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Synergistic anti-*Staphylococcus aureus* activity of amoxicillin in combination with *Emblica officinalis* and *Nymphae odorata* extracts

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ABSTRACT

Objective: To evaluate the antibacterial activity of *Emblica officinalis* Gaertn (*E. officinalis*; Family: Euphorbiaceae) seed and *Nymphae odorata* Aiton (*N. odorata*; Family: Nymphaeaceae) stamen extracts, alone and in combination, and in combination with amoxicillin (Ax) against *Staphylococcus aureus (S. aureus)*. **Methods:** Antibacterial activity of ethanolic extracts of amla, *E. officinalis*, seed (AMS; 500 μg) and sapla, *N. odorata*, stamen (SAP; 500 μg) for 12 methicillin-resistant *S. aureus* (MRSA) isolates was determined following agar diffusion; in order to assess the combined antibacterial activity, AMS (250 μg) plus SAP (250 μg) were considered. The Ax (10 μg) activity alone and in combination with AMS (250 μg), and SAP (250 μg) was determined by disk diffusion. The zone diameters of inhibition (ZDIs) for the agents were recorded, and growth inhibitory indices (GIIs) were calculated. **Results:** The MRSA isolates (n=12) had AMS (500 μg) and SAP (500 μg) ZDIs of 12-19 mm and 21-24 mm, respectively. The ZDIs (range 24-27 mm) increased by 3-4 mm due to combined action of AMS (250 μg) and SAP (250 μg) indicating synergy between extracts for MRSA (GII 0.634-0.742). The MRSA isolates were resistant to Ax (ZDI: 8-11 mm), which in combination with AMS and SAP had synergistic effect, both due to increased ZDI [mean±SD=(3.5±0.577) mm] and GII (0.631-0.894). **Conclusions:** The data suggest that the plants, *E. officinalis* and *N. odorata* alone or in combination, are promising in the development of phytomedicines, which may be used, alone or in combination with the antibiotic, Ax, against MRSA infection.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a wide spread nosocomial pathogen, and the prevalence of MRSA strain in communities has been reported too[1]. Several authors reported MRSA strains showing resistance to multiple antibiotics, and development of such multidrug resistance resulted therapeutic limitation[1, 2]. Considering the fact, the multidrug resistance in MRSA, researchers studied on the antibacterial properties of various plants against gram negative as well as gram positive bacterial strains including *Staphylococcus aureus (S. aureus)*[3-7], but there is scanty report on antibacterial activity of amla *(Emblica officinalis)* against *S. aureus*[8], and the only report has been documented on antibacterial activity of sapla *(Nymphae odorata)* against two plant pathogenic bacteria, *Erwinia carotovora* and *Agrobacterium tumefaciens*[9]. Moreover, the interactions between the extracts of *Nymphae odorata (N. odorata)* and *Emblica officinalis (E. officinalis)*, and with antibiotic have not been documented earlier, though the fruit of *E. officinalis* is highly valued in traditional Indian medicine[10], and in Unani medicine the dried fruits of amla are used to treat haemorrhage, diarrhoea and dysentery, and the *N. odorata* is one of the most easily recognized of all the aquatic food plants in our part of the globe. Synergistic antibacterial activity of antibiotic cefuroxime and plant, *Rosmarinus officinalis*, extract against MRSA has been reported earlier by Jarrar *et al.*[11]. Herein, the *in vitro* antibacterial activities of *E. officinalis* and *N. odorata* alone and in combination were studied against clinical isolates of MRSA; the anti-MRSA activities of amoxicillin (Ax) alone and in combination with the plant extracts were studied as well.

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2. Materials and methods

2. 1. Bacterial strains

The methicillin-resistant *S. aureus* (MRSA; *n*=12), isolated from throat swab samples from the patients (having throat infection), who attended the Calcutta School of Tropical Medicine, India for treatment, during February 2007 and January 2009, were utilized in the study. A single isolate was obtained from each of the 12 samples collected from throat infection cases (*n*=12). The *S. aureus* ATCC 25923 strain was used as the control.

2. 2. Plant extract preparation

The *E. officinalis* Gaertn (Family: Euphorbiaceae) fruits and *N. odorata* Aiton (Family: Nymphaeaceae) flowers were collected locally, in Kolkata, India, and respectively, the seeds and stamens were taken out, and the extracts were prepared following the protocol mentioned earlier[4], using 50 gram of each of the dried materials and ethanol as the extractant. The ethanolic extracts of amla *(E. officinalis)* seed (AMS) and sapla (*N. odorata)* stamen (SAP) were stored at 4 ℃ in 50 % ethanol, and were utilized within one week; the extracts were prepared freshly when needed.

2. 3. Agar diffusion susceptibility

The antibacterial activities of the two extracts (SAP and AMS) were assessed against 12 MRSA isolates following agar diffusion technique[12]. Briefly, the extracts, each alone and in combination, were dropped on the three of the four properly marked sectors, such as AMS, SAP, and ASM+SAP, on Mueller-Hinton agar (MHA) plates, each of which were seeded with 10^8 CFU. The concentrations used in the study were 500 μg (25 μL) for each of the extracts, AMS and SAP, and in order to assess the combined antibacterial activity, AMS (250 μg) plus SAP (250 μg) were considered[13]. Each of the control sectors, on the plates, contained 25 μL of 50 % ethanol that results no zone diameter of inhibition (ZDI) to ZDI of 6 mm for the test bacterial isolates, and, therefore, the sensitivity to the plant extracts for the MRSA strains were considered with ZDI ≥ 7 mm, which has also been considered earlier by Nascimento *et al.*[14].

2. 4. Disk diffusion susceptibility

The Ax susceptibility for the isolates was determined by disk diffusion method as described earlier by Bauer *et al.*[15], using10 μg Ax disks (Hi-Media, Mumbai, India). To determine the combined effect with Ax, AMS and SAP, 250 μg each, were added to 10 μg Ax disks placed on the surface of MHA plate inoculated with 10^8 CFU. Antibacterial activity of SAP and AMS alone were also recorded using 250 μg for each.

2. 5. Interpretation of the results

The antibacterial activity of the agents was expressed by measuring ZDI due to the action of the plant extracts, alone or in combination, and Ax alone and in combination with the plant extracts, after 24 h incubation at 35 ℃.

Considering the concentrations of the agents used in susceptibility tests, the effect between the two antimicrobial agents was considered synergistic by the increase of ZDI from combined action compared to the average ZDI obtained due to single action of the two components; when zone diameter is less in combination, the interaction is defined as antagonistic, and additive when no change in ZDI in combination. The growth inhibitory indices (GIIs) were calculated following the formula:

GIIs= ZDI in combination / total of ZDIs of the two agents in single action

in order to corroborate the synergistic activity (as has been defined in terms of increment of ZDI mentioned above) of the antibiotic in combination with the plant extracts, and synergy between the plants extracts. The synergistic, additive and antagonistic activities, if any, in between any two of the antimicrobial agents were defined with GIIs > 0.5, 0.5 and < 0.5.

2. 6. Statistical analysis

The χ^2 test was employed in order to compare the antibacterial activities (in terms of ZDIs) of AMS and SAP, alone and in combination, and Ax alone and in combination with AMS and SAP, against MRSA; a *P*- value of ≤ 0.001 was considered significant.

3. Results

The agar diffusion test results for 12 MRSA isolates that were exposed to AMS and SAP alone and in combination are depicted in Figure 1. All the MRSA isolates showed sensitivity to AMS and SAP having ZDIs of 12-19 mm [mean ± SD=(14.83 ± 3.07) mm] and 21-24 mm [mean ± SD=(22 ± 1.044) mm], respectively. When SAP was used in combination with AMS, the ZDI for the isolates ranged 24-27 mm [mean ± SD=(25.5± 1.2) mm].

The disk diffusion test results for a total of 12 MRSA isolates are presented in Figure 2. The isolates showed resistance to Ax [ZDI: 8-11 mm; mean ± SD=(9.25±1.36) mm]. The Ax ZDI increased up to 13-17 mm [mean ± SD=(15.75 ± 1.36) mm] with the addition of AMS (250 μg) to the standard Ax discs (10 μg), and with SAP (250 μg) the Ax ZDI increased up to 12-16 mm [mean ± SD=(14.33 ± 1.61) mm].

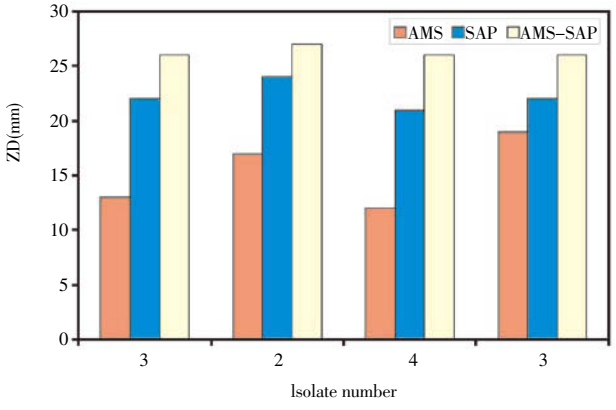

Figure 1. ZDI due to the action of *E. officinalis* seed (AMS) and *N. odorata* stamen (SAP) ethanolic extracts alone and in combination for 12 MRSA isolates.

The concentrations of each of the extracts were 500 μg when used alone, and 250 μg when used in combination.

The GII, considering the activity of AMS and SAP alone and in combination, is represented in Table 1; the GII ranged in between 0.634 and 0.742. The GII from Ax–SAP and Ax–AMS combinations ranged from 0.631 to 0.714 and from 0.684 to 0.894, respectively (Table 1).

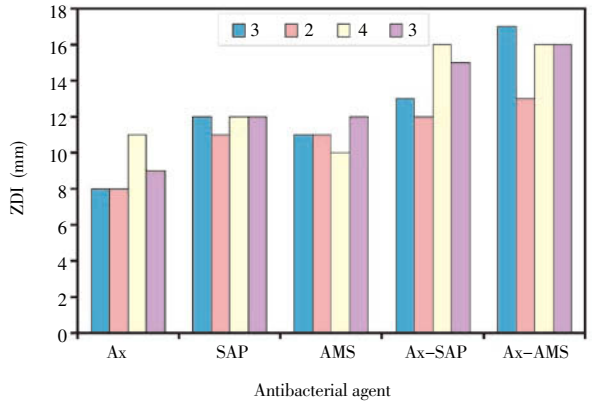

Figure 2. ZDI of amoxicillin (Ax; 10 μg-disc) alone and in combination with *E. officinalis* seed (AMS; 250 μg) and *N. odorata* stamen (SAP; 250 μg) ethanolic extracts for 12 MRSA isolates. Numbers within the figure indicate the number of isolates showing ZDIs of the agents.

Table 1

Growth inhibitory indices of combined antibacterial activity of amoxicillin and plant extracts for 12 MRSA isolates (n = number of isolates)

n (%)	GII from combination		
	AMS–SAP	Ax–SAP	Ax–AMS
3 (25.00 %)	0.742	0.650	0.894
2 (16.67 %)	0.658	0.631	0.684
4 (33.33 %)	0.727	0.695	0.762
3 (25.00 %)	0.634	0.714	0.762

GII = growth inhibitory index, AMS = ethanolic extract of amla (*E. officinalis*) seed, SAP = ethanolic extract of sapla (*N. odorata*) stamen, Ax = amoxicillin.

4. Discussion

The medicinal plants are important elements of indigenous medical systems in country like India, where the use of different parts of various medicinal plants to cure specific ailments has been in vogue from ancient times, and interest in medicinal plants has revived as a consequence of current problems associated with the use of antibiotics[16–18]. In recent years, different reports, from different countries have been published showing the antimicrobial activities of various medicinal plants[4, 7, 13]. In the present study, AMS and SAP showed excellent antibacterial activity against nosocomial MRSA isolates.

The anti bacterial activity of *E. officinalis* has been documented by earlier authors. Saeed and Tariq[19] reported antibacterial activities of *E. officinalis* aqueous infusions, having ZDIs 10.23–20.6 mm, against a large number of gram negative bacterial strains causing urinary tract infections. The ethanolic extract of *E. officinalis* leaf showed strong activity against both gram negative and gram positive bacteria including *S. aureus* (ZDI 9 mm), as reported by Nair and Chanda[8]. But the antibacterial activity of *N. odorata* has not been studied earlier against clinical bacteria including *S. aureus*. However, the anti-*S. aureus* activity of other medicinal plants has earlier been reported. Abu-Shanab *et al*[6]. reported anti-MRSA activity of *Syzygium aromaticum* (seed), *Cinnamomum cassia* (bark), *Salvia officinalis* (leaf), *Thymus vulgaris* (leaf), and *Rosmarinus officinalis* (leaf) showing ZDIs 22–38 mm. Chandarana *et al*.[7] reported, against *S. aureus*, the antibacterial activity of *Zingiber officinale*, *Curcuma amada* and *Curcuma longa*, of which the first two extracts showed ZDIs 11.5–12.33 mm and 9.5–11mm, respectively, while third one was found ineffective against *S. aureus*. The ethanolic extract of *Rhus coriaria* seed against clinical bacterial strains of MRSA, multi-drug resistant (MDR) *Pseudomonas aeruginosa(P. aeruginosa)*, *E. coli*, *Proteus vulgaris* and *Klebsiella pneumoniae* produced ZDIs 15–25 mm, as reported by Abu-Shanab *et al*.[20]. Herein, the SAP exhibited stronger anti-MRSA activity [ZDI:mear±SD=(22±1.044) mm] than AMS [mean ZDI:mean±SD=(14.83±3.07) mm], and there was significant difference in between the antibacterial efficacy of the two extracts (P<0.001).

Several authors studied the antibacterial activities of various plant extracts in combination with each other. Chandarana *et al*.[7] showed highest antibacterial activity of *Z. officinale*– *C. longa* combination (ZDI:14.5 mm) followed by *Z. officinale*– *C. amada* combination, against *S. aureus*, while the *C. longa*–*C. amada* combination was ineffective. The combinations of ethanolic extracts of *S. officinalis* with *R. officinalis* and of *R. officinalis* with *T. vulgaris* on bacterial species tested exhibited a higher effect than that of any individual extract[6]. The ethanolic and methanolic extracts of *Rhus coriaria* (seed) respectively had ZDIs 16 mm and 15 mm, against clinical isolate of *P. aeruginosa*, and both kinds of extracts of *Thymus vulgaris* (leaf) produced 6 mm ZDI, while in combination, the two ethanolic extracts (ZDI 21 mm) and the methanolic extracts (ZDI 21 mm) had stronger antibacterial potentialities[13]. In the present communication, it is interesting to note that there was synergistic antibacterial activity between AMS and SAP against MRSA; the increment of ZDI by 3–4 mm (mean 3.5 ± 0.577 SD) due to SAP–AMS combination compared to the average ZDI obtained due to single action of the two components supported the view. Significant difference was found in between the anti-MRSA activity of SAP or AMS alone and SAP–AMS combination (P<0.001). Moreover, the GII of 0.634–0.742 strongly supported the fact of synergistic activity between SAP and AMS against MRSA, in the present study.

The rampant and indiscriminate use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains, and such loss of clinical efficacy of previously effective first-line antibiotics results shifting of antibiotic treatment regimen to second-line or third-line antibiotics that are often more expensive with many side effects[21]. Based upon the fact the ability of crude extracts of plants to accelerate the activity of antibiotics has been studied by some researchers. Darwish *et al*.[22] reported to improve the efficacy of gentamicin (GM) and chloramphenicol (CM) against *S. aureus* by the use of some Jordanian plant materials. Ahmad and Aqil[23] reported that crude extracts of Indian medicinal plants demonstrated

synergistic interaction with tetracycline and ciprofloxacin against extended spectrum β-lactamase producing MDR enteric bacteria. Against *S. aureus*, plants like *Syzygium aromaticum*, *Psidium guajava* and *Cymbopogon citrates* presented highest synergism rate with antibiotics, while *Zingiber officinale* and *Allium sativum* showed limited synergistic capacity, as reported by Betoni *et al*[24] and Mahboobi *et al*[25] reported that the combination of clove, lavender and geranium oils showed the most inhibitory effect and strong synergy with GM against MDR *P. aeruginosa*. The tea extract showed synergistic activity with CM and other antibiotics like GM, methicillin and nalidixic acid against bacterial strains excluding *S. aureus*[26]. The effect of combinations of the acetone extract of Garcinia kola seed and some antibiotics including Ax showed synergistic interactions against gram positive organisms including *S. aureus*[27]. Synergy between cefuroxime and *R. officinalis* extract against MRSA isolates has also been reported[11]. In the present investigation, the combinations of Ax (one of the conventional antibiotics used against *S. aureus* infection) and, SAP and AMS were investigated for possible synergistic interactions. The synergy was detected for both Ax-SAP and Ax-AMS combinations, and the significant differences were found between the activity of Ax alone and Ax-SAP combination ($P<0.001$), and Ax alone and Ax-AMS combination ($P<0.001$). The presence of synergy between Ax and both SAP and AMS has also been supported by GII of > 0.5, as has been recorded in the present study.

Thus, the present findings suggest that the synergy with Ax observed in this study was attributable to such compounds, showing antibacterial activity, present in the crude extracts of the plants *E. officinalis* and *N. odorata*. The plants used in the present study might be potential source of non-antibiotic drugs that might potentially improve the performance of antibiotics against MRSA infection. An elucidation of the mechanism of action of the compounds must be followed by toxicity (though the fruits of *E. officinalis* and parts of *N. odorata* are consumed by humans) and *in vivo* tests in order to determine the applicability and dose of such compounds in combination therapy; before that the extracts may be useful at least for topical application due to MRSA infection.

Conflict of interest statement

We declare that we have no conflict of interest.

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