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ANTIFUNGAL ACTIVITY OF THYMOL ON PLANKTONIC AND BIOFILM CELLS

Chaimae Imane Sennouni, Mounia Oukouia, Imane Jabeur, Fouzia Chami, Adnane Remmal*

Address(es):
University Sidi Mohamed Ben Abdallah, Faculty of Science of Fes, P.O. Box 1796, Atlas, Fez, Morocco; Tel: (+212) 535 64 23 98; Fax: (+212) 535 64 25 00; Email: support.fsdm@usmba.ac.ma.

*Corresponding author: chaimaeimane.sennouni@usmba.ac.ma

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ABSTRACT

The purpose of this study was to evaluate the antifungal effects of thymol on fungi planktonic and biofilm cells. Firstly, the antifungal activity of a thymol-based product on reducing fungi load in poultry drinking water samples was assessed *in vitro* using the plate count method. Samples were treated by increasing concentrations of thymol (1, 2 and 4 $g.l^{-1}$ of product). Reduction is about one log unit for 1 $g.l^{-1}$, about two log units for 2 $g.l^{1}$, and a total desperation for 4 $g.l^{-1}$. Secondly, the antifungal activity was tested on biofilms formed on coiled pipes using an experimental arrangement simulating animals watering conditions in poultry farming. Two concentrations (1 and 2 $g.l^{-1}$) were used in two ways, preventive and curative treatment. The first one consisted in preventing fungal development on pipes and the biofilm formation. The second consisted in treating biofilms that have already settled on pipes. The plat count method was used to check the antifungal activity of thymol. Data of this experiment data have shown that thymol may prevent *Candida albicans* and *Aspergillus niger* proliferation in the experimental arrangement. After just a week, a significant reduction ($P<0,05$) was shown with the concentration 1 $g.l^{-1}$. The reduction was much important when the product was used at a concentration of 2 $g.l^{-1}$ (about tree log units). For curative treatment, results showed a significant reduction ($P<0,05$) of *C. albicans* load after only 24 hours with 1 $g.l^{-1}$ (about one log unit). With this same concentration, reduction is more important over time for both spices (about four log units after a week). A significant reduction (more than two log units) was showed in just 24 hours of treatment with the concentration of 2 $g.l^{-1}$. This work offers an alternative solution to chemical biocides, which can treat water and disinfect distribution system in poultry farming.

Keywords: thymol, biofilm, drinking water, *A. niger*, *C. albicans*

INTRODUCTION

Poultry provides different products to humans such as meat, and eggs, which may pose a risk for human pathogen exposure **(Meurens *et al.*, 2021)**. Breeders do not realize the importance of the microbiological safety of drinking water used for animals watering **(Stojanov *et al.*, 2015)**. Different infectious diseases of livestock are transmitted through contaminated water with pathogenic microorganisms. These latter survive for different periods and raise the health risk for animals. Among these, we find fungi, despite the low knowledge base concerning the occurrence of fungi in drinking waters, compared to bacteria **(Afonso *et al.*, 2021)**. Fungi were infrequently considered pathogenic microorganisms in drinking water.

In poultry farming, the water used for watering is either surface or underground water. Generally, surface water could be more contaminated by fungi than groundwater **(Hageskal *et al.*, 2009)**, due to direct contact with soil, organic material, and air. The simultaneous presence of pathogens in the water necessarily exerts a form of continuous stress on the immune system of farm animals as demonstrated by **Olkowski, 2009** and **Dennery *et al.*, 2012**. In addition, fungi produce some toxic compounds called mycotoxins, which have severe chronic and acute effects on animal and human health **(Zain, 2011; Al-gabr *et al.*, 2014)**. Mycotoxins are toxic to different animal groups in low concentrations **(Bennett, 1987)**. The negative effects of mycotoxins on chicken performance have been demonstrated in numerous studies. They can reduce the feed intake and body weight gain and increase the liver and kidney weights of broilers. Buccal-oral ulceration, plaque formation, and fertility problems were also observed after the consumption of food rich in mycotoxins **(Smith *et al.*, 1992; Brake *et al.*, 2000)**. They also, affect the taste and odor of the water **(Pereira *et al.*, 2010)**.

On the other hand, microorganisms adhere themselves to various surfaces (like tanks and pipes in poultry farming) and form biofilms which are protected by an extracellular polymeric matrix **(Costerton et *al.*, 1995; Flemming *et al.*, 2010)**. The high humidity levels and favorable temperature in farm facilities make favorable conditions for fungi development. Biofilms in water supply systems can represent temporary or permanent problems **(Wingender *et al.*, 2011; Stojanov *et al.*, 2017)**. These are the cause of numerous poultry diseases causing considerable damage and financial losses in the poultry industry **(Fulleringer *et al.*, 2006; Lorin *et al.*, 2017)**.

Bacteria, viruses, and parasites are the most frequently studied groups **(Mahmud *et al.*, 2019; Wen *et al.*, 2020)**, while fungi can be considered as a serious chronic problem in drinking water distribution systems **(Hageskal *et al.*, 2007)**. Living in a biofilm, fungi become extremely tolerant to antifungals and disinfectants **(Donlan *et al.*, 2002)**.

Recent publications from our laboratory have previously reported *in vitro* and *in vivo* the antifungal activity of some essential oils and their major compound, especially phenolic compounds such as thymol **(Oukhouia *et al.*, 2017; Jabeur *et al.*, 2018)**. The antifungal activity of essential oils against biofilms has been demonstrated in several works **(Souza *et al.*, 2016; Serra *et al.*, 2018; Gabriel *et al.*, 2018).**

The major aim of the present work was to evaluate firstly the antifungal activity of a natural alternative (NP EAU DE BOISSON (NPEB)) *in vitro* on different drinking water samples. Secondly, to assess the antifungal effect of this product on biofilms formed in an experimental arrangement simulating water supply system in poultry farming. The NPEB is a thymol-based product capable of replacing the chemical product, currently used in the poultry industry.

MATERIALS AND METHODS

Antifungal agent

In this experiment, the thymol-based product (NPEB) (15% of thymol) was used to treat animals watering samples and fungi biofilm formed on coiled pipes. Thymol was obtained from the *Origanum compactum*. To provide stability and solubility, other excipients have been added to thymol. The product was produced by the Industrial Laboratory of Veterinary Alternatives (LIAV, LLC) in Morocco.

Antifungal activity of thymol in different water samples

Water samples are used as drinking water in different poultry farms. They were collected in sterile bottles from the surface water Tank (T), water tower (WT) (coming from a river), and four different groundwater points (W1, W2, W3, and W4). They were transported in a cooler at 4°C directly to the laboratory and analyzed within 24 hours of arrival. Different concentrations of the thymol (1, 2, and 4 $g.l^{-1}$ of NPEB) were added to each water sample. A negative control was also prepared.

Sabouraud nutritious medium supplemented with Chloramphenicol (Biokar) was used for the antifungal test, using the plate count method. The medium was

prepared and sterilized according to the manufacturer's instructions. 100 µl of each sample was brought onto a solid medium and evenly spread over the agar surface with a sterile glass spreader. The inoculated dishes were incubated at 27°C for three to five days.

Antifungal activity of thymol on fungal biofilms formed on coiled pipes

NPEB was used at two concentrations (1 and 2 $g.l^{-1}$) and in two ways, preventive and curative treatment. The first one consisted in preventing the development of fungi on pipes and biofilm formation. NPEB has been incorporated in tank 1 in the permanent presence of the fungal load for 21 days. The second treatment consisted in treating biofilms that have already settled on pipes: after a week of circulation of water contaminated by fungal, tanks and pipes have been washed with sterile water and supplied with two liters of water treated with NPEB. The total treatment time was one week for the concentration 1 $g.l^{-1}$ and 24 h for the concentration 2 $g.l^{-1}$. For both manipulation, preventive and curative, the control arrangement contained only water loaded with the fungal load.

Organisms and growth conditions

The fungal strains used in this work, *Candida albicans* and *Aspergillus niger*, were isolated in our laboratory from a water facility in poultry farming. The Fod strain identification was done using a conventional method based on the colony's macroscopic characteristics and the microscopic appearance of conidia.
A volume of 50 µl containing *C. albicans* strain stored in 20% glycerol was added to 9 ml of sterile Sabouraud nutritious medium supplemented with Chloramphenicol (Biokar), the same as another volume of 40 µl containing *A. niger*. Tubes were incubated for 48 h at 30°C for *C. albicans* and during 5 days at 25°C for *A. niger*. Then, a microdrop (10 µl) was transferred to a Malassez chamber for microscopic counting. Cells were counted in 10 different fields using standard techniques. The fungal load obtained was 4 10^7 $cfu.ml^{-1}$ for *C. albicans* and 10^6 $conidia.ml^{-1}$ for *A. niger*.

Experimental arrangement

In this study, the fungal biofilm was experimentally formed on one cm diameter and two meters length PVC coiled pipes, which link two tanks (Fig. 1). The first tank (N°1) contains two liters of aqueous nutrient solution (5 g of Sabouraud medium supplemented with Chloramphenicol (Biokar) and 18 g of NaCl) contaminated with fungal. The second tank (N° 2) is a recovery tank. To favor biofilm formation, a tap at the bottom of tank N°1 ensured the passage of a low water flow through the pipe. The coiled pipe promoted water stagnation and fungal development. In addition, the room ambient temperature was adjusted using electric heating to 30°C.

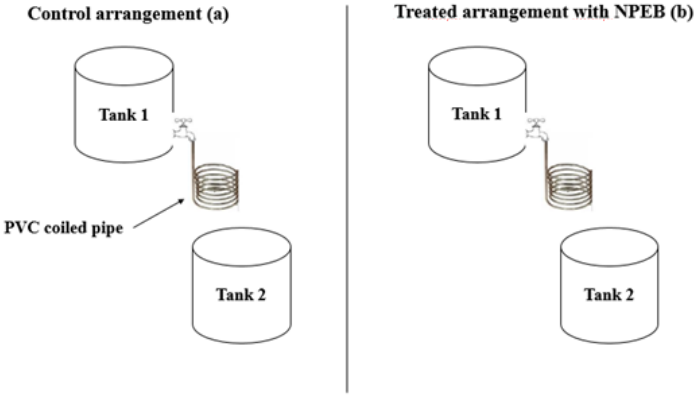

Figure 1 Experimental arrangement. ***Tank (1)*** *contains 2 liters of aqueous nutrient solution contaminated with fungal load (4 10^7 $cfu.ml^{-1}$ of C. albicans or 10^6 conidia/ml of A. niger);* ***Tank (2)*** *is used to collect the water from the tank (1);* ***(a)*** *Control arrangement: contains only water contaminated with fungal;* ***(b)*** *Treated arrangement: contains water contaminated with C. albicans and A. niger + NPEB at 1 $g.l^{-1}$or 2 $g.l^{-1}$*

Evaluation of fungi's load after preventive or curative treatment with NPEB

Firstly, pipes have been rinsed twice with sterile distilled water to ensure removing loosely associated or planktonic cells. Then, a 3rd rinse followed by stirring was made to recover the germs adhering to the inner pipes and a water sample was collected for analysis.
Fungal load enumerations: Analysis was carried out in triplicate using the plate count method. The medium used for the cultivation and enumeration of *C. albicans and A. niger* was the Sabouraud medium supplemented with Chloramphenicol (Biokar). 100 µl of each sample was transferred onto the center of the agar plate and then spread evenly over the entire surface with a sterile glass spreader. The plates were then incubated for three to five days at 27°C for *A. niger* detection and 48 hours at 30°C for *C. albicans* detection.

Statistical analyses

The results are presented by the means and their standard error. The data were analyzed by the T-test using SigmaStat 4.0. The results of the experiment were considered significant at P<0,05.

RESULTS AND DISCUSSION

Effect of the antifungal activity of thymol in different water samples

The Antifungal activity of thymol in different water samples is shown in table 1. A high fungal load characterizes samples collected from GP1, GP4, T, and WT. While samples collected from GP2 and GP3 were free, treatment with NPEB at the concentration 1 $g.l^{-1}$, of both samples GP1 and GP4, caused a complete elimination of the fungal load. A significant reduction (P <0.01) in the fungal load of sample T was also obtained with the concentration of 1 $g.l^{-1}$. An even more significant reduction (P <0.01) was noted after treatment with 2 $g.l^{-1}$ and the disappearance was total after treatment with 4 $g.l^{-1}$. A non-significant (P>0.05) reduction in sample WT load was obtained for the concentrations of 1 $g.l^{-1}$, unlike concentration 2 $g.l^{-1}$ (P <0.05). A total elimination of the fungal load in sample WT was observed after treatment with 4 $g.l^{-1}$.

Antifungal activity of thymol on fungal biofilms formed on coiled pipes

Effect of preventive treatment with NPEB on C. albicans and A. niger load

Figure 2 shows the evolution of *C. albicans* and *A. niger* loads, during the three weeks of treatment with NPEB in the treated pipes water compared to the controls. A significant reduction (P<0.05) in the *C. albicans* load was noted after treatment with both concentrations 1 and 2 $g.l^{-1}$ of NPEB, unlike the untreated control pipes where the load remained higher.
During the three weeks of treatment, the burden of *A. niger* was also reduced for pipes treated with NPEB compared to the control. The difference is significant (P<0.05) after seven days of treatment for both tested concentrations. However, treatment with 2 $g.l^{-1}$ led to a greater reduction in the load (about three log units).

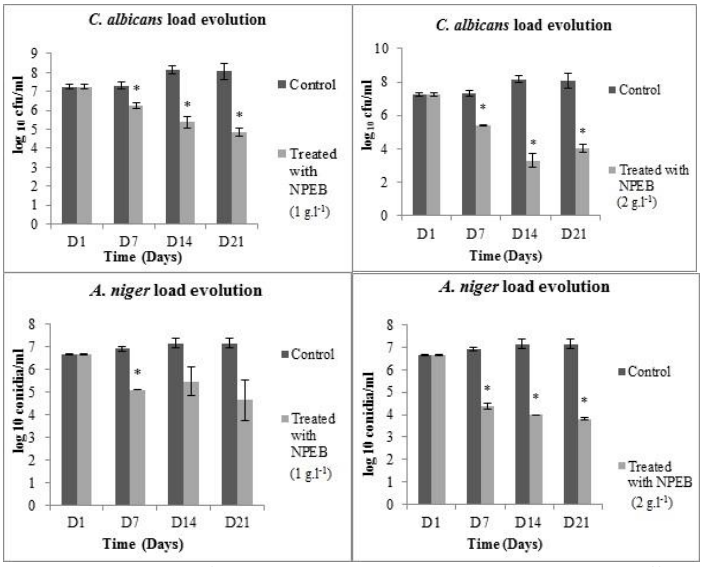

Figure 2 Evolution of *C. albicans* and *A. niger* loads using two different concentrations of NPEB (1 and 2 $g.l^{-1}$). Values are means (n=3) ± SEM (Standard error of the mean) (*indicates a significant difference between the control group and the treated group at P<0.05 according to t-test, D (Day))

Table 1 Variation of the fungal load in different samples depending on the thymol concentration

	Fungal load ($\log_{10}$ Cells/ml)					
	(GP1)	*(GP2)*	*(GP3)*	*(GP4)*	*(T)*	*(WT)*
Control	2.36 ± 0.23	0 ± 0	0 ± 0	2.21 ± 0.17	3.48 ± 0.06	**2.8 ± 0.13**
1 g.l^{-1} of NPEB	0 ± 0	0 ± 0	0 ± 0	0 ± 0*	2.11 ± 0.07**	**1.9 ± 1.26**
2 g.l^{-1} of NPEB	0 ± 0	0 ± 0	0 ± 0	0 ± 0*	1.74 ± 0.06**	**0.56 ± 0.75***
4 g.l^{-1} of NPEB	0 ± 0	0 ± 0	0 ± 0	0 ± 0*	0 ± 0**	**0 ± 0***

Values are means (n=6) ± SEM (Standard error of the mean); Comparison with control: * P <0.05; ** p<0.01

GP1 (Groundwater point N° 1): Well located in a poultry farm in the province of El Jadida in Morocco; GP2 (Groundwater point N° 2): Well located in the rural commune of Dayat Aoua in Morocco; GP3 (Groundwater point N° 3): Well located in the rural commune of Kariat Ba Mohammed in Morocco; GP4 (Groundwater point N° 4): Well located in the rural commune of Ain Aicha in Morocco; T (Tank): Water basin supplied from the Oued de Sebou located in a poultry farm in the province of Taounate in Morocco; WT (Water tower): Water tower supplied from the Oued de Sebou located in a poultry farm in the province of Taounate in Morocco

Effect of curative treatment with NPEB on *C. albicans* and *A. niger* load

The *C. albicans* and *A. niger* load evolution in infected pipes water after treatment with NPEB compared to untreated control pipes is represented in figure 3. Treatment with both concentrations (1 and 2 g.l^{-1}) caused a significant reduction (P<0.05) in the *C. albicans* load. Treatment with 2 g/l for only 24 hours resulted in a reduction of around tree log units, greater than the one obtained with 1 g/l for seven days. Concerning the *A. niger* load, a reduction in treated pipe water became significant (P<0.05) after 48 hours of treatment with the concentration 1 g.l^{-1}. Whereas for the concentration 2 g.l^{-1}, the reduction was very significant (P<0.05) after only one hour of treatment.

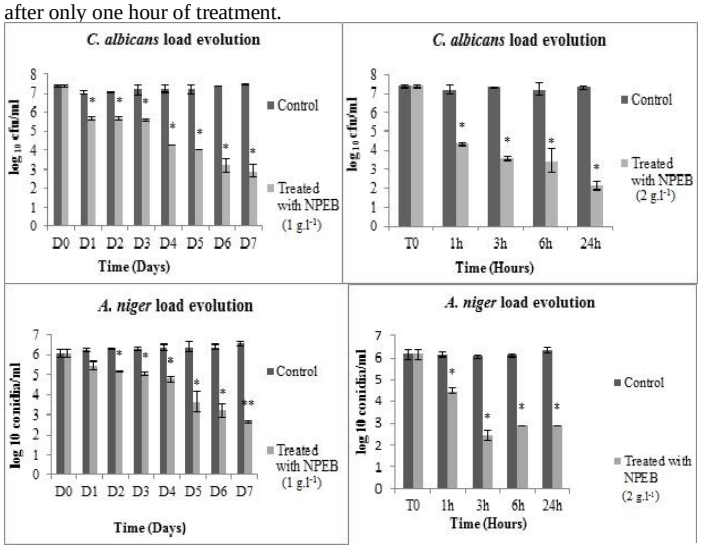

Figure 3 Evolution of *C. albicans* and *A. niger* loads using two different concentrations of NPEB (1 and 2 g.l^{-1}). Values are means (n=3) ± SEM (Standard error of the mean) (*indicates a significant difference between the control group and the treated group at P<0.05 according to the t-test; **indicates a significant difference between the control group and the treated group at P< 0.01 according to t-test; D (Day))

In this study, we choose to test the effect of a developed product based on thymol, on the treatment of fungal planktonic in drinking water and fungal biofilms formed in water pipes and distribution systems. There are multiple mechanisms of action of thymol. It acts on the cell membrane, impairing the permeability of substances. A denaturation of enzymes and modification of proton driving forces, due to changes in pH and electric potential, can also be caused by thymol (**Stammati *et al.*, 1999**). The antifungal effect of thymol has been demonstrated on several species (**Decastro *et al.*, 2015; Saker *et al.*, 2018**). Obtained results of the first experiment show that four of six of the tested water samples, used as drinking water in different poultry farms, have a significant fungal load (around 10^2 to 10^3 cfu.ml^{-1}). The treatment of these samples with the concentration of 1 g.l^{-1} of NPEB provided a significant reduction in the fungal load. An increase in the concentration of the product leads to a decrease in the load. The growth inhibition was obtained at the concentration of 4 g.l^{-1}. There were not other similar research that have tested the effect of essential oils or majority compounds product-based (specifically thymol) on the fungal load of water with whose we could compare our results. However, a study conducted by **Jafri *et al.*, (2020)** has demonstrated the antifungal activity of *Thymus vulgaris* and thymol against the Candida strains. Planktonic Minimum inhibitory concentration (MIC) of *T. vulgaris* and thymol were exhibited ranging from 1.56- 50 µg/ml against the test strains of *Candida albicans* and *Candida tropicalis.*

The effect obtained after using the NPEB on planktonic fungi prompted us to test the effect of adhering fungi on water tanks and pipes. A diversity of mold and yeast species have been isolated from the drinking water system. Among these are potentially pathogenic, allergenic, and toxigenic species. Genus *C. albicans and A. niger* were chosen in this experiment because they were the most frequently isolated mold and yeast (investigation results). In addition, *Candida* and *Aspergillus* species are the most common genera of fungi associated with water (**Chandra *et al.*, 2001; Gonçalves *et al.*, 2006**). Problems linked to the presence of fungal biofilms in water distribution systems in poultry farming have been cited in previous work (**Stojanov *et al.*, 2017**). Several researchers proved that *C. albicans* biofilm is responsible for many infections. It can resist in biofilm for long periods and on different surfaces. In addition, *C. albicans* resists a diversity of antibiotics and antifungal products (**Sanglard *et al.*, 1996; Douglas, 2003**; **Theraud *et al.*, 2004**). Normally, disinfection processes are periodically performed between production rounds to remove the biofilm. The more often reported problem is the disinfection efficiency decline. A study carried out by **Theraud *et al.*, (2004)** demonstrated that eight out of nine antiseptics and disinfectants' overall agents were ineffective against *Candida* growing in biofilms. Generally, microorganisms come from the water source (underground or surface water), adhere, and accumulate on the inner surface of water distribution systems to proliferate and contaminate animals during the breeding period (**Scwab *et al.*, 2004**). To approach this problem, an experimental arrangement simulating the conditions of animals watering in poultry farming was developed. In this experiment, a PVC coiled pipe, linking the two tanks, allows a low flow of water rate and ensures water stagnation.

Our data have shown that NPEB added to water may prevent *C. albicans* proliferation in tanks and pipes. After just a week, a significant reduction (approximately one logarithmic unit) was shown when the initial tank, containing a *C. albicans* load (10^7 cfu.ml^{-1}), was treated with NPEB at the concentration of 1 g.l^{-1}. The reduction was much more important when the product was used at a concentration of 2 g.l^{-1}, since it was reduced by about two logarithmic units in a week. This observation led us to check whether the NPEB would also be able to combat *C. albicans* already attached to pipes. The obtained results showed after only 24 hours that the treatment at 1 g.l^{-1} allows a reduction of the load by approximately one logarithmic unit. After 7 days, the burden continued to decrease over time to reach approximately four logarithmic units. The use of NPEB at 2 g.l^{1} showed in just 24 hours of treatment, a reduction of three logarithmic units.

Based on this finding, thymol may prevent the formation of *C. albicans* biofilm on water systems. In addition, both concentrations (1 and 2 g.l^{-1}) of NPEB can eliminate already-formed biofilms. Similar results have been obtained with *A. niger*.

CONCLUSION

Disinfection programs of poultry production houses performed to remove biofilm are usually done in absence of animals because of the products used toxicity. On the contrary, thymol has no acute or chronic toxicity and can be used in an animal's presence. In addition to managing biofilm in poultry drinking water, it can improve animals' zootechnical performances and be a growth promoter. Due to the effectiveness and biocompatibility of products extracted from medicinal plants, they can be one alternative strategy to control microorganisms in poultry farming. According to the obtained results, thymol exercises a significant antifungal action on poultry drinking water, which will be beneficial to chicks and positively affect their zootechnical performance. On the other hand, thymol allows adhesion prevention and the proliferation of fungal species in drinking water systems. At the same time, it removes already-formed biofilms. In addition to demonstrating a high antifungal activity, this work offers an alternative solution to chemical biocides used in poultry farming to disinfect the distribution system of water.

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Mohamed Ben Abdallah. The product used in this experiment was produced by the LIAV and RDAA contributed to this work through its expertise in research and development.

Data Availability: The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest: No conflict of interest declared.

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