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# Mechanistic insights into the mode of action of anticandidal sesamol

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ABSTRACT

Previously we have deciphered the antifungal effect of sesamol (Ses), a phenolic compound obtained from sesame oil, against human fungal pathogen *Candida albicans*. To gain deeper insights into the possible mechanisms involved, transcription profiling was done in presence of Ses which revealed various targets through which Ses was barricading the growth of *C. albicans*. We observed that Ses perturbs membrane integrity confirming our previous observations and displayed disrupted plasma membrane ATPase activity. We further investigated that Ses leads to inhibited morphological transition, biofilm formation and epithelial cell adhesion which are significant virulence attributes required for pathogenesis. Interestingly, Ses also causes amendment in iron homeostasis as revealed by hypersensitivity under iron deprivation, ferroxidase assay to estimate iron levels and concomitant upregulation of *FTR2*, a high affinity iron transporter. Finally we assessed that Ses causes defect in mitochondrial functioning and DNA repair mechanism. Together, being source of consumable natural product, further studies on Ses are warranted so that it can be exploited as effective antifungal agent.



## 1. Introduction

From ages, fungal pathogens are exploiting the human host by causing superficial to deep seated fungal infections. *Candida albicans* in particular is a major threat among all fungal infections causing high mortality worldwide and is most common culprit for nosocomial fungal infections. The opportunist *C. albicans* becomes pathogenic among immunocompromised patients such as AIDS, cancers, organ transplantations [1]. *C. albicans* consists of explicit virulence traits that endure against the current antifungal therapy. Moreover, antifungal drug resistance is another illustrious dilemma faced by the current antifungals [2] hence searching novel compounds from natural sources having limited side effects is the need of hour.

Sesamol (Ses) is a natural phenolic compound isolated from sesame oil and already reported as potential anticandidal agent against *C. albicans* [3]. Recently acute oral toxicity in C57BL/6 mice was studied which shows that Ses is not toxic [4]. The present study aimed to expand our knowledge and understanding of the mechanistic action of Ses against most prevalent human fungal pathogen, *C. albicans*. The transcriptional profiling in response to Ses exposes several potential targets such as membrane homeostasis, morphogenetic switching, biofilm formation, cell adhesion, iron homeostasis, mitochondria and DNA repair.

## 2. Materials and methods

All Media chemicals YEPD (Yeast Extract Peptone Dextrose), nutrient broth, yeast nitrogen base w/o amino acid and ammonium sulphate (YNB w/o amino acid and ammonium sulphate), agar, horse serum, N-Acetyl glucosamine, osmium tetroxide ($OsO_4$), hexamethyldisilizane (HMDS), glutaraldehyde, propidium iodide were purchased from Himedia (Mumbai, India). Sodium chloride (NaCl), di-sodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, di-potassium hydrogen orthophosphate, D-glucose, methanol, dimethyl sulphoxide (DMSO) were obtained from Fischer Scientific. Tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from SRL, Mumbai. Sesamol (Ses) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.1. Growth media and strains used in this study

All the strains of *C. albicans* (Table S1) were cultured in YEPD broth with the composition of yeast extract 1% (w/v), peptone 2% (w/v) and dextrose 2% (w/v). For agar plates, 2% (w/v) agar was

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added to the media. The cells were freshly revived on YEPD broth and transferred to agar plate before each study to ensure the revival of the strains.

### 2.2. Spot assay

Spot assays for all strains were determined using a method as described earlier [3,5]. Briefly, for the spot assay 5 μL of fivefold serial dilutions of each yeast culture (each with cells suspended in normal saline to an $OD_{600}$ nm of 0.1) was spotted onto desired media plates in the absence (control) and presence of the Ses. Growth was not affected by the presence of solvent (methanol/water 2:1) used in the study (data not shown). Growth difference was measured after incubation at 30 °C for 48 h. The growth media and concentrations of drugs used in this study are specified in figure legends.

### 2.3. RNA isolation

Isolation of RNA from *C. albicans* was carried out by using combination of Trizol and Qiagen RNeasy mini kit with DNase treatment. The cells were diluted into 50 ml fresh YEPD broth at $OD_{600}$ of 0.1 ($10^6$ cells $ml^{-1}$) in absence and presence of Ses (552 μg $ml^{-1}$) and grown at 30 °C till $OD_{600}$ of 1.0. Cells were harvested by transferring the cells into a centrifuge tube. Three separate experimental replicate cultures of each condition were used. Lysate was prepared by Trizol, chloroform was added to lysate and samples were subject to centrifugation. The supernatant was taken from previous step and after adding ethanol to the supernatant, the mixture was loaded on Qiagen column and Manufacturer's guidelines were followed from DNase treatment step. Purity (Ratio of 260/280 and 260/230) and Concentration was assessed by NanoDrop 1000. Integrity of RNA was assessed on Agilent Bioanalyzer 2100.

### 2.4. cDNA synthesis and hybridization

For RT-PCR the cDNA was converted with the cDNA synthesis kit (RevertAid H minus first strand cDNA synthesis kit) from the RNA isolated by trizole method from the exponentially growing culture of *C. albicans*. The samples for Gene expression were labeled using Agilent Quick-Amp labeling Kit (p/n5190-0442). 500 ng each of total RNA were reverse transcribed at 40 °C using oligo dT primer tagged to a T7 polymerase promoter and converted to double stranded cDNA. Synthesized double stranded cDNA were used as template for cRNA generation. cRNA was generated by in vitro transcription and the dye Cy3 CTP(Agilent) was incorporated during this step. The cDNA synthesis and in vitro transcription steps were carried out at 40 °C. Labeled cRNA was cleaned up using Qiagen RNeasy columns and quality assessed for yields and specific activity using the Nanodrop ND-1000. 1000 ng of labeled cRNA sample were fragmented at 60 °C and hybridized on to a Genotypic designed *Candida albicans*_8 × 15K (AMADID: 026377). Fragmentation of labeled cRNA and hybridization were done using the Gene Expression Hybridization kit (Agilent Technologies, In situ Hybridization kit, Part Number 5190-0404). Hybridization was carried out in Agilent's Surehyb Chambers at 65° C for 16 h.

### 2.5. Scanning and data analysis

The hybridized slides were washed using Agilent Gene Expression wash buffers (Agilent Technologies, Part Number 5188-5327) and scanned using the Agilent Microarray Scanner (Agilent Technologies, Part Number G2600D). Data extraction from images was done using Feature Extraction software Version 11.5.1.1 of Agilent. Images were quantified using Feature Extraction Software (Version-11.5, Agilent). Feature extracted raw data was analyzed using GeneSpring GX Version 12.0 software from Agilent. Normalization of the raw data was done in GeneSpring GX using Quantile method (Quantile normalization makes the distribution of expression values of all samples similar in an experiment.) After, normalization all statistical parameters of the sample i.e., mean, median and percentile of all samples will be identical. It works well with reducing variance between arrays. Differential expression patterns were identified among the samples. Significant genes up regulated fold >1.5 ($log_2$) and down regulated < −1.5 ($log_2$) in the test samples with respect to control sample were identified. Statistical student T-test and *P* value among the replicates was calculated based on volcano plot algorithm. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns. The Significant Functional classification of differentially regulated genes was performed using GeneSpring GX software gene ontology. Microarray data used in this study is fully described in GEO and the raw as well as normalized data files have been deposited under accession number GSE83080.

### 2.6. RT-PCR

For validation of the microarray results, reverse transcriptase (RT) PCR was done as described in the RevertAid H Minus kit (Invitrogen). Briefly, 5 μg isolated RNA was DNase treated at 37 °C for 30 min and reaction was terminated by adding 1 μl of 25 mM EDTA and incubated at 65 °C for 60 min. RNA was subsequently primed with oligo $(dT)_{18}$ for cDNA synthesis at 42 °C for 60 min. Reverse transcription reaction was terminated by heating at 70 °C for 5 min. The synthesized cDNA product (2 μl) was directly used for PCR amplification reaction (50 μl) using gene specific forward and reverse primers (Table S2). The amplified products were gel electrophoresed and the densities of bands (for genes of interest) were measured and quantified by normalizing to that of the constitutively expressed actin gene (*ACT1*).

### 2.7. PM-ATPase mediated proton pumping

The proton pumping activity of *C. albicans* was estimated by monitoring the glucose-induced acidification of the external medium due to pH changes as previously reported [5]. Overnight cultures of *C. albicans* were grown in YEPD broth for 18 h at 30 °C. The cells were collected by centrifugation at 3000 × g for 5 min at 4 °C and washed with sterile distilled water and 50 mM KCl (pH 6.5). The washed cells were resuspended in 40 ml of 50 mM KCl (pH 6.5) and incubated at 4 °C overnight to deplete their carbon reserves. The carbon-starved cells were harvested by centrifugation, and approximately 1.0 g wet weight of the pellet was resuspended in 40 ml of 50 mM KCl (pH 6.5). To a 40 ml aliquot of the cell suspension, Ses at $MIC_{80}$ was added to obtain the required concentration and mixed well, and the volume was adjusted to 45 ml with 50 mM KCl. The cell suspension was incubated at room temperature with gentle stirring for 10 min, and then 5 ml of 20% glucose (final concentration, 55 mM) was added and the pH of the external medium was monitored at regular intervals for 60 min at indicated time points.

### 2.8. Intracellular pH (pHi)

Intracellular pH was measured as described previously [5]. Mid-log phase cells grown in YEPD medium were harvested and washed twice with distilled water. Cells (0.1 g) were suspended in 5 ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$. Desired