Bactericidal activity of acetone extract of Alpinia galanga on multidrug resistant clinical isolates of Enterococcus faecalis

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Abstract

Introduction: The objective of the study was to evaluate the inhibitory activity of five traditional Indian medicinal herbs against multidrug resistant clinical isolates of E. faecalis. Methods: Sixty five E. faecalis derived from clinical specimens were tested for their resistance pattern to 7 antibiotics by disc diffusion method. Extracts from 5 medicinal plant parts were obtained and tested for inhibitory activity against multi drug resistant clinical E. faecalisisolates. The minimum bactericidal concentration (MBC) for the active plant extract exhibiting highest activity towards the susceptible isolateswas determined by macro broth dilution method followed by sub culturing on agar plate. Results: All the clinically derived E. faecalis isolates were resistant to two or more antibiotics. Acetone extract of Alpinia galanga inhibited 67.69% of E. faecalis and concentration of <5.0mg/ml was found to exert bactericidal activity against multi drug resistant isolates. Conclusion: Acetone extract of A. galanga exhibit bactericidal activity against multi-drug resistant clinical isolates of E. faecalis and hence could be potential phytotherapeutic candidate.

Keywords: A. galanga, drug resistance, E. faecalis, inhibition, medicinal plants

Introduction

India is a land of rich heritage for employing medicinal plants in treating illness among its diverse indigenous cultures. Several of these folklore medicines, employing herbs and medicinal plants are validated through scientific research and made accessible to the global population. Therapy through plant products benefits its users by providing generalised immunostimulatory and immunomodulatory effects apart from abating the side effects of antibiotics and monetary expenditure for cure [1, 2].

Enterococci are one among the leading nosocomial pathogens commonly associated with urinary tract infections, blood stream infections and catheter related infections. Of more than a dozen species in the genus, Enterococcus faecalis is involved in 70 – 80% of clinical infections [3]. Infections by multidrug resistant E. faecalis encompassing even the last line drug of choice are emerging around the globe, accounting for increase in morbidity and mortality rates [4,5].

E. faecalis is an ideal candidate which deserves a new phytochemical therapeutic agent considering their significance in nosocomial infections and antibiotic resistance. This study unearths the inhibitory activity of five traditional Indian medicinal plants against clinical isolates of E. faecalis. The study is novel and innovative as there are no existing literatures proving the inhibitory potential of acetone extract of Alpinia galanga and its minimalbactericidal concentration (MIC) against drug resistant clinical isolates of E. faecalis.

Materials and Methods

Place of study and type of study: The test-tube lab research study was carried in the Microbiology laboratory of Rajah Muthiah Institute of Health Sciences to demonstrate the inhibitory activity of traditional medicinal plants on multi-drug resistant E. faecalisisolated from clinical specimens.

Isolation of E. Faecalis: The clinical specimens- urine, blood and pus submitted for bacteriological investigation to the Microbiology laboratories at Rajah Muthiah Institute of Health Sciences were employed for
the study. The specimens were inoculated on routine
diagnostic culture media and Pfizer’s selective
enterococcus agar (Hi-Media Laboratories, India).
Following aerobic incubation of the inoculated culture
plates, colonies morphologically resembling
Enterococcus were characterized and identified as E.
faecalis by standard microbiological and biochemical
procedures [6]. The isolates were stored at -20 °C in
20% glycerol Brain Heart Infusion broth (Sisco
Research Laboratories Pvt. Ltd., India) until further use.

Detection of antibiotic resistance for clinical E.
faecalis isolates: E. faecalis obtained from clinical
specimens were studied for their antibiotic resistance
pattern by disc diffusion test and the results were
interpreted according to CLSI, 2012 [7]. The antibiotics
(Hi-Media Laboratories, India) tested includes
Penicillin (10 units), Ampicillin (10 μg), Gentamicin
(high content) (120 μg), Streptomycin (high content)
(300 μg), Ciprofloxacin (5 μg), Erythromycin (15 μg)
and Vancomycin (30 μg).

Plant extracts employed for study: The plants were
chosen based on i) their documented use as antimicrobials in literature and ii) traditional use of
these plants among the regional folklore against
infections [8]. The plants employed in the study (Table
1) were harvested in Cuddalore district and taxonom-
ically identified. Crude extracts from the plants were
extracted with solvents mentioned in Table 1 as per the
method described by Eloff [9]. The extracts were stored
in the form of dried powder at -20 °C until it was used
for further biological evaluation.

Determination of inhibitory activity of the plant
extracts by disk diffusion test: The plant extracts were
tested for their inhibitory activity against the 25
multidrug resistant E. faecalis isolates derived from
clinical specimens. A single colony from overnight
growth on blood agar plates were inoculated in Brain
heart infusion broth and incubated at 37 °C for 4 h.

This bacterial suspension was adjusted to 0.5 Mc
Farland Standard and used as inoculum to obtain a lawn
culture on Mueller Hinton agar plates (Sisco Research
Laboratories Pvt. Ltd., India). Discs (6 mm in diameter)
obtained from Whatmann No.1 filter paper was soaked
with the plant extracts reconstituted with 20% DMSO
(to obtain a concentration of 5mg/ml) and placed on the
inoculated plates. Suitable controls were included.
Following incubation at 37 °C for overnight period,
the plates were evaluated for the presence of zone of
inhibition around the discs. The extracts were considered
to exhibit anti-E. faecalis activity if a inhibition zone
>10mm was observed was found around the extract
impregnated disc.

Determination of the minimum bactericidal
concentration (MBC) of A. galanga against E.
faecalis isolates: Macrobath dilution test was
employed to determine the minimum inhibitory
concentration (MIC) of A. galangaon susceptible
multidrug resistant E. faecalis isolates according to the
protocol of CLSI, 2012 [7]. Extract obtained from A.
galanga was diluted with Mueller Hinton broth in test
tubes to obtain a final concentration of 2.0, 2.5,3.0, 3.5,
4.0, 4.5mg/ml. E. faecalis adjusted to 1.5 x 10⁶ CFU/ml
was used as inoculum at a quantity of 1ml on the tubes
containing A.galanga extract. Mueller Hinton broth
containing E. faecalis suspension alone and A. galanga
extract without the inoculum were included as controls.
The minimum concentration of A. galanga extract
which inhibited the visible growth of E. faecalis was
considered as MIC value. The MBC of the extract was
determined by spot inoculating 10 μl of the suspension
from macrobath dilution tubes lacking the growth of E.
faecalis, on Mueller Hinton agar plates. Following
incubation at 37 °C for overnight period the minimum
concentration of the extract that produced no visible
colonies was recorded as the MBC value for the strain
tested.

Results

Isolation of E. faecalis from clinical specimens: Sixty
five isolates of E. faecalis was isolated from urine, pus,
blood and ascitic fluid at the isolation rate of 44%, 28%,
16% and 12% respectively.

Antibiotic resistance pattern of clinical E. faecalis
isolates: Antimicrobial resistance pattern for E.faecalis
isolates showed 72% of isolates to be high-level
gentamicin resistant and 68% of isolates demonstrated
resistance to 2 antibiotics - erythromycin and
ciprofloxacin. None of the isolates were found
vancomycin resistant (fig. 1).

The clinical isolates of E. faecalis were found to be
resistant to a combination of 2 or more antibiotics (fig.
2). Combined resistance to three antibiotics was the
frequent observation and was seen among 35% of
isolates. However, none of the isolates were found
resistant to all the 7 antibiotics used in the study.

Plant extracts used and their inhibitory activity
against E. faecalis: Of the 5 plant extracts studied for
their inhibitory activity against 65 antibiotic resistant
clinical isolates of E. faecalis by disc diffusion test,
A.galanga was found to inhibit 67.69% of isolates
followed by *M. fragrans* which inhibited 47.69% of isolates (fig.3). Since, *A. galanga* exhibited inhibitory activity on the majority of the *E. faecalis* isolates used in our study, this extract and the strains inhibited by the extract were chosen for further studies.

**Minimum bactericidal concentration (MBC) of *A. Galanga* extract for the test isolates:** The 44 *E. faecalis* isolates which were inhibited by *A. galanga* extractas determined by disc diffusion test was further studied for the MBC value of *A. galanga* extract required to inhibit them. A concentration of 3.0 mg/ml of *A. galanga* extract was found to be the MBC value for a majority of *E. faecalis* isolates (36.3%). MBC value for more than 50% of our isolates was $\leq 3.0$ mg/ml (table 2).

**Discussion**

In our study, *E. faecalis* was commonly isolated from urine, followed by pus and blood samples. The predominance of this pathogen in urinary tract infections and pyogenic infections are reported widely [10, 11]. The 1st line drug of choice for treating enterococcal infections is with a synergistic combination of beta lactam and aminoglycoside antibiotics [12]. In our study, 42% and 27% of isolates exhibited resistance to beta lactam class of antibiotics – penicillin and ampicillin respectively. Further, high-level resistance to aminoglycosides- gentamicin and streptomycin was noted with 72% and 46% of isolates respectively. With the prevalence of higher resistance rates towards the synergistic antibiotic combinations and the occurrence of multidrug resistance among our clinical isolates, the treatment options for infections demands the role of 2nd and last line drug of choice which are also noted for their adverse effects [10, 13].

The isolation of multidrug resistant *E. faecalis* from clinical specimens in our geographical locality makes the situation imperative to find an alternate treatment option. The phytochemicals of the extracts among the 5 plants selected in the study have proven antibacterial activity against common Gram positive and Gram negative pathogens [8, 14,15]. In our study, the extracts from the five selected plants exhibited inhibitory activity on 13.84% - 67.69% of *E. faecalis* isolates with *A. galanga* demonstrating inhibition on a majority of isolates. *A. galanga* is widely cultivated medicinal herb in India and the traditional medicinal systems have used the rhizome for diuretic, expectorant, carminative purposes and in treating tubercular glands, bronchitis, kidney and heart diseases [16]. The chief chemical constituent of rhizome is flavonoid which exerts antibacterial activity through inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of the attachment and biofilm formation, inhibition of the porin on the cell membrane, alteration of the membrane permeability and attenuation of the pathogenicity [17, 18].

The inhibitory activity of *M. fragrans* succeeds *A. galanga* in our study, and was active on 47.69% of *E. faecalis*. Methanol extracts of *M. fragrans* was earlier reported to have strong antibacterial activity against multi-drug resistant *S. typhi* [19]. Extracts from *A. graveolens*, *B. serrate* and *H. indicus* inhibited 9%, 41.53% and 18.46% of our isolates. Literatures citing the inhibitory activity of these medicinal plants against several Gram positive, Gram negative bacterial pathogens, yeast and fungi are recorded earlier [20 – 22].

The acetone extract of *A. galanga* employed in our study proved to exhibit inhibitory activity on *E. faecalis*. However previous studies indicate ether, ethyl acetate, aqueous extract and essential oil of *A. galanga* for significant antibacterial activity against *S. aureus* and *S. pyogenes* [23]. In our study, the MBC of acetone extract of *A. galanga* for the clinical isolates of *E. faecalis* ranged between 2.0 – 4.5 mg/ml, and the concentration of 3.0 mg/ml of the extract inhibited 36.3% of isolates.

This concentration was found to be lesser when compared tothe study of Okonogi *et al* wherein 8mg/ml was the MBC of essential oil of *A. galanga* against *S. aureus* ATCC 25923 [24]. In another study involving human pathogens, an active component 1′-acetoxy chavicol acetate from the ethyl acetate extract of *A. galanga* demonstrated antibacterial activity against *P. acnes* with the MIC and MBC values of 156 and 312 μg/ml [25]. A study by Warit *et al* showed the extract of *A. galanga* and its major component S-enantiomer of acetoxy chavicol acetate exhibited anti-tubercular activity on clinical isolates at 2.0 μg/mL [26].

**Conclusion**

As an attempt to elude from the repercussion of antibiotic usage for pathogens which exhibit multiple drug resistance, an extensive search is made towards herbal therapy. The study documents the inhibitory activity of acetone extract of *A. galanga* to be superior compared with other medicinal plants for their anti-*E. faecalis* activity. Further, bactericidal activity on the majority of the common Gram positive and Gram negative pathogens is demonstrated.
activity was demonstrated at concentration of <5.0 mg/ml against all the multidrug resistant isolates used in our study. This substantiates the efficacy of A. galanga as an alternative therapeutic option to multiple drug resistant E. faecalis.

Table-1: Plant extracts used for the study

| Plant (scientific name) | Family       | Parts used | Solvent for extract preparation |
|-------------------------|--------------|------------|--------------------------------|
| Anethum graveolens      | Apiaceae     | Fruits     | Water                          |
| Alpinia galanga         | Zingiberaceae| Rhizome    | Acetone                        |
| Boswellia serrata       | Burseraceae  | Gum resin  | Methanol                       |
| Hemidesmus indicus      | Asclepiadaceae| Root       | Chloroform                     |
| Myristica fragrans      | Myristicaceae| Seeds      | Methanol                       |

Table-2: MBC of A. galanga extract for E. faecalis

| MBC values of A. galanga (mg/ml) | No. of E. faecalis isolates inhibited by the extract (Percentage of isolates) (n=44) |
|---------------------------------|---------------------------------------------------------------------------------|
| 2.0                             | 2 (4.5)                                                                         |
| 2.5                             | 6 (13.6)                                                                        |
| 3.0                             | 16 (36.3)                                                                       |
| 3.5                             | 14 (31.8)                                                                       |
| 4.0                             | 4 (9.1)                                                                          |
| 4.5                             | 2 (4.5)                                                                          |

Fig-1: Antibiotic resistance pattern of clinical E. faecalis isolates

Fig-2: Percentage of E. faecalis isolates demonstrating resistance to a combination of 2 or more antibiotics
Fig-3: Inhibitory activity of plant extracts on *E. faecalis* isolates

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Authors Contributions
R. Arularasi Aberna and K.Prabhakar contributed to the conception and the design of the experiment.
R. Arularasi Aberna performed the experimental analysis, interpretation and manuscript writing.
K.Prabhakar critically evaluated the paper.

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