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Short communication

Unveiling the mode of action of antibacterial labdane diterpenes from *Alpinia nigra* (Gaertn.) B. L. Burtt seeds

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ABSTRACT

The labdane diterpene, (E)-labda-8(17), 12-diene-15, 16-dial (compound **A**) and its epoxide analogue, (E)-8β, 17-Epoxylabd-12-ene-15, 16-dial (compound **B**) were isolated from the seeds of *Alpinia nigra* for the first time. The antibacterial activities of both compounds were evaluated against three Gram-positive and four Gram-negative bacteria, and flow cytometric analysis revealed that these compounds caused significant damage to the bacterial cell membranes. Further, field emission scanning electron microscope imaging and cell leakage analysis confirmed that the labdane diterpenes were responsible for bacterial cell membrane damage and disintegration. Our findings provide new insight into the broad-spectrum effects of two natural labdane diterpenes that may be useful in the future development of herbal antibiotic products.

1. Introduction

Resistance of bacterial pathogens to antibiotics is escalating day by day and imposing an alarming threat to mankind. It has been observed that the Gram-negative and Gram-positive bacteria isolated from hospitalized and ambulatory patients possess increasing resistance to one or multiple antibiotic classes [1,2]. Many critical infections like listeriosis, yersiniosis, enteric fever and other food borne diseases caused by a wide range of bacteria are a major health concern in both developed and developing countries [3–5]. Consequently, there is a greater need to discover and identify natural antibacterial agents from available bioresources as potent future antibacterial therapeutics [6,7].

Alpinia nigra (Gaertn.) B. L. Burtt (family: Zingiberaceae), locally known as "Tora" in North East India, is one of the 250 diverse species of the genus *Alpinia*, distributed through tropical and subtropical climates of Asia and the Pacific [8]. Several natural compounds were identified from the genus *Alpinia* and the majority of them have shown remarkable biopharmaceutical applications [9]. Various health problems like intestinal parasitic infection, gastric ulcers, irregular menstruation, bone weakness and jaundice are known to be cured by ethnomedical uses of *A. nigra* in different states of North East India [10]. Besides these, *A. nigra* has now been established as a notable antihelminthic source towards the possible remedy against intestinal helminth infection [11]. In recent years, extensive work on the genus *Alpinia* has revealed its chemical complexity, diverse bioactive molecules and their antibacterial properties [9], but the species *A. nigra* is still unexplored even though it has versatile ethnomedical uses.

In current study potential bioactive compounds were isolated from the mature seeds of *A. nigra* that were further investigated by different spectral and analytical techniques so as to unveil the mechanism of action as bactericidal agents against tested bacteria.

2. Results and discussion

2.1. Chemistry

On phytochemical investigation, a labdane diterpene dialdehyde (compound **A**) and its epoxide derivative (compound **B**) were isolated and identified as (E)-labda-8(17), 12-diene-15, 16-dial and (E)-β, 17-epoxylabd-12-ene-15, 16-dial respectively (Fig. 1). The structures of the compounds were elucidated by spectroscopic techniques, and the data obtained (NMR, MS and optical rotation) are in agreement with previous published reports [12,13]. The natural occurrence of labdane diterpenes is prevalent in Zingiberaceae

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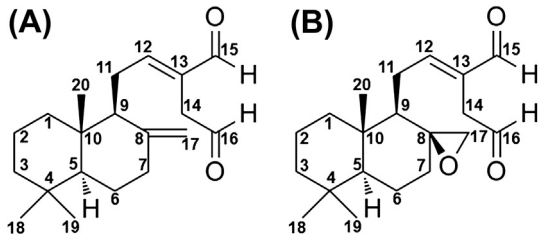

Fig. 1. Chemical structure of (E)-labda-8(17), 12-diene-15, 16-dial (A) and (E)-8β, 17-Epoxylabd-12-ene-15, 16-dial (B).

family, noticeably in *Alpinia* genus [12]. Among the members of *Alpinia*, compound A was first identified from *Alpinia speciosa* [14] and later from many other species [15,16], whereas, compound B was reported only in *Alpinia galanga* [13,15]. However, this is the first time that these two labdane diterpenes were isolated from the seeds of *A. nigra* and further explored for their broad-spectrum bactericidal activities.

2.2. Antibacterial activity

The antibacterial activities [minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)] of the two compounds were evaluated against seven pathogenic bacteria. The MIC and MBC for bacterial strains were in the range of 3.375–25 μg/ml (Table 1). Compound **B** showed the greatest antibacterial activity over compound A in terms of MIC and MBC (except the MBC of *Salmonella paratyphi*). Furthermore, it was observed that of the pathogens tested, *Staphylococcus aureus* (MIC = 3.375 and MBC = 6.75 μg/ml) and *Yersinia enterocolitica* (MIC and MBC = 3.375 μg/ml) were most sensitive to compound **B**.

2.3. Flow cytometric investigation

Flow cytometric (FC) analysis was carried out to assess the effect of compound **A** and **B** on bacterial cell membrane integrity. The cell damaging effects of both compounds were tested at their respective MICs for each bacterial strain. Flow cytometric histograms and median fluorescence intensity (MFI) of propidium iodide (PI) stained bacteria are shown in Fig. 2. The vehicle control (cells treated with ethanol) showed minimal changes in relative fluorescence intensity with respect to untreated control cell populations (Fig. 2a–g). While, the positive control (heat killed bacteria) showed significant increases in relative fluorescence intensity for all of the test bacteria (Fig. 2a–g) and confirmed the cell damage or death. Increases in fluorescence similar to those observed for the positive controls were also observed when the bacterial cells were treated with compounds **A** and **B**. MFI values indicated that treatment of bacteria with compounds **A** and **B** caused significant (Tukey's test, $p < 0.001$) increases in fluorescence intensity compared to the vehicle control for all seven bacterial strains, with compound **B** having a significantly more pronounced effect than compound **A**, especially for the Gram-negative bacterial strains (Tukey's test, $p^{E.\ coli} = 0.001$, $p^{S.\ paratyphi} = 0.038$, $p^{E.\ coli\ \text{enterotoxic}} = 0.009$, and $p^{Y.\ enterocolitica} < 0.001$).

Table 1
The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (μg/ml) of two isolated compounds from *A. nigra* seeds against selected Gram-positive and Gram-negative bacteria.

Tested microorganism	MIC (μg/ml)		MBC (μg/ml)	
	Compound **A**	Compound **B**	Compound **A**	Compound **B**
Gram (+)ve				
S. aureus ATCC 6538	12.5	3.375	25.0	6.75
B. ceresus ATCC 11778	12.5	6.75	12.5	6.75
L. monocytogenes ATCC 19115	25.0	12.5	25.0	12.5
Gram (−)ve				
E. coli ATCC 25922	25.0	12.5	50.0	12.5
S. paratyphi MTCC 735	12.5	6.75	12.5	12.5
E. coli enterotoxic MTCC 723	25.0	6.75	25.0	6.75
Y. enterocolitica MTCC 859	12.5	3.375	25.0	3.375

2.4. Field emission scanning electron microscopy (FESEM) study

The most susceptible Gram-positive bacterium (*S. aureus*) and Gram-negative bacterium (*Y. enterocolitica*) were examined by FESEM to observe morphological changes caused by treatment with compound **A** and **B** (Fig. 3). FESEM images of untreated *S. aureus* showed intact, smooth cell surface with defined cell features (Fig. 3a), whereas membrane disintegration and prominent damage of the cell wall was observed in bacterial cells treated with each compound (Fig. 3b, c). Similarly, membrane damaging effects of each compound were revealed in treated *Y. enterocolitica* cells (Fig. 3e, f) in comparison to the characteristic features of the intact untreated bacterial cells (Fig. 3d). These findings indicate that both compounds cause lysis of bacteria by degrading bacterial cell walls and confirm results that have been described previously [17,18].

2.5. Effect of extracts on bacterial cell membrane

Nucleotides and their constituent building blocks (purines, pyrimidines, pentose and inorganic phosphate) are known to leak from compromised bacterial cells and the levels of leakage of these moieties were determined by measuring the optical density (OD) at 260 nm using UV/VIS spectrophotometer. It was observed that when the values of OD_{260} were plotted, the amount of low molecular weight metabolites increased with increasing time of exposure due to continuous release of cellular materials through compromised cell membrane of treated bacterial strains as compared to controls (Fig. 4). Similar observations of bacterial cell damage and subsequent leakage have been reported previously [19,20]. Also, the epoxide analogue (compound **B**) was found significantly more effective than compound **A** considering the results of cell leakage at 8 and 16 h treatments (Tukey's test, $p < 0.05$). Our findings are in agreement with an earlier published report describing the role of plant derived terpenes towards cell membrane damage of diverse pathogenic bacteria [18].

3. Conclusions

In the current study we have isolated two bioactive labdane type diterpenes from the seeds of *A. nigra* for the first time. Both compounds were characterized by spectroscopic techniques and their antibacterial properties were evaluated. FESEM images clearly revealed that the diterpenes caused extensive damage to the cell membranes of *S. aureus* and *Y. enterocolitica* respectively. The compromised state of the bacterial membranes was further evaluated by flow cytometry and cell leakage analysis which confirmed the strong bactericidal action of two isolated labdane diterpenes.

4. Experimental

4.1. Chemistry

4.1.1. Plant material

The seeds of *A. nigra* were collected from IIT Guwahati (IITG) campus (26°12.476′N to 91°41.965′E) during the period of