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ANALYZING THE MECHANISM OF ANTIBACTERIAL PROPERTIES OF METHANOLIC EXTRACTS OF A HIMALAYAN HERB *SAUSSUREA GOSSYPIPHORA*

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Key words*: Saussurea gossypiphora, Antibacterial activity, Bactericidal, Time-kill kinetic studies, Intracellular material.*

Abstract– In the present study, the Anti-bacterial activity of methanolic flower extracts of a Himalayan herb, *Saussurea gossypiphora* has been studied. The antibacterial activity of *S. gossypiphora* extracts was examined against Gram-positive and Gram-negative bacteria. The extracts inhibited Gram-positive bacteria, especially *Staphylococcus epidermidis* and *Bacillus cereus*. Hence these two strains were selected for further studies. Minimum Inhibitory Concentration of the extracts was assessed and observed to be 100 mg/ml for *S. epidermidis* and 125 mg/ml for *B.cereus*. Time-kill kinetic studies indicated bactericidal effect of the extract on both strains. Scanning electron microscopy of the strains when treated with the extracts revealed morphological changes in the cells i.e., shrinkage of cells, damage and disruption of cell wall and cell membrane. Further analysis revealed leakage of DNA and proteins from the bacteria upon treatment with the extracts. In conclusion, *S. gossypiphora* extracts have significant bactericidal activity against Gram-positive bacteria which results in disruption of the microbial membrane, leading to the leakage of intracellular material that results in microbial death. Thus the extracts can be further explored for their antimicrobial activity.

INTRODUCTION

Infectious diseases remain a major health concern, accounting for 41% of the global disease burden measured by Disability-Adjusted Life Years (DALY's). A key issue worsening this situation is the widespread emergence of bacterial resistance to antibiotics, which presents a serious threat to global public health through both epidemics and pandemics Hemeg *et al.* (2020). Even though pharmaceutical industries have developed numerous new antibiotics, microbial resistance has risen. Bacteria generally have the genetic capacity to transfer and develop resistance to therapeutic agents Gislene G. F. Nascimento *et al.* (2000). Some species of *Staphylococcus* and *Streptococcus* cause respiratory and skin infections, while *Pseudomonas* and members of the *Enterobacteriaceae* family cause gastrointestinal, urogenital diseases, and wound infections. These microorganisms are completely resistant to older antibiotics Wasihun *et al.* (2023).

According to the World Health Organization (WHO), herbal medicines derived from medicinal plants are the most effective for treating diseases and offering primary health care to about 75% of people in developing nations. From 1983 to 1994, nearly 78% of the drugs approved by the FDA were either herbal or semi-synthetic products derived from botanical sources Burman *et al.* (2018). As a result, there is increasing interest in investigating biologically active compounds from plant species traditionally used in herbal medicine. These plant-derived compounds are being explored as potential new sources of antibacterial and anti-fungal agents. The antimicrobial properties of plants are mainly due to their ability to produce a diverse range of secondary metabolites with complex structures that possess antimicrobial activity.

Saussurea genus consists of 410 species which are found in regions with cold climate all over the world. In India, there are only 62 species. Of these 37 species are native to the Indian Himalayan region (IHR). Plants belonging to this species possess medicinal properties. *Saussurea gossypiphora,* commonly known as the snowball, is a perennial herbaceous species. According to traditional Chinese medicine, it is known to have significant medicinal properties for example, in treatment of menstrual

disorders, gynecological disorders and hysteria. Its roots yield essential oils which are used in making perfumes. The wool of the herb is applied to fresh cuts for rapid treatment of wounds. It is typically found at altitudes ranging from 4300 to 5600 meters Bisht and Purohit (2010); Kala *et al.* (2006); Tiwari *et al.* (2010). The present study deals with analysis of antibacterial activity of crude methanolic flower extracts of *S. gossypiphora.*

MATERIALS AND METHODS

Collection of plant material

Saussurea gossypiphora (Sg) flowers were purchased from a local vendor of herbals in Hyderabad, India. The plant material was checked for any contaminants such as unsafe foreign materials, stones, poisonous chemical residues, etc. The collected plant material was washed with sterile water and allowed to air dry at 25-30 ^{0}C and the dried flowers were powdered by maceration.

Extraction of *S. gossypiphora*

10 g of the dried flowers of *S. gossypiphora* were powdered and dissolved in 100 ml of methanol and incubated in an orbital shaker incubator at 37 ^{0}C with constant shaking (150 rpm) and after 72 h of incubation the extract was filtered through Whatman No. 1 filter paper. The extract was dried by using a rotor evaporator (Equitron, Roteva, India) at 60 ^{0}C under reduced pressure to remove the solvent and the crude dried extract was collected. The crude dried extract was re-suspended in methanol to obtain a final concentration of 5 mg/ml and 200 mg/ml. The collected crude extract was preserved at -20 ^{0}C until further use Olivia *et al*., (2021).

Bacterial Strains

The bacterial strains used in the study are *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 49134), *Bacillus subtilis*, *Bacillus cereus* (ATCC14579), *Escherichia coli* (ATCC 25922) and *Proteus vulgaris* (ATCC 49132).

Preparation of inoculum

The medium used to test microorganisms was nutrient broth (Hi-media, India). For the activation of the bacterial strains, cultures were revived from the stock sub-cultured into the fresh nutrient broth and incubated at 37 ^{0}C overnight. The suspension was serially diluted with sterile broth to obtain approximately 10^7 CFU/ml and compared with 0.5 McFarland turbidity standards. McFarland number 0.5 standard was prepared by dissolving 0.05 ml of Barium Chloride Dihydrate ($BaCl.2H_2O$) with 9.95ml of 1% Sulfuric Acid (H_2SO_4) to ensure the bacterial density. This was stored in an air-tight bottle and used to standardize the approximate number of bacteria in suspension whenever required.

Agar Well Diffusion Method

A modified agar well diffusion method was employed for the determination of the antibacterial activity against gram-positive and gram-negative bacteria. Test organisms included in this study were six laboratory reference bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Bacillus subtilis, Escherichia coli, Proteus vulgaris*). Overnight-grown bacterial strains with an inoculum size of (10^7 CFU/ml) of bacteria, compared with 0.5 McFarland turbidity standards were used in the study Abalaka *et al.* (2012). Briefly, 100 µl of the bacterial suspension (10^7 CFU/ml) was transferred on the surface of a sterile nutrient agar plate by using a micro-pipette and was swabbed by rotating plate, to uniformly disperse bacteria throughout the media surface, using a sterile cotton swab. The swabbed nutrient agar plates were left for 15 minutes to adhere to bacteria on the surface of the media. Later, the sterilized cork borer of 6 mm diameter was used to create wells on swabbed nutrient agar plates. At the time of creating wells on media for different test bacteria, the cork borer was sterilized by using alcohol and exposed to Bunsen burner flames. The created wells were filled with 80µl of S.*gossypiphora* extracts at a concentration of 50, 100, and 250 mg/ml, and the negative control methanol was used respectively. After all the wells were filled, the Petri plates were placed in the refrigerator at 4 °C for 15 min to facilitate diffusion of extracts in the media. Followed by incubation of Petri plates at 37 °C for 24 h in the incubator (BioTechnics India). The zone of inhibition was measured and recorded (Abew *et al*. (2014), Ohikhena *et al.* (2017), Suurbaar *et al*. (2017).

Determination of Minimum Inhibitory Concentration

Stock solutions of methanolic flower extract of *S. gossypiphora*, concentrations ranging from 5 mg/ml to 200 mg/ml were prepared to check MIC against *Staphylococcus epidermidis* and *Bacillus cereus* using

the microdilution method described (Agyare *et al.,* 2013). Briefly, to 100 µl of 2 x nutrient broth 20 µl of inoculum (1 × 10^7 CFU/ml) was added in each well of 96 well plates and then treated with 20 µl of different concentrations of the extract, after incubating at 37 ^{0}C for 24h 20 µl of MTT dye was added and again incubated for 30 minutes and checked for purple coloration which indicates growth whereas, white or pale yellow indicates no growth. 1mg/ml ampicillin was used as positive control. The experiment was carried out in triplicates.

Time kill Assay

Time-kill studies of methanolic flower extract of S. *gossypiphora* were carried out as described previously Theresa Appiah *et al.,* (2017)**.** 20 ml of the overnight broth cultures with an inoculum size of 10^7 CFU/ml was used to determine time kill kinetics of the bacteria. *S. epidermidis* and *B.cereus* were treated with 100 and 125 mg/ml of the extract respectively and 50 µL of the treated culture was aliquoted at specific time intervals such as 0, 1, 2, 3, 4, 5, 6, 12 and 24 h, respectively, and spread plated on sterile Muller Hinton agarplates and incubated at 37 ^{0}C for 24 h. Controls for each bacteria were also maintained. The experiment was done in triplicates.

Scanning Electron Microscopy Analysis

A modified scanning electron microscopy (SEM) as described previously Rehman, *et al.,* (2022), was performed to examine the morphological changes of each representative strain of *Staphylococcus epidermidis* and *Bacillus cereus.* 20 mL of overnight grown culture with an inoculum size of 1×10^7 CFU/mL (Muller Hinton broth) was treated with minimum inhibitory concentrations of *S.gossypiphora* extracts and incubated at 37 ^{0}C on a shaker incubator (180 rpm). After completion of each specific time interval (0, 6, 12, and 24 h) the cells were pelleted by centrifugation at 8000 rpm for 5 min and the bacterial pellet was collected and washed twice with phosphate-buffered saline. The cells were then fixed in 2.5% glutaraldehyde in PBS for 2 h at room temperature. After incubation, the cells were again rinsed twice with PBS for 15 min and subsequently dehydrated in increasing concentrations (30 - 100%) of ethanol solution for 10 min. Subsequently, the samples were coated with gold in a sputter coater (E-1010, Hitachi) and analyzed under a scanning electron microscope (S-3700N, Hitachi) in secondary electron detection mode. The working distance and the step-up voltage were adjusted to obtain a suitable magnification.

Determination of Nucleotide and Protein Leakage by Cell Leakage Assay

Leakage of nucleic acids and protein from the bacterial cells in response to treatment with *S. gossypiphora* extracts was analyzed as per previously described protocol Alayande *et al.* (2017). 20 ml overnight cultures of *S. epidermidis* and *B.cereus* which were serially diluted to 10^7CFU/ml were treated with minimum inhibitory concentration of *S.gossypiphora* and incubated for 24 hat 37 ^{0}C in a shaker incubator. After 24 h incubation, the sample was centrifuged at 3000 rpm for 5mins, supernatant was collected and filtered through 0.2 µm pore size membrane filters. Leakage of nucleic acid and protein from the bacterial cytoplasmic membrane was determined by measuring the absorbance of filtrates at 260 and 280 nm wavelength and calculated by using the following formula. Filtrate was diluted to 1 in 100 for OD reading.

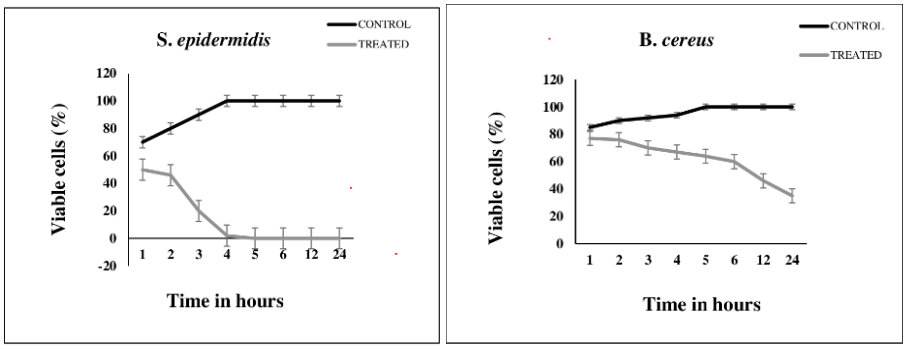

Fig. 1. Time kill kinetic studies of *S. epidermidis* and *B. cereus.* Cell viability of bacterial cells treated with minimum inhibitory concentration of methanolic flower extracts of *S. gossypiphora* was calculated at various time points. X-axis shows percentage of viable cells and Y-axis shows time intervals in hours.

ds DNA concentration = 50 mg/ml × OD × Dilution factor

Protein concentration = 1.55× A280 £- 0.76×A260

RESULTS

Determination of Antibacterial Activity using the Agar Well Diffusion Method

The antibacterial activity of crude extract of *S. gossypiphora* was assessed at 50, 100 and 250 mg/ml. The results of the zone of inhibition are given in Table 1. As the highest zone of inhibition was measured against *S. epidermidis* and *B. cereus* these two strains were selected for further studies. However, the methanolic extract of *S. gossypiphora* could not inhibit the growth of *E. coli* and *P. vulgaris*.

Determination of Minimum Inhibitory Concentration of methanolic extract of *S. gossypiphora*

As there was no significant Zone of Inhibition (ZOI) observed for Gram-negative bacteria, only Gram-positive bacteria were studied for assessment of MIC. Among them, *S. epidermidis* and *B.cereus* were used as they displayed the highest ZOI. Absorbance values after incubation with MTT reagent showed the MIC of methanolic flower extract of *S.gossypiphora* for *S. epidermidis* at around 100 mg/ml, whereas for *B. cereus* it was observed between 125 to 150 mg/ml.

Time kill kinetic studies of methanolic flower extract of *S. gossypiphora*

The time-kill kinetic studies against test organisms showed a significant effect against *S.epidermidis*, as the growth in the first hour of the extract treated sample was 20£¥ less when compared to its untreated control whereas 2nd and 3rd hours noted more than 60 and 90 £¥decrease, whereas in 4th and 5thhour it was significant and there was no growth. Comparatively, in untreated controls a full lawn was observed since 3rd hour of treatment. For *B.cereus* there was no significant decrease for the first two hours and from 3rd hour number of colonies started decreasing gradually by either 5 or 10% but not

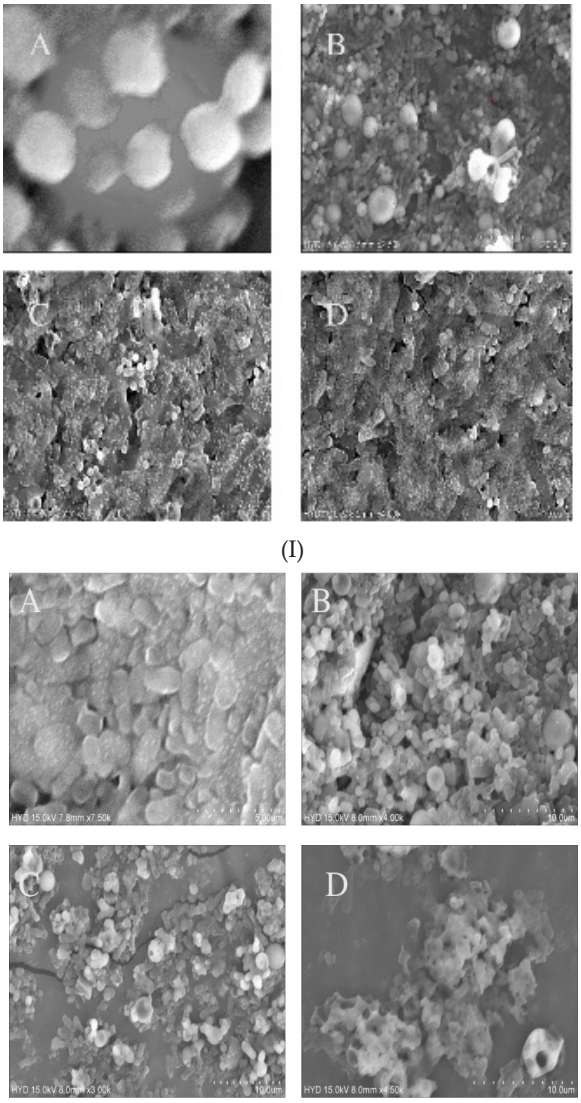

Fig. 2. Scanning electron micrographs of *S. epidermidis (I)* and *B. cereus (II)* treated with minimum inhibitory concentration of methanolic flower extract of *S. gossypiphora.* (A) 0 h (B) 6 h (C) 12 h and (D) 24 h treatment.

Table 1. Determination of Antibacterial Activity of methanolic extracts of *S. gossypiphora* using agar well diffusion method.

S. No.	Concentration of MESGD (mg/ml)	*S. aureus* (cm)	*S.epidermidis* (cm)	*B.cereus* (cm)	*B. subtilis* (cm)
1	50	1.3	1.5	1.3	1.4
2	100	1.6	1.9	1.5	1.5
3	250	1.8	2.1	1.9	1.7
4	Control	£-	£-	£-	£-

more, eventually by the 24th hour it showed a more than 50% reduction. Whereas, in untreated controls, by 4th hour large number of colonies were observed on the culture plates and by the 5th hour a complete lawn was formed. These results show that the methanolic flower extract of *S. gossypiphora* shows a significant bactericidal effect specifically against gram-positive bacteria (Figure 1).

Scanning electron microscopy of Bacteria exposed to extracts

SEM micrographs of the bacterial cells treated with MIC concentrations of the extract showed prominent variations in morphology compared to untreated controls. A change in the bacterial structure of both the strains, *S. epidermidis* and *B. cereus* (Figure 2) was observed upon 6 h exposure to the extracts. By the end of 12 h incubation the cell walls of the bacteria were disrupted, cells shrunk insize and leakage of internal components was also observed. At the end of 24 hours, all the treated bacterial cells were observed to have lost their membrane integrity and cytoplasmic components, which lead to total cell death.

Determination of Nucleotide and Protein Leakage by Cell Leakage Assay'

The amount of DNA and protein leakage caused upon exposure to extracts was analyzed in *S. epidermidis and B.cereus.* DNA leakage in S. *epidermidis* was observed but in B. *cereus* 40 µg/ml of DNA leakage was detected. The amount of Pprotein leakage was measured as 50 and 13 µg/ml for *B.cereus* and *S. epidermidis* respectively.

DISCUSSION

Several studies have previously reported that medicinal and aromatic plants can be used for their anti-microbial properties Kengne, *et al.* (2021). The present study reported the anti-microbial activity of *S. gossypiphora* against various gram-positive and gram-negative bacteria such as (*S. epidermidis, S. aureus, B. subtilis, B. cereus, E. coli*) by using agar well diffusion assay. However, the Methanolic flower extract of *S.gossypiphora* showed the highest activity against *S. epidermidis* and *B. cereus* which are both Gram positive bacteria.

A modified broth dilution method was utilized to determine the minimum inhibitory concentration (MIC) of the extract. This approach addresses the limitations of the agar diffusion test, such as the inability of some extracts to diffuse into the agar and the challenge of distinguishing between bactericidal and bacteriostatic effects. The MIC results indicated that the extract inhibited gram-positive bacteria with a MIC of 100 mg/ml for *S. epidermidis* and 125 mg/ml for *B. cereus.* However, the MIC of flower extract of *S. gossypiphora* via broth dilution method has not been reported in previous studies.

Time-kill kinetic studies are important as they provide information about the pharmacodynamics of the antibacterial agent Raveesha, (2021). In this study, time-kill kinetic studies indicate that methanolic flower extract of *S. gossypiphora* exhibited bactericidal actions. There are few or no reports on the time-kill kinetic studies of methanolic flower extract of *S. gossypiphora* Appiah, *et al* (2017). The SEM images indicate that *S. epidermidis* and *B. cereus* strains treated with extracts have morphological changes in the cells such as cell shrinkage, deformity, and roughened surface. It was further observed that the extracts of *S. gossypiphora* induced leakage of biological substances absorbing at 260 and 280 nm, probably nucleic acid and protein derivatives by *S. epidermidis and B. cereus*, at concentrations equal to the MIC of the extracts.

CONCLUSION

Overall, these findings confirm that the methanolic flower extracts of *S. gossypiphora* disturbs the microbial membrane, leading to the leakage of intracellular material that results in microbial death, corroborating their microbicidal effect. Similar mechanisms of bacterial death have been reported in earlier studies with other plant extracts. The findings obtained against the gram-positive bacteria thus support the traditional application of methanolic extract of *S. gossypiphora* as an antibacterial agent.

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Conflict of Interest- None

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