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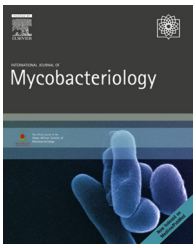

Full Length Article

Antimycobacterial mechanism of vanillin involves disruption of cell-surface integrity, virulence attributes, and iron homeostasis

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ABSTRACT

Objective/Background: Tuberculosis (TB) remains a global threat, claiming one-third of the population annually. The ever increasing emergence of multidrug-resistant TB (MDR-TB) is the major impediment to effective anti-TB therapy. Under such circumstances, deciphering the antimycobacterial potential of natural compounds has gained considerable prominence. This study evaluated the antimycobacterial activity of vanillin (Van), a natural food-flavoring agent and preservative, along with its potential mechanisms of action.

Methods: Drug susceptibilities were performed using broth microdilution, spot, and filter-disc assays. Membrane damage was studied by nitrocefin hydrolysis and electron microscopy. Virulence attributes were assessed by biofilm formation and cell adherence. Iron availability was estimated by enzymatic (ferroxidase) assay.

Results: We found that the antimycobacterial activity of Van against *Mycobacterium smegmatis* (a surrogate of *Mycobacterium tuberculosis*) is 125 μg/mL. Additionally, we observed disruption of membrane homeostasis in the presence of Van, as revealed by enhanced membrane permeability and transmission electron microscopy images showing a disturbed cell envelope. Concomitant with our findings, we also observed that Van leads to enhanced drug susceptibility to membrane targeting known anti-TB drugs. Furthermore, Van affects significant virulence traits of Mycobacterium by inhibiting biofilm formation and cell adhesion. Finally, we observed that Van disrupted iron homeostasis as displayed by hypersensitivity to iron deprivation.

Conclusion: The results established for the first time that Van could be an effective antimycobacterial agent that could be exploited further in treating mycobacterial infections.

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Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) remains one of the major enemies to humanity. In 2015, approximately 9.6 million people suffered with TB, of which 1.5 million died [1]. Although TB is generally regarded as a curable disease, the surplus use of anti-TB drugs has led to emergence of multidrug-resistant TB (MDR-TB), which occurs via several mechanisms, including overexpression of drug-efflux pumps, alteration of membrane permeability, drug modifications, and target alternation [2]. Therefore, it is extremely pertinent to search for novel and cost-effective regimes with fewer side effects and superior efficacy over available drugs. Natural compounds are one of the alternative emerging approaches that have gained considerable interest for combating the problem of MDR-TB [3,4].

Among the natural compounds, phenolic derivatives are of considerable interest based on their various properties, such as antimicrobial, antioxidant, anticarcinogenic, antimutagenic, and anti-inflammatory activities [5]. Several studies reported the antimycobacterial potential of various phenolic compounds. For example, a lignan phenolic compound, mesodihydroguaiaretic acid isolated from *Larrea tridentate*, inhibits the alpha subunit of MTB coenzyme A transferase, an enzyme responsible for both 1- and 2-methylnaphthalene- and geraniol-degradation pathways [6]. Similarly, chebulic acid produced by *Terminalia chebula* is potent to the DNA gyrase enzyme from wild-type and mutant strains of *Mycobacterium* [7]. In previous studies, the antimycobacterial activity of tricyclic diphenol ether engelhardion and 7-methyljuglon was also reported against MTB [8,9].

Vanillin (Van) is a natural phenolic aldehyde purified from seed pods of *Vanilla planifolia* that belong to the Orchidaceae family. It has a pleasant smell, tastes of vanilla, and is used as a flavoring agent and aroma in food, beverages, and pharmaceuticals. It exhibited antimicrobial activity [10], antioxidant activity [11,12], hypolipidemic activity [13], and anticarcinogenic activity [14]. Additionally, some studies reported antifungal activity of Van against the medicinally important yeasts *Cryptococcus neoformans* and *Candida albicans* [15]. Furthermore, the antibacterial effects of Van mixtures with cinnamon and clove essential oils in controlling *Escherichia coli* 15:*h7* and *Listeria monocytogenes* in milk were demonstrated [16]; however, no antimycobacterial activity of Van has been reported.

Here, for the first time, we established the antimycobacterial activity of Van against *M. smegmatis*, a surrogate of MTB. We observed that Van administration led to altered cell-surface integrity and increased susceptibility to known first-line anti-TB drugs. We also showed the effect of Van on biofilm formation and adhesion of *M. smegmatis*, which are major virulence traits. Van also disturbed iron homeostasis and DNA-repair mechanisms.

Materials and methods

Materials

All media chemicals, Middlebrook 7H9 broth, Middlebrook 7H10 agar, albumin/dextrose/catalase (ADC), and oleic ADC (OADC) supplements were purchased from BD Biosciences (San Jose, CA, USA). Tween-80, nitrocefin, ethambutol (EMB), isoniazid (INH), and Van were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethidium bromide (EtBr), crystal violet, and ferrozine were purchased from Himedia (Mumbai, India). Dimethyl sulfoxide (DMSO), potassium chloride (KCl), sodium chloride (NaCl), disodiumhydrogen orthophosphate (Na_2HPO4), potassium dihydrogen orthophosphate (KH_2PO_4), glycerol, ferrous sulphate ($FeSO_4$) and ascorbic acid (AA) were obtained from Thermo Fischer Scientific (Waltham, MA, USA). Methanol was purchased from Merck (Kenilworth, NJ, USA). Sodium acetate was purchased from Qualigens Fine Chemicals (Mumbai, India).

Bacterial strains and culture conditions

M. smegmatis mc^2155 was grown in Middlebrook 7H9 (BD Biosciences) broth supplemented with 0.05% Tween-80 (Sigma-Aldrich), 10% ADC (BD Biosciences), and 0.2% glycerol (Thermo Fischer Scientific) in 100-mL flasks and incubated at 37 °C. Cultures were subsequently grown on Middlebrook 7H10 (BD Biosciences) agar media supplemented with 10% (v/v) OADC (BD Biosciences) for solid agar allowing growth for 48 h at 37 °C. Stock cultures of log-phase cells were maintained in 30% glycerol and stored at −80 °C.

Drug-susceptibility testing

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined using the broth-dilution method described previously [17] according to Clinical and Laboratory Standards Institute guidelines [18]. Briefly, 100 μL of Middlebrook 7H9 broth was placed in each well of a 96-well plate, followed by the addition of the drug with the remaining media and subsequent serial dilution. Cell suspension (100 μL in normal saline at an optical density at 600 nm [OD_{600}] of 0.1) was added to each well, followed by incubation at 37 °C for 48 h. The MIC (Genetix, Biotech Asia Pvt. Ltd.) values were evaluated by observing the OD_{600} in a microplate reader.

Spot assay

Spot assays for the strains were determined using a method described previously [17]. Briefly, 5 μL of fivefold serial dilutions of each *M. smegmatis* culture (each with cells suspended in normal saline to an OD_{600} of 0.1) was spotted onto Middlebrook 7H10 agar plates in the absence (control) or presence of the drugs. Growth difference was measured after incubation at 37 °C for 48 h.

Filter-disc assay

The filter-disc assay was performed as described previously [17]. The drugs were spotted in a volume of 5–10 μL at the indicated concentrations, and the diameters of the respective zones of inhibition were measured after incubation of the plates for 48 h at 37 °C.

Membrane-permeability assay

The β-lactamase activity associated with the permeabilization of *M. smegmatis* was determined by measuring the

hydrolysis of nitrocefin by whole cells as described previously [17]. Briefly, cells were grown overnight at 37 °C in the absence (control) or presence of Van at its subinhibitory concentration with continuous shaking. Cells were then equilibrated with cold 1× phosphate buffered saline (PBS; pH 7.4). Nitrocefin was added to a final concentration of 0.25 mg/mL to the aliquot of cells (2 mL) in 1× PBS (pH 7.4), and hydrolysis was monitored at 485 nm for 60 min using a double-beam spectrophotometer (VSI-501; VSI Electronics, Punjab, India).

Transmission electron microscopy

Treated and untreated cells of *M. smegmatis* were observed using transmission electron microscopy (TEM: JEOL JEM-1011; JEOL, Delhi, India). Cells at an OD_{600} of 0.1 were seeded to the media in the absence (control) and presence of Van and incubated for 24 h at 37 °C. Sample preparation and analysis were performed using previously described methods [17]. Briefly, cells were harvested in PBS, fixed with 2.5% glutaraldehyde in 0.1% phosphate buffer for 1 h at room temperature (20 °C), washed with 0.1 M phosphate buffer (pH 7.2), and post-fixed with 1% Osmium tetroxide (OsO4) in 0.1 M phosphate buffer for 1 h. Cells were then dehydrated with ethanol, dried and coated with gold, and observed at a magnification of 10,000×.

Biofilm formation

M. smegmatis biofilm-forming potential was qualitatively and quantitatively analyzed using the microtiter-plate method as described previously, with some modifications [19,20]. Briefly, *M. smegmatis* cultures were grown overnight at 37 °C in Middlebrook media, followed by transfer of 100 μL of the media in each well of the 96-well plate with or without the addition of the drug. Cultures that had reached an OD_{600} of 0.1 were diluted (1:100) using Middlebrook media, and 100 μL of each diluted culture was pipetted into each well of a 96-well flat-bottom microtiter plate and incubated at 37 °C for 48 h. The wells were rinsed with water, and 125 μL of 0.1% solution of the crystal violet was added. Plates were incubated for 10 min, followed by washing with distilled H_2O three times and observation under a light microscope at 100×. For quantitative assays of the biofilm, 200 μL of 95% ethanol was added to each crystal violet-stained well, and plates were incubated for 10 min at room temperature. Contents of each well were mixed by pipetting, followed by transfer of 125 μL of the crystal violet/ethanol solution from each well to a separate well of an optically clear flat-bottom 96-well plate. The OD_{600} was measured using a spectrophotometer. The antibiofilm capability of Van-treated cells was measured as percentage of inhibition/reduction. For the cell-adhesion assay, the same procedure was followed, except that treated and untreated cells were grown to an OD_{600} of 1.0, and after washing the nonadhered cells, they were directly quantified using crystal violet staining.

Adherence of Mycobacterium on human buccal epithelial cells

The cell-adherence assays were modified from previous methods [21,22]. Epithelial cells were obtained from the mouth cavity of healthy people and washed two to three times with PBS by centrifugation at 1048 x g for 5 min at 4 °C. The pellets were then resuspended in PBS to obtain an OD_{600} of approximately 0.5. Bacterial cells were grown in Middlebrook 7H9 broth in the presence or absence of Van at subinhibitory concentrations (15 μg/mL) and incubated overnight at 37 °C. The culture was adjusted to allow for absorbance at 650 nm of approximately 0.5. The bacteria were then washed twice in PBS and centrifuged for 10 min at 10,000 rpm and resuspended in PBS. The test was performed by taking equal volumes of buccal epithelial cells (OD_{600} = 0.5) and bacterial suspensions that were mixed and incubated with shaking (120 rpm) at 37 °C for 2–3 h. After incubation, two drops of trypan blue solution (0.4%) and carbol fuchsin (3–5 μL) were added to each tube, and the mixture was gently shaken. The stained suspension (10 μL) was transferred to a Neubauer chamber and examined under light microscopy.

Ferroxidase assay

Whole-cell protein was extracted, and protein concentration was determined using the Lowry method in preparation for the ferroxidase assay according to a method previously described [23,24]. Bacterial cells were grown in Middlebrook 7H9 broth in the presence or absence of Van at subinhibitory concentrations (15 μg/mL). The assay was performed using ferrous sulphate as the electron donor and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a chelator to specifically detect the ferrous iron remaining at the end of the reaction. Each assay mixture (0.2 mL) contained 50 mM Na-acetate buffer (pH 5) and 0.1–0.3 μg of protein extract. The reaction was started by addition of 0.2 mM $FeSO_4$, and samples were quenched by adding 3.75 mM ferrozine. Fe(II) oxidation was determined by measuring the absorbance of residual Fe(II)-ferrozine at 570 nm.

Results

Antimycobacterial activity of Van

To detect the antimycobacterial activity of Van, three independent types of drug-susceptibility assay, including measurement of MIC using the broth-dilution method, spot assay, and filter-disc assay, were performed. The broth-dilution method revealed that Van showed antimycobacterial activity against *M. smegmatis* at 125 μg/mL (Fig. 1A). This result was further confirmed by performing spot assays (Fig. 1B) and filter-disc assays (Fig. 1C). All the susceptibility tests correlated with each other, and it was observed that *Mycobacterium* showed susceptibility to Van at a concentration of 125 μg/mL.

Assessment of growth following Van treatment

For subsequent biochemical assays and to study the effects of Van treatment, we needed to determine a subinhibitory concentration of Van that would affect normal cellular functions of *Mycobacterium* without causing cell death. To address this issue, growth curves were obtained in the absence (control) and presence of Van, and we observed that growth was partially affected at 15 μg/mL Van (Fig. 2A). To confirm the above concentration, a spot assay was performed using 15 μg/mL

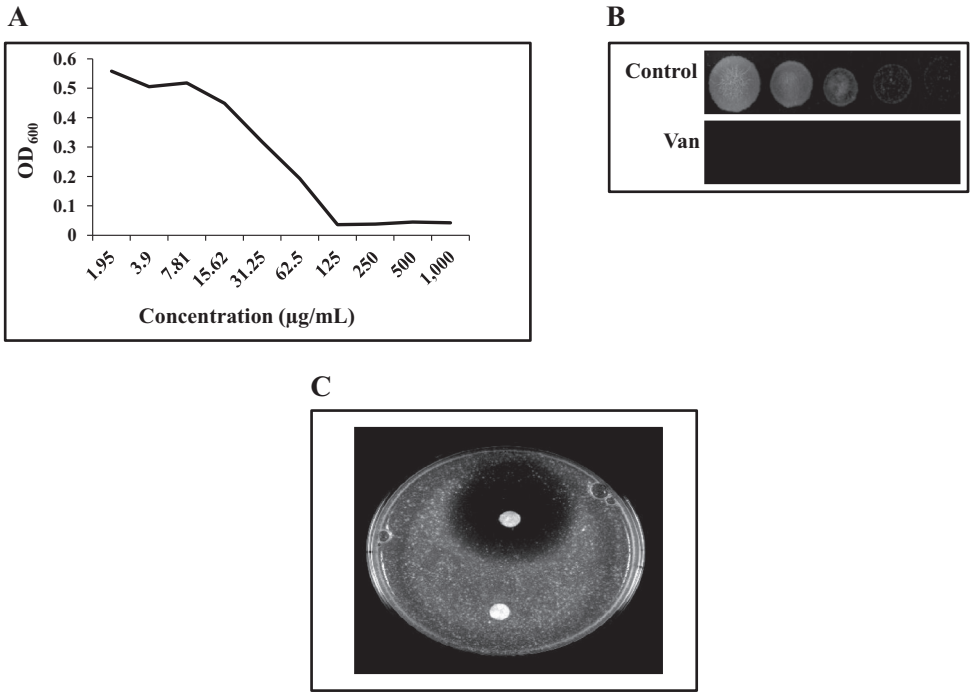

Fig. 1 – Antimycobacterial activity of Van against *Mycobacterium smegmatis*. (A) Broth-microdilution assay to determine the MIC_{80} of *M. smegmatis* in the presence of Van. (B) Spot assay of *M. smegmatis* in the absence (control) or presence of Van (125 μg/mL). (C) Filter-disc assay against and their zone of inhibition in the absence (control) or presence of Van (125 μg/mL). Discs were spotted with solvent (DMSO) as control. *Note:* DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; Van, vanillin.

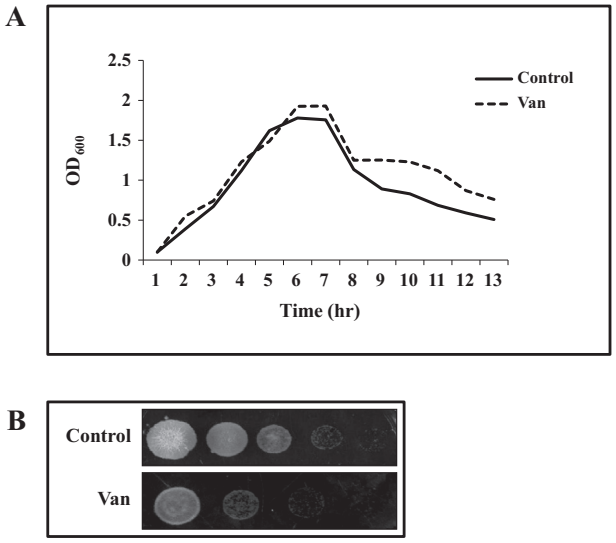

Fig. 2 – Assessment of growth in the presence of Van. (A) Growth curve of *Mycobacterium smegmatis* in the absence (control) or presence of Van (15 μg/mL). (B) Spot assay of *M. smegmatis* in the absence (control) or the presence of subinhibitory concentrations of Van (15 μg/mL). *Note:* Van, vanillin.

Van, resulting in partially inhibited growth of *M. smegmatis* (Fig. 2B). Therefore, we selected 15 μg/mL as the subinhibitory Van concentration for further experiments.

Effect of Van on cell-surface integrity

To study the effects of Van treatment on membrane integrity, we performed a membrane-permeability assay. Our results showed enhanced membrane permeability due to hydrolysis of nitrocefin in the presence of Van compared with permeability measured in untreated cells (control), indicating distortion of the *M. smegmatis* membrane (Fig. 3A). Disrupted membrane integrity was further observed by TEM, which clearly showed that Van disrupted the *M. smegmatis* cell envelope (Fig. 3B). Furthermore, we also tested the effects of Van in combination with known anti-TB drugs, including INH and EMB, which target membranes. We performed a broth-microdilution assay and observed that the drug-susceptibility associated with both of the anti-TB drugs (INH and EMB) was enhanced (from 4 μg/mL to 1 μg/mL for INH, and from 0.25 μg/mL to 62.5 ng/mL for EMB) in the presence of Van (Fig. 3C).

Effect of Van virulence attributes

Van inhibits biofilm formation

To study the effects of Van treatment on biofilm formation in *M. smegmatis*, we performed a biofilm-formation assay using qualitative and quantitative methods involving crystal violet dye. For qualitative testing, biofilm was visualized by staining control and Van-treated biofilms with crystal violet dye and observing considerable reduction in biofilm formation in the Van-treated samples (Fig. 4A). This result was validated by quantitative analysis through solubilizing the stained crystal

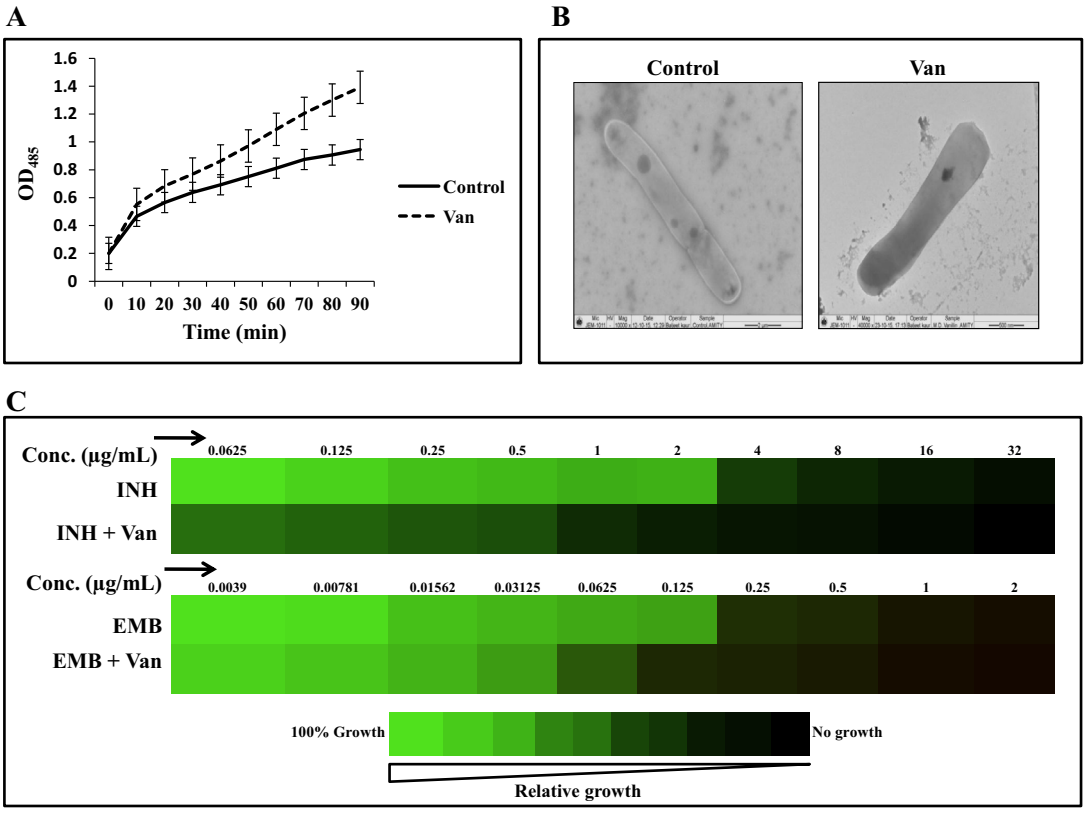

Fig. 3 – Effect of Van treatment on cell-surface integrity. (A) Membrane-permeability assay for *Mycobacterium smegmatis* cells grown in the absence (control) or the presence of Van (15 μg/mL). Mean of OD_{485} ± SD of three independent experiments are depicted on the *Y*-axis with respect to time (min) on *X*-axis ($p < .05$). (B) Transmission electron micrograph images of *M. smegmatis* cells grown in the absence (control) or presence of Van (125 μg/mL), showing disruption of membrane integrity. (C) Broth-microdilution assay of *M. smegmatis* with known anti-TB drugs (INH and EMB) in the presence of Van. Data were quantitatively displayed with color (see color bar), where each shade of color represents relative optical densities of the cell. The minimum drug concentration that inhibits growth by 80% relative to the drug-free growth control is indicated as MIC_{80}. *Note:* Conc., concentration; EMB, ethambutol; INH, isoniazid; MIC, minimum inhibitory concentration; OD, optical density; SD, standard deviation; Van, vanillin.

violet in 95% ethanol and measuring the absorbance at 600 nm, which confirmed that biofilm formation was significantly inhibited (64.96%) in the presence of Van relative to biofilm formation observed in control (Fig. 4B).

Van inhibits cell adherence to polystyrene and buccal epithelial cells

We tested the effects of Van treatment on cell adherence to both polystyrene and human epithelial cells. We observed that Van treatment resulted in diminished cell adherence of *M. smegmatis* on polystyrene microtiter surfaces (Fig. 4C). Interestingly, we also observed that Van treatment inhibited adherence of *Mycobacterium* on buccal epithelial cells compared with adherence on untreated cells (Fig. 4D).

Van disrupts iron homeostasis

We then assessed the effects of Van treatment on iron availability. We performed spot assays with ferrozine, which is an iron chelator, and observed that *M. smegmatis* were hypersensitive in presence of Van to iron deprivation as compared with control cells (Fig. 5A). To confirm this observation, we performed a ferroxidase enzymatic assay to estimate the nonoxidized ferrous iron present in the reaction mixture. As expected, we found significantly reduced ferroxidase activity by *M. smegmatis*, suggesting that free iron levels were reduced in the presence of Van (Fig. 5B). Furthermore, we investigated the effects of Van treatment under alkaline pH conditions by performing a spot assay. Unexpectedly, we observed no difference in the growth of *M. smegmatis* at alkaline pH compared with that observed at physiological pH, even in the presence of Van (Fig. 5C).

Effects of Van treatment on genotoxicity and redox status

We determined whether the antimycobacterial mechanisms associated with Van are also associated with effects on DNA repair. We performed spot assays on *M. smegmatis* using EtBr, which is a known DNA-damaging agent, at concentrations that resulted in no appreciable growth defects. We observed that Van treatment exhibited a moderate effect on DNA-repair mechanisms, as cells were susceptible to damage resulting from EtBr administration (Fig. 6A). Furthermore, we assessed whether the observed genotoxicity due to Van

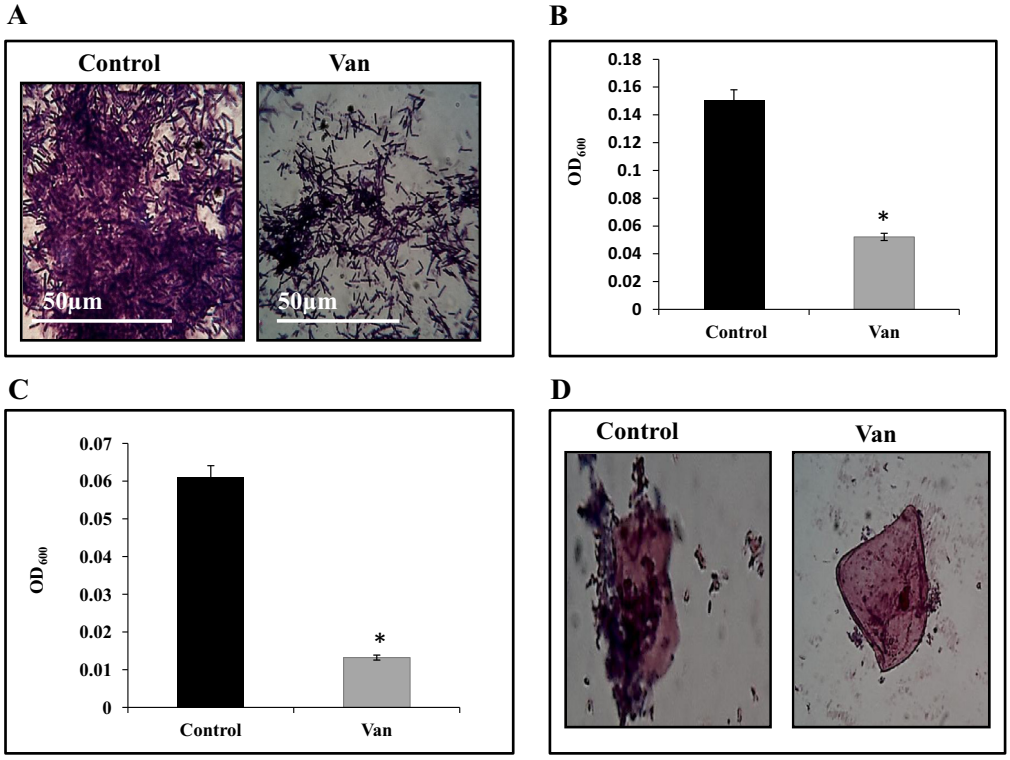

Fig. 4 – Effect of Van treatment on virulence traits of *Mycobacterium smegmatis*. (A) Crystal violet staining showing the biofilm formation in the absence (control) and presence of Van (15 μg/mL). (B) Effect of Van (15 μg/mL) on biofilm formation of *M. smegmatis* depicted as a bar graph and quantified using crystal violet dye. Mean OD_{600} ± SD of three independent experiments are depicted on the Y-axis. (C) Effect of Van (15 μg/mL) on cell adhesion on microtiter polystyrene surface of *M. smegmatis* quantified using crystal violet dye. Mean OD_{600} ± SD of three independent experiments are depicted on the Y-axis. (D) Effect of Van (15 μg/mL) on cell adherence of *M. smegmatis* with human oral epithelial cells. Left panel depicts untreated cells adhered to human buccal epithelial cells and right panel depicts Van-treated (15 μg/mL) *M. smegmatis* cells not adhered to epithelial cells. *Note:* OD, optical density; SD, standard deviation; Van, vanillin. $^{*}p < .05$.

treatment was associated with alterations in cellular redox states. We performed a spot assay in the presence of a known antioxidant (ascorbic acid) and observed that cells were hypersensitive to Van treatment when grown at MIC_{80}; however, this hypersensitivity could not be rescued, even upon supplementation with the antioxidant (Fig. 6B).

Discussion

In the era of increasing MDR-TB burden, discovery of novel antimycobacterial compounds preferably from natural sources due to their safety profiles has become a renewed source of interest. This study aimed to establish the antimycobacterial activity of Van, a natural food-flavoring agent, against *M. smegmatis*, a surrogate of MTB. All three independent methods of measuring drug susceptibility confirmed that Van exhibited antimycobacterial activity at 125 μg/mL. Deeper insights into the possible modes of action revealed multiple targets for Van.

Mycobacterium contains unique cell-surface architectures comprised of peptidoglycan attached to arabinogalactan, which is covalently linked to mycolic acid. The mycolic acids are the main components of mycobacterial cell walls and attractive targets of many anti-TB drugs. Moreover, several natural compounds were reported to possess antimycobacterial activity against *Mycobacterium* through membrane targeting. Epigallocatecin gallate is a compound found in green-tea extract and is capable of damaging the cell wall in *M. smegmatis* [25]. Similarly, another study showed three compounds, butein, isoliquirtigenin, and 2,2′,4′-trihydroxy chalcone, derived from *Rhus verniciflua* and *Dalbergia odorifera*, respectively, which inhibit mycolic acid biosynthesis in MTB [26]. Therefore, we investigated the effect of Van treatment on membrane integrity using different methods.

First, we performed membrane-permeability assays using nitrocefin, a chromogenic cephalosporin substrate containing an amide bond in the β-lactam ring, which is hydrolyzed by β-lactamase, normally localized to the bacterial periplasm. Membrane permeability was considerably enhanced in presence of Van (Fig. 3A). Similarly, TEM images revealed that Van disrupted the cell envelope of *M. smegmatis* (Fig. 3B). To substantiate the membrane- perturbing effects, we tested the effects of Van treatment in combination with known membrane-targeting anti-TB drugs INH and EMB. Enhanced drug susceptibilities for both the tested drugs in presence of Van (Fig. 3C) reinforced our findings that Van targeted the membrane integrity of *M. smegmatis*.

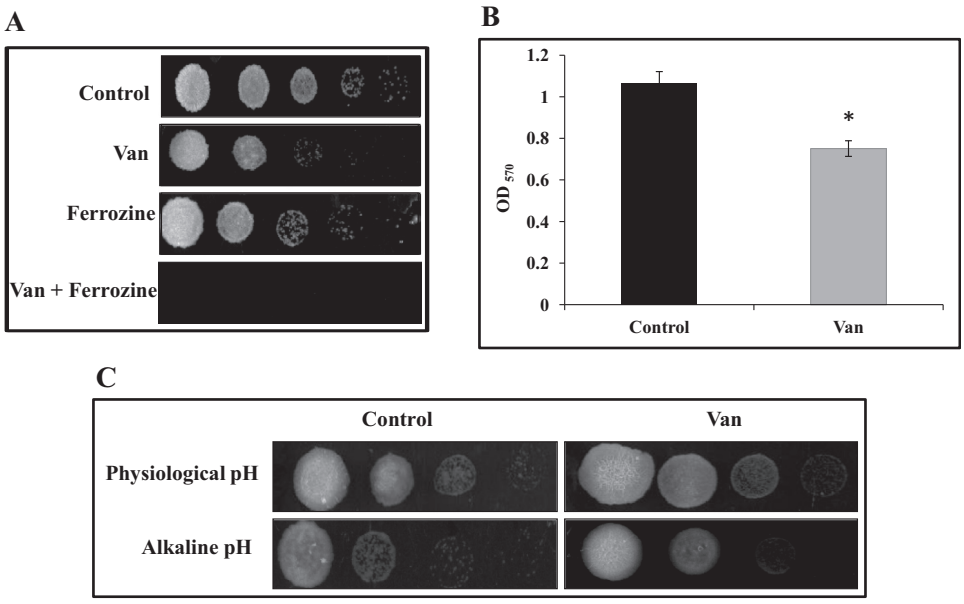

Fig. 5 – Effects of Van treatment on iron homeostasis. (A) Spot assay of *Mycobacterium smegmatis* with iron chelator ferrozine (2.5 mM) in the absence (control) or presence of Van (15 μg/mL). (B) Effect of Van treatment (15 μg/mL) on iron availability in *M. smegmatis* depicted as a bar graph and quantified by ferroxidase assay. Mean OD_{570} ± SD of three independent experiments are depicted on the Y-axis. (C) Spot assay of *M. smegmatis* in the absence (control) or presence of Van (15 μg/mL) at physiological and alkaline pH 10. *Note:* OD, optical density; SD, standard deviation; Van, vanillin. $^{*}p < .05$.

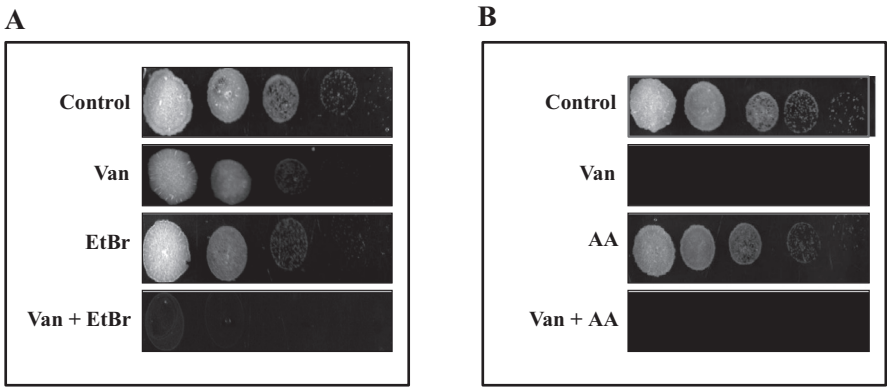

Fig. 6 – Effect of Van treatment on genotoxicity and redox status of *Mycobacterium smegmatis*. (A) Spot assay showing hypersensitivity of *M. smegmatis* to Van treatment (15 μg/mL) in the presence of EtBr (4 μg/mL), a DNA-damaging agent. (B) Spot assay demonstrating hypersensitivity of *M. smegmatis* in the presence of Van (MIC_{80}) that could not be reversed following addition of the antioxidant ascorbic acid (5 mM). AA, ascorbic acid; EtBr, ethidium bromide; MIC, minimum inhibitory concentration; Van, vanillin.

Biofilms are formed by the adhesion of bacterial cells to the cell surface through extracellular polymeric substances. This is a major virulence factor that contributes to resistance against various antimycobacterial drugs. Further, inhibited biofilm formation (Fig. 4A and B) led us to examine the effects of Van treatment on cell adherence, because it is the primary step for biofilm formation. Interestingly, we observed that Van treatment diminished *M. smegmatis* cell adherence to polystyrene microtiter surfaces (Fig. 4C). We subsequently verified these results using human buccal epithelial cells. For the infection process, bacterial pathogens express various molecules capable of promoting attachment of bacteria to host epithelial cells. The adhesion molecules interact with host-cell receptors or soluble proteins, thereby acting as a link between the bacteria and the host. Therefore, this is a critical step prior to invasion and pathogenesis. We observed that Van treatment inhibited the adherence of *Mycobacterium* on epithelial cells compared with untreated cells (Fig. 4D). These results confirmed that Van is a potent inhibitor of virulence attributes associated with *M. smegmatis*, resulting in inhibition of biofilm formation and cell adherence.

Iron is a metal essential for the survival of living organisms, including *Mycobacterium*. It is essential for the activity of different enzymes involved in wide ranges of metabolic processes, including DNA synthesis and cell respiration. The availability of free iron in the mammalian host is extremely

low; therefore, *Mycobacterium* compete with the host for limited iron to establish successful infection. Our group previously reported that iron deprivation affects susceptibility of *Mycobacterium* to known anti-TB drugs, and that iron availability is a key factor in considering antimycobacterial drug susceptibilities [17,27]. Moreover, we established that Van treatment hinders iron homeostasis in other prevalent human fungal pathogens, such as *Candida albicans* (unpublished data). The above findings necessitated to study the effects of Van treatment on iron homeostasis in *Mycobacterium*, revealing that Van treatment disrupted iron homeostasis (Fig. 5A and B).

Alkaline pH also mimics iron deprived condition [17]. In the body, iron exists in two states: insoluble Fe(III) and soluble Fe(II). In the case of alkaline pH, iron is predominantly present in the insoluble form, thereby representing an iron-deprived condition [28]. Moreover, when *Mycobacterium* infects humans, it encounters anatomically diverse niches that include alkaline-pH environments. Therefore, we studied the effect of Van treatment at alkaline pH; however, we observed no difference in the growth of *M. smegmatis* at alkaline pH compared with that observed at physiological pH, even in the presence of Van (Fig. 5C). This suggests that Van specifically targeted iron and not pH-dependent pathways in *Mycobacterium*.

During the course of infection through inhalation, *Mycobacterium* encounter alveolar macrophages in the lungs. Macrophages are phagocytic cells involved in the production of reactive oxygen and nitrogen species during phagocytosis or stimulation with various agents. Macrophages can cause different types of DNA damage, including oxidation, depurination, deamination, and methylation, which are responsible for single- and double-strand breaks [29]. In our study, we confirmed that antimycobacterial mechanisms associated with Van were also associated with effects on DNA repair (Fig. 6A). We also determined that genotoxicity following Van treatment was not associated with any alterations in the redox state of the cell (Fig. 6B), ruling out the possibility that antimycobacterial mechanisms associated with Van treatment involved Reactive Oxygen Species (ROS) production. However, further studies are needed to validate the above hypothesis.

Conclusion

Natural compounds, such as Van, that may be used as antimycobacterial drugs to overcome MDR-TB have become emerging sources of interest. Our results suggested that Van is active against *Mycobacterium* and could be a promising candidate for the development of new antimycobacterial agents.

Conflicts of interest

None to declare.

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