IN VITRO COMPARISON BETWEEN ANTIBACTERIAL ACTIVITY OF CATHARANTHUS ROSEUS AND NYCTANTHES ARBORTRISTIS ON ANTIBIOTIC RESISTANT STAPHYLOCOCCUS AUREUS STRAIN

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ABSTRACT

Among the important plants mentioned in AYURVEDA (an Indian Sanskrit literature), Catharanthus roseus (PERIWINKLE) and Nyctanthes arbortristis (SIULI) are used in folk medicine. The antibacterial activity of the ethanolic extracts of these two leaves was screened against antibiotic resistant Staphylococcus aureus, a cause of nosocomial and post-surgical wound infections. This study aims to evaluate the possibility of the presence of any novel bio-active antibacterial agent(s). The test organism, S. aureus has developed drug resistance against all commonly used antibiotics. Ethanolic extracts of the leaves of these plants were used in agar well diffusion assay and effective zone of inhibition was found at the concentration of 0.5gm/ml. Further, MIC value of C. roseus and N.arbortristis extract against S. aureus was 125mg/ml and 250mg/ml respectively and the pattern of inhibition of growth of the organisms were observed as bacteriostatic. This study indicates a future for designing potentially active antibacterial agents from common Indian herbs like C. roseus and N. arbortristis.

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INTRODUCTION

Resistance to antimicrobial agents such as antibiotics is emerging worldwide in a variety of organisms and multiple drug resistant organisms thus pose serious threats to treating infectious diseases [1]. *Staphylococcus aureus* (colloquially known as "Staph aureus") is one of the major resistant pathogens, found on the mucous membranes and the human skin of around a third of the world population and it is extremely adaptable to antibiotic pressure. CA-MRSA (Community-acquired MRSA) has now emerged as an epidemic that is responsible for rapidly progressing fatal diseases including necrotizing pneumonia, sepsis and necrotizing fasciitis [2]. Plant derived antimicrobial agents have received considerable attention in recent years. WHO report depicts that, plant based products or its derivatives accounts for nearly 28% of drugs available in the market [3]. Actually plant based phytochemicals play a critical role in diversity-oriented synthesis (DOS) of natural product-like pharma-compounds [4]. Medicinal plant-based drugs have the advantage of being simple, effective and exhibit broad spectrum activity [5]. Hence, global attention has shifted towards finding new chemicals, specifically herbas, for the development of new drugs. These natural products can provide unique elements of molecular diversity and biological functionality, which is indispensable for novel drug discovery [6]. India is endowed with about 47000 species of plants and is the 8th richest country in the world in terms of biodiversity [1]. Of these, 8000 species are known to have medicinal properties, of which, 2500 plant species belonging to more than 1000 genera, are used in Indian systems of medicine [1]. *Catharanthus roseus* is an important medicinal plant of family *Apocynaceae*. It is cultivated mainly for its anticancer activities [7]. This plant possesses known antifungal, antidiabetic, anticancer and antiviral activities [8]. However, in spite of extensive survey of literature on *C. roseus* which shows various aspects of studies, the proper mechanism of action for damaging or inhibiting growth of microorganisms is not clearly known. *Nyctanthes arbor-tristis* Linn. commonly known as Harsinghar or Night Jasmine is another well known medicinal plant. Different parts of *N. arbor-tristis* are known to possess medicinal activities and are used for treating various ailments by rural, mainly tribal people of Orissa and Bihar, along with its use in Ayurveda, Siddha and Unani systems of medicines. Juice of the leaves is used as digestive, antidote to reptile venoms, mild bitter tonic, laxative, diaphoretic and diuretic [9, 10, and 11]. Traditionally the powdered stem bark is given in rheumatic joint pain, in treatment of malaria and also used as an expectorant [10]. Leaf extracts was found to have antimicrobial activity [12]. There is no such report of successive mode of action of these plant extracts on multi drug resistant pathogen like *S. aureus*.

The present study was carried out to evaluate the antimicrobial effects of ethanolic leaf extracts of *C. roseus* and *N. arbor-tristis* against antibiotic resistant *S. aureus* strain. We would also try to understand the mechanism of interaction between the plant product(s) and bacterial plasma membrane. The inhibiting activities of the two different herbal product(s) were also compared by agar well diffusion (AWD) test, determination of MIC values and growth curve experiments.

MATERIALS & METHODS

Media used for the experiment- Nutrient Agar Media (Himedia,Mumbai,India) ,10% NaCl Media (2.8gm of dehydrated Nutrient Agar Media (Himedia) and 10 gm NaCl was dissolved in 100 ml of distilled water and sterilized), Mueller–Hinton Agar and broth (Himedia,Mumbai,India).

Collection of leaves and preparation of extracts-

Fresh leaves of *C. roseus* (periwinkle,“Rosea” variety) and *N. arbor-tristis* (siuli) were collected from the compound of our institution, washed and dried in shade and extracted with ethanol. Ethanol extract using leaves [13,14] -50gm of fresh leaves were crushed by the help of mortar and pestle. The mixture was dissolved in 100ml ethanol for 48 hours, and then filtered. The concentrated solution (0.5gm/ml) was stored at 4°C.

Collection of bacterial strains-

Clinical strains of *S. aureus* were collected from Ramkrishna Mission Seva Pratishthan Hospital, Kolkata. Reference strain of *S. aureus* (ATCC - 25923) was collected from NICED, Kolkata. The isolated strains were maintained in the laboratory. Confirmation of *S. aureus* was done by Gram staining, growth on 10% NaCl plate and catalase test. Antibiotic resistance of the strains were confirmed by, growth on NA plate, containing ampicillin (concentration-0.25mg/ml) and azithromycin (concentration-0.25mg/ml).

Antibacterial screening test-

Agar well diffusion - 20ml of temperate MHA media along with 0.25ml of inoculum were mixed well and poured in the plates. After setting of the agar plate, 1cm diameter holes were cut. 100µl of the extract added to each hole and plates are kept for overnight incubation at 37°C. Then the resulting zones of inhibition were measured in millimetre scale.

Minimum inhibitory concentration –

The MIC was determined using Mueller–Hinton broth (MHB; Mumbai, Himedia), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [6]. A stock extract solution of 0.5gm/ml was prepared and serial dilution was performed using this stock solution. The concentration range became 0.25gm/ml – 0.00187 gm/ml. The inoculum were added to each of the test tubes and kept for 18hours incubation at 37°C. After that OD was measured at 530nm.

Growth curve of *S. aureus* in presence of *C. roseus* and *N. arbor-tristis* -

25ml of Mueller Hinton broth was taken in 5 finger flasks and inoculated in following manner, Blank: 25ml M.H broth , A. 25ml broth + 0.5ml *S.aureus*, B. 25ml broth + 6.25ml *C. roseus* extract (0.5gm/ml), C. 25ml broth + 6.25ml *N. arbor-tristis* extract.
(0.5gm/ml), D. 25ml broth + 0.5ml S.aureus suspension + 6.25ml C. roseus extract (0.5gm/ml), E. 25ml broth + 0.5ml S.aureus suspension + 6.25ml N. arbor-tristis extract (0.5gm/ml), F. 25ml broth + 0.5ml S.aureus suspension + 1ml ampicillin (5mg/ml). After mixing the contents the finger flask was placed at water bath shaker and temperature maintained at 37°C. After each 30min interval the OD were noted. It was continued till the OD reached to a constant value. Taking the time at X-axis and OD at Y-axis the growth curve were drawn which shows patterns of growth.

Interaction between bacterial cell wall and plant extracts -

Isolated single colonies of different strains were treated with ethanolic extract of C. roseus and N. arbor-tristis individually. After 4 hours (as indicated by Growth Curve experiment), smear was placed on slides by taking one loop of liquid culture and Gram staining was carried out and observed under the microscope.

Colony Count Experiment –

After performing the growth curve experiment, bacterial colonies were counted, taking 1µl (1:10 dilution) sample from each tube.

Comparison between the antibacterial effects of C. roseus and N. arbor-tristis extracts with that of ampicillin –

An agar well diffusion experiment was carried out with graded doses of ampicillin, along with the C. roseus and N. arbor-tristis extracts in the same plate. A standard curve was drawn by plotting the concentration of ampicillin along the X-axis and the diameter of the respective zones of inhibition produced along the Y-axis. From this standard curve, the concentration of the ethanolic leaf extracts which produces a zone of inhibition equivalent to that produced by ampicillin is determined.

Statistical analysis –

Data are expressed as mean ±SEM unless otherwise indicated. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by post hoc Newman-Keuls multiple comparison test of significance using GraphPad Prism 3.02 software.

RESULTS & DISCUSSION

Our study was conducted to compare the antimicrobial activities of the leaf extracts of C. roseus and N. arbor-tristis against antibiotic resistant S. aureus strain. Organic ethanolic extracts exhibited potent antibacterial activity.

Determination of Zone of Inhibition-

Application of the two ethanolic leaf extracts in agar well diffusion study produced a visible zone of inhibition (Figure-1a), against the clinical multidrug resistant S.aureus strain at the concentration of 0.5gm/ml. This comparative study is significant (Figure-1b) for the identification and isolation of novel bio-active compound from two natural plants.

Effect of C. roseus extract.  Effect of N. arbor-tristis extract.

Figure-1a : Effect of C. roseus & N. arbor-tristis against S. aureus in agar well diffusion
Figure 1b: Effects of *C. roseus* & *N. arbortristis* leaf extracts against clinical strain of *S.aureus*. Values were expressed as Mean±SEM. *** p<0.001. Ext-Extract.

**Determination of MIC value**

The minimum inhibitory concentration is found to be ≥0.125gm/ml for *C. roseus* (ethanol extract), whereas the minimum inhibitory concentration is found to be ≥0.25gm/ml for *N. arbortristis* (ethanolic extract) against the clinical strain of *S.aureus*

**Evaluation of bacterial growth by Growth Curve Experiment**

The pattern of growth curve indicates the bacteriostatic effect of the components, present in the ethanolic extracts of these two plants. The inhibitory action of *C. roseus* (Figure-2) extract starts almost half an hour prior in comparison with *N. arbortristis* (Figure-2) extract. After 6 hours the growth of multidrug resistant *S. aureus* become completely inhibited individually by these two herbs.

Figure-2 Growth curve of *S. aureus* in presence of *C. roseus* and *N. arbortristis* leaf extract (O.D. measured at 530 nm).

**Results for colony count experiment**

After performing the growth curve experiment, bacterial colonies were counted, taking 1µl (1:10 dilution) samples from each tube. Culture treated with *C. roseus* extract produces significantly (Figure-3a) lesser number of colonies compared to *N. arbortristis* extract (Figure-3b).
Figure -3a. Number of *S.aureus* colonies in presence *C. roseus* and *N. arbortristis* leaf extract.

3b. Bacterial colony count along with two different extracts.

Values were expressed as Mean±SEM. *** p<0.001. Ext-Extract.

**On comparison between the antibacterial activity of *C. roseus* and *N. arbortristis* extract with that of ampicillin-**

It was found that the diameters of the zones of inhibition, produced by treatment with *C. roseus* and *N. arbortristis* leaf extracts and different concentrations of a standard antibiotic i.e. ampicillin, 168µg ampicillin produced a zone of inhibition equivalent to that produced by *C. roseus* extract (500 mg /ml) whereas 64µg is the ampicillin concentration, which correlate with *N. arbortristis* (500 mg /ml) extract (Figure-4).

![Image of graph showing zone diameter vs concentration of ampicillin](image)

**Figure-4 : Comparison between effective doses of ethanolic leaf extract of *C. roseus* and *N. arbortristis* along with ampicillin.**
Gram-Staining Results –
On treatment with the two leaf extracts, a large number of *S. aureus* cells were found to be clumped together, where as the untreated cells showed normal morphology (Figure-5).

![Gram staining of clinical S.aureus strain in absence of C. roseus leaf extract](image1)

![Gram staining of clinical S.aureus strain in presence of C. roseus leaf extract.](image2)

![Gram staining of clinical S.aureus strain in presence of N. arbortristis leaf extract.](image3)

Figure- 5: Results of Gram staining, after treatment with *C.roseus* and *N. arbortristis*.

CONCLUSION
As most bacteria nowadays have become resistant to commonly used antibiotics, attempts are being made worldwide to isolate compounds having antimicrobial activity from diverse plant resources [15], so that they may be used as an alternative to costly antibiotics for curing diseases caused by antibiotic resistant bacteria and also to prevent the rapid spread of antibiotic resistance. Results of this study demonstrate the antimicrobial activity of ethanolic leaf extracts of *C. roseus* and *N. arbortristis*, against the test antibiotic-resistant *S. aureus* strain. Ethanolic leaf extract of *C. roseus* produces a greater zone of inhibition compared to that of *N. arbortristis*, showing that it is much more effective than *N. arbortristis* against antibiotic resistant *S. aureus* strain. Clinical strains are more sensitive than the reference strain, to both these extracts. These extracts show a bacteriostatic effect, which is supported by the pattern of growth curve. The cell walls of bacterial cells are being denatured on treatment with both the leaf extracts. Thus from this study, it may be concluded that certain natural plant products like “PERIWINKLE” and “SIULI” leaves have antimicrobial activity against *S.aureus* and may be therefore be used as an alternative to conventional antibiotics. However, these studies need to be replicated before making conclusive statements regarding these findings. Future studies need to be done to isolate the actual bio-active compounds from the whole leaf extracts of these two plants which are responsible for the antimicrobial activity. Side effects and toxicity of these plant products should also be investigated both in vitro and in vivo so that they may be used as an alternative to the conventional, costly antibiotics.

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Competing Interests:
The authors declare that they have no conflict of interest.

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