Evaluation of Antimicrobial Potential of Some Indian Ayurvedic Medicinal Plants

Mrinmoy Nag, Pulok k Mukherjee *, Rajarshi Biswas, Joydeb Chanda, Amit Kar

ABSTRACT
Introduction: Stereospermum suaveolens Roxb., Viscum articulatum Burm., Annona squamosa, Capsicum annuum cayenne, Ananas comosus Merrill. are used for the management of microbial infection in Ayurveda. The present study was designed to standardize the extract of S. suaveolens bark (SSB), V. articulatum aerial part (VAAP), A. squamosa leaf (ASL), C. annuum fruit (CACF), A. comosus fruit (ACF) and performed antibacterial activity. Methods: The antibacterial activity of the five extracts were evaluated against certain bacteria such as B. subtilis, B. cereus, S. aureus (gram positive); E. coli, S. typhi, and P. aureuginosa (gram negative) by disc diffusion method, time course assay, pH sensitivity assay and minimum inhibitory concentration (MICs) through broth micro-dilution method. Results: The plants extracts VAAP, ASL, and CACF showed potent inhibitory activity against S. aureus with MIC 728, 742, and 698 µg ml⁻¹, respectively, while CACF showed inhibitory activity against B. subtilis with MIC 690 µg ml⁻¹. The results further demonstrated that the inhibitory activity of CACF against E. coli with MIC 760 µg ml⁻¹. P. aureuginosa was inhibited by ASL and CACF with MIC 1100 and 1120 µg ml⁻¹, respectively. The ASL showed notable MBC against the tested microorganism. Moreover, all extracts were completely inactivated bacterial strains (except B. cereus, S. typhi) within 2-10 h of exposure, determined by time course assay. Conclusion: The outcomes of our study elucidate that standardized extracts of A. comosus, A. squamosa, C. annuum, S. suaveolens, and V. articulatum may be used as natural antimicrobial agents. Key words: Antibacterial, Ananas comosus Merrill, Annona squamosa, Capsicum annuum cayenne, Stereospermum suaveolens Roxb, Viscum articulatum Burm.

INTRODUCTION
The quest for new antimicrobial lead is a global challenge, as microbes are becoming resistant to the conventional antimicrobials as drug resistance is a natural phenomenon and microbes may develop resistance even without any exposure to a drug.¹⁻³ One way to overcome this problem of drug resistance is by developing new leads from natural resources. Now a day’s many antibacterial agents are available, which are costly, have toxicity and yielded drug-resistance mutants. Therefore, it needs to find cost effective readily available natural anti-microbial agents, with minimum side effects.

*Stereospermum suaveolens* Roxb. (Family: Bignoniaceae) is commonly known as Trumpet. Various parts of the plant are used in the treatment of diabetes, diuretic, pain, fever, inflammations, hiccups, leprosy and asthma. The root is used in the preparation of Ayurvedic formulation known as Dashmula.⁴⁻⁶ The plant bark contains sterekunthal B, stereohenols A and B, lapachol, dehydro-a-lapachone, apigenin. The major constituents are lapachol and apigenin have several pharmacological activities, including antimicrobial, antiviral, anti-inflammatory, antiparasitic, leishmanicidal and anticancer.⁷⁻⁹ *Viscum articulatum* Burm. (Family: Loranthaceae) is an ethnomedical plant are commonly known as mistletoe.⁹ In Ayurveda, the plant parts are used in “Kapha”, “Vata”, diseases of the blood, ulcer, epilepsy and biliousness.¹⁰⁻¹¹ The plant parts are also used in urinary tract infection, low back pain, dysentery, uterine bleeding and to treat weakness.¹² The plant contains triterpenoids (α-amyrin, lupeol, betulin, betulinic acid and oleanolic acid). Among them betulinic acid, betulin and oleanolic acid exhibit antimicrobial activity.¹²

*Annona squamosa* (Family: Annonaceae) English name is custard apple, sugar apple or Sweetsop. The plant is traditionally used for the treatment of epilepsy. The plant is used in the treatment of dysentery, cardiac problems, worm infestation, cough, conjestion, hemorrhage, diarrhoea, fever, thirst, bronchitis, helminthiasis, dropsy, painful malignant tumours and ulcers.¹³ Ayurvedic practitioners use stem and leave extract as an indigenous
Antimicrobial screening of Indian Medicinal Plants

Traditional medicinal plants have been used for the treatment of various ailments. In the present study, a total of 30 species of medicinal plants were collected from different regions of India and their antimicrobial activities were evaluated. The plants were identified and authenticated by experts from the Department of Pharmacognosy, Jadavpur University, Kolkata. The voucher specimens were deposited in the herbarium of the Department of Pharmacognosy, Jadavpur University, Kolkata, India.

The antibacterial assay of crude extracts and their biomarkers were performed by disc diffusion method. The antibacterial activity was evaluated against both gram-positive and gram-negative bacteria. The results revealed that the crude extracts and their biomarkers had significant antibacterial activity against the tested bacterial strains.

Materials and methods

Chemicals and reagents
All the chemicals and reagents were of analytical grade. The antibacterial assay was performed with a 600-controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) spectrophotometer, and a Rheodyne 7725i injector having 20 µl loop. Quantitative estimation was performed with Empower 2 software programs using the external calibration method.

Plant material collection and extraction

The Stereospermum suaveolens bark (SSB), Viscum articulatum aerial part (VAAP), Annona squamosa leaf (ASL), and Cassia angustifolia cayenne fruit (CACF) were collected from different regions of India. The voucher specimens were deposited in the herbarium of the School of Natural Product Studies (SNPS), Jadavpur University, Kolkata, India. After authentication, the samples were air-dried and ground to a fine powder using a mechanical grinder. The powder was soaked in 95% methanol at room temperature (25°C) for successive extraction. The whole extract was collected, filtered, and the solvent was evaporated to dryness under reduced pressure and temperature (45°C) using an Eyela Rotary Evaporator (Japan).

Bacterial strains and culture condition

Gram positive (Bacillus cereus ATCC 14579, Staphylococcus aureus ATCC 29213) and gram negative (Escherichia coli ATCC 25922, Salmonella typhi MTCC 734, and Pseudomonas aeruginosa ATCC 9027) bacteria were selected as standard strains as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI), formerly known as the National Committee for Clinical Laboratory Standards. For experimental purpose bacterial cultures were maintained on Nutrient agar (NA) or Nutrient Broth (NB) (Himedia, Mumbai, India) at 4°C and subculture in every 4 weeks.

Disc diffusion method

The antibacterial assay of crude extracts and their biomarkers was performed by disc diffusion method. Concisely, 10 ml of sterilized Muller Hinton Agar (MHA) (pH 7.2 ± 0.2) was poured into sterile Petri dishes (9 cm in diameter, Borosil) and allowing 2 µl of each test sample (5 µl of individual plant extract, 10 µl of each standard) to spread evenly over the surface. The plates were incubated at 37°C for 24 h and the zone of inhibition was measured.

HPLC analysis

The HPLC analysis was performed with a 600-controller pump, a multiple-wavelength ultraviolet-visible detector, and a Rheodyne 7725i injector having 20 µl loop. Quantitative estimation was performed with Empower 2 software programs using the external calibration method. Membrane filters of 0.45 µm pore size (Millipore) were used for filtration of the mobile phase and 0.45 µm syringe filters (NYL) were used for the filtration of the sample.

Antibacterial assay

Preparation of stock solution of antibiotic, plant extracts and their biomarkers

Stock solution of ampicillin and streptomycin (Sisco Research Laboratory, Mumbai, India) were used as a concentration of 10 µg/ml (w/v). DMSO 1% (v/v) was used as a solubilizing solvent for test samples and also used as control to evaluate the antibacterial assay. Stock solution of individual plant extracts (Stereospermum suaveolens, Viscum articulatum, Annona squamosa, Cassia angustifolia cayenne and Ananas comosus) were prepared and the final concentration of each plant extract was 5000 µg/ml, freshly prepared stock solution and requisite different concentration for the bacterial tests were prepared from this stock solution.
was taken as an average of three measurements at different directions. All experiments were performed in triplicate.

**Determination of minimum inhibitory concentration (MICs) and minimum bactericidal concentration (MBCs)**

MICs values were determined by broth micro-dilution method suggested by the CLSI. Briefly, microbial cultures were prepared by suspending one isolated colony from each base plate in 5ml of MHB. After 24 h of proper incubation period, the suspensions were diluted in to get the final inoculum population (5×10⁵ CFU/ml) by using 0.5 Mac Farland standard. Colony morphology and gram stain procedure were adopted for checking of accuracy of mother culture throughout the test. 96-well microtiter plates were used for two fold serial dilutions of test samples using known stock solution with MHB. An equal volume of bacterial inoculums was added to each well on the microtiter plate consist of 0.05 ml of serial dilutions of compound which was incubated at 37 ± 2°C for 24 h. MICs values were defined as the lowest concentration of substance that inhibits visible growth of bacteria in media. Bacterial growth was displayed by the presence of turbidity and a pellet on the well bottom. MICs were determined presumptively as the first well, where no pellet appeared. It was calculated by comparing the absorbance of sample wells with the control wells with the help of Spectra-max M5 (USA) at 405 nm wavelengths. The MBC was determined by adding 50 μl of the suspensions from the wells in 25 ml fresh MHB. These suspensions were re incubated at 37°C for 48 h. The MBC was determined as the lowest concentration of extract which inhibited the complete growth (100%) of microorganisms.

**pH sensitivity assay**

The effect of pH on antibacterial activity of the plant extract was determined by pH sensitivity assay. Overnight broth cultures of B. subtilis, B. cereus, S. aureus, E. coli, S. typhi, and P. aeruginosa with different pH range (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) were prepared by using 0.1N HCl and 5M NaOH and swabbed on MHA plates with the corresponding pH. The antibacterial activity was analyzed by disc-diffusion method. Ampicillin and streptomycin were used as positive control for gram negative and gram positive bacteria, respectively.

**Time course assay**

The rapidity and duration of antibacterial activity was determined by time-kill analysis. Overnight broth cultures of bacterial strains were adjusted to the concentration of 5x10⁶ CFU/ml and were treated with plant extracts (MIC×2). Control tubes were also prepared without plant extract. Then 100 μl of sample was taken and plated on MHA plates at regular time intervals (0, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h). The plates were incubated at 37°C for 24 h and CFU was calculated. All the determinations were done in triplicates.

**RESULTS AND DISCUSSION**

**Determination of selected phytomarkers in plant extract by RP-HPLC method**

The mean R₅ was observed, 7.96 ± 0.06 (for lapachol), 6.34 ± 0.06 (for apigenin), 21.5 ± 0.04 (for oleanolic acid), 11.23 ± 0.05 (for gallic acid), 5.11 ± 0.03 (for capsaiacin), 5.83 ± 0.05 min (for ferulic acid) by comparing between standard and extract chromatograms. Chromatograms has been represented in supplementary file (1S-10S). The calibration range of lapachol, apigenin, oleanolic acid, gallic acid, capsaiacin and ferulic acid was found to be 10-100, 1-80, 10-800, 10-1000, 1-80, 1-100 μg/ml respectively, with the linear equation Y = 26513X + 62826, Y = 23838X + 58264, Y = 19462X + 16172, Y = 51474X + 13792 and Y = 83252X + 10246 with coefficient of determinants (r²) of 0.996, 0.993, 0.995, 0.996 and 0.994 respectively. The amount of lapachol and apigenin, oleanolic acid, gallic acid, capsaiacin and ferulic acid found in SSU, VAAP, ASL, CACF and ACF was 1.42% and 0.46%, 1.96%, 0.50%, 3.12%, 1.05% (w/w), respectively.

**Antibacterial activity**

**MIC and MBC of plant extracts**

Five plant extract tested against six bacterial (E. coli, S. aureus, B. subtilis, S. typhi, B. cereus, P. aeruginosa) strains showed significant inhibitory activity with MIC bellow 2000 µg ml⁻¹ (Table 1). All the five plant, SSU, VAAP, ASL, CACF and ACF showed inhibitory activity against S. aureus with MIC 935, 728, 742, 698, 892 μg ml⁻¹ (Table 1), while ASL and CACF showed inhibitory activity against bacterial strains (B. subtilis) with MIC 812 and 690 µg ml⁻¹ respectively (Table 1). The results further demonstrated that the inhibitory activity of VAAP, ASL, CACF and ACF against E. coli with MIC 920, 802, 760 and 792 μg ml⁻¹, respectively. Where as ASL and CACF inhibited gram negative bacterial strain (P. aeruginosa) with MIC 1100 and 1120 μg ml⁻¹ (Table 1) respectively. Hence, the results indicated that CACF showed potent (15.2 mm) antibacterial activity against S. aureus. Whereas, the SSU, VAAP, ASL, CACF and ACF plant extract possesses moderate to poor degree (10-15 mm) of antibacterial activity against 3 strains S. aureus, B. subtilis, E. coli. The % inhibition of the SSB, VAAP, ASL, CACF and ACF against the tested bacterial strains are shown in Figure 1 (A-E). The minimal bactericidal concentration assay, using 2- to 3-fold MIC, presented that at lower concentrations of the plant extract had bacteriostatic activity, but at higher concentrations had bacteriocidal activity (Table 2), due to the presence of one or more active principle in the extract.

### Table 1: Zone of inhibition and MIC value of the plant extracts

<table>
<thead>
<tr>
<th>Name of the Plant Extract</th>
<th>Name of the Bacteria</th>
<th>MIC (µg/ml)</th>
<th>ZOI (mm)</th>
<th>MIC (µg/ml)</th>
<th>ZOI (mm)</th>
<th>MIC (µg/ml)</th>
<th>ZOI (mm)</th>
<th>MIC (µg/ml)</th>
<th>ZOI (mm)</th>
<th>MIC (µg/ml)</th>
<th>ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. suaveolens</td>
<td>S. aureus</td>
<td>935</td>
<td>9.2 ±0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V. articulatum</td>
<td>B. subtilis</td>
<td>728</td>
<td>11.±0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. squamosa</td>
<td>B. cereus</td>
<td>742</td>
<td>12.±0.06</td>
<td>812</td>
<td>13.3±0.05</td>
<td>-</td>
<td>-</td>
<td>802</td>
<td>10.±0.03</td>
<td>-</td>
<td>1100</td>
</tr>
<tr>
<td>C. ammum cayenne</td>
<td>E. coli</td>
<td>698</td>
<td>15.±0.06</td>
<td>690</td>
<td>12.8±0.08</td>
<td>-</td>
<td>760</td>
<td>12.±0.02</td>
<td>-</td>
<td>-</td>
<td>1120</td>
</tr>
<tr>
<td>A. comosus</td>
<td>S. typhi</td>
<td>892</td>
<td>8.2±0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>792</td>
<td>13.1±0.04</td>
<td>-</td>
<td>-</td>
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</table>

**Statistical analysis**

Data expressed as mean Inhibition zone diameter ± SEM. The results recorded were statistically analyzed by one way ANOVA using GraphPad InStat Version 5.0 (GraphPad Software, Inc., USA).
Table 2: MBC value of the plant extracts

<table>
<thead>
<tr>
<th>S.I. No.</th>
<th>Plant extract</th>
<th>S. aureus</th>
<th>B. subtilis</th>
<th>B. cereus</th>
<th>E. coli</th>
<th>S. typhi</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. suaveolens</td>
<td>1870</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>V. articulatum</td>
<td>1456</td>
<td>-</td>
<td>-</td>
<td>1840</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>A. squamosa</td>
<td>1484</td>
<td>1624</td>
<td>-</td>
<td>1604</td>
<td>-</td>
<td>2200</td>
</tr>
<tr>
<td>4</td>
<td>C. annuum cayenne</td>
<td>2094</td>
<td>2070</td>
<td>-</td>
<td>2280</td>
<td>-</td>
<td>3360</td>
</tr>
<tr>
<td>5</td>
<td>A. comosus</td>
<td>2230</td>
<td>-</td>
<td>-</td>
<td>1980</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 1A** : Percentage of inhibition curve of *Viscum articulatum* extract.

**Figure 1B** : Percentage of inhibition curve of *Stereospermum suaveolens* extract.

**Figure 1C** : Percentage of inhibition curve of *Annona squamosa* extract.

**Figure 1D** : Percentage of inhibition curve of *Capsicum annuum* cayenne extract.

**Figure 1E** : Percentage of inhibition curve of *Ananas comosus* extract.
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Figure 2A : Effect of pH against *Staphylococcus aureus*.
Figure 2B : Effect of pH against *Bacillus subtilis*.
Figure 2C : Effect of pH against *Escherichia coli*.
Figure 2D : Effect of pH against *Pseudomonas aureugenosa*.
Figure 3A: Time course assay of the plant extracts on Staphylococcus aureus.
Figure 3B: Time course assay of the plant extracts on Bacillus subtilis.
Figure 3C: Time course assay of the plant extracts on Escherichia coli.
Figure 3D: Time course assay of the plant extracts on Pseudomonas aureugenosa.
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Figure 1S: RP-HPLC chromatogram of Lapachol and Apigenin

Figure 2S: RP-HPLC chromatogram of Stereospermum suaveolens extract

Figure 3S: RP-HPLC chromatogram of Oleanolic acid

Figure 4S: RP-HPLC chromatogram of Viscum articulatum extract

Figure 5S: RP-HPLC chromatogram of Gallic acid

Figure 6S: RP-HPLC chromatogram of Annona squamosa extract

Figure 7S: RP-HPLC chromatogram of Capsaicin

Figure 8S: RP-HPLC chromatogram of Capsicum annuum cayenne extract
Antimicrobial screening of Indian Medicinal Plants extracts (MBC) exhibited bactericidal effect on S. aureus and C. annuum, L. leaves using high-532 C. annuum, S. suaveolens that the studied plant extracts completely reduces the reproducing capability within 2 h and 4 h (Figure 3A and 3B), respectively. Whereas on the test organisms. Out of five plants extracts cayenne Treatment with both are showed highest zone of inhibition 18.6 mm and 19.5 mm (Figure 2A). The effect of pH on inhibition zone of the plant extracts increasing the pH of the medium. The highest zone of inhibition (18.4 mm) The antibacterial activity of plant extracts increased gradually with Time course assay Treatment with S. suaveolens, V. articulatum, A. squamosa, C. annuum cayenne and A. comosus extracts (MBC) exhibited bactericidal effect on the test organisms. Out of five plants extracts A. squamosa and C. annuum cayenne completely inactivated S. aureus and B. subtilis population within 2 h and 4 h (Figure 3A and 3B), respectively. Whereas S. suaveolens, V. articulatum and A. comosus inactivated S. aureus population within 4 h (Figure 3A). In case of E. coli, the two plant extracts (V. articulatum and A. squamosa) completely inactivated bacterial population within 4 h and other two plant extracts (C. annuum cayenne and A. comosus) inactivated bacterial population within 2 h (Figure 3C). While, A. squamosa and C. annuum cayenne completely inactivated P. aeruginosa population with in 10 h (Figure 3D). These results suggest that the studied plant extracts completely reduces the reproducing capability of bacterial strains (S. aureus, B. subtilis, E. coli, P. aeruginosa) within 2-10 h of exposure (Figure 3A-3D).

CONCLUSION
The results of the experiments clearly suggested that A. comosus, A. squamosa, C. annuum, S. suaveolens, V. articulatum extracts has potential antibacterial agents against S. aureus, B. subtilis, E. coli and P. aeruginosa. A. squamosa, and C. annuum possessed most potent antimicrobial activity among the five tested plant extract. This scientific exploration will help to identify effective antimicrobial agents from medicinal plants, which may be clinically investigated for the treatment of infectious diseases.

ACKNOWLEDGEMENT
The authors are thankful to Jadavpur University for providing the opportunity to carry on Research work.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

REFERENCES

Figure 9S : RP-HPLC chromatogram of Ferulic acid

Figure 10S : RP-HPLC chromatogram of Ananas comosus extract

Effect of pH on the antibacterial activity
The antibacterial activity of plant extracts increased gradually with increasing the pH of the medium. The highest zone of inhibition (18.4 mm) against S. aureus was observed at pH 9 with C. annuum cayenne extract (Figure 2A). The effect of pH on inhibition zone of the plant extracts against S. aureus, B. subtilis, E. coli, P. aeruginosa are shown in Figure 2 (A-D). The growth of bacteria in the control discs loaded with DMSO, unaffected by the changes in pH. In case of Ampicillin and Streptomycin both are showed highest zone of inhibition 18.6 mm and 19.5 mm respectively, at pH 9 compared with other pH ranges.

Time course assay
Treatment with S. suaveolens, V. articulatum, A. squamosa, C. annuum cayenne and A. comosus extracts (MBC) exhibited bactericidal effect on the test organisms. Out of five plants extracts A. squamosa and C. annuum cayenne completely inactivated S. aureus and B. subtilis population within 2 h and 4 h (Figure 3A and 3B), respectively. Whereas S. suaveolens, V. articulatum and A. comosus inactivated S. aureus population within 4 h (Figure 3A). In case of E. coli, the two plant extracts (V. articulatum and A. squamosa) completely inactivated bacterial population within 4 h and other two plant extracts (C. annuum cayenne and A. comosus) inactivated bacterial population within 2 h (Figure 3C). While, A. squamosa and C. annuum cayenne completely inactivated P. aeruginosa population with in 10 h (Figure 3D). These results suggest that the studied plant extracts completely reduces the reproducing capability of bacterial strains (S. aureus, B. subtilis, E. coli, P. aeruginosa) within 2-10 h of exposure (Figure 3A-3D).

CONCLUSION
The results of the experiments clearly suggested that A. comosus, A. squamosa, C. annuum, S. suaveolens, V. articulatum extracts has potential anti-bacterial agents against S. aureus, B. subtilis, E. coli and P. aeruginosa. A. squamosa, and C. annuum possessed most potent antimicrobial activity among the five tested plant extract. This scientific exploration will help to identify effective antimicrobial agents from medicinal plants, which may be clinically investigated for the treatment of infectious diseases.


