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INVESTIGATION OF ANTIMICROBIAL ACTIVITY OF *LALLEMANTIA ROYLEANA*

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ABSTRACT:

Lallemantia royleana is a member of the Lamiaceae or Labiateae family.Its seeds were used as cooling, diuretic, and sedative; as a soothing agent during urinary troubles, fever, common cold, intestinal troubles. It was also used as cephalic astringent, cardiac tonic, carminative and for cough.Using a Soxhlet apparatus, methanol was used to extract the *Lallemantia royleana* seeds. The resulting dried powder was then mixed with DMSO at a concentration of 1 mg/ml. By employing the well diffusion technique, these methanolic extracts were assessed for their antimicrobial activity. The test was run on various fungus and bacterial strains. Five fungal strains and ten bacterial strains were examined for antimicrobial activity. In the current investigation, a crude methanolic extract made from *Lallemantia royleana* seeds was used.Various doses of the methanolic seed extract revealed various-sized zones of inhibition. The methanolic extracts of *Lallemantia royleana* showed the most promising anti-bacterial activity against Gram-positive *Staphylococcus epidermidis* and *S.aureus*. Also, gram-negative bacteria like *Proteus vulgaris* showed great antibacterial action. The current study additionally assessed the crude methanolic extract of *Lallemantia royleana* seeds' anti-fungal properties. It was observed that it significantly inhibited the growth of *Candida albicans* and *Saccharomyces cervisiae*. *L. royleana's* crude methanolic seed extract can be used to treat human pathogenic illnesses as a powerful antibacterial agent. In order to effectively combat fungal diseases, *L. royleana's* crude methanolic seed extract can be employed.

Keywords: Antimicrobial, antibacterial, antifungal, *Lallemantia royleana*.

INTRODUCTION:

Iranian Jews use *Lallemantia royleana* as a key therapeutic herb. However, it is grown in North India for its incredibly beneficial and mucilaginous seeds, which are frequently utilized as cures for a variety of illnesses [1]. It is also believed to be closely linked to the Verbenaceae family, but recent phylogenetic analyses have revealed that several genera formerly thought to be members of the Verbenaceae family really belong to the Lamiaceae. Verbenaceae's main genera are more closely linked to Lamiales' members than to Lamiaceae's core genera. 6900–7200 species are found in 233–263 genera in the expanded Lamiaceae family [2]. Common names for *Lallemantia royleana* include Balangu (in both

English and Hindi), Tukhm-e-balanga (in Hindi), and Balangu Shirazi (in Urdu). The plants are herbaceous, annual, biennial, or perennial, glabrous, or white-haired. The leaves are sessile, sub-entire, or petiolate. The flowers are axillary verticillasters with six petals, and the bracts are either ciliate or awned crenate. The pedicel is firm, flattened, and upright. The calyx is tubular, 15-veined, straight, closed after anthesis, and has a posterior tooth that is wider than the other teeth with a thicker fold in the tooth sinus [3, 4]. The plant is also employed as a cleaner. The plant is one of the main components of an ointment used to treat skin cancer in Chinese medicine. Different plant components are used to cure fever, rheumatic pain, and intercostal pain in Ivory Coast, Burkina Faso, Gabon, and Tanganyika. Pneumonia is treated with leaf and root decoctions. In India, the plant is also used to make herbal brain tonics [5].

MATERIALS AND METHODS:

Collection of plant material

The plants were procured from Unani practitioners. Plant parts were separated, cleaned, washed with water and air-dried. The dried plants stored in air-tight containers until used.

Preparation of plant extract

Different plants were prepared as organic solvent extracts. With a few minor alterations, the extracts were made in accordance with Alade and Irobi (1993). 30 g of powdered plant material was soaked in 100 ml of distilled water for 72 hours at room temperature and in darkness to create the aqueous extract [6]. At set intervals, the mixture was frequently stirred. The mixture was then concentrated in vacuo after being filtered using Whatman Filter Paper No. 1. Methanol was used as the organic solvent in this study. The produced extracts were kept in aliquots and kept at 4 °C until needed.

Test strains Bacterial strains

The bacterial strains that were used for the experiments were purchased .The bacterial strains used in the study are as follows:

Bacterial Stains- *Bacillus cereus , Bacillus subtilis , Enterobacter aerogenes, Eschericia coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Staphylococcus epidermidis* .

Fungal Stains- *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Candida albicans, Saccharomyces cervisiae*.

Culture media Composition of different media

The culture media used for anti-microbial susceptibility testing of the bacterial and fungal strains in the present study are as follows:

- Nutrient Broth
- Nutrient Agar
- Potato Dextrose Broth
- Potato Dextrose Agar

A common microbiological growth medium for the ordinary cultivation of undemanding bacteria is nutrient agar. Because it maintains its solidity even at quite high temperatures, it is beneficial. Additionally, the surface of the nutrient agar supports the growth of tiny colonies of bacteria. The bacteria in nutritional broth grow in the liquid and are perceived as a soupy

material rather than as distinct clumps. Typically, nutrient agar contains 0.5 g of peptone, 0.3 g of beef extract, 1.5 g of agar, and 0.5 g of sodium chloride [7]. The final volume is made up of 100 ml of distilled water. At 37°C, the pH is set to neutral (6.8). With the exception of leaving out the agar, the composition and preparation of nutritional broth are both the same.

Common microbiological media produced from potato infusion and dextrose (corn sugar) include potato dextrose agar (PDA) and potato dextrose broth (PDB). The most popular medium for cultivating fungus and bacteria that attack living plants or decompose dead plant tissue is potato dextrose agar. Boiling 300g of sliced (washed but unpeeled) potatoes in water for 30 minutes can be used to make potato infusion. The broth should then be decanted or strained through cheesecloth. To make the suspension a total of one liter, distilled water is added. After adding 20g of dextrose and 20g of agar agar powder, the medium is autoclaved at 15 psi for 15 minutes to sterilize it. Yeasts like *Candida albicans* and *Saccharomyces cerevisiae*, as well as molds like *Aspergillus niger*, can both be cultivated on PDA [8].

Preparation of Inoculum Growth Method

At least three to five well-isolated colonies with the same morphological type are chosen from an agar plate culture before the growth procedure is carried out. The growth is transferred into a tube holding 4 to 5 ml of nutrient broth by touching the top of each colony with a loop. The broth culture is incubated at 37°C for the entire night until it meets or exceeds the 0.5 McFarland criterion for turbidity. To get a turbidity visually equivalent to the 0.5 McFarland standard, the turbidity of the actively growing broth culture is adjusted using sterile saline or broth. As a result, an approximate suspension was produced [9]. The inoculum tube and the 0.5 McFarland standard can be visually compared to a card with a white background and striking black lines. However, proper lighting is required.

Preparation of Turbidity standard

A $BaSO_4$ turbidity standard, corresponding to a 0.5 McFarland standard, or its optical equivalent (such as a latex particle suspension), should be used to standardize the inoculum density for a susceptibility test. One method for creating a $BaSO_4$ 0.5 McFarland standard is as follows:

To maintain a suspension, a 0.5-ml aliquot of 0.048 mol/L of $BaCl_2$ is introduced to 99.5 ml of 0.18 mol/L of H_2SO_4 (1% v/v) with steady stirring. By measuring the absorbance with a spectrophotometer with a 1-cm light path and a matching cuvette, the right density of the turbidity standard should be confirmed. For the 0.5 McFarland standard, the absorbance at 625 nm should be between 0.008 and 0.10. Transfer the Barium Sulfate suspension in 4 to 6 ml portions into screw-cap tubes the same size as those used for bacterial inoculum growth or dilution. These tubes need to be well sealed and kept at ambient temperature and in darkness. Before each usage, the barium sulfate turbidity standard should be agitated ferociously in a mechanical vortex mixer and checked for a uniformly murky appearance. If big particles are noticed, the standard needs to be changed. Avoid using a vortex mixer when gently inverting latex particle suspensions [10]. The densities of the barium sulfate standards should be checked every month, or they should be replaced.

Preparation of dried filter paper discs

The preparation of 6 mm-diameter discs, which are then put in a Petri dish and sterilized in a hot air oven, uses Whatman filter paper no. 1. Later, the experiments used the sterilized discs.

Antibiotic discs

Antibiotic discs are paper discs with antibiotic impurities that are used in antibiotic testing. The antibacterial and antifungal discs were obtained from Hi-Media along with the antibiotic discs. Amoxycillin (30 mcg), Chloramphenicol (30 mcg), Doxycycline (30 mcg), Gentamicin (10 mcg), Nalidixic acid (30 mcg), and Tetracycline (30 mcg) were the six antibacterial discs utilized, each of which had a distinct potency. Both Nystatin (100 mcg) and Clotrimazole (10 mcg) were utilized as antifungal discs. In general, cartridges comprising paper discs made commercially for susceptibility testing are packaged to provide the proper anhydrous conditions.

Discs were stored as follows:

Until needed, store the containers in a no frost-free freezer at -14°C or below or refrigerate at 8°C or below. One to two hours prior to use, the unopened disc containers should be taken out of the freezer or refrigerator so they can acclimate to room temperature. The quantity of condensation that develops when warm air comes into touch with cold discs is reduced by this process. A cartridge of discs should be stored in a tightly sealed, desiccated container after being taken out of its sealed packaging. When employing a disc-dispensing device, it must have a secure cover and a sufficient amount of desiccant. Before using, the dispenser must warm up to room temperature. When the indicator changes color, replace the desiccant to prevent excessive moisture [11]. The dispensing device housing the discs must always be kept chilled while not in use. You can only use discs that are still inside the manufacturer's expiration window, which is noted on the label. Discs must be thrown away after their expiration date.

Antibiotic Susceptibility Test

A Kirby-Bauer technique susceptibility test for antibiotics was conducted. Antibiotic-impregnated wafers are used in the Kirby-Bauer antibiotic testing (also known as KB testing or disk diffusion antibiotic sensitivity testing) to determine which bacteria are susceptible to which antibiotics. On agar plates, a known quantity of bacteria is cultivated while being exposed to thin wafers that carry the appropriate antibiotics. If the bacteria are susceptible to a specific antibiotic, a zone of clearing (also known as a zone of inhibition) surrounds the wafer where bacteria are unable to grow. This is used to estimate the bacteria's susceptibility to that specific antibiotic combined with the rate of drug diffusion. Smaller minimum inhibitory concentrations (MIC) of antibiotics for certain bacteria generally correspond with larger zones. Using this knowledge, one may select the best antibiotics to treat a certain ailment[12].

A sterile cotton swab should be dipped into the corrected suspension within 15 minutes of modifying the turbidity of the inoculum suspension. Multiple rotations are made with the swab before it is firmly placed against the tube's interior wall above the fluid level. This clears the swab of extra inoculum. A sterile agar surface on a nutritional agar plate is streaked with the swab to inoculate the dried surface. To achieve a uniform dispersion of the inoculum, streaking is performed two more times, turning the plate by about 60 degrees each time. The rim of the agar is swabbed as a last step. For three to five minutes, the lid is kept slightly ajar to allow the inoculum to properly set [13].

Evaluation of anti-bacterial activity of plant extracts

Both the Disc diffusion method and the agar well diffusion method were used for the anti-bacterial assay. 0.5 ml (10^5 CFU/ml) of diluted inoculums of the test organism were applied to plates of nutritional agar. For well diffusion, 8 mm-diameter wells were punched into the agar medium and filled with various plant extract concentrations. At 37°C, the plates were incubated overnight[14]. By assessing the zone of inhibition against the test organism, antibacterial activity was assessed. The diameter of the zone of inhibition is measured, compared to the recommended antibiotics, and the results are then evaluated.

Dilution Susceptibility Testing Methods

The smallest anti-microbial concentration required to prevent or eradicate the bacteria is found using dilution susceptibility testing procedures. Antimicrobials can be diluted in media such as broth or agar to achieve this [15]. Antimicrobials are examined using two fold $\log_2$ serial dilutions.

Evaluation of anti-fungal activity of plant extracts

Both the classic Disc diffusion method and the agar well diffusion method were used for the anti-fungal experiment. On Potato dextrose agar (PDA) plates, 0.5 ml (105 CFU/ml) of diluted inoculums of the test organism were applied. For well diffusion, 8 mm-diameter wells were punched into the agar medium and filled with various plant extract concentrations. At 38°C, the plates were incubated for 48 hours. By assessing the zone of inhibition against the test organism, antifungal activity was assessed. The diameter of the zone of inhibition is measured, compared to the recommended antibiotics, and the results are then evaluated [16].

RESULTS AND DISCUSSION:

Anti-bacterial Activity of *Lallemantia royleana*

The petri dishes were used to create the bacterial culture plates after being filled with nutritional agar and allowed to set for 30 minutes. After the agar had set, bacterial culture (5×10^4 bacteria/petridish) was applied. Following an overnight incubation at 37°C, the zone of inhibition was measured in order to evaluate the anti-bacterial activity of the methanolic plant extracts. Various doses of the methanolic seed extract revealed various-sized zones of inhibition. With the exception of *Klebsiella punemoniae*, which exhibited very little or no antibacterial action, the methanolic extract of *L. royleana* seeds showed antibacterial activity against practically all bacterial strains. When 500 g of the methanolic seed extract from *L. royleana* was utilized, *Proteus vulgaris* and *Bacillus cereus* showed the highest levels of bacterial growth inhibition, with extensive zones of inhibition in each case (Table 1).

The various concentrations of the seed extract revealed various-sized zones of inhibition. With increasing plant extract concentrations, it was seen that the zone of inhibition for *E. coli* was expanding. At a concentration of 100 g, the zone of inhibition was not visible, but when the concentration was increased to 200 g and the zone was 6 mm, it was visible. At increasing concentrations, the zone of inhibition grew, measuring 10 mm at 400 g of methanolic seed extract of *L. royaleana* and 15 mm in diameter at 500 g of methanolic seed extract of *L. royaleana*.

The zone of inhibition was discovered in all doses of methanolic seed extract of *L. royaleana* when *Enterobacter aerogenes* was utilized. When the zone of inhibition was measured, it was

discovered to be 6 mm, 7 mm, 8 mm, 10 mm, and 13 mm at concentrations of 100 g, 200 g, 300 g, and 500 g of methanolic seed extract of *L. royalena*, respectively. Additionally, it demonstrated how the crude extract's content increased along with the diameter of the bacterial growth that was being prevented.

The methanolic seed extract of *L. roayaleana* was tested against *Bacillus cereus* at four different doses, and it was discovered that there was no zone of inhibition at 100 g, while the zone of inhibition was determined to be 10 mm at 200 g. The zone of inhibition was shown to be expanding with an increase in the concentration of methanolic seed extract. *Bacillus subtilis* was also discovered to be sensitive to the methanolic seed extract and had shown inhibitory zones at various concentrations of methanolic seed extracts of *L. royaleana*, with the larger diameter being found to be 12 mm and 15 mm at 300 g and 500 g concentrations of methanolic seed extract of *L. royaleana*, respectively. At 100 g, 200 g, 300 g, and 500 g of seed extract, the zones where bacterial growth was inhibited were determined to be 6 mm, 10 mm, 13 mm, and 15 mm.

The zone of inhibition was also discovered in *Kebsiella puemoniae* at various methanolic seed extract concentrations; however, it was only discovered at a concentration of 500 g of *L. royaleana*'s methanolic seed extract, where the zone of inhibition had an 11 mm diameter. This demonstrates that the bacteria are less sensitive and that there was little to no growth inhibition.

One of the most sensitive species, *Proteus vulgaris*, displayed extensive zones of inhibition when exposed to *L. royaleana* methanolic seed extract at various concentrations. At 500 g of methanolic seed extract, the biggest zone of inhibition in this bacteria was discovered to be 16 mm in diameter. At 100g, 200g, 300g, and 500g concentrations of methanolic seed extract of *L. royaleana*, the zones measured 8 mm, 9 mm, 14 mm, and 16 mm (Table 1).

Psuedomonas aeroginosa growth was observed to be inhibited in zones measuring 6 mm, 8 mm, 10 mm, and 12 mm at various concentrations of 100 g, 200 g, 300 g, and 500 g of methanolic seed extract. Similar to other bacteria, the pattern of the zone of inhibition was seen to increase with higher seed extract concentrations.

While *Salmonella typhinurium* did not exhibit a zone of inhibition at a 100 g concentration of *L. royaleana*'s methanolic seed extract, a zone of inhibition with an 8 mm width was seen at a 200 g concentration of the extract. At seed extract concentrations of 300 g and 500 g, the zones of inhibition were 11 mm and 13 mm, respectively.

With increasing concentrations, *Staphylococcus epidermidis* likewise exhibited growth inhibition against seed extract, with the zones of inhibition expanding. At 100g, 200g, 300g, and 500g of seed extract concentration, the zones of inhibition were found to be 8 mm, 11 mm, 14 mm, and 17 mm in diameter, respectively. All of the bacterial strains utilized in the current investigation had rather broad zones of inhibition, demonstrating that S. epidermidis is extremely susceptible to the methanolic seed extract of *L. royaleana*.

Comparative results were also obtained with *Staphylococcus aureus*, which was likewise discovered to be sensitive and to exhibit a sizable zone of inhibition with seed extract at various concentrations. Zones of inhibition were discovered at concentrations of 100 g, 200 g, 300 g, and 500 g of methanolic seed extract of *L. royaleana*, respectively, as shown in Table 1.

Figure 1 shows the comparisons between all bacterial strains and the antibiotic discs used as a

positive control.

Table 1: Anti-bacterial activity of *Lallemantia royleana* methanolic seed extract atdifferent concentration

S. No.	Bacterial strain	Zone of inhibition (mm)			
		100 (µg)	200 (µg)	300 (µg)	500 (µg)
1	*Escherichia coli*	-	6	10	15
2	*Enterobacter aerogenes*	6	8	10	13
3	*Bacillus cereus*	-	10	12	15
4	*Bacillus subtilis*	6	10	13	15
5	*Klebsiella pneumoniae*	-	-	-	11
6	*Proteus vulgaris*	8	9	14	16
7	*Psuedomonas aeroginosa*	6	8	10	12
8	*Salmonella typhinurium*	-	8	11	13
9	*Staphylococcus epidermidis*	8	11	14	17
10	*Staphylococcus aureus*	10	11	15	16.5

*** All experiments were carried in triplicates and the standard deviation was calculated.**

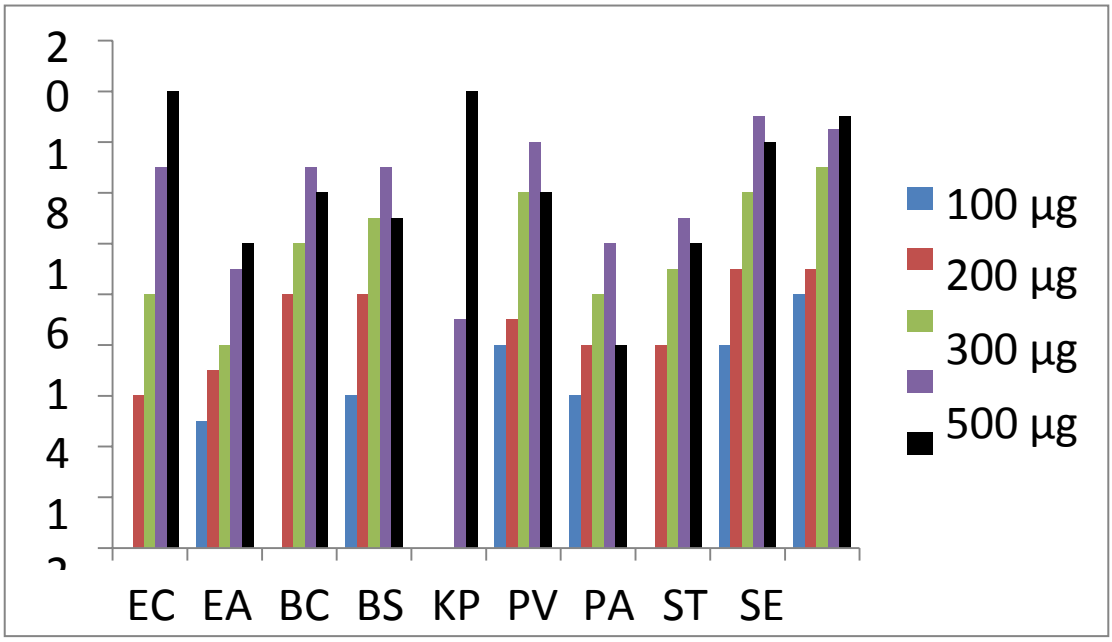

Fig. 1: The comparative results of all the anti-bacterial activity of the methanolicseed extract of *Lallemantia royleana* against the bacterial strains.

Antifungal activity of *Lallemantia royleana*

To perform antifungal activity in the current investigation, a methanolic extract of *Lallemantia royleana* seed was made in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. The seed extract underwent antifungal testing, and when examined in the well diffusion assay, significant zones of inhibition were seen. After 72 hours of incubation at

37^{o}C in an incubator, the zones were evaluated by measuring the width of the zone of inhibition, and the anti-fungal discs acquired from Hi.-Media were used as positive controls. Different concentrations of *Lallemantia royaleana* methanolic seed extract, including 100 g, 200 g, 300 g, and 500 g, were used to evaluate the fungal strains. The crude plant extract was found to suppress the growth of almost all fungus strains.

At a concentration of 200 g, the methanolic seed extract of *Lallemantia royaleana* inhibited the growth of Aspergillus flavus, whereas at a concentration of 100 g, there was no zone of inhibition. However, it was shown that as the concentration of seed extract increased, so did the diameter of the zone of inhibition. At 200 g and 300 g of methanolic seed extract of *Lallemantia royaleana*, the zones of inhibition were measured to be 6 mm and 8 mm, respectively. At 500 g of *Lallemantia royaleana* methanolic seed extract, the zone of inhibition measured 13 mm (Table 2). In the current investigation, it was discovered that Aspergillus niger was resistant to the methanolic extract from *Lallemantia royleana* seeds. At concentrations of 100 g, 200 g, and 300 g of methanolic seed extract of *Lallemantia royaleana*, no zone of inhibition was seen. At 500 g of seed extract, the fungal growth was seen to be suppressed, with a zone of inhibition measuring 4 mm in diameter.

Table 2:Anti-fungal activity of *Lallemantia royleana* methanolic seed extract at different concentration

S. No.	Fungal strains	Zone of inhibition (mm)			
		100 (µg)	**200 (µg)**	**300 (µg)**	**500 (µg)**
1	*Aspergillus flavus*	-	6	8	13
2	*Aspergillus niger*	-	-	-	4
3	*Aspergillus parasiticus*	-	4	8	12
4	*Candida albicans*	6	8	12	14
5	*Saccharomyces cervisiae*	9	10	12	15

***All experiments were carried in triplicates and the standard deviation was calculated.**

Similar to this, it is observed that *Aspergillus parasiticus* growth was inhibited at comparatively higher concentrations of *Lallemantia royaleana*'s methanolic seed extract. At 200 g and 300 g, the zone of inhibition diameter was only 4 mm and 6 mm, which is relatively low, whereas at 500 g, the zone of inhibition obtained was up to 12 mm.

The methanolic extract of *Lallemantia royleana* seed significantly inhibited the growth of the Candida albicans strain. At concentrations of 100 g, 200 g, 300 g, and 500 g of methanolic seed extract of *Lallemantia royaleana*, the zones of inhibition were measured to be 6 mm, 8 mm, 12 mm, and 14 mm, respectively. At 500 g of concentration, the zone of inhibition was comparable to the positive control.

When examined in an agar-well diffusion assay with methanolic seed extract from *Lallemantia royaleana*, Saccharomyces cervisiae was discovered to be the most sensitive of all the fungal strains and to produce bigger zones of inhibition. When compared to the antifungal discs employed as a positive control, the potency of the seed extract was found to be equivalent. When the concentration of the seed extract was increased, the zone of inhibition was found to be approximately 12 mm at 300 g and 15 mm at 500 g of methanolic seed extract of *Lallemantia royaleana*, which was the same as found in the positive control (Table 2). The zone of inhibition was found to be 9 mm at 100 g and 10 mm at 200 g in diameter. Figure 2 displays the findings of the comparison.

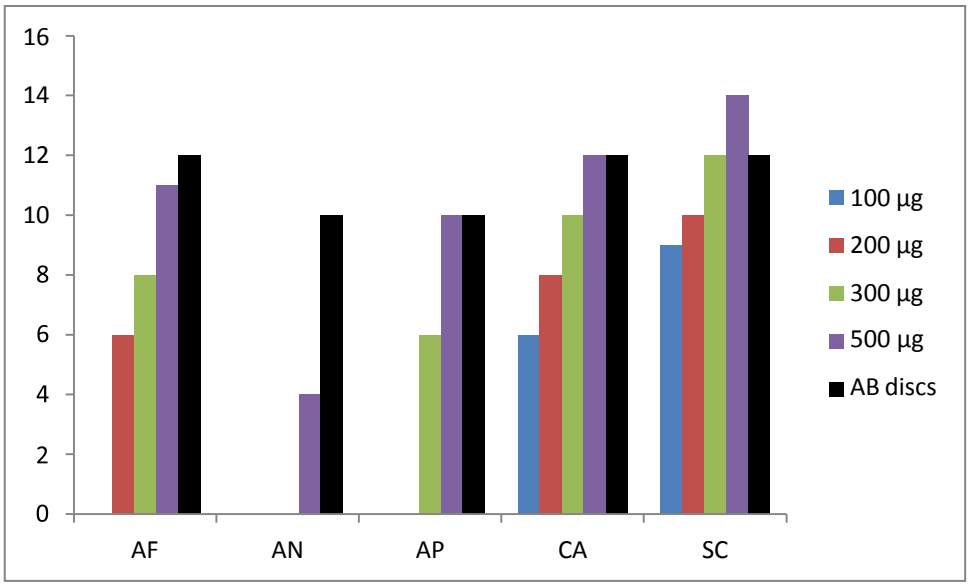

Fig. 2: The comparative results of all the anti-fungal activity of the methanolic seed extract of *Lallemantia royleana* against the fungal strains.

CONCLUSION:

The antibacterial activity of the methanolic extract of *Lallemantia royleana* seeds against various Gram-positive and Gram-negative bacteria was assessed in the current investigation. When compared to conventional antibiotics, the crude extract demonstrated good action against these bacteria. Gram-positive bacteria were more susceptible than other strains to the methanolic extract of *Lallemantia royleana*. The methanolic extracts of *Lallemantia royleana* showed the most promising anti-bacterial activity against Gram-positive *Staphylococcus epidermidis* and *S.aureus*. Also, gram-negative bacteria like *Proteus vulgaris* showed great antibacterial action. At 500 g, the *L. royleana* methanolic seed extract demonstrated the greatest ability to prevent the growth of bacterial cells, with the diameter of the inhibition zone measuring between 11 and 17 mm. Higher concentrations of the methanolic seed extract of *L. royleana*, i.e., 200 g concentrations, inhibited the development of the bacteria *E. coli, B. cereus,* and *Salmonella typhimurium.* Similarly, *Klebsiella pnueumoniae* showed resistance against the crude methanolic seed extract of *L. royleana*, and the inhibition in the growth of bacterial cells was found when the seed extract was used at a concentration of 500 g. This inhibition was not significant as compared to *S. epidermis, S. aureus, P. vulgaris, E. coli,* and *B. subtilis*, where the inhibition of growth in the bacterial cell was found to be comparable to the standard antibiotic discs used. In order to effectively

combat bacterial infections, *L. royleana*'s crude methanolic seed extract can be employed. It was discovered that it significantly inhibited the growth of *Candida albicans* and *Saccharomyces cervisiae*. The highest growth inhibition of fungal cells by the *L. royleana* methanolic seed extract was observed at 500 g, and the diameter of the inhibition zone was reported to range from 4 to 15 mm. When used against *Aspergillus niger*, the methanolic seed extract of *L. royleana* was least sensitive, with the diameter of the zone of inhibition being less than 5 mm. When the crude extract of the seed of *L. royleana* was utilised at a higher concentration, i.e., 200 g, the activities of the methanolic seed extract of *L. royleana* in *A. flavus* and *A. parasiticus* were observed. Comparable to the typical anti-fungal discs employed in the current study, the fungal growth was observed to be inhibited.

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