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Antioxidant and Antibacterial Properties of Opuntia spp. Extracts and Their Role in Enhancing Probiotic Enterococcus Durans from Honeybee (Apis Mellifera Meda)

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Abstract

The objective of this study was to investigate the antioxidant and antibacterial activities of Prickly Pear Cactus extracts and their effects on stimulating the probiotic activity of Enterococcus Durans isolated from the honey stomach of honeybees (Apis Mellifera Meda). After extraction of fresh fruit, the phenolic and flavonoid contents and antioxidant activity were determined using Folin Ciocalteu reagent, colorimetric aluminum chloride, and 2-Diphenyl-1-Picrylhydrazyl (DPPH) assays. Lactic Acid Bacteria (LAB) were isolated from the honey stomach using selective media, and their antimicrobial compounds were extracted. The antimicrobial activity of cactus extracts and crude bacteriocins against Gram-positive bacteria was evaluated by the well diffusion method. Finally, the effect of a mixture of cactus extracts and bacteriocins on the probiotic activity of the isolated bacteria was assessed. The average antioxidant activity was 38.6 ± 3.5 mg/ml. All extracts exhibited antimicrobial activity against pathogenic organisms, with Enterococcus Durans showing the highest activity; Bacillus cereus was the most sensitive bacterium. The responsible compounds in the cactus inhibited the growth of pathogenic bacteria and enhanced the activity of LAB to varying degrees.

Keywords: Antioxidant activity, Prickly pear cactus, Enterococcus Durans, Apis Mellifera Meda, Probiotic activity.

1 | Introduction

Lactic Acid Bacteria (LAB), including enterococci, are a group of Gram-positive microorganisms that play important roles in human and animal health due to their probiotic potential [1]. These bacteria are known to

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colonize the gastrointestinal tract and contribute to maintaining microbial balance, enhancing host immunity, and preventing the growth of pathogenic microorganisms. LAB produces a wide variety of bioactive compounds, including organic acids (lactic acid, formic acid), ethanol, hydrogen peroxide, and fatty acids, which inhibit the growth of harmful bacteria and improve food safety. Many LAB strains also produce bacteriocins and bacteriocin-like peptides, which are ribosomally synthesized antimicrobial peptides with narrow- or broad-spectrum activity against Gram-positive bacteria [2]. In addition, some LAB produce other antimicrobial peptides and enzymes that contribute to food preservation and offer potential health benefits.

Cacti (family Cactaceae, subfamily Opuntioideae) comprise more than 200 species worldwide and are highly adaptable to diverse ecological and climatic conditions [3], [4]. Among them, the prickly pear cactus (Opuntia spp.) is native to the Americas and is widely cultivated in arid and semi-arid regions due to its drought resistance and nutritional value [5]. Today, Opuntia species are distributed globally and provide a rich source of vitamins, minerals, dietary fibers, and bioactive compounds [6]. Prickly Pear Fruit (PPF) contains 20–40 mg/100 g fresh weight of ascorbic acid, titratable acidity of 0.03%–0.12%, and pH values ranging from 5.0 to 6.6. The total soluble solids content of PPF (12%–17%) is higher than that of many common fruits, such as prunes, apricots, and peaches [7]–[9].

PPFs are a significant source of natural antioxidants, including vitamin C, betalains, polyphenols, flavonoids, and taurine, which help neutralize free radicals and reduce oxidative stress in biological systems. Additionally, the fruits contain pectin and mucilaginous polysaccharides, which are useful as natural thickeners and stabilizers in food processing [10]–[14]. Due to their diverse bioactive compounds and high ecological adaptability, Opuntia species have attracted attention for their potential applications in functional foods, nutraceuticals, and natural preservatives.

Recent research has focused on natural phytochemicals from fruits, vegetables, seeds, and herbs as potential antioxidants and functional food ingredients due to their multiple biological activities, including antimicrobial, anti-inflammatory, and immunomodulatory effects [15]–[17]. Combining natural plant extracts with probiotic bacteria has emerged as a promising approach to enhance the growth, activity, and efficacy of probiotics, leading to improved gut health and resistance against pathogens.

Therefore, the present study aimed to evaluate the antioxidant activity and antimicrobial efficacy of methanolic, ethanolic, and aqueous extracts of Opuntia fruits against selected pathogenic bacteria, including Staphylococcus aureus, Methicillin-Resistant Staphylococcus Aureus (MRSA), Bacillus subtilis, and B. cereus, in vitro. Moreover, the study investigated the potential of these extracts to stimulate the growth and probiotic activity of Enterococcus Durans isolated from the honey stomachs of honeybees (Apis Mellifera Meda), providing insight into their potential application in functional foods and probiotic formulations.

2 | Materials and Methods

2.1 | Total Flavonoid Determination

The total flavonoid content of PPF extracts was determined using the colorimetric aluminum chloride method [18]. Briefly, 0.5 ml of a 1:10 g/ml PPF extract in methanol was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. The reaction mixture was allowed to stand at room temperature in the dark for 30 min. Absorbance was measured at 415 nm using a UV-Vis spectrophotometer (2100, Unico, USA). Total flavonoid content was expressed as mg quercetin equivalent per gram of dry mass (mg QE/g).

2.2 | Total Phenol Determination

Total phenolic content was measured using the Folin-Ciocalteu method [19]. A 0.5 ml aliquot of PPF extract (1:10 g/ml) or gallic acid standard was mixed with 5 ml of Folin–Ciocalteu reagent (diluted 1:10 with distilled water) and 4 ml of 1 M aqueous Na_2CO_3. The mixture was allowed to react for 15 min at room temperature,

and absorbance was recorded at 765 nm. Total phenolic content was expressed as mg gallic acid equivalent per gram of dry mass (mg GAE/g). All measurements were performed in triplicate.

2.3 | Determination of the Scavenging Effect on 2-Diphenyl-1-Picrylhydrazyl Radicals

The free radical scavenging activity of the Opuntia fruit methanolic extract was determined using the method [20]. The assay is based on the reduction of the stable 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical, which results in a decrease in absorbance as the purple color of the DPPH solution fades. Different concentrations of the PPF extract (0.1–1.0 mg/ml) were prepared in methanol. An aliquot of 0.1 mL of each extract concentration was mixed with 3.9 mL of a 0.1 mM DPPH solution in methanol. The mixture was incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer.

The percentage of radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \left(\frac{\text{A control} - \text{Asample}}{\text{Acontrol}}\right) \times 100,$$

where Acontrol is the absorbance of the DPPH solution without extract, and Asample is the absorbance in the presence of the extract. All experiments were performed in triplicate, and results were expressed as mean ± Standard Deviation (SD).

2.4 | Extraction of Plant Material and Antibacterial Activity Assay

Fresh PPFs (30 g) were washed with distilled water, peeled, chopped, and homogenized. The homogenate was extracted by maceration with 300 ml of solvent (1:10 w/v) using methanol, ethanol, or distilled water. Extractions were carried out on a rotary shaker at room temperature (ca. 22–25 °C) for 24 h in the dark. The extracts were filtered through Whatman No. 1 filter paper and centrifuged at 4000 rpm for 20 min to remove particulate matter. The supernatants were collected and concentrated under reduced pressure using a rotary evaporator at 40 °C (for organic extracts) and lyophilized (for aqueous extracts) or dried to constant weight. The dried extracts were stored at −20 °C until use. Prior to antibacterial testing, the extracts were reconstituted in their respective solvents and sterilized by passage through a 0.22 μm syringe filter.

Antibacterial activity was evaluated by the agar well diffusion assay following CLSI/Bauer-Kirby guidelines with minor modifications. Briefly, test strains (MRSA, PTCC 25923), Bacillus subtilis (PTCC 1023), Bacillus cereus (PTCC 1247), and Staphylococcus aureus (PTCC 1431) were obtained from the microbial culture collection of the scientific and industrial research organization of Iran. Bacterial suspensions were prepared in sterile saline and adjusted to a 0.5 McFarland standard (~1.5 × 10^8 CFU/ml). Mueller-Hinton agar plates were inoculated by evenly spreading the bacterial suspension over the surface with a swab. Wells of 6 mm diameter were punched into the agar, and 50 μl of each extract concentration (e.g., 10, 25, 50, and 100 mg/mL) was dispensed into the wells. A solvent control (methanol/ethanol/water) and an antibiotic positive control (e.g., vancomycin 30 μg) were included on each plate. Plates were incubated at 37 °C for 18–24 h [21]. The diameter of the inhibition zones was measured in mm, and results were reported as the mean ± SD of three independent experiments (each performed in triplicate).

All experiments involving MRSA were conducted in a BSL-2 laboratory in accordance with institutional biosafety guidelines. Data were analyzed using SPSS software (version X). One-way ANOVA was performed to compare means, followed by Duncan's multiple range test for pairwise comparisons. Differences were considered statistically significant at $p < 0.05$.

2.5 | Isolation and Identification of Lactic Acid Bacteria

LAB were isolated from the honey stomachs (crop) of worker honeybees on selective media. Samples were inoculated on de Man, Rogosa, and Sharpe (MRS) agar and M17 agar (Merck, Darmstadt, Germany) and

incubated at 37 °C for 72–96 h under anaerobic conditions using CO_2-generating gas pack [22]. Preliminary identification of isolates was based on morphological and biochemical characteristics, including Gram staining, catalase activity, motility, and carbohydrate fermentation profiles. Colonies that were Gram-positive, catalase-negative, non-spore-forming, and non-motile were considered potential LAB strains. Further identification was performed using 16S rRNA gene amplification and sequencing. The obtained sequences were analyzed using the BLAST program against the NCBI GenBank database to confirm the taxonomic identity of the isolates.

2.6 | Extraction and Partial Purification of Bacteriocin

The extraction and purification were performed in accordance with the methods given by [23], [24] with some modifications. The Enterococcus Durans isolates were propagated in the MRS broth at 37 °C for 48 h under microaerophilic conditions. After incubation, the cultures were centrifuged at 8000 rpm for 20 min at 4 °C to remove the bacterial cells. The supernatant was carefully collected and filtered through Whatman No. 1 filter paper to obtain the crude bacteriocin extract.

The crude supernatant was first evaluated for antimicrobial activity using the agar well diffusion assay against selected indicator strains. For partial purification, the bacteriocin was precipitated by ammonium sulfate saturation. The crude extract was brought to 70% saturation by the gradual addition of solid ammonium sulfate at pH 4.0 with continuous stirring at 4 °C for 24 h. The mixture was then centrifuged at 8000 rpm for 30 min, and the resulting protein pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.0). The partially purified bacteriocin was stored at −20 °C until further use [25].

2.7 | Assay of Isolates for Antimicrobial Activity

The purified compounds from LAB were subjected to their antimicrobial activity against common pathogens involved in food spoilage. The organisms selected include Bacillus subtilis, Staphylococcus aureus, MRSA, and Bacillus cereus. The antimicrobial activity against these organisms was determined by the well diffusion agar method under aerobic conditions. The purified samples were added to the wells at 25 µl, 50 µl, 75 µl, and 100 µl concentrations. The plates were then incubated at 37°C for 24 hours. After 24 hours, the activity was measured by the presence of an inhibitory zone.

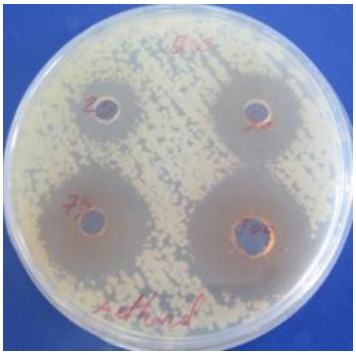
2.8 | Evaluation of the Effect of Opuntia Extracts for Stimulating the Probiotic Activity of Enterococcus Durans

The stimulatory effect of Opuntia fruit extracts (ethanolic, methanolic, and aqueous) on the probiotic activity of Enterococcus Durans was evaluated using the agar well diffusion method. Equal volumes (1:1, v/v) of each Opuntia extract and a freshly prepared suspension of E. durans were mixed. The mixture was added into wells of 6 mm diameter in nutrient agar plates containing the indicator pathogens (Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, and MRSA). Different volumes of the mixture (25 µl, 50 µl, 75 µl, and 100 µl) were applied into separate wells. Plates were incubated at 37 °C for 24 h under aerobic conditions. The probiotic stimulation and antimicrobial synergistic activity were assessed by measuring the inhibition zone diameters (in mm) formed around each well. Each test was performed in triplicate, and the results were expressed as mean ± SD.

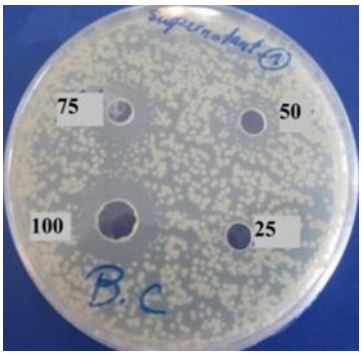
3 | Results

The results obtained in this study demonstrated that the total flavonoid content of the Opuntia fruit extract, expressed as quercetin equivalent, was 128.4 ± 0.9 $mg.g^{-1}$. The total phenolic content, determined using the Folin Ciocalteu reagent and expressed as gallic acid equivalent, was 641.2 ± 9.6 $mg.g^{-1}$. Antioxidant capacity was assessed using the DPPH radical scavenging assay, a widely used method for evaluating the free radical-scavenging properties of natural compounds. The average antioxidant activity of the Opuntia fruit methanolic extract was 38.6 ± 3.5 $mg.ml^{-1}$, indicating a moderate free radical scavenging potential.

Statistical analysis was performed using one-way ANOVA in SPSS. Mean comparisons were conducted using Duncan's multiple range test at a significance level of $P < 0.05$. The analysis of variance revealed significant differences ($p < 0.05$) among the extracts in phenolic, flavonoid, and antioxidant content. All Opuntia extracts exhibited antimicrobial activity against the tested pathogenic bacteria. Among the evaluated solvents, the ethanolic extract demonstrated the strongest antibacterial effect, particularly against MRSA, Bacillus cereus, and Bacillus subtilis, followed by the methanolic extract, which showed high inhibition against B. cereus and B. subtilis (*Fig. 1a*).



a.



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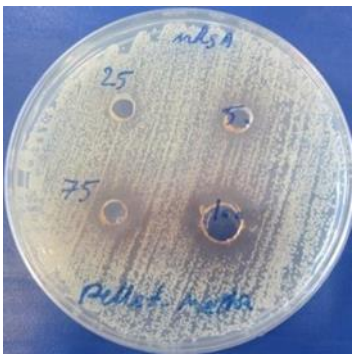

c.

Fig. 1. The inhibition zone against bacteria, a. The Opuntia methanol extract effect against Bacillus subtilis, b. Initial supernatant effect of Enteroccus Durans against Bacillus cereus, and c. The effect of Enterococcus Durans antimicrobial compound, along with extracts, against methicillin-resistant staphylococcus aureus.

The aqueous extract exhibited comparatively lower antimicrobial activity, especially at lower concentrations, with the highest inhibition observed against B. cereus (27.6 mm) and the lowest against MRSA (13.6 mm) (*Fig. 2*).

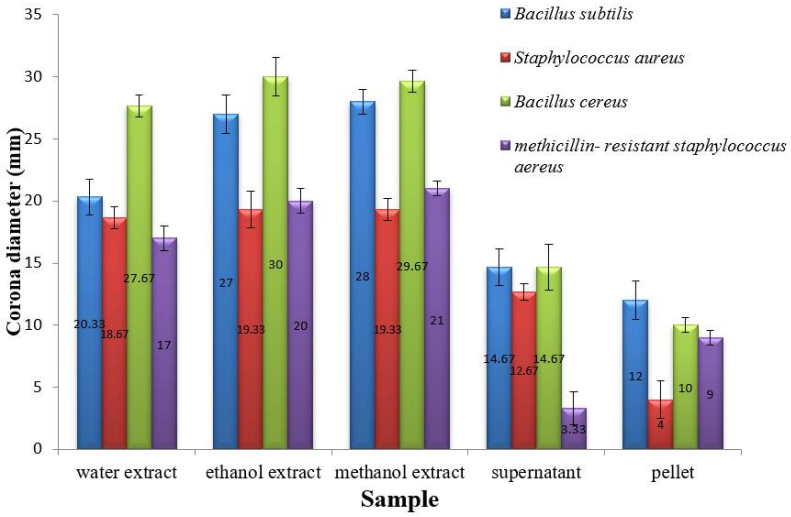

Fig. 2. The Opuntia extract effect against bacteria, Bacillus cereus, methicillin-resistant Staphylococcus aureus, Bacillus subtilis, and Staphylococcus aereus.

From the honey stomach samples, three LAB isolates with coccoid morphology were selected based on biochemical characterization. All isolates were Gram-positive, catalase-negative, H_2S-negative, indole-negative, and non-motile. 16S rRNA gene sequencing and BLAST analysis identified isolate 2016 as Enterococcus Durans strain M.D.E MRS4-10, isolate 2012 as Enterococcus faecium strain PON94 (99% similarity), and isolate 2017 as E. faecium strain HB2003 (100% similarity).

All Enterococcus isolates exhibited inhibitory effects against the tested pathogens, with isolate 2016 (E. Durans) showing the highest antibacterial activity, with an average inhibition zone of 13.2 mm (*Table 1*).

Table 1. Activity of partially purified bacteriocin against pathogenic strains.

Pathogen LAB	Bacillus Cereus	Bacillus Subtilis	Staphylococcus Aereus	Methicillin-Resistant Staphylococcus Aereus
(2016 strain) Enterococcus Durans	15.3 mm	15 mm	12.5 mm	10 mm
(2012 strain) Enterococcus faecium strain PON94	12.2 mm	10.66 mm	11 mm	12 mm
(2017 strain) Enterococcus faecium strain HB2003	12 mm	9.5 mm	10 mm	11.33 mm

As shown in *Fig. 1.b*, the antimicrobial compounds produced by E. Durans were most effective against B. subtilis and B. cereus. Among the pathogens, MRSA was the most sensitive, while B. Cereus was the most resistant to E. Durans.

The combination of crude bacteriocin from E. Durans with Opuntia extracts enhanced the antibacterial activity, particularly against B. subtilis and B. cereus (*Fig. 3*). However, the aqueous extract of Opuntia did not significantly stimulate the antimicrobial activity of the isolated Enterococcus strain.

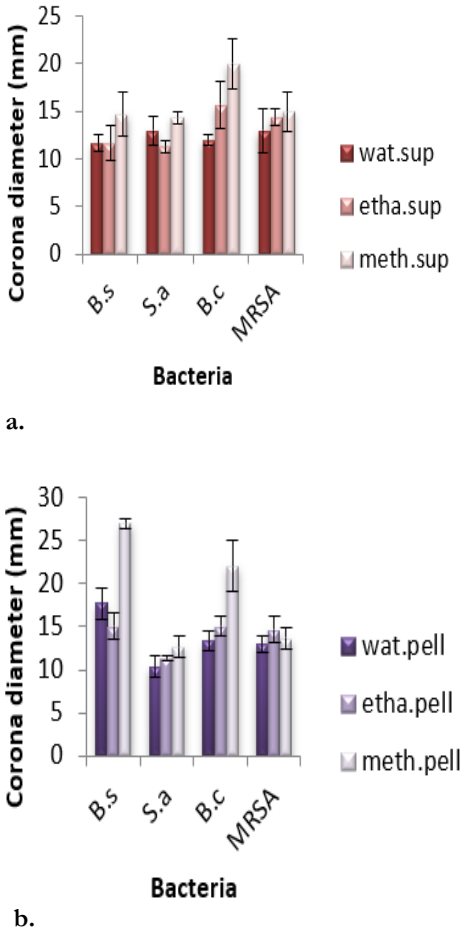

Fig. 3. a. Effect of the supernatant, and b. partial-purified bacteriocin with Opuntia extracts against pathogenic bacteria.

4 | Discusion

The antimicrobial activity or inhibition of pathogenic strains observed in this study may be attributed to the production of several metabolic compounds such as organic acids (e.g., lactic and acetic acids), hydrogen peroxide, diacetyl, and bacteriocins [26], [27]. It is therefore plausible that this antimicrobial effect is further enhanced by the bioactive constituents present in Opuntia extracts. These effects are likely associated with the presence of plant-derived phenolic compounds, ascorbic acid, and betalains. Although the precise antimicrobial mechanisms of phenolic compounds have not yet been fully elucidated, they are generally recognized for their broad antibacterial properties [28].

Previous studies have reported the antimicrobial potential of cactus (Opuntia spp.) against both Gram-positive and Gram-negative pathogenic bacteria, and the findings of the present work are consistent with these reports [29], [30]. Specifically, the ethanolic extract exhibited the strongest antibacterial activity, producing an inhibition zone of 30 mm against Bacillus cereus at a concentration of 100 µl, whereas the methanolic extract showed a comparable effect, with an inhibition zone of 29.6 mm at the same concentration.

The partially purified bacteriocin, in combination with the methanolic Opuntia extract, exhibited the highest antibacterial effect against Bacillus subtilis, while the crude bacteriocin from Enterococcus Durans, combined with the ethanolic extract, displayed the lowest inhibitory activity against MRSA (10 mm inhibition zone at 100 µl). The use of the salt-saturation method for partial purification may have removed some antimicrobial components, which could explain the reduced or unchanged activity in some treatments. Nevertheless, the

antimicrobial activity notably increased after partial purification with ammonium sulfate, particularly in the methanolic and ethanolic extracts.

Overall, all Opuntia extracts exhibited a synergistic effect on the antimicrobial activity of Enterococcus Durans. Exposure of both crude and partially purified bacteriocins to Opuntia extracts significantly enhanced the antibacterial efficacy of Enterococcus Durans, suggesting that the cactus fruit contains bioactive compounds capable of stimulating bacteriocin production or activity. These findings indicate that Opuntia fruit possesses strong medicinal potential and can be utilized to promote health, enhance probiotic antimicrobial activity, and possibly support natural antibiotic production.

The results confirmed that Opuntia extracts were effective against Bacillus subtilis, Staphylococcus aureus, Bacillus cereus, and MRSA. This broad-spectrum activity may be attributed to the presence of various phytochemicals with potent antimicrobial properties in the cactus extracts.

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