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Stimulation of Phagocytosis in Alveolar Macrophages and Resistance against Infection in Mice by the Aqueous Extract of Rhizomes of *Aframomum danielli*, Schum (Zingiberaceae)

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Abstract

Background: Alveolar Macrophages (AMs) play important role in preservation of lungs from infectious diseases development through a series of activities including phagocytosis. The present study examined the effects of aqueous extract of *Aframomum danielli* rhizomes on the AMs functions.

Methods: Extract was investigated for microbe's ingestion, bacteria killing and reactive oxygen and nitrogen species production in alveolar macrophages. Extract was investigated for repository, suppressive and curative treatments of *S. aureus* infection in mice.

Results: The extract of *Aframomum danielli* augmented (approximately threefold) the ingestion of *Candida albicans* by phagocytes. Furthermore, extract concentration-dependently increased the killing ability of *S. aureus* of mice AMs. Moreover, the extract significantly stimulated the production of O_2, H_2O_2 and nitric oxide (NO) by mice AMs. In addition, extract make mice to become more resistant to *S. aureus* infection. Finally, aqueous extract of *A. danielli* dose dependently increased reduced the bacteremia density in Dex-mice in suppressive, curative and repository treatments.

Conclusion: Together the results showed that aqueous extract of *A. dalnielli* promotes the phagocytosis of microbes by alveolar macrophages and thereby may contribute to respiratory tract prevention from bacterial colonization.

Keywords: *Aframomum danielli*; Alveolar macrophages; Phagocytosis; Immunostimulation

Introduction

In developing nations, where, malnutrition and infectious diseases remain a challenge, a stimulation of body defense mechanisms is of high interest. Malnutrition and infectious diseases contribute to cause immunodeficiencies.

Immunomodulating drugs are therefore necessary to control various opportunistic infections such *Staphylococcus aureus* infection [1-4]. In respiratory tract, alveolar macrophages are frequently target of immunomodulating drugs to boost the resistance against infections [5,6].

Medicinal plants such as *Pterocarpus erinaceus*, *Barleria prionitis*, *Pseudocedrela kotschyi*, *Ficus septica* have been claimed to possess immunomodulatory activities [7-10]. Plant such as *Aframomum danielli* is cited in control of diseases related to immune disorders, however it is not been studied on alveolar macrophages despite its common use in management of respiratory tract infections [11-14].

As, alveolar macrophages, constitute the first line of defence against microorganisms carried out by alveolar macrophages, the present investigation is designed to assess the effect of aqueous extract of *A. danielli* rhizomes on alveolar macrophages functions through evaluation of phagocytosis and killing of *Staphylococcus aureus* in mice [15].

Materials and Methods

Study design and setting

The study protocol was approved by the Ethics Committee for animal use in research studies of the laboratory of the Department of Biological Sciences, University of Bamenda, Cameroon.

The study was conducted at the Institute of Agricultural Research for Development (IRAD) of Bambui, Cameroon. All procedures involving animal were in accordance with the Principles of Laboratory Animal Care [16].

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Experiment animals

Male albinos white mice (Mus musculus) originated from Laboratoire National Veterinaire (LANAVET) were used for isolation of macrophages and *in vivo* tests. They were housed in plastic cages with softwood shavings and chips as beddings, with free access to pellet diet and clean drinking water. Animals were acclimatized to the working environment 1 week before the beginning of the experiment. At end experiment, they were anesthetized by a mixture of ketamine chloride (80 mg/kg) and xylazine chloride (3.2 mg/kg) injected intraperitoneally.

Microbe strains and infection

Clinical isolates of *C. albicans* and *S. aureus* used were got from the laboratory of Biochemistry, University of Dschang, Cameroon. They were administered to mice intravenously via the tail or intranasal (1×10^8 microorganisms/animal).

Plant collection and extraction

Aframomum danielli specimen was collected from Bambili, Cameroon. The plant was identified by a taxonomist at the Department of Biological Sciences and confirmed by the National herbarium Yaounde, Cameroon. Plant rhizomes were collected, chopped into small pieces, washed with clean water and air dried at 38°C. They were powdered and boiled in distilled water for 1 hour. After filtration, using Whatman paper No 1, the filtrate was evaporated at 35°C in the oven and the resulting dried product was collected and used after dissolution in PBS.

Isolation of Alveolar Macrophages

Alveolar macrophages (AMs) were obtained by lavage of lungs from five mice. Prior to the date of isolation (20 h), mice were given a single S.C. dose of saline of dexamethasone disodium phosphate (Dex, Sigma Chemical) of 20 mg/kg. The lungs were excised and washed 5 times with RMPI-1640 using brief massage. Collected cells were estimated to about 5-10 × 10^6 cells (80% macrophages) for the 5 mL lavage fluid. After centrifugation (10 min, 1800 tr/min) at 37°C, cell pellet was suspended in complete RMPI-1640 medium (medium supplemented with 5% bovine serum fetal, 100 units/mL penicillin and 100 µg/mL streptomycin). The number of cells was counted using a malassez hemocytometer. The viability of the cells (≥ 98%) was determined by trypan blue (Difco) exclusion assay.

Slide methods for evaluating the phagocytosis

Human blood (0.4 mL) was obtained by finger prick method on a sterile glass slide and incubated at 37°C for 25 min to allow clotting. Later, the clot was carefully removed, and the slide was slowly drained with sterile PBS, taking care not to wash the adhered phagocytes. Next, the slide was flooded with 1 mL of extract and incubated at 37°C for 20 minutes. Slides were once more flooded with 1 mL of suspension of opsonized *C. albicans* prepared in normal saline solution mixed with human serum (ratio 8:2) and incubated at 37°C for 30 minutes. Finally, the slide was drained fixed methanol and stained with giemsa stain (the procedures were repeated and the slide stained after 1 hour).

The mean number of phagocytosed cells on the slide was determined microscopically for 100 phagocytes using morphological criteria. This number was taken as the Phagocytic Index (PI) and was compared with basal PI of controls. Plant effect expressed in percentage was calculated using the following equation:

$$\text{Stimulation (\%)} = PI_{(test)} - PI_{(control)} \times 100/PI_{(control)}$$ [17]

Bacteria killing assay

The killing assay for *S. aureus* was adapted from a method of measurement of intracellular killing of bacteria [18]. AMs were cultured in T75 flasks (NUNC) for 48 hours in RMPI-1640 with the extract or levamisole (4 µg/mL) as control positive. Later, they were cultured in triplicate in a 96-wells microplate with a blank well containing the medium (2×10^7 macrophages/mL). To each well, it was added 100 µL bacterial suspension in RPMI contenaing 3% Normal Human Serum (NHS) and incubated at 37°C under gentle shaking for 90 min. Bacteria was added in ratio macrophages/bacteria of 1:4 and the final volume of the mixture was 150 µL per well. From each well, 100 µL of the culture was collected and transferred to other microplate, immediately after adding the bacterial suspension (T_0) and 60 min after (T_{60}), and placed on ice. At 90 min (T_{90}), the initial microplate was also placed on ice.

Serial 10-fold dilutions were made in ice-cold H_2O of the three cultures and plated on LB agar plates. After overnight culture at 37°C the numbers of colonies were counted. The killing was reflected by a decrease in number of colonies at T_{60} and T_{90} relative to the number of colonies at T0. The killing percent was calculated using the following formula:

% Killing = 100 × {1-(Number of colonies at T_{60} or T_{90})/(Number of colonies at T_0)} [19]

Cell culture and ROS assay

The purified alveolar macrophages (2×10^5 cells/well in 96 well tissue culture plate) of Dex-treated mice were incubated with RMPI 1640 containing 1 µg/mL lipopolysaccharide (LPS) and extract at different concentrations for 24 hours at 37°C. Macrophages from Dex-untreated mice were used as positive control. Nitric oxide (NO) and hydrogen peroxide (H_2O_2) were determined in the macrophage culture supernatants, while superoxide anion (O_2^-) was measured in cells by colorimetric methods. The activity of the extract was determined and expressed as percentage of stimulation.

Measurement of AM superoxide anion synthesis

The assay used was the ability of the superoxide produced by macrophages to reduce yellow nitroblue tetrazolium (NBT) to blue formazan [20]. To the cells, 50 µL of NBT solution (1 $mg.ml^{-1}$) was added and incubated for 1 h. Then, washed 3 times with PBS 1X and cells were fixed with methanol 100% for 3 min. After fixation, cells were washed twice with 70% methanol and allowed to air dry. The formazan produced was solubilized by adding 120 µL of 2M KOH and 140 µL 100% DMSO. Absorbance was read at 630 nm using KOH/DMSO as blank. The optical densities were used as follows to determine the effect of the extract:

$$\text{\% activity} = 100 \times \{(OD_{sample} - OD_{control})/OD_{control}\}$$

Measurement of AM NO oxide production

The nitrite/nitrate content, indicative of NO production by iNOS, was monitored by the Greiss reagent assay [9]. In 96-well plates, supernatant samples (100 µL) were mixed with 100 µl Greiss reagent, consisting of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-napthyl) ethylenediamine dihydrochloride (1:1 vol/vol) and incubated for 10 min. The change in absorbance was monitored at 545 nm and the optical densities were used to determine the % activity.

$$\text{\% activity} = 100 \times \{(OD_{sample} - OD_{control})/OD_{control}\}$$

Measurement of AM hydrogen superoxide release

H_2O_2 was analyzed using the colorimetric method [21]. To 50 µL 10 mM $FeSO_4 \cdot 7H_2O$ (dissolved in 1 mM HCl) and 25 µL 2.5 mM KSCN (dissolved in PBS) were added to 100 µL of the supernatant. Optical densities were measured in a microplate reader at 490 nm wavelength. Additionally calibration curve were assessed. Optical densities of RPMI 1640 containing various H_2O_2-concentrations (0-200 µM f.c.) and 20 µL TCA (50%) were measured and used for standard curve for determination of the H_2O_2 release in tested well. The % activity was determined as follows:

% activity = 100 × {$(H_2O_2)_{sample}$ - $(H_2O_2)_{control}$)/$(H_2O_2)_{control}$}

Evaluation of the effect of extract on death rate induced by *S. aureus* infections in dexamethasone-treated mice.

Mice (Mus musculus) were treated with dexamethasone 20 mg.kg^{-1}, 20 hours prior to infection. Later, they were infected with lethal inocula 1 × 10^8 *S. aureus* and daily treated P.O. with extract for 5 days. Levamisole (25 mg/kg) was administered to the control. Mice were observed for 28 days. From day 6 post treatment, the survival percentage and survival time for each mouse was recorded. The percentage of survival was calculated as follows:

% Survival = 100 × (Number of survival/Total number of group)

Antibacterial activity tests of extract in repository, suppressive and curative treatments against *S. aureus* in mice.

Swiss albino mice (18-25 g) were randomly divided into different groups of 5 mice per cage for the various treatments as described in Table 1. Extract, levamisole and distilled water were orally and daily administered by gavage for 7 days. Animal were intra-nasally inoculated and *S. aureus* (1 × 10^8 microorganisms in PBS) was used as microorganisms. Nasal lavages were obtained as described by Bogaert *et al.* and the lavage product was inoculated on LB agar [22]. The bacteremia level was assessed by counting the number of colonies 24 hours of incubation at 37°C.

Statistical analysis

All statistical analyses were done on GraphPad Prism version 5.02. Data were expressed as mean ± Standard Deviation (SD). ANOVA test was used to analyze the difference among various treatments with Least Significance Difference (LSD) at 0.05 followed by student Newman-keuls's post-test.

Results

Stimulation of *C. albicans* ingestion by human peripheral blood phagocytes

Results as presented in Table 2 showed that the extract, concentration-dependently and time-independently, increased the phagocytosis of opsonized *C. albicans* ($p<0.05$). Used of heat-inactivated serum for opsonisation did not change the extract-mediated enhancement of phagocytosis (data not shown), indicating complement independency.

Table 1. Animal grouping for repository, suppressive and curative treatment.

Test	Groups	Treatment
Extract-repository antibacterial activity test	Group 1	Treatment with distilled water + Infection + Nasal lavage
	Group 2	Treatment with 25 mg/kg Lev + Infection + Nasal lavage
	Group 3	Treatment with 100 mg/kg extract + Infection + Nasal lavage
	Group 4	Treatment with 200 mg/kg extract + Infection + Nasal lavage
	Group 5	Treatment with 400 mg/kg extract + Infection + Nasal lavage
Extract-suppressive antibacterial activity test	Group 1	Infection + Treatment with distilled water + Nasal lavage
	Group 2	Infection + Treatment with 25 mg/kg Lev + Nasal lavage
	Group 3	Infection + Treatment with 100 mg/kg extract + Nasal lavage
	Group 4	Infection + Treatment with 200 mg/kg extract + Nasal lavage
	Group 5	Infection + Treatment with 400 mg/kg extract + Nasal lavage
Extract-curative antibacterial activity test	Group 1	Infection + 3 days of observation + Treatment with distilled water + Nasal lavage
	Group 2	Infection + 3 days of observation + Treatment with 25 mg/kg Lev + Nasal lavage
	Group 3	Infection + 3 days of observation + Treatment with 100 mg/kg extract + Nasal lavage
	Group 4	Infection + 3 days of observation + Treatment with 200 mg/kg extract + Nasal lavage
	Group 5	Infection + 3 days of observation + Treatment with 400 mg/kg extract + Nasal lavage

Table 2. Effect of *A. danielli* rhizomes aqueous extract on alveolar macrophages phagocytosis by slide method using *Candida albicans*.

Variables	Mean Percentage of Stimulation ± SD	
Concentration of extract (µg/ml)	**30 min Extract**	**60 min Extract**
Control	0	0

40	5.92 ± 4.57	10.62 ± 7.31
80	8.00 ± 6.33	19.11 ± 12.63**
160	13.42 ± 5.26*	40.21 ± 15.20***
320	21.20 ± 13.30**	45.00 ± 21.36***

Values expressed are mean ± standard deviation of triplicate measurements. Mean values with asterisks were significantly different from the control (% stimulation was taken as zero) ($p<0.05$).

Stimulation of killing of *S. aureus* by Alveolar macrophages

Aqueous extract of *A. dalnielli* increased significantly ($p<0.05$) the intracellular bacteria killing activity of Dex-treated macrophages compared to untreated macrophages (Table 3).

The % killing of extract-treated macrophages was concentration dependent at any time point with comparable percent to those of macrophages pre-incubated with levamisole at 160 µg/mL and 320 µg/mL.

Table 3. Effect of the extract of the rhizomes of *A. dalnielli* on the percentage of killing of *S. aureus* of Dex-treated mice alveolar macrophages.

Variables	**Percentage of *S. aureus* killing**	
Incubation period	**60 min**	**90 min**
Control		
Medium	8,5 ± 16,7[a]	6,62 ± 8,15[a]
Levamisole (4 µg/mL)	63,26 ± 26,4*[a]	82,35 ± 9,49***[b]
Concentration of extract (µg/mL)		
40	24,56 ± 8,8[a]	22,11 ± 11,11[a]
80	47,64 ± 3,0*[a]	49,36 ± 5,31*[a]
160	60,41 ± 1,3*[a]	79,51 ± 9,17**[b]
320	66,76 ± 2,5**[a]	84,17 ± 4,14***[b]

Data are Mean ± SD of the results of three separate experiments given as the percentage killed *S. aureus* performed in triplicate. Means values with asterisks were significantly different from the percentage killed *S. aureus* by untreated macrophages. Letters a and b indicates a time-dependent difference.

Effect of *A. dalnielli* on production of reactive oxygen species

Data in the present study (Table 4) showed that extract of *A. danielli* elicited a dose-dependent significant ($P<0.05$) increase of the production of superoxide anion production, nitric oxide and hydrogen peroxide by macrophages of Dex-treated mice compared to untreated macrophages.

Table 4. Effect of aqueous extract of *A. danielli* rhizomes on production of superoxide anion production, nitric oxide and hydrogen peroxide by macrophages of Dex-treated mice.

Variables	**Free-DM**		**Macrophages of Dex-treated mice**			
	LPS	**LPS**	**Concentrations of extract (mg/ml) + LPS**			
	1 µg/ml	**1 µg/ml**	**40**	**80**	**160**	**320**
Superoxide anion	131.15 ± 12.2[a]***	32.8 ± 12.01	39.63 ± 3.79[b]ns	60.67 ± 6.24[b]*	89.83 ± 6.01b***	128.65 ± 7.21[a]***
Nitric oxide	60.25 ± 6.12[a]***	5.73 ± 3.27	6.49 ± 2.07[b]ns	9.74 ± 1.68[b]ns	38.27 ± 3.13b***	55.64 ± 4.56[a]***

Hydrogen superoxide	95.31 ± 12.23^{a***}	19.32 ± 7.12	24.15 ± 4.03^{b}ns	36.51 ± 4.38^{b*}	52.75 ± 9.40b***	85.16 ± 14.58^{a***}

Values expressed are mean ± standard deviation of triplicate measurements. Mean values with asterisks were significantly different from the control (effect of LPS on macrophages Dex-treated mice) (p<0.05). Letters indicates the difference compared to the macrophages of free Dex-treated mice.

Protection of dexamethasone-treated mice against *S. aureus*

Treatment with aqueous extract of A. dalnielli showed significant resistant of mice against *S. aureus* (Figure 1). At 21 days, all saline water treated mice dead while 40%, 60% and 90% survived and were cured in groups treated with 100, 200 and 400 mg/kg extract (P<0.05).

Treatments of experimentally induced *S. aureus* infection in mice

Aqueous extract of *A. danielli* at 100, 200 and 400 mg/kg, in repository treatment as well as in suppressive and curative treatment, caused a dose-dependently suppression of bacteremia against *S. aureus* reducing the density of bacteremia compared to control (Figure 2). Similarly, levamisole caused significant suppression of bacteremia density in repository, suppressive and curative treatment.

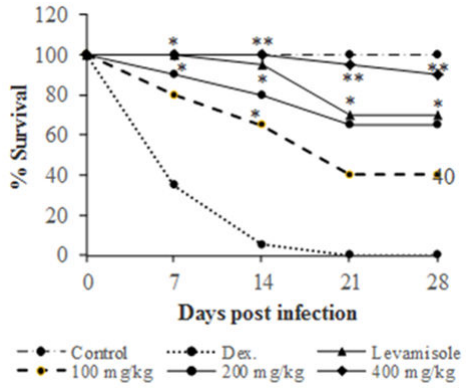
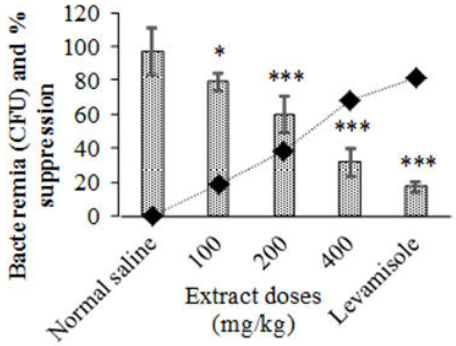
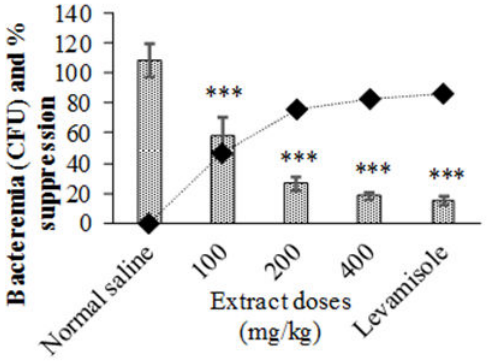


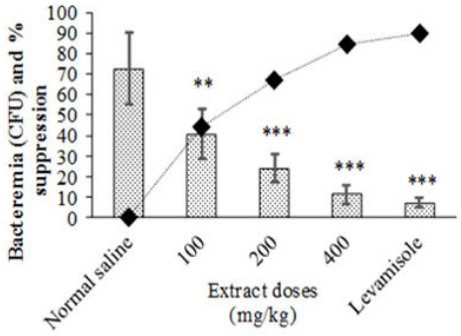
Figure 1. Extract treatment induced resistance against *S. aureus* in Dex-treated mice. Data are mean of the results of two independent experiments. Control represents untreated mice. *p<0.05 for treated mice vs. mice treated with dexamethasone by t-test.



(A)



(B)



(C)

Figure 2. Repository (A), suppressive (B) and curative (C) effects of aqueous extract of *A. danielli* rhizomes against *Staphylococcus aureus* in mice. Values expressed are mean ± standard deviation. Mean values with asterisks were significantly different from the negative control at p<0.05. Chart and curve represent respectively bacteremia density and % suppression.

Discussion

In a therapeutic dose 0.05 mg/kg, dexamethasone results in reduction of AMs as well as depression of their phagocytic activity [5]. It inhibits the functions of blood leucocytes and tissue macrophages by modulating the release of cytokines, inflammatory mediators and ROS generation, avoiding that the animal's own immunologic response to the disease cause intense tissue damage and further aggravation of the condition [15,23]. In the present study, our analysis provided clear evidence that the extract of *A. dalnielli* increases level of microorganism's ingestion by human blood cells as well as stimulates the killing of *S. aureus* by alveolar macrophages of Dex-treated mice. This demonstrates that the extract of *A. dalnielli* the extract stimulates the activities the phagocytic activities AMs.

Dexamethasone, in the 0.05 mg/kg therapeutic dose, was described to reduce the oxidative metabolism of healthy calves' alveolar macrophages [15]. Here, we found that AMs of Dex-treated mice produced more of superoxide anion, hydrogen superoxide and nitric oxide when treated with the extract *A. dalnielli* rhizomes.

this might justify the effectiveness of the extract shown in bacterial killing as reported by numerous studies [24].

Reactive oxygen species are generated in macrophages mainly by following mechanisms: xanthine oxidase-dependent, respiratory chain and NADPH oxidase-(NOX-) dependent pathways [25]. Extract of *A. dalnielli* has increased ROS production suggesting that it might be acted by activating one of these pathways. Macrophages NO production was significantly increased by extract of *A. dalnielli*. As NO produced by iNOS in macrophages and neutrophils serves an important pathological and immunological role, this suggest that extract *A. dalnielli* rhizomes might activate iNOS in macrophages as it is inhibited by dexamethasone [26].

In vivo test, extract significantly increased survival percent of Dex-treated mice infected with *S. aureus*, reducing the bacteraemia level. This suggests that the extract of *A. dalnielli* rhizomes might act not only on alveolar macrophages but it could affect peripheral blood polymorphonuclear or mononuclear phagocytes. In curative, suppressive and prophylactic treatment against nasal infections by *S. aureus* in Dex-treated mice, the extract of *A. dalnielli* significantly exhibited suppressive, prophylactic and curative activities confirming that extract stimulate the phagocytosis in alveolar macrophages.

Conclusion

The findings of this study supported by the traditional uses, sustain that *A. danielli* rhizomes are important in control of pulmonary infections via its stimulatory effect on macrophages, when *S. aureus* is known as one of the most common causes of bacterial lung infections in normal hosts.

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Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

OM was participated for conception of ideas, experiments, data analysis and interpretation, and preparing the manuscript. GKO was also participated for conducting the experiments. TC was participated for conception of ideas. KA was participated for conception of ideas. All authors read and approved the final manuscript.

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