ANTISAPROLEGNIA ACTIVITY OF METHANOLIC EXTRACT OF *ZINGIBER OFFICINALE* IN BUNNEI (*BARBUS SHARPEYI*) HATCHERY

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ABSTRACT

Usage of chemicals such as malachite green to disinfect fish eggs in aquaculture has been banned in many countries around the world due to its environmental negative impact. This study aimed to evaluate the *Zingiber officinale* rhizomes extract as an antifungal agent during incubation period of Bunnei eggs and comparison of its effects with malachite green. For this purpose 24 hours after fertilization, methanolic extract of *Zingiber officinale* rhizomes in four groups with a concentration of 500, 750, 1000, and 1250 mg/L and three replications per concentration in a (10 minute) bath every 12 hours and malachite green group with a concentration of 0.1 mg/L for (6 minutes) bath was used twice a day and control group without antifungal agents under the same physiochemical conditions as other treatments were tested. The present study showed that the highest percentage of fungal infection was 41.82±0.5 % for the control group and the lowest 0.3±0.5 % was obtained for the treatment of 1250 mg/L of *Zingiber officinale* rhizomes. Also, the results showed that there were significant differences between treatments of different concentrates of *Zingiber officinale* rhizomes and with malachite green group in fungal infection, percentage of fertilization and survival do not exist (P>0.05.) No apparent deformity and abnormality were observed in the hatched larvae of Bunnei, so the extract of *Zingiber officinale* rhizomes with concentration 500 mg /L can be used to disinfect Bunnei eggs during Incubation period and safe material for humans and the environment.

Key words: Bunnei eggs, methanolic extract, *Zingiber officinale*

التأثير المضاد للفطريات لمستخلص جذور الزنجبيل الكحولي أثناء فترة حضانة بيض سمك البني

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المستخلص

ان استخدام الملخايت الأخضر كمطهر لبيوض الاسماك في المزارع محظور في الوقت الحالي في العديد من البلدان حول العالم لتاثيره الضار على المحيط . الهدف من هذه الدراسة هو تقييم مستخلص جذور نبات الزنجبيل كعامل مضاد للفطريات خلال فترة حضانة بيض البني ومقارنة تأثيره مع الملخايت الاخضر. تم معالجة البيوض بعد 24 ساعة من الإخصاب بالمستخلص الميثانولي لجذور الزنجبيل في أربع مجموعات بتركيز 500 ,750, 1000 و 1250 ملجم / لتر بواقع ثلاث مكررات لكل تركيز في مغطس (10 دقائق) كل 12 ساعة ومعاملة الملاخايت الاخضر بتركيز 0.1 مجم / لتر في مغطس (6 دقائق) مرتين في اليوم ولم تستخدم المعاملة الضابطة العوامل المضادة للفطريات بنفس الظروف التي تم اختبارها مع المعاملات الأخرى. أظهرت نتائج الدراسة الحالية أن أعلى نسبة إصابة بالفطريات كانت 41.82 ±0.5 للمعاملة الضابطة وأقل نسبة كانت 0.3 ± 0.5 تم الحصول عليها في معاملة 1250 ملجم / لتر من جذور الزنجبيل. كما أظهرت النتائج وجود فروق معنوية بين معاملات التراكيز المختلفة لجذور الزنجبيل ولم يلاحظ أي تشوه واضح في يرقات البني بعد الفقس، لذلك يمكن استخدام مستخلص جذور الزنجبيل بتركيز 500 ملجم / لتر لحماية بيض البني خلال فترة الحضانة من الاصابة الفطرية وهي آمنة للإنسان والبيئة.

الكلمات المفتاحية: مستخلص جذور الزنجبيل, مستخلص كحولي, بيض البني.

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INTRODUCTION

Fungal disease one of the most important detrimental factors in the aquaculture industry and considered the main problem during Fish eggs incubation (9). Saprolegniosis is fungal infections and the most important fungal disease infects fish and their eggs in aquaculture industry (18). It is estimated that this disease causes extinction of several fish species like salmon, European eels and catfish. Also in tilapia fish cause losses in hatcheries and In Japan, 80% of Kohu salmon from *Saprolegnia parasitica* are extinct (15). Bunnei is the local name of *Barbus Sharpeyi* which an important species in the Tigris and Euphrates basins and marshes of southern Iraq due to the negative effects of the marsh drainage program, which had started in 1992, their populations dropped dramatically and reduced the natural spawning of this fish, this species is in danger of extinction (4,20). *Saprolegnia* zoospores can attached and penetrate to the cell wall of dead cells also spread from infected eggs the healthy eggs (9**).** Malachite green is used in treatment of fungal infections in fertilized eggs and due to its adverse effects like carcinogenicity and negative environmentally impact the use of this substance has receded in many countries (12) It has also been recorded that residues of Green malachite in fish grown from eggs treated with this material so it's important to replace malachite green with an effective antifungal agent with less side effects (6) this led researchers to find more natural and eco-friendly compounds. Plant metabolites as a huge and valuable source of bioactive compounds (23,16). One of these plants that can be mentioned is *Zingiber officinale* rhizomes, which considered as antioxidant sources and its antimicrobial properties against bacteria and fungi have been exposed to The ethanolic and methanolic extracts of *Zingiber officinale* rhizomes(11,22),results of other studies confirmed that extract of this plant have a better antimicrobial effect compared with other prepared extracts have shown (17) . The aim of this study was to evaluate the antifungal effects of different concentrations of methanolic extracts of *Zingiber officinale* rhizomes during incubation period of Bunnei eggs.

MATERIAIS AND METHODS

Methanolic extract of *Zingiber officinale* rhizomes was prepared according to the method achieved by (8), grind *Zingiber officinale* rhizomes after drying then For every 1 gram of powdered plant we added 10 ml of methanol to dissolve the powder. The solution was placed on a shaker with 120 rpm for 24 hours. After that, the solution was filtered with sterile Whatman filter paper. The extract was concentrated by rotary apparatus and placed them at laboratory temperature to evaporate the solvent. After preparing methanolic extract of *Zingiber officinale* rhizomes. The desired research work achieved in the first half of March 2021 for five days in Al-wahda fish hatchery south of Baghdad (Fig.1).So that after ovulation of female fish and artificial insemination, desired number of eggs per liter was obtained, fertilized eggs were cultured in incubators. All the factors of physics and Chemical test environment including pH, temperature and water salinity daily Measured and recorded. Fertilized eggs divided into six Experimental groups, three replications for each group 500m g / L treatments, 750,1000 and 1250 methanolic extracts of *Zingiber officinale* rhizomes , malachite green in the amount 0.1 mg / L and the control group without the use of any disinfectant. The amount of eggs for each of the treatments was considered to be 1000 ±5 eggs /L. After 24 hours from the incubation time to determine the percentage of fertilization, eggs sampling was randomly done and percentage Fertilization was estimated according to the following formula (2):

(percentage Fertilization %)= number of fertilized eggs/ total number of eggs ×100

Disinfecting treatment achieved by sinking the incubated eggs in bath for 10 minutes with use concentrations of 500m g / L, 750,1000 and 1250 for the Methanolic extract of *Zingiber officinale* rhizomes every twice daily the eggs were siphoned from the jar into the pan and air currents are blown through it was set up inside the jar. Malachite green treatment at intervals of 12 hours with a concentration of 9 ppm (14) for 6 minutes were done per day. Also, three jar incubators (control group) control with full supervision in accordance

with all the conditions of other treatments Tested. Because fish larvae does not have much swimming power and cannot get to the part The higher they are, the more likely they are to suffocate. For this reason the eggs after blinking, it is transferred from jar incubators into trucks and separate the hatched larvae from the eggs, the disinfection treatment continued in pans and aeration flow was performed. After complete hatching of the eggs, the number of fungal infected eggs in the treatments were calculated and according to the equation:

Percentage of hatching after collecting the fries of each of the treatments:

were calculated according to (1):

Calculation of hatching rate:

Fertilization rate % = amount of fertilized eggs / total amount of eggs × 100

Hatching rate % = amount of hatching fries / amount of fertilized eggs× 100

Figure 1. incubators in Al-Wahda fish hatchery.

After hatching eggs, larvae of each treatment were kept up in separate trays. The purpose of this is separation the larvae were related to each of the treatments so that in case of any change in body shape, the effect of each treatment on the appearance of larvae determined.

RESULTS AND DISCUSSION

In this research, all physicochemical factors as temperature, salinity, Water pH were measured, pH = 7.5, average salinity was 166 mg/L, dissolved oxygen was 12 mg/L the mean water temperature during the incubation period 18±2 ^{0}C was obtained. The results related to the percentage of fertilization are shown in (Tab.1). It is clear according to the results, the highest percentage of fertilization for malachite green treatment at the rate of 80.54±3.3 and the lowest for the control group the rate was 80.12±3.7. However, a statistical comparison of these results shows the percentage of fertilization between treatments of *Zingiber officinale* rhizomes with malachite green group and also did not show a significant difference with the control group (P>0.05). According to the results related to the percentage of fungus shown in (Tab.1) the highest percentage of fungal infection for the control group was 41.82±0.5 And the lowest for the treatment of 1250 mg / L *Zingiber officinale* rhizomes extract at the rate of 0.3±0.5 was obtained. Comparison of relevant statistical results indicates that the percentage fungal infection Difference between *Zingiber officinale* rhizomes extract treatments and malachite green group doesn't show significance (p> 0.05). It was also found that the amount Percentage of fungal infection in *Zingiber officinale* rhizomes extract and Malachite Green group treatments respectively Significance was less than the control group (p <0.05). The results of the hatching percentage (Tab.1) show that among the studied groups the highest percentage of hatching in the malachite green group 45.7± 3.5 % and the lowest for the control group 21.7± 1.1 % was obtained. Statistical studies show that Percentage of hatching between treatments of *Zingiber officinale* rhizomes extract with malachite green group and also the percentage of hatching in the treatments of 500 mg / L and 750 *Zingiber officinale* rhizomes extract does not show a significant difference with the control group (p>0.05) It was found that the percentage of hatching in 1000 mg / L and 1250 treatments *Zingiber officinale* rhizomes extract and malachite green group were significantly higher than the control group (P≤0.05).

Table 1. percentage of fertilization

Treatment	Control Mean ±S.D	Malachite green Mean ±S.D	500 mg Mean ±S.D	750mg Mean ±S.D	1000 mg Mean ±S.D	1250mg Mean ±S.D
Fertility rate	80.12±3.7a	80.5±3.3a	80.2±1.7a	80.2±2.2 a	80.7±0.9a	80.7±0.7a
Percentage of infected eggs	41.82±0.5a	3.3±1.9b	3.2±0.9b	2.3±1.7b	1.1±0.1b	0.3±0.5b
Percentage of non-infected eggs	79.7±5.5b	93.5±0.7a	90.5±0.2a	98.1±0.4a	98.4±0.9a	99.8±0.2a
hatching Eggs percentage	21.7± 1.1b	45.7± 3.5a	50.90± 0.8ab	54.3± 2.3ab	40.6± 2.1a	41.4± 0.7a

*** percentage of fungal infected eggs, percentage of non-fungal eggs and percentage of hatching in different treatments similar letters in each row indicate no significant difference between groups**

number of healthy and lost eggs in all treatments showed in (Tab.2) statistical comparison of results related to the number of infected eggs indicate the average number of fungal infected eggs between treatments *Zingiber officinale* rhizomes extract with malachite green group did not show a significant difference, It was also found that the number of fungal infected eggs in the treatments of *Zingiber officinale* rhizomes extract and malachite ($P<0.05$). Examining the results of the number of lost eggs, it was found that Between *Zingiber officinale* rhizomes extract treatments together and with malachite green group as well number of eggs lost in 500m g / L treatments and 750 *Zingiber officinale* rhizomes extract Does not show a significant difference with the control group ($p>0.05$) It was found that the number of eggs lost in the 1000 mg / L treatments And 1250 *Zingiber officinale* rhizomes extract and malachite green group were significantly less than The control group.

Table 2. Mean of dead, hatched and fungal infected eggs of Bunnie fish during five days of incubation and treatment with methanolic *Zingiber officinale* rhizomes extract (similar letters in each row represent no significant difference between the groups).

Treatment	Control	Malachite green	500 mg	750mg	1000 mg	1250mg
Total count eggs	1000±5	1000±5	1000±5	1000±5	1000±5	1000±5
Number of dead eggs	744±7.5a	350±4.3b	472±9.4ab	522±8.6ab	521±22.4b	505±10.5b
Number of hatched eggs	104±15.4b	544±8.6a	496±12.2ab	470±18.4ab	464±11.7a	485±8.8a
Number of infected eggs	152± 8.4a	32± 7.3b	36± 3.5b	20± 1.5b	18± 2.6b	15± 0.7b

According to the results of healthy eggs number up to the hatching stage between the *Zingiber officinale* rhizomes extraction treatments and malachite green group as well as number of healthy eggs in treatments 500 and 750 mg/L *Zingiber officinale* rhizomes extract showed a significant difference with the control group in addition to the number of healthy eggs in treatments 1000 and 1250 *Zingiber officinale* rhizomes extract and malachite green group were significant more than the control group were ($p<0.5$). monitoring eggs of each of the treatments until hatching in the tray and larvae belonging to each of the treatments were kept separately for up to three days after hatching was examined but no apparent deformation in no larvae were observed for any of the treatments. Figure 2 show different embryonic stages and larvae during this study.

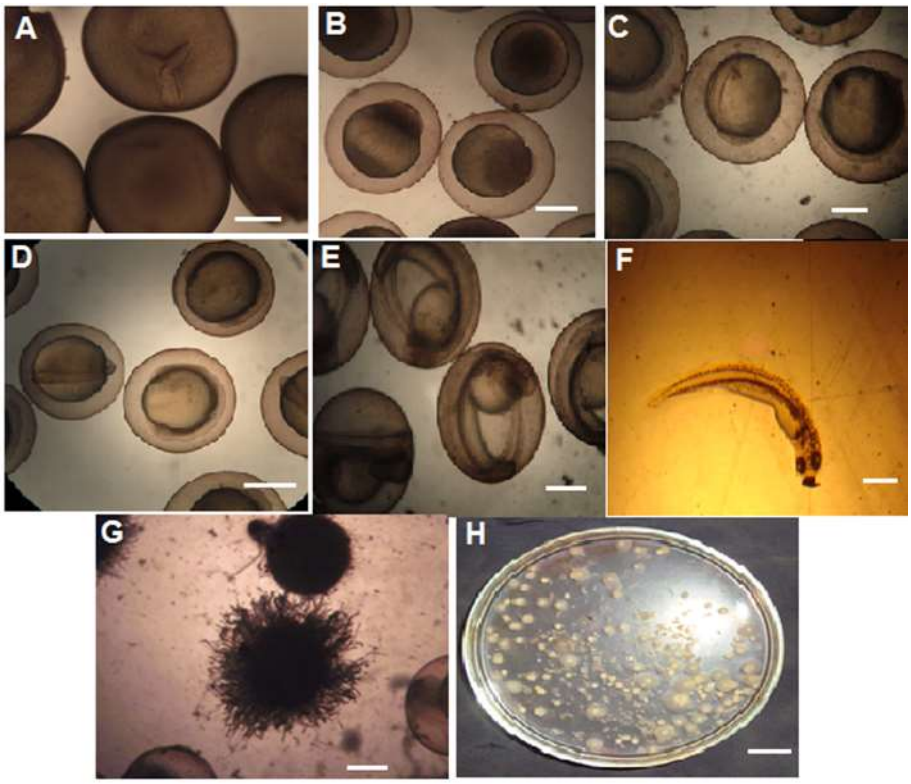

Figure 2. Embryonic stages during Bunnei eggs incubation,(A): fertilize eggs at 0 time of incubation,(B):eggs after 12 hours,(C):eggs after 24 hours,(D):eggs after 48 hours ,(E):eggs after 72 hours,(F):eggs after 120 hours,(G): infected dead eggs after 48 hours of incubation. scale bar for mention photos = 50 µm, (H): infected eggs in Petri dish

Water molds, especially the Saprolegniaceae considered a serious problem of fish breeding centers and it reduce the production of these centers which reduces fish growth and damages its gills (1,19) as well as a ban on the use of chemicals such as malachite green to find a suitable alternative medicine such as usage of herbal ingredients or compounds that have the less environmental impact. In the present study, to obtain a percentage of fertilization approximately equal to different treatments as well as the same test conditions for every treatment, result between the percentage of fertilization in *Zingiber officinale* rhizomes, malachite green and control treatments there were no significant difference with control. In a similar study by (3) compared the antifungal effects of formalin, malachite green and potassium permanganate were applied to Persian sturgeon during which due use of fertilized eggs for all treatments was 77٪. The results showed that the highest rate of fungal infected eggs related to the control group was 41.82±0.5 significantly differences with the treatments of *Zingiber officinale* rhizomes extract and malachite green. The reason for this difference can be not using any disinfectant in the control group the lower rate was 0.3±0.5 of infected eggs in the treatment of 1250 mg/L *Zingiber officinale* rhizomes extract. It was found that the reason could be the use of high concentrations of *Zingiber officinale* rhizomes extract. It can also be acknowledged that the methanolic extract of *Zingiber officinale* rhizomes has a significant antifungal effect compared to the material it is common like malachite green due to its antiseptic properties. In a similar study the anti-fungal effect of *Sama* plant extract against *Rhizoctonia solani, F. oxysporum Phytophthora drechsleri, Bipolaris sorokiniana.* According to the results of this study, all concentrations in this study have an inhibitory effect of raw *Zingiber officinale* rhizomes extract it was found that this issue reflects the wide range of antifungal effects of the *Zingiber officinale* rhizomes extract is one of the most eco-friendly and alternative chemicals consumptionof high persistence of many pesticides in nature which causes environmental pollution (21,10) plant extract decomposes more rapidly than chemicals such as malachite green After returning to the

environment, it does not cause environmental pollution and also no harm from *Zingiber officinale* rhizomes extracts to consumer as human or other creatures. Researchers (5) approved the effectiveness of the phytase enzyme as a feed additive on growth routine and survival rate against the Saprolegniasis in common carp. Essential oils of plants that contain karyofylene have anti-inflammatory properties they are good antimicrobial and antifungal can be stated because crude plant extract is made up of karyofylene which has significant antimicrobial and antifungal properties (2). Based on the results of this study, the control group has the lowest percentage Hatching 21.7± 1.1 and in *Zingiber officinale* rhizomes treatment also between treatments *Zingiber officinale* rhizomes extract, 1250m g/L treatment has the highest percentage of hatching 50.90±0.8 which can be considered the appropriate concentration of plant extract in this treatment, compared to other treatments. During the study similar evaluation of the effects of two *Terminalia catappa* ethanolic extracts (27 ℃ and 80 ℃) against *S. prasitica* on fish eggs by (9) .In another study by (22) found that essential oils of *Zataria multiflora* in dietary intake significantly enhanced respiratory burst activity of blood neutrophils and indicates that when common carp's health is at risk due to heat stress, administration of *Zataria multiflora* and *Eucalyptus globulus* essential oils may prove useful. Another study of *eucalyptus* essential oil in the control of fungal infections in fish eggs from 4-year-old rainbow lizard hatcheries *eucalyptus* in doses of 50 ppm, 25ppm, and 100 ppm in terms of antifungal properties significantly differentiated with the control. In comparison, the percentage of hatching was also treated with 25 ppm 9.15٪ had the highest percentage of hatching (2). larvae produced from each of the extract treatments were examined for appearance during which no deformity or body shape was observed in the larvae(13). During a similar study to investigate the effect of egg bath with malachite green in creating anomalies and changes in appearance observed in silver Bunnei larvae, no significant relationship no relationship were observed between malachite green and the degree of deformity of the vertebrate, malachite green as a chemical also changes the appearance of the form The body of the larvae does not grow, but it can be stated that it does not change its appearance The larva in *Zingiber officinale* rhizomes extract, which is a safe plant material it can be considered as one of the advantages of this plant material. However, in research conducted between *Zingiber officinale* rhizomes extract treatments in terms of percentage fungal infections in all four concentrations of 500 mg/L, 750 mg/L, 1000 mg/L and 1250 mg/L were significantly different from the control group but within treatments, no significant difference was observed in malachite green, which confirms the appropriate effect and the optimal antifungal coating of this extract in the control of fungal infection in The incubation stage of Bunnei fish eggs (7). On the other hand, according to the difference between the different concentrations of the extract in terms of fungal control no significance can be observed due to economic aspects and problems due to the high volume consumption of the extract, the concentration of 500m g / L as the concentration Optimal antifungal substance of the extract in the form of a bath for 10 minutes and twice Recommended per day in the control of fungal infections of Bunnei fish eggs.

CONCLUSION

The findings of this study suggest that crude *Zingiber officinale* rhizomes extract helps control Saprolegniasis in aquaculture. The effectiveness of these products in tank water needs to be tested further as well as their use in aquaculture supplies.

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