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Efficacy of Some Aromatic Plant Extracts on Treating the Eggs of the Common Carp (*Cyprinus carpio* L.) Against Fungal Infection in Comparison with Traditional Fungicide Malachite Green

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Abstract: The current study was carried out in Basrah Prawn Hatchery during the period from 23 March 2016 till 15 April 2016. Water extracts of four species of aromatic plants: garlic *Allium sativum* L., eucalyptus *Eucalyptus camaldulensis* Deh., mint *Mentha spicata* L. and ginger *Zingiber officinale* Roscoe were used as alternative materials instead of malachite green to control the pathogenic fungus *Saprolegnia parasitica* on eggs of the common carp *Cyprinus carpio*. The reason of using these substituted materials used here is to avoid the carcinogenic effect of malachite green for producers, consumers and abnormalities malformations of fish fries, contamination of the aquatic environment and economic losses resulted from malformation of fish larvae. The results showed significant differences in the sterilization ratio with the studied plants according to species and concentration of the extract in comparison with that of malachite green. The results showed that with application of 100% of the stock solution (full strength of garlic extract), the activity of treatment reached 96%, while lowest activity of sterilization reached 90% with using 100% ginger extract as compared to malachite green (99%). The number of alive fish larvae differs according to the species of the plant; the highest number of alive fish larvae (18694 larvae) was achieved with ginger, while the lower number (12320) was with eucalyptus in comparison with malachite green (13878). No such malformation in the larvae was occurred when treated with garlic, ginger and mint, while just 1% (non-significant) of malformation occurred with eucalyptus in comparison with malachite green (7%).

Keywords: Common carp's eggs, Hatchery, Fungi, Treatment, Aromatic plants, Malachite green.

Introduction

In recent years many fish hatcheries have been created in Iraq for providing fish fries and fingerlings for ponds and cages, but these hatcheries suffer from many problems causing degradation of the production (Khalil I. Salih, Pers. Comm.). One of those problems is the

fungal infection that existed seasonally on the eggs during artificial reproduction (Eakaphun *et al*., 2003). The main reason of these diseases got from freshwater fungi of the Saprolegniaceae in the fresh waters, which belong to Oomycetes, saprophytic fungi on detritus, and represented by *Saprolegnia parasitica*, a common causative of saprolegniasis on fishes and which causes high economic losses (Durborow *et al.*, 1991).

Malachite green is a toxic material tentatively used as a stain, but at low concentration it is used as anti-parasite, antifungal and antibacterial against infection of fishes and their eggs (Due *et al*., 1998). It is considered as carcinogenic and mutagenic matter and no longer used by FDA (Food and Drug Administration) for food fishes (Moghaddam *et al.*, 2004). It causes congenital malformation for fish embryos as well as considered as carcinogenic and an expensive item (Meinelt *et al.*, 2001). Many chemicals were approved activity in prevention or treatment of fungal infection of fishes. However, on the other side, malachite green is considered as dangerous for aquatic organisms and the environment owing to its toxic effect, carcinogenic, mutagenic and malformation for fish embryos (Doerge *et al.*, 1998). Many countries refused to use this material in the aquatic environment, and alternative number of studies tends to find safety treatments which do not harm the environment. One of these safety approaches was using some plant extracts and their oils that repeatedly and historically used for treatment of human diseases and as antimicrobials at the same time (Al-Mayah, 2013).

Aromatic plants are defined as plants that totally or partly containing active materials which are useful for treatment of humans or animals. They are common in the flowering plants especially in dicotyledons, but restricted in some families such as Solanaceae, Papaveraceae and Lamiaceae (Al-Mayah, 2013). The garlic is known as important item against fungi, bacteria and parasites (Al-Jubouri and Al-Naemi, 2008).

Eucalyptus has antifungal activity owing to its content of A, B sideroxylon (Singh *et al*., 1997). The mint contains special aromatic oil called "Peppermint oil" which has a unique smell and hard taste (Al-Shahat, 1988; Al-Ameri and Mohammed, 2006). It has a role in treatment of number of microbial diseases due to its antibacterial properties (Al-Jeburi, 1994). Ginger is used as therapeutic material for sea sickness, inflammations, rheumatism nausea in the digestive system and as an antibacterial agent (Azu and Onyeagba, 2007).

The current study aims to replace the malachite green with safe substances to human health, find inexpensive alternative materials available throughout the year to sterilize fish eggs, reduce economic losses in produced larvae due to malformations caused by the use of malachite green, and use of aquatic plant extracts from locally available aromatic plants for treatment of *Saprolegnia* that infects fish eggs during the artificial propagation process.

Materials and Methods

Four species of aromatic plants were collected from the local markets and gardens in Basrah governorate. Table (1) shows the scientific names, local names and the part of plant which is used. The plants were classified by Dr. Taha Y. Al-Edany, College of Agriculture, University of Basrah. They were dried in a well-aerated shaded place at room temperature with continuous stirring to prevent their decomposition. The plants were grinded and put in paper bags, labeled and kept at room temperature until use (Al-Niaeem, 2006). Plant materials were water extracted according to the method described by Twaij *et al*. (1983). An amount of 25 gm of dried powder of plants was placed in a 500 ml conical flasks, then 250 ml of distilled water were added. The mixture was placed in blender at room temperature for three hours, then centrifuged for 15 minutes and the clear extract was completed with distilled water to 250 ml of four concentrations of each extract (25%, 50%, 75% and 100% of the stock solution). A concentration of 0.5 gm/ l of

malachite green was applied as indicated by FAO (1985).

The eggs were collected from each incubator and examined by microscope to confirm the transmission of fungal infection from infected eggs to healthy eggs (Singhal *et al.*, 1987). The fungus identification followed Muhsin (1977) and confirmed by Dr. Abdul-Hafiz Al-Duboon at the Microbiology Laboratory, Department of Marine Biology, Marine Science Centre, University of Basrah. After positive finding of the fungus, 10 ml of infected eggs were placed inside each incubator and left for 12 hours for permitting the fungus to infect the eggs.

The eggs were treated by using a 50 ml syringe ending with narrow plastic tube to reach the bottom of the brood bottle. The extracts were pumped with slowing water flow to the lowest possible speed to permit the sterilizing material to stay with the eggs for an appropriate period. The period of holding plant extracts and the malachite green with eggs was about 40-45 seconds each time. The first sterilization was 12 hours after laying eggs in the brood. The sterilization was continued every six hours with watching eggs hatching. Sterilization was stopped when the first larva was observed (FAO, 1985; Singhal *et al*., 1987). Four replicates were used for both stock solution and malachite green, and replicated twice for each of the other concentrations, as well as leaving one of the brood vases without sterilization.

Randomized samples of eggs were collected from each incubator and each concentration of sterilizing materials. A total of 100 eggs were examined from each replicate to investigate fungal infection and to count the percentage of sterilized eggs to determine the efficacy of the sterilizing materials using a dissecting microscope. The percentage of infected and uninfected eggs was calculated according to Al-Shaikh and Rabee (1993), as in the following equation:

Percentage of sterilization = (number of uninfected eggs/ number of examined eggs) x 100

The number of larvae produced was determined by taking a known-sized sample from the incubator's water containing the larvae after closing the water and allowing the larvae to distribute in the water column of the incubator in a homogeneous manner, then counting the number of larvae in the representative sample and multiplying the number in the size of incubator water. The process of estimation was repeated three times. The number of larvae was estimated as in the following equation:

Number of larvae in each incubator = Number of larvae in a given volume of incubator water × volume of water in the incubator.

In addition, 100 larvae were taken by siphoning from each treatment and replicate to get the percentage of deformed larvae as in the following equation:

Percentage of deformed larvae = (number of deformed larvae/ total number of larvae) x 100

The IPM SPSS Statistics version 22 was used to analyze the results and to extract the correlation between the concentrations and the sterilization ratio. Significant differences were also compared with the revised least significant difference (RLSD) at a significance level of 0.95.

Table (1): Names of studied plants of and their part used.

Common name	Scientific name	The used part
Garlic	***Allium sativum* L.**	**bulbs**
Eucalyptus	***Eucalyptus camaldulensis* Deh.**	**leaves**
Mint	***Mentha spicata* L.**	**leaves**
Ginger	***Zingiber officinale* Roscoe**	**rhizomes**

Results

All fungal smears, taken from infected eggs from all incubators and all concentrations, were identified as *Saprolegnia parasitica* Coker 1923. The percentage of sterilization for fish eggs varied according to type of the extracts and their concentrations. When eggs were treated with a 25% concentration of stock solution, percentage of sterilization was 48% in treatment with eucalyptus and garlic, 43% with mint and 36% with ginger. Statistical analysis proved that sterilization with 25% concentration of extracts of all plants was significantly less than that of malachite green, as shown in Fig. (1).

At the concentration of 50% of the stock solution, the highest level of sterilization was obtained by using the water extract of the garlic (71%), followed by the eucalyptus (56%), then the mint (48%), while the lower level of sterilization was with the ginger (47%). The statistical analysis showed that sterilization at a concentration of 50% for all extracts was significantly less than that of malachite green, as shown in Fig. (2).

The statistical analysis showed that the highest rate of sterilization at 75% of the stock solution used was the extract of eucalyptus (86%), garlic (84%), mint (74%), and ginger (73%) which all were significantly less than the sterilization of malachite green, as shown in Fig. (3).

By applying 100% of the stock solution, the highest rate of sterilization was with employment of water extract of garlic (96%), eucalyptus (95%), mint (93%), and the lowest rate of sterilization was with extract of ginger (90%). The rate of sterilization with malachite green was 99%, and the rate of infection in non-sterile eggs was 73%. The statistical analysis showed no significant differences in sterilization at 100% concentration compared with malachite green sterilization for all extracts used, as shown in Fig. (4). Statistical analysis also showed a high positive correlation between sterilization and the concentration of water extract of plants.

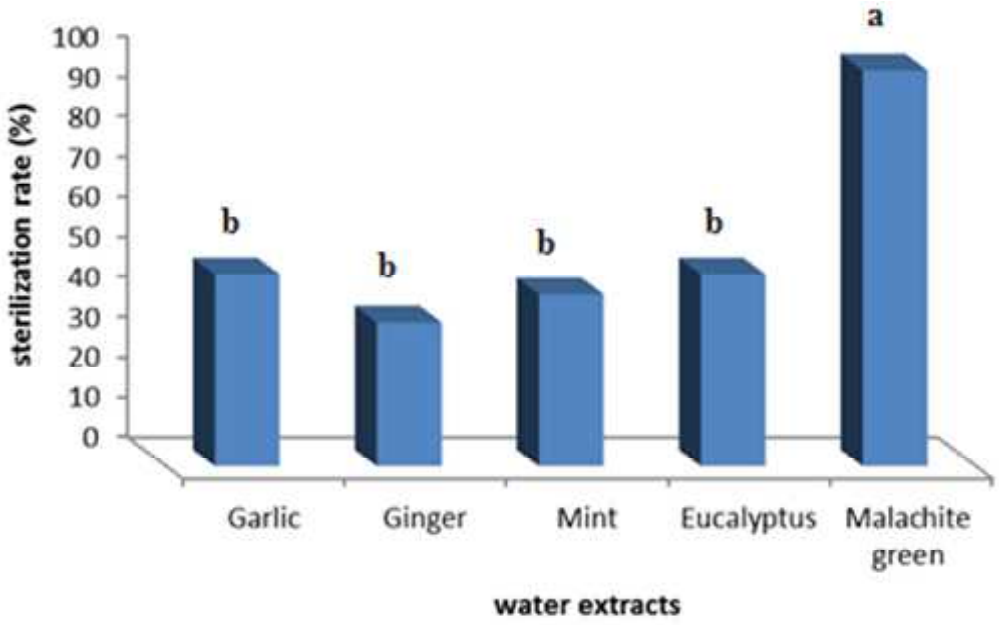

Fig. (1): The sterilization rate in water extracts of plants (25% of the stock solution) compared to malachite green. The same letters means significantly no differences among different treatments.

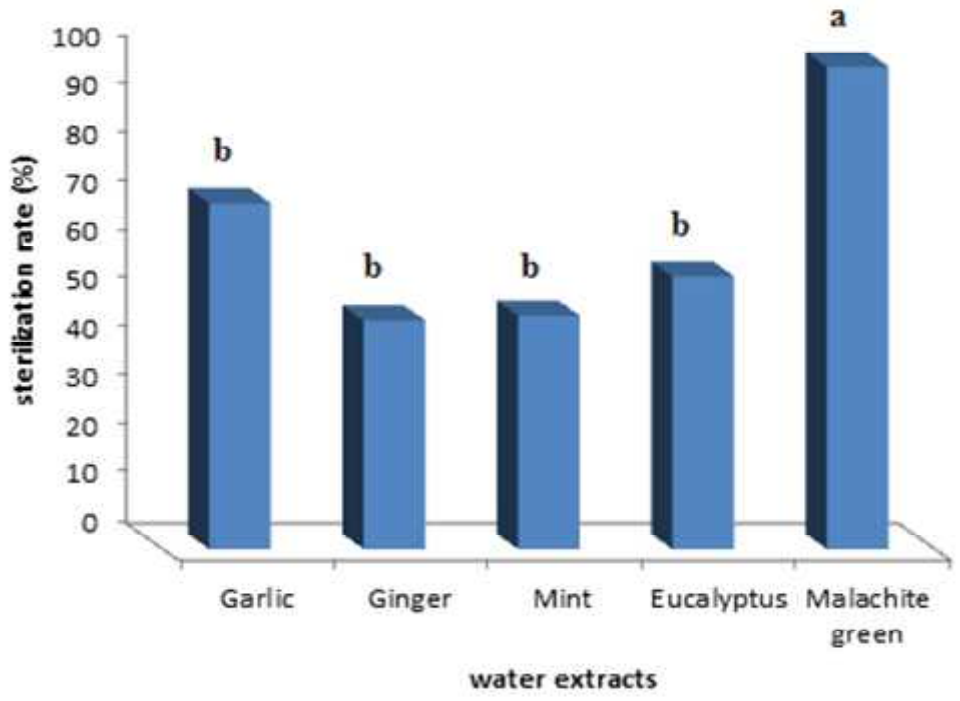

Fig. (2): The sterilization rate in water extracts of plants (50% of the stock solution) compared to malachite green. The same letters means significantly no differences among different treatments.

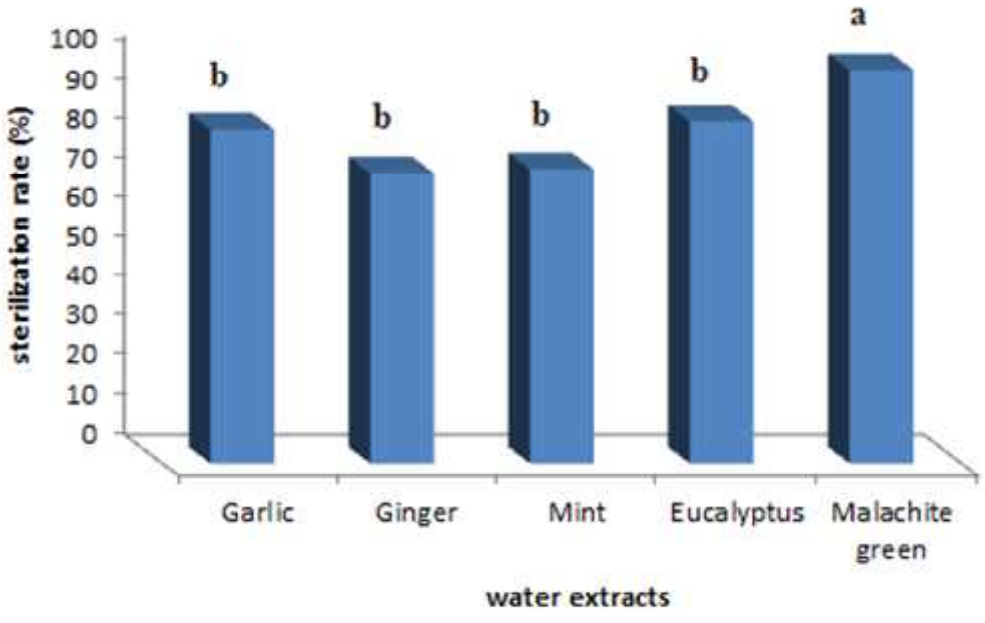

Fig. (3): The sterilization rate in water extracts of plants (75% of the stock solution) compared to malachite green. The same letters means significantly no differences among different treatments.

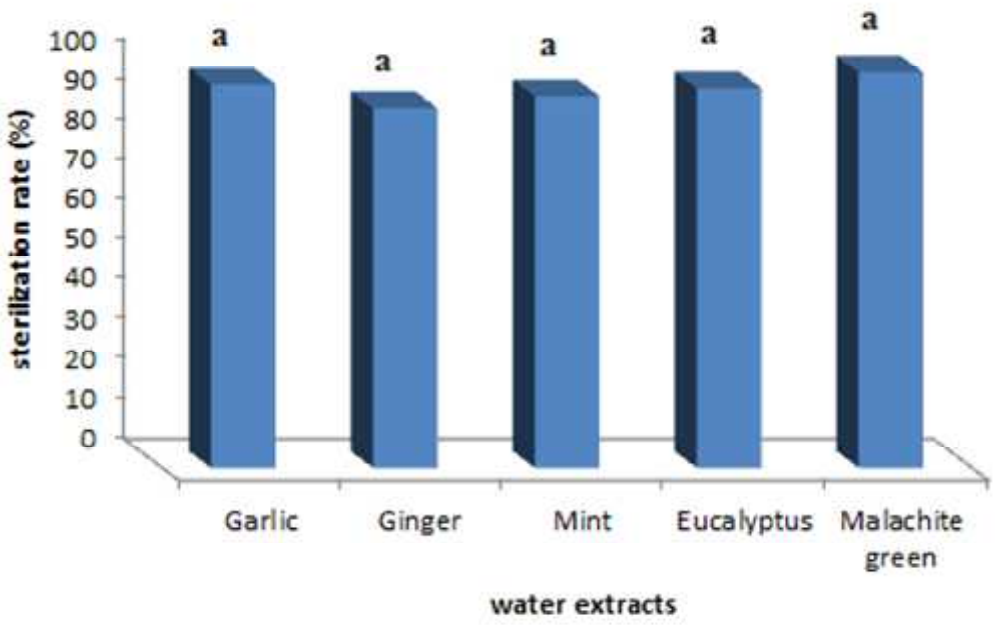

Fig. (4): The sterilization rate in water extracts of plants (100% of the stock solution) compared to malachite green. The same letters means significantly no differences among different treatments.

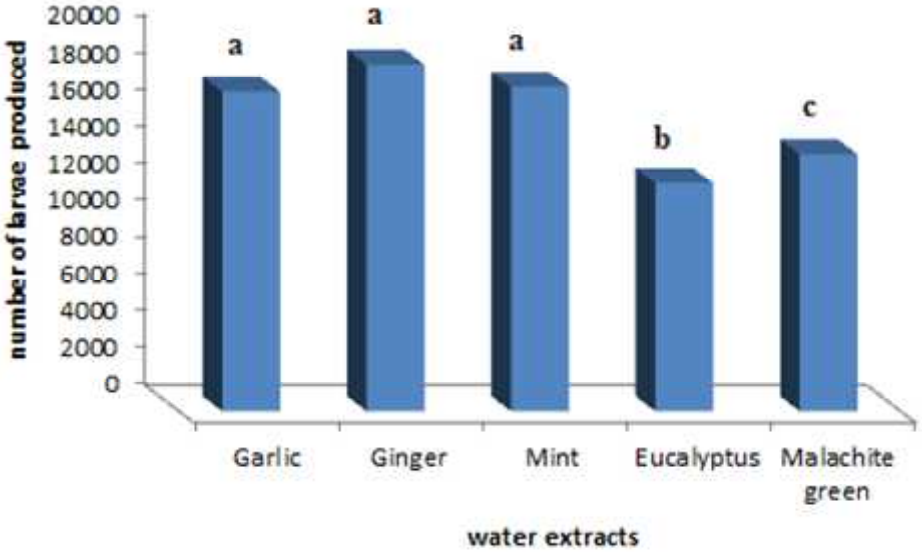

Fig. (5): The number of larvae produced when using different plant extracts compared to malachite green. The same letters means significantly no differences among different treatments).

The number of produced larvae differs according to the material used in egg sterilization. The highest number of larvae produced was with the application of ginger extract (18694 larvae) followed by mint extract (17500 larvae), then garlic (17228 larvae), malachite green (13,878 larvae) and ultimately eucalyptus (12320 larvae). Statistical analysis showed that number of larvae produced by the use of ginger, mint and garlic extract was significantly higher

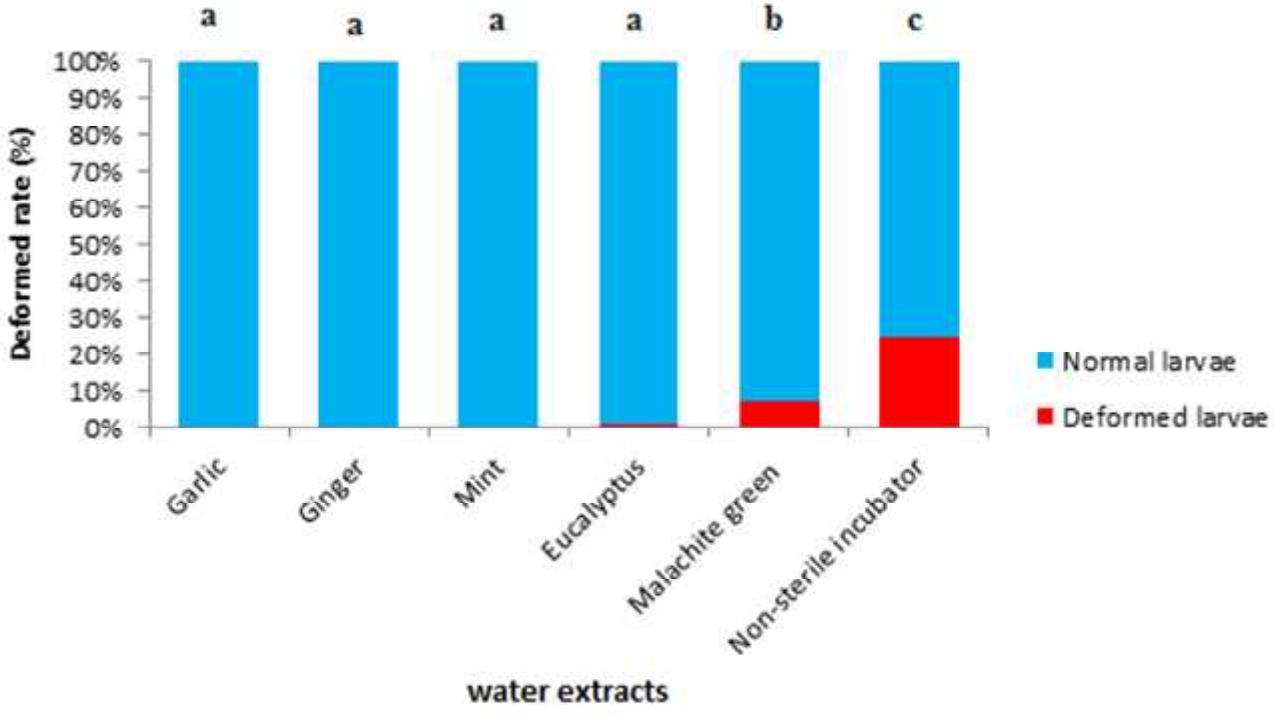

Fig. (6): The percentage of natural fish larvae and deformed larvae produced from different plant extracts compared to malachite green and non-sterile incubator. The same letters means significantly no differences among the treatments.

than the number when malachite green was used. Number of larvae produced by applying eucalyptus extract was significantly lower than the number of larvae produced with application of malachite green. Fig. (5) showed the relationship between the number of produced larvae and the materials used to sterilize the eggs.

The results showed no abnormalities in larvae treated with garlic, ginger and mint, while the percentage of malformations in larvae treated with malachite green was 7%, eucalyptus achieved 1% larvae, while 25% larvae of non-sterilized incubator. The statistical analysis showed significant differences in the number of deformed larvae between eucalyptus treatment and non-sterile incubator compared to malachite green treatment as indicated in Fig. (6).

Discussion

The results showed that there is an inhibitory effect of all water extracts of the examined plants on the studied fungi. This is due to the presence of effective inhibitory substances in these plants on fungus. Garlic bulbs contain saponins, glycosides, isothiocyanate, volatile oils (Allicine, Alliin, Allimine and Indol) and sulfur compounds (Al-Shamma, 1990; Dutta *et al*., 2005; Al-Mayah, 2013). Ginger rhizomes contain phenols, alkaloids, resins, saponins, tannins, flavones, steroids, gingerol, linalol, camphenol and olego-resin-gingerol (Chevalier, 1990; Reddy and Seetharam, 2009; Mohammad, 2012; Al-Mayah, 2013). Leaves of eucalyptus contain phenols, cineol, eucalyptol, tannins, piperitone and citronellal (Al-Mayah, 2013), while mint leaves contain many volatile oils, including menthol, menthone, pinene, lemonin, cineole and carvon (Al-Dury, 2012; Al-Mayah, 2013).

The active substances mentioned above are considered to be the most important antifungal agents, which cause membrane disruption, that affects the metabolic efficiency of the cells and thus inhibit them (Hili *et al*., 1997). They also interfere with the walls of fungal cells as well as with the DNA of the fungus causing inhibition. The active substances of the extracts act as inhibitors of microbial enzymes as well as rupture of cell

membranes, their association with DNA (Draughon, 2004; Benkeblia, 2005; Unver *et al*., 2009), and their attachment to the membranes or fungal cells walls and their association with lipophilic compounds, thus rupturing the walls of these cells and thereby inhibiting the growth of fungi (Cowan, 1999). These substances also act to reduce oxygen uptake and inhibit the manufacture of fats, proteins and nucleic acids and thus affect the production of energy, as well as the oxidation of proteins and amino acids in the cellular membrane (Weiss and Fintelmann, 2000). They also affect fat metabolism by influencing the effectiveness of the 3-hydroxy-3methyl glutase enzyme, which is responsible for the construction of mevalonic acid, which paves the way for the construction of sterols and inhibits the synthesis of the enzyme acetyl CoA (Singh *et al*., 2008). Therefore, they prevent the formation of fatty acids, sterols and many other compounds, thus breaking the fungal cell membrane and thus inhibiting the growth of fungi. Hussein (1981) indicated that these extracts caused the cytoplasmic mass within the fungus and caused malformations of the edge of mycelium and reduced diameter, resulting in weak growth of fungi colonies.

The exposure of fish eggs to fungal infection in the hatcheries during incubation, especially *Saprolegnia* species, is common in the hatcheries (Bruno and Wood, 2011; Kalatehjari *et al*., 2015). It is a common fungus in fish hatcheries which causes large losses (Mustafa, 1999). Subasinghe and Sommerville (1988) explained that the cause of eggs loss is that scratches in the membrane of the chorion leading to the infection of these eggs by fungus through these scratches and thus they loss. The mycelia also cover the eggs and thus prevent oxygen to reach the eggs (Bauer *et al*., 1977). Therefore, the hatchery owners apply protective steps to reduce the infection with this fungus, such as the use of uncontaminated water, or the removal of adherents of eggs and the withdrawal of dead eggs (Mhaisen, 1983).

The results showed that the highest percentage of sterilization was achieved with application of 100% of the stock solution of the water extracts of garlic, which may be due to the content of garlic of saponin, which has a greater effect on fungi than both alkaloids and phenolic compounds (Chakravarty, 1976). The results showed that the concentrations of 25, 50 and 75% of garlic extract were less significant than the malachite green, but the 100% extracts gave sterilization rate closer to that of malachite green. This is due to the high concentration of active substances in the extract with a concentration of 100% (Hili *et al*., 1997). The obvious effect of plant extracts at high concentrations on fungi cause shrinkage of the colony (Hernandez *et al*.,1994; Lee *et al*.,1999; Sawangjaroen and Sawangjaroen, 2005; Thanaboripat *et al*., 2007; Ali *et al*., 2012), while lower concentrations cause dispersion of the active materials away from fungal cells, resulting in a decrease in the inhibitory effectiveness of the material used (Hili *et al*., 1997), or that the low concentration of active substances makes the substance of non-therapeutic benefit (Al-Zubaidy, 1998). Thanaboripat *et al*. (2007) found that the low concentrations of water extracts did not inhibit fungi but, on the contrary, could promote fungal growth, while Sultan and Antar (2010) found that the effect of low concentrations is greater than that of high concentrations, which they interpreted to increase the resistance of fungi to active compounds. Salman *et al.* (2010) explained that certain compounds when diluted might be free and have more power of penetration and effectiveness in the cells while these compounds become concentrated and more complex at high concentrations.

Number of larvae produced by the artificial reproduction of common carp in fish hatcheries varies according to several factors, including environmental factors such as temperature, pH, dissolved oxygen and salinity (Al-Gezali, 2010), the quality of eggs and sperms and genetic characteristics of the mothers (Al-Gypore, 2011), or the production capacity of the hatcheries, their design and the efficiency of their workers (Al-Hameary, 2011). The number of larvae produced for each treatment was variable. This may not necessarily be due to the use of a particular

substance in the sterilization, but the number of produced larvae was strongly correlated with the mothers feed (both quantity and quality), essentially, amino acids, vitamins, carbohydrates and minerals which are important priorities for ovulation and the production of large-size eggs containing high nutrient stock (Naif, 2005). The size of eggs and success of the hatching process are related to the age and weight of the females (Alikhunhi, 1966). The large females produce larger eggs and the hatching rate is greater and therefore, the chances of survival are greater. Number of eggs is related to the size of females where mother fishes give more than 200,000 eggs/ kg of fish (Hepher and Pruginin, 1981).

Many antifungal agents are used to reduce the infection with *Saprolegnia* to avoid high financial losses in the hatcheries. These substances may be natural, such as extracts of medicinal and aromatic plants that containing active substances (Al-Zubaidy, 1998; Al-Tamimi, 2001; Yaseen, 2004; Al-Niaeem and Al-Yassein, 2009) or the use of certain antifungal chemicals (Barnes *et al*., 2000; Rahkonen and Koski, 2002; Svobodova and Kolarova, 2004; Khomvilai *et al.*, 2006). Some of these substances have side effects that appear during or after treatment in fishes, environment or the consumers. Some substances can lead to fish loss (Meinelt *et al*., 2001), or may affect important organs of the fish's body such as the gills, liver and kidneys (Helfrich and Smith, 2000).

The results showed that no distorted larvae were obtained when using any plant extracts except that of eucalyptus. However, the percentage was very low and not significant. The cause of deformation may be due to the use of randomly inbreeding culture. Undesirable appearance of fishes may be due to increased gene recurrence and the possibility of the emergence of transgenic traits on the recessive gene and then the emergence of a mutilated offspring that is what happened in the Iraqi breeding herds (Naif, 2005; Al-Abadi, 2015), which may happen with use of malachite green which is routinely incorporated in sterilization of fish eggs.

In the case of the use of malachite green, the percentage of deformities jumped to 7%. This is due to the fact that this material is known to cause deformation of fish larvae. Duijn (1973) found that the toxicity of this substance increases with exposure to light. Woynarovich and Horvath (1980) confirmed that malachite green is highly toxic to larvae if used with high concentrations, while Mustafa (1999) found that it is difficult to control constant concentration of this material during the sterilization process. Lilley and Inglis (1997) concluded that the chemical effectiveness of this substance and its effect on fishes changed with the changing temperature of water. Many researchers have confirmed that malachite green is characterized by serious disadvantages and the most important is the occurrence in deformations or genetic mutations as well as its carcinogenic effects (Hoffman and Meyer, 1974; Herwig, 1979; Alderman, 1985; Mustafa, 1999), as well as its effect on the growth of fish eggs (Mhaisen, 1983).

Conclusions

Water extracts of the studied plants (garlic, mint, eucalyptus and ginger) proved their efficacy as safe materials to be successfully used in sterilization of eggs of fishes in hatcheries. These plants have high efficacy to be substitute substances instead of a highly toxic malachite green, as well as the advantage that the produced fish larvae did not suffer from malformation and the survival rate reach 100%.

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