

# Biological Evaluation of Endophytic Fungus, *Chaetomium globosum* JN711454, as Potential Candidate for Improving Drug Discovery

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**Abstract** The main objective of this research work focused on investigating the biological and chemical aspects of endophytic fungus *Chaetomium globosum*, for pharmaceutical purposes to improve the drug discovery process. The endophytic *C. globosum* was isolated from healthy leaves of Egyptian medicinal plant *Adiantum capillus-veneris* collected from Saint Katherine Protectorate, Sinai, Egypt. The identification of *C. globosum* was on the basis of classical and molecular taxonomy. Gene encoding for 18S rRNA was partially sequenced, submitted to the GenBank and got the accession number JN711454, to resolve the phylogenetic relations with fungal ancestor using phylogenetic tree. To explore the biosynthetic power of endophytic *C. globosum* JN711454, the fungus was cultivated over five different media, oatmeal, rice, yeast malt glucose, potato dextrose agar (PDA) and Czapek's dox media, for 3 weeks at 30 °C, followed by extraction with different solvents, ethyl acetate (EA), and methanol. The ethyl acetate extract of *C. globosum* cultivated on PDA

medium was the most potent extract. It showed strong antioxidant activity with EC<sub>50</sub> 11.5 µg/ml, potent anticancer activity with 55 % toxicity toward HepG-2 cells at 100 µg/ml and 66 % cytotoxicity to FGC<sub>4</sub> cells at 250 µg/ml, promising butyrylcholinesterase inhibitory activities (>85 %), and moderate antimicrobial and stopped the attachment of HSV-2 virus to VERO cells. The metabolomic profiling of PDA–EA extract using LC–MS revealed the presence of several metabolites to which the observed bioactivities could be attributed. Here we report for the first time inhibitory activity of endophytic *C. globosum* JN711454 secondary metabolites to butyrylcholinesterase, one of neuro hydrolase enzymes that play a major role in development of Alzheimer's disease.

**Keywords** *Adiantum capillus-veneris* · Alzheimer · Cytotoxicity · Endophytic *Chaetomium globosum* · LC–MS · Metabolomics

## Introduction

Endophytes are microbes (mostly fungi) that colonize inter- and/or intracellular spaces of apparently healthy plant tissues without causing any apparent harm or symptomatic infection to their host. Endophytes represent an abundant and dependable source of bioactive agents with potential for exploitation in pharmaceutical applications and considered relatively underexploited sources of novel bioactive agents. All plants on our planet may serve as a reservoir of untold and large numbers of endophytes [1, 2]. The great biodiversity and promising bioactivities of endophytic isolates from medicinal plants, indicate the importance of excessive investigation of endophytic strains. The crucial approach for searching on novel natural

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products will help in conservation of medicinal plants and maintenance of environmental biodiversity [2].

Saint Katherine Protectorate (SKP), Sinai, is natural reservoir of wild fauna and flora, has especial environmental characters as well as it contains wide diversity of Egyptian medicinal plants, and considered as an inexhaustible resource of bioactive endophytic strains that have not yet been well exploited [2]. The medicinal plant, *Adiantum capillus-veneris*, is a species of ferns of genus *Adiantum*, which is widely distributed throughout the world and considered one of target species in SKP [3, 4]. It has many applications in folk medicine as tonic, analgesic, diuretic, stimulant, emollient, purgative, demulcent, and excessively used in treatment of cold, fever, cough, bronchial disorders, skin diseases, tumor, jaundice, and hepatitis. Also, antimicrobial, anti-inflammatory, and hypoglycemic activities of *A. capillus-veneris* were reported [4, 5]. However, little is known about endophytes harbored inside the healthy tissues of *A. capillus-veneris*, so our target was isolation, identification, and biological evaluation of endophytes associated with this medicinal plant; we succeeded in isolation of the endophytic fungus, *Chaetomium globosum*.

The genus *Chaetomium* is one of the most common endophytic ascomycetes of family Chaetomiaceae. Members of this family are characterized by production of wide range of secondary metabolite with significant biological activities. The endophytic *C. globosum* has been reported from different niches as prolific producers of bioactive agents. The previous studies of endophytic *C. globosum* strains have resulted in the isolation of several bioactive compounds, such as indole derivatives, chaetoglobosins [6, 7], globosumones [8], globosuxanthone [9], cochliodes [10], azaphilones [11, 12], chaetoglocins [13].

The bioactivity of the endophytic fungus *C. globosum* isolated from medicinal plant *A. capillus-veneris* from SKP, Sinai, Egypt will be discussed. Also, we will shade some light on the different cultivation conditions for maximum production of these active fungal metabolites. The metabolomic profiling of secondary metabolites based on LC–MS technique will be used as useful tools for prediction of active compounds which could be responsible for such bioactivities.

## Materials and Methods

### Plant Material

The medicinal plant *A. capillus-veneris* (family Adiantaceae) was collected from SKP, Sinai, Egypt, in September 2010. Only apparently healthy and disease free plant was collected in order to minimize the presence of pathogenic and saprophytic microbes. The plant was stored in separate

plastic bags at 4 °C in an ice box until isolation procedures [2].

### Isolation of Endophytic Fungus

The endophytic fungus *C. globosum* was isolated from fresh leaves of *A. capillus-veneris* by following the method that described in Selim et al. [2].

### Identification of Endophytic Fungus

Taxonomic identification of endophytic isolate was performed on the basis of morphological characters of fungal culture, colony, or hyphae, and the characters of reproductive structure of endophytic fungus, according to identification keys [14].

Genetic identification using molecular taxonomy was performed to validate and confirm the morphological identification of *C. globosum* and to study the phylogeny. Specific PCR primers were used to amplify 18S rDNA region of endophytes and for partial sequencing of gene encoding Ribosomal RNA [15].

### Extraction of Genomic DNA

The extraction of genomic fungal DNA from *C. globosum* was performed by following the general instructions in [16] and GenELute Genomic DNA extraction Kit (Sigma-Aldrich). About 15 mg of fungal mycelial mat were scraped with a sterile nipper from fresh culture growing on PDA medium at 28–30 °C for 15 days and grinded vigorously with sterile sea-sand. Grinded mycelial cells were suspended in lysis buffer T/STL buffer, vortexed strongly and incubated at 55 °C in water bath for 60 min, then incubated at –80 °C for 24 h. After that, frozen cells were leaved at room temperature for 30 min to allow sudden shock to cells, then centrifugation at 12,000g/5 min. The pellet was resuspended in 200 µl of lysozyme solution, vortexed and incubated at 55 °C for 60 min and centrifuged to remove supernatant. Finally, 20 µl of the proteinase K solution was added to the sample, followed by 200 µl of lysis solution C, vortexed (about 15 s), and incubated at 55 °C for 10 min.

For precipitation of proteins and DNA, 200 µl of protein precipitation solution (PPS) were added to lysate and kept in ice for 5 min then centrifuged at 12,000g/5 min. The supernatant was transferred to a new Eppendorf tube using a blunt-ended pipette tip. The chromosomal DNA was precipitated by the addition of ethanol, then centrifugation at 12,000g/5 min. The DNA pellets were transferred to a new Eppendorf tube, and rinsed with 70 % ethanol, centrifuged at 12,000g/5 min, and then dried under vacuum. This protocol was successful in producing amplifiable

DNA from most of fungi [17]. The extracted DNA pellet was then dissolved in a suitable volume (100 µl) of TE buffer (100 mM NaCl, 1 mM EDTA, 100 mM Tris–HCl, pH 8.00) and kept at 2–8 °C for short term storage, and at –20 °C for long term storage [15].

#### Amplification of Genomic DNA (PCR)

Amplification of 18S rDNA gene was done by polymerase chain reaction (PCR) using universal fungal specific primers designed to amplify about 530 bp fragment of conserved region of ribosomal DNA. The forward fungal specific primer was EF4f (5'-GGAAGGG [G/A] TGTA TTTATTAG-3'), and the reverse fungal specific primer was Fung5r (5'-GTAAAAGTCCTGGTTCCC-3') [17].

Amplification reactions (PCR mixture) were performed in 50 µl polymerase buffer containing 30 pmol of each primer, 10 ng of template DNA, 200 µM dNTPs and 2.5 units of *Taq* polymerase. The PCR thermal cycle was carried out for 30 cycles at 94 °C for 1 min for denaturation step, followed by primer annealing at 50 °C for 1 min and at 72 °C in the extension step for 2 min. After completion, the PCR product was examined using agarose gel electrophoresis by running 5 µl of the PCR product in 1.0 % (w/v) agarose gel with 1 × tris/borate electrophoresis buffer (TBE). The gel bands were stained with ethidium bromide (0.5 µg/ml). The gel was photographed using gel documentation UV transilluminator, and the DNA bands on gel were compared with reference marker [18]. The amplified DNA was purified using QIA quick PCR amplification reagent (Qiagen).

#### Sequencing of Amplified DNA Fragment

The purified PCR products sequenced directly with the same PCR primers, using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit including *Taq* polymerase and ABI PRISM 377 DNA Sequencer (Perkin Elmer).

#### Sequence Alignment and Phylogenetic Analysis

Analysis of sequence was performed using the basic sequence alignment BLAST program in comparison with available data from NCBI GenBank databases (website: [www.ncbi.nlm.gov/blast](http://www.ncbi.nlm.gov/blast)) to assess the DNA similarities. The nucleotide sequence of active endophytic fungus was submitted to the GenBank and assigned accession number JN711454.

The sequence of endophytic fungal isolate was aligned with representative sequences of reference taxa (sequences available from GenBank including members from other orders) to resolve phylogenetic relationships and to root cladograms. Multiple sequence alignment and molecular

phylogeny were performed using BioEdit software [19]. The phylogenetic tree was displayed using the TREEVIEW program [20].

#### Fermentation and Extraction of Fungal Secondary Metabolites

In order to exploit and explore the full biosynthetic potential of *C. globosum* JN711454, the secondary metabolites production was examined over different reported standard media to enhance the biosynthetic power of *C. globosum*. Fresh culture of *C. globosum*, grown on potato dextrose agar (PDA) medium [9], for 7 days, was inoculated into 100 ml of (a) Natural media: oatmeal [21] and rice medium [10]; (b) semi natural media: yeast malt glucose (YMG) [22], and PDA [9]; (c) synthetic medium: Czapek's Dox (Cz) [23]. The inoculated media were incubated at 30 °C, statically for 3 weeks. After incubation period, the mycelial mat and media were extracted with different organic solvents, ethyl acetate (EA) and methanol (MeOH), using ultrasonic bath for 30 min. The extraction procedures were performed in triplicate. All of the organic fractions were combined and concentrated to dryness under reduced vacuum to obtain crude fungal extracts.

#### Biological Evaluation of Fungal Metabolites

The variations of metabolites production were monitored by different bio-assays to select the most suitable medium for fermentation, and to select the most suitable extracting organic solvent. The different crude extracts of endophytic fungus, *C. globosum* JN711454, grown on different standard media were investigated for their antimicrobial, antioxidant, butyrylcholinesterase inhibitory and cytotoxic activity. All biological experiments were done in triplicates.

#### Antimicrobial Activity

The antimicrobial activity of the extracts were tested against five Gram positive bacteria (*Bacillus cereus* ATCC 14579, *Bacillus megaterium*; clinical local isolate, *Bacillus subtilis* NRRL-B-4219, *Micrococcus luteus* NRRL-B-287, *Staphylococcus aureus* ATCC 29213), four Gram negative bacteria (*Alcaligenes faecalis* B-170, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10131, *Pseudomonas aeruginosa* ATCC 27953), four yeasts (*Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750, *Saccharomyces cerevisiae* ATCC 2180-1A), and four filamentous fungi, clinical local isolate (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Penicillium* sp.). The test microbes; including clinical local isolates were identified

and obtained from Chemistry of Natural and Microbial Products Department, National Research Center (NRC), Egypt. The assay was performed using disc diffusion method at concentration of 500 µg/disk, as previously mentioned [2, 24].

#### *In Vitro Antioxidant Activity*

The antioxidant activities of the extracts were estimated using free radical scavenging (FRS) model and determination of total phenolic contents of extracts.

**Free Radical Scavenging (FRS) Model** The FRS assay was performed, as described in [25], with some modifications. One milligram of ethyl acetate of each of the fungal extracts were dissolved in 1 ml DMSO to prepare stock solution of 1,000 µg/ml. DPPH (0.004 mg) was dissolved in 100 ml of methanol HPLC grade to prepare 0.004 % solution, and stored in dark until use. Different concentrations (5–25 µg) of reference standard compounds (Vitamin C and Quercetin) were prepared.

In each well of a 96-well plate, 20 µl of stock solutions (samples-or-standard) was added followed by addition of 180 µl of methanolic solution of DPPH (0.004 %) to reach the final maximum concentration of tested samples 50 µg/ml. The reaction mixture was incubated for 30 min, and at the end of incubation time the plate was measured at  $\lambda = 540$  nm by microplate Reader. Negative controls were done to correct the absorbance of colored extracts to avoid the interference. Blank was measured by replacing 20 µl of samples by 20 µl of dissolving agent (DMSO). The assay was run in triplicate and repeated at least once for active extracts. The radical scavenging activity of fungal extracts and calibrator can be calculated from the following equation:

$$\% \text{ of scavenging activity} = \frac{[(A_{\text{Blank}} - A_{\text{Sample}})/A_{\text{Blank}}] \times 100}{100}$$

where  $A_{\text{Blank}}$  is the absorbance of reaction mixture without test sample (DPPH only), and  $A_{\text{Sample}}$  is the absorbance of reaction mixture in presence of test samples.

**Estimation of Total Phenolic Contents Using Folin Assay** The total phenolic contents of extracts were estimated colorimetrically using Folin assay, according to method that described in [26] with some modifications. The sample preparations were similar to FRS-assay. Folin–Ciocalteu reagent was diluted (1:9) with distilled water. Sodium carbonate solution (20 %) was prepared, filtered, and used.

Gallic acid (GA) was used as the standard phenolic compound, and series of GA concentrations (3.125–100 µg/ml)

were prepared for standard curve. Briefly, 10 µl of stock samples solutions were mixed with 40 µl of diluted Folin–Ciocalteu reagent in 96-well plate and incubated at room temperature for 3–5 min followed by addition of 50 µl of 20 % sodium carbonate. The reaction mixture was incubated in dark for 30 min, and absorption was determined spectrophotometrically at  $\lambda = 690$  nm using microplate reader. The standard gallic acid solutions were treated in the same manner as for samples and standard curve was obtained using various concentrations of GA. The total phenolic contents were calculated from standard curve and were expressed as mg/ml in comparison to GA.

#### *In Vitro Inhibitory Activity of Butyrylcholinesterase Using Ellman Assay*

The butyrylcholinesterase inhibitory activity was measured spectrophotometrically, according to Ellman method mentioned in [27], with some modifications, using Cholinesterase BTC/DTNB lyophilized kit (BEN Biochemical Enterprise, Italy). The butyrylthiocholine (BTC, 7 mmol/l) was used as substrate and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB, 0.25 mmol/l) was used as coloring agent in the assay. The BTC and DTNB were prepared by dissolving the powders in pyrophosphate buffer (>20 mmol/l, pH 7.7). Butyrylcholinesterase enzyme was obtained from the Biochemistry Laboratory, National Egyptian Center for Clinical and Environmental Toxicology Research (NECTR), Faculty of Medicine (Kasr Al Ainy), Cairo University. Samples were dissolved in DMSO, and diluted with distilled water to prepare stock solution (1,000 µg/ml, in 10 % DMSO). The reaction mixture contained 1,000 µl of DTNB, 100 µl of test extracts, and 100 µl of enzyme, the reaction mixture of test extracts incubated at 37 °C with the enzyme for 15 min to initiate inhibition of enzymes (test samples + DTNB + enzymes). The reaction was initiated by the addition of 100 µl of substrate (BTC). The hydrolysis of BTC was monitored at 405 nm, and the butyrylcholinesterase activity was determined by measuring the change in absorbance at time intervals (3 times, each 30 s). Samples that inhibited the enzyme activity greater than 85 % or more were regarded as active. Also, the inhibitory of 10 % of DMSO was measured. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition of BChE} = \frac{[(A_{\text{Blank}} - A_{\text{Sample}})/A_{\text{blank}}] \times 100}{100}$$

where  $A_{\text{Blank}}$  is the activity of the enzyme without test extracts, and  $A_{\text{Sample}}$  is the activity of enzyme in presence of test extracts.

### *In Vitro Cytotoxicity (Anticancer)*

The cytotoxicity of *C. globosum* metabolites was performed using neutral red uptake (NRU) assay and sulforhodamine B (SRB) assay.

**Neutral Red Uptake (NRU) Assay** The cytotoxicity of *C. globosum* extracts against rat hepatoma cancer cell line (FGC<sub>4</sub>) was determined by colorimetric neutral red uptake (NRU) assay according to modified method [25]. Rat hepatoma cancer cell line (FGC<sub>4</sub>, ATCC<sup>®</sup>) was maintained in humidified atmosphere (CO<sub>2</sub> incubator, Shellab, 5 % CO<sub>2</sub>, 37 °C) as monolayer cultures in nutrient mixture F-12 Ham medium (Lonza, Belgium) supplemented by 10 % FBS, 2 mM L-glutamine, 1 % MEM non essential amino acids, and antibiotic/antimycotic mixture (0.25 µg/ml amphotericin B, 100 U Penicillin and 100 µg/ml streptomycin sulfate).

The preliminary screening of all samples was based on dilution of the fungal samples stock solution in culture medium to give final concentration of 250 µg/ml for all of extracts. Exponentially growing monolayers of FGC<sub>4</sub> cells (at about 75–85 % confluency) in 24-well cell culture plates was treated with either DMSO (vehicle control) or the *C. globosum* extracts. Treated plates were incubated for 72 h before being subjected to cytotoxicity measurement by NRU. The absorbance was read on Fluostar Optima microplate reader (BMG LABTECH, Germany) at 540 nm. Cell free blank was used for background correction of the absorbance. The viability was calculated as a percentage relative to absorbance value of control after subtracting the contribution from cell-free blank. The optical density of the test wells after 72 h period of exposure to test extract is  $T_i$ , the optical density at time zero is  $T_0$ , and the control (untreated cells) optical density is  $C$ .

Percentage cell growth is calculated as

$$\frac{(T_i - T_0)/(C - T_0)}{\times 100} \text{ for concentrations at which } T_i \geq T_0$$

$$\frac{(T_i - T_0)/T_0}{\times 100} \text{ for concentrations at which } T_i < T_0.$$

\* On basis of the previous experiments results, EA extract of PDA medium was selected for further excessive biological evaluation for antiviral and anticancer activities on different human cancer cell lines.

**Sulforhodamine B (SRB) Assay** The growth inhibitory effects of EA extract of *C. globosum* cultivated on PDA medium were tested against five human cancer panel cell lines [CaCO-2 (colon), HepG2 (liver), MCF-7 (breast), TK10 (renal), and UACC62 (melanoma)], using a sulforhodamine B (SRB) assay, according to method that described in [28] with some modifications; all cell lines

were obtained from ATCC. The human cell lines were routinely maintained as monolayer cell cultures in RPMI medium or minimum essential Eagle medium. Both media were supplemented by 5 % fetal bovine serum, 2 mM L-glutamine, and 50 µg/ml gentamicin antibiotic.

For the cytotoxicity experiment, the cells (3–19 passages) were inoculated in 96-well microtiter plates at plating densities of 7–10,000 cells/well and were incubated for 24 h. After 24 h, one plate was fixed with trichloroacetic acid to represent a measurement of the cell population for each cell line at the time of drug addition. The other plates were treated with the experimental samples which were previously dissolved in DMSO as 10,000 µg/ml stocks, and diluted in medium to a final concentration 100 µg/ml. Cells without samples served as controls. Blank wells contained complete medium without cells, emetine was used as a reference standard control at 10 µM. The plates were incubated for 48 h after addition of the EA extract. At the end of the incubation period, the cells were fixed to the bottom of each well with cold 50 % trichloroacetic acid, washed, dried, and dyed with SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris–base for optical density determination at a wavelength 540 nm using a microplate reader. Optical density measurements were used to calculate the net percentage cell growth, as mentioned before.

### *In Vitro Antiviral Activity Using EPTT*

Herpes simplex virus type-2 (HSV-2) was chosen as an example for DNA viruses, and vesicular stomatitis virus (VSV) was chosen as an example of RNA viruses to evaluate the antiviral activity of EA extract of endophytic fungus *C. globosum* cultivated on PDA. Viruses were obtained from the Holding Company for Biological Products and Vaccines (VACSERA), Egypt. The antiviral bioassay is carried out by the end point titration technique (EPTT). The technique depends on the detection of the visible cytopathogenic effects (CPE) that is produced on thin confluent monolayer of VERO cells, after inoculation separately with the virus.

Assessment of the antiviral activity was accomplished, according to aspects described in [29, 30] with some modifications. EPTT was used to determine in vitro inhibition of CPE and reproducibility of HSV-2 and VSV on monolayer VERO cells infected with viruses and treated with safe concentration of fungal extract estimated from cytotoxicity assay. Confluent monolayers of VERO cells grown in 96-well plates were treated with safe concentration of test extracts for 24 h, then treating buffers were decanted and HSV-2 and VSV were serially tenfold diluted and dispensed to reciprocal wells to allow viral infections. Virus control and tissue culture control were considered in

order to evaluate the CPE; the plates were incubated at 37 °C with 5 % CO<sub>2</sub>. Infected cells were examined daily by means of microscopy for detecting CPE for a week. Virus suspensions were titrated to determine the virus titer in treated and non treated cells, where the virus titers are the smallest amount of virus capable of producing a reaction in the host cells. The antiviral activity depend on the ability of the extracts to inhibit the produced CPE and expressed by the difference in viral titer between treated and non treated cells (CPE inhibition%) and reveal the antiviral activity percent.

$$\begin{aligned} &\% \text{ of antiviral activity in form of CPE inhibition } \% \\ &= [1 - (\text{CPE}_{\text{test}}/\text{CPE}_{\text{control}})] \times 100. \end{aligned}$$

### Metabolomic Profiling

Metabolomic profiling for promising biological active EA extract of *C. globosum* JN711454 cultivated on PDA medium was done using LC–MS. Electro-spray Ionization mass (ESI-MS) spectra for crude EA extract was measured using a Waters Q-TOF micro LC–MS/MS instrument (Waters) with Waters Cap-LC system with integrated photodiode array and ESI ion spray source (Korea University, Seoul, South Korea). The samples were dissolved in MeOH for HPLC (1 mg/ml) for injection into the HPLC-ESI-MS system. Separation through LC-column was performed using gradient separation from 20 to 100 % acetonitrile/water over a period of 10 min with a flow rate 1 ml/min, and monitored by absorption at 254 nm with a photodiode array detector. Positive and negative ionization modes were detected with mass scan range 100–1,400 amu.

## Results and Discussion

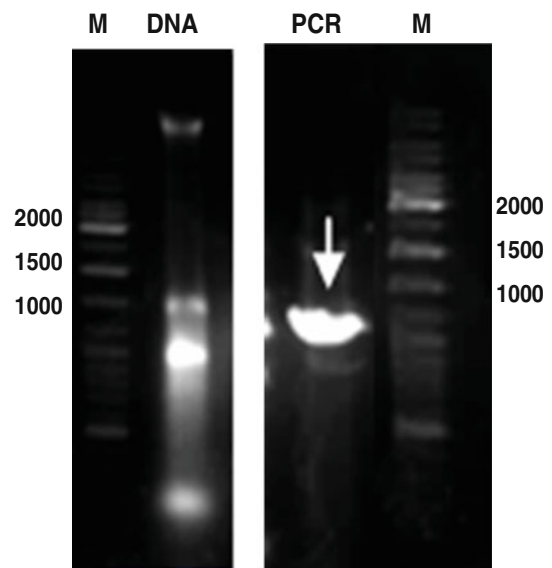
Endophytic fungi represent an important source for improving drug discovery process, where they may possess the steadiness antimicrobial, anticancer, antiviral, and antioxidant activities [1, 31, 32]. Through our screening program for promising biological active endophytes from Egyptian medicinal plants, the endophytic fungus *C. globosum* was isolated from fresh leaves of medicinal plant *A. capillus-veneris*. The preliminary identification of endophytic fungus was performed according to classical mycology on bases of morphological features of fungus colony, while the identification of majority of endophytic fungi to the species level is very difficult using their microscopic characteristics. Therefore, a confirmation of morphological identification with modern molecular biology method was needed [15].

For accurate identification and confirmation of the morphologically identified endophytic strain, *C. globosum*, the most reliable molecular typing technique based on partial sequencing and analysis of the 18S rDNA was used.

The genomic DNA of *C. globosum* was isolated, the 18S rDNA was amplified by PCR using universal forward and reverse primers (EF4f and Fung5r, respectively), primer pair EF4f–Fung5r was adopted for the assessment of the ecology of various fungi and amplifying genomic DNA of wide range of fungi, but other eukaryotic or bacterial DNA was not amplified [17]. PCR product was examined using agarose gel electrophoresis (Fig. 1). The PCR product was sequenced. The sequence was about 530 bp fragment of the rDNA region of endophytic fungus. Sequence analysis of 18S rDNA of tested isolate with available NCBI GenBank database showed that endophytic fungus associated with *A. capillus-veneris* belong mainly to ascomycetes group (GeneBank accession number FJ393436), with high identity (98 %) to *Chaetomium* sp. (GeneBank accession number AB489068), and revealed high close similarity (98 %) with species *C. globosum* (GeneBank accession number DQ234257). The molecular identification of endophytic *C. globosum* using partial sequencing of 18S rDNA gene confirmed our morphological identification of endophytic strain. The nucleotide sequence of active endophytic fungus was submitted to the GenBank and assigned accession number JN711454.

### Phylogenetic Analysis

Alignment of partial 18S rDNA gene sequence with standard reference taxa in the Blast GeneBank database with highest percentage of identity (multiple sequence alignment) and molecular phylogeny were performed using



**Fig. 1** Gel electrophoresis profiles of 18S rDNA region amplified from genomic DNA of morphologically identified endophytic fungus *Chaetomium*, where (M) is the 100 bp DNA ladder (DNA marker), Lane genomic DNA for target endophytic isolate, PCR product (around 530 bp)

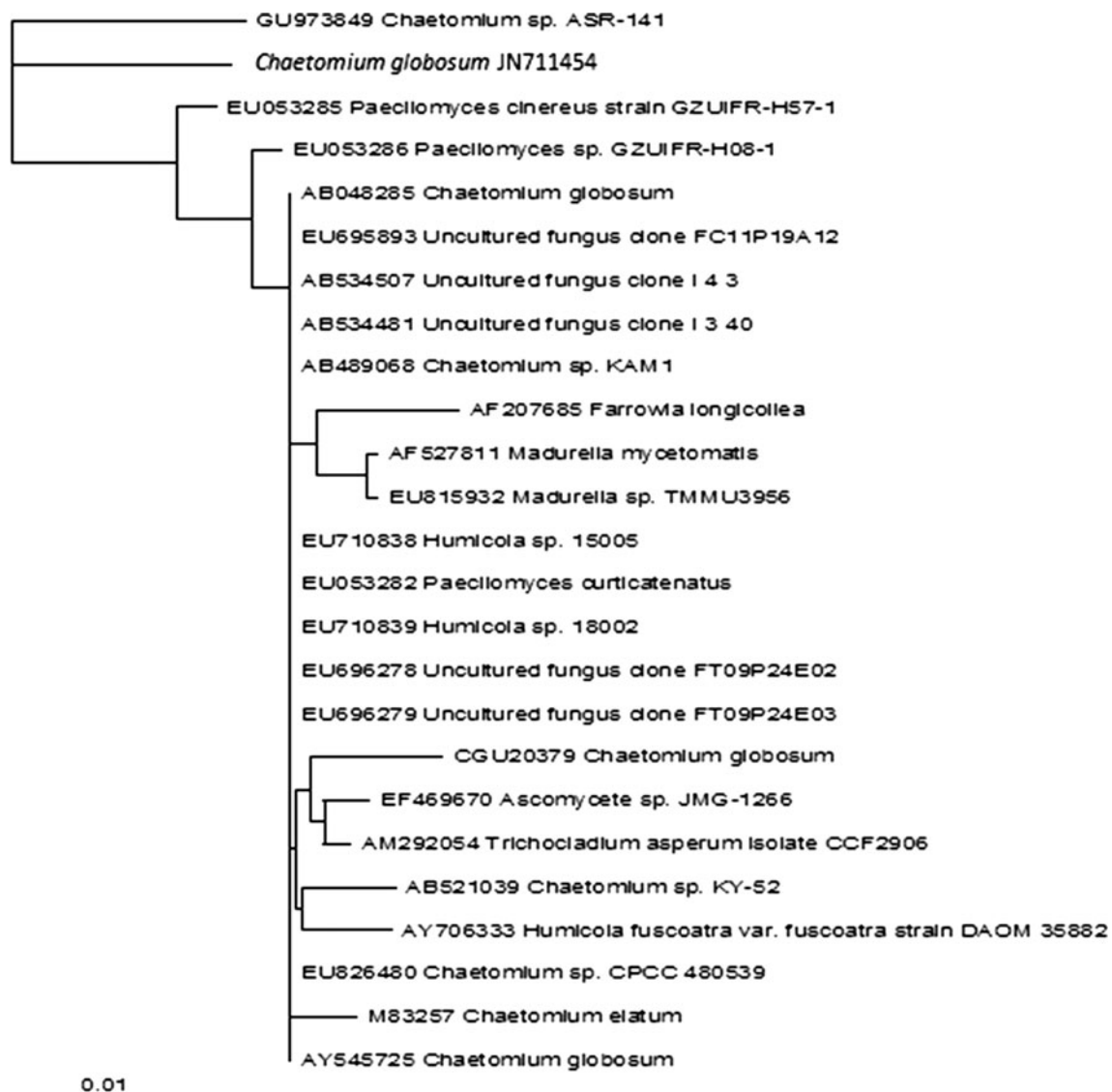
BioEdit software to resolve phylogenetic relationships. The phylogenetic tree (Fig. 2) was displayed using the TREEVIEW program. The sequences of close relatives were obtained from GenBank to reconstruct the phylogenetic tree and to resolve the phylogenetic relations with ancestor.

In conclusion, the molecular identification results (partial sequencing of 18S rDNA gene and phylogenetic analysis) were in agreement with morphological observations and matched the microscopic identification of endophytic fungus *C. globosum*.

#### Selection of Fermentation Media for Endophytic Fungus *C. globosum*

In natural product discovery programs from endophytes, the typical procedures include the cultivation of endophytic

fungi on various selective or non-selective media for examining the biosynthetic power of endophytic fungi and then extracting the fungal metabolites by different organic solvents; in order to further explore the active media with targeting active principles by appreciate selective solvent. The biosynthesis of endophytic secondary metabolites is greatly affected by the medium composition and by the extracting solvent [33, 34]. Our pharmaceutical target is focusing on improving drug discovery process for potent and promising anticancer, antioxidant, antimicrobial, and anti-Alzheimer agents. The crude extracts of endophytic *C. globosum* JN711454, cultivated on different media (rice and oatmeal as a natural media; YMG and PDA as a semi natural media; and Cz as a synthetic media) and extracted with different organic solvents (EA and MeOH), were tested for antimicrobial activity on different microbial test



**Fig. 2** The phylogenetic tree based on partial sequencing of 18S rDNA gene showing relationship neighbor-joining between endophytic fungus *Chaetomium globosum* and other closely related sequences on NCBI GenBank reference taxa

strains, for antioxidant activity, for inhibitory activity of butyrylcholinesterase, and for antitumor activity on liver cancer cell line (rat hepatoma cancer cell line FGC<sub>4</sub>).

### Antimicrobial Activity

The antimicrobial activity of endophytic *C. globosum* was reported against wide range of pathogenic bacteria and fungi, especially against plant pathogens [13, 22, 35], which motivated us to examine antimicrobial activity of *C. globosum*, but against human pathogens to improve the search for potent antibiotics against drug resistant microbes [1]. The antimicrobial activity of endophytic fungus *C. globosum* extracts; cultivated on different media, were measured against broad range of pathogenic bacteria (Gram positive and Gram negative), yeasts, and filamentous fungi. The antimicrobial activity was performed using disk diffusion assay at concentration of 500 µg/disk; inhibition zones were measured in mm and are listed in table (1). The antimicrobial results revealed that activities were mainly related to ethyl acetate (EA) extracts of PDA and YMG (with higher activity for YMG extract), and to methanol (MeOH) extract of PDA; against different pathogenic gram positive and gram negative bacteria and against pathogenic yeasts. None of *C. globosum* extracts showed antifungal activity. Here, we could see that *C. globosum* JN711454 exhibited an obvious antibacterial activity compared with the antifungal activities, and this is may be due to the similarities in eukaryotic characteristics between the endophytic fungi and the test fungi [36].

### In Vitro Antioxidant Activity

The FRS model for scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability. The DPPH<sup>•</sup> (2,2-diphenyl-1-picrylhydrazyl radical) is a stable free radical which can be used to assess the radical scavenging activity. The radical state of DPPH characterized by deep purple color (absorbs light at wave length range  $\lambda = 515\text{--}540$  nm). In the presence of a hydrogen/electron donor (antioxidant agent), the DPPH<sup>•</sup> reacts with free radical scavenging antioxidant agent and reduced to molecular formula (DPPHH). The DPPHH has yellow color with no absorbance at the wave length ( $\lambda = 515\text{--}540$  nm). The decrease in absorption intensity is proportional to the number of electrons captured, and hence proportional to scavenging power [26, 37, 38].

The antioxidant activities using FRS assay was measured at 50 µg/ml of *C. globosum* extracts; cultivated on different media, samples that showed 50 % or more antioxidant activity at concentration 50 µg/ml relative to control were considered active. The EA extract of PDA showed the high antioxidant activity with 97 % scavenging

of DPPH, while EA extracts of oatmeal, YMG, and rice media showed also potent antioxidant activity with 91, 90, 89 % scavenging activity, respectively (Fig. 3). All MeOH extracts showed weak antioxidant activity, except MeOH extract of rice medium, which showed 80 % antioxidant activity.

To determine the EC<sub>50</sub> (the effective concentration that cause 50 % scavenging of DPPH), the antioxidant activities for serial dilutions ranging from 3.125 to 100 µg/ml of EA extract of endophytic fungus *C. globosum* cultivated on PDA medium, were measured. The EC<sub>50</sub> was shown to be 11.5 µg/ml EC<sub>50</sub> (Fig. 4).

The determination of the total phenolic contents of *C. globosum* extracts cultivated over different media will provide complete image of the most suitable medium for maximum antioxidant activity, where the lifespan and stability of a fungal microorganism to the environmental factors is tightly connected with the state of the cell antioxidant defense components (phenolic compounds). The phenolic compounds seem to have an important role in antioxidant activity, which is emphasized in several reports. The mechanism of action of phenolic compounds is related to presence of hydroxyl groups, which confer scavenging ability of fungus [26].

The total phenolic contents of different *C. globosum* extracts were measured at 100 µg/ml, and expressed as gallic acid (GA) equivalents (µg eq. GA per 100 µg/ml of *C. globosum* extracts), by using standard curve of GA (Fig. 5) with  $R^2 = 0.994$ . The total phenolic content of different *C. globosum* extracts (Fig. 6) varied from 10.23–36.98 µg per 100 µg/ml of tested extracts. The maximum total phenolic contents were expressed in EA extracts of YMG, PDA, and oatmeal media and on MeOH extract of oatmeal medium, which in agree with DPPH assay, and reveal that phenolic compounds in *C. globosum* JN711454 extracts could be responsible for antioxidant activity. But the total phenolic content of rice medium is small in comparison to its antioxidant activity using DPPH assay, and this could be related to presence of another moiety (not phenolic compounds) of compounds responsible for such antioxidant activity like, flavonoids.

The oxidation stress induced by reactive oxygen species (ROS) can result in cell membrane disintegration, membrane protein damage, and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury, and cardiovascular disease [39]. Although the body possesses such defense mechanisms, as enzymes and antioxidant metabolites, which arrest the damaging properties of ROS [40, 41], continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage [42]. Therefore, antioxidants

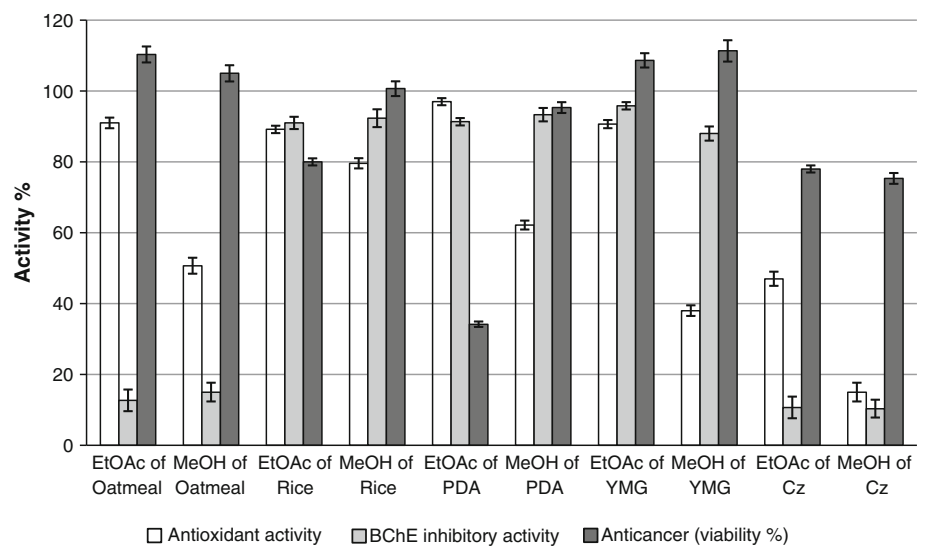


**Table 1** Antimicrobial activities of different fractional extracts of endophytic fungus *C. globosum* JN711454 cultivated on different media, against different pathogenic test microorganisms

Pathogenic test microorganisms	EA extracts					MeOH extracts				
	Oat	Rice	PDA	YMG	Cz	Oat	Rice	PDA	YMG	Cz
<b>Gram +ve bacteria</b>										
<i>Bacillus cereus</i>	–	–	11	15	–	–	–	11	–	–
<i>Bacillus megaterium</i>	–	–	10	14	–	–	–	9	–	–
<i>Bacillus subtilis</i>	8	–	13	16	–	–	–	13	–	–
<i>Micrococcus luteus</i>	–	–	10	14	–	–	–	9	–	–
<i>Staphylococcus aureus</i>	–	–	12	11	–	–	–	13	–	–
<b>Gram –ve bacteria</b>										
<i>Alcaligenes faecalis</i>	18	–	11	17	19	–	–	10	–	–
<i>Escherichia coli</i>	–	–	11	14	–	–	–	10	–	–
<i>Klebsiella pneumonia</i>	–	–	12	17	–	–	–	12	–	–
<i>Pseudomonas aeruginosa</i>	–	–	13	16	–	–	–	13	–	–
<b>Yeast</b>										
<i>Candida albicans</i>	11	–	11	13	–	–	–	12	–	–
<i>Candida parapsilosis</i>	–	–	17	11	–	–	–	14	–	–
<i>Candida tropicalis</i>	–	–	8	11	–	–	–	–	–	–
<i>Saccharomyces cerevisiae</i>	–	–	18	10	–	–	–	17	–	–
<b>Filamentous fungi</b>										
<i>Aspergillus flavus</i>	–	–	–	–	–	–	–	–	–	–
<i>Aspergillus niger</i>	–	–	–	–	–	–	–	–	–	–
<i>Aspergillus terreus</i>	–	–	–	–	–	–	–	–	–	–
<i>Penicillium</i> sp.	–	–	–	–	–	–	–	–	–	–

Inhibition zones were in mm

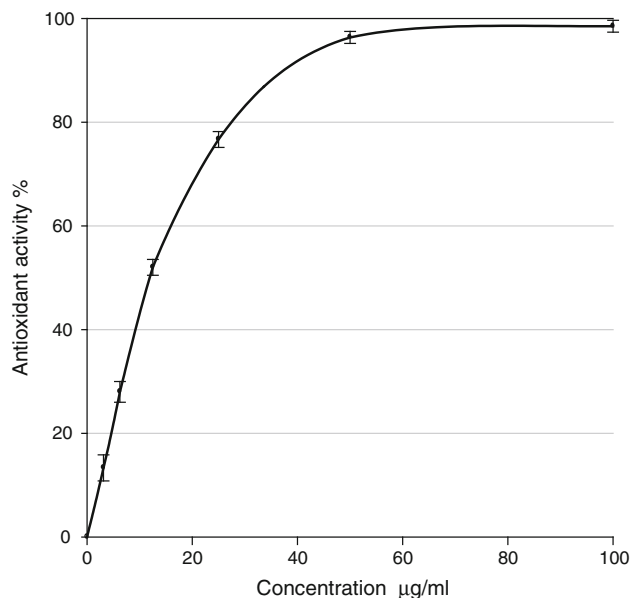
**Fig. 3** The percentage of antioxidant activity (white bars), BChE inhibitory activity (gray bars), and anticancer activity (black bars) of different extracts of *C. globosum* on different cultivation media. Error bars correspond to standard deviation (mean ± SD of triple determination). Antioxidant activity was expressed as scavenging% of DPPH. Anticancer activity was expressed as viability% of cancer hepatoma cell line (FGC4) after treating with extracts and the viability% is relative to the 100 % viability of non-treated control



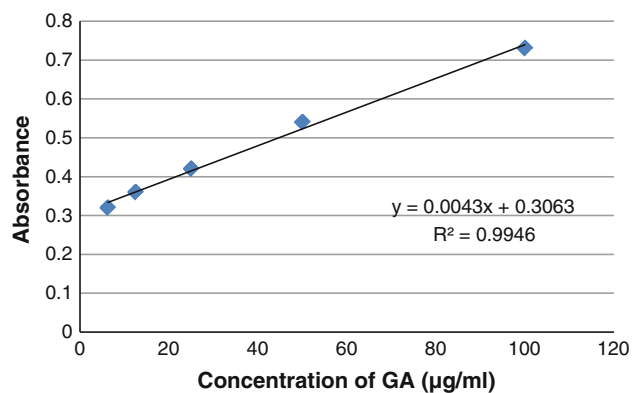
with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases, in which oxidants or free radicals are implicated [43]. For these reasons, the major concern to the medical community is the search for novel natural antioxidant resources to minimize oxidative damage to living cells.

**In Vitro Inhibitory of Butyrylcholinesterase Activity**

Alzheimer disease (AD) is a progressive, and neurodegenerative disorder resulting in impaired memory and behavior, and characterized by the formation of amyloid peptide fibrils that eventually lead to death of brain cells,



**Fig. 4** Antioxidant activities for serial dilutions of ethyl acetate extract of *C. globosum* cultivated on PDA medium, with  $EC_{50}$  11.5 µg/ml. Error bars correspond to standard deviation (mean  $\pm$  SD of triple determination)



**Fig. 5** Standard curve of Gallic acid (GA) to measure total phenolic contents in extracts of *C. globosum*.  $R^2 = 0.994$  and the absorbance ( $Y$ ) = 0.004 GA (µg/ml) + 0.306

and consequently, loss of the memory. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are hydrolases that play a key role in cholinergic transmission by catalyzing the rapid hydrolysis of the neurotransmitter acetyl- and butyl-choline [44]. One of the most promising approaches for treating AD is to enhance the acetylcholine and butylcholine levels in the brain using AChE and BChE inhibitors. The brain of mammals contains two major forms of cholinesterases, AChE and BChE. In human brain, BChE is found in neurons and glial cells as well as in neuritic plaques and tangles, and its level shows some increase in AD patients, while AChE activity decreases progressively in the brain of AD patients [45]. Nature is a

rich source of biological and chemical diversity, and various natural products have been recognized as inhibitors of AChE and BChE enzymes from microbial origin [46]. The recent approach for the treatment of Alzheimer's disease by the blockage of choline degradation, target BChE to find potent inhibitors of enzyme.

Ellman assay for measuring butyrylcholinesterase activity, depend on the ability of BChE enzyme to catalyze the hydrolysis of the substrate butyrylthiocholine (BTC), forming butyrate and thiocholine. The thiocholine reacts with coloring agent (Ellman's reagent) 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) forming colored 5-thio-2-nitrobenzoate which is detected spectrometrically at  $\lambda = 412$  nm. Thus, concentration shifts of thiocholine were monitored by spectroscopic methods after addition of test extracts, and the decrease in absorbance is proportional to inhibition of enzymes activity [27], and we consider extracts that show 85 % inhibitory activity or more is active.

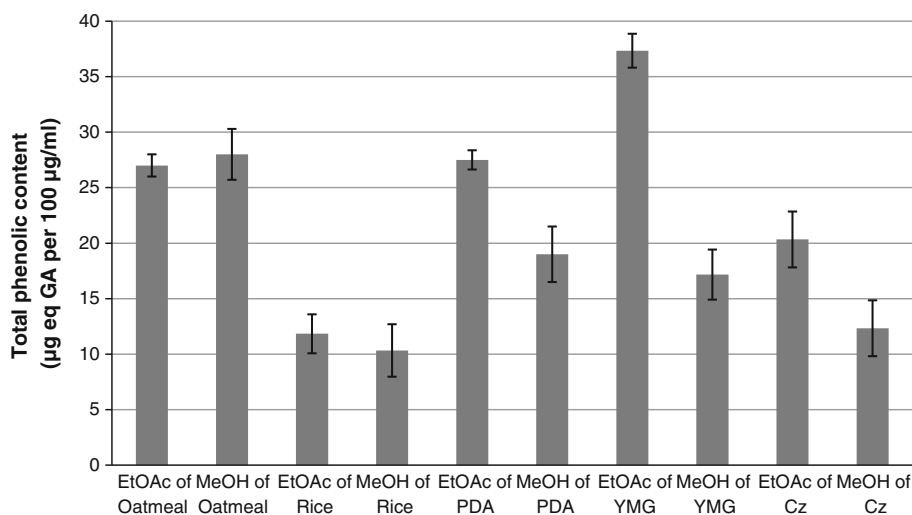
The activity of BChE enzyme was evaluated for *C. globosum* extracts using Ellman assay, and the inhibitory activity present is summarized in Fig. 3. The results revealed that most of extracts showed inhibition of BChE activity (>85 %), except for EA and MeOH extracts of oatmeal and Cz media, which showed no activity against enzyme. This is the first report of inhibitory activity of *C. globosum* extracts to BChE enzyme, which could open the way for finding novel agent(s) that could be used in pharmaceutical industry as anti-Alzheimer disease.

#### In Vitro cytotoxic activity

Neutral red (3-amino-7-dimethylamino-2-methylphenazine) is a supravital dye which is taken up and accumulated in the lysosomes of viable cells [47]. Neutral red is a cationic dye that readily penetrates cell membranes by non ionic diffusion, accumulating intracellularly in the lysosomes. Any changes in the cell surface or the lysosomal membrane will lead to irreversible lysosomal fragility. Thus, the action of a toxin results in decreased uptake and binding of neutral red. This property can be utilized to assess cell viability. The cytotoxic activity of *C. globosum* was reported [6–11], so for preliminary screening the anticancer activity was measured at 250 µg/ml against rat hepatoma cell line FGC<sub>4</sub>, the extract that cause mortality of 50 % or more of cancer hepatoma cell line FGC<sub>4</sub> was considered cytotoxic to cancer cells. Figure 3 shows the viability percent of hepatoma cell line FGC<sub>4</sub> after treatment with *C. globosum* extracts. Only, ethyl acetate extract of PDA medium showed 34 % viability with 66 % cytotoxicity to hepatoma FGC<sub>4</sub> cell line.

As conclusion, we could see that medium compositions affect greatly on secondary metabolites production and

**Fig. 6** Total phenolic content of different extracts of *C. globosum* on different cultivation media, expressed as  $\mu\text{g eq. GA per } 100 \mu\text{g/ml}$ . Error bars correspond to standard deviation (mean  $\pm$  SD of triple determination)



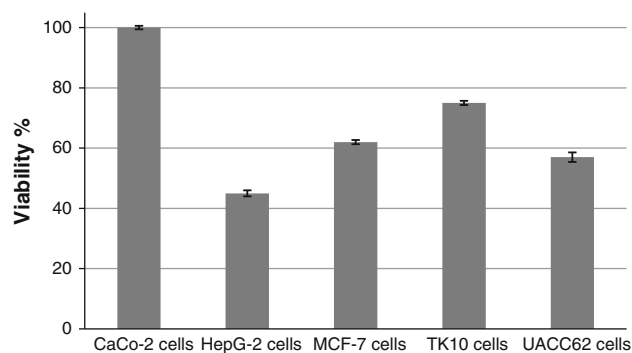
could favor production of some metabolites over the others. This is clear through, antimicrobial, antioxidant, and anticancer activities, where only PDA and YMG media favor production of antimicrobial agents and changing medium composition lead to change in the phenolic content profile in comparison to DPPH activity. Also, in anticancer activity against FGC<sub>4</sub> cell line, only PDA medium favor production of cytotoxic metabolites.

On basis of the previous bioactivities (cytotoxicity, antimicrobial, scavenging of DPPH, estimation of total phenolics content, and inhibitory of butyrylcholinesterase), the EA extract of *C. globosum* JN711454 cultivated on PDA medium was selected for further biological evaluation tests for antiviral activity and cytotoxicity against different human cancer cell lines, where it show the potent cytotoxicity toward FGC<sub>4</sub> cancer cells and antioxidant activities, with >90 % inhibition to BChE enzyme and moderate antimicrobial activity in compare to other extracts. It is premise that EA extract of *C. globosum* cultivated on PDA medium was the most promising extract with a multiple roles.

#### In Vitro Anticancer Activity Against Human Cancer Cell Lines

The SRB assay was developed to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye sulforhodamine B to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilized for measurement [28].

The ethyl acetate extract of PDA medium was measured at lower concentration (100  $\mu\text{g/ml}$ ) using SRB assay against five human cancer cell lines [colon adenocarcinoma (CaCO-2), hepatocellular carcinoma (HepG-2), breast



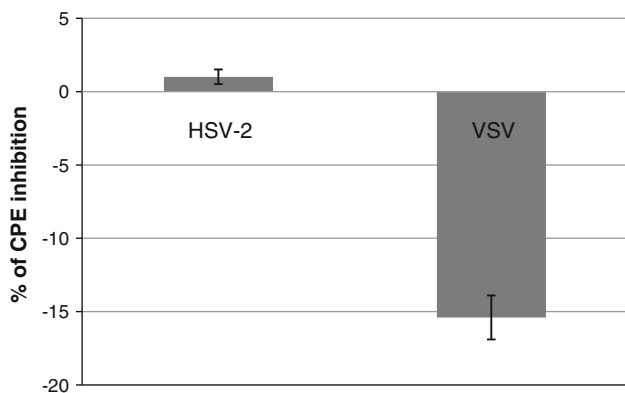
**Fig. 7** Differential response of human cancer cell lines to 100  $\mu\text{g/ml}$  of EA extract of endophytic fungus *C. globosum* cultivated on PDA media. Error bars correspond to standard deviation (mean  $\pm$  SD of triple determination). The viability% is relative to the 100 % viability of non-treated control

carcinoma (MCF-7), renal carcinoma (TK10), and melanoma carcinoma (UACC62)]. The crude ethyl acetate extract of PDA medium showed 55, 43, 38, and 25 % cytotoxicity to HepG-2, UACC62, MCF-7, and TK10 cells, respectively, while it produced no cytotoxicity on CaCO-2 cells (Fig. 7).

The cytotoxicity results indicate the premise that screening of beneficial endophytic fungi possesses enhanced anticancer capabilities [48], and the capability of EA extract of *C. globosum* to induce in vitro apoptosis to cells of liver cancer, where it produced toxicity to both human and rat cancer cell lines (HepG-2 and FGC<sub>4</sub>), but further histopathological and biochemical examinations are required for complete evaluations of EA extract of *C. globosum*.

#### In Vitro Antiviral Activity

The antiviral bioassay is carried out by the end point titration technique (EPTT); the technique depends on the



**Fig. 8** Antiviral Activity (in form of % of CPE inhibition) of EA extract of endophytic fungus *C. globosum* cultivated on PDA media against HSV-2 and VSV. Error bars correspond to standard deviation (mean  $\pm$  SD of triple determination)

detection of the visible cytopathogenic effects (CPE) that is produced on thin confluent monolayer of VERO cells, after inoculated separately with the virus. The CPE of infected cells by HSV-2 include enlargement, rounding, and refractile of cells, then cells detach from the growth surface leaving cleared areas. Stranding of the cytoplasm (greatly elongated and thin appearance) may be pronounced, and cell fusion may be evident. While, VSV produces CPE by 24–48 h with two forms of observed CPE, the most common CPE was cytoplasmic granulation after which cells become rounded, take on a refractile appearance, and undergo lytic degradation. The second type of CPE associated with VSV infection is formation of multinucleated giant cells [30].

In vitro cytotoxicity assay revealed that the 50  $\mu$ g/ml of EA extract of PDA medium was safe concentration for in vitro antiviral assay against DNA virus (HSV-2) and

**Fig. 9** Metabolomic profiling using LC–MS, chromatogram of EA extract of *C. globosum* JN711454 showing MS of different compounds

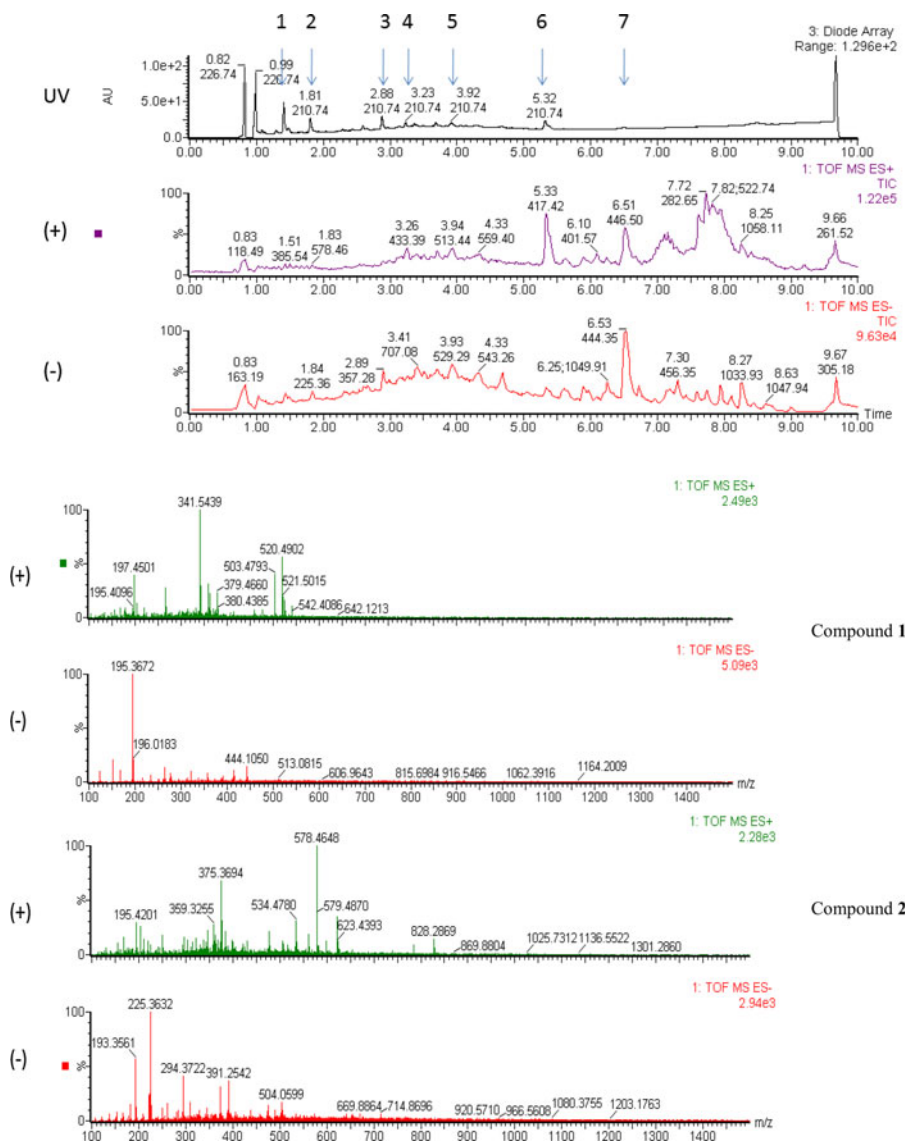
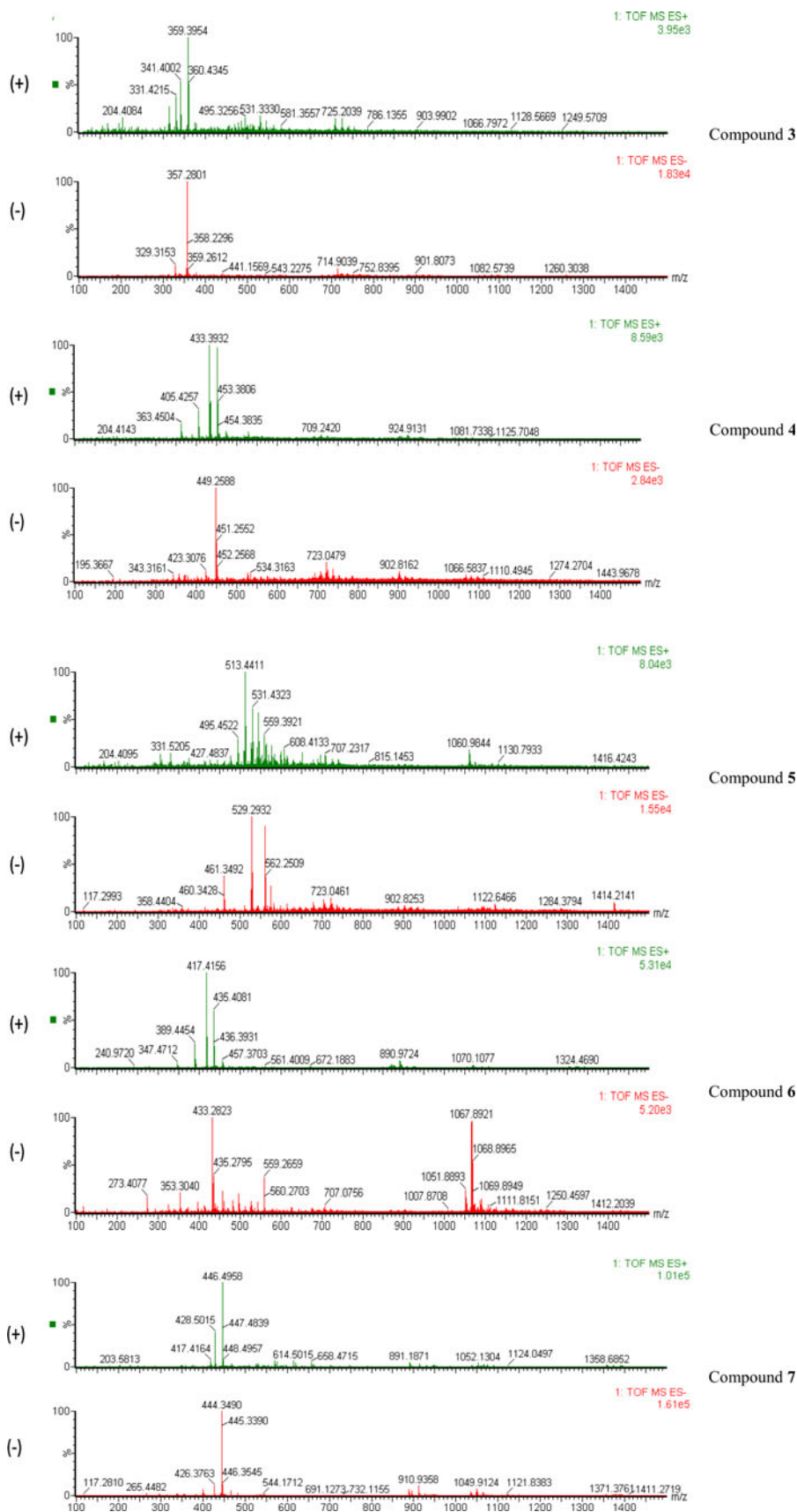


Fig. 9 continued



RNA virus (VSV). The VERO cells were treated with safe concentrations of fungal extract for 24 h before viral infection to examine the effect of EA extract of *C. globosum* on ability of HSV-2 and VSV viruses to adhere to cells.

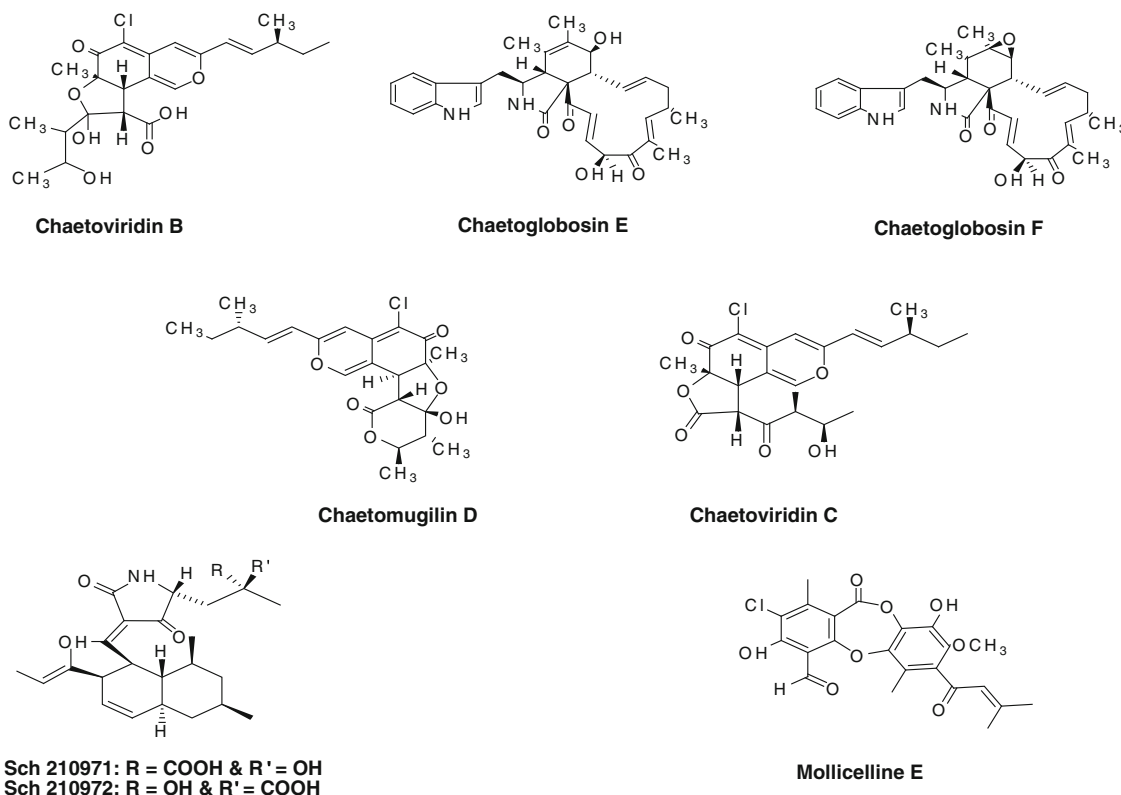
The results of antiviral activity (Fig. 8) of EA extract of *C. globosum* expressed in form of inhibition percent of cytopathogenic effects (% of CPE inhibition). The negative signs of % of CPE inhibition referred to increase in virus titer in treated cells in compare to control (non treated cells), which indicate that VSV adhered to cells and replicated, this means that *C. globosum* extract is inactive completely against VSV. The positive signs indicated that the extracts in somewhere active, where they stopped adhering machinery of virus to cells and stopped the reproducibility machinery of virus in compare to control, which indicate that EA extract of *C. globosum* cultivated on PDA medium is active against HSV-2. Here, for the first time we report the antiviral activity of *C. globosum* as active extract against herpes simplex virus.

#### Metabolic Profiling of EA Extract of *C. globosum* Grown on PDA Medium

As a result of the promising biological activities observed, it became essential to study the metabolomic profile of the EA extract of *C. globosum* grown on PDA medium. The

LC–MS of the EA extract revealed the presence of several metabolites; clearly we could determine the mass spectrum of 7 compounds (Fig. 9). From ESI mass spectral data of LC–MS experiment, we could expect that compound 4 could be chaetoviridin B, as deduced from the molecular ion  $[M]^+$  at  $m/z$  452 [49], and the ESI-MS of compound 5 displayed a molecular ion  $[M]^+$  at  $m/z$  530, so it may be chaetoglobosin E- or -F [6]. Compound 6 was assigned to be chaetomugilin D- or -chaetoviridin C, where its ESI mass spectrum showed a molecular ion  $[M]^+$  at  $m/z$  434 [11, 49]. The molecular mass of compound 7 revealed a molecular ion  $[M]^+$  at  $m/z$  445 and/or 446, so it may be one of chemokine receptor CCR-5 inhibitors, Sch 210971 and Sch 210972 for  $m/z$  445 -or- mollicelline E for  $m/z$  446 [50, 51]. Metabolomic study using LC–MS profile revealed that endophytic fungus *C. globosum* JN711454 is a rich source by different moieties of secondary metabolites (Fig. 10) and most of its compounds were related to chlorinated azaphilones, indol cytochalasins alkaloids, and depsidones.

The previous reports of azaphilones, cytochalasins and depsidones compounds indicated that they are characterized by wide range of bioactivities, which agree and support the bioactivities results of our strain *C. globosum* JN711454 [6, 7, 11, 51]. The antimicrobial and cytotoxicity results could be attributed to one or more azaphilones, and cytochalasins compounds, while the antioxidant activity



**Fig. 10** Chemical Structure of expected compounds in EA extract of *C. globosum* JN711454 from LC–MS experiment

may be related to phenolic depsidones compounds. Chaetoviridin B have been reported as antimicrobial agent [35], while compounds chaetomugilin D and chaetoglobosin E and F show cytotoxicity against different cancer cell lines [6, 11], as well as mollicelline E which shows cytotoxicity and antimalarial activity [51]. A more extensive detailed study is required to identify the remaining compounds, and to isolate, purify, and elucidate the chemical structural of pure compounds with investigating of their biological activities, which could be novel bioactive agents and to which the observed biological activities could be attributed.

## Conclusion

The endophytic fungus *C. globosum* JN711454, isolated from medicinal plant *A. capillus-veneris*, possess broad spectrum of in vitro bioactivities and rich by secondary metabolites, so it could be considered as a promising source for improving drug discovery process through investigation of its pure active principles.

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