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Antioxidant, Anti-proliferative, Cytotoxicity, and Antiviral Studies on *Chaetomium interruptum* and *Chaetomium laterale*

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WITH the rise of drug-resistant diseases, there is a growing demand for innovative medicines that do not rely on chemotherapeutic medications. It has been believed that medicinal plants provide an ideal habitat for various bioactive microbial communities. The medicinal plant endophytes *Chaetomium interruptum* and *Chaetomium laterale* were examined for their antioxidant, antiviral, cytotoxic, and anticancer properties in this study. Chemigraphic separation and identification of metabolic components were employed in the chromatographic analysis of *Chaetomium interruptum* ethyl acetate and petroleum ether extracts. Phytochemical assessment of the extracted metabolites indicated the presence of phenolics, esters, alcohols, and hydrocarbons in both extracts. The key fundamental isolated compounds were nine ester compounds, acetyl tributyl citrate (ATBC) of (35.29 and 45.91%), hexadecanol of (8.35%), phenol compounds of (18%), 2,4-ditert-butylphrenol of (13.40%), and alcohols of (8.3%). The fungal extracts exhibited radical-scavenging capacity, antiproliferative effect against three human carcinoma cell lines, and minimal toxicity towards normal cells. Cellular shape and morphology alterations were captured in the hepatocellular carcinoma cell line after being treated with *C. interruptum* ethyl acetate extract. *In vitro*, the mild to moderate cytopathic inhibition effectiveness of both fungal extracts against Herpes Simplex Virus, type 1, was detected. Genus *Chaetomium* is still a valuable source of species in biomedical applications.

Keywords: Biomedical, phytochemical, endophytes, fungi.

1. Introduction

In the present decay, many fatal human diseases have emerged mainly due to fast urbanization and industrialization, such as cancer and various viral mortal infections. The severe development of these critical disorders compelled the world to use chemical medication, which in turn, potentially exacerbates human health issues. Accordingly, many investigations were conducted to replace chemical treatments with biological control, which at the same time is safe for human health, the same as the objective of this study. Microbial communities that inhabit plants with medicinal properties are valuable bio-resources for solving and fighting such aggravated, deadly diseases. Endophyte fungi have the fermentative ability to produce valuable bioactive metabolites, which are significant for human and host plant survival and the economy (**Oteino et al., 2015; Lata et al., 2018; Xu et al., 2023**).

Endophyte fungi, promising for pharmaceutical purposes: Bio-efficient fungi are an outstanding reservoir source of many beneficial drugs able to suppress a wide diversity of human diseases, including severe infections. Medicinal-plant endophyte fungi are important sources of biotechnology employment in medicine, agriculture, and industry (**Kaur et al., 2021; Nisa et al., 2015**). Many of these endophytic fungi possess notable biomedical disciplines; they are agents of antimicrobial, antiproliferative, antioxidant, cholesterol-inhibiting, and antidiabetic properties (**Kannan et al., 2017a; Eskander et al., 2020; Elkhouly et al., 2021**). Endophyte-plant fungi are a valuable source of various medicinal and other useful chemicals like peptides, flavonoids, alkaloids, terpenoids, steroids, azadirachtin, VOCs, and more (**Mishra et al., 2021; Kumar and Prasher, 2023; Macias-Rubalcava and Garrido–Santos, 2022**). These associative endophytes were documented by (**Kumar et al., 2013; Qadri et al., 2013; Salini et al., 2014; Jha et al., 2023**) to behave as immunomodulatory and substantial antibacterial and antifungal factors that are applied in agricultural issues biocontrol strategies. These organisms have been represented as hidden treasures of microbial biocontrol metabolites (**Jha et al., 2023**). Interestingly, phytochemical secondary metabolic substances of such fungi are supportive agents of the biomedical properties of their host plants (**Arora and Mahajan, 2018**).

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Endophyte *Chaetomium* species, the hidden treasure of bio remedial compounds: *Moringa oleifera* Lam application in the pharmaceutical sector is expansive because of its valuable therapeutic characteristics, which include anticancer and antiproliferative effects, antidiabetic properties, antihypertensive attributes, and anti-asthmatic capabilities (**Neelam et al., 2021**). *Moringa oleifera* endophyte species, *Aspergillus fumigatus, Fusarium, Nigrospora, C. laterale,* and *C. interruptum* confirmed an antibacterial property against bacterial diseases (**Kaur et al., 2020a; Hemeda et al., 2022**). *Chaetomium* species have been regarded as biotechnological tools across many disciplines; they introduced new potential still sources in the biomedical field, as our study documented and supported. The *C. globosum* antibacterial potential was observed by **Kaur et al. (2020 b)**; the authors isolated fungal metabolites via the Gas Chromatography-Mass Spectrometry (GC-MS) technique. In the study of **Elkhateeb et al. (2021)**, an effective antiproliferative impact of *Chaetomium* species was detected against tumour PC-3 cell line. Some biological properties of *C. globosum*, such as antibacterial, cytotoxic, antiviral, antiproliferative, and others, were also confirmed (**Yang et al., 2021; Kumar and Prasher, 2023**). Relative putdown of human colorectal adenocarcinoma (HCT-8) and breast cancer cells (MCF-7 and MDA-MB-231) were obtained by *C. globosum* 7951 regarding a pyridine benzamide (novel) and epipolythiodioxopiperazine (ETP) (**Wang et al., 2019**); notable cytotoxic bioactivity of pyridine benzamide towards lung cancer cells (H460) was also detected in the survey. *C. globosum* was studied (**Wang et al., 2019**), and it was found that the fungus fermented over 200 compounds like steroids, terpenoids, anthraquinones, orsellides, tetramic acids, benzo-quinones, xanthones, and others. Regarding health hazards due to oxidative stress, there is a significant need for natural, safer, and more effective antioxidants (**Zhao et al., 2021**). Several bioactive scavenging antioxidants of endophyte fungi were studied, such as flavonoids, phenolic acids, tannins, melanin, lignin, and phenylpropanoids (**Kumar and Prasher, 2023; Bora and Davi, 2023**). It was elucidated that the plant hosts handle stress with the support of their inhabitant fungi valuable antioxidants. The scavenging activity of the ethyl acetate extract of endophyte *Aspergillus fumigatu*s was also detected (**Bora and Davi, 2023**).

The bioactive compounds of plant-endophytic fungi also involve a potential remedial antiviral property; thus, this consideration is largely still. A recorded scarcity of information about this field is only available (**Adeleke and Babalola, 2021**). The primary conflict issues challenged during the survey process are due to the unsuitability, ineffectiveness, or absence of antivirals. Most survey investigations for metabolic substances depend on screening methods (**Adeleke and Babalola, 2021**). Several endophyte fungi antivirals were involved in the study of (**Liu et al., 2019**), which included two new substances from *Cytonaema spp*., cytonic acid A and B, and new human cytomegalovirus protease inhibitors; investigators also studied the endophyte anti-HIV-1 protease hinnuliquinone.

2. Materials and Methods

2.1 Experimental site

All tests were conducted at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University. Associate Professor Ahmed Khalafallah from the Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt, assisted with the statistical analysis.

2.2 Endophyte fungal strains

Chaetomium interruptum and *Chaetomium laterale* were propagated by **Hemeda et al. (2022)** from leaves and stems of the medicinal plant *Moringa oleifera*, identified using the 18S rRNA gene sequence and GenBank database via BLAST (http://www.ncbi.nlm.nih.gov.blast). The two species were deposited in the Gene Bank under accession numbers ON782293 and ON778729.

2.3 Chemical materials

- Crystal violet, trypan blue, ascorbic acid, and dimethyl sulfoxide (DMSO). All were obtained from Sigma (St. Louis, MO., USA).

- Crystal violet/methanol mixture (1% w/v); it is diluted in double-distilled water (ddH_2O) and filtered with Whatman No.1 filter paper.

- L-glutamine, gentamycin, 0.25% trypsin-EDTA, RPMI-1640, Fetal bovine serum, Dulbecco's Modified Eagle Medium (DMEM), and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer solution. All were obtained from Lonza (Belgium).

- Trypsin–Hank's balanced salt solution (0.8 L of distilled water + 7.999 g NaCl + 0.048 g anhydrous $MgSO_4$ + 0.14 g $CaCl_2$ + 0.4 g KCl).

- DPPH radical methanol solution of 0.004% (w/v). It was maintained after preparation at a temperature of 10°C in the dark.

2.4 Cell line

The test Vero cell lines were human PC-3 (prostate cancer), HepG-2 (hepatocellular carcinoma), HCT-116 (colon cancer), and normal African green monkey kidney. The cell lines were gained from the American Type Culture Collection (ATCC) / Manassas, VA, USA.

2.5 Test virus type

Herpes simplex virus type 1 (HSV-1) GHSV-UL46.

2.6 Fungal metabolites extraction

Following **Hemeida (2023)**, partially purified extracts, petroleum ether, and ethyl acetate were separated from *C. laterale* and *C. interruptum*.

2.7 Scavenging free radical assay

The antioxidant activity of fungal extracts was assessed via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity assay.

2.8 DPPH free radicals scavenging capacity (antioxidant activity)

In this antioxidant activity evaluation, freshly prepared DPPH solution was supplemented with different concentrated fungal methanol solutions (40 µl/3 ml DPPH). The absorbance value was tested at 517 nm using a UV-visible spectrophotometer (Milton Roy, Spectronic 1201) and the absorbance decrease was followed at 1-minute intervals until the absorbance was stabilized at 16 min The absorbance of the antioxidant-free DPPH radicals of the reference compound, ascorbic acid (positive control) and negative control (sample-free), in addition to the DMSO-treated control, was evaluated.

The percentage of the free radical scavenging capacity of the sample was estimated considering the positive control. The DPPH free radicals Percentage Inhibition (PI) was evaluated through the following equation:

$$PI = [\{(AC - AT) / AC\} \times 100]$$

where AC is the control absorbance at time zero (t = 0 min) and AT is the absorbance of the sample containing DPPH after 16 min (t = 16 min) (**Zhao et al., 2021**).

The concentration needed to suppress the DPPH radicals by 50% (IC_{50}) was deduced from the graphic plots of the dosage response curve; the values of IC_{50} were elucidated via Finney software; the outcome values are expressed in µg/ml (**Elbatrawy et al., 2015**). The antioxidant activity was signified as the IC_{50} value of DPPH radical scavenging capacity.

2.9 Cell line propagation

1% L-glutamine, HEPES buffer, DMEM with 10% heat-inactivated fetal bovine serum (FBS), and 50 µg/ml gentamycin were used to propagate the cancer (HepG2, HCT-116, and PC3) cell lines. At 37°C, the cell lines were incubated in a 5% CO_2 humidity-controlled environment; the cell lines had to be re-cultured two times/week. In the confluent cell phase, the cells were treated with Trypsin–Hank's balanced salt solution and gathered (**Kaur et al., 2021**). To check the cell viability, the suspension of cells was centrifuged for 15 min at 150 rpm, the supernatant was eliminated, DMEM (5 ml) was provided to the cell pellets, and the cell number was counted using a hemocytometer.

2.10 Antiproliferative potential assay

The antiproliferative activity of the two fungal extracts was evaluated against the cancer cell lines (HepG2, HCT-116, and PC3). The cancer cell line at 1×10^4 cell concentration was divided into 96-well plates containing 100 µl/well growth medium; after 24 h, a fresh medium with the tested concentration was added. Twofold-serial extract dilutions were added to the confluent monolayer cells; the dilution mixtures were divided by a multichannel pipette into flat-bottomed microtiter plates of 96 wells (Falcon, NJ, USA). In 5% CO_2 at 37°C for 48 h, the microtiter plates were incubated in a humid environment incubator. After freezing the extract samples, control cells were propagated in the presence of a test fungal extract-free sample and with DMSO or DMSO-free. There were no notable effects on the screening results at low DMSO concentrations (up to 0.1%). To check the cell viability via the colourimetric method (**Mosmann et al., 1983**), media were aspirated when the incubation time was ended, 1% solution of crystal violet was provided into wells, incubated for at least 30 min, the stain was rinsed carefully with tap water, and a 30% acetic acid glacial was supplemented in the wells and mixed thoroughly. To estimate the viable and non-viable cell numbers (not stained cells and stained cells, respectively), a microplate reader detected the absorbance at 490 nm wavelength (SunRise, TECAN, Inc., USA).

2.11 Cytotoxicity potential assessment

This assessment was estimated according to **Kaur et al. (2021)**. The absorbance was determined, and the percentage of cell proliferation was evaluated by the equation [At / Ac] x 100%, where (At) is the mean absorbance value of treated wells while (Ac) is that of control wells. After treatment with the test compound, the survival curve for cancer cell lines was plotted regarding the drug concentration and survival relationship (**Al-Salahi et al., 2015**). The inhibitory concentration necessary to produce a toxic effect on 50% of the intact cells is abbreviated as IC_{50}. GraphPad Prism (San Diego, CA., USA) software was applied for each concentration to estimate the IC_{50} value from the dose-response curve (**Al-Salahi et al., 2015**). The ratio of the CC_{50} (i.e. 50% cytotoxic concentration of the sample toward normal cell line) to the IC_{50} for cancer cell selective index (SI) was also considered for fungal extracts.

Alternations in cell shape and morphology were tracked as affected by *C. interruptum* ethyl acetate extract against HepG2 cells were tracked by the inverted microscope, and photos were captured.

2.12 Antiviral potential assessment

The cytopathic effect of the inhibition test was performed to estimate the antiviral effectiveness that supports studying a definite biological function inhibition in mammalian susceptible cells (**Hu and Hsiung, 1989**). In the 96-well microtiter plate, 10,000 Vero monolayer cells were propagated till the well bottom adherence at 37°C in a 5% CO_2 humidified incubator for 24 h. The microtiter plates were washed with fresh DMEM and then tested with 104 potions of the herpes simplex 1 virus (HSV-1). The propagated plates were treated with the extract of serial twofold dilutions (500, 250, 125, 62.5, …, 1.95 µg/ml fresh preservation medium) and incubated for 48 h at 37°C. The untreated controls of the Vero and infected cells were also performed with the tested free extract. Via the inverted microscope, the wells were evaluated every 24 hours until complete virus-induced cytopathic influences were obtained in the control wells. The Vero cell protection is attained due to the tested extract's action on the cytopathic effect suppression. In medicine, acyclovir is a herpes viral drug; so, it was used as the positive control in this test.

After the end of the incubation time, the media were aspirated, and the cells in the examined microtiter plates were stained with crystal violet (1%) for 30 min and rinsed with water. The test plates were desiccated, and the acetic acid glacial (30%) was provided in all wells and mixed. The plate's optical density (OD) was detected via the microplate reader at 590 nm (Al-Salahi *et al.*, 2015). The viral inhibition rate was evaluated as the following formula: [(ODtv − ODcv) / (ODcd − ODcv)] × 100%, where ODtv, ODcv and ODcd indicated the OD of extract-treated virus-infected cells, control virus and cell control, respectively. Modelling STATA software was applied to follow the outcomes, and then the graphics were plotted, and the dose inhibited 50% (EC_{50}) of the viral infection was evaluated. Based on the ratio of (CC_{50}) 50% cytotoxic concentration to the EC_{50}, the (SI) selectivity index was also evaluated. The selectivity index is necessary to decide if the extract has an appropriate antiviral influence that overcomes its toxicity level. The SI is a useful remedial index in predicting if a compound needs further screening. It was mentioned that the compounds of SI values = 2 or more are considered bio-effective (**Al-Salahi *et al.*, 2015**).

2.13 Low-pressure liquid chromatography-mass spectrometry (LC-MS) analysis technique

Metabolites from the *C. interruptum* ethyl acetate extract were separated and identified using the Aria TLX-1 LC system paired with a TSQ Quantum Ultra and an electrospray ionization (ESI) source (Thermo Scientific, Austin, TX, USA). Then, the relative percentages of each separated substance were determined.

2.14 The Aria TLX-1 system

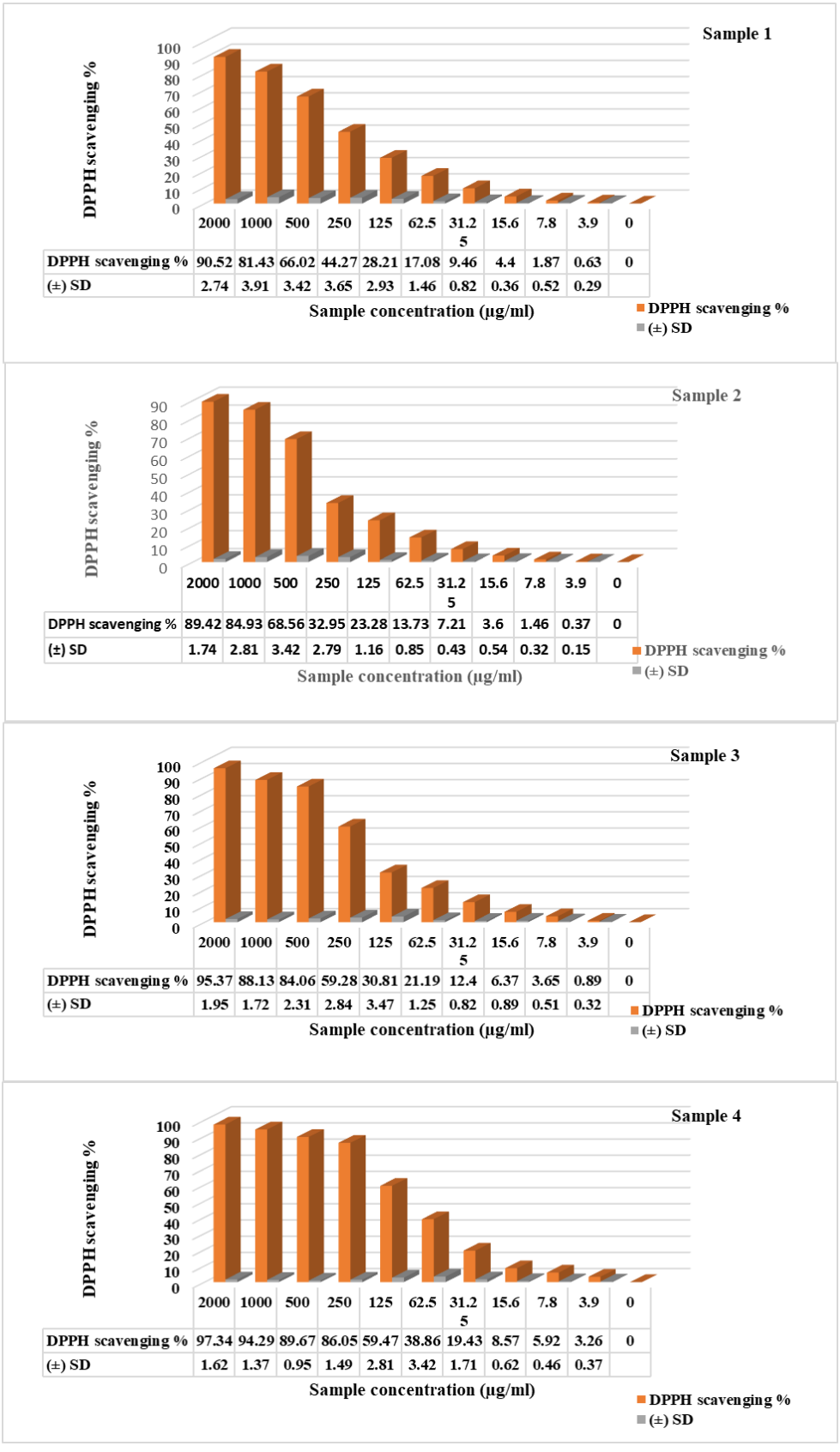
A C18 HRPLC column (4.6 × 150 mm, 5 µm particle size) with Thermo Scientific Cyclone™ MAX TurboFlow column of 0.5 × 50 mm size was applied.

2.15 Parameters applied for mass spectrometry technique

1- Positive ion polarity mode; 2- Vaporizer of 500°C temperature; 3- Capillary 300°C Temperature; 4- Sheath (N_2) Gas Pressure of 60 units; 5- Auxiliary (N_2) Gas Pressure of 55 units; 6- Full Scan Type, 50-2500 Mass range.

2.16 Statistical analysis

The variation in the responses of each examined factor to the fungal extracts was assessed by the (ANOVA 1) one-way analysis of variance regarding SPSS software (SPSS, 2012).



Sample concentration (µg/ml)	2000	1000	500	250	125	62.5	31.25	15.6	7.8	3.9	0
DPPH scavenging %	90.52	81.43	66.02	44.27	28.21	17.08	9.46	4.4	1.87	0.63	0
(±) SD	2.74	3.91	3.42	3.65	2.93	1.46	0.82	0.36	0.52	0.29	

Sample concentration (µg/ml)	2000	1000	500	250	125	62.5	31.25	15.6	7.8	3.9	0
DPPH scavenging %	89.42	84.93	68.56	32.95	23.28	13.73	7.21	3.6	1.46	0.37	0
(±) SD	1.74	2.81	3.42	2.79	1.16	0.85	0.43	0.54	0.32	0.15	

Sample concentration (µg/ml)	2000	1000	500	250	125	62.5	31.25	15.6	7.8	3.9	0
DPPH scavenging %	95.37	88.13	84.06	59.28	30.81	21.19	12.4	6.37	3.65	0.89	0
(±) SD	1.95	1.72	2.31	2.84	3.47	1.25	0.82	0.89	0.51	0.32	

Sample concentration (µg/ml)	2000	1000	500	250	125	62.5	31.25	15.6	7.8	3.9	0
DPPH scavenging %	97.34	94.29	89.67	86.05	59.47	38.86	19.43	8.57	5.92	3.26	0
(±) SD	1.62	1.37	0.95	1.49	2.81	3.42	1.71	0.62	0.46	0.37	

Fig. 1. Fungal extract dose-response curve displaying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity.

(1-2): ethyl acetate metabolites of *Chaetomium laterale* and *Chaetomium interruptum*

(3-4): petroleum ether metabolites of *Chaetomium laterale* and *Chaetomium interruptum*

3. Results

3.1 Antioxidant DPPH free radicals scavenging potential

In Fig. 1, the two species of Chaetomium displayed DPPH free radical inhibition of more than 90% at 2000 µg/ml extract concentration. The IC50 values were established for ascorbic acid, ethyl acetate extract of C. laterale and C. interruptum, and petroleum ether extract of C. laterale and C. interruptum; they were 13.9 ± 0.43, 315.8 ± 16.7, 373.4 ± 21.9, 209.1 ± 14.2, and 96.3 ± 4.8 µg/ml, respectively.

The recorded low IC50 results indicated a high DPPH scavenger potential. The C. interruptum petroleum ether extract displayed a high capacity as a free radical inhibitor (DPPH scavenger).

3.2 Antiproliferative and cytotoxic potential

Chaetomium extracts showed an antiproliferative potential against the three lines of cancer cells at a high concentration of 1000 µg/ml, and an inhibition of (83 - 97.5%). It was noticed that a high inhibition potential of *Chaetomium* extracts was observed in the HepG2 cell line. Worthwhile, the three carcinoma cells showed less cytotoxic susceptibility against *Chaetomium* extracts compared with the Cisplatin reference drug (**Fig. 2**). In Significance, the fungal extracts exhibited low cytotoxicity against Vero cells (CC_{50} = 128.4 ± 8.8; 148.1 ± 9.5; 436.3 ± 39.1 and 372.8 ± 23.4 µg/ml for extract samples 1, 2, 3 and 4, respectively). As shown in **Table 1**, (SI) values towards HepG-2, PC3 and HCT-116 were 16.91, 11.51 and 8.2. The ethyl acetate extract of the *C. laterale* exhibited a high selective inhibition value (≥ 2) against cancer cell lines.

Table 1. Anti-proliferation activity in terms of (IC_{50}) of the fungal extracts against HepG2, HCT-116 and PC-3 cells and the selective inhibitory index (SI).

Extract Number	CC50[#] (µg/ml)	HepG2		HCT-116		PC-3	
		IC50[#] (µg/ml)	SI[##]	IC50[#] (µg/ml)	SI[##]	IC50[#] (µg/ml)	SI[##]
1	128.4± 8.8	43.9±2.7	2.92	89.5±4.1	1.43	112.3±6.9	1.14
2	148.1± 9.5	66.3±3.9	2.23	107.8±8.3	1.37	124.7±7.8	1.19
3	436.3± 39.1	25.8±2.4	16.91	53.2±2.9	8.20	37.9±2.3	11.51
4	372.8± 23.4	103.4±5.1	3.61	329.7±21.4	1.13	232.1±8.9	1.61
Cisplatin[###]	**79.4 ± 7.2**	**6.3 ± 0.9**	**12.60**	**8.4±0.8**	**9.45**	**15.6±1.3**	**5.09**

(1-3): ethyl acetate metabolites of *Chaetomium laterale* and *Chaetomium interruptum* respectively
(2-4): petroleum ether metabolites of *Chaetomium laterale* and *Chaetomium interruptum* respectively
[#] IC_{50}, 50% inhibitory concentration; CC_{50}, 50% cytotoxic concentration toward a normal cell line; [##] SI: selectivity index
[###] Cisplatin: a reference anticancer standard drug for evaluating metabolites' efficiency

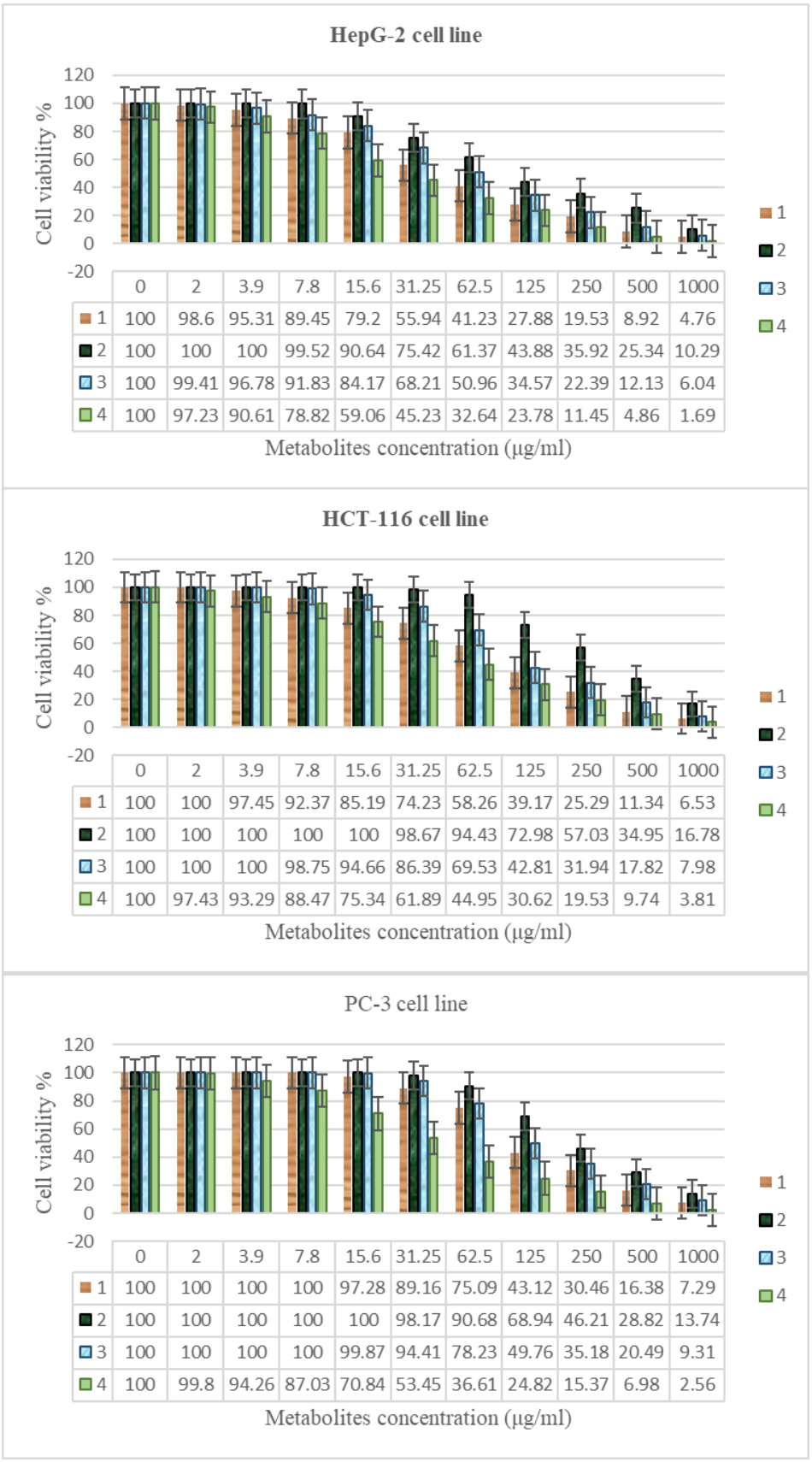


Fig. 2. Fungal extract cytotoxic effects against tumour cell lines, HepG2, HCT-116 and PC-3 via MTT assay.

(1-3): ethyl acetate metabolites of *Chaetomium laterale* and *Chaetomium interruptum*
(2-4): petroleum ether metabolites of *Chaetomium laterale* and *Chaetomium interruptum*
Cisplatin: a reference anticancer standard drug for evaluating metabolites' efficiency

3.3 Morphological studies via inverted microscopy

The inverted microscope was applied to follow the changes in cell shape and morphology formed by the ethyl acetate extract of *C. interruptum* effect against hepatocellular carcinoma (HepG2) cells; images were taken as shown in **Fig. 3.** The control group cancer cells displayed cellular outgrowth represented in adhesive cell growth with a distinct polygonal shape typical of HepG2 cells and very few round cells. Notable alterations in HepG2 cell morphology were captured after 24 h of the fungal extract treatment at different concentrations. A decrease in the adhesive capacity of the cancer cells with a turning toward a more spherical morphology was also noted. The population of floating smaller cells increased in the medium, while a few cell numbers remained attached at elevated concentrations after 48 h of incubation. The treatment of HepG2 cells with different extract concentrations resulted in observable alterations in cell morphology and reduced adhesion to the substratum. Finally, the cell number decreased, contrary to the untreated cells, which exhibited a polygonal structure. A moderate inhibition impact on the cells of the HepG2 line was attained at 15.6 µg/ml of extract concentration. A low cytopathic impact towards the HepG2 cell line was detected by the tested extract doses (7.8 and 3.9 µg/ml) as illustrated in **Fig. 3**.

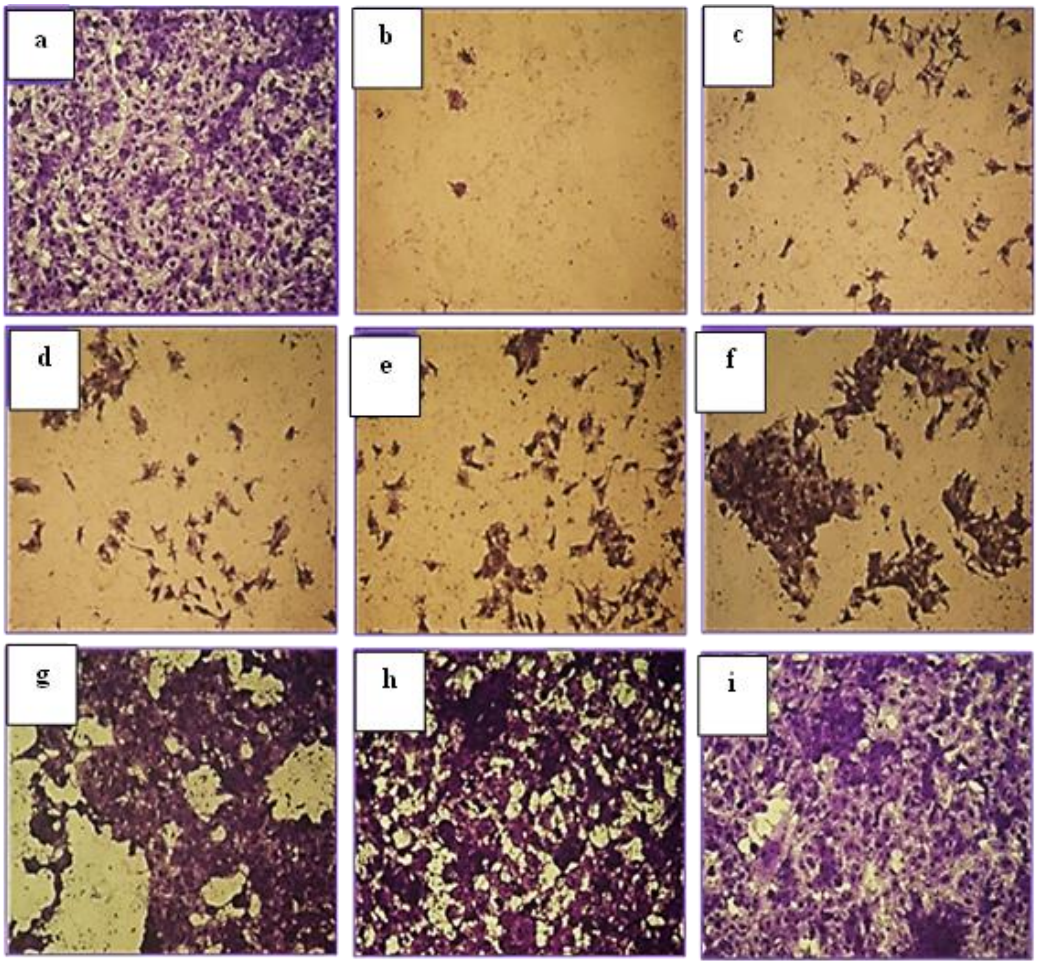


Fig. 3. Morphological characteristics of HepG2 cells treated with or without *Chaetomium* metabolites under an inverted microscope.

Panel (a) indicates the control of HepG2 cells after 48 h of incubation

Panels (b, c, d, e, f, g, h, and i) indicate HepG2 cells treated with different concentrations of *Chaetomium interruptum* metabolites (500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 µg ml^{-1}, respectively). Magnification: ×40

3.4 Antiviral potentiality

The outcomes of the *in vitro* screening of ethyl acetate and petroleum ether extracts showed weak to moderate anti-HSV-1 potential (**Tables 2 & 3**). The extract samples (1 and 2) EC_{50} values were 83.6 and 317.8 µg/ml,

matching a remarkable effect compared to the EC_{50} value of acyclovir (3.2 µg/ml). At the same time, samples (3 and 4) verify relatively low potential towards HSV-1. Table 3 illustrates that the most distinctive antiviral impact was established by sample (1) against HSV-1 *in vitro* at noncytotoxic concentrations, considering the EC_{50} and SI values. It was observed in **Table 2** that sample (1) is still less active than the acyclovir drug reference. However, samples (3 and 4) had a minimal insignificant effect against HSV-1.

Table 2. Antiviral activity of the fungal extract against HSV-1 via an MTT assay in Vero cells.

Sample concentration (µg/ml)	Inhibition (%)			
	1	**2**	**3**	**4**
1000	83.62 ± 1.94	74.23 ± 2.97	24.87 ± 0.91	13.94 ± 1.82
500	76.34 ± 2.75	62.91 ± 1.85	15.38 ± 0.74	6.59 ± 0.67
250	65.49 ± 3.47	53.67 ± 2.31	7.12 ± 0.54	2.36 ± 1.42
125	57.80 ± 2.68	40.95 ± 1.72	3.21 ± 0.37	0
62.5	46.02 ± 2.91	28.63 ± 2.89	0	0
31.25	33.89 ± 2.47	20.45 ± 0.73	0	0
15.6	24.65 ± 1.39	8.71 ± 0.87	0	0
7.8	12.97 ± 0.85	3.92 ± 0.84	0	0
3.9	5.26 ± 1.42	1.53 ± 0.39	0	0
2	2.30 ± 0.65	0.74 ± 0.28	0	0
0	0	0	0	0
EC_{50}	83.6	214	Weak	Weak

(1-2): ethyl acetate metabolites of *Chaetomium laterale* and *Chaetomium interruptum*
(3-4): petroleum ether metabolites of *Chaetomium laterale* and *Chaetomium interruptum*
The inhibitory effect of the fungal metabolites was expressed as the percentage of Vero cell survival
The data are presented as the means ± standard deviations of three separate experiments performed with six replicates

Table 3. Antiviral activity of fungal extract against HSV-1 in terms of CC50, EC50 (µg/ml).

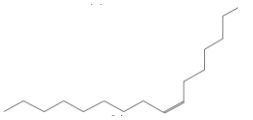
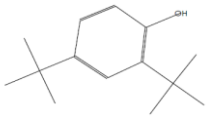
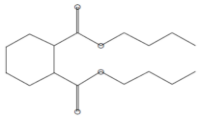
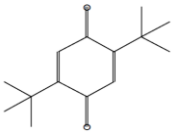
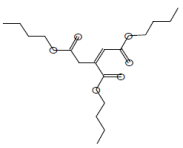
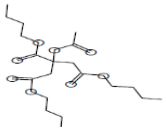
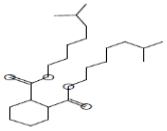
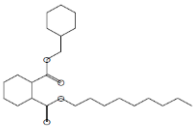
Tested metabolites	EC_{50}[#] (µg/ml)	CC_{50}[¶] (µg/ml)	SI[##] (CC_{50}/EC_{50})
1	83.6 ± 4.2	128.4± 8.8	1.53
2	-	148.1± 9.5	-
3	317.8 ± 19.6	436.3± 39.1	1.38
4	-	372.8± 23.4	-
Acyclovir[###]	3.2 ± 0.4	546.1 ± 39.5	170.6

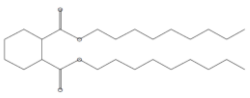
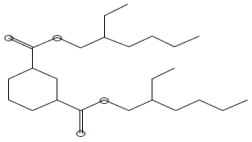
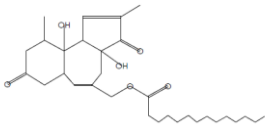
(1 - 3): ethyl acetate metabolites of *Chaetomium laterale* and *Chaetomium interruptum* respectively
(2 - 4): petroleum ether metabolites of *Chaetomium laterale* and *Chaetomium interruptum* respectively
[#]EC_{50}, the 50% effective inhibitory concentration; [¶]CC_{50}, the 50% cytotoxic concentration toward normal cell line; [##]SI: selectivity index; [###]Acyclovir: reference antiviral standard drug

3.5 *Chaetomium* extracts LC-MS analysis

C. interruptum ethyl acetate extract was separated into distinctive fragment ions, as identified by the LC-MS and system software (**Table 4 and Fig. 4**). Eight phytochemical groups were detected; nine ester compounds, acetyl tributyl citrate (ATBC), hexadecanol, phenol compounds, 2,4-ditert-butylphrenol,1-8, alcohols, and other compounds were included. On the other hand, in **Table 5** and **Fig. 5**, the *C. laterale* ethyl acetate extract detached mainly esters. Alcohols, phenols, and hydrocarbons were also represented, while amides, fatty acids, and anthocyanins were matched in small amounts.

Table 4. The identified metabolites of *Chaetomium interruptum* ethyl acetate extract via LC-MS Chromatography.

Peak Number	Retention Time (min)	Separated Compounds	Relative ratio (%)	Chemical Formula	Molecular Weight	Chemical Formula
1	16.11	1-Tetradecene	1.45	$C_{14}H_{28}$	196	
2	20.94	7-Hexadecene	4.18	$C_{16}H_{32}$	224	
3	22.75	2,4-Di-tert-butylphenol	13.40	$C_{14}H_{22}O$	206	
4	26.59	1-Hexadecanol	8.35	$C_{16}H_{34}O$	242	
5	31.89	1-Nonadecene	5.67	$C_{19}H_{38}$	266	
6	34.94	Phthalic acid, dibutyl ester	0.88	$C_{16}H_{22}O_4$	278	
7	35.93	2,5-di-tert-Butyl-1,4-benzoquinone	1.21	$C_{14}H_{20}O_2$	220	
8	36.60	1-Docosene	3.21	$C_{22}H_{44}$	308	
9	37.13	cis-13-Octadecenoic acid, methyl **ester**	1.23	$C_{19}H_{36}O_2$	296	
10	37.69	Phthalic acid, butyl 2-ethyl butyl **ester**	1.71	$C_{18}H_{26}O_4$	306	
11	38.48	Diphenylamine, n-ethyl-4-nitro-	1.62	$C_{14}H_{14}N_2O_2$	242	----
12	40.13	Decanedioic acid, dibutyl **ester**	4.40	$C_{18}H_{34}O_4$	314	
13	40.75	1-Propene-1,2,3-tricarboxylic acid, tributyl ester = Tributyl aconitate	2.17	$C_{18}H_{30}O_6$	342	
14	40.88	1-Heneicosyl formate (Ketone)	1.16	$C_{22}H_{44}O_2$	340	
15	43.04	Acetyl tributyl citrate (ATBC)	35.29	$C_{20}H_{34}O_8$	402	
16	48.49	Diisooctyl phthalate	0.67	$C_{28}H_{46}O_4$	390	
17	49.96	1,2-Cyclohexanedicarboxylic acid, cyclohexyl methyl nonyl ester	1.64	$C_{24}H_{42}O_4$	394	

Peak Number	Retention Time (min)	Separated Compounds	Relative ratio (%)	Chemical Formula	Molecular Weight	Chemical Formula
18	50.53	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	0.96	$C_{26}H_{48}O_4$	424	
19	50.68	1,4-Benzenedicarboxylic acid, bis (2-ethyl hexyl) ester	0.78	$C_{24}H_{44}O_4$	390	
20	51.79	(3a,10a-Dihydroxy-2,10-dimethyl-3,8-dioxo-3,3a,4,6a,7,8,9,10,10a,10b-decahydrobenzo[e]azulen-5-yl) methyl myristate	1.73	$C_{31}H_{48}O_6$	516	

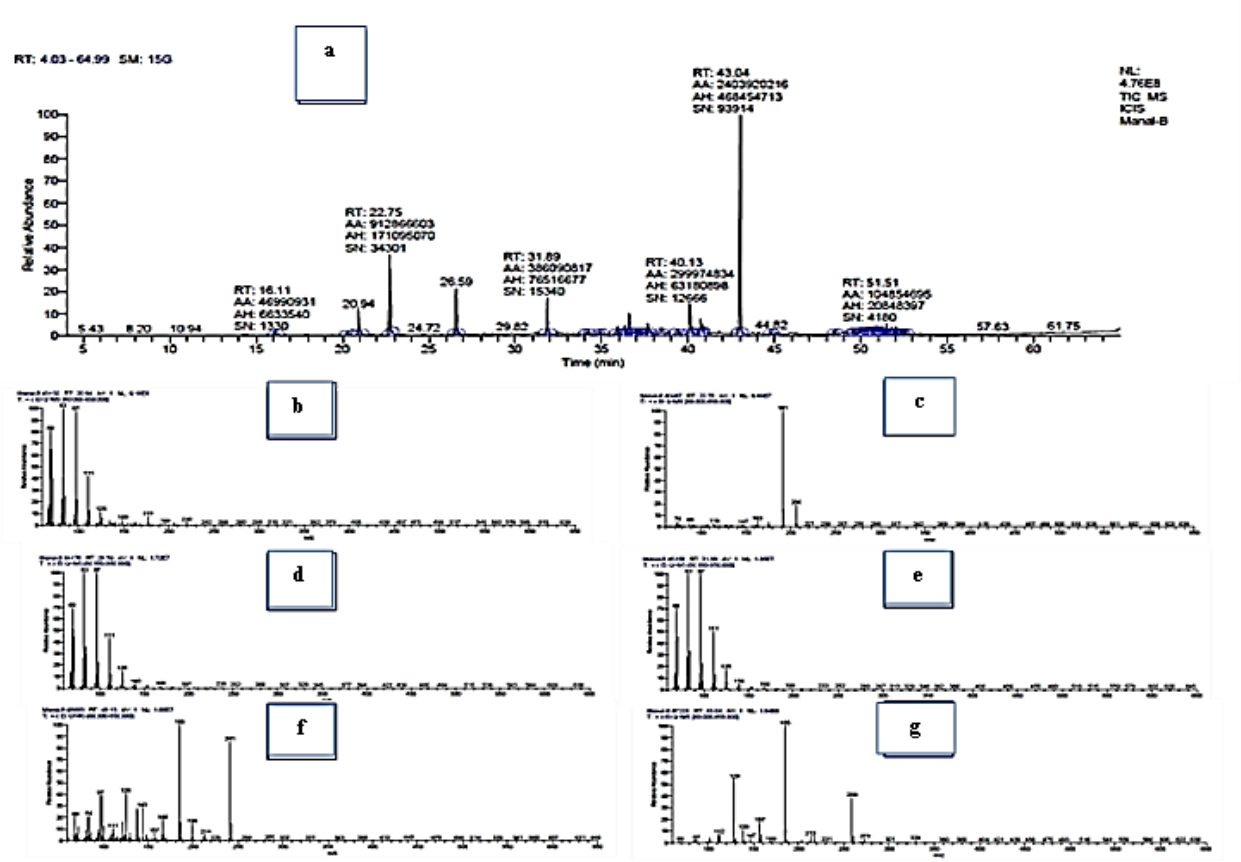
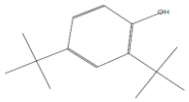
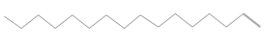
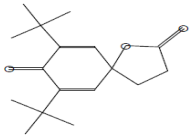
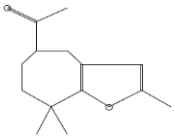
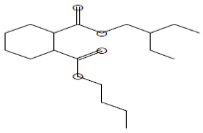
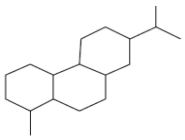
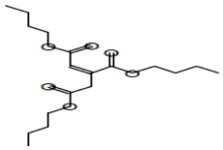
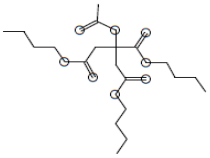
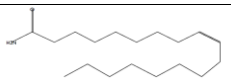
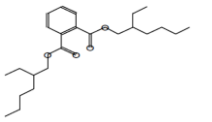


Fig. 4. Chromatographic analysis of the ethyl acetate extract obtained from *Chaetomium interruptum*.

(a) LC-MS total ion chromatogram visualizing all separated peaks; (b, c, d, e, f) Mass spectrum of the peak formed at retention times of 22.75, 26.61, 43.11, 43.82, and 48.52 min, respectively

Table 5. The identified metabolites of Chaetomium interruptum ethyl acetate extract via LC-MS Chromatography.

Peak Number	Retention Time (min)	Separated Compounds	Relative ratio (%)	Chemical Formula	Molecular Weight	Chemical Formula
1	20.94	1-Hexadecanol	1.70	$C_{16}H_{34}O$	242	
2	22.75	2,4-Di-tert-butylphenol	5.91	$C_{14}H_{22}O$	206	
3	26.6	1-Hexadecene	4.06	$C_{16}H_{32}$	224	
4	31.89	1-Octadecanol	3.67	$C_{18}H_{38}O$	270	
5	35.93	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	0.63	$C_{17}H_{24}O_3$	276	
	OR	1-(2,8,8-Trimethyl-5,6,7,8-tetrahydro-4H-cyclohrepta [b] furan-5-yl) teprenone		$C_{14}H_{20}O_2$	220	
6	36.34	9-Eicosene	0.88	$C_{20}H_{40}$	280	
7	36.61	1-Nonadecene	2.18	$C_{19}H_{38}$	266	
8	37.13	9-octadecenoic acid, methyl ester	0.54	$C_{19}H_{36}O_2$	296	
9	37.69	Phthalic acid, butyl 2-ethyl butyl ester	0.73	$C_{18}H_{26}O_4$	306	
10	38.48	Phenanthrene, 1,2,7,8,8a,9,10,10a-octahydro-2,2,7,7-tetramethyl-, trans	0.73	$C_{18}H_{26}$	242	
11	40.76	1-Propene-1,2,3-tricarboxylic acid, tributyl **ester**	3.15	$C_{18}H_{30}O_6$	342	
12	43.11	Acetyl tributyl citrate = 1,2,3-Propanetricarboxylic acid, 2-(acetyloxy)-, tributyl **ester**	45.91	$C_{20}H_{34}O_8$	402	
13	43.82	Hexanedioic acid, bis (2-ethylhexyl) **ester**	19.65	$C_{22}H_{42}O_4$	370	

Peak Number	Retention Time (min)	Separated Compounds	Relative ratio (%)	Chemical Formula	Molecular Weight	Chemical Formula
14	45.83	9-Octadecen**amide**	0.54	$C_{18}H_{35}NO$	281	
15	48.52	1,2-benzene dicarboxylic acid (**ester**)	9.43	$C_{24}H_{38}O_4$	390	

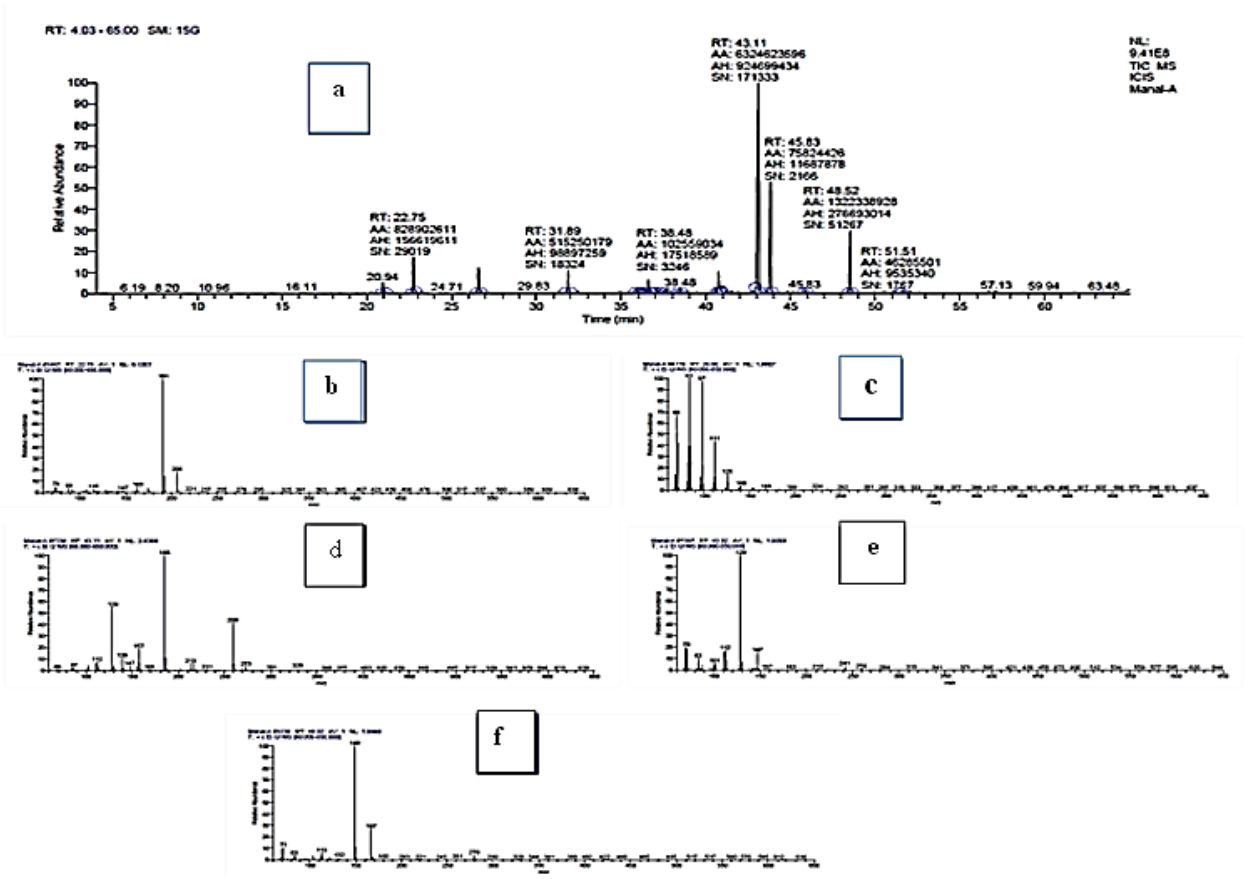


Fig. 5. Chromatographic analysis of the ethyl acetate extract detached from *Chaetomium laterale.*

(a) LC-MS total ion chromatogram revealing all separated peaks; (b, c, d, e, f) Mass spectrum of the peak formed at retention times 22.75, 26.61, 43.11, 43.82, and 48.52, respectively

4. Discussion

4.1 Chaetomium, a challenging biomedical and therapeutic source

C. interruptum and *C. laterale* phytochemical analyses were not set up to date in former studies, nor as a biomedical or therapeutic agent. In the current study, both species uncovered the potential ability to serve as new dependable bioactive sources in biomedical and therapeutic issues in the human population.

The present survey focused on the two bioactive endophytic fungi, *C. laterale* and *C. interruptum*, which were isolated by **Hemeda *et al.* (2022)**; the properties of phytochemistry and biology of both species were studied. Current and former studies have verified many important bioactive features of endophyte fungi metabolites; they have proved various challenging applications in medicine, such as anticancer, antimicrobial, antiviral, antitumour, antidiabetic, antioxidant potential, and cholesterol-inhibiting (**Kannan *et al.*, 2017a; Eskander *et al.*, 2020; Elkhouly *et al.*, 2021**). These organisms are valuable sources of fundamental and unique therapeutic

economic biomolecules (**Vijayalakshmi *et al.*, 2016**). Likewise, **Mubeen *et al.* (2020)** reported that *Moringa oleifera* endophytes can induce bioactive metabolites, which may introduce advanced potential more than those produced by the host plant. The endophyte fungi have been described as " *A Treasure House of Bioactive Compounds of Medicinal Importance* " (**Gouda *et al.*, 2016**).

4.2 An antioxidant, *Chaetomium*

C. globosum crude extract displayed notable DPPH free radicals scavenging antioxidant potential with 74.44 µg/ml of IC_{50} and a fundamental inhibition rate of 89.88 (**Kumar and Prasher, 2023**). Likewise, the *C. globosum* crude extract, which inhabits *Moringa oleifera,* showed a high scavenging antioxidant capacity (**Kaur *et al.*, 2020b**). On the other hand, the radical scavenging potential of the crude ethyl acetate extract of endophyte fungus *Pestalotiopsis neglecta* was detected effectively against 2,2 ′- diphenyl-1-picrylhydrazyl, for which the IC_{50} value was 36.6 µg ml^{-1} (**Almustafa and Yehia, 2023**); this was inharmonious with the outcomes of the present study since at a dose of 2000 µg/ml, the IC_{50} value achieved was 96.3 µg/ml, indicating further than 90 repressions of DPPH radicals. This can be attributed to the presence of phenolic and flavonoid compounds, which serve as main and secondary antioxidants, successfully suppressing the peroxidation of lipids. **Mahdi et al. (2014)** also reported that the *Moringa oleifera Chaetomium sp*. crude extract had high total polyphenolic content and scavenging antioxidant capacity. Another study documented similar effects with phenolic and flavonoid chemicals of endophyte metabolites (**Elfita *et al.*, 2021**). The reductive and enhanced electron patron potentials of *Chaetomium sp*. in the free radical stability had been attributed to the high phenol content produced by the fungus organism (**Yadav et al., 2014**).

4.3 *Chaetomium*, a potential cancer fighter

One of the important life-threatening global issues is cancer, which requires critical attention and challenge; how to fight through biological tools rather than chemical treatments for human safety. Findings obtained in the present study revealed the potential anticancer efficacy of the tested fungal extracts, which outlined hepatocellular melanoma (HepG2 cell line) inhibition. Carcinoma cells displayed lower vulnerability to the cytotoxic efficiency of the fungal extracts. Sample 3 documented a high SI against HepG-2, PC3, and HCT-116 cell lines because of its low toxicity toward Vero normal cells. **Results of Valderrama et al. (2016**) were mostly in agreement with the present study, which reported a high SI and low toxicity toward normal cells. The cancer cell lines, MCF-7 and HEPG-2, were tested for the cytotoxicity of *C. globosum* extract using EtOAc by Hani and Eman, 2015; the authors verified inhibitory effects on the survival and proliferation of the two cell lines and documented no significant difference detected between the tested cell lines considering their IC_{50}, in which relatively aligned with the current presentation outcomes. The BT-549 and SKOV-3 cell lines were also tested by **Wen et al. (2022)**; the endophyte fungal cytotoxicity registered IC_{50} value scopes of 0.16 - 1.97 µg/ml and 0.12 - 1.76 µg/ml. Vice versa was in the current study, the IC_{50} values registered a higher level against the examined carcinoma cell lines as follows: 25.8±2.4, 53.2±2.9 and 37.9±2.3 µg/ml, respectively.

4.4 Antiviral Potentiality of *Chaetomium*

Viruses have been a major global health concern for decades, and this problem has only grown worse in recent years. Natural bioactive compounds with promising therapeutic potential could one day provide a management solution to the problem of viral illness (**Techaoei et al., 2020**). The anti-HSV impact of the petroleum ether and ethyl acetate metabolite extracts varied in our study. According to the results obtained *in vitro*, the ethyl acetate metabolites derived from *C. laterale* and *C. interruptum* significantly affected HSV-1, with EC_{50} values of 83.6 and 317.8 µg/ml, respectively. The impact of acyclovir, which showed an EC_{50} value of 3.2 µg/ml, was comparable to these outcomes. Based on their EC_{50} and SI values, the ethyl acetate extract of *C. laterale* showed the highest effectiveness level on HSV-1 in the *in vitro* investigation, even at concentrations that were not lethal. Additionally, neither *Chaetomium* species' petroleum nor extract metabolites had much of an effect on HSV-1. Endophyte fungi's antiviral efficacy hasn't been thoroughly investigated in earlier references. Meanwhile, **Liu et al. (2019)** found that endophytic fungi in the phyllosphere (leaves) of *Quercus coccifera* manufacture hinnuliquinone, an antiviral drug, and that it inhibits the activity of HIV-1 protease.

4.5 *Chaetomium* metabolites hold a medical bioactivity

The results of this experiment showed that there are eight distinct categories for the ethyl acetate metabolite extracts obtained from *C. interruptum*. Nine compounds, or approximately half of the total contents evaluated, are part of the ester group, that had identified within the examined groupings. The percentage of phenol compounds is 18% while the alcohol percentage is 8.3%. However, much of the ethyl acetate metabolite extract from *C. laterale* was esters (>80%), with the remaining 10% being a mix of alcohols, phenolic chemicals, and hydrocarbons. There was a notable dearth of ingredients such as amides, fatty acids, and anthocyanins. Previous

references provided evidence that the present study procedures are trustworthy and consistent with the above-mentioned results. A minimum of 400 species spread worldwide comprise the endophyte fungal genus *Chaetomium*, as revealed in the literature review by **Rao et al. (2023**). Researchers have found that the genus *Chaetomium* may form over 500 variable chemical compounds with broad bioactivity varieties and chemical structures, such as anti-inflammatory, antiproliferative, antioxidant, antibacterial, phytotoxic, and plant growth inhibitory activities. The current study's findings are consistent with those of **Rao et al. (2023)**, who studied the phytochemicals of *Chaetomium* crude extract. Many diverse chemicals, such as steroids, azaphilones, cytochalasins, polyketides, diketopiperazines, anthraquinones, and alkaloids, were identified and successfully isolated in the **Rao et al. (2023)** study. To isolate *Chaetomium*'s primary compounds, **Kumar and Prasher (2023)** applied ethyl acetate as a solvent. The presence of alkaloids, flavonoids, phenolics, terpenes, and saponins was confirmed in a preliminary study of these extracts, applying phytochemical analysis. The natural bioactivity of many *Chaetomium* species was also studied by **Elkhateeb et al. (2021)**; and several bioactivities of *C. globosum* were similarly reported by **Wang et al. (2019); Yang et al. (2021); Kumar and Prasher (2023)**.

4.6 The key chemicals in the *Chaetomium* extract

Several common compounds were detected in the chromatographic experiment that screened the ethyl acetate crude extract of *C. interruptum*. The percentage of the separated chemicals included acetyl tributyl citrate (ATBC) at 35.29, 2,4-ditert-butylphenol (2,4-DBP) at 13.4, 1-hexadecanol at 8.35, 1-nonadecene at 5.67, decanedioic acid, dibutyl ester at 4.4, and 7-hexadecane at 4.18. Analyzing the crude extract of *Chaetomium* sp. by GC-MS revealed the presence of many components with bioactive capabilities, including antioxidants and antibacterial substances, in line with the results of **Elkhateeb et al. (2021)**. Bis (2-ethylhexyl) phthalate, 5-isopropyl-2-methylcyclohexane, 2,6-octadienal, 3,7-dimethyl-, D-carvone, 2-propenal, 3-phenyl, hexadecane, 1,2-benzene dicarboxylic acid, and other similar chemicals are included in the **Elkhateeb et al. (2021)** study. The endophytic fungus *Daldinia eschscholtzii* was used to purify the inhibitor quorum sensing (2,4-DBP QS) by **Mishra et al. (2020**). Their results are consistent with our analysis because they also found that 2,4-DBP inhibits the production of biofilms and pathogenic factors mediated by QS. They also found that when 2,4-DBP is used with antibiotics that have therapeutic value, it has a multiplicative effect. The GC-MS analysis in the study of **Aja et al. (2014)** determined the chemical composition of the methanolic extract of the *Moringa oleifera* leaves. One of the chemicals that was successfully isolated and identified was 1-hexadecanol. Due to its lack of toxicity in animal studies, this fatty acid alcohol is thought to be non-toxic and harmless. This study also found 1-hexadecanol; however, this time it was in an ethyl acetate solvent. The tested endophytic fungi fermented hexanedioic acid, which had significant antioxidant and antibacterial properties, as reported by **Harikrishnan et al. (2021)**. Several compounds with different peaks and retention durations were identified in the present study by chromatographic separation of the crude extract obtained from *Chaetomium* sp. A thorough GC-MS study of the crude *C. globosum* extract was conducted by **Kumar and Prasher (2023)**; they were exposed to several chemical components, most often flavonoids and phenolics. These chemicals included squalene, butanoic acid, 2-methyl-, hexadecenoic acid, 2-propanone, 1-phenyl-, 5-oxo-pyrrolidine-2-carboxylic acid methyl ester, and 9,12-octadecadienoic acid (z). Verifying this chemical in the extract, their identification was a relief.

4.7 Acetyl tributyl citrate (ATBC), significant remedial potential

To determine how ATBC affects gene expression and steroid production in human adrenal H295R cells, **Strajhar et al. (2017)** experimented to identify possible endocrine-disrupting substances; the authors applied the OECD validation test 456 guideline to cover a cell line and observed testosterone and estradiol production changes. **Takeshita et al. (2011)** investigated the activation of the steroid and xenobiotic receptor (SXR), also known as the pregnane X receptor (PXR), by various compounds, including ATBC. The current study revealed that CV-1 monkey kidney fibroblasts showed an increase in the expression of SXR following ATBC treatment. The cells displayed this effect following co-transfection with a reporter gene and a lethal SXR expression vector. It was observable that another cell line, which naturally expresses SXR, a fatal colon epithelial cancer, exhibited increased expression of the metabolic enzyme CYP3A4. ATBC treatment resulted in increased CYP3A4 levels in mortal liver cells. The results indicate that ATBC has a positive impact on cellular metabolism. **Rasmussen et al. (2017a, b)** evaluated the SXR receptor expression and its transcription target in the ovary. The authors postulated that ovarian follicles modulate CYP3A4 activity, which would explain their effect on steroid levels. Peroxisome proliferation is the main mechanism ATBC can affect the liver, leading to enlarged liver cells, elevated liver weight, and even enzyme induction (**Rasmussen et al., 2017b**). Still, there is a lack of available data regarding the acute inhalation toxicity or lethality of ATBC. The study matched mortality in neither the male nor the female rats. Mice were given 25,000 mg/kg of ATBC as a single dosage.

In the present issue, *C. interruptum and C. laterale* had not been the subject of any prior biological or phytochemical investigations, according to the reviewed literature. It has been found, however, that both species can serve as dependable sources of bioactive metabolites that can successfully treat important human medical problems.

Conclusions

The *Chaetomium* fungal species could be a pivotal key for ingredients in advancing novel bioactive compounds throughout biomedical and pharmaceutical applications and diverse domains. In this study, the *Chaetomium* species extracts showed an antiproliferative potential against different cancer cells while exhibiting low cytotoxicity against normal cells. The extracts also displayed a high antioxidant DPPH scavenger potential (i.e. high free radical inhibitor). They showed weak to moderate anti-HSV-1 activity—the chemical extracts of the two *Chaetomium* species contain many variable bioactive known components towards microbial pathogens and other human diseases. Our findings call for more toxicological and *in vivo* investigations to fully investigate and confirm the purified active compounds' potential as significant medicinal treatments.

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