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Full Length Article

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

Sharda Sharma, Rahul Pal, Saif Hameed *, Zeeshan Fatima* *

Amity Institute of Biotechnology, Amity University Haryana, Gurgaon, India

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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.

* Corresponding authors at: Amity Institute of Biotechnology, Amity University Haryana, Amity Education Valley, Gurgaon (Manesar) 122413, India.

E-mail addresses: saifhameed@yahoo.co.in (S. Hameed), drzeeshanfatima@gmail.com (Z. Fatima).

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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

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