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Original Article

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. aureugenosa* (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. aeruginosa* 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.

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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, hiccup, leprosy and asthma. The root is used in the preparation of Ayurvedic formulation known as Dashmula.[4-6] The plant bark contains sterekunthal B, stereochenols A and B, lapachol, dehydro-α-lapachone, apigenin. The major constituents are lapachol and apigenin have several pharmacological activities, including antimicrobial, antiviral, anti-inflammatory, antiparasitic, leishmanicidal and anticancer.[7-8]

Viscum articulatum Burm. (Family: Loranthaceae) is an ethnomedicinal 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.[9] 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, constipation, hemorrhage, diarrhoea, fever, thirst, bronchitis, helminthiasis, dropsy, painful malignant tumours and ulcers[13] Ayurvedic practitioners use stem and leave extract as an indigenous

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uterotonic drug. The major active constituents of the leaves are anonaine, borneol, carvone, β-Caryphyllene, eugenol, farnesol, geraniol, higemamine, isocorydine, limonine, linalool acetate, menthone, α-pinene, β-pinene, rutin, and gallic acid.[14-16]

Capsicum annuum (Family Solanaceae) is extensively used in food industry as natural flavouring and coloring agent and having more than 30 species.[17-19] Among them *Capsicum annuum* cayenne one of the most popular species found in India. Capsicum has several therapeutic properties as topical analgesic, tonic, antiseptic, carminative and counters irritant property and also used for the treatment of inflammation, rheumatism, arthritis, neuralgia, itching, lumbago, spasms, obesity, cardiovascular and gastrointestinal diseases. The major active secondary metabolite found in capsicum fruit is capsaicin. Capsicum fruit contains health-promoting metabolites, such as carotenoids, ascorbic acid (vitamin C), vitamin A and capsaicinoids[19]

Ananas comosus (L.) Merrill., (Family: Bromeliaceae) native to Central and South America, and can be found in Hawaii, Philippines, Caribbean, Malaysia, Thailand, Australia, Mexico, Kenya, South Africa and China. In China, the Pineapple cortexes were used as alexipharmic, antitussive and antidiarrheal agents. Juice of the leaves is used for the control of hiccoughs and vermifuge. The ripe pineapple fruit juice used as antiscorbutic, cholagogic, diaphoretic, refrigerant, and treatment of jaundice.[20-21] The fruit juice contains ferulic acid, which is a phenolic acid having many activities, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, anti-cancer and anti-obesity activities. It also protects coronary disease, decreases cholesterol level and enhances sperm viability.[22-23]

Ample of evidences suggested that *Stereospermum suaveolens* Roxb., *Viscum articulatum* Burm., *Annona squamosa*, *Capsicum annuum* cayenne, *Ananas comosus* Merrill. are widely used in Ayurveda for treatment of microbial infection. The present study was designed for the evaluation of antimicrobial activity five plant extracts. Additionally, standardization has been performed by RP-HPLC to correlate the activity with phyto-constituents.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents were of analytical grade. Cell culture grade DMSO, HPLC grade of methanol, acetonitrile, water (Milli-Q) and glacial acetic acid were purchased from Merck Ltd. (Mumbai, India). Standard lapachol, apigenin, oleanolic acid, gallic acid, ferulic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Nutrient Agar (NA) and Muller Hinton Agar (MHA) obtained from Himedia, India. Ampicillin and streptomycin were purchased from Sisco Research Laboratory, India.

Plant material collection and extraction

The *Stereospermum suaveolens* bark (SSB), *Viscum articulatum* aerial part (VAAP), *Annona squamosa* leaf (ASL), *Capsicum annuum* cayenne fruit (CACF), *Ananas comosus* fruit (ACF) were collected from the North and South Bengal region in the month of December and July 2013. Further, collected plant sample was identified and authenticated by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, Emerald, Tamilnadu, India. After authentication, sample specimen was deposited in the Herbarium of the School of Natural Product Studies (SNPS), Jadavpur University, Kolkata, India for future reference. The vide voucher specimen number are SNPS-JU/2013/1462, SNPS-JU/2013/1463, SNPS-JU/2013/1467 and SNPS-JU/2013/1468 for CACF, SSB, VAAP, ASL, respectively.

The plant SSB, VAAP, ASL, CACF were dried under shade and pulverized by using a mechanical grinder to make a coarse powder. Then 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) by using Eyela Rotary Evaporator (Japan). The yield of methanol extract of SSB, VAAP, ASL and CACF was found to be 13.21%, 11.35%, 12.98% and 10.45% (w/w) respectively. All dried methanol extract was stored at 4°C for further use.

The stalk (central core) of the pineapple (ACF) was separated from the fleshy fruits. The flesh of the fruit portion was then cut into small pieces and pulverized by using a mechanical grinder. The juice was filtered through a white cloth to remove the fibrous materials. The filtrate was centrifuged for 10 min to remove insoluble materials. The obtained clear supernatant was filtered again through what man filter paper. Further, the clear supernatant was lyophilized to make fine powder and stored at -20°C.

HPLC analysis

The HPLC system (Waters, Milford, MA, USA) used for the analysis was equipped with a 600-controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) detector equipped with an in-line degasser AF2489 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, India) were used as a concentration of 10 µg/ml (w/v). DMSO 1% (v/v) was used as 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, Capsicum annuum* cayenne *and Ananus comosus*) were prepared and the final concentration of each plant extract was 5000 µg ml^{-1}, freshly prepared stock solution and requisite different concentration for the bacterial tests were prepared from this stock solution.

Bacterial strains and culture condition

Gram positive (*Bacillus subtilis* ATCC 11774, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 29213), and gram negative (*Escherichia coli* ATCC 25922, *Salmonella typhi* MTCC 734, *and Pseudomonas aureugenosa* ATCC 9027) bacteria were selected as standard strains as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI), formerly called National Committee for Clinical Laboratory Standards[24] 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 were performed by disc diffusion method.[24] Concisely, 10 ml of sterilized Muller Hinton Agar (MHA) (pH 7.2 ± 0.2, at 25°C) were applied in to the surface of sterile Petri dishes (9 cm in diameter, Borosil) and allowing them to settle for base plate preparation. 100 ml of test bacterial suspension (5×10^5 CFU/ml) were poured to each base plate and cotton swab (Himedia). 20 ml of different concentrations of each test sample (50-2000 mg/disc) were soaked with sterile paper discs (6 mm). The air-dried discs were placed on each base plate and incubated at 37 ± 2°C for 24 h. Ampicillin and streptomycin were used in 20 mg/disc concentration range as positive control for gram negative and gram positive microorganisms respectively. The inhibition of zones around the discs was determined as the diameter (mm) of bacterial growth inhibition. The zone of inhibition

was taken as an average of three measurements at different directions.[25] 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.[24] 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 (5×10^5 CFU/ml) by using to 0.5 Mac Farmland 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.[26] 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.[27] Overnight the broth cultures of *B. subtilis*, *B. cereus*, *S. aureus*, *E. coli, S. typhi, and P. aureugenosa* 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.[28] Overnight broth cultures of bacterial strains were adjusted to the concentration of 5×10^5 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.

Statistical analysis

Data expressed as mean Inhibition zone diameter ± SEM. The results recorded were statistically analyzed by one way ANOVA using Graph-Pad InStat Version 5.0 (GraphPad Software, Inc., USA).

RESULTS AND DISCUSSION

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

The mean R_t 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 capsaicin), 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, capsaicin 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 (r^2) of 0.996, 0.993, 0.995, 0.996 and 0.994 respectively. The amount of lapachol and apigenin, oleanolic acid, gallic acid, capsaicin and ferulic acid found in SSB, 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, SSB, 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 SSB, 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*. While *S. aureus*, *P. aeruginosa* had weak activity (7-10 mm). 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

Name of the plant extract	Name of the Bacteria											
	S. aureus		*B. subtilis*		*B. cereus*		*E. coli*		*S. typhi*		*P. aeruginosa*	
	MIC (µgml^{-1})	ZOI (mm)	MIC (µgml^{-1})	ZOI (mm)	MIC (µgml^{-1})	ZOI (mm)	MIC (µgml^{-1})	ZOI (mm)	MIC (µgml^{-1})	ZOI (mm)	MIC (µgml^{-1})	ZOI (mm)
S. suaveolens	935	9.2 ±0.02	-	-	-	-	-	-	-	-	-	-
V. articulatum	728	11.4±0.05	-	-	-	-	920	10.1±0.06	-	-	-	-
A. squamosa	742	12.3±0.06	812	13.3±0.05	-	-	802	10.9±0.03	-	-	1100	9.2±0.01
C. annuum cayenne	698	15.2±0.06	690	12.8±0.08	-	-	760	12.4±0.02	-	-	1120	8.4±0.02
A. comosus	892	8.2±0.05	-	-	-	-	792	13.1±0.04	-	-	-	-

Table 2: MBC value of the plant extracts

S.l. No.	Plant extract	Bacteria MBC (µg ml^{-1})					
		S. aureus	*B. subtilis*	*B. cereus*	*E. coli*	*S. typhi*	*P. aeruginosa*
1	*S. suaveolens*	1870	-	-	-	-	-
2	*V. articulatum*	1456	-	-	1840	-	-
3	*A. squamosa*	1484	1624	-	1604	-	2200
4	*C. annuum cayenne*	2094	2070	-	2280	-	3360
5	*A. comosus*	2230	-	-	1980	-	-

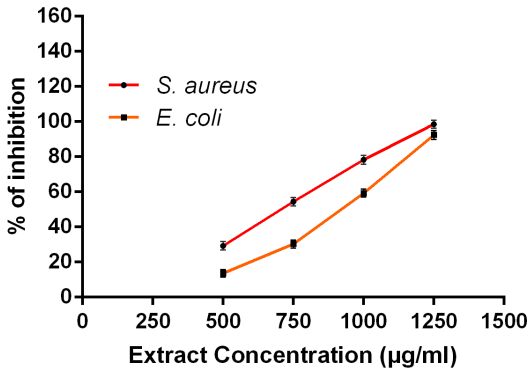


Figure 1A

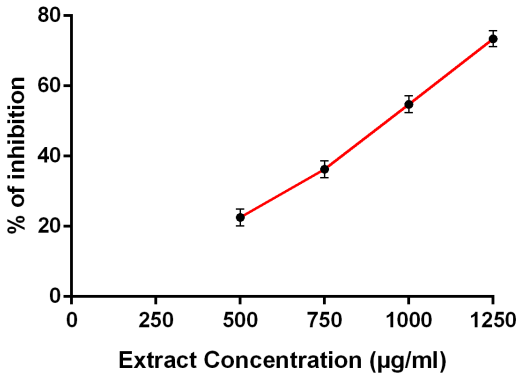


Figure 1B

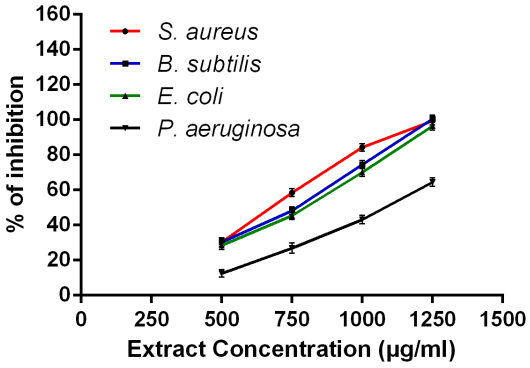


Figure 1C

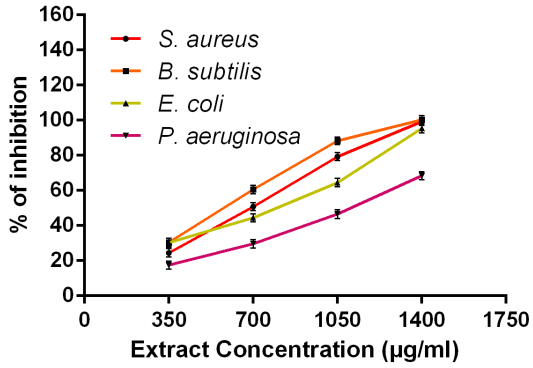


Figure 1D

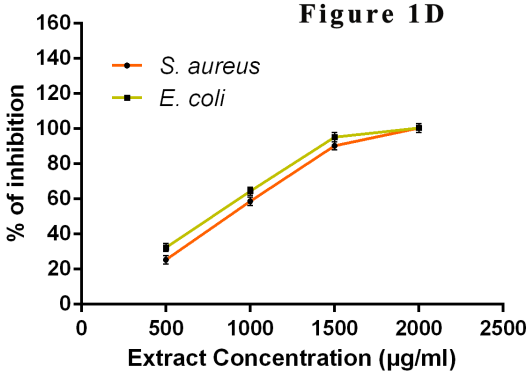


Figure 1E

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.

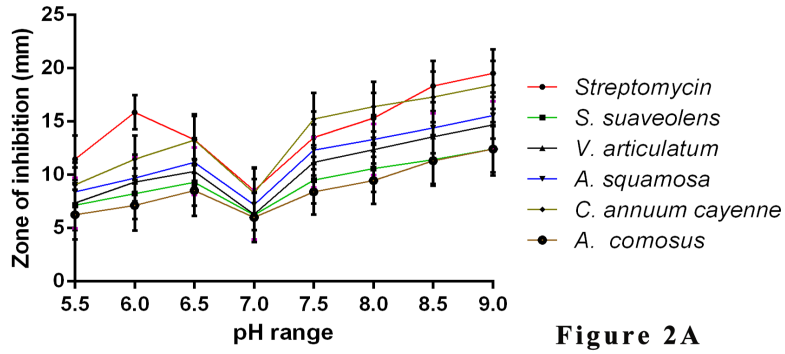
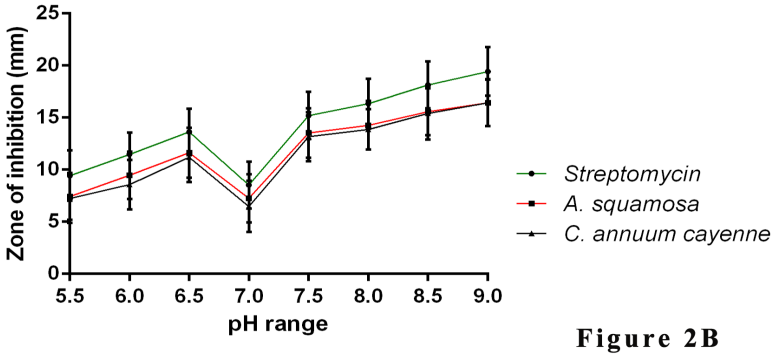


Figure 2A



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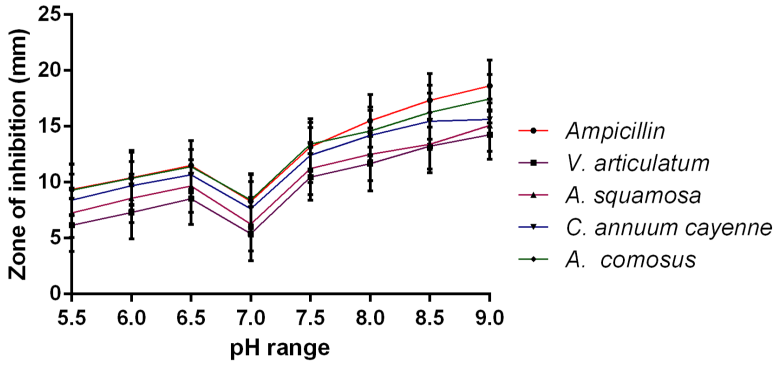

Figure 2C

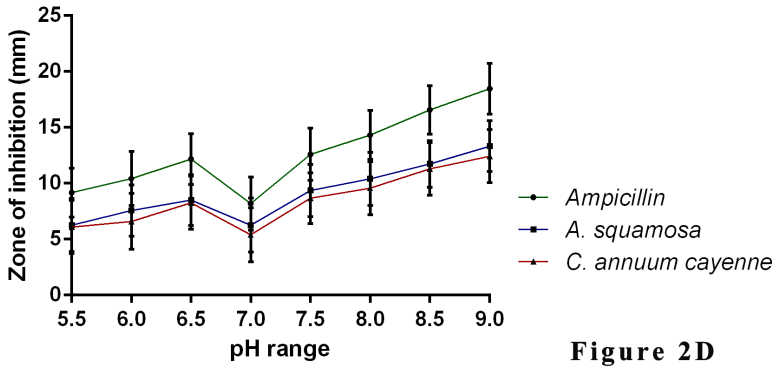

Figure 2D

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*.

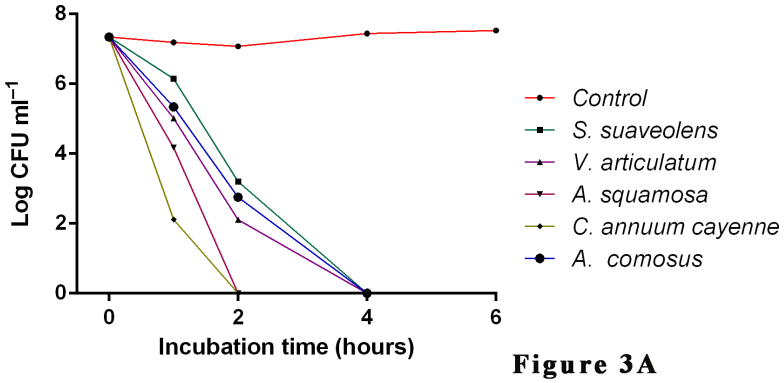

Figure 3A

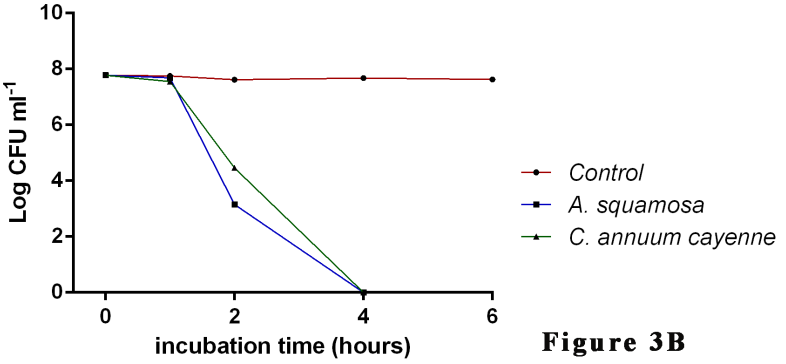

Figure 3B

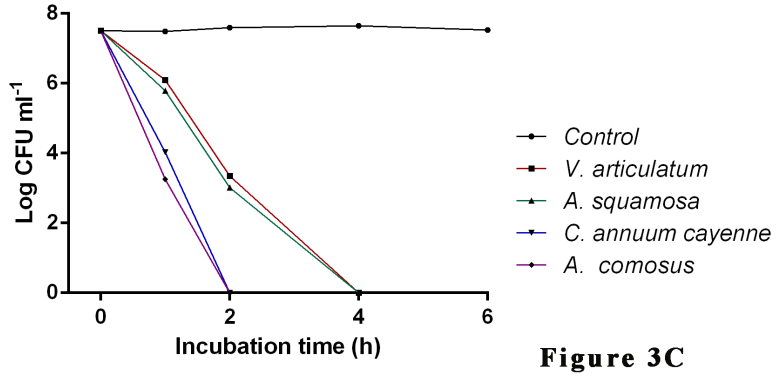

Figure 3C

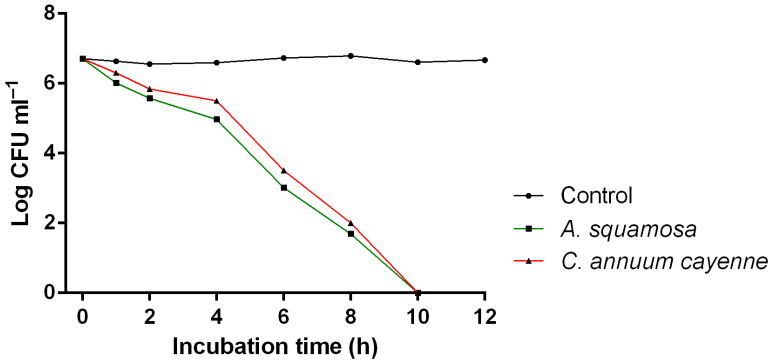

Figure 3D

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*.

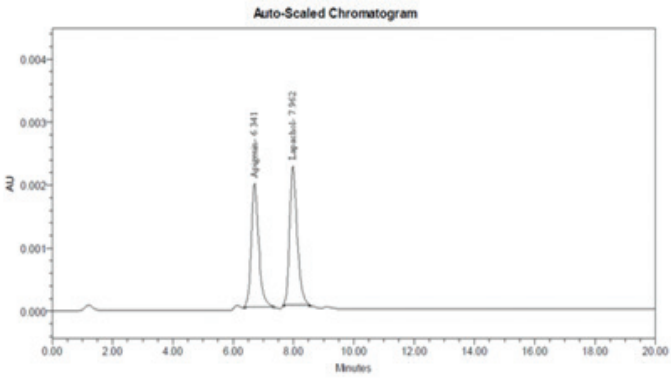

Figure 1S : RP-HPLC chromatogram of Lapachol and Apigenin

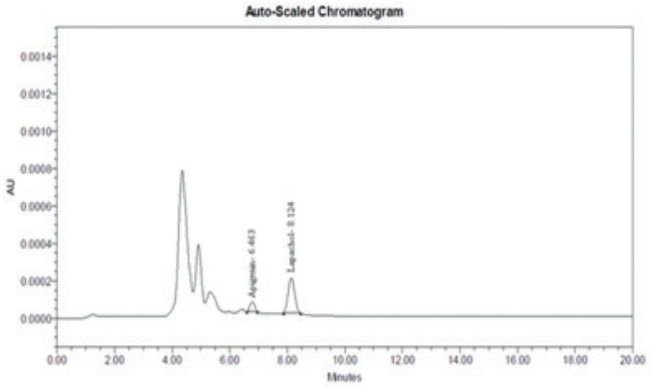

Figure 2S : RP-HPLC chromatogram of *Stereospermum suaveolens* extract

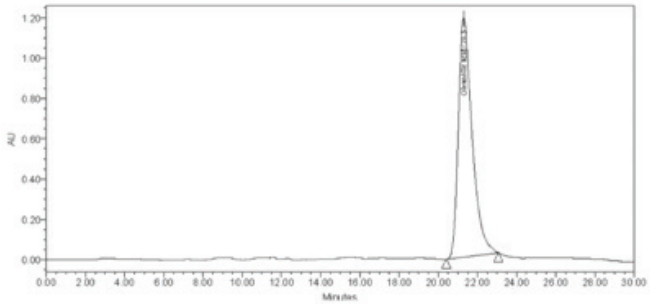

Figure 3S : RP-HPLC chromatogram of Oleanolic acid

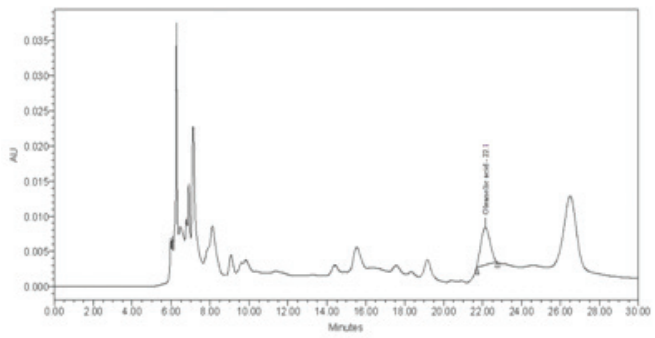

Figure 4S : RP-HPLC chromatogram of *Viscum articulatum* extract

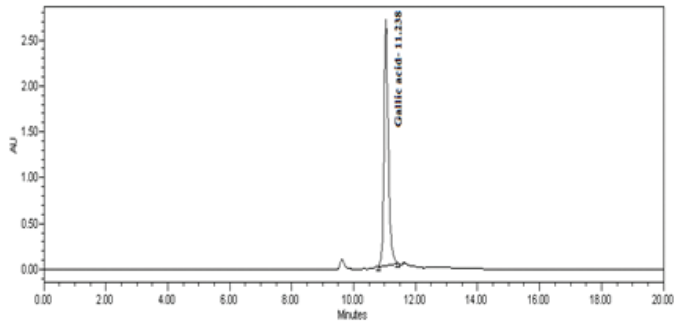

Figure 5S : RP-HPLC chromatogram of Gallic acid

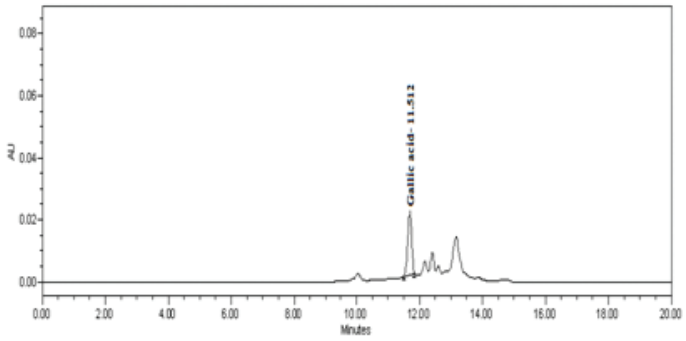

Figure 6S : RP-HPLC chromatogram of *Annona squamosa* extract

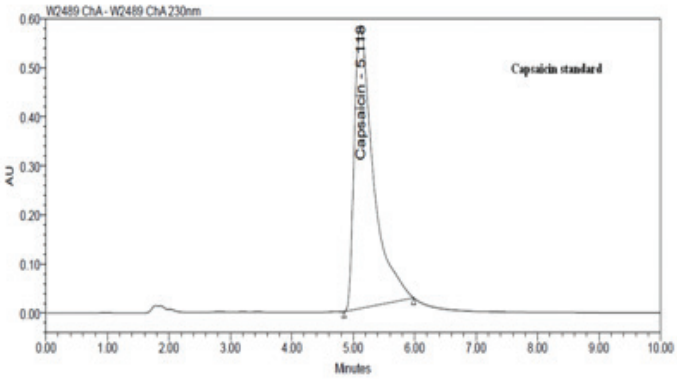

Figure 7S : RP-HPLC chromatogram of Capsaicin

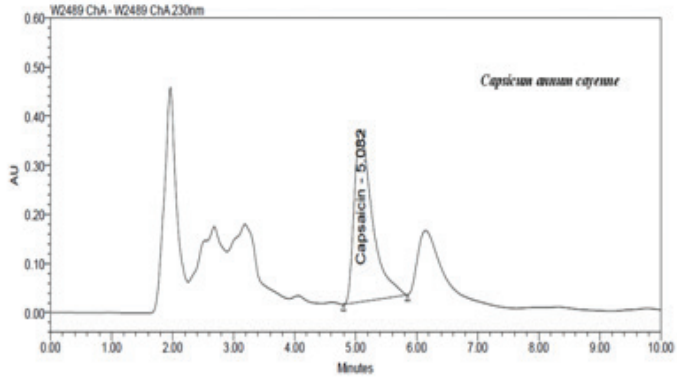

Figure 8S : RP-HPLC chromatogram of *Capsicum annuum* cayenne extract

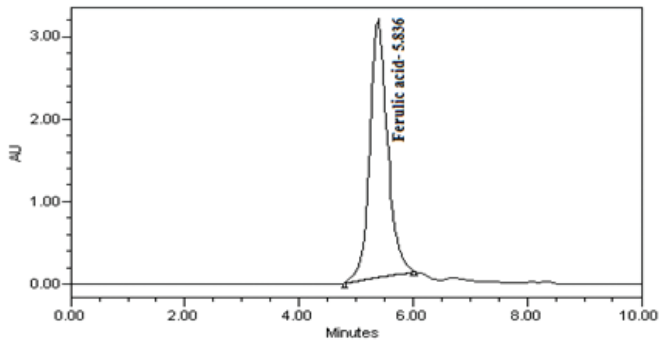

Figure 9S : RP-HPLC chromatogram of Ferulic acid

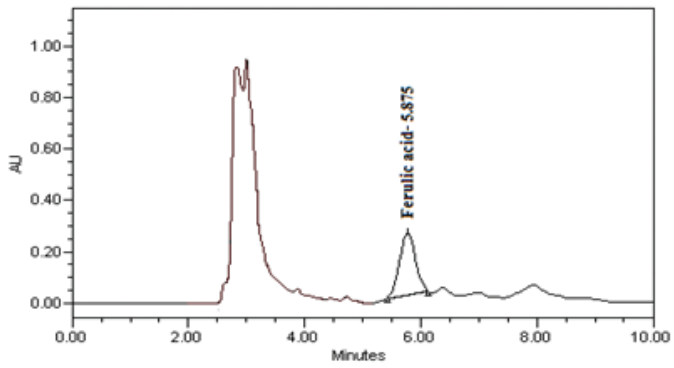

Figure 10S : RP-HPLC chromatogram of *Ananas comosus* extract

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 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.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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