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Original Research Article

Evaluation of Cytotoxicity and Antimicrobial Activity of Flavipin Against *Acinetobacter baumannii*, Methicillin-Resistant *Staphylococcus aureus*, and *Candida albicans*

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ABSTRACT

Flavipin, a metabolite produced by *Aspergillus* species is a potent antibiotic, antifungal, anticancer, and nematocidal agent. The present study was conducted to investigate the cytotoxicity and antimicrobial activity of flavipin produced by *Aspergillus flavus*. Eighty isolates of *Aspergillus* species were screened for the presence of flavipin. Macroscopic, microscopic, and molecular characterizations were performed to identify the *Aspergillus* species. Flavipin extract was prepared, fractionated, and the concentration was determined in the selected isolates. The antimicrobial activity of flavipin was tested against *Candida albicans*, *Acinetobacter baumannii*, and methicillin-resistant *Staphylococcus aureus* (MRSA). The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) were determined. A cytotoxicity test of flavipin was conducted on MCF-7 and WRL-68 cell lines. The results of the antibacterial activity of flavipin against MRSA and *A. baumannii* showed a wide zone of inhibition of 35 and 30 mm, respectively. Flavipin exhibited the highest antifungal activity against *C. albicans*, with inhibition zones of 44 mm in diameter. The MICs of flavipin for both *A. baumannii* and MRSA were 31.2 µg/mL, while the MIC of flavipin for *C. albicans* was 62.5 µg/mL. The MBC values of flavipin for MRSA and *A. baumannii* were 62.5 and 125 µg/mL, respectively, while the MFC of flavipin against *C. albicans* was 125 µg/mL. The IC_{50} of flavipin was 52.34 µg/mL for the cancer cell line MCF-7 and 298.1 µg/mL for the normal cell line WRL-68. The findings of the study revealed that flavipin produced by *Aspergillus flavus* has antimicrobial and cytotoxic properties.

***Keywords*:** *Acinetobacter baumannii*, Antimicrobial activity, *Aspergillus flavus*, *Candida albicans*, Flavipin, IC_{50}, Methicillin-resistant *Staphylococcus aureus*.

Introduction

Bioactive compounds are primarily responsible for the activity patterns of endophytic fungi. Also, bioactive compounds exhibit a wide range of biological actions in their pure form, including antifungal, antibacterial, anticancer, antioxidant, antiviral, anti-inflammatory, anti-parasitic, and immunosuppressive characteristics.[1] Flavipin produced by *Aspergillus terreus*, *Aspergillus flavus*, *and Epicoccum nigrum* has a potent antimicrobial activity. It has been shown that flavipin had a positive effect on the biological control of *Monilinia* spp. Consequently, flavipin in addition to its anti-fungal action, also demonstrated antagonistic action against numerous phytopathogenic bacteria.[2] Flavipin showed high antimicrobial activity against MRSA and *Staphylococcus aureus*.[3] It appeared to be very powerful against some *Chlorella* species, while it has a mild inhibitory effect against *Bacillus megaterium*.

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Flavipin has been reported to have phytotoxic properties.[4] It has been found that flavipin inhibited the germination of *Botrytis allii* conidia,[5] the oogonia of *Pythium intermedium*, the zoospores of *Phytophthora cinnamomi*, and the mycelial growth of *Pythium* species, as well as *Phytophthora* species.[6] Furthermore, it inhibited the growth of various fungi, including *Monilinia laxa* and *Saccharomyces cerevisiae*.[7] The toxicity of conventional drugs is a constant issue in successful chemotherapies. As a result, developing medications with potent anticancer activities while being less hazardous is a top priority. Flavipin inhibits cancer cell motility, suggesting that it might be used to reduce cell invasiveness in patients with breast cancer.[8] The present study was aimed at evaluating the cytotoxicity and antimicrobial activity of flavipin extracted from *Aspergillus* species.

Materials and Methods

Collection of bacterial and fungal isolates

The collection of fungal isolates took place from October 2021 to January 2022. These isolates were obtained from different sources, such as soil, water, air, plants (endophytic fungi), and some laboratories at Maysan University and the Central Health Department. Eighty isolates of *Aspergillus* were used in this study to investigate the presence of flavipin (a secondary metabolite). *Acinetobacter baumannii* and MRSA were used in this study. These bacteria were obtained and identified in the medical microbiology laboratory at the College of Medicine, Al-Nahrain University, Iraq. Also, *Candida albicans* from clinical samples were previously isolated, identified, and deposited in the medical mycology laboratory at the College of Medicine, Al-Nahrain University, Iraq. The study was conducted from October 2021 to June 2022.

Identification of Aspergillus species

The growth morphology of colonies and microscopic characterization were evaluated daily according to standard laboratory procedures to identify the *Aspergillus species*.[9,10,11] For the molecular identification of producer isolates, DNA was isolated from fungi using a DNA extraction kit (Presto™ Mini gDNA Yeast Kit, Genaid, USA) following the manufacturer's instructions. The identification procedure was carried out according to the method described by Alshehri (2020).[11] Universal primers for ITS1 and ITS2 were used for molecular identification. The NCBI/BLAST search program was employed to find the most closely related sequences in the GenBank database.

Preparation and fractionation of flavipin extract

Fungal isolates were cultured on a potato dextrose agar PDA (200 g of potato, 20 g of dextrose, 20 g of agar/ 1L of H_2O) for 5 to 10 days at 25 °C. Eight pieces (5 mm in diameter) of mycelial agar plugs were obtained from the edge of early endophytic fungal cultures and inoculated into each flask (250 mL Erlenmeyer flask) with 100 mL of potato dextrose broth (PDB). The culture was incubated on a mechanical shaker (150 rpm) at 25 ±1 °C for 12 days. To separate the culture broth and mycelia, the broth culture was filtered. The culture broth was extracted three times with 1 L of ethyl acetate. A rotary evaporator was used to mix and dry the extracted ethyl acetate. All extracts were stored at 4°C until analysis. The purification of flavipin was achieved using silica gel column chromatography. The 25 g of silica (60-120 mesh) was activated in a 90–120ºC oven for 24 hours before being suspended in 25 mL of methanol and packed firmly onto the column chromatography (36 x 1.5 cm size) without air bubbles. The extract of flavipin was concentrated at room temperature for 24 hours before being placed on silica gel column chromatography using ethyl acetate and dichloromethane (v/v, 1:1) as solvents. The crude extract was eluted with ethyl acetate and dichloromethane at a rate of 20 mL/hour, and four fractions were obtained.

Determination of flavipin concentration from selected isolates

Flavipin concentration was determined using the standard curve method as described by Ye *et al.* (2013).[12] The standard curves were created by serially diluting flavipin (0, 20, 40, 60, 80, 100, and 120 µg/ml). A UV-visible spectrophotometer was used to estimate its optical density at 264 nm. A standard curve was plotted by graphing absorbency vs. standard concentration. The unknown flavipin concentrations in samples were determined using the equation: Y = 0.032X, where Y is the absorbance or optical density obtained with a UV-visible spectrophotometer and X is the flavipin concentration.[12]

Preparation of the flavipin concentrations for antimicrobial tests

For antibacterial and antifungal tests, a 500 µg/ml stock solution was first dissolved into DMSO and then made up with either Muller Hinton or Sabouraud Dextrose Broth, respectively. The stock was also prepared in twofold serial dilutions (250, 125, 62.5, 31.2, 15.6, and 7.8 µg/mL) according to the Clinical and Laboratory Standard Institute (CLSI),[13] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[14] The minimum inhibitory concentrations (MIC) were calculated as the lowest concentrations of flavipin at which no bacterial or fungal growth was visible in broth serial dilution, or, in the case of the resazurin broth test, at which the color changed from blue to pink.[15]

Inoculum preparation of Candida albicans, Acinetobacter baumannii, and MRSA

At least 2-3 colonies of *C. albicans* were inoculated into 3 ml of normal saline (0.85%) after 24 hours of SDA plate culture. The turbidity was adjusted to 3 x 10^8 CFU/ml using a 1.0 Macfarland tube. A few colonies of *A. baumannii* and MRSA were transferred from overnight nutrient agar plates to 5 ml of normal saline (0.85%). The turbidity was measured at 1.5 x 10^8 CFU/mL, which was equal to 0.5 Macfarland solution.[16]

Determination of the antimicrobial activity of flavipin

The antimicrobial effect of flavipin on *albicans*,[17] *A. baumannii*, and MRSA was evaluated using the agar disk diffusion and the agar well diffusion methods. *Candida albicans* was activated on SDA, while *A. baumannii* and MRSA were activated on nutrient agar plates. The cultures were incubated at 37°C for 48 and 24 hours, respectively. The microbial inoculum was then added to the sterile Müller-Hinton agar surface plates with a sterile cotton swab. A sterilized cork borer was used to puncture 5 mm wells into Müller-Hinton agar plates. Each well received 50 mL of flavipin with a concentration of 50 µg/ml, while distilled water was used as a negative control. The plates were then incubated for 48 and 24 hours at 37°C, respectively. For *C. albicans*, amphotericin B (20 µg), ketoconazole (10 µg), fluconazole (10 µg), and nystatin (100 units) were selected as positive controls.[18] The positive controls for *A. baumannii* and MRSA were cefixime (30 µg), ceftriaxone (30 µg), ticarcillin/clavulanic acid (75/10 µg/disk), and azithromycin (30 µg).[19]

Determination of the minimum inhibitory concentration (MIC)

Resazurin was acquired in the form of a powder and prepared in accordance with the manufacturer's instructions. It was dissolved in 50 mL of PBS (phosphate-buffered saline; pH 7.4) and vortexed. The test was performed using a final volume of 1:10 (15). Resazurin was filtered with a 0.22-micron filter and kept at 4 °C for up to 2 weeks.[20] For this test, 100 µL of bacterial suspension turbidity matching the McFarland 0.5 standard (1.5 x 10^8 organisms/mL) was administered to each selected well. The resazurin assay (resazurin microtiter plate assay, or REMA) was used to demonstrate the minimum inhibitory concentrations (MICs) of flavipin against *Acinetobacter baumannii*, MRSA, and *Candida albicans* by using a 96-well microtiter plate with lid, sterile distal water (100 µL) as a negative control was placed in the well in Row A and broth (100 µL) with an active bacteria as a positive control in row B (Figure 1). Six concentrations of flavipin (250, 125, 62.5, 31.2, 15.6, and 7.8 g/mL) were prepared, and 100 µgL was added to the wells in Rows C to E. Both fungus and bacteria were distributed equally in these Rows (C, D, and E). Each row of wells received 20 µL of the resazurin dye solution, which was then incubated at 37 °C overnight. The isolates had begun to grow when their blue color changed to pink. A similar approach was performed to estimate the MIC of flavipin against *C. albicans*, except that SD broth was used instead of Mueller-Hinton broth for the growth of the fungal isolate, and the density was equal to 1.0 Macfarlane tube adjusted at 3 x 10^8 CFU/ml. The MIC of the fungus and bacteria was established by adding 20 µL of resazurin to each well. For 2-4 hours, the reduction of resazurin was examined for a change in colour (from blue to pink). Resorufin, a pink-fluorescent dye, is produced when the non-fluorescent blue dye resazurin interacts with metabolically active cells.[21, 22]

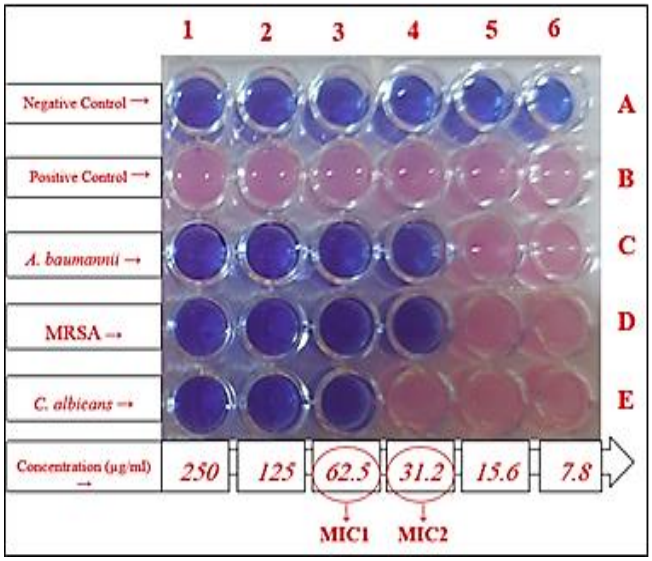

Figure 1: *Candida albicans* (column 3, row E), MIC2 represents the MIC of flavipin for both *Acinetobacter baumannii* (column 4, row C) and Methicillin-resistant *Staphylococcus aureus* (column 4, row D).

Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The MBC and MFC were obtained by sub-culturing the solutions of the wells from the MIC and above concentrations of the serial broth microdilution. The sample with no obvious growth was sub-cultured by uniformly distributing a suspension on a separate plate on an MHA medium for fungi and bacteria, then incubating them at 37° C for 24 hours. MBC and MFC were evaluated by sub-culturing wells on new agar plates with no colour change and incubating them at 37°C for 16 to 20 hours. The lowest dose that produced no observable growth after incubation was chosen as the MBC and MFC.[15,20,22,23]

Determination of flavipin cytotoxicity by using the MTT assay

The goal of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test is to determine viable cells at a reasonably high throughput (96-well plates) without the need for complex cell counting.[24] MCF-7 cells (breast cancer cell line) were cultured in 96-well plates. For 24 hours, the plates were incubated at 37°C with 5% CO_2. The medium was then removed, and the flavipin was treated in the wells at concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml). The production of formazan crystals, which resulted in a purple tint, demonstrated the presence of living cells. The sample was transferred to a spectrophotometer cuvette, and DMSO was used as a blank to measure the OD readings at 569 nm. These readings were analyzed statistically, and the data were obtained by averaging three experiments. Every procedure that was applied to cancer cells (MCF-7 cell line) was also applied to non-mutagenic fetal hepatocytes (WRL-68 cell line). [25, 26] The viability percentage of cell lines was calculated according to the following formula:

Viability (%) = (optical density of sample/optical density of control) x 100

Statistical analysis

The statistical analysis applied in this study was performed using Microsoft Office Excel (2016) and the Statistical Package for Social Sciences (SPSS; version 21.0) software. This program was used to assess the impact of different research factors. Descriptive statistics were calculated using the mean and standard deviation (SD). An analysis of variance (ANOVA) was conducted, and a significance level of $p \leq 0.05$ was considered significant. The MTT test results and IC_{50} values were analyzed by the GraphPad Prism 8 program, utilizing the inhibitor against a normalized response technique.

Results and Discussion

Out of 80 isolates of *Aspergillus*, one isolate (*Aspergillus flavus*) was a producer of flavipin. A PDA medium was used to isolate desirable fungal colonies. Cultural and microscopic characteristics were used to identify these isolates. The colonies of *A. flavus* were olive to lime green in color and spread rapidly. This species was biseriate in general, with rough conidiophores and smooth conidia. The isolated compound from *Aspergillus flavus* showed significant antibiotic action against a variety of disease-causing microbes, raising severe public health issues.[27] The present study reveals that flavipin can be produced from *Aspergillus flavus*. The results of sequencing were achieved by using the Basic Local Alignment Search Tool (BLAST) program, which revealed that the sequence of the local isolate (*Aspergillus flavus*) was identical to *Aspergillus flavus* strain ND36 with 99.46% and accession number MG659630.1. This result was consistent with the findings of Lloyd in 1969, which demonstrated that flavipin can be produced by *Aspergillus flavus*.[28] The macroscopic and microscopic characteristics of the species were in agreement with the findings of different studies, such as Okayo *et al.* in 2020, Saif *et al.* in 2021, and Nazir *et al.* in 2020.[29,30,31]

For the molecular detection of *A. flavus*, partial sequencing of ITS PCR products using ITS1 and ITS4 universal primers was used. The BLAST analysis of the resultant partial nucleotide sequences was done using the National Center for Biotechnology Information (NCBI) database. The amplified PCR product for *A. flavus* was 565 bp. These results were in agreement with the findings of several studies,[11,32,33] which reported that the ITS amplicons for these *Aspergillus* species ranged in size from 565-613 bp. The sequence of *A. flavus* showed the highest sequence similarity with *A. flavus* strain ND36, and its "Expect value" (E-value) was 0.0. This strain (ND36) was submitted by Nleya *et al.* (2021).[34] Calibration curves were initially made by estimating the synthesis of flavipin using its serial dilutions (standard). These results agree with Ye *et al*.'s study in 2013, which used a standard curve to detect the production of flavipin.[12] A study by Madrigal *et al.* (1991) also used a standard curve to estimate the concentration of flavipin.[35]

Flavipin was found to be effective against a wide variety of plant-pathogenic fungi in many studies,[5,6,7,36,37,38] but, to the best of our knowledge, no specific study has been completed to assess the antimicrobial activity of flavipin against resistant *C. albicans* till date. Flavipin showed the strongest effective antifungal activity, with inhibition zones of 44 mm in diameter (Figure 2), while clotrimazole, miconazole, and econazole had the lowest antifungal activity, with inhibition zones of 10, 8, and 7 mm, respectively. These *C. albicans* isolates, on the other hand, were resistant to amphotericin-B (20 µg), fluconazole (10 µg), ketoconazole (10 µg), and nystatin (100 units). Flavipin's antibacterial effect against two bacterial isolates (MRSA and *Acinetobacter baumannii*) revealed a zone of inhibition against both isolates. The measurements were 35 and 30 mm, respectively (Figure 3). Positive controls included cefixime (30 µg), ceftriaxone (30 µg), azithromycin (30 µg), and ticarcillin/clavulanic acid (75/10 µg)/disk, while distilled water served as a negative control. The inhibition zones of ceftriaxone and cefixime against MRSA were within the range of resistance (≤19 mm) as shown in Figure 3A. The inhibition zone of ceftriaxone against *Acinetobacter baumannii* was hardly noticeable. CLSI classified these bacteria as resistant to ceftriaxone in the zone of inhibition of ≤13 mm (Figure 3B).[39] To the best of our knowledge, this is the first study that has been done to evaluate the antibiotic activity of flavipin, a substance produced by *A. favus*, against resistant *Candida albicans*, MRSA, and *A. baumanni*.

The colour produced by the test isolates was compared to the colour produced in the positive control wells (Row B). As shown in Figure 3, the blue-colored wells indicated that microbial growth was inhibited, while the pink-colored wells indicated that growth occurred. The results showed that the MICs of flavipin for *C. albicans* were 62.5 and 31.2 µg/mL for *Acinetobacter baumannii* and MRSA, respectively (Column 4, Rows C and D, respectively), while the MIC of flavipin for *C. albicans* was 62.5 µg/mL (Column 3, Row E). In the current study, the MIC value against MRSA was different from that found by Flewelling *et al.* in 2015, at which the MIC of flavipin against MRSA was 510 µg /mL.[3]

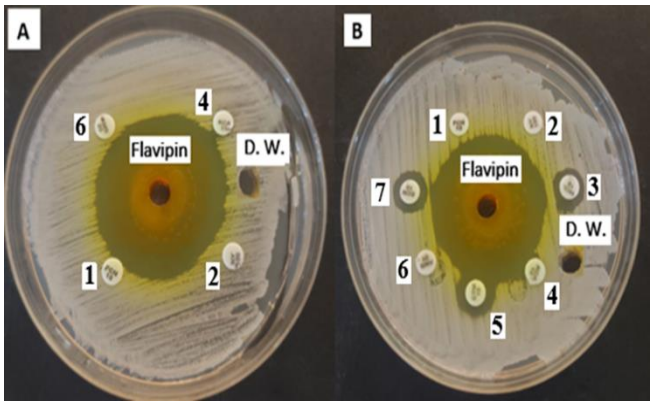

Figure 2: Antifungal activity of flavipin and other antimycotics.

A: the inhibition zone of flavipin observed in the middle of the agar plate and surrounded with antifungal discs as a positive control; D.W: Distilled water; B: Clear inhibition zone in a middle plate surrounded by other antifungal discs with some inhibition zones; 1: Fluconazole; 2: Nystatin: 3: Miconazole; 4: Ketoconazole; 5: Clotrimazole; 6: Amphotericin-B; 7: Econazole.

Table 1: Minimum bactericidal concentration (MBC) and minimum fungicidal concentration of flavipin against test organisms

Number of isolates	Isolate	Concentration (µg/mL)
1	*Acinetobacter baumannii*	125
1	Methicillin-Resistant *Staphylococcus aureus*	62.5
1	*Candida albicans*	125

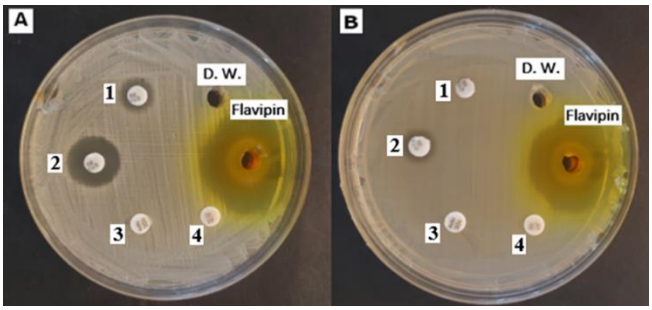

Figure 3: Antibacterial activity of flavipin.
A: Inhibition zone of flavipin against Methicillin-Resistant *Staphylococcus aureus* with 4 important antibiotic discs as a positive control and distilled water as a negative control; B: Flavipin inhibition zone against *Acinetobacter baumannii* with the same controls mentioned above; 1: Cefixime; 2: Ceftriaxone; 3: Ticarcillin/clavulanic acid; 4: Azithromycin

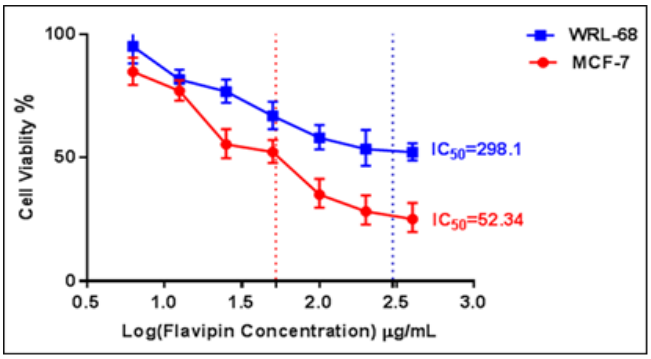

Figure 4: Cytotoxic effect of flavipin against MCF-7 and WRL-68 cell lines.

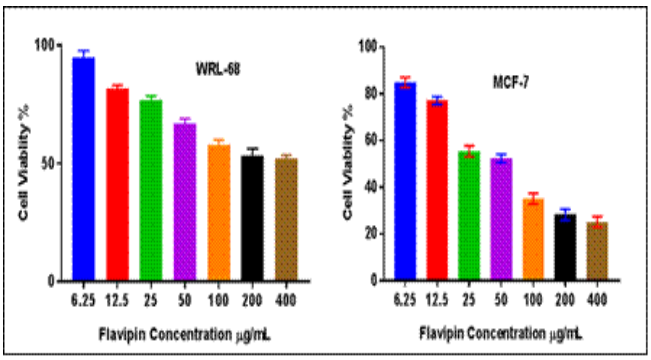

Figure 5: Cytotoxic effect of flavipin.
The concentrations of flavipin ranged from 6.25 to 400 µg/ml.

These variations might result from the different approaches used to determine the MIC of flavipin against the test isolate. In the present study, REMA was carried out to detect the MICs of flavipin for every selected isolate, including MRSA by using a 96-well microtiter plate. A micro broth dilution antibiotic susceptibility assay that was modified from McCulloch *et al*. (2011),[41] was used in the studies of Flewelling *et al*. (2015),[3] and Flewelling *et al*. (2013).[40] Harwoko *et al*. (2020) evaluated antibiotic activity against *Acinetobacter baumannii* using the broth microdilution technique according to the Clinical and Laboratory Standards Institute (CLSI)'s recommendations.[42,43] The growth inhibition zone (in mm) around the disk was determined, and the lowest concentration of the test substances that did not inhibit growth was termed the minimum inhibitory concentration (MIC).[43] As demonstrated in Table 1, the flavipin MBC values for MRSA and *A. baumannii* were 62.5 and 125 µg/mL, respectively, while the MFC of flavipin against *C. albicans* was 125 µg/mL. To the best of our knowledge, no studies that determined the MBC and MFC of flavipin against these two isolates of resistant bacteria and fungus have ever been reported. However, only a small number of studies have been done to assess the antibacterial activity of specific compounds against the above-mentioned pathogens. These compounds were either considered flavipin-derived alkaloids [43] or produced by the same fungi from which flavipin was isolated.[44] The most recent study reported that the MIC of this substance against *C. albicans* was 40 µg/mL.

According to Figure 4, the MCF-7 cell line's 50% inhibitory concentration (IC_{50}) was 52.34 µg/mL ($logIC_{50}$=1.719), but the IC_{50} for the WRL-68 cell line was 298.1 µg/mL (logIC50=2.474). Using the MTT assay, the cytotoxic activity of flavipin was investigated against two types of cell lines: a breast cancer cell line (MCF-7) and a non-mutagenic fetal hepatocyte (WRL-68) as a normal cell. The IC_{50} for the MCF-7 cell line was 52.34 µg /mL, while the IC_{50} for the WRL-68 cell line was 298.1 µg/mL (Figure 5). These findings are similar to the report of Kumar *et al.* (2019), who showed that flavipin suppressed the growth of MCF-7 cancer cells in a dose-dependent manner with an IC_{50} value of 54 µg/ml, but was less sensitive (IC50 = 78.89 µg/ml) against normal cell lines.[45] Figure 5 displays a dose-dependent pattern of cytotoxicity beginning from the lower concentration (6.25 µg/mL) to the high concentration level (400 µg/mL) of flavipin, where a strong inhibition was observed (52.24% in WRL 68 cell line, and 25.18% inhibition in MCF-7 cells). Flavipin cytotoxicity thus increased with increasing concentrations and showed the highest cytotoxic activity on cancer cell lines (MCF-7) compared with fetal hepatocyte cell lines (WRL-68). Hanieh *et al*.,[46] proved that flavipin inhibited the growth and motility of breast cancer cells. Epicorazine A (an alkaloid derived from flavipin) was highly cytotoxic to mouse lymphoma cell lines and moderately cytotoxic to leukemia and human lymphoma cell lines, according to research by Harwoko *et al*. (2019).[43] According to the study of Senthil *et al*. (2019), [45] flavipin was shown to increase caspase-3, 8, and 9 levels in malignant cells, demonstrating that apoptosis was caused by both intrinsic and extrinsic pathways. In light of the information that was readily available, it was absolutely necessary to determine whether or not Flavipin was effective against a wide variety of pathogenic bacteria. The usage of applied molecular techniques, such as PCR, It was implemented in many areas of medicine, [47-71] and as a result, it is highly suggested in order to achieve accurate outcomes pertaining to alternative antibiotics in the context of pathogenic microorganisms.

Conclusion

The findings of this study reveal that flavipin produced by locally isolated *A. flavus* has strong antimicrobial activity against *A. baumannii*, MRSA, and *C. albicans*. Also, it has a higher cytotoxic activity against cancer cell line compared to normal cell line. Further studies are therefore recommended on the antimicrobial activity of flavipin against medically important pathogens, such as multi-drug-resistant (MDR) and extensively drug-resistant (XDR) bacteria and fungi.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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