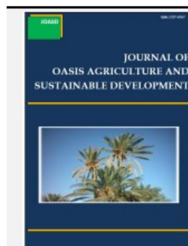

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Polysaccharides from Tossa jute (*Corchorus olitorius* L.) leaves: Extraction, antioxidant and antibacterial activities

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Abstract

The biological proprieties of mucilaginous polysaccharides (PSc) extracted from Tossa jute leaves (*Corchorus olitorius* L.) using an ethanol precipitation method were studied. The results showed that PSc had higher total polyphenols and flavonoids and greater antioxidant activities. At 1.5 mg/ml PSc, the antioxidant activities were about 90% against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH•) and 78% against lipid peroxidation. The PSc with a FRAP assay at the same concentration showed an effective protection against hydroxyl radicals and DNA breakage. Furthermore, the extracted PSc had a wide spectrum of antibacterial activities against all bacteria tested (Gram+ and Gram-). The overall data suggested that this natural PSc may be used as a competitive antioxidant and antimicrobial additive in food and in medicinal preparations.

1. INTRODUCTION

Chemical reactions, free radicals and some redox reactions can be a source of oxidative damage of macromolecules in cell structures and biomolecular functions causing numerous diseases (Kil et al., 2009). Synthetic antioxidants (butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)) are used in food to delay these damages. However, these additives may, for example, cause liver damages (Lin, & Tang, 2007; Namki, 1990). Natural antioxidants as substitutes for synthetic antioxidants are being investigated to prevent human disease risks from free radicals and from various diseases risks such as cancer, heart disease and arthritis (Andrade et al., 2009).

Polysaccharides (PS) are widely distributed in plants and algae (Ignat, 2012). Natural sources of PS are normally nontoxic and can act as beneficial bioactive agents in food and which have received increased attention for their benefits (Lovegrovea et al., 2017). They have a broad spectrum of biological activities, such as antibacterial, anticoagulant, anti-inflammatory, anticancer and anti-oxidative activities (Raposo et al., 2013; Majdoub et al., 2009; Challouf et al., 2011).

Tossa jute (*Corchorus olitorius* L.) cultivated throughout tropical Asia and Africa, is one of the traditional plants that has the potential to be used for medicinal purpose. Their leaves are consumed for their nutritive and medicinal values. Several studies reported that Tossa jute had antiviral, antibacterial and antioxidant activities (Ramadevi, & Ganapaty, 2011; Barku et al., 2013; Ben Yakoub et al., 2018), due to its high amounts of vitamin E, β-carotene, ascorbic acid, α-tocopherol, glutathione and phenolic compounds (Furumoto et al., 2002; Zeghichi et al., 2003; Azuma et al., 1999). C. olitorius leaves

also contain a large amount of mucilaginous polysaccharides (PSc) (Ohtani et al., 1995), which had interesting functional proprieties and enhanced the appearance, texture and flavor of yogurt (Hussein et al., 2011).
Nevertheless, there is a lack of information about PSc antioxidant and antimicrobial activities. The biological activities of PSc were investigated for antioxidant and antibacterial activities in vitro.

2. MATERIALS AND METHODS

2.1. Plant material

C. olitorius fresh leaves were harvested in August 2017 from the experimental field of the Arid and Oases Cropping Laboratory, Arid Lands Institute, Gabes, Tunisia, with an arid climate characterized by a mean rainfall <150 mm/year. Immediately after harvest, leaves were washed with tap water and shade-dried at room temperature (25–27 °C) to reach a constant weight. Thereafter, the dried leaves were powdered in a Moulinex blender (Moulinex, Écully, France), labeled, and preserved in tight glass at -20 °C in the dark and used within 8 wk.

2.2. Extraction of PSc

The extraction procedure was done by macerating 20 g of leaves powder in 400 ml of distilled water at 95 °C for 20 min (Zhu et al., 2008). The maceration was done in a water bath equipped with a magnetic stirrer. The aqueous macerate was dialyzed for 48 h at 4 °C against double-distilled water (changed twice daily), using a dialysis membrane having a nominal cut-off of 14 kDa (Spectra/Por™ Co., Thermo Fisher Scientific, San Jose, CA, USA), to eliminate excessive salts. Subsequently, the dialysate was deproteinized for 8 h using an alkaline protease, Purafect® added at 1% (m/v) after pH adjustment to 10.0 with NaOH. The solution was kept for proteolysis at 50 °C for 24 h and then the mixture was centrifuged at 5000 g for 30 min at 4 °C (MPW-350R, MPW, Warsaw, Poland). The supernatant was precipitated with absolute ethanol (v:2v) at 4 °C for 24 h, as described by Abdelhedi et al. (2016).
The precipitate (pellet), containing PSc was recovered by centrifugation (5000 g, 30 min, 4 °C), while the supernatant was concentrated with vacuum at 40 °C to evaporate the ethanol. Thereafter, frozen PSc were freeze-dried (Christ ALPHA 1-2 LD, Bioblock Scientific, Illkrich-Cedex, France) and stored at -20 °C in the dark and used within 3 wk (Fig. 1).

Characterization of C. olitroius extracts

2.3. Polysaccharide yield

The yield of polysaccharides form C. olitroius leaves (PSc) was calculated using equation 1:

Psc extraction Yield (%,W/W) =
(Weight of dried polysaccharides / Weight of dried leaves) x 100
(1)

2.4. The total polyphenols (TPC) and total flavonoids contents (TFC)

The TPC in PSc was estimated and expressed as mg GA(E)/g (Javanmardi et al., 2003). Briefly, 100 µl of each extract was mixed with 100 µL 1 N Folin-Ciocalteu reagent. The mix was incubated for 2 min in the dark at room temperature. Then, 800 µl of sodium carbonate (5%) was added. After 20 min of incubation at 40 °C in the dark, the absorbance was measured at 760 nm. A blank was prepared by adding 100 µl of the sample solution to 100 µl of distilled water.

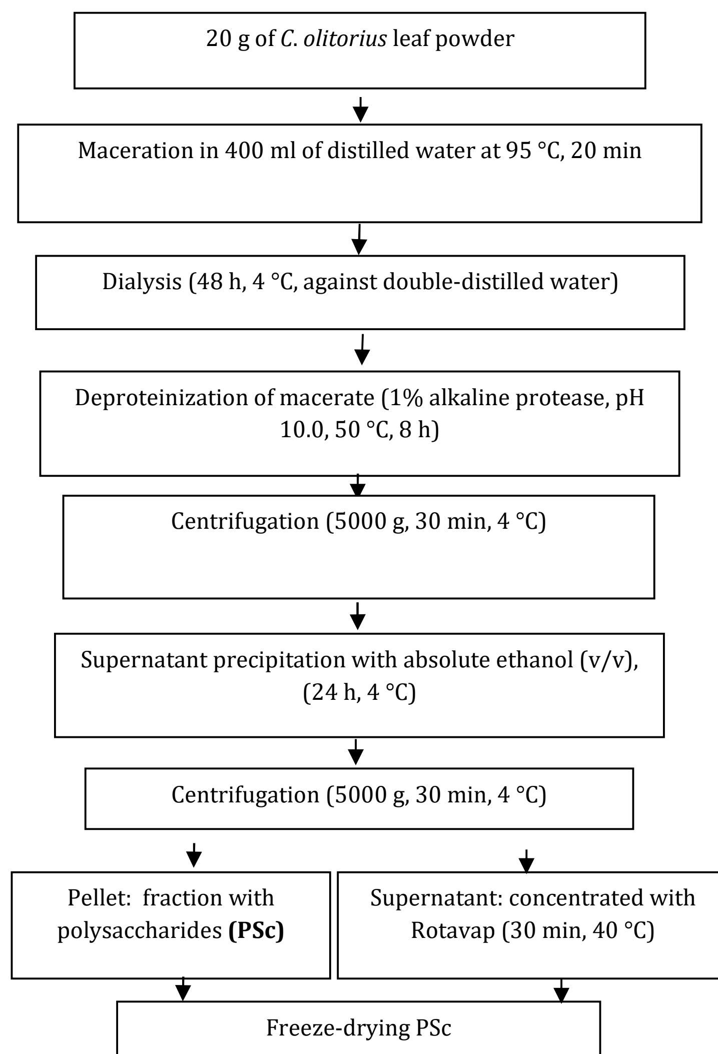

Fig. 1. Diagram of polysaccharides (PSc) from Tossa jute (*C. olitorius* L.) leaves.

The TFC were expressed as mg QE/g using the aluminum chloride colorimetric method reported by Quang, and Jong-Bang (2011). Briefly, 250 µl of PSc were added to 1 ml of distilled water and 0.15 ml, 15% sodium nitrite solution followed by incubation for 6 min in the dark at room temperature after which 0.075 ml of 10% aluminum chloride was added. The mixture allowed to stand for 5 min at room temperature before 1 ml of NaOH (4%) was added. The absorbance of the reaction mixture was measured at 510 nm.

2.5. Antioxidant activities

The antioxidant activities of PSc were measured using the ferric reducing antioxidant power assay (FRAP), scavenging activity against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH•), β-carotene, lipid peroxidation and DNA nicking assays.

2.6. Ferric Reducing Antioxidant Power (FRAP) Assay

The ability of samples to reduce iron (III) was determined using the method of Yildirim et al. (2001). PSc were prepared at different concentrations (0.05, 0.1, 0.25, 0.5, 0.75 and 1.5 mg/ml), then 0.5 ml was mixed with 1.25 ml of potassium phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of 1% potassium ferricyanide solution. After incubation for 20 min at 50 °C, 1.25 ml of 10% trichloroacetic acid (TCA) was added. The supernatant (1.25 ml) obtained using centrifugation (3000 g, 10 min, 5 °C) was mixed with 1.25 ml of distilled water and 0.25 ml of ferric chloride (0.1%) added. The absorbance of PSc and BHA used as a reference was measured after incubation for 10 min at 700 nm. The results of the reducing power were shown as EC50 values, which were defined as the samples concentration having 0.5 of the initial absorbance value. Lower EC50 values reflected better antioxidant activity.

2.6. Free radical scavenging activity on DPPH

The radical scavenging capability of extracts on DPPH free radicals was determined as described previously by Bersuder et al. (1998). PSc solutions (500 µl of 0.05 to 1.5 mg/ml) were mixed with 375 µl of absolute ethanol and 125 µl of DPPH• solution (0.2 mM in ethanol). The reduction of DPPH• radical was measured at 517 nm after incubation in the dark for 60 min at room temperature. The scavenging activity was calculated using equation 2:

$$\text{Scavenging activity (\%)} = ((A_{control} + A_{blank} - A_{sample})/A_{control}) \times 100 \quad (2)$$

Where Acontrol: the absorbance of the control reaction, Ablank: the absorbance of the blank reaction and Asample: the absorbance of the sample reaction.

2.7. Inhibition of linoleate-autoxidation model system

Inhibition activity of in vitro lipid peroxidation of PSc was determined by assessing their ability to inhibit oxidation of linoleic acid in an emulsified model system (Osawa and Namiki, 1985). Briefly, freeze-dried PSc at different concentrations (0.02 to 1.5 mg/ml) were dissolved in 2.5 ml of 50 mM phosphate buffer (pH = 7.0) and added to 2.5 ml of 50 mM linoleic acid in ethanol (95%). Distilled water was then added to adjust the volume to 6.25 ml. After 10 days of incubation at 45 °C in the dark, the degree of oxidation was evaluated by measuring the ferric thiocyanate by mixing a 0.1 ml aliquot with 4.7 ml of 75% ethanol. The mixture was reacted with 0.1 ml of 30% ammonium thiocyanate followed by the addition of 0.1 ml of 20 mM ferrous chloride solution in 3.5% HCl. The control reaction was done without sample. The percentage of color development was measured at 500 nm and the degree of oxidation inhibition was expressed using equation 3:

$$\text{Lipid peroxidation inhibition (\%)} = (1-(A_{sample}/A_{control})) \times 100 \quad (3)$$

2.8. DNA nicking assay

The ability of the PSc to protect CRIITMTOPO plasmid (Invitrogen, Carlsbad, CA, USA) DNA against OH was measured using the DNA nicking assay using the protocol of Lee et al. (2002). The reaction between 10 µl of each samples (PSc prepared at 2 and 1 mg/ml) and 2 µl of pGapZαA® plasmid DNA (0.5 µg/well) for 10 min at room temperature was followed by the addition of 10 µl of Fenton's reagent (3 mM H_2O_2, 50 µM L-ascorbic acid and 80 µM $FeCl_3$). The mixture allowed to react for 5 min at 37 °C. After incubation, the reaction mixtures were loaded on 1% agarose gel (Serva GmbH, Heidelberg, Germany) and the closed circular, linear, and relaxed forms of DNA were then observed using a 1% (w/v) agarose gel electrophoresis using 5 µl ethidium bromide staining (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) (voltage 100 A). Images

of the ethidium bromide stained DNA agarose gel was obtained using an AlphaImager TM Gel Documentation (Vilber Lourmat, France).

2.9. Antibacterial activity

Microbial strains

Eight pathogenic bacteria strains, already available in the laboratory, were used for antibacterial assessment of the PSc. Cells were grown in liquid Luria-Bertani (LB) broth (a liter of medium contained 10 g tryptophan, 5 g yeast extract and 10 g sodium chloride) (Sigma Aldrich, USA) (Bertani, 1951) and stored long-term in inoculated sterile LB in 15% (v/v) glycerol in cryotubes at -80 °C (up to 5 yr)
Three Gram-positive bacteria: Staphylococcus aureus (ATCC 25923), Micrococcus luteus (ATCC 4698) and Bacillus cereus (ATCC 11778) and 5 Gram-negative bacteria: Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC 13883), Salmonella enterica (ATCC 43972) and Salmonella typhimurium (ATCC 19430) along with Enterobacter sp. were tested.
For each bacteria, a curve of colony forming units (CFU) as a function of absorbance (at 600 nm) was plotted and used to estimate the number of colony. Once they reached ~106 CFU, the bacteria culture was stopped and used for the antibacterial activity study.

Agar diffusion method

Cultures suspensions (200 µl) of the microorganisms (106 CFU/ml forming units) of bacterial cells were spread on LB-agar media already poured in the Petri dishes. Then, 60 µl of PSc (25 and 50 mg/ml), were loaded into wells (6 mm diameter) in the agar layer using sterile Pasteur pipettes. Gentamycin (30 µg/well) was used as the positive control. Thereafter, the Petri dishes were kept for 1 h at 4 °C. Then, they were incubated for 24 h at 37 °C (Berghe, & Vlietinck, 1991). The antimicrobial activity was evaluated by determining the inhibition zone of growth (diameter expressed in mm) around the wells, using a ruler (minimum resolution ~1 mm).

2.10. Statistical analysis

All the results are expressed as mean ± standard deviation (SD). Statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) ver. 17.0 software (Professional edition, SPSS Inc., Hong Kong, China). Mean (triplicate analyses) comparisons were carried out using one-way analysis of variance (ANOVA) and Duncan's multiple range tests ($p<0.05$).

3. RESULTS AND DISCUSSIONS

3.1. PSc antioxidant activity assessment

The antioxidant capacities of PSc were evaluated for their FRAP, DPPH•, lipid peroxidation inhibition and bleaching DNA nicking.

3.2. FRAP assay

FRAP assay is based on electron-transfer reactions and is commonly used for the analysis of antioxidants. The presence of antioxidants in the sample causes the reduction of Fe3+/ferricyanide complex to the Fe2+ form (Liu et al., 2012). The ability of samples to reduce yellow ferric tripyridyltriazine complex (Fe (III)-TPTZ) to blue ferrous complex (FE (II)-TPTZ) by the action of electron donating antioxidants depends on the reducing power of the sample. The antioxidant capacities of PSc and BHA (used as a reference) at different concentrations are shown in Fig. 2.a.
Based on the OD values, PSc had an effective reducing power and PSc showed a significant activity in a dose-dependent manner between samples. The values increased with the increasing concentration of the sample in the range of 0.05 and 1.5 mg/ml to reach their maximal absorbance at 0.75 mg/ml. PSc with respective EC50 values of 60 µg/ml. Nevertheless, PSc had a lower activity than BHA, which reached maximal OD at 1.5 mg/ml. The data indicated that PSc could act as an electron donor that reacted with free radicals to convert them to more stable products and thereby terminate radical chain reactions. The reducing power of PSc was higher than PS extracted from leaves of Russula vinosa (Liu et al., 2014) and Gynura procumbens (Li et al., 2017).
DPPH• radical scavenging activity
The DPPH• radical scavenging activity assay measures the capacity of the extract to donate hydrogen or to scavenge free radicals (Hseu et al., 2008). The scavenging activity of PSc and BHA on DPPH• radical was measured and data are shown in Fig.2.b.

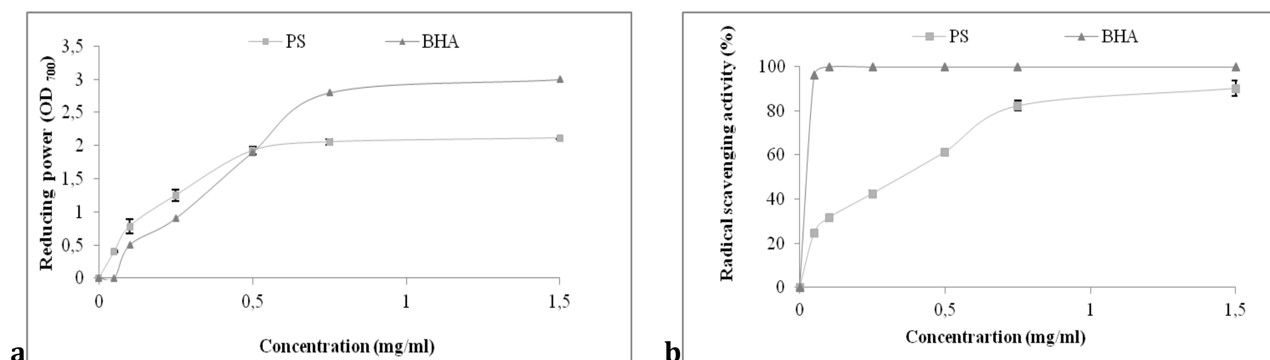

Fig. 2. Antioxidant properties of PSc as a function of their concentrations (mg/ml); (a): Reducing power assay, (b): DPPH• Radical scavenging activity.

Results indicated that the increasing concentration of PSc from 0.05 to 1.5 mg/ml induce significant increasing of DPPH• radical scavenging activity. A noticeable activity was observed at 1.5 mg/ml ($p<0.05$). Nevertheless, PSc had lower activity than BHA, which reached its maximum activity at 0.1 mg/ml. These results confirmed that PSc had a noticeable activity on scavenging free radicals, which was comparable to those extracted from carrot peels (Ghazala et al., 2015) and watermelon rinds at 1.2 mg/ml (Ben Romdhane et al., 2017). However, DPPH• radical scavenging activity of PSc was higher than PS extracted from Chuanminshen violaceum (Dong et al., 2016) and Gynura procumbens leaves (Li et al., 2017).

Nevertheless, PSc provided a better inhibition than those extracted from watermelon rinds (Ben Romdhane et al., 2017), Lilium lancifolium leaves (Xua et al., 2016), while, less efficient than polysaccharides from *Cyclocarya paliurus* (Xie et al., 2015).

3.3. Inhibition of linoleate-autoxidation

The lipid peroxidation inhibition activity of PSc is shown in Fig. 3. PSc had a significant effect on inhibiting lipid peroxidation, in a concentration-dependent manner ($p<0.05$). This inhibitory effect increased suggesting that PSc had an effective lipid peroxidation inhibition capacity. These results are higher than those reported by Ma et al. (2012) in PS from Inonotus obliquus leaves, which showed a peroxidation inhibition capacity of about 87.8% at 5 mg/ml.

3.4. DNA nicking assay

Fig. 4 shows the antioxidant activity of PSc using the DNA nicking assay. Line 1 corresponds to the native DNA with its nicked circular, linear and supercoiled forms. Line 2 represents the reaction between the plasmid DNA and the Fenton's reagent without sample. This incubation led to the loss of the three bands which was explained by the complete degradation of the supercoiled form of the plasmid. The presence of PSc (lines 3 and 4) that was reacted with Fenton's reagent, provided protection of the plasmid DNA. Furthermore, the PSc added at 2 mg/ml, showed the highest protective effect with significant conservation of the supercoiled DNA band intensity. However, at

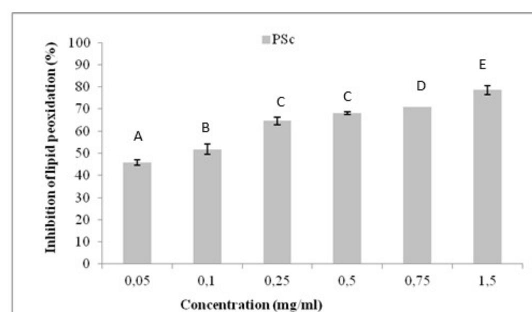

Fig. 3. Inhibition of linoleic acid peroxidation (%) of PSc as a function of their concentrations (mg/ml). Data showed as mean ± SD (n = 3), different letters mean significant differences (*p*<0.05) between the activities of different PSc concentrations.

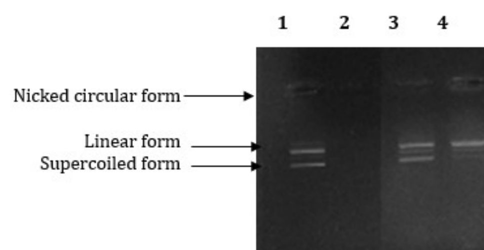

Fig. 4. Gel electrophoresis pattern of pGapZαA®DNA incubated with Fenton's reagent in the presence and absence of PSc; lane 1: native DNA; lane 2: DNA incubated with Fenton's reagent; lanes 3 and 4, Fenton's reagent+ DNA+ PSc at 2 and 1 mg/ml, respectively.

1 mg/ml, PSc showed a partial protection against hydroxyl radical induced DNA damage. These results were consistent with those obtained by Ben Romdhane et al. (2017), who showed a strong protection of PS from watermelon against hydroxyl radicals' induced DNA breakage but at higher concentration (5 mg/ml) compared to the present study.

3.5. Antibacterial assay

The antibacterial activity of the extracted PSc were tested at 25 and 50 mg/ml against 8 bacteria. As can be seen in Table 1, the inhibitory activity of PSc with different bacterial species depended on the sample. The increase of the sample concentration increased inhibition against all bacteria. PSc showed a significantly greater and wider spectrum of activities, inhibiting the development of all bacteria.

Table 1. Antibacterial activity of PSc at 25 and 50 mg/ml against Gram-positive and Gram-negative bacterial strains.

Inhibition zone diameters (mm)	PSc	
Extract concentration (mg/ml)	**25**	**50**
S. aureus	16 ± 1 [a]	31 ± 1 [b]
M. luteus	8 ± 0 [a]	16 ± 1 [b]
B. cereus	8 ± 0 [a]	17 ± 1 [b]
E. coli	14 ± 2 [a]	25 ± 2 [b]
K. pneumoniae	9 ± 1 [a]	17 ± 2 [b]
S. typhimurium	8 ± 1 [a]	17 ± 2 [b]
Enterobacter sp.	7 ± 1 [a]	15 ± 1 [b]
S. enterica	5 ± 1 [a]	14 ± 0 [b]

Data of inhibition zone diameters are expressed in mm and given in mean ± SD;
[a,b,c,d] Different letters on the same line indicate significant differences ($p<0.05$)

S. aureus and E. coli were the most sensitive bacteria to PSc at 50 mg/ml. However, the highest bacterial resistance against PS from leaves of potatoes peels was for Gram-positive bacteria, particularly, B. panis and S. aureus (Ben Jeddou et al., 2016). This inhibitory effect of polysaccharides on bacteria cell viability can be explained by the alteration of membrane integrity and permeability (He et al., 2010).

4. CONCLUSION

PSc was extracted using ethanol precipitation. The TPC, the TFC, the antioxidant and the antibacterial activities of PSc were investigated. Results showed that, based on different activities, PSc had a greater ability to prevent continuous production of radicals by donating electrons; preventing lipid peroxidation and the propagation of free radicals. All the bacterial strains were sensitive to PSc.

These functional PSc may, therefore, be considered as natural preservatives against food-borne pathogens that may be useful in foods and for protecting human health.

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