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Secondary metabolites of rhizospheric fungal isolate *Aspergillus carneus* ABRF4 regulate the antibacterial and anti-proliferative activity against cancer cells

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Abstract. *Sahu MK, Yeeravalli R, Das A, Jha H. 2023. Secondary metabolites of rhizospheric fungal isolate* Aspergillus carneus *ABRF4 regulate the antibacterial and anti-proliferative activity against cancer cells. Nusantara Bioscience 15: 137-142.* The medicinal capabilities of plants are influenced by soil chemistry, genotype, and climate. Many biotic and abiotic factors affect soil composition. Microorganisms constituting the soil microflora indicate a mutualistic relationship with plant rhizospheric region, and they play an important role in plant secondary metabolite production, yield, and efficacy. They are the major resources for structurally unique bioactive natural metabolites. The aim of the present study was to evaluate the bioactivity of the secondary metabolite extracted from the rhizospheric fungal isolate *Aspergillus carneus* ABRF4) isolated from the Achanakmar Biosphere Reserve, Chhattisgarh, India. The fungal secondary metabolites were extracted using several solvents by soxhlet extraction techniques. The crude and partially purified column fractions of *A. carneus* ABRF4 were characterized by Gas Chromatography-Mass Spectroscopy (GC-MS), Fourier Transform Infra-Red (FTIR) spectrum, and Thin Layer Chromatography (TLC). Results showed that the acetonitrile fraction had an antimicrobial activity with the variable zone of inhibition against human pathogens such as *Bacillus circulans* (MTCC-7906), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus (*MTCC-96), and *Ralstonia eutropha* (MTCC-2487). The crude extracts and the identified secondary metabolite, trans- 1,3-dimethyl-Cyclohexane, possess different anti-proliferative activity against human tissue-specific cancer cell lines, including breast cancer (MDA-MB-231, MDA-MB-468, and MCF-7), liver cancer (HepG2), lung cancer (A-549), and prostate cancer (DU-145) suggesting a potential therapeutic application of the isolated rhizospheric fungi.

Keywords: Antimicrobial activity, anti-proliferative activity, *Aspergillus* sp., fungus

INTRODUCTION

The rhizosphere is the region around the plant roots with higher nutrients, so it has a higher microbial diversity than other soil areas. The plant roots benefit from several microorganisms residing in the rhizospheric region. These microorganisms create a microenvironment that inhibits the growth of pathogenic microorganisms. Rhizospheric microbes also impact the host plant's physiology and metabolism by changing the metabolic flux. They improve nitrogen fixation and subsequent nutrient uptake, including micronutrients and humus. It was previously reported that rhizospheric microbes might induce and modify the aromatic secondary metabolite pathways (Retnowati et al. 2018; Kong and Liu 2022). The rhizospheric microorganisms use the nutrients from the roots. The soil microenvironment, composition, temperature, and moisture affect its diversity. Fungi, such as mycorrhiza or free-living rhizospheric fungi, dominated the rhizospheric microbiome. The seasonal variation and anthropogenic activities may also affect the growth and rhizospheric fungi diversity. It has an impact on the growth of crops and medicinal plants. The Rhizospheric fungi reside in healthy living root tissue of medicinal plants with mutual beneficial effects. They produce biologically active secondary metabolites. The secondary metabolites of the Rhizospheric fungi have been reported to have a broad spectrum of applications in biotechnology, pharmaceuticals, food, and agriculture (Radhika and Rodrigues 2010; Ferris and Tuomisto 2015). They provide a natural and efficient alternative to conventional and xenobiotic chemicals and products. Rhizospheric fungi must synthesize metabolites to compete with co-emerging organisms, hosts, and pathogens to colonize the host and for nutrition. Fungus produces metabolites that may kill bacteria and regulate the metabolism of other fungi and plant cells (Zeilinger et al. 2016). These metabolites might be used to defeat diseases and cancer cells. They may control the growth of cancer cells or kill the cancer cells.

Exploring the resources of various functional chemicals is crucial as the primary sources of structurally distinct bioactive natural metabolites with broad therapeutic potential, including alkaloids, enzopyranones, benzoquinones, flavonoids, phenols, steroids, terpenoids, and tetralones (Pandit et al. 2018). They are a good source of biological substances with therapeutic potential, such as

antibacterial (Takahashi and Nyvad 2008), antifungal (Nicoletti et al. 2007), antiviral (Parshikov et al. 2015), and anticancer, cholesterol-lowering agents (Noman et al. 2021). Using morphological, microscopic, and the ITS 18S rDNA sequences, the Aspergillus strain (Isolate No. ABRF4) that was isolated from the Achanakmar Biosphere Reserve, Chhattisgarh, India, was identified as *Aspergillus carneus*. Previous research showed that compounds from *A. carneus* are effective against Gram-positive bacteria (Özkaya et al. 2018). The current investigation was conducted to determine the antimicrobial activity of *A. carneus* against four pathogenic bacteria, i.e., *Bacillus circulans* (MTCC-7906), *Bacillus subtilis* (MTCC 441), *Ralstonia eutropha* (MTCC-2487) and *Staphylococcus aureus* (MTCC-96), and proliferative activity against several cancer cell lines.

MATERIALS AND METHODS

Ethics statement

Ethics approval and consent to participate are not applicable as this study did not involve human participants or animals.

Chemicals and reagents

Chemicals and reagents used in this study were: Potato Dextrose Agar, Potato Dextrose Broth, and Czapekdox Agar (Himedia, India). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'azinobis (3-ethyl benzothiazole-6-sulphonic acid) (ABTS+), acarbose, potassium persulfate, were purchased from Sigma-Aldrich, USA. All reagents and chemicals were analytical grades. In addition, all the solvents (toluene, chloroform, ethyl acetate, methanol, and acetonitrile) used for extraction were HPLC grade.

Fungal isolation and identification

As previously described, a pure fungal strain ABRF4 was isolated from the rhizospheric soil of Achanakmar Biosphere, Bilaspur, CG, India (Radhika and Rodrigues 2010). It was identified as *A. carneus*.

Source of microorganisms for therapeutic studies

The antibacterial activity of fungal metabolites was tested against four pathogenic bacterial strains and one yeast isolate. Bacterial strains and yeast were obtained from microbial-type culture collection (MTCC, CSIR-IMTECH, Chandigarh, India). Bacteria isolates and yeast used in this study were *B. circulans* (MTCC-7906), *B. subtilis* (MTCC 441), *R. eutropha* (MTCC-2487), *S. aureus* (MTCC-96). All bacterial cultures were cultivated overnight on Nutrient Agar Medium (NA) slants and kept at 4°C as described previously (Ferris and Tuomisto 2015; Sahu et al. 2020; Sahu et al. 2022).

Fermentation

The pure fungal strain was inoculated into 500 mL of Yeast Extract Sucrose Broth (YESB) medium in a 1,000 mL Erlenmeyer and cultured for 14 days at 28°C. The culture was filtered using laboratory filter paper after incubation. The antibacterial and anti-proliferative activity of the column-purified fractions of the fungal extract was investigated.

Selection of optimum culture medium

To evaluate the best media for secondary metabolite growth, development, and production, fungal isolate ABRF4 was cultivated for 15 days in five different fungal growth media. Five media used in this study were CzapekDox Broth (CDB), CzapekDox Yeast Broth (CDYB), Malt Extract Broth (MEB), Potato Dextrose Broth (PDB), and Yeast Extract Sucrose Broth (YESB) (Sahu et al. 2022). Fungal culture achieved maximum growth and secondary metabolites production in YESB medium incubated at 28°C for 15 days.

Screening

At post-incubation, culture media was added with 1.5% tween-80 and shaken for 30 min at ambient temperature for homogeneous cell wall disruption of the fungal biomass and subsequent release of intracellular secondary metabolites into media. As described previously, the fungal biomass was screened for intracellular and extracellular secondary metabolites (Alkhulaifi et al. 2019). The filtrates were collected and concentrated up to 20% of their original volume (v/v) using a vacuum evaporator at 50 ± 5°C. The residue was concentrated and stored before diluting it as a working solution in sterile distilled water for further studies.

Extraction

Fungal biomass was extracted using ethanol on a soxhlet extractor, followed by filter sterilization before concentration and drying off the ethanol extract.

The ethanol extract was purified by further procedures to obtain active metabolites. Briefly, 1g of dry extract was solubilized in ethanol (1:1 w/v) and subjected to adsorption chromatography on a glass column packed with silica gel (60-120 mesh size) in toluene. Next, elution was carried out by standard method with increasing polarity of toluene, chloroform, ethyl acetate, methanol, and acetonitrile. Fractions obtained from each solvent were collected and subjected to spectrophotometric evaluation. Finally, a rotatory evaporator further concentrates selected fractions. Since the preliminary study results showed that the maximum activity was obtained in the ethanolic extracts, it was further analyzed. The crude ethanolic extract was mixed with toluene (1:1 w/v) and subjected to adsorption chromatography on a glass column packed with silica gel (60-120 mesh size) with different eluents according to the increasing polarity of toluene, chloroform, ethyl acetate, methanol, and acetonitrile. In addition, TLC and Gas Chromatography-Mass Spectroscopy (GC-MS) methods were used to identify active secondary metabolites in the extracts.

Purification of ethanolic extract

Thin layer chromatography (TLC)

The ethanol extract was transferred on a TLC plate and developed using an 18:80:2 (v/v) solvent system of

acetonitrile, water, and acetic acid. The chromatogram profile was examined under UV at 365nm to determine the Rf value. The fractions with similar TLC profiles were grouped, and a single spot on the TLCs indicated that the compounds were relatively pure (Azerang et al. 2019).

Identification of compound using spectroscopic methods

Fourier Transform Infrared (FTIR) spectroscopy

A Fourier Transform Infra-Red Spectrometer (I05 Nicolet Avatar 370, ThermoScientific, USA) was used to determine the infrared spectra of the pure compound at room temperature. Pure chemical (5 mg) was combined with spectroscopic grade KBr (95 mg) for pellet manufacturing. The IR spectra were captured in transmission mode at a 4000-400 cm^{-1} frequency range. A KBr pellet with no sample was employed as the control (Sahu et al. 2020).

Gas Chromatography-Mass Spectroscopy (GC-MS)

The samples were analyzed using GC-MS (Shimadzu GC-MS-QP2020; Kyoto, Japan) for qualitative and quantitative analysis utilizing the electron impact ionization (70 eV) method and mass spectra (Shimadzu GC-MS-QP2020; Kyoto, Japan). First, the compounds were identified by comparing their relative index to the mass spectra of standards in the National Institute of Standards and Technology's (NIST; Gaithersburg, Maryland, United States) GCMS library. Then, the proportion of the compound was calculated based on the peak area.

Bioactive properties of the isolated compound

In all the tests, the antimicrobial and anti-proliferative properties of extract and pure compounds from the strain ABRF4 were assessed in triplicates with appropriate blanks and controls.

Assessment of antibacterial activity of the fungal extract

The antibacterial activity of the fungal extract and fractions was determined using the agar well diffusion method (Balouiri et al. 2016) against human pathogenic bacteria, i.e., *Bacillus circulans* (MTCC-7906), *B. subtilis* (MTCC 441), *S. aureus* (MTCC-96), and *R. eutropha* (MTCC-2487). The vehicle control was ethanol, while the positive control was streptomycin (1 mg/mL) (Sahu and Jha 2020a,b).

Anti-proliferative activity

The cytotoxicity of the fractions was assessed using the Sulforhodamine B assay in various human tissue-specific cancer cell lines, including breast cancer (MDA-MB-231, MDA-MB-468, and MCF-7), liver cancer (HepG2), lung cancer (A-549), prostate cancer (DU-145) and primary control cell line (HEK-293). Briefly, 5×103 cells/ well were seeded onto a 96-well plate. After a 24 h incubation period, cells were treated for 48 h with increasing concentrations (1, 10, 100, and 300 mg/mL) of methanol, and ethanol fractions, followed by fixation and SRB staining of treated cells and vehicle controls. Doxorubicin was used as a positive control. A multimode reader (Perkin Elmer, Germany) was used to detect the optical density at 510 nm, and IC_{50} was calculated using GraphPad Prism 9.0 (Manupati et al. 2017).

Statistical analysis

All experiments were carried out in triplicate. Therefore, all the statistical data are the average of triplicate determination ± SD.

RESULTS AND DISCUSSION

Determination of secondary metabolites using TLC, GC-MS analysis, and FTIR

A fungus isolated from the Achanakmar Biosphere of Chhattisgarh was identified as *A. carneus* (isolate no. ABRF4) based on macroscopic and microscopic properties and the ITS 18S rDNA sequences (Sahu and Jha 2020a,b). Its secondary metabolites were screened for their bioactivities. The ethanolic extract of fungal biomass was fractionated using various solvents ranging from polar to non-polar solvents, i.e., acetonitrile, ethyl acetate, methanol, chloroform, and toluene. Fractions were tested for biological activity.

ABRF4 ethanolic extract (Spot A) and fractions of ethyl acetate and acetonitrile produced fluorescent spots under UV light (Spots B and C). The R_f values for Spots A, B, and C were 0.451, 0.351, and 0.283, respectively.

Ethyl acetate extract of *A. carneus* ABRF4 had maximum activity. Therefore, it was further spotted on TLC to isolate and purify the compounds (Figure 1.A). The results showed the presence of four peaks in purified fractions (Figure 1.B). A unique peak in the ethyl acetate fraction was found at 1190 cm^{-1}, which represented C–OH stretching and C–C stretching due to esters. COO- symmetric stretching was suggested by absorption at 1449.19 cm^{-1}, whereas alkane and amine group stretching was indicated by absorption at 2979.32 cm^{-1}. The IR spectrum revealed the presence of an aromatic ring replacing the ester bond, clearly indicating the predicted functional group of cycloalkanes structure (Figure 1.C-F). The GC-MS of the ethyl acetate fraction revealed the presence of cyclohexane, 1,3-dimethyl-, trans (Molecular weight 112 Da) (Table 1, Figure 1.G). This secondary metabolite has been hypothesized as the bioactive compound, while the remaining compounds are either primary metabolites or chemical compounds not involved in biological activity.

Antibacterial activity

The acetonitrile, ethyl acetate, methanol, chloroform, and toluene fractions of *ABFR4* isolate were tested for antibacterial activity at 100 µg/mL. It inhibited the growth of *B. circulans* (MTCC-7906), *B. subtilis* (MTCC-441), *R. eutropha* (MTCC-2487) and *S. aureus* (MTCC-96) (Figure 2). A clearance zone indicated antibacterial activity. The diameter of inhibition against *S. aureus* was 17.18 ± 1.3 mm, and the diameter against *R. eutropha* was 15.95 ± 1.03 mm. The ethyl acetate fraction had the highest antibacterial activity (Table 2).

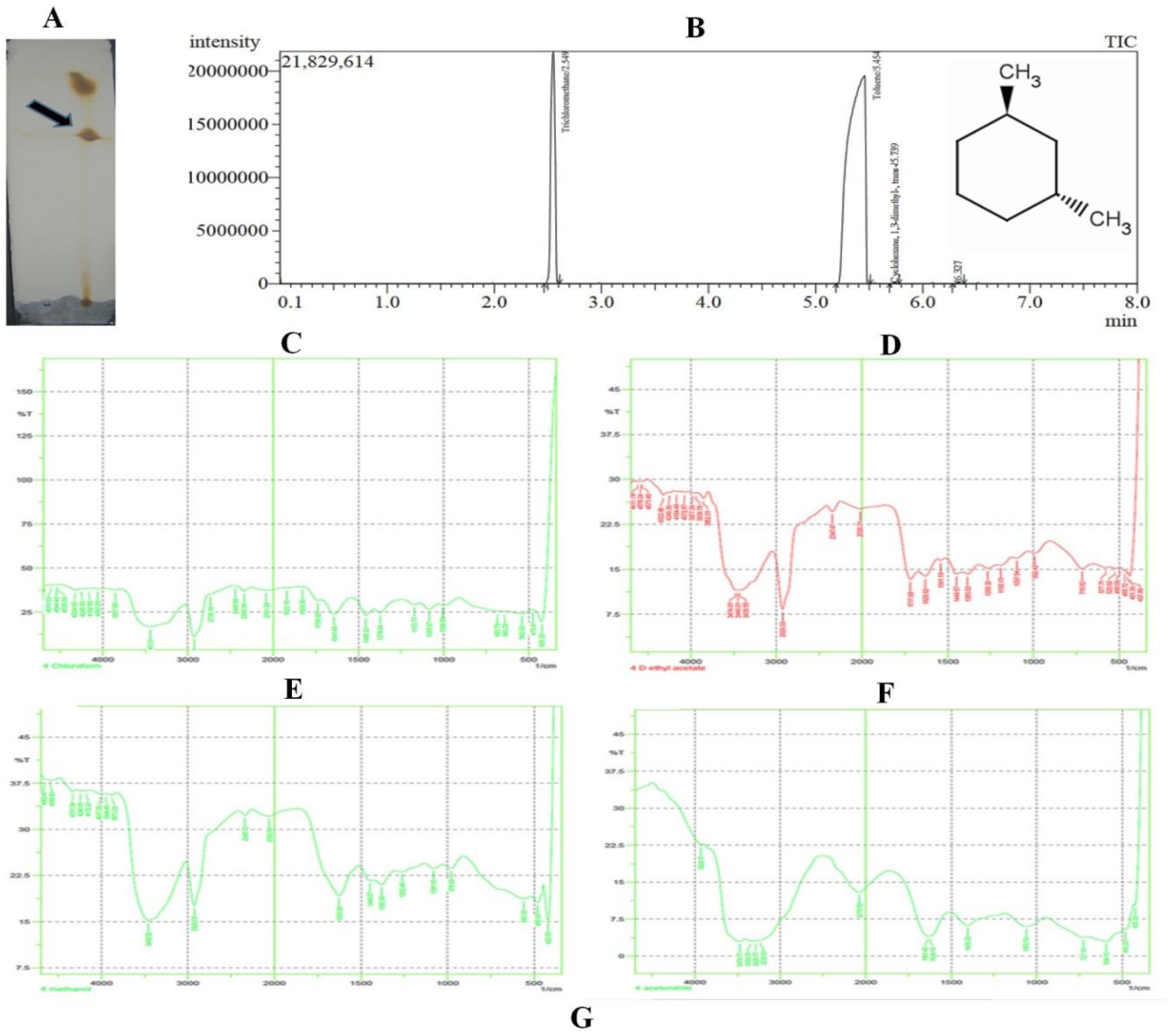

Figure 1. A-G. Chromatographic and spectroscopic analysis of the purified compound from *Aspergillus carneus* ABRF4

Table 1. Retention time of *Aspergillus carneus* (ABRF4) secondary metabolites and their respective retention time obtained by GCMS

Peak	Retention time	Area	Area percent	Name
1	2.549	59467953	21.73	Trichloromethane
2	5.454	213778647	78.11	Toluene
3	5.739	198449	0.07	Cyclohexane, 1,3-dimethyl-, trans-
4	6.327	256562	0.09	
		273701611	100	

Table 2. Antibacterial activity of *Aspergillus carneus* (ABRF4) activity

Bacterial pathogen	Zone of inhibition [a] Column fraction of ethanolic extract of *Aspergillus carneus* (ABRF4)					Positive[≠] control	Negative[≠] control
	Toluene	Cholororm E.	Acetate	Methanol	Acetonitrile	Streptomycin	Ethanol
B.circulans (gram +ve)	Nd	11 ± 1.3	15.4 ± 1.25	Nd	16.16 ± 1.1	24.28±1.25	Nd
B.subtilis (gram +ve)	Nd	Nd	14.7 ± 1.15	Nd	15.81± 1.8	24.21±0.93	Nd
S. aureus (gram +ve)	Nd	Nd	17.18 ± 1.3	Nd	17.16 ± 0.2	24.62±1.05	Nd
R. eutrophae (gram –ve)	Nd	Nd	Nd	Nd	15.95 ± 1.0	25.33 ± 1.7	Nd

Note: Nd: not detected. The superscript letters are significantly different ($p < 0.05$). [a]Inhibition zone excluding disc. [≠]6 mm disc as standard

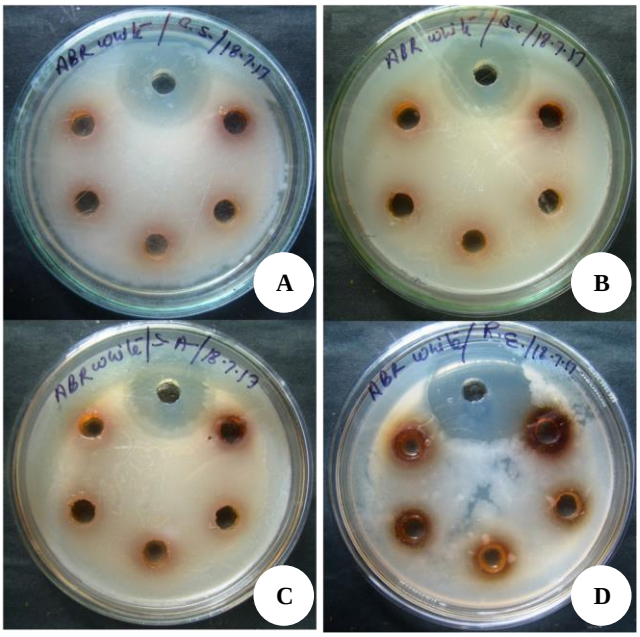

Figure 2. Antimicrobial activity of *Aspergillus carneus* ABRF4 against A. *Bacillus subtilis*, B. *Bacillus circulans*, C. *Staphylococcus aureus*, D. *Ralstonia eutropha*

Determination of anti-proliferative activity

SRB assay was used to test all three fractions of the fungal isolate ABRF4 against tissue-specific cancer cell lines. Fraction A (ethyl acetate fraction) exhibited relatively moderate anti-proliferative activity against lung cancer cell line, A-549, breast cancer cell lines, MCF-7, and MDA-MB-468, with an IC_{50} of 14.69, 15.01, and 18.93 µg/mL, respectively. Fraction B (methanol fraction) had an IC_{50} of 14.3, 15.66 µg/mL against the breast cancer cell line, MDA-MB-231, and prostate cancer cell line, DU-145, respectively. Finally, fraction C (ABRF4, ethanol fraction) exhibited an IC_{50} of 11.83, 12.75, and 13.82 µg/mL against DU-145, MDA-MB-231, and HepG2, respectively. These three fractions showed an IC_{50} value approximately five times higher than the control primary cell line, HEK-293, suggesting these fractions had anti-proliferative activity (Table 3).

Discussion

The fungus *A. carneus* isolate-ABRF4 was identified based on morphological and sequencing similarity. Extracellular and intracellular extracts were tested for their bioactivities. Intracellular extracts had higher anti-proliferative activity than extracellular extracts (data not shown). The fractions were investigated against Gram-positive and Gram-negative bacteria. The results showed that the fractions of ethyl acetate, methanol, and acetonitrile inhibit the growth of both Gram-positive and Gram-negative bacteria. The TLC profile of the ethyl acetate fraction revealed a single spot with an Rf value of 0.5, like alternariol-like compounds isolated from fungi (Wei et al. 2017; Tulsawani et al. 2020). Trans-1,3-dimethyl-cyclohexane was one of the metabolites identified in the fraction that might have biological activity. Therefore, it needs further analysis. FTIR was used to validate the identity of the compound and its structural group. Subsequently, the GC-MS of ethyl acetate fraction confirmed the presence of trans-1,3-dimethyl-cyclohexane. Esters exhibit antibacterial activities (Tomar et al. 2019). Cyclohexane and its derivatives have various biological activities such as antioxidant, anticancer, cytotoxic, analgesic, anti-inflammatory, and anti-thrombin activities (Modak et al. 2011; Shoaib et al. 2021). The previous study by Lallo et al. (2014) showed 3-acetyl-4-benzoyl-1-benzoyloxymethyl-1,6-diepoxycyclohexan-2,3,4,5-tetrol has cytotoxic activity against pancreatic and breast cancer but not against normal cell lines. The 1,1-disubstituted cyclohexane-1-carboxamides also exhibited anticancer activity against various cancer cell lines, including MCF-7, HepG2, A549, and HTC-116. The compounds were considered potent apoptotic inducers interfering with extrinsic and intrinsic apoptotic pathways (Abd-Allah and Elshafie 2018; Abd-Allah et al. 2019). The results of the present study showed that trans-1,3-dimethyl-cyclohexane isolated from ethyl acetate fraction of *A. carneus* ABRF4 has anti-proliferative properties by inducing an apoptotic regulation in the cancer cells.

Table 3. IC_{50} value of *Aspergillus carneus* (ABRF4) fractions against various cancer and non-cancerous cell lines

***Aspergillus carneus* (ABRF4) HPLC fractions**	**IC50a (µg/mL)**						
	MCF-7b	**MDA-MB-468c**	**MDA-MB -231d**	**DU-145 e**	**HEPG-2f**	**A-549 g**	**HEK-293h**
Fraction A	15.01± 0.35	18.93 ± 2.12	41.87 ± 1.98	53.22± 2.73	25.67± 3.73	14.69±2.76	90.82± 1.93
Fraction B	44.28 ± 1.10	29.74± 2.42	14.31 ± 1.53	15.66 ± 2.47	57.61± 2.82	79.58± 2.92	82.09± 5.45
Fraction C	19.26± 1.48	22.48 ± 3.26	12.75 ± 0.02	11.83 ± 0.01	13.82± 1.89	18.67 ± 0.55	77.3± 1.28
Doxorubicin (µM) Positive control	5.78 ± 0.44	0.77 ± 0.06	1.33 ± 0.02	0.74 ± 0.15	0.90 ± 0.07	2.98± 0.25	37.95± 4.50

Note: Fraction A: n-valeric acid (methanol), B: ethyl oleate (methanol), C: Cyclohexane, 1,3-dimethyl-, trans- (Ethanol). [a]50% inhibitory concentrations and mean ± SEM of IC_{50} (µg/mL) values of different fractions represent the mean of three replications. [b]Luminal-A ($ER^+/PR^+/Her2^-$) breast cancer. [c]Basal (low claudin) Triple-negative ($ER^-/PR^-/Her2^-$) breast cancer. [d]Basal Triple negative ($ER^-/PR^-/Her2^-$) breast cancer. [e]Moderate metastatic potential (PSA^+) Androgen-independent prostate cancer. [f]Liver hepatocellular carcinoma. [g]Adenocarcinoma human alveolar basal epithelial cells lung cancer. [h]Non-cancerous primary human embryonic kidney cells.

In conclusion, the *A. carneus* ABRF4 contains several potent secondary metabolites, including trans-1,3-dimethyl-cyclohexane (Molecular weight 112 Da), which has anti-proliferative activity suggesting its potential therapeutic application.

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