

RESEARCH ARTICLE

Antibacterial Activity of Chitosan, Some Plant Seed Extracts and Oils Against *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

In this present study, various concentrations (0.5, 1, and 2%) of chitosan extracted through the chemical methods from the shells of crayfish (*Astacus leptodactylus*), pink shrimp (*Parapenaeus longirostris*), blue crab (*Callinectes sapidus*) shells, methanolic extracts of black cumin (*Nigella sativa* L), flaxseed (*Linum usitatissimum*), Chaste Tree (*Vitex agnus-castus* L.) and black cumin and flaxseed oil were tested in vitro for their antibacterial activities against two pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*, using the disk diffusion method. On the other hand, were Erythromycin and florfenicol were used as a positive control. The negative control was Acetic Acid and cotton oil. Antimicrobial activity of chitosan is affected by different intrinsic and extrinsic factors. Chitosan source, molecular weight, deacetylation degree, viscosity, and solvent material, pH, ionic strength, metal ions, and bacteria cultures. In the present study, all four extracted chitosans were showed different antimicrobial effects on two different types of bacteria, while there are not any antibacterial effect of aqueous extracts and oils of the three plants seeds that used in this study. This is the first report concerning the antimicrobial activity of chitosan compared to some plant seed extracts and oils against *Escherichia coli* and *Staphylococcus aureus*. Furthermore, results showed the chitosan of this species might be an alternative as an antimicrobial agent for the pharmaceutical industry.

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Introduction

Currently, there is intensely scientific awareness that has contributed to the development of research on natural products, to the increase in knowledge about the close relationship between the chemical structure of a certain compound and its biological properties. (Cheng et al., 2009). The natural products of animal or plant origin which have long been used as an alternative source of antimicrobials and it is thought that their influences on the environment are few and can be used as biological control agents instead of antibiotics

to control bacteria that have the development of bacterial resistance to synthetic antibiotics (Viegas, and Bolzani, 2006). For these reasons, natural sources are important substances for the study of their biological strong uses as safe sources that act as new antimicrobial agents of different groups of microorganisms.

Chitosan (poly-b- 1.4-2-amino-2-deoksi-b-D-glucopyranose) is a non-toxic natural biopolymer of animal origin modified from chitin (b-1,4-poly-N-acetyl-D-glucosamine) by deacetylation process by alkaline treatments (e.g. with NaOH)

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from squid pens, cell walls of some fungi, exoskeletons of insects and Crustacea (lobsters, shrimp, and crab) shells which is the main structural component of their shells. Can be obtained about 20-30% chitin from Crustacea shells (Seo, 2006). Chitin and chitosan are naturally copolymers found together. They are well known to have specific properties of being environmental friendly due to its biodegradability and because of that, Chitin and chitosan have received increased attention as one of the promising renewable polymeric materials for various applications. These biopolymers offer a wide range of unique and useful applications in many sectors and in various industries including biochemistry, biotechnology, bio pharmaceuticals, agriculture, veterinary, cosmetics, biomedical, dentistry, environmental protection, textile, packaging, purification of water and waste treatment, food products, etc. (Muzzarelli, 1985; Al-Manhel, 2018; Boonlertnirun, 2017).

Throughout recent years, considerable attention has been paid to the antimicrobial activity of chitin, chitosan and their derivatives against various groups of microorganisms, including bacteria, fungi, yeast and viruses (Rabea et al., 2003; Hongpattarakere, and Riyaphan, 2008; Küçükgülmez, et al., 2013).

Medicinal plants and plant derivatives (intact plant, essential oils, extracts, powder, and phytochemicals) are used worldwide as an important traditional medicine source in the treatment of a variety of diseases (Phillipson, 1994). WHO (1996) has listed 21,000 plants that have medicinal uses around the world (Noor et al, 2013). In addition to being cheap to produce, the use of plant products for disease control has many advantages; they are biodegradable and readily available; Pathogenic bacteria can be combated with successful plant products without harmful side effects and environmental hazards (Ray et al., 2004).

Antimicrobial activity of plant derivatives (has been extensively researched against a number of microorganisms due to the ability to control microorganisms including gram-negative and gram-positive bacteria. Nevertheless, the advent of multidrug-resistant bacteria presents a threat to the treatment of infections, so it is evident the need to find new substances with antimicrobial properties for use in combating these microorganisms (Hemaiswarya et al., 2008).

The aim of the study was to investigate the antibacterial activity of chitosan extracted through the chemical methods from the shells of crayfish (*Astacus leptodactylus*), pink shrimp (*Parapenaeus longirostris*), blue crab (*Callinectes sapidus*), warty crab (*Eriphia verrucosa*), methanolic extracts of black cumin (*Nigella sativa* L), flaxseed (*Linum usitatissimum*), chaste tree (*Vitex agnus-castus* L.) and flaxseed oils against to pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* compared to the antibiotics used as control.

Materials and Methods

Materials

In this study, crayfish (*Astacus leptodactylus*), from Germeçtepe Dam Lake (Kastamonu), were bought from local

seller and transferred to the laboratory and stored in refrigerator conditions until used. Shrimp (*Parapenaeus longirostris*) processing waste sample was obtained from the fish processing facility in İzmir/Aegean Region. It was confirmed that all shells were from a single species of shrimp (*P. longirostris*). Blue crab (*Callinectes sapidus*) bought from local fishermen from the Antalya/ Mediterranean Sea were used. The shells of the warty crab (*Eriphia verrucosa*) which collected from the İnebolu/Kastamonu/Black Sea shores as a fishing waste also were used in this study.

The ripe seeds of black cumin (*Nigella sativa* L), flaxseed (*Linum usitatissimum*), Chaste Tree (*Vitex agnus-castus* L.) were Collected from the market of Kastamonu.

Methods

Preparation of chitosan

To remove soluble organics, adherent proteins, and other impurities under running warm tap water, shells were washed then collected and boiled for one hour in the water bath to extract the tissue and then dried in the oven for two hours at 160 °C to make them more fragile and break down the crystalline chitin structure according to Mukherjee (2001) method. The dried shells were gradually ground into a fine powder using a standard grinder (Model KU-2, PredomMesko, SkarzyskoKam., Poland). To the remove the calcium carbonate which is the main inorganic component of the shells, diluted hydrochloric acid with a range from 1/10 to 1/30 (w/v) at concentrations ranged from 1 to 2 M was used and the reaction time was from ten to one hundred twenty min under continuous stirring of 150 rpm at room temperature. The decalcified shells were collected on a 250 mm sieve, washed with tap water then rinsed with deionized water, and oven-dried overnight at 80 °C. (No and Meyers., 1995). Similar experimental conditions for the demineralization of dried shells were applied. The concentration of sodium hydroxide varied from 0.5 to 5 M, the time of reaction ranged from 10 to 40 min and the temperature varied from 45 to 65 °C after that the material was filtered, washed and dried. And the chitin residue was mixed with acetone at a solid/solvent ration of 1:10 (w/v) for 10 min, filtered, dried for 2 h at room temperature, followed by bleaching with 0.315% NaOCl for 5 min at the same solid/solvent ration, this process was performed as described by (No and Meyers 1995). Chitin conversion to chitosan involved deacetylation using (Kurita et al., 2003) process. Briefly, suspension of 1 g of chitin in 50 mL of aqueous sodium hydroxide and mixed for 3 to 5 hours at 90 to 100 °C in a water bath under constant stirring. at the end of this process, the solid was filtered, washed with 80% alcohol then dried for overnight at 80 °C. The final product (chitosan) was dissolved in acetic acid to prepare concentrations of 0.5%, 1%, and 2% then kept in refrigerator until used.

Preparation of plant seeds extracts

The ripe seeds of black cumin (*Nigella sativa* L), flaxseed (*Linum usitatissimum*), chaste tree (*Vitex agnus-castus* L.) were collected from the market of Kastamonu and extracted by using an aqueous methanol extraction method according to (Pakravan et al., 2012), with some modification (Bilen et al.,

2016) as follows; the ripe seeds were ground in a mechanical grinder to a fine powder and 50 g of the ground seeds was added to 1 liter of 40% methanol (Sigma-Aldrich) and the mixture was allowed to stand at room temperature for 3 days and was shaken every day. After 3 days seeds extract was filtered through filter paper (Whatman filter No 1) and the filtration was collected and evaporated in a rotary evaporator at 55-65 °C for removing alcohol from the extract. The final product (crude) was dissolved in hot distilled water to prepare concentrations of 0.5%, 1%, and 2% then kept at 4 °C until used.

Preparation of plant seed oils

The essential oils of different seeds of black cumin (*Nigella sativa* L), flaxseed (*Linum usitatissimum*), were isolated by cold press oil machine of ripe seeds. Both oils, cotton oil were used to prepare three concentrations 0.5%, 1% and 2% and stored in refrigerated.

Preparation of bacteria

Antimicrobial activity tests were carried out against the bacteria pathogenic bacteria gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*) were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 6 min, the pellet was suspended in double-distilled water (which has been adjusted to 0.5 McFarland standard).

Disc diffusion method

The disc diffusion method for antimicrobial testing was carried out according to the standard method by Bauer (1966) to assess the presence of antibacterial activities of the chitosan, plant seeds extracts and oils. The sterile SS and BHI agar were poured on to the petri dishes and the media was allowed to solidify. The bacterial colonies turbidity was adjusted to match 0.5 McFarland standard (1.0×10^8 CFU ml⁻¹) and the suspension of the bacteria was spread over the agar having a sterile glass swab.

The plates were dried for 15 minutes and then used for the antibacterial activity test. The discs placed on the agar surface by disc dispenser then impregnated with 20 µl of mother solution and diluted concentration as described above.

Each plate comprises of ten discs. Positive control, which is a standard commercial antibiotic disc, negative control, and treated discs. The standard antibiotic discs were erythromycin (15µg ml⁻¹) and florfenicol (30µg ml⁻¹), were used as a positive control. The negative control was acetic acid and cotton oil.

The plate was then incubated at 37°C for 24 hours. After the incubation, the plates were examined for inhibition zone. The inhibition zone were then measured using calipers and recorded. The test were repeated two times to ensure reliability.

Results

The effect of different chitosan concentration on antimicrobial activity

The effect of chitosan extracted from crayfish (CH1), pink shrimp (CH2), blue crab (CH3) and warty crab (CH4) at different tested concentrations against *Escherichia coli* and *Staphylococcus aureus* are shown in (Table 1).

Data indicated that chitosan markedly could inhibit the growth of both the gram-negative bacteria tested in varying ratios. However, the inhibitory effects differed depending on the types of chitosan, bacteria, and also concentration.

It was observed that the antimicrobial activity of all concentrations of extracted chitosan (CH1 and CH2) against *E. coli*. was much higher than to *S. aureus* with inhibition zone diameter between 9.00 and >160.00 mm and between 7.00-9.90 mm for *E. coli* and *S. aureus* respectively except (CH4) for *S. aureus* in 1% and 2% concentration with inhibition zone diameter <7.00mm.

It could be reported that (CH3) and (CH4) 0.5% could inhibit the growth of *E. coli* more than (CH1) and (CH2) 0.1% compared to other concentrations. Where it has been reported the lowest antimicrobial activity to *E. coli* was observed in 0.2% chitosan from all Crustaceans.

The lowest antimicrobial activity against *S. aureus* was observed in (CH1) and (CH4). The both result of 0.5% are 7.00-8.9mm, 1% concentration inhibition zone diameter was 7.00-8.9 mm and <7mm and 2% was same inhibition zone diameter <7mm both, respectively. While no differences were recorded between the remaining groups, even though they recorded anti-bacterial activity against *S. aureus*.

Inhibition zone diameter changed with different extracted chitosan concentrations from different crustaceans against *E. coli* it was determined 16.00mm in *E. coli* in (CH1) 0.5% and 1% concentrations, (CH2) in 2% concentration, and (CH3) and (CH4) in 1% concentration. While it was determined between 14.00-16.00 mm in (CH2) 0.5% concentration and (CH3), (CH4) in 1% and 2% concentrations, the inhibition zone diameter was observed between 9.00-10.90 mm in (CH1) and (CH2) in 2% concentration.

Inhibition zone diameter in *S. aureus* and it was determined between 9.00mm and 10.90mm in all chitosan concentrations except (CH1) in 2% and (CH4) 1% and 2% were determined as <7.00 mm while it was determined between 7.00-8.90 mm in (CH2) 2% concentration

Acetic acid used as a negative control group had antimicrobial effects on *E. coli*. and *S. aureus* (11.00-13.90 mm of zone diameter).

Table 1. Antimicrobial activity of different concentrations of chitosan samples extracted from the wastes of Crayfish (CH1), Pink Shrimp (CH2), Blue Crab (CH3), and Warty Crab (CH4) against *Escherichia coli* and *Staphylococcus aureus* using disc diffusion method

Sample	Bacteria and inhibition zone (mm)		
	Concentration	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
CH 1	0,5 %	+++++	++
	1 %	+++++	++
	2 %	+++	-
CH 2	0,5 %	++++	++
	1 %	+++++	++
	2 %	+++	+
CH 3	0,5 %	+++++	++
	1 %	++++	++
	2 %	++++	++
CH 4	0,5 %	+++++	++
	1 %	++++	-
	2 %	++++	-
Positive control	Erythromycin. 10µl	+++++	+++++
	Florfenicol. 10µl	+++++	+++++
Negative control	Acetic Acid	++++	++++

Results were interpreted in terms of the diameter of the inhibition zones: (-), <7.00 mm; (+), 7.00-8.90 mm; (++) , 9.00-10.90mm; (+++), 11.00-13.90 mm; (++++), 14.00-16.00 mm. (+++++), >16.00 mm.

The effect of different plant seeds extract on antimicrobial activity

The effect of black cumin (Ext1), flaxseed (Ext2), chaste tree (Ext3) extracts at different tested concentration against *Escherichia coli* and *Staphylococcus aureus* antimicrobial activity are shown in (Table 2).

The results, which observed in this study indicated that compared to the control (erythromycin and florfenicol) no antibacterial activity was recorded for any concentration of the plant extracts against *E. coli* and *S. aureus*.

The inhibition zone diameter was observed <7.00mm in all concentrations of differences plant seeds extracts (Table 2).

Table 2. Antimicrobial activity of black cumin (Ext 1), flaxseed (Ext 2), Chaste Tree (Ext 3) extracts against *Escherichia coli* and *Staphylococcus aureus* using disc diffusion method

Sample	Bacteria and inhibition zone (mm)		
	Concentration	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Ext 1	0,5 %	-	-
	1 %	-	-
	2 %	-	-
Ext 2	0,5 %	-	-
	1 %	-	-
	2 %	-	-
Ext 3	0,5 %	-	-
	1 %	-	-
	2 %	-	-
Positive Control	Erythromycin. 10µl	+++++	+++++
	Florfenicol. 10µl	+++++	+++++

Results were interpreted in terms of the diameter of the inhibition zones: (-), <7.00 mm; (+), 7.00-8.90 mm; (++) , 9.00-10.90 mm; (+++), 11.00-13.90 mm; (++++), 14.00-16.00 mm; (+++++), >16.00 mm.

The effect of different plant seeds oil on antimicrobial activity

The effect of black cumin (Oil 1) and flaxseed (Oil 2) oils at different tested concentration against *Escherichia coli* and

Staphylococcus aureus antimicrobial activity are shown in (Table 3).

Both plant seeds oils showed weak antibacterial activity against both pathogenic bacteria and were found to be

concentration-dependent. Even though both showed slightly clearer activity against *E. coli* but were not considered when compared to the control.

Inhibition zone diameter in *E. coli* and it was determined between 11.00-13.90mm in (Oil 1) in 3%. Whilst, the diameter of the inhibition zone was between 9.00-10.90 in (Oil 2) 0.5% concentration and it was determined between 7.00-8.90 mm in (Oil 1) in 2% and (Oil 2) in 1% and 2% concentration.

Inhibition zone diameter in *S. aureus* and it was determined between 7.00-8.90mm in all plant seed oil concentrations except (Oil 1) in 0.5 % and 1% were determined as <7.00 mm.

While cotton oil was used as a negative control group and had no antimicrobial effects on *E. coli*. and *S. aureus* (<7.00 mm of zone diameter).

Table 3. Antimicrobial activity of black cumin (Oil 1), flaxseed (Oil 2) oil against *Escherichia coli* and *Staphylococcus aureus* using disc diffusion method

Samples	Bacteria and inhibition zone (mm)		
	Concentration	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Oil 1	0,5 %	+++	-
	1 %	++	-
	2 %	+	+
Oil 2	0,5 %	++	+
	1 %	+	+
	2 %	+	+
Positive Control	Erythromycin. 10µl	+++++	+++++
	Florfenicol. 10µl	+++++	+++++
Negative Control	Cotton Oil 10µl	-	-

Results were interpreted in terms of the diameter of the inhibition zones: (-), <7.00 mm; (+), 7.00-8.90mm; (++) , 9.00-10.90 mm; (+++), 11.00-13.90 mm; (++++), 14.00-16.00 mm. (+++++), >16.00 mm.

In this study, erythromycin (15µg ml⁻¹) and florfenicol (30µg ml⁻¹) as antibiotics were used to compare the antimicrobial activity of chitosan, plant seed extract, plant seed oil at different concentrations. In the disk diffusion assays, both antibiotics demonstrated the antimicrobial effect >16.00 mm on both bacteria (Tables 1, 2, 3).

Discussion

In the present study, the effects of different concentrations (0.5%, 1%, 2%) of chitosan, plant seeds extract and oils as antibacterial against pathogens bacteria were examined, furthermore to know whether extraction of chitosan from different crustaceans affected the antibacterial activity.

The results of the effects of chitosan clearly showed that a good antibacterial activity was exhibited by chitosan from blue crab (CH3) and warty crab (CH4) than the chitosan from other crustaceans against pathogens bacteria which used in this study and the increasing of the concentrations led to improve the activity (Table 1).

Several studies were conducted in a variety of conditions on the effect of chitosan and its various derivatives as a potential antimicrobial agent on the different types of bacteria. (Tsai et al., 2004; Jeon et al., 2001; Yang et al., 2005).

Besides, our findings showed that the effects of chitosan on two bacteria were different for each concentration and source. In agreement with this finding, (Küçükgülmez, 2012) who reported that chitosan demonstrated different effects as

antimicrobial agent suggested that these differences may be due to many factors. Such as the chitosan concentration, molecular weight, the solvent, and the bacteria type. (Seo et al., 2008; Tajik et al., 2008), also reported that the chitosan concentration affected the antibacterial activities and indicated that the high concentration of chitosan increased the antimicrobial activity against *E. coli* and *S. aureus*.

According to (Zheng and Zhu, 2003) the possible mechanisms for antimicrobial activity, the mechanism of the effect of chitosan on the *S. aureus* is that chitosan can be formed a polymer membrane on the surface of the cell, which prevents the nutrients from entering the cell and thus its death. While it affects to the *E. coli* by reaching the chitosan into the cell by pervasion. Because chitosan will adsorb and flocculate the electronegative material inside the cell, it disturbs and destroys the bacteria's physiological activities. (Benhabiles, et al 2012).(Darmadji and Izumimoto, 1994) mentioned in their study the possible causes of the chitosan effect on various types of bacteria and it may be due to its interaction with membranes or components of the bacteria cell wall, which increases the membrane's permeability and the leakage the materials out the cell.

The reason may also be the high capacity of chitosan to bind to water molecules, which inhibit the activity of enzymes and thus the bacterial cell death. Moreover, chitosan can absorb nutrients from the medium, which contributes to preventing the growth of bacteria. (Küçükgülmez, 2012).

In agreement with the present results (Seo et al. 2008; Tajik et al., 2008), were reported that the chitosan

concentration affected the antibacterial activities and indicated that the high concentration of chitosan increased the antimicrobial activity against (*E. coli* and *S. aureus*).

The results in Tables 2 and 3 showed the antibacterial activity of the plant seeds extract and Oils against *E. coli* and *S. aureus*.

The results showed in the (Table 2) that no antibacterial activity was exhibited by different plant seeds extract, while black cumin oil showed some antibacterial activity, especially the concentration 0.5% against *E. coli* with inhibition zone between 9.00-13.90mm. Unlike the aqueous extract of the same plant seeds, which did not show any antibacterial effect the inhibition zone diameter was observed <7.00mm, this applies to the flax seeds extract and oil as well. This is probably due to the reason that Al-Reza et al, (2010) indicated that the chemical composition of the bacterial membrane which enables it to interact well with the compounds present in the essential oils of the seeds, which leads to the decomposition of the cell membrane and consequently its inevitable death. While it repels the water component of the aqueous extract and prevents penetration of the target bacterial cell wall (Calsamiglia et al, 2007). Phytochemicals in the black cumin oils appeared to be more potent than that of the extract, and the oils produced a low level of inhibition of bacterial growth when these results compared with the Essential oils from *Mentha spicata* against *S. aureus* which did not show any activity allows concluding that it does not have antimicrobial activity Škrinjar, and Nemet, (2009). In agreement with this finding, (Singh et al 2005a) Both essential oil and extract from black cumin seeds were found to be ineffective against *Escherichia coli* and *Salmonella typhi* at all the tested concentrations in their study. Also, Singh, et al (2005b) have reported a lower antimicrobial activity of the extracts of pepper and ginger using acetone, which may be attributed to lower volatility of essential oil or extract components.

Conclusion

It can be generally concluded that the obtained results indicated the possibility of using the chitosan as natural sources were recommended to be used against the screened bacterial species. Moreover, further investigations in this area, in particular with regard to the effect of these natural compound on bacterial resistance mechanisms are warranted.

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