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Shivkrupa D Halbandge
DST-FIST & UGC-SAP Sponsored School of Life Sciences, SRTM University, NAAC Accredited with 'A' Grade, Nanded, Maharashtra, India

Supriya P Mortale
DST-FIST & UGC-SAP Sponsored School of Life Sciences, SRTM University, NAAC Accredited with 'A' Grade, Nanded, Maharashtra, India

Ashwini K Jadhav
DST-FIST & UGC-SAP Sponsored School of Life Sciences, SRTM University, NAAC Accredited with 'A' Grade, Nanded, Maharashtra, India

Kiran Kharat
Department of Biotechnology, Deogiri College, Aurangabad, Maharashtra, India

Sankunny Mohan Karuppayil
DST-FIST & UGC-SAP Sponsored School of Life Sciences, SRTM University, NAAC Accredited with 'A' Grade, Nanded, Maharashtra, India

Correspondence
Sankunny Mohan Karuppayil
DST-FIST & UGC-SAP Sponsored School of Life Sciences, SRTM University, NAAC Accredited with 'A' Grade, Nanded, Maharashtra, India

Differential Sensitivities of various growth modes of *Candida albicans* to sixteen molecules of plant origin

Shivkrupa D Halbandge, Supriya P Mortale, Ashwini K Jadhav, Kiran Kharat and Sankunny Mohan Karuppayil

Abstract
Candida albicans is opportunistic fungal human pathogen. *Candida* infections are becoming serious concern in immuno-comprised patients. Targeting of virulence factor can be an effective strategy to combat against *C. albicans* infections. Invasive growth is considered as crucial factor involved in virulence of *C. albicans.* Studies regarding drug sensitivity of invasive mode of growth are lacking. Sixteen molecules of plant origin were analyzed to test their efficacy against invasive, peripheral filamentous and total growth of *C. albicans*. Out of sixteen molecules, only four molecules i.e. Carvacrol, Geraniol, Eugenol and Geranyl acetate were specific inhibitors of invasive growth and their MIC values are presented in this communication. Fourteen molecules showed inhibitory activity against peripheral filamentous growth. Toxicity studies were done using human erythrocytes. These molecules showed negligible hemolytic activity at their MIC values. In addition, these molecules inhibited cell cycle division cycle. These molecules may be of use in developing novel drugs against candidiasis.

Keywords: *Candida albicans*, phytomolecules, invasive growth, peripheral filamentous growth, candidiasis, cell cycle, hemolytic activity.

Introduction
The human opportunistic fungal pathogen, *C. albicans* contributes to 50–60 % cases of candidiasis in immunocompromised patients. Currently, only four classes of antifungal drugs are used for the treatment of these infections which include polyenes, azoles, pyrimidines, and echinocandins [5]. Prolonged use of antifungals leads to development of resistance towards the drug in use [25]. Two strategies are considered for the drug development i.e. looking for new inhibitors which specifically target the fungal cells or repositioning of existing drugs [9].
C. albicans exhibits considerable morphological plasticity. This plasticity is characterized by its ability to reversibly transform to different morphotypes like yeast, psuedohyphae and true hyphae. It can also form biofilms on biotic as well as abiotic surfaces. The biofilm forms are intrinsically resistant to most of the antifungal drugs used currently. This has posed a serious challenge to drug developers and physicians, necessitating the continued search for effective antibiofilm agents. A number of studies are available on the potential of molecules of natural origin as antibiofilm agents. Some workers have established MIC and MFC values for libraries of small molecules [15, 29]. The molecular modes of action of some of molecules are studied [6, 16]. Yeast to hyphal form morphogenesis is a virulence factor which is studied in detail [31]. The sensitivity of yeast to hyphal morphogenesis to small molecules of plant origin is reported [26, 29]. Targeting of virulence factors for the development of drug without killing organism is considered as a novel paradigm in antifungal drug development [9].
C. albicans cells can respond to surface topography of semisolid surfaces by growing into the growth medium [17]. Invasive growth form is an important virulence factor in *C. albicans*. Considerable insight into the invasive mode of growth has come from studies using the *S. cerevisiae* model [13, 14]. Researchers have widely used the agar model system to study invasive growth that may mimic some of the features that occurs during the host pathogen interaction [14]. The molecular basis of invasive growth in *C. albicans* is extensively investigated by Kumamoto and others [4, 17, 35].
Various workers have reported the efficacy of molecules of plant origin against virulence factors such as yeast to hyphal form morphogenesis, biofilm formation, adhesion, ergasterol biosynthesis and cell cycle progression [12, 26, 28, 34]. Terpenoids are the main components present in volatile and essential oils. We have limited information on the drug sensitivity of invasive and peripheral filamentous forms of *C. albicans*. Not much information is available on MIC values of various drugs and molecules against invasive growth of *C. albicans* except few studies [24, 32]. In this communication we are reporting the sensitivity of the *in vitro*

invasive growth (IG) and peripheral filamentous growth (PFG), total growth (TG) of *C. albicans* to a small library of molecules. Cell cycle inhibitory properties of phytomolecules are also reported. Additionally, toxicity studies are done using hemolytic assay.

2. Materials and methods

2.1. Phytochemicals and Antifungal standards

Two standard antifungal drugs were used in this study i.e. Fluconazole (Forcan, Cipla Pvt. Ltd, Mumbai, India) and Amphotericin B (LYKA Labs LTD, Ankleshwer, Gujarat, India). Citral, Citronellal, Geranyl acetate, β-Citronellol, Geraniol, Linalool, Nerol, t, t-Farnesol, Carvacrol, β-pinene, Terpinolene, limonene were purchased from Sigma-Aldrich Chemicals Ltd, Mumbai, India. B-ionone, α-pinene, Thymol, and Eugenol were obtained from Himedia Chemicals Ltd, Mumbai, India. Dimethyl sulphoxide (Himedia Chemicals Ltd, Mumbai, India) was used to dissolve phytochemicals at a final concentration of 1 %.

2.2. Culture media

Yeast extract-Peptone-Dextrose (YPD) (1 % Yeast extract, 2 % Peptone, and 2 % Dextrose) were prepared and used for inoculum preparation. All the components were obtained from Himedia Chemicals Ltd, Mumbai, India. RPMI-1640 medium (w/L-glutamine w/o sodium bicarbonate), pH 7, buffered with 165 mM 3-[N-morpholine] propane sulphonic acid was filter sterilized. All the ingredients of spider medium (1 % Mannitol, 1 % Nutrient broth, 0.2 % KH_2PO_4) were obtained from Himedia Chemicals Ltd, Mumbai, India.

2.3. Inoculum Preparation

C. albicans strain ATCC 90028 was obtained from the Institute of Microbial Technology, Chandigarh, India and maintained on Yeast extract-Peptone-Dextrose (YPD) agar slants at 4 °C. A Single colony from YPD agar plates were inoculated in fifty ml of YPD and incubated at 30 °C in shaking incubator for 24 h. Cells from the activated culture were harvested by centrifugation for 5 min at 2000 g, washed three times, and resuspended in PBS buffer (10 mM Phosphate buffer, 2.7 mM Potassium chloride, and 137 mM Sodium chloride, pH 7.4).

2.4. Agar invasion assay

The agar invasion assay was done as per Gimeno *et al*. (1992). Solid spider medium plates were prepared with varying concentrations of phytochemicals ranging from 0.007 to 4 mg/ml. Plates without test molecules were maintained as control. Flucanazole and Amphotericin B were used as standard antifungal agents in this study. *C. albicans* with cell density 1×10^4 cells ml^{-1} were starved by keeping for agitation for 1 hour in distilled water. Starved cells were spread on different concentrations of spider plates. These plates were incubated at 37°C for 4 days. To study the filamentous growth on spider medium, these colonies were examined. Filamentous colonies were counted. A colony protruding at least 20 filaments from periphery was defined as 'peripheral filamentous colony' (Zucchi at al., 2010). Non-invaded cells were removed by washing away by rubbing the agar surface with gloved hand while rinsing the plate under running water. Invasive cells were seen as visible patches on the surface of agar after washing. Colonies noticeable after washing was referred as 'invasive colony'. Invasion of the colony was confirmed by microscopic examination of the cells remaining after washing. Populations of invasive cells were confirmed by light photomicroscopy by removing a piece of agar containing invasive cells and crushing the agar piece between a coverslip and a microscope slide. Agar invasion was assayed by calculating percentage of invasive colonies.

Percentage of invasion= No. of colonies after washing / No of colonies before washing×100

2.5. Cell cycle analysis of *C. albicans*

Cell cycle analysis of *C. albicans* ATCC 90028 was determined as described earlier [34]. Cells were harvested, washed and kept for starvation for one hour. 10^3 cfu/ml of cells were inoculated to each flask containing fifty ml of RPMI with MICs concentration of these phytochemicals. Control was maintained by inoculating starved cells into the fifty ml RPMI flask lacking phytochemicals. All the flasks were incubated at 30^0 C for three hours. Cells were harvested from these flasks by centrifugation for five minutes at 2000 g. These cells were washed twice with PBS and fixed with 70 % ethanol at room temperature for 30 min. These cells were resuspended in 500 µl of 50 mM Tris with ten µg of RNase A and kept for incubation for 2 hours at 37^0 C. After incubating the samples were treated with protease (5 mg/ml pepsin in 0.05 M HCl) for one hour at 25^0 C. This step was done to reduce the cellular clumps. Cells were harvested and resuspended in 0.5 ml of FACS buffer (200 mM Tris/HCl pH 7.5; 200 mM NaCl; 78 mM $MgCl_2$). These samples were treated with propidium iodide (1 mg/ml) and kept for incubation for 30 min. Sonication was done for 10 s before analysis. Samples were analyzed using FACS Calibur cytometer (Becton-Dickinson, San Jose, CA, USA).

2.6. Hemolytic activity

The hemolytic activity of phytochemicals was determined using mammalian red blood cells [1]. Human Erythrocytes were collected. EDTA (1–2 mg/ml) was added in tube containing erythrocytes as anti-coagulant. The erythrocytes were harvested by centrifugation (Heraeus Megafuge 40, Thermo Fisher Scientific Inc., MA) for ten minutes at 634 × g at 20°C, and washed three times in saline. 10 % (v/v) erythrocytes/saline suspension was prepared by adding saline to pellet. The 10 % suspension was diluted 1:10 in saline in Eppendrof tube. From different dilution of test molecule prepared in saline, 100 µl of different concentration of test molecules were added in triplicates to 100 µl of cell suspension. 1 % Triton x -100 was used as negative control and to achieve total hemolysis. Saline without molecule was maintained as positive control. The Eppendrof tubes were incubated for 1 h at 37°C and centrifuged for 10 min at 634 × g at 20°C. From the supernatant fluid, 150 µl was added to a flat-bottomed microtiter plate (Himedia Chemicals India Ltd., India), and the absorbance was measured at 450 nm. Percent hemolysis was calculated using following equation:

Percentage hemolysis = [(Abs414 nm in the compound solution – Abs414 nm in PBS)/ (Abs414 nm in 0.1% Triton X-100 – Abs414 nm in Saline)] x 100

Statistical analysis

Values presented are the means with standard deviations, obtained from three different observations. Values in the control and treatment groups for various molecules as well as the results obtained in agar invasion and hemolytic assays were compared using the Student's t-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Phytochemicals retards peripheral filamentous growth of *C. albicans*

The effect of phytochemicals on various modes of growth of *C. albicans* was studied. *C. albicans* exhibited different modes of growth on solid media plate i.e. Peripheral filamentous growth (PFG) and Invasive growth (IG). To investigate the effect of phytochemicals on peripheral filamentous growth, cells were grown on solid spider medium to induce hyphal morphogenesis and phytochemicals were tested against PFG. PFG was characterized by the presence of fila mentation at the periphery of colonies. This growth was visible under a dissection microscope.

Out of sixteen molecules tested, fourteen inhibited PFG in a concentration dependant manner. PFG was inhibited by two antifungals drugs (Fluconazole and Amphotericin). All the phytomolecules tested except Thymol and β- ionone inhibited PFG resulting into smooth colony lacking fila mentation at the periphery (in the range of 0.007 to 0.5 mg/ml). Among these molecules, Carvacrol, Eugenol, Geraniol, Geranyl acetate, Citronellal, Nerol, Fernesol and Citral were found to be most potent inhibitors of PFG (in the range of 0.007 to 0.031 mg/ml) (Table 01; Figure 02). This was followed by the inhibitory activity of citronellal, Linallol, Limonene, β-Pinene (in the range of 0.062 to 0.5 mg/ml). Terpinolene and α-Pinene less effective against PFG (in the range of 1 to 2 mg/ml) (Table 01; Figure 02).

3.2. Phytochemicals inhibit invasive growth of *C. albicans*

Invasive growth was also visible under a dissection microscope. Invasive cells forms filaments and penetrate to agar. Invasive growth assay was carried out by using solid spider medium to induce the invasive growth. Sixteen molecules were tested against IG, out of that, only four molecules were effective against IG. IG was inhibited by Carvacrol, Eugenol, Geraniol, Geranyl acetate and two antifungal drugs i.e. Fluconazole and Amphotericin B. IG was insensitive to one dozen of the molecules and MIC could not be achieved, including molecules like Farnesol and Linallol which are highly effective against yeast to hyphal form conversion (Table 01). The MIC for invasive growth was at 0.062 mg/ml for Carvacrol, Geraniol and Eugenol and 0.5 mg/ml for Geranyl acetate (Figure 01). These molecules allowed yeast colony growth but retarded invasive growth (Figure 01). As such these molecules are considered as specific inhibitors invasive growth.

3.3. Inhibition of total growth by phytomolecules

Total growth inhibition of *C. albicans* colonies were observed in response to 14 molecules. Carvacrol, Eugenol, Geraniol, Fernesol and Citral exhibited most potent activity in the range of 0.031 to 0.125 mg/ml against TG. Geranyl acetate, β-Citronellal, Nerol, Citronellal, Linallol, Thymol was effective against TG (in the range of 0.5 to 2 mg/ml). Terpinolene, Limonene, α –Pinene and β-Pinene did not inhibit growth and inhibition could not be achieved upto 4 mg/ml. (Table 01). In general the sensitivity of FG, IG and TG were in the following order: PFG > IG > TG. PFG is most sensitive followed by IG and TG. All the growth modes were sensitive to the antifungal tested.

3.2. Cell cycle arrest by phytochemicals

Fourteen phytomolecules were tested for their cell cycle inhibitory properties against *C. albicans*. Treatment with these molecules resulted in significant accumulation of cells in different phases of cell cycle division. Out of fourteen molecules tested, three i.e. Terpinolene, Limonene and Eucalyptol did not significantly affect cell cycle. *C. albicans* cells treated with molecules such as Linallol, β- pinene, α-pinene, Thymol, and Geraniol induced cell cycle arrest in G1 phase whereas Eugenol, Citronellal, β-Citronellol and Nerol arrested cell cycle in S phase of cell cycle division (Figure 03, Table 02). Carvacrol and β-ionone treated *C. albicans* showed cell cycle inhibition in G2M and S phase (Figure 03). Out of these 14 molecules, four molecules such as β- Ionone, α-Pinene, Thymol and Nerol were found as good inhibitors of cell cycle arrest in *C. albicans* (Figure 03).

3.3. Hemolytic activity of phytochemicals

Hemolytic activity of fifteen molecules was evaluated. Causing hemolysis can limits clinical application of these molecules for treatment as they can lead to the lysis of erythrocytes of humans. Hemolytic activity was tested as it can give the idea about its cytotoxicity to mammalian cells. The hemolytic activity of phytomolecules were evaluated in the concentration range of 0.0625 to 1 mg/ml. Out of fifteen molecules tested, thirteen molecules such as Citral, Citronellal, Geranyl acetate, β-Citronellol, Geraniol, Linalool, Nerol, Carvacrol, β-pinene, Terpinolene, Limonene, β-ionone and Eugenol showed negligible hemolysis (ranges from 1- 2 %) at the concentration 0.0625 mg/ml while α-pinene showed 27 % of hemolysis at the same concentration (Figure 04). These molecules did not show considerable hemolysis at the concentration ranges from 0.0625 to 0.5 mg/ml except Terpinolene and α-pinene. At higher concentrations i.e. 1 mg/ml, seven molecules such as Carvacrol, Citronellol, Eucalyptol, Nerol, Eugenol, Linallol and Geraniol did not cause significant hemolysis (ranges from 1-7 %) while remaining eight molecules showed significant hemolysis (Figure 04).

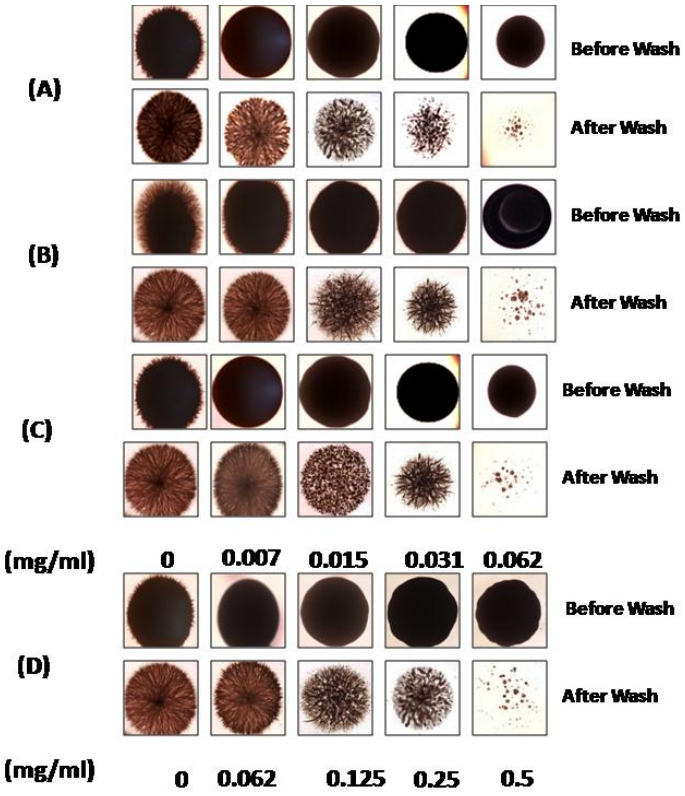


Fig 1: Inhibition of invasive growth of *C. albicans* ATCC 90028 by four phytochemicals (A) Eugenol (B) Carvacrol (C) Geraniol (D) Geranyl acetate Cells of *C. albicans* ATCC 90028 were incubated on the surface of spider medium with the indicated concentrations of phytomolecules for 4 days at 37^0C. Representative colonies were photographed at 4X power. Colonies were washed off the surface of agar. Invaded cells remaining on the surface of the agar were photographed at 4 X power after wash.

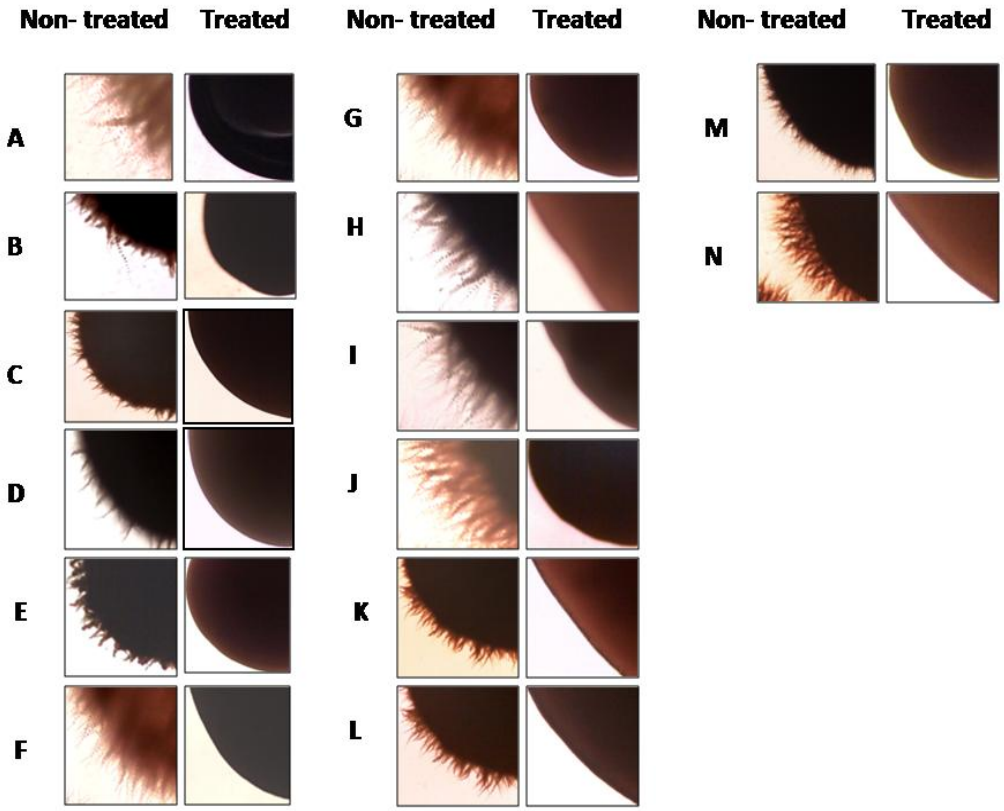
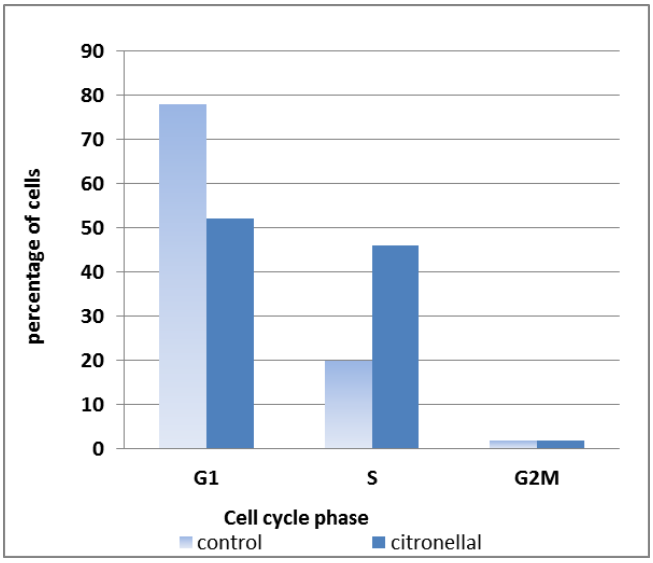
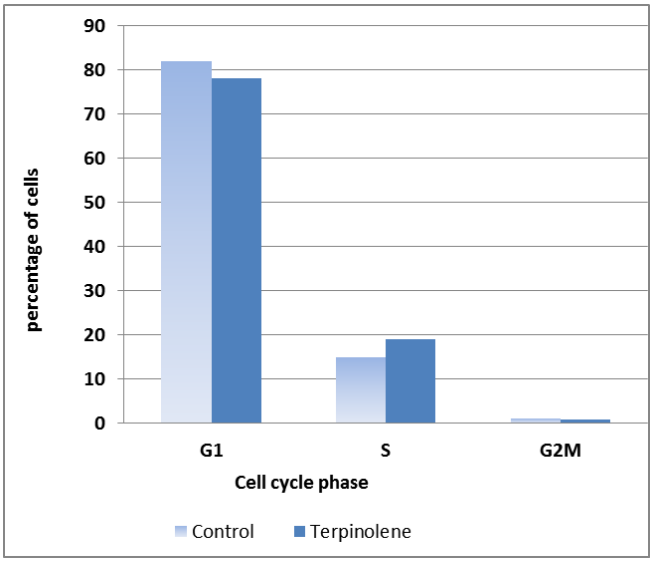


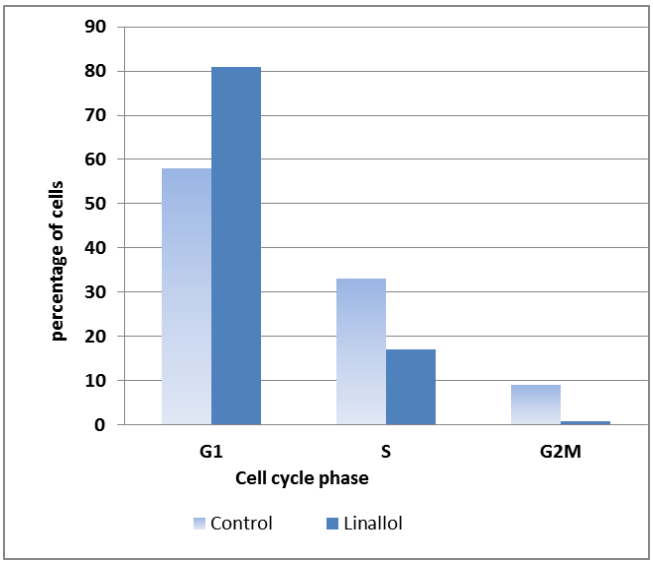
Fig 2: Inhibition of peripheral filamentous growth of *C. albicans* ATCC 90028 by different phytochemicals (A) Non-treated and treated with Carvacrol (0.031 mg/ml) (B) Non-treated and treated with Eugenol (0.007 mg/ml) (C) Non-treated and treated with Geraniol (0.007 mg/ml) (D) Non-treated and treated with Geranyl acetate (0.031 mg/ml) (E) Non-treated and treated with β- Citronellol (0.015 mg/ml) (F) Non-treated and treated with Nerol (0.015 mg/ml) (G) Non-treated and treated with Citronellal (0.062 mg/ml) (H) Non-treated and treated with Linallol (0.062 mg/ml) (I) Non-treated and treated with Terpinolene (2.00 mg/ml) (J) Non-treated and treated with Fernesol (0.015 mg/ml) (K) Non-treated and treated with Limonene (0.5 mg/ml) (L) Non-treated and treated with α - Pinene (1.00 mg/ml) (M) Non-treated and treated with β- Pinene (0.5 mg/ml) (N) Non-treated and treated with Citral (0.015 mg/ml)


percentage of cells
90
80
70
60
50
40
30
20
10
0
G1
S
G2M
Cell cycle phase
control
citronellal

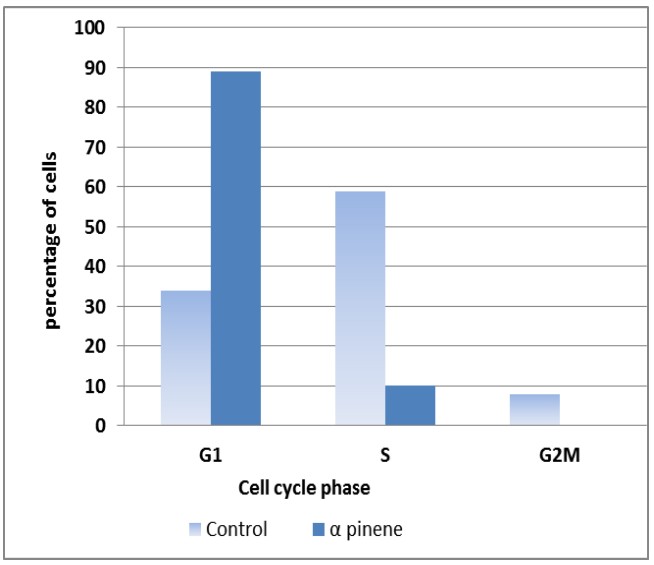
(A)


percentage of cells
90
80
70
60
50
40
30
20
10
0
G1
S
G2M
Cell cycle phase
Control
Terpinolene

(B)


percentage of cells
90
80
70
60
50
40
30
20
10
0
G1
S
G2M
Cell cycle phase
Control
Linallol

(C)


percentage of cells
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90
80
70
60
50
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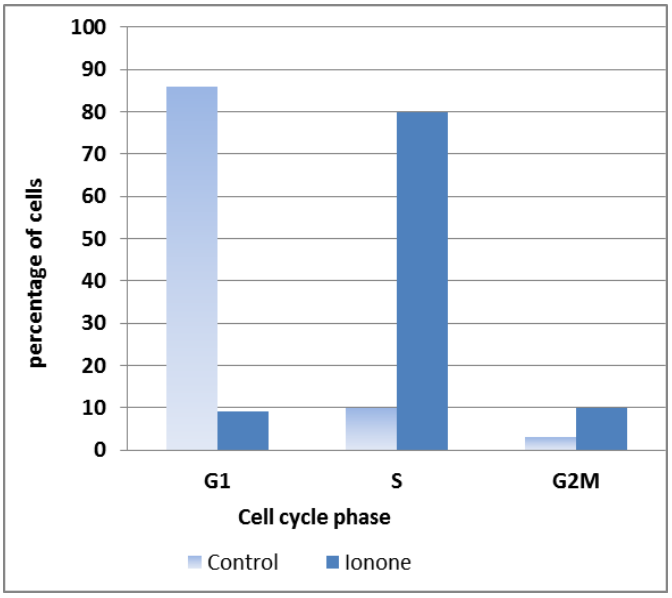
30
20
10
0
G1
S
G2M
Cell cycle phase
Control
α pinene

(D)


percentage of cells
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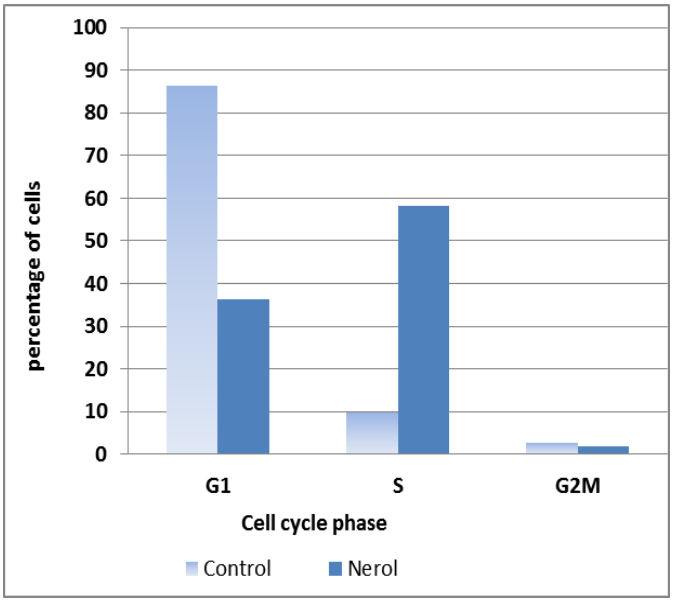
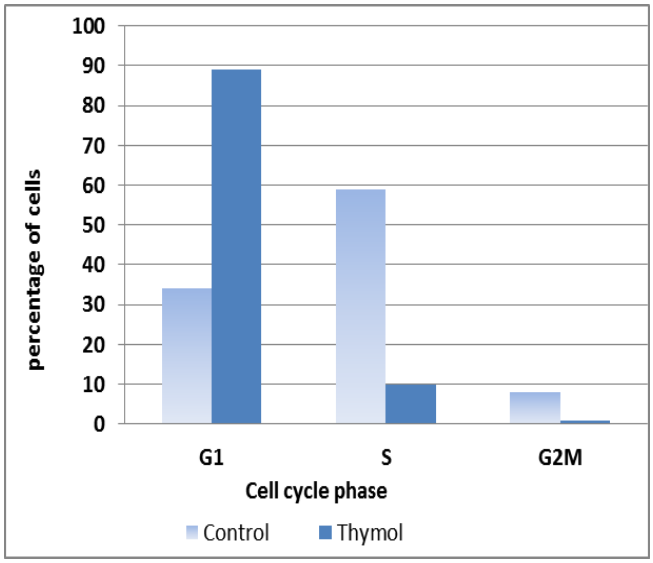
90
80
70
60
50
40
30
20
10
0
G1
S
G2M
Cell cycle phase
Control
Ionone

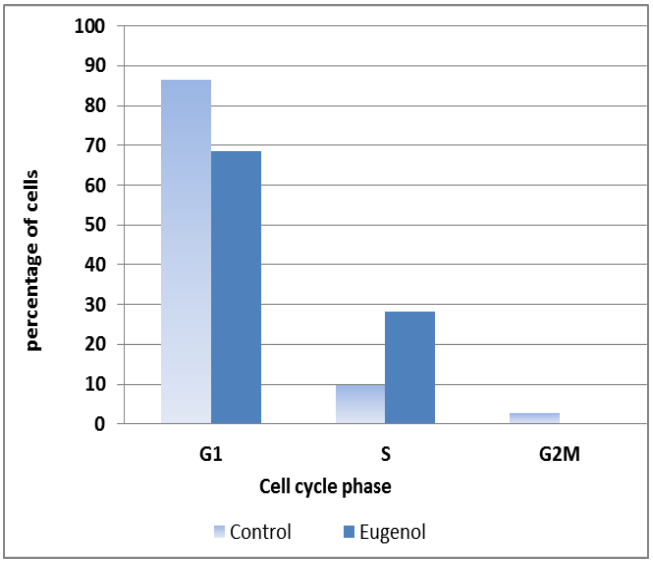
(E)


percentage of cells
100
90
80
70
60
50
40
30
20
10
0
G1
S
G2M
Cell cycle phase
Control
Nerol

(F)


100
90
80
70
60
50
40
30
20
10
0
percentage of cells
G1
S
G2M
Cell cycle phase
Control
Thymol

(G)


100
90
80
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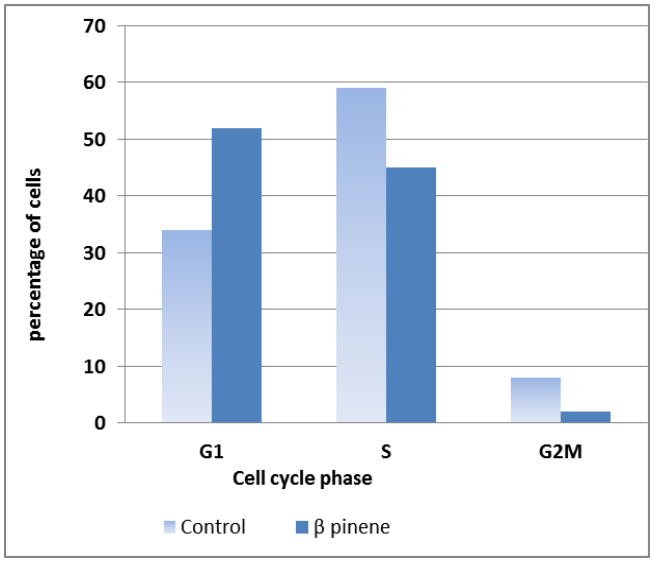
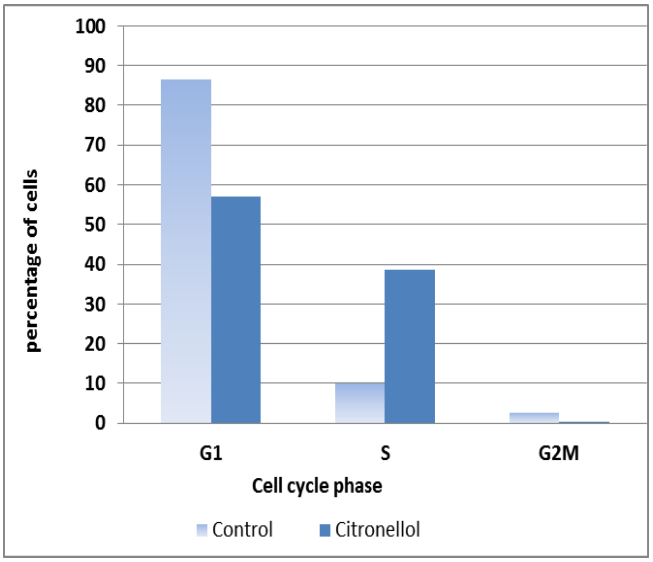
50
40
30
20
10
0
percentage of cells
G1
S
G2M
Cell cycle phase
Control
Eugenol

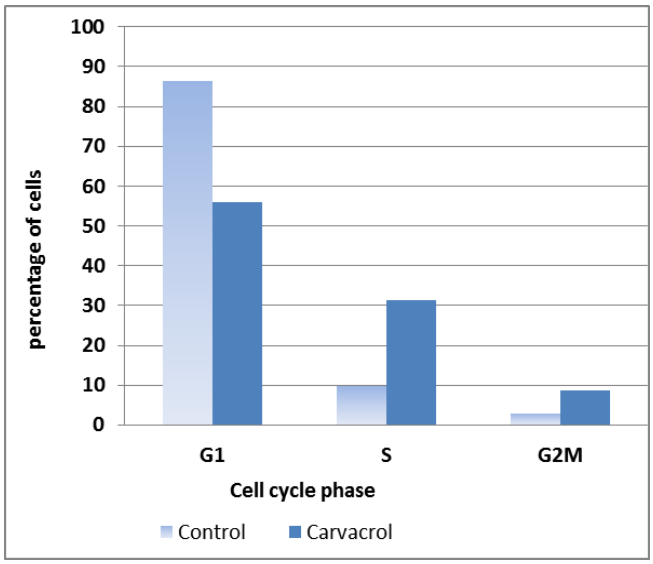
(H)


70
60
50
40
30
20
10
0
percentage of cells
G1
S
G2M
Cell cycle phase
Control
β pinene

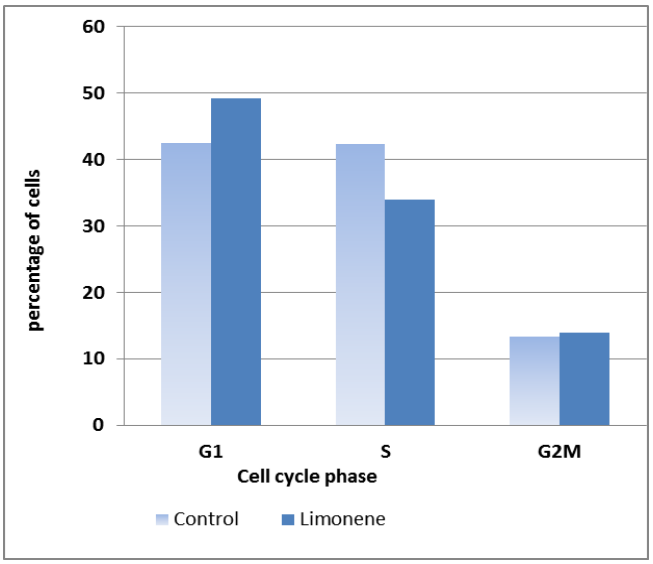
(I)


100
90
80
70
60
50
40
30
20
10
0
percentage of cells
G1
S
G2M
Cell cycle phase
Control
Citronellol

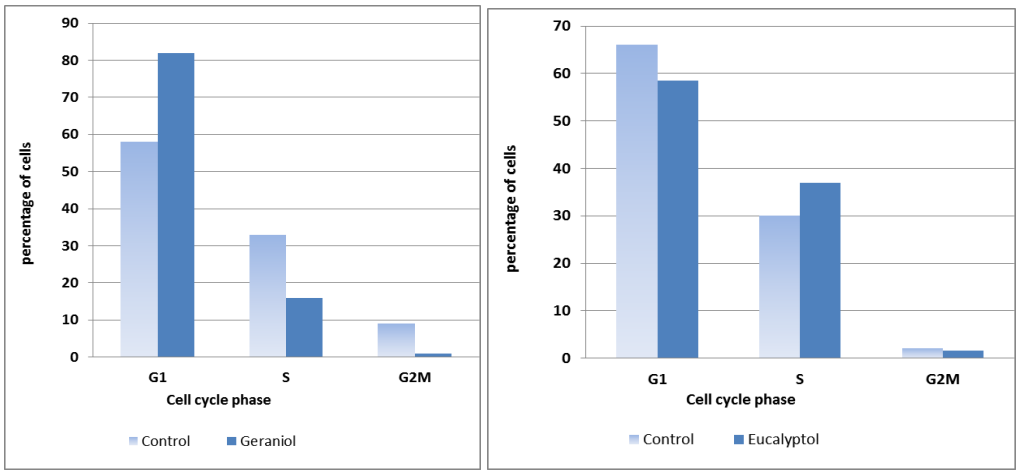
(J)


100
90
80
70
60
50
40
30
20
10
0
percentage of cells
G1
S
G2M
Cell cycle phase
Control
Carvacrol

(K)

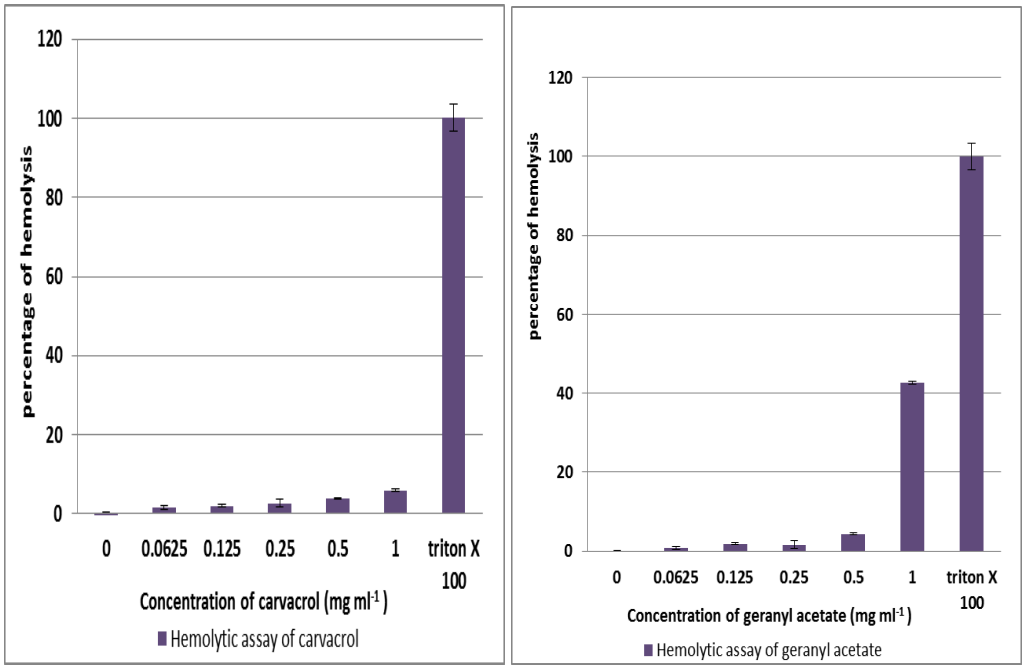

60
50
40
30
20
10
0
percentage of cells
G1
S
G2M
Cell cycle phase
Control
Limonene

(L)

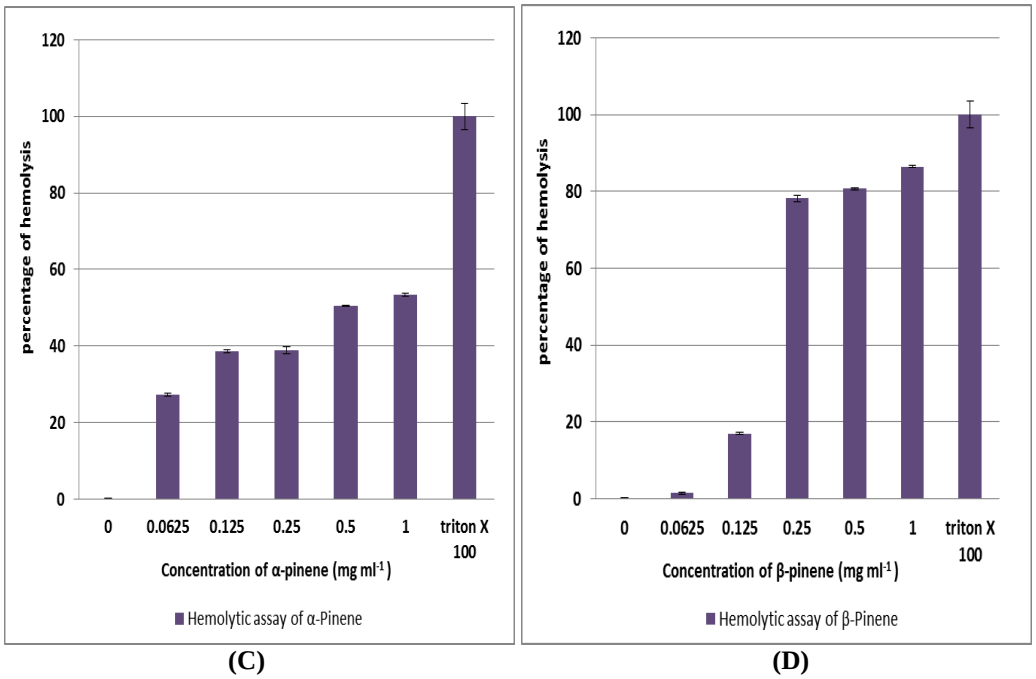


(M) **(N)**

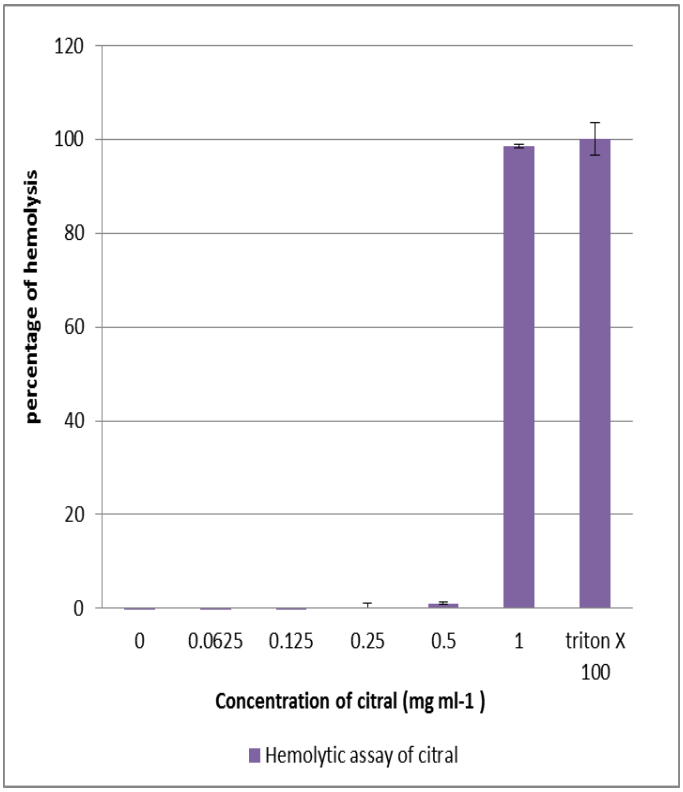
Fig 3: Effect of different phytomolecules on cell cycle progression of C. albicans 90028 Compared with control group (A) Citronellal (B) Terpinolene (C) Linallol (D) α Pinene (E) β Ionone (F) Nerol (G) Thymol (H) Eugenol (I) β Pinene (J) Citronellol (K) Carvacrol (L) Limonene (M) Geraniol (N) Eucalyptol



(A) **(B)**



(C) **(D)**


120
100
80
60
40
20
0
percentage of hemolysis
0
0.0625
0.125
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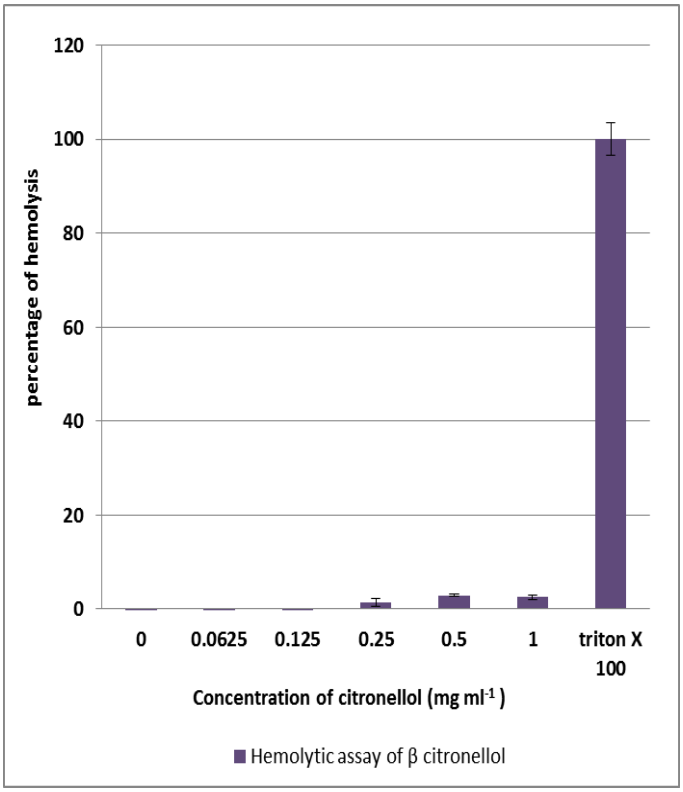
1
triton X 100
Concentration of citral (mg ml-1)
Hemolytic assay of citral

(E)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percentage of hemolysis
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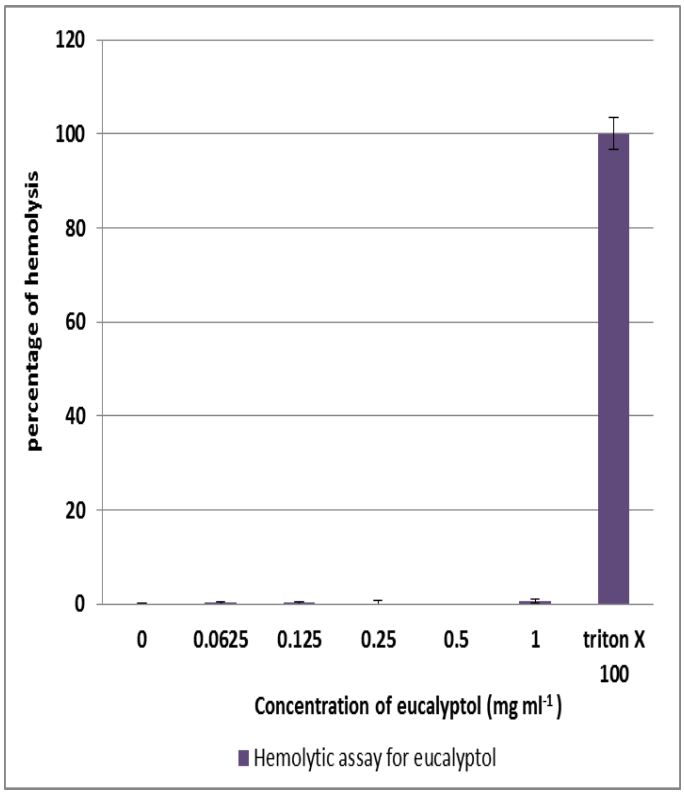
triton X 100
Concentration of citronellol (mg ml-1)
Hemolytic assay of β citronellol

(F)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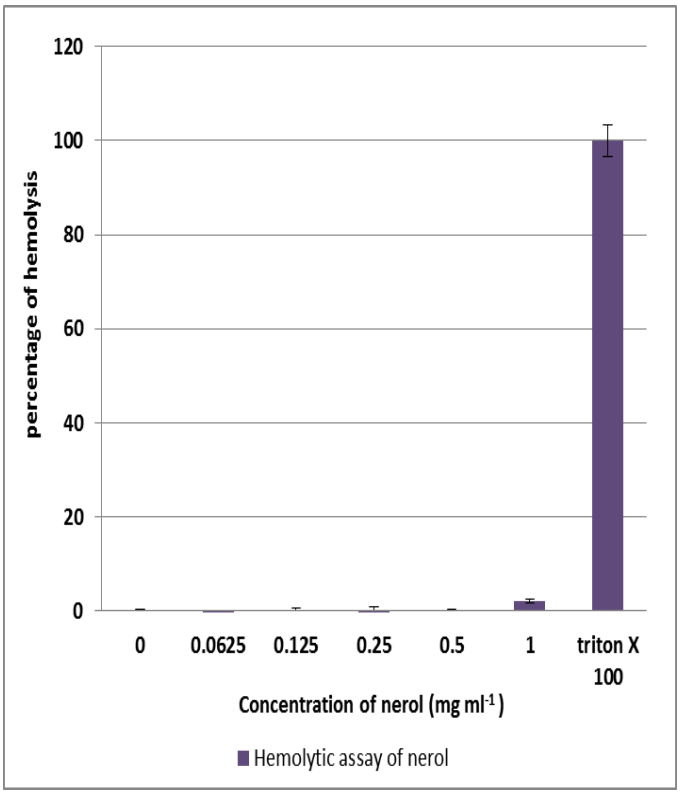
Concentration of eucalyptol (mg ml-1)
Hemolytic assay for eucalyptol

(G)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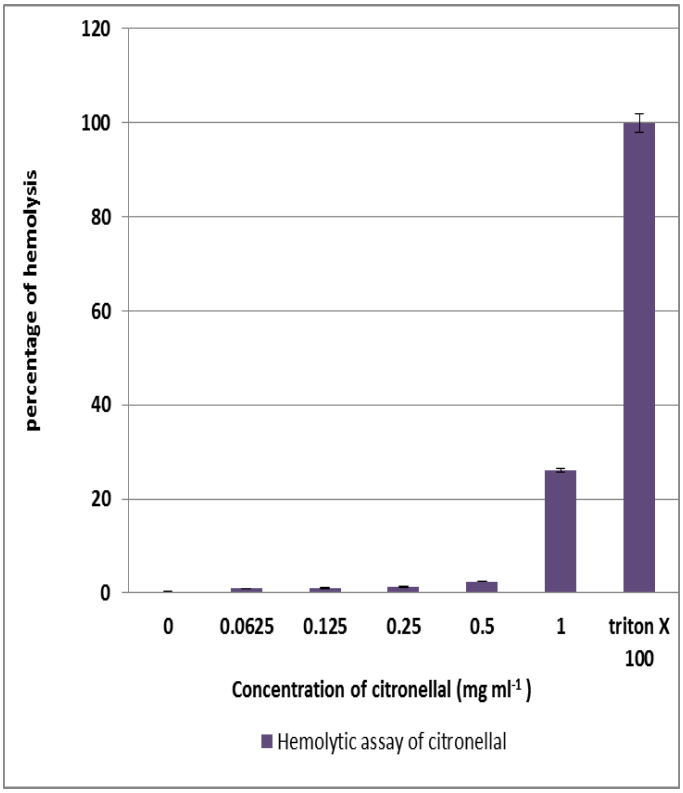
Concentration of nerol (mg ml-1)
Hemolytic assay of nerol

(H)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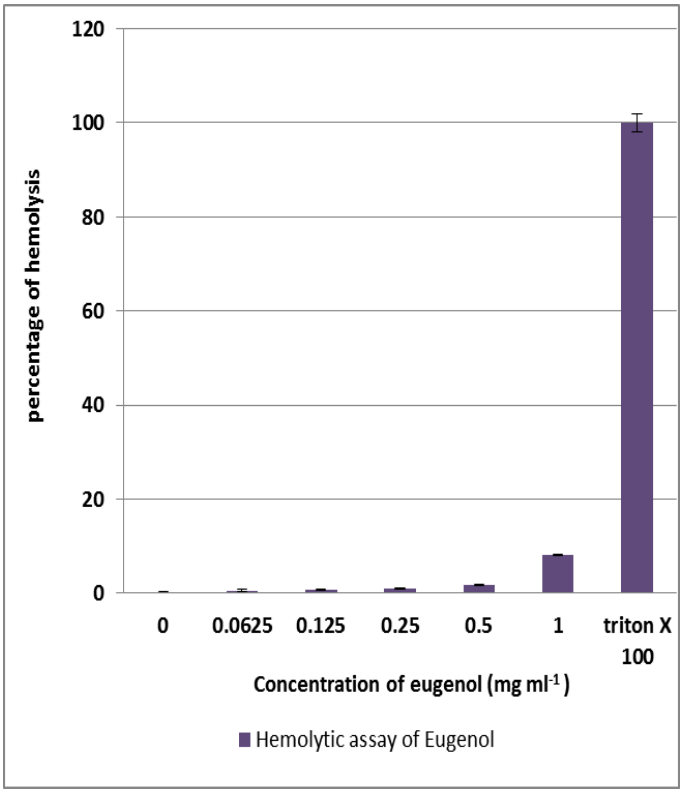
triton X 100
Concentration of citronellal (mg ml-1)
Hemolytic assay of citronellal

(I)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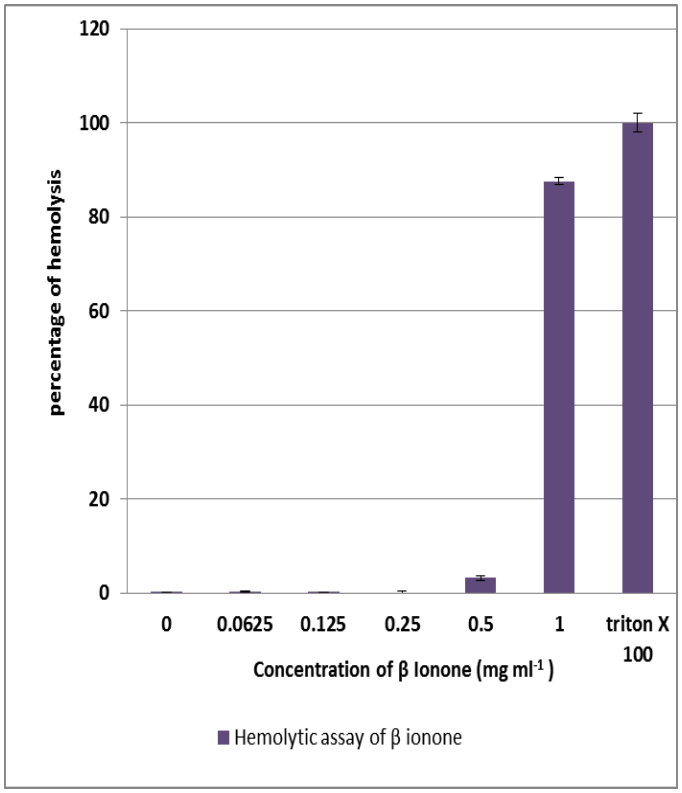
Hemolytic assay of Eugenol

(J)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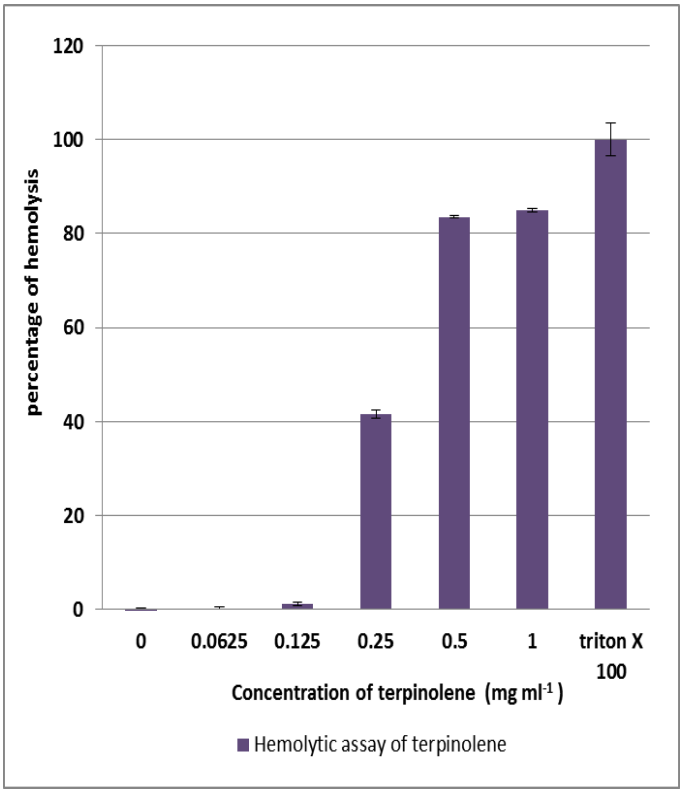
Concentration of β Ionone (mg ml-1)
Hemolytic assay of β ionone

(K)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percentage of hemolysis
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0.25
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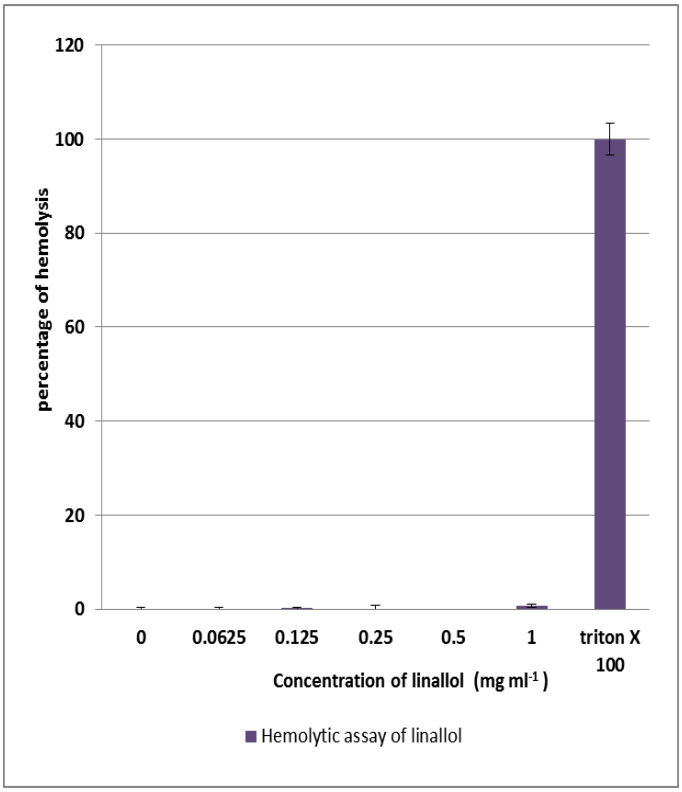
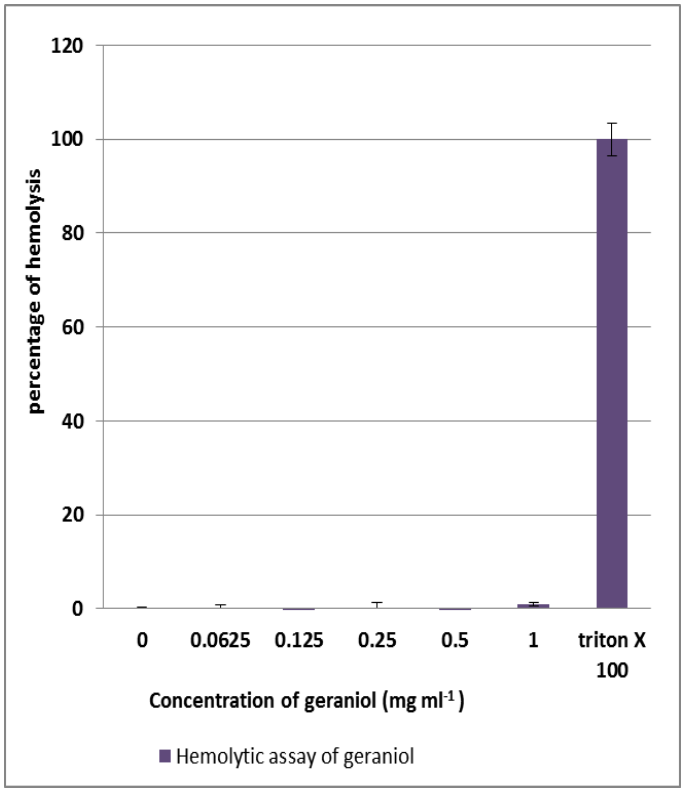
1
triton X 100
Concentration of terpinolene (mg ml-1)
Hemolytic assay of terpinolene

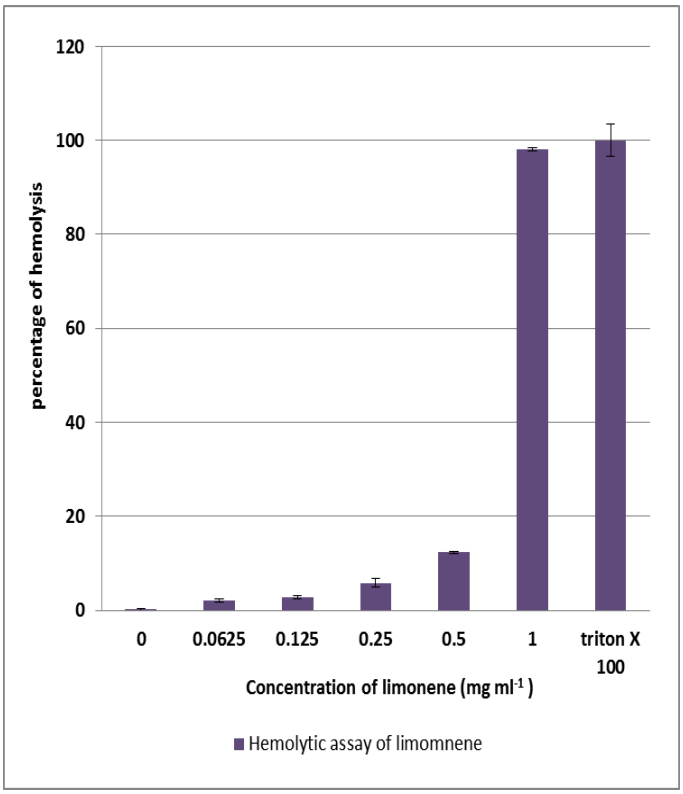
(L)



(M)



(N)



(O)

Fig 4: Hemolytic activity of different phytochemicals: (A) Carvacrol (B) Geranyl acetate (C) α Pinene (D) β pinene (E) Citral (F) Citronellol (G) Eucalyptol (H) Nerol (I) Citronellal (J) Eugenol (K) β Ionone (L) Terpinolene (M) Linallol (N) Geraniol (O) Limonene. Hemolysis was measured by absorbance reading at 450 nm and compared with hemolytic activity of Triton X-100. Error bars are showing mean ±SD of three experiments.

Table 1: Differential Sensitivities of multiple growth modes of *Candida albicans* ATCC 90028 to phytochemicals

Phytomolecules	MIC in mg/ml for Peripheral filamentous growth (PFG)	MIC in mg/ml for Invasive growth (IG)	Concentration in mg/ml for Total growth (TG) inhibition
Carvacrol	0.031	0.062	0.125
Eugenol	0.007	0.062	0.125
Geraniol	0.007	0.062	0.125
Geranyl acetate	0.031	0.5	1
β-Citronellol	0.015	NA	1
Nerol	0.015	NA	0.5
Citronellal	0.062	NA	1
Linallol	0.062	NA	1
Terpinolene	2.00	NA	NA
Farnesol	0.015	NA	0.031
Limonene	0.5	NA	NA
β Ionone	NA	NA	0.125
α Pinene	1	NA	NA
β Pinene	0.5	NA	NA
Citral	0.015	NA	0.125
Thymol	NA	NA	1
Flucanazole	0.125	0.25	2
Amphotericin B	0.25	0.25	1

Values indicated are MICs (Minimum Inhibitory Concentration) were the concentrations at which >50% inhibition of growth was obtained. These values (i.e. growth in control and treated with molecules) were compared using the Student's t –test, and a P value of < 0.05 was considered significant. (NA: MIC not achieved at highest concentration tested i.e. 4 mg/ml).

Table 2: Cell cycle arrest in *C. albicans* ATCC 90028 by different phytochemicals. Values indicated are the percentage of cell population accumulated in specific phases of cell cycle division.

Phytomolecules	Percentage of cell population arresting in G1 Phase	Percentage of cell population arresting S in Phase	Percentage of cell population arresting G2M in Phase
Terpinolene		04	
Limonene	07		
Eucalyptol		07	
Linallol	23		
Geraniol	24		
Eugenol		20	
Carvacrol		21	06
Citronellol		29	
β- Pinene	18		
Citronellal		26	
α - pinene	65		
β –ionone		70	07
Nerol		48	
Thymol	65		

4. Discussion

Inhibition of virulence factors such as yeast to hyphal form transition, biofilm formation and invasive growth are considered as a novel paradigm for antifungal drug development [2, 16]. This morphogenetic transformation is inhibited by various phytomolecules and small molecules. Studies done in our laboratory has identified a number of components of essential oils especially terpenoids as inhibitors of growth, yeast to hyphal form conversion and biofilm formation in *C. albicans* [28, 29]. The MIC values of a number of phytochemicals against yeast to hyphae transition are established by various workers [1]. However, studies related to drug sensitivities of various growth modes on solid medium of *C. albicans* remains neglected.

C. albicans exhibits different modes of growth on hyphal inducing solid medium. These different modes of growth forms include peripheral filamentous form and invasive form [35]. Filamentous growth in fungi is a differentiation process that can be triggered by various external stimuli. One of which is nutrient limitation. In our study, we used nutrient limiting media i.e. spider media (carbon deprivation media) which may induces hyphae formation through Efg1 pathway involving the Gpr1 G-protein-coupled receptor and its G-alpha subunit Gpa2 [19]. Filamentous growth may contribute to many of the pathogenic aspects of fungal pathogens such as host- cell attachment, invasion into tissues and virulence [10]. Peripheral filamentous colony is considered as colony which protrudes at least 20 filaments from its periphery [35]. Molecules such as Citral, Citronellal, Geranyl acetate, β-Citronellol, Geraniol, Linalool, Nerol, t, t-Farnesol, Carvacrol, β-pinene, Terpinolene, Limonene, α-pinene, and Eugenol inhibited filamentous growth in the range of 0.07 to 0.125 mg/ml (Table 01; Figure 02). Treatment with these resulted in colonies with smooth periphery and inhibited formation of radial colony. Radial colony usually indicates presence of mycelia cells while smooth colonies indicates yeast cell inside [20] Peripheral filamentation may help *Candida* to move away from metabolites like alcohol produced by the colony [8].

Invasive growth could be another antifungal target. Extensive studies by Gimeno *et al* (1992) and Zucchi *et al* (2010) provided considerable information on this mode of growth, based on *in vitro* studies [14,35]. Interestingly, there are no studies on molecules inhibiting invasive mode of growth except few studies [24]. We are reporting efficacy of sixteen phytochemicals of inhibition of IG *in vitro* model (Table 01; Figure 01). These molecules were selected based in their efficacy against *C. albicans* [29]. Another subtle mode of growth studied was filamentous mode of growth of *C. albicans* on solid spider medium plates. MIC values were established against IG, FG and total growth inhibition. Significantly, only four molecules inhibited invasive growth. Carvacrol, Eugenol and Geraniol showed most potent activity against invasive growth at the concentration 0.062 mg/ml while Geranyl acetate exhibited its effect against invasive growth at the concentration at 0.5 mg/ml (Table 01, Figure 01). Interestingly, these molecules allowed yeast phase growth but inhibit invasive growth. Rest of the twelve molecules failed to inhibit IG. This is very surprising. The reason for the resistance of IG is not known. IG was sensitive to Flucanazole and Amphotericin B. In addition, on the treatment with the higher concentration of phytochemicals *C. albicans* showed total growth inhibition suggesting that these molecules may be showing fungicidal activity. These three types of growth exhibited different sensitivity in the following order. Peripheral Filamentous growth > Invasive growth > Total growth inhibition

Invasive growth and filamentous growth may help *C. albicans* cells to move away from nutrients depleted or toxic metabolites environment of colonies in search for nutrients. Both could be target for drugs. More in detailed studies are required on the four molecules identified in this study. Dozens of molecules identified were not effective against IG and are reported as effective inhibitor of yeast to hyphae transition and biofilm formation [29]. Many of the phytochemicals are known to induce cell cycle arrest in eukaryotes [3, 21]. Cell cycle inhibition was exhibited by few of the molecules

(Figure 03). Geraniol was found to inhibit cell cycle in G_1 cycle whereas Eugenol and Carvacrol induced cell cycle arrest in S phase of cell cycle (Figure 03).

The exact mechanism of antifungal activities of these molecules is not clear. The inhibitory activity of terpenoids to *C. albicans* may be due to membrane damage and inhibition of oxidative phosphorylation and respiratory chain [22]. Carvacrol exerts anticandida activity by interfering with endoplasmic reticulum integrity resulting into the endoplasmic reticulum stress and activation of unfolded protein response [6]. Carvacrol also exerts antifungal effect by altering calcium homeostasis [27], ergasterol biosynthesis [1] and plasma membrane [7]. Eugenol is also known for its excellent fungicidal activities. It inhibits H^+ATP_{ase} activity of *C. albicans* cells that leads to intracellular acidification and cell death [1]. Eugenol inhibits amino acid permeases that are involved in dual transport of aromatic and branched chain amino acids in the yeast cytoplasmic membrane that may lead to leakage of cell contents [11]. In a study carried in *Saccharomyces cerevisiae*, Eugenol mediates alteration in Ca^{2+} homeostasis that leads to elevation of Ca^{2+} which a protective mechanism exerted by *S. cerevisiae* to protect against Eugenol toxicity [30]. Geraniol exerts ant candida effect by interfering with cell membrane that leads to increase in the rate of leakage of potassium ions. It does not show any prominent action on cell wall and erg sterol binding indicating that its probable mechanism does not involve action on cell wall [18].

The plant molecules selected in the study caused differential inhibition of various mode of growth of *C. albicans*. Based on these studies it can be hypothesized that the mode of action of phytochemicals against PFG and IG may be quite different. Molecular studies may reveal the mechanism of action of the four molecules identified as specific inhibitors of IG.

Inhibitors of invasive growth Carvacrol, Geraniol, Geranyl acetate and Eugenol produced negligible hemolysis at their MICs value of invasive and peripheral filamentous growth. Similarly, other molecules tested did not show considerable hemolysis at their MIC of peripheral filamentous growth. Suggesting that, these compounds may not cytotoxic at this concentration (Figure 04).

To conclude, this study for the first time report differential sensitivity of phytomolecules to different modes of growth of *C. albicans*. Different threshold concentrations are identified for different modes of growth. These molecules inhibited cell cycle at different stages of cell cycle. Cell cycle inhibition may be one of the major modes of cell cycle inhibition through which these molecules inhibit growth and morphologies like yeast to hyphal form transformation and invasive growth. Carvacrol, Eugenol, Geraniol and Geranyl acetate are the molecules that can be considered for drug development against candidiasis since they exhibit negligible hemolytic properties at their MIC values. However, *in vivo* and toxicity studies are required to be done to for further development.

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