The impact of chitin isolation process sequences on the physicochemical properties and antimicrobial activity of the resultant chitosan compounds

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ABSTRACT

Three chitosan (CS) compounds were prepared by deacetylation (DA) of chitin isolated from shrimp shell using different sequences of isolation process steps which include deproteinization (DP), demineralization (DM) and decolorization (DC). The chitosan compounds produced were DPMCA, DMPCA and DCMPA (the letter sequences indicate the sequential processes used to prepare chitosan). The impact of alternate steps sequence through chitin isolation process on the physicochemical properties and biological activity of resultant chitosans compared with a commercial chitosan compound was investigated. The results indicated that no differences in the solubility of the three chitosan compounds in 1% acetic acid. When the isolation process started with DC step, a chitosan with high average viscosity molecular weight of 4.26 x 10^5 Da and high degree of deacetylation (DDA) 87.2% was obtained compared with DMPCA and DPMCA. The antibacterial assessment of resultant chitosan compounds were performed against *Corynebactrium spp.* and *Erwinia amylovora* and the results are expressed as a minimum inhibitory concentration (MIC). In general, the results indicated that all chitosan compounds have a good antibacterial activity, however the bacterium of *Corynebactrium spp* was more sensitive to chitosan compounds than *E. amylovora* where the MIC in average was 650 µg ml^{-1} and 2150 µg ml^{-1}, respectively. The antifungal assessment was also performed against fungi of *Fusarium culmorum*, *Aspergillus niger* and *Rizoctonia solani*. The results are expressed as an effective concentration that inhibits 50% of mycelia growth (EC_{50}). The result demonstrated a good and closer antifungal activity for the three chitosan compounds, however, DCMPA chitosan has low antifungal activity compared with other two chitosan compounds with EC_{50} values ranged between 2339 to 3430 µg ml^{-1} for all tested chitosan compounds.

INTRODUCTION

After cellulose, chitin is the second most abundant natural biopolymer found in nature (No *et al.*, 1989). It is linear homopolymer of β (1-4) linked 2-acetamido-2-deoxy-D-glucose. It is mainly used as a raw material to produce chitin-derived products, such as chitosan, chito-oligosaccharides and glucosamine.

Chitosan is a natural carbohydrate polymer derived by deacetylation (DA) of chitin. It is a non-toxic, biodegradable and biocompatible polymer (Muzzarelli, 1977). Over the last several years, chitinous polymers, especially chitosan, has received attention as one of the promising renewable polymeric materials for their extensive applications such as pharmaceutical and biomedical industries in wastewater treatment, in food industries (Knorr, 1984; Subramanian, 1978). In agriculture, chitosan has been widely employed. It has been reported to induce many defense-related responses in plants (Cabrera *et al.*, 2006; El Ghaouth *et al.*, 1992; Hofgaard *et al.*, 2005) and to possibly has a dual mode of action by direct affecting fungal growth as well as inducing defense related mechanisms in plants (Peter *et al.*, 1998).

Earlier studies by No *et al.*, (2000); Cho *et al.*, (1998); Wu and Bough (1978) have demonstrated that the physicochemical characteristics of chitosan affect its functional and biological properties, which also differ due to crustacean species and preparation methods. Several procedures have been developed and proposed by many researchers over the years for

preparation of chitosan from different crustacean shell wastes. Some of these formed the basis of chemical processes for industrial production of chitosan (Domard and Rinaudo, 1983 ; Batista and Roberts, 1990).

Traditional isolation of chitin from crustacean shells involves three sequential steps: demineralization (DM, removing calcium carbonate/phosphate), deproteinization (DP), and decolorization (DC, removing mainly astaxanthin) No et al., (1989).. Chitin is converted to chitosan by deacetylation (DA). Isolation steps may be shortened, depending on intended applications of chitosan. We aim in this research to investigate the influence of using different sequences of chitin isolation process steps on the physicochemical properties and biological activities of resultant chitosan after deacetylation of chitin

MATERIALS AND METHODS

Materials and Instruments: The raw material used as chitin source was shrimp shell wastes obtained from seafood industries of Alexandria city, Egypt. After separation of tail and head the shell were dried and ground to obtain particles between 20 to 40 mesh. Nutrient broth (NB) (5g peptone, 3g beaf extract and 1L sterile distilled water pH 5.9) were purchased from loba chemical co. Potato Dextrose Agar (PDA) media were prepared in laboratory using 300g of potatoes, 20g of Dextrose and 15g of agar per 1L. Hydrochloic acid, sodium hydroxide, acetic acid, sodium hypochloride and sodium chloride were obtained from Algomhoria chemical co. Egypt and used without further purification . For viscosity determination, Ostwald viscometer was used to determine the intrinsic viscosity. For IR-measurements, Shimadzu FT-IR Prestige (Shimadzu Corporation). was used.

Tested microorganisms: Two bacteria species of *Corynibacterium spp.* and *E. amylovora* were grown in NB medium. And three fungi species *F. culmorum* , *A. niger* and *R.solani* grown on PDA at 24°C in the dark, All microorganisms were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

Chitosans preparation : Chitosan compounds were produced according to the methods of No and Meyers (1995) and No *et al.*, (2000). Depending upon the production sequence, The following steps procedure were carried out, shells particle were demineralized with 1 N HCl for 30 min at room temperature with constant stirring at solid-to-solvent ratio of 1:15 (w/v) and then filtered under vacuum. The residue was washed for 30 min with tap water and oven-dried. Shells particle were deproteinized with 3.5% NaOH solution for 2 hr at 65°C with constant stirring at a solid to alkali solution ratio of 1:10 (w/v) and then filtered under vacuum. The residue was washed for 30 min with tap water and oven-dried. Shells particle were decolorized with acetone for 10 min and dried for 2 hr at ambient temperature, followed by bleaching with 0.315 % (w/v)sodium hypochloride (NaOCl) solution for 5 min at ambient temperature with a solid to solution ratio of 1:10 (w/v) and then filtered under vacuum. The residue was washed for 30 min with tap water and oven-dried. Deacetylation was achieved by autoclaving at a pressure of 15 psi for 60 min at 121°C using 50% concentrated NaOH solution with a solid-to-solvent ratio of 1:10 (w/v). The resulting chitosans were collected, washed as described previously.

Characterization of chitosan:

Viscosity measurement and molecular weight determination: Intrinsic viscosity of chitosan in 0.1M acetic acid - 0.2 M sodium chloride as the solvent was measured using an Ostwald capillary Viscometer in a constant-temperature water bath at 25 ± 0. 1°C. A series of diluted solutions viscosities were measured for each chitosan compounds. These measurements were then mathematically related to the intrinsic viscosities, [η], of each

chitosan compounds. Since the relationship of averaged molecular weight (M_v) to intrinsic viscosity has been established empirically, the average viscosity molecular weight for each chitosan compound was then calculated employing the equation of Mark-Houwink-Kuhn-Sakurada (MHKS) $M_v = ([\eta]/k)^{1/\alpha}$, where constants of "k" and "α" are 1.81×10^{-5} and 0.93, respectively (Anthonsen, *et al.*, 1993; No et al.,2003; Roberts & Domaszy, 1982).

Degree of deacetylation (DDA) : The FT-IR spectra were measured in KBr pellets in the transmission mode in the range of 400–4000 cm^{-1} using Shimadzu spectrophotometer. The DDA of chitosan compounds were calculated from the IR spectra according to Brugnerottoa *et al.*, (2001). Intensity of the bands at 1320 cm^{-1} and 1420 cm^{-1} were chosen to measure the DDA. As probe and internal reference band respectively, The computation equation is given below:

$$DDA = 100 - [31.92 \times (A_{1320}/A_{1420}) - 12.20] \quad r = 0.990$$

Solubility: Percentage of solubility of chitosan was determined at a 0.5% chitosan concentration in 1% acetic acid for 60 min with shaking at room temperature. Then, the solution was immersed in a boiling water bath for 15 minutes, cooled to room temperature and centrifuged for 10 min. The supernatant was decanted. The undisolved particles were washed in distilled water (25ml) then centrifuged. The supernatant was removed and undisolved pellets dried at 60 °C for 24hr. Finally, weighed the undisolved particles and percentage of solubility was calculated. Nitrogen content was estimated using the Kjedahl method (AOAC 976.06, 1995). Ash content was determined using procedure 942.05, as outlined by AOAC (1995).

Antibacterial activity assay: The Minimum Inhibitory concentration (MIC) of different chitosan compounds were estimated according to Marques *et al.*, (2008) by incubation the tested bacteria in nutrient broth (NB) media overnight. The bacterial culture was distribute into screw-capped tubes (3 replicates; 3 ml each) and mix with an aliquot a solution contain a range of concentration of chitosan compounds prepared in 1 % acetic acid and adjust the pH to 5.9 before mixing. The final volume was adjusted by sterilized nutrient broth and incubate the mixture at 37 °C for 24 h and detect the presence of turbidity, the concentration of the polymer was effective at inhibiting growth will appear clear. The viable cell in the clear solution was tested by formation of colony on nutrient broth agar after incubation at 37 °C for 24 h. The minimum inhibitory concentration (MIC) was the lowest concentration of antimicrobial required to inhibit the visible growth of the bacterium after incubation (Marques *et al.*, 2008).

Antifungal activity assay: The antifungal activity of chitosan samples was tested using the radial growth technique method (Torgeson 1967). Chitosan compounds were dissolved in 1% acetic acid and the pH was adjusted to ≈5.9 before mix with media. Mycelial discs (5 mm) of each pure culture were placed in the center of Petri plates (9 cm diameter) containing PDA with different chitosan concentration. Control Petri plates contained 1% acetic acid in PDA with pH adjusts to ≈5.9. The test plates were incubated in the dark at 25 °C. Growth measurement was recorded when the growth on the control reaches the edge of the plate. Inhibition percentage of mycelia growth was calculated as follows:

$$\text{Mycelia growth inhibition (\%)} = [(DC - DT)/DC] \times 100$$

Where DC and DT are average diameters of fungal colony of control and treatment, respectively. The EC_{50} with its corresponding 95% confidence limits was estimated by probit analysis (Finney, 1971).

Statistical analysis: Results are depicted as mean ± S.D. from three measurements. Significance between the mean values was calculated using ANOVA one-way analysis. Mean separations were performed by Waller-Duncan K-ratio t Test (WDK) test. Probability values $p < 0.05$ were considered significant. The log dose–response curves allowed determination of the concentration at which 50% of mycelial growth was reduced (EC_{50}) in an in vitro antifungal assay were analyzed with probit analysis (Finney, 1971) using ldp line software.

RESULTS AND DISCUSSION

Chemical composition of shrimp shell. Chemical composition of shrimp shell on dry weight basis which used as a source of chitin is shown in Table 1. It is indicated that the shell is an excellent sources of chitin which form about 24.29 % the crude protein is 27.42% and ash is 45.32 % of shrimp shell. No *et al.*, (1989) reported that there were 16.9% crude protein, 23.6% chitin, 63.6% ash, and 24.8% calcium in crawfish shells. Crustacean shell mainly consists of 30-40% protein, 30-50% calcium carbonate, and 20-30% chitin (Johnson and Peniston 1982). These portions vary with species and season (Green and Mattick 1979).

Table 1: Chemical composition of shrimp shell.

Composition	Means (%) ± standard deviation [a]
Crude protein [b]	27.42 (±0 .06)
Ash content	45.32 (±1.18)
Chitin content	24.29 (±0.45)

[a] (n = 4).Dry weight bases.

[b] Crude protein = (total nitrogen - chitin nitrogen) x 6.25.

Yield of chitosan compounds. The yield was calculated as the dry weight of chitosan obtained from 100g of dried shrimp shell powder in three replicate in each process sequences, as shown in Table 2. Chitosan yields ranged from 17.55 – 18.84 %. The highest yields were obtained from DCMPA, followed by DMPCA and DPMCA (conventional process sequence, control). Results indicated that there is no significance in the yield of the chitosan (DMPCA and DPMCA) but they are significantly lower than the chitosan types (DCMPA) which has the highest yield 18.84 % compare to other chitosan compounds. Fernandez-Kim (2004) used different modified procedure in isolation of chitin from crab shell and converts it to chitosan through deacetylation process. The yield of chitosan compounds were ranged from 16.8 – 18.8 % of initial crab shell and the follow sequence DMPCA, DPMCA have the lowest yield 16.7 and 16.8 %, respectively, on other hand, the DCMPA had yield about 18.3 % of initial shrimp shell.

Table (2): Yield of shrimp chitosan compounds using modified process during preparation.

CS compounds [b]	Yield [a]
DMPCA	17.67[b] (±0.09)
DPMCA ©	17.55[b] (±0.09)
DCMPA	18.84[a]. (±0.10)

[a] Means ± standard deviation (n = 3). Means with different letters indicate significant differences at $P<0.05$, according to Waller Duncan K-ratio t Test (WDK) test.
© Control (traditional method).
[b] DPMCA = (deproteinized + demineralized + decolorized + Deacetylated);
DMPCA = (demineralized + deproteinized + decolorized + Deacetylated)
DCMPA = (decolorized + demineralized + deproteinized + Deacetylated);

Characteristics of chitosans. The physicochemical characteristics of various chitosans prepared under different isolation processes were determined. Results are shown in Table 3. The nitrogen content of the shrimp shell chitosan compounds were varied between 7.38 % and 7.43% on dry and ash-free basis but no significantly difference between sequences of production were observed in nitrogen content. Only the commercial chitosan had the significantly high value of nitrogen content 7.59 % this may be explained by high deacetylation value.

It is well known that chitosan is insoluble in wat9er at neutral and basic pH-values due to the presence of NH_2 group. The solubility of chitosan compounds ranged from 92.9 % to 96.85 %, as shown in Table 3.

Several researchers observed changes in the nitrogen contents of chitosan from various crustacean species. Shepherd *et al.*, (1997) found squid pen chitosan and crawfish chitosan have 7.5 % - 7.2 % of nitrogen content, respectively however Cho *et al.*, (1998) found 7 % nitrogen content in shrimp chitosan. Rout (2001) indicated that crawfish chitosan has 7.3 % of nitrogen content. No and Meyers (1995) reported that the nitrogen content were 7.06 % and 7.97 % in shrimp and crab shell chitosan, respectively.

Ash measurement is an indicator of the effectiveness of the demineralization (DM) step for removal of calcium carbonate. The ash content in chitosan compounds were ranged between 0.28% to 0.35% with on significantly difference between difference production sequences while the commercial chitosan has significantly higher ash content (0.74%) value than prepared ones as shown in Table 3. The ash content in chitosan is an important parameter some residual ash of chitosan may affect its solubility, consequently contributing to lower viscosity, or can affect other more important characteristics of the final product. A high quality grade of chitosan should have less than 1% of ash content (No and Meyers, 1995).

Table 3: Physicochemical characterization of chitosan products prepared under various step sequence.

CS compound [e]	(DDA) [g]	MW (x10^5 Da) [d]	N % [b]	Ash % [a]	Solubility % [a,b]
DMPCA	87	3.81	7.38[b] (±0.025)	0.29[b] (±0.07)	96.85[a] (±1.86)
DPMCA	85	3.29	7.37[b] (±0.022)	0.28[b] (±0.10)	95.63[a] (±1.75)
DCMPA	87	4.26	7.39[b] (±0.027)	0.35[b] (±0.12)	92.90[a] (±2.48)
Acros	90	3.56	7.59[a] (±0.02)	0.74[a] (±0.10)	94.10[a] (±2.35)

[a] Means ± standard deviation (n = 3). Means with different letters within columns indicate significant differences at $p<0.05$, according to Waller-Duncan K-ratio t Test (WDK) test .

[b] measured in (1% acetic acid as a solvent at 0.5 % conc .

[d] molecular weights deduced from intrinsic viscosity using ($K=1.81x10^{-5}$ and $\alpha = 0.93$) as MHKS constant

[g] determined by IR Method according to Brugerotto *et al.*, (2001)

[e] DPMCA = (deproteinized + demineralized + decolorized + Deacetylated);
DMPCA = (demineralized + deproteinized + decolorized + Deacetylated)
DCMPA = (decolorized + demineralized + deproteinized + Deacetylated);
Across = commercial chitosan.

The result show no significantly differences between various chitosans and commercial chitosan. The same observation was obtained by Fernandez-Kim S (2004) which found that chitosan compounds produced from crab shell using alternative process sequence have solubility ranged between 93.3 to 94.3% with no significance differences in solubility between different modified process sequences.

The average viscosity molecular weights of chitosan compounds ranged from 3.29 x10^5 Da to 4.26 x10^5 Da while, commercial chitosan sample has a Mw of 3.56 x10^5 Da. The chitosan prepared from the shrimp shell show variations in their average viscosity molecular weight that seem to be related to alternating process sequences during isolation, since the condition of every step DC, DP and DM through isolation were constant. Beginning of isolation process by DC step also showed affect on the Mw of chitosan compared to chitosan sample obtained by DC step applied after DM and DP steps.

The DDA of chitosan samples obtained through modified step sequences in the isolation process from shrimp shell was ranged between 85% to 87% while, the commercial chitosan has DDA of 90%. Also the beginning of isolation process by DC step gives final chitosan with high DDA (87%). The same observation has been reported by Fernandez-Kim (2004) who obtains a chitosan with high DDA when isolation process began with DC compared to beginning with DP or DM steps.

Antibacterial activity of chitosan compounds. The antibacterial activity of chitosan samples was examined in vitro against two plant pathogenic bacteria *Corynibacterium spp.* and *E. amylovora* and the result was expressed as MIC as shown in Table 4. The results indicated that all of chitosan compounds exhibited higher activity toward *Corynibacterium sp.* than *E. amylovora* . All chitosan compounds exhibited a good antibacterial potency against *Corynibacterium spp.* with MIC values ranged between 650 and 700 µg ml^{-1} but the antibacterial activity of all chitosan compounds against *E. amylovora* were lower (MIC values ranged between 2050 and 2250 µg ml^{-1}).

Table 4: The MIC ($\mu g\ ml^{-1}$) of chitosan compounds against *E. amylovora* and *Corynebactrium spp.*

CS compounds [a]	MIC ($\mu g\ ml^{-1}$)	
	Corynebactrium spp.	*E. amylovora*
DMPCA	700	2250
DPMCA	700	2200
DCMPA	650	2050
Acros	600	2100

[a] DPMCA = (deproteinized + demineralized + decolorized + Deacetylated).

DMPCA = (demineralized + deproteinized + decolorized + Deacetylated).

DCMPA = (decolorized + demineralized + deproteinized + Deacetylated).

Across = commercial chitosan.

Chitosan inhibits the growth of a wide variety of bacteria (Lim and Hudson 2003 and No *et. al.,* 2002). Moreover, chitosan has several advantages over other type of disinfectants because it possesses a higher antibacterial activity, a broader spectrum of activity, a higher killing rate, and a lower toxicity toward mammalian cells (Franklin and Snow, 1981 and Takemono, *et al.,*1989)

The extent of chitosan antimicrobial action is influenced by several intrinsic (associated with the constitution of the macromolecule) and external factors such as: molecular weight, DDA, pH, temperature, target microorganism, concentration and chitosan batch, for example Lim and Hudson (2003); No *et al.,* (2002); and No *et al.,* (2006) reported that the MIC of chitosan ranged from 5 to 100mg L^{-1} depending on the species of bacteria and Mw of chitosan samples. The antimicrobial activity of native chitosan is higher at around pH≈6 (compared to at pH≈7.5), when most amino groups remain protonated (Stossel and Leuba, 1984)

Chitosan showed relatively stronger bactericidal effects for gram-positive bacteria than for gram-negative bacteria in the presence of 0.1% chitosan, as observed by Jeon *et al.,* (2000) and No *et al.,* (2002). This finding is in agreement with our result since *Corynibacterium spp.* consider Gram-positive bacterium and *E. amylovora* consider gram-negative bacterium.

Antifungal activity of chitosan compounds.

The antifungal activity of chitosan compounds was in vitro examined against three plant pathogenic fungi *F. culmorum*, *A. niger* and *R. solani,* The result was expressed in term, The Medium Effective Concentration (EC_{50}), which is the effective concentration that inhibit 50% of mycelia growth, EC_{50} of different chitosan compounds against the three plant pathogenic fungi are shown in Table 5.

The EC_{50} value differed with the use of different sequence through preparation of chitosan through isolation process. The experimental result indicated that DPMCA has the highest antifungal activity whereas the DCMPA chitosan has the lowest activity as shown in Table 5. The result matches with Xu *et al.,* (2007) which investigate *in vitro* antifungal activity of oligochitosan against nine phytopathogenic fungi *Phytophthora capsici, Verticillium dahlia, A. solani, Botrytis cinerea, Colletotrichum orbiculare, Exserohilm turcicum, F. oxysporum, F. graminearum* and *Pyricularia oryzae*. They found that oligochitosans were more effective than original chitosan in inhibiting mycelia growth of *P.capsici.*

Table 5: The antifungal activity of chitosan compounds against *F. culmorum*, *A. niger* and *R. solani.*

CS compounds [a]	EC_{50} (µg ml^{-1})		
	F. culmorm	*A.niger*	*R.Solani*
DMPCA	2573	2727	2682
DPMCA	2339	2375	2480
DCMPA	3430	3350	3172
Acros	2748	2540	2840

[a] DPMCA = (deproteinized + demineralized + decolorized + Deacetylated).

DMPCA = (demineralized + deproteinized + decolorized + Deacetylated).

DCMPA = (decolorized + demineralized + deproteinized + Deacetylated).

Across = commercial chitosan.

Several authors have been studied the antifungal activity of chitosan compounds against a wide range of plant pathogenic fungi. Benhamou *et al.*, (1994) indicated that chitosan derived from crab shell at concentration of 500 and 1000 mg L^{-1} was effective in reducing disease incidence caused by *F.oxysporum* f. sp. *Radicislycopersici.* At the same manner El-Ghouth *et al.*, (1994) revealed that chitosan was effective in inhibiting mycelia growth of *P. aphanidermatum* completely at a concentration of 400 mg L^{-1} while, at aconcentration of 100 mg L^{-1} it cause a 75 % reduction of the mycelia dry weight. Our result in agreement with El-Ghouth *et al.*,(1992) which found that with increasing in the chitosan concentration (750-6000 mg. L^{-1}) the radial growth of *A.alternata, B.cinerea, Colletotricum gleosporioides* and *Rizopus stolonifer* were decreased.

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الملخص العربى

اجريت هذة الدراسة لاختبار تاثير التغيير فى تسلسل العمليات التى تجرى على قشور الجمبرى المعذولة من متبقيات مطاعم مدينة الاسكندرية وذلك خلال استخلاص مركب الكيتين الذى يتم تحويلة فيما بعد من خلال المعاملة بمحلول قلوى مركز من NaOH وتم اختبار التغيير فى ترتيب المعاملات والتى تتمثل فى خطوة ازالة البروتين (DP) خطوة ازالة الاملاح المعدنية (DM) خطوة ازالة الصبغات (DC) وبعد الحصول على الكيتين يتم اجراء خطوة ازالة الاسيتيل (DA) للحصول على الكيتوزان. تم الحصول على ثلاث مركبات من الكيتوزان اخذت الاختصارات الاتية تبعا لترتيب الخطوات التى اجريت لتحضيرها (DPMCA – DMPCA - DCMPA) وتم مقارنتها من حيث بعض الخواص الفيزيوكياوية (نسبة الرماد – المحتوى النيتروجينى – الذوبان – درجة عدم الاسئلة – متوسط الوزن الجزينى مقاسا بطريقة اللزوجة الجوهرية) والنشاط البيولوجى لها كمضادات بكتيرية وفطرية ضد بعض الممرضات النباتية مع عينة تجارية من الكيتوزان وكانت النتائج المتحصل عليها توضح عدم وجود اختلافات بين الثلاث مركبات كيتوزان المتحصل عليها من حيث الزوبان ونسبة الرماد والمحتوى النيتروجينى الا ان العينة التجارية كانت اعلى فى محتوى الرماد والنيتروجين من الكيتوزان المحضر. وكانت عينة الكيتوزان DCMPA الاعلى فى درجة عدم الاستلة ومتوسط الوزن الجزينى فى حين كانت عينة الكيتوزان DPMCA اقلهم . واظهرت عينات الكيتوزان المتحصل عليها نشاط ضد البكتيريا المختبرة وهى *Corynebactrium spp.* و*Erwinia amylovera* وكان نشاط مركبات الكيتوزان المختبرة اعلى بدرجة كبيرة ضد *Corynebactrium spp.* مقارنة بنشاطة ضد *Erwinia amylovera* كما اظهرت المركبات المختبرة نشاط ضد الفطريات المختبرة وهى, *Fusarium culmorum* , *Aspergillus niger* , *Rizoctonia solani*. وكانت النتائج متقاربة الا ان DPMCA كان اكثرهم كفائة ضد الفطريات الثلاثة المختبرة.