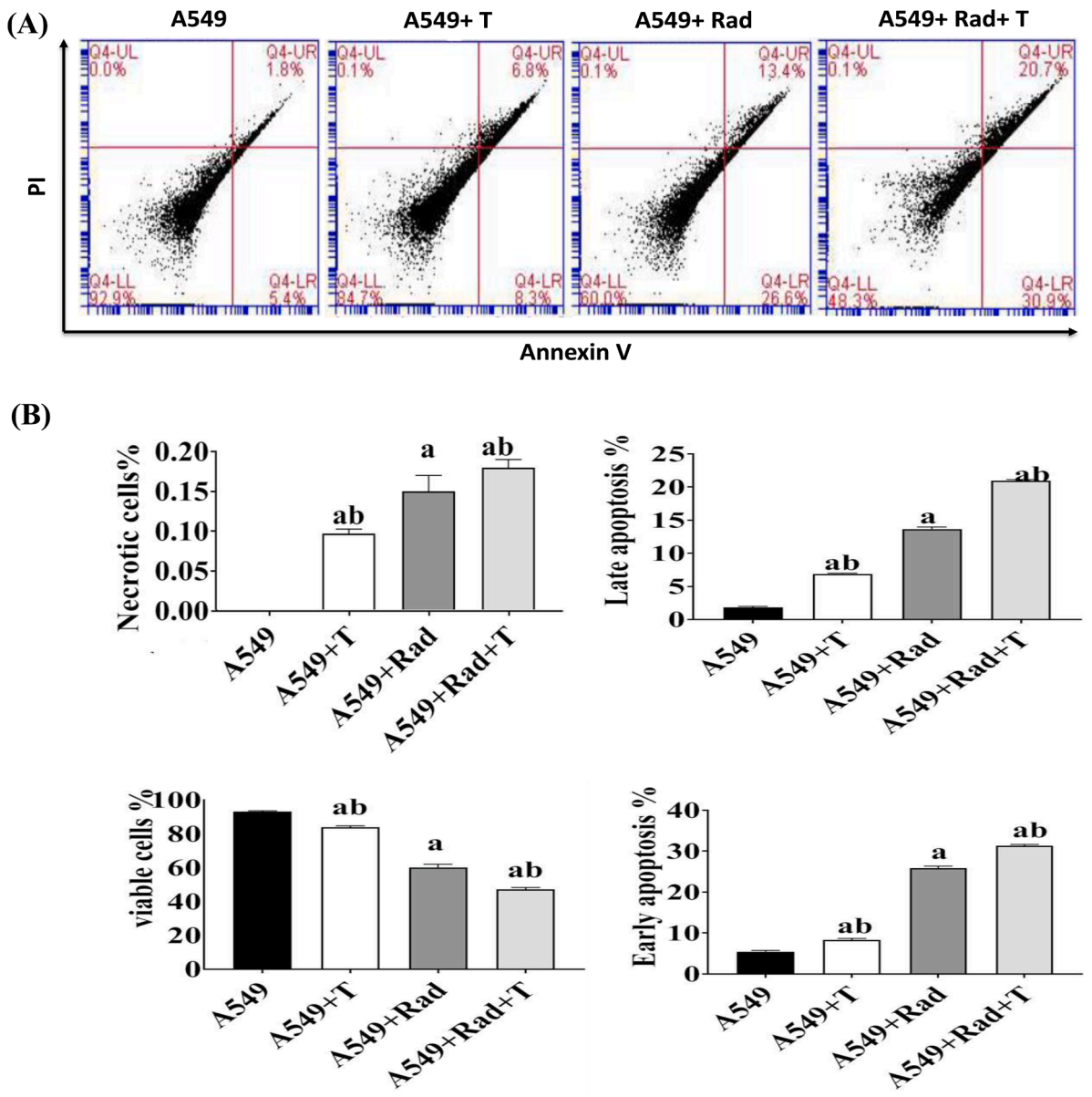

Fig. 8. Effects of *Mucor bainieri* MK-Bee-2 crude extract and ionizing radiation treatment on apoptosis of A549 cells (A and B). Each value represents the mean ± SEM ($n = 3$). Columns denoted with "a" significant from A549 cancer cells, "b": significant from A549+Rad, at ($p < 0.01$). Statistical analysis was carried out using one way analysis of variance (ANOVA). A549, untreated A549 cells control; A549+*T*, A549 cells treated with Mucor bainieri MK-Bee-2 extract; A549+Rad, A549 cells treated with radiation; A549+Radiation+*T*, A549 cells treated with *Mucor bainieri* MK-Bee-2 extract and radiation.

radiation exposure virtually increased the apoptosis. Thus, it can be inferred that the crude extract sensitizes lung cancer cells to radiation-induced cell death [58]. The combination of radiotherapy with the fungal extract as an apoptosis inducer showed synergistic antitumor effects. Given the pressing clinical needs, the development of original approaches in cancer treatment to arrive new level of success in the continuous battle against cancer required searching deeply into the relatively tiny details. One of the interesting tricky points is the relation between crude extract and gamma irradiation. This combination is promising. Thus, we are confident that this unique endeavor of combination will remain a positive footmark in cancer research. In the future, *in vivo* studies and clinical trials with this combination will warrant the effectiveness of the current approach.

5. Conclusion

In conclusion, *Mucor bainieri* MK-Bee2, a fungal species, was isolated for the first time from honeybees. This fungus produces various metabolites which have wide biological activities including antimicrobial, antioxidant, and antitumor. The crude extract of this fungus showed antibacterial against Gram-positive and Gram-negative bacteria, as well as *Candida albicans*. Furthermore, the extract showed potential antioxidant activities when compared to ascorbic acid, suggesting that the fungal crude extract has a potential activity against cancer cells. Where, MTT assays, morphological observations, and flow cytometry analyses indicated that the extract could selectively inhibit A549 cell growth by inducing apoptosis. This study indicates that the *Mucor bainieri* MK-Bee2 extract could be a novel source for promising anticancer agents against human lung and hepatocellular carcinoma. But, future studies are required to investigate the mechanism of apoptosis induced by each fungal metabolite for better action and long-term safety.

Authors' contributions

M.H.K. research conceptualization; M.H.K., M.H.S., M.A.E., S.E.E., N.M. E., and M.H.S. performed research, analyzed data, and wrote the paper; M.H.K. and M.A.E. drafted the manuscript. All authors reviewed and approved the manuscript.

Research involving humans and animals statement

This research does not involve human or animal study.

Informed consent

Not applicable.

Funding

This research did not receive any funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We would like to express our appreciation to Botany and Microbiology Department, Faculty of Science, Al-Azhar University and Zoology and Entomology Department, Faculty of Science (Girls), Al-Azhar University for supporting this study.

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