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Effects of vanillin on cell wall integrity, vacuole homeostasis, biofilm formation, and immune evasion in *Candida auris*

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A B S T R A C T

Background and Purpose: *Candida auris* has presented a serious global health threat over the past decade due to its intrinsic multidrug resistance and frequent misidentification with closely related *Candida* species. Therefore, alternative therapeutic strategies involving natural compounds are urgently needed. This study aimed to evaluate the antifungal efficacy of Vanillin (Van), a food flavoring agent with known medicinal properties, against *C. auris* and elucidate its underlying mechanisms of action.

Materials and Methods: Cell wall analysis assessed compositional changes in chitin, glucan, and mannan by calcofluor white, aniline blue, and Concanavalin A-fluorescein conjugate staining, respectively. Vacuole integrity was examined by vacuolar acidification and morphology through Quinacrine and FM4-64 staining, respectively. Crystal violet staining and metabolic activity by MTT assay and biomass measurement were used to evaluate biofilm formation. The THP-1 macrophage model was used to study immune evasion and macrophage-mediated killing, while the *Caenorhabditis elegans* model was used to test antifungal efficacy and survival outcomes.

Results: The Van treatment induced significant changes in the cell wall composition of *C. auris*, affecting the levels of chitin, glucan, and mannan. Vacuole homeostasis was disrupted, indicated by impaired acidification and altered morphology. Biofilm formation was significantly reduced, as indicated by decreased staining, metabolic activity, and biomass. Immune evasion was attenuated, leading to increased macrophage-mediated killing in the THP-1 cell model. In *C. elegans*, Van treatment significantly enhanced nematode survival following *C. auris* infection.

Conclusion: The Van exhibited promising antifungal activity against *C. auris* by targeting cell wall integrity, vacuolar function, biofilm formation, and immune evasion. Given its established safety profile and *in vivo* efficacy in nematode models, further studies in mammalian systems (e.g., murine models) are warranted to validate its therapeutic potential.

Keywords: Biofilm, *Candida*, cell wall, *C. elegans*, macrophage, vacuole

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Introduction

Candida auris has presented a formidable challenge over the past decade, despite being the most recently identified *Candida* species. It has already caused numerous outbreaks worldwide and particularly affects patients with immunocompromised conditions [1]. Since its inception in 2009, *C. auris* has attained the status of superbug and is classified by the World Health Organization as a critical priority pathogen [2]. Although it is another *Candida* species, its epidemiology, genetics, antifungal resistance, virulence, and host adaptation are found to be unique [3, 4].

Misidentification of *C. auris* with other closely related *Candida* species and the presence of intrinsic drug resistance against current antifungals are the major factors associated with high mortality rates [5, 6]. Additionally, it possesses novel abilities to persist on the surfaces and get transmitted easily [7]. *Candida auris* also displays the ability to form biofilms and adapt well to evade host immune responses [8, 9]. Therefore, there exists an unequivocal challenge for the identification of new antifungal compounds against the superbug, *C. auris*.

Synthesis of new antifungal drugs is not only time-consuming and costly but also difficult due to the resemblance between *Candida* and human cellular machinery, as both are eukaryotes. Another feasible approach is to identify natural compounds with potential antifungal activity that are cost-effective and exhibit negligible toxicity, followed by structural modifications of the lead compound to generate more potent derivatives.

Vanillin (Van) is a naturally occurring food flavoring agent with "generally regarded as safe" status [10]. Chemically, it is a phenolic aldehyde with the name 4-hydroxy-3-methoxybenzaldehyde. In addition to its usage in the food and cosmetic industry, Van also possesses antimicrobial activities. For instance, synthetic bioactive derivatives of Van were demonstrated to have antimicrobial and anti-quorum-sensing activities [11]. A study has shown the antifungal properties of Van derivatives against numerous fungi of

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clinical interest, such as *Candida albicans*, *Cryptococcus neoformans*, and *Trichophyton rubrum* [12]. Similarly, nanocomposites with Van have also been shown with commendable antifungal activities against *C. albicans* and *Candida glabrata* [13].
Mechanisms of action of Van against *C. albicans* are also documented [14-16]. However, no study has yet demonstrated the antifungal potential of Van against *C. auris*. Therefore, considering the commercial and industrial significance of Van, this study aimed to replenish the existing literature about the therapeutic potential of Van against C. auris with the mode of action. Accordingly, it aimed to uncover the effect of Van on cell wall remodeling, vacuole functioning, biofilm formation, and immune escape mechanisms in *C. auris*.

Materials and Methods

All media chemicals, yeast extract, peptone, and dextrose (YEPD) and brain heart infusion (BHI) media were obtained from Himedia (India). Agar, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, sodium hydroxide, and dimethyl sulfoxide (DMSO) were obtained from Fischer Scientific (USA). Crystal violet, Calcofluor-white (CFW), Van, aniline blue, and Phorbol 12-myristate 13-acetate were obtained from Sigma Chemical Co. (USA). Thiazolyl blue (MTT) was obtained from Sisco Research Laboratories Pvt. Ltd., India. Moreover, FM4-64 and quinacrine dihydrochloride were purchased from Invitrogen (USA) and Merck (Germany), respectively.

Growth media and strain

The *C. auris* CBS10913T from Clade II was used in the study and grown in YEPD broth with the composition of yeast extract 1% (w/v), peptone 2% (w/v), and dextrose 2% (w/v) or for agar plates 2 % (w/v) agar.

Antifungal susceptibility assays

Minimal inhibitory concentration (MIC) and spot assay were performed for antifungal susceptibility.

Minimum inhibitory concentration

A broth microdilution assay was conducted based on the method described by the Clinical and Laboratory Standards Institute [17-19]. At least 90% growth inhibition criteria were used to define the MIC_{90}.

Spot assay

The drop dilution method was employed to perform the spot assay by the previously defined method [20]. The YEPD plates were spotted with 5 µl of fivefold serial dilutions of *C. auris* in the presence of a subinhibitory concentration of Van, and growth was recorded after 2 days at 30 °C.

Static/Cidal determination assay

The *C. auris* cells at an initial density of 0.1 OD_{600} were grown at 30 ˚C in the presence of Van at the MIC. The next day, 100 µl cultures were re-inoculated in the absence of Van at 30 ˚C for 24 h. The static-cidal activity of Van was assessed by measuring the OD_{600} with a spectrophotometer (VSI Electronics Pvt Ltd/model no.VSI-501) [20].

Cell wall staining (Chitin, β-1,3-glucan, and mannan)

Candida auris exponential phase cells 2.5×10^6 from the control group, and cells treated with Van (150µg/ml) were harvested and fixed with 4% paraformaldehyde for 30 min in phosphate-buffered saline (PBS). The cells were washed and stained for mannan with 50 µg/ml Concanavalin A-fluorescein conjugate, 100 µg/ml of AB for β-1,3-glucan, and 5 µg/ml of CFW for chitin, and then were further incubated for another half an hour at room temperature. A fluorescence microscope (Nikon, Japan) was used to see the fluorescence-stained cells at 100x magnification [21, 22].

Vacuolar morphology and acidification

Overnight *C. auris* cells from the control group, and cells treated with Van (150µg/ml) were harvested in PBS and resuspended in fresh YEPD for 4 h. For morphology and acidification, the cells were washed and stained with 40 µM FM4-64 and 10 µl of the Quinacrine solution (10 mg/ml; Molecular Probes), respectively, and incubated for 15 min at room temperature. A fluorescence microscope (Nikon, Japan) was used to see the fluorescence-stained cells at 100x magnification [23, 24].

Biofilm formation

Crystal violet staining

The biofilm formation was quantified by crystal violet staining. In the first step, the biofilm was formed, and after completing the incubation for 48 h, the biofilm was washed twice with 200 µl PBS, and each biofilm was stained by using 0.4% of crystal violet solution. After this, all biofilms were washed with 300 µl sterile distilled water four times, immediately followed by destaining via 95% ethanol (100 µl). Next, the biofilm was airdried for 45 min and visualized in a light microscope [25].

Biofilm metabolic activity

To estimate biofilm metabolic activity, about 50 µl tetrazolium salt and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were added to each well and incubated at 37 °C. After 5 h, 200 µl of dimethyl sulfoxide was added to solubilize the MTT formazan product, and absorbance was measured at 450 nm to depict the metabolic activity [15].

Biofilm biomass quantification

Biofilm biomass was measured by our previously described method [15, 20]. The total biofilm biomass was calculated by subtracting the difference in the weight of the silicon square after biofilm formation from the pre-weight silicon square before biofilm formation.

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Macrophage infection and killing assay

Macrophages derived from THP-1 cell lines were infected with *C. auris* for 4 h at a multiplicity of infection ratio of 5:1 (yeast: host) in the presence of amikacin (200 g/ml). After washing the cells with PBS, they were resuspended by RPMI media supplemented with 10% fetal bovine serum. After 24 h of infection, monolayers were gently washed thrice with PBS, aspirated, and the supernatant was collected. Serial dilutions of the lysates were done and plated on agar plates for colony-forming units (CFUs) counting after two days at 30 °C [20, 22].

Caenorhabditis elegans studies

The standard Kaplan Meier method was used for *Caenorhabditis elegans* nematode studies [20, 26]. The L4-stage young adult worms (50-60) were transferred to BHI medium in the presence of Van at sub-MIC, seeded from an *Escherichia coli* OP50 lawn, and nematode survival was observed for up to 7 days. For the *C. elegans* co-infection liquid assay, adult nematodes were pre-infected with *C. auris* for 2 h on BHI agar medium containing ampicillin (100 mg/ml). The worms were incubated at 25 ˚C and scored as alive or dead for up to 7 days, and images were captured using a stereomicroscope at 10x magnification. For visualization of intestinal persistence, worms were infected overnight in *C. auris* lawns. Cells were stained after 2 days with CFW, and images were captured at 100x magnification by a fluorescence microscope (Nikon, Japan).

Statistical analysis

Each experiment was performed at least thrice (n=3), and the mean ± SD was calculated to report the results. Statistical significance was performed using Student's t-test, with $p < 0.05$ considered statistically significant.

Results

Vanillin depicts effective antifungal activity against Candida auris

First, the MIC and MFC were determined to show the *in vitro* antifungal activity of Van. It was found that the growth of *C. auris* was inhibited by 90% at a 200 µg/mL concentration, depicting its MIC (Figure 1A). The MIC results were also validated by a spot assay that confirmed the estimated MIC (Figure 1B). To determine the MFC, a spectrometric assay was performed, where it was observed that growth resumed on day 2 when the culture treated with Van at the MIC was reinoculated. However, no growth resumption was observed at 400 µg/mL of Van, indicating the MFC of Van (Figure 1C). For the conduction of other biochemical assays, a sub-inhibitory concentration of 150 µg/mL was selected based on a growth curve experiment (data not shown), which partially inhibited *C. auris* growth.

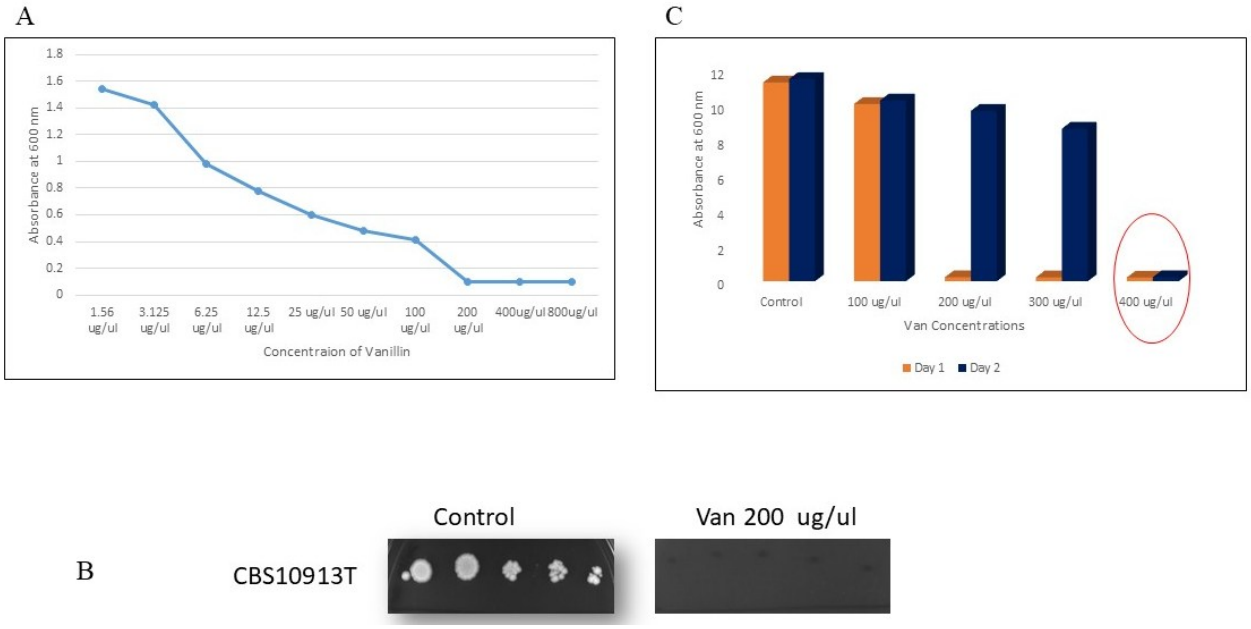


Figure 1. Antifungal activity of Van. **A.** Broth microdilution assay was used to determine the MIC_{90} of *Candida auris* in the presence of Vanillin. Y-axis depicts the absorbance measured at 600 nm, and the x-axis depicts the Van concentrations in ug/ml. The minimum Van concentration depicted by the arrow that inhibits growth by 90% relative to the drug-free growth control is indicated as MIC_{90}. **B.** Spot assay of C. auris in the absence and presence of Van at MIC_{90}. **C.** Bar graph depicting the revival of untreated *C. auris* cells and non-revival of Van-treated (minimum fungicidal concentration) cells in yeast extract, peptone, and dextrose media.

Vanillin confers cell wall compositional changes and alteration in vacuole homeostasis

Major components of the *C. auris* cell wall comprise chitin, β-1,3-glucan, and mannan. Impact of Van was systematically investigated using specific fluorescent dyes. Chitin and β-1,3-glucan were stained with CFW and aniline blue, respectively, while mannan was stained with fluorescein-conjugated Concanavalin A. In the presence of Van, chitin and β-1,3-glucan exhibited increased fluorescence intensity, compared to untreated control cells, indicating an altered or exposed cell wall structure (Figure 2A and B). Conversely, mannan staining was markedly

reduced upon Van treatment, suggesting possible degradation or masking of mannan components (Figure 2C).

To further assess the effect of Van on vacuolar function, both vacuole morphology and acidification were examined. The FM4-64, a lipophilic dye that is internalized through endocytosis and stains the vacuolar membrane, revealed normal ring-like staining in control cells, indicative of intact vacuolar morphology. In contrast, Van-treated cells displayed a diffuse and irregular staining pattern, suggesting vacuolar deformation (Figure 3A). Vacuolar acidification was evaluated using quinacrine, a weak base that accumulates in acidic vacuoles and fluoresces upon protonation. Control cells showed strong fluorescence, confirming proper acidification. However, cells treated with Van exhibited significantly reduced fluorescence, indicating elevated vacuolar pH and disrupted acidification (Figure 3B).

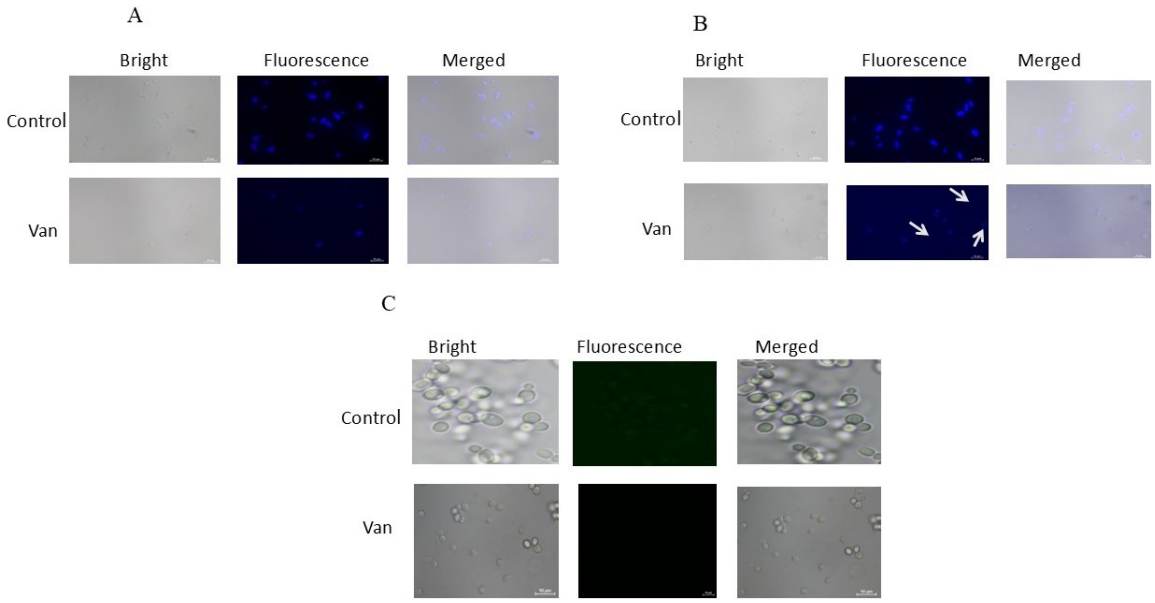


Figure 2. Effect of Vanillin on cell wall composition of *Candida auris*. **A.** chitin (calcofluor white staining) scale bar=10µm, **B.** β1,3-glucan (aniline blue staining) scale bar =10µm, and C. mannan (Concanavalin A staining) scale bar =20µm.

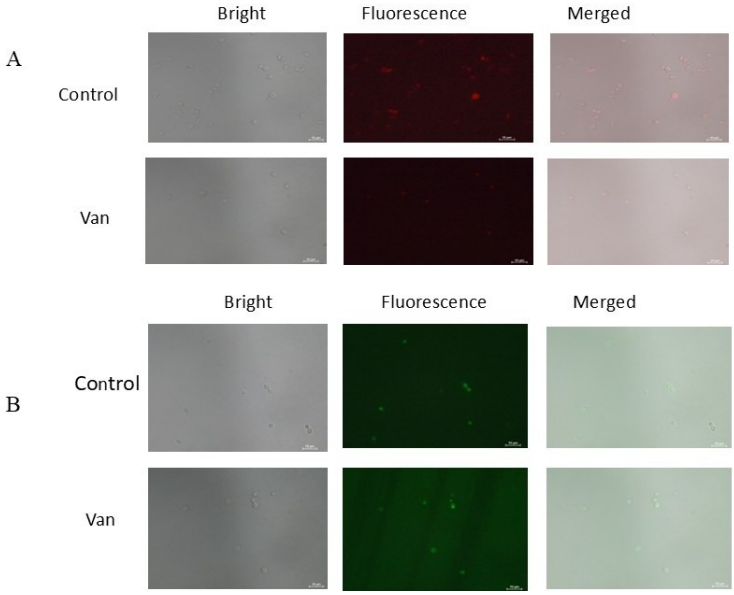


Figure 3. Effect of Vanillin on vacuole homeostasis of *Candida auris*. **A.** Vacuole morphology of control and Van-treated *C. auris* observed by FM4-64 staining. Scale bar =20 µm. **B.** Vacuole acidification of control and Van-treated *C. auris was* observed using quinacrine staining. Scale bar = 20 µm.

Vanillin impairs biofilm formation in Candida auris

The impact on biofilm-forming ability in the presence of Van was initially assessed with the help of crystal violet staining, which demonstrated less stained matrix (Figure 4A). Furthermore, the biofilm metabolic activity was estimated by MTT assay as described in the methods section. It was observed that metabolic activity was diminished considerably by 41% in the presence of Van (Figure 4b). Additionally, the biomass of the biofilms was measured in the presence of Van and was found to be considerably reduced by 32% (Figure 4C).

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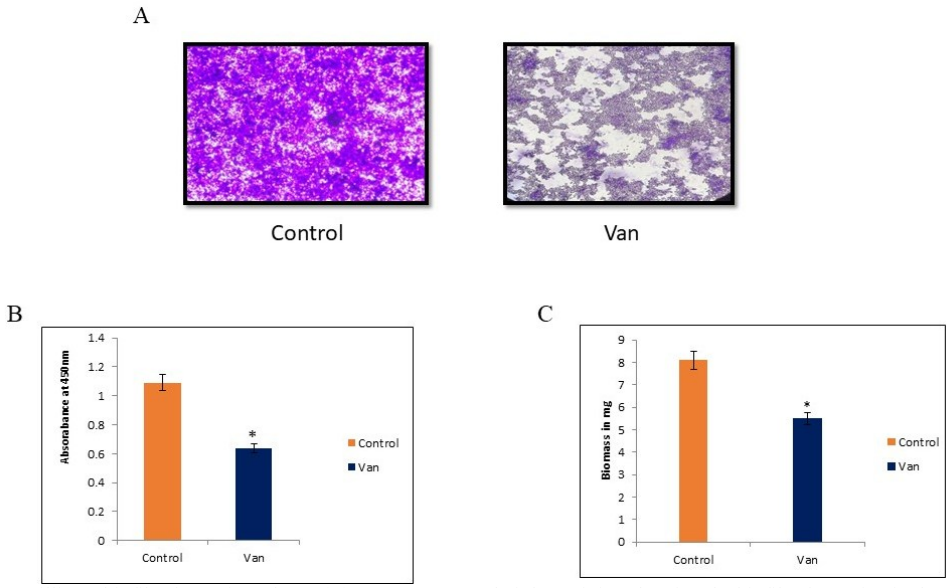


Figure 4. Effect of Vanillin on biofilm formation of *Candida auris*. **A.** Crystal violet staining showing the biofilm formation in the absence and presence of Van. **B.** Effect of Van on biofilm metabolic activity, shown as a bar graph and quantified using the MTT assay. The mean optical density at 450 nm ± SD of three independent sets of experiments is depicted on the Y-axis. * depicts *p* value <0.05. **C.** Effect of Van on biofilm biomass formed on silicone sheets. Mean of dry weight ± SD of three independent sets of experiments is depicted on the Y-axis. * depicts $p < 0.05$.

Vanillin impedes immune evasion of Candida auris by enhanced macrophage-mediated killing

Intracellular killing of the fungal cells was studied with the help of THP-1 cell lines. The cell lines were treated appropriately for differentiation into macrophages and subsequently infected with *C. auris* as described in the methods. Killing of fungal cells was indirectly estimated by counting the CFU, which displayed a significant reduction (Figure 5A). The untreated cells displayed around 282 CFU log 105/ml, contrary to Van-treated cells with only 63 CFU log 105/ml (Figure 5B).

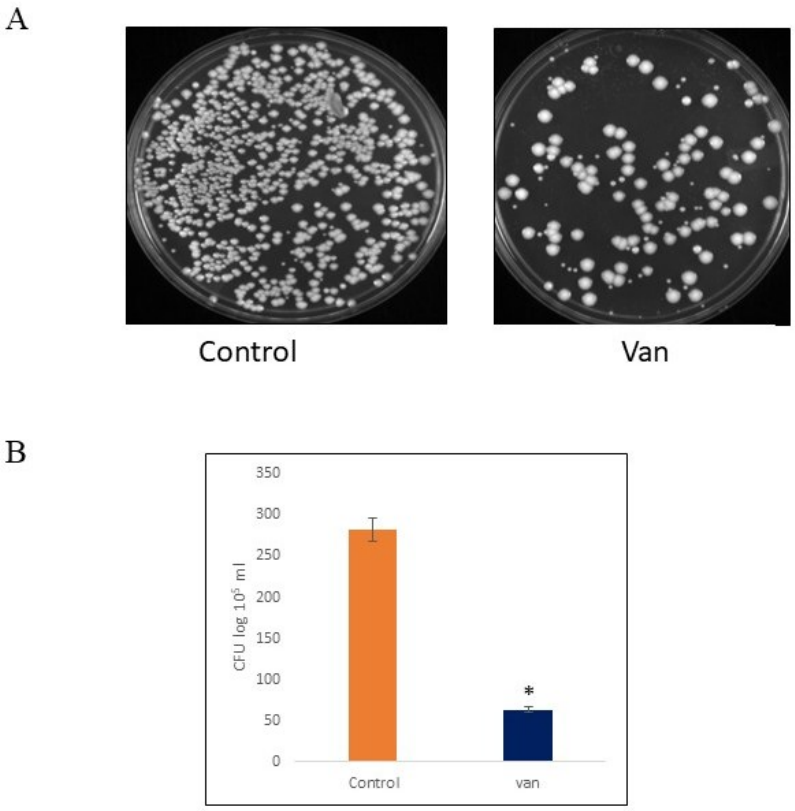


Figure 5. Effect of Van on macrophage killing ability. **A.** Colony forming unit image of control and Vanillin-treated *Candida auris* after 24 h of infection at the dilution 10-5 and magnification 40x. **B.** Bar graph represents colony-forming unit log105/ml on the y-axis after 24 h of infection with control and Van-treated *C. auris,* depicting the killing of fungi by macrophages. Data are expressed as mean ± SD of three independent sets of experiments.

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Vanillin prolongs the Candida auris-infected Caenorhabditis elegans survival

Lastly, the *in vivo* therapeutic efficiency was also demonstrated in the nematode model. The host toxicity for Van was initially evaluated in *C. elegans*, which showed no appreciable growth difference between the control (78%) and treated worms (66%) for up to one week (Figure 6A). Furthermore, upon *C. auris* infection, the survival rate of the worms was monitored for one week, and an enhanced survival rate was observed in the presence of Van (70%) compared with the control group (51%) (Figure 6B). Additionally, the CFW staining of the infected worms also displayed diminished blue fluorescence in the intestinal regions, compared to the untreated control with enhanced fluorescence (Figure 6C).

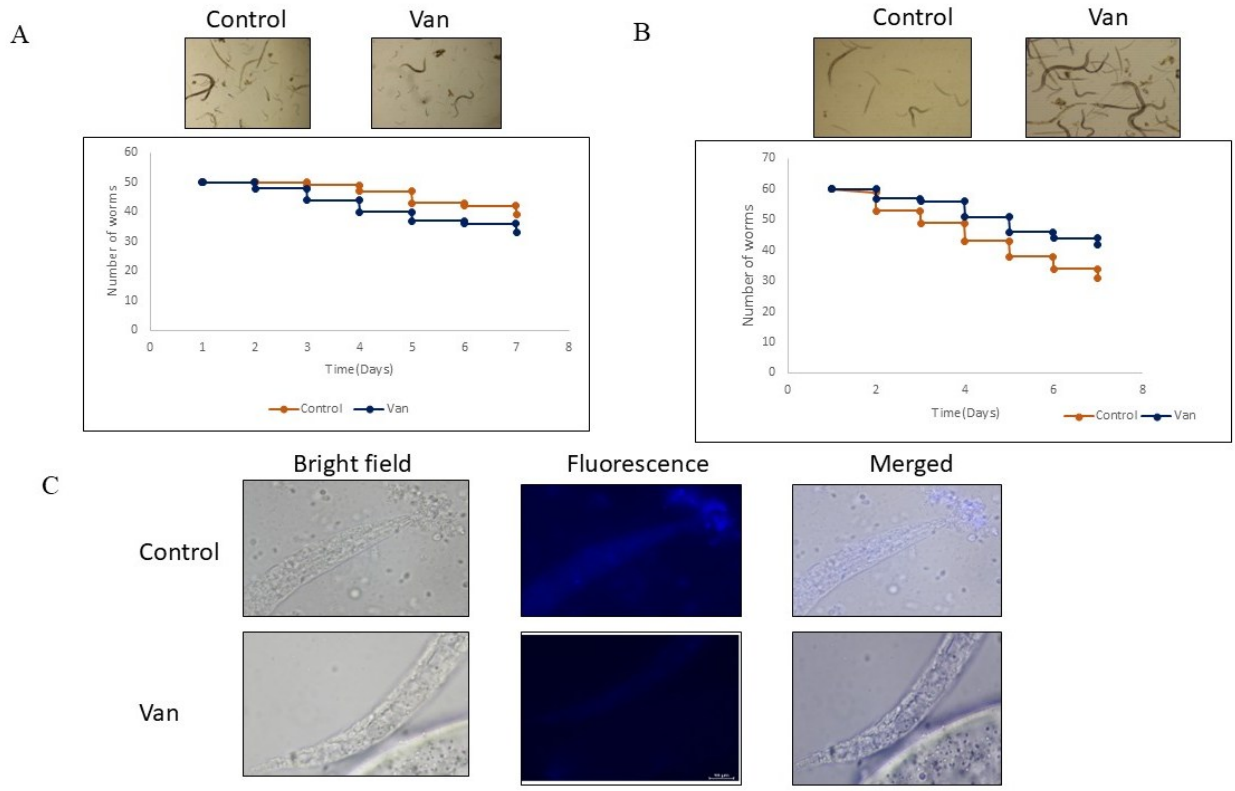


Figure 6. *In vivo* studies of Vanillin on nematode model *Caenorhabditis elegans*. **A.** Toxicity test of Van on *C. elegans* depicted by Kaplan–Meier curve showing the number of *C. elegans* in the presence of Van at sub- minimum inhibitory concentration. Worm survival was determined based on movement. Toxicity of Van was studied on nematodes by determining survival rates for up to 7 days. **B.** Kaplan–Meier curve number of *C. auris*-infected *C. elegans* in the presence of Van for up to 7 days. **C.** Intestinal persistence of *C. auris* represented by the calcofluor white-stained *C. auris* visualized in the intestine of *C. elegans* treated with Van, showing a lesser fungal burden. Magnification 100x.

Discussion

Although *C. auris* has been recently discovered, it is considered a global threat and has been designated as one of the critical priority fungal pathogens by the World Health Organization [2]. Under such pressing circumstances, it becomes inevitable to identify alternative novel antifungal therapeutic options. Natural compounds have always been considered safe alternatives with minimal toxicity concerns [27-29]. Hence, the present study elucidated the antifungal action of Van, a natural food flavoring agent.

This study demonstrated the antifungal activity at 200 µg/ml (Figure1A and B), depicting the MIC of Van. Although fungistatic at the MIC, it was found that the minimum fungicidal concentration (MFC) of Van was 400 µg/ml (Fig. 1C). Few natural compounds have already been demonstrated to possess fungicidal activity. For instance, ibrexafungerp showed fungicidal activity against *C. albicans*. Similarly, Geraniol, another monterpenoid, displayed activity against *C. albicans* and *C. auris,* respectively [28, 30].

To investigate the mechanism of antifungal action, the cell wall composition was first examined, as it is absent in humans and serves as an effective drug target, being the primary point of contact during infection and providing protection, rigidity, and shape [31]. The cell wall comprises a distinct composition with an outer mannan layer followed by β-glucan and chitin [32, 33]. These components serve to offer immunogenic, antifungal, and permeability activities [34]. Significant remodelling of the cell wall was observed in the presence of Van, where alterations in all the above components were noticeable (Figure 2). Furthermore, there are studies that suggest a linkage of fungal vacuole functionality and cell wall compositional changes [35-37]. Therefore, vacuole function was assessed based on two attributes, namely morphology and acidification, and both were found to be abrogated in the presence of Van (Figure 3).

Candida auris is known to contaminate medical devices and colonize human skin for transmission of infection. Biofilm formation is a major factor contributing to its persistence, leading to hindered therapeutics and increased resistance [38-40]. Hence, the biofilm formation ability was studied in the presence of Van, which was disrupted considerably (Figure 4). This observation could also be attributed to the fact that changes in cell wall composition are known to affect biofilm formation [41]. Particularly, the β-glucan component of the cell wall is incorporated into the biofilm matrix, preventing the drug from reaching the target and rendering the biofilm drug-resistant [42, 43]. Therefore, the inhibition of biofilm formation by Van could be due to the reduction of cell wall β-glucan observed in the present study.

Cell wall components are known to play a key role in the host-pathogen interaction to establish successful infection [44, 45]. In fact, β-glucan is the primary component of the cell wall that elicits an immune response against the invading fungi [46-48]. Macrophage-mediated killing of the intracellular pathogen as a defence response is the foremost step [48, 49]. Low CFU/ml in the presence of Van (Figure 5) suggested attenuated infectivity of *C. auris* or an immune evasion mechanism. This observation is particularly noteworthy, as the reduced β-glucan observed in the presence of Van could contribute to the decreased infectivity. The macrophages may not be able to recognize the reduced exposed glucan; hence, they cannot evade the immune response as efficiently as in the absence of Van.

Finally, *in vivo* efficacy of Van was also demonstrated in a nematode model using *C. elegans*. This was achieved by assessing the non-toxicity of Van on the nematode (Figure 6A) along with the enhanced survival rate of the nematode after *C. auris* infection (Figure 6B). Additionally, reduced *C. auris* intestinal persistence in the nematode was observed upon CFW staining (Figure 6C). The *Caenorhabditis elegans* model has already been demonstrated to be useful for the study of the pathogenesis of *C. auris* [50, 51].

Conclusion

The present study described various targets for Van in *C. auris* without unravelling the chronology of the mode of action; nevertheless, the biological targets, such as the cell wall, vacuole, and biofilm, are of considerable pharmacological interest. The encouraging result from this study provides adequate advocacy for the promising antifungal potential of Van. Additionally, exploiting new derivatives of Van could reveal novel compounds that may hold therapeutic promise. Further animal studies are warranted for the evaluation of the effective therapeutic window of Van to treat *C. auris* infections and subsequent clinical trials. As driven by the increasing prevalence of drug resistance in *C. auris* to the current antifungals, there is an urgent need for safe, effective, and broad-spectrum agents with strong pharmacological profiles.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Authors' contributions

A. K. was responsible for data curation, formal analysis, investigation, methodology, validation, visualization, and roles/writing of the original draft. K. G. contributed to data curation, formal analysis, and methodology. Z. F. handled conceptualization, project administration, resources, supervision, validation, and writing, review and editing. S. H. contributed to conceptualization, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, roles/writing of the original draft, and writing, review and editing.

Financial disclosure

None to declare.

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