SUPPLEMENTARY INFORMATION

Therapeutic potential of labdane diterpene isolated from *Alpinia nigra*: Detailed hemato-
compatibility and antimicrobial studies

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Abstract

(E)-labda-8(17), 12-diene-15,16-dial has been isolated from the seeds of *Alpinia nigra* that is unsuitable for oral administration evident from *in silico* studies. The present investigation therefore deals with understanding the effect of this compound on RBCs for intravenous administration. No prominent hemolytic effect of compound at a concentration of ≤ 0.4 mg/ml was found whereas higher concentrations perforated RBC membrane. The molecule showed remarkable inhibitory potential against Gram negative bacteria (concentration ≥ 0.025 mg/ml) causing cell lysis. In case of pathogenic yeast *Candida albicans* although growth was inhibited (concentration ≥ 0.0025 mg/ml) growth kinetic study revealed that the diterpene significantly delayed the growth (concentration 0.005 – 0.020 mg/ml) by preventing substrate uptake and was able to extend its lag phase in a dose-dependent manner. This study tries to unveil the mechanism of action of this diterpene on microorganisms with differential cell wall compositions.

Keywords: *Alpinia nigra*, *Candida albicans*, Gram negative bacteria, Labdane diterpene, RBCs

Experimental

**Isolation of compound**

Preparation of organic extracts from the seeds of *A. nigra* and isolation of the compound was carried out as previously described (Chakrabartty et al. 2018).

**In silico studies**

Lipinski’s rule

The molecular properties and drug likeliness of the compound was examined on the basis of ‘Lipinski’s rule of five’ using Molinspiration server (http://www.molinspiration.com/)

**Hemolytic assay**

Quantitative hemolytic activity assays was performed for different concentrations of labdane diterpene (0.2 mg/ml - 1 mg/ml). Approximately 2% ethanol in 1X PBS was used as carrier solvent for all the sample preparations.

**Quantitative hemolytic assay**

**RBC sample preparation**

RBCs were isolated from whole blood as per the protocol of Zohra and Fawzia (Zohra & Fawzia 2014) with some modifications. Briefly, fresh blood [O group] was drawn from healthy volunteers after obtaining informed consent. The whole blood was centrifuged at 2000 rpm for 10 minutes and 20 % erythrocyte suspension was prepared in 1X PBS (pH 7.4).

**Assay**

Hemolytic activity of samples was tested *in-vitro* using spectrometry. The different test samples were mixed with erythrocyte suspension in 1:1 ratio and incubated at 37°C for 40 mins. 1X PBS was used as
negative control and 0.5% Triton X as positive control. After incubation, the cells were spun down and the supernatant was collected. Absorbance reading taken at 540 nm using multimode microplate reader (Tecan, Infinite M – 200 pro, Switzerland) and the results presented as percentage hemolysis or hemolytic ratio calculated using the following formula (Shrestha et al. 2016):

\[
\% \text{Lysis} = \frac{[As - An]}{[Ap - An]} \times 100
\]

where As: Absorbance of test sample
An: Absorbance of negative control
Ap: Absorbance of positive control

**Microscopic analysis**

*Field emission scanning electron microscopy (FESEM)*

Erythrocyte suspension (20%) for higher magnification imaging using FESEM were prepared as mentioned earlier with minor modification (Hortolà 1992). In brief, treated and untreated RBCs were washed with 1X PBS (pH 7.4) and fixed in 2.5% glutaraldehyde solution for 24 h at 4°C. Cells were washed twice with 1X PBS and were dehydrated with graded ethanol from 10% to 100%. Samples were coated with gold film in a Polaron sputter coater. The images were acquired with field emission scanning electron microscope (Carl Zeiss Ultra 55) at 3.0 kV power.

**Antimicrobial studies**

*Microbial cultures*

The antibacterial activity of labdane diterpene was evaluated against two Gram negative bacteria: *Escherichia coli* enterotoxic (MTCC 723) and *Salmonella paratyphi* (MTCC 735). The bacterial strains were grown and maintained as previously described (Kesari et al. 2010) on nutrient agar plates. The biological potential of the isolated compound was also tested against *Candida albicans* (NCCPF no. 400035) and the culture was maintained on YPD (1% yeast extract, 2% peptone and 2% dextrose) agar plates. Before each experiment the microbes were sub-cultured twice in their respective broths (nutrient broth for bacteria and YPD broth for *C. albicans*) to ensure their active growth.

*Sample preparation*

A stock concentration of labdane diterpene (2 mg/ml) was prepared in 5% DMSO (SRL, India) in 1X PBS (pH 7.2; prepared from its constituent salts procured from HiMedia, India) and further diluted in 1X PBS. The dilutions of the test samples were prepared for labdane (0.4 mg/ml – 0.006 mg/ml for bacterial culture and 0.020 mg/ml- 0.000312 mg/ml for fungal culture).

*FESEM*
FESEM was used to visualize the changes in the morphology of the microbial cells before and after treatment with the compound and positive control, kanamycin (0.025 mg/ml; Sigma, India). The microbial cells were washed with freshly prepared 50mM phosphate buffer (pH 7.2), fixed with 2.5% (v/v) glutaraldehyde (HiMedia, India) in PBS and rinsed again with the same buffer. The specimen was dehydrated with increasing grades of ethanol from 10% to 100%. The specimens were then, coated with gold and analyzed through FESEM (Carl Zeiss Ultra 55), as previously described (Ghosh et al. 2013).

Growth kinetics

Bacteria

Growth curve

Growth curve analysis was carried out in 96-well micro-titre plates (Eppendorf - cell culture) as per protocol (Sekse et al. 2012) with some modifications. Briefly, around 100 µL of bacterial culture (adjusted to 10⁶ cells) was added to corresponding well and to this the samples were added so that the final concentrations mentioned above are obtained. Antibiotic kanamycin (Sigma, India) (0.025 mg/ml) was used as positive control and 5% DMSO in 1X PBS was used as solvent control. Plates were incubated at 37°C in shaking incubator (Orbitek, India) and absorbance values were recorded at regular intervals of 2 hrs for 24 hrs using multimode microplate reader (Tecan, Infinite M – 200 pro, Switzerland). The observed optical density values were plotted against time to obtain the growth pattern.

Viability assessment of bacteria

2,3,5-triphenyl tetrazolium chloride (TTC) assay is the simple method to assess the cell viability. In presence of live cells, TTC is reduced to triphenyl formazan by the respiratory linked dehydrogenase enzymes that require NAD or NADP (Alkinsnson et al. 1950). The development of red color due to formation of formazan salt is the indicator for presence of live cells and its intensity is directly proportional to number of live cells.

For cell viability analysis after treatment with compound, around 100 µL of bacterial culture adjusted to 10⁶ cells was added to corresponding microfuge tubes (Eppendorf) and to this the samples were added so that the final concentrations mentioned above are obtained. Tubes were incubated at 37°C in shaking incubator for 12 hours. Kanamycin (0.025 mg/ml) was used as positive control and 1X PBS was used as solvent control. After incubation, the cells were spun down and washed with 1X PBS twice. Then the 1% TTC solution (SRL, India) was added to the tubes to a final concentration of 2.5 mM and incubated in dark for 8 hours at 25°C. The formazan salts that resulted were solubilized using 1% SDS in 50% methanol for 30 minutes at 60°C. The cells were again spun down and supernatant was collected. The absorbance values were recorded at 485 nm and the results were represented as relative viability percentage (Castro-concha et al. 2012). Relative viability percentage is calculated using the formula:
Relative Viability = \( \frac{[A_s - A_n]}{[A_p - A_n]} \times 100 \)

- \( A_s \): Absorbance of test sample
- \( A_n \): Absorbance of negative control
- \( A_p \): Absorbance of positive control

**Fluorescence microscopy**

Extent of bacterial cell damage and lysis after treatment with the different samples was also determined using fluorescence microscopy. Freshly inoculated bacterial cells were treated with the samples for 6 hrs at 37°C. Post incubation, the cells were collected and washed with 1X PBS twice. Antibiotic kanamycin (Sigma, India; 0.025 mg/ml) was used as positive control and 1X PBS was used as solvent control. Cells were later fixed with glutaraldehyde (2.5% v/v) for 3 hrs followed by washing with PBS twice. Fixed cells were then, stained with propidium iodide (PI, Sigma, India) for 10 mins. Cells were visualized with inverted fluorescent microscope (100X; Olympus) and images were captured to observe the average number of stained cells. Cells were counted using Image J software.

**Fungus**

**Minimum effective concentration determination**

The isolated compound was screened to evaluate the minimum effective concentration required to inhibit the growth of *C. albicans* using agar well diffusion method as previously described (Magaldi et al. 2004). Different dilutions of the compound were prepared in DMSO (0.020 mg/ml – 0.000312 mg/ml). Flucanozole at a concentration of 0.020 mg/ml and 5% DMSO were used as positive and vehicle controls respectively.

The minimum effective concentration was also determined by micro broth dilution method in 96 – well micro-titre plate as per the protocol (Hood et al. 2010) with some modifications. Two fold serial dilution of the compound was prepared in DMSO ranging from 0.010 – 0.000156 mg/ml and added to each individual well. Around 50 µl of *Candida* suspension (adjusted to \( 10^6 \) cells/ml) was added and the plates were incubated at 28°C for 18hrs. Results were analyzed with multimode microplate reader (Tecan, Infinite M – 200 pro, Switzerland) at 660nm and the lowest concentration at which fungal growth was inhibited was considered as the minimum effective concentration, which was reported as the mean of triplicate experiments.

**Raman spectroscopy**

Characterization of the fungal cells before and after treatment with the compound was also performed by using Raman spectroscopy. Untreated *Candida* cells were used as negative control. About 3ml of fungal
cultures at exponential growth phase (10^8 CFU/ml) were centrifuged at 8000 rpm for 5 min. Cells were
washed and suspended in sterile distilled water. The sample was put on clean coverslip, allowed to dry at
37ºC and subjected to Raman spectrophotometer (Horiba LabRam HR). The decrease in the intensities of
Raman shift indicates fall in the number of microorganism due to inhibition of growth upon treatment
(Das et al. 2013).

Cell leakage analysis
In order to confirm the membrane damaging efficacy of the compound, cell leakage analysis was
performed by monitoring the absorbance at 260 nm of the cell supernatants. C. albicans was inoculated in
YPD broth (1% yeast extract, 2% peptone, 2% dextrose) and treated with the different concentrations of
the compound. The cultures were incubated for 4, 8, 12, 16, 20 and 24 hrs respectively. The samples were
centrifuged at 5000 rpm for 5mins in order to separate the fungal cells from low molecular weight
metabolites such as nucleotides, amino acids and inorganic ions which are known to leak from the cells
after membrane damage. Finally, the level of released material from the bacterial cell was determined by
measuring the optical density of the supernatant at 260nm using UV-Vis (Gene Quant 1300, India)
spectrophotometer. The average of triplicate experiments was used for the plot.

Dissolved oxygen analysis
Oxygen uptake for growth of the C. albicans cells before and after treatment with the compound was
performed using biological dissolved oxygen monitor (BOD; Hach, USA). DO in media is a direct
measurement of the bacterial respiration and reflects the growth state of microorganism (Krishnaraj et al.
2010).

Growth curve
The YPD medium was used for the growth of C. albicans and treatment was given with different
concentrations of labdane (0.020 mg/ml – 0.0000312 mg/ml). A 1% v/v inoculum of active fungal culture
was added to 50ml media in 250 ml Erlenmeyer flasks. The flasks were incubated in a shaker incubator at
28ºC and 180rpm. Flucanozole (>98% purity; procured from Sigma) at a concentration of 0.020 mg/ml
and 5% DMSO were used as positive and vehicle controls respectively. The measurement of growth and
glucose uptake was done as previously described (Dutta et al. 2015) and absorbance was taken using
UV/Vis spectrophotometer (AnTech, India).

Substrate uptake
The uptake of glucose by C. albicans was obtained by colorimetric assay using GOD-POD kit as
previously described (Krishnaveni et al. 1984).

Statistical analysis
All experiments were set up in a completely randomized design and with a minimum of three replicates. All the graphs were plotted as mean of the observed values using Origin 8.5. The statistical analysis was carried out using SPSS Statistics 17.0 ($R^2 \geq 0.96$). Significance of analysis for mean values of each experiment were compared to the means of corresponding vehicle control at respective time points using one-way ANOVA followed by Tukey’s test ($p < 0.05$).

**Supplementary Figures**

**Figure S1 (a)** Percentage lysis of erythrocytes caused by labdane (0.4 to 0.6 mg/ml); inset shows the percentage lysis of solvent and positive control; FESEM images of RBCs treated with (b) 1X PBS (c) Triton X, (d) labdane (0.8 mg/ml); inset shows pores on RBC membrane
Figure S2 (a) FESEM images of *E. coli* (a, d and g) and *S. paratyphi* (b, e and h) treated with 0.006 mg/ml of labdane, positive control and 0.050 mg/ml of labdane respectively; untreated, flucanozole and labdane treated *C. albicans* (c, f and i) respectively.

Figure S3 (a) Percentage of viable Gram negative bacteria upon treatment with labdane; inset shows percentage viability of positive and solvent control; Fluorescent microscopic images of *E. coli* (b-e) and *S. paratyphi* (f-i) treated with positive control, solvent control, 0.006 and 0.050 mg/ml of labdane respectively.
Figure S4 Growth profile (a) and substrate uptake (b) respectively, of C. albicans at different concentrations of labdane diterpene in the presence of fixed initial substrate concentration (2% dextrose)

Supplementary Results

In silico studies

Lipinski’s rule

Lipinski’s rule of five states that a drug is orally active if it fulfils all of the following criteria: not more than 10 hydrogen bond acceptors, not more than 5 hydrogen bond donors, molecular weight to be below 500 Dalton, and the partition coefficient (log P) should be less than 5 (Lipinski et al. 1997). The calculation obtained shows that labdane diterpene satisfies all but one clause of the Lipinski’s rule, suggesting that this compound is not suitable for oral administration (Table S1). The log P value of this compound exceeds 5 indicating its higher lipophilicity. However, the log P value of labdane diterpene satisfies the claims of the ‘modified Lipinski’s rule of five’ (Ghose et al. 1999); hence it becomes a potential “oral” therapeutic drug molecule.

Table S1 Lipinski parameters for isolated labdane diterpene

| Parameters          | Predicted Values |
|---------------------|------------------|
| H-bond acceptor     | 2                |
| H-bond donor        | 0                |
| Molecular weight    | 302.45           |
| Log P               | 5.36             |
| Violations          | 1                |

* log P is the logarithm of partition coefficient between water and 1-octanol, indicates lipophilicity, should be less than 5
**Bacterial growth curve**

It is seen that labdane diterpene is highly effective in inhibiting the growth of Gram negative bacteria upto a period of 26 hrs (**Figure S5 a and b**). The inhibition of labdane at higher concentrations is comparable to that of the positive control. However, low concentrations of the compound (0.012 mg/ml and 0.006 mg/ml) are not able to stop the growth of *E. coli*. In case of *S. paratyphi*, the lag phase of the bacteria is extended upto 16hrs of treatment after which it resumes its normal growth pattern. Whether the compound kills the bacteria or merely prevents its growth and extends its lag phase need to be investigated further.

![Figure S5 (a-b) Growth curves of E. coli and S.paratyphi respectively treated with different concentrations of labdane](image)

**Candidical activity**

*Minimum effective concentration determination*

The minimum effective concentration was found to be 0.000312 mg/ml for *C. albicans* till 12 hrs of incubation (**Table S2**). The minimum effective concentration of the compound by microbroth dilution method was also found to be 0.0003125 mg/ml (Data not shown).
Table S2 Inhibitory efficacy of different concentrations of labdane diterpene dialdehyde against *C. albicans*

| Sr. No. | Concentration of labdane diterpene (mg/ml) | Zone of inhibition (mm) |
|---------|-------------------------------------------|-------------------------|
| 1       | 0.020                                      | 15.95±0.5               |
| 2       | 0.010                                      | 14.35±1.85              |
| 3       | 0.005                                      | 12.1±1.1                |
| 4       | 0.0025                                     | 8.25±1.4                |
| 5       | 0.00125                                    | 6.11±0.55               |
| 6       | 0.000625                                   | 3.07±1.25               |
| 7       | 0.0003125 µg/ml                            | 2.0±1.02                |

Zone of inhibition of flucanozole (0.020 mg/ml) is 21.25±1.25 mm

**Raman spectroscopy**

Raman spectroscopy gives detailed information about the composition of the fungal cell and provides a fingerprint region below 1800 cm⁻¹ (Movasaghi et al. 2007). Sharp peaks at 646 cm⁻¹ and 687 cm⁻¹ corresponds to biochemical element amino acids. Peaks from 719-792 cm⁻¹ are attributed to the nucleotides: nitrogenous bases and O-P-O stretch of DNA (Chouthai et al. 2015). Peaks at 948 cm⁻¹ and 957 cm⁻¹ correspond to glucose. The sharp peak at 1315 cm⁻¹ corresponds to nucleic acid and amide III band of tryptophan. The peak at 1443 cm⁻¹ is attributed to δCH₃ group. Moreover, a sharp decline in almost all prominent peak intensities of *Candida* cells after treatment with the compound signifies extensive cell damage (**Figure S6**). This decrease in intensity after treatment with the compound might be due to due to lack of cell growth as cell damage or destruction was not observed.
Figure S6 Raman spectra of *C. albicans* showing candicidal activity of labdane diterpene

*Cell leakage analysis*

Nucleotides and their constituent building blocks (purines, pyrimidines etc.) are known to leak from compromised fungal cells and levels of release of these moieties are determined by measuring optical density at 260 nm. It was observed that the amount of low molecular weight metabolites remained constant with increasing time of compound treatment (*Figure S7*). This indicated that there was no release of metabolites from the yeast cells; the amount of released metabolites being comparable to the untreated cells. This further added to the observation that no cell lysis or surface pore formation occurred upon compound treatment; hence cell materials were not released.

Figure S7 Absorbance of cell materials at 260 nm releasing from *C. albicans* after treatment with labdane diterpene at 0, 4, 8, 12, 16, 20 and 24hrs

*Dissolved oxygen analysis*
Respiration activity of the pathogen, *C. albicans*, was performed to elucidate the possible mode of action of the labdane diterpene. In our present study, we observe that the amount of dissolved oxygen remains constant in the treated cells when compared with untreated fungal culture. Amount of dissolved oxygen in the media reduces <0.7 mg/l in the untreated cells whereas in the treated cells, it remains almost equal to its initial concentration (>7mg/l) (Figure S8). The respiration rate and as a result the growth of the *Candida* cells is inhibited under the effect of labdane diterpene. This can be attributed to the inhibitory activity of labdane diterpene on certain membrane proteins or enzymes like ATP-binding cassette (ABC) transporters, acetylcholine receptor and protein transferases and result in delay in growth of the fungal cells (Seo et al. 2012).

![Figure S8 Changes in the respiratory activity of *C. albicans* after treatment with labdane diterpene](image)

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