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Extract of Microbial Isolates From the Western Region of the Kingdom of Saudi Arabia and their Applications in the Field of Health

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Abstract

Objectives: In a previous study, the presence of *Aspergillus* species in high-salt soil was investigated. However, it was hypothesized that the addition of a protein source to the soil could reveal other fungal species. This study aimed to test this hypothesis by repeating the methodology used in the previous study, but with the inclusion of a protein source.

Methods: The ribosomal DNA (ITS) region was sequenced to identify the fungal isolate. The results showed that the isolate belonged to a different genus within the *Aspergillus* species, specifically *Aspergillus sydowii*. The strain was named WIS and recorded in the gene bank with the accession number OR262340.1. The antibacterial activity of the isolated fungus was evaluated by measuring the inhibition zone diameter after exposing pathogenic bacteria to the fungus. Additionally, the anti-cancer activity of the isolate was tested on six different cell lines, including breast cancer, hepatic cancer, and colorectal cancer. To further support the evidence of the bioactivity of this fungus, liquid chromatography-mass spectrometry (LCMS) was conducted on the fungal extract to identify its secondary medicinal metabolites. Additionally, an in-silico study was performed to predict the toxicity, pharmacokinetic properties, and safety profiles of each molecule.

Results: *Aspergillus sydowii* exhibited antibacterial activity against both gram-positive and gram-negative bacteria. It also demonstrated excellent anti-cancer activity on all tested cell lines.

Conclusion: Future studies should focus on investigating the antimicrobial activities of this fungus against a wider range of pathogenic bacteria, particularly multidrug-resistant strains. Furthermore, it would be beneficial to study the genetic changes in bacteria and cancer cells treated with these fungal extracts at the genetic level.

Keywords: Molecular identification, marsh soil, antibacterial, anti-cancer, fungal isolates, PCR identification, liquid chromatography, in-silico studies

Introduction

Biotic and abiotic factors are crucial in determining the composition of organisms in soil environments.[1] Contrary to earlier beliefs, studies have shown that environments such as deserts have a diverse range of microbial life.[2] Saudi Arabia, with more than half of its landmass being desert, has been the subject of previous studies on microbial diversity in Sabkha and deserts.[3]

Antimicrobial resistance (AMR) and cancer are two health issues that are on the rise globally.[4,5] Despite the increase in these problems, significant progress has been made in the field of research to find a solution to these issues.

Bacterial resistance to antibiotics has become a challenge for doctors, with some bacteria developing immunity to multiple types of antibiotics.[4] This has led to an increase in morbidity and mortality rates, especially for gram-negative bacterial diseases. Fungi have been used extensively in medical fields since the discovery of the bioactivity of Penicillium on bacterial growth.[6]

Cancer is one of the leading causes of mortality globally, affecting both high-income and low-income countries.[5] Although cancer mortality is lower in high-income countries, cancer incidence is on the rise worldwide due to various risk factors.[7-9] It is estimated that cancer incidence and mortality rates will continue to increase in the coming years due to population growth, age, and exposure to carcinogens. Therefore, scientific efforts must be combined to limit the spread of this disease and find a cure for it.

A recent study by a research team from Princess Nourah bint Abdulrahman University found that some fungi may have anticancer and antimicrobial activity.[3] Our research team's previous study identified fungal and bacterial species in the western region of Saudi Arabia. In this study, we aim to test the biological activity of the extracts of these fungi against cancer and bacterial growth and the possibility of using fungal extracts as a future cancer drug.

Materials and Methods

Fungal Isolation and Morphological Characterization

To investigate the microflora of Sabkha soil in the Makkah region of Saudi Arabia, thirty soil samples were collected from Rabigh City. Samples were taken from a depth of 5 to 20 cm and transported to the laboratory in plastic bags. Grounded chicken meet was added to the soil samples. The isolation of fungi was carried out using the dilution and blotter method, followed by incubation at 25 ± 2°C for 7 days.[10] Pure colonies were then preserved on Potato Dextrose Agar (PDA) slants at 4°C for future use. Traditional characterization techniques, including colony texture assessment on specific media such as Sabouraud dextrose agar and Czapek-Dox agar supplemented

with chloramphenicol, were employed for species identification. Surface, reverse colony, and microscopic observations were also conducted.[10]

Physicochemical Investigations

The soil samples underwent examination using a digital camera to analyze particle topographies, including color, shape, and morphology. pH analysis was performed using a pH meter (model HI98107), while moisture content (MC) was determined according to Cruz-Romero et al. (2004) and Jackson (1958).[11] Electrical conductivity (EC) for each soil sample was measured using an EC-meter (Matter Toledo-AG).

Microscopic Study of Isolates

The exterior properties and colors of the fungal colonies were examined for identification purposes. The presence of fungal biofilms on the crystal surface of the substrate was observed using a Scanning Electron Microscope (SEM). Sample preparation involved fixation, drying, and gold coating. Finally, the samples were observed using an SEM (JEOL 7500FA JEOL, Peabody, MA, USA) at 10 kV.*2.4.*

Molecular Characterization of the Isolates

DNA extraction, amplification, and sequencing

A 2 µl aliquot of Potato Dextrose Broth (PDB) (himedia, mumbai, India) was poured into PDA tubes and vortexed to disperse the spores. The spore PDB mixtures were then added into flasks containing 100 ml of PDB. The flasks were kept undisturbed at room temperature for two to three days. The mycelium was harvested by filtration, frozen at −80°C for 30 minutes, lyophilized, and stored at −80°C. The mycelium was ground in liquid nitrogen with a sterile mortar to obtain mycelium powder.

DNA was extracted from 20mg of mycelium powder using a DNeasy plantmini Kit. The DNA quantity and quality were checked by electrophoresis on 0.8% agarose gel and visualized with Ethidium bromide under UV transillumination.

The internal transcribed spacer (ITS) region of ribosomal DNA was amplified by PCR with the ITS1-F (5′-CTTGGTCATT TAGAGGAAGTAA-3′) and ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′) primers.[12,13]

PCR amplification was carried out in a final volume of 50 µl, containing 2 µl of DNA, 0.5 mm of each primer, 150 mm of dNTp, 1u of Taq DNA polymerase (promega), and PCR reaction buffer.

Amplification was carried out in a thermal cycler with an initial denaturation of 3 mins at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The amplified product was checked by electrophoresis on 1% agarose gel and visualized with Ethidium bromide under UV transillumination based on the manufacturer's instructions. The PCR product was purified using an Exo SAPIT kit (USB corporation, Amersham place, UK, under license from GE healthcare).

Sanger sequencing was carried out at Beijing Genomic Institute (BGI), Hong Kong, China. The sequence was compared to the NCBI database using the BLAST software. The obtained sequences were aligned in Ugene (Okonechnikov et al. 2012)[14] using the T-Caffee algorithm (https://www.ebi.ac.uk/, EMBL-EBI, Cambridgeshire, UK) and a phylogenetic tree was generated using iTOL, an interactive tree tool (http://itol.embl.de/index. shtml)(Letunic and Bork 2011).[15]

Antimicrobial Assay

The antibacterial investigation was done for the fungi isolates against tested bacteria using agar disk diffusion assay. Each fungal isolate was cultured on Sabouraud dextrose agar with chloramphenicol 1% plate, for seven days, at 28°C. Then, disks (15 mm diameter) were cut from the Sabouraud dextrose agar and placed to the top of different media plate that previously inoculated with bacteria: Blood agar (BA), Nutrient agar (NA) and Sabouraud dextrose agar (SDA). The plate was incubated at 37°C for seven days. Antimicrobial activity was evaluated by visualization followed by the measurement of inhibition zones (in mm).

Statistical Analysis

Data are expressed as the mean ± SD of three replicates.

Anti-cancer

Cell culture

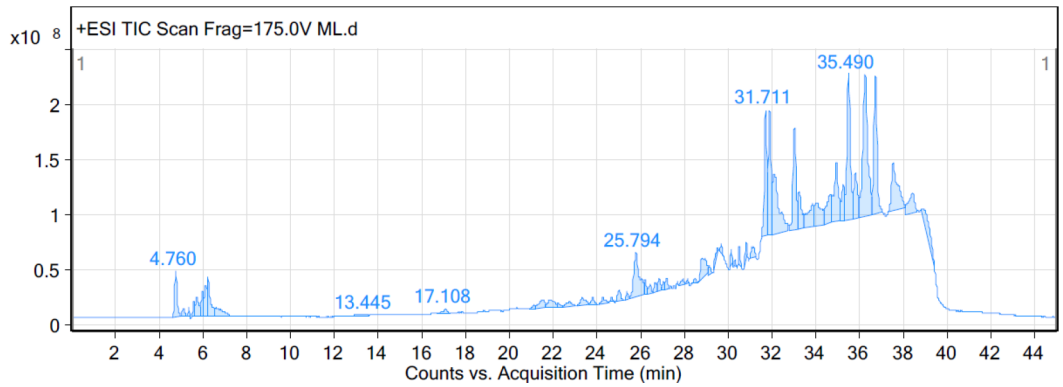
Six different cell lines were used in this study. The hepatoma cell line, HepG2, Colorectal cancer cell lines, HCT8 and HCT116, and the human drug-resistant metastatic breast cancer, MDA-MB-231, were purchased from ATCC, United States. Whereas epithelial-like breast carcinoma cells KAIMRC1 (Ali et al. 2017) and triple-negative KAIMRC2 (Ali et al. 2021) were isolated and characterized at King Abdullah International Medical Research Center (KAIMRC), Riyadh, Saudi Arabia. All these cell lines were grown in advanced DMEM containing 10% fetal bovine serum, 1% L-glutamine, and 1% antibiotics (Pen/Strep).[16,17] Cells were cultured for at least 24 h before any experiment in 96-well plates at the density of 10,000 cells per well.

Cell Proliferation Assay (MTT)

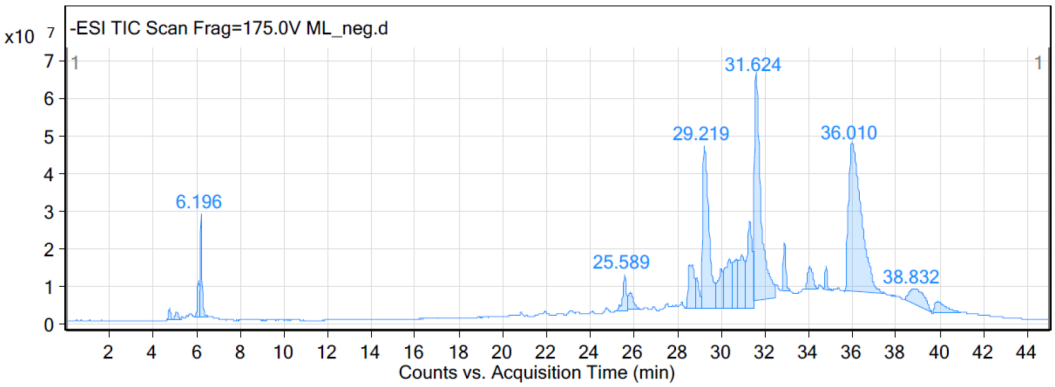
The extract, *Aspergillus sydowii isolate,* was examined for its anticancer activity against the above-mentioned cell lines. The cells were treated with various doses of each substance, ranging from 0 to 1000 µg/ml. Doxorubicin, an anthracycline chemotherapy drug, and Mitoxantrone, an anthracenedione antineoplastic agent, were used as positive controls for cytotoxicity, and cells treated with DMSO only served as negative control. After 48 hours, the cells were treated with 5 mg/ml of MTT solution. Following 3 hours at 37°C of incubation, the media was aspirated, and 100 µL of the DMSO solvent was added to each well. After shaking the plate on an orbital shaker for 45 mins, the absorbance was measured at OD 590 nm. Half-maximal inhibitory concentration (IC_{50}) values for each chemical were determined using the dose-response curve in GraphPad Prism 8. Each experiment was performed in triplicates.

Identification of Secondary Medicine Metabolites using QTOF-LCMS

The Ethanolic extract of the *Aspergillus* ***sydowii*** was subjected to total ion current spectra (TIC) raw data (See Figure 1), the data-analysis program Mass Hunter (Agilent Technologies) qualitative and quantitative analysis software has been used.



Elector spray ionization total ion chromatogram (Positive Mode) of *Aspergillus sydowii* extract



Elector spray ionization total ion chromatogram (Negative Mode) of *Aspergillus sydowii* extract

Fig. 1 **Elector spray ionization total ion chromatogram (Positive, and Negative Mode) of *Aspergillus sydowii* extract.**

After conducting a mass screening on the below spectrum (Figure 1) we have summarized the following, Chemical features were extracted from the LC-MS data using the Molecular Features Extraction (MFE) algorithm and the recursive analysis workflow. Features have been extracted by screening the detected nodes at various retention time per minutes, with a minimum intensity of 6,000 counts and aligned with previously detected compounds considering adducts ($[M+H]^+$, and $[M-H]^-$).

In-silico Predictions and Studies

In-silico Predictions for Secondary Metabolites

The secondary metabolites contained within the fungal samples were subjected to identification, utilizing a library of 28 chemical structures. These structures were subsequently employed in computational predictions to assess the biological anti-cancer activity, pharmacokinetic properties, and safety profiles of each molecule. The in-silico predictions were conducted using the Simplified Molecular Input Line Entry System (SMILES) representations, which are summarized in supplementary Table S1. Furthermore, the chemical structures of all the metabolites can be observed in Figure 2.

Utilization of pass online web server for anticancer activity predictions

To predict the anti-cancer activity of the identified metabolites, the 2D chemical structures were inputted into the PASS Online web server (http://www.way2drug.com/passonline). The results are presented as a probability score (P_a), where a score greater than 0.7 indicates potent anti-cancer properties, while a score between 0.7 and 0.5 suggests moderate anti-cancer activity if tested experimentally.[18]

Pharmacokinetic Parameters and Oral Bioavailability Predictions

Utilizing the SwissADME online web (http://www.swissadme.ch/), multiple pharmacokinetic parameters were evaluated for the identified metabolites, including lipophilicity, molecular weight, solubility, insolubility, insaturation, and flexibility. These parameters were predicted and analyzed to determine the probability of the metabolite to be orally active. The results were represented as a radar plot, where the properties included in the colored zone are within the acceptable range for orally active drugs.[19]

Assessment of organ and endpoint toxicity

To determine the potential toxicity of identified metabolites, we employed the ProTox-II webserver (https://tox-new.charite.de/protox_II/). This powerful tool enabled us to predict the organ and endpoint toxicity of these metabolites, including hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity. This approach was crucial in ensuring the accuracy of our predictions for these molecules, providing a comprehensive assessment of their toxicity profiles.[20]

Table S1. **The Simplified Molecular Input Line Entry System (SMILES) for the secondary metabolites identified in *Aspergillus sydowii*, *Aspergillus flavus*, and *Aspergillus welwitschiae***

Aspergillus sydowii	
Name of Metabolite	**Smiles**
1. Diorcinolic acid	CC1=CC(=CC(=C1C(=O)O)OC2=CC(=C(C(=C2)C)C(=O)O)O)O
2. β-d-glucopyranosyl Aspergillusene A	CC(C)CC/C=C(\C)/c1ccc(cc1O)CO[C@H]2C[C@H]([C@@H]([C@H](O2)CO)O)O
3. Violaceol II	CC1=CC(=C(C(=C1)O)OC2=CC(=CC(=C2O)O)C)O
4. Cordyol C	CC1=CC(=CC(=C1)OC2=CC(=CC(=C2O)O)C)O
5. Cyclo-(L-Phe-L-Trp)	C1=CC=C(C=C1)CC2C(=O)NC(C(=O)N2)CC3=CNC4=CC=CC=C43
6. 7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone	CC(O)CC1=CC(=O)C2=C(O1)C=C(O)C=C2C
7. n-acetylserotonin glucuronide	CC(=O)NCCC1=CNC2=C1C=C(C=C2)OC3C(C(C(C(O3)C(=O)O)O)O)O
8. Shornephine A methyl ester	CC(C)(C=C)C1(C2=C(C(=CC=C2)O)NC1=O)CC(C(=O)OC)NC(=O)C(CC3=CC=CC=C3)O
9. 1-methylpyrogallol	CC1(C=CC=C(C1O)O)O
Aspergillus flavus	
Name	**Smiles**
1. Aflavinine	CC1CCC2(C(CCC(C23C1C(=C(CC3)C(C)C)C4=CNC5=CC=CC=C54)O)C)C
2. Dihydro-24-hydroxyaflavinine	C[C@](CC1)([C@@H](C2)C)[C@@](CC3)(C[C@H]2O)[C@]([C@@H]1C)([H])[C@](C4=CC5=CC=CC=C5N4)([H])[C@@H]3C(C)(O)C
3. Phomaligin A	CCC(C)C(=O)C1=C(C(C(=O)C(=C1OC)C)(C)O)NCCO
4. Hydroxysydonic acid	CC(C)(CCCC(C)(C1=C(C=C(C=C1)C(=O)O)O)O)O
5. Gregatin B	CCC=CC=CC1(C(=O)C(=C(O1)C)C(=O)OC)C
6. Pulvinulin A	CC/C=C/C=C/[C@](OC1CCCCC)(C)C(C1C(OC)=O)=O
7. Chrysogine	CC(C1=NC2=CC=CC=C2C(=O)N1)O
8. Aspergillic acid	CCC(C)C1=CN=C(C(=O)N1O)CC(C)C
9. Aflatoxin B1	COC1=C2C3=C(C(=O)CC3)C(=O)OC2=C4C5C=COC5OC4=C1
10. Aflatoxin G1	COC1=C2C3=C(C(=O)OCC3)C(=O)OC2=C4C5C=COC5OC4=C1
Aspergillus welwitschiae	
Name	**Smiles**
1. Atromentin	C1=CC(=CC=C1C2=C(C(=O)C(=C(C2=O)O)O)C3=CC=C(C=C3)O)O)O
2. Fonsecin B	CC1(CC(=O)C2=C(C3=C(C=C(C=C3C=C2O1)OC)OC)O)O
3. Funalenone	CC1=CC(=O)C2=C(C(=C(C3=C2C1=C(C=C3O)O)O)OC)O

(Continued)

Table S1. **The Simplified Molecular Input Line Entry System (SMILES) for the secondary metabolites identified in *Aspergillus sydowii*, *Aspergillus flavus*, and *Aspergillus welwitschiae*—Continued**

Aspergillus welwitschiae	
Name	**Smiles**
4. Rubrofusarin	CC1=CC(=O)C2=C(C3=C(C=C(C=C3C=C2O1)OC)O)O
5. Aurasperone E	CC1=CC(=O)C2=C(C3=C(C=C(C=C3OC)OC)C(=C2O1)C4=C(C5=C(C6=C(C=C5C=C4OC)OC(CC6=O)(C)O)O)OC)O
6. Aurasperone D	CC1=CC(=O)C2=C(O1)C=C3C=C(C(=C(C3=C2O)OC)C4=C5C(=C(C6=C4C=C(C=C6O)OC)O)C(=O)C=C(O5)C)OC
7. Aurasperone C	CC1(CC(=O)C2=C(C3=C(C(=C(C=C3C=C2O1)O)C4=C5C(=C(C6=C4C=C(C=C6OC)OC)O)C(=O)CC(O5)(C)O)OC)O)O
8. Nigerone	CC1=CC(=O)C2=C(C3=C(C=C(C=C3OC)OC)C(=C2O1)C4=C5C(=C(C6=C4C=C(C=C6OC)OC)O)C(=O)C=C(O5)C)O
9. αβ-dehydrocurvularin	CC1CCCC=CC(=O)C2=C(CC(=O)O1)C=C(C=C2O)O

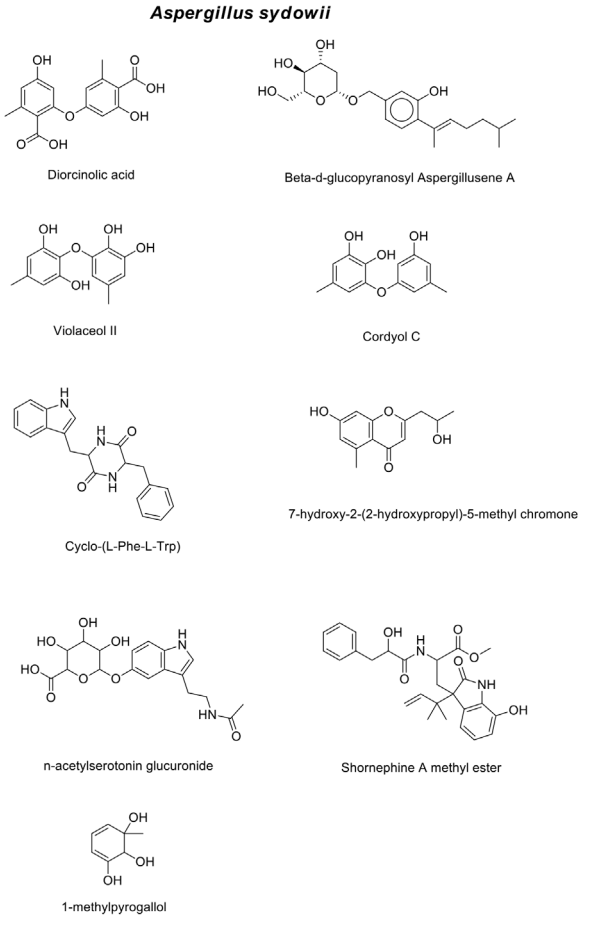


Fig. 2 **The chemical structures of secondary metabolites identified in *Aspergillus sydowii*.**

A

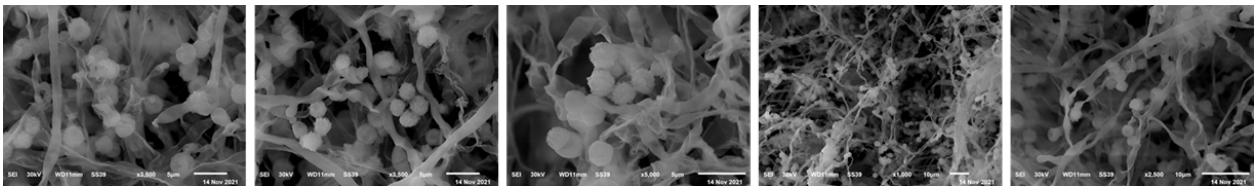


Fig. 3 **Scan electron microscope images showing characteristic features of *Aspergillus* conidia.**

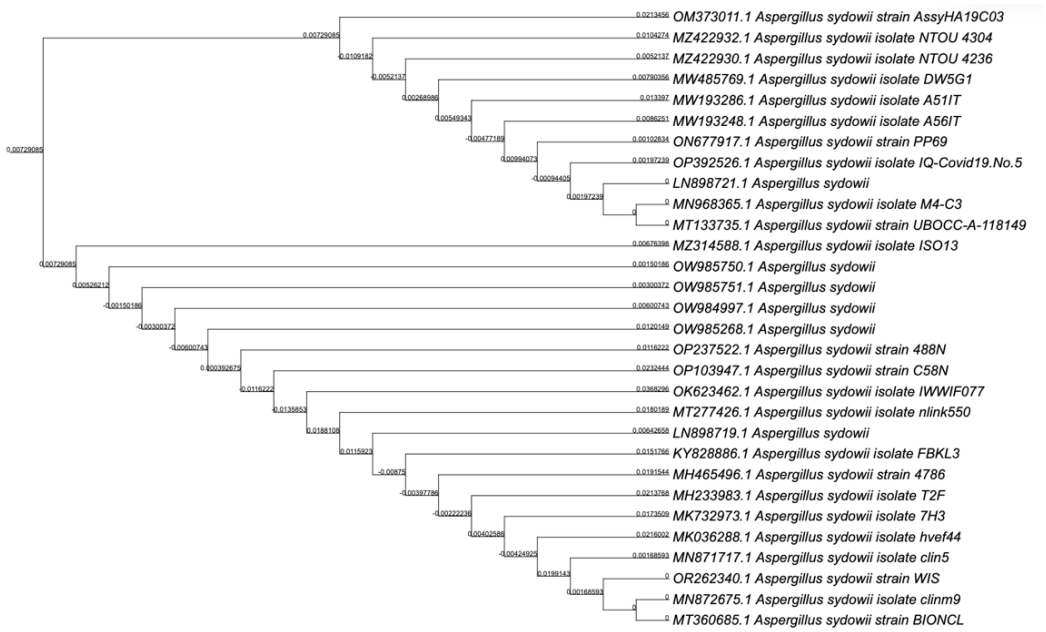


Fig. 4 **Phylogenetic tree showing the position of *Aspergillus sydowii* strain WIS (with acc. no. OR262340.1) and the closest related strains based on (ITS) region sequences.**

Assessment of blockage of hERG K⁺ channels

The potential toxicity of the identified metabolites was assessed using the Pred-hERG 5.0 web server (http://predherg.labmol.com.br/), which predicts the cardiotoxicity of compounds by evaluating the blockage of hERG K^+ channels. The results were presented as probability predictions, distinguishing between blocker and non-blocker for each molecule.[21]

Results

Macroscopic Results of Isolates

Conidia morphology-based characterization is used in fungi taxonomy.[22]

Unquestionably, SEM offers a more precise method for studying conidial ornamentation, enabling a more precise and truthful differentiation between the various types of ornamentation. As the isolate belonged to *Aspergillus* species, similarity of its conidia's to *Aspergillus* species conidia was obvious under SEM (Figure 3).

Molecular identification of Fungal Strains

Based on molecular identification, there were found that the selected isolates WIS belonged to o with 100% identity and 100% coverage as revealed by BLAST. The strain was named WIS and given the accession no. OR262340.1. The phylogenetic tree indicated the occurrence of three clusters (Figure 4). *Aspergillus sydowii* strain WIS belonged to a cluster together with thirty strains of *A. sydowii*. The closest two clusters also included *A. sydowii* strains.

Table 1. **Antibacterial activity using fungi *Aspergillus sydowii* growth against pathogenic bacteria evaluated by the inhibition zone diameter in mm**

Growth condition	Gram-negative *E. coli*	Gram-positive *S. aureus*
Sabouraud dextrose agar	10.9 ± 0.1	30.9 ± 0.1
Nutrient agar	23.5 ± 0.5	29.8 ± 0.2
Blood agar	11.7 ± 0.2	9.5 ± 0.5

Mean significant at $P < 0.05$. No effect (NE).

Antimicrobial Assay

The variable degree of antifungal activities against pathogenic bacteria showed (Table 1 and Figure 5). *Aspergillus sydowii* against Gram-Positive bacteria *S. aureus* showed inhibition zone on three types of media, the maximum inhibition zone on Sabouraud dextrose agar about 30.9 mm while the minimum inhibition zone on Blood agar is 9.5 mm. *Aspergillus sydowii* showed the maximum inhibition zone on Nutrient agar about 23.5 mm.

Estimation and Analysis of Cell Viability

Cells were exposed to various drug concentrations ranging from 0 to 1000 µg/ml to evaluate the anti-cancer potential of the extracts. The MTT assay was used to determine cell viability after a 48-h incubation period. In addition, to evaluate growth inhibition, Mitoxantrone, and Doxorubicin were used as positive controls. We chose six different cell lines ranging from Breast Cancer, Hepatic cancer, and colorectal cancer to diversify our findings. These cell lines are unique and have distinct genomic and proteomic landscapes. Moreover, newly developed Saudi-specific KAIMRC1, $ER^+/PR^+/HER2^-$, and KAIMRC2 triple-negative cell lines were used to target the local population. The extract of *Aspergillus sydowii* showed excellent anti-cancer activity on all the cell lines, Data is summarized in (Table 2) and graphs are shown in (Figure 6).

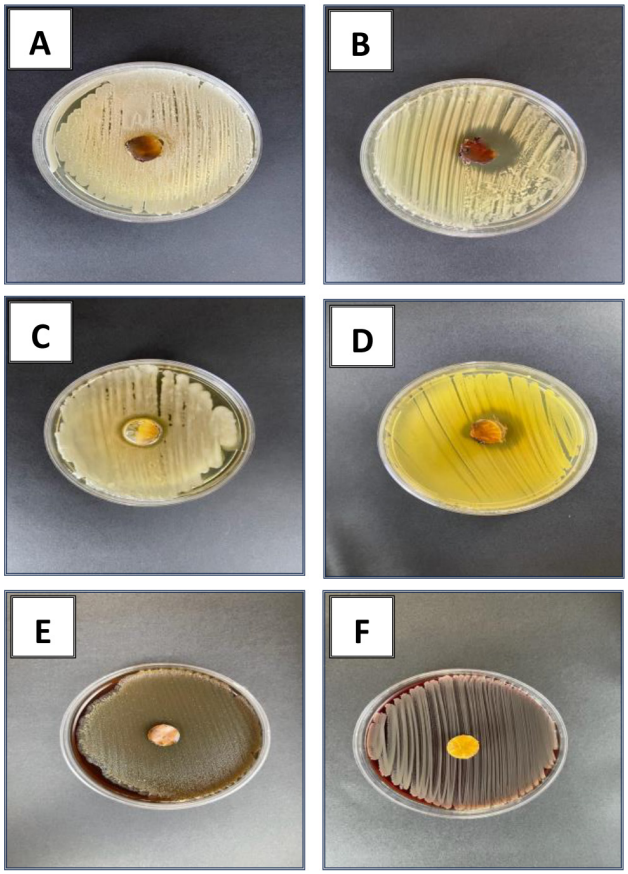


Fig. 5 **Antimicrobial activity against Gram-positive bacteria ($^+$ve) *Staphylococcus aureus* (*S. aureus*) and Gram-negative bacteria ($^-$ve) *Escherichia coli* (*E. coli*) of *Aspergillus sydowii* on three different media, Sabouraud dextrose agar (SDA), Nutrient agar (NA) and Blood agar (BA). A ($^-$ve on SDA); B ($^+$ve on SDA); C ($^-$ve on NA); D ($^+$ve on SDA); E ($^-$ve on BA) and F ($^+$ve on BA).**

Identification of Herbal Medicine Metabolites using QTOF-LCMS

LCMS of Aspergillus sydowii

The tentatively identified compounds are Diorcinolic acid (Takenaka et al. 2003), β-d-glucopyranosyl Aspergillusene A (Tullberg, Grøtli, and Luthman 2006), Violaceol II (Song et al. 2013), Cordyol C (Bunyapaiboonsri et al. 2007), yclo-(L-Phe-L-Trp) (Huang et al. 2014), 7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone (Huang et al. 2014), n-acetylserotonin glucuronide (Wu et al. 2016), Shornephine A methyl ester (Orfali et al. 2021), 1-methylpyrogallol (Orfali et al. 2021) Means m/z implies measured m/z (Table 3) (Figure 1).[23-29]

Results for In-silico Predictions

Anti-Cancer Activity Predictions

The computed anti-cancer activity of the identified metabolites was assessed using the PassOnline web server, a powerful tool for predicting the potential anti-cancer properties of compounds. This analysis aimed to determine the therapeutic benefits of the identified metabolites and their potential for developing new anti-cancer drugs from natural sources. The results, summarized in Table 4, revealed that only one metabolite, the 1-Methylpyrogallol, exhibited significant anti-cancer activity from *Aspergillus Sydowii*, indicated by a P_a (Probability of Activity) value of 0.854., suggesting that this fungus could be a potential source for discovering highly active anti-cancer agents.

Pharmacokinetic Parameters Assessment

In this section, we present the findings obtained from the utilization of the SwissADME online web server, a powerful tool for evaluating the pharmacokinetic properties of the identified metabolites. Through this comprehensive analysis, we were able to assess various pharmacokinetic parameters and determine the likelihood of the metabolites being orally active. Table 5 illustrates the results of this analysis, where most of the metabolites demonstrated pharmacokinetic properties within the recommended range for lipophilicity, molecular weight, solubility, insolubility, insaturation, and flexibility. Notable metabolites exhibiting favorable properties include β-d-glucopyranosyl Aspergillusene A,

Table 2. **The IC50 (µg\ml) values of different extracts against six cancer cell lines using MTT cell viability assay**

#	Extracts	IC_{50} (µg/ml)											
		KAIMRC1		KAIMRC2		MDA MB 231		HEPG2		HCT8		HCT116	
		IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2
1	*Aspergillus sydowii*	48.56	0.81	68.33	0.72	27.38	0.85	121.7	0.93	134.5	0.73	55.25	0.74
2	Doxorubicin	0.1234	0.9	3.102	0.92	0.7421	0.9	57.33	0.73	0.6473	0.9	8.134	0.92
3	Mitoxantrone	0.09807	0.94	0.05587	0.83	0.1726	0.91	0.491	0.94	0.1389	0.91	0.443	0.9

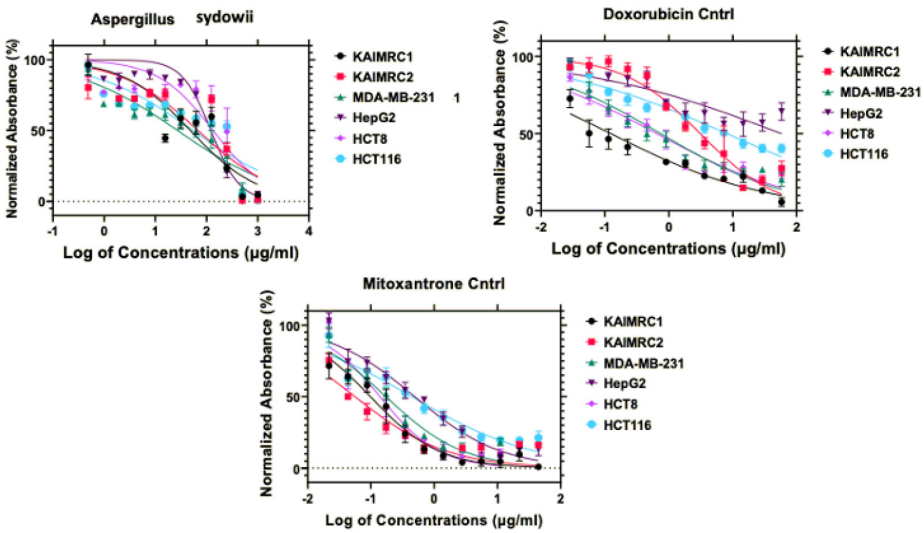


Fig. 6 **MTT assay on six cell lines. The extract of *Aspergillus sydowii*, along with Doxorubicin and Mitoxantrone were used. X-axis shows the log of concentrations of the extracts in µg/ml and Y-axis shows Normalized absorbance in %.**

Table 3. **Compounds (M1–M9) tentatively identified from of *Anastatica hierochuntica***

Peak no.	Rt (min)	$[M+H]^+$	$[M-H]^-$	Err PPM	Molecular formula	Tentative identification	Literature review of the compounds
Peak A (M1)	(17.128–17.474)	318.0739	317.0643	–0.50	$C_{16}H_{14}O_7$	Diorcinolic acid	(Takenaka et al. 2003)[24]
Peak B (M2)	(18.375–18.806)	380.4750	379.3817	– 0.45	$C_{21}H_{32}O_6$	β-d-glucopyranosyl Aspergillusene A	(Tullberg et al. 2006)[25]
Peak C (M3)	(25.048–25.765)	262.2671	261.2557	–0.60	$C_{14}H_{14}O_5$	Violaceol II	(Song et al. 2013)[26]
Peak D (M4)	(26.058–26.257)	246.2680	245.2571	–0.55	$C_{14}H_{14}O_4$	Cordyol C	(Bunyapaiboonsri et al. 2007)[27]
Peak E (M5)	(26.432–26.785)	333.4890	332.2977	–0.45	$C_{20}H_{19}N_3O_2$	Cyclo-(L-Phe-L-Trp)	(Huang et al. 2014)[28]
Peak F (M6)	(29.484–29.650)	234.0892	233.0877	–0.35	$C_{13}H_{14}O_4$	7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone	(Huang et al. 2014)[28]
Peak G (M7)	(29.754–29.821)	394.4572	393.9232	–0.30	$C_{18}H_{22}N_2O_8$	n-acetylserotonin glucuronide	(Wu et al. 2016)[29]
Peak H (M8)	(32.004–32.368)	466.5576	465.4931	–0.60	$C_{26}H_{30}N_2O_6$	Shornephine A methyl ester	(Orfali et al. 2021)[30]
Peak I (M9)	(33.225–33.761)	142.1521	141.6544	–0.50	$C_7H_{10}O_3$	1-methylpyrogallol	(Orfali et al. 2021)[30]

Table 4. **The predicted probabilities of anti-cancer activity for the identified metabolites in *Aspergillus sydowii*, *Aspergillus flavus*, and *Aspergillus niger* fungi samples**

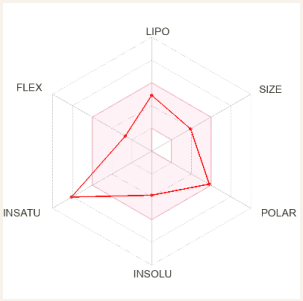
Anti-cancer activity for metabolites	P_a	P_i
A) *Aspergillus Sydowii*		
1. Diorcinolic acid	0.398	0.031
2. β-d-glucopyranosyl Aspergillusene A	0.691	0.028
3. Violaceol II	0.661	0.004
4. Cordyol C	0.458	0.023
5. Cyclo-(L-Phe-L-Trp)	0.483	0.007
6. 7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone	0.553	0.056
7. N-acetylserotonin glucuronide	0.563	0.015
8. Shornephine A methyl ester	0.393	0.016
9. 1-Methylpyrogallol	0.854	0.006

7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone, 1-methylpyrogallol. However, some of the remaining metabolites displayed violations in one or more parameters. Further analysis and refinement may be necessary to optimize their pharmacokinetic properties for potential use as anti-cancer agents. Overall, these findings contribute valuable information to the development of new orally active anti-cancer drugs sourced from natural compounds.

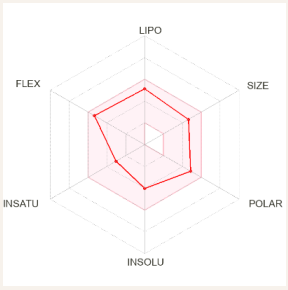
Toxicity Assessment

Multiple toxicity parameters for the identified metabolites were thoroughly assessed to ensure their safety and suitability for anti-cancer applications. A comprehensive evaluation of toxicity was conducted, and the results are summarized in Table 6. None of the metabolites analyzed, exhibited a predicted hepatotoxicity. Likewise, none of the metabolites were found to possess a carcinogenicity. Immunotoxicity analysis revealed that two metabolites, n-acetylserotonin, and

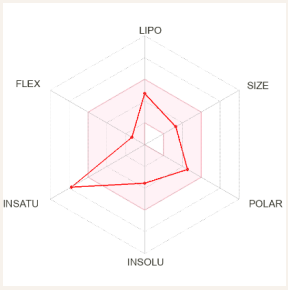
Table 5. **Pharmacokinetic properties for the identified metabolites using swissADME webserver**



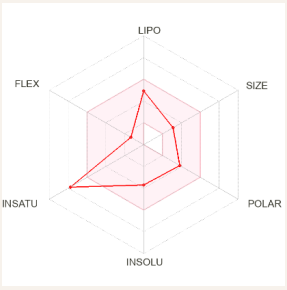
Diorcinolic acid



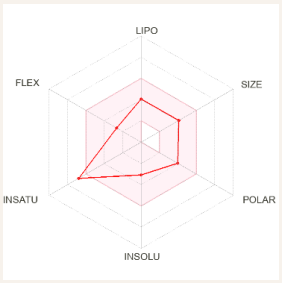
β-d-glucopyranosyl Aspergillusene A



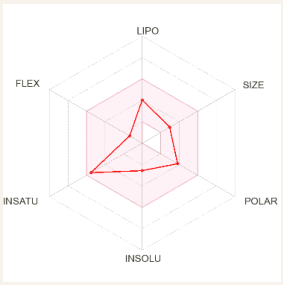
Violaceol II



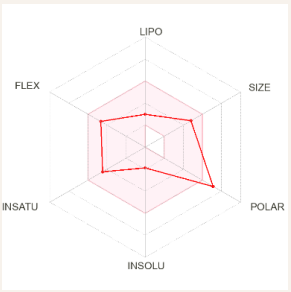
Cordyol C



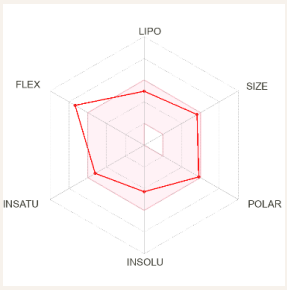
Cyclo-(L-Phe-L-Trp)



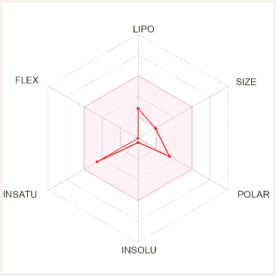
7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone



n-acetylserotonin glucuronide



Shornephine A methyl ester



1-methylpyrogallol

Table 6. **Comprehensive toxicity assessment of the identified metabolites using Protox II webserver**

Metabolite number	Classification				
	Organ toxicity (% Probability)	Toxicity endpoint (% Probability)			
	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
	Aspergillus sydowii				
Diorcinolic acid	Inactive (0.56)	Inactive (0.82)	Inactive (0.98)	Inactive (0.79)	Inactive (0.89)
β-d-glucopyranosyl Aspergillusene A	Inactive (0.89)	Inactive (0.72)	Active (0.88)	Inactive (0.82)	Inactive (0.85)
Violaceol II	Inactive (0.71)	Inactive (0.65)	Inactive (0.96)	Inactive (0.71)	Inactive (0.94)
Cordyol C	Inactive (0.72)	Inactive (0.59)	Inactive (0.95)	Inactive (0.72)	Inactive (0.95)
Cyclo-(L-Phe-L-Trp)	Inactive (0.66)	Inactive (0.66)	Inactive (0.98)	Inactive (0.71)	Inactive (0.93)
7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone	Inactive (0.66)	Inactive (0.57)	Inactive (0.97)	Active (0.51)	Inactive (0.85)
n-acetylserotonin glucuronide	Inactive (0.82)	Inactive (0.63)	Active (0.52)	Inactive (0.66)	Inactive (0.64)
Shornephine A methyl ester	Inactive (0.61)	Inactive (0.63)	Inactive (0.99)	Inactive (0.65)	Inactive (0.59)
1-methylpyrogallol	Inactive (0.81)	Inactive (0.58)	Active (0.74)	Inactive (0.61)	Inactive (0.73)

β-d-glucopyranosyl Aspergillusene A, exhibited significant activity, with a probability range of 0.52 to 0.88. Furthermore, only one metabolite (7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone) displayed mutagenic potential, while none of the metabolites were found to be cytotoxic. These findings provide valuable insights into the safety profile of the identified metabolites for further development as anti-cancer agents.

Prediction of Cardiac Toxicity

To ensure a comprehensive toxicity assessment of the identified metabolites, it was crucial to evaluate their potential to block the hERG channel, which is known to cause cardiac toxicity. In addition to evaluating organ and endpoint toxicity, a cardiac toxicity assessment was conducted using a pred-hERG web server. This analysis aimed to assess the metabolites' ability to inhibit the hERG cardiac potassium channel and potentially lead to adverse cardiovascular effects. Remarkably, all of the metabolites showed no signs of cardiac toxicity (Table 7).

Discussion

The presence of *Aspergillus* species in high-salt soil has been previously investigated. However, it was hypothesized that adding a protein source to the soil could reveal other fungal species. This study aimed to test this hypothesis by replicating the methodology used in the previous study, but with the inclusion of a protein source. The fungal isolate obtained from the soil was identified as *Aspergillus sydowii* based on the sequencing of the ribosomal DNA (ITS) region, which supported the previous hypothesis.

The results of the anti-bacterial tests showed that *Aspergillus sydowii* exhibited antibacterial activity against both gram-positive and gram-negative bacteria. Additionally, the isolate was tested for its anti-cancer activity on six different cell lines, including breast cancer, hepatic cancer, and colorectal cancer. The results revealed that *Aspergillus sydowii* demonstrated excellent anti-cancer activity on all tested cell lines.

Table 7. **Evaluation of Cardiac Toxicity in the Identified Metabolites using the pred-hERG Webserver**

Metabolite name	Activity on hERG channel	Confiability %
Aspergillus sydowii		
1. Diorcinolic acid	Non-blocker	93.5
2. β-d-glucopyranosyl Aspergillusene A	Non-blocker	70.0
3. Violaceol II	Non-blocker	69.49
4. Cordyol C	Non-blocker	64.7
5. Cyclo-(L-Phe-L-Trp)	Non-blocker	91.86
6. 7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone	Non-blocker	92.18
7. n-acetylserotonin glucuronide	Non-blocker	60.69
8. Shornephine A methyl ester	Non-blocker	68.08
9. 1-methylpyrogallol	Non-blocker	99.66

These findings suggest that *Aspergillus* sydowii, has potential as a source of antibacterial and anti-cancer agents. Further research is needed to explore the specific compounds responsible for these activities and their potential applications in medicine and agriculture.

Regarding the secondary metabolite in *Aspergillus sydowii*

Peak A (M1) the appeared m/z value at retention time (17.128–17.474) minutes, with $[M+H]^+$ m/z in positive mode 318.0739 with $[M-H]^-$ m/z in nigative mode 317.0643 daltons and molecular formula of $C_{16}H_{14}O_7$ were correlated with the parent compound Diorcinolic acid.[23]

Peak B (M2) the appeared m/z value at retention time (18.375–18.806) minutes, with $[M+H]^+$ m/z in positive mode 380.4750 with $[M-H]^-$ m/z in negative mode 379.3817 Daltons and molecular formula of $C_{21}H_{32}O_6$ were correlated with the parent compound β-d-glucopyranosyl Aspergillusene A.[24]

Peak C (M3) the appeared m/z value at retention time (25.048–25.765) minutes, with $[M+H]^+$ m/z in positive mode 262.2671 with $[M-H]^-$ m/z in negative mode 261.2557 Daltons and molecular formula of $C_{14}H_{14}O_5$ were correlated with the parent compound Violaceol II.[25]

Peak D (M4) the appeared m/z value at retention time (26.058–26.257) minutes, with $[M+H]^+$ m/z in positive mode 246.2680 with $[M-H]^-$ m/z in negative mode 245.2571 Daltons and molecular formula of $C_{14}H_{14}O_4$ were correlated with the parent compound Cordyol C.[26]

Peak E (M5) the appeared m/z value at retention time (26.432–26.785) minutes, with $[M+H]^+$ m/z in positive mode 333.4890 with $[M-H]^-$ m/z in negative mode 332.2977 Daltons and molecular formula of $C_{20}H_{19}N_3O_2$ were correlated with the parent compound cyclo-(L-Phe-L-Trp).[27]

Peak F (M6) the appeared m/z value at retention time (29.484–29.650) minutes, with $[M+H]^+$ m/z in positive mode 234.0892 with $[M-H]^-$ m/z in negative mode 233.0877 Daltons and molecular formula of $C_{13}H_{14}O_4$ were correlated with the parent compound 7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone.[27]

Peak G (M7) the appeared m/z value at retention time (29.754–29.821) minutes, with $[M+H]^+$ m/z in positive mode 394.4572 with $[M-H]^-$ m/z in negative mode 393.9232 Daltons and molecular formula of $C_{18}H_{22}N_2O_8$ were correlated with the parent compound n-acetylserotonin glucuronide.[28]

Peak H (M8) the appeared m/z value at retention time (32.004–32.368) minutes, with $[M+H]^+$ m/z in positive mode 466.5576 with $[M-H]^-$ m/z in negative mode 465.4931 Daltons and molecular formula of $C_{26}H_{30}N_2O_6$ were correlated with the parent compound Shornephine A methyl ester.[29]

Peak I (M9) the appeared m/z value at retention time (33.225–33.761) minutes, with $[M+H]^+$ m/z in positive mode 142.1521 with $[M-H]^-$ m/z in negative mode 141.6544 Daltons and molecular formula of $C_7H_{10}O_3$ were correlated with the parent compound 1-methylpyrogallol.[29]

Discussion for the Bioinformatic Tests in the Study

The results of the in-silico predictions provide valuable insights into the potential of identified metabolites as anti-cancer agents. The analysis conducted using the PassOnline

web server revealed that 1-Methylpyrogallol from *Aspergillus Sydowii* exhibited significant anti-cancer activity, indicating that this fungus could be a promising source for discovering highly active anti-cancer agents.[30]

The pharmacokinetic properties of the identified metabolites were assessed using the SwissADME online web server. Most of the metabolites displayed favorable properties within the recommended range for lipophilicity, molecular weight, solubility, insaturation, and flexibility. Notable metabolites with favorable properties include β-d-glucopyranosyl Aspergillusene A, 7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone, and 1-methylpyrogallol.[30]

Toxicity assessment was conducted to ensure the safety and suitability of the identified metabolites for anti-cancer applications. None of the metabolites showed predicted hepatotoxicity or carcinogenicity. Immunotoxicity analysis revealed significant activity for n-acetylserotonin and β-d-glucopyranosyl Aspergillusene A. Only one metabolite, 7-hydroxy-2-(2-hydroxypropyl)-5-methyl chromone, displayed mutagenic potential, while none were found to be cytotoxic. These findings provide valuable insights into the safety profile of the identified metabolites.[31]

Furthermore, a cardiac toxicity assessment was conducted using the pred-hERG web server to evaluate the metabolites' potential to inhibit the hERG cardiac potassium channel. Remarkably, all of the metabolites showed no signs of cardiac toxicity.[30]

Overall, these results contribute valuable information to the development of new orally active anti-cancer drugs sourced from natural compounds. The identified metabolites with significant anti-cancer activity, favorable pharmacokinetic properties, and low toxicity offer promising potential for further research and optimization as anti-cancer agents.[31]

List of Abbreviations

Items	Meaning
PCR	Polymerase Chain Reaction
AMR	Antimicrobial Resistance
EC	Electrical Conductivity
SEM	Scanning Electron Microscopy
WIS	Women In Science
ITS	Internal Transcribed Spacer
SDA	Sabouraud Dextrose Agar
NA	Nutrient Agar
BA	Blood Agar
KAIMRC	King Abdullah International Medical Research Center
LCMS	Liquid Chromatography–Mass Spectrometry
BGI	Beijing Genomic Institute
DMSO	Dimethyl Sulfoxide
MFE	Molecular Features Extraction
TIC	Total Ion Current Spectra

Supplementary Materials

Not applicable.

Author Contributions

Conceptualization, S.G., W.A., R. S and B.S.; methodology, S.G.,W.A,R. S, and B. S; software, S.G.,W.A,R. S and B. S; validation, S.G.,W.A,R. S, and B. S; formal analysis, S.G.,W.A,R. S, and B. S; investigation, S.G.,W.A,R. S, and B. S; resources, S.G., W.A., R. S, and B. S; data curation, S.G.,W.A,R. S, and B. S, writing—original draft preparation, S.G., W.A., R. S, and B. S; writing—review and editing B. S; visualization, B. S; supervision, B. S; project administration B. S; funding acquisition, B. S All authors have read and agreed to the published version of the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

Institutional Review Board Statement

Not applicable.

Data Availability

The data presented in this study are available on request from the corresponding author.

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Principal Investigator

None.

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