

# Antimicrobial and antioxidant effects of phlorotannin coating in spoilage prevention of tomato during storage

## Abstract

The microbial flora present on vegetables and fruit is commercially significant as they cause spoilage and potential health risks in consumers. Synthetic chemicals used to combat food microbes posed serious health and environmental hazards leading to the search for natural alternatives. In this study, phlorotannins from three Indian brown seaweeds, *Sargassum myriocystum*, *Turbinariaornata*, and *Padina tetrastromatica* were evaluated for antimicrobial, antioxidant, and fruit spoilage prevention potentials. The crude phlorotannin of *Padina* with the highest content ( $0.114 \pm 0.0005 \text{ g kg}^{-1}$ ) and antioxidant activity ( $92.65 \pm 3.43\%$ ) was purified by column chromatography. The *Padina* water fraction I with the highest antioxidant potential was characterized by Fourier Transform Infra-Red spectroscopy and a 5% solution was applied as a coating on tomatoes segregated into control chilled (CC), treated chilled (TC), control non-chilled (CNC) and treated non chilled (TNC). Spoilage parameters were studied over twenty days, using a swab and spread plate methods to assess the microbial load on tomatoes. Compared to the control sets, the treated sets exhibited a low number of microbial colonies and retained a fresh look and yellow color. The antibacterial and antioxidant properties of phlorotannin coating as a natural alternative for preservatives, effectively reduced bacterial load, inhibiting the early decay of tomatoes.

**Keywords:** antimicrobial, phlorotannins, antioxidant, *padinatetrastromatica*, food spoilage, postharvest storage

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**Abbreviations:** CC, control chilled; TC, treated chilled; CNC, control non-chilled; TNC; treated non-chilled; TPC, total phenolic content; WF, water fraction; MF, methanol fraction; UV, ultraviolet; GA, gallic acid; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; SD, standard deviation; FTIR, fourier transform infrared; KBr, potassium bromide; CFU, colony forming units; DMSO, dimethyl sulfoxide; AP, aqueous phase; GAE, gallic acid equivalent

## Introduction

The surface of vegetables and fruit harbor a variety of microbes including bacteria and fungi. The diversity and population of these species depend on several factors such as the farming environment, type of plant cultivars, postharvest handling, transportation, and storage conditions.<sup>1</sup> These microbes slowly degrade the crop causing early spoilage during storage. They may be pathogenic or non-pathogenic, and some are even resistant to antibiotics. On consuming raw vegetables and fruit, these organisms enter the human gut leading to disease outbreaks and allergies.<sup>2</sup> Many techniques are available for the spoilage prevention of easily perishable commodities. The use of antimicrobial compounds, fungicides, and synthetic chemical preservatives is a common practice. These preservatives compromise the organoleptic characteristics and nutritional quality of the crop.<sup>3</sup> In the course of time, such chemicals caused serious health and environmental impacts which stirred up the research community to discover natural alternatives for the preservation of easily spoiled vegetables and fruit.

Brown seaweeds are treasure houses of numerous commercially significant compounds employed in diverse fields. Phlorotannins are

phenolic compounds exclusive to brown algae, and form the largest group of secondary metabolites identified in algae.<sup>4</sup> They are polymers of phloroglucinol units formed in the acetate-malonate pathway, as a part of the defense mechanism in algae and are broadly classified into three as fucols, phlorethols and fuhals, and eckols and carmalols.<sup>5,6</sup> The molecular weight ranges from 126-6500 Da.<sup>7</sup> Several biological activities have been reported for phlorotannins, viz, antioxidant, anti-inflammatory, antimicrobial, antiprotozoal, antifungal, antimalarial, anticancer,<sup>8</sup> antidiabetic,<sup>9</sup> and anti-arthritis.<sup>10,11</sup> Hence they are used as ingredients in drugs, animal feeds, diet supplements, fertilizers, nutraceuticals, and cosmetics.<sup>12</sup>

There are numerous reports on the antimicrobial and antioxidant activities of methanolic extracts and phlorotannins from brown algae.<sup>13,14</sup> Phlorotannins purified from the various *Phaeophyceae* members like *E. bicyclis*, *E. kurome*, *H. fusiformis*, and *E. cava* have exhibited superior antioxidant properties with 2-10 folds increased free radical scavenging activity than catechin,  $\alpha$ -tocopherol and ascorbic acid.<sup>15</sup> The methanol extracts of *Sargassum wightii* and *Turbinariaornata* have polyphenols responsible for their antioxidant and antibacterial activities.<sup>16</sup> The phlorotannin-rich extracts of *Cystoseira nodicaulis*, *Cystoseira tamariscifolia*, and *Cystoseira usneoides* were found to possess antibacterial and antifungal properties.<sup>17</sup> The bacteriostatic and bactericidal effects of phlorotannins from *Ascophyllum nodosum* against rumen microbes in cattle and of phlorotannins from *Turbinaria conoides* and *Padina gymnospora* on *E. coli* has been reported.<sup>4</sup> On the other hand, an attempt to use phlorotannin as a coating on fruit or vegetables to protect them from decay has not yet been undertaken. In this context, the present study aimed to extract phlorotannins from the three Indian

brown seaweeds, *Sargassum myriocystum*, *Turbinariaornata*, and *Padina tetrastrum*, use it as a coating on tomatoes and to evaluate its antimicrobial and antioxidant activities to assess the spoilage prevention potential.

## Materials and methods

### Materials

The chemicals and analytical grade reagents were purchased from Hi-Media and Sisco Research Laboratories, Mumbai and Chennai, India.

### Collection of seaweeds

Fresh, healthy specimens of the seaweeds were collected from the Kilakarai coast (latitude 9°14' N and longitude 78°50' E), Gulf of Mannar located in the Southeast coast of Tamil Nadu, India. The algae were identified by experts in the Centre for Advanced Studies in Botany, University of Madras, Tamil Nadu, India. After washing thoroughly in tap water to remove all epiphytes, sand particles, and associated fauna, the seaweeds were shade dried for five days, followed by oven drying for 12 h at 60°C. The material was ground to a coarse powder using mixer-grinder (Philips HL 1643/04 Vertical Mixer Grinder, India).

### Preparation of seaweed extracts

Twenty grams of algal powder was extracted with 250 mL of 80% methanol, stirred on an incubator shaker (Lark Innovative Fine Teknowledge, Chennai, India) at 28°C for 24 h. The extract was filtered and the process was repeated thrice using 100 mL of fresh 80% methanol. The combined extracts were filtered using a funnel and Whatman filter paper No.1 and concentrated using a rotary evaporator (Lark Innovative Fine Teknowledge, Chennai, India) operated at ≤35°C, to evaporate off the methanol. The total phenolic content (TPC) and the antioxidant activity of the resulting aqueous solution were determined. The aqueous filtrate of *Padina* that showed the maximum content and activity was transferred into a clean separating funnel and partitioned by adding one volume of dichloromethane. The solution was vigorously shaken and left to stand for 10 min for phase separation. The bottom dichloromethane layer containing lipids was discarded and the partitioning procedure was repeated thrice using fresh 20 mL dichloromethane. The partitioned aqueous layer was collected into a pre-weighed 20 mL scintillation vial and evaporated residual dichloromethane using a rotary evaporator operated at ≤35°C, to obtain a polyphenolic extract which was stored at -20°C.

### Preparation of phlorotannin-enriched fraction

Silica gel (60-120 mesh size; Sisco Research Laboratories, Chennai, India) column chromatography was carried out with the above extract to obtain phlorotannin-enriched fractions. A column chromatography tube loaded with silica gel slurry was preconditioned with 12 mL methanol followed by 18 mL deionized water. The extract was dissolved in 10 mL deionized water and sonicated for 10 min for proper dissolution. This sample was loaded onto the preconditioned column cartridge using a glass Pasteur pipette. The column cartridge was eluted with 20 mL deionized water. The resulting eluant was collected as 10 mL each into two glass vials (water fraction I & II or WF I & WF II). Then the column cartridge was eluted with 30 mL methanol and collected the eluant as 10 mL each into three vials (methanol fraction I, II & III or MF I, MF II & MF III). The water fractions and methanol fractions were concentrated on a rotary

evaporator operated at ≤35°C, lyophilized (Mini-lyodel, Delvac Pumps Pvt. Ltd., Chennai, India), sealed in glass vials and stored at -20°C.

### Estimation of total phenolic content (TPC)

The concentration of phenolics in algal extracts, water fractions, and methanol fractions were determined using spectrophotometric method.<sup>18</sup> The absorbance was determined using a UV-visible spectrophotometer (Shimadzu, UV-1700, Japan) at  $\lambda_{max}$  = 765 nm. The samples were prepared in triplicate and the mean value of absorbance was obtained. The standard used was Gallic acid. The phenolic content was expressed in terms of Gallic acid equivalent (g of GA per kg of extract).

### In-vitro antioxidant activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The DPPH radical scavenging assay was carried out.<sup>19</sup> 100 µL each of algal extracts, water, and methanol fractions were tested. Absorbance was taken at 516 nm in spectrophotometer after 15 min, using methanol as blank. The free radical scavenging activity was calculated as:

$$\% \text{ Antiradical activity} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} * 100$$

Each experiment was carried out in triplicate, and results are expressed as mean % antiradical activity ± SD.

### Reducing power assay

Briefly, the reducing power of the extracts was evaluated.<sup>20</sup> The absorbance of all samples was measured at 700 nm. L-ascorbic acid was used as a standard. The % of reducing power was calculated by the same equation above.

### Fourier transform infrared (FT-IR) spectroscopy

The WF I that exhibited the highest total phenolic content and the highest antioxidant activity was characterized by Perkin Elmer System One (PerkinElmer Pvt. Ltd., Maharashtra, India) FTIR between 450-4000 cm<sup>-1</sup>, at 64 scans, 4 cm<sup>-1</sup> wavenumber, and resolution transmission mode under standard environmental conditions. Sample (2 mg) was ground evenly with approximately 100 mg KBr until particles measured <2.5 mm in size. The FTIR spectra were obtained and the signals were compared to a background spectrum collected from the KBr alone at room temperature.

Assessment of antimicrobial activity and spoilage prevention potential of phlorotannin (WF I) on tomato (Modified method of de Corat et al.<sup>21</sup> The assessment of antimicrobial activity and spoilage prevention potential of *Padina* phlorotannin (WF I) on tomato was carried out by the modified method of de Corato et al.<sup>21</sup>

### Sample collection and processing

The fresh tomatoes selected for antimicrobial and shelf-life studies, procured from a local farm in Chennai, Tamil Nadu, India, were collected in sterile polythene ziplock bags to avoid handling contamination and transported to the laboratory. Replicate samples were collected from discrete fruit. The sample was ensured to be not stocked. Since tomatoes are intrinsically fragile, prone to physical damage and have a limited shelf life, it was taken care of to obtain the freshest fruit possible. Before testing, the sample homogeneity was ensured by placing the samples in large, sterile ziplock bags and

mixing gently before being segregated. The microorganisms present on tomatoes being naturally-occurring microflora might be distributed heterogeneously from fruit to fruit. This allowed even distribution of microbial load or species composition of the initial inoculum. Even though there were chances for variation in species composition among the samples due to factors such as seasonal variation, a region of production, variety of produce, etc., the microflora of these samples were expected to remain fairly similar, with the largest uncontrollable variable being the overall microbial load.

### Sample treatment

Eight quarts of tomatoes (about 4 kg) were procured and the worst 5% that was discolored, physically damaged, or visually molded were discarded. The selected ones were rinsed in distilled water, dried properly, and transferred to sterile ziplock bags and mixed slowly to evenly distribute any initial microbes present on the fruit. Four quarts of tomatoes were retained as a control experiment set, while the other four quarts were coated thrice with 5% phlorotannin solution on drying. Out of the four quarts of control tomatoes, two quarts were placed in an open sterile plastic tray for observations of the non-chilled treatment, and the other two quarts were placed in sterile zip lock bags and stored in the refrigerator for the chilled treatment. The same was repeated for the treated sets of tomatoes also. Out of the two quarts, quart was for weight loss calculations and the documentation of color, turgor, and overall organoleptic quality. The remaining quart was used for microbial counts.

### Sampling process

From test day 1, in every third day that followed, for 20 days, the samples were examined visually for organoleptic qualities like brown spots, soft spots, wilting/loss of turgor, visible indicators of microbial growth, etc. and weight was noted. The photos were taken for visual documentation of the sample condition. This data was subsequently correlated with the microbial data obtained. Once the organoleptic studies were done, the samples were returned to their corresponding test environment and the samples for microbial enumeration were prepared.

### Plating process

The plating of the bacterial load on tomato samples was done by the swab/ streak method, and spread plate method. All the required glassware and medium components were autoclaved at 121°C at 103.42 kPa for 120 min to sterilize. All the techniques were done aseptically.

### Swab/Streak method

Bacterial samples were taken from the tomato surface using pre-autoclaved, sterile cotton swabs rubbed over randomly chosen sample surface for about 1-2 min and the microbe loaded swab was used to streak the agar plates. Proper care was taken not to cut the agar surface. The aseptically prepared plates were incubated in an inverted position at 37°C for 24–48 h for microbial growth.

### Spread plate method

Ten milligrams of flesh from each set of sample was weighed in sterile conditions and homogenized in 10 mL sterile peptone water (0.1%) using pestle and mortar for 5 min. One milliliter of each sample was serially diluted tenfold in sterile distilled water up to 10<sup>-5</sup> dilution, from which 0.1 mL at 10<sup>-4</sup> dilution was spread over the nutrient agar

plate using glass L-rod spreaders. The aseptically prepared plates were incubated at 37°C for 24 – 48 h for the appearance of colonies. After 48 h incubation, the bacterial colonies were counted by plate counting method.

### Total plate count of bacteria (CFU mL<sup>-1</sup>)

The microbial load in each set of tomato was determined as CFU mL<sup>-1</sup> using the formula:

$$CFU\ mL^{-1} = [(No.\ of\ colonies \times dilution\ factor) / volume\ of\ inoculum]$$

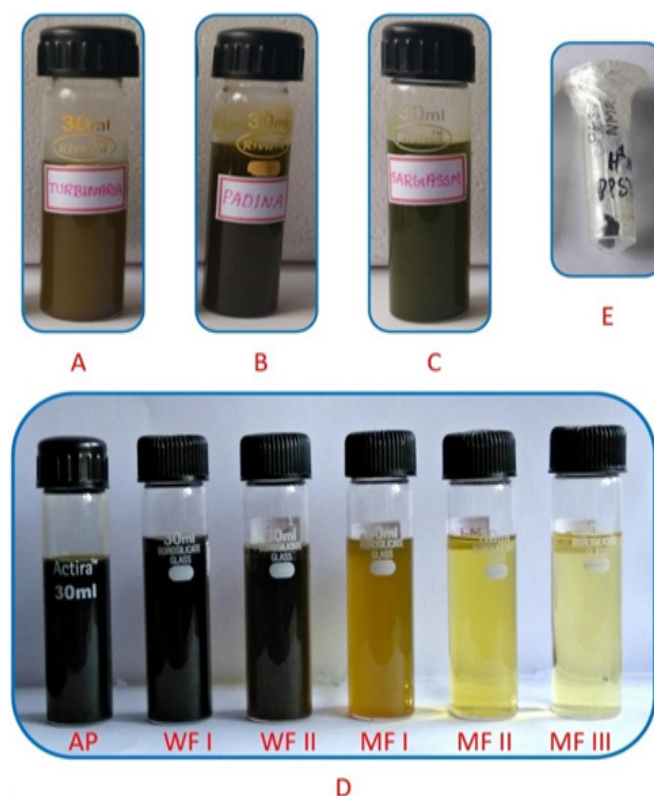
### Antimicrobial activity of phlorotannin

The antimicrobial activity was evaluated by agar well diffusion method. The microbial colonies isolated from the above plates were carefully transferred aseptically using a loop into the nutrient broth (50 mL) and incubated overnight at room temperature. The following day, the bacterial inoculum was uniformly spread using an L – rod spreader on to nutrient agar plates and allowed to dry. Then wells were made in the seeded plates with the help of a cork-borer. 50µL distilled water was added to the first well as negative control, while 50µL tetracycline was loaded to the second well as positive control. The phlorotannin was loaded to the remaining two wells in a concentration of 50µL and 100µL respectively. The systems were incubated under aerobic conditions for 24 h at room temperature. After incubation, the zone of inhibition was measured in millimeters.

Similarly, the antimicrobial activity of phlorotannin against two Gram-positive bacteria (*Bacillus cereus*, *Micrococcus luteus*) and three Gram-negative bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*) was evaluated by the same method. The microorganism was activated by inoculating a loopful of the strain in nutrient broth (50 mL) and incubated overnight at room temperature. Then the bacterial inoculum was uniformly spread using a sterile cotton swab on a sterile Petri dish with Mueller-Hinton agar. Thereafter wells were made in the seeded plates with the help of a cork-borer. 10µL of tetracycline was loaded to the first well as positive control. DMSO (Dimethyl sulfoxide) (1 mL) was dissolved in 9 mL distilled water and used as a negative control. Phlorotannin was loaded to the remaining three wells in a concentration of 30, 60, and 90µL respectively. The plates were incubated for 24 hours at room temperature under aerobic conditions. After incubation, the inhibition of bacterial growth was measured in millimeters.

### Results

The three seaweeds *S. myriocystum*, *T. ornata*, and *P. tetrastrum* were extracted thrice with 80% methanol to obtain the crude extracts (Figure 1). The total phenolic content, and the antioxidant activity of the extracts, were evaluated, and the Padina extract with the highest content and activity was further processed. The crude extract was partitioned thrice with equal volumes of dichloromethane and the aqueous phase (AP) was collected and concentrated which was subjected to silica gel column chromatography to obtain two water fractions (WF I & WF II) and three methanol fractions (MF I, MF II & MF III) (Figure 1). The total phenolic content, as well as the antioxidant activity of these fractions, was also evaluated. The WF I which had the maximum content and activity was dialyzed and lyophilized to yield the phlorotannin which was further used to treat tomatoes to elucidate the antimicrobial and vegetable spoilage prevention potentials.

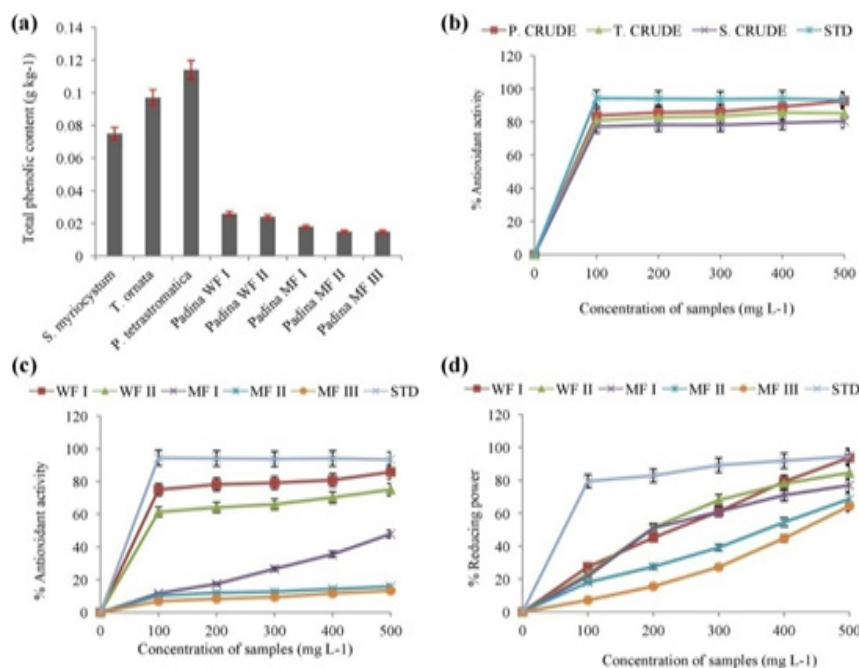


**Figure 1** Algal extracts and fractions: A) *Turbinaria* crude extract, B) *Padina* crude extract, C) *Sargassum* crude extract, D) Fractions of *Padina* crude extract on silica gel column chromatography: AP, aqueous phase after partitioning with dichloromethane; WF, water fraction; MF, methanol fraction, E) Lyophilized phlorotannin.

### Total phenolic content (TPC)

The highest phenolic content was found in *Padina* extract ( $\text{GAE} = 0.114 \pm 0.0005 \text{ g kg}^{-1}$ ) followed by *Turbinaria* ( $\text{GAE} = 0.097 \pm 0.0005$

$\text{g kg}^{-1}$ ) and *Sargassum* ( $\text{GAE} = 0.075 \pm 0.000 \text{ g kg}^{-1}$ ) extracts. The column fractions of *Padina* extract exhibited a decreasing order in the total phenolic content, the water fraction I (WF I) having the highest ( $\text{GAE} = 0.026 \pm 0.000 \text{ g kg}^{-1}$ ) (Figure 2).



**Figure 2** (a). Total phenolic content (TPC) in  $\text{g kg}^{-1}$  Gallic acid Equivalents of the crude extracts of *Sargassum*, *Turbinaria*, *Padina*, water fractions, and methanol fractions of *Padina* crude extract; (b). Percentage DPPH radical scavenging activity of crude extracts and standard ascorbic acid (STD); (c). Percentage DPPH radical scavenging activity of water fractions, methanol fractions, and standard ascorbic acid (STD); (d). Percentage reducing the power of water fractions, methanol fractions, and standard ascorbic acid (STD). (Mean values  $\pm$  SD;  $n=3$ ).



## Antioxidant activity of crude extracts and fractions

### DPPH radical scavenging activity

The Figure 2 illustrates the percentage DPPH radical scavenging potential of the various concentrations (100–500 $\mu$ L) of crude methanolic extracts of the three algae and the water fractions and methanol fractions of *Padina*. The antioxidant activity of the standard ascorbic acid has been given for reference. The highest activity was exhibited by 500 $\mu$ L crude *Padina* extract ( $92.65 \pm 3.43\%$ ) while the least was in 100 $\mu$ L crude *Sargassum* extract ( $77.17 \pm 1.24\%$ ). In case of *Padina* fractions, the activity was the highest in 500 $\mu$ L of water fraction I (WF I) ( $85.76 \pm 3.99\%$ ), and the least in 100 $\mu$ L of methanol fraction III (MF III) ( $6.88 \pm 2.56\%$ ). The average activity of the standard ascorbic acid was  $93.87 \pm 0.35\%$ .

### Reducing power assay

The reducing power assay was conducted with the fractions of crude *Padina* extract. The highest activity was observed in the 500 $\mu$ L of water fraction I (WF I) ( $93.81 \pm 26.39\%$ ), while the least was in 100 $\mu$ L of methanol fraction III (MF III) ( $7.21 \pm 22.99\%$ ). Overall the reducing power of each fraction showed an increase with the increase in concentration, and the same pattern was observed in case of fractions as a whole, i.e., the activity of WF I > WF II > MF I > MF II > MF III (Figure 2).

### Fourier transform infra-red (FT-IR) spectroscopy

The water fraction I (WF I) was characterized using Perkin Elmer System One FTIR. The FT-IR spectral study is preferred because of its non-destructive method, easiness to perform and the meager quantity requirement of the sample. The FT-IR spectrum (Figure 3) showed the following significant wavelengths corresponding to phlorotannins, previously reported. The strong band at 3405  $\text{cm}^{-1}$  denotes the stretching vibrations of O-H groups. The signal at 2924  $\text{cm}^{-1}$  corresponds to the C-H stretch vibration. The band at 662  $\text{cm}^{-1}$  was attributed to the stretching vibration of the benzene ring containing aromatic substitution.<sup>22</sup> The band at 1453  $\text{cm}^{-1}$  corresponds to the aromatic nuclei. The band at 1037  $\text{cm}^{-1}$  corresponds to the glycosidic linkage vibrations of C-O-C and C-O-H, indicating the presence of some carbohydrates in the sample.<sup>23</sup>

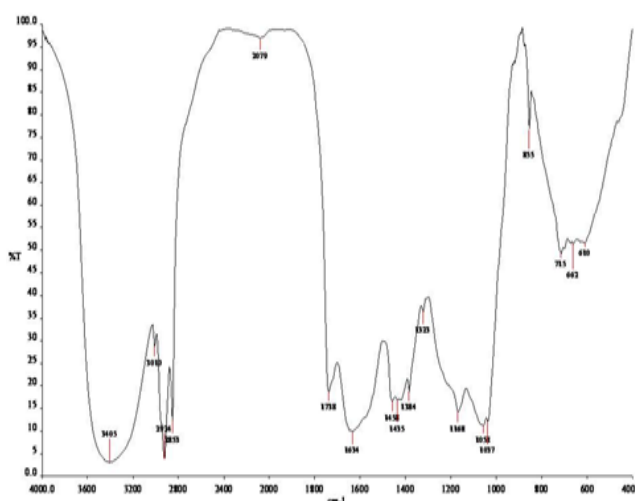


Figure 3 FT-IR spectrum of phlorotannin from *Padina tetrastrumtica*.

## Assessment of antimicrobial activity and spoilage prevention potential of phlorotannin (WF I) on tomatoes

The role of antioxidant and antimicrobial properties of phlorotannin in spoilage prevention and shelf life enhancement in an easily perishable vegetable like tomato was assessed. Figure 4 shows the various stages in the ripening and spoilage of tomatoes, and the treated and control set of tomatoes. The organoleptic properties recorded are given in Table 1.

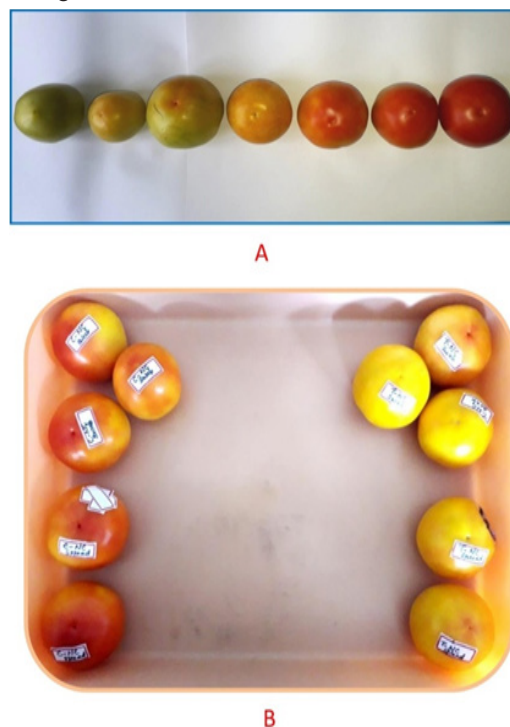


Figure 4 A - Various stages in the ripening and spoilage of tomatoes; B - The control set of tomatoes (left) and the treated set of tomatoes (right).

From the table, it is clear that the spoilage and the change in all parameters were initiated on 6th observation day after treatment, gradually leading to a slow decay process. Visible indicators of microbial spoilage were absent during the study period, but the presence and extent of microbial load on samples were elucidated using plating techniques. One important observation was that the color of treated tomatoes turned yellow from green but remained yellow throughout the study. Whereas the untreated set of tomatoes turned red quicker. There was a slight weight loss from the third observation day, and it gradually increased with time. The microbial load on unwashed, untreated tomatoes was the highest. The treated tomatoes exhibited a higher microbial load than the control at the beginning of the experiments. This may be due to the dampness of the phlorotannin solution used to treat the tomatoes. On the second observation day, swab plates of treated batch showed microbial growth than the control plate, and vice versa in spread plates. The trend of microbial growth reversed on the third observation day when the control batch of both chilled and non-chilled set exhibited a higher microbial load than the treated batch of the chilled and non-chilled set. On the fourth observation day also, the microbial growth was higher in control chilled and non-chilled batches than in treated batches of tomatoes. There was a gradual increase in the microbial load in all sets of samples, but lesser in treated sets. The chilled sets of tomatoes

showed early color changes and an indication of spoilage than the non-chilled sets stored in open lab space. The high antioxidant and antimicrobial properties of phlorotannins might be preventing the color change and early spoilage in tomatoes. The treated tomatoes retained fresh appearance as compared to their untreated counterparts. There were four colonies observed in the plates: white rhizoid raised

colony, reddish circular flat colony, pale white circular flat colony, and a yellow circular flat colony. The red, white, and pale white colonies were Gram-negative, whereas the yellow colony was Gram-positive. The colony-forming units (CFU) or cells per milliliter of the samples on each observation day was calculated using the formula (Table 2). The CFU mL<sup>-1</sup> range from 106 to 108 cells mL<sup>-1</sup> indicates spoilage.

**Table 1** Organoleptic/ sensory indicators observed in control and treated tomatoes on every third day after treatment, for twenty days. (x) Indicates absence & (□) indicates the presence of a property. CC, control chilled; CNC, control non-chilled; TC, treated chilled; TNC, treated non-chilled

Observation days	Experiment sets	Soft spots	Brown Spots	Color changes	Loss of turgor	Texture/ Hardness	Odors	Visible	Weight	Weightloss (%)
						Indicators of microbial spoilage				
1st day	CC	x	x	x	x	x	✓	x	174.94	0
	CNC	x	x	x	x	x	✓	x	275.39	0
	TC	x	x	x	x	x	✓	x	183.06	0
	TNC	x	x	x	x	x	✓	x	209.52	0
2nd day	CC	x	x	x	x	x	✓	x	174.94	0
	CNC	x	x	x	x	x	✓	x	275.39	0
	TC	x	x	x	x	x	✓	x	183.06	0
	TNC	x	x	x	x	x	✓	x	209.52	0
3rd day	CC	x	x	✓	✓	✓	✓	x	174.62	5
	CNC	x	x	✓	✓	✓	✓	x	274.27	9
	TC	x	x	✓	x	x	x	x	183.01	6
	TNC	x	x	✓	x	x	x	x	206.48	8
4th day	CC	x	x	same	✓	same	✓	x	173.58	9
	CNC	x	x	same	✓	same	✓	x	274.12	2
	TC	x	x	same	x	same	✓	x	182.98	8
	TNC	x	x	same	x	same	✓	x	204.51	9
5th day	CC	x	x	same	same	same	same	x	173.02	1
	CNC	x	x	same	same	same	same	x	273.99	6
	TC	x	x	same	same	same	same	x	182.95	6
	TNC	x	x	same	same	same	same	x	204.48	1
6th day	CC	x	✓	✓	same	✓	✓	x	172.96	1
	CNC	x	✓	✓	same	✓	✓	x	273.92	8
	TC	x	✓	✓	same	✓	✓	x	182.89	6
	TNC	x	✓	✓	same	✓	✓	x	204.32	6
7th day	CC	x	same	same	x	same	✓	x	172.73	9
	CNC	x	same	same	x	same	✓	x	273.58	4
	TC	x	same	same	x	same	✓	x	182.74	6
	TNC	x	same	same	x	same	✓	x	204.23	1
8th day	CC	x	✓	✓	✓	same	✓	x	172.57	5
	CNC	x	✓	✓	✓	same	✓	x	273.25	6
	TC	x	✓	✓	✓	same	✓	x	182.82	6
	TNC	x	✓	✓	✓	same	✓	x	204.12	1

**Table 2** Colony-forming unit (CFU mL<sup>-1</sup>) of the samples at 10<sup>-4</sup> dilution on 1st, 2nd, 3rd, and 4th observation days

Type of Plates	Colony forming unit (CFU mL <sup>-1</sup> )			
	Day 1	Day 2	Day 3	Day 4
Control Chilled	5.68×10 <sup>5</sup>	5.94×10 <sup>5</sup>	4.26×10 <sup>6</sup>	4.63×10 <sup>6</sup>
Treated Chilled	4.20×10 <sup>5</sup>	4.82×10 <sup>5</sup>	3.09×10 <sup>6</sup>	3.29×10 <sup>6</sup>
Control	6.12×10 <sup>5</sup>	6.54×10 <sup>5</sup>	5.17×10 <sup>6</sup>	5.37×10 <sup>6</sup>
Non-Chilled				
Treated	5.63×10 <sup>5</sup>	5.81×10 <sup>5</sup>	3.92×10 <sup>6</sup>	4.16×10 <sup>6</sup>
Non-Chilled				

### Antimicrobial activity of phlorotannin

The microbial colonies isolated from the plates were developed into broth cultures and the phlorotannin was directly tested for its antimicrobial activity using agar well diffusion assay. The zone of inhibition of various microbes has been given in Table 3. The highest antimicrobial activity (39 mm diameter) was observed in 100µL of WF I against the red colony, while the least activity (1.3 mm diameter) was observed in the 100µL of WF I against yellow colony. In accordance with these values, it was observed in the agar plates that in course of time, the red colonies had disappeared in the treated tomatoes, while the white and yellow colonies were present but in lesser amounts than in the control batch. These findings hence point towards the efficient antimicrobial activity of phlorotannins in the prevention of early spoilage in tomatoes. The inhibition of fungal components was however not studied. The phlorotannin also exhibited antimicrobial activity at higher concentrations against all the five microbes tested (Table 4).

**Table 3** Zone of inhibition (including 8 mm borer diameter) of various concentrations of *Padina* phlorotannin against vegetable spoilage microbes in tomato (Note: PC, positive control; NC, negative control)

Conc. of PadinaPhlorotannin	Zone of Inhibition (mm)		
	Microbe A	Microbe B	Microbe C
	(red)	(yellow)	(white)
PC	44	2.6	3.7
NC	-	-	-
30	33	-	1.7
50	27	-	2.5
70	36	-	3.1
100	39	1.3	3.3

**Table 4** Zone of inhibition (including 8 mm borer diameter) of various concentrations of *Padinaphlorotannin* against selected food borne microbes (Note: PC, positive control; NC, negative control)

Algal extract	<i>Padina tetrastrum</i> phlorotannin (Zone of inhibition(mm))				
	PC	NC	30	60	90
Concentration (µL)					
<i>Escherichia coli</i>	16	-	-	12	13
<i>Klebsiella pneumoniae</i>	15	-	-	10	12
<i>Pseudomonas aeruginosa</i>	18	-	11	14	16
<i>Micrococcus luteus</i>	13	-	11	13	13
<i>Bacillus cereus</i>	30	-	12	15	17

### Discussion

In brown algae, the total phenolic content estimation corresponds to the content of phlorotannin in them, as phlorotannins form their main phenolic constituent. Phlorotannins are highly hydrophilic and exist abundantly in marine brown algae as an integral component of their cell wall as well as in vesicles called physodes.<sup>24</sup> The phlorotannins stored in physodes are soluble, while that of cell walls are insoluble, forming complexes with proteins and alginic acid.<sup>23</sup> In previous studies, the phlorotannin levels reported were nearly 20% in Fucales and 30% in Dictyotales (dry weight). The content of cell-wall bound phlorotannins is usually much lower than the amount of soluble/free phlorotannins.<sup>25</sup> The amounts found in this study was 0.15, 0.48, 0.57, 0.13, 0.12, 0.09, 0.075, and 0.075% in Sargassum, Turbinaria, Padina, WF I, WF II, MF I, MF II and MF III respectively which is considerably lower (i.e., ≤0.1% of dry matter). This difference in yield may be attributed to the factors like species, geographic location of collection site, period collection, environmental factors, and the extraction and purification techniques employed. Also, phlorotannins being hydrophilic and polar are more soluble in polar solvents like water, methanol, ethanol, etc., and a multi-component solvent system like 80% methanol effectively extracts out phlorotannins.

The antioxidant activity observed in crude Padina extract was comparable to that shown by the ascorbic acid, which further supports the findings previously published. The overall activity of water fractions was higher compared to the methanol fractions, due to higher amounts of phlorotannins in water fractions (especially WF I) as the polarity of water is more than methanol. The antioxidant property of the phlorotannins obtained from brown seaweeds has already been reported worldwide. Free radical scavenging property is crucial for any drug candidate molecule or food industry targeted molecule for imparting its benefits effectively. The antioxidant property of phlorotannins has been reported to be comparable with that of many commercially available antioxidant compounds like ascorbic acid and α-tocopherol.<sup>26</sup> It has been reported that the aqueous extract of Padina tetrastrum and Padina gymnospora from Mandapam coast in Tamil Nadu, showed higher TPC which corresponded with higher DPPH scavenging of the extracts. The present findings correlated with this report. It was also noted that polar solvent extracts of brown algae exhibited high antioxidant properties, and the chemical composition analysis of these extracts revealed the presence of radical scavengers such as phlorotannins and fucoxanthin.<sup>27</sup> The high activity observed for Padina may be due to the abundance of

low molecular weight phlorotannin polymers, which are known to be powerful antioxidants. The low degree of polymerization allows more number of free hydroxyl units which in turn allows a higher level of hydrogen donation and consequent stabilization of free radicals.<sup>28</sup> Phlorotannins purified from the brown algae such as *E. bicyclis*, *E. kurome*, *H. fusiformis*, *U. pinnatifida*, and *E. cava* have displayed potent antioxidant effects comparable to antioxidants like ascorbic acid and  $\alpha$ -tocopherol as reported earlier.<sup>15</sup>

The antimicrobial study of algal phenolics has been reported specifically against *Staphylococcus aureus* and *Bacillus* spp. The methanol and diethyl ether extracts of brown seaweeds like *Padina gymnospora*, *Turbinaria ornata*, *Turbinaria conoides*, *Dictyotadichotoma*, *Ascophyllum nodosum*, and *Ecklonia cava*, containing phlorotannins were usually reported with higher antimicrobial activity against a wide range of bacterial pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Bacillus cereus*, *Listeria monocytogenes* etc.<sup>4</sup>

The use of seaweed extracts to deal with the postharvest loss of fruit started around the 2000s. A commercial seaweed extract (Cytolan Star®) was found to improve the quality of orange fruit (cv. Valencia) during storage and maintained for a longer period. Thereafter the use of seaweed extracts for preventing microbial spoilage and pathogenic attack on crops was investigated further. The ethanolic extract of the red alga *Solieria robusta* was found to inhibit five fruit spoiling fungi *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *P. funiculosus*, and *Phytophthora infestans*, and also the growth of *Macrophomina phaseolina*, *R. solani*, and *Fusarium solani*. Besides, the seaweed extracts also stimulated growth in strawberry by preventing pathogenic attacks and physiological hazards both in vivo and under storage.<sup>21</sup>

## Conclusion

The three brown algae were found to be valuable sources of phlorotannins, reported with a wide range of biological activities. In this study, *P. tetrastrumatica* exhibiting the highest total phenolic content showed effective antimicrobial and antioxidant potential against food spoilage microbes. The promising results of bacterial growth inhibition and inactivation by purified *Padina* phlorotannin and the lack of studies carried out in real foods open a novel research niche with a high application at food industrial level. This is the first report on the direct application of phlorotannin from *P. tetrastrumatica* on tomatoes for spoilage prevention. The algal product requires further elaborate exploration of its stability and properties for its direct incorporation in food or use as a natural food preservative. Consequently, the twofold use of algal bioactive as an antimicrobial and antioxidant in food products has good prospects in the preservation of easily perishable vegetables. It helps in the development and innovation of sustainable food products that are as natural as possible. Also, the phlorotannins being water-soluble, its coating can be easily removed before consumption, unlike the other preservatives that require chemicals or specific solutions for their removal. Hence this suggests that further research may give insights into the proper utilization of macroalgae like *P. tetrastrumatica* for the largescale extraction of a natural and powerful antioxidant and antimicrobial agent like phlorotannin for application in foods, both as a preservative and a nutraceutical.

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## Conflicts of interest

The authors declare that there was no conflict of interest.

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