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Evaluation of Inhibitory Effect (Against Biofilm Forming Yeast and Cancer Cell Lines) of Two Novel Compounds Obtained Through Endophytic Fungal Transformation of Clotrimazole

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Abstract: Fifteen clotrimazole-resistant endophytic fungal cultures were screened for their ability to transform clotrimazole (antifungal and antitumor drug), only two cultures viz., *Macrophomina phasolina and Chaetomium globosum* transformed clotrimazole into two of its metabolites M1and M2 respectively basing on HPLC analysis. M1 and M2 were characterized using of mass spectrometry, infrared spectrometry and nuclear magnetic resonance spectrometry as 5, 5-diphenyl-5H-imidazole [2, 1-α] iso indole and 5-acetoxy-2-chloro-3-hydroxyphenyl) and (1H-imidazol-1-yl) methylene) bis (4, 1-phenylene) di-acetate respectively. Out of seventy two clotrimazole- resistant Candida isolates 45 (62.5%) were biofilm producers including the strongest biofilm forming isolate (C. *albicans*16). Treatment of clotrimazole resistant- C. *albicans*16 cells with M2 compound affected cell wall integrity, cell membrane permeability and metabolic activity as indicated using transmission electron, inverted-phase contrast and fluorescence microscopy with triple-staining method. M1 compound had higher antitumor potency against tumor mammalian cell lines: MCF-7 cells (breast cancer cell line) and HCT-116 (colon cancer cell line) than clotrimazole as indicated by the concentrations that inhibit cell survival by 50% (IC50). Treatment with M1 compound induced late stage of apoptosis in clotrimazole resistant-HepG2 (liver cancer cell line) cells, including cell shrinkage, chromatin condensation, presence of apoptotic bodies and fragmented nuclei as indicated using inverted- phase contrast and fluorescent microscopy. The potent activities of M1 and M2 against significant biofilm forming *C. albicans*16 and different tumor cell lines respectively were agreed with their structure activity relationship and did not accompanied with hemolytic activity against human erythrocyte.

Key words: Biotransformation · Endophytic fungi · Clotrimazole · Biofilm and antitumor

INTRODUCTION

Clotrimazole (Ctz) is a well known topical and intravaginal imidazole antifungal agent, it has been available for over 30 years and considered the prescribed treatment for candidiasis and that may be due to Ctz is cheap, more effective than nystatin and safer than other therapies such as amphotericin-B, fluconazole and flucytosine [1]. *Candida* species, the most common fungal pathogens of humans, is associated with high morbidity and mortality in immunocompromised patients [2]. Candida biofilms on implanted medical devices and host epithelial cell surfaces favor establishment of infection [3], biofilm related infections show different properties from those of planktonic cells, such as, increased resistance to antimicrobial agents especially to azole drug including clotrimazole, multiple drug resistance and tolerance to the host immune defenses, biofilms formed by *C. albicans* may be up to 2000 fold more resistant to antifungal drugs than the planktonic cells [4]. Although clotrimazole showed potentially useful antitumor activity which recognized as an inhibitor of glycolysis (The primary energy source in most cancer cells) as demonstrated by Penso and Beitner [5], there is a need to enhance the efficacy of potentially useful anticancer agents like Ctz and to develop new compounds capable of activity against tumor cells refractory to apoptosis since the latter property usually correlates with

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drug resistance [6]. Biotransformation is a useful technique to perform some modifications in the chemical structure of both active and inactive materials by using microorganisms or their enzymes, furthermore this technique is an important route for introducing chemical functions into inaccessible sites of molecules and there by to produce rare structures [7]. The biotransformation process provides a number of advantages over chemical synthesis, this process can be carried out under mild conditions like temperature and without the need of high pressure and extreme conditions, thus reducing undesired byproduct, energy needs and cost [8], a molecule can be modified by transforming functional groups, with or without degradation of carbon skeleton, such modifications result in the formation of novel and useful products not easily prepared by chemical methods [9]. Endophytic microorganisms are able to produce necessary enzymes for the colonization of plant tissues and to use, at least *in vitro*, most plant nutrients and components [10], therefore more recently, endophytes have received attention as biocatalysts in the transformation process of natural products and drugs, due to their ability to modify chemical structures with a high degree of stereospecificity and to produce known or novel enzymes that facilitates the production of compounds of interest. Although the high potential of these microorganisms, studies using endophytes in the field of biotransformation are still limited [11].

MATERIALS AND METHODS

Microorganisms: Fifteen endophytic fungal cultures were selected from The Regional Center for Mycology and Biotechnology (RCMBC) based on their resistance to clotrimazole at concentration (100 mg/l). The fungal cultures, namely: *Alternaria alternata, Auropasidium pullulanum, Bipolaris sp., Chaetomium globosum, Cladosporium herbarum, Dreschlera portulacae, Epicoccum nigrum, Fusarium equiseti, Macrophomina phasolina, Merothecium verrucaria*, *Mucor racemosus, Rhizopus stolonifer, Torula utilis, Trichoderma viride* and *Trichothecium roseum*. Stock cultures were maintained on potato dextrose agar slants at 4°C and subcultured for every 3 months.

Biotransformation Assay: Biotransformation was performed using two-stage fermentation protocol as demonstrated by Prasad *et al*. [12], in the first stage, fermentation was initiated by inoculating a 250 ml culture flask consists of 50 ml of liquid medium, the liquid medium used contain (per liter) glucose (20 g), peptone (5 g), yeast extract (5 g), K_2HPO_4 (5 g) and sodium chloride (5 g). The pH of the broth was adjusted to 6. The media was inoculated with a 5mm disc cut off culture obtained from freshly grown potato dextrose agar plates. The flasks were incubated at 120 rbm and 25°C for 48 h. Second stage cultures were initiated in the same media using an inoculum of 1 ml of first stage culture per 20 ml of medium in a 100-ml culture flask. The second stage cultures were incubated for 24 h and the substrate clotrimazole (obtained from SIGMA 23593-75-1) in dimethyl formamide was added to give a final concentration of 100 mg/l. Culture controls consisted of a fermentation blank in which the microorganisms was grown under identical conditions and no substrate was added. Substrate controls comprised of clotrimazole added to the sterile medium was incubated under similar conditions. Each culture was studied in triplicate. The cultures were extracted with three volumes of ethyl acetate; the combined organic extracts were evaporated.

High Performance Liquid Chromatography (HPLC): The resultant residues were examined by HPLC for detecting the presence of new metabolites (new peaks other than clotrimazole peak). The HPLC system consisted of GBC-LC1110 HPLC pump (Germany) equipped with GBC-LC1210 UV/VIS detector (Germany), a GBC-LC1445 injector (3.1) and a Chromatography data system software (Swinchrome-Germany) was used. Chromatographic separation was performed on 5 μm HPLC column (Kromacil- Germany). The mobile phase consisted of a mixture of acetonitrile and water (65:35, v/v) with pH conditioned by phosphoric acid to 3.5. At a flow rate of 1ml/ min and detection at 220nm, the total time of analysis was less than 10 min [13].

Separation of the Transformation Products by Thin-Layer Chromatography (TLC): TLC was used to separate new metabolites alone from clotrimazole, new metabolites produced in filtered culture broth evidenced from HPLC analysis were extracted with ethyl acetate, Active ingredients were separated from excipients and other drugs by TLC on a precoated silica gel. Plate with a solvent system of n-hexane-chloroform-methanol-diethylamine (50:40:10:1 v/v). The separated metabolites were visualized under short-wave UV light at 220 nm. Preparative TLC is used for analytical separations of larger quantities of materials; the compounds to be separated are often applied as long streaks, developed and then recovered by scraping the adsorbent from the plate and eluting with methanol [14].

Hemolytic Activity Assay: The cytotoxic activity of clotrimazole, M1, M2 and amphotericin B was assayed by determining the hemolytic activity against 50% suspension of fresh human erythrocytes at 414 nm with a microtiter ELISA plate reader. Hemolytic rates of zero and 100% were determined in a PBS (35mM phosphate buffer/150mM NaCl, pH 7.4) alone and 0.1% Triton X-100, respectively. The percentage of hemolytic activity was calculated by using the following equation [15]:

Hemolytic percentage = [(Abs 414 nm in PBS – Abs 414 nm in the sample solution)/(Abs 414 nm in PBS -Abs 414 nm in 0.1% Triton X-100)] x 100.

Biofilm Formation Ability Assay: The present study was conducted from 2011 to 2013. A total number of 70 clotrimazole-resistant Candida isolates were isolated from patients attending at The Culture and Sensitivity Unit-The Regional Center for Mycology and Biotechnology-Azhar University. The yeast strains were isolated from different clinical specimens like urine, blood, pus and wound swab etc. All the microbial strains were identified at the Regional Center for Mycology and Biotechnology (RCMB), microtitre plate biofilm assay was used to detect microbial attachment to an a biotic surface as described by Merritt *et al*. [16], 100µl of each Candida culture was inoculated into three wells in a microtiter plate. The plates were incubated at 37°C for 48 hours and then the wells were washed twice to remove planktonic cells. Microbial cells which were adhered to the wells were subsequently stained with 125µl of 0.1% crystal violet solution, then microtiter plates were then inverted and tapped vigorously on tissue paper to remove any excess liquid that allowed visualization of the attachment pattern, this surface associated dye was solubilized by adding 200µl of 95% ethanol then measured at a wavelength of 545nm in an ELISA reader. Isolates were classified into four groups as non adherent, weakly adherent, moderately adherent and strongly adherent according to biofilm optical density as detected by Stepanovi *et al*. [17].

Effect of New Compounds (M1 and M2) on Clotrimazole Resistant-Strongest Biofilm Forming Isolate (*Candida albicans*-16): Steps described by Ramage *et al.* [18] were used to detect the effect of M1 and M2 against *Candida albicans*-16, Briefly, a cell suspension of *Candida albicans*-16 ($1x10^6$ yeast cells per ml) was prepared in RPMI medium 1640/20 mM MOPS (pH 7.0), this cell suspension was introduced into polystyrene wells or Petri dishes and incubated at 37°C without agitation, which allowed the cells to attach to the surface of the Petri dish and form the biofilm structure, then M1 and M2 were added at final concentration 8 mg/l (safe concentration without hemolytic activity) and incubated at 37°C then biofilms formation were examined using CKX41 inverted microscope during different incubation periods (3, 6, 12, 24 and 48 hours), photography was performed with an Olympous camera with the 20x objective.

Examination of M2-treated C. *albicans*-16 Cells by Transmission Electron Microscopy (TEM): *C. albicans*-16 cells treated with clotrimazole and M2 for 48 hr. were prepared for TEM as previously described by Nett *et al*. [19], following fixation in 4% formaldehyde and 2% glutaraldehyde, cells were postfixed with 1% osmium tetroxide and 1% potassium ferricyanide, stained with 1% uranyl acetate, dehydrated in a graded series of ethanol concentrations and embedded in Spurr's resin. Sections (70 nm) were cut, placed on copper grids, post stained with 8% uranyl acetate in 50% methanol and Reynolds' lead citrate and analyzed by TEM (Jeol-Jem 1010 Japan). The cell wall width of different reference and treated biofilm cells were measured using LEICA, AS-V4 software.

Examination of M2-treated C. *albicans*-16 Cells by Fluorescence Microscopy: A triple-staining method was used to visualize the effect of M2 compound against *C. albicans*-16 cells. The following fluorochromes were used: (I) Ethidium bromide (EB) is double-stranded DNA-binding dye (ii) the vital dye 5-Cyano-2,3-ditolyl-2H-tetrazolium chloride (CTC) was used to stain viable micro-organisms (iii) Calcofluor White (CFW) is a fluorescent dye to view cell wall. After C. *albicans*-16 cells were treated with 8 µg/ml of clotrimazole and M2 compound, the cell suspension was placed in the wells of 24 well plates and then the plate was incubated for 24h with agitation. Both CTC and EB were made up as stock solutions in water at a concentration of 6 mg/ml and 10 mg/ml respectively. The plate contains clotrimazole and M2 treated yeast cells were divided into four groups each group contain three wells (i): wells incubated with 90µl of CTC (6 µg/ml) for 90 min (ii): wells incubated with 100µl of CFW (20 µg/ml) for 30 min (iii): wells incubated with 10µl of EB(10 µg/ml) for maximum 10min. The plate was incubated at 37°C with agitation (150 rpm). After incubation 25µl of each group placed on slide and then slides were examined by fluorescent microscope (LEICA-DM5000B). Images were recorded digitally using LEICA–DFC280 fixed camera.

Cytotoxicity Assay: The cytotoxic activity was evaluated by the crystal violet staining (CVS) method described by Saotome *et al*. [20] and modified by Itagaki *et al*. [21]. Mammalian cell lines: MCF-7 cells (breast cancer cell line), HepG2 (liver cancer cell line) and HCT-116 (colon cancer cell line) were cultured in a 96-well tissue culture microplate, 24 h after incubation, they were treated decreasing concentrations (8000-0 ng/ml) of clotrimazole, M1 and M2. After the 48 h incubation period, the viable cells yield was determined by a colorimetric method. In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using distilled water. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly. The quantitative analysis (colorimetric evaluation of fixed cells) was performed by measuring the absorbance in an automatic microplate reader at 595 nm. The effect on cell growth was calculated as the difference in absorbance percentage in presence and absence of (clotrimazole, M1 and M2) and illustrated in a dose-response curve. The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) was obtained from this dose-response curve. The standard antitumor drug used was vinblastine sulfate.

Morphological and Nuclear Changes of Clotrimazole-Resistant HepG2 Cells after Treatment with M1: Clotrimazole-resistant HepG2 cells were plated in 24-well plates and treated with 15.3 ng/ml (IC_{50} concentration) of M1. Morphological changes were evaluated after 48 hours of treatment using CKX41 inverted microscope. In order to detect nuclear changes of clotrimazole - resistant HepG2 cells after treatment with 15.3 ng/ml of M1 for 48 hours and then incubated with 1µg/mL solution of acridine orange/ethidium bromide (AO/EB) (Sigma Chemical Co.) dissolved in DMEM culture medium. Cells were observed under a fluorescence microscope (LEICA-DM5000B). Images were recorded digitally using LEICA–DFC280fixed camera.

RESULTS AND DISCUSSION

Biotransformation Results: Out of fifteen fungal species screened, two cultures viz., *Macrophomina phasolina* and *Chaetomium globosum* transformed clotrimazole into two of its metabolites M1and M2 respectively basing on the observation of new peaks other than clotrimazole peak in HPLC analysis (Fig. 1). The metabolites were identified and characterized with the help of the Mass Spectrometry (Fig. 2 A&B), Infrared Spectrometry (Fig. 2 C&D) and Nuclear Magnetic Resonance Spectrometry (Fig. 2 E&F). The analytical data indicated that the metabolites were:

M1 (5, 5-diphenyl-5H-imidazole [2, 1-α] Isoindole): ^{1}HNMR δ (ppm): 6.4-7.8 (m, 15H, Ar-H), Mass spectrum of this compound reveals molecular ion peak m/z (%) M^+, 309 consistent with the expected structure, this structure is less than the M^+ of clotrimazole by 36.5 (HCl).

M2(4,4'-((5-acetoxy-2-chloro-3-hydroxyphenyl)(1H-imidazol-1- yl)methylene)bis(4,1-phenylene) diacetate): I.R. (cm^{-1})= 1739, 1700(CO), 3409(OH),^{1}HNMR δ (ppm) = 2.2, 2.6 (2s, 9H, $3CH_3$), 6.4 (s, 1H, OH), 6.8-7.5 (m, 13H. Ar-H), Mass spectrum m/z (%), M^+ = 534.

The proposed pathway of the metabolite formation by *Macrophomina phasolina* and *Chaetomium globosum* is shown in Fig. 3.

Hemolytic Activity: The toxicity of M1 and M2 was evaluated toward mammalian cells by measuring their hemolytic activity against human erythrocytes. Many antifungal such as Amphotericin B acts on fungal cell membranes by binding to ergosterol and has the capability of binding to the cholesterol in mammalian cell membranes, which is associated with toxicity problems in human [22], as shown in Table 1 & Fig. 4 amphotericin B had significant hemolytic activity against human red blood cells, clotrimazole revealed hemolytic activity at concentration more than 8 µg/ml. however, M1 and M2 exhibited no hemolytic activity at any concentration. This indicated that M1 and M2 might be used in clinical use for human diseases, without cytotoxicity.

Biofilm Formation by Candida Strains: As a result of collection of clotrimazole-resistant clinically isolated Candida, out of 72 Candida isolates 53 were *Candida albicans*, 12 were *Candida tropicalis*, 1 was *Candida dubliensis*, 4 were *Candida krusei* and 2 were *Candida glabrata*. Amongst 72 isolated Candida, 45(62.5%) were biofilm producers, 39/45(86.67%) were *Candida albicans*, 4/45(8.89%) were *Candida tropicalis*, 2/45(4.44%) were *Candida krusei*, these results are consistent with previous analyses of clinical strains demonstrated by Shin *et al*. [23] and show that the potential for biofilm formation varies widely among strains. Isolates were classified into four groups as non forming biofilm (NF), weakly forming biofilm (WF), moderately forming biofilm (MF) and strongly forming biofilm (SF) as visualized in Fig. 5 according to their attachment to microtitre plate

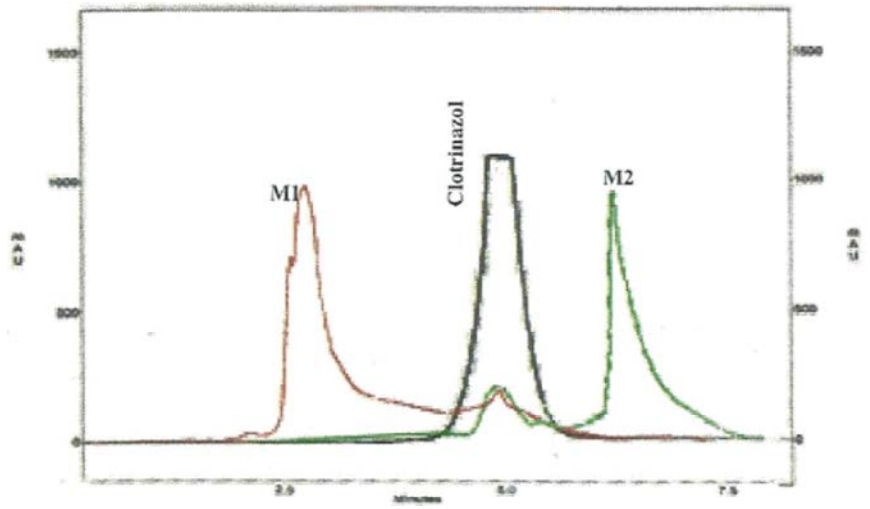

Fig. 1: HPLC chromatogram showing (in red) new metabolite M1 in culture broth of *M. phasolina* (in green) new metabolite M2 in culture broth of *C. globosum* (in grey) clotrimazole in substrate control broth

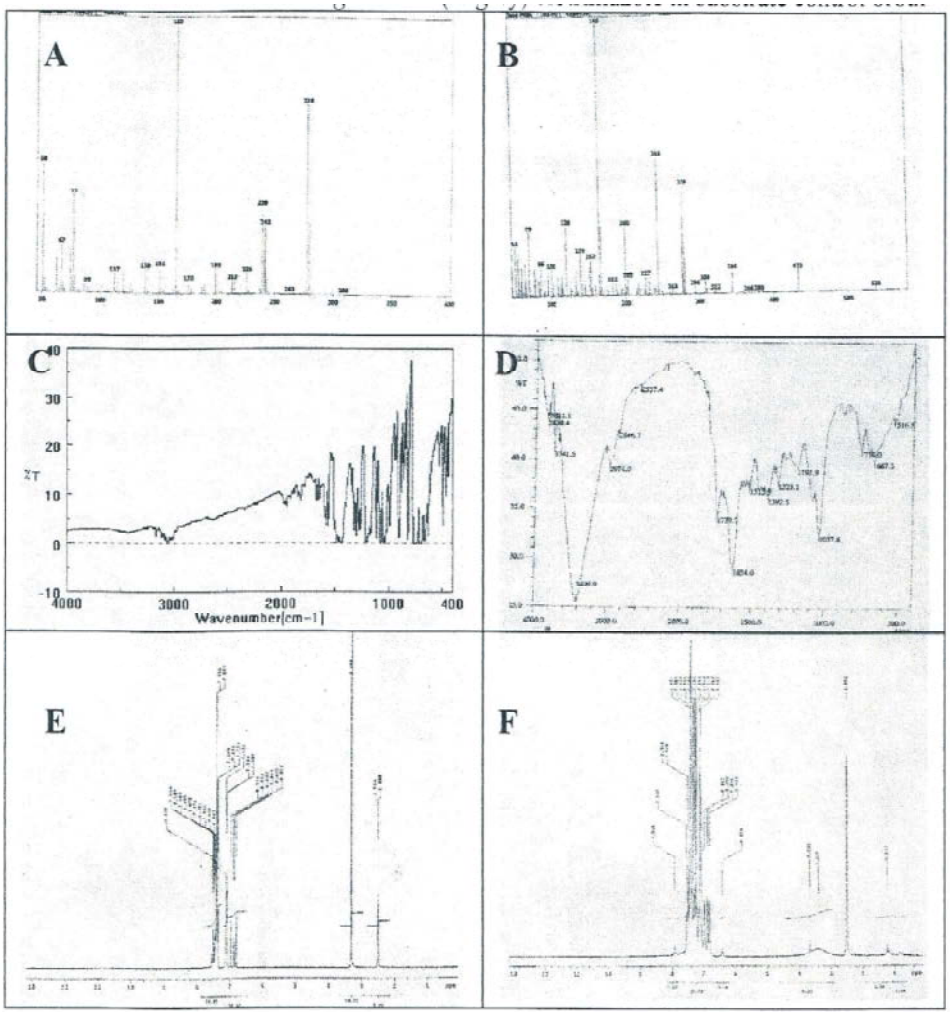

Fig. 2: (A and B) Mass spectra of M1 and M2 respectively, (C and D) Infrared spectra of M1 and M2 respectively and (E and F) Magnetic Resonance spectra of MI and M2 respectively

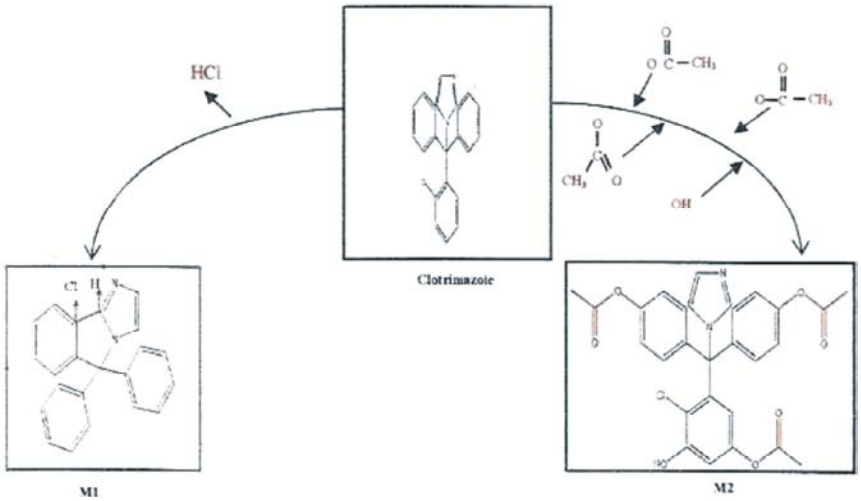

Fig. 3: Proposed metabolic pathway of clotrimazole (left hand) *M. phasolina* and (right hand) *C. globosum*

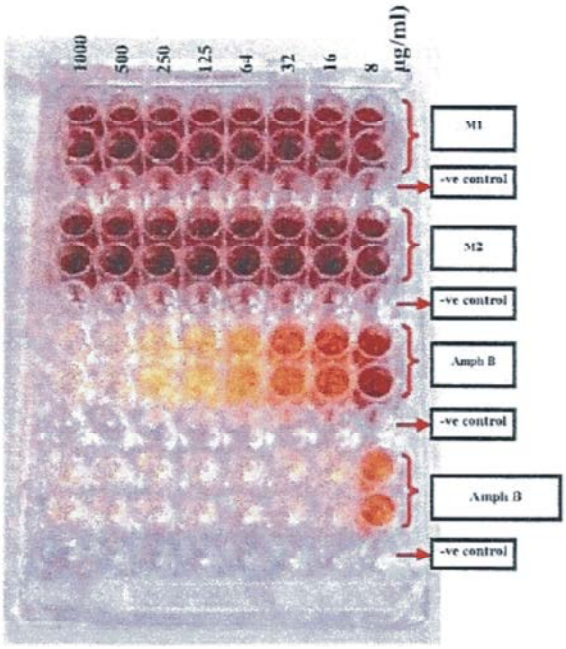

Fig. 4: Hemolytic activity of M1, M2, clotrimazole and Amphotericin B against human erythrocytes

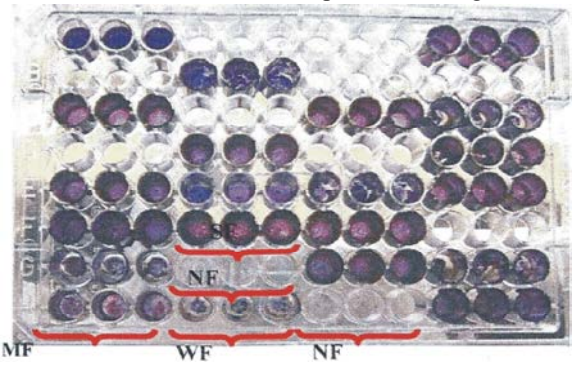

Fig. 5: Microtitre plate biofilm for detection of microbial attachment, NF: Non Forming Biofilm, WF: Weakly Forming Biofilm, MF: Moderately Forming Biofilm and ST: Strongly Forming Biofilm

Table 1: Hemolytic activity of clotrimazole, M1 and M2 against human erythrocytes

	(µg/ml)							
	100	500	250	125	64	32	16	8
Sample con.	Hemolysis %							
Clotrimazole	100	100	97.3	85.22	62.42	46.63	32.62	0
M1	0	0	0	0	0	0	0	0
M2	0	0	0	0	0	0	0	0
Amphotercin B	100	100	100	100	100	100	94.25	62.43

Table 2: Incidence (%) of yeast isolates according to their biofilm forming ability.

	Biofilm formation			
	Non	Weak	Moderate	Strong
Organisms	Number (%)			
C. albicans	53 (73.6)	14 (19.44)	12 (16.67)	8 (11.11)	19 (26.39)
C. tropicalis	12(16.67)	8 (11.11)	1 (1.39)	2 (2.78)	1 (1.39)
C. dubliensis	1 (1.39)	1 (1.39)	0 (0)	0 (0)	0 (0)
C. glabrata	2 (2.78)	2 (2.78)	0 (0)	0 (0)	0 (0)
C. krusei	4 (5.56)	2 (2.78)	1 (1.39)	1 (1.39)	0 (0)
Total	72 (100)	27 (37.5)	14 (19.44)	11(15.28)	20 (27.78)

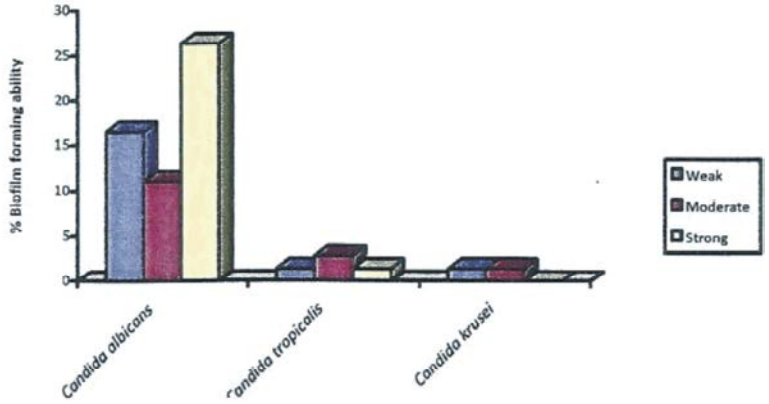

Fig. 6: Histogram represents percentage of biofilm producing strains amongst *Candida* strains

biofilm as demonstrated by Stepanovi *et al*. [17] who detected that biofilm forming organisms are grouped into weak group (OD = 0.003 to 0.006), moderate group (OD = 0.006 to 0.012) and strong group (OD > 0.012). So the incidence (%) of yeast isolates according to their biofilm forming ability (optical density-data not shown) was revealed in Table 2 & Fig. 6.

Inhibitory Evaluation of Clotrimazole, M1 and M2 Against the Strongest Biofilm Forming Isolate: The strongest biofilm forming isolate- *C. albicans* 16 (according to its highest optical density) was incubated on polystyrene surfaces in RPMI medium under biofilm-forming conditions, when clotrimazole and M1 were separately added, the cells in the wells adhered to the plastic surface (Fig. 7 A, F at 3h of incubation), proliferated and produced hyphae (Fig. 7B, C, G and H at 6 and 12h of incubation, respectively) and ultimately produced a three-dimensional biofilm structure (Fig. 7 D, E, I and J at 24-48h of incubation). These stages of biofilm development have been described previously by Ramage *et al*. [18] and Chandra *et al*. [24]. In other wells, M2 was added at the same time that clotrimazole and M1 were added. After 3h of incubation in the presence of M2, cells adhered to the plastic surface, as in the case of clotrimazole and M1 (Fig. 7K). However, proliferation of the cells was reduced in descending order (Fig. 7L, M and N at 6, 12 and 24h of incubation), respectively. Even after 48 h of incubation, the three-dimensional structure typical of a mature biofilm did not form (Fig. 7O).

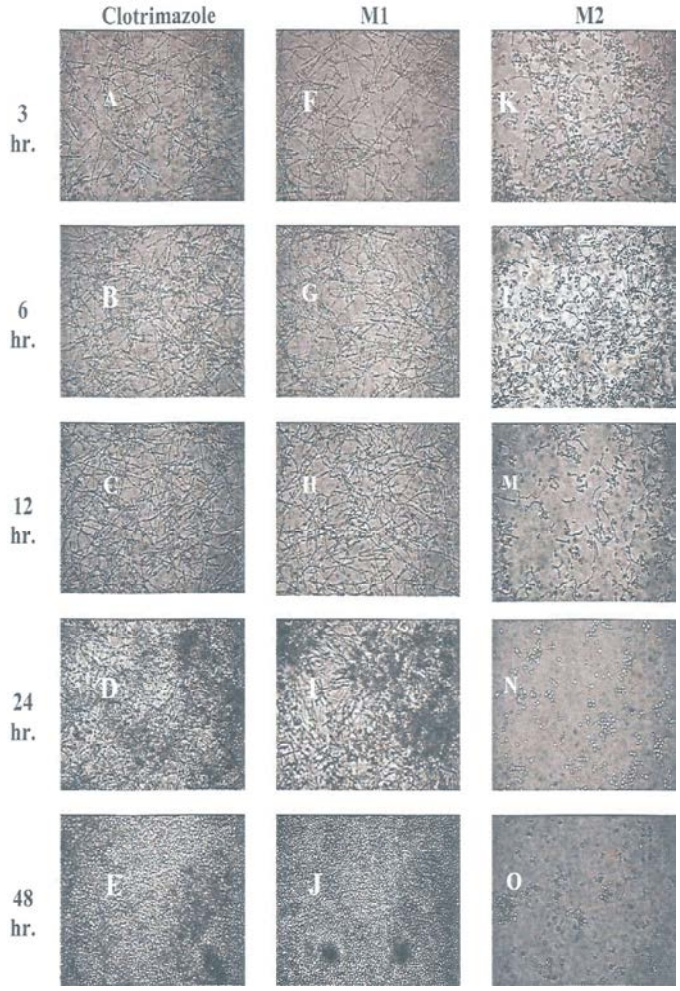

Fig. 7: Biofilm develpment of *C. albicans*16 after treatment with (A-E) clotrimazole, (F-J) M1 and (K-O) M2, at different incubation periods (3, 6, 12, 124 and 48 hrs.) recpectively)

The biofilm formation is commonly associated with antifungal resistance as demonstrated by Nobile and Mitchell [25], *C. albicans* biofilms are notorious for their high resistance to antifungal drugs, resistance to both azole drugs and amphotericin B has been documented [26]. Fortunately, the unexpected finding that M2 inhibits biofilm formation of clotrimazole-resistant clinically isolated *C. albicans* 16 may be due to M2 reduced the growth of the cells and prevented them from acquiring typical biofilm-associated resistance.

Examination of *C. albicans*16 by Transmission Electron Microscopy (TEM): The effect of M2 at 8µg/mL against clotrimazole –resistant *C. albicans*16 cells was visualized using TEM. The ultra structure of M2 treated *C. albicans*16 cells was altered, more specifically the whole cell shape was modified significantly and there is an irregulation of cell content (Fig. 8B) compared to cells treated with clotrimazole (Fig. 8A). Cell walls appeared thinner after treatment with M2 (Fig. 8D) compared to that of clotrimazole treated cells (Fig. 8C). Measurements demonstrated that the cell wall width for M2 treated cells was approximately half that measured for the clotrimazole treated cells (Fig. 9).

Examination of *C. albicans*16 by Fluorescent Microscopy (FM): Cell wall integrity, cell membrane permeability, DNA (leakage) and metabolic activity of clotrimazole and M2 treated *C. albicans* 16 cells were achieved by their exposition to triple staining with the fluorescent probes EB, CFW and CTC. The results obtained with the triple-staining approach are illustrated in Figure (10). No appreciable fluorescent signal was discerned in cells treated with M2 stained with CTC indicating absence of

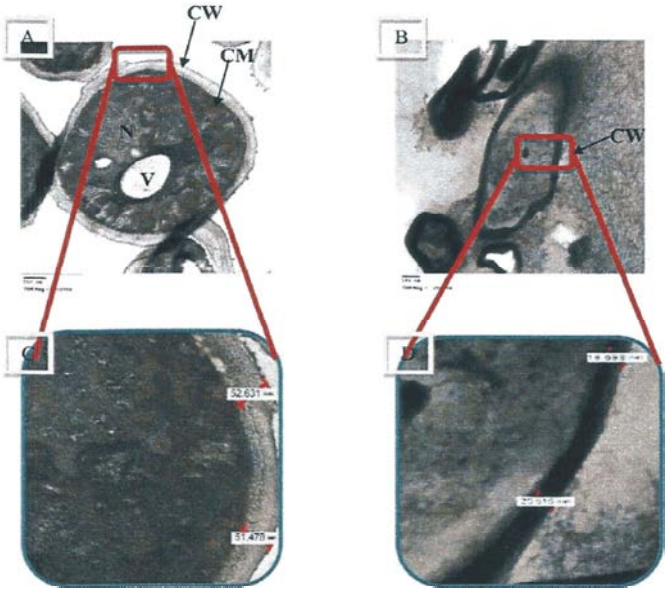

Fig. 8: TEM images of the *C. albicans*16 cells ultra structure after treatment with (A) clotrimazole and (B) M2. Cell wall width after treatment with (C) clotrimazole and (D) M2. Cell wall (CW), Cell membrane (CM), (N) Nucleus and (V) vacuole. Scale bar = 500 nm. Cell walls widths were measured using LAS 0.4

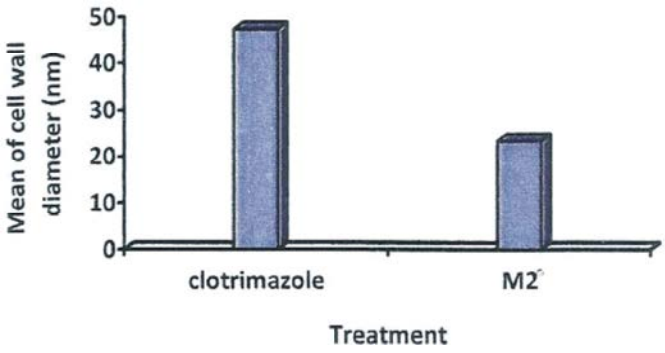

Fig. 9: Histogram represents Cell wall width after treatment with clotrimazole and M2

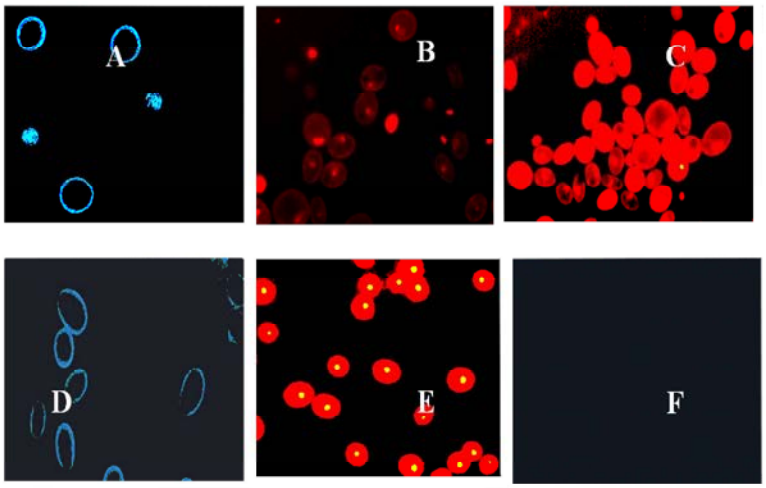

Fig. 10: (A,B and C) clotrimazole treated *C. albicans* 16 cells stained with CFW, EB and CTC respectively,: (D, E and F) M2 treated *C. albicans* 16 cells stained with CFW, EB and CTC respectively

metabolic activity (Fig. 10 F) comparing to bright red fluorescent of clotrimazole treated cells (Fig. 10 C). CTC is a mono tetrazolium redox dye, which is practically colourless and non fluorescent unless it is exposed to an electron-transport chain. This readily reduces the compound to an insoluble red fluorescent CTC-formazan salt. Therefore the presence of red CTC-formazan fluorescence indicates the presence of metabolically active (respiring) yeast [27]. At concentration of 1 µg/ml EB enters quickly cells with leakage cell membrane and produces bright red cells with bright orange fluorescence of DNA content [28], thus the obvious red fluorescent cells with bright orange DNA fluorescence indicated that M2 treated cells is accompanied by the leakage of cytoplasmic components but not DNA content. (Fig. 10E) on the other hand, faint red fluorescent signal was discerned in clotrimazole treated cells (Fig. 10B) indicated that these cells became permeable to few small molecules of EB resulted in slight alteration in the permeability of cell membranes of clotrimazole treated cells. The pale and irregular blue fluorescent detect in the cell walls of M2 treated *C. albicans* 16 cells (Fig. 10A) comparing to manifest blue fluorescent visualized in cell walls of clotrimazole treated cells (Fig. 10D) detecting that the cell wall integrity have been affected after treatment with M2 since the intensity of CFW fluorescence of yeast cells was found to be an accurate reflection of the cell wall chitin content and subsequently cell wall integrity as demonstrated by Walker *et al*. [29].

Taken together TEM and FM results addressed the question of whether and to what extent M2 affects *C. albicans* 16 cells, the data suggest that the global changes and subsequently sensitivity of yeast cells is closely related to(i): highly compromised cell membrane and absence of metabolic activity which are complementary with each other for detecting cell death, since compromised cell membrane not always results in cell death it may has a different outcome because it depends on the extent of membrane damage as in the case of membrane permeation of *E. coli* caused by temporin L (a small cationic and amphiphilic peptide)but did not led to bacterial cell death according to their metabolic activity detected by CTC [30] (ii): alteration of cell wall integrity, this finding is in the same line with the finding of Nett *et al*. [31], who demonstrated that *C. albicans* biofilm matrix production and drug resistance pathways intersect with the cell wall integrity, this connection helps explain how pathogens in a multi cellular biofilm community are protected from anti-infective therapy, briefly affecting cell wall integrity led to rendering resistant biofilm *C. albicans* matrix more susceptible to a variety of stressors.

Cytotoxic Activity Against Tumor Cell Lines: The cytotoxic results revealed that clotrimazole and M1 were cytotoxic in a dose-dependent manner to all tumor cell lines evaluated except for HepG2 which was resistant to clotrimazole but not for M1 (Table 3 & Fig. 11). On the other hand, no cytotoxic activity had been demonstrated on different cell lines treated with M2 (Data not shown). The cytotoxic effect of clotrimazole was very potent in the sub-micrograms range against MCF and HCT (Table 3 & Fig. 11). These results are in accordance with the previous results of Penso and Beitner [32] and Furtado *et al*. [33], who demonstrated that clotrimazole (CTZ) have promising antitumor activity against colon carcinoma and breast carcinoma, respectively, Although different cell lines were more sensitive to M1 than to clotrimazole indicating that M1 has higher potency against tumor cells than clotrimazole (Table 3) as indicated by the concentrations that inhibit cell survival by 50% (IC50) comparing to the vinblastine sulphate- reference drug with IC50 values (460, 260 and 120 ng/ml) against MCF, HCT and HepG2 respectively (data not shown).

Morphological and Nuclear Analysis: Clotrimazole resistant HepG2 cells treated with M1 presented significant morphological changes including, cellular shrinkage and irregularities in the plasma membrane and bleb formation on the cell surface (Fig. 12B) compared with clotrimazole treaded HepG2 cells (Fig. 12A). Unfortunately morphological method may characterize apoptosis but it cannot rule out other types of cell death. Therefore, it is very important to analyze cell death using other fluorescent DNA binding dyes such as acridine orange/ethidium bromide staining [34]. Staining cells with fluorescent dyes, including acridine orange and ethidium bromide, is used in evaluating the nuclear morphology of mammalian cells. Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity [35]. Clotrimazole and M1 treated- HepG2 cells were cultured in complete media and stained with AO/EB respectively. Clotrimazole treated- Cells stained green represent viable cells (Fig. 12C), whereas M1 treated reddish or orange staining cells represents late stage apoptotic activity, as shown in Fig. 12D, HepG2 cells

Table 3: *In vitro* cytotoxic activities of clotrimazole (CTZ) and M1 on different cell lines:

Lin Celle	MCF (treated with CTZ)	MCF (treated with M1)	HCT (treated with CTZ)	HCT (treated with M1)	HepG2 (treated with CTZ)	HepG2 (treated with M1)
Sample Con. (ng/ml)[b]	Cell viability %± S.D[a]					
8000	3.18 ± 0.23	3.78 ± 1.21	4.64±0.29	4.97± 1.12	100±0.82	3.12 ± 1.41
4000	4.93 ± 0.45	4.22 ±0.21	7.85±0.87	6.83± 2.38	100±0.64	3.98± 2.24
2000	6.58 ± 0.16	6.13 ± 1.33	9.37±0.92	8.16± 4.56	100±0.82	5.32 ± 2.96
1000	11.97 ± 1.5	8.96 ± 0.98	13.75±0.72	10.88± 5.32	100±0.82	8.73± 1.41
500	14.72 ± 0.9	11.68 ± 0.25	18.24±1.20	14.75± 4.04	100±0.12	12.90± 1.25
250	20.93 ± 1.3	13.87 ± 0.36	22.97±0.67	16.89± 3.96	100±0.55	14.82 ± 2.14
125	31.28 ± 0.6	17.92± 0.42	31.84±1.44	21.74± 3.96	100±0.21	17.46 ± 1.33
62.5	40.52 ±1.23	23.86 ± 2.21	39.78±2.60	30.91± 3.96	100±0.44	22.34± 1.44
31.25	49.63 ± 1.4	35.08 ± 2.21	51.86±2.32	39.85± 3.96	100±0.33	36.12 ± 1.52
15.6	58.12 ±1.22	47.98 ± 2.21	60.94±3.20	50.36± 3.96	100±0.55	49.64 ± 2.15
7.8	65.27 ±0.47	56.85 ± 2.21	67.53±5.65	59.78± 3.96	100±0.48	58.38± 1.33
3.9	74.48 ± 1.2	63.98 ± 2.21	78.19±2.86	66.13± 3.96	100±0.26	64.56 ± 1.22
0	100.0 ± 2.1	100.0± 2.21	100.00±1.97	100.0± 3.96	100±0.37	100 ± 2.12
[b]IC_{50}	30.1ng/ml	13.8 ng/ml	39.2 ng/ml	16.1 ng/ml	--	15.3 ng/ml

[a]: Standard deviation, [b]:nanogram / millimeter

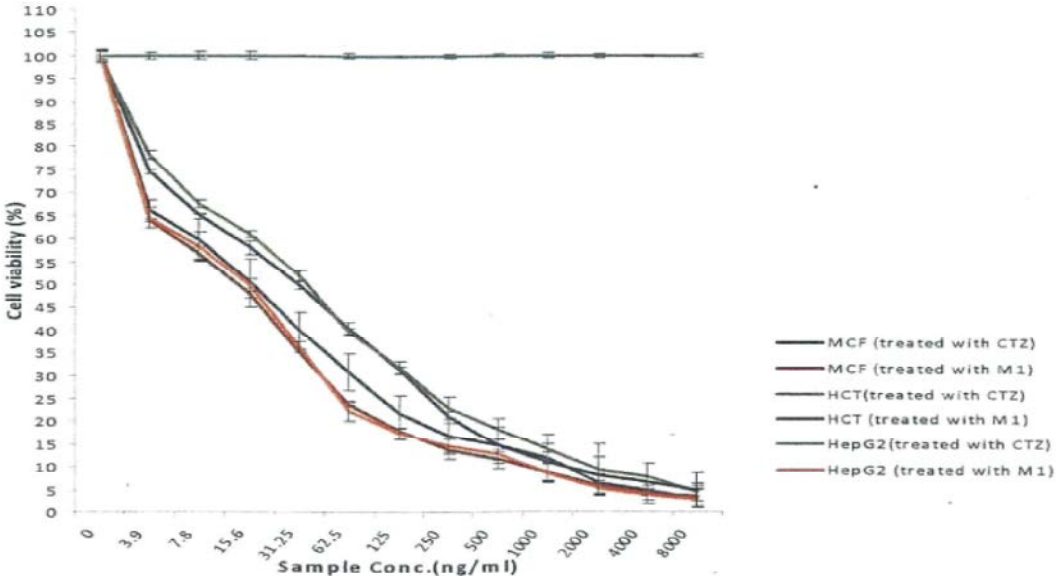

Fig. 11: Cell viability % of clotrimazole and M1agains different cell lines

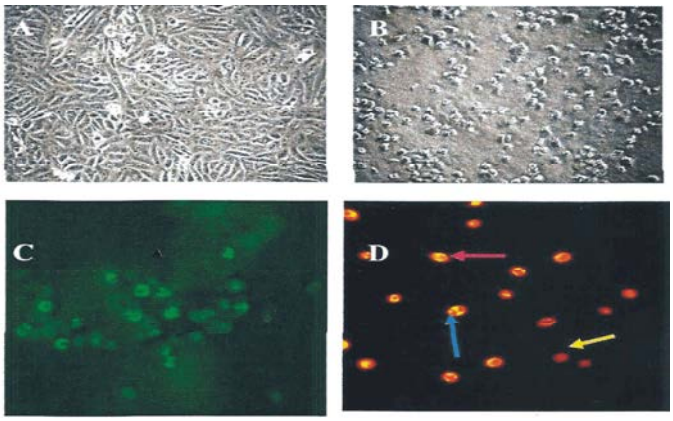

Fig. 12: (A-B) Photomicrographs from inverted microscopy of clotrimazole treated-HepG2 tumor cell (A) and M1 treated-HepG2 tumor cells. (C-D): Fluorescent micrographs revealed viable clotrimazole treated-HepG2 cells (C), M1 induced apoptosis (D) including, chromatin condensation (pink arrow), presence of apoptotic bodies (yellow arrow), and fragmented nuclei (blue arrow) Amplification of 20x

treated with M1 showed changes in nuclear morphology, including chromatin condensation, presence of apoptotic bodies and fragmented nuclei which are indicative of apoptosis as demonstrated by Gabrie *et al*. [35].

Structure Activity Relationship (SAR): M2 compound (as a result of biotransformation Process of clotrimazole which led to hydroxylation and acetylating of clotrimazole) exhibited potent antifungal activity against significant biofilm forming clotrimazole resistant *Candida albicans* and that may be due to acetylated azoles are potent anti candidiasis agents against many *Candida* species as demonstrated by Kanagarajan *et al*. [36]. M1 compound (as a result of biotransformation Process of clotrimazole which led to elimination of HCl from clotrimazole and form indole ring) exhibited potent anticancer more than clotrimazole and against resistant -clotrimazole. Indole is consisting of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring; indole is a popular component of fragrances and the precursor to many pharmaceuticals. Compounds that contain an indole ring (indoles) have potent anticancer activity [37]. Notably, many marketed standard anticancer drugs are indoles such as: vinblastine (antitumor standard drug), vinorelbine and vindesine [38], vincristine and mitraphylline [39].

CONCLUSION

Actually the point of this study came after noticing increasing numbers of Candida isolates causing fungal infection (which have been isolated from patients attending at the Culture and Sensitivity Unit, the Regional Center for Mycology and Biotechnology, Al Azhar University, Cairo, Egypt based on the request of the doctors treating them), unfortunately the sensitivity of these isolates to different antifungal drugs came disappointing, since most of these isolates were multi drug resistant, including clotrimazole despite being the most descripted antifungal for candida treatment by doctors and therapists (previous review). Results of the current study shed light on the importance of including endophytic fungi in the screening approach for novel drugs through biotransformation process as a novel alternative method to obtain such compounds. Unfortunately, Echinocandins are the only class of cell wall inhibitor still in development, with caspofungin receiving approval for use in treating refractory infections caused by *Aspergillus* species while echinocandins are not effective against all human pathogenic fungi. For example, caspofungin is relatively ineffective against the important human yeast pathogens [40, 41], So Searching for compounds not only have antifungal activity against planktonic candida cells but also have the ability to prevent them from acquiring typical biofilm-associated resistance refractory to cell wall integrity is needed and that could be useful for development of new drugs to reduce the incidence of antifungal resistant -associated infections. FM with triple-staining method and AO/EB shedding light on the mode of action of M2 and M1 respectively and proposing a valuable, quick and accurate approach to study the modes of action of active compounds.

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