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ORIGINAL ARTICLE

Time Kill Assay and Bactericidal Mechanism of Action of Ethanolic Flowers Extract of *Sphaeranthus indicus*

Nazish Mumtaz[1], Muhammad Arif Asghar[2,]*, Syed Baqir Shyum Naqvi[3], Muhammad Asif Asghar[4], Muhammad Liaquat Raza[5], Ahad Abdul Rehman[6]

[1] Department of Pharmaceutics, Faculty of Pharmacy, Benazir Bhutto Shaheed University, Lyari, Karachi, Pakistan

[2] Department of Pharmaceutics, Faculty of Pharmacy, Jinnah Sindh Medical University, Rafiqui H.J. Shaheed Road, Karachi-75510, Pakistan

[3] Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, Karachi, Sharea Madinat Al-Hikmah, Muhammad Bin Qasim Avenue, Karachi-74600, Pakistan

[4] Food and Feed Safety Laboratory, Food and Marine Resources Research Centre, PCSIR Laboratories Complex, Shahrah-e-Salimuzzaman Siddiqui, Off University Road, Karachi-75280, Pakistan

[5] Department of Clinical Sciences, Biomedical Centre – BMC, Lund University Hospital, Lund, Sweden

[6] Department of Pharmacology, Faculty of Pharmacy, Jinnah Sindh Medical University, Rafiqui H.J. Shaheed Road, Karachi-75510, Pakistan

Authors' Contributions
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Address of Correspondence Author:
m.arifasgher@hotmail.com

ABSTRACT

Objective: The purpose of the study was to identify the bactericidal mechanism of action and time kill assay of a medicinal plant *Sphaeranthus indicus* Linn against some highly resistant Gram positive and Gram negative clinical pathogens.

Methods: Antimicrobial action of this plant was compared with four different antibiotics using disk diffusion method. The MIC and MBC of the extract were measured by broth dilution method. The bacterial killing mechanism of extract was examined using SEM technique.

Results: It was observed that the zones of inhibition produced by the plant extract were higher than that of some well-reputed antibiotics. The values of MIC and MBC indicated that the extract had strong inhibitory and bactericidal activity against tested isolates. The plant extract achieved a more drop in growth profile of *S. pneumonia*, *S. typhi*, *E. coli*, and *P. aeruginosa* within 20 mins. After 12 h exposure with extract, the SEM images of *S. pneumonia* showed the destruction of cell membrane and cells were completely lysed.

Conclusion: It is concluded that ethanolic flowers extract of *S. indicus* has promising bactericidal potential at very low concentration. Its time killing was extremely faster especially against *S. pneumonia*. Its flowers extract produced bactericidal action through a cell membrane disruption of bacteria.

Keywords: Antimicrobial activity, mechanism of action, *Sphaeranthus indicus*, *Streptococcus pneumonia*, time kill assay.

INTRODUCTION

Medicinal plants play an enormous role as a most effective antimicrobial agent because of its outstanding antimicrobial activity. According to the World Health Organization (WHO), medicinal plants have been used as a traditional medicine by more than 80% of the world's population. The chemical constituents obtained from medicinal plants are used to treat various infectious as well as chronic diseases [1].

Those communities who survive in rural areas tribes specifically depends on various plants as a source of making household implements, fire and shade, construction of dwellings, food, forage, sleeping mats, and as a source of herbal medicine. In many developing countries, medicinal plants are used as a traditional remedy in a wide range of their rural areas [2]. It has been claimed by traditional healers that plant-based medicines are cost-effective as compared to other modernistic medicines. Non affording people in different developing countries such as village isolated people, farmers and native societies use herbal medicines for the treatment of different infections [3].

Among different medicinal plants, *Sphaeranthus indicus* is of great importance which belongs to family *Asteraceae*. It is used worldwide to cure different human ailments and conditions such as dermatitis, bronchitis, rheumatism, and asthma [4]. *S. indicus* Linn is known to be called "Boddasoram" in Telugu while in English it is known to be called 'East Indian globe thistle'. It is widely cultivated in different regions of India and other sub-tropical regions [5].

The juice of this herbaceous plant has shown powerful biological action to treat epileptic convulsions, dysentery diseases of the spleen while flowers of *S. indicus* have powerful depurative and tonic properties [6]. Furthermore, the knowledge regarding its antimicrobial time assay and killing mechanism against different microorganisms is insufficient. In order to promote *S. indicus* as a bactericidal plant different strains of Gram positive, Gram negative and a fungal specie were kept for observation to determine its activity. Therefore, the aim of this study was to investigate the bacteriostatic and bactericidal actions of flowers extract of *S. indicus* against some highly resistant clinical pathogens such as *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pneumonia*, Methicillin-resistant *S. aureus, Escherichia coli*, *Pseudomonas aeruginosa*, and a fungal specie *Candida albicans* were used. The killing kinetics and mechanism of microbial killing by flowers extract of *S. indicus* were examined using a broth dilution method at a minimum bactericidal concentration (MBC) of each bacterial strain.

MATERIALS AND METHODS

Collection of Antimicrobial Agents

Flowers of *S. indicus* were collected from local market of Karachi Pakistan during the winter season. The voucher specimen was submitted in Faculty of Pharmacy, Hamdard University Karachi-Pakistan and flowers were identified and confirmed by a Pharmacognosist and Meritorious Professor Dr. Ghazala H. Rizwani. Antibiotics discs namely ciprofloxacin, imipenem, tetracycline, ampicillin and an antifungal agent *i.e*. Amphotericin-B were purchased from Musaji Adam and Sons (OXOID, UK) distributor.

Collection of Different Pathogenic Strains

The different clinical isolates including *Salmonella typhi* (LT 0266), *Staphylococcus aureus* (LT 0674), *Bacillus subtilis* (LT 0312), *Streptococcus pneumonia* (LT 0048), Methicillin-resistant *S. aureus (MRSA) (LT 0011), Escherichia coli* (LT 0023), *Pseudomonas aeruginosa* (LT 0178), and a fungal specie *Candida albicans* (LT 0019) were obtained from Hamdard University Hospital and Kutiyana Memon Hospital Pathological Laboratories in Karachi-Pakistan. The obtained pathogenic strains from the hospitals were identified on the basis of their cultural, morphological and biochemical reaction by pathologists.

Preparation of Plant Extract

Extract of *S. indicus* flowers was prepared according to the method used in our previous study [7]. Fresh and healthy flowers of *S. indicus* were washed properly with water which was followed by the distilled water. The flowers of *S. indicus* were shaded, desiccated and turned into the powdered form using an electric blender. The 200 g of powder of *S. indicus* flowers was soaked into 1600 mL of ethanol using Soxhlet extractor. The obtained extract was filtered and desiccated using rotary vacuum evaporator to obtain a crude extract of flowers of *S. indicus*.

Determination of Antimicrobial Activity

Antimicrobial activity of *S. indicus* flowers was studied using a well reputed standard disc diffusion method. This method was approved by the Clinical Laboratory and Standard Institute (CLSI) for testing of different bacterial strains [8]. It is the most commonly used method in many clinical settings to identify antibacterial susceptibility since 1940. Four different

antimicrobial discs of different concentrations were used namely ciprofloxacin (10 µg/disc), imipenem (10 µg/disc) tetracycline (30 µg/disc), ampicillin (2 µg/disc), Amphotericin-B (20 µg/disc) and the ethanolic extract of plant disc (6 mm in diameter). These discs were placed on the preinoculated Mueller Hinton Agar (MHA) plates with respective cultures and were incubated at 37 °C for 24 hrs. Inhibition zones of different antimicrobial agents were measured and recorded.

Determination of Minimum Inhibitory and Bactericidal Concentrations

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of ethanolic extract of *S. indicus* were estimated using the broth dilution method [9]. All tests were performed in nutrient broth and solubilized the extracts using Tween 20 as a medium. Different concentrations of plant extract were prepared ranged from 0.5 to 100 mg/mL by serial dilution technique. The concentration of each isolate was adjusted to 1×10^8 cfu/mL. The inoculated plates were incubated at 37 °C for 24 h. The MIC value defined as the minimum concentration of the extract at which the tested isolates does not exhibit visible growth. The growth of tested isolates was determined by the turbidity in the test tube. The broth was incubated in Tyramide Signal Amplification (TSA) system at 37 °C overnight. The MBC refers to the minimum concentration of the extract at which isolates were killed completely.

Killing Kinetics Assay

The time kill study of ethanolic extract of *S. indicus* was conducted at the flowers extract concentrations of plant equal to MBC of all collected bacterial strains. The cells of tested isolates were grown to logarithmic phase for 1 h in nutrient broth prior to the exposure of the ethanolic extract of *S. indicus*. The concentration between 6 to 8 log cfu/mL for each isolate was used. The bacterial cultures were incubated at 37 °C in a shaker until no viable bacterial cells were found. The viability of bacterial cells was measured using 50 µl of already known dilutions of the inoculated culture strain on to the TSA system. Then plates were incubated for 48 h and considered as having no growth was observed. The bacterial cells count plates with 20 to 200 colonies were utilized for cfu counts. The bactericidal kinetic plot was constructed between Log cfu/mL and time in min.

Morphology of *S. pneumonia* Cells

The morphology of bacterial cells at initial and after exposed to *S. indicus* extract was studied according to the modified method of Yousra *et al.* using scanning electron microscopy (SEM) technique [10]. The suspension of bacteria was placed into a membrane filter and dried before and after treated with extract. Then culture was fixed with glutaraldehyde (2.5% in phosphate buffered). Next, the isolates were stained for 1 h with OsO_4 (1% in phosphate buffered) and dehydrated with methanol and water. The gold coated membrane was analyzed with SEM.

RESULTS

Antimicrobial Activity

The antimicrobial actions of different well known antimicrobial agents and one of the crude ethanolic flower extract of *S. indicus* against highly resistant microbes are best expressed in Table **1** and **2** respectively. Among the tested clinical pathogens *S. pneumonia*, *S. typhi*, *B. subtilis*, and *E. coli* were highly sensitive to the ethanolic extract.

Table 1. Zone of inhibitions of different antimicrobial agents against highly resistant clinical pathogens.

Microorganisms	Zone of inhibitions in mm				
	Ciprofloxacin	Imipenem	Tetracycline	Ampicillin	Amphotericin-B
S. typhi	11.7 ± 1.419	17.2 ± 0.173	17.1 ± 0.462	16.5 ± 0.224	NA*
S. aureus	10.1 ± 0.415	18.2 ± 0.215	23.2 ± 0.141	16.5 ± 0.774	NA
B. subtilis	14.2 ± 0.626	23.0 ± 1.804	17.8 ± 0.124	15.0 ± 0.587	NA
S. pneumonia	14.2 ± 0.581	16.8 ± 2.274	18.0 ± 1.627	17.2 ± 1.187	NA
MRSA	13.2 ± 1.291	19.4 ± 0.675	19.4 ± 1.572	15.7 ± 1.744	NA
E. coli	16.2 ± 0.824	19.4 ± 1.378	23.7 ± 0.419	14.0 ± 1.287	NA
P. aeruginosa	16.3 ± 1.195	18.2 ± 0.810	17.3 ± 1.187	17.4 ± 0.618	NA
C. albicans	—	—	—	—	15.3 ± 1.841

Results are mean ± standard deviation.
N = 21 (*E. coli*); 14 (*K. pneumonia*); 10 (*P. aeruginosa*); 14 (*B. subtilis* and *MRSA*); 13 (*S. aureus*); 18 (*S. typhi*); 7 (*C. albicans*)
*No Activity

It was observed that the zones of inhibition produced by the plant extract were higher than that of some well-reputed antibiotics.

The values of MIC and MBC of ethanolic flower extract of *S. indicus* Linn showed conclusive results in screening test of differently selected pathogens (Table **3**). The results of MIC and MBC tests gave the evidence that extract of *S. indicus* had strong inhibitory as well as bactericidal activity against different pathogenic strains such as *S. pneumonia*, *S. typhi*, *B. subtilis*, *MRSA* and *S. aureus*. It was found that MIC and MBC tests of plant extract showed identical results *i.e*. 1.0, 1.0, 1.0, 2.0 and 2.0 mg/mL respectively. The results of the statistical analysis are presented in Table **4**.

Table 2. Antimicrobial activity of ethanolic extract of *Sphaeranthus indicus* against clinical isolates at different concentrations.

Microorganisms	Zone of inhibitions in mm			
	5 mg/mL	10 mg/mL	15 mg/mL	20 mg/mL
S. typhi	16.3 ± 0.472	21.5 ± 0.680	23.1 ± 1.375	24.1 ± 2.105
S. aureus	12.1 ± 0.195	15.2 ± 1.216	16.6 ± 1.412	18.8 ± 1.292
B. subtilis	14.1 ± 1.722	18.2 ± 1.142	21.0 ± 0.563	27.9 ± 0.990
S. pneumonia	16.8 ± 0.131	20.9 ± 1.092	23.3 ± 1.247	28.2 ± 0.329
MRSA	12.3 ± 1.624	17.5 ± 1.180	19.4 ± 1.034	21.1 ± 2.154
E. coli	13.1 ± 1.212	17.0 ± 1.023	23.0 ± 1.125	23.7 ± 1.036
P. aeruginosa	9.4 ± 1.241	12.7 ± 1.220	15.7 ± 0.774	16.9 ± 2.674
C. albicans	8.1 ± 1.717	13.1 ± 0.591	17.4 ± 0.970	24.6 ± 1.255

Results are mean ± standard deviation.
N = 21 (*E. coli*); 14 (*K. pneumonia*); 10 (*P. aeruginosa*); 14 (*B. subtilis* and *MRSA*); 13 (*S. aureus*); 18 (*S. typhi*); 7 (*C. albicans*)

Table 3. MIC and MBC of ethanolic extract of *S. indicus*.

Microorganisms	MIC* (mg/mL)	MBC** (mg/mL)
S. typhi	1.0 ± 1.315	1.0 ± 0.721
S. aureus	2.0 ± 0.875	2.0 ± 1.752
B. subtilis	1.0 ± 1.612	1.0 ± 1.970
S. pneumonia	1.0 ± 1.580	1.0 ± 2.240
MRSA	2.0 ± 1.375	2.0 ± 0.987
E. coli	2.0 ± 0.640	4.0 ± 1.027
P. aeruginosa	4.0 ± 0.941	8.0 ± 1.770
C. albicans	3.0 ± 1.413	6.0 ± 2.870

Results are mean ± standard deviation
*Minimum inhibitory concentration
**Minimum bactericidal concentration

Table 4. Statistical analysis of ANOVA and Post Hoc Tukey's test.

Microorganisms	One-way ANOVA (Level of significance*)	Multiple comparisons with ethanolic extract of *S. indicus* (Level of significance*)				
		Ciprofloxacin	Imipenem	Tetracycline	Ampicillin	Amphotericin-B
S. typhi	0.031	0.025	0.017	0.031	0.018	0.000
S. aureus	0.022	0.021	0.024	0.013	0.025	0.000
B. subtilis	0.010	0.027	0.038	0.016	0.010	0.000
S. pneumonia	0.018	0.013	0.008	0.015	0.031	0.000
MRSA	0.031	0.016	0.021	0.019	0.024	0.000
E. coli	0.013	0.011	0.012	0.014	0.016	0.010
P. aeruginosa	0.013	0.011	0.013	0.012	0.003	0.000
C. albicans	0.011	0.000	0.000	0.000	0.000	0.034

*P<0.05

Antimicrobial Killing Kinetics

After exposure to the ethanolic flower extract of *Sphaeranthus indicus*, the growth profile of each tested microorganisms at MBC are presented in Figure **1**. This killing kinetics study was carried out over a period of 48 hours against highly resistant pathogenic strains. The ethanolic flower extract of *S. indicus* achieved a more drop in growth profile of *S. pneumonia*, *S. typhi*, *E. coli*, and *P. aeruginosa* within 20 mins. Furthermore, it was also observed that after exposure of all tested microorganisms to ethanolic flower extract of *S. indicus*, the microorganisms showed stationary growth phase after 2 hrs.

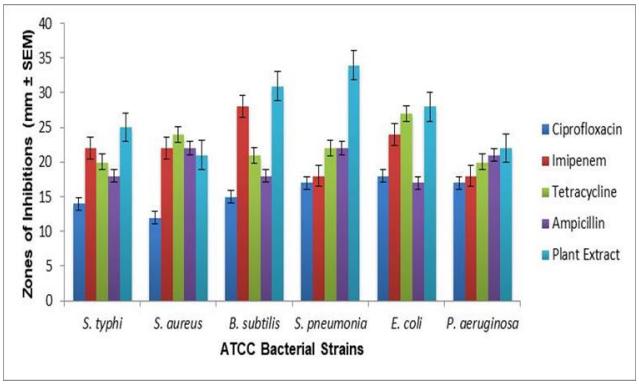

Figure 1. Antibiotic and plant extract susceptibility of ATCC microbial strains.

Morphology Study of *S. pneumonia*

Our study findings demonstrated that *S. pneumonia* showed the most sensitive to *S. indicus* extract among all tested isolated. According to Centers for Disease Control and Prevention (CDC) report, lower respiratory diseases are the 3rd leading cause of death worldwide and pathogenic strain of *S. pneumonia* is commonly responsible for causing serious lower respirato6ry infections [11]. Therefore SEM study was performed on *S. pneumonia* to examine its morphology. Under the SEM study of *S. pneumonia* cells, it was observed that the bacterial cells got shrunk within a few hours after exposure to extract of *S. indicus* at different time intervals. Figure **2A** shows the normal cells of *S. pneumonia* at the primary stage of exposure to extract while after 6 h, the decline in cell growth occurred (Figure **2B**). After treatment to 12 h with plant extract, the complete death of the cells observed as shown in Figure **2C**.

In recent trends, different novel chemotherapeutic medicinal agents are developed using plant extracts. The *in vitro* antimicrobial activity studies on these plants extracts are the essential step to achieve this goal. Many medicinal uses of flower extract of *S. indicus* were reported such as anti-cancerous, antimicrobial, antioxidant and anti-inflammatory [12-14]. However, the study on killing kinetics and killing mechanism of *S. indicus* was not reported yet.

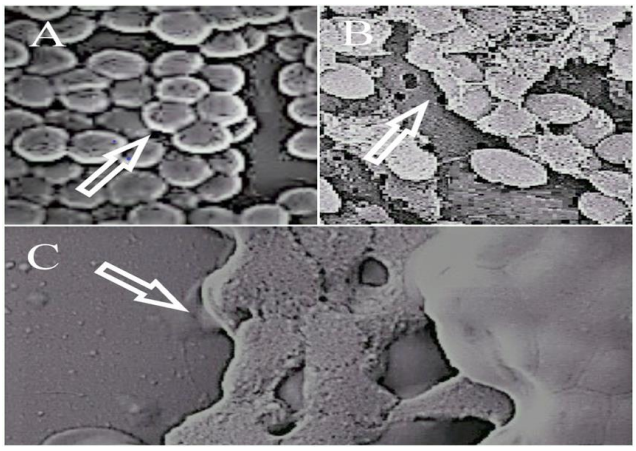

Figure 2. Morphology of *S. pneumonia* at initial stage (A), after 6 hours (B), and 12 hours (C), exposed to methanolic extract of *S. indicus* at *K. pneumonia* MIC.

In the present study, the ethanolic flowers extract of *S. indicus* showed significant antibacterial and antifungal activity against *S. typhi*, *S. aureus*, *B. subtilis*, *S. pneumonia*, *MRSA*, *E. coli*, *P. aeruginosa* and *C. albicans*. The obtained results reflect that growth inhibitory effects of plant extract against most of the microorganism were much greater as compared to four well reputed marketed antibiotics. Furthermore, the statistical results of ANOVA and Post Hoc Tukey's tests proved that the significant differences found between the antimicrobial activity antibiotics and plant extract. Moreover, the extraction of plants constituents using different solvents is very important in obtaining the antimicrobial activity of plant extract [15]. The variability in antimicrobial activity of plant extract observed between microorganisms suggests that the antimicrobial activity of the extract was mediated by a composition of different antimicrobial constituents.

Differences found in MIC and MBC values among different microorganisms may be due to the difference in morphology and composition of microbial cells. Our findings also indicated that the more concentrated extract produced greater growth inhibitory effects due to the greater amount of antimicrobial constituents in the extract.

The activity of *S. indicus* extract against different pathogenic strains showed in a significantly increased

in a number of killed bacteria at the initial period as indicated in Figure **1**. It was observed that the ethanolic flowers extract of *S. indicus* killed all tested microorganisms within 60 mins. The results of killed kinetics study showed that the extract produced bactericidal effects more quickly against Gram-negative organisms as compared to Gram-positive organisms except for *S. pneumonia*. The extract of *S. indicus* killed *S. pneumonia* up to 6 logs cfu/mL within 20 mins. Moreover, the time for the complete killing of *S. pneumonia* by *S. indicus* extract was only 30 mins.

Many researchers reported the bacterial killing mechanism of the different plant using SEM technique [16, 17]. The SEM study was performed on *S. pneumonia* after exposure to the extract at a different time by examining the changes in morphology of bacterial cells in order to identify the possible killing mechanism of *S. indicus* extract. The appearance of the completely lysed cells of *S. pneumonia* was obviously shown in Figure **3**.

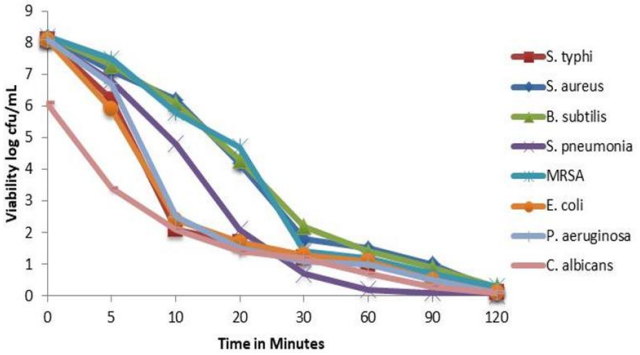

Figure 3. Growth profile of different isolates at different time interval after exposure to methanolic extract of *S. indicus* at MBC of each microbe.

The SEM images showed that the cells of *S. pneumonia* had undergone significant cytological and morphological alterations. The similar alterations in the morphology of *MRSA* and *E. coli* were observed after treated with *Enteromorpha intestinalis* and *Alpinia galanga*, respectively [18, 19]. After 12 h exposure with *S. indicus* extract, the cells of *S. pneumonia* were morphologically lysed and collapsed. This could be suggested that the extract of *S. indicus* had a high binding affinity with the lipopolysaccharide on a cell membrane of *S. pneumonia*. This interaction of extract with lipopolysaccharide altered the morphology of the bacterial cell membrane.

CONCLUSION

Hence on the light of achieved findings, it is concluded that ethanolic flowers extract of *Sphaeranthus indicus* have promising antimicrobial potential. The results show that the plant extract had strong bacteriostatic and bactericidal potency against different highly resistant microorganisms. The lower MIC and MBC values of extract indicated the greater potency for bactericidal actions. According to the SEM images, it is suggested that *S. indicus* extract produced bactericidal action through a morphological alteration in the cell membrane of bacteria.

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