Synergistic Antioxidant and Antibacterial Effects of the Combination of Chitosan from Shrimp Shells with Polyphenols from *Cynara cardunculus var. scolymus* (L.)

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Abstract

Natural compounds are often endowed with biological properties of health, food and pharmalogical interest. Chitosan from crustacean shells and polyphenols from artichoke heart, Cynara cardunculus var. scolymus (C. scolymus), are examples of natural compounds that are preferable to chemical substances as food preservatives. The current study investigates the antioxidant and antibacterial properties of chitosan extracted from shrimp shells waste, both alone and combined with polyphenols of C. scolymus. Shrimp shells were washed, dried, ground, and the issued flour was demineralized and deproteinised to prepare chitin. Chitosan was obtained by deacetylation of chitin and then analyzed on the basis of its moisture, ash, protein, molecular weight and degree of deacetylation. The polyphenols of C. scolymus were extracted using water and methanol as solvents. Total phenolic and flavonoid contents of C. scolymus were determined using spectrophotometry. DPPH and β-carotene bleaching tests were used to assess the antioxidant activity of chitosan and C. scolymus extracts. The antibacterial activity of chitosan and C. scolymus extracts against five pathogenic strains and two beneficial strains was determined. Results have shown that chitosan extraction yield was about 5.80 ± 1.09 %, and it contains 4.43 ± 0.57, 1.32 ± 0.62 and 0.18 ± 0.04 % of moisture, ash and protein, respectively. Its molecular weight and its degree of deacetylation were about 859.48 ± 21.62 KDa and 75%, respectively. The combination of chitosan and C. scolymus extracts was more effective on DPPH radical scavenging capacity and β-carotene degradation prevention ($P<0.05$) than those of chitosan or C. scolymus extracts used separately. Furthermore, this combination significantly inhibits the growth of the five pathogenic bacteria. This

suggests that chitosan, in association with C. scolymus polyphenols might be used to prevent food spoilage.

Keywords: - Chitosan; *Cynara cardunculus* var. *scolymus*; Polyphenols; Shrimp shell waste; antioxidant; antibacterial.

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Introduction

Food products are subjected to various chemical and microbial spoilage, under normal manipulation, processing and storage conditions, resulting in various changes in their characteristics and rendering the food undesirable or inappropriate for human consumption [1]. The oxidation process is the main cause of chemical food spoilage, occurring during food processing, cocking and storage. Thus, it produces rancid, off-odours and off-flavours, which lower the product's customer acceptability. The polyunsaturated fatty acids are mostly affected by oxidation, which may reduce the nutritional value, food quality and the shelf life of the oxidized product. It generates free radicals, peroxides and other toxic metabolites that are harmful to consumer's health [2].

On the other hand, microbiological deterioration occurring during food storage and distribution is a major issue that affects food quality and safety while shortening shelf life. Furthermore, the development of pathogenic microorganisms in food is associated with infections and/or intoxications [3].

Synthetic food preservatives agents have been used for decades to prevent food spoilage despite their negative health effects increased numbers of multi-drug resistant bacteria. Consumer awareness is forcing manufacturers to gradually replace them with natural antioxidant and antibacterial compounds [4]. Therefore, it is important to look for new useful natural compounds to preserve food from microbial spoilage and oxidation.

Chitosan and polyphenols are two examples of natural compounds with antioxidative and antibacterial properties [5]. Chitosan is a functional ingredient that may be used in food products to improve shelf life and promote consumer health [6]. It is a linear cationic polysaccharide polymer composed of β-(1–4)-linked N-Acetyl-D-Glucosamine [7]. Chitosan is made by partial alkaline deacetylation of chitin from shrimp waste, insects, fungi, or molluscs, in which acetyl groups are changed into amino groups [6]. Recently, this polymer has attracted considerable interest because of its favourable characteristics such as biodegradability, biocompatibility, safety, and non-allergenicity. In addition, it has many biological properties such as antioxidant, antibacterial, antidiabetic, anti-cancer, anti-obesity and neuroprotective effects, among others [8].

Polyphenols are a group of secondary plant metabolites that may be used as natural preservatives in food. In addition to being healthy, they can enhance the shelf life of perishable products [9]. The Mediterranean plant Cynara cardunculus var. scolymus (artichoke) belonging to the Asteraceae family, is one of the richest sources of bioactive phenolic compounds including gallic, caffeic and coumaric acids [10, 11]. Artichoke is rich in nutrients such as proteins, lipids, carbohydrates, vitamins and minerals and has good sensory properties [10]. It has been reported that C. scolymus

extracts have many therapeutic effects such as anticancer, hepatoprotective, antibacterial, antioxidant, anti-HIV activities, and can inhibit cholesterol biosynthesis and lipid oxidation [12, 13].

To our knowledge, the potential synergistic effect of chitosan and polyphenols on antioxidant and antibacterial activity has not been investigated. The present study was designed and run in order to complete this lack while valorizing shrimp shell waste on one hand, and to explore the synergistic effect of the combination of their chitosan content with artichoke heart polyphenols in trapping free radicals and inhibition of growth of bacteria responsible for food poisoning, on the other hand.

Materials and Methods

Chemicals and reagents

All reagents used in this study were obtained from Sigma-Aldrich (St Louis, Mo USA).

Cynara scolymus L. (artichoke)

Fresh mature artichoke was purchased from a local market in Chlef (West Algeria: 36° 10' 0.001'' N 1° 19' 59.999'' E) during the harvest season. The external bracts and leaves have been removed. The artichoke heart was cut into small parts and washed in a 0.2% (V/V) citric acid solution before dried in an oven at 40°C. Samples were milled using an electric grinder, and the obtained powder was stored at 4°C in flasks until extraction.

Shrimp shell wastes

Shrimp shells wastes were collected from local restaurants in Mostaganem (West Algeria: 36° 8' 26.664'' N 1° 19' 50.124'' E) and stored in plastic bags at -18°C until extraction.

Bacterial strains

Five reference strains belonging to the American Type Culture Collection (Staphylococcus aureus ATCC 29213, Bacillus subtilis ATCC 6633, Listeria monocytogenes ATCC 7644, Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 25922, as well as two beneficial bacterial strains (Bifidobacterium animalis ssp lactis Bb12 provided by CHR Hansen, Hoersholm, Denmark and Lactobacillus rhamnosus LbRE-LSAS from our laboratory collection) were used in the present study to investigate the antibacterial properties of Chitosan, artichoke heart polyphenol extracts and the combination of chitosan-artichoke heart polyphenols.

Extraction of polyphenols from artichoke heart

Two different polyphenol extracts were prepared from the powdered artichoke heart: methanolic and aqueous extracts. 10 g of the powdered artichoke were mixed with 100 mL of methanol (98°) and kept on a rotary shaker for 2h. The extract was filtered on N°1 Watman paper and concentrated in a rotary evaporator14].

The artichoke heart aqueous extract (AqE) was obtained using the protocol described by Dhanami et al. [15]. Briefly, 5 g of the powdered artichoke was gradually added to 50 mL of distilled water, and the mixture was stirred for 14h in a rotary shaker. The filtration of the mixture was followed

by a concentration of the extract using a rotary evaporator. The obtained extracts were separately stored in dark flasks at 4°C.

Determination of the total phenolic content of artichoke heart extracts

The total phenolic contents of methanolic and aqueous extracts of artichoke hearts were determined by the Folin–Ciocalteau method [16]. 200 mL of the extract (1 mg/mL) were mixed with Folin-Ciocalteu reagent (1.5 mL) (diluted 10-fold) and 1.5 mL of 6% (w/v) sodium bicarbonate solution. The tubes were incubated at room temperature in the dark for 90 min before measuring the absorbance at 725 nm using a UV–visible spectrophotometer. The concentration of polyphenols in the samples was derived from a standard calibration curve of gallic acid. The total polyphenol content of the extracts was expressed as mg of Gallic Acid Equivalent (GAE) per 100g of extract.

Determination of the total flavonoid content of artichoke heart extracts

The total flavonoid content of methanolic and aqueous extracts of artichoke heart was determined by the method described by Woisky and Salatino [17]. 1 mL of the extract (1 mg/mL) was added to 4 mL of the mixture of distilled water, 0.3 mL of 10% (w/v) aluminium chloride and 0.3 mL of 5% (w/v) sodium nitrite. After 5 min at room temperature, 2 mL of 1M sodium hydroxide was added to the solution which was diluted with 10 mL of distilled water. Absorbance was measured at 510 nm. Flavonoid content of the extracts was calculated using a standard quercetin (QE) calibration curve, and expressed as mg QE per 100g of extract.

Chitosan extraction and characterization

Raw material preparation

The discarded shrimp shells were removed from the flesh and rinsed with tap water before being dried at room temperature until consistent weight and crushed to a powder.

Chitin preparation

Chitin was obtained by demineralization and deproteinization of the shell waste powder as described by Youcefi and Riazi [18]. Shrimp shell waste powder was slowly added to 2M HCl in a ratio of 1/15 (w/v) and 12h stirred at room temperature for its demineralization. Then, the acid was removed by filtration. The sample was washed continuously until the water pH was neutral and then dried overnight at 30°C. The deproteinization step was carried out using a 3% (w/v) NaOH solution in a 1/10 (w/v) ratio between the demineralized shell waste and solvent. The mixture was then heated to 90°C and stirred for 1h. The obtained chitin was filtered and washed continuously until the water pH was neutral, and then dried overnight at 50°C.

Deacetylation of chitin

Deacetylation of chitin involves removal of acetyl groups to produce crude chitosan and was run according to the method described by Youcefi and Riazi [18]. Chitin was added to a 50% (w/v) NaOH solution at a ratio of 1:20 (w/v), and heated at 60°C for 8h with stirring. The mixture was then filtered and the obtained chitosan was water-washed until pH neutrality. Then, chitosan was dried overnight at 50°C, grounded into a fine powder, and bottled.

Characterization of chitosan

Moisture and ash contents, molecular weight, presence of protein and degree of deacetylation of chitosan were determined.

Moisture content determination

The moisture content (MC) of chitosan was determined by gravimetric method as described by Olafadehan et al. [19]. A previously weighed sample of chitosan was dried at 100 °C to constant weight. The moisture content (%) was calculated using the following formula:

$$MC\ (\%) = ((W-w)/W) \times 100 \quad (1)$$

Where W and w are the weights of the chitosan sample, before and after drying, respectively.

Ash content determination

The ash content of chitosan (AC) was determined by the method of Olafadehan et al. [19]. 1 g of chitosan was placed in a pre-weighed ceramic crucible and heated to 600°C in a muffle furnace for 2h. The crucible was weighed after 30 min of cooling in a desiccator. This procedure was continued until ash was obtained (constant weight). The ash content (%) was calculated using the following formula:

$$AC\ (\%) = [W1 / (Wc \times((100-MC)/100))] \times 100 \quad (2)$$

Where W1 and Wc are the weights of the residue and the chitosan sample, respectively. MC is the moisture content of chitosan (%).

Protein content determination

The protein content of chitosan was determined by the method of Lowry et al. [20]. The Bovine Serum Albumin (BSA) was used as a standard. Briefly, chitosan was dissolved in an acetic acid solution (0.1 M) at a concentration of 1 mg/mL. After addition of the Lowry solution, the absorbance of the mixture was measured at 720 nm.

Protein concentration of chitosan was calculated using the standard protein calibration curve.

Viscosity average-molecular weight

The viscosity average-molecular weight (Mw) of the extracted chitosan dissolved in a mixture of 0.1 M acetic acid/0.1 M sodium acetate was determined by the method described by Sugiyanti et al. [21] using a viscometer (Brookfield Digital viscometer SNB-1, USA). The viscosity average-molecular weight was calculated using the Mark-Houwink parameters as follows:

$$[\eta] = k.\ (Mw).\ \alpha \quad (3)$$

Where [η] is the intrinsic viscosity, Mw is the Viscosity average-molecular weight, k and α are constants (k = 0.078 cm3.g-1 and α = 0.76).

Degree of deacetylation (DD)

The extracted chitosan was identified by Fourier transform infrared (FTIR) spectroscopy (Bruker Alpha-T) in the 400 to 4000 per cm range. To do so, 10g of chitosan was mixed with 100 g of potassium bromide (KBr) and compressed to prepare salt disks (10 mm diameter). The disks were conditioned in a desiccator placed in an oven at 80°C for 16 h before being analyzed by spectral reading. The absorbance at 1655 and 3450 cm^{-1} was used to calculate the DD according to the following formula:

$$100-((A1655\ cm^{-1})/(A3450\ cm^{-1}))/1.33\ X\ 1OO \quad (4)$$

Antioxidant activity

DPPH radical scavenging activity

The free radical DPPH scavenging activity of artichoke heart extract, chitosan and their association was determined according to the method of Blois [22]. Artichoke heart extract and chitosan were used either separately or combined together at the same volumes (1 mL) and same concentrations of 2, 4, 6, 8, and 10 mg/mL. The chitosan sample (1 mL), artichoke heart extract (1 mL) or both combined was added to 1 mL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, and incubated 30 min in the dark at room temperature. The absorbance was measured against blank at 517 nm, and the inhibition rate of DPPH was calculated using the following formula :

$$I\ (\%) = (1-(A_{blank}–A_{sample})/A_{blank})) \times 1\ 00 \quad (5)$$

Where: I (%): inhibition rate of the DPPH radical

Ablank: absorbance of the control; Asample: absorbance of the sample.

The concentration of chitosan or artichoke heart polyphenol extract providing 50 % inhibition rate of DPPH (IC50: mg/mL) was obtained from the algorithm of the graph plotted inhibition rate against compound concentration.

β -Carotene-linoleic acid assay

The β-carotene bleaching essay was performed according to the method of Kelin and Tepe [23]. A mixture of 0.5 mg of β-carotene, 1 mL of chloroform, 25 µL of linoleic acid and 200 mg of tween 40 was prepared. Then, chloroform was completely removed by vacuum evaporation. The obtained mixture was diluted with 100 mL of oxygen-saturated distilled water with a vigorous shaking to form an emulsion.

2.5 mL of the resulting emulsion were dispersed in different test tubes, 350 µL of artichoke heart extract, chitosan (2mg/mL) or an equivalent mixture of artichoke extract and chitosan (V/V) were added to the mixture and allowed to stand for 48h at room temperature. The absorbance of the mixtures was measured against control at 490 nm. The inhibition rate of β-carotene bleaching (I%) was calculated according to the following formula:

$$I\ (\%) = (A_{control} – A_{sample})/A_{control}) \text{ X } 100 \quad (6)$$

Where: $A_{control}$: absorbance of the control. A_{sample}: absorbance of the sample.

Determination of antibacterial activity and minimum inhibitory concentration (MIC)

The antibacterial activity of artichoke extracts, chitosan or their combinations was evaluated against five pathogenic and two beneficial bacterial strains using agar disc diffusion method. Bacterial inocula were prepared by inoculating a 16h bacterial preculture in Muller–Hinton broth (MHB; Fluka) at 37°C. The density of overnight cultures was adjusted to the turbidity of 0.5 McFarland Standard (equivalent to 106 CFU/mL) using MHB. Sterile filter paper discs were impregnated with 20 µL of artichoke heart extract, chitosan or their combination (1/1) and placed on the inoculated plates. The Petri dishes were incubated 24h at 37 °C, and then the inhibition zone diameters (mm) were measured.

The minimum inhibition concentration (MIC) is the lowest concentration of antimicrobial agent at which no visible bacterial growth was observed after 24h of incubation at 37°C. Variable volumes of artichoke heart extract, chitosan or an equivalent mixture of both compounds (1/1) were added in tubes in order to obtain gradient geometric progression equal to 2 and from 0 to 50 mg/mL. After what, 1 mL of each dilution was mixed with 10 µL of a bacterial suspension (108 CFU/mL) and 19 mL of MHB. The Final concentrations obtained correspond to 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 and 0.01 mg/mL. All tubes were incubated for 24 h at 37°C. The bacterial growth was observed visually after inoculation onto the MHB agar.

Statistical analysis

Each experiment was repeated three times, and the values are mean ± standard deviation (SD) of the three measurements. The Analysis of variance (ANOVA) was carried out using the Past software version 3.19. The results that correlate to a P value <0.05 are considered statistically significant.

Results

Total phenolic (TPC) and flavonoid (TFC) contents in artichoke extracts

The determination of total phenolic compounds and flavonoid contents in methanolic and aqueous extracts of heart artichoke showed significant differences (P<0.05) between the two extracts (Figure 1). The amount of total polyphenols extracted with methanol from heart artichoke (159.42 ± 5.24 mg AGE/g) is higher than that obtained with water (142.38 ± 3.45 mgAGE/g); while total flavonoid content of methanolic extract (89.87 ± 2.95 mgQE/g) was lower than that of aqueous extract (118.64 ± 2.74 mgQE/g).

Previous studies [24, 25] have reported that artichoke is a good source of polyphenols and flavonoids. These compounds include cafeic acid and its mono and dicaffeoylquinic derivative acids, which are available to humans in their diet, as well as a small amount of luteolin and apigenin glycoside. Thereby, Punzi et al. [26] reported that the heart, leaves, external bracts and stems of artichoke contain 1446 ± 15, 1343 ± 25, 907 ± 37 and 774 ± 28 mg/kg fresh weight of total phenolic compounds, respectively. Less value of total polyphenol content (528 µg GAE/g) was observed in a typical variety of artichoke (i.e Carciofo di Montoro) of southern Italy ([27], than that obtained in the present study. Durmus et al. [28] have observed that frozen artichoke heart contains 814.70 ± 58.98 mg AGE/100g of total polyphenols.

The difference between the polyphenol and flavonoid extraction yields observed in this experiment and those reported by other studies would be probably due to the nature of the solvents used as well as to the variety and origin of the plant material used [29].

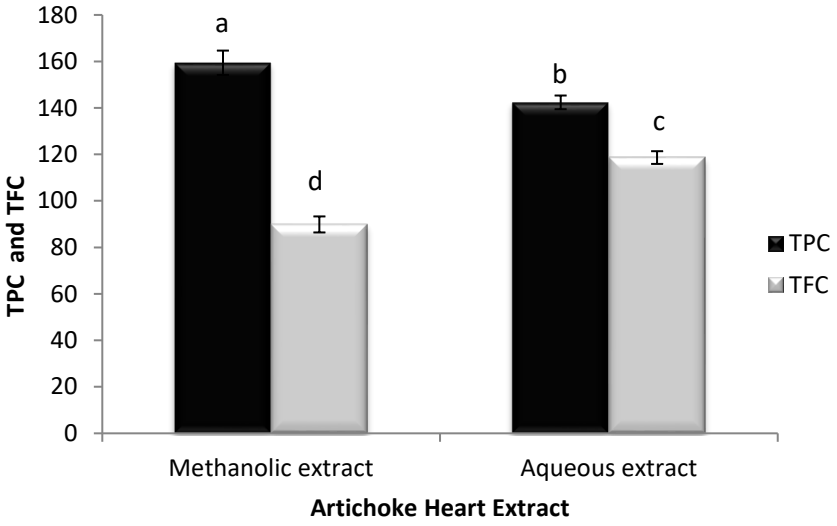

Figure 1. Total phenolic (TPC: mg Gallic acid equivalent) and flavonoid (TFC: mg Quercetin equivalent) contents of methanolic and aqueous extracts of artichoke heart. Values represent the mean ± SD of 3 measurements (n = 3). Alphabetic letters indicate significantly different values P<0.05.

Characterisation of chitosan

After pre-treatment of the shrimp shell wastes followed by demineralisation, deproteinization and deacetylation steps, a white, colourless and tasteless powder, representing the chitosan, was obtained. Shrimp shell chitosan extraction yield obtained in this study (5.80 ± 1.09 %) is lower than that reported by Youcefi et al. [5] (10.73 ± 0.94 %) or for crab shells by Bolat et al. [30] (7.25 %); but is similar to that of crab shells (5.90 ± 0.61 %) as shown by Sujianti et al. [21]. These differences might be due to the composition of crustacean exoskeletons, which depends on the source, species, age, gender, season and environmental conditions [31, 32].

Figure 2. Aspect of chitosan extracted from shrimp shell waste.

Table 1. Physicochemical properties of chitosan

Parameter	Moisture content (%)	Ash content (%)	Protein content (%)	Molecular weight (KDa)	Degree of deacetylation (%)
Result	4.43±0.57	1.32±0.62	0.18±0.04	859.48±21.62	75

The low ash and protein contents of chitosan obtained here reflects the efficacy of demineralisation and deproteinization which removed almost all the minerals and proteins. The low ash content of chitosan can be due to the low content of calcium carbonate in shrimp shells as has been reported by Youcefi et al. [5]. According to Narudin et al. [33], the residual ash content of chitosan affects its solubility and viscosity and high residual ash content indicates the less efficient demineralization of the shells waste, probably due to the low HCl concentration used in the demineralization. Youcefi et al. [5] have reported that chitin occurs naturally in association with proteins, and some of this chitin can be extracted by mild methods, but other portion is not readily extracted.

The results of this study have shown that the Molecular weight of chitosan (859.48±21.62 KDa) determined herein is lower than that found by Youcefi et al. [5] (1414.33 ± 16.99 kDa) and slightly higher than that reported by Sujianti et al. [21] (ranged from 557 to 690 KDa). Molecular weight of chitosan depends on the source of raw material and the method of extraction [5, 21]. Chitosan deacetylation degree registered in the present study (75%) is similar to those determined by Olafadehan et al. [19] for shrimp (81.79%) and crab (81.94%) shells.

Antioxidant activity

DPPH radical scavenging activity

Antiradical activity of artichoke extracts, chitosan and their different combinations is illustrated by the respective increasing IC50 values reported in Figure 3: chitosan-MetE (0.642 mg/mL), chitosan-AqE (1.143 mg/mL), chitosan (1.728 mg/mL), MetE (2.003 mg/mL), and AqE (3.117 mg/mL). DPPH radical scavenging activity of ascorbic acid (IC50 = 0.071 mg/mL) outperformed that of chitosan, artichoke extracts, and their combinations ($P<0.05$). The combination of artichoke extracts and chitosan outperformed chitosan or artichoke extracts alone in terms of free radical scavenging capacity ($P<0.05$).

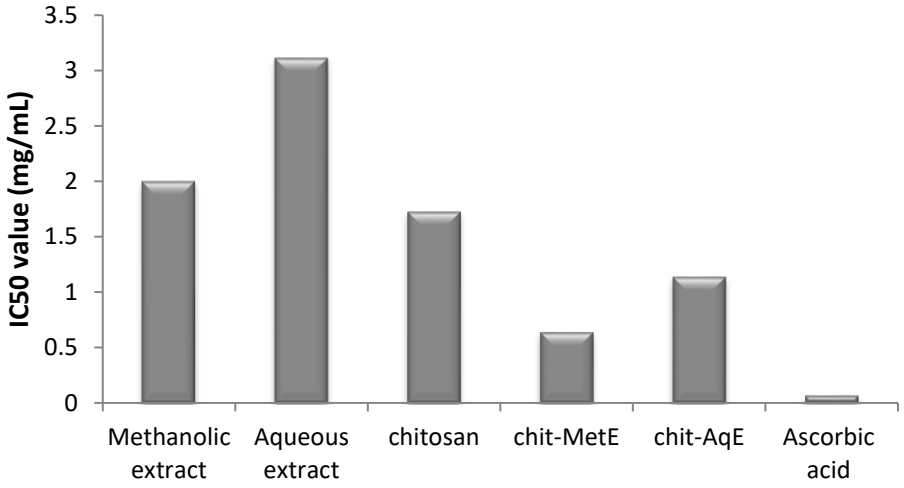

Figure 3. IC50 (mg/mL) values of artichoke extracts, chitosan and their combination in comparison with ascorbic acid as a standard. Values represent the mean ± SD of 3 measurements (n = 3). Alphabetic letters indicate significantly different values at $p<0.05$.

β-carotene bleaching

Figure 4 shows the antioxidant capacity of artichoke extracts, chitosan and their combination as determined by the β-carotene bleaching method. In the absence of an antioxidant, the free radicals generated upon the abstraction of a hydrogen atom from linoleic acid attack the β-carotene molecule, causing it to lose its characteristic yellow colour. The presence of an antioxidant can reduce the β-carotene bleaching.

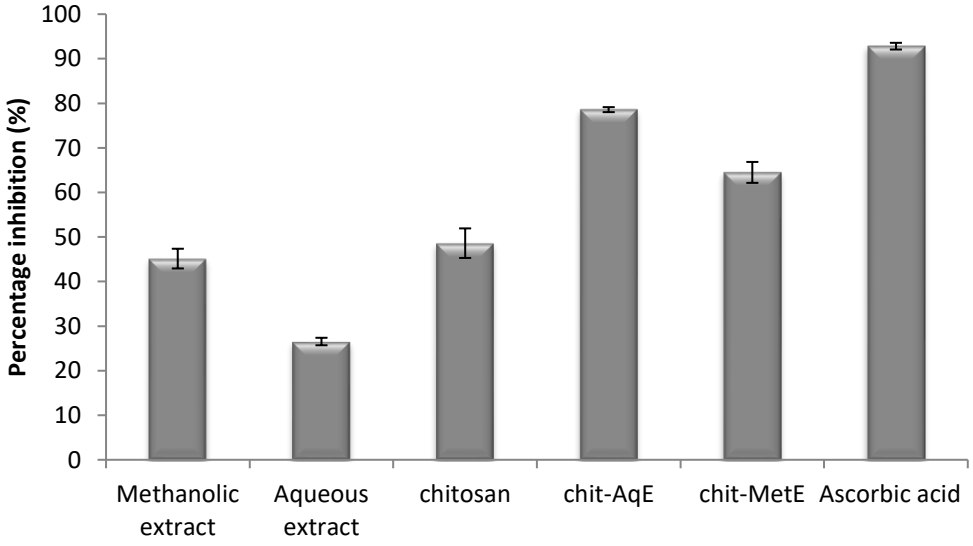

Figure 4. Inhibition rate of β-carotene bleaching by artichoke extracts, chitosan and their different combinations in comparison with ascorbic acid as a standard. Values represent the mean ± SD of 3 measurements (n = 3). Alphabetic letters indicate significantly different values at $p<0.05$.

In the present study, the highest inhibition rates of β-carotene bleaching ($P<0.05$) were obtained when chitosan was combined with heart artichoke phenolic extracts. The inhibition rate registered with the chitosan/methanolic polyphenol extract and chitosan/aqueous extract combinations were 87.58 ± 0.56 and 64.49 ± 2.35 %, respectively. Moderate inhibition rate of β-carotene bleaching was recorded when chitosan (48.61 ± 3.32 %) and methanolic polyphenol extract (45.15 ± 2.21 %) were used separately. In addition, aqueous extract of artichoke heart polyphenol extract had less inhibition effect of β-carotene bleaching (26.54±0.84%). Nevertheless, no extract, neither chitosan nor its different combinations with polyphenol extracts outperformed the capacity of ascorbic acid in inhibition of lipid peroxidation (92.82 ± 0.75 %).

In agreement with the results of the present study, the free radical scavenging capacity of chitosan was evaluated between 3.7 and 16.8 % with a concentration of shrimp shell chitosan of 1 to 2 mg/mL by Trung and Bao [34]; while those recorded by Rajalakshmi et al. [35] and Youcefi and Riazi [18] with 1 to 5 mg/mL and 0.5 to 2 % (p/v) of chitosan, respectively, amounted to more than 60% and ranged from 37.66 to 62.66 %, respectively. Prabu and Natarajan [36] found that the DPPH radical scavenging activity of chitosan extracted from a swimmer crab (Podophthalmus Vigil) used at concentrations of 0.1 to 10 mg/mL ranged from 18.08 % to 55.56 %; while Park et al. [37] reported that chitosan neutralises various free radicals through the action of its nitrogen at the C-2 position. Xia et al. [38] suggested that the nitrogen of amino groups has a single pair of electrons that can react with a proton released from acidic solution to form ammonium (NH_4^+) groups. Thus, the free radicals respond with the hydrogen ion of the NH_4^+ to form a stable molecule.

Inhibition of lipid peroxidation by 1 to 2 mg/mL chitosan ranged from 1.7 to 15.1 % according to Trung and Bao [34]; while Kurniasih et al. [39] evaluated the inhibition of linoleic acid oxidation by chitosan using the ferrothiocyanate method.

Mahmoudi and Mahmoudi [40] investigated the DPPH radical scavenging activity of different part of C. scolymus (heart, stem, choke and brackets) and found lower IC50 values than those observed in the present study (0.025 to 0.031 mg/mL). These IC50 values were positively correlated ($r = 0.66$) with total phenolic content of artichoke parts.

Eldin Awad et al. [41] observed a significant difference in the antioxidant activity of aqueous and ethanolic artichoke extracts as determined by the DPPH radical scavenging test (IC50: 247.30 and 130.54 µg/mL for aqueous and ethanolic extracts, respectively). The same authors reported that artichoke by-products are good sources of phenolic compounds and potent free radical scavengers.

The enhanced antioxidant activity of chitosan-artichoke extracts combinations can be attributed to an interaction between chitosan and phenolic chemicals found in artichoke extracts. Therefore, Li et al. [42] reported that chemical modification of chitosan coumarins significantly increased its antioxidant activity... These researchers observed that inhibition of lipid peroxidation, iron chelating, and hydroxyl (•OH) radical scavenging of the synthesized chitosan derivatives (a combination of chitosan and coumarins) exhibited a remarkable improvement over chitosan.

The therapeutic efficiency of chitosan-polyphenols conjugates has been investigated for the treatment of various diseases caused by peroxydes and has yielded good and notable results [43].

Antibacterial activity and MIC determination

As can be seen in table 2, the pathogenic bacterial strains have been inhibited at different degrees. The polyphenol methanolic extract of artichoke heart is more effective in inhibition of pathogenic strains than the aqueous extract. The inhibition zone diameters of bacterial strains by the methanolic and the aqueous extracts ranged from 6.0 ± 0.57 to 19.5 ± 0.57 mm and from 6.0 ± 0 to 14.5 ± 0.16 mm, respectively. This result may be related to the higher total phenol content of methanolic extract comparatively to the aqueous extract, as well as to the difference in the composition of the two extracts. Our findings are in agreement with those of Demir and Agaoglu [44] who reported that *cynara sclolymus* extracts strongly inhibited foodborne pathogen growth, which could be related to their richness in polyphenols and flavonoids. These bioactive molecules most likely act through their ability to modulate the permeability of cell membranes and the cell wall rigidity, and may link to soluble proteins and enzymes to form different complexes inducing irreversible damages and thus inhibiting the cell functions [45, 46].

Table 2. Diameters of the inhibitory zones (mm) of pathogenic and beneficial strains by the chitosan and artichoke heart extracts, and their combination.

Bacterial strain	Chitosan	Methanolic extract	Aqueous extract	Combination chit-MetE	Combination chit-AqE
Staphylococcus Epidermis	14.5±0.28^{b}	08.33±0.57^{a,b}	6.0±0^{a}	16.66±1.15^{b}	10.33±0.86^{b}
Pseudomonas Aerugénosa	12.5±0.5^{b}	15.5±0.16^{b}	8.0±0.33^{ab}	19.16±0.28^{c}	20.00±0^{c}
Listeria monocytogenes	17.0±0.57^{c}	19.5±0.57^{c}	14.5±0.16^{b}	37.5±0.33^{e}	29.5±0.66^{d}
Staphylococcus Aureus	32.5±0.5^{e}	18.0±0.57^{c}	NE	39.0±0.86^{f}	12.5±0.33^{b}
Escherichia Coli	10.0±0.16^{b}	6.0±0.57^{a}	9.5±0.5^{ab}	18.0±0.33^{c}	13.5±0.16^{b}
Salmonella Typhimurium	15.5±0.28^{b}	14.0±0.16^{b}	10.0±0.28^{b}	22.5±0.5^{c}	20.5±0.66^{c}
Bif. animalis subsp lactis	NE	NE	NE	NE	NE
Lactobacillus Plantarum	NE	NE	NE	NE	NE

NE: No Effect, Different letters show statistical difference ($p<0.05$).

Chitosan strongly inhibited the growth of Gram-positive strains compared to Gram-negative strains. The inhibition zone diameters of *Staphylococcus epidermis*, *Staphylococcus aureus* and *Listeria monocytogenes* by chitosan were about 14.5 ± 0.28, 32.5 ± 0.5 and 17.0 ± 0.57 mm, respectively; while those of the Gram-negative strains ranged from 10.0 ± 0.16 to 15.5 ± 0.28 mm. This observation is in agreement with that of previous studies of Prabu and Natarajan [36], and Alqahtani et al. [47].

Several mechanisms of action involved in the antimicrobial effect of chitosan have been reported. According to Prabu and Natarajan [36], the electrostatic interaction between the positively charged C-2 amino sites of chitosan and the negatively charged components of the microorganisms such as lipopolysaccharides and proteins induces significant damages to the structure of the cell's outer membrane; which results in the loss of cell constituents and cell death. Additionally, chitosan may bind to essential nutrients decreasing their availability to microorganisms [48].

The present study evidenced the increase in the inhibition zone diameters of foodborne pathogenic strains when chitosan is associated with methanolic or aqueous artichoke heart polyphenol extracts. These inhibition zone diameters ranged from 10.33 ± 0.86 to 39.0 ± 0.86 mm in such synergistic association; while beneficial strains were not inhibited by chitosan or artichoke heart polyphenol extracts or their association. The minimal inhibitory concentrations of chitosan and phenolic extracts on the pathogenic strains are reported in table 3.

Table 3. Minimal inhibitory concentrations: MIC (mg/mL) of pathogenic beneficial strains with the chitosan, artichoke extracts and associations chitosan-artichoke extract.

Bacterial strain	Chitosan	Methanolic extract	Aqueous extract	Combination chit-MetE	Combination chit-AqE
Staphylococcus Epidermis	3.12	3.12	6.25	0.78-0.78	1.56-1.56
Pseudomonas Aerugénosa	6.25	0.78	6.25	0.39-0.39	0.78-0.78
Listeria monocytogenes	1.56	0.78	1.56	0.19-0.19	0.09-0.09
Staphylococcus Aureus	0.39	1.56	-	0.09-0.09	0.39-0.39
Escherichia Coli	3.12	3.12	3.12	0.09-0.09	0.39-0.39
Salmonella Typhimurium	0.78	1.56	1.56	0.09-0.09	0.19-0.19

It has been noticed that the bacterial strains tested showed more sensitivity to chitosan and artichoke heart extracts when used in association than when used alone. Therefore, it has been observed that at low concentration, association of chitosan and methanolic artichoke heart extract was sufficient

to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli* and Salmonella typhimurium. The growth of *Listeria monocytogenes* was inhibited by chitosan and aqueous artichoke heart extract at a concentration of 0.09 mg/mL. However, when used separately, high concentrations of chitosan and artichoke heart extracts were required to inhibit pathogenic strain growth. The MIC values observed ranged between 0.39 and 6.25 mg/mL for chitosan used alone; and 0.78 to 3.12 mg/mL and between 1.56 and 6.25 mg/mL for methanolic and aqueous aqueous artichoke heart extracts used separately, respectively.

According to Pattnaik et al. [43], antioxidant activity of chitosan is limited because of its low solubility and the non-availability of hydrogen donor capacity. Therefore, chitosan has been associated with artichoke polyphenols to overcome the limitations of both chitosan and polyphenols, along with increasing the potential synergistic effects of their combination for therapeutic applications.

Liskova et al. [49] observed that polyphenol enriched chitosan hydrogels exert high antibacterial activity. Effectiveness of association of chitosan with curcumin, galic acid, catechin and quercetin has been reported by Queiroz et al. [50]; while therapeutic efficacy of chitosan and polyphenols was attributed to the presence of an active ammonium group in the chitosan and phenolic hydroxyl groups of polyphenols [40].

However, further research must identify and quantify the phenolic chemicals found in artichoke heart extracts. More precise methodologies should be used in future study to elucidate the processes involved in improving the antioxidant and antibacterial activity of chitosan and polyphenol combinations.

Conclusion

Chitosan and polyphenols are well-known as antioxidants and antimicrobials; however, both have inherent limitations that reduce their therapeutic effectiveness in the biological environment. Therefore, the combination of chitosan and polyphenols emerges as a potential candidate to overcome the disadvantages of both compounds and bring synergistic health benefits. This study highlights the efficacy of synergistic antioxidant and antimicrobial effect of the association of chitosan and artichoke heart extract. This suggests that the combination of chitosan and phenolic compounds of *C. Scolymus* may be an attractive approach in food preservation.

Authors Contribution

The first and second authors (Nabil Bouziane and Meryem Sadoud) equally performed the experiments on the chemical characterization of chitosan, the determination of antioxidant and antimicrobial activities. They also wrote the manuscript. Extraction and dosage of artichoke polyphenols were made by Sarah Bouamar, Meryem Belabbas and Djahira Hamed who were also involved in the antioxidant activity experiment. Extraction of chitosan was done by Nawal Boukezzoula and Abdenour Benguendoz who participated to the determination of antimicrobial activity of the extracts. Ali Riazi contributed to the design of the study and to the supervision of the experiments. He carried out the statistical validation of the results and he was also responsible for writing the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare no conflict of interests.

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