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Major Article

Insights into the intracellular mechanisms of citronellal in *Candida albicans*: implications for reactive oxygen species-mediated necrosis, mitochondrial dysfunction, and DNA damage

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Abstract

Introduction: Citronellal (Cit) possesses antifungal activity and has possible implications for reactive oxygen species (ROS) generation in *Candida albicans*. In this study, the effects of Cit on ROS generation and the mechanisms by which Cit exerts anti-*Candida* effects were examined. **Methods**: A 2′,7′-dichlorodihydrofluorescein diacetate assay was used to assess oxidative damage. Cell necrosis was determined by flow cytometry after FITC-Annexin V staining. Mitochondrial function was studied based on mitochondrial potential, metabolic activity (MTT assay), and phenotypic susceptibility on a non-fermentable carbon source. Membrane intactness and DNA damage were estimated by a propidium iodide (PI) uptake assay and 4',6-diamidino-2-phenylindole (DAPI) staining. **Results**: ROS generation was enhanced in response to Cit, leading to necrosis (2%). Additional hallmarks of cell death in response to Cit, such as mitochondrial membrane depolarization and DNA damage, were also observed. Cit treatment resulted in dysfunctional mitochondria, as evidenced by poor labeling with the mitochondrial membrane potential-sensitive probe rhodamine B, reduced metabolic activity (61.5%), and inhibited growth on a non-fermentable carbon source. Furthermore, Cit induced DNA damage based on DAPI staining. These phenotypes were reinforced by RT-PCR showing differences in gene expression (30-60%) between control and Cit-treated cells. Finally, PI uptake in the presence of sodium azide confirmed non-intact membranes and suggested that Cit activity is independent of the energy status of the cell. **Conclusions**: Cit possesses dual anticandidal mechanisms, including membrane-disruptive and oxidative damage. Taken together, our data demonstrated that cit could be used as a prominent antifungal drug.

Keywords: Reactive oxygen species. Necrosis. Mitochondria. DNA. Propidium iodide. *Candida albicans.*

INTRODUCTION

The opportunistic pathogen *Candida albicans* is normally a commensal organism in humans, but in immunocompromised conditions, such as in patients with AIDS, diabetes, organ transplantation, and cancer, it causes mucosal, cutaneous, or invasive mycosis[1]. Prolonged usage of antifungals has recently, in some instances, led to the emergence of multidrug-resistant strains of *Candida*[2]. In addition, high costs and severe toxicity along with stagnation in the development of new drugs have become major impediments to effective antifungal therapy. Thus, phytochemical research has steadily increased, owing to their safety profiles and low side effects, with promising results[3].

Citronellal (Cit) is a monoterpenoid (3,7-dimethyloct-6-en-1-al) found in *Cymbopogon* plants. It gives the characteristic lemon-scented aroma to citronella oil. The anticandidal potential of Cit has been demonstrated; it primarily targets fungal cell membranes and inhibits virulence traits, such as morphological shifts and biofilm formation, and it may promote the generation of ROS[4]. This study aimed to add to the existing literature about ROS generation in Cit-treated cells and elucidate other potential targets of Cit in *C. albicans*. The results confirmed that Cit increases ROS levels and elicits cell necrosis, mitochondrial dysfunction, and deoxyribonucleic acid (DNA) damage in *C. albicans*. We also showed that the energy status of the cell is not important for the antifungal mechanisms of Cit.

METHODS

All media chemicals, Yeast Extract Peptone Dextrose (YPD), and propidium iodide (PI) were purchased from Himedia (Mumbai, India). Citronellal, DCFDA (2,7-dichlorofluorescein diacetate), DAPI (4',6-diamidino-2-phenylindole), the Annexin V–FITC Apoptosis Detection Kit, and Zymolyase 2000T,

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primerswere obtained from Sigma Chemicals (St. Louis, MO, USA) and IDT (San Diego, CA, USA). Rhodamine B was purchased from CDH (Delhi, India) and sodium azide (NaN_3) was purchased from Fischer Scientific (Waltham, MA, USA).

Growth media and strains

The laboratory *Candida* strain SC5314 was used; its whole genome was sequenced in 2004 and it is considered the most widely used laboratory strain. It was cultured in YPD broth with yeast extract 1% (w/v), peptone 2% (w/v), and dextrose 2% (w/v). For agar plates, 2% (w/v) agar was added to the media. *Candida* strains were stored in 30% (v/v) glycerol stock at -80°C. The cells were freshly revived twice on YPD broth and transferred to agar plates. The cells were grown overnight at 30°C on agar plates before each analysis to ensure the revival of the strains.

Phenotypic susceptibility assay

Phenotypic susceptibility assays were performed as described elsewhere[4]. Briefly, for the spot assay, 5µL of fivefold serially diluted yeast cultures (cells suspended in normal saline to an OD_{600} of 0.1 (1×10^6 cells mL^{-1}) were spotted on YPD plates in the absence (control) and presence of Cit (250µg mL^{-1}). Growth was not affected by the presence of a solvent (dimethyl sulfoxide) (data not shown). Growth differences were measured after incubation for 48h at 30°C.

RT-PCR

Ribonucleic acid (RNA) was isolated and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described earlier[5]. In brief, cells were diluted in 50mL of fresh YPD broth at OD_{600} of 0.1 (10^6 cells mL^{-1}) in the absence (control) and presence of Cit (250µg mL^{-1}) and grown at 30°C until reaching OD_{600} of 1.0. RNA isolation was performed by the TRIzol method and reverse-transcriptase (RT) PCR was performed using the RevertAid H Minus Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Briefly, 5µg of isolated RNA was DNase-treated at 37°C for 30 min and the reaction was terminated by adding 1µL of 25mM 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 was terminated by heating at 70°C for 5 min. The synthesized cDNA product (2µL) was directly used for PCR amplification (50µL) using gene-specific forward and reverse primers The amplified products were separated by electrophoresis and the densities of bands (for genes of interest) were measured and normalized to that of the constitutively expressed actin gene (*ACT1*).

Analysis of apoptotic markers

Candida albicans spheroplasts were prepared as described earlier[5]. Briefly, Cit-treated (MIC_{80}) cells were incubated with Zymolyase (100U/g wet weight) at 37°C for 3h and vortexed to remove the cell wall. Spheroplast preparation was monitored by adding 0.2% sodium dodecyl sulfate to lyse the enzyme-digested cells and visualized under a microscope (Olympus, Mumbai, India). Cell necrosis was determined by the externalization of phosphatidyl serine (PS), an apoptotic marker, using a Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit. (Sigma Chemicals). Cells were analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA) using a 488-nm excitation wavelength and a 515-nm band pass filter for FITC detection and a filter of >560nm for PI detection. A total of 10,000 events were counted at the flow rate. Data analysis was performed using Summit v4.3 (Beckman Coulter).

Fluorescence microscopy

Experimental cultures were grown in the presence of Cit at 30°C for 3h with shaking. Cells were harvested and washed three times with phosphate-buffered saline (PBS) stained with PI (1µg mL^{-1}), an indicator of membrane damage, DCFDA (1µg mL^{-1}), an indicator of ROS generation, Rhodamine B (0.5µg mL^{-1}), an indicator of mitochondrial membrane potential ($\Delta\Psi m$), and DAPI (2µg mL^{-1}), an indicator of DNA damage, for 20 min in the dark[6-8]. For PI experiments, *Candida* cells were pre-incubated with 5mM NaN_3 for 60 min. Subsequently, the unbound dye was removed by washing with the same buffer. Specimens were then examined under a Cos Lab fluorescence microscope (Ambala Cantt, India).

Hemolytic activity assay

The hemolytic activity of Cit was assayed as described elsewhere[9], with modifications. In brief, fresh human red blood cells (hRBCs) collected in the presence of an anti-coagulant from a healthy volunteer (author donated voluntarily) were washed three times in PBS. The drug was added to the suspension of red blood cells (4%, v/v) in PBS to a final volume of 1mL and incubated at 37°C for 35 min. The samples were then centrifuged for 2 min at 2000rpm, and the release of hemoglobin was monitored by measuring the absorbance (A_{sample}) of the supernatant at 540nm. For negative and positive controls, hRBCs in PBS (A_{blank}) and in 1% (final concentration v/v) Triton X-100 were used, respectively. The percentage of hemolysis was calculated according to the following equation: $\text{Hemolysis (\%)} = [(A_{sample} - A_{blank})/(A_{Triton} - A_{blank})]*100$.

RESULTS

Cit induces ROS generation, leading to necrosis in *Candida albicans*

We first confirmed the effect of Cit on ROS production in *C. albicans*. This was achieved using DCFDA, an oxidant-sensitive probe, to assess ROS generation; in this analysis, increased fluorescence within cells indicates enhanced ROS levels[6]. As expected, the fluorescence in Cit-treated cells was considerably higher than that in untreated cells. The amplified fluorescence intensity was reverted when the treated cells were incubated with an antioxidant, such as ascorbic acid (**Figure 1A**). Differential expression of genes associated with oxidative stress, i.e., *SOD2* (Superoxide dismutase 2), *CAP1* (Adenylate Cyclase Associated Protein 1), and *GPX2* (Glutathione Peroxidase 1), was quantified by RT-PCR and these results were consistent with those for ROS levels (**Figure 1B**).

We further determined whether Cit-mediated ROS accumulation leads to apoptosis or necrosis in *Candida* cells.

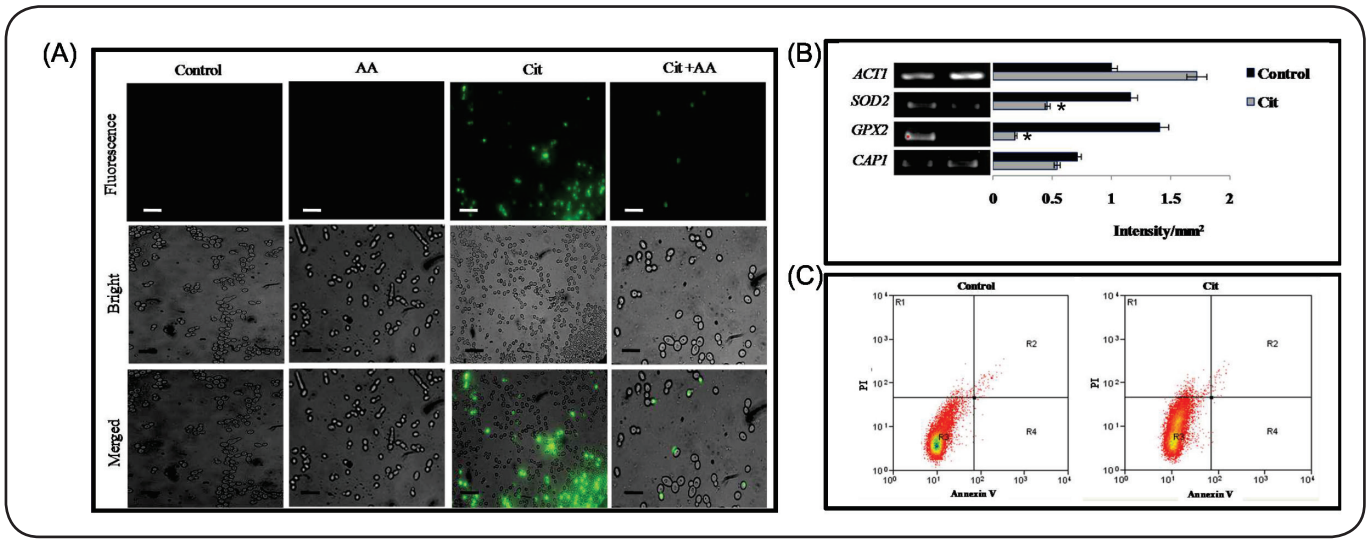

FIGURE 1 - Effect of Citronellal on ROS generation and necrosis. **(A)** Fluorescent microscopy of DCFDA for the detection of increased ROS in the presence of Cit, and reversion to normal levels after treatment with **AA**. Scale bar depicts 20µm. **(B)** RT-PCR of differentially regulated genes in response to Cit. The left panels show transcript levels of *SOD2*, *CAP1*, and *GPX2* in Control cells (**Lane 1**) and Cit-treated cells (250µg/mL) (**Lane 2**). The right panel shows the quantitation (density expressed as intensity/mm^2) of the respective transcripts normalized against constitutively expressed *ACT1* transcripts. **(C)** Measurement of apoptosis or necrosis by tracking FITC and PI in the absence (control) or presence of Cit (1mgmL^{-1}). Cit treatment showed necrosis in 2% of cells**. AA:**ascorbic acid**; Cit:** Citronellal; **ACT1:** Actin 1; **SOD2:** superoxide dismutase 2; **GPX2:** glutathione peroxidase 1**; CAP1:** adenylate cyclase associated protein 1**; ROS**: Reactive Oxygen Species; **DCFDA:** 2',7' –dichlorofluorescin diacetate; **RT-PCR**: Reverse Transcriptase Polymerase Chain Reaction; **FITC:** Fluorescein isothiocyanate ; **PI**: Propidium Iodide.

FITC labeling indicates the presence of late apoptotic cells, while PI labeling confirms the presence of necrotic cells[7]. Our flow cytometry analysis revealed that almost 2% cells underwent necrosis following Cit treatment, with no apoptosis (**Figure 1C**).

Cit leads to dysfunctional mitochondria

We subsequently evaluated the role of mitochondria in Cit-induced cell death. We examined mitochondrial membrane potential (ΔΨm) by employing a Rhodamine B probe[8]. Exposure to Cit resulted in substantial hyperpolarization of mitochondria in *C. albicans,* confirming mitochondrial dysfunction (**Figure 2A**). Next, we studied the activity of mitochondrial dehydrogenases, which are indicators of the metabolic activity of functional mitochondria[10]. Our results showed that Cit had inhibitory effects on the activity of mitochondrial dehydrogenases in *C. albicans* (**Figure 2B**). Finally, even at a non-lethal Cit concentration (250µg mL^{-1}), cells were unable to grow when non-fermentable glycerol was provided as the sole carbon source (**Figure 2C**).

Anticandidal activity of Cit is independent of energy status

NaN_3 blocks intracellular ATP by inhibiting cytochrome oxidase[11]. The effect of the cellular energy status was evaluated by estimating PI uptake, which binds to nucleic acids only in injured membranes, as in Cit-treated cells. We observed that cell viability was substantially reduced in the presence of Cit, regardless of the presence of NaN_3 (**Figure 3A**). Moreover, the downregulation of *NPC2* (NPC Intracellular Cholesterol Transporter 2), a gene involved in sterol transport, further confirmed the loss of membrane function (**Figure 3B**).

Cit induces DNA damage

To confirm DNA damage, we utilized the DAPI staining method. DAPI binds to AT sites within the minor groove of DNA, where its fluorescence can be assessed as a direct indicator of DNA damage. During ROS formation, the permeability of the dye increases, resulting in deep blue nuclear fluorescence. *C. albicans* cells exposed to Cit exhibited enhanced fluorescence, indicating DNA damage (**Figure 4A**). We further explored whether DNA damage in the presence of Cit was related to defects in DNA repair mechanisms. Based on RT-PCR, the downregulation of *MSH6* (MutS protein homolog 2) was involved in DNA repair (**Figure 4B**).

Hemolytic activity of Cit

The hemolytic activity of Cit against human erythrocytes is a major parameter related to the antifungal toxicity against human cells. Accordingly, hRBCs were isolated and hemolysis was evaluated in the presence of Cit. We observed only 15% hemolysis at the MIC of Cit in comparison to Triton X (control), showing 100% hemolysis (**Figure 5**).

DISCUSSION

The results of this study elucidate the oxidative stress mechanism induced by Cit. The induction of oxidative stress contributes to the anticandidal activity of Cit, possibly via the augmentation of excessive ROS production[4]. Our fluorescence microscopy-mediated DCFDA assay confirmed that ROS production is enhanced in the presence of Cit (**Figure 1A**). Moreover, *SOD2*, *CAP1,* and *GPX2* were downregulated (**Figure 1B**) in cells treated with Cit, validating that the

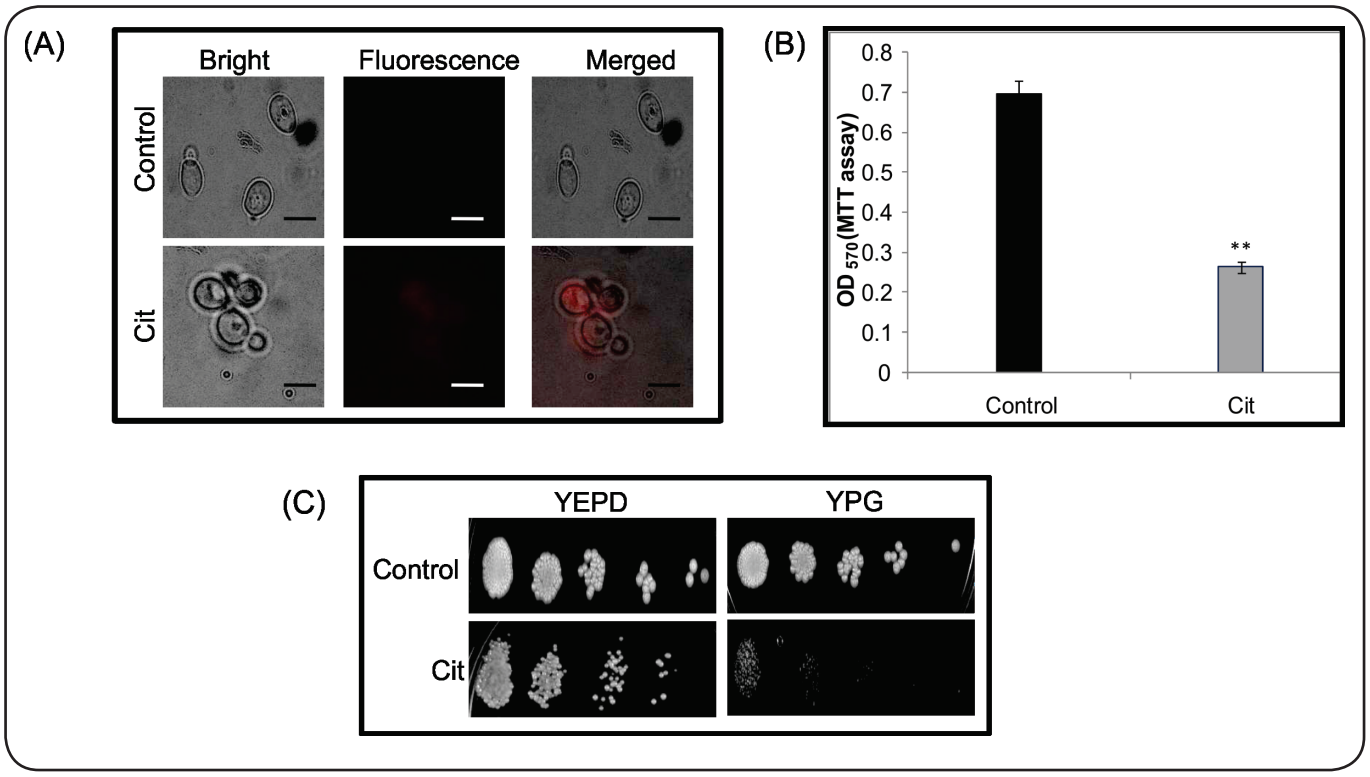

FIGURE 2 - Effect of Citronellal on mitochondrial functioning. **(A)** Fluorescent microscopy of Rhodamine B for the analysis of mtΨm in the presence of Cit. Control shows the quenching of fluorescence in comparison to Cit-treated cells, which show clear fluorescence, suggesting a change in the mitochondrial membrane potential. Scale bar depicts 10 µm. **(B)** Effect of Cit (250µgmL^{-1}) on mitochondrial activity in *Candida. albicans* based on an MTT assay. Mean OD_{570} of three independent sets of experiments is depicted on the *Y*-axis. **(C)** Phenotypic susceptibility assays in the absence (control) and presence of Cit (250µgmL-1) in YPD (fermentable carbon source) and YPG (Yeast Extract-Peptone-Glycerol) media (non-fermentable carbon source). **YEPD:** Yeast extract Peptone Dextrose; **YPG:** Yeast Extract Peptone Glycerol; **Cit**: Citronellal; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PI:** Propidium Iodide**.**

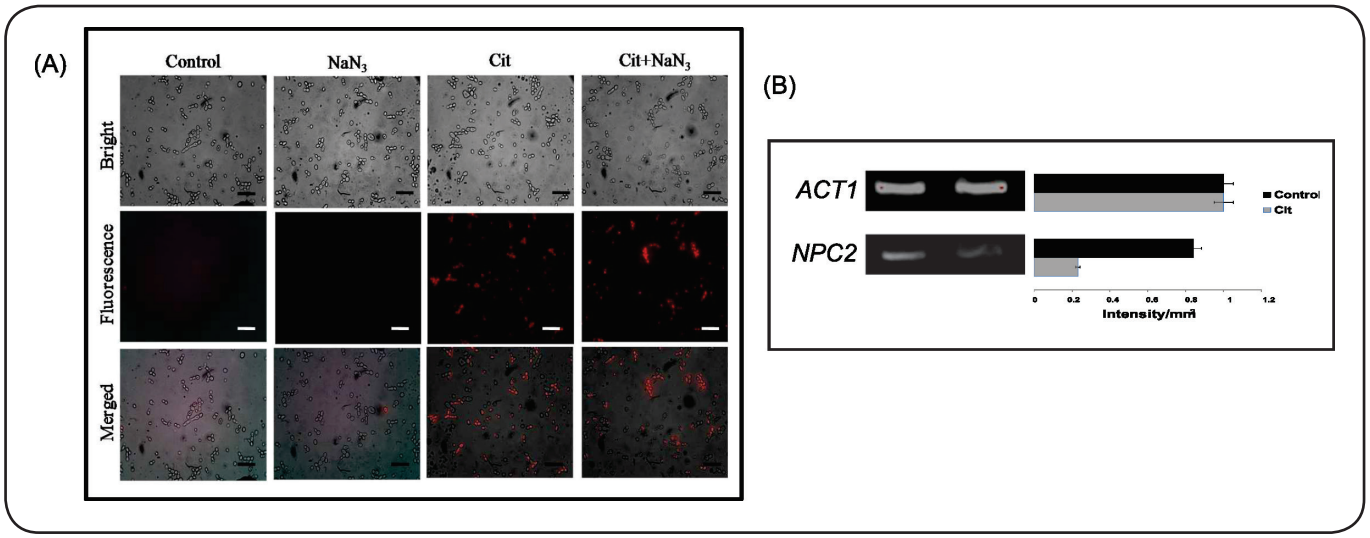

FIGURE 3 - Effect of energy depletion on anticandidal activity of Citronellal. **(A)** Fluorescent microscopy of PI for the detection of membrane damage in the presence of Cit. Scale bar depicts 25µm. Cells were pre-incubated with 5mM NaN_3 at 30°C for 60 min. Fluorescence in NaN_3+Cit-treated cells suggests that antifungal activity of Cit is energy-independent. **(B)** RT- PCR of *NPC2* in response to Cit. The left panels show transcript levels of *NPC2* in Control cells (**Lane 1**) and Cit-treated cells (250µg/mL) (**Lane 2**). The right panel shows the quantitation (density expressed as intensity/mm^2) of the respective transcripts normalized against constitutively expressed ACT1. **NaN3:** Sodium Azide**; Cit:** Citronellal; ***ACT1****:* Actin 1; ***NPC2*****:** NPC Intracellular Cholesterol Transporter **2; RT-PCR:** Reverse Transcriptase Polymerase Chain Reaction.

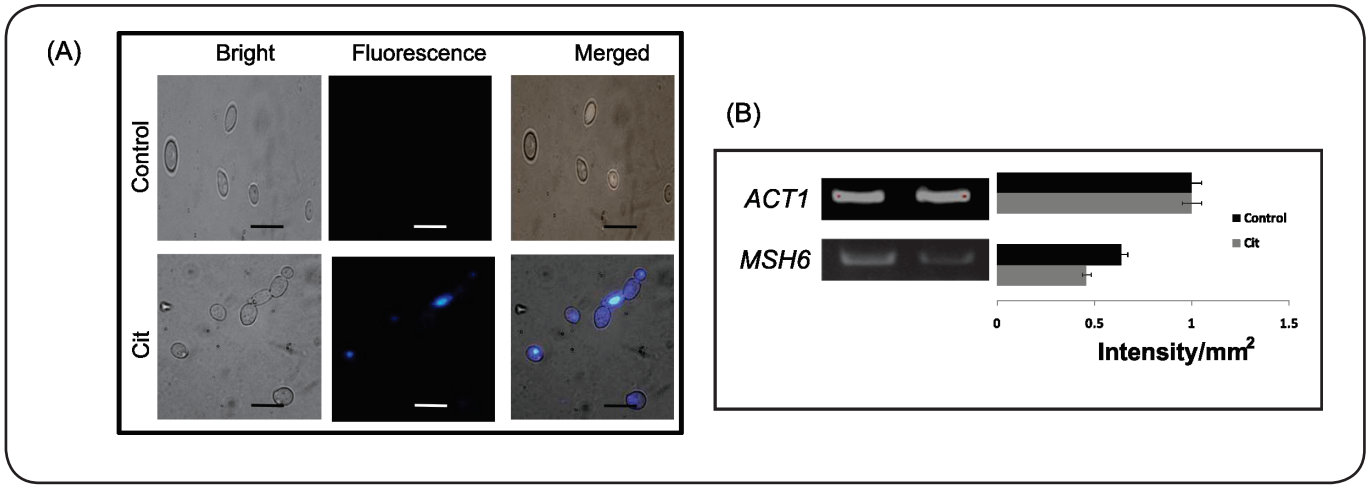

FIGURE 4 - Effect of Citronellal on DNA damage. **(A)** Fluorescent microscopy of DAPI for the detection of DNA damage in the presence of Cit. Scale bar depicts 10µm. **(B)** RT-PCR of *MSH6* in response to Cit. The left panel shows transcript levels of *MSH6* in Control cells (**Lane 1**) and Cit-treated cells (250µg/mL) **(Lane 2**). The right panel shows the quantitation (density expressed as intensity/mm^2) of the respective transcripts normalized against constitutively expressed *ACT1* transcripts. ***ACT1****:* Actin 1**;** ***MSH6*****:** MutS protein homolog 2; **DNA:** deoxyribonucleic acid**; DAPI:** 4',6-diamidino-2-phenylindole; **Cit:** Citronellal**; RT-PCR:** reverse transcriptase polymerase chain reaction.

effects of Cit against *C. albicans* involve ROS production. Sod2p (superoxide dismutase 2 protein) is a manganese-dependent mitochondrial protein that plays an essential role in protection against oxidative damage[12]. Further, Cap1p, which is homologous to *Saccharomyces cerevisiae* Yap1p, is responsible for the activation of genes related to oxidative stress. When ROS accumulates, Cap1p oxidizes and accumulates in the nucleus, further activating genes carrying Cap1 response elements in their promoters[13]. However, Yap1p is not directly oxidized, but needs a glutathione peroxidase (Gpx)-like protein to transduce Yap1p signaling by creating a disulfide bond between Gpx3p and Cys36 (evolutionarily conserved redox-sensitive residues) and Yap1p with Cys598[14]. It is well-known that ROS accretion plays a pivotal role in the necrosis of yeast cells[6]. For instance, natural compounds, such as melittin, silymarin, and silibinin, induce apoptotic pathways via ROS generation[15-17]. Thus, we further checked whether Cit-mediated ROS accumulation also leads to apoptosis or necrosis in *Candida* cells. A flow cytometry analysis confirmed that Cit treatment leads to necrosis in approximately 2% cells (**Figure 1C**).

A hallmark of yeast cells involved in ROS generation and apoptosis is dysfunctional mitochondria[18]. Since mitochondria are key organelles governing apoptotic events, we evaluated their role in Cit-induced cell death. To access the functionality of mitochondria, we first studied the loss of ΔΨm, a distinctive feature of cells undergoing apoptosis. Moreover, it plays a role in ATP synthesis via mitochondrial oxidative phosphorylation. Thus, ΔΨm is a sensitive indicator of the energy status of mitochondria, which is determined by an electrochemical gradient maintained by the electron transport chain[19]. Our results confirmed that exposure to Cit resulted in significant hyperpolarization of mitochondria in *C. albicans,* providing evidence for mitochondrial dysfunction (**Figure 2A**). Cell

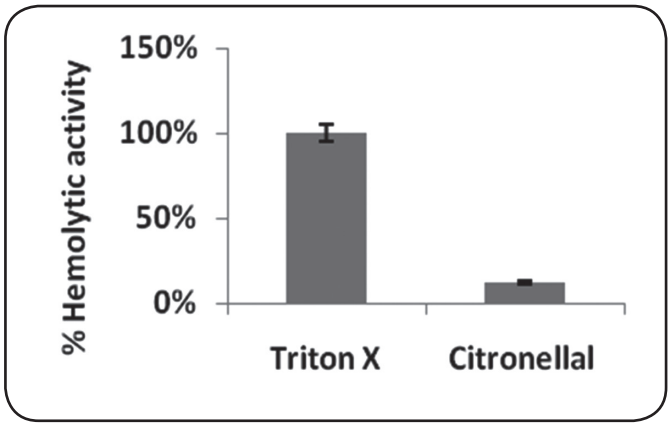

FIGURE 5 - Hemolytic activity of Citronellal against human erythrocytes. Hemolysis was determined by recording absorbance at 450nm in Cit-treated cells and cells treated with 1% Triton X-100 (reference for 100% hemolysis). Data are presented as the means of triplicate experiments. **Cit:** Citronellal.

death in yeast also correlates with altered ΔΨm stemming from oxidative damage caused by ROS accumulation[20]. Next, using an MTT assay, we found that the metabolic activity of functional mitochondria was inhibited (**Figure 2B**) in the presence of Cit[10]. The impaired functionality of mitochondria was further apparent when we compared the growth of Cit-treated cells on non-fermentative and fermentative carbon sources, indicating hypersensitivity in non-fermentable medium (**Figure 2C**). These results confirmed that Cit leads to dysfunctional mitochondria.

The observation of dysfunctional mitochondria prompted us to further examine the effect of ATP depletion on the activity of Cit. Enhanced PI uptake, as evidenced by increased fluorescence (**Figure 3A**), and the downregulation of *NPC2*, a gene involved in sterol transport (**Figure 3B**), in the presence of Cit indicate a non-intact membrane. Thus, these results not only confirm that

Cit leads to membrane disruption, but also revealed that Cit activity was unaffected by NaN_3, suggesting that its antifungal effects are mediated by cellular functions, irrespective of energy consumption.

DNA damage is another key feature of cells involved in ROS generation and apoptosis[21]. Moreover, Cit induces hypersensitivity in the presence of a DNA damaging agent[4]. Based on DAPI staining, in the presence of Cit, cells showed enhanced fluorescence indicating DNA damage (**Figure 4A**) as well as the downregulation of *MSH6,* a gene involved in DNA repair (**Figure 4B**). These results confirm that Cit induces DNA damage in *C. albicans*; however, whether DNA damage mediated by Cit occurs via the generation of ROS remains to be validated. Finally, we assessed the toxicity of Cit against mammalian cells, and we observed negligible toxicity in comparison to the positive control (**Figure 5**).

Taken together, in addition to membrane disruption, the primary mode of anticandidal action for Cit, our results confirmed other effects of Cit, including ROS generation, necrosis, mitochondrial dysfunction, and DNA damage. Although the link between these mechanisms of disruption is unclear, these findings suggest that Cit could trigger these events after membrane damage against *C. albicans*. Further studies are warranted to effectively employ phytotherapeutics, such as Cit, for the treatment of *Candida* infections.

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Conflict of interest

The authors declare that there is no conflict of interest.

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REFERENCES

1. Singh S, Fatima Z, Hameed S. Predisposing factors endorsing *Candida* infections. Infez Med. 2015;23(3):211-23.
2. Tanwar J, Das S, Fatima Z, Hameed S. Multidrug resistance: an emerging crisis. Interdiscip Perspect Infect Dis. 2014; doi: 10.1155/2014/541340.
3. Patwardhan B, Vaidya AD. Natural products drug discovery: accelerating the clinical candidate development using reverse pharmacology approaches. Indian J Exp Biol. 2010;48(3):220-7.
4. Singh S, Fatima Z, Hameed S. Citronellal-induced disruption of membrane homeostasis in *Candida albicans* and attenuation of its virulence attributes. Rev Soc Bras Med Trop. 2016 49(4):465-72.
5. Ansari MA, Fatima Z, Hameed S. Anticandidal effect and mechanisms of monoterpenoid, perillyl alcohol against *Candida albicans*. PLoS One. 2016;11(9):e0162465.
6. Menezes RA, Amaral C, Batista-Nascimento L, Santos C, Ferreira RB, Devaux F, et al. Contribution of Yap1 towards *Saccharomyces cerevisiae* adaptation to arsenic-mediated oxidative stress. Biochem J. 2008;414(2):301-11.
7. Eisenberg T, Carmona-Gutierrez D, Büttner S, Tavernarakis N, Madeo F. Necrosis in yeast. Apoptosis. 2010;15(3):257-68.
8. Reungpatthanaphong P, Dechsupa S, Meesungnoen J, Loetchutinat C, Mankhetkorn S. Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug-sensitive and resistant cells. J Biochem Biophys Methods. 2003;57(1):1-16.
9. Asthana N, Yadav SP, Ghosh JK. Dissection of antibacterial and toxic activity of melittin: a leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity. J Biol Chem. 2004;279(53):55042-50.
10. Adam-Vizi V, Tretter L. The role of mitochondrial dehydrogenases in the generation of oxidative stress. Neurochem Int. 2013;62(5):757-63.
11. Wilson DF, Chance B. Azide inhibition of mitochondrial electron transport. I. The aerobic steady state of succinate oxidation. Biochim Biophys Acta. 1967;131(3):421-30.
12. Luk E, Yang M, Jensen LT, Bourbonnais Y, Culotta VC. Manganese activation of superoxide dismutase 2 in the mitochondria of *Saccharomyces cerevisiae*. J Biol Chem. 2005;280(24):22715-20.
13. Komalapriya C, Kaloriti D, Tillmann AT, Yin Z, Herrero-de-Dios C, Jacobsen MD, et al. Integrative model of oxidative stress adaptation in the fungal pathogen *Candida albicans*. PLoS One. 2015;10(9):e0137750.
14. Rodrigues-Pousada CA, Nevitt T, Menezes R, Azevedo D, Pereira J, Amaral C. Yeast activator proteins and stress response: an overview. FEBS Lett. 2004;567(1):80-5.
15. Lee J, Lee DG. Melittin triggers in *Candida albicans* through the reactive oxygen species-mediated mitochondria/caspase-dependent pathway. FEMS Microbiol Lett. 2014;355(1):36-42.
16. Yun DG, Lee DG. Silibinin triggers yeast apoptosis related to mitochondrial Ca2+ influx in *Candida albicans*. Int J Biochem Cell Biol. 2016; 80:1-9.
17. Yun DG, Lee DG. Silymarin exerts antifungal effects via membrane-targeted mode of action by increasing permeability and inducing oxidative stress. Biochim Biophys Acta. 2017;1859(3):467-74.
18. Bhat AH, Dar KB, Anees S, Zargar MA, Masood A, Sofi MA, et al. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. Biomed Pharmacother. 2015;74:101-10.
19. Brand MD, Chien LF, Ainscow EK, Rolfe DF, Porter RK. The causes and functions of mitochondrial proton leak. Biochim Biophys Acta. 1994; 1187(2):132-9.
20. Xu C, Wang J, Gao Y, Lin H, Du L, Yang S, et al. The anthracenedione compound bostrycin induces mitochondria mediated apoptosis in the yeast. FEMS Yeast Res. 2010;10(3):297-308.
21. Rowe LA, Degtyareva N, Doetsch PW. DNA damage-induced reactive oxygen species (ROS) stress response in Saccharomyces cerevisiae. Free Radic Biol Med. 2008;45(8):1167-77.