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Original article

Antimicrobial activity of polyphenolic compounds from *Spirulina* against food-borne bacterial pathogens

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ABSTRACT

Food-borne drug-resistant bacteria have adverse impacts on both food manufacturers and consumers. Disillusionment with the efficacy of current preservatives and antibiotics for controlling food-borne pathogens, especially drug-resistant bacteria, has led to a search for safer alternatives from natural sources. *Spirulina* have been recognized as a food supplement, natural colorant, and enriched source of bioactive secondary metabolites. The main objectives of this study were to isolate polyphenolic compounds from *Spirulina* and analyze their antibacterial potential against drug-resistant food-borne bacterial pathogens. We found that fraction B of methanol extract contained a high quantity of polyphenols exhibiting broad spectrum antimicrobial effects against drug-resistant food-borne bacterial pathogens. Potential secondary metabolites, such as benzophenone, dihydro-methyl-phenylacridine, carbanilic acid, dinitrobenzoate, propanediamine, isoquinoline, piperidin, oxazolidin, and pyrrolidine, were identified by gas chromatography and mass spectrophotometry (GCMS). These metabolites are active against both gram-positive and gram-negative pathogens. Our work suggests that phenolic compounds from *Spirulina* provide a natural and sustainable source of food preservatives for future use.

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1. Introduction

Food safety is a global issue with a significant impact on human health. Annually, over 2 billion people are affected by food-related illnesses that increase food-borne outbreaks globally (Uyttendaele et al., 2016). To regulate food-borne illnesses, food and health authorities must control pathogenic microorganisms. Food spoilage and pathogenic microbes are controlled by chemical preservatives or antibiotics widely used by the food industry (Enzo et al., 2007). Globally, regulatory restrictions have greatly increased due to negative impacts of synthetic preservatives or antibiotics. On the other hand, due to the limited effective life span of antimicrobials, emerging resistant microbes have increased the pressure to develop alternative compounds to combat food-spoiling pathogenic microbes (Silva and Lidon, 2016). Organic food preservatives from natural sources, consisting of secondary metabolites known

as polyphenols, have strong antimicrobial and antioxidant properties (Kuan et al., 2019).

The practice of using natural secondary metabolites from plants in food preservation has gained attention due to the incipient threat of bacterial pathogens that are more permissive to food processing and preservation methods, as well as resistant to antibiotics. The negative health impacts of chemical synthetic preservatives have prompted the food industry to search for alternative natural preservatives from food-grade sources to enhance safety and food quality (Pedan et al., 2019). *Spirulina* is a free-floating filamentous cyanobacteria growing naturally in warm aquatic environments. Around the world, it is largely cultivated in its natural habitat, ponds and lakes. Traditionally, *Spirulina* was used in Asian, African, and Mexican communities for direct consumption from the beginning of the sixteenth century (De Morais et al., 2015). Consumption of *Spirulina* as a functional food has been recently increasing in western countries. *Spirulina* is an excellent source of bioactive secondary metabolites apart from carbohydrates, protein vitamins, minerals, essential fatty acids, or dietary fiber, and so it is widely used as nutraceutical supplement in the food industry (Karolina et al., 2018). Bioactive secondary metabolites from *Spirulina* include polyphenols, phenolic acids, tocopherols, and linolenic acid. These metabolites exhibit anticancer, anti-inflammatory, antioxidant, antidiabetic, neuroprotective, and hepatoprotective activities (Mazur-Marzec et al., 2015).

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Spirulina has been listed by the US Food and Drug Administration under the category “generally recognized as safe” (GRAS), and it was also endorsed by the Intergovernmental Institution Against Malnutrition (IIMSAM) (Mathur, 2018). The biomass of *Spirulina* is a rich source of phenolic compounds involved in redox mechanisms, reducing reactions, and oxygen quenching. However, the ability of these phenolic compounds to combat food pathogens has not been extensively studied for the purpose of food preservation. Therefore, the present study aimed to analyze the antimicrobial activity of *S. platensis* fractions against food-borne pathogenic isolates to find alternative strategies for food preservation.

2. Materials and methods

2.1. Chemicals and food-borne bacterial pathogens

All chemicals were of analytical grade: methanol, acetic acid, osmium tetroxide, and glutaraldehyde (Sigma Aldrich, St. Louis, Missouri, USA), uranyl acetate (Agar Scientific, UK), and Mueller Hinton Broth (Hi-media, Mumbai, India) were purchased. Drug-resistant (DR) food-borne bacterial pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Streptococcus pyogenes* were isolated from our previous research (Krishnamoorthy et al., 2018). The ATCC strains *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) were used as control bacterial strains.

2.2. Collection of algal biomass

Food-grade *S. platensis* powder was prepared using *S. platensis* cultured in-house. The original microalgae *S. platensis* were obtained from the culture collection of algae at the University of Texas at Austin, USA (UTEX NO. LB2340). This strain was grown in the laboratory according to methods described before (Chang et al., 2013), using Zarrouk medium (Zarrouk, 1966). Outdoor cultures were carried out according to a separate methodology described before (Al-Homaidan, 2002). Harvesting was done by filtration through nylon filters (150–200 mesh). After collection, *S. platensis* was rinsed with deionized water and dried overnight in an oven at 80 °C. The dried biomass was ground well and sieved using a standard metal mesh (100 mm pores), then stored in a desiccator to avoid moisture absorption.

2.3. Extraction of bioactive compounds

Bioactive compounds from *S. platensis* were extracted via the extraction protocol described below. Freeze-dried *Spirulina* biomass was homogenized with methanol–water–acetic acid (30:69:1 v/v/v) and kept in a water bath at 70 °C for 4 h with constant shaking (Ludmila Machu et al., 2015), then treated with 80% methanol (70 °C for 1 h), 70% acetone (30 °C for 1 h) and 100% methanol at 20 °C for 4 h with constant shaking. The extract was centrifuged at 2800 xg for 10 min at room temperature, supernatant was filtered with Whatman filter paper, and solvent was evaporated using a rotary evaporator and condensed further. Part of this extract (8 g/column) was subjected to column chromatography (silica gel) and eluted with n-hexane (n-Hex), chloroform (CHCl₃), ethyl acetate (EtOAc), and methanol (MeOH) in increasing polarity to give 13 fractions of 200 mL each; these were combined based on thin layer chromatography (TLC) into two fractions: fraction A (n-Hex: CHCl₃ (100:0, 4:1, 3:2, 1:4, 0:100)) and fraction B (CHCl₃: EtOAc (4:1, 3:2, 1:4, 0:100) and EtOAc: MeOH (4:1, 3:2, 1:4, 0:100)). Both fractions were subjected to total polyphenols estimation (Aryal et al., 2019) and antimicrobial activity analysis.

2.4. Minimal inhibitory (MIC) and minimal bactericidal concentration (MBC)

The MIC and MBC were determined by the microdilution method as described by the Clinical and Laboratory Standards Institute. Initially, fraction (A and B) stock solution concentrations of 100 mg mL⁻¹ in Muller Hinton Broth (MHB) were used. The stock concentration was then diluted to yield final test concentrations ranging from 0.12 to 250 µg mL⁻¹. Finally, for each strain, 10 µl of adjusted inoculum (3 × 10³ CFU mL⁻¹) was added to each respective well to produce a final working volume of 200 µl/well. The 96-microwell plates were incubated at 37 °C for 24 h. The MIC was determined as the concentration that showed no visible growth, while MBC was determined by sub-culturing 20 µl from each well and demonstrating no growth on MH agar. MHB broth, bacterial inoculum, and 1% DMSO were used as negative control, and ciprofloxacin was used as a positive control.

2.5. GC–MS analysis

The fraction demonstrating antimicrobial activity was subjected to GCMS analysis. The samples were injected into GC–MS system (Agilent 6890 N, Santa Clara, CA, USA) fitted with a column (CP-Sil 5 CB column; HP-5 ms; film thickness 0.25 µm; internal diameter 0.25 mm) and coupled to a mass-selective detector (MS 5973 N). Ultra-purity helium (99.9%) was used as the carrier gas with flow rate 1.0 mL/min. The sample was injected with split ratio 1.0, column temperature 50 °C, injection temperature 250 °C, total flow rate 6.4 mL/min, column flow rate 1.69 mL/min, mass spectra detected ACQ mode at scan speed 2000, start m/z 40.0, and end m/z 1000.0.

2.6. Electron microscopic analysis

2.6.1. Scanning electron microscopy (SEM) analysis

The morphological changes in gram-positive and gram-negative treated and untreated cells were assessed by JSM–7600 field emission SEM (Joel, Japan). Prior to observation, the cells were buffered with (cacodylate buffer pH 7.2) 3% glutaraldehyde for 30 min, then rinsed twice with buffer and treated with Zetterquist's osmium tetroxide for 30 min. The fixed samples were dehydrated with an ascending series of ethanol for 10 min at each concentration. All the specimens were dried under vacuum and sputter-coated with a palladium gold thin film. The specimens were viewed with FE SEM in high-vacuum mode at 5 kv.

2.6.2. Transmission electron microscopy (TEM) analysis

The treated and untreated (control) bacterial cell suspension were centrifuged at 2000 rpm for 10 min. The cell pellet was transferred in a sterile Eppendorf tube and fixed with 2.5% glutaraldehyde and 0.1 M sucrose in a 0.1 M phosphate buffer at pH 7.4, and then post-fixed with 1% osmium tetroxide for 12 h. Cells were rinsed twice in the buffer, followed by centrifugation for buffer removal. Cells were rinsed with ultra-purified water and stained with 1% uranyl acetate for 2 h. Then, the specimens were dehydrated using an ascending series of ethanol (50%, 70%, 95%, and 100% ethanol) and treated with propylene oxide for 20 min. The dried cell blocks were infiltrated by a mixture of 1:1 (v/v) propylene oxide and eponate 12 resin for 1 h at 37 °C, then by a mixture of 1:2 (v/v) polypropylene/resin overnight at room temperature on a rotator.

Finally, cells were infiltrated in 2 changes of 100% eponate 12 resin over 2 to 6 h at 37 °C. Following infiltration, plastic capsules were used to embed the tissue blocks, which were then polymerized for 12 h at 60 °C. Ultra-thin sections (70 nm) were prepared using an ultramicrotome (Leica EM UC6, Leica Microsystem GmbH,

Vienna, Austria). Sections were stained in 2% aqueous uranyl acetate for 20 min, washed with distilled water, stained in Reynold's lead citrate for 15 min, and washed again with distilled water. After air drying, TEM images of the cells were obtained using a JEOL transmission electron microscope.

3. Results and discussion

3.1. Total phenolic content

Phenolic compounds are large molecules consisting of an aromatic chain with a hydroxyl group, and they are considered a major source of anti-microbial activity (Barnes et al., 2013). The phenolic content of *Spirulina* is influenced by several factors, such as geographical origin, as well as environmental, physiological, and nutritional variations (Marinho Soriano et al., 2006). The condensed MeOH extract of food-grade *Spirulina* was subjected to silica gel column chromatography, and 13 fractions were collected. Based on TLC as mentioned above, the collected samples were divided into fractions A and B. The highest amount of phenolic compounds (44.48 ± 1.71 mg GAE/g) was found in fraction B, with less (7.80 ± 0.15 mg GAE/g) in fraction A. It is generally understood that the quantity of phenolic content is influenced by the type of solvents and conditions during extractions (Robards, 2003).

3.2. Antimicrobial activity

The column fractions (A and B) were analyzed against a wide range of food-borne pathogenic isolates, and results are presented in Table 1. Many researchers consider an MIC value of < 0.1 mg mL⁻¹ to indicate significant antimicrobial potential in bioactive secondary metabolites (Kueete, 2010; Ríos and Recio, 2005). Based on this criterion, fraction B had significant antimicrobial activity against both gram-positive and gram-negative samples of eight tested bacterial strains, with MIC value between 7.8 and 62.5 µg mL⁻¹. However, DR gram-negative strains are more sensitive (MIC 31.2 and MBC 62.5 µg mL⁻¹) DR gram-positive bacterial strains (MIC 62.5 and MBC 250 µg mL⁻¹). At this concentration, fraction A did not show any activity against the tested strains.

Recent reports suggest that the difference between MIC and MBC could be related to bioactive compounds altering the permeability of cell membranes, having the ability to interact with intracellular molecules such as mRNA and DNA, and inhibiting cell functions (Alves et al., 2013), which is in agreement with our reports. Moreover, the control strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213), which are non-drug-resistant, showed MIC (7.8 µg mL⁻¹) and MBC (15.6 µg mL⁻¹) at the least concentrations tested. The obtained phenolic compounds had higher activity against a majority of gram-negative pathogens at lower concentrations.

Furthermore, other studies report that the fraction B exhibits anti-microbial activity at concentrations > 100 µg mL⁻¹ against

different strains of DR gram-negative bacilli such as *E. coli*, *P. mirabilis*, *A. baumannii*, *P. aeruginosa*, and *K. pneumonia* (Orhan et al., 2010; Kueete et al., 2009; Alves et al., 2013). From the obtained data, it is evident that fraction B had more effective activity against food-borne bacterial pathogens than commercial antibiotics commonly used in the food industry. This could be explained by the fact that the high level of phenolic compounds in fraction B interacts in a synergistic manner at lower concentrations.

3.3. GC-MS analysis

In order to identify the phenolic compounds present in fraction B, GC-MS analysis was used. The GC-MS chromatogram (Fig. 1) shows the presence of nine bioactive compounds (Table 2) identified according to mass fragmentation patterns of compounds corresponding to NIST library entries. These compounds mainly contained a phenol-carbonyl-phenol skeleton. Among the nine compounds, benzophenone chloroacetyl hydrazine (diphenyl-methylene) (28.43%), propanediamine 2-butyl (18.09%), dihydro-methyl-phenylacridine (16.51%), isoquinoline (8.21%), piperidin-1-dihydrodibenzo (7.17%), and carbanilic acid, methyl-2-chloroethyl ester (6.57%) were found to be major compounds. Pyrrolidine (5.41%), oxazolidin (4.96%), and dinitrobenzoate (4.95%) were minor compounds present in fraction B.

Benzophenone is a natural polyphenol that has been reported as a bioactive secondary metabolite exhibiting significant antimicrobial and anti-fungal activities against a wide range of clinical pathogens (Shi-Biao Wu et al., 2014; Wang et al., 2010). In the last 15 years, benzophenone has been found in plants and fungi (Cuesta-Rubio et al., 2005; Kumar et al., 2013). In the current study, we have reported for the first time the presence of benzophenone in *Spirulina*. We assume that this compound could also be used at lower concentrations than the commercial antibiotic ciprofloxacin. against drug-resistant bacterial pathogens. Moreover, dinitrobenzoate, propanediamine, isoquinoline, piperidin, oxazolidin, and pyrrolidine act as bio-preservatives that maintain the organoleptic properties and safety of perishable food from microbial spoilage or the oxidation process (Callemien and Collin, 2009; Pedan et al., 2019). Synergistic effects between phenolic compounds in Fraction B may enhance their bioactive potential compared to the pure single compounds alone. Fraction B exhibits broad-spectrum activity against resistant bacterial pathogens and can serve as alternative to synthetic food preservatives.

3.4. Morphological and anatomical changes to the bacterial cell induced by phenolic compounds

The morphological changes caused by polyphenols in fraction B were examined in *E. coli* and *S. aureus* (gram-negative and gram-positive bacteria) using SEM. As shown in Fig. 2a and 3a, untreated bacterial cells appear regularly round-shaped and rod-shaped, with clear and intact cell walls. After treatment, cell morphology

Table 1
Antibacterial activity of column fractions of *Spirulina*. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values. CIP, Ciprofloxacin.

#	Bacterial Strains	Fraction A		Fraction B		CIP	
		MIC (µg mL ⁻¹)	MIC (µg mL ⁻¹)	MIC (µg mL ⁻¹)	MBC (µg mL ⁻¹)	MIC (µg mL ⁻¹)	MBC (µg mL ⁻¹)
1	<i>E. coli</i> (ATCC 25922)	–	–	7.8	15.6	15.6	31.2
2	<i>E. coli</i>	–	–	31.2	62.5	62.5	125
3	<i>K. pneumoniae</i>	–	–	31.2	62.5	62.5	125
4	<i>P. aeruginosa</i>	–	–	31.2	62.5	62.5	125
5	<i>S. aureus</i> (ATCC 29213)	–	–	7.8	15.6	15.6	31.2
6	<i>E. faecalis</i>	–	–	62.5	250	62.5	125
7	<i>S. aureus</i>	–	–	62.5	250	62.5	125
8	<i>S. pyogenes</i>	–	–	62.5	250	62.5	125

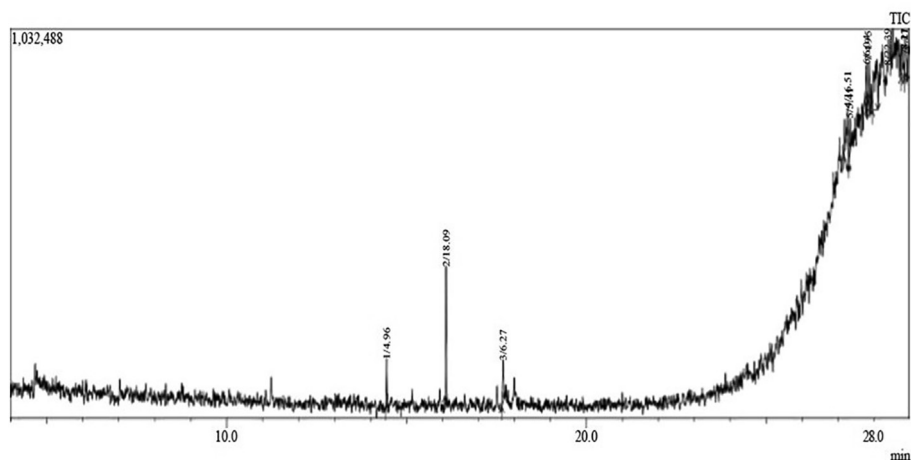


Fig. 1. Gas chromatography and mass spectrophotometry profile of fraction B.

Table 2

GCMS profile of phenolic compounds found in fraction B.

	Compound name	RT	weight	Peak area %
1	Oxazolidin-2-one	14.442	139	4.96
2	Propanediamine	16.100	130	18.09
3	Carbanilic acid	17.683	213	6.27
4	Phenylacridine	27.267	271	16.51
5	Pyrrolidine	27.333	327	5.41
7	Dinitrobenzoate	27.867	268	4.95
8	Benzophenone (Diphenylmethylene)	28.083	272	28.43
9	Piperidine	28.767	320	7.17
10	Isoquinoline	28.858	238	8.21

changed dramatically in both cells, as shown in Fig. 2b, 2c, 3b, and 3c. Almost all margins of the cell membrane were wrinkled and appeared with irregular boundaries due to lack of cellular integrity. It can be concluded that the phenolic compound acted on bacterial cells and caused cell death. (See Fig. 3)

The above results were further analyzed with respect to intracellular and anatomical changes in both bacterial cell types. The *S. aureus* treated and untreated cells were captured during the cell division process. In untreated cells, cell walls appeared thick and consisted of several layers of peptidoglycan (Fig. 4a). Cells appeared round with well-defined morphology, and cells in the process of cell division exhibited a distinct cross-wall that completely separated the two daughter cells and compacted the cytoplasm. The gram-negative bacterial cells (*E. coli*) appeared in diverse shapes with thin cell walls and dense cytoplasm (Fig. 5a). All the untreated cells displayed clear and compact cytoplasmic membrane, dense cytoplasm, homogenous electron density of genetic material, and at the margin of the cytoplasm, numerous granule-like objects consistent with ribosomes. In both cells, after

treatment with phenolic compounds, damage was observed in the ultra-structure (Fig. 4b and 5b).

The TEM micrograph confirmed the synergistic effects of the phenolic compounds in fraction B on food-borne bacterial pathogens. Cells undergoing cell division appeared malformed, with septa that were completely lysed or that failed to form at all. All treated cells appeared extensively damaged, or at least having lysed cell walls. Depletion of intracellular contents with heterogeneous electron density and scattered ribosomes were also observed. In gram-negative cells, spherical shape without cell membrane or envelope was observed, and hyper-hydrated cytoplasmic regions appeared electron-lucent with leakage of intracellular material on the surface.

3.5. Conclusions

According to the obtained results, antibacterial activity could be ascribed to the different behavior of polyphenols, present in fraction B, that act against more than one target site of drug-

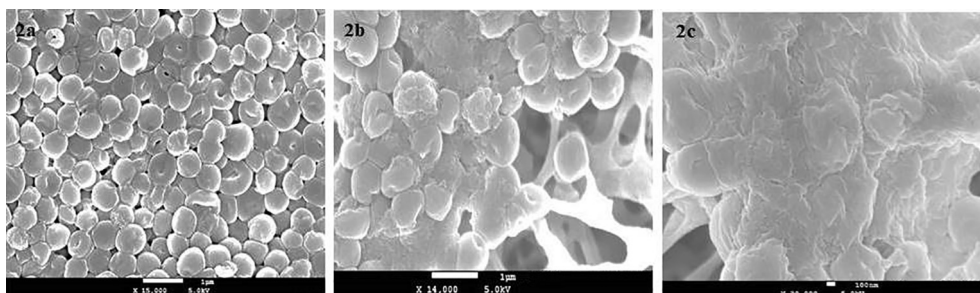


Fig. 2. Scanning electron microscopic image illustrating the effects of polyphenols from *Spirulina* on *S. aureus*. a untreated and b & c treated bacterial cells.

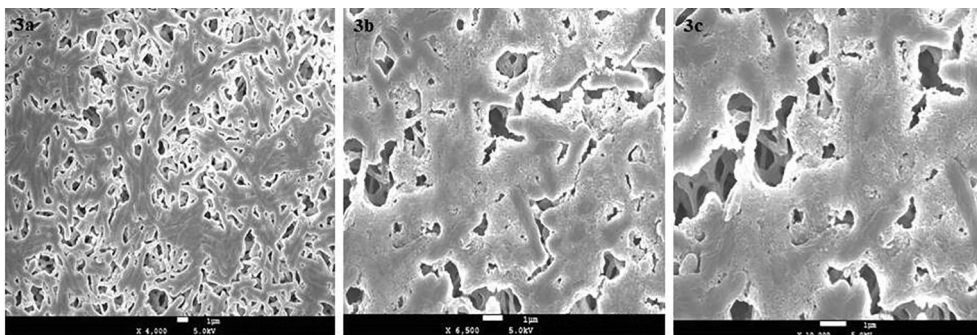


Fig. 3. Scanning electron microscopic image illustrating the effects of polyphenols from *Spirulina* on *E.coli*. a) untreated and b & c) treated bacterial cells.

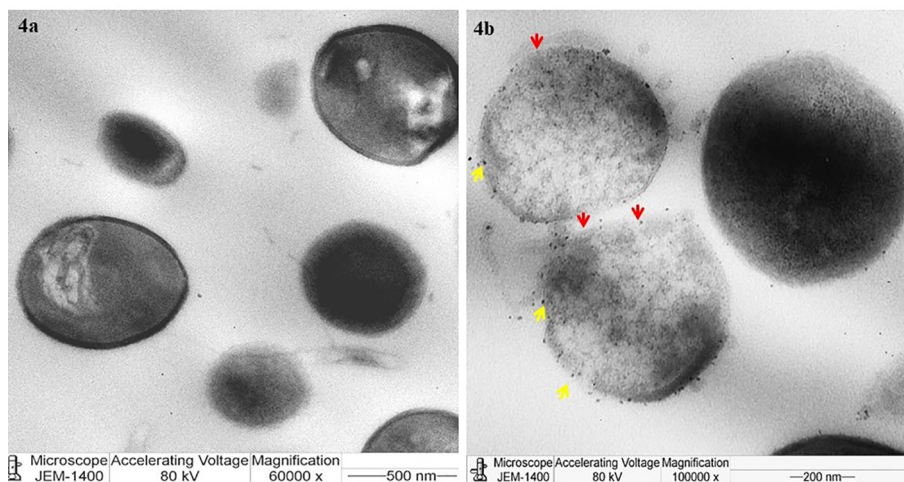


Fig. 4. Transmission electron microscopic micrographic illustration of a) untreated and b) treated gram-positive (*Staphylococcus aureus*) bacterial cells. Red arrows – disintegrated cell wall and cell membrane; yellow arrows – scattered ribosomes.

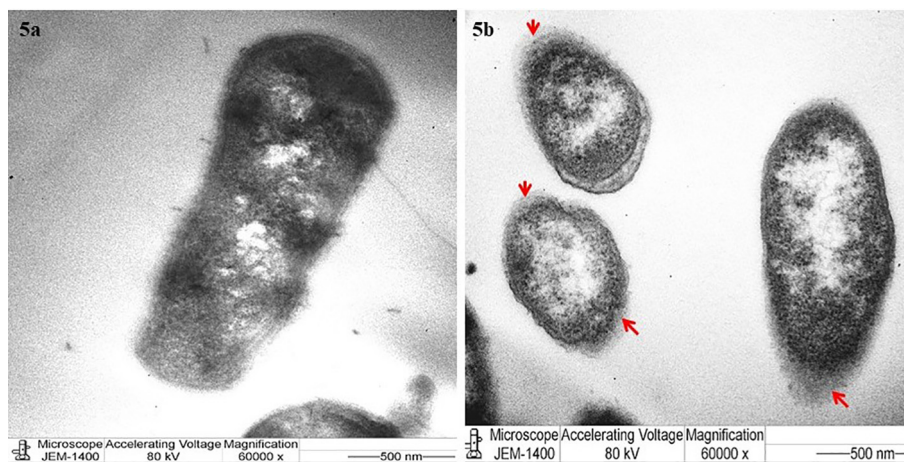


Fig. 5. Transmission electron microscopic micrographic illustration of a) untreated and b) treated gram-negative (*Escherichia coli*) bacterial cells. Red arrows – disintegrated cell wall and cell membrane.

resistant food pathogens. Fraction B may act as a potential food preservative to reduce food contamination, especially for drug-resistant bacteria, and to extend the shelf-life of food materials. The present work also proposes that fraction B from *Spirulina* has

potential to contain a new food preservative or anti-microbial agent against drug resistant food pathogens. However, further research work is required to fully understand the interaction of the fraction contents with food components.

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