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Research Article **Microbiology**

Antibacterial and antifungal activity of chitosan against *Bacillus cereus and Aspergillus niger* isolated from some Egyptian canned and fast food

Saida Mohamed Amer [1], **Azza Abd El-Rahman Mostafa**[2] **Maha Mahmoud Azab**[1] **and Mostafa Fathi Shaaban**[1]

1 Botany and Microbiology Department, Faculty of Science, Tanta University, Tanta, Egypt.
2 Biological and Environmental Departmental Science, Faculty of Home Economics, Al-Azhar University, Tanta, Egypt.
*** Correspondence**: Saida M. Amer
E-mail: Saida_amer2014 @yahoo.com

KEY WORDS

Canned food, Fast food, *Aspergillus niger, Bacillus cereus*, Biochemical tests, Minimum inhibition concentration

ABSTRACT

A total of (213) canned food samples comprising of Tuna &Sardines, Juices, Tomatoes pasts, Jam and Beef were randomly collected from super stores and local markets in Tanta city from Awlad Ragab, Fatthallah, Munshwi, Casion. Also 24 fast food samples collected from local cafeterias and restaurants in Tanta city from Al Gaan, Abu Deshish, Al Baraka, Abu Owaf. All canned food samples were within expiry date, none of which is bloated, leaking and/or physically damaged. Samples were investigated for some bacteria and fungi using specific media and incubated for suitable incubation period. The results revealed that species of microbes isolated in this study namely *Bacillus subtilis, Bacillus cereus, Bacillus atrophaeus., Staphylococcus saprophyticus, Enterococcus faecalis, Staphylococcus epidermides, E. coli, Klebsiella, Salmonella , Aspergillus niger, Penicillium notatum, Candida tropicalis* and *Saccharomyces cerevisiae.* Biochemical tests were performed for all isolates to know the most common isolates (*Bacillus cereus* and *Aspergillus niger*). Antimicrobial activities of chitosan were investigated against the most common isolates *B. cereus* and *A. niger*. Results revealed that chitosan has antibacterial activity towards *Bacillus cereus*. The minimum inhibition concentration (MIC) for chitosan was 6.25 µg/ml with mean diameter of inhibition zone 8 mm. Also chitosan has antifungal activity toward *Aspergillus.niger* with minimum inhibition concentration (MIC) 30mg/ml and percent of fungal growth inhibition 16.7%.

Introduction

Canned foods are foods that packed in hermetically sealed containers and become sterile through packing**.** Canning leads to kill harmful microbes in food, however, if canning processing were performed improperly, canned food become available media for microbial contamination by different microbes which may be harmful for consumers if they increase in number or leads to toxicity. This contamination may occur during preprocessing, processing or after processing, may be due to physical causes like defective containers, improperly closed cans or bad packing or transportation (1). Microbes that may contaminate canned food are mainly of spore forming genera like *Bacillus, Clostridium* and *Desulfotomaculum* (2).

Fast food are foods which prepared in cafeteria or related food restaurants and immediately consumed, this fast food may contain food eaten raw like salads, spices which are favorable media for microbial contamination by pathogenic and spoilage microbes especially in crowded restaurants and from suppliers, so to improve safety of these food products, the associated stuff need to be sure for good manufacturing practices from food suppliers and food workers **(3)**.

A lot of food borne diseases and related illnesses caused by *Campylobacter* spp*., nontyphoidal Salmonella* and pathogenic *E.coli* that are colonize gastrointestinal tract of most animals raised for human consumption **(4)**. Food contamination by pathogenic fungi considered one of most difficult challenges that face food safety as these fungi may produce mycotoxins that cause many health diseases **(5).** Contamination in food industry by storage fungi like *Aspergillus* and *Pencillium* is of great concern because of secondary metabolites produced by these fungi such as mycotoxins that has a bad effect on human health **(6)**. *Aspergillus niger* is a saprophytic and filamentous fungus lives in variable habitats like soil, forage, organic debris and other food products causing much plant diseases **(7).** The most important mycotoxigenic fungi that contaminate food and feed are black *Aspergilla*, caused decay of fruits, vegetables, nuts, beans and cereals. The most important features that encourage its growth are fast growth, pH tolerance, tolerance variable environments.

Natural compounds are compounds produced by living plant, animal, or microorganism naturally which may be have antimicrobial or biological activity **(8).** Natural antimicrobials have given more important due to the increase in bad effects for chemical preservatives, despite that this chemical preservatives are approved for human consumption at acceptable level but there is increase in human diseases related to worldwide increase in utilizing this chemical preservative, also the antimicrobial resistance toward this chemical preservatives increase from microbial strain to other **(9,10)**. Plants produce different secondary metabolites that have antimicrobial activity towards pathogenic and spoilage microbes **(11)**. So there is increase interest for production of natural antimicrobial to inhibit microbial growth and increase shelf life of products **(12, 13)**. Natural antimicrobial in food safety gained much more attention in food industry and for consumers **(14).** The best antimicrobial agents for food preservation which are natural and biodegradable like biodegradable chitosan, so chitosan and chitosan based film or polymer can be used for food preservation because it have shown antimicrobial properties **(15 , 16).**

Chitosan is the second most abundant polysaccharide in nature after cellulose. It is a direct polysaccharide comprising of (1, 4) - connected 2-amino-deoxy-β-D-glucan, is a deacetylated derivative of chitin. In addition to being a successful antimicrobial agent, chitosan is nontoxic, biodegradable, bio practical and biocompatible. Chitosan with high molecular weight result in poor solubility at neutral pH and high solution viscosity, these properties limit its use in food, cosmetics, agriculture and health industry **(17).** Many researches show that the antibacterial activity of chitosan effective than antifungal activity **(18, 19)**.

Material and methods

Sample collection

About (213) samples of a canned foods comprising of five different categories of canned food (Juices, Jam, sardines & Tuna, tomatoes Pastes and Beef) were examined. Samples within the expiry date as

indicated on the container were randomly collected from super markets and shopping malls in Tanta city. Samples were taken to the laboratory for analysis. The information on the container/labels was recorded to include manufacture and expiry dates, manufacturer's address, also 24 fast food samples were collected from local cafeteria and restaurant in Tanta city. The fast food samples were transformed in sterile container within few hours to the microbiology laboratory at Faculty of Science, Tanta University according to **(20)**. The samples were investigated for bacteria and fungi associated with human heath according to standard methods reported by **(21).**

Food samples preparation and analysis

For canned food prior to analysis, the surface of the container was cleaned with 70% ethanol and tincture of iodine. Containers were opened near the flame of the Bunsen burner to avoid contamination. For fast food, samples were taken from restaurant in sterile plastic bags in Ice-Box, according to **(20)**.

Isolation, purification and identification of bacteria:

From each sample 25 g was aseptically weighed and macerated in sterile bag with 225 ml of sterile buffered peptone water. Two fold serial dilutions were carried out using sterile buffered peptone water as diluents. From each dilution 1 ml was plated using the pour plate methods of **(22)** into following growth media:

MacConkey agar medium: Differential and selective media used to distinguish lactose fermenting from non-lactose fermenting **(23).**

Salmonella-Shigella agar medium: SS Agar (Salmonella Shigella Agar) is a differential selective media used for the isolation of *Salmonella* and some *Shigella* species from food and pathological specimens **(23)**.

Mannitol salt agar: A selective and differential media for the isolation of pathogenic *Staphylococci* **(24, 25*).***

Mannitol egg yolk polymyxin agar for pathogenic *Bacillus* and *Staph*: Used to isolate and enumerate *B.cereus* from foods, recommended by APHA **(26).**

MacConkey sorbitol agar base w/ Rhamnose: Selective and differential media for detection and isolation of *E.coli* forms from various samples clinical, dairy, food, water, pharmaceuticals etc. **(27).**

Buffered peptone water: Buffered Peptone Water used in recovery of injured cells that may be sensitive to low pH or temperature **(28).**

Nutrient agar: Non-selective media used for purification of microorganisms **(29).**

L. mono Differential Agar Base: Selective and differential media used for isolation of *Listeria monocytogenes* from food and animal feeds which is adopted by ISO Committee **(30)**.

Muller Hinton agar: Used as a test medium for antimicrobial susceptibility testing **(31)**.

All plates were incubated for 24-48 hr at temperature suitable for microbial growth, at the end of incubation time characteristics colonies on plates were gram stained, purified by repeated subculture and stored on agar slants and glycerol water until further biochemical characterization.

Biochemical identification

Bacteria isolates were identified according to **(32)**, (**33**) and methods described in (**34).**

Isolation, purification and identification of fungi

From each food sample 25 g was aseptically weighed and macerated in sterile bag and 225 ml of sterile buffered peptone water was added. Two fold serial dilutions of **(35)** were carried out using sterile buffered peptone water as diluents. From each dilution 1ml was plated using the pour plate methods of (**22)** on sabourd dextrose agar plate. Plates incubate for 5 day at 25-30^{o}C. after incubation, the plates examined macroscopically and microscopically, Purification of yeast colonies were achieved by streaked methods, isolated yeast cell investigated under microscope, maintain on sabourd dextrose agar slants at 4ºC for short period storage or mixed with glycerol water and store at -18 for long time preservation **(36).** Fungi were isolated from sabourd dextrose agar and preserved on agar slant at 4ºC, also fungal spore preserved on sterile saline water for long period at 4ºC for further investigations.

Sabourd dextrose agar: employed to determine microbial contamination in food, cosmetics, and clinical specimens **(37).**

Yeast identification: Yeast was identified accordingto **(38).**

Fungal identification: Fungi were identifiedaccording to **(39, 40, 41).**

Antibacterial activity of chitosan solution against *Bacillus cereus* by disc diffusion and micro dilution method

Muller Hinton agar plate inoculated by 0.1ml of *Bacillus Cereus* $1.5*10^8$ cfu/ml (0.5Macfarland), let for 4hr at 4 °C, 6mm sterile disc impregnated with 50µl of (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 µg/ml) concentration of 1% acetic acid chitosan solution applied to muller hinton agar plate and incubated for 18-24hr at 35°C. The tests were triplicate and the mean result of diameter zone was record, 1% acetic acid solution used as negative control, tetracycline with concentration 30 µg/ml used as positive control. Diameter of inhibition zone was determined as described by Kirby-Bauer disc diffusion method **(42).** Serial dilution of chitosan solution was prepared by micro dilution method using microtitre plate to obtain minimum inhibition conc. (MIC) of chitosan against *Bacillus cereus* **(42).**

Antifungal activity of chitosan solution against *Aspergillus niger* by agar dilution growth method:

The antifungal activity of chitosan against *Aspergillus niger* was determined by agar dilution growth method as described by **(43)**. chitosan solution dissolved in acetic acid were prepared with (120,60,30,15,7.5,3.75 mg/ml) concentration added to melted sabourad dextrose agar, sterilize, thoroughly mixed and pour into sterile petri plate at 45ºC, plugs of 6mm from 3-4 days fungal mycelium cut from edge of active growing colony were inoculated in the center of agar plate and incubated at 25ºC for 5 days, control cultures were prepared by 1%acetic acid as negative control and fluconazole 75mg/ml as positive control, radial growth were measured after incubation for 5 days and compared to controls, results were expressed as the percentage of hyphal growth inhibition (44), the lowest concentration show fungal growth inhibition is the (MIC). All tested were performed triplicate and the results were analyzed statistically.

Statistical analysis

Statistical analysis and analysis of the present study was conducted using the mean, standard deviation and ANOVA.

Results and Discussion

Isolation, characterization and identification of bacterial isolates:

Bacterial isolates from canned and fast food were indicated in Table 1and 2, also biochemical test used for identification of bacterial isolates was shown on Table 3.

Table 1: Percentage abundance of bacterial isolates in canned food

Bacterial isolates	Juices (n=65)	Tomato pastes (n=45)	Jam (n=15)	Tuna& sardine (n=49)	Beef (n=39)	Total number of samples(n=213	Percentage frequency
Bacillus cereus	8	8	4	25	13	58	27.2%
Bacillus subtilis	14	9	5	12	4	44	20.6%
Bacillus atrophaeus	0	0	0	0	0	0	0%
Staph.saprophyticus	0	1	0	0	0	1	0.46%
Staph.epidermis	1	0	1	2	3	7	3.3%
Enterococcus faecalis	1	2	1	0	1	5	2.3%
E.coli	0	0	0	0	0	0	0%
Klebsiella	0	0	0	0	0	0	0%
Salmonella	0	0	0	0	0	0	0%

0: absent

Results of bacterial isolation from canned food in Table 1 showed that *Bacillus cereus* is the most isolated bacteria with percent 27.2%, then *Bacillus subtilis* 20.6, *Staph.epidermis* 3.3%, *Enterococcus faecalis* 2.3% and *Staph.saprophyticus 0.46%.*

Results in Table 2 showed that the most isolated bacteria from fast food was *Bacillus cereus* with percent 87.5 %, then *Klebsiella* 50%, *E.coli* 33.3%, *Bacillus atrophaeus* 33.3%, *Salomonella 20.8% and Bacillus subtilis 4.1%.*

Table 2: Percentage abundance of bacterial isolates in fast food

Bacterial isolates	Falafel (n=3)	Liver (n=3)	Tuna (n=3)	Egg (n=3)	Sogeh (n=3)	Cheese (n=3)	Beef Burger (n=3)	Chicken Burger (n=3)	Chicken nugts (n=3)	Total number of samples (n=24)	Percentage frequency
	Sandwich										
Bacillus cereus	3	3	3	3	3	0	2	2	2	21	87.5%
Bacillus subitils	0	0	0	0	0	0	1	0	0	1	4.1%
Bacillus atrophaeus	0	2	3	3	0	0	0	0	0	8	33.3%
Staph.saprophyticus	0	0	0	0	0	0	0	0	0	0	0%
Staph.epidermis	0	0	0	0	0	0	0	0	0	0	0%
Enterococcus faecalis	0	0	0	0	0	0	0	0	0	0	0%
E.coli	3	0	0	0	3	0	0	1	1	8	33.3%
Klebsiella	3	0	0	0	3	0	2	2	2	12	50%
Salmonella	3	0	0	0	2	0	0	0	0	5	20.8%

0: absent

Biochemical identification of Bacteria

Table 3: Biochemical characteristics of bacterial isolates of fast and canned food

Bacterial isolates	Oxidase	Catalase	Indole	Methyl red	VP	Citrate	Glucose	Lactose	Sucrose	Mannitol
Bacillus cereus	+	+	_	_	+	+	+	_	_	_
Bacillus subtilis	_	+	_	_	+	+	+	_	+	+
Bacillus atrophaeus	_	+	_	_	+	+	+	_	+	+
Staph.saprophyticus	_	+	_	+	+	ND	+	+	+	+
Staph.epidermis	_	+	_	+	+	ND	+	+	+	_
Enterococcus faecalis	_	_	ND	ND	+	ND	+	+	+	+
E.coli	_	+	+	+	_	_	+	+	_	_
Klebsiella	_	+	_	_	+	+	+	+	+	+
Salmonella	_	+	_	+	_	+	+	_	_	+

+: positive, -: negative ND: not detected

Results of biochemical tests for bacterial isolates from canned and fast food in Table 3 showed that all isolates were negative for oxidase test except for *Bacillus cereus* was positive, also all isolates were positive for catalase except for *Enterococcus faecalis* was negative. For indole test, all isolates were negative except *E.coli* was positive and *Enterococcus faecalis* not detected. Methyl red test result showed that *Staph. epidermis, Staph. saprophyticus, E.coli and klebsiella* were positive. while, all *Bacillus* species and *klebsiella* were negative. V.P (Voges-Proskauer) test showed that all isolates were positive except *E.coli* and *Salmonella* were negative. Sugar fermentation test for glucose, sucrose, lactose, mannitol and citrate showed various results between isolates as showed in Table 3.

Isolation, characterization and identification of fungal isolates

Yeast identification

Biochemical identification of yeast isolates were shown in Table 4. Yeast was identified according to **(38).**

Biochemical identification of yeast isolates

Table 4: Biochemical tests for yeasts isolates of fast and canned food.

Yeast isolates	Sugar fermentation*									
	Inositol	Xylose	Glucose	Sucrose	Lactose	Maltose	Sorbitol	Trehalose	Cellobiose	Raffinose
Candida tropicalis	-	+	+	+	-	+	+	+	-	-
Saccharomyces cereviseae	–	–	+	+	-	+	-	-	-	+

+: positive; -: negative; *fermentation means production of gas independent of pH changes.

Sugar fermentation tests for yeast isolates from canned and fast food showed that Candida tropicalis was positive for xylose, glucose, sucrose, maltose, sorbitol, trehalose and negative for inositol, lactose, cellobiose and raffinose. Also Saccharomyces cereviseae was positive for glucose, sucrose, maltose, raffinose and negative for inositol, xylose, lactose, sorbitol, trehalose and cellobiose.

Fungal identification:

Fungal isolates in canned food showed in Table 5 and fungal isolates in fast food showed in Table 6. Fungi were identified according to **(39, 40, 41).**

Table 5: Percentage abundance of fungi in canned food

Yeast isolates	Juices (n=65)	Tomato pastes (n=45)	Jam (n=15)	Tuna& sardine (n=49)	Beef (n=39)	Total number of samples(n =213)	Percentage frequency
Candida tropicalis	3	0	0	0	0	3	1.4%
Saccharomyces cereviseae	1	0	0	0	0	1	0.47%
Fungal isolates							
A. niger	9	2	3	0	0	14	6.6%
Penicillium notatum	0	1	0	0	0	1	0.47%

0: absent

Table 5 showed that fungal isolates from canned food were *Aspergillus niger* with percent 6.6% then *candida tropicalis* 1.4%, *Saccharomyces cereviseae* and *Penicillium notatum* were 0.47%.

Table 6: Percentage abundance of fungi in fast food

Yeast isolates	Falafel (n=3)	Liver (n=3)	Tuna (n=3)	Egg (n=3)	Sogeh (n=3)	Cheese (n=3)	Beef Burger (n=2)	Chicken Burger (n=2)	Chicken nugts	Total number of samples(n=24)	Percentage frequency
	Sandwich										
Candida tropicalis	0	0	0	0	0	0	0	0	0	0	0 %
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0	0	0	0 %
Fungal isolates											
A. niger	0	0	0	0	0	0	0	0	0	0	0 %
Penicillium notatum	0	0	0	0	0	0	0	0	0	0	0 %

0: absent

Table 6 showed that no fungal isolates were obtained from fast food survey.

Antibacterial activity of chitosan solution against Bacillus cereus by disc diffusion and micro dilution method

Antibacterial activity, MIC of chitosan solution against Bacillus cereus was indicated in Table 7 by Kirby Baur agar and micro-dilution method. Figure 1 showed antibacterial activity of chitosan by disc diffusion method. Results from Table 7 indicated that chitosan has antibacterial activity against Bacillus cereus with diameter of inhibition zone from 8mm to 13mm. Also, antibacterial activity increase with increase chitosan concentration then activity decrease due to increase viscosity of chitosan solution so it is difficult to diffuse through agar media. This result agrees with research's that indicated chitosan can inhibit the growth of a wide range of bacteria **(45)**.

Table 7: Antibacterial activity of chitosan solution against *B. cereus* by disc diffusion method with determination of MIC value by micro dilution method

Chitosan solution (µg/ml) in 1% acetic acid solution	Inhibition zone diameter (mm)± SD; n=3	Turbidity in microtitre plate
Blank (acetic acid 1%)	6.5	Turbidity
Positive control (tetracyclin 30 µg)	12.60	No turbidity
800	10	No turbidity
400	13	No turbidity
200	12	No turbidity
100	12	No turbidity
50	12	No turbidity
25	10	No turbidity
12.5	8	No turbidity
6.25	8	No turbidity(MIC)
3.12	6.5	Turbidity
1.56	6.5	Turbidity

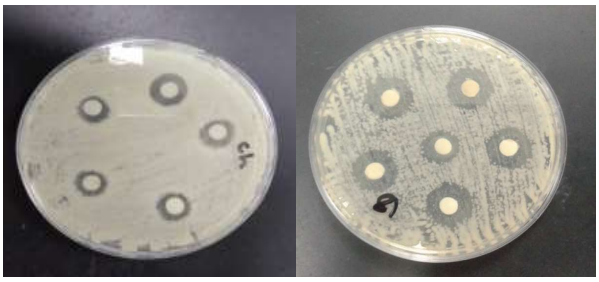

Fig 1: Antibacterial activity of chitosan solution against *Bacillus cereus* by disc diffusion agar method

Antifungal activity of chitosan solution against *Aspergillus niger* by agar dilution growth method

Antifungal activity of chitosan solution against *A.niger* was indicated in Table 8, results showed that with increase chitosan concentration, antifungal activity increase but activity is less than fluconazole which has antifungal activity. Figure 2 showed antifungal activity of chitosan after incubation for 5 days. These results were approved by several researches that indicated antimicrobial activities of chitosan against abroad range of microorganisms **(46).**

Table 8: Antifungal activity of chitosan against *Aspergillus niger* growth with percent of fungal growth inhibition

Treatment concentration (mg/ml)	Mean radial diameter (cm)	Percent inhibition (%)
Negative control (water)	9 ± 0.0	00.0
Positive control (Fluconazole 75mg/ml)	1.5 ± 0.1	83.3
120	3 ± 0.1	66.7
60	4.5 ± 0.1	50.0
30	7.5 ± 0.1	16.7
15	9 ± 0.1	00.0
7.5	9 ± 0.1	00.0
3.75	9 ± 0.1	00.0

±SD; n=3

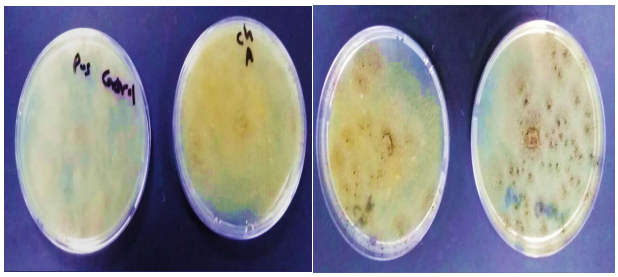

Fig. 2: Hyphal growth inhibition with various chitosan conc**.**

Conclusion

Chitosan has antibacterial and antifungal activity against the most isolates from canned and fast food which are *Bacillus cereus* and *Aspergillus niger,* this activity can be further developed for preparation of natural and safe antimicrobial agents for food preservation to reduce harmful effects of chemical and synthetic products on human health.

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دراسة النشاط المضاد للكيتوزان ضد بكتريا الباسيليس سيريس وفطرالاسبرجلس نيجر المعزولة من بعض الاطعمة المصرية المعلبة والسريعة

أ.د/ سعيدة محمد عامر[1] ,ا.د/ عزة عبدالرحمن مصطفي [2], د./ مها محمود عزب [1]، مصطفى فتحي شعبان[1]

1 قسم النبات- كلية العلوم – جامعة طنطا- طنطا - مصر

2 كلية الاقتصاد المنزلي – جامعة الازهر - طنطا – مصر

هدفت هذه الدراسة الي معرفة تاثير الكيتوزان علي اكثر انواع البكتريا والفطريات المعزولة من الاغذية المعلبة وسريعة التحضير حيث تم فحص 213 عينة اطعمة معلبة عبارة عن (تونة وسردين وعصائر وصلصة طماطم ومربي وبيف) تم تجميعها من سوبر ماركت (اولاد رجب وفتح الله والمنشاوي واوكازيون) وكذلك تم تجميع 24عينة طعام سريعة التحضيرمن مطاعم (الجعان وابودشيش والبركة وابوعوف) من مدينة طنطا وقد تم التاكد من كل العينات المعلبة انها في فترة الصلاحية ولايوجد اي عيوب تصنيعية. تم عزل الميكروبات باستخدام الاوساط الغذائية المناسبة حيث وجد ان اكثر انواع البكتريا شيوعا هو الباسيليس سيريس واكثر انواع الفطريات شيوعا هو الاسبرجلس نيجر وقد تم تعريف البكتريا بالاختبارات البيوكيميائية وكذلك تم تعريف الفطريات بالشكل الظاهري وتحت الميكروسكوب. تم اختبارالنشاط الميكروبي للكيتوزان علي بكتريا الباسيليس سيريس وكان اقل تركيز اظهر تثبيط للنمو البكتيري هو 6.25 ملجرام/ملي وقطر منطقة التثبيط 8 مللي وكذلك كان للكيتوزان نشاط مضاد لفطر الاسبرجلس نيجر وكان اقل تركيز أحدث تثبيط لنمو الاسبرجلس نيجر هو 30 ملجرام /ملي ونسبة تثبيط النمو الفطري 16.7%.الخلاصة تشير هذه الدراسة الي ان يمكن استخدام الكيتوزان كمادة حافظة لتاثيرها المضاد للنمو الميكروبي ولتقليل الاثر السيئ علي صحة الانسان من المواد الحافظة الكيميائية.