

## **Contribution on some medicinal plants and drugs as antifungal against fungal fish diseases**

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### **Abstract**

This study was conducted to investigate the antifungal effect of some Medicinal plants were garlic (raw, powder and oil) and clove (grain, powder and oil) and the drugs Nystatin, Clotrimazole and Fluconazole were used in this study. The tested fungi were (*Saprolegnia* spp., *Cladosporium* sp., *Chrysosporium* sp., *Scopulariopsis brevicaulis*, *Aspergillus versicolor* sp., *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus glaucus*, *Aspergillus wentii*, *Rhodotorulla* sp., *Penicillium* sp., *Syncephalastrum* sp., *Aspergillus tamarii*., *Aspergillus flavus*, *Trichoderma* sp., *Acremonium* sp., *Rhizopus* sp., *Fusarium* sp., *Phoma* sp., *Absidia* sp., *Aspergillus candidus*, *Alternaria* sp., *Aspergillus terreus*, *Aspergillus fumigatus* and *Paecilomyces* sp.), which previously isolated from diseased fish. Raw Garlic was efficient against *Absidia* sp., while Garlic powder had positive effect against *Aspergillus flavus*, *Aspergillus ochraceus*, *Fusarium* sp., *Rhizopus* sp. and *Absidia* sp. Clove grain had positive effect against *Fusarium* sp., *Rhizopus* sp. and *Absidia* sp., on the other hand, clove powder affected in *Fusarium* sp., *Rhizopus* sp. and *Absidia* sp. Clotrimazole, Nystatin and Fluconazole, had positive effect against all tested fungi.

**Key word:** antifungal, fungal fish diseases, medicinal plant, Garlic, Clove, Clotrimazole, Nystatin and Fluconazole.

### **Introduction**

It is argued that fish when available, contain protein levels of 17-20% as well as minerals and vitamins. Moreover, aquaculture integrated into the existing farming system has been shown to improve both food and income security with little or no external input (Wetengere, 2010). Increase in aquaculture has been accompanied by

outbreaks of disease from an ever-increasing range of pathogens. Fungal infections are mainly secondary to environmental stressors exist in water environment and may result in economic losses to farming projects due to increased mortality, poor weight gain, besides low market value (**Rajinikanth et al., 2010**).

The different species of molds (*Saprolegnia* spp., *Aspergillus* spp., *Penicillium* sp., *Mucor* sp., *Rhizopus* sp., and *Cladosporium* sp.) which isolated from different localities in Egypt caused tail and fin rot syndrome and skin lesions in tilapia species and Nile catfish (**Marzouk et al., 1990**). Saprolegniasis was a worldwide serious mycotic winter fresh water disease often affects wild and cultured fishes (**Osman et al., 2010**). Hyphae of *Saprolegnia* sp. may invade deep tissues of fish and penetrate the vital organs even the central nervous system (**Zaki et al., 2003**). *Saprolegnia delica* and *Dictyuchus carpophorus* (the greatest fungal populations) were the most dominant isolated zoosporic fungal species where they were highly occurred especially at the hyper-polluted waters with the heavy metals (**Ali, 2007**).

(**Refai et al., 2010**) Isolated moulds in *Oreochromis* species belonged to the following genera: *Saprolegnia*, *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, *Scopulariopsis*, *Paecilomyces* and *Curvularia*.

The change in environmental temperature increases the rate of transmission of fungal disease between fish (**Hunter et al., 1980**). The effect of elevated ammonia and carbon dioxide levels combined with low dissolved oxygen concentration. They proved that increase in the mortality rate of Channel cat fish in an experimental infection with fungi under the above mentioned condition (**Walters and Plumb, 1980**). Overcrowding and bad handling result in significantly higher mortalities due to the flourishing fungal infection (**Hosmer, 1980**).

The medicinal plants are currently used in commercial aquaculture as growth promoting substances, nutrients and antimicrobial agents for prevention and control of fish diseases (**Galina et al., 2009**). Garlic (*Allium sativum*) had been agreed upon as an antibiotic for fungal associated diseases (**Ghahfarokhert et al., 2006** and **Shalaby et al., 2006**). Clove oil possesses antibacterial, antifungal and antiviral properties (**Chaieb et al., 2007**). Clove is considered an antifungal herb (**Steven, 2010**).

Nystatin was discovered as antifungal drug since that time the drug was used for treatment of yeast infections specially candidiasis (**Brown and Hazen, 1949**). Clotrimazole, a tritylimiazole derivative is a new drug with a wide spectrum of antifungal activity against dermatophytes, candida and other yeast species, Aspergilli, and many of the organisms responsible for subcutaneous and systemic mycotic infections and some Gram-positive bacteria (**Clayton and Connor, 1973**).

This study concerned with the control or prevention of fungal fish diseases using some drugs and medicinal plants *in vitro*.

### **Material and methods**

#### **Fungi;**

(*Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus* sp., *Fusarium* sp. and *Absidia* sp.,) previously isolated from clinically diseased *O. niloticus* in the Fish Disease Department CLAR were used in this study.

#### **Media;**

Czapek's Yeast Extract Agar (CYA), Malt Extract Agar (MEA), Oatmeal Agar (OA), Potato Dextrose Agar (PDA) and Sabouraud's Dextrose Agar (SDA) were used for fungus growth and storage and Czapek's Extract Agar medium for sensitivity test of tested fungi to medicinal plants and synthetic drugs.

#### **Plants and drugs;**

Medicinal plants which used in this study were garlic (*Allium sativum*) in three forms (raw (slices), powder (dissolved in distilled water) and oil (dissolved in Ethanol)) and clove (*Syzygium aromaticum*) also in three forms (grain (used as it is), powder dissolved in water and oil dissolved in Ethanol) which collected from the market. Ethanol was used as control. Antifungal drugs Nystatin 100.000 units/ml (Mycostatin, Squibb, Egypt), Clotrimazole 10 mg/ml, (Dermatin, Pharco Pharmaceuticals, Alexandria) and Fluconazole 150 mg/capsule (Fungican, Pfizer, Egypt) (Table 1) also were examined its antifungal effect against tested fungi.

**Table (1) Concentration and symbol of the tested antimycotic drugs discs for testing the antimycotic susceptibility of the tested fungi.**

Disc	Diffusible amount of antibiotic
Nystatin	100 I.U
Clotrimazole	50 µg
Fluconazole	50 µg

### **Preparation method of antifungal drugs**

Fluconazole a fresh solution was prepared for antifungal examination by dissolving the substance in Dimethyl formamide (DMF). Nystatin and Clotrimazole dissolved in distilled water. 1 mg of antifungal of Clotrimazole was dissolved in 1 ml of distilled water. 2000 I.U. of Nystatin dissolved in 1 ml distilled water. 1 ml of Fluconazole dissolved in 50 ml dimethyl formamide. dimethyl formamide was used as control.

### **Testing the antifungal susceptibility by disc diffusion technique**

In this well-known procedure, agar plates are inoculated with standardized inoculums of the test microorganism  $(0.4-5) \times 10^4$  cfu/ml (Heatley, 1944). Then, filter paper discs (about 6 mm in diameter) containing the tested medicinal plants and synthetic drugs at a desired concentration, are placed on the agar surface using disk diffusion method. The Petri dishes are incubated at  $30 \pm 2^\circ\text{C}$  for three days. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones were measured (mm).

## **Results and Discussion**

### **1- Garlic (*Allium sativum*):**

The results from table (2) showed the inhibition zones of garlic in three forms (raw, powder and oil) against tested fungi (photo, 2, 3 and 4). Raw garlic had antifungal effect only against *Absidia sp* and gave inhibition zone (20 mm). Garlic powder had antifungal effect against *Aspergillus flavus*, *Aspergillus ochraceus*, *Fusarium sp.*, *Rhizopus sp.* and *Absidia sp.*. The highest effect of garlic powder was against *Absidia sp.* (30 mm) inhibition zone in diameter. Garlic powder gave the same diameters of inhibition zones with the other tested fungi *Aspergillus flavus*, *Fusarium sp.*, *Rhizopus sp.* and *Aspergillus Ochraceus* (10 mm) and had no effect against *As. niger*. Garlic oil had

no effect against the all tested fungi. This result may match with results of other researchers used garlic extracts by adding it to grass of fishes but by improvement of immune system of fish to resist fungal infection. Some approaches suggested that garlic extract exerts its effect by the oxidation of thiol groups present in the essential proteins, causing inactivation of enzymes and subsequent microbial growth inhibition (**Bokaeianet al., 2010, Belguith *et al.*, 2010 and Elias and Abd El Ghany, 2008**).

## 2- Clove

Clove in grain form had positive effect as antifungal against *Absidia* sp., *Fusarium* sp. and *Rhizopus* sp. The highest effect was against *Fusarium* sp. and *Rhizopus* sp. giving the same inhibition zone (30 mm) in diameter, followed by *Absidia* sp. (25 mm). Clove grains had no effect on the other tested fungi. Also clove powder had positive effect against *Absidia* sp., *Fusarium* sp. and *Rhizopus* sp. It gave high inhibition growth of *Absidia* sp. forming inhibition zone (35 mm) in diameter, and the same inhibition zone in diameter (30 mm) with *Fusarium* sp. and *Rhizopus* sp.. Clove oil had no effect against the all tested fungi. Also This result may match with results of other researchers used clove oil which used as a bath for short time for one hour with certain doses that was recorded by (**Abd El-Ghany *et al.*, 2009**) by adding it to grass of fishes but by improvement of immune system of fish to resist fungal infection where clove contain Eugenol compound which have antifungal effect. This result matches with (**Velisek *et al.*, 2006**) who recorded that Eugenol can be used as an antimycotic agent in warm water fish aquaculture. Also in case of treatment with clove oil, no clinical signs or mortalities were detected, and (**Suliman *et al.*, 2007**) found also that, the highest inhibitory effect of clove bud oil was found against *Aspergillus niger*.

## 3- Nystatin

From table (2) Nystatin had positive effect against all tested fungi by inhibiting the fungal growth. Nystatin gave the highest inhibition zone with *Absidia* sp. 17 mm in diameter, the *Rhizopus* ap. and *Fusarium* sp.15 mm of each. Nystatin had the same antifungal effect against *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger* (10 mm) in diameter.

#### 4- Clotrimazole

Clotrimazole was highly effective against the tested fungi by inhibiting its growth giving inhibition zones ranged from 10 to 50 mm in diameters. The highest inhibition zone was 50 mm in diameter against *Aspergillus ochraceus*, after that 40 mm against *Absidia* sp.. Nystatine had inhibition zones 25, 15, 10 and 10 mm in diameters against *Aspergillus flavus*, *Aspergillus niger*, *Fusarium* sp and *Rhizopus* sp. respectively.

#### 5- Fluconazole

Data from table (2) showed the positive effect of Fluconazole against all tested fungi. Fluconazole gave the highest inhibition zone 40 mm in diameter against *Fusarium* sp and *Rhizopus* sp. Fluconazole gave inhibition zones 20, 20, 19 and 20 mm in diameters against *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus niger* and *Absidia* sp. respectively.

Measurements of antifungal drugs included the curing effect; the size of inhibition zone about a disc saturated by the antifungal or well contains antifungal, failure of the fungus to grow in liquid or solid media or even in vivo experiments using mouse or golden hamster, as (Emmons *et al.*, 1979) recorded.

Our findings matched with that reported by (Kassem, 1982) who concerning molds, *Aspergillus flavus* was highly sensitive to Econazole followed by Clotrimazole, Triconazole, Ketoconazole, Miconazole, Nystatin while amphotericin B had the lowest activity. As for *Fusarium* sp. Fluconazole showed highest inhibition zone diameter (40 mm) followed by Nystatin (15 mm) and Clotrimazole (10 mm). these results matches with that reported by (Kassem, 1982) who indicated that Econazole had the highest activity, followed by Miconazole, Ketoconazole, Triconazole, amphotericin B, Nystatin and Clotrimazole while the fungus was resistant to 5-Fc.

Generally the interpretation of results of susceptibility testing of antifungal drugs *in vitro* may present problems, one factor is that the development of standaralized procedures is still in early stage and at present each institution carrying out such tests uses its own preferred methods so that the comparisons of results is difficult. Another factor is the difference of each strain in its susceptibility to antifungal drugs.

**Table (2) Antimycotic sensitivity test of saprophytic molds to synthetic drugs and medicinal plants.**

Antifungal Agents  Tested Molds	A mean zone diameter of inhibition (mm) / 5 days / 30°C											
	Raw garlic	Garlic powder	Garlic oil		Grain clove	Clove powder	Clove oil		Nystatin	Clotrimazole	Fluconazole	
			Oil+ethanol	Ethanol			Oil+ethanol	Ethanol			Flu+DMF	DMF
<i>As. flavus</i>	-	10	-	-	-	-	-	-	10	25	20	-
<i>As. ochraceus</i>	-	10	-	-	-	-	-	-	10	50	20	-
<i>As. niger</i>	-	-	-	-	-	-	-	-	10	15	19	-
<i>Fusarium</i> sp.	-	10	-	-	30	30	-	-	15	10	40	-
<i>Rhizopus</i> sp.	-	10	-	-	30	30	-	-	15	10	40	-
<i>Absidia</i> sp.	20	30	-	-	25	35	-	-	17	40	20	-



**Fig. (1)** Clotrimazole effect on *A. flavus*



**Fig. (2)** Fluconazole effect on *A. flavus*



**Fig.(3)** Nystatin effect on *A. flavus*



**Fig.( 4)**Garlic powder effect on *A. flavus*



**Fig. (5)** Clotrimazole effect on *A. ochraceus*

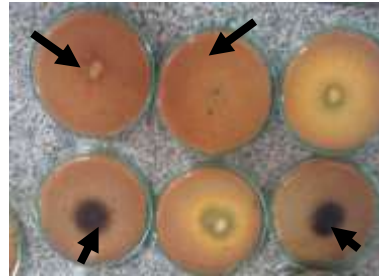


**fig.(6)**Fluconazole effect on *A. ochraceus*





**Fig. (7)** Garlic powder effect on *A. ochraceus*



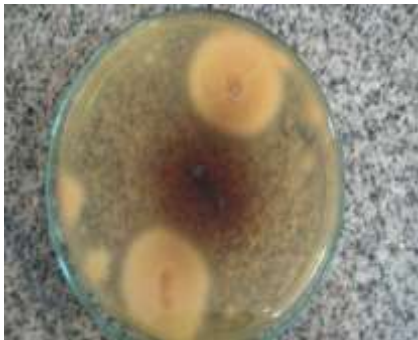
**Fig. (8)** Negative effect of raw garlic, Garlic Oil, clove grain, clove powder and Clove oil on *A. ochraceus*



**Fig. (9)** Nystatin effect on *A. niger*



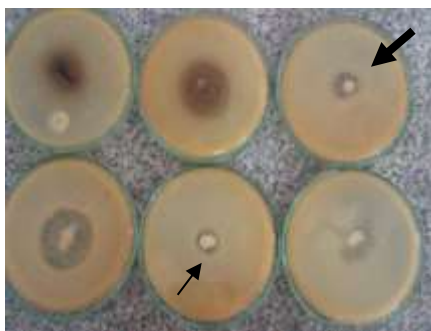
**Fig.( 10)** Fluconazole effect on *Fusariumsp.*



**Fig.( 11)** Clove grain effect on *Fusariumsp.*



**Fig. (12)** Clotrimazole effect on *Fusarium sp.*



**Fig. (13)** Clotrimazole and garlic powder have the same effect on *Fusarium* sp.



**Fig. (14)** Fluconazole effect on *Rhizopus* sp.



**Fig. (15)** Nystatin effect on *Rhizopus* sp.



**Fig. (16)** Garlic powder effect *Rhizopus* sp.



**Fig. (17)** Clotrimazole effect on *Absidia* sp.



**Fig. (18)** Clove powder effect on *Absidia* sp.



**Fig. (19)** Clove grain effect on *Absidia* sp.



**Fig. (20)** Nystatin effect on *Absidia* sp.

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## الفطريات المرتبطة بالبلطي النيلي المجمع من مزارع سمك العباسة وحساسيتها تجاه النباتات والأدوية المضادة للفطريات

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### الملخص العربي

تربية الأحياء المائية لا تزال واحدة من أسرع قطاعات إنتاج الأغذية نمواً، ومن المقرر أن تلعب دوراً رئيسياً في تلبية الطلب المتزايد على المنتجات السمكية التي تعتبر مصدر آخر للبروتين، فالبلطي النيلي من أسماك المياه العذبة الأكثر اقتصاداً في مزارع العباسة، واحده من أهم مزارع الأسماك في مصر.

(١) تم فحص "٤٣٠ سمكة" من أسماك البلطي النيلي بأوزان مختلفة تم تجميعها من ثلاث مزارع مختلفة لأسماك البلطي بالعباسة أثناء الفترة من أكتوبر ٢٠١٤ إلى يوليو ٢٠١٥. وقد خضعت هذه الأسماك للفحص الأكلينيكي والفطري والهستوباثولوجي.

(٢) الفطريات المعزولة من الأسماك المختلفة أشتملت على الأنواع التالية :

سيروليجنيا والكلادوسبوريم والكريسوسبوريم والاسكوبولاريوبسيس بريفيكالس والأسبراجلس فيرسي كلرجروب والأسبراجلس أوكراشيس والأسبراجلس نيجروالأسبراجلس جلاكس والأسبراجلس ونيتاي والرودوتورولا والبنيسيليم والسيسيفالسترم والأسبراجلس تاميراي والألسبراجلس فلافس والتريكوديرما والأكريمونيوم والريزوبس والفيوزاريوم والفوما والأبسيديا والأسبراجلس كانديس والألترناريا والأسبراجلس تيريس والأسبراجلس فيوميجاتس والباسيلومييسز فارينوسيز.

(٣) أوضحت هذه الدراسة أن معدل الإصابة بالفطريات في الأسماك التي تم فحصها كان (٤,٣٠٪) بين أسماك البلطي النيلي.

(٤) أوضحت الدراسة أن معدل الإصابة بالفطريات طوال العام كان (٣,١٨٪) و(٢,١٢٪) و(٦,١٠٪) و(٩,٩٪) و(٩,١٪) للبنيسيليم والأسبراجلس فلافس والأسبراجلس تاميراي والرودوتورولا والأسبراجلس نيجر على التوالي، يليها (٦,٧٪) للأسبراجلس فيرسي كلر والتريكوديرما، يليها (٦,٨٪) لكل من كلادوسبوريم وريزوبس، يليها (٦,١٪) و(٥,٣٪) للسيسيفالسترم والفيوزاريوم على التوالي، يليه (٤,٥٪) للكريسوسبوريم والأسبراجلس ونيتاي والأبسيديا والأسبراجلس تيريس والسيروليجنيا، يليها (٣,٨٪) و(٣,٣٪) و(٢,٢٩٪) لكلا من أسبراجلس أوكراشيس وفوما وسكوبولاريوبسيس بريفيكالس، يليها (١,٥٪) لكلا من الأسبراجلس جلاكس والألترناريا، يليها في النهاية أقل معدل للإصابة (٠,٧٪) لكل من الأكريمونيوم والأسبراجلس كانديس والأسبراجلس فيوميجاتس و الباسيلومييسز فارينوسيز.

(٥) بالنسبة لمعدل الإصابة في فصول السنة المختلفة فقد أوضحت النتائج ان أعلى معدل إصابة خلال فصل الصيف بمعدل (٦,٤٦٪)، بينما أقل معدل للإصابة كان (١,١٧٪) في فصل الربيع.

٦) تمت المعالجه أو اختبار حساسية تلك الفطريات معمليا تجاه بعض من النباتات مثل الثوم والقرنفل في صورهم المختلفه من زيت أو حب أو مسحوق مجفف حيث كان الثوم فعال في حال الأبيديا فقط وفي حاله الثوم البودر كان فعال في حالة الاسبراجيلس فلافس والأسبراجيلس أوكراشيس والفيوزاريوم والريزوبس والأبيديا بينما زيت الثوم لا يؤثر على كل الفطريات المختبره . ولكن القرنفل الحب كان فعال في حالة الفيوزاريوم والريزوبس والأبيديا كذلك القرنفل البودر كان فعال في حاله الفيوزاريوم والريزوبس والأبيديا ولكن زيت القرنفل أعطى نتيجة سلبية في كل الفطريات المختبره. وباستخدام بعض العقاقير الطبيه ذات المواد الفعاله ضد النمو الفطري مثل الكلوترايمزول والنيستاتين والفلوكونازول كان فعال تجاه كل الفطريات. وكان تأثيرات تلك النباتات وكذلك العقاقير علي النمو الفطري ملحوظ على الفطريات المختبره مكونا منطقه شفافة أو عديمه اللون تعني منع نمو الفطر بها حيث كانت أكبر فاعليه للكلوترايمزول على الأسبراجيلس أوكراشيس, وكانت أكبر فاعليه للفلوكونازول على الفيوزاريوم والريزوبس بنفس القدر, أما النيستاتين فكان تأثيره الأكبر على الأبيديا.