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Study on synergistic and antimicrobial activity of certain *Bifidobacterium bifidum* strains and plant extracts against clinical isolates

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ABSTRACT

To evaluate the synergistic and antimicrobial activity of certain *Bifidobacterium bifidum* strains and methanolic extracts of *Withania somnifera (Aswagandha)* and *Aloe vera* plant extracts against clinical isolates from veterinary hospitals by using well diffusion method. The results obtained from the combined testing of *Bifidobacterium adolescentis* 236 strain and methanolic plant extracts of *Withania somnifera* showed significantly higher antibacterial/synergistic activity against isolates of *E.coli* and *Staphylococcus aureus* with an average zone of inhibition of 19.0 mm and 20.0 mm respectively, whereas against *Klebsiella pneumoniae* and *Salmonella typhimurium* showed resistance/lower zone of inhibition with an average zone of inhibition 13.0 mm and 14.0 mm, respectively. Further, it was also observed that the *Bifidobacterium bifidum* strains and *Withania somnifera* and *Aloe vera* plants methanolic extracts exhibited excellent antibiotic activity when they were tested alone against the clinical pathogens. However, *Bifidobacterium bifidum* strains 229 and 232 tested in combination with methanolic extracts of *Withania somnifera* and *Aloe vera plant* extracts exhibited significantly low antibacterial activity/zone of inhibition against clinically isolated pathogens. The present study specifies that the *Bifidobacterium adolescentis* 236 strain and methanolic extract of *Withania somnifera* extracts exhibited good antibacterial activity when they are used in combination, such combinations can be further tested as an alternative to antibiotics for effective prophylactic measures and also for treatment of contagious diseases caused by pathogenic microorganisms.

Keywords: *Bifidobacterium bifidum*, Plant extracts, Synergistic activity, Probiotics, LAB.

INTRODUCTION

In recent years, antibiotics have been used excessively for therapeutic purposes, especially for treating diseases caused by clinical isolates found in various samples collected from animals. This has resulted in the development of multiple drug resistance in clinical isolates, leading to infectious diseases in humans [1]. The effectiveness of antimicrobials worldwide is declining due to their rapid accumulation and overuse. The slow identification of new antibiotics has led to the emergence of antimicrobial resistance (AMR) as a major public health concern. This has also led to a rapid increase in the development of newer, more expensive drugs, alongside older, frequently used classes of drugs [2]. Recent trends have shown a decrease in susceptibility of most bacterial strains to multiple antibiotics, posing a major health security challenge and a serious threat in clinical practices [3]. The reduced susceptibility of currently available antibiotics to human pathogens has prompted the search for new alternatives [4]. In order to control diseases in animals and humans, the World Health Organization (WHO) has recommended the development and use of eco-friendly alternative methods.

Currently, there are several alternatives to antibiotics available for the treatment of infectious diseases. According to the World Health Organization [5], medicinal plants are excellent sources of a variety of drugs [6]. In recent times, plant-derived products are being used to treat many illnesses in humans and animals, as a part of the Indian traditional system of medicine. Nowadays, most researchers are primarily focused on developing alternative antibiotics from plant and microbial extracts, essential oils, pure secondary metabolites, and newly synthesized molecules [7]. Bifidobacterial strains are known for their strong antibacterial properties against harmful microorganisms. They achieve this by producing organic acids, peptides, and bacteriocins [8, 9]. Utilizing plant extracts and *Bifidobacterium bifidum* bacterial strains, which have well-established antimicrobial properties, could be highly significant in treating infectious diseases caused by microbial pathogens.

In developing countries, the drugs used for the treatment of diseases are highly expensive, and also these are causing side effects [10]. To address the issue of antibiotic resistance, a multifaceted approach is necessary. This includes educating people about reducing the use of antibiotics and promoting

alternative methods. However, it's important to note that no single alternative method can replace all uses of antibiotics. A variety of specific and general methods are needed to prevent and treat diseases. Therefore, it is essential to search for cost-effective alternatives to antibiotics. In our current study, we evaluated the antagonistic/synergistic activity of methanolic extracts from *Withania somnifera*, *Aloe Vera, and Bifidobacterium bifidum* bacterial strains against clinical isolates.

MATERIALS AND METHODS

Plant material Collection

The plant materials were gathered from various locations in Hyderabad. The collected samples were initially cleaned to remove any dirt particles and then disinfected. Subsequently, the samples were dried in an oven and crushed into a powdered form using a mortar and pestle.

Extracts preparation

The extraction process involved soaking 25 grams of crushed plant material in 100 ml of methanol for 72 hours, followed by filtration. The filtered extracts were then evaporated in a water bath at 40°C to obtain the methanolic extract. The remaining crude extracts were weighed after evaporation and dissolved in dimethyl sulphoxide at a standard ratio of 1 ml dimethyl sulphoxide per milligram of extracts. The extracts were then transferred to centrifuge tubes and stored in a refrigerator at 4 °C. [11].

Chemicals and Biological

The lactic acid bacterial strains *Bifidobacterium bifidum* 229, *Bifidobacterium bifidum*-232, and *Bifidobacterium adolescentis*236 were sourced from the National Dairy Research Institute (NDRI, Karnal) and the National Institute of Nutrition (NIN, Hyderabad). The media and chemicals were obtained from Himedia Laboratories, India and were prepared using standard procedures. The Bifidobacterium bifidum bacterial strains were sub-cultured three times before use in sterile de Mann Mclean Rogosa Sharpe broth (MRS) with 1% inoculums and then incubated at 37 °C for 48 hours.

Procurement and maintenance of clinical pathogens

The procurement and maintenance of clinical pathogens involved isolating various pathogenic microorganisms from different clinical samples collected at the veterinary clinical complex. These microorganisms were then characterized using standard microbiological procedures in the department. The isolated pathogens were sub cultured three times at 37 °C for 24 hours before use. One milliliter of culture was inoculated into the nutrient broth and then incubated at 37 °C for 24 hours. The overnight cultures were subsequently used to evaluate the antibacterial activity of plant extracts and *Bifidobacterium bifidum* bacterial strains using a well diffusion method.

Antibacterial activity by *Bifidobacterium bifidum* bacterial strains

The study aimed to assess the antibacterial activity of *Bifidobacterium bifidum* bacterial strains and various plant extracts using the agar well diffusion method as described by [12]. Overnight cultures of pathogenic microorganisms including *Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium*, and *Staphylococcus aureus* were grown in nutrient broth. A lawn of these pathogenic organisms was prepared by spreading the cell suspension on MRS agar plates using a sterile cotton swab. The plates were then dried and wells of uniform size (7.0 mm diameter) were created using a sterile cork borer. Each well was filled with 50 µl of lactic acid bacterial inoculum from MRS broth and then incubated at 37 °C for 36-48 hours. After incubation, the diameter (in mm) of the inhibition zone around each well was measured.

Antibacterial activity by plant extracts

The antimicrobial activity of methanolic extracts from *Withania somnifera* and Aloe vera was tested against various pathogenic bacteria using the agar well diffusion method [13, 14]. Isolated pathogenic microorganisms (200 µl) were aseptically spread on the surface of nutrient agar plates using cotton swabs. A 7 mm diameter well was aseptically punched on each agar plate using a sterile cork borer. Then, 50 µl of plant extracts were introduced into the wells. The plates were left in a laminar flow for 30 minutes for pre-diffusion of the extracts to occur and were then incubated at 37 °C for 24 hours. The presence of a zone of inhibition was considered an indicator of antimicrobial activity, and its average diameter was measured in millimeters. To evaluate the efficiency of the methodology and compare the potential antibacterial effect of the crude extracts tested, a negative control well with 50 µl of the extracting solvent and a positive control with a standard antibiotic disc were included. The standard antibiotics used in this assay were Tetracycline (T 30 µg, Oxoid), Vancomycin (V 30 µg, Oxoid), and Sulfamethoxazole trimethoprim. (SXT 25 µg, Oxoid).

Antibacterial activity of plant extracts against *Bifidobacterium bifidum* bacterial strains

Bifidobacterium bifidum bacterial strains were cultivated overnight in MRS broth. Then, a layer of these bacterial strains was spread over the surface of MRS agar plates using a sterile cotton swab to create a lawn. After allowing the plates to dry, a sterile cork borer was used to create uniform 7.0 mm diameter wells in the agar. Each well was filled with 50 µl of plant extract and then the plates were incubated at 37°C for 36-48 hours. Following the incubation period, the diameter (in mm) of the inhibition zone around each well was measured.

Antagonistic/synergistic activity of plant extracts, *Bifidobacterium bifidum* bacterial strains combined against clinical isolates

Pathogenic microorganisms including *Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium*, and *Staphylococcus aureus* were cultured overnight in a nutrient broth. A layer of these pathogenic organisms was spread on the surface of nutrient agar plates using a sterile cotton swab to create a lawn of indicator strain. The plates were then allowed to dry, and uniform wells with a diameter of 7.0 mm were cut into the agar using a sterile cork borer. Each well was filled with 25 µl of *Bifidobacterium bifidum* bacterial inoculum from MRS broth, 25 µl of plant extract (1:1), and then incubated at 37°C for 36-48 hours. After incubation, the diameter (in mm) of the inhibition zone around each well was measured.

RESULTS

The study found that the three *Bifidobacterium bifidum* strains demonstrated strong antimicrobial activity against various test pathogens when used individually (Table 1). *Bifidobacterium bifidum*-229 exhibited effective antibacterial activity against the tested pathogens, with a range of inhibition zones measuring from 14mm to 16mm. *Bifidobacterium bifidum* 232 inhibited the growth of clinically isolated pathogens, with inhibition zones ranging from 13mm to 15mm. Among the three strains, *Bifidobacterium adolescentis* 236 showed significant antibacterial properties, inhibiting the growth of all pathogenic microorganisms isolated from different clinical samples, with inhibition zones ranging from 14mm to 18mm.

The study tested methanolic plant extracts against pathogenic microorganisms isolated from clinical samples using a well diffusion method. The extract of *Withanea somnifera* plant showed a higher zone of inhibition against *Staphylococcus aureus* (18mm) (gram-positive) compared to other organisms such as *E. coli* (14mm), *S. typhimurium* (15mm), and *K. pneumoniae* (16mm) (gram-negative). The methanolic extract of *Aloe vera* also exhibited a good zone of inhibition against *E. coli* (15mm), *K. pneumoniae* (14mm), *S.*

typhimurium (13mm) (gram-negative), and *Staphylococcus aureus* (13mm) (gram-positive). (Table 2.)

The present study found that all *Bifidobacterium* lactic acid bacterial strains, when combined with methanolic extracts of *Withania somnifera* and *Aloe vera*, demonstrated resistance to all tested clinical isolates using the well diffusion method. However, *Bifidobacterium adolescentis* 236 showed good synergistic activity against all the clinical isolates when combined with methanolic extracts of *Withania somnifera*. Furthermore, it was observed that *Bifidobacterium adolescentis* 236 exhibited a higher zone of inhibition/synergistic activity against *Staphylococcus aureus* (20mm) (gram-positive) compared to *E. coli* (16mm), *Klebsiella pneumonia* (14mm), and *Salmonella typhimurium* (13mm) (gram-negative). (Table 3).

Table 1: Antimicrobial activity against *Salmonella typhimurium, Klebsiella pneumoniae, Eschrestia coli* and *Staphylococcus aureus*

Name of the organisms	*Bifidobacterium bifidum 229*	*Bifidobacterium bifidum 232*	*Bifidobacterium adolescentis 236*
S. typhimureum	16	13	14
K. pneumonea	14	14	16
E. coli	15	15	18
S. aureus	15	14	15

Table 2: Antibacterial activity of different solvent extracts from *Withanea somnifera* and *Aloe Vera* against clinical isolates (zone of inhibition in mm)

Name of the organisms	*Withanea somnifera* (root)	*Aloe Vera* (leaf)
Salmonella typhimureum	15	12
Klebsialla pneumonea	16	14
Escheria coli	14	16
Staphylococcus aureus	18	10

Table 3: Antagonistic /synergistic activity of *Bifidobacterium bifidum* bacterial strains in combination with methanolic extracts of *Withanea somnifera* and Aloe *vera* against clinical isolates (zone of inhibition in mm) Note: R = Resistant

Name of the organisms	*Withanea* + *B. bifidum 229*	*Withanea*+ *Bifidobacterium adolescentis* 236	*Withanea*+ *Bifidobacterium bifidum-232*	*Aloe Vera*+ *B. bifidum 229*	*Aloe Vera*+ *Bifidobacterium adolescentis* 236	*Aloe Vera*+ *B. bifidum* 2362
E. coli	R	16.0mm	R	R	12mm	R
S. typhimureum	10mm	13mm	R	R	10mm	R
S. aureus	R	20mm	R	R	13mm	10mm
K. pneumonea	R	14mm	12mm	R	12mm	R

DISCUSSION

The *Bifidobacterium bifidum* probiotic strains used in the study have shown good antibacterial activity against the clinical isolates. These findings are in line with those of [15]. According to [16], slight variations in the antagonistic activity of *Bifidobacterium bifidum* strains against tested clinical pathogenic organisms may result from differences in the production of organic acids, peptides, bacteriocins, and other antimicrobial compounds. Recent reports have also suggested that *Bifidobacterium* may boost the immune system. Additionally, *Bifidobacterium bifidum* may inhibit the growth of pathogenic microorganisms by lowering the pH [12]. The antimicrobial effect of organic acids is attributed to the undissociated form of acid, which penetrates the membrane and releases hydrogen ions in the neutral cytoplasm, thereby inhibiting vital cell functions [16].

Methanol extracts of *Withanea somnifera* exhibited more inhibitory activity against tested pathogenic microorganisms. The present study observed that the zone of inhibition against *S. aureus, E. coli, K. pneumonia,* and *S. typhimureum* was 18mm, 14mm, 16mm, and 15mm respectively and these findings were in accordance with the findings of [17], and contradictory with findings of [18] Owais *et al.*, 2005. The differences in the zone of inhibition against different clinical isolates might be due to the difference in bacterial strains, different geographical areas from where the plant was obtained, and the methodology employed [17].

"The extracts of *Aloe vera* demonstrated significant antibacterial activity against all the tested pathogenic microorganisms, except for *Staphylococcus aureus* and *S. typhimureum*, with a low zone of inhibition. These findings are in contrast to those of [19,20]. Additionally, our study found that the plant extracts from *Withanea somnifera* exhibited superior antibacterial activity against clinical isolates when compared to the plant extracts of Aloe vera."

In the present study, it was found that testing *Bifidobacterium bifidum* strains with methanolic extracts of *Withania somnifera* and *Aloe vera* resulted in low or minimal antibacterial activity against most clinical isolates. However, when *Bifidobacterium adolescentis* 236 was tested in combination with methanolic extracts of *Withania somnifera*, significant synergistic activity against clinical isolates was observed, which was significantly higher than testing the components alone for their antibiotic activity.

CONCLUSION

The present study showed that *Bifidobacterium bifidum* strains, methanolic extracts of *Withania somnifera*, and *Aloe vera* have effective antibacterial properties when used alone and in combination. Additionally, the study found that combining *Bifidobacterium adolescentis* 236 with methanolic extracts of *Withania somnifera* resulted in strong synergistic activity against clinical isolates. These combinations could potentially be developed into natural drugs for treating infectious diseases caused by pathogenic microorganisms. Furthermore, this research may help us understand the connections between traditional remedies and modern medications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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